The role of nitric oxide and somatic action potentials in a GluR1 independent LTP

by

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DECLARATION

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ABSTRACT

Studies in GluR1 knockout mice have shown that neocortical LTP consist of both pre- and post-synaptic components that rely on nitric oxide and GluR1 respectively (Hardingham and Fox, 2006). Given that GluR1 knockout also show hippocampal LTP (Hoffmann et al., 2002) I hypothesised that the residual LTP might depend on nitric oxide. I have found that hippocampal LTP can be induced in GluR1 knockout with purely orthodromic stimuli in mature mice (>8weeks) and that a theta-burst protocol was effective at inducing LTP while 100Hz stimulation was not. I found that only theta-burst stimulation produced reliable post-synaptic spikes, while 100Hz stimulation produced relatively few spikes. Inhibition of post-synaptic somatic spikes with local TTX application prevented LTP in the GluR1 knockout mice. Theta-burst induced LTP in GluR1 knockout was almost entirely nitric oxide dependent and involved both nitric oxide synthase 1 and nitric oxide synthase 3 isoforms. Finally, I also found that somatic spike production was also necessary for a nitric oxide dependent form of LTP in wild-type mice, which made up approximately 50% of the potentiation at 2 hours post-tetanus. I conclude that nitric oxide dependent LTP can be produced by physiologically relevant theta-burst stimuli because this protocol evokes reliable action potentials. Since this form of activity occurs during learning it could be relevant to memory formation in GluR1 knockout and wild-type mice.

This PhD thesis is dedicated to my parents

Liz and Geoff Phillips

Thank you for all the love, support and help you have given me in everything I have chosen to do.

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Abbreviations

[Ca2+]	Calcium Concentration
7-CIK	7- chlorokyurenic acid
AA	Arachidonic acid
ACSF	Artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANOVAs	Analysis of variance
AP	Action Potentials
CaMKII	Ca2+/calmodulin-dependent protein kinases
cAMP	Cyclic adnosine monophosphate
CED	Cambridge Electronic Design
cGMP	Cyclic guanosine monophosphate
CNQX	6-cyano-7-nitro-quinoxaline- 2,3 -dione
CNS	Central nervous system
CV	Coefficient of variation
DAA	D-amino-adipate
D-AP5	D-2-amino-5-phosphonovalerate
DG	Dentate gyrus
DNQX	6,7-dinitro-quinoxaline-2,3 -dione
eNOS	Endothelial nitric oxide synthase
EPSP	Excitatory post synaptic potential
fEPSP	Field excitatory post synaptic potential
GABA	y-amino-butyric acid
GC	guanylyl cyclase
GFP	Green Florecent protein
GluR1	AMPA receptor subunit 1
GluR2	AMPA receptor subunit 2
GluR3	AMPA receptor subunit 3
GluR4	AMPA receptor subunit 4
HFS	High frequency stimulation
I	Current
iGluRs	Ionotropic glutamate receptors
iNOS/NOS2	Inducible nitric oxide synathase
IO	Input Output
KA	Kainic acid
KAR	Kainate receptors
L-NAME	N (G)-nitro-L- arginine methyl ester
L-NNA	NG-Nitro-L-arginine
LTD	Long-term depression
LTP	Long Term Potentiation
mGluR	Metabotropic glutamate receptors
NBQX	2-3 -dihydroxy-6- nitro-7-sulphmoyl-benzo(F)quinoxaline
NMDA	N-methyl-D-aspartic acid
NMDAR	NMDA Receptor
nNOS/NOS1	Neuronal nitric oxide synthase
NO	Nitric Oxide
NOS	Nitric Oxide synthase

OLM	Oriens lacunosum moleculare
PKA	Protein kinase A
PKC	Protein kinase C
PPF	Paired-pulse facilitation
PPR	Paired-pulse ratio
PSD	Postsynaptic density
S	Serine
S1	Stimulating electrode 1
S2	Stimulating electrode 2
SNAP	Soluble NSF Attachment Protein
SNARE	Soluble NSF Attachment Protein Receptors
T	Threonine
TBS	Theta burst stimulation
TLE	Temporal lobe epilepsy
TM	Transmembrane
TTX	Tetrodotoxin
V	Voltage

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1 Introduction

Synaptic plasticity is a widely studied phenomenon that is likely to be involved in learning and memory. To understand how our memories are formed is a fascinating scientific goal and the discovery that repetitive electrical stimulation could produce persistent and stable changes in neuronal activity has fuelled the drive towards this goal for over 30 years.

The key finding was the identification of a form of synaptic plasticity that became known as long-term potentiation (LTP) (Bliss and Lomo, 1973). The implications that synapses could function in a flexible, or plastic, fashion has spread to groups of researchers working on many different brain or CNS areas in a variety of animals but has always remained closely associated with the mammalian hippocampus where the discovery was first made. The hippocampal formation has long been understood to be an area of the brain involved in processing sensory information into a form that is then available to memory processes such as recall or remembering. In other words, the hippocampal formation is responsible for "laying down" memories or forming the memory "traces" of our experiences. It was quite justifiable then, that the finding of an experimental protocol that could change the output of neurons in the hippocampus generated so much speculation and research into possible mechanisms of memory formation.

1.1 Activity-Dependent Modifications

In 1973 Tim Bliss and Terje Lomo reported that very short bursts of high frequency stimulation applied to an afferent input produced a persistent increase in the output of the target neurons that greatly outlasted the duration of the stimulus. This important phenomenon was named long-term potentiation (LTP) and is of interest as it is likely to be part of the normal function of certain brain regions and may be involved in the formation of certain forms of memory. Long-term potentiation has been demonstrated in many areas of the mammalian brain including hippocampus, amygdala, prefrontal cortex, motor cortex, visual cortex, and in human neocortex (Bliss & Lomo, 1973; Gerren & Weinberger, 1983; Clugnet & LeDoux, 1990, Aroniadou & Teyler, 1991; Baranyi *et al.,* 1991; Chen *et al.,* 1996). Bliss and Lomo induced LTP in the dentate gyrus of the hippocampal formation of anaesthetised rabbits by stimulation of the

perforant pathway. The hippocampal formation has a number of anatomically distinct areas with excitatory pathways and local inhibitory circuits and is described briefly below.

1.2 The hippocampal formation

1.2.1 Anatomy and organization

The hippocampal formations are situated beneath the cortices of the temporal lobes and are found bilaterally. They lie lateral to the thalamus and medial to the temporal horns of the lateral ventricles (Figure 1.1). The gross anatomy of the hippocampal formation is an elongated structure, bending in a c-shaped manner, its long axis is known as the septotemporal axis and its short axis is known as transverse (as reviewed Amaral and Witter, 1989). The hippocampal formation can be divided into four main regions, dentate gyrus (DG), Ammon's horn (*Cornu Ammonis* CA1-4), subicular complex (subiculum, presubiculum and parasubiculum) and the entorhinal cortex (Blackstad, 1956; Lorente de No, 1934; Ramon and Cajal, 1911; Rose, 1926). The dentate gyrus and Ammon's horn form the hippocampus proper where these two cell body layers fold around one-another. The hippocampus is named after its shape, as it resembles a sea-horse (*Hippo* - horse, *kampus* - sea). Under low magnification the cell body layers of the hippocampus are visible in a transverse slice (see Figure1.1).

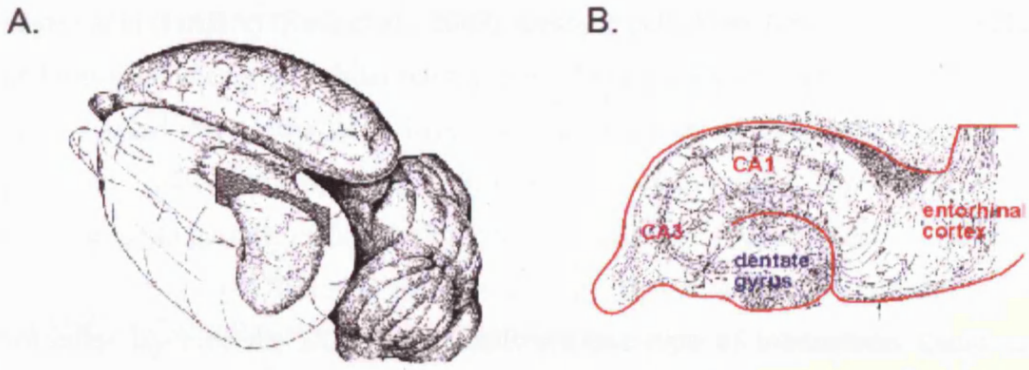


Figure 1.1: The hippocampal formation.
A) The location ofthe hippocampal formation in rodent brain. B) Transverse cross-section of the hippocampal formation
http ://www.neuroscience.bham.ac.uk/neurophysiology/research/hippocampus.htm.

The hippocampus receives inputs from entorhinal cortex, septum, amygdala, thalamus and hypothalamus to name a few and it is thought that the hippocampus is a region of integration or "supramodal association cortex" (Cooper & Lowenstein, 2001)

1.2.1.1 Dentate Gyrus

The DG (*fascia dentata*) can be divided into three main areas, the granule cell layer, molecular cell layer and polymorphic layer or hilus (Blackstad, 1956). The granule layer is named after the principal cells of the DG whose somata reside in this region; granule cells. The molecular layer houses the apical dendrites of the granule cells and the hilus supports the granule cell axon bundles, also known as mossy fibers, and a number of inhibitory interneurons (as reviewed by O'Keefe et al., 1979).

The granule cells receive glutamatergic inputs from the axons of the perforant pathway originating in the entorhinal cortex (Andersen et al., 1966a; Lomo, 1971). The perforant path is subdivided into medial and lateral pathways which bring afferent inputs from differing regions of the entorhinal cortex (Amaral and Witter, 1989). Mossy fibers leave the DG via the hilus, forming synapses with proximal dendrites of CA3 pyramidal cells (Blackstad and Kjaerheim, 1961; Hamlyn, 1961, 1962; Laatsch and Cowan, 1966). Granule cells are glutamatergic (Andersen et al., 1966a), however, recent evidence suggests that these terminals co-release *y*-amino-

butyric acid (GABA) (Ruiz et al., 2003). Granule cell axons form several branches in the hilus and synapse with hilar interneurons. Mossy cells are found in this region and are so named due to the large irregular-shaped spines on their proximal dendrites. Ipsilateral (associational) and contralateral (commissural) inputs from these interneurons to granule cells contribute to the regulation of granule cell excitability. A number of other GABA-ergic interneurons are also found in the polymorphic and molecular layer of the DG. Basket cells are one type of interneuron found in the granule cell layer of the DG.

1.2.1.2 Structure of the CA3

The strata of the CA region consist of the *stratum oriens*, *stratum pyramidale*, *stratum lucidum*, *stratum radiatum* and *stratum lacunosum moleculare*. The principale cell type found in the CA3 region is the glutamatergic pyramidal neuron, which possess a triangular soma. Dendrites from the cells in the cell body layer project apically to the *stratum lucidum, radiatum* and *lacunosum molecular.* Basal dendrites project into the *stratum oriens*. As discussed previously, the main intrinsic input to the CA3 is the mossy fibers from the DG granule cells. Association fibers are other excitatory inputs in the CA3 which come from other CA3 cells and synapse with basal and apical dendrites (Lorente de No, 1934; Ramon Cajal, 1911). CA3 afferent inputs also arise in the septum, however these inputs are mostly cholinergic and GABA-ergic (Amaral and Witter, 1989).

Pyramidal cells in all parts of the CA3 give rise to outputs to the CAl. One branch leaves the hippocampus via the fornix to the contralateral hippocampus (commissural pathway) and the other, the Schaffer collateral axons, synapse with the proximal dendrites of CAl pyramidal cells, in the *stratum radiatum* of the same hippocampus (Andersen et al., 1966b). Collaterals of the CA3 cells also project to dendrites of CAl GABA-ergic interneurons providing feed-forward inhibition to CA1 (Zimmer, 1971).

1.2.1.3 Structure of the CA1

The CAl afferent inputs arise primarily from CA3 efferents arising from the ipsilateral hippocampus via the Schaffer collaterals and contralaterally via the commissural fibers (Gottlieb and Cowan, 1973). The CA1 also receives efferent activation from the septal nuclei; these inputs are cholinergic (Dudar, 1977; Storm-Mathisen, 1977), and are thought to play a role in regulating the hippocampal theta rhythm (Sotty et al., 2003). Dopaminergic inputs have also been identified in the CA1. Monoamine inputs arising in the medial raphae to the CA1 pass via septal areas or amygdale (Storm-Mathisen, 1977). Dopamine is thought to regulate the excitability of the CA1 (Gribkoff and Ashe, 1984) and hyperactivity of hippocampal dopamine has been associated with schizophrenia (Krieckhaus et al., 1992).

CA1 pyramidal neurons are found in the cell body layer (*stratum pyramidale*) and are smaller, have fewer spines and are less densely packed than their counterparts in the CA3. CAl cell dendrites are polarised and the apical dendrites project to the *stratum radiatum* and distally in the stratum lacunosum moleculare. The basal dendrites project to the *stratum oriens*. The axons of pyramidal cells of the CAl are heavily branched and project into the alveus, a thin layer of axons running along the cortical side of the *stratum oriens*. Axons of the CAl cells synapse either with inhibitory interneurons within CAl, which regulate the excitatory tone of the CAl layer, or project to the subiculum (Finch and Babb, 1981) and entorhinal cortex (Swanson et al., 1978). CAl axons have also been shown to project further to the perirhinal and frontal cortex and also the septum.

Interneurons are another cell type found in the CAl, these are GABA-ergic and hence inhibitory. One interneuron may affect firing of up to a thousand other pyramidal cells (Buhl et al., 1995), acting to finely tune the activity of the CAl . The CAl interneuron population is diverse in dendritic and axonal arborisation. Interneurons are identified by their firing patterns and specific expression of neuropeptides (e.g. somatostatin, cholecystokinin) and Ca^{2+} binding proteins (e.g. calbindin and parvalbumin). Cell types include fast spiking basket cells and OLM (*oriens lacunosum moleculare*) interneurons. Basket cell stomata are located in the pyramidal cell layer of the CAl

and dendrites receive inputs from the Schaffer collaterals and commissural fibers, efferents synapse axosomatically with pyramidal cells in a feed forward manner. This means that interneurons receive input in series with the pyramidal cell and the activity of pyramidal cells are regulated based upon this information. OLM interneurons are an example of feedback regulation of pyramidal cells. This means that the OLM cells receive input from the cell they are regulating and feedback to the cell based upon the information it receives. Somata of OLM interneurons are found in the *stratum oriens*, receiving inputs from the CAl pyramidal cells. Axons pass through the *stratum radiatum* and cell body layer to synapse with pyramidal cell dendrites in the *lacunosum moleculare* (for a more comprehensive review see Freund and Buzsaki, 1996)

1.2.1.4 The trisynaptic circuit

Intrinsic connections of neurons within the hippocampus form a trisynaptic circuit. The circuit starts with the perforant pathway from the entorhinal cortex, which synapses onto granule cells in the dentate gyrus. The mossy fibers of the granule cells project onto the CA3 pyramidal cells whose Schaffer collaterals then synapse with CAl pyramidal cells. To complete the circuit the axons of the CAl cells then continue to the subiculum/entorhinal cortex (Finch and Babb, 1981). A summary of the intrinsic connections in the hippocampus can be seen in Figure 1.2. The trisynaptic circuit is thought to be critical for learning and hence this is a good preparation to study the synaptic mechanisms of learning and memory. The structure and function of the hippocampus appears to be similar in rodents, including rats and mice, with that of primates (Squire et al., 1992), making the rat hippocampus a valid choice for investigation of memory function.

Within the hippocampus there are local networks of connections formed by inhibitory interneurons and principal neurons. However even if only the excitatory connections are considered, the actual extent of connections is much more complex than the trisynaptic pathway suggests, although the "direction of flow" of information is overall the same. Not only does the perforant path form synapses in dentate gyrus

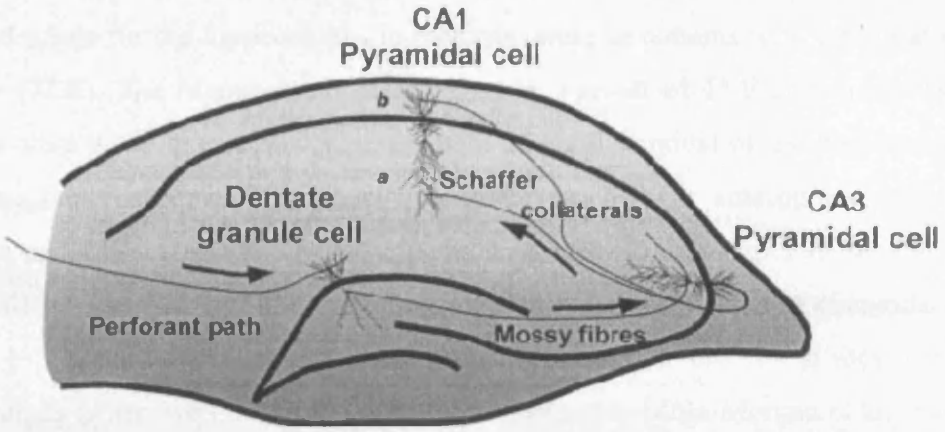


Figure 1.2: Trisynaptic pathway.
A simplified view of the connectivity in the hippocampus. Connections and the flow of information are considered unidirectional, starting from sensory input arriving at the entorinal cortex. An axon pathway from layer II of the entorinal cortex perforates the subiculum and terminates in the dentate gyrus. Dentate gamule cells have axons known as mossy fibres that form synaptic connections in CA3. The axons of CA3 pyramidal cells form the axon pathway known as the Schaffer collaterals which terminate on CA1 pyramidal cells on the apical or basal dendrites (a and b)

but there are also projections from layer II of the entorhinal cortex to CA3. The entorhinal cortex has axons extending from the lateral and medial portions of layer III directly to CAl and subiculum. These connections are formed along the septotemporal axis at different positions along the CA3-to-CA1 and CA1-to-subiculum boundaries depending on their origin. Associational connections are formed in dentate gyrus and CA3. The associational connections of CA3 and the Schaffer collaterals project along the septotemporal axis rather than being confined to more two-dimensional projections (Johnston & Amaral, 1999).

1.2.2 Function of the hippocampus

Much of the evidence relating to the function of the hippocampus points to a role in storing and processing memories. These are specifically thought to be related to memories of a spatial context (Mishkin, 1978; Squire and Zola-Morgan, 1991). The hippocampus may also however be involved in other types of memory, such as declarative memory (recollection of facts) (Squire et al., 1992), formation of new memories and consolidation of memories. Much of the early literature exploring the function of the hippocampus focused on the effects of damage to the hippocampus

from human studies or through ablation studies in animals. The first study that proposed a role for the hippocampus in memory, were in patients with temporal lobe epilepsy (TLE). The hippocampus is damaged as a result of TLE and in the 1950s attempts were made to treat severe cases with bilateral removal of the hippocampus. However, the consequence of these resections resulted in anterograde amnesia, (Scoville and Milner, 1957). In these patients, memories from prior to the surgery could still be recalled and understanding and IQ remained unaltered (Scoville and Milner, 1957), but new memories could not be formed, this lead to the idea that the hippocampus is involved in the forging of new memories (Zola-Morgan et al., 1986). The loss of a single hippocampus does not result in amnesia, suggesting a single hippocampus can perform the function of the absent one (Penfield and Milner, 1958; Scoville and Milner, 1957). A major problem with interpreting the effects of resection of the hippocampus is that the area surrounding the hippocampus is also damaged (as reviewed by Jarrard, 1995). Aspiration, electrolytic lesion or thermocoagulation are the conventional techniques for generation of hippocampal lesions *in vivo*. The use of ibotenic acid injections is now used in preference to these methods, as it results in selective destruction of the hippocampus and minimizes damage to the cortex surrounding the hippocampus (Jarrard, 1995).

1.2.3 Role of the hippocampus in spatial learning

The removal of the hippocampus in rats results in impaired learning and utilization of spatial and contextual information. This is based on the results of poor performance in spatial tasks (Mishkin, 1978; Squire and Zola-Morgan, 1991), including the spatial radial maze and rewarded attention task tests (Bouffard and Jarrard, 1988; Morris et al., 1990). Rats with hippocampal lesions perform as well as control rats in non spatial memory dependent tasks (Jarrard, 1993), suggesting the importance of the hippocampus specifically in spatial memory. In 1971, it was suggested that neurons of the hippocampus coded for a cognitive map, a neural representation of the external environment (O'Keefe and Dostrovsky, 1971). This group identified cells in the hippocampus that fired in a particular location, regardless of direction of travel. These cells were later found to be CA1 and CA3 pyramidal cells (O'Keefe, 1979) and are now referred to as "place cells". It now seems unlikely that the map codes for a

specific spatial map, but more likely encodes relative position and this aids animals in solving navigational tasks. The position in space at which a place cell fires is known as the place field. The closer the cell is to the place field the more the place cell fires. It has been shown that each place cell has differing place fields corresponding to different places i.e. one cell may fire when a rat is in one corner of a room, a second may fire in an overlapping section of the room. Each place cell can have multiple place fields, i.e. the same neuron could have a place field in two different rooms. This enables each neuron to map for a number of locations, showing the role of place cells more in navigation than in coding for a map *per se*. Place cells can be re-mapped in new environments and the long term maintenance of the place field relies on NMDA receptor (NMDAR) activation, suggesting a link between spatial learning and memory with LTP and LTD (Leutgeb et al., 2005).

The Morris water maze is a task that was specifically developed to study spatial learning and memory (Morris., 1984). In these experiments, a submerged platform is placed into a tank of water containing milk. This means that the rodent cannot see the platform, as the milk makes the water opaque. During the task the rat uses spatial cues to learn where the platform is located relative to them. Under control conditions, the length of time taken to find the platform decreases as the rat learns where the platform was positioned. Selective blockade of NMDARs in the hippocampus by D-(2R)-amino-5-phosphonovaleric acid (D-AP5) resulted in a loss of learning in the water maze (Davis et al., 1992). This provides another link with learning and memory in the hippocampus with the molecular mechanisms of LTP and LTD. Research has shown that there are more place cells in the dorsal than in the ventral hippocampus, indicating that this region may be more important in the regulation of spatial memory (Hock and Bunsey, 1998; Jung and Larson, 1994; Moser et al., 1993; Moser, 1995; O'Keefe and Dostrovsky, 1971). This principle is supported by human studies made on London taxi drivers, whose vocation requires the extended use of spatial learning (Ghaem et al., 1997; Maguire, 1997; Maguire et al., 1998). Not only do taxi drivers have larger hippocampi than control subjects, their hippocampi are selectively enlarged in the posterior region of the hippocampus, (Maguire et al., 2000). These data provide evidence for a role of the dorsal hippocampus in spatial learning.

1.3 Glutamate receptors

Glutamate was first discovered as an excitatory neurotransmitter in 1954 when it was shown that application of sodium glutamate or sodium aspartate induced motor cortex seizures (Hayashi T., 1954). Glutamate is regarded as the principla excitatory neurotransmitter in the mammalian central nervous system (CNS). It is released from the presynaptic nerve terminal and actives both ionotropic glutamate receptors (ligand-gated ion channels) and metabotropic glutamate receptors (mGluRs) which are positioned in the postsynaptic membrane (Hollmann and Heinemann, 1994). Beneath these receptors there is a large group of scaffolding and anchoring proteins known as the postsynaptic density (PSD).

Ionotropic glutamate receptors (iGluRs) mediate fast excitatory neurotransmission (milliseconds) whereas mGluRs are coupled to G-proteins and mediate signalling in the hundreds of milliseconds timescale. Glutamate receptors are involved in synaptic plasticity (LTP and LTD) which are thought to underlie learning and memory. A number of different receptors exist within the ionotropic glutamate receptor family and are classified by their pharmacology and sequence homology (Bettler and Mulle, 1995; Dingledine et al., 1999). iGluRs activated by N-methyl-D-aspartate (NMDA) are known as NMDA receptors (NMDARs) (Curtis et al., 1972) and non-NMDA receptors are iGluRs which are not activated by NMDA. ά-amino-3- hydroxy-5-methyl-4-isoxazoleproprionate (AMPA) receptors (AMPARs) and kainate receptors (KARs) make up the non-NMDA receptor subclass (as reviewed by Bettler and Mulle, 1995).

1.3.1 iGluR topology

The basic iGluR subunit structure is similar through out all subgroups of receptors. Early research suggested that AMPA subunits form pentamers (Brose et al., 1993; Ferrer-Montiel and Montal, 1996; Hollmann and Heinemann, 1994; Wenthold et al., 1992). However, it is now generally accepted that receptors are formed as multimeric

assemblies of four subunits around a central pore (Mano and Teichberg, 1998; Nusser et al., 1998; Rosenmund et al., 1998)

The N-terminal domain of iGluRs are extracellular and hydrophobicity plots have suggested that there are four transmembrane domains (TM) (Bennett and Dingledine, 1995). TM2 forms a re-entrant loop in the membrane (Bennett et al., 1995), and consequently the C-terminal domain is intracellularly located. The long extracellular loop between TM3 and TM4 associates with the C-terminal segment of the N-terminal domain at residues T686 and E402 respectively to form the glutamate-binding domain (Tygesen et al., 1995). This domain forms a clamshell like structure, through a conserved amino acid sequence, which binds glutamate in a non-covalent manner (as reviewed by Dingledine et al., 1999). Single subunits assemble into a tetramer and the TM2 regions form the pore through which the ions may pass. iGluR differ from many other receptors as their ligand does not bind to the interface between two receptor subunits, but to a single subunit. Upon agonist binding a conformational change occurs in the receptor to allow ions to permeate the channel. The direction of flow of ions is determined by the Nernst equation which calculates the equilibrium between electrical and chemical gradients. In neurons at their resting potential, Ca^{2+} and Na^{+} will pass into the cell and K^{+} will pass out of the cell.

$$V = \frac{RT}{ZF} \ln \frac{Co}{Ci}$$

This equation can be used to calculate the reversal potential (V) for ions, where R = gas constant, T = absolute temperature, Z = valence, F = Faradays constant and Co and Ci is the extra- and intracellular concentration of ions respectively.

The functional diversity between glutamate receptor subunits is also increased by various post-transcriptional RNA modifications (see below).

1.3.2 NMDARs

NMDARs as discussed above form tetrameric receptors. Three NMDAR subunits have been identified: NMDAR subunit (NR) 1, NR2 and NR3. There are four NR2 subunits (NR2A-D) and two NR3 subunits (NR3A and B), each coded for by single genes (as reviewed by Cull-Candy et al., 2001; Dingledine et al., 1999; Hollmann and Heinemann, 1994). Each functional NMDAR contains two NR1 subunits plus two NR2 or NR3 subunits. In addition each NMDAR subunit can undergo alternative splicing resulting in a large number of NMDAR isoforms (Durand et al., 1992) and hence a large variation in Ca^{2+} permeability (Michaelis, 1998) and deactivation kinetics (Stern et al., 1992; Wyllie, 1992)

1.3.2.1 iGluR expression

The expression profile of all iGluR subunits are both spatial and temporally regulated (Durand and Zukin, 1993). NR1 subunits are expressed ubiquitously in the CNS (Moriyoshi et al., 1991), however, NR2 subunits exhibit a more variable spatiotemporal expression pattern. NR2A mimic the NR1 expression, increasing with age, however NR2B expression is ubiquitous in embryonic stages and decreases with age (Monyer et al., 1994; Stocca and Vicini, 1998). NR2C subunits are expressed at high levels in cerebellar granule cells (Monyer et al., 1992) and NR2D expression is limited to the embryonic and juvenile diencephalon and brain stem (Watanabe et al., 1993). Recent evidence has shown that NMDAR subunit composition determines the synaptic location of the receptors (Rumbaugh and Vicini, 1999; Tovar and Westbrook, 1999) i.e. NR2B-containing receptors are found extrasynaptically and whereas NR2A-containing receptors are generally found at synaptic locations (Momiyama, 2000) (the importance of this finding will be discussed below).

1.3.2.2 NMDA coincidence detection

NMDARs are distinct from AMPARs and KARs in that they require binding of two co-agonists, glutamate and glycine, to be activated (Kleckner and Dingledine, 1988)

At resting membrane potentials there is a blockade of the ion pore by Mg^{2+} (Ault et al., 1980; Mayer et al., 1984) which prevents ions from passing through the channel

even when activated. NMDARs only pass current when the cell is depolarised and the Mg^{2+} block is relieved (Nowak et al., 1984). This depolarisation is usually caused by the sustained activation of AMPARs or KARs. NMDARs are therefore regarded as "coincidence detectors", which require both ligand-binding and postsynaptic depolarisation. The coincidence detection of the NMDAR is central to

synaptic plasticity and activation of silent synapses (see below).

1.3.2.3 Ca^{2+} permeability

Both NMDARs and non-NMDARs are permeable to K^+ and Na^+ when open. At resting potentials Na^+ moves into the cell and K^+ out, down their respective electrochemical gradients as determined by the Nerst equation. Furthermore, activated NMDARs are highly permeable to Ca^{2+}. Intracellular $[Ca^{2+}]$ is tightly regulated in all cells. The reason for the strict concentration maintenance is due to the fact that Ca^{2+} modulates the activity of a multitude of intracellular processes. In presynaptic terminals Ca^{2+} activates neurotransmitter release (Augustine et al., 1985a, b) and postsynaptic enzymes e.g. kinases and phosphatases. Ca^{2+} also regulates channel opening, gene expression and protein synthesis (as reviewed by Levitan & Kaczmarek, 1997). In excessive amounts, Ca^{2+} is highly toxic and excess glutamate can lead to Ca^{2+} dependent toxicity. This is known as glutamate-mediated excitotoxicity and was first described by Lucas and Newhouse in 1957 and is now a phenomenon associated with cerebral ischemia (Gagliardi, 2000). The permeability of each NMDAR is regulated by the positioning of an asparagine residue in the TM2 domain (Cull-Candy et al., 2001). Depolarisation and subsequent Ca^{2+} entry through NMDARs leads to activation of intracellular cascades in the postsynaptic cell, which are required for the expression of LTP and LTD.

1.3.2.4 Pharmacology

NMDARs were first characterized by the excitatory effects of the agonist NMDA (Curtis et al., 1972). NMDARs are also activated by aspartate and glutamate. Examples of competitive antagonists, which compete for the glutamate binding site include D-amino-adipate (DAA) (Biscoe et al., 1977) and the most potent and

selective NMDAR antagonist to date D-2-amino-5-phosphonovalerate (D-AP5) (Davies and Watkins, 1982). Zn^{2+}, polyamine toxins and MK-801 are all NMDAR channel blocking antagonists and are therefore activity dependent (Hood et al., 1989; Paoletti et al., 1997; Williams, 1997a, b; Woodruff et al., 1987). Endogenous polyamines, spermine and spermidine, and exogenous polyamine toxins e.g. argiotoxin have both been shown to antagonise NMDARs, however not all results have been consistent. The conflict in results may be a consequence of the difference between intracellular and extracellular presence of the polyamines. NMDARs can also be antagonized by Zn^{2+} and polyamines, at a second extracellular site which is in the proximity of the glycine binding site on NR1 subunits. At this site protons may also antagonise NMDARs. The glycine binding site found on the NR1 subunit is antagonized by glycine and also D-serine. This site is antagonized by 1-aminocyclobutane-1-carboxylic acid (ACBC) and also 7- chlorokyurenic acid (7-CIK).

1.3.2.5 NMDAR regulation by phosphorylation

Phosphorylation of NMDARs results in increased NMDAR responses (Cull-Candy & Brickley, 2000) and activation of NR1 and NR2 has been shown to be increased following phosphorylation by PKC, due to a reduction in block and increased open channel probability (Chen and Huang, 1992). Although NR1 has a three potential sites of phosphorylation (S890, S896 and T879), none of these appear to be phosphorylated by PKC (Tingley et al., 1997). Phosphorylation of NMDARs by other S/T kinases has also been demonstrated. S879 of NR1 is phosphorylated by PKA, but this does not alter channel function (Tingley et al., 1997). CaMKII phosphorylates S1303 of NR2B, but its function remains unknown (Omkumar et al., 1996). NMDARs are also subject to phosphatase activity. Dephosphorylation of NMDARs by calcineurin/PP2b shortens channel opening times (Lieberman and Mody, 1994) and phosphatases PP1 and PP2a cause a reduction in channel open probability (Wang & Salter, 1994). This evidence suggests that NMDAR function is regulated by phosphatase and kinase activity.

1.3.3 AMPARs

AMPARs mediate the majority of the fast excitatory transmission in the CNS and were first cloned in 1991 following screening of rat brain cDNA expressed in Xenopus oocytes (Hollmann et al., 1991). In hippocampal CA1 pyramidal neurons the fast component of the EPSC is mediated by AMPARs. AMPARs are composed of four basic subunits, GluR1-4 (Boulter et al., 1990; Hollmann & Heinemann, 1994; Keinanen et al., 1990) all coded for by separate genes. AMPARs have been shown to form homomers and heteromers and more than one receptor combination can exist within a cell. It has been proposed that GluR1/2 and G1uR2/3 heteromers and GluR1 homomers are the most common AMPAR isoforms found in the hippocampus (Wenthold et al., 1996). In principal neurons, G1uR2 is present in most receptors (Jonas & Burnashev, 1995), but 8% of AMPARs form homomeric GluRl receptors (Wenthold et al., 1996). Further experiments suggest that under steady state conditions more GluR1 (perhaps complexed with GluR2) is expressed at the plasma membrane and that 50% of G1uR2/3 complexes are present in intracellular reserve pools during development (Hall & Soderling, 1997; Lee et al., 2001). Wenthold et al. 1996, proposed that the different receptor combinations may regulate AMPAR intracellular targeting and synaptic expression.

AMPAR subunits are synthesised in the rough endoplasmic reticulum (ER) (Ayalon & Stern-Bach, 2001). Subunits are thought to dimerise, then tetramerise in the ER (Greger et al., 2003). As receptors mature through the ER glycosylation occurs and adaptor proteins are thought to attach to the receptor, which then traffic complete receptors to their functional sites at synapses. It appears that GluR1/2 receptors are inserted extrasynaptically then translocated to the synapse, however the GluR2/3 receptors can be inserted directly into the synapse (Malinow & Malenka, 2002).

1.3.3.1 Expression

Expression of AMPARs is selective and differential (Martin et al., 1993) and is especially high in the hippocampus. GluR1, G1uR2, G1uR3, GluR4 are all expressed in pyramidal cells (Martin et al., 1993), with GluRl, G1uR2 and GIuR3 predominating in mature hippocampal neurons (Wenthold et al., 1996). Expression of GluR2

increases from P3-P20 by 67-96% (Pickard et al., 2000). Relative to other subunits this increase is very great, suggesting the GluR2 content of AMPARs increases dramatically in postnatal development (Zhu et al., 2000). GluR4 subunit expression peaks during development and in immature hippocampus they complex with GluR2 to form functional receptors (Zhu et al., 2000).

A role for 'placeholders' or 'slots' for AMPARs at synapses has been proposed (Barry & Ziff 2002; Malinow et al., 2000). The molecular composition of these placeholders is not known but the complex may contain anchoring proteins and glutamate receptor interacting proteins. 4.1N, CaMKII, actinin and actin have been proposed to act as a slot complex for GluR1 (Lisman & Zhabotinsky, 2001). Another protein suggested for a slot is PSD-95 as its over-expression increases AMPARs number at synapses (Bredt & Nicoll, 2003).

1.3.3.2 Recycling and degradation

The half life of AMPARs at synapses is suggested to be less than one hour (Beique & Andrade, 2003; Ehrlich & Malinow, 2004). It is accepted that AMPARs are loosely held at the plasma membrane in comparison with NMDARs and that AMPARs are constitutively recycled. AMPAR internalisation from the cell surface occurs via clathrin-mediated dynamin dependent mechanism (Blanpied et al., 2002; Carroll et al., 2001; Carroll et al., 1999a; Lai et al., 1999; Lee et al., 2002b; Lin et al., 2000; Wang & Linden, 2000). In cultured hippocampal neurons 60-70% of GluR1 is expressed on membrane and 40-50% of GluR2/3 are on cell surface (Martin et al., 1993). Internalised AMPARs can follow a number of different pathways. AMPARs can either be sorted into a lysosomal compartment for degradation or to endosomal compartment ready for recycling (Ehiers, 2000; Lin et al., 2000). Following internalisation of AMPARs, it is unclear what directs the fate of AMPARs. Some groups have suggested that the fate may depend on the subunit composition or the phosphorylation state (Ehlers, 2000). A change in expression of AMPARs at the synapse is also thought to be critical in the expression of plasticity (Ehiers, 2000; Lin et al., 2000; Luscher et al., 1999; Shi et al., 2001) and following ischemic trauma,

may also contribute to glutamate-mediated toxicity (Pellegrini Giampietro et al., 1992).

1.3.3.3 AMPAR post transcriptional modifications

AMPAR functional diversity is further increased by alternative splicing and/or messenger ribonucleic acid (mRNA) editing of subunits. The flip/flop splice variation occurs in all AMPAR subunits at the extracellular loop between TM3 and TM4. Although only a few amino acids are changed between the two sequences the resulting AMPARs have very different properties (Monyer et al., 1991; Sommer et al., 1990). The flop version is highly expressed embryonically and postnatally (Monyer et al., 1991), the flop variant, however, is rarely expressed in tissue before P8 (Monyer et al., 1991). By P14 flip and flop variants are equally expressed. It has been suggested that the difference in kinetic properties between these channels in some way contributes to development of synapses (as reviewed by Molnar and Isaac, 2002). Intracellular C-termini of GluR2 and 4 are also subject to alternative splicing. G1uR2 has two splice variants $GluR2_{long}$ and $GluR2_{short}$ (Kohler et al., 1993). A short version of GluR4 has also been found in the cerebellum and is referred to as GluR4c (Gallo et al., 1992).

Another modification of AMPARs is the Q/R editing of G1uR2 in the hydrophobic TM2 region. DNA is coded faithfully, but mRNA is edited. This results in the reading of mRNA as CIG (CTG) which codes for an arginine residue instead of CAG which formerly coded for a glutamine (Q) residue at site 607 (Keinanen et al., 1990; Sommer et al., 1991). 99% of G1uR2 is edited at this site (Seeburg and Osten, 2003). The Q/R editing site is situated in the lining of the channel pore (Sommer et al., 1991) rendering any channel containing a G1uR2 subunit impermeable to Ca^{2+} ions. This results in a decreased single channel conductance and a linear current-voltage relationship (Boulter et al., 1990). GluRl, GluR3 and GluR4, remain unchanged at the Q/R site, which means channels lacking GluR2 are Ca^{2+} permeable and, due to block of the channel by intracellular polyamines at positive membrane potentials, have inwardly rectifying current-voltage relationships. It has recently been proposed that Q/R editing controls channel function and trafficking of AMPARs (Greger et al,

2003; Greger et al, 2002). Unedited AMPARs are easily trafficked from the ER to the membrane surface whereas edited forms appear to be held in intracellular stores (Greger et al., , 2003), suggesting that edited AMPARs may undergo regulated delivery to the synapse. Embryonically a significant fraction of G1uR2 is unedited, however, postnatally, the majority of G1uR2 is edited (Burnashev & Rozov, 2000). The reasons for this are not understood and the physiological role of unedited G1uR2 is unclear.

1.3.3.4 Pharmacology

AMPARs can be activated by a number of compounds, in rank order of potency these are quisqualate > AMPA> glutamate> kainate, with AMPA being the most selective (Bleakman & Lodge, 1998). The first AMPAR antagonists used were derivatives of quinoxalinedione. These compounds are selective and competitive AMPAR antagonists and examples include 6-cyano-7-nitro-quinoxaline- 2,3 -dione (CNQX), 6,7-dinitro-quinoxaline-2,3 -dione (DNQX) and 2-3 -dihydroxy-6- nitro-7-sulphmoyl-benzo(F)quinoxaline (NBQX). These are the standard blocking tools for AMPARs (Honore et al., 1988).

1.3.3.5 Regulation by phosphorylation

The C-terminal domain of AMPARs have been identified to have consensus sequences for protein kinases, PKA, PKC and CaMKII, however not all of these sites have been shown to be phosphorylated *in vivo*. GluR1 has a PKA phosphorylation site at S845 (Roche et al., 1996). S831 can be phosphorylated by either PKC (Roche et al., 1996) or CaMKII (Roche et al., 1996). The C-terminal of GluR1 shows little homology with other AMPAR subunits and hence these sites do not exist on G1uR2-4. On GluR2 S880 can be phosphorylated by PKC (Matsuda et al., 1999) and G1uR4 at S842 by PKC, PKA or CaMKII (Carvaiho et al., 1999). The functional relevance of AMPAR phosphorylation will be discussed in later sections.

1.3.3.6 Protein-protein interaction sites

The glutamate receptor C-terminus is important due to its intracellular location. From trafficking through the Golgi matrix until positioned in the postsynaptic membrane, it is constantly exposed to the cytoplasm, which allows many proteins to interact with the receptor throughout its journey in the cell. Most AMPAR interacting proteins appear to do so with its extreme C-terminal domain, where the PDZ consensus sequence exists. Examples of PDZ domain-containing proteins which can interact with the consensus sequence are synapse-associated protein 97 (SAP97) (Leonard et al., 1998) with GluRl and glutamate receptor interacting protein (GRIP) (Dong et al., 1999; Srivastava et al., 1998) and protein interacting with C kinase- 1 (PICK-1) (Dev et al., 1999; Xia et al., 1999) with G1uR2. The C-terminal region of GluR2 has another protein binding region where NSF (Nishimune et al., 1998) and AP2 (Lee et al., 2002a) can bind. In addition, stargazin binds close to the transmembrane spanning regions of all AMPARs (Chen et al., 2000).

1.4 Long-term synaptic plasticity and LTP

Synaptic connections are plastic meaning that the efficiency of information transfer across the synapse can decrease and increase. This is thought to underlie learning and memory (Bliss & Collingridge, 1993; Eccles, 1964; Hebb, 1949; Kandel, 1997). In 1949, Donald Hebb proposed that a correlation of pre and postsynaptic activity would lead to a strengthening of the connection between two neurons (Hebb, 1949). It wasn't until 1973 that a candidate process for Hebbian synaptic plasticity was demonstrated experimentally. A brief high-frequency stimulus (HFS) through the perforant path in the hippocampus was shown to induce a long-lasting increase in synaptic strength in dentate granule cells (Bliss & Lomo, 1973). This was to become known as long-term potentiation (LTP). Based on Hebb's theory it was hypothesised that plasticity could occur as a change in presynaptic or postsynaptic activity or both. Presynaptically, changes in probability of neurotransmitter release and the number of release sites may contribute to plasticity, whereas postsynaptically, plasticity could be expressed as a change in the number of neurotransmitter receptors or their single channel conductance (as reviewed by Nicoll & Malenka, 1999).

1.4.1 The basic properties of LTP

LTP is thought to be the cellular correlate of learning and memory (Alkon & Nelson, 1990; Kandel, 1997; Morris, 1989) as memories are stored for great lengths of time and LTP is a cellular process shown to last for days and weeks *in vivo* (Bliss & Lomo, 1973; Bliss & Gardner-Medwin, 1973) and when LTP is inhibited with NMDAR antagonists types of learning can be prevented (Tan et al., 1989; Ward et al., 1990). LTP can be reversed by low frequency stimulation (LFS) (depotentiation) (Fujii et al., 1991; Staubli & Lynch, 1990). LTP has now been described in many brain regions including visual cortex (Artola & Singer, 1987), barrel cortex (Crair & Malenka, 1995) and cerebellum (Aizenman & Linden, 2000). LTP is cooperative (McNaughton et al., 1978), associative (Levy & Steward, 1979) and input specific (Andersen et al., 1977; Lynch et al., 1977). There is a threshold for LTP induction, which is determined by the intensity and pattern of stimulation, if the intensity of afferent stimulation is not sufficient LTP will not be induced (McNaughton et al., 1978). This is termed cooperativity. In the event of a weak tetanus coinciding with another weak stimulus in a convergent pathway, LTP may be induced; this is associativity (Levy & Steward, 1979; McNaughton et al., 1978). Input specificity denotes that LTP will only occur in the stimulated pathway, as opposed to unstimulated pathways (Andersen et al., 1977; Lynch et al., 1977).

1.4.2 LTP induction mechanisms

Synaptic plasticity is induced by specific patterns of synaptic activity, including trains of high and low frequency stimulation, pairing stimulation trains with postsynaptic depolarisation and pairing of pre and postsynaptic activity (spike-timing dependent plasticity). LTP can be induced in slices by tetanic stimulation, with the delivery of 50-100 stimuli at 100 Hz (as reviewed by Bliss & Collingridge, 1993). A theta-burst stimulation protocol, several bursts at 100 Hz separated by a 200 ms burst interval also induces LTP (Larson & Lynch, 1989). This protocol is often used instead of a tetanic stimulation as the theta burst protocol mimics patterns of bursting found in the hippocampus (Otto et al., 1991).

In 1986, several groups demonstrated that pairing of a postsynaptic depolarisation with a presynaptic activation is a method of LTP induction (Kelso et al., 1986; Sastry et al., 1986; Wigstrom et al., 1986). This supported Hebb's theory that both cells are required to be active in order to generate a synaptic change. However it was not fully understood how this co-activation was detected at the synapse. There was a requirement for a coincidence detector and something which could detect a change from both pre and postsynaptic sites. NMDARs, as discussed previously, require L glutamate and glycine binding, paired with postsynaptic depolarisation to remove the Mg^{2+} block from the channel pore (Ascher & Nowak, 1988). This made the NMDAR an ideal candidate as the coincidence detector as it detects the presynaptic release of L-glutamate and postsynaptic depolarisation, as a consequence of repetitive AMPAR stimulation.

The properties of LTP are hence explained; if the cooperativity threshold is not achieved, the presynaptic terminals do not release sufficient L-glutamate to cause postsynaptic depolarisation to release the Mg block and hence no LTP will follow. However, upon sufficiently strong stimulation, L-glutamate will activate AMPARs. AMPAR activation leads to depolarisation of the postsynaptic terminal and release of the NMDAR Mg^{2+} block, which in the presence of L-glutamate results in the postsynaptic activation of NMDARs. Associativity is described in a similar way to cooperativity; only simultaneous afferent stimulation originates from another source of inputs. The contribution from these 'helper' afferent inputs integrates to produce sufficient postsynaptic depolarisation to unblock NMDARs and allow LTP induction. Input specificity arises as a result of the fact that the NMDARs are coincidence detectors and may only be activated as a result of L-glutamate release and Mg^{2+} block relief, without this LTP cannot occur. As discussed above, the requirement for cooperativity and associativity are a function of the need for postsynaptic membrane depolarisation. This was demonstrated in 1986 by Kelso et al. who found that LTP could be induced by pairing a weak stimulus (1 Hz) with postsynaptic depolarisation. These methods are now commonly referred to as pairing protocols (Kelso et al., 1986).

The role for NMDARs as a coincidence detector for LTP was confirmed when antagonism of NMDARs was found to inhibit LTP induction. Antagonists which acted at the L-glutamate site, D-AP5 and MK801 (Coan et al., 1987; Collingridge et al., 1983) and those at the glycine site, 7-chlorokynuretic acid (Bashir et al., 1990) blocked LTP. NMDAR independent LTP has also been described in the dentate gyrus, producing the same effects but via different mechanisms (Wang et al., 1997; Wang et al., 1996). This thesis will focus on NMDAR dependent LTP at the CA3-CA1 synapse in the hippocampus; therefore the discussion of the LTP mechanisms will focus on NMDAR-dependent LTP.

Recent evidence suggests that the subunit type of NMDARs is important for the induction of LTP. NR2A-containing receptors are thought to mediate LTP whereas NR2B-containing receptor activation is thought to result in LTD (Liu et al., 2004; Massey et al., 2004). NR2A-containing receptors are positioned in the postsynaptic density and have relatively fast kinetics (Monyer et al., 1994; Rumbaugh & Vicini, 1999; Stocca & Vicini, 1998; Tovar & Westbrook, 1999), resulting in the magnitude and timing of the influx of Ca^{2+} which is required for LTP (Malenka, 1991). The NR2B-containing receptors are thought to be located extrasynaptically and generate slower Ca^{2+} currents (Monyer et al., 1994; Rumbaugh & Vicini, 1999; Stocca & Vicini, 1998; Tovar & Westbrook, 1999) required for LTD induction (Artola & Singer, 1993; Cummings et al., 1996; Lisman, 1989; Nishiyama et al., 2000). However, this research has not been supported by work from other groups (Berberich et al., 2005; Weitlaufet al., 2005), therefore this hypothesis is controversial.

1.4.3 Role of postsynaptic Ca^{2+} in LTP

Spines are specialised areas of the dendrite which receive most excitatory inputs in mammalian neurons (as reviewed by Nimchinsky et al., 2002). Spines have a low endogenous buffering capacity for Ca^{2+} which allows for large and rapid changes in $[Ca^{2+}]$ (Sabatini et al., 2002). Changes in $[Ca^{2+}]$ play an essential role in LTP and LTD (Zucker, 1999) and is required to activate enzymes involved in modifications of synaptic strength (as reviewed by Bliss & Collingridge, 1993; Kemp & Bashir, 2001).

An early yet important study identified the role of Ca^{2+} in LTP using intracellular application of EGTA, a Ca^{2+} chelator, to block LTP (Lynch et al., 1983). In agreement with this study, an NMDAR-dependent increase in postsynaptic Ca^{2+} was observed following tetanic stimulation (Regehr & Tank, 1990). More recent evidence however, has suggested that Ca^{2+} released from intracellular stores enhances LTP (Emptage et al., 1999), and is further confirmed by studies showing that drugs which block the release of Ca^{2+} from stores, but not NMDARs, block LTP (Bortolotto & Collingridge, 1993). Increasing intracellular Ca^{2+} concentration by flash photolysis has also been shown to result in an increase of AMPAR-mediated excitatory postsynaptic current (EPSC) amplitude (Malenka et al., 1988). All of these studies demonstrate a role for Ca^{2+} signalling in NMDAR-dependent LTP.

Many groups have attempted to estimate the intracellular increase in $[Ca^{2+}]$ during induction of plasticity, however changes in dendritic $[Ca^{2+}]$ ranging from 1-40μM have been published (Franks & Sejnowski, 2002; Muller & Connor, 1991; Neveu & Zucker, 1996b; Petrozzino et al., 1995; Regehr & Tank, 1990; Sabatini et al., 2002; Yang et al., 1999; Yuste et al., 1999). The large variation in the reported changes are partly due to a lack of sensitivity in the Ca^{2+} compounds used for these experiments and the inability to differentiate between the Ca^{2+} in the spine and that in the local dendritic area. Considering that a change in Ca^{2+} is required for both LTP and LTD, it was also difficult to determine whether the Ca^{2+} changes in response to LTP and LTD induction protocols were different (Neveu & Zucker, 1996a; Neveu & Zucker, 1996b). Lisman et al, in 1989 first proposed that a large rapid increase in Ca^{2+} was required for LTP. It is now widely accepted, that the amplitude and duration of the intracellular Ca^{2+} increase are key factors in determining the direction of plasticity and the cellular machinery recruited (Yang et al., 1999) The rise in $[Ca^{2+}]$ is thought to be required to last for 2.0- 2.5 s to generate LTP (Malenka et al., 1992) and restriction of the change in Ca^{2+} to the spine is thought to account for input specificity and is referred to as Ca^{2+} compartmentalisation (Ismailov et al., 2004; Teyler et al., 1994).

1.5 LTP expression mechanisms

LTP research during the 1990s was characterized by intense debate as to whether NMDAR-dependent LTP is expressed presynaptically or postsynaptically. Many experiments relied on classical electrophysiological approaches, such as paired-pulse facilitation, failures analysis, or quantal analysis. Unfortunately, no clear consensus has emerged owing to the difficulty of obtaining accurate measurements, coupled with an incomplete understanding of the requirements for successful quantal analysis at these synapses. Over the years considerable evidence has accumulated in favour of both presynaptic and postsynaptic mechanisms.

1.5.1 Postsynaptic mechanism of expression.

It is now thought that the number of receptors at a synapse can be postsynaptically modified by regulation of:

- Intracellular trafficking of receptors
- Insertion of receptors into the plasma membrane (exocytosis)
- Stabilisation of receptors in the membrane
- Removal of receptors from the plasma membrane (endocytosis)
- Recycling/degradation of available receptors

The expression of postsynaptic LTP is characterised by an increase in the AMPAR-mediated synaptic currents, which is thought to be a result of a postsynaptic change in either AMPAR number or function. AMPARs can be modulated by phosphorylation, altering receptor conductance (Derkach et al., 1999), open probability (Banke et al., 2000) or acting on proteins which cause an alteration in membrane targeting (as reviewed by Malinow & Malenka, 2002). AMPARs can be directly phosphorylated at 12 distinct sites (as reviewed by Song & Huganir, 2002).

1.5.1.1 CaMKII phosphorylation and LTP

GluR1 contains two residues of interest for serine/threonine (S/T) kinase phosphorylation, serine (S) 845 and S831, and phosphorylation at both of these sites

potentiates AMPAR function (Barria et al., 1997a; Roche et al., 1996). Ca^{2+} calmodulin protein kinase (CaMKII) phosphorylates GluR1 at S831 (Barria etal., 1997a; Roche et al., 1996). Activated CaMKII results in an increase in glutamatergic transmission at resting membrane potentials (McGlade-McCulloh et al., 1993), and S831 phosphorylation potentiates AMPAR function via an increase in single channel conductance (Derkach et al., 1999). It has also been shown that an increase in AMPAR conductance occurs during LTP (Benke et al., 1998). An increase in phosphorylation of GluR1 at S831 also occurs during LTP induction (Barria et al., 1997b; Lee et al., 2000) and CaMKII activity is required for the synaptic incorporation of GluR1 in LTP (Esteban et al., 2003).

CaMKII is an enzyme activated by a Ca^{2+} complex, which forms when intracellular Ca^{2+} levels rise. Once activated, CaMKII can then phosphorylate target proteins. CaMKII can also phosphorylate itself forming a memory of previous Ca^{2+} calmodulin binding (autophosphorylation), therefore leading to sustained phosphorylation of targets even when the $[Ca^{2+}]$ has returned to normal (Braun & Schulman, 1995). This was an attractive model for the encoding of long-term memory (Lisman & Goldring, 1988) as sustained intracellular increases in $[Ca^{2+}]$ result in cellular damage (Pellegrini-Giampietro et al., 1992).

The first evidence to suggest a role for CaMKII in LTP came in 1989, when inhibitors of the enzyme were shown to block LTP (Malenka et al., 1989). The development of an CaMKII knock-out mouse supported a role of CaMKII in LTP, as no LTP was observed in the CA1 of these mice (Silva et al., 1992). Postsynaptic application of the catalytic fragment of CaMKII was also shown to result in a potentiation of synaptic transmission which occluded LTP (Ledo et al., 1995). Autophosphorylation of CaMKII occurs following LTP (Barria et al., 1997b; Fukunaga et al., 1995) and a point mutation of CaMKII at T286, which prevents autophosphorylation, resulted in mice that exhibited no LTP and impaired spatial learning (Giese et al., 1998). These findings further support the role for CaMKII phosphorylation of GluR1 and potentiation of synaptic transmission in LTP.

AMPAR phosphorylation has been suggested to determine receptor fate by controlling entry into differing intracellular trafficking pathways. In a set of experiments CaMKII was shown to be required for AMPAR delivery to the synapse (Esteban et al., 2003; Hayashi et al., 2000). However, Esteban et al., (2003) proposed that this process also requires PKA, which allows AMPARs to be available for insertion and CAMKII triggers some other process perhaps intracellular machinery, e.g. ras signalling system, which results in the insertion of AMPARs (Esteban et al., 2003).

1.5.1.2 PKC phosphorylation and LTP

S831 on GluR1 can be phosphorylated by protein kinase C (PKC) and S845 by cAMP-dependent protein kinase (PKA) (Roche et al., 1996). Bath application of phorbol esters (PKC activators) has been shown to result in an increase in synaptic transmission (Malenka et al., 1986), but the importance of these findings were not immediately clear as PKC has many substrates, both pre and postsynaptically. However, the intracellular injection of the catalytic subunit of PKC resulted in increased synaptic responses (Hu et al., 1987), suggesting that PKC may regulate synaptic transmission. PKC has been shown to phosphorylate S831 of GluR1 (Roche et al., 1996) and PKC activity is elevated following LTP induction (Akers et al., 1986). In some cases, PKC inhibitors have been shown to prevent LTP induction (Lopezmolina et al., 1993; Lovinger et al., 1987). However, one experiment found that LTP was reversed by a PKC inhibitor which acted at the PKC catalytic site, but not by an inhibitor that blocked the PKC activation site (Malinow et al., 1988), suggesting that LTP may be maintained by a constitutively active form of PKC.

In this regard there is some evidence that a constitutively active form of PKC, PKMζ is involved in LTP maintenance. PKMζ was shown to be activated specifically following LTP (Sacktor et al., 1993). PKMζ is found in the hippocampus, including the CA1 (Naik et al., 2000). Concentrations of this protein increase dramatically and specifically 30 minutes after LTP induction (Sacktor et al., 1993), suggesting a role in the maintenance of LTP as opposed to the induction of LTP. PKMζ has been shown to be necessary and sufficient for the enhanced synaptic transmission during LTP (Ling et al., 2002) and is required to maintain the late phase of LTP (Serrano et al.,

2005). It was thought that the activation of PKMζ may reflect the hypothesis of autophosphorylation of CaMKII following LTP. However, recent evidence has shown that PKMζ is not a cleavage product but a product of its own gene (Hemandez et al., 2003), thus providing a direct link between LTP and gene transcription. The activation of PKMζ has also been demonstrated to be involved in memory formation as expression and specific activation of a mouse PKMζ gene results in enhanced memory (Drier et al., 2002).

1.5.1.3 PKA phosphorylation and LTP

Increasing intracellular [Ca^{2+}] is one of many ways of increasing intracellular concentrations of cyclic adnosine monophosphate (cAMP) which activates cAMP-dependent protein kinase (PKA) (reviewed by Nguyen & Woo, 2003). PKA phosphorylates S845 of GluR1 (Roche et al., 1996) which results in increased AMPAR-mediated transmission as a result of increased peak open probability of the ion channels (Banke et al., 2000). Application of forskolin (an adenylyl cyclase activator) results in an increase in AMPAR-mediated synaptic transmission in heterologous cells (Greengard et al., 1991), which was found to be blocked by PKA inhibitors (Carroll et al., 1998). This increase was not observed with GluRl S845A mutations (Roche et al., 1996). Tetanus-induced LTP results in an increase in cAMP (Roberson & Sweatt, 1995), NMDAR activation has been shown to activate PKA (Roberson & Sweatt, 1996) and research demonstrated that PKA is activated during LTP (Frey et al., 1993; Hu et al., 1987; Roberson & Sweatt, 1996). It appears however that S845 is phosphorylated under basal conditions (Mammen et al., 1997) and therefore phosphorylation of S845 by PKA may be necessary, but not sufficient for LTP (Esteban et al., 2003). Phosphorylation of GluR1 is also not sufficient to drive GluR1 into synapses, however blocking phosphorylation does prevent receptor insertion (Esteban et al., 2003).

1.5.1.4 Role for AMPAR trafficking in LTP

The major current hypothesis for postsynaptic expression mechanism for LTP is an increase in AMPAR number. This was first proposed by Lynch & Baudry in 1984

(Lynch & Baudry, 1984). Subsequent work indicated that following LTP induction there was an increase in AMPAR-mediated responses when compared with NMDAR-mediated responses (Kullmann, 1994). From this study, it was proposed that some synapses in a neuron do not include active AMPARs. These synapses would be functionally silent at resting membrane potentials due to the absence of AMPARs and the voltage dependence of NMDARs. Such 'silent synapses' were proven to exist in 1995 (Isaac et al., 1995; Liao et al., 1995). Furthermore, it was discovered that these synapses may become functional at resting membrane potentials in response to a pairing protocol, due to the activation of NMDARs, leading to the appearance of functional AMPARs (Isaac et al., 1995; Liao et al., 1995). The discovery of LTP at silent synapses was the first evidence of a role for AMPAR trafficking in LTP. This mechanism, however, is still under scrutiny, as one group has proposed a fusion pore hypothesis which suggests that variation in the presynaptic fusion pore can affect the amount of glutamate released. This has been shown to alter AMPAR-mediated response but not NMDAR-mediated response (Choi et al., 2003; Renger et al., 2001). This theory suggests that the amount of glutamate in the synaptic cleft is regulated by varying the size of the pore through which glutamate is released from the presynaptic terminal. A second group has suggested that the spill-over of neurotransmitter to other synapses may be enough to activate NMDARs but not AMPARs and explain the increase in AMPA currents following LTP (Asztely et al., 1997; Kullmann, 2003; Kullmann & Asztely, 1998; Kullmann et al., 1999; Rusakov & Kullmann, 1998).

1.5.1.5 Role of membrane fusion in rapid regulation of AMPAR number

AMPARs are inserted into the plasma membrane in an exocytosis-dependent manner. Exocytosis is the process whereby intracellular membrane-bound vesicles fuse with the plasma membrane (as reviewed by Lin & Scheller, 2000). This process results in two key outcomes, firstly allowing vesicle contents to be released from the cell, (e.g. exocytosis at pre-synaptic terminal for neurotransmitter release) and secondly inserting vesicle membrane and its associated proteins into the plasma membrane. This could be a mechanism for insertion of AMPAR at synapses during LTP (Lledo et al., 1998). The delivery of AMPARs to synapses is thought to occur via two pathways, regulated (activity-dependent) and constitutive exocytosis (as reviewed by

Malinow & Malenka, 2002; Passafaro et al., 2001; Shi et al., 1999). Regulated exocytosis is triggered by an increase in intracellular [Ca^{2+}] (Becherer et al., 2003).

SNAP and SNAREs are essential proteins for membrane fusion events (as reviewed by Lin & Scheller, 2000). AMPARs have been shown to be inserted into the PSD as a result of NMDAR activation in a SNARE-dependent manner (Lu et al., 2001). SNAREs are involved in the activity-dependent exocytosis of AMPARs in LTP because blocking their effects with tetanus toxin (an inhibitor of one SNARE, synaptobrevin) prevents LTP (Lledo et al., 1998; Lu et al., 2001). The role for SNAP in LTP has been suggested following experiments using the intra-CA1 infusion of an antisense SNAP-25 into the CAl. This was found to impair spatial memory, contextual fear memory and LTP (Hou et al., 2004).

1.5.1.6 Subunit specific regulation of AMPARs during LTP

As mentioned previously, studies suggest that AMPARs can be trafficked to the membrane following LTP induction. It has previously been shown that GluR1 knockout mice lack LTP, suggesting an absolute requirement for GluR1 in LTP (Mack et al,, 2001; Zamanillo et al., 1999). Interestingly, whereas the loss of LTP in CA1 was observed after various stimulation protocols—e.g., tetanic stimulation (1 x 100 Hz or 4 x 100 Hz "field" LTP) or 3-minute pairing of postsynaptic depolarisation and low-frequency Schaffer collateral stimulation ("cellular" LTP)—a protocol using theta-burst frequency of pairing pre- and postsynaptic activity induced a slow-onset LTP that reached normal levels within ~20 minutes after induction. This was the first evidence that two forms of LTP coexist at the Schaffer collateral to CAl synapses: one dependent on and the other independent of GluR1 - containing AMPA receptors (Hoffman et al., 2002). Similarly, at the perforant path to dentate gyrus granular cell synapses, GluR1 -independent LTP was detected in adult GluR1 knockout mice (Zamanillo et al., 1999); this indicates that in wild- type animals LTP at these synapses is at least partly GluR1 -independent.

Molecular mechanisms underlying synaptic plasticity in the hippocampus also change during development. In contrast to adult GluR1-deficient mice, juvenile (2-week-old) knockout mice have normal field and cellular LTP in Ca1 (Jensen et al., 2003b). This "juvenile" LTP may rely on expression of other AMPA receptor subunits, such as $GluR2_{long}$ (Kolleker et al., 2003). The notion that GluR1-dependent function is not critical in juvenile mice is consistent with the observation that hippocampal LTP in adult GluR1 knockout mice can be rescued by transgenic forebrain-specific expression of GFP-tagged GluR1 with an onset from the second to the third postnatal week (Mack et al., 2001). This indicates that a GluR1-independent form of synaptic plasticity is sufficient for development of normal hippocampal synaptic circuits. Later in development, it has been suggested that the GluR1 dependent plasticity becomes the dominant form of LTP (Jensen et al., 2003b).

Malinow's group have used recombinant receptors tagged with green fluorescent protein (GFP), combined with electrophysiology and high resolution imaging to track AMPAR insertion following LTP induction (Hayashi et al., 2000; Shi et al., 2001; Shi et al., 1999). The number of tagged GluR1 AMPARs at synapses were shown to be increased following tetanic stimulation, which was inhibited by NMDAR antagonists (Shi et al., 1999). An increase in AMPARs at the postsynaptic membrane was also observed following LTP induction using a pairing protocol or co-expression of active CaMKII. However, mutation of the PDZ domain on GluR1 prevented this increase in transmission (Hayashi et al., 2000). This suggests that not only are GluR1 -containing AMPARs inserted into the postsynaptic membrane following LTP induction, but also that this is mediated by a PDZ interaction with GluR1. Other work has also supported these ideas (Passafaro et al., 2001).

These studies led to the development of a model for the delivery of AMPARs to the synapse during plasticity and under basal conditions. The hypothesis is that the sites for protein-protein interaction on the C-terminal tail of AMPARs regulate the synaptic delivery of AMPAR. GluR1/2 heteromers are delivered to the synapse in an activity dependent manor regulated by interactions with the C-terminal domain of GluR1. Whereas GluR2/3 heteromers are thought to replace GluR1/2 at the synapse in a

constitutive manner, which is controlled by the GluR2 C-terminal domain (as reviewed by Malinow & Malenka, 2002; Shi et al., 2001).

1.5.2 Presynaptic mechanisms of expression.

There are several ways, not mutually exclusive, in which LTP could be expressed presynaptically.

- Increase in the number of release sites (either within a presynaptic terminal or as a result of the formation of new terminals)
- Increase in the number of vesicles released per impulse
- Alteration in the amount of L-glutamate stored in or released from each vesicle
- Increase in the probability of release (Pr) in response to the invading action potential

The studies that have measured the amount of L-glutamate released before and after LTP cannot distinguish between these possibilities or other alterations that affect the extracellular concentration of L-glutamate. However, there is little evidence that the uptake or diffusion of L-glutamate is affected during LTP. Several lines of evidence are consistent with an increase in the probability of transmitter release. Using minimal stimulation to activate a putative single release site, Stevens and Wang (1994) showed that LTP in area CA1 was associated with a decrease in failure rate without change in potency (the mean amplitude of responses excluding failures). Bolshakov and Siegelbaum (1995) also reported an increase in Pr in the same region in neonatal rats. Consistent with the idea of an increase in Pr, a few studies have detected a decrease in paired-pulse facilitation (PPF) during LTP (Schulz et al., 1994; Kleschevnikov et al., 1997). However, many studies have observed no changes in PPF hence suggesting a postsynaptic locus of expression. Direct evidence for a presynaptic mechanism in cultured hippocampal neurons was obtained by measuring exocytotic-endocytotic cycling with antibodies against the synaptic vesicle protein synaptotagmin (Malgaroli et al., 1995).

A novel mechanism proposed by Tsien and colleagues involves an LTP-dependent modification of the fusion pore such that more L-glutamate is released from a fused vesicle (Choi et al., 2000). Under basal conditions the amount of L-glutamate released may be so small as not to elicit a detectable AMPAR-mediated postsynaptic response. Glutamate concentration may, however, be high enough to activate NMDARs, thus providing a potential explanation for silent synapses (see below). LTP then modifies the release machinery to enable a greater amount of L-glutamate discharge from each fused vesicle.

Another direct way of estimating the probability of glutamate release is to measure the rate of de staining of synaptic vesicles loaded with the styryl dye FM 1-43. Activity dependent destaining was more rapid following induction of LTP, an effect that was blocked by NMDA receptor antagonists (Stanton et al., 2005). An intriguing example of presynaptic unsilencing has been provided by Ma et al. (1999), who showed that in hippocampal cultures treated with a cAMP analogue (a treatment that induces a late, protein synthesis-dependent form of LTP), there was a dramatic increase in the number of active boutons taking up the dye FM1-43.

Long-term potentiation is associated with an increase in phosphorylation of the presynaptic protein GAP-43 (Routtenberg and Lovinger, 1985; Gianotti et al., 1992). Other studies examined glutamate-induced LTP in cultured hippocampal neurons and found an increase in presynaptic boutons associated with clusters of the presynaptic proteins synaptophysin (Antonova et al., 2001) and synuclein (Liu et al., 2004b).

Changes in Pr at single synapses following induction of LTP in pyramidal cells of areas CA1 and CA3 has been assessed using Ca^{2+} indicators to monitor synaptic events at single visualised spines in organotypic hippocampal cultures (Emptage et al., 2003). Emptage et al. argued that the probability of evoking a Ca^{2+} transient in the visualised spine, PCa, provides an accurate measure of the probability of transmitter release, Pr, at that synapse, since manipulations that reflect an increase in Pr, such as paired pulse facilitation, also result in an increase in PCa, whereas manipulations that decrease Pr, such as bath application of adenosine, lead to a decrease in PCa. In the

majority of spines examined, induction of LTP by high-frequency stimulation was accompanied by an increase in PCa at the imaged spine, whereas in experiments in which LTP was blocked by D-AP5, or in which the threshold for LTP was not reached, no change in PCa was seen.

1.5.2.1 Retrograde signalling is required for presynaptic expression mechanisms.

The trigger for the induction of LTP in the CA1 – CA3 pathways is the entry of calcium through activated NMDA channels located on the postsynaptic cell. Evidence from a variety of techniques and preparations points to a presynaptic component to the expression of LTP. Early evidence for increased transmitter release in LTP led Bliss and Dolphin (1984) to postulate that a diffusible retrograde signal is released from the postsynaptic site of induction to interact with the presynaptic terminal and in some manner stimulate transmitter release. Most attention has focused on two candidates for this signal, the unsaturated membrane fatty acid arachidonic acid (AA) and the gas nitric oxide (NO) synthesized from L-arginine by NO synthase (NOS). Other potential retrograde messengers have been considered in the past including platelet-activating factor (Miller et al., 1992) and carbon monoxide (Stevens and Wang, 1993). Other potential diffusible messengers are brain-derived trophic factor (BDNF) and an endogenous endocannabinoid, 2-AG, which appears to act as a retrograde mediator of heterosynaptic LTD at inhibitory synapses in area CAl (Chevaleyre and Castillo, 2003). Intersynaptic communication via membrane-spanning adhesion molecules is another way in which signals could be communicated in a retrograde direction (Murai and Pasquale, 2004).

1.5.3 Arachidonic acid

Arachidonic acid (AA) is produced by the action of a Ca^{2+} dependent enzyme, phospholipase A2, on membrane phospholipids. In 1987, Piomelli et al. (1987) suggested that AA, or one of its metabolites, might serve as a synaptic retrograde messenger in LTP on the basis of experiments in Aplysia that identified lipoxygenase metabolites of arachidonic acid as a novel class of second messenger. A year later,

Bockaert and his colleagues made the significant observation that activation of NMDA receptors in cultured striatal neurons led to the release of AA into the culture medium (Dumuis et al., 1988). The first evidence that the AA cascade played a role in LTP followed in the same year in a report that an inhibitor of AA production blocked chemically induced LTP in area CA1 and the dentate gyrus (Williams and Bliss, 1988). Subsequent studies, reviewed in Bliss et al. (1990), demonstrated that AA satisfies several of the criteria for a retrograde messenger: (1) LTP is accompanied by an increase in the concentration of AA in a postsynaptic membrane fraction and an increase in the extracellular concentration of AA; (2) inhibitors of phospholipase A_2 block induction of LTP; (3) the application of arachidonic acid to active hippocampal synapses causes delayed but persistent potentiation of evoked responses. Nevertheless, these results, although pointing to a role for arachidonic acid in LTP, do not compel the conclusion that it is a retrograde messenger as no one has shown that extracellular scavengers of AA block induction of LTP. What is the presynaptic mode of action of AA that leads to an increase in transmitter release? Moreover, some of the evidence can be explained by other known properties of AA, including inhibition of glutamate uptake into glia (Barbour et al., 1989) and its facilitatory action on NMDA currents (Miller et al., 1992). Evidence that a 12(S)-lipoxygenase metabolite of AA, 12(S)-HPETE, mediates the induction of mGluR dependent LTD in area CA1 of young rats has been presented by Bolshakov and colleagues (Feinmark et al., 2003). It remains to be seen whether 12(S)-HPETE satisfies all the criteria for a retrograde messenger, including activity-dependent up regulation in the postsynaptic cell.

1.5.4 Nitric oxide

By contrast to conventional transmitters, which are synthesized well before release and are stored in lipid vesicles, when NO is used as a neurotransmitter it is synthesized immediately before use. NO synthesis is triggered by an increased intracellular Ca^{2+} concentration, which activates the enzyme NOS via Ca^{2+}/ calmodulin, leading to an immediate production of NO (Bredt and Snyder 1992). Two isoforms of NOS, NOS-I (nNOS) and NOS-III (eNOS), are found in the nervous system (Feldman et al., 1993).

By contrast to conventional transmitters, which are water insoluble and cannot cross lipid membranes, NO is both water and lipid soluble, and so after it is synthesized, it freely diffuses from cell to cell. This allows NO to act on a wider array of targets than do conventional transmitters. The potential targets of NO are increased further by an additional difference between NO and conventional transmitters. Whereas conventional transmitters act on target neurons by binding to specific membrane-bound receptors, NO is a free radical that affects target neurons via chemical interactions with any molecule that is particularly reactive with a free radical.

The most prominent natural target of NO is soluble guanyl cyclase (sGC), whose activation produces cyclic GMP (cGMP) when NO binds to a heme group in the enzyme (Bredt and Snyder 1989). cGMP in turn activates cGMP-dependent protein kinase (PKG), which may affect additional second messenger systems. cGMP can also directly activate other protein kinases, such as the cyclic AMP-dependent kinase (PKA) (Miller 2000). Thus, NO is similar to conventional transmitters that act via second messengers to activate protein kinases, which may in turn affect transcription factors and protein synthesis. However, NO differs from conventional transmitters in that it may also affect many additional targets. NO is often covalently bonded to cysteines of target proteins (S-nitrosylation), and NO thereby affects their activity (Ahern et al., 2002). NO can directly affect the core complex of proteins involved in synaptic vesicle and cell membrane interactions and thereby promote exocytosis, perhaps via S-nitrosylation (Meffert et al., 1996).

An additional mechanism by which NO affects cells is via ADP-ribosylation. In this process, NO activates ADP-ribosyl transferases, which cause ADP-ribose moieties to be transferred onto proteins (particularly onto arginine and cysteine residues) from NAD+. This changes the activity of the proteins (Schuman et al., 1994). NO release in the nervous system has widespread modulatory effects on other neurons and thereby can modulate the levels of release of many other transmitters (Prast and Philippu 2001).

The techniques used to examine NO transmission and its role in LTP differs somewhat from those used to examine other transmitters. Because NOS activity leads to an immediate release of NO, NO transmission is often examined by applying a NOS inhibitor, thereby preventing NO release. The inhibitor is usually an inactive analogue of the amino acid L-arginine, which NOS converts to citrulline and NO. In addition, because NO diffuses freely, and reacts widely, NO transmission can be blocked by treatment with an exogenous substance that actively reacts with NO and thereby scavenges NO and prevents it from reacting with biological target molecules.

1.5.4.1 Nitric oxide and LTP

Attempts to block induction of LTP with inhibitors of NO synthase have had variable success. *In vitro* (Cummings et al., 1994) and *in vivo* (Bannerman et al., 1994) experiments failed to confirm initial reports that inhibition of NO synthase blocked the induction of LTP (Bohme et al., 1991; Schuman and Madison, 1991; Haley et al., 1992). In experiments in hippocampal slices, inhibition of LTP by NO synthesis inhibitors was seen at room temperatures but not at higher, more physiological temperatures (Williams et al., 1989). There is also disagreement about whether the application of NO, in combination with weak synaptic activation, produces LTP; two groups reported that it did (O'Dell et al., 1991b; Bon and Garthwaite, 2003), whereas a third found that it did not (Murphy et al., 1994).

Knockout by homologous recombination of either of the two genes encoding the two isoforms of NO in hippocampal neurons fails to block LTP. However, LTP is compromised, though not abolished, in a double knockout of both genes (Son et al., 1996).

Evidence linking postsynaptically generated NO to enhanced transmitter release has emerged from a study on cultured hippocampal neurons, in which NO released by synaptic stimulation led to accelerated vesicle endocytosis via a cGMP dependent pathway (Micheva et al., 2003). An interesting insight from this work is that NO acts only on stimulated terminals, consistent with the possibility that it could selectively affect neighbouring active terminals, in addition to the terminal(s) whose activity was

responsible for evoking its release from postsynaptic cell(s). The observed changes were blocked by NMDA receptor antagonists, establishing the likelihood that NO was produced postsynaptically and was thus acting as a genuine diffusible retrograde messenger.

In a provocative study, Madison and Schuman (1994) made the intriguing observation that intracellular injection of NO synthase inhibitors blocked LTP when it was induced by pairing of single stimuli with depolarisation of the impaled cell but not when it was induced by high-frequency trains. Their explanation of this dissociation is as follows *'When pairing is used to induce LTP, the paired cell itself is the sole source of the putative retrograde messenger'*. If it is NO (or a messenger downstream of NO synthesis), intracellular application of an NO synthase inhibitor would be expected to block LTP, as observed. However, when LTP is induced by a tetanus, the surrounding cells are subject to LTP and themselves make and release the putative retrograde messenger. This could act on the single cell in which NO synthesis has been shut down. Thus, aided and abetted by its neighbours, terminals afferent to this cell can share in the potentiation induced by the high-frequency train (see chapter 6 for detailed discussion of NO and LTP)

1.5.4.2 Mechanism of action

There are conflicting reports on the mechanisms of NO action. A number of studies have shown that inhibition of PKG can block LTP and that selective activators of PKG can produce LTP (Zhuo et al.,1994a; Son et al.,1998), implicating the cGMP second messenger cascade. The most convincing evidence in favour of this hypothesis is that in cultured hippocampal neurons, intracellular injection of a PKG blocker prevents LTP when it is injected into the presynaptic neuron but not the postsynaptic neuron. In addition, intracellular injection of an isoenzymes of PKG into the presynaptic neuron but not the postsynaptic neuron elicited LTP (Arancio et al.,2001). However, there are reports that blocking the cGMP second messenger pathway, either via pharmacological agents or via mutations of key genes, fails to block LTP (Schuman et al.,1994; Selig et al., 1996; Kleppisch et al., 1999). These studies found that NO induces ADP-ribosylation, and pharmacological inhibitors of ADP-

ribosylation blocked LTP (Schuman et al., 1994; Kleppisch et al., 1999), suggesting that NO induces its effects via ADP-ribosylation of proteins. The seemingly contradictory evidence on whether the cGMP cascade is involved in LTP may be resolved by studies showing that the effects of analogues of cGMP are highly sensitive to experimental conditions (Son et al., 1998) and that the effects of NO on LTP differ considerably between different animal strains and species, at different stages of development, and as a result of small differences in the protocols used (Holscher 2002; Blackshaw et al., 2003).

Additional studies examined the targets on which the cGMP cascade acts. These studies found that PKG activates phosphodiesterases, which degrade cGMP, and thereby lowers its concentration. This effect is necessary for LTP (Monfort et al., 2002). The use of NO in this system takes advantage of the ability of the substance to diffuse across cell membranes and to activate second messenger cascades that initiate maintained changes in cellular properties. However, conventional transmitters activating second messenger cascades could produce similar effects, by building reciprocal connections between the presynaptic and postsynaptic neurons. Effects on adjacent presynaptic neurons arise from the unconventional features of NO.

In addition to acting presynaptically as a retrograde transmitter, NO also acts postsynaptically. At least two postsynaptic effects have been identified. One effect leads to an enhancement of LTP in some experimental protocols (Ko and Kelly 1999). A second effect is to suppress LTP, by depressing NMDA receptor-mediated synaptic transmission. This effect is much slower than the presynaptic effect and may occur via S-nitrosylation of postsynaptic proteins (Murphy and Bliss 1999). Simultaneous presynaptic and postsynaptic effects at different timescales and via different mechanisms could explain the problems in identifying precise effects of NO.

More recent data have shown that NO has additional modulatory effects on LTP. When LTP is initiated by pairing NO application with a presynaptic tetanus, blocking NOS for some minutes prior to the tetanus and for 15 minutes after it can nonetheless block LTP, despite the presence of an exogenous source of NO (Bon and Garthwaite

2003). This effect is dependent on the cGMP second messenger cascade. These data have been interpreted as showing that the background release of NO is a critical factor in modulating synapses that undergo LTP.

1.5.5 Structural modification as a result of LTP

Another postsynaptic modification thought to be important in controlling the changes in synaptic transmission following induction of plasticity is the structural modification of dendritic spines (as reviewed by Matus, 2005). Advances in fluorescence imaging and electron microscopy have allowed visualisation of spines and hence changes in their physical appearance following LTP induction to be observed. It has been shown that spines increase in size and filopodia extend following LTP (Engert & Bonhoeffer, 1999; Maletic-Savatic et al., 1999) and it has been suggested that smaller weaker spines preferentially undergo LTP (Matsuzaki et al., 2004). The shape of spine heads may change within a few seconds (Fischer et al., 1998), utilising the actin cytoskeleton, NMDARs, and calmodulin (Hosokawa et al., 1995; Maletic-Savatic et al., 1999; Matsuzaki et al., 2004). This process is also thought to be Ca dependent (Emptage et al., 1999; Korkotian & Segal, 1999) and the size of the spine determines the elevation and duration of postsynaptic changes in $[Ca^{2+}]$ (as reviewed by Tada & Sheng, 2006). Matsuzaki et al. show that the volume of a spine head is proportional to the number of postsynaptic AMPARs (Matsuzaki et al., 2001). Experiments have also shown that over-expression of GluR2 can result in a change in spine size and density in hippocampal neurons (Passafaro et al, 2003).

LTP has been shown to cause an increase in synapses which in turn leads to their perforation (Geinisman, 1993; Geinisman et al., 1991; Schuster etal., 1990). Use of immunogold labelling suggests that larger PSD contain more AMPARs (Nusser et al., 1998; Takumi et al., 1999) and perforated synapses contain more AMPARs than simple ones (Desmond & Weinberg, 1998). With this in mind, it is also known that the splitting or budding of spines can occur (Edwards, 1995), which may then both be activated by a single presynaptic bouton. It is postulated that induction of LTP results

in perforation of synapses which then leads to the budding of synapses (Toni et al., 1999). In addition, 2-photon and confocal microscopy experiments have been used to demonstrate that LTP results in the formation of de novo spines on dendritic shafts (Engert & Bonhoeffer, 1997; Sorra et at,, 1998). However one hypothesis does not rule out the other. The detailed mechanisms underlying the changing morphology of spines following LTP induction are as yet unclear. (For a comprehensive review of proposed mechanisms see Tada & Sheng, 2006). The discovery of structural plasticity is important as it indicates that LTP not only encourages the development of spines, but also that the size of the spine can dictate the amount of plasticity which occurs at that synapse.

1.5.6 Maintaining LTP

LTP is thought to be maintained as a result of protein synthesis and gene expression and LTP beyond four hours is critically dependent on protein and mRNA synthesis (Frey et at., 1996; Frey et at., 1993; Frey et al, 1988; Frey & Morris, 1997; Krug et at., 1984; Nguyen et at., 1994; Nguyen & Kandel, 1996; Otani & Abraham, 1989). Late-phase LTP has been shown to be inhibited by anisomycin (protein synthesis inhibitor) both *in vivo* and in vitro (Frey et al., 1988; Krug et al., 1984) and by actinomycin D (mRNA synthesis inhibitor) (Frey et at., 1996). Localised protein synthesis has been suggested to occur in dendrite shafts due to the presence of spine associated polyribosomes (Tone & Steward, 1996).

The mechanisms underlying how the cell can regulate late-phase LTP and delivery of newly synthesised proteins are still under debate. However, there is one prominent hypothesis: that the induction of LTP results in the 'tagging' of synapses for the specific delivery of de novo proteins (Frey & Monis, 1997). Synaptic tags are thought to mark activated synapses to sequester plasticity related proteins in order to stabilise the temporary changes induced by LTP induction (as reviewed by Frey & Monis, 1998). There are two hypothesised ways in which the synapse could be tagged, either by a change in the spine size (Rusakov et al., 1995) or by a phosphorylated kinase (Osten et al., 1996; Sajikumar et al., 2005). PKM has been proposed to be a marker for LTP, as concentrations of this protein increase dramatically and specifically 30

minutes after LTP induction (Sacktor et al., 1993) and is necessary for late phase LTP (Serrano et al., 2005).

1.6 Aims

The aim of this thesis is to identify if LTP can be induced in adult GluR1 knockout mice using standard induction protocols, and to identify the properties of these induction protocols that mediate any differences. The locus of expression of GluR1 independent LTP will also be investigated. The GluR1 knockout mice will then be used as a tool to investigate the role that NO plays in LTP in the hippocampus. Finally, using GluR1/NOS1 and GluR1/NOS3 double knockout mice the NOS isoforms mediating the NO component of LTP will be investigated.

Methods

1.7 Animals

Experiments were performed on brain slices from adult mice aged P45-64 (6-9 weeks) for the intracellular experiments and P57-64 (8-9 weeks) for the extracellular experiments. Mice were AMPA receptor subunit 1 (GluR1) knockout mice, NO synthase isoform 1 (NOS-1) knockout mice, NO synthase isoform 3 (NOS-3) knockout mice and wild-type littermates bred into a C57BL/6 background and maintained the colony as heterozygotes. Experimental null mutants and wild-type littermates were bred from heterozygote crosses (cousin mating). Double knockout animals were created by breeding heterozygous single knockout until double heterozygous males and females were produced. Double knockout were produced by mating double heterozygous animals, or on a few occasions by mating GluR1-/-NOS -/+ mice with double heterozygotes. The GluR1 knockout mice were kindly supplied by Rolf Sprengel (Heidelberg) via the Rawlins lab in Oxford. The αNOS-1 and NOS-3 knockout were obtained from Jackson labs (Bar Harbour, ME). The genotyping was kindly performed by Phil Blanning by PCR using primers ordered from MWG (Ebersberg, Germany). The following primer sequences were used, for NOS-1: (oIMR13) 5′ CTT GGG TGG AGA GGC TAT TC 3′; oIMR14 5′AGG TGA GAT GAC AGG AGA TC 3′; (oIMR406) 5′ TCA GAT CTG ATC CGA GGA GG 3′; (oIMR407) 5′ TTC CAG AGC GCT GTC ATA GC 3′. For NOS-3: (oIMR94) 5′ TGG CTA CCC GTG ATA TTG CT 3′; (oIMR1823) 5′ ATT TCC TGT CCC CTG CCT TC 3′; (0IMR1824) 5′GGC CAG TCT CAG AGC CAT AC 3′. Jackson labs supplied both NOS-1 and NOS-3 primer sequences. For the GluR1 knockout we used (1005) 5' AAT GCC TAG TAC TAT AGT GCA CG 3'; (MH60) 5' CAC TCA CAG CAA TGA AGC AGG AC 3'; (3Int3) 5' CTG CCT GGG TAA AGT GAC TTG G 3'. Rolf Sprengel supplied primer sequences for the GluR1 knockout.

1.8 Slice preparation

1.8.1 Dissection procedure

Brain slices were prepared from mice aged between 8 and 10 weeks. Mice were sacrificed by the dislocation of the neck, according to the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (UK). The brain was quickly removed and immediately immersed in ice-cold artificial cerebrospinal fluid (ACSF) for 3 minutes. The ACSF contained (in mM) 124 NaCl, 2.3 KCl, 2 $CaCl_2$, 1.3 $MgSO_4$, 1 NaH_2PO_4, 26 $NaHCO_3$, and 11 D-glucose, and was constantly oxygenated with 95% O_2/5% CO_2.

1.8.2 Mounting and Sectioning of the Brain

A block of brain tissue containing the hippocampus was cut out from the whole brain and affixed to a metal stage with cyanoacrylic glue, and immersed in a tissue chamber constantly cooled to 4 °C. Coronal slices (400 μm) were cut using a vibratome and 3–4 slices containing hippocampus were harvested and placed in an incubation chamber in ACSF at 27–28 °C and kept oxygenated. The slices were left to recover for at least 2 hours before recording.

1.9 Electrophysiological Recordings

1.9.1 Recording Preparation

During electrophysiological experiments the brain slices were placed in the recording chamber and continuously perfused with ACSF at a flow rate of approximately 6 ml/min. The slices were secured in the chamber by a platinum "horse shoe" with two nylon strands stretched across. All recordings were made at 32 °C. To maintain this temperature constantly the stock ACSF was heated in a water bath and also the perfusion tubing passed through a heat exchange bath just before it arrived at the recording chamber. A thermometer probe was placed in the recording chamber to monitor the final bath temperature. The ACSF was constantly removed from the chamber by suction using a vacuum pump.

1.9.2 Recording Field Excitatory Post Synaptic Potentials (fEPSPs)

Field EPSPs were evoked by stimulation of the Schaffer collateral-commissural fibres in the stratum radiatum with two stainless steel monopolar stimulating electrode with a tip impedance of 0.5 MΩ (Micro Probe, Inc). The frequency and amplitude of the stimulation was controlled via a Master 8 connected to an isolated stimulator (Model DS2 Digitimer Ltd England). The stimulating electrodes were held in place with coarse manipulators (Narishige, Japan) attached to a magnetic stand (Narishige, Japan) on the air table surrounded by a custom built Faraday cage. Field excitatory postsynaptic potentials (fEPSP) were recorded in the CA1 region of the hippocampus via a carbon fibre electrode (figure 2.1). The electrode was connected to a head stage (NL-100 Digitimer Ltd) and then amplified 500 times (NL104 Digitimer) before being digitised at 3kHz (National Instruments Ltd) and recorded on a PC using Signal (CED). The recording chamber also contained a chlorided silver pellet that was connected to the head stage as a floating earth. The recording electrode was moved into position using a micromanipulator (Narishigi, Japan) mounted to the air table, which enabled movement in the x, y and z axes.

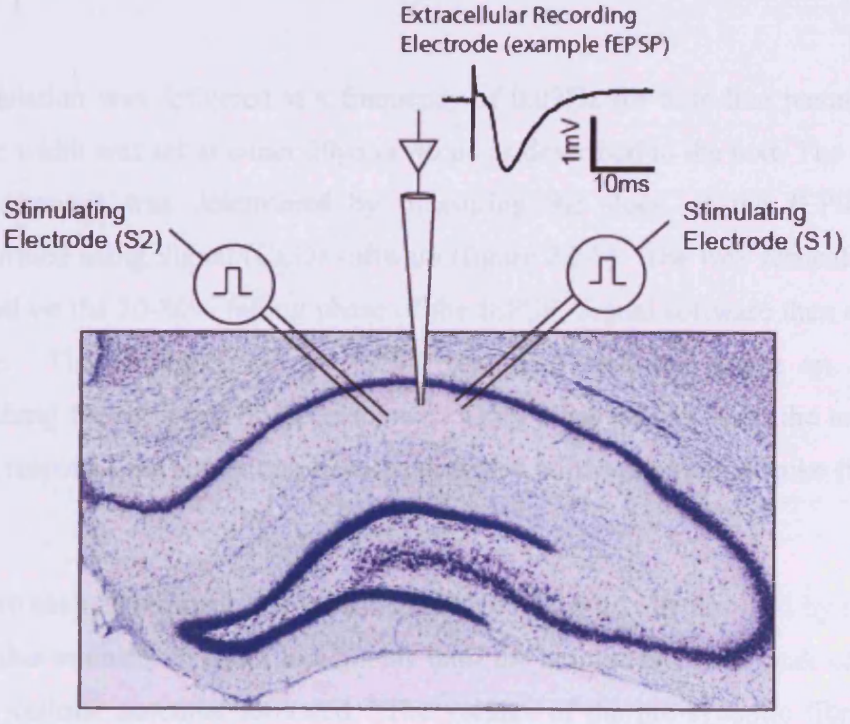


Figure 2.1 Schematic of a hippocampal slice recording set up

The two recording electrodes (S1 and S2) were moved into place into the centre of the *stratum radiatum* between the subiculum and the CA3 region (figure 2.1). The S1 electrode was placed approximately equidistant from the molecular and pyramidal layers on the CA3 side of the recorded cell. To ensure pathway independence, the stimulating electrodes were placed at slightly different depths in the stratum radiatum. The S2 electrode was placed either higher or lower than S1 (in alternate experiments) and was always located on the subiculum side of the recorded cell. Pathway independence was assessed by looking for paired pulse facilitation (PPF) between the two pathways. Stimuli were delivered to the two pathways with an interval of 70ms. The amplitude at a given pathway was compared between naïve conditions and after stimulation of the other pathway. No change in the amplitude represents pathway independence. Since pathway independence was achieved in 98% of experiments (independence n = 85 from n = 87), if the two stimulating electrodes were placed in slightly different layers of *stratum radiatum* I did not perform this test on all occasions.

Stimulation was delivered at a frequency of 0.02Hz for base line recordings and the pulse width was set at either 20μs or 40 μs as described in the text. The magnitude of the response was determined by measuring the slope of the fEPSP. This was performed using Signal (CED) software (figure 2.2A). The two vertical cursors were placed on the 20-80% falling phase of the fEPSP, Signal software then calculated the slope. The amplitude of the fEPSP was also measured using an active cursor searching for the peak of the response. Only slope was used in the analysis as the peak response can sometimes become distorted with a population spike (figure 2.2 A).

Before each experiment, the maximum fEPSP slope was determined by increasing the stimulus intensity in small increments until the amplitude of the peak of the negative extra cellular potential saturated. The voltage of the pre-synaptic fibre stimulation was then adjusted to evoke fEPSPs that were 40% of the maximal slope for LTP experiments (figure 2.2 B).

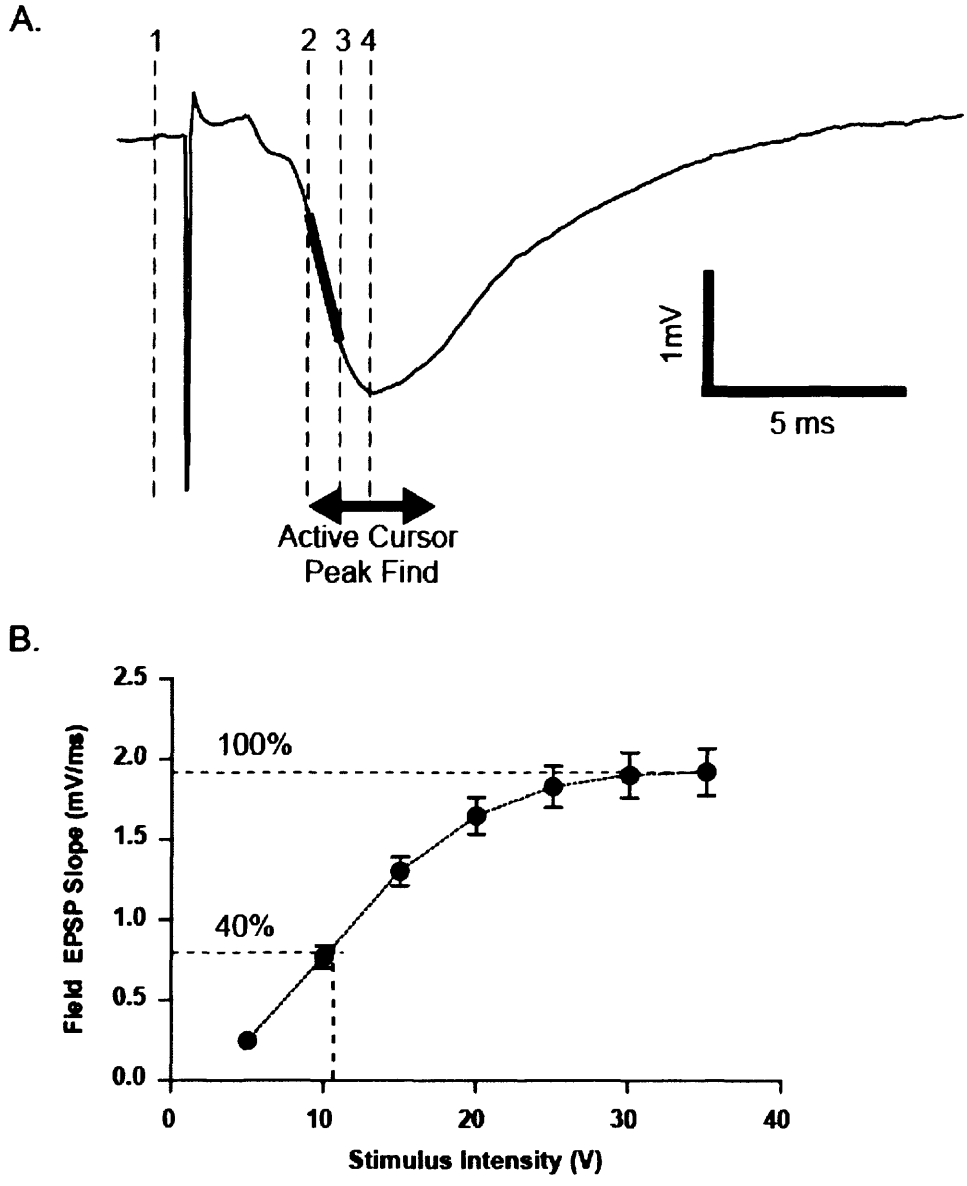


Figure 2.2

A, schematic to show how a fEPSP is measured. Dotted lines indicate four Signal (CED) cursors. Cursor 1 is static and measures the baseline, cursor 2 and 3 are placed on the linear falling slope of the fEPSP and measures the slope of the fEPSP (dark line), cursor 4 is in active mode searching for the peak of the fEPSP within a defined search window. B. Input output relations ship showing the maximum response (100%) and the 40% response. In this case the stimulus intensity would be set to ~11V for base line recordings.

1.9.3 Whole-cell Somatic Recordings

Recordings were made in a submerged chamber perfused with ACSF at 32°C. To enable the results from the intracellular recordings to be compared directly to the extracellular recordings it was important to keep the stimulus strength constant between studies. This was achieved by recording an extracellular input output curve before the intracellular recording was made. Intracellular recordings were then obtained from CA1 cells that were directly above the fEPSP recording electrode, perpendicular to the stratum radiatum. CA1 pyramidal cells were visualized using an Olympus Optical BH2 video microscope (Tokyo, Japan), using DIC optics.

Micropipettes, 1-2μm tip size, tip resistance 3-5MΩ were pulled from borosilicate filamented glass capillaries using a horizontal puller (Sutter). Pipettes were filled with intracellular solution containing in mM: 110 K-gluconate, 10 KCl, 2 $MgCl_2$, 0.3 Na_2ATP, 0.03 Na_2GTP, and 10 HEPES corrected to pH 7.3 (290 mOsm). 20μM QX314 was included in the electrode filling solution for one set of experiments as described in results. Intracellular solution was made as a stock solution, aliquoted and then stored at -80°C and defrosted on ice on the day of the experiment to prevent degradation of the solution during multiple freeze-thaw cycles. An anti-vibration table was required to prevent loss of recordings caused by surrounding vibrations (Isostation, Newport, USA).

Conventional bridge-balance recording were employed in all experiments, using an Axopatch 2B amplifier (Axon Instruments). Once the electrode resistance had been offset, the resistance at the tip of the electrode was monitored with a 100ms long 0.05nA current pulse from the amplifier. Positive pressure was firstly applied as the electrode entered the solution, and then more positive pressure applied as the electrode entered the brain slice, which ensured a clean electrode tip as it touched on the chosen cell. The positive pressure also enabled an easy passage for the electrode through the extracellular matrix around the cell. On touching the cell, the positive pressure was then taken off the electrode; gentle suction typically produced a high resistance seal of 1-4 GΩ between the electrode tip and the cell membrane. Recording electrode capacitance transients were compensated for using the pipette

capacitance compensation functions on the patch clamp amplifier after formation of the seal. It was important to do this to prevent errors in resistance assessment during subsequent whole cell recording. Further suction ruptured the cell membrane and the whole-cell patch clamp configuration was achieved. Depolarising pulses of injected current were used to elicit action potentials at the soma and the shape/frequency of the action potentials were used to help confirm the identity of the cell.

Once whole cell configuration had been achieved, recording began. A protocol including a 0.05nA current step for 10ms and a stimulation to evoke EPSPs through the stimulation electrodes was applied at a frequency of 0.2Hz and the resulting responses were recorded and analysed online. The sweep length recorded was for 1s. The current step was used to estimate the input resistance. These values along with membrane potential were displayed on line using signal (CED) and were used to monitor the health of the cell. During the current step the amplitude of the plateau following the relaxation of the capacitance transient was used to estimate the membrane resistance of the cell using Ohm's law (voltage = current x resistance). Whole-cell recordings were made at post break in resting membrane potential (average V_m of –64 ± 3 mV for wild-type, –68 ± 4 mV for GluR1 knock-outs) in the current clamp mode but discarded if the input resistance changed by >20%. Accepted input resistances were between 100 and 300 MΩ. Responses were amplified (Axoclamp 2B), filtered at 4-5kHz, digitized at 10kHz (CED 1401) and recorded using Signal (CED).

1.9.4 Measurement of Evoked Synaptic Amplitudes

For analysis of synaptic responses, Signal software (CED) was used to extract the amplitudes of individual synaptic events from raw sweeps of data. These were measured as differences between two mean voltages taken from a 10ms window shortly before the EPSP (base line) and at the peak of the EPSP. The base line window was set using a static cursor and the peak was then found using an active cursor searching for the peak of the response (figure 2.3). The noise of the recordings was also measured using the same length window, but at a set distance away from the EPSP (typically 100ms).

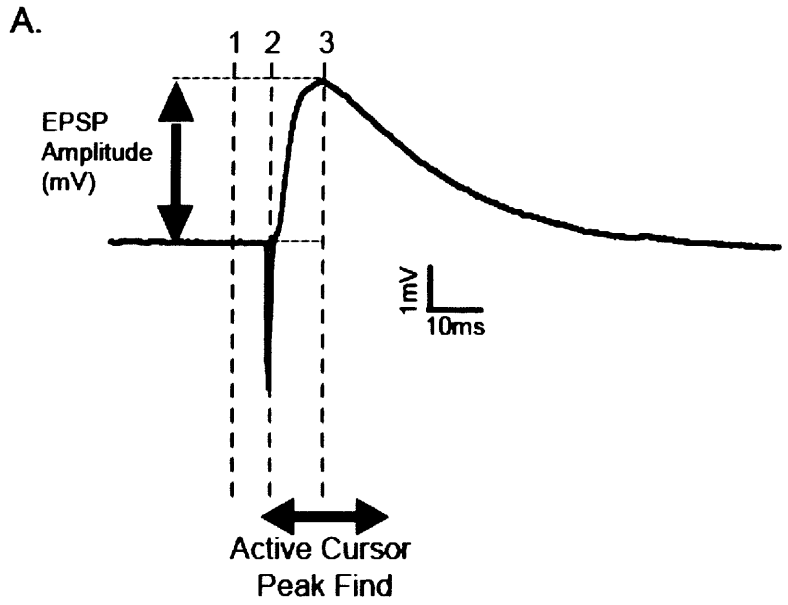


Figure 2.3
A, Schematic to show how a EPSP is measured. Dotted lines indicate three Signal (CED) cursors. Cursor 1 is static and measures the baseline, cursor 2 is triggered with the stimulus and measures the time of the stimulus artifact, cursor 3 is in active mode searching for the peak of the EPSP within a defined search window (eg, start = cursor 2, finish = cursor 2+ 200ms).

1.9.5 Tetrodotoxin applications

When indicated in the text, 10μM Tetrodotoxin (TTX) dissolved in ACSF was pressure-applied through a patch pipette positioned near the soma, under visual guidance. After achieving the whole cell recording configuration the TTX pipette was carefully positioned next to the soma of the cell. To initiate the flow of TTX, pressure was applied to the pipette using a syringe attached by a length of tubing. To stop the flow a very slight negative pressure was applied. The flow of the solution was monitored by using 0.1% Fast Green in the TTX pipette and optimized to avoid TTX diffusion into the *stratum radiatum* (Figure 2.4). The blockade of somatic spikes was monitored by the injection of a current step to induce action potentials. If the pipette was positioned correctly then pressure application of TTX quickly (~5 seconds) blocked the action potentials. On all occasions the effect of TTX pressure application on baseline EPSP amplitude was identified and if any change occurred the TTX electrode was repositioned or the cell was discarded.

A.

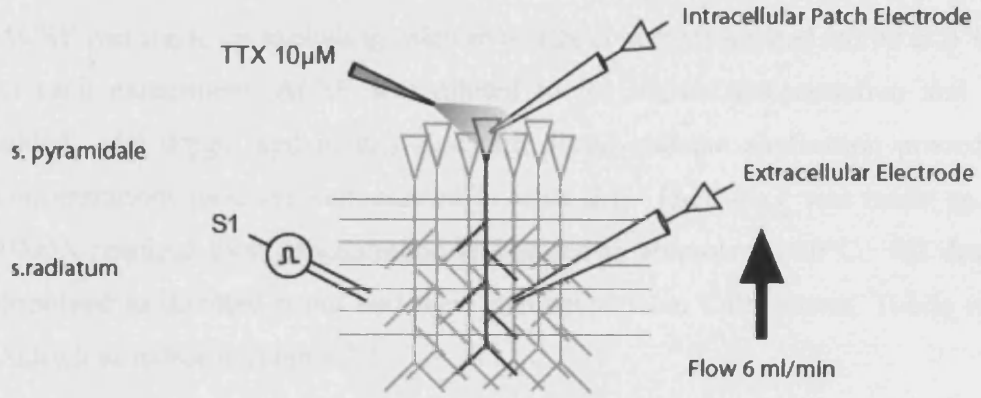


Figure 2.4 Local somatic TTX applications.
A, Schematic diagram of the experimental set up. Action potential generation and propagation can be blocked by local pressure application of TTX (10μM) to the soma. The slice is positioned so that TTX does not perfuse on the Stratum Radiatum.

1.9.6 LTP Induction

After recording base line synaptic responses for 20 min in the field recording experiments or 10 minutes during intracellular recordings, LTP was induced by one of four different protocols;

100Hz stimulation for 1 second repeated 3 times at 0.05Hz at baseline voltage, 20μs pulse width (low intensity).

100Hz stimulation for 1 second repeated 3 times at 0.05Hz at baseline voltage, 40μs pulse width (high intensity).

Theta burst stimulation (TBS) that consisted of 3 trains at 0.02Hz, each train consisting of 10 bursts at 5Hz and each burst consisting of 4 pulses at 100Hz, delivered at baseline voltage, 20μs pulse width (low intensity).

Theta burst stimulation (TBS that consisted of 3 trains at 0.02Hz, each train consisting of 10 bursts at 5Hz and each burst consisting of 4 pulses at 100Hz, delivered at baseline voltage, 40μs pulse width (high intensity).

1.10 Drugs

ACSF was made up excluding calcium at 10x concentration and stored at 4°C. Prior to each experiment, ACSF was diluted to the correct concentration and calcium added. All drugs used in this study are listed and the application procedure and concentrations used are summarised in table 2.1. Each drug was made up to 100-1000X required final concentration and stored in aliquots at -20°C. All drugs were dissolved in distilled water and were purchased from Calbiochem, Tocris or Sigma Aldrich as indicted in table 2.1.

Table 2.1

Drug	Action	Supplier	Concentration	Application
D-AP5	NMDAR antagonist	Tocris	50μM	Bath applied; slice pre-incubated for 5 minutes prior to LTP induction.
AIP	CaMKII inhibitor	Calbiochem	1μM	Extracellular; Bath applied, slice pre-incubated for 5 minutes prior to LTP induction Intracellular; Applied via patch pipette.
L-NNA	NOS inhibitor	Tocris	100 μM	Bath applied; slice pre-incubated for 5 minutes prior to LTP induction.
L-NAME	NOS inhibitor	Tocris	100 μM	Bath applied; slice pre-incubated for 5 minutes prior to LTP induction.
TTX	Inhibitor of Na^+ channel conductance.	Sigma	10μM	Locally applied to the soma via pipette.
QX314	Inhibitor of Na^+ channel conductance	Sigma	0.2mM	Applied via patch pipette.

1.10.1 Data Analysis

Responses were normalized with respect to the baseline and averaged into 1 min bins, data groups expressed as mean ± S.E.M. For extracellular recordings data comparisons were made between test and control pathways at the time points given. For intracellular recordings data was compared within pathway between baseline and 40 – 45 minutes post LTP induction. ANOVAs were then run to test for effects of genotype and drug application, together with any interaction terms using Graphpad

software. Post hoc Bonferroni corrected t tests were then used to investigate the origin of effects further. For tests of statistical significance, the reported values' are in the form T or $F_{(\text{degree of Freedom})}$ = Value, P = significance level.

1.10.2 1/CV^2 analysis

Changes in the CV (Coefficient of variation of a distribution) can be used to probe the locus of any changes in synaptic strength (Malinow and Tsien, 1990). The mean amplitude and variance were measured for the whole control period plus stable periods of at least 50 (and usually 100) stimuli after the induction of LTP. At least two separate noise measurements were taken for each EPSP, from non-overlapping parts of the baseline, to calculate the mean noise SD. This noise SD was then subtracted from the EPSP SD using the equation;

$$(\text{EPSP SD})^2 = (\text{SD of combined EPSP + noise})^2 - (\text{noise SD})^2$$

Two periods of data were studied using $1/CV^2$, the control period and the last 10 minutes of potentiation. We calculated the squared coefficient of variation (CV^2) from the variance and mean amplitude (CV^{-2} = mean^2/variance), normalized the mean amplitude and CV^{-2} values to the control period, and plotted values for the two time periods (Malinow and Tsien, 1990).

2 Characterisation of LTP produced by theta burst and high frequency stimulation

2.1 Introduction

The most predominant and extensively studied form of LTP is the NMDA receptor dependent LTP seen in the CA1 region of the hippocampus. This form of LTP requires glutamate to bind to the NMDA receptor at the same time as the cell is sufficiently depolarised to relieve the Mg^{2+} block. This can be achieved in the in vitro slice preparation by using a number of different stimulation protocols. Probably the most commonly used protocol in field potential recordings, where no direct control of the postsynaptic membrane is possible, is the so called high-frequency tetanus (HFS), which is a train or trains of 50–100 stimuli at 100Hz. The intensity that these stimuli are given at is generally between 30-50% of the maximum fEPSP, although many other permutations have been used (e.g. EPSP set 1mV or 50% of the magnitude to where a population spike appeared). Typically one train of HFS produces a sub maximal potentiation, while if 3 or more trains are given (separated by 10 – 20 sec) the LTP reaches a saturated level. LTP induced by HFS is effective at producing a very stable potentiation that can last for over 3 hours or as long as the experimenter can keep the slice healthy.

Due to the complexity of the processes that affect postsynaptic depolarisation during tetanus-induced LTP, many investigators have evoked LTP in a simpler and more defined way by using what is termed a "pairing protocol". This protocol requires control of the postsynaptic cell potential, so is generally used in intracellular or whole cell patch clamp studies. "Pairing" circumvents many of the complexities of synaptically induced postsynaptic depolarisation, by simply imposing a depolarisation current injection through the microelectrode (Gustafsson et al., 1987) or by voltage clamp, using the somatic patch electrode (Malinow and Tsien, 1991). Standard pairing protocols use a much lower frequency of synaptic stimulation (0.1-2 Hz) (Colino *et al.*, 1992), (Malinow, 1991); (Perkel *et al.*, 1993) than during tetanic stimulation (100Hz). This produces an extra simplification of avoiding the transient forms of presynaptic plasticity that occur during high-frequency stimulation. A consequence of using low-frequency stimulation is that the depolarisation during

pairing must be long. Typically, this depolarisation is applied for more than one minute (Colino et al. 1992; Manabe et al. 1992; Otmakhov et al. 1997).

The requirement for long depolarisation raises the question of whether pairing protocols are comparable to the LTP induced by tetanic stimulation, or whether there are fundamental differences. One difference is the size of the LTP induced by these different protocols. The standard pairing protocol produces a very large LTP, that is often over 400% and can sometimes be 1,000% (Malinow 1991; Otmakhov et al. 1997). In contrast, the LTP induced by tetanic stimulation and field recording methods typically shows an LTP that is <200% and is often only 150%.

HFS and "pairing" have both been criticised as they are both very artificial manipulations of cells and do not mimic naturally occurring firing patterns of neurons *in vivo*. For example, it is not certain that hippocampal neurons in the living animal fire at 100 Hz for a full second, making standard HFS protocols questionable. Pyramidal cells in CA1 more commonly fire short (30- to 40-ms) bursts of three to four spikes (Kandel and Spencer 1961; Ranck 1973), with the bursts being repeated at the theta burst stimulation frequency (Green et al. 1960). This theta burst frequency was also seen in EEG recordings of animals engaged in learning related behaviours (Grastyan et al. 1959; Vanderwolf 1969; Bland). (See introduction for more detailed discussion).

To try to recreate the firing properties that were observed *in vivo* during learning behaviours, a theta burst stimulation protocol was developed that also produced a robust LTP (Larson et al., 1986). It was shown that LTP could be optimally induced if short (40ms) 100Hz bursts were repeated at 200ms intervals (5Hz or theta burst stimulation), while longer or shorter burst intervals (10, 0.5, and 0.1Hz) produced small or no LTP. The effectiveness of producing LTP using theta burst stimulation seems to be related to the refractory period of the IPSPs. The stimulation of hippocampal afferents not only initiates EPSPs in pyramidal cells, but also recruits IPSPs in these cells by means of feed-forward activation of interneurons (Alger and Nicoll 1982). These feed-forward IPSPs become refractory for 200-500 ms thereafter

(McCarren and Alger 1985). Hence, the theta burst stimulation interburst interval of 200 ms delineates a period when IPSPs are difficult to recruit. Repeated application of brief bursts of stimuli, at the theta burst stimulation frequency, allows for more effective temporal summation of EPSPs in the absence of the strong feed-forward inhibition that would otherwise reduce excitatory transmission.

Given that theta burst stimulation protocols appeared effective in inducing LTP, studies were also conducted addressing whether theta burst stimulation protocols were also effective at generating long-lasting LTP. Nguyen *et al.* (1994) tested theta burst stimulation protocols (3 groups of theta burst stimulation: 15 bursts of 4 pulses at 100 Hz; pulse width 50 μs; interburst interval 200 ms) in CA1 mouse hippocampal slices and found that the LTP response (EPSP slope, ~ 170% of baseline) was maintained out to 180 min post stimulation, whereas LTP induced by 60 Hz for 1 second had decayed back to near-baseline levels by this time point. Unfortunately, Nguyen and Kandel did not test and compare the common 100 Hz HFS protocol in this study. The differences between HFS and theta burst stimulation were looked at directly in a well controlled study by Hernandez et al., (2005). They showed that when higher numbers of pulses were given in the induction (200 -300 pulses) a larger LTP was seen in theta burst stimulation LTP compared to HFS.

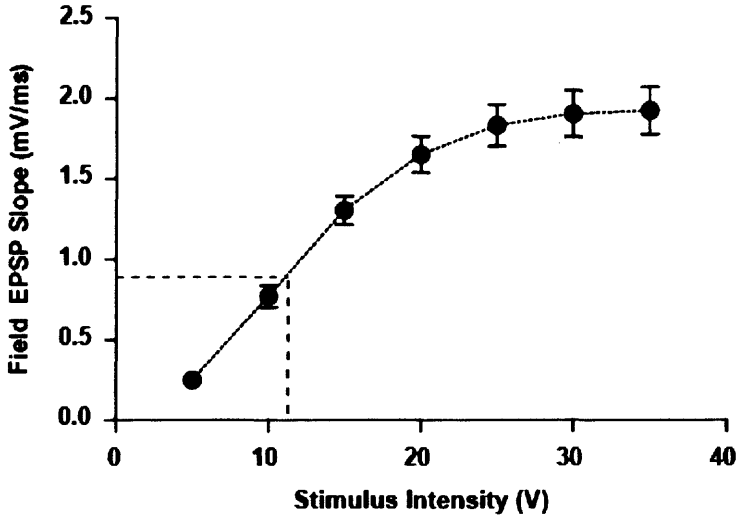
Although the Hernandez study looked at the differences between theta burst stimulation and HFS using different numbers of stimuli, there have been no studies looking at the effect of stimulus intensity between the two protocols. In the following chapters I have used HFS and theta burst stimulation at high and low stimulus intensities to look at LTP in wild-type and different knockout animals. It was therefore important to identify if these two protocols produced differing magnitudes of LTP in wild-type animals when the stimulus intensity was changed. The aim of this introductory experimental chapter is to characterise LTP in my experimental set up and to see if there are any differences between the LTP induced using a high and low stimulus intensity in both theta burst stimulation and HFS.

2.2 Results

2.2.1 Input output relationship.

Field EPSPs were evoked in control and test pathways with a 10μs square voltage step applied at 0.05Hz alternately through two stainless steel monopolar electrodes located in stratum radiatum (test = S1 control = S2). At the beginning of each experiment a detailed input output curve was generated from both the control and test pathways (Figure 3.1). The stimulus intensity was gradually increased in 5V steps until the fEPSP slope saturated. Figure 3.1B shows an example of fEPSPs that saturate at 35V. There was no significant difference between the IO curves for S1 and S2 pathways and therefore the data pooled in Figure 1 (maximum fEPSP slope in S1 = 1.92 ± 0.15 $mV.ms^{-1}$ S2 = 1.95 ± 0.18 $mVms^{-1}$). A two way ANOVA test showed a significant interaction of voltage ($p < 0.01$) but no significant interaction of pathway ($p > 0.05$). The distance between the stimulating and recording electrodes can have significant effects on both the I/O relationship and on the magnitude of LTP (Kopanitsa et al., 2006). Care was therefore taken to keep the distance between the stimulating electrodes and the recording electrodes constant between experiments at ~1mm. The voltage intensity was then set to produce a fEPSP that was 40% of maximum that could be evoked.

A.



B.

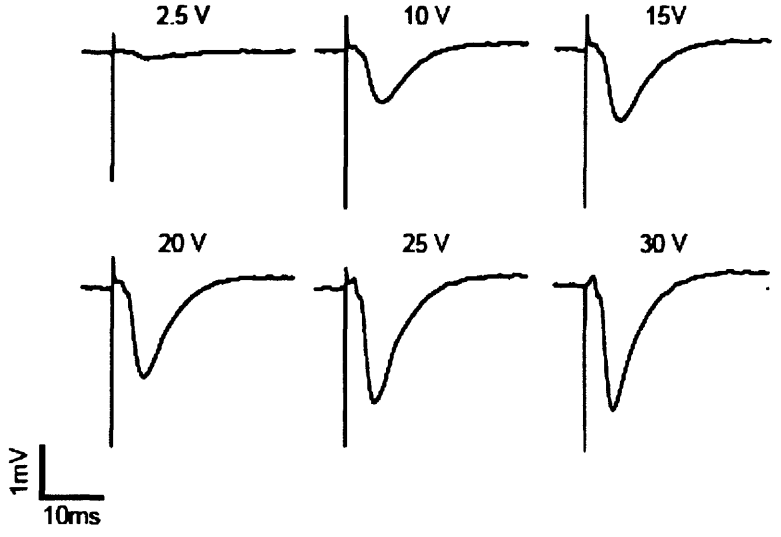


Figure 3.1 Input output relationship
A, Input/output response in wild-type, the stimulus intensity was gradually increased in 5V steps until the fEPSP slope saturated. B, Example traces illustrating the size of the fEPSP generated.

2.2.2 Control LTP

In field EPSP recordings, before any LTP was induced, a baseline period was recorded for at least 20 minutes to check that the response was stable. LTP was induced in the S1 pathway by either HFS or TBS. The S2 pathway was not stimulated during this period. The amount of LTP was measured 55-60 min after the tetanus and expressed as % change from the baseline.

One train of HFS (1s at 100Hz at test pulse width) produced a sub maximal LTP (Figure 3.2A). If three subsequent trains were given at 20 second intervals a robust and stable LTP was observed (Figure 3.2). The potentiation at 60 minutes post tetanus was 153 ± 8.9 % (n = 12). No effect was seen in the S2 control pathway (101 ± 2%, n = 12). One train of TBS (4 pulses at 100Hz, repeated 10 times at 5Hz) also produced a sub maximal LTP, which largely decayed down to base line within 20 minutes (Figure 3.3A). If 3 trains of TBS were given, a stable LTP could be produced with a potentiation (at 60 minutes post tetanus) of 163.5 ± 8.3% (n = 11). When 3 trains of TBS were given to the S1 pathway a very small and transient depression of the S2 pathway was observed in some cases (Figure 3.3A, B). This depression was very short lasting with the EPSP returning to base line within 1 minute after the tetanus. This observation is likely to be due to a transient increase in GABA release in the slice, as it has been shown that TBS can cause a small short lasting potentiation of GABA IPSCs (Perez et al., 1999). Thus LTP can reliably be induced in the experimental set up using two standard protocols.

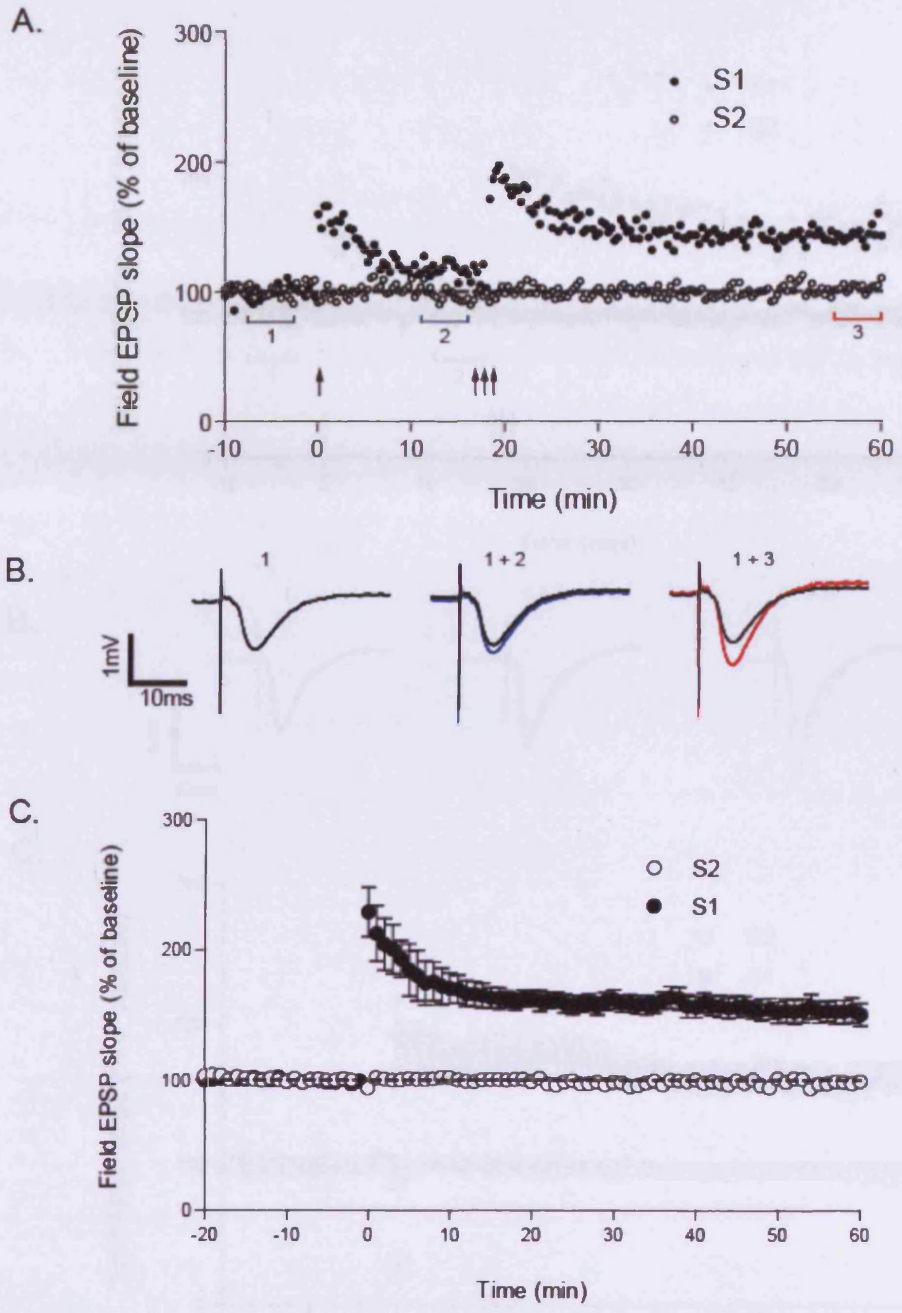


Figure 3.2 Extracellular field LTP induced using a 100Hz protocol.
A Example experiment, showing that 1 train of 100Hz stimulation produces a sub maximal LTP while 3 trains can induce a larger magnitude potentiation B, Example traces taken at time points indited by the bars. C, Group data (n = 12) showing that three trains of 1s 10Hz stimuli produce a long lasting LTP. Closed circles indicate the tetanised pathway, open circle indicate the un-tetanised control pathway.

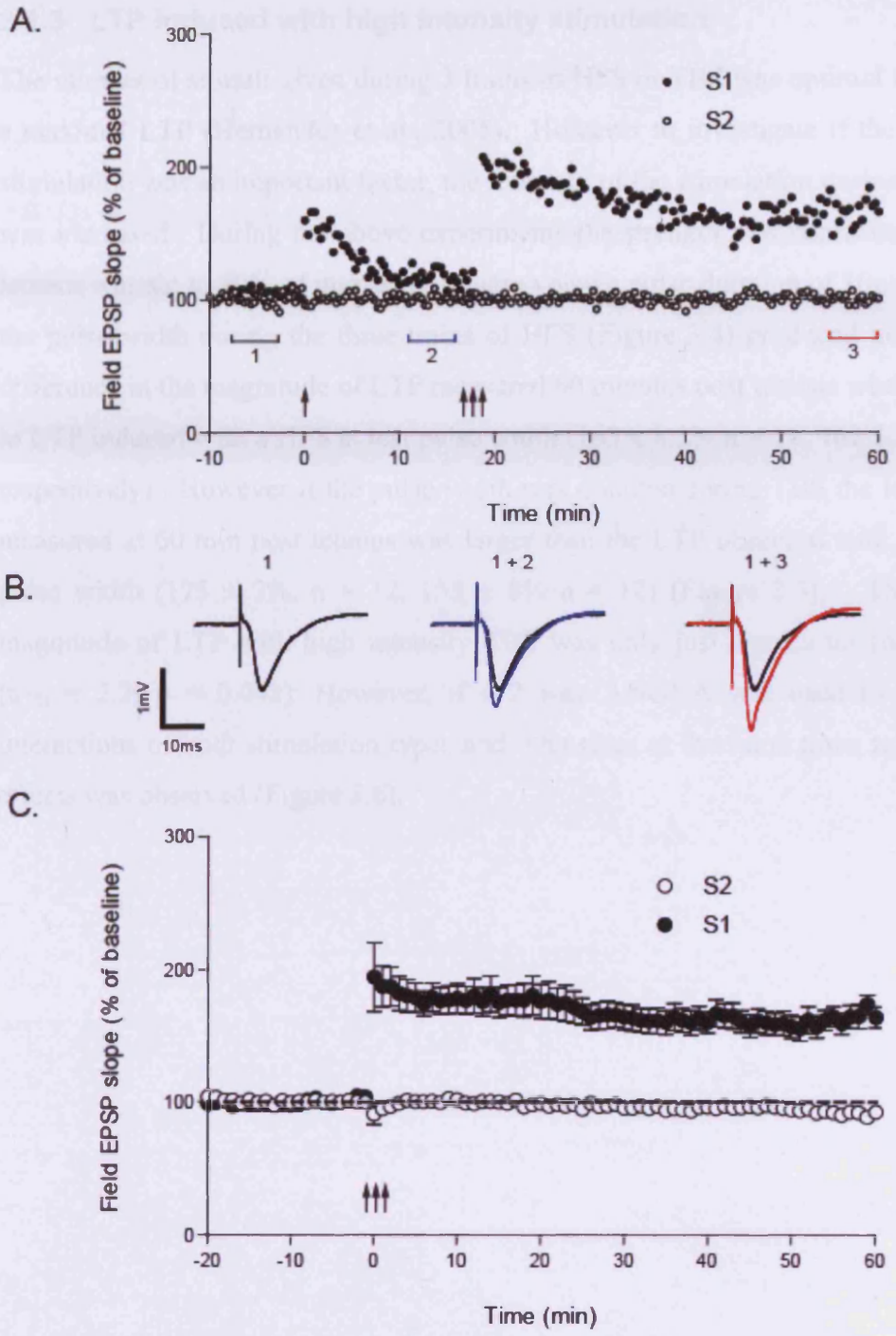


Figure 3.3 Extracellular field LTP induced using a theta burst protocol.
A Example experiment, showing that 1 train of theta burst stimulation produces a sub maximal LTP while 3 trains can induce a larger magnitude potentiation B, Example traces taken at time points indicated by the bars. C, Group data (n=11) showing that three trains of theta burst stimulation produce a long lasting LTP. Closed circles indicate the tetanised pathway, open circle indicate the un-tetanised control pathway. Theta burst protocol applied as indicated by the arrows.

2.2.3 LTP induced with high intensity stimulation

The number of stimuli given during 3 trains of HFS or TBS was optimal for inducing a maximal LTP (Hernandez et al., 2005). However to investigate if the strength of stimulation was an important factor, the intensity of the stimulation during the tetanus was increased. During the above experiments the strength of stimulation during the tetanus was set to 40% of max using square voltage pulse duration of 10μs. Doubling the pulse width during the three trains of HFS (Figure 3.4) produced no significant difference in the magnitude of LTP measured 60 minutes post tetanus when compared to LTP induced with a HFS at test pulse width (163 ± 8.2% n = 12, 162 ± 11% n = 11 respectively). However if the pulse width was doubled during TBS the level of LTP measured at 60 min post tetanus was larger than the LTP observed with TBS at test pulse width (175 ± 7%, n = 12, 158 ± 8% n = 12) (Figure 3.5). The increased magnitude of LTP with high intensity TBS was only just significant (paired t test) ($t_{(22)}$ = 2.2, p = 0.042). However, if a 2 way ANOVA was used to look at the interactions of both stimulation types and intensities at the same time, no significant effects was observed (Figure 3.6).

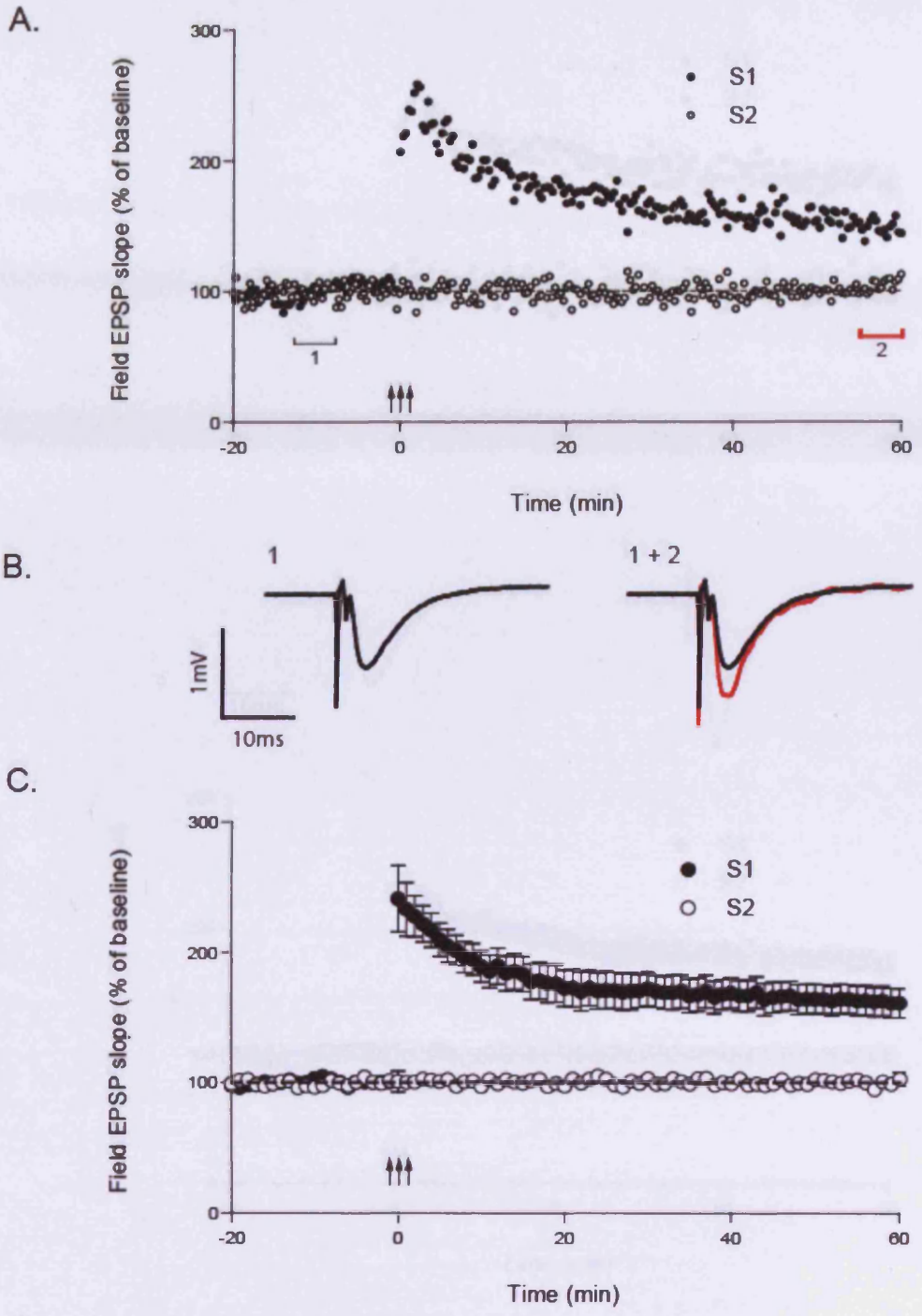


Figure 3.4 The magnitude of LTP is unaffected by increasing the intensity of the stimulation during the 100Hz tetanus.
A Example experiment, three trains of 100Hz stimulation at double pulse intensity was given at the time indicated by the arrow B, Example traces taken at time points indicated by the bars. C, Group data (n=12), no change in the magnitude of the LTP is observed if a stronger intensity 100Hz stimulation is applied.

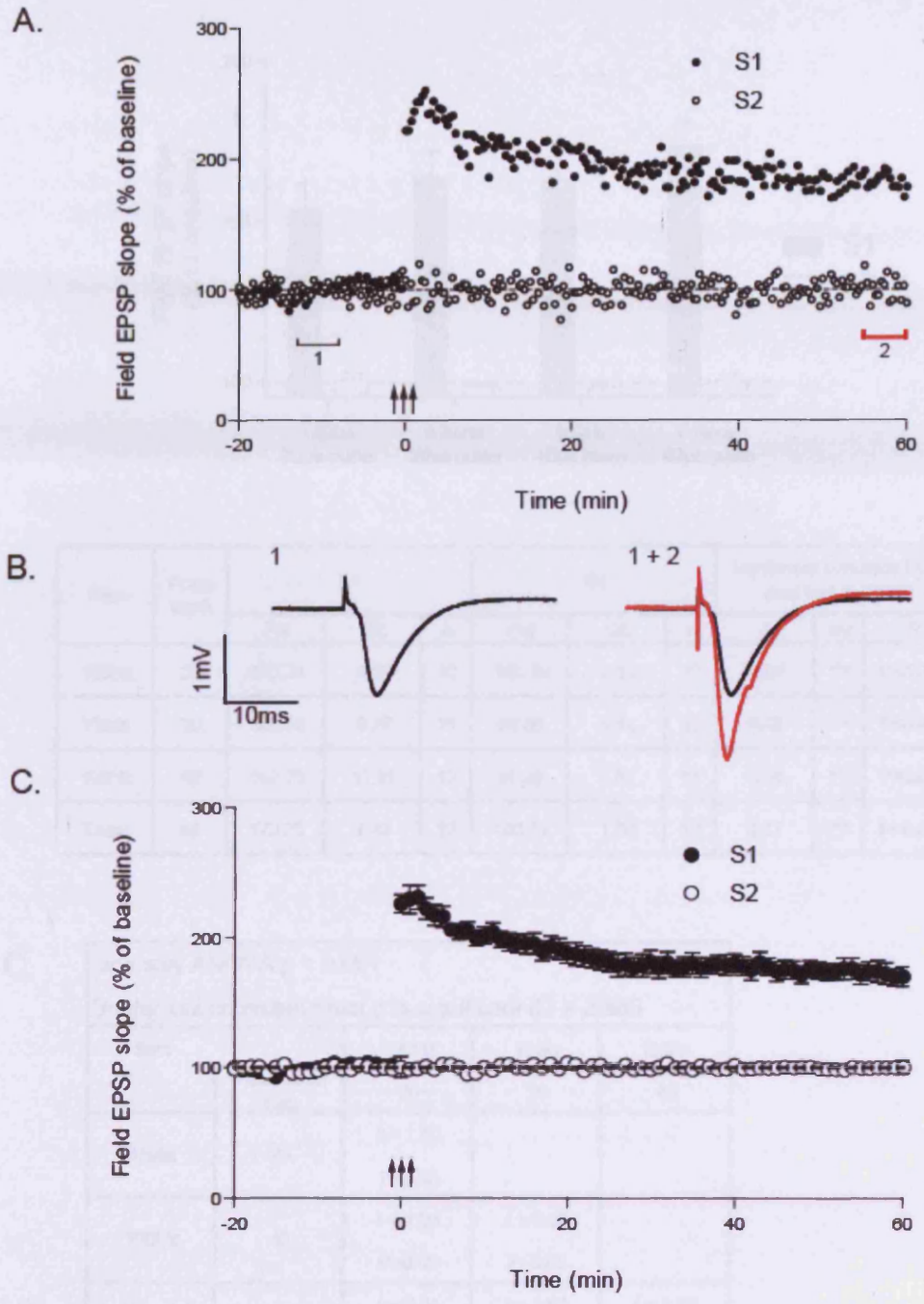
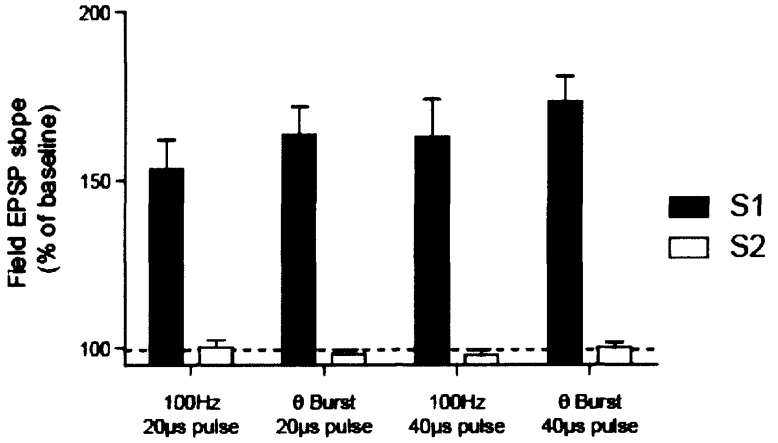


Figure 3.5 The magnitude of LTP is slightly larger if the intensity of the stimulation during the theta burst is increased.
A, Example experiment, three trains of theta burst stimulation at double pulse intensity were given at the time indicated by the arrows B, Example traces taken at time points indicated by the bars. C, Group data (n = 12), doubling the pulse width during theta burst slightly increases the magnitude of the LTP.

A.



B.

Stim	Pulse width	S1			S2			Bonferroni corrected t-test post test (t>2.885)		
		Pot	SE	n	Pot	SE	n	t	sig	P
100Hz	20	153.21	8.89	12	100.19	2.19	12	5.92	***	P<0.001
Theta	20	163.56	8.27	11	98.08	1.44	10	6.68	***	P<0.001
100Hz	40	162.75	11.31	12	97.90	1.67	11	6.94	***	P<0.001
Theta	40	173.25	7.43	12	100.11	1.60	12	8.17	***	P<0.001

C.

One way ANOVA $p < 0.001$

Bonferroni corrected t-test p is significant if $t > 2.885$

Stim	Pulse width (us)	100Hz 20	Theta 20	100Hz 40
Theta	20	t = 1.10 P>0.05		
100Hz	40	t = 1.04 P>0.05	t = 0.08 P>0.05	
Theta	40	t = 2.24 P>0.05	t = 1.03 P>0.05	t = 1.15 P>0.05

Figure 3.6 Summary data showing the magnitude of the LTP at 60 minutes after the tetanus. A, bars Indicate the magnitude of the LTP in the potentiated S1 pathway and the un-potentiated S2 pathway, after 100Hz and theta burst stimulation at both high and low intensity. B, Table showing the raw data. C, Results from the ANOVA reveal no effect of either protocol or intensity.

2.2.4 Two pathway LTP

The above set of experiments show that HFS at both high and low intensities, and theta burst stimulation at low intensities, gave a similar magnitude of LTP (~160%). However high intensity TBS seems to produce a slightly larger potentiation (not a significant increase). To establish that there was indeed a difference between TBS and HFS at the high intensities, a 2 pathway protocol was used. LTP was induced in the S1 pathway with one protocol, and then again in the S2 pathways using a different protocol, allowing a within slice comparison to be made in each experiment. Figure 3.7 shows an example experiment. LTP was first induced in the S1 pathway with a high intensity TBS at time point 0 and then again in the S2 pathway after 15 minutes (Figure 3.8 shows the group data). There was no significant difference between the magnitude of LTP in the S1 pathway (177 ± 13%, n = 12) compared to the S2 pathway (169 ± 14 %, n = 12) 60 minutes post tetanus using paired statistics (paired t-test $t_{(11)} = 0.45$, $p > 0.05$,). In a separate experiment TBS was again given to the S1 pathway, but this time a HFS was given to the S2 pathway after 15 minutes (Figure 3.9 and 3.10). The S1 pathway (TBS) produced a LTP at 60 min post tetanus of 174 ± 8 % (n = 8), while in the S2 pathway (HFS) the LTP was only 158 ± 12% (n = 8). This difference in the magnitude of LTP was highly significant ($t_{(7)} = 3.54$ $p = 0.001$). To confirm that the difference was not just due to the second pathway showing slightly lower levels of LTP irrespective of protocol, six experiments were performed in reverse, with the S1 pathways receiving a HFS, and the S2 pathway receiving TBS. A similar result was observed (Figure 3.10B), the level of LTP in S1 (HFS 162 ± 6%, n = 6) was significantly smaller when compared to S2 (TBS 178 ± 9% , n = 6) using a paired t test ($t_{(5)} = 1.67$, $p < 0.05$). These results indicate that a high intensity stimulus seems to only increase the magnitude of LTP when TBS is used and not using a HFS.

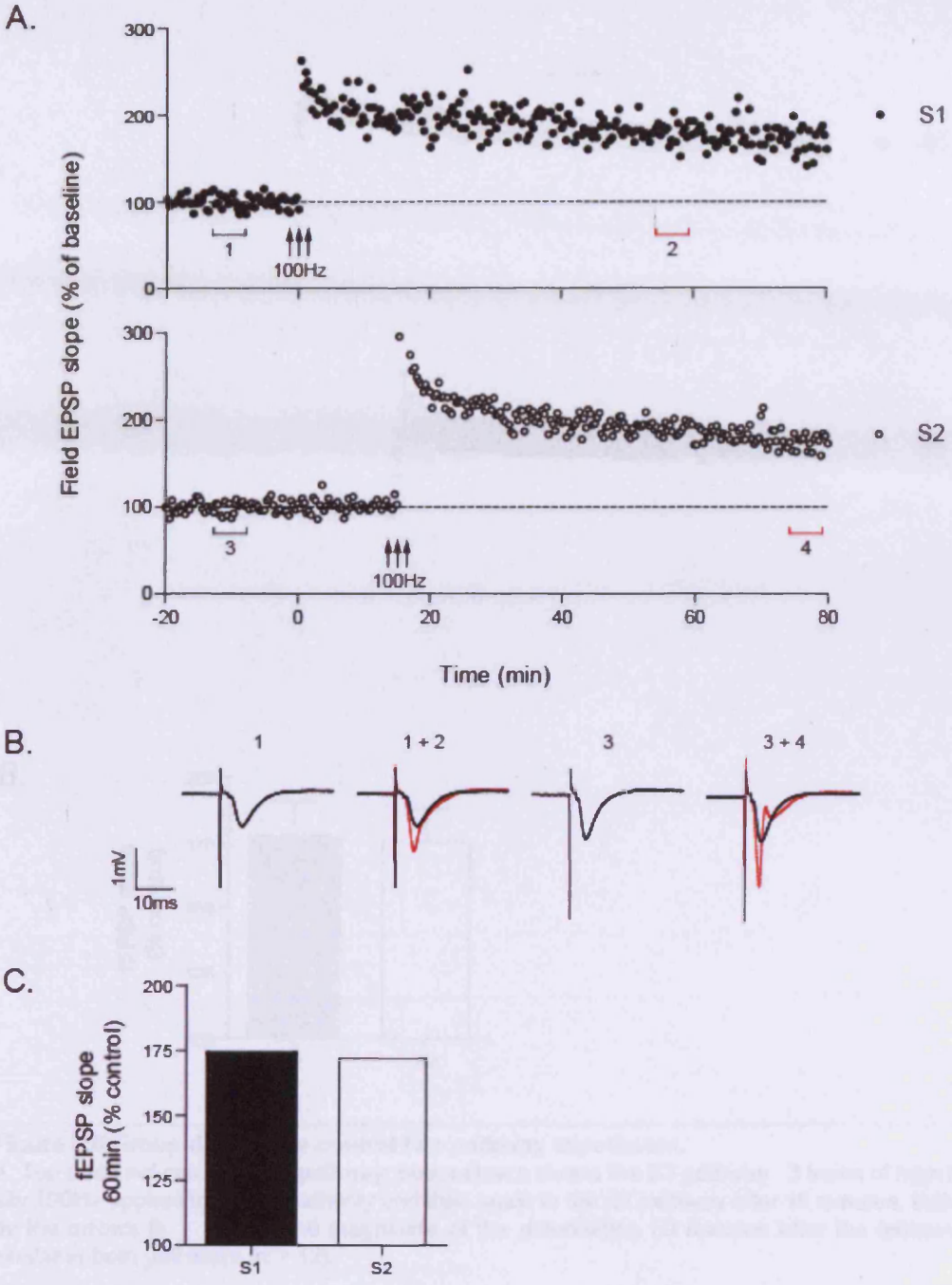


Figure 3.7 Example of a control two pathway experiment.
A, Top trace indicates the S1 pathway; bottom trace shows the S2 pathway. 3 trains of high intensity 100Hz applied to the S1 pathway and then again to the S2 pathway after 15 minutes, indicated by the arrows. B, example traces at time points indicated by the bars. C, The magnitude of the potentiation 60 minutes after the tetanus was similar in both pathways.

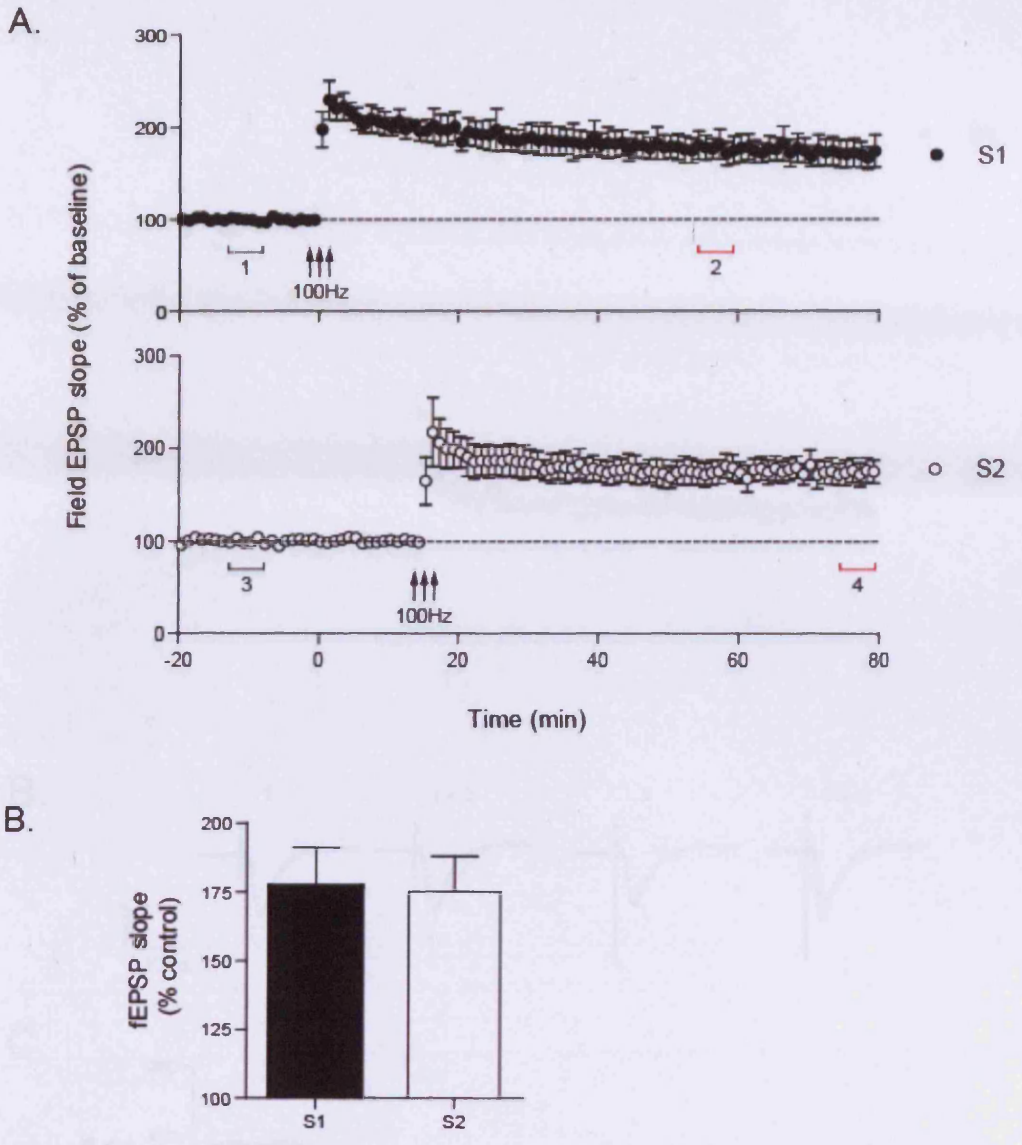


Figure 3.8 Group data of the control two pathway experiment.
A, Top trace indicates the S1 pathway; bottom trace shows the S2 pathway. 3 trains of high intensity 100Hz applied to the S1 pathway and then again to the S2 pathway after 15 minutes, indicated by the arrows (n = 12). B, The magnitude of the potentiation 60 minutes after the tetanus was similar in both pathways (n = 12).

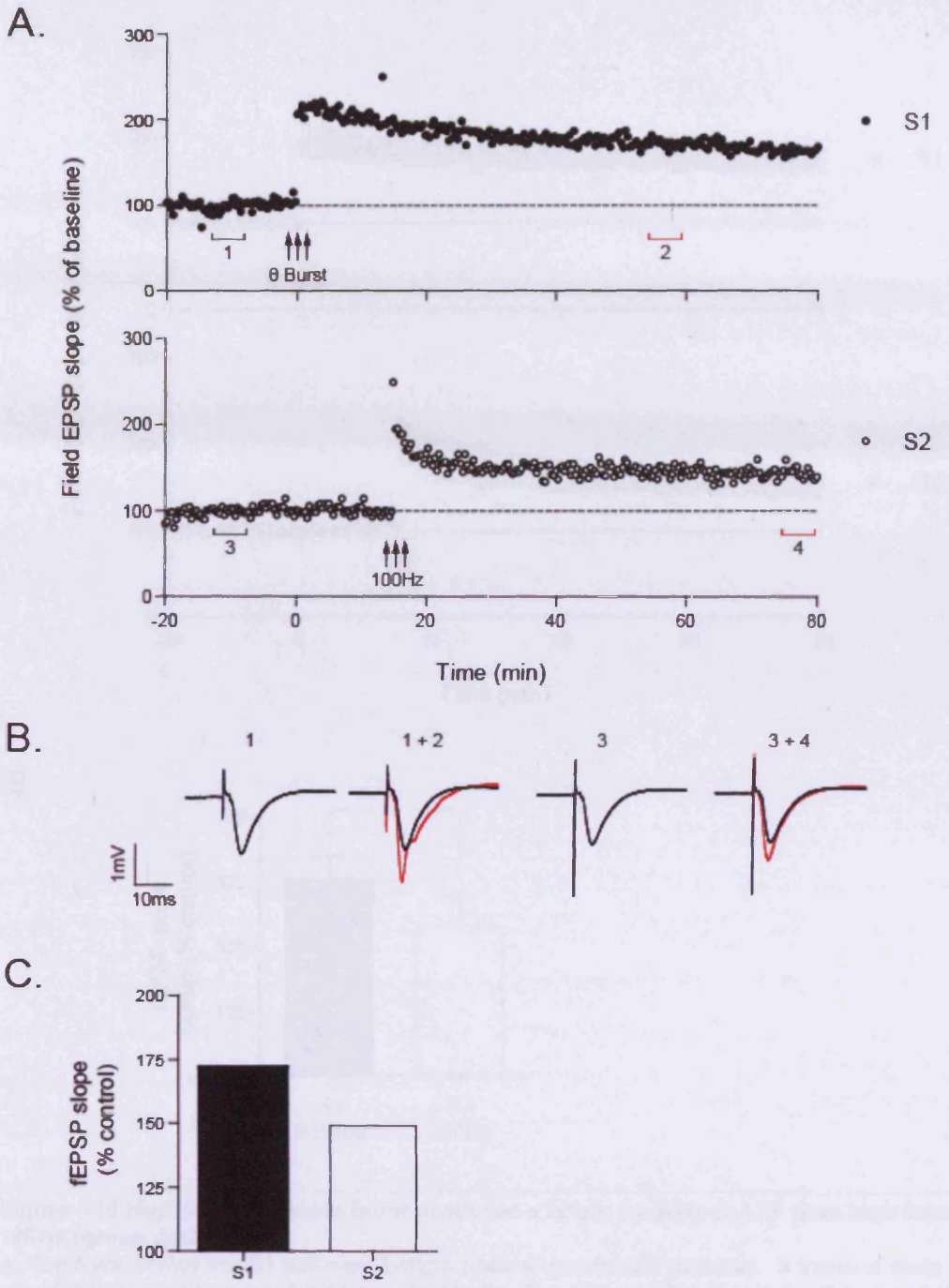
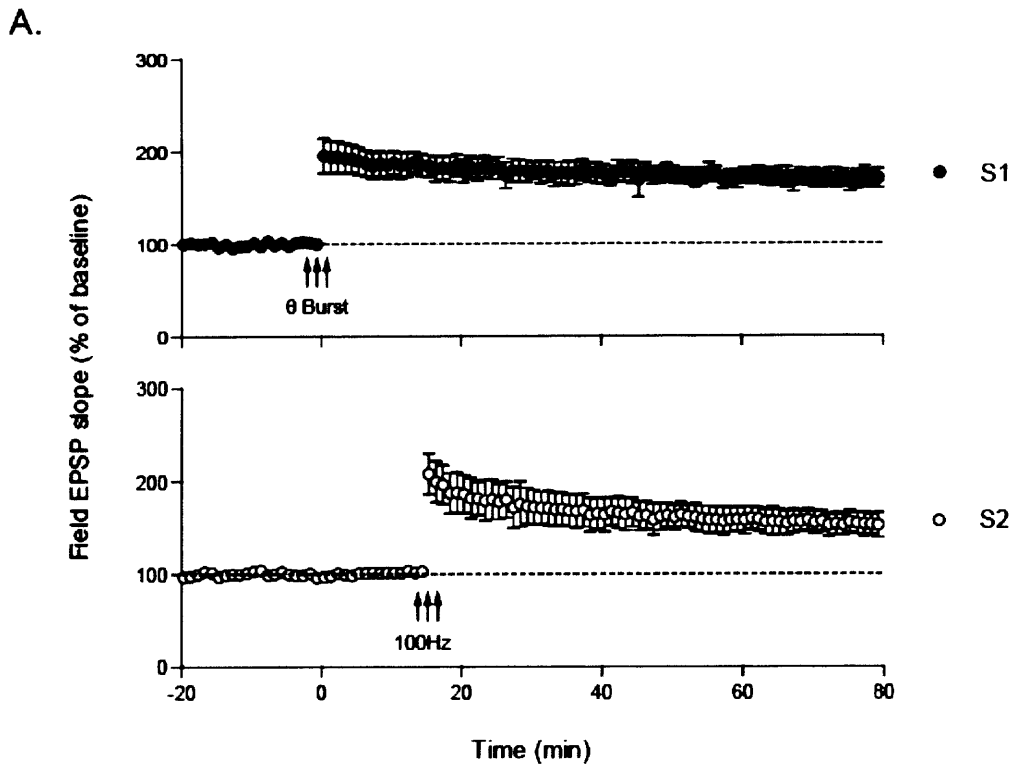


Figure 3.9 High intensity theta burst produces a larger magnitude LTP than high intensity 100Hz

A, Top trace indicates the S1 pathway; bottom trace shows the S2 pathway. 3 trains of theta burst stimulation at double pulse duration applied to the S1 pathway, then after 15 more minutes 3 trains of 100Hz stimulation at double pulse duration applied to the S2, at times indicated by the arrows. B, example traces at time points indicated by the bars. C, the magnitude of the potentiation 60 minutes after the tetanus was larger after theta burst stimulation when compared to 100Hz stimulation.



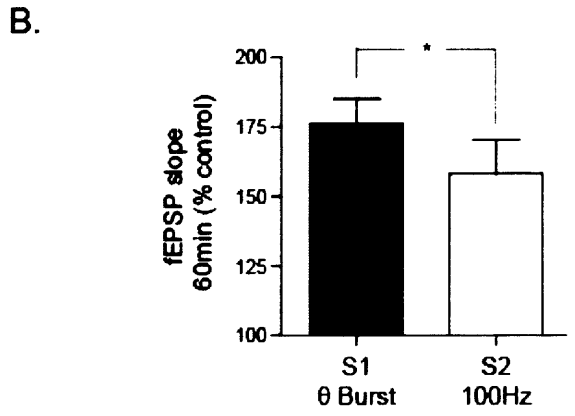


Figure 3.10 High intensity theta burst produces a larger magnitude LTP than high intensity 100Hz (group data).

A, Top trace shows the S1 pathway; bottom trace shows the S2 pathway. 3 trains of theta burst stimulation at double pulse duration applied to the S1 pathway, after 15 minutes 3 trains of 100Hz stimulation at double pulse duration applied to the S2 pathway, at times indicated by the arrows (n = 8). B, The magnitude of the potentiation 60 minutes after the tetanus was significantly larger after theta burst stimulation when compared to 100Hz stimulation (n = 8, $p < 0.05$).

2.2.5 Occlusion of LTP

The results above indicate that LTP induced by a high intensity TBS is larger than LTP induced by high intensity stimulation at 100Hz. This raised the question: "is the expression mechanism of LTP different between these two protocols"? To address this issue an experiment was performed to identify if these two forms of LTP can occlude each other. LTP was first induced in the S1 pathway using a high intensity TBS. Once the potentiation had reached a stable level (~30 – 40 min post tetanus) the baseline was reset by reducing the stimulus intensity in order to exclude any ceiling effect. After 20 minutes of collecting a new baseline, high intensity 100Hz tetanus was then given to the same S1 pathway (Figure 3.11). The effect of 100Hz stimulation (96 ± 4%, n = 3) was not significantly different from the control S2 pathway (92 ± 6%, n = 3) (paired t-test $t_{(2)}$ = 1.59 p = 0.25) after the priming theta burst stimulation LTP. In contrast, when high intensity TBS was applied after LTP induced by stimulation at 100Hz (Figure 3.12), this stimulation still elicited a small, but significant, long-lasting synaptic potentiation. The level of potentiation was smaller (112 ± 8, n =3) compared to control experiments without the priming 100Hz (171 ± 16 % n =12) however it was significantly different from the S2 control pathway (94 ± 3% paired t-test, $t_{(2)}$=6.3 p=0.02). There was also a clear reduction in the early phase of this potentiation (which slowly increased with time). These experiments indicate that LTP induced by high intensity TBS saturates the LTP and is expressed, at least in part, via similar mechanisms to 100Hz stimulation. High intensity 100 Hz on the other hand is not saturating, as a further potentiation can be seen if TBS is applied. It is possible that this further potentiation is mediated by an independent mechanism.

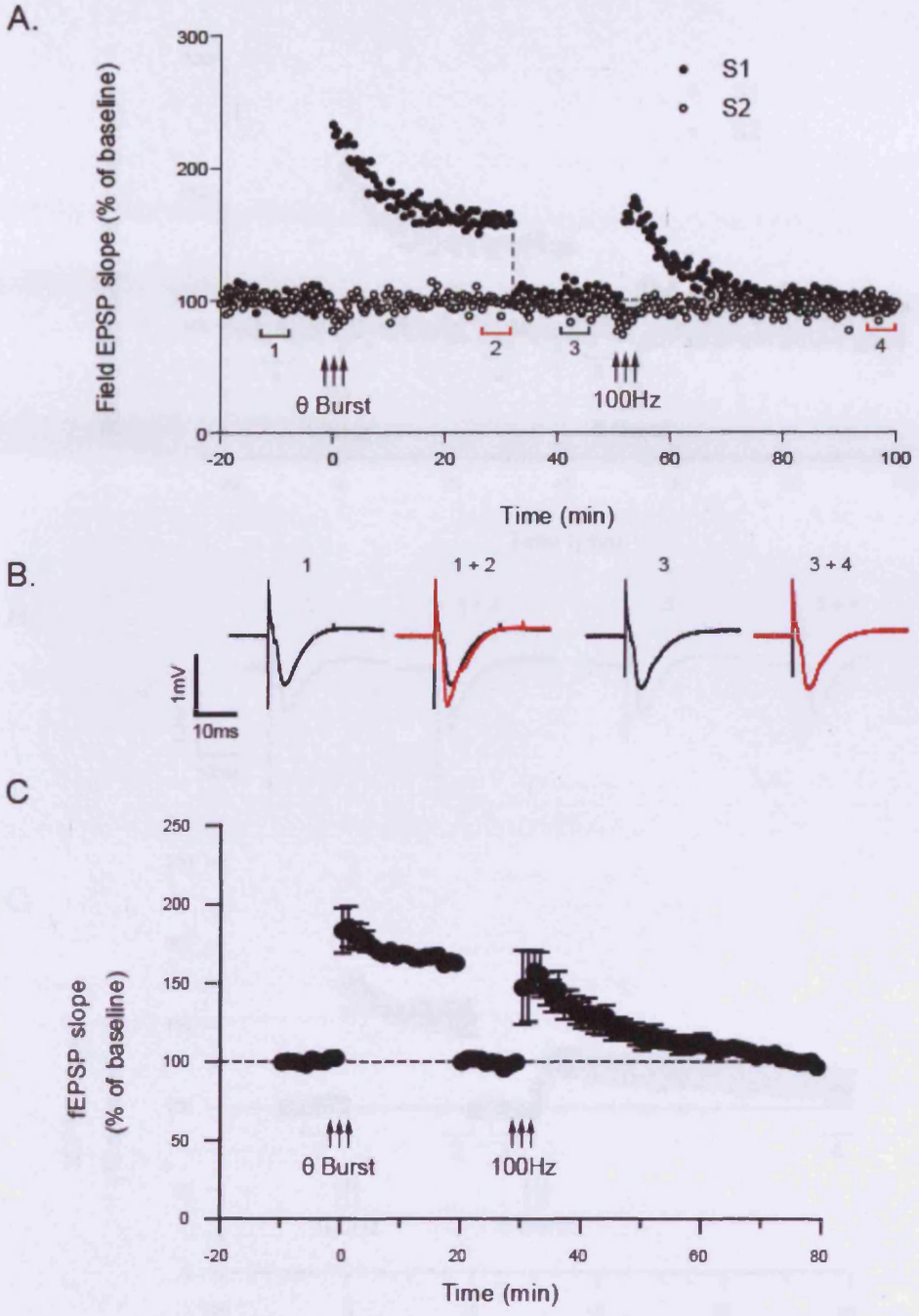


Figure 3.11 Occlusion of the LTP induced using 100Hz by a preceding high intensity theta burst.

A, 3 trains of theta burst (double pulse duration) were initially applied at time point zero, after 25 minutes the fEPSP was reset to base line by reducing the voltage of the stimulus pulse. A second control period was recorded and then 3 trains of 100Hz stimulation (double pulse) were applied to the same pathway. B, example traces at time points indicated by the bars. C, Average data shows that 100Hz LTP was fully occluded by the preceding theta burst (n = 3).

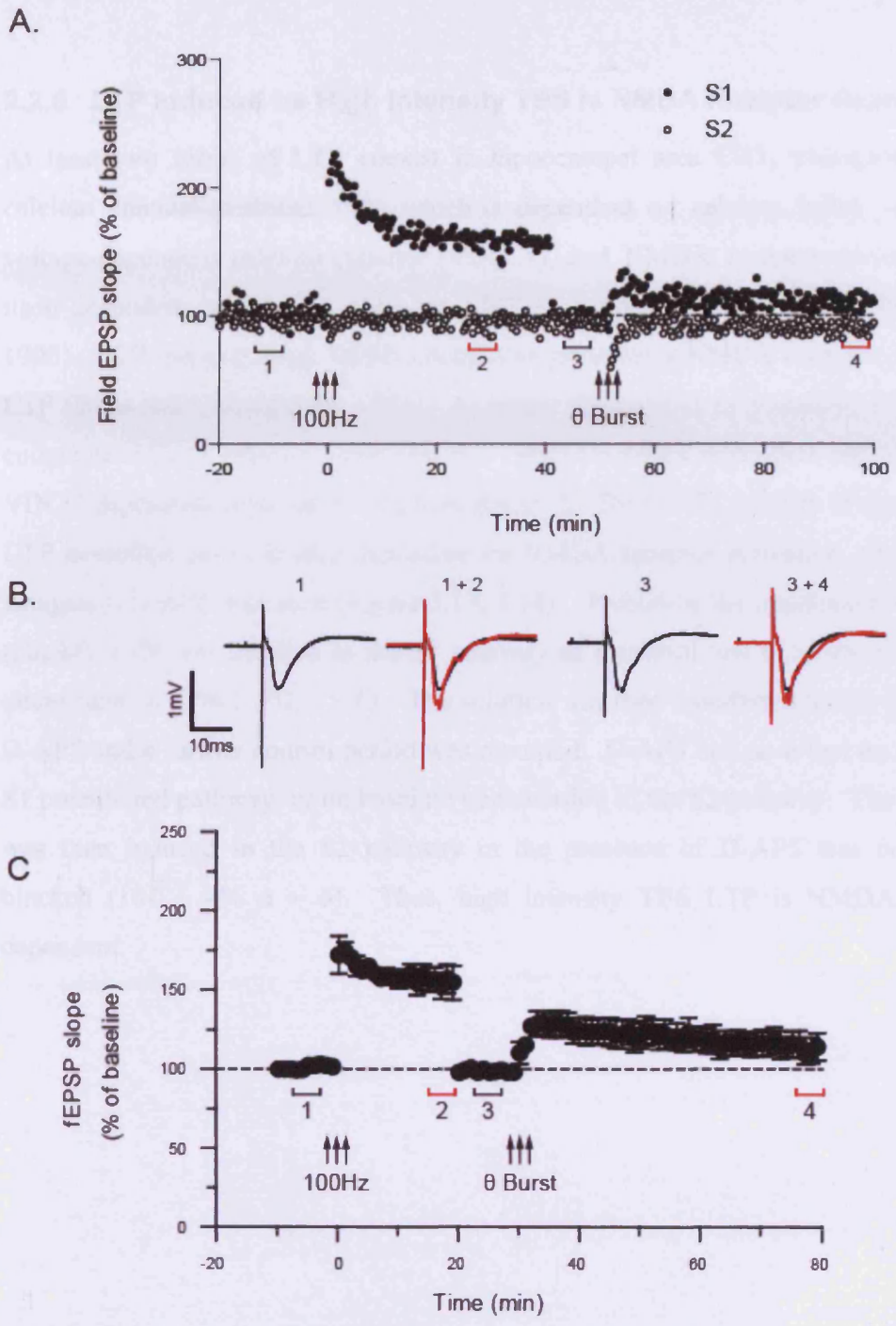


Figure 3.12 Theta burst LTP is not fully occluded by a preceding 100Hz.
A, 3 trains of 100Hz (double pulse duration) were initially applied at time point zero, after 40 minutes the fEPSP was reset to base line by reducing the voltage of the stimulus pulse. A second control period was recorded and then 3 trains of theta burst stimulation (double pulse duration) were applied to the same pathway. B, example traces at time points indicated by the bars. C, Average data shows that theta burst LTP was not fully occluded by the preceding 100Hz (n = 3).

2.2.6 LTP induced be High Intensity TBS is NMDA receptor dependent.

At least two forms of LTP coexist in hippocampal area CA1; voltage-dependent calcium channel-mediated LTP, which is dependent on calcium influx via L-type voltage-dependent calcium channel (VDCCs), and NMDA receptor-mediated LTP, itself dependent on calcium influx via NMDA receptor channels (Grover and Teyler, 1990). LTP induced using 100Hz stimulation produces a NMDA receptor dependent LTP (Bliss and Collingridge, 1993). At higher frequencies of stimulation (200Hz) a compound LTP is induced, consisting of a NMDA receptor dependent component and VDCC dependent component (Zakharenko et al., 2003). To identify if the maximal LTP described above is also dependent on NMDA receptor activation, the selective antagonist D-AP5 was used (Figure 3.13, 3.14). Preceding the application of D-AP5 (50μM), LTP was induced in the S1 pathway as a control and to allow within slice quantification (176 ± 5%, n = 6). The solution was then transferred to one containing D-AP5 and a further control period was recorded. D-AP5 had no effect on either the S1 potentiated pathway, or on baseline transmission in the S2 pathway. The LTP that was then induced in the S2 pathway in the presence of D-AP5 was completely blocked (104 ± 4% n = 6). Thus, high intensity TBS LTP is NMDA receptor dependent.

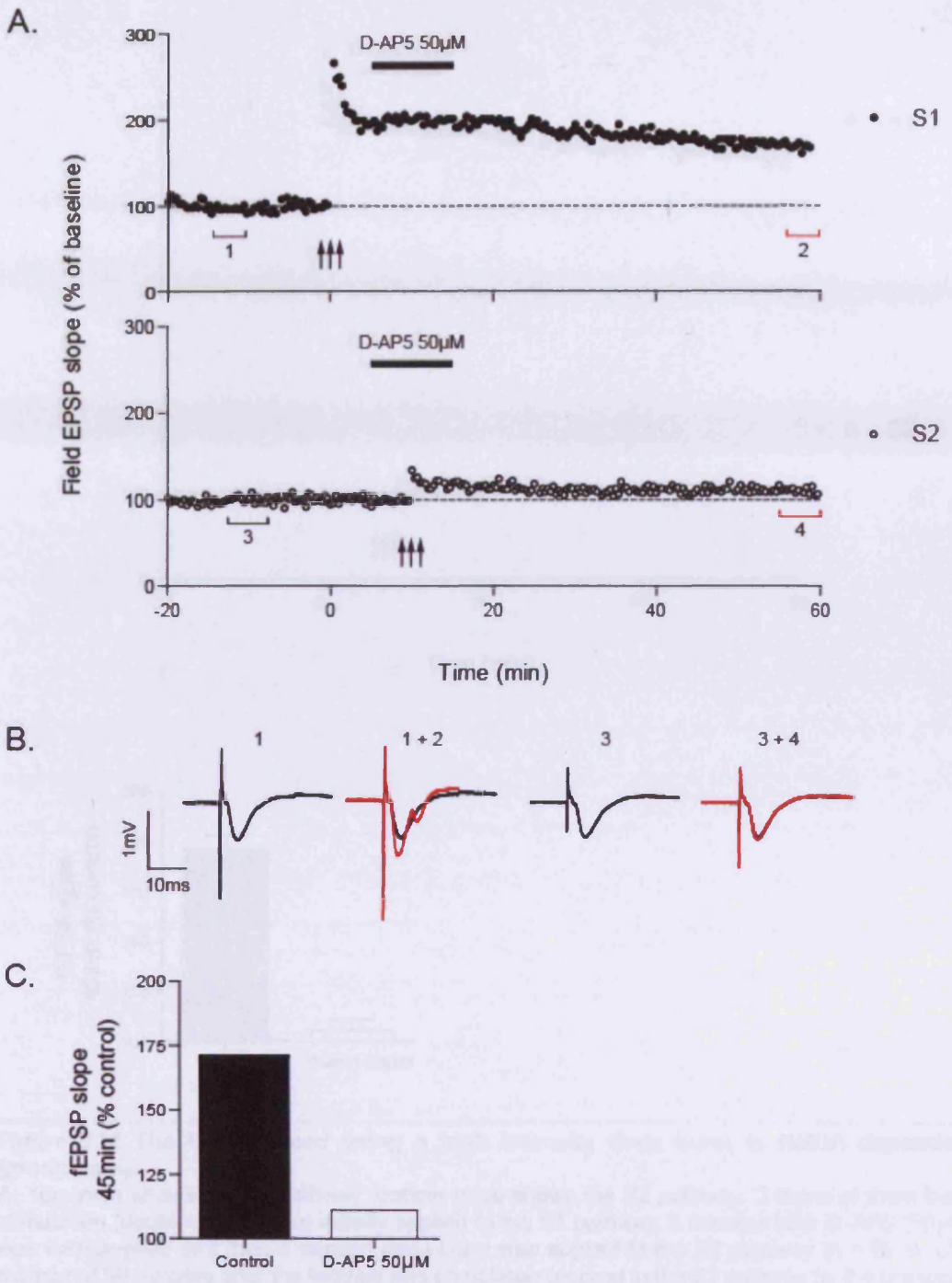
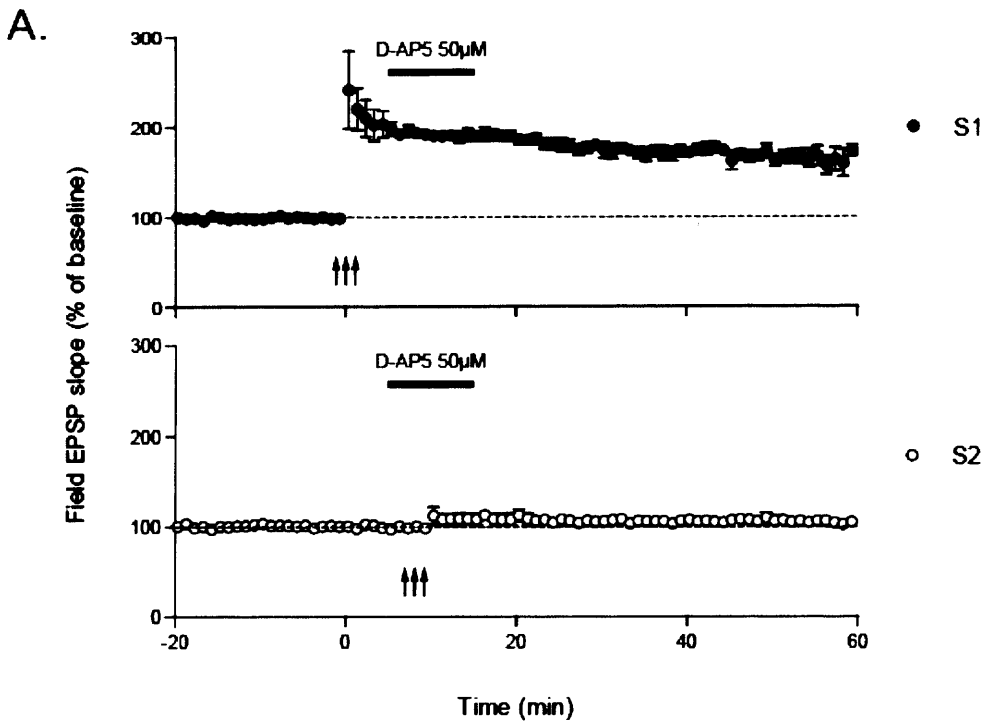


Figure 3.13 The LTP induced using a high intensity theta burst is NMDA receptor dependent (example experiment).
A, Top trace shows the S1 pathway; bottom trace shows the S2 pathway. 3 trains of theta burst stimulation (double pulse duration) were initially applied to the S1 pathway, 5 minutes later D-AP5 (50μM) was bath applied and then a second theta burst was applied to the S2 pathway. B, example traces at time points indicated by the bars. C, LTP measured 60 minutes after the tetanus was completely blocked in the S2 pathway in the presence of D-AP5.



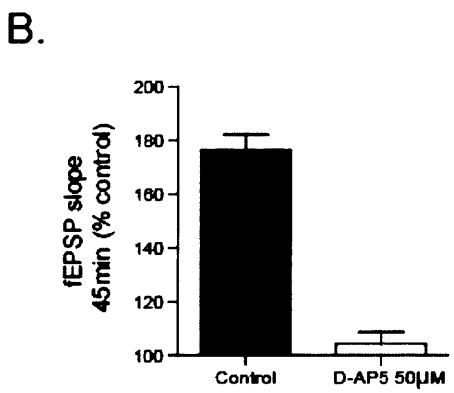


Figure 3.14 The LTP induced using a high intensity theta burst is NMDA dependent (group data).
A, Top trace shows the S1 pathway; bottom trace shows the S2 pathway. 3 trains of theta burst stimulation (double pulse) were initially applied to the S1 pathway, 5 minutes later D-AP5 (50μM) was bath applied and then a second theta burst was applied to the S2 pathway (n = 6). B, LTP measured 60 minutes after the tetanus was completely blocked in the S2 pathway by the presence of D-AP5 (n = 6).

2.3 Discussion

The aim of this introductory experimental chapter was to characterise the LTP protocols that I have used throughout my thesis, and specifically to identify if any differences can be observed between them. I used two standard protocols to induce LTP at the *Schaffer collateral* CA1 synapse; theta burst and also 100Hz stimulation. I went on to identify if the strength of stimulation used has any effect on the time course, basic pharmacology or magnitude of the potentiation induced.

The main findings from this chapter are that three trains of 100Hz stimulation, given at test intensity (40% of max), produce an LTP that could not be further potentiated, even if the 100Hz stimulation was repeated at a stronger intensity (double pulse width). The magnitude of this potentiation was similar to the magnitude of the LTP induced using three trains of theta burst stimulation if it was applied at the test intensity. However, a larger magnitude (NMDA receptor dependent , CAMKII sensitive) LTP could be induced if theta burst stimulation was given at a higher intensity. This larger magnitude LTP seems to be expressed, at least in part, through an independent mechanism as it was reduced, but not fully occluded, by a preceding 100 Hz stimulation.

HFS (1sec at 100Hz) and theta burst stimulation (4 at 100Hz, repeated 10 at 5Hz) are probably the two of the most common LTP induction protocols used in fEPSP recording in the hippocampus. However, the intensity that they are given at, and the number of times they are repeated, varies considerably depending on the question the experimenter is asking. For example in experiments looking at protein synthesis in late-LTP, the standard HFS has to be repeated 3 or 4 times to induce this long lasting potentiation. On the other hand in experiments looking at signalling cascades directly downstream of Ca^{2+} entry, one train of HFS may be sufficient. It is also probably true that the choice of protocols favoured by a particular laboratory relies more on tradition than any clearly demonstrated superiority. The factor influencing my decision on the number of pulses given during a burst, and the number of times these bursts were repeated, stemmed from my ultimate goal of being able to identify

LTP in the GluR1 knockout. Therefore, to give me every chance of achieving this, I wanted to produce a maximal form of LTP. Another important aspect was to use a "standard" protocol so that any results obtained could be directly compared to other published work.

The observation that a 1 sec 100Hz burst repeated 3 times produces a saturating LTP is widely accepted, and hence the observation that increasing the stimulus intensity does not affect the magnitude further is not surprising. In a parametric study of induction protocols in which theta burst stimulation was compared with 100Hz stimulation, it was concluded that the major factor controlling the magnitude of LTP was the number of stimuli in a train, rather than the pattern of stimulation (Hernandez et al., 2005). My results are then somewhat surprising, as a similar level of LTP was produced during theta burst stimulation compared to 100Hz, even though the theta burst protocol has almost a third fewer pulses during the burst (theta 120 pulses, 100Hz 300 pulses). This discrepancy can be explained by my observation that a larger magnitude LTP can be produced if the intensity of the pulse[1] during the theta burst stimulation is increased. Therefore, if more pulses are given it is likely that later pulses in the train would become progressively enhanced due to a post tetanic potentiation (PTP) from the preceding pulse. Effectively, the later pulses in a train are given at a "higher intensity" and therefore a larger magnitude LTP is observed. This effect would be particularly pronounced in the Hernandez study during the higher pulse numbers tested (200 – 300). These were given in trains of 100 pulses every 10sec, and since a single train is able to produce a large PTP independently, then even more enhancement of the later pulses would occur in the second or third train. These

[1] Intensity in some studies is taken to mean the repetition rate and not the pulse intensity as is the case here.

results show that it is not just the number of pulses, but also the intensity that they are given at during the theta burst, that determines the magnitude of LTP.

Theta burst stimulation is suggested to be optimal in producing LTP in the hippocampus (Bayazitov et al., 2007; Larson and Lynch, 1989; Larson et al., 1986; Staubli et al., 1999) and the reason for this effect has been clarified (Davies et al., 1991). The initial trains activate feed forward, GABA interneurons, leading to $GABA_A$ and $GABA_B$ mediated hyperpolarisation in the pyramidal cell, but importantly also to activation of presynaptic $GABA_B$ auto receptors. The latter produce a transient reduction in GABA release that is maximal at around 100-200ms. Thus the second train produces much less GABA mediated hyperpolarisation, with consequent enhancement of the voltage dependent NMDA receptor mediated current. Minimal patterns of stimulation of this kind are far more likely to occur naturally than the longer trains of hundreds of stimuli during 100Hz stimulation. The high intensity protocols are also likely to be relevant *in vivo*, as the fEPSP recorded in the slices were only ~ 1mV in amplitude. Since fEPSP of over 3.5mV have been recorded in the CA1 region of freely moving animals (Buzsaki et al., 1987) this "high intensity" might not be that high in the intact animal.

The larger magnitude LTP induced by high intensity theta burst stimulation, is expressed at least in part via some common mechanism to the LTP induced using 100Hz as, although theta burst LTP was not full occluded by 100Hz stimulation, it was significantly reduced. It also shares a common induction mechanism, as both the 100Hz and high intensity theta burst LTP, was NMDA receptor dependent and required the activation of CAMKII. However, the residual potentiation in the occlusion experiment implies that ~30% of the LTP induced using a high intensity theta burst, is expressed via an independent mechanism to the LTP induced using either low intensity theta burst, or high intensity 100Hz stimulation. Consistent with these studies, a compound LTP, induced with a theta burst protocol with two distinct expression mechanisms, has also recently been described (Bayazitov et al., 2007). By using a transgenic mouse strain that expresses a pH sensitive fluorescent VAMP2 marker in neurons, pre-synaptic activity and plasticity could be monitored directly.

The presynaptic component of LTP was only induced using the theta burst stimulation, and not by 100Hz stimulation. This component of LTP however did not require NMDA receptor activation (Bayazitov *et al.*, 2007).

In conclusion, I have shown that LTP can be reliably recorded in my experimental set up using two standard induction protocols. I have also shown that if a high intensity stimulus is used during the theta burst protocol, the LTP induced is expressed via two seemingly different mechanisms, but with common induction mechanisms. The LTP induced by 100Hz stimulation, however, is unaffected by the intensity that the stimulus is applied at. The reasons for this and its importance will be considered further in the next chapter.

3 LTP in GluR1 knockout mice

3.1 Introduction

The mechanisms involved in NMDA receptor dependent LTP have been debated over the last 20 years and, most controversially, whether changes occur pre or post-synaptically. The most commonly cited changes that could occur post-synaptically are the trafficking of new AMPA receptors into the membrane, increasing the single channel conductance of AMPA receptors, or increasing the sensitivity of the receptor to glutamate. Pre-synaptic changes would involve alteration of the mode or probability of vesicle release, via some retrograde message.

In the early 90s two papers were published that seemed to provide strong evidence that the expression of NMDA receptor dependent LTP in the hippocampus was expressed presynaptically (Bekkers and Stevens, 1990; Malinow and Tsien, 1990). Both these authors found that LTP was associated with a decrease in the coefficient of variation (CV). The basis of CV analysis is that changes in CV, or more specifically $(CV)^{-2}$, are independent of quantal size (q), and only depend on changes in presynaptic factors n (number of release sites) and p (probability of release). Admittedly, q may be dependent on both pre- and postsynaptic factors, for example the number of molecules released by a specific vesicle and the availability of postsynaptic receptors, however it is generally treated as a postsynaptic indicator. The decrease in the CV after LTP, described by the two groups, appeared to only be possible via a presynaptic modification, and could not be explained by a change in the number, sensitivity, or conductance, of AMPA receptors. Malinow & Tsien, also showed that following LTP there was a decrease in the number of failures of transmission, which they again attributed to an increase in probability of release after LTP (Malinow and Tsien, 1991). At the time, these two studies appeared to provide convincing evidence for a presynaptic expression mechanism of LTP.

One of the requirements for postsynaptic induction of LTP but presynaptic expression, was the need for a retrograde message to travel from the conditioned postsynaptic cell to modify presynaptic function. These two papers led to an

explosion of publications trying to identify and characterize the retrograde messenger. As discussed in the introduction, NO was the most popular candidate, and led to over 100 publications in the 90s alone. However, in 1994 Kullmann suggested an alternative postsynaptic explanation for the results described by Malinow and Tsien (Kullmann, 1994). The decrease in failures could be explained if a population of synapses lacked functional AMPA receptors, while having the normal complement of NMDA receptors, and that during LTP these synapses would acquire AMPA receptor function.

A series of experiments were performed to test more directly the existence of these "silent synapses" (Isaac et al., 1995). Using minimal stimulation techniques, Isaac et al. found a population of synapses that contained no detectable AMPA component, but had a normal NMDA component. Furthermore, an LTP-inducing protocol rapidly switched on these silent synapses via AMPA receptor insertion. Liao *et al.* (1995) obtained virtually identical results. A number of studies then went on to identify that these "silent synapses" were actually missing AMPA receptors, and that they were inserted into the synapse in an activity dependent fashion (Isaac, 2003). This process of activity dependent trafficking of AMPA receptors has received a lot of attention and has lead to the development of a model where, after LTP induction, GluR1 containing AMPA receptors are phosphorylated via a number of different kinases tagging them for delivery into the synapse (see Introduction for detailed discussion).

Further evidence for the above model and in particular highlighting the importance of the GluR1 subunit, was a study by Zamanillo et al., (1999). This group managed to produce a knockout mouse that lacked the GluR1 subunit. These GluR1 knockout mice could not produce any LTP, suggesting this subunit is critical for plasticity. In a follow up study they showed that this deficit in LTP could be rescued if GFP-GluR1 was genetically inserted into the knockout mice with a viral vector. This result was confirmed by Jensen et al, who showed that while in juvenile GluR1 knockout mice a small amount of LTP is evident, LTP is absent in the adult animal (Jensen et al., 2003). The results from these knockout studies, and from the evidence of all the receptor trafficking studies, left the possibility of a presynaptic expression mechanism

unlikely. This was confounded by the lack of any agreement in the literature on NO, with some labs reporting totally opposite findings (see later discussion). Due to this, research into NO's involvement in LTP stalled, without any real agreement of results.

Several lines of evidence however do not fit with a purely post synaptic expression mechanism of LTP. Although it is clear that AMPA channels are added to the synapse during LTP (Hayashi et al., 2000; Petralia et al., 1999), you do not see the corresponding increase in quantal size (the standard postsynaptic indicator) that would be expected if this was the only mechanism occurring (Sokolov et al., 2002). This group, among others, have also reported decreases in failures and also increases in both probability of release and quantal content in adult animals. As discussed above, this could be explained via postsynaptic mechanisms if silent synapses are present. However, although silent synapses have been shown to be common in young animals, their frequency is known to decrease with development, and in adult animals they are thought to be rare (Petralia et al., 1999). This suggests that in adult animals at least there must be presynaptic changes occurring after LTP. Evidence for an increase in presynaptic function has come from the use of FM dyes. These dyes can be loaded into vesicles, and are then released from the presynaptic terminal when the vesicles fuse with the presynaptic membrane. Zakharenko et al., (2001) used this technique and showed that there was enhanced transmitter release after LTP induced by TBS or multiple 200Hz tetani, but not after a single 100Hz tetanus. This increase in transmitter release developed slowly, being apparent at 30min after the tetanus and larger at 1 hour. These results not only implicate presynaptic changes in LTP, but importantly also suggest that these changes may be dependent on the type of protocol used to induce LTP.

One of the lines of evidence that has been cited repeatedly for a wholly postsynaptic expression mechanism in LTP, has been the GluR1 knockout studies mentioned above. These studies all used the standard 100Hz induction protocol to induce LTP. However, would the same result have been observed if a different protocol had been used? Hoffman et al., (2002) looked at this by inducing LTP in the GluR1 knockout animals using a spike timing theta burst pairing protocol. This protocol uses

extracellularly stimulated sub threshold EPSPs paired with somatic action potential, generated by current injection at the soma, repeated at the theta burst stimulation frequency. Using this protocol Hoffman et al., showed that LTP could be induced in the GluR1 knockout animals and that this LTP developed slowly over time, reminiscent of the FM dyes study discussed above. Hoffman et al., made no attempt to identify the locus of this GluR1 independent LTP, but it is tempting to hypothesize, based on the FM dye results, that this LTP is expressed presynaptically.

One of the reasons the pre versus post debate has lasted so long, is that many laboratories have tried to show that one model excludes the other, and have not entertained the possibility that it could be a combination of both. In a recent paper Lisman and Raghavachari, (2006) have tried to reconcile the different findings from seemingly contradictory studies by linking both pre and postsynaptic changes into one "unifying model" of LTP. In support of this argument, Hardingham et al, suggested that the LTP in layer II/III of the barrel cortex was dependent on a combination of presynaptic and postsynaptic mechanisms (Hardingham et al., 2007). The two components of the LTP could be separated by manipulating GluR1 and NO synthase. While the GluR1 subunit was responsible for the post-synaptic component of LTP in the neocortex, the presynaptic component was dependent on postsynaptic NO synthase.

Using the GluR1 knockout mice as tools to dissect out the pre and postsynaptic components of LTP in the CA1 region of the hippocampus could be a very useful tool to try to clarify the confusion in the NO literature. One possible confounding factor in the results could be that the different protocols and different experimental conditions used may have changed the balance of presynaptic versus postsynaptic expression, and hence the level of NO dependence. However, before this is possible it was important to identify if a GluR1 independent LTP could be expressed using a "standard" protocol in field recording experiments. The aim of this chapter is to identify if a GluR1 independent LTP can be produced using extracellular field recording, using one of the four different standard protocols discussed in the previous chapter.

3.2 Results

3.2.1 Baseline transmission is unaffected in the GluR1 knockout mouse.

No differences were observed in the synaptically evoked fEPSPs in slices taken from wild-type mice (15 animals, n = 34) or GluR1 knockout mice (14 animals, n = 36). The extracellular recorded fEPSP had the same wave shape, and there was no significant difference in the input output response (Figure 4.1A). A two way ANOVA for intensity and genotype indicate that there was no effect of genotype ($F_{(6,294)} = 1.68$, $p = 0.2$) or any interaction of genotype with intensity ($F_{(6,294)} = 1.29$, $p = 0.3$). At the higher intensities there appears to be a slight reduction in the size of the fEPSP. However, if a standard t-test is used to look at the largest difference there is still no significant difference between the wild-type and GluR1 knockout animals ($t_{(68)} = 1.4$, $p = 0.2$). The fEPSP could be blocked by the presence of the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM) , in slices from wild-type ($n = 3$) as well as GluR1 knock-out mice ($n = 2$), which demonstrates that there is still AMPA receptor-mediated neurotransmission in the absence of GluR1.

3.2.2 Presynaptic function is unaffected in the GluR1 knockout mouse.

Short-term synaptic plasticity was investigated to identify if any presynaptic compensation had occurred due to the lack of GluR1 in the knockout animals. Changes in paired pulse facilitation in extracellular recordings have previously been shown to be affected in animals that have deficits in presynaptic function (Huang et al., 2005). Paired-pulse facilitation, at all inter stimulus intervals tested (20–300 ms), was unaffected in the GluR1 knock out animals (3 animals, $n = 4$, Figure 4.1B,C) compared with wild-type animals (3 animals, n = 4). A two way ANOVA for paired pulse interval and genotype indicate that there was no effect of genotype ($F_{(1,90)} = 0.53$, $p = 0.46$) or any interaction of genotype with paired pulse interval ($F_{(14,90)} = 0.37$, $p = 0.98$). For example, at an inter stimulus interval of 60 ms the paired pulse facilitation ratios were 1.48 ± 0.09 and 1.45 ± 0.1, respectively (simple t-test ($t_{(6)} = 0.22$, $p = 0.8$).

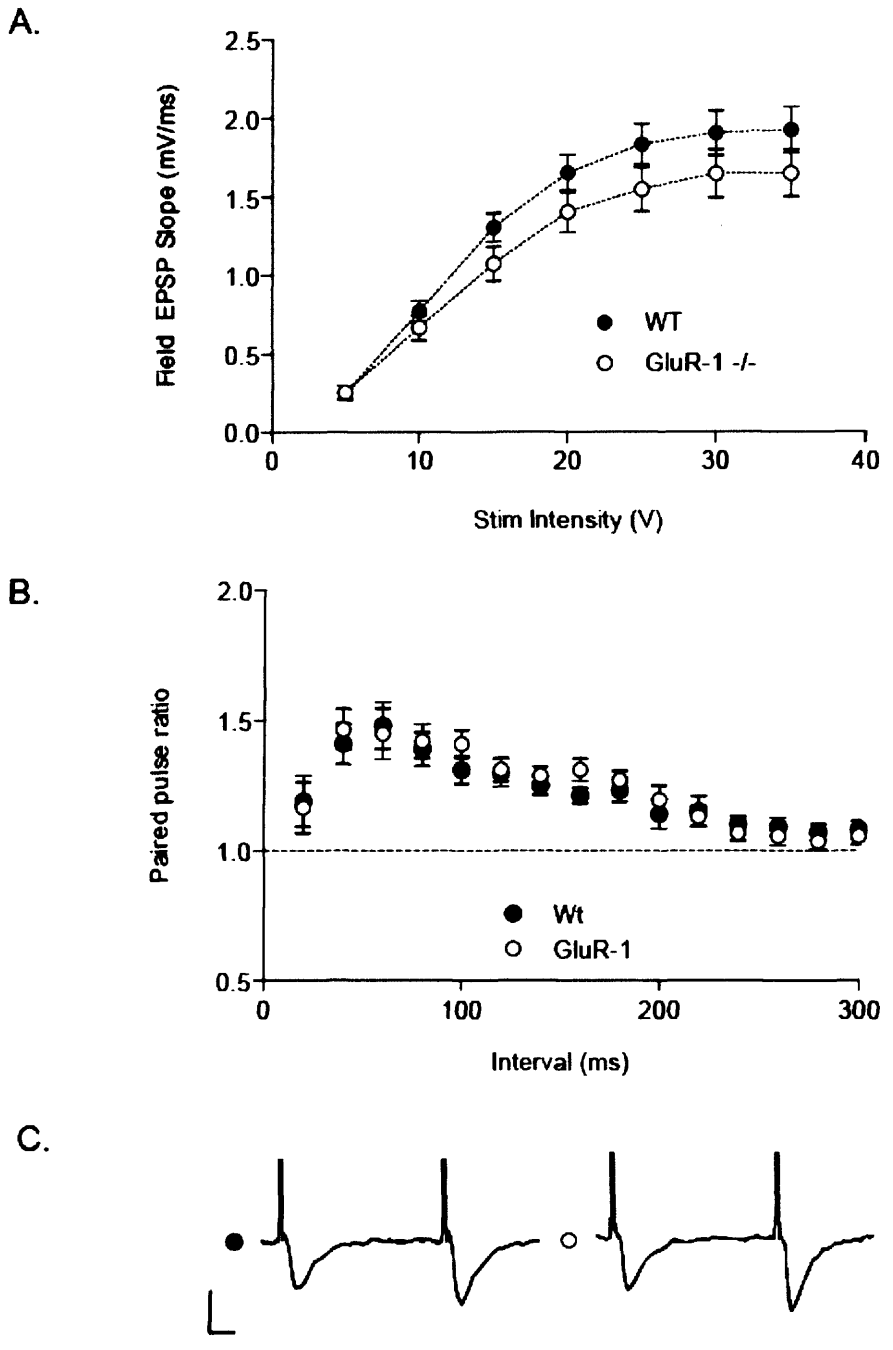


Figure 4.1 Basal synaptic transmission and presynaptic function are unaffected in the GluR1 knockouts.

A, Input output curves for wild-type (•) and GluR-1-/- (○) mice show no difference in baseline transmission ($p > 0.05$). B, Paired pulse facilitation in the WT (•) is identical to that observed in the GluR-1-/- (○) mice ($p > 0.05$) C, Example traces showing the facilitation of the fEPSP at a inter pulse interval of 70 ms in the wild-type (left) and GluR-1-/- mice (○). Scale bar 1mV, 1ms.

3.2.3 GluR1 Independent LTP is dependent on the induction protocol used.

In agreement with previously published data (Zamanillo et al., 1999) three trains of 100Hz stimulation at test pulse width failed to produce any LTP in the GluR1 knockout animals (6 animals, n = 10, Figure 4.2). The normalised slope of the fEPSP in the test S1 pathway, measured 60 minutes post tetanus (96 ± 4 %), was not significantly different from the control (S2) un tetanised pathway (95 ± 2 %) (Bonferroni corrected paired t-test ($t_{(10)}$ = 1.45, p = 0.2). One might argue that this deficit was due to inefficient tetanisation in the GluR1 knockout animals, although this is unlikely, as there was no significant difference in the input/output response (see above). To rule out this possibility, the experiment was repeated with a higher intensity stimulation than that used during the three 100Hz bursts. This was achieved by doubling the pulse width from 20μs to 40μs. As described in chapter 3, doubling the pulse width had no effect on the level of LTP induced in wild-type animals (153 ± 9 %, 162 ± 11% respectively). Unsurprisingly this was also the case in the GluR1 knockout animals, where doubling the pulse width still failed to produce any LTP (7 animals, n = 10, Figure 4.4). The normalized slope of the fEPSP in the test S1 pathway (101 ± 4 %) was not significantly different from the control (S2) untetanised pathway (103 ± 3%) (Bonferroni corrected paired t-test ($t_{(9)}$ = 0.192, p = 0.8) . Thus the standard 100Hz stimulation at both a high and low intensity failed to produce any LTP in the GluR1 knockout animals. To identify if this was also the case for other "standard" extracellular LTP protocols, the theta burst stimulation protocol (described in chapter 3) was also used in these animals.

TBS applied at test pulse width also failed to produce a significant LTP in the GluR1 knock out animals (7 animals, n = 11, Figure 4.3). There was no significant difference between the S1 (98 ± 6%) and the S2 (101 ± 2 %) pathways (Bonferroni corrected paired t-test ($t_{(9)}$ = 0.6 p = 0.5). However if the TBS was given at the higher intensity double pulse width, a slowly rising form of LTP was expressed in the S1 pathway (171 ± 16 %) that was highly significant ($t_{(11)}$ = 8.1, p = < 0.001) when compared to the control S2 pathway (101 ± 1 %, Figure 4.5). The magnitude of the GluR1 independent LTP (171 ± 16%) was similar to the magnitude of LTP observed in wild-type animals (173 ± 7 %), (Bonferroni corrected t-test $t_{(23)}$ = 0.05, p >0.05)

when measured 60 minutes after the tetanus. The LTP in the knockout mice is markedly slower in its expression when compared to wild-type, this difference is clearly illustrated by subtracting the LTP in the GluR1 knockout from the LTP in wild-type (Figure 4.5C, solid line). To identify if this difference in time course was significant, the data from the two genotypes were binned into 10 minute epochs and then ran versus genotype using a two way ANOVA. There was a highly significant interaction of genotype vs time ($F_{(5,125)} = 7.2$, $p < 0.0001$) and a Bonferroni post hoc test revealed that this was due to a significant difference between wild-type and knockout mice only during the first 10 minute period after the tetanus ($t_{(26)} = 3.1$ $p < 0.05$, Figure 4.5).

This slowly developing LTP in the GluR1 knockout is similar to the LTP described by Hoffman et al. (2002) and Jensen et al., (2003). However, in this study LTP was produced in extracellular recordings using a "standard" protocol which relied on purely orthodromic stimulation and the slices were taken from adult animals (< P56). Induction of LTP was dependent not only on stimulus intensity, but also on stimulus protocol, as neither theta burst stimulation with a 10μs stimulus pulse-width, nor 100Hz stimulation with a 20μs stimulus pulse-width caused LTP.

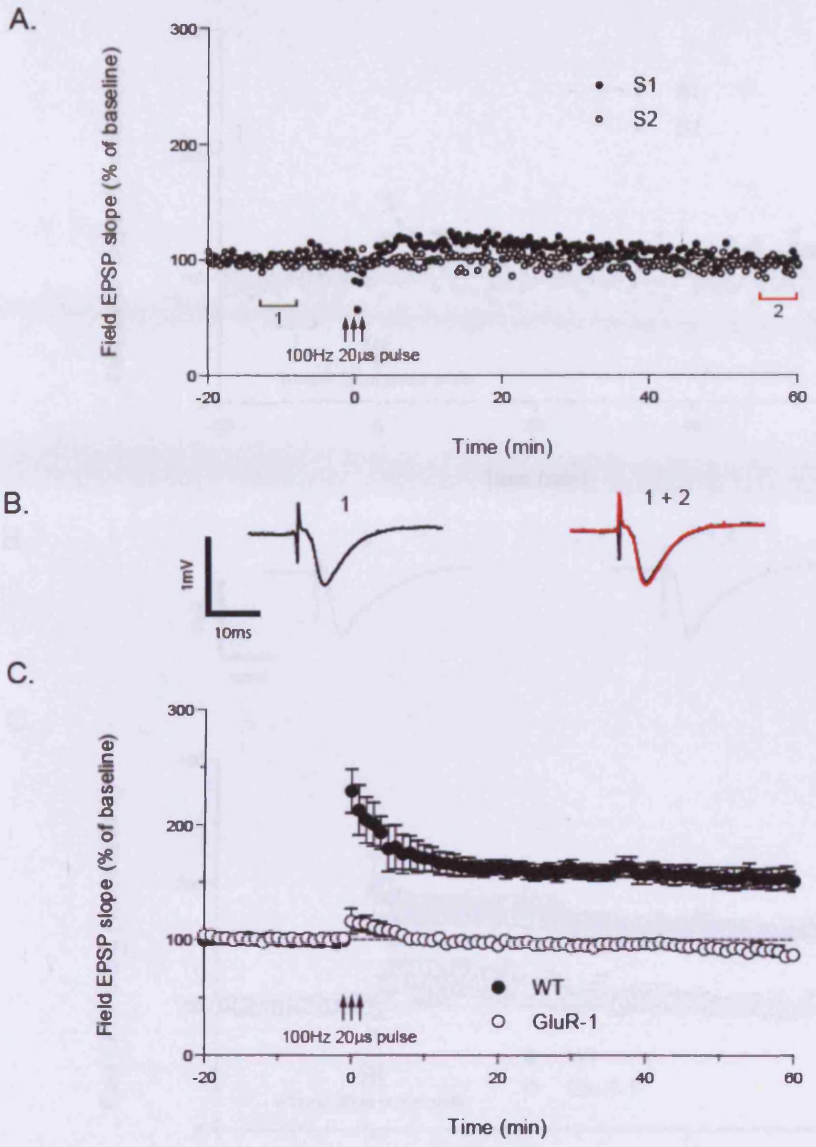


Figure 4.2 LTP induced by three trains of low intensity 100Hz is absent in the GluR1 knockout.
A, Example experiment in the GluR-1^{+} mice. B, example traces at time points indicated by the bars. C, In GluR-1^{+} mice (○),100Hz stimulation delivered at low intensity (test pulse-width 20µs) produces no significant potentiation of the fEPSP (n = 10), compared to a highly significant potentiation in WT mice (●) (n =12).

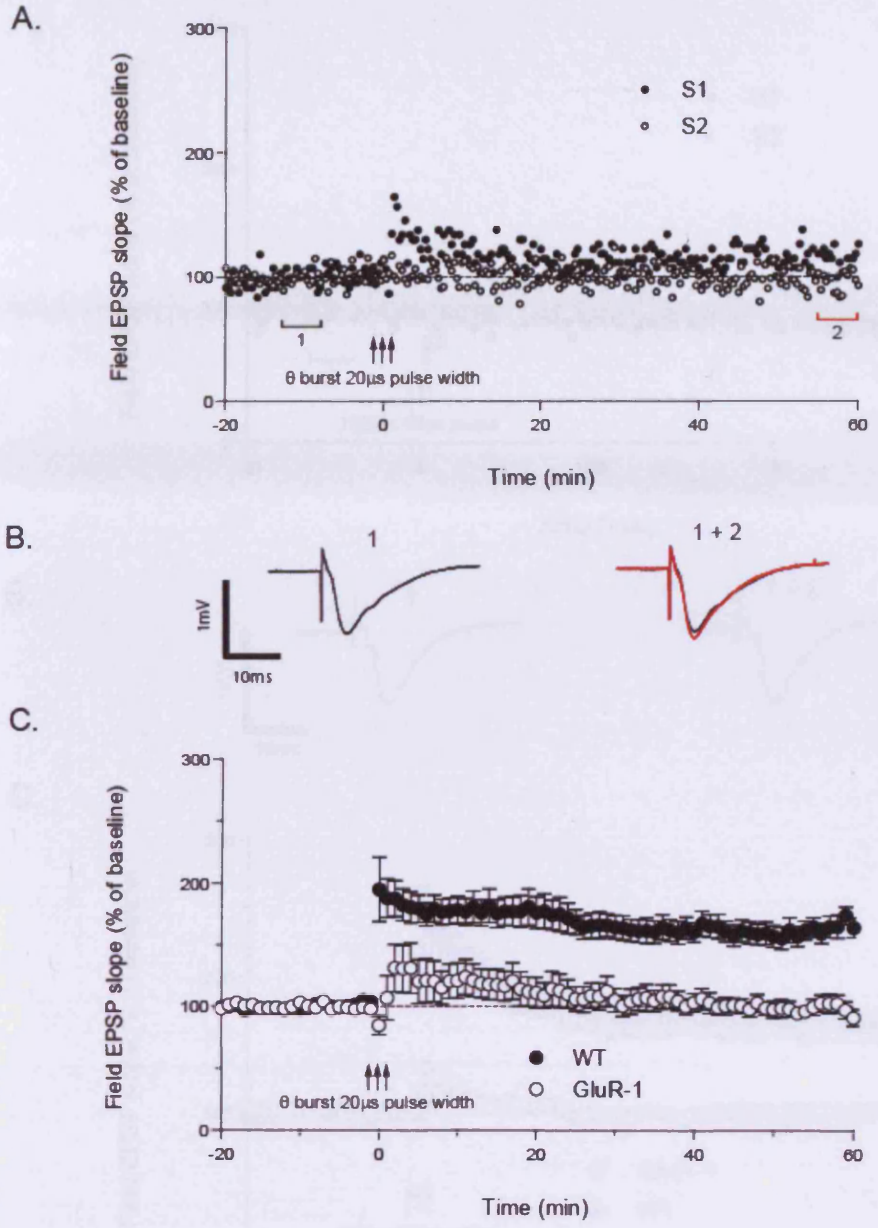


Figure 4.3 LTP induced by three trains of low intensity TBS is absent in the GluR-1 knockout.

A, Example experiment in the GluR-1^{+} mice. B, example traces at time points indicated by the bars. C, In GluR-1$^{-/-}$ mice (○),TBS stimulation delivered at low intensity (test pulse-width 20μs) produces no significant potentiation of the fEPSP (n = 10), compared to a highly significant potentiation in WT mice (●) (n = 12).

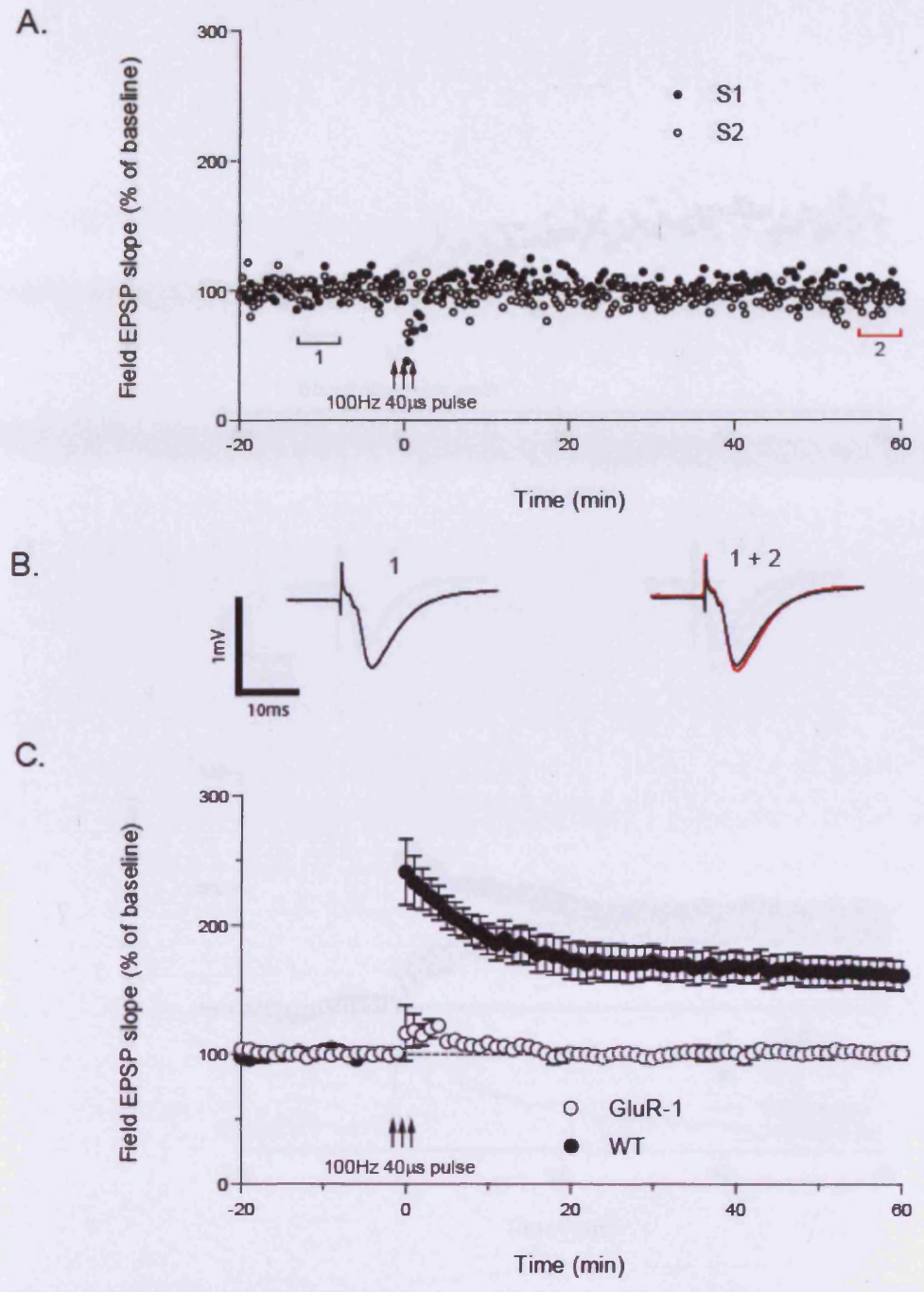


Figure 4.4 LTP induced by three trains of high intensity 100Hz stimulation is absent in the GluR-1 knockouts.

A, Example experiment in the GluR-1$^{+/-}$ mice. B, example traces at time points indicated by the bars. C, In GluR-1$^{+/-}$ mice (○),100Hz stimulation delivered at high intensity (double test pulse-width 40µs) produces no significant potentiation of the fEPSP (n = 10), compared to a highly significant potentiation in WT mice (●) (n = 12).

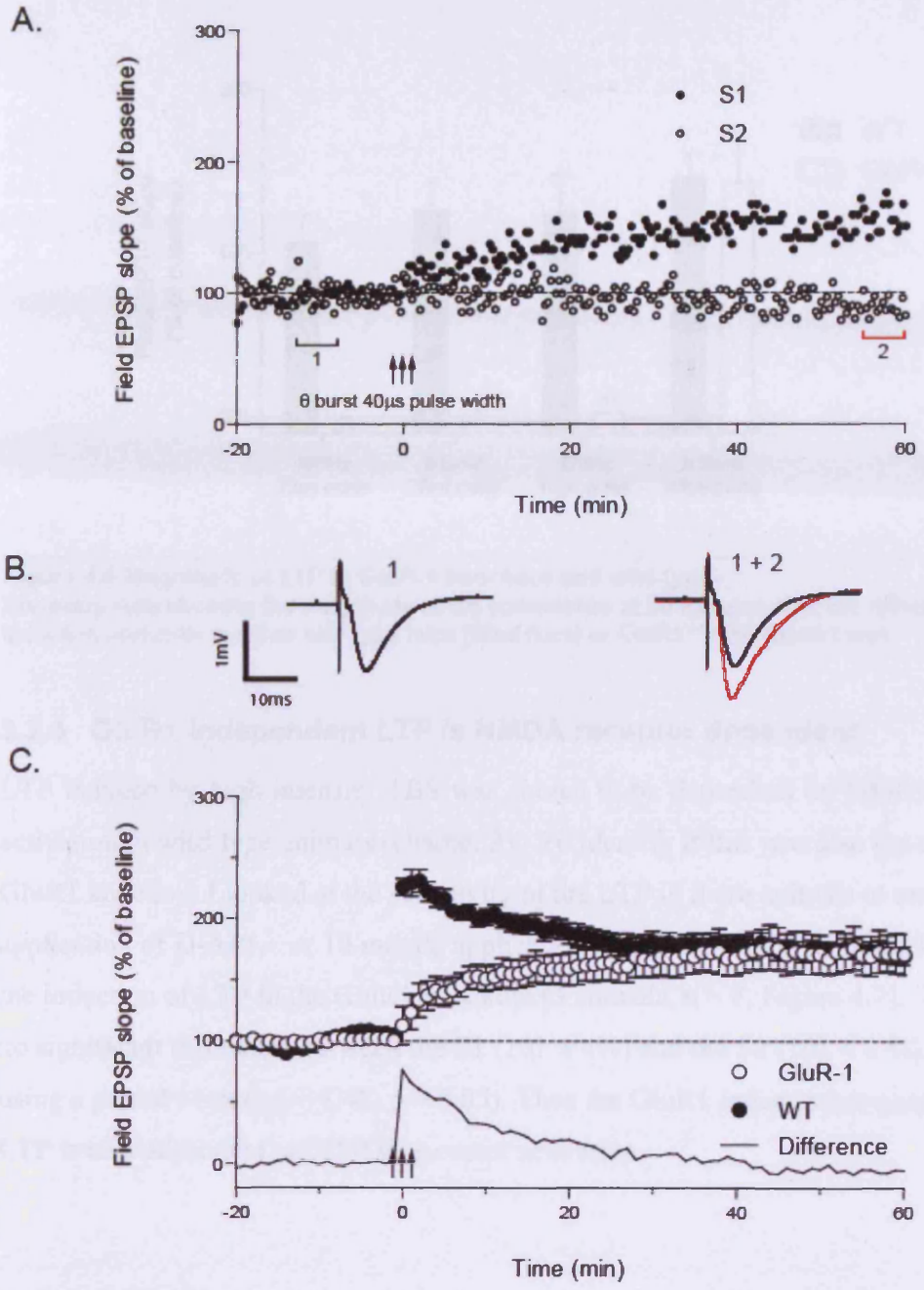


Figure 4.5 High intensity TBS produces GluR-1 independent LTP.
A, Example experiment in the GluR-1$^{-/-}$ mice. B, example traces at time points indicated by the bars. C, High intensity TBS produces a slowly rising form of potentiation in GluR-1$^{-/-}$ mice (○) (n = 12) that is indistinguishable from the potentiation in WT mice (●) at 60 min (n = 12). Solid line represents the difference between the two genotypes.

A.

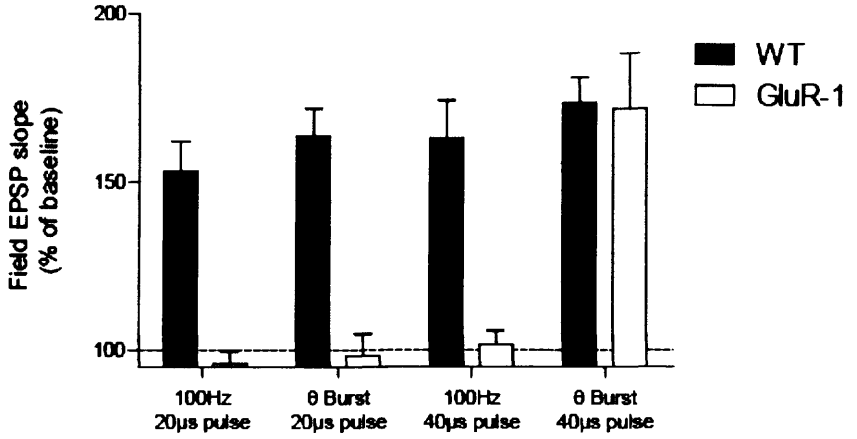


Figure 4.6 Magnitude of LTP in GluR-1 knockout and wild-type.
Summary data showing the magnitude of the potentiation at 60 minutes after the different induction protocols in either wild-type mice (filled bars) or GluR1$^{-/-}$ mice (open bars).

3.2.4 GluR1 Independent LTP is NMDA receptor dependent.

LTP induced by high intensity TBS was shown to be dependent on NMDA receptor activation in wild-type animals (chapter 3). To identify if this was also the case in the GluR1 knockout I looked at the sensitivity of the LTP in these animals to extracellular application of D-AP5. A 10 minute application of D-AP5 50µM completely blocked the induction of LTP in the GluR1 knockout (3 animals, n = 7, Figure 4.7). There was no significant difference between the S1 (101 ± 4%) and the S2 (105 ± 3 %) pathways using a paired t-test ($t_{(7)} = 1.48$, $p > 0.05$). Thus the GluR1 independent component of LTP is also dependent on NMDA receptor activation.

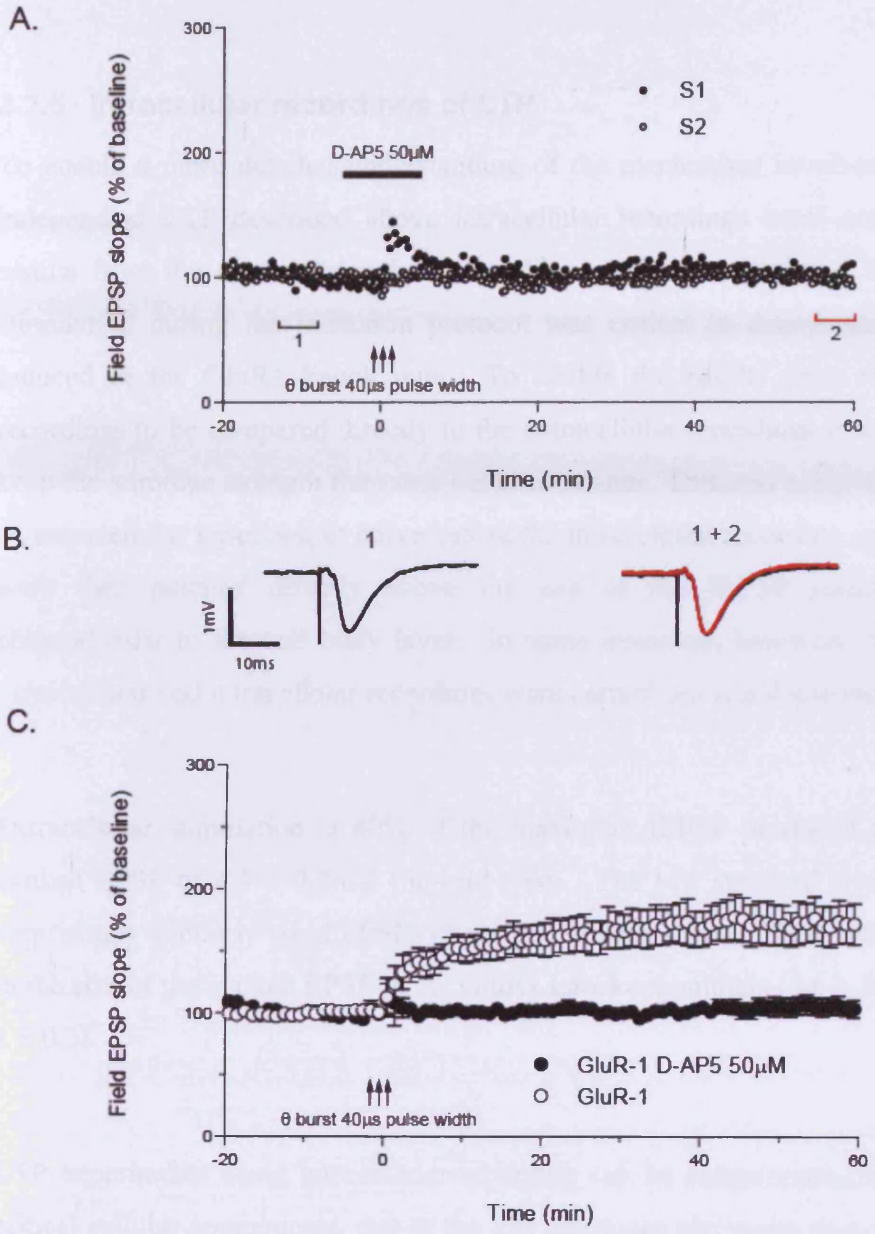


Figure 4.7 The GluR-1 independent LTP induced using a high intensity theta burst is NMDA receptor dependent.
A, Example experiment in the GluR-1$^{-/-}$ mice, 50µM D-AP5 was applied during induction of LTP at time indicated by the solid bar. B, example traces at time points indicated by the bars. C, High intensity TBS produces a slowly rising form of potentiation in GluR-1$^{-/-}$ mice (○) (n = 12) that was completely blocked by the presence of 50µM D-AP5 during induction (●) at 60 min (n = 7).

3.2.5 Intracellular recordings of LTP

To enable a more detailed understanding of the mechanism involved in the GluR1 independent LTP described above intracellular recordings were undertaken. The results from the extracellular field recording studies revealed that the intensity of stimulation during the induction protocol was critical in determining if LTP was induced in the GluR1 knock outs. To enable the results from the intracellular recordings to be compared directly to the extracellular recordings it was important to keep the stimulus strength the same between studies. This was achieved by recording an extracellular input/output curve before the intracellular recording was made. Cells were then patched directly above the site of the fEPSP recording electrode perpendicular to the cell body layer. In some instances, hardware permitting, dual extracellular and intracellular recordings were carried out simultaneously.

Extracellular stimulation at 40% of the maximum fEPSP produced an intracellular evoked EPSP of 4.3 ± 0.2mV (in wild-type). The low standard error indicates the surprisingly similarly sized EPSPs observed between slices. There was no difference in the size of the evoked EPSP in the GluR1 knockout animals (4.6 ± 2mV, $t_{(43)} = 0.2$, $p = 0.3$).

LTP experiments using intracellular recording can be compromised by wash out of critical cellular components, due to the low resistance electrodes that are used in this technique, a problem that is enhanced at higher temperatures. To minimize washout of LTP only 5 minutes of control data was recorded before LTP was induced. Patching and holding CA1 cells from adult mouse slices (>P56) is technically challenging, especially at higher temperatures (32°C), therefore recordings were carefully monitored and discarded if input resistance changed by more than 20% and if the resting membrane changed by ±5mV. LTP could be reliably produced from intracellular recordings in adult wild-type mice, by high intensity TBS (that was stable for as long as the cell could be kept healthy (~45 -100min)). The magnitude of LTP using intracellular recordings was 199 ± 17 % (n = 23) at 45 minutes post

tetanus. In agreement with the field recording experiments, high intensity TBS could also produce a reliable LTP in the GluR1 knockout (188 ± 20%, n = 21, Figure 4.8) that was similar in magnitude to wild-type LTP (199 ± 17%), ($t_{(42)}$ = 0.43, p = 0.7). The time course of the potentiation was also similar, with a delayed early component which then developed slowly over time.

3.2.6 GluR1 independent LTP is dependent on postsynaptic CaMKII activation.

In field recording experiments, the induction of LTP by high intensity TBS was blocked by AIP, a specific inhibitor of CaMKII (chapter 3). To identify if the induction of GluR1 independent LTP was also dependent on CaMKII, and to identify if CaMKII was acting presynaptically or postsynaptically, a series of experiments with AIP included in the intracellular electrode were performed. In the field recording experiments a version of AIP was used that contained a linked amino acid sequence that increases cell permeability. In this set of experiments the non cell permeable version of AIP was used, to limit its action to the postsynaptic cell being recorded. 50μM AIP included in the patch electrode, completely blocked the induction of LTP in the GluR1 knockout (n = 10, Figure 4.9). There was no significant difference between the EPSP measured 45 min post tetanus (103 ± 7 %) and the EPSP measured before the tetanus (98 ± 2%) using a paired t-test ($t_{(9)}$=1.5, p = 0.2). These results indicate that the induction of GluR1 independent LTP still requires activation of postsynaptic CaMK-II.

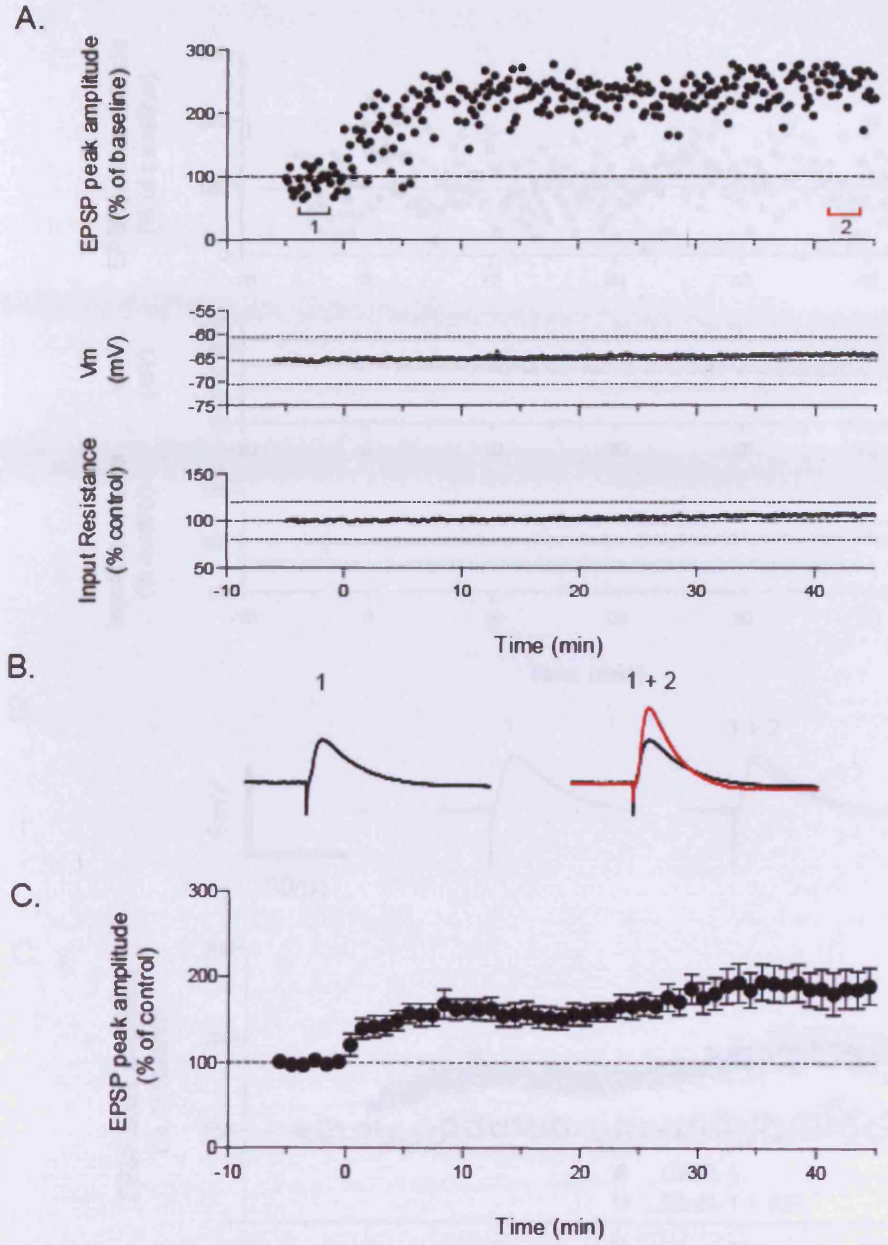


Figure 4.8 The GluR-1 independent LTP could also be observed using whole-cell somatic recordings.

A, An example experiment in the GluR-1$^{-/-}$ mice, lower traces indicate that the membrane voltage (Vm) and input resistance was stable through out the recording period. Dashed lines indicate the point at which a recording was considered unstable and would have been stopped (20% input resistance, 5mV membrane potential). B, Example traces at time points indicated by the bars. C, Average data showing that a slowly rising GluR-1 independent LTP could also be induced using whole cell recordings (n = 21).

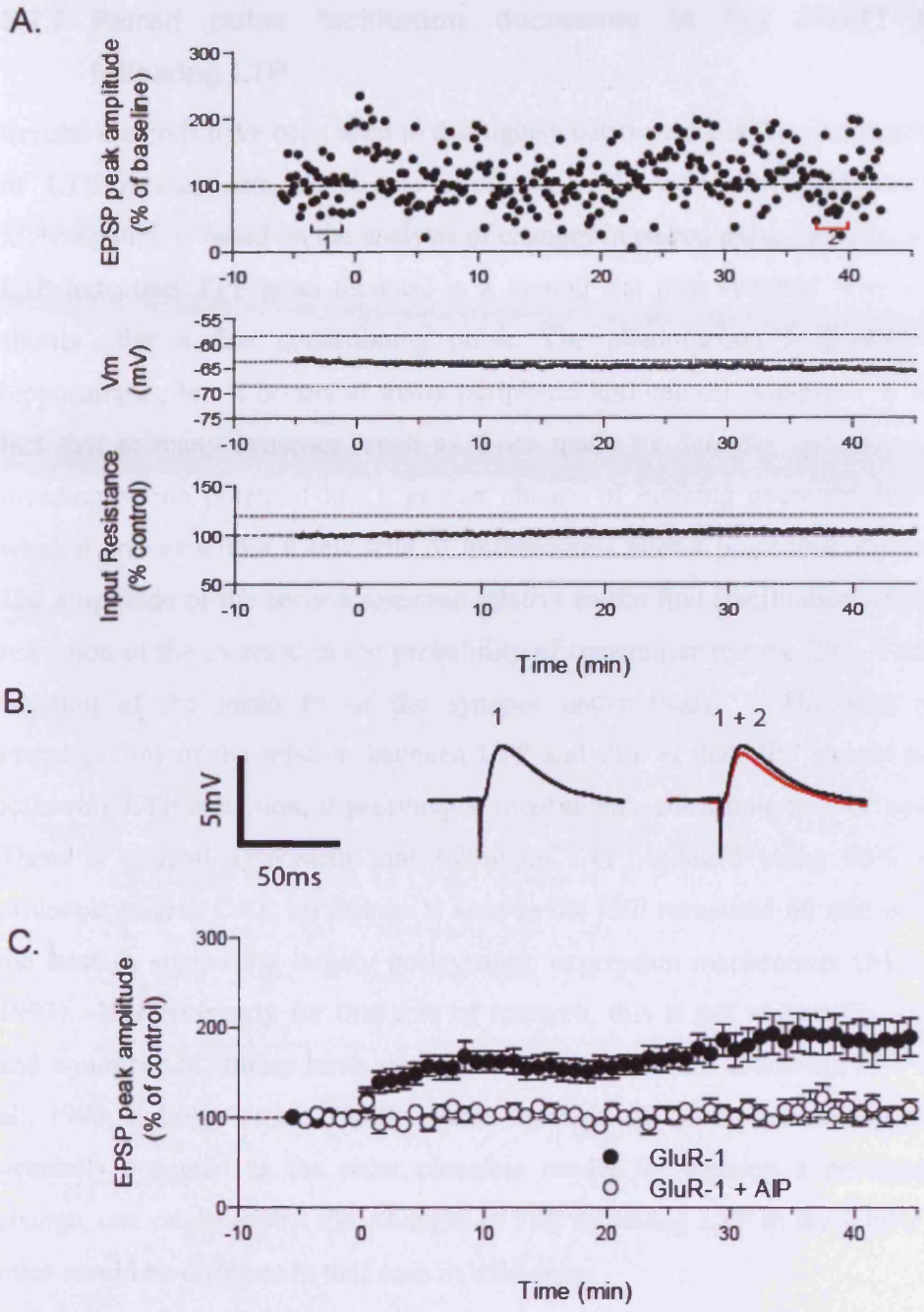


Figure 4.9 Postsynaptic CaMKII inhibition blocked the GluR-1 independent LTP.
A, An example experiment in the GluR-1-/- mice with 50μM AIP included in the patch pipette, lower traces indicate that the membrane voltage (Vm) and input resistance was stable through out the recording period (>20% input resistance, > 5mV membrane potential). B, example traces at time points indicated by the bars. C, Average data showing the LTP in the GluR-1-/- mice was completely blocked if 50μM AIP was included in the patch pipette (○) (n = 10).

3.2.7 Paired pulse facilitation decreases in the GluR1 knockout following LTP.

Several methods have been used to distinguish between pre and postsynaptic locations of LTP mechanisms. A simple approach, used more than 20 years ago by McNaughton, is based on the analysis of changes in paired pulse facilitation following LTP induction. PPF is an increase in a second test post synaptic response, elicited shortly after a first conditioning pulse. The phenomenon is prominent in the hippocampus, but it occurs at many peripheral and central synapses. It reflects the fact that at many synapses, such as those made by *Schaffer collateral* axons, an invading action potential has a greater chance of evoking neurotransmitter release when it arrives within a few tens of milliseconds after a preceding action potential. The amplitude of the second response relative to the first (facilitation ratio) is then a reflection of the increase in the probability of transmitter release (Pr). Facilitation is function of the mean Pr of the synapse under study. The idea underlying investigations of the relation between LTP and PPF is that PPF should be changed following LTP induction, if presynaptic mechanisms contribute to LTP maintenance. There is general agreement that following LTP, induced using HFS or pairing protocols in area CA1, no change is seen in the PPF measured 60 min or more after the tetanus, suggesting largely postsynaptic expression mechanisms (Manabe et al., 1993). Unsurprisingly for this area of research, this is not universally agreed upon and a number of studies have argued for a change in PPF following LTP (Schulz et al., 1994; Sokolov et al., 1998). Since insertion of GluR1 containing receptors is generally accepted as the most plausible model for causing a postsynaptic gain change, one might expect that changes in PPF following LTP in the GluR1 knockout mice would be different to that seen in wild-type.

In agreement with other studies when PPF was monitored before, and 40 minutes after, induction of LTP in wild-type and then averaged over 20 slices, no change associated with LTP was apparent (Figure 4.10A Control = 1.62 ± 0.28, LTP = 1.55 ± 0.33, $p > 0.05$). However in individual slices there were sometimes significant decreases in PPF, yet in others there were increases in PPF. Figure 4.10B illustrates the variability of these changes; six cells show an increase (Figure 4.10C), six cells show no change, while eight show a decrease in the PPF after LTP (Figure 4.10D). In

the GluR1 knockout on the other hand, there was much less variability (Figure 4.10B right panel), with only one cell increasing (Figure 4.10C right), four remaining unchanged while 16 cells had a decrease in the PPF following LTP (Figure 4.10D right). Indeed when all 21 slices were averaged there was a significant decrease in the PPF following LTP (PPF (Control) = 1.64 ± 0.22, PPF (LTP) = 1.47 ± 0.21, paired t-test $t_{(20)} = 2.9$, $p < 0.01$).

The initial PPF ratio has been shown to be inversely related to the magnitude and sign of the change in PPF following LTP in the hippocampus (Kleschevnikov et al., 1997; Schulz et al., 1995). In agreement with these data I also saw an inverse correlation between the initial PPF ratio and the change in PPF after LTP, both in wild-type ($r = 0.47$, $n = 19$, $p < 0.05$) and GluR1 knockout ($r = 0.55$, $n = 21$, $p < 0.01$) (Figure 4.11A). In the GluR1 knockout there was also a negative correlation between the change in the PPF ratio and the magnitude of LTP at 45 minutes ($r = 0.52$, $n = 21$, $p = 0.03$) (Figure 4.11B) and a positive correlation between the initial PPF ratio and the magnitude of LTP ($r = 0.47$, $n = 21$, $p = 0.01$) (Figure 4.11C). However neither of these correlations were apparent in the wild-type. The initial PPF, or the change in PPF, failed to predict the magnitude of LTP in wild-type, presumably due to additional postsynaptic mechanisms involving GluR1, while in the knockout these postsynaptic mechanisms are not available and possibly presynaptic mechanisms are more dominant.

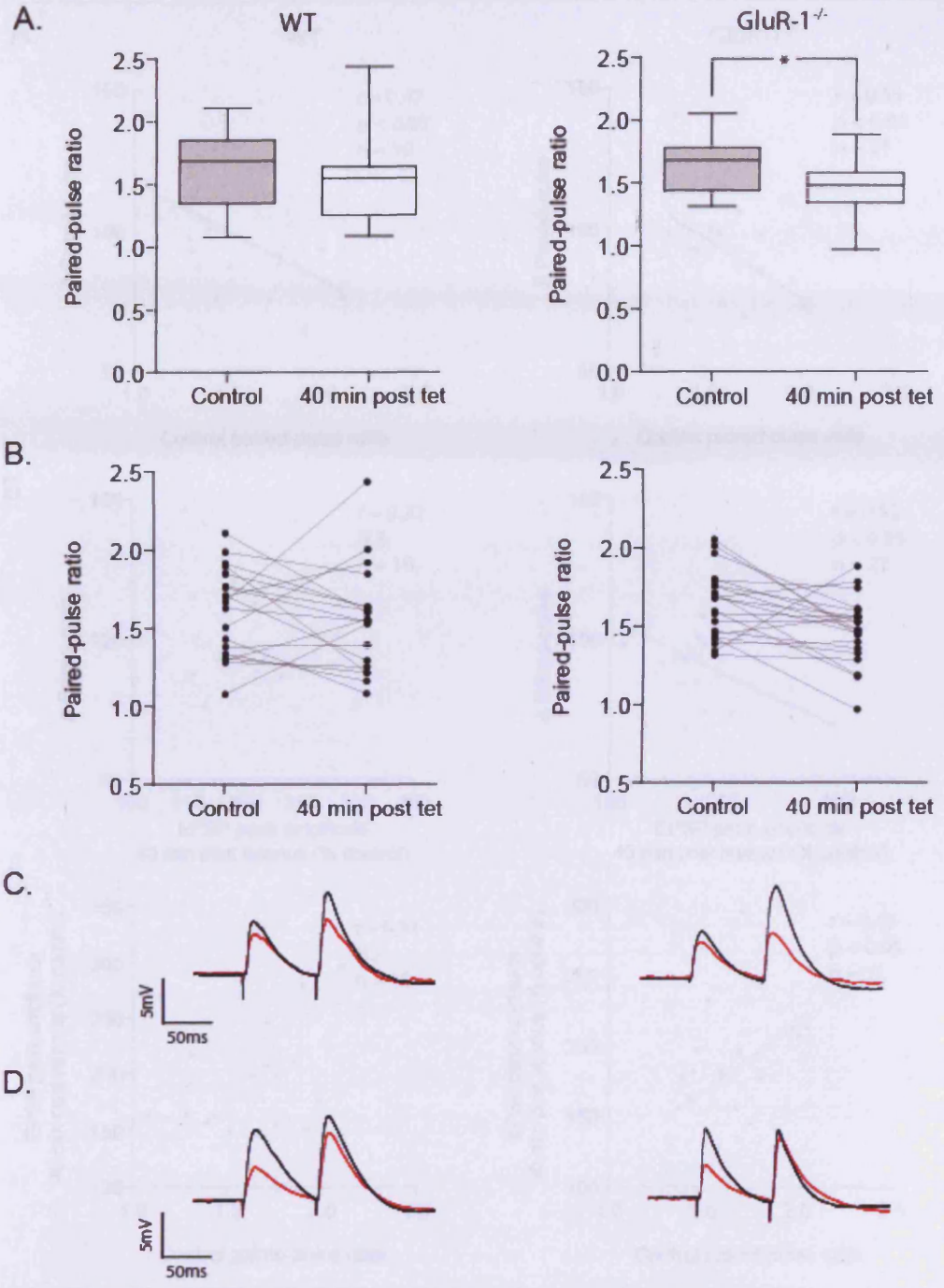


Figure 4.10 The locus of plasticity in wild-type and GluR1 knock-out
A, Changes in paired pulse facilitation (PPF, 75 ms inter pulse interval) 40 minutes after LTP induction is highly variable in wild type (left box plot), Less variability is observed in the GluR-1 knockout (right box plot) and there is a significant decrease in the PPF 40 minutes after LTP induction. B, Individual experiments showing the variability in the wild-type (6 substantially increase, 6 do not change and 8 decreased) (left) and in the GluR-1 knockout (1 substantially increased, 4 do not change and 16 decrease) (right). C, A trace showing an example of a response that showed no change in the PPF ratio measured 45 minutes after the tetanus in both wild-type (left) and GluR-1 knockout (right), red = control, black = potentiated response. D, A trace showing an example of a response that showed a decrease in the PPF ratio measured 45 minutes after the tetanus in both wild-type (left) and GluR-1 knockout (right), red = control, black = potentiated response.

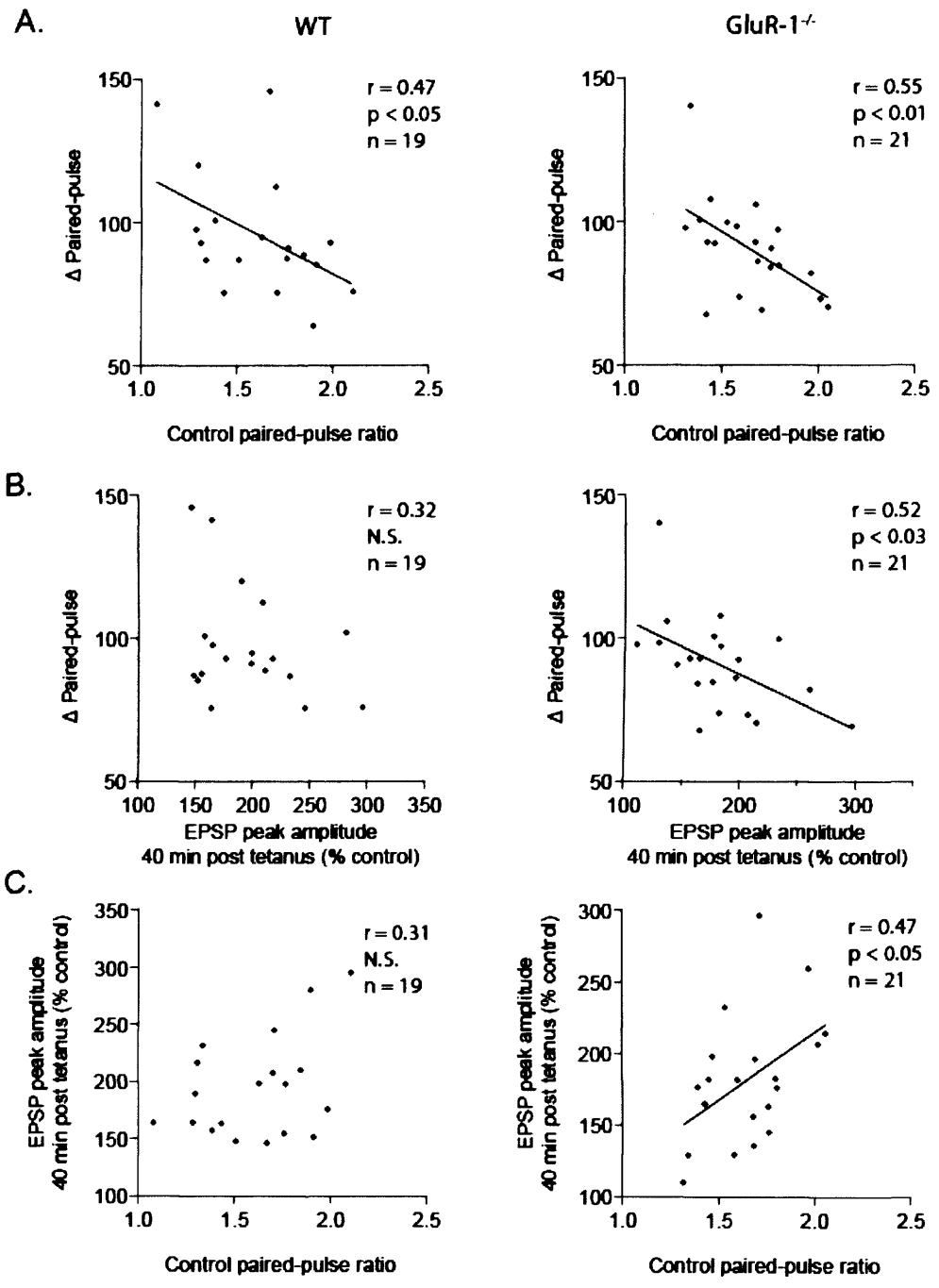


Figure 4.11 Correlations between initial PPF ratios, changes in PPF after LTP and magnitude of LTP suggests that modifications occurring in the GluR-1 knockout following LTP are associated with more change in PPR than in wild-type.
A, The initial PPF ratio gives a good indication of whether the PPF ratio will change after theta burst stimulation in both WT and GluR-1 knockouts. EPSPs with high initial PPF ratios (i.e .low release probabilities) are more likely to show a reduction in the PPF ratio after the tetanus. B, In the GluR-1 knockout the level of LTP is inversely correlated with the change in the PPF ratio (but not in wild-type). C, In the GluR-1 knockout the initial pre tetanic PPF ratio is a very good indictor of the size of the LTP (but not in wild-type). In wild-type connections with both low and high PPF ratios potentiate to the same degree, while in the GluR-1 knockout the level of LTP is correlated with the initial PPF of the EPSP.

3.2.8 $(CV)^{-2}$ analysis is consistent with a presynaptic expression of LTP in the GluR1 knockout mice,

The paired-pulse data discussed above suggests that LTP induced using a high intensity theta burst stimulation, is expressed both postsynaptically via GluR1 and also presynaptically via a GluR1 independent mechanism. To try and add weight to this argument I used another approach that has historically been used to identify the locus of LTP expression. $(CV)^{-2}$ analysis was originally developed at the neuromuscular junction, and is a function that uses the variance of postsynaptic currents to estimate quantal parameters. At the neuromuscular junction the number of quanta released by a nerve impulse is well described by a binomial distribution. The binomial fit of the data thus gives estimates of q, n, and p. As mentioned earlier, the variables n and p are viewed as measures of presynaptic function, whereas q is usually considered to reflect postsynaptic function. The coefficient of variation (CV) of a simple binomial distribution is a parameter that is dependent only on n and p; $CV = \sqrt{[(1-p)/np]}$. Therefore CV is determined by variables that are measurements of presynaptic function, and are independent of q. If this assumption is taken CV can be compared before and after alterations in synaptic efficacy, to determine whether the change is mediated by a presynaptic process, reflected in a change in CV. Alternatively, postsynaptic changes would not be accompanied by changes in CV (Martin 1966).

$(CV)^{-2}$ is a closely related statistical parameter to CV and was used in a seminal study by Malinow and Tsien, (1991) to identify the locus of LTP expression (see Introduction). $(CV)^{-2}$ is equal to the $mean^2/variance$ (M^2/σ^2) where M is the mean amplitude of the synaptic current and σ^2 is the variance of the response around the mean. Typically on a plot of normalized $(CV)^{-2}$ versus amplitude (Figure 4.12C), the mechanism is regarded as postsynaptic when the experimental points fall on line I, and presynaptic if on or above line II, whereas both sites are involved if the result is between these two lines (area III). However, the interpretations of the computed results depend upon assumptions about the release process and hence care must be taken when drawing conclusions. In the case of Malinow and Tsien, who were using

minimal stimulation and assumed that single inputs were being activated, the experimental points falling in region III would be taken to imply a presynaptic locus. On the other hand (Bekkers and Stevens, 1990), were using a stronger stimulation and hence stimulating multiple inputs would assume a postsynaptic locus for the same area on the plot.

The amplitudes of the EPSPs varied from stimulus to stimulus, even though stimulus intensity was kept constant (Figure 4.12A). The variance in the amplitude of the EPSP was reduced after LTP in both wild-type and in the GluR1 knockout. The mean and variance of EPSP amplitudes were calculated in two 10 minute bins, one during the control period, and one at 45 minutes after the high intensity TBS (see methods) and then plotted against the normalized mean amplitude. There was a certain amount of heterogeneity in the trajectories of the $(CV)^{-2}$ in both wild-type and GluR1 knockout, with points falling above and below the diagonal line. In the wild-type six cells fell above, four on, and eight below the diagonal line, while in the GluR1 knockout 15 cells fell above and only three below the diagonal. When the data was averaged over all the trials, the trajectory of the plot in the GluR1 knockout was significantly steeper than in the wild-type (wild-type slope = 1.07 ± 0.05, GluR1 knockout slope = 1.3 ± 0.07, t-test $t_{(34)} = 2.5$, $p < 0.05$). In wild-type the trajectory of the line was effectively superimposed on the line of unity. In the GluR1 knockout on the other hand, the trajectory was significantly steeper than the diagonal, implying a purely presynaptic expression mechanism. The three cases in the GluR1 knockout that showed trajectories into zone III of the plot imply that there could still be a small residual postsynaptic component of the LTP, even though the GluR1 component is absent.

The conclusions of the paired pulse analysis are in general agreement with the $(CV)^{-2}$ analysis in that it also indicated a predominantly presynaptic component to LTP in the GluR1 knockout, and a mixed presynaptic / postsynaptic locus in the wild-type.

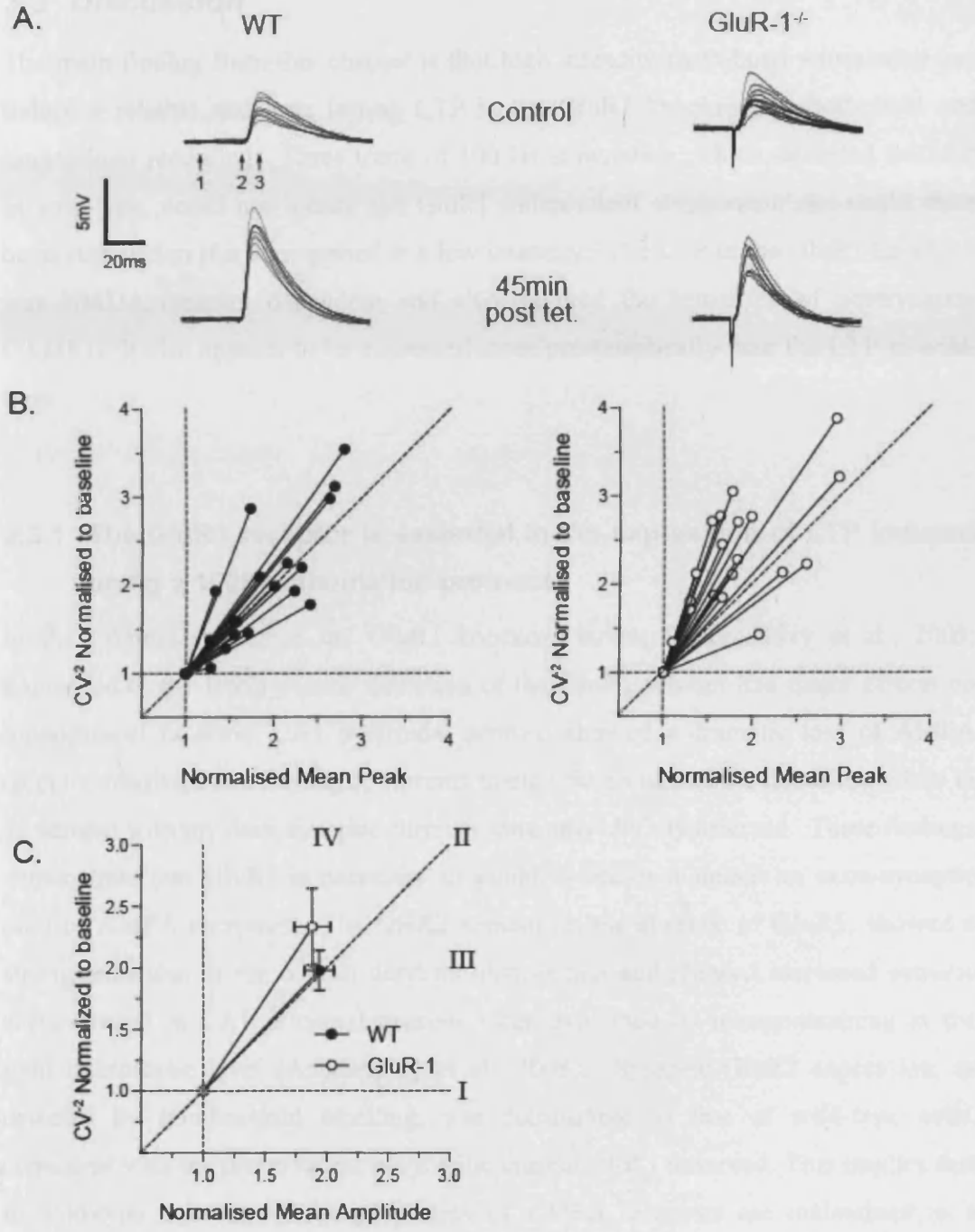


Figure 4.12 The locus of plasticity in wild-type and GluR1 knockout using CV^{-2} analysis. A, Traces illustrating the variance in the amplitude of the EPSPs Arrows 1 + 2 indicate the time point at which the SD of the noise was calculated, and arrow 3 where the SD of the peak EPSP was calculated. B, Plots for the individual experiments illustrate the heterogeneity in the change in $1/CV^{-2}$ in the wild-types while in the GluR-1 knockouts the majority fall above the unity line. C, The average normalised mean response versus CV^{-2} trajectory is significantly more vertical for GluR1 knockout than wild-type. The origin (1,1) represents the baseline condition while the points to the right are taken at 40 minutes after LTP induction. Both CV^{-2} and mean are normalized to unity.

3.3 Discussion

The main finding from this chapter is that high intensity theta burst stimulation can induce a reliable and long lasting LTP in the GluR1 knockout, in both field and intracellular recordings. Three trains of 100 Hz stimulation, which saturated the LTP in wild-type, could not induce the GluR1 independent component, nor could theta burst stimulation if it was applied at a low intensity. The LTP in the GluR1 knockout was NMDA receptor dependent and also required the activation of postsynaptic CAMKII. It also appears to be expressed more presynaptically than the LTP in wild-type.

3.3.1 The GluR1 receptor is essential in the expression of LTP induced using a 100Hz stimulation protocol.

In the original studies in the GluR1 knockout mouse (Andrasfalvy et al., 2003; Zamanillo et al., 1999) genetic depletion of the GluR1 subunit had major effects on hippocampal function. CA1 pyramidal neurons showed a dramatic loss of AMPA receptor-mediated extra-synaptic currents in both the soma and the dendrites, while in agreement with my data, synaptic currents were only slightly affected. These findings demonstrate that GluR1 is necessary to establish and/or maintain an extra-synaptic pool of AMPA receptors. The GluR2 subunit, in the absence of GluR1, showed a strong reduction in the overall dendritic distribution and showed increased somatic accumulation in CA1 principal neurons when examined by immunostaining at the light microscope level (Andrasfalvy et al., 2003). Synaptic GluR2 expression, as detected by immunogold labelling, was comparable to that of wild-type cells, consistent with the preservation of synaptic currents that I observed. This implies that in wild-type animals a large proportion of AMPA receptors are maintained as a reserve pool at extra-synaptic sites and are not immediately participating in synaptic signal transduction.

This reserve pool of AMPA receptors seems to be critical in the expression of adult LTP induced using 100 Hz protocol. In agreement with my data Zamanillio et al.

(1999) showed that LTP was completely absent in the adult GluR1 knockout using this protocol. Although these data suggest that it is the GluR1 subunit that is critical, since GluR2 was also reduced in the extra-synaptic regions, it is possible that both subunits contribute to the expression in wild-type. A specific role for the GluR1 subunit was however suggested from studies examining the function of GluR1 C-terminal phosphorylation. First, experiments in hippocampal slice cultures suggested that PKA phosphorylation of GluR1 C-tail ser 845 is necessary for synaptic insertion of GluR1-containing AMPA receptors after LTP induction (Esteban et al., 2003); and secondly gene-targeted mice carrying a mutation of GluR1 serine 845 showed reduced 100 Hz induced LTP despite normal levels of extra-synaptic AMAP receptors (Lee et al., 2003). These studies provide evidence for a specific role of the GluR1 subunit in 100 Hz induced LTP at CA1 synapses.

The finding from the original studies in the GluR1 knockout animals lead to a popular theory for the expression of LTP. LTP would be expressed by an activity dependent increase in the number of GluR1 containing AMPA receptors available to respond to a constant amount of synaptically released glutamate. These receptors could be inserted from an intracellular compartment and/or diffuse laterally from the extra synaptic regions described above.

3.3.2 Theta burst produces a GluR1 independent component to LTP.

Although the above model can account for many of the findings of 100 Hz induced LTP it cannot explain my observation that if a high intensity theta burst is used during induction, a GluR1 independent component can be identified in these knockout. This LTP seems to have a reduced component in the first 20 minutes; but is indistinguishable from the LTP induced in wild-type after 60 minutes. Hoffman et al., (2002) described a similar LTP in these knockouts using a theta burst pairing protocol. In this protocol sub threshold EPSP are paired with somatic current injection at the soma to produce back propagating action potentials and these pairings were then repeated at the theta frequency. They proposed that the two components of wild-type LTP, one requiring GluR1 for its expression and the other independent of GluR1, were mediated by different spatial and temporal Ca^{2+} signals as shown by

differing sensitivities to intracellular Ca^{2+} buffers. They suggested that the two mechanisms may operate in parallel and independently from each other, and that the induction may be such that the GluR1 dependent component is preferentially activated by local Ca^{2+} influx depending on the detailed spatiotemporal Ca^{2+} changes in dendritic spines and shaft, while the GluR1 independent component is sensitive to global Ca^{2+}. While my data cannot confirm this hypothesis, they suggest that the expression mechanisms diverge after the increase in intracellular Ca^{2+} has occurred, as the LTP in the GluR1 knockout was blocked with NMDA antagonists and also required postsynaptic CAMKII activation.

3.3.3 The early component of GluR1 independent LTP is reduced

Although the magnitude of the LTP in the GluR1 knockout was similar 60 minutes after the tetanus, the early phase of the potentiation was significantly smaller. Assuming that theta burst LTP is not only activating the GluR1 independent mechanism, but also the GluR1 dependent mechanisms, this observation suggests that insertion of GluR1 containing receptors tails off after ~ 20 minutes also during LTP induced using 100Hz. Since the LTP induced using 100Hz is totally absent in the knockout, the GluR1 dependent mechanism must be replaced with another process that stabilizes the potentiation after ~20 minutes. This process must also be dependent on the initial insertion of the GluR1 containing receptors, as without them no LTP can be produced. An explanation for this observation was provided by Shi et al., (2001) who provided direct evidence for two distinct mechanisms by which AMPA receptors can be delivered to the synapses. They showed that there was delivery of GluR1/GluR2 AMPA receptors following LTP, thereby effecting synaptic enhancement. However in addition to the GluR1/GluR2 insertion, additional proteins were also delivered in tandem to the synapses during plasticity. These proteins could then serve as placeholders or slots that could be filled with non-synaptic GluR2/GluR3 receptors to stabilize the potentiation. Shi et al., suggested that these GluR1/GluR2 receptors may leave the "slots" slowly over days (Shi et al., 2001) , however my evidence from the GluR1 knockout suggests that these GluR1/GluR2 leave within ~20 minutes.

Additional evidence that the GluR1 containing receptors are replaced within 25 minutes was provided in a slightly controversial study by Plant et al., (2006). They took advantage of the unique properties of GluR2-lacking AMPARs and measured AMPAR-mediated excitatory postsynaptic currents (EPSCs) at two membrane potentials before and after LTP induction. As expected, when synapses do not contain GluR2-lacking AMPARs, AMPAR EPSCs showed no inward rectification during basal synaptic transmission. Shortly after LTP induction however, outward synaptic currents hardly changed, while inward EPSCs were enhanced, suggesting that LTP was caused by addition of GluR2-lacking AMPARs. By 25 minutes after LTP induction, the outward synaptic currents had increased, restoring rectification to its pre-LTP basal value. LTP was also sensitive to blockade by philanthotoxin shortly after LTP induction, while the toxin had no effect when added before, or 30 minutes after, LTP induction. These observations are consistent with the transient insertion of GluR2-lacking AMPARs into the synaptic plasma membrane after LTP induction, and their gradual replacement by GluR2-containing AMPARs over about 25 minutes. The source of contention in the study was that these GluR2 lacking receptors are presumably GluR1 homomers. Thus the presence of such GluR2-lacking receptors implies that recently potentiated synapses have a new source of Ca^{2+} influx when these receptors are activated, and hence could help stabilize the potentiation further. Plant et al., went further and suggested that this Ca^{2+} influx is itself essential for LTP maintenance. Philanthotoxin applied within a few minutes of LTP induction reduced synaptic potentiation, but not just acutely, as expected if the blocked AMPARs were still being gradually replaced with GluR2-containing ones. Instead, philanthotoxin reversed the LTP, returning the EPSCs to basal levels, suggesting that the transiently available GluR2-lacking AMPARs must be activated for LTP to persist. To test this idea further, they induced LTP, but then stopped synaptic stimulation for 15 minutes, reasoning that this is functionally similar to toxin block. They found that 15 minutes of inactivity was sufficient to reverse the potentiation and return synaptic strength to pre-LTP levels. Although this theory is interesting, two recent publications have failed to replicate the effect (Adesnik and Nicoll, 2007; Grey et al., 2007)

3.3.4 GluR1 independent LTP seems to be expressed presynaptically.

Possibly one of the most interesting observations from this study is that while the LTP in wild-type is not associated with any change in the PPF ratio, a significant decrease in the PPF ratio is seen in the GluR1 knockout. This implies that LTP in the knockout has a larger proportion of the plasticity expressed presynaptically compared to wild-type. This argument is strengthened by the $(CV)^{-2}$ analysis, which showed a more vertical trajectory in the plot of the GluR1 knockout compared to wild-type, again implying a larger presynaptic locus if the GluR1 subunit is absent. Although the evidence provided by PPF and $(CV)^{-2}$ analysis is suggestive rather than conclusive, and a change in PPF is not a wholly reliable guide to a presynaptic locus, using this technique in the GluR1 knockout is strengthened for a number of reasons. Firstly, I am using the technique to compare two different genotypes, and therefore not taking the absolute level of change as an indication of locus, but comparing the change between the two. Secondly, the main criticism of these techniques is that one has to assume all synapses are initially homogeneous with respect to release probability and hence synaptic facilitation. This assumption however may not be true in the hippocampal excitatory synapses, as postsynaptic incorporation of AMPARs at silent synapses makes release sites detectable that were previously undetectable. It has been suggested therefore that LTP may affect the PPF ratio of AMPAR EPSPs by a purely postsynaptic mechanism (Gasparini et al., 2000; Hessler et al., 1993; Poncer and Malinow, 2001; Rosenmund et al., 1993). However in this study I was using adult animals, where silent synapses are thought to be rare (Isaac et al., 1997; Petralia et al., 1999; Takumi et al., 1999) and even if silent synapses were present, the un-silencing requires the activity dependent insertion of GluR1 receptors which are obviously lacking in the knockout.

Previous studies of the relationship between LTP and short-term facilitation of synaptic transmission in wild-type have yielded apparently conflicting results. Whereas the majority of groups have reported no overall change in short-term facilitation following the induction of LTP, (Anwyl et al., 1989; Asztely et al., 1996; Buonomano, 1999; Foster and McNaughton, 1991; Gustafsson et al., 1988; Manabe et al., 1993; Muller and Lynch, 1989; Schulz et al., 1994; Selig et al., 1999), a few groups have found a reduction in PPF which persisted for variable periods (Grover,

1998; Kleschevnikov et al., 1997; Schulz, 1997; Sokolov et al., 1998; Voronin et al., 1990; Wang and Kelly, 1997). There are a number of likely explanations for the discrepancy between these studies. Firstly, I have shown that the choice of induction protocols seems to be critical in the expression of this GluR1 independent presynaptic component of LTP. Therefore protocols such as 100Hz, that do not induce this presynaptic mechanism and seem to be expressed solely postsynaptically via GluR1 insertion, would not produce any corresponding changes in PPF. Experimental evidence that supports this hypothesis comes from a number of studies that have used two different techniques to directly visualize presynaptic activity during LTP (Bayazitov et al., 2007; Zakharenko et al., 2003; Zakharenko et al., 2001). All three studies showed that a slowly developing presynaptic component of LTP could be induced using TBS, while the LTP induced using either 100Hz or 50Hz stimulation was not associated with any presynaptic changes. Secondly, it is possible that the discrepancy between the different studies can be accounted for by differences in the initial levels of PPF due to different experimental conditions, as it is known that there is a large heterogeneity in the basal properties of different synapses (Dobrunz and Stevens, 1997; Hessler et al., 1993; Rosenmund et al., 1993). Previous studies have shown that variations in initial transmitter release probabilities (0.03–0.99) can account largely for the variability in PPF at CA3 to CA1 synapses (Dobrunz and Stevens, 1997; Turner et al., 1997).

The initial PPF ratio gives an indication of the value of p for the synapse or synapses under investigation. Synapses with values of p equal or close to one will have no PPF, or even slight paired pulse depression, as p cannot increase as it is already close to unity. Synapses with low values of p will have the scope to increase and will show greater PPF. The initial PPF ratio, and hence p, has been shown to be inversely related to the magnitude and sign of the change in PPF after LTP (Kleschevnikov et al., 1997; Schulz et al., 1995). Cells in which the initial amount of PPF is large (i.e. initial p is low) are more likely to show a decrease in PPF after LTP, whereas cells in which the initial amount of PPF is small (i.e. initial p is high) tend to show increases in PPF after LTP. In agreement with these results I also saw an inversely related correlation between the initial PPF ratio and the change in PPF after LTP. An even stronger correlation was seen in the GluR1 knockout.

If an additional presynaptic modification is occurring following theta burst stimulation then one might predict that the magnitude of the change in PPF would be inversely related to the magnitude of the LTP expressed. Indeed this has been reported in a number of studies using a high frequency tetanus (Kleschevnikov et al., 1997; Schulz et al., 1994) although disputed in others (Manabe et al., 1993; Muller and Lynch, 1989). I replicated this result in the GluR1 knockout, which showed a strong inverse correlation between the change in PPF and the magnitude of LTP. However I failed to replicate this correlation result in the wild-type.

These groups also reported a relationship between the initial release probability of a synapse, indicated by the initial PPR, and the magnitude of the LTP. They are suggesting that at low probability synapses, with low initial PPF ratios, there was more scope for presynaptic modifications in p and hence a higher likelihood of producing larger levels of plasticity. These results were again replicated in the GluR1 knockout where a very strong positive correlation was seen between the initial PPF ratio and the magnitude of LTP at 45 minutes. However once again there was no correlation evident in the wild-type, and in fact there were three cases with almost maximal LTP and very low initial PPF ratios. The initial PPF or the PPF change failed to predict the magnitude of LTP in wild-type, presumably due to a postsynaptic mechanism of LTP expression reliant on GluR1. In the knockout these mechanisms are not available and hence the initial level of p and the PPF ratio change is a good indicator of the magnitude of the potentiation.

In summary I have shown that high intensity TBS can induce LTP in the adult GluR1 knockout, while 100Hz stimulation was ineffective. The LTP in the GluR1 knockout seemed to be expressed more presynaptically than the LTP in wild-type as shown by changes in PPF and $1/CV^{-2}$. This suggests that TBS can induce two components of LTP, one that is expressed presynaptically and one that is expressed purely postsynaptically via insertion of GluR1 containing receptors. However both forms require the activation of NMDA receptors and CAMKII. Interestingly 100Hz stimulation can only induce the GluR1 dependent post synaptic component of LTP.

The LTP in the GluR1 knockout was completely prevented by postsynaptic blockade of CaMKII and is expressed at least in part presynaptically; this suggests that a retrograde message might be required at these synapses.

4 The role somatic action potentials in the induction of GluR1 independent LTP

4.1 Introduction

In the previous section I showed that a GluR1 independent LTP could be induced in both field and intracellular recordings using a "typical" induction protocol that relied on purely orthrodromic stimulation. It was clear that the type and strength of the induction protocol used was critical in determining whether plasticity could be expressed in the GluR1 knockout. While 100Hz stimulation at either a low or high intensity failed to elicit any plasticity, TBS could induce plasticity if the stimulus strength during the induction was increased. This observation raises the obvious question, what is occurring during the high intensity TBS, that is not occurring in either low intensity TBS, or low and high intensity HFS, to induce this GluR1 independent mechanism? The importance of this question is emphasised by the observation that high intensity TBS also produces a larger magnitude LTP in wild-type (chapter 3) and seems to be expressed both postsynaptically via GluR1 and presynaptically via some other mechanism (chapter 4). Determining what is uniquely different about this induction protocol could therefore help to clarify the parameters that are important in shifting the balance of LTP expression from postsynaptic to presynaptic or a combination of both. This could then shed light on the reasons why different laboratories have seen such contradictory results over the role that NO plays in LTP.

Although this is the first report of a GluR1 independent LTP using a standard induction protocol in adult animals (Phillips et al., 2005; also at the same meeting Romberg et al., 2005), it is also possible to induce this form of LTP in the hippocampus (Hoffman et al., 2002) and barrel cortex (Hardingham and Fox, 2006) if a spike timing dependent protocol (STDP) is used.

The initial study that paved the way for the use of spike pairing protocols was performed by Stuart et al., (1997) who showed unequivocally that the action potential in cortical pyramidal cells propagates back into the dendritic tree. This discovery was extremely important, as it identified an associative signal at the synapse that could

link presynaptic and postsynaptic firing. It was then demonstrated that these back propagating spikes could function as an associative signal for LTP induction in two publications in 1997. Markram et al., (1997) utilized cell pair recording in neocortical slices to show that when postsynaptic spikes were paired with small bursts of sub-threshold excitatory postsynaptic potentials (EPSPs), LTP of subsequent single EPSPs was produced. This phenomenon was associative, in that neither the EPSP bursts nor the postsynaptic spiking alone was sufficient to induce LTP. A detailed study revealed that when the onset of the EPSP either preceded or followed the onset of the spike with an interval of $\geq$100 ms no alteration in synaptic strength was produced. When the interval was reduced to 10 ms, LTP was induced when the EPSP preceded the spike, and LTD was induced when this order was reversed. At a similar time, this same basic finding was made using recordings of hippocampal Schaffer collateral–CA1 synapses by Magee and Johnson, who also showed that LTP induced in this manner could be blocked by bath application of NMDA receptor antagonists (Magee and Johnston, 1997). Magee and Johnston also demonstrated that LTP induction could be blocked by local application of tetrodotoxin (TTX) specifically to the region of the dendrite between the soma and the activated synapses. Thus in this system somatic spikes that fail to propagate to the site of activated synapses cannot contribute to LTP induction.

These initial findings have since been confirmed and extended using several different preparations, including hippocampal CA3–CA3 cell pairs in cultured slices (Debanne et al., 1998), and cultured hippocampal pyramidal neurons (Bi and Poo, 1998). In each case LTP was induced by paired activity in which spikes followed EPSPs by 10 ms and LTD was induced when this order was reversed. The forms of LTP and LTD described above depend upon the activation of the NMDA receptor and subsequent Ca^{2+} entry. The arrival of the back propagating spike during the EPSP functions to depolarise the postsynaptic membrane strongly and thereby facilitate Ca^{2+} flux mediated by the NMDA receptor. In contrast, when the spike precedes the EPSP, the EPSP coincides with the after hyperpolarisation, which may reduce NMDA receptor-mediated Ca^{2+} influx.

A number of Ca^{2+} imaging studies that have examined the interaction between back propagating spikes and EPSPs in dendrites appear to support this model. (Yuste and Denk, 1995) used two-photon microscopy to show that while both bursts of sub-threshold EPSPs and back propagating spikes could produce Ca^{2+} transients in dendritic spine heads of hippocampal CA1 pyramidal cells, simultaneous spikes and EPSPs produced a supralinear Ca^{2+} transient. Several subsequent studies have confirmed this basic finding using both two-photon (Koester and Sakmann, 1998; Schiller et al., 1998) and confocal (Magee and Johnston, 1997) microscopy. An important extension of this finding was that pairing in which postsynaptic spikes preceded EPSPs resulted in a sublinear Ca^{2+} transient (Koester and Sakmann, 1998).

While spike timing dependent plasticity (STDP) elegantly satisfies Hebbian learning rules, the model is complicated by the need to repeat the pairing during the induction multiple times. Moreover in most studies there is a specific range of repetition rates that must be used for successful induction. At frequencies below 10Hz, LTP cannot be induced. This repetition rate requirement suggests that something more than simply a pairing of EPSPs and back propagating action potentials (bAPs) is necessary for the induction of LTP. Another complication is that in most STDP experiments, the EPSP is sub threshold, and the back-propagating spike is triggered non-physiologically by current injection at the soma. Golding et al., showed that if the spikes were generated by supra threshold EPSPs (the authors suggest this is more physiological than current injection) then local TTX application at the soma did not block the induction of LTP at distal synapses (Golding et al., 2002). They showed that local dendritic spikes were sufficient to provide the depolarisation needed for LTP induction and that action potentials back propagating from the soma were not required. This was not only applicable at the distal synapses of the perforant path, where back propagating action potentials show strong attenuation with distance, but also at the proximal Schaffer collateral inputs to CA1 where back propagating action potentials are much larger and more reliable.

According to Hebb's theory, pre and postsynaptic firing is required for synaptic weight change and hence the level of interest in STDP;

"When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth type process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased" (Hebb,1949).

However, although there is an absolute requirement for postsynaptic activation in LTP induction, it is not necessary that this activation takes the form of spiking, e.g. mossy fibre LTP, heterosynaptic LTP and LTD and mGluR dependent LTP, in addition firing is not even necessary at the CA1 synapses. Many protocols for inducing LTP in this pathway do fire action potentials (e.g. the above STDP and some, but not all, forms of pairing). However brief HFS stimulation that does not evoke postsynaptic spiking is still sufficient to induce LTP (Douglas et al., 1982; Lee, 1983), as is high-frequency stimulation accompanied by postsynaptic application of a drug to inhibit spikes via blockade of postsynaptic voltage-gated Na^+ channels (Gustafsson et al., 1987; Thomas et al., 1998). Pairing induced LTP is also not blocked by intracellular injection of lidocaine derivatives such as QX314 that block spiking (Gustafsson et al., 1987; Harsanyi and Friedlander, 1997; Kelso et al., 1986). According to Thomas et al., 1998, intracellular QX314 prevents LTP induced by low frequency TBS trains, but has no effect on the efficacy of high-frequency trains. Primed burst stimulation can also readily induce LTP in vitro at stimulus strengths that are below threshold for spike firing (Davies et al., 1991). In some circumstances, even low frequency trains can induce LTP without firing the postsynaptic cell (Krasteniakov et al., 2004) but see (Pike et al., 1999). LTP can also be induced by repetitive pulses of glutamate targeted by two photon excitation of a caged precursor to a single spine, a protocol that does not fire the postsynaptic cell (Matsuzaki et al., 2004). All this evidence leads to the conclusion that postsynaptic somatic action potentials are not an absolute requirement for LTP at CA1 synapses, at least in vitro. Although it is experimentally possible to bypass the postsynaptic action potential, it may be that in a physiological context the only way that sufficient depolarisation can be obtained is by postsynaptic firing.

Previously, spike timing dependent protocols were the only way of identifying a GluR1 independent LTP in the hippocampus, while more standard orthrodromic stimulation protocols were ineffective. Since I have shown that LTP can be induced using a high intensity TBS protocol, but not by either high or low intensity HFS, I hypothesised that the differences in the protocols' effectiveness at inducing LTP in the GluR1 knockout might be related to the effect these different protocols have on postsynaptic spiking.

4.2 Results

4.2.1 Probability of postsynaptic action potential firing is highly variable between induction protocols.

High intensity TBS (40μs pulse duration) could induce LTP in the GluR1 knockout, while low intensity TBS (20μs pulse duration) was ineffective. The intensity delivered to the extracellular stimulating electrode was routinely changed by increasing or decreasing the voltage of a 20μs pulse (e.g. in the input output responses). During standard LTP experiments the intensity of stimulus is never changed and the induction protocol is generally given at the test intensity (in my case this was set to 40% of max). This is useful as it prevents mistakes being made when resetting the intensity back to control values after the induction protocol. However the stimulus intensity had to be adjusted during the induction in high intensity TBS and HFS and it was more accurate, to change the duration of the pulse rather than the voltage of the stimulus.

When the stimulating voltage is being applied in pulses of constant voltage the relation between the voltage and the duration of the pulse that gives the same response yields the distinctive strength-duration curve (Figure 5.1A) (Ranck, 1975). The curve is generally described by two parameters, the rheobase[2] and the chronaxie[3]. The rheobase is defined as the lowest stimulus amplitude (*I* or *V*) needed for activation and relates to an infinitely large pulse width. The chronaxie is defined as the pulse width corresponding to twice the rheobase. Reported values of the chronaxie in the Schaffer

[2] The root word "rheo" means current and "base" means foundation: thus the rheobase is the foundation, or minimum, current (stimulus strength) that will produce a response.

[3] The root word "chron" means time and "axie" means axis: chronaxie, then, is measured along the *time* axis and, thus, is a Duration that gives a response when the nerve is stimulated at twice the rheobase strength.

collaterals are rare and variable, but are generally between 50 and 200μs (Tekkok et al., 1998). The stimulus pulse widths used in the LTP experiments are well below these values (10-20μs) and therefore the responses are highly dependent on changes in pulse width. To determine what effect doubling the pulse width had on the evoked responses, input output curves were measured during single (20μs) and double (40μs) pulse width voltages (Figure 5.1B). At low voltage intensities (5-20 Volts) doubling the pulse width shifted the input output curve to the left. For example, at a stimulus intensity of 10 Volts the slope of the fEPSP was increased by 58% from 0.77 ± 0.07V/s (n = 36) with a 10μs pulse width to 1.23 ± 0.11 V/s (n = 6) with a 20μs pulse width. However, doubling the pulse width had no effect on the maximal response.

During the intracellular LTP experiments the test intensity was set by initially recording a extracellular field input/output response perpendicular to the patched cell. The voltage of the 10us pulse was then adjusted to elicit a 40% maximal fEPSP (7.2 ± 3.2 V). This protocol produced surprisingly small variations in the size of the intracellular recorded EPSP between slices and condition (Figure 5.1C). As mentioned previously, there was also no difference in the peak amplitude of the evoked EPSPs between wild-type (4.3 ± 0.2 mV) and GluR1 knockout (4.6 ± 0.2 mV). Doubling the pulse width at this voltage increased the peak amplitude of the evoked EPSPs from 4.4 ± 0.2 mV to 8.0 ± 0.3mV in wild-type and from 4.4 ± 0.2mV to 7.8 ± 0.5mV in the GluR1 knockout, approximately an 80 % increase (Figure 5.1C).

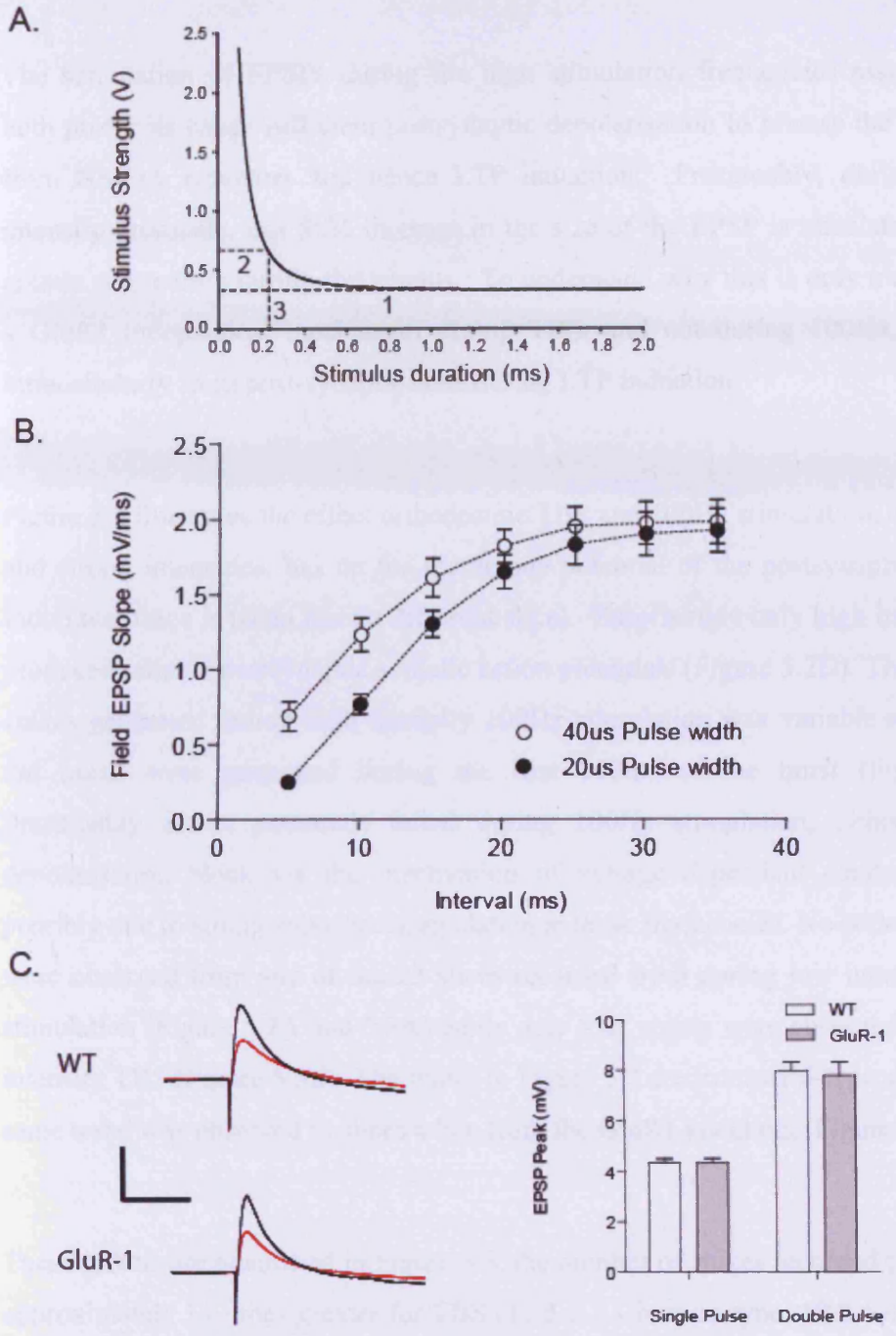


Figure 5.1 The effect of doubling the pulse width on synaptic responses.
A, Example of a strength-duration curve, the rheobase (line 1) is the minimum stimulus strength that will produce a response. This is the voltage to which the strength-duration curve asymptotes. In the example above, this value is 0.35 V. The chronaxie, is the stimulus duration that yields a response when the stimulus strength is set to exactly 2 x rheobase (line 2). In the example above, the chronaxie is 0.22 ms (line 3) adapted from; http://www.unm.edu. B,IO curves at low (20μs pulse width) and high intensity pulses (40μs pulse width). C, Intracellular responses generated by a stimulus at 40% of max, using a 20μs pulse width (red) or a 40μs pulse width (black). Bars indicate the group data, no difference in the evoked response is seen at this stimulation intensity between wild-types or GluR-1 knockouts.

The summation of EPSPs during the high stimulation frequencies associated with both protocols cause sufficient postsynaptic depolarisation to release the Mg^{2+} block from NMDA receptors and hence LTP induction. Presumably, during the high intensity protocols, this 80% increase in the size of the EPSP is translated into even greater summation during the tetanus. To understand why this is only translated into a GluR1 independent mechanism during TBS, and not during 100Hz, I recorded intracellularly from post-synaptic cells during LTP induction.

Figure 5.2 illustrates the effect orthodromic TBS and 100Hz stimulation, at both weak and strong intensities, has on the membrane potential of the postsynaptic cell (each individual trace is taken from a different slice). Surprisingly only high intensity TBS produced reliable postsynaptic somatic action potentials (Figure 5.2D). The number of spikes generated during high intensity 100Hz stimulation was variable and any that did occur were generated during the first 200ms of the burst (Figure 5.2C). Presumably action potentials failed during 100Hz stimulation, either due to a depolarisation block via the inactivation of voltage dependant conductance's, or possibly due to strong spike accommodation at these frequencies. No action potentials were observed from any of the 23 slices recorded from during low intensity 100Hz stimulation (Figure 5.2A and 5.3A) while only four spikes were observed during low intensity TBS (Figure 5.3B). The traces in Figure 5.2 are from wild-type slices but the same trend was observed in slices taken from the GluR1 knockout (Figure 5.3).

These effects are quantified in Figure 5.3, the number of spikes recorded per train was approximately 13 times greater for TBS (17.5 ± 3.1 in wild-type, 19.2 ± 4.3 in GluR1 knockout) than for 100Hz stimulation (1.3 ± 0.6 in wild-type, 2.8 ± 1 in GluR1 knockout), using the same intensity stimulation in each case (40% of maximum fEPSP I/O response, 40μs pulse-width). The data did not pass the normality test required for parametric ANOVAs, so a Kruskal-Wallis nonparametric ANOVA was used to show that this difference was highly significant (wild-type, $n = 16$, $p < 0.0001$, GluR1, $n = 16$, $p < 0.0001$). Fewer stimulus pulses are given during TBS trains (40 pulses per train) compared to 100 Hz trains (100 pulses per train), I therefore also

calculated the probability of an action potential being generated per stimulus. This calculation showed that, when the number of stimuli given during the train was taken into account, the difference in probability of spike generation during theta burst in comparison to 100Hz stimulation was even greater. The spike probability (per stimulus) was 40 fold greater for TBS (44 ± 8% in wild-type, 53 ± 6% in GluR1 knockout) than for 100Hz stimulation (1 ± 6% in wild-type, 2 ± 7% in GluR1 knockout). Consequently, applying more presynaptic stimuli during a 100Hz protocol actually produced many times fewer spikes than with the TBS protocol.

On average, TBS using a double pulse-width produced more than one spike per burst (mean = 1.75) and therefore essentially produced a number of complex spikes in the post-synaptic cells (Figure 5.2D). From the 44 cells recorded there were on average four complex bursts per train and all showed at least one burst per train. Single pulse-width TBS produced far fewer spikes (mean = 0.22 spikes per burst) and rarely produced complex spikes (2 from 16 cells).

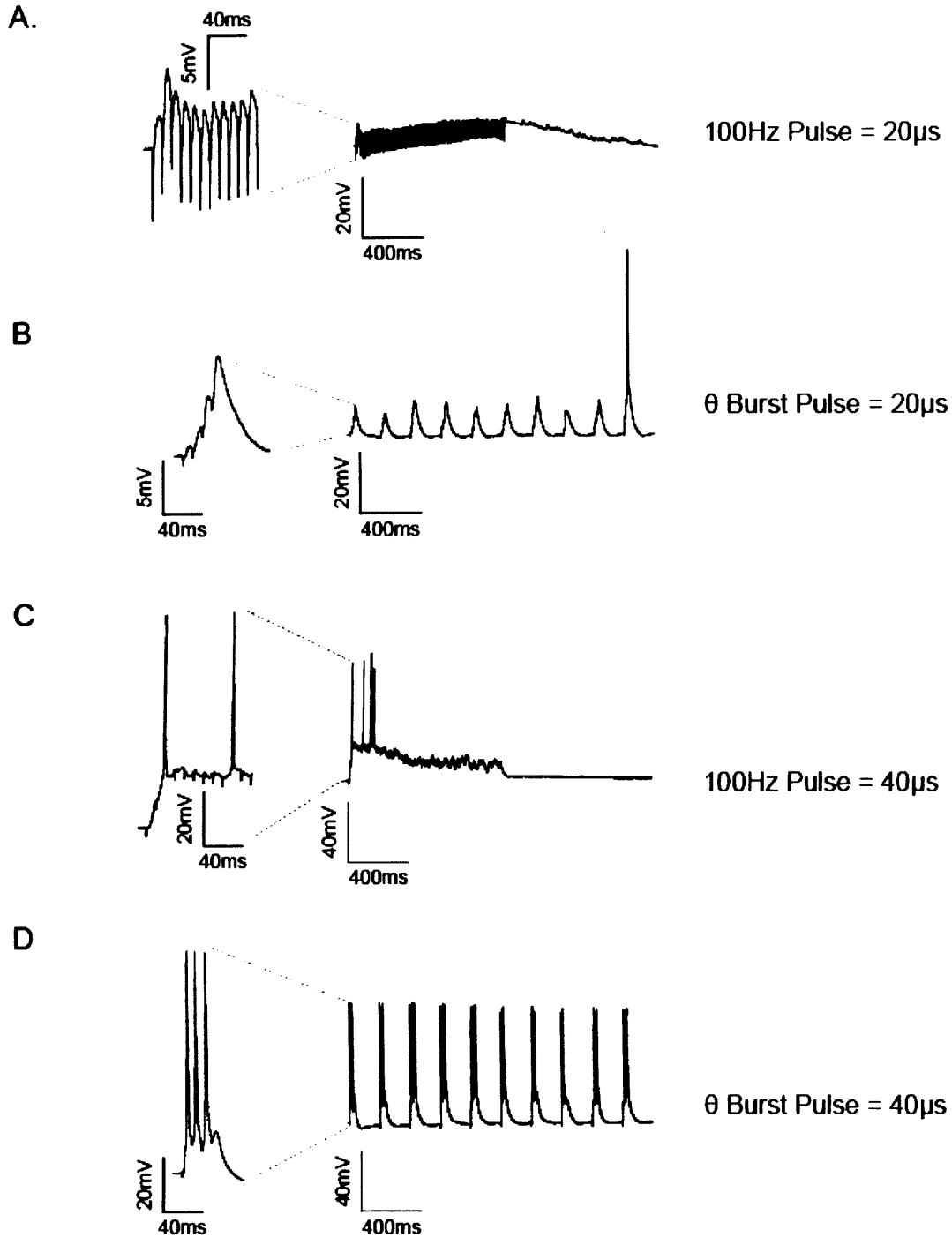


Figure 5.2 Number of spikes during induction differs significantly between protocols. Example traces of intracellular recording during A, HFS at low intensity, B, TBS at low intensity, C, HFS at high intensity, D, TBS at high intensity. Left (panels) are the first 100ms of each burst.

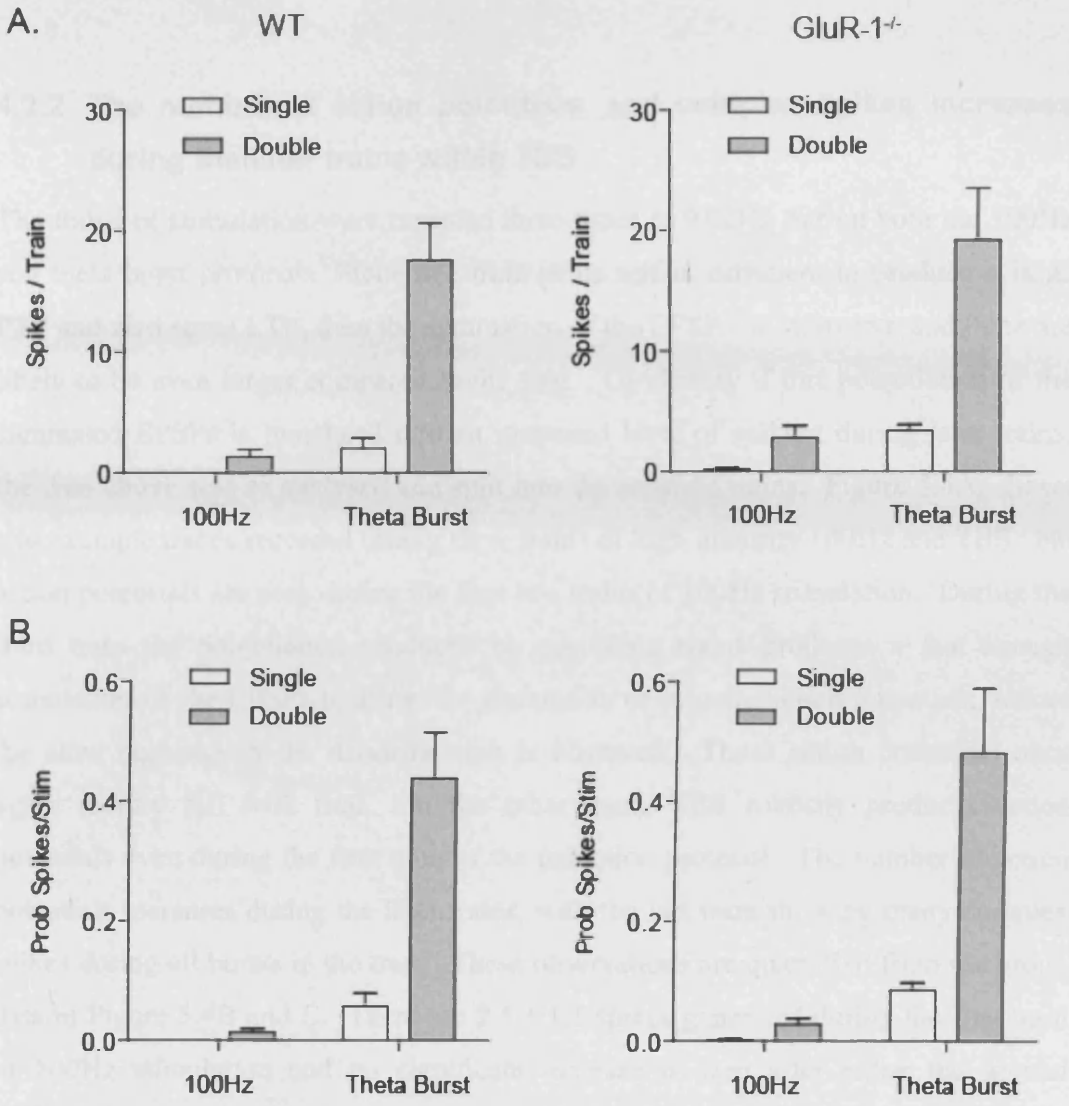


Figure 5.3 Number of spikes during induction differs significantly between protocols.
A. The total number of spikes per train is significantly greater during high intensity (grey bars) TBS compared to low intensity (white bars) HFS , high intensity HFS or low intensity TBS. The same trend is observed in the GluR-1 knockouts (right). B, Probability of generating a spike per stimulus applied is significantly greater during high intensity (grey bars) TBS compared to low intensity HFS (white bars), high intensity HFS and low intensity TBS and again this is the same in the GluR-1 knockouts (right).

4.2.2 The number of action potentials, and complex spikes increases during the later trains within TBS.

The trains of stimulation were repeated three times at 0.02Hz during both the 100Hz and theta burst protocols. Since one train in its self is sufficient to produce a large PTP and also some LTP, then the summation of the EPSPs in trains two and three are likely to be even larger compared to the first. To identify if this potentiation of the summated EPSPs is translated into an increased level of spiking during later trains, the data above was re analysed and split into the separate trains. Figure 5.4A, shows two example traces recorded during three trains of high intensity 100Hz and TBS. No action potentials are seen during the first two trains of 100Hz stimulation. During the third train the potentiation produced by preceding trains produces a fast enough summation of the EPSPs to allow the generation of somatic action potentials, before the slow decrease in the depolarisation is observed. These action potentials once again rapidly fail with time. On the other hand, TBS robustly produces action potentials even during the first train of the induction protocol. The number of action potentials increases during the later trains, with the last train showing many complex spikes during all bursts in the train. These observations are quantified from the group data in Figure 5.4B and C. There are 2.5 ± 1.3 spikes generated during the first train of 100Hz stimulation and no significant increase is seen after either the second (3.6 ± 2.4) or third (4.9 ± 2.4) trains (Kruskal-Wallis nonparametric ANOVA, $KW_{(33)} = 3.655$, $p > 0.05$). However, there is a highly significant increase in the number of spikes after successive trains of TBS ($KW_{(33)} = 46.58$, $p < 0.0001$). There are twice the number of spikes in the second train (12.3 ± 3.1) and 5 times as many in the last train (27.4 ± 6.2) compared to the first (5.2 ± 1.3).

This huge increase in the number of action potentials is also reflected by the 10 fold increase in the number of complex spikes (ISI < 7ms) seen during later trains of TBS (1^{st} train = 1.0 ± 0.5, 3^{rd} train = 9.8 ± 0.8, $KW_{(33)}=45.9$, $p < 0.0001$, Figure 5.3C). High intensity 100 Hz stimulation on the other hand rarely produced any complex spikes (0.8 ± 0.4 per train) and only a slight increase in their occurrence was observed during later trains (1^{st} train = 0.2 ± 0.9, 3^{rd} train = 1.3 ± 0.3, $KW_{(33)}=13.1$, $p < 0.01$, Figure 5.4C).

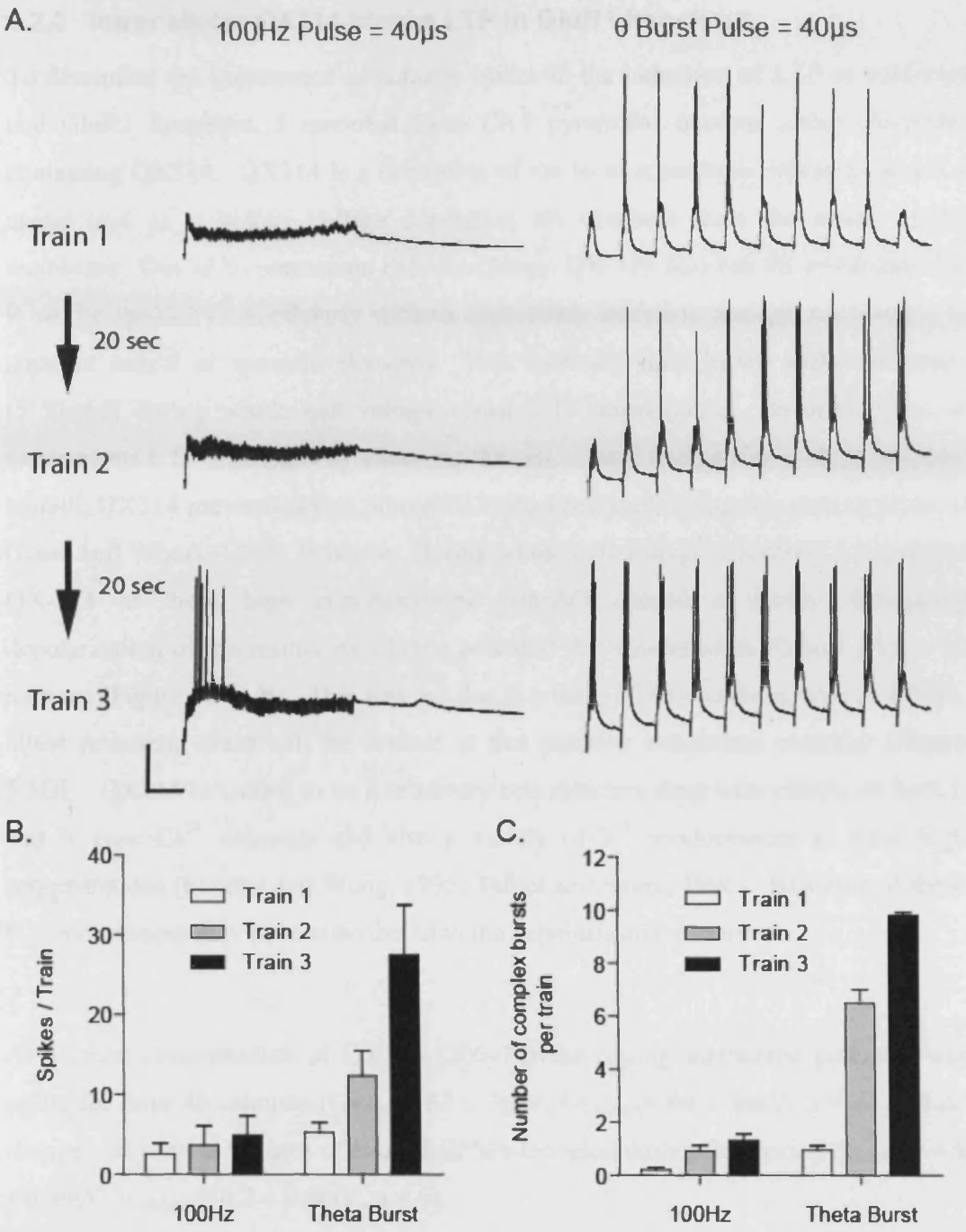


Figure 5.4 The number of action potentials, and complex spikes increases during the later trains of TBS.

A. An example trace showing the intracellular responses that are generated during the three trains of high intensity 100Hz stimulation (left) and TBS (right). TBS robustly produces action potentials even during the first train of the induction protocol. The number of action potentials increased during the later trains, with the last train showing many complex spikes during all bursts in the train. B. Average data showing the number of action potentials generated during each train for either high intensity 100Hz or TBS. C, Quantification of the number of complex spikes (ISI < 7 ms) generated during the different protocols and trains.

4.2.3 Intracellular QX314 blocks LTP in GluR1 knockout.

To determine the importance of somatic spikes in the induction of LTP in wild-type and GluR1 knockout, I recorded from CA1 pyramidal neurons using electrodes containing QX314. QX314 is a derivative of the local anaesthetic lidocaine and is a useful tool as it blocks voltage dependent Na channels from the inside of the membrane. Due to its permanent cationic charge, QX-314 also has the advantage that it can be applied intracellularly without appreciable diffusion through membranes to adjacent neural or synaptic elements. It is routinely used in the high mM range (5-50mM) during whole cell voltage clamp LTP experiments. In these types of experiments LTP is induced by clamping the cell at 0mV and pairing with presynaptic stimuli, QX314 prevents action potentials being generated during this pairing protocol (Isaac and Wheal, 1993). However, during whole cell voltage recording, I found that QX-314 at these high concentrations (10mM) caused a slowly developing depolarisation of the resting membrane potential that plateaued at +20mV after ~ 40 minutes (Figure 5.5 A B). This was not due to a toxic effects on the neuron as EPSPs, albeit reversed, could still be evoked at this positive membrane potential (Figure 5.5D). QX314 is known to be a relatively non selective drug with effects on both L and N type Ca^{2+} channels and also a variety of K^+ conductances at these high concentrations (Perkins and Wong, 1995; Talbot and Sayer, 1996). Blocking of these K^+ conductances may have contributed to the depolarisation observed.

At a lower concentration of QX314 (200μM) the resting membrane potential was stable for over 40 minutes ($t_{0\text{-}5min}$ = 67 ± 3mV, $t_{35\text{-}40min}$= 64 ± 6mV, n = 6) and no change was seen in the size of evoked EPSPs recorded during this period ($t_{0\text{-}5min}$ = 4.8 ± 0.3mV, $t_{35\text{-}40min}$= 4.2 ± 0.5mV, n = 6).

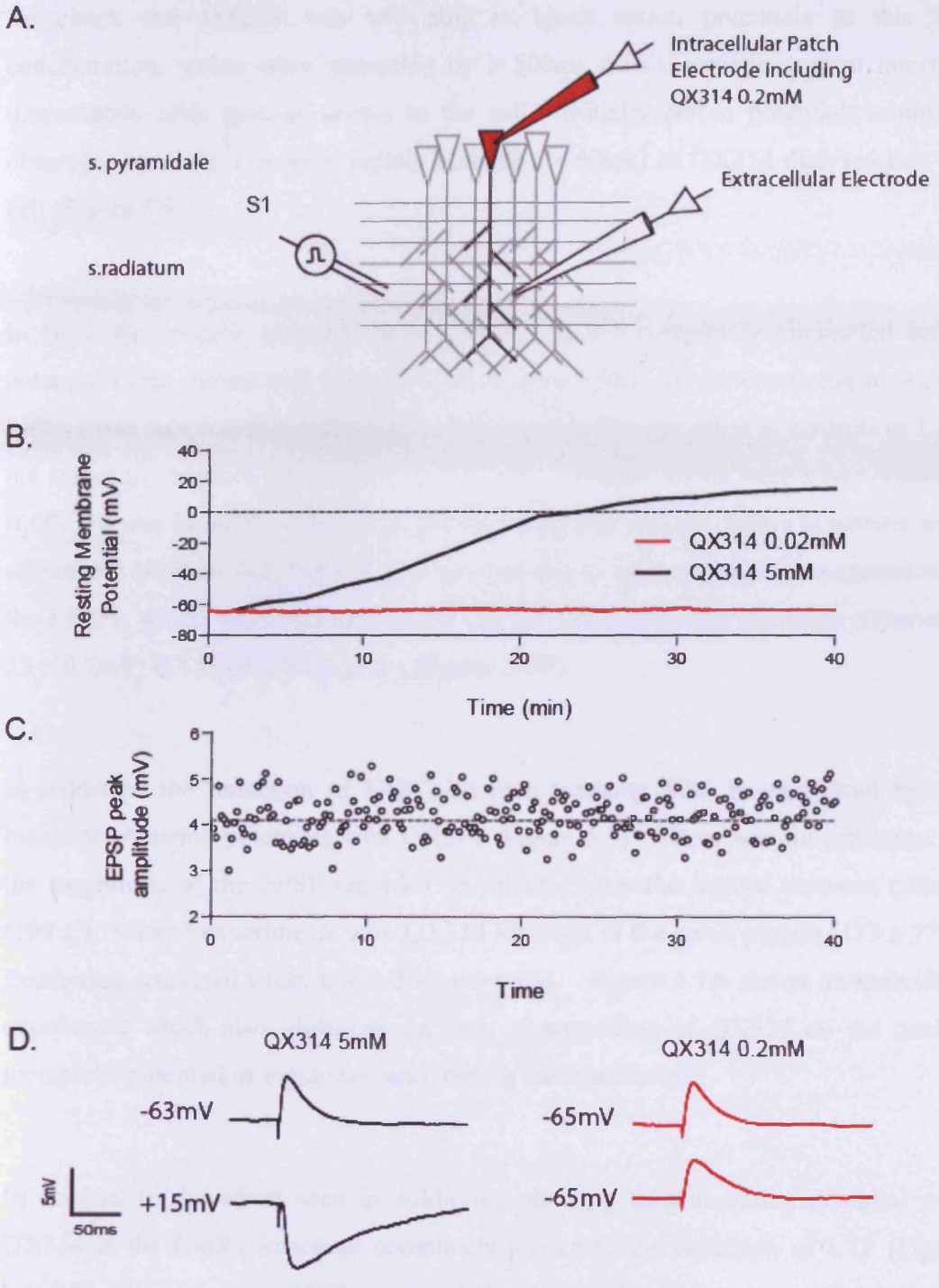


Figure 5.5 Effects of QX314 included in the patch pipette.
A. Diagram showing the experimental set up, QX314 0.2mM was included in the patch pipette, and dual intracellular and extracellular recordings were performed. B, 5mM QX314 caused a slowly developing reversal of the membrane potential in voltage recordings, lowering the concentration to 0.2mM prevented this effect. C, Evoked EPSPs were stable for over 40 minutes if 0.2mM QX314 was included in the patch pipette. D, Example traces taken during the first 5 minutes (top) and after 45 minutes (bottom) using either 5mM (left) or 0.2mM QX314 (right).

To check that QX314 was still able to block action potentials at this low concentration, spikes were generated by a 500ms 2.5nA somatic current injection immediately after gaining access to the cell. Initially action potentials could be observed, however they were rapidly blocked (> 60sec) as QX314 dialysed into the cell (Figure 5.6A).

In 10 of the 19 cells recorded from, 200μM QX-314 completely eliminated action potential firing during high intensity TBS (Figure 5.6B). In the remaining nine cells spiking was substantially reduced from 19.2 ± 4.3 spikes per burst in controls to 1.5 ± 0.4 spikes per burst in cells containing QX314. Complex bursts were never observed if QX314 was included in the patch pipette. Although somatic action potentials were effectively blocked with QX314 this was not due to lack of temporal summation of the EPSPs, which were unaffected with QX314 included in the electrode (Control = 22 ± 0.7mV; QX314 = 23 ± 1.1mV) (Figure 5.6C).

In wild-type the induction of LTP with high intensity TBS is unaffected by the blockade of action potentials with QX314 (Figure 5.7). There was no difference in the magnitude of the EPSP recorded 45 minutes after the tetanus between control (199 ± 17%) and experiments with QX314 included in the patch pipette (173 ± 27%, Bonferroni corrected t-test, $t_{(19)} = 0.96$, $p > 0.05$). Figure 5.7A shows an individual experiment which also illustrates the lack of any effect of QX314 on the resting membrane potential or input resistance during the experiment.

In contrast to the effect seen in wild-type, blocking somatic action potential with QX314 in the GluR1 knockout completely prevented the induction of LTP (Figure 5.8, 5.9). The size of the EPSP measured 45 minutes after the tetanus (103 ± 3%) was not significantly different from pre tetanic control values (99 ± 1% paired t-test $t_{(18)} = 0.77$, $p > 0.05$). The experiments on the different genotypes with and without QX314 were interleaved and therefore a 2 way ANOVA was used to identify if the effect was significant. The results of the ANOVA show that there was a highly significant interaction between QX314 and genotype ($F_{(1,74} = 10.05$, $p < 0.001$), a post hoc Bonferroni corrected t-test revealed that this was because the GluR1

knockout only showed significant LTP in the absence of QX314 ($t_{(18)} = 3.03$, $p < 0.01$), while wild-type showed LTP irrespective of the presence of QX314 ($t_{(19)} = 1.68$, $p > 0.05$).

As QX314 is membrane impermeable, LTP should only be blocked in the cell being recorded from, and hence responses recorded from the overall population should still be potentiated. This would provide a convenient inter experimental control to show that LTP could have been generated within the slice if somatic spikes had not been blocked. I tested this prediction by simultaneously recording intracellular EPSPs and extracellular fEPSP. Figure 5.8 shows an example experiment with dual intra- and extra-cellular recordings in GluR1 knockout. High intensity TBS was given at time zero and induced a slowly developing potentiation of the extracellular recorded fEPSP (Figure 5.8A). However, there was no increase in the simultaneously recorded intracellular EPSP if somatic spikes were blocked with QX314 (Figure 5.8B). Figure 5.9 shows the group data, while the potentiation of the fEPSP measured 45 minutes after the tetanus (167 ± 3%) was significantly different from the pre-tetanic control (102 ± 3%, paired t-test $t_{(6)} = 1.97$, $p < 0.05$), no significant increase in the intracellularly recorded EPSP was observed (pared t-test $t_{(18)} = 0.77$, $p > 0.05$).

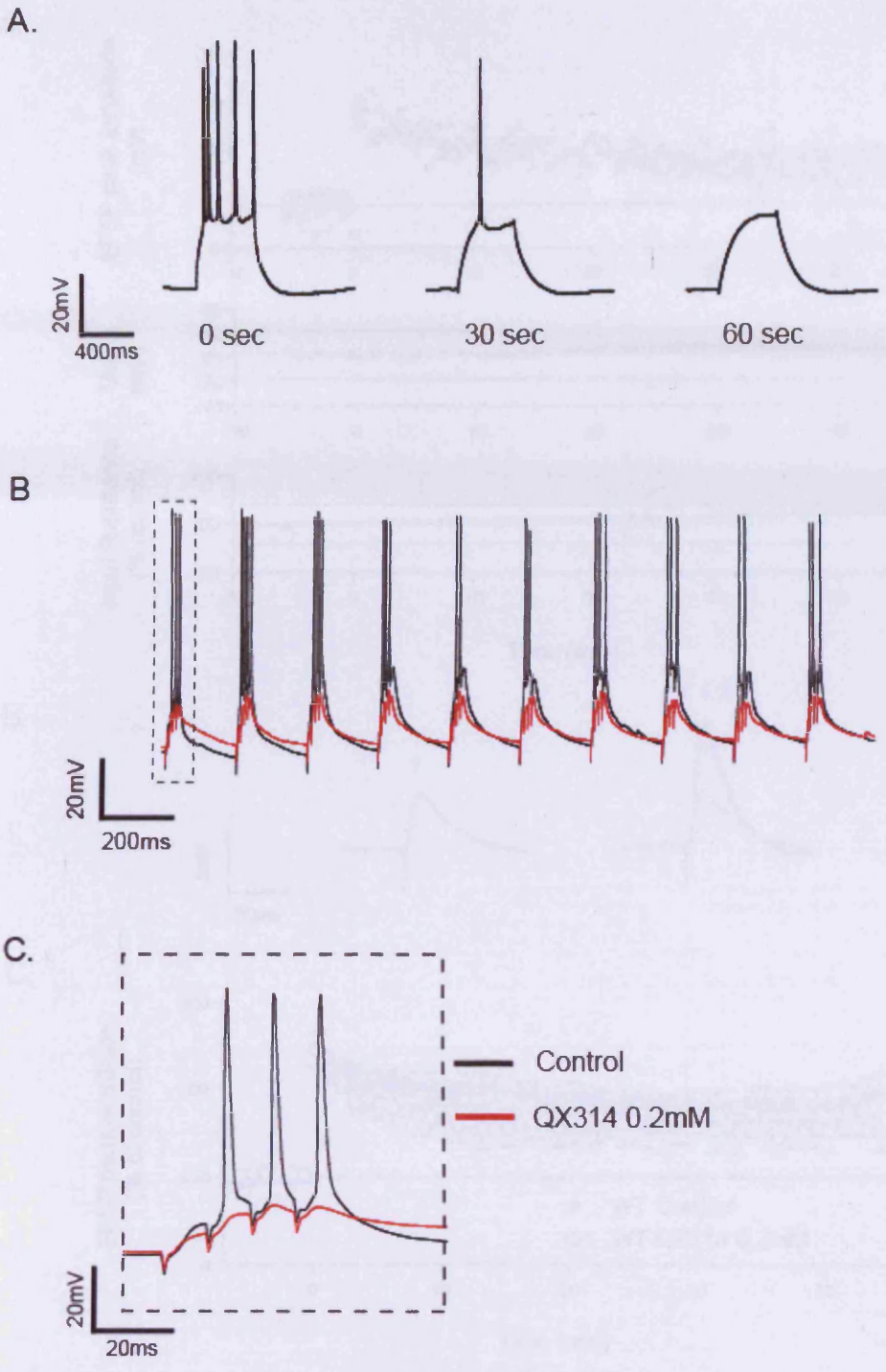


Figure 5.6 QX314 0.2mM blocks somatic action potentials.
A, Spikes generated by depolarizing current injection (2.5nA, 500ms), are blocked by QX314 (0.2mM). B, Spiking during high intensity TBS (black line) is blocked when QX314 is included in the patch electrode (red line). C, EPSP summation is unaffected by the inclusion of QX314 in the electrode (control black line, QX314 red line).

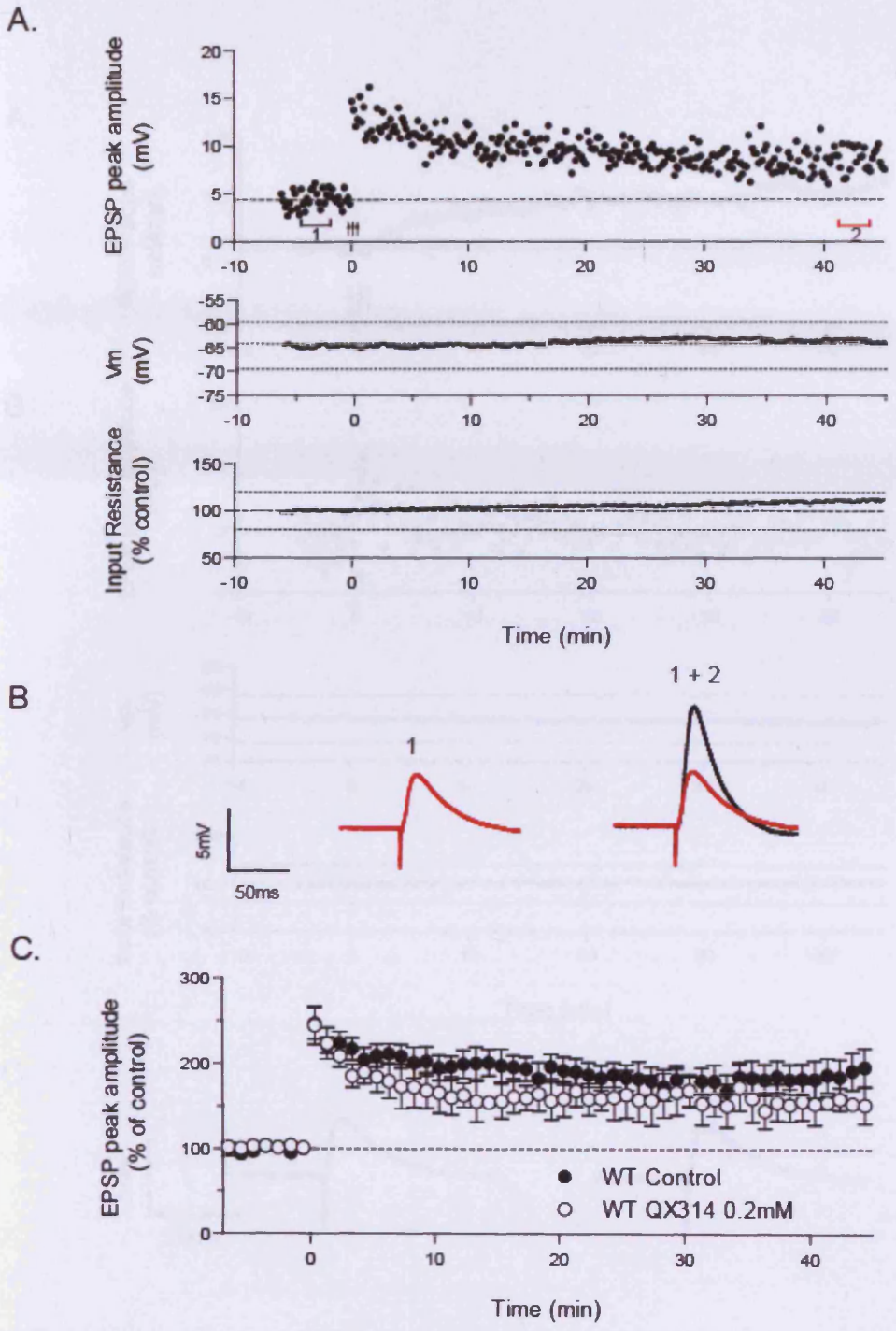


Figure 5.7 Blocking action potentials with QX314 0.2mM reduces but does not block the induction of LTP in wild-type.
A, Example experiment in wild-types with QX314 included in the patch pipette. B, Example traces at time points indicated by the bars. C, Averaged data showing that LTP is reduced compared to control (•) (n =10) but can still be induced if somatic action potentials are blocked with QX314 (○) (n = 10).

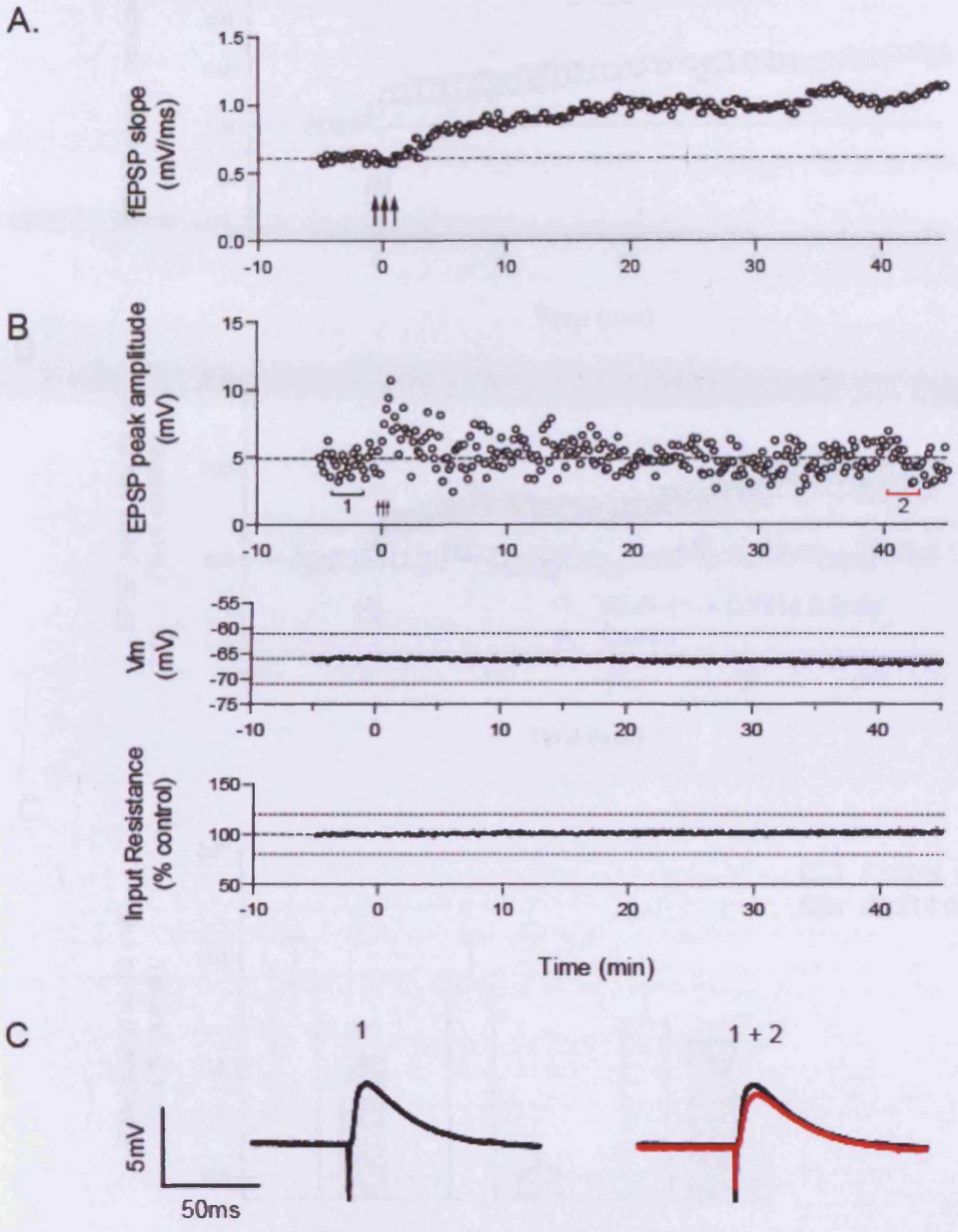


Figure 5.8 Dual field and intracellular recordings show that QX314 blocks the plasticity in GluR-1 knockout (example experiment).
Example experiment of a simultaneously recorded intracellular and extracellular EPSP in slices from GluR-1 knockout. A, The field response is potentiated by the TBS, indicating that plasticity was possible in the slice. B, If somatic spikes are blocked in a single cell with QX314 included in the patch pipette then LTP can not be induced in the GluR-1 knockout. C, Example trace of intracellular recorded EPSPs at times indicated by the bars.

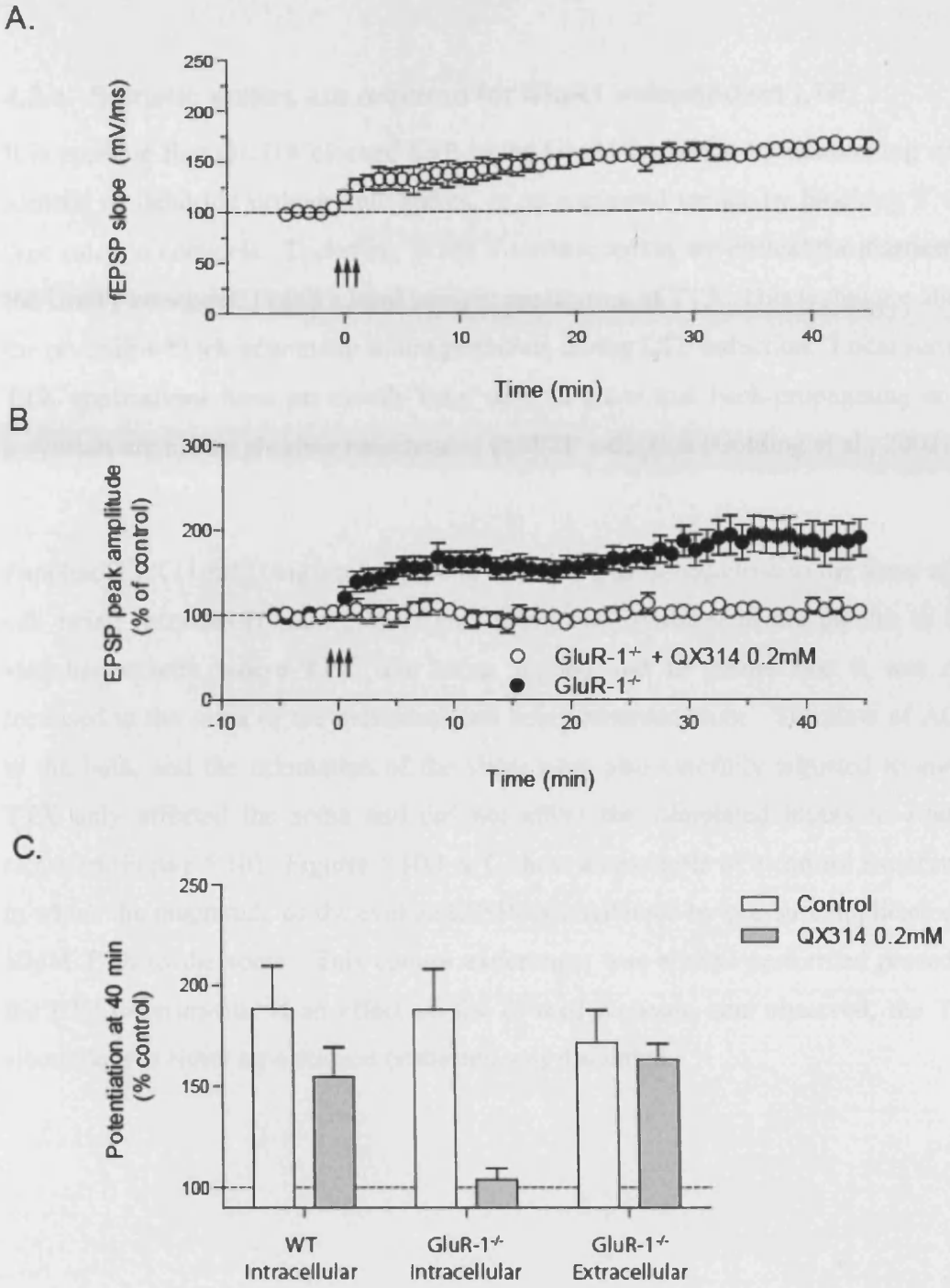


Figure 5.9 Dual field and intracellular recordings show that QX314 blocks plasticity in GluR-1 knockout (group data).
A, Control field recording experiment showing that LTP was possible in the slice if somatic spikes are blocked in a single postsynaptic cell (n = 7). B, Postsynaptic blockade of somatic action potentials with QX314 completely prevented the induction of LTP in the GluR-1 knockout (n = 9). C, Bars showing the magnitude of the potentiation at 45 minutes after the TBS in dual intracellular and extracellular recordings with (open bars) and with out (grey bars) somatic spikes blocked

4.2.4 Somatic spikes are required for GluR1 independent LTP.

It is possible that QX314 blocked LTP in the GluR1 knockout by eliminating either somatic or dendritic orthodromic spikes, or as suggested earlier by blocking T or L type calcium channels. Therefore, to see if somatic spikes are critical for plasticity in the GluR1 knockout, I used a local somatic application of TTX. This technique allows the reversible block of somatic action potentials during LTP induction. Local somatic TTX applications have previously been used to show that back-propagating action potentials are not an absolute requirement for LTP induction (Golding et al., 2002).

I applied TTX (10μM) via a micropipette carefully positioned close to the soma of the cell being recorded (Figure 5.10). Fast green was included in the pipette to help visualise exactly where TTX was being applied and to ensure that it was only localised to the soma of the individual cell being recorded from. The flow of ACSF in the bath, and the orientation of the slice, were also carefully adjusted to ensure TTX only affected the soma and did not affect the stimulated inputs to stratum radiatum (Figure 5.10). Figures 5.10B & C show an example of a control experiment in which the magnitude of the evoked EPSPs is unaffected by pressure application of 10μM TTX to the soma. This control experiment was always performed preceding the LTP experiments. If an effect on the evoked response was observed, the TTX electrode was either repositioned or the cell was discarded.

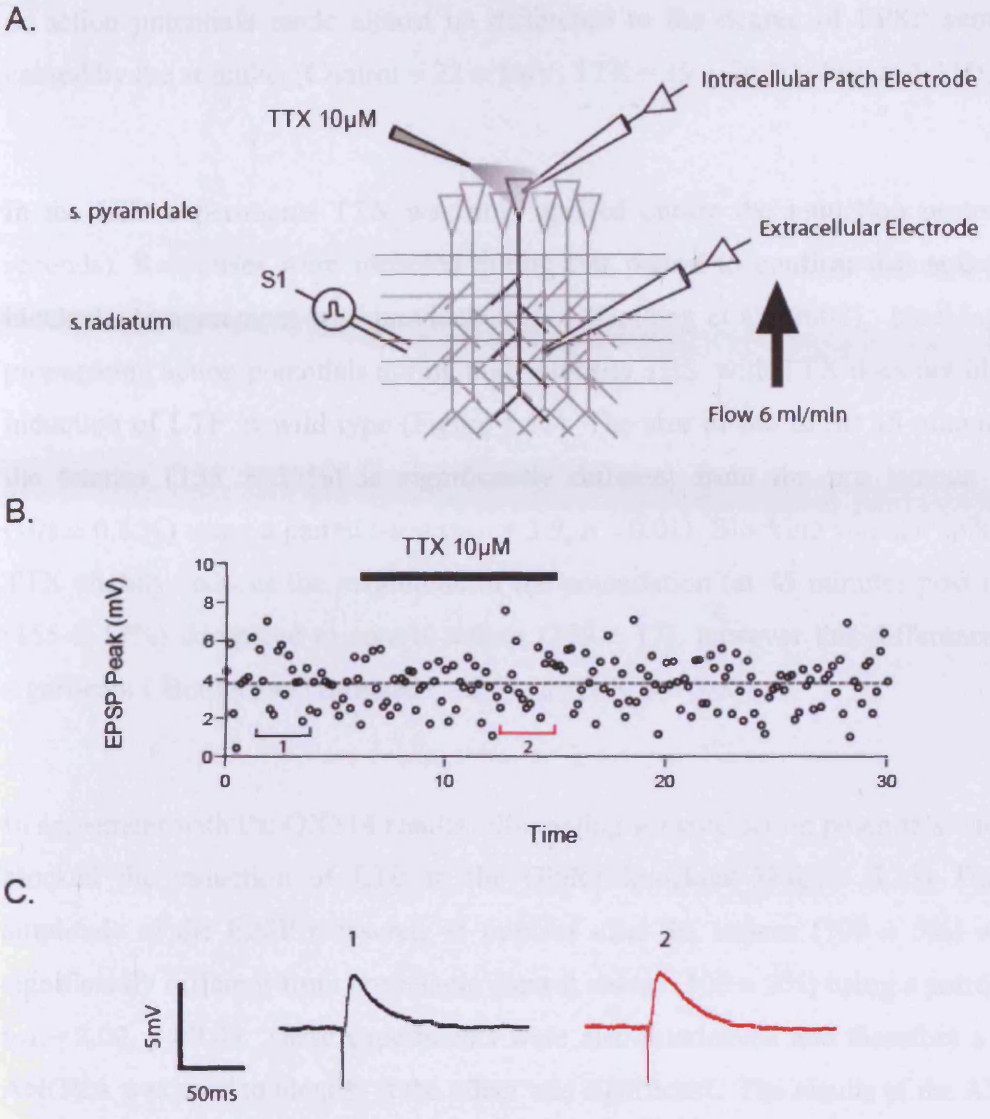


Figure 5.10 Local somatic TTX applications.
A, Schematic diagram of the experimental set up. Action potential generation and propagation can be blocked by local pressure application of TTX (10μM) to the soma. The slice is positioned so that TTX does not perfuse on the *stratum radiatum*. B, Local somatic perfusion of TTX does not affect the magnitude of evoked EPSPs. TTX applied at time indicated by the bar. C, Example traces showing that the size and time course of the evoked EPSP is unaffected by local TTX applications.

Local TTX application at the soma was able to block action potentials induced by a 500ms 2.5nA somatic current injection (Figure 5.11A). This block could be reversed if the pressure application of TTX was stopped, it took between 2-3 minutes for the TTX to fully wash out and spiking to resume. Somatic action potentials were also fully eliminated during high intensity TBS (Figure 5.11B). Once again the blocking

of action potentials made almost no difference to the degree of EPSP summation caused by the stimulus (Control = 22 ± 1mV, TTX = 19 ± 2mV) (Figure 5.11B, C).

In the LTP experiments TTX was only applied during the induction protocol (60 seconds). Responses were recorded during this period to confirm that spikes were blocked. In agreement with previous studies (Golding et al., 2002), blocking back-propagating action potentials during high intensity TBS with TTX does not block the induction of LTP in wild-type (Figure 5.12). The size of the EPSP 45 minutes after the tetanus (155 ± 17%) is significantly different from the pre tetanus control (101 ± 0.8 %) using a paired t-test ($t_{(11)} = 3.9$, $p < 0.01$). Blocking somatic spikes with TTX slightly reduces the magnitude of the potentiation (at 45 minutes post tetanus) (155 ± 17%) compared to control values (199 ± 17), however this difference is not significant (Bonferroni corrected t –test $t_{(34)} = 1.8$, $p > 0.05$).

In agreement with the QX314 results, eliminating somatic action potentials with TTX blocked the induction of LTP in the GluR1 knockout (Figure 5.13) The peak amplitude of the EPSP measured 45 minutes after the tetanus (109 ± 5%) was not significantly different from pre-tetanic control values (105 ± 2%) using a paired t-test $t_{(14)} = 2.02$, $p > 0.05$. These experiments were also interleaved and therefore a 2 way ANOVA was used to identify if the effect was significant. The results of the ANOVA show that there was a significant interaction between TTX treatment and genotype ($F_{(1,64)} = 5.08$, $p < 0.05$). A post hoc Bonferroni corrected t-test revealed that this was because the GluR1 knockout only showed significant LTP in the absence of TTX ($t_{(36)} = 5.25$, $p < 0.001$), while wild-type showed LTP irrespective of the presence of TTX ($t_{(35)} = 1.68$, $p > 0.05$).

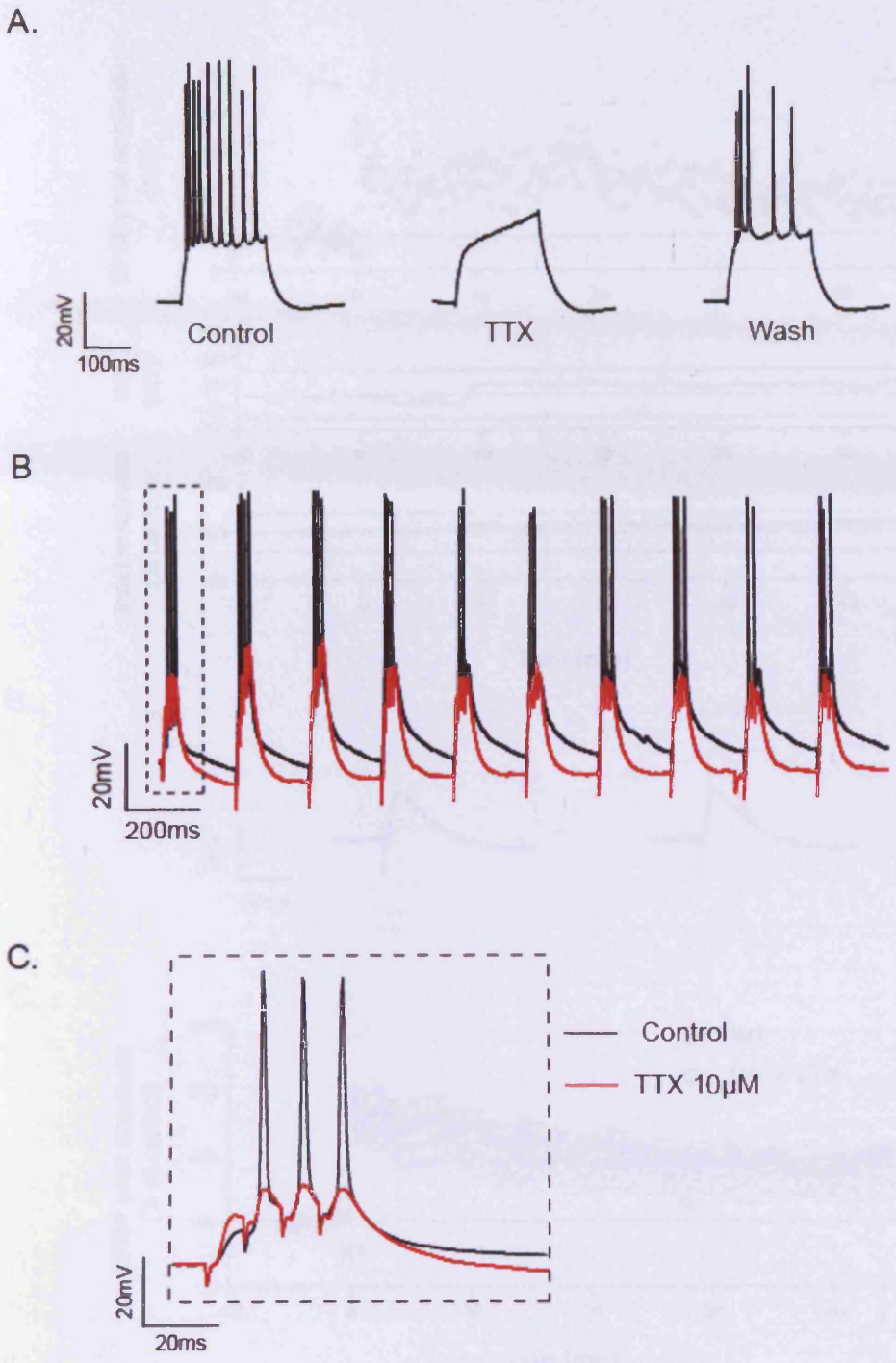


Figure 5.11 Local TTX blocks somatic action potentials.
A, Example trace illustrating how spikes generated by a depolarizing current injection (2.5nA, 500ms), can be reversibly blocked by the local somatic application of TTX (10μM). B, Spiking during high intensity TBS (black line) is blocked if TTX is perfused on the soma (red line). C, EPSP summation during the TBS is unaffected by somatic TTX application.

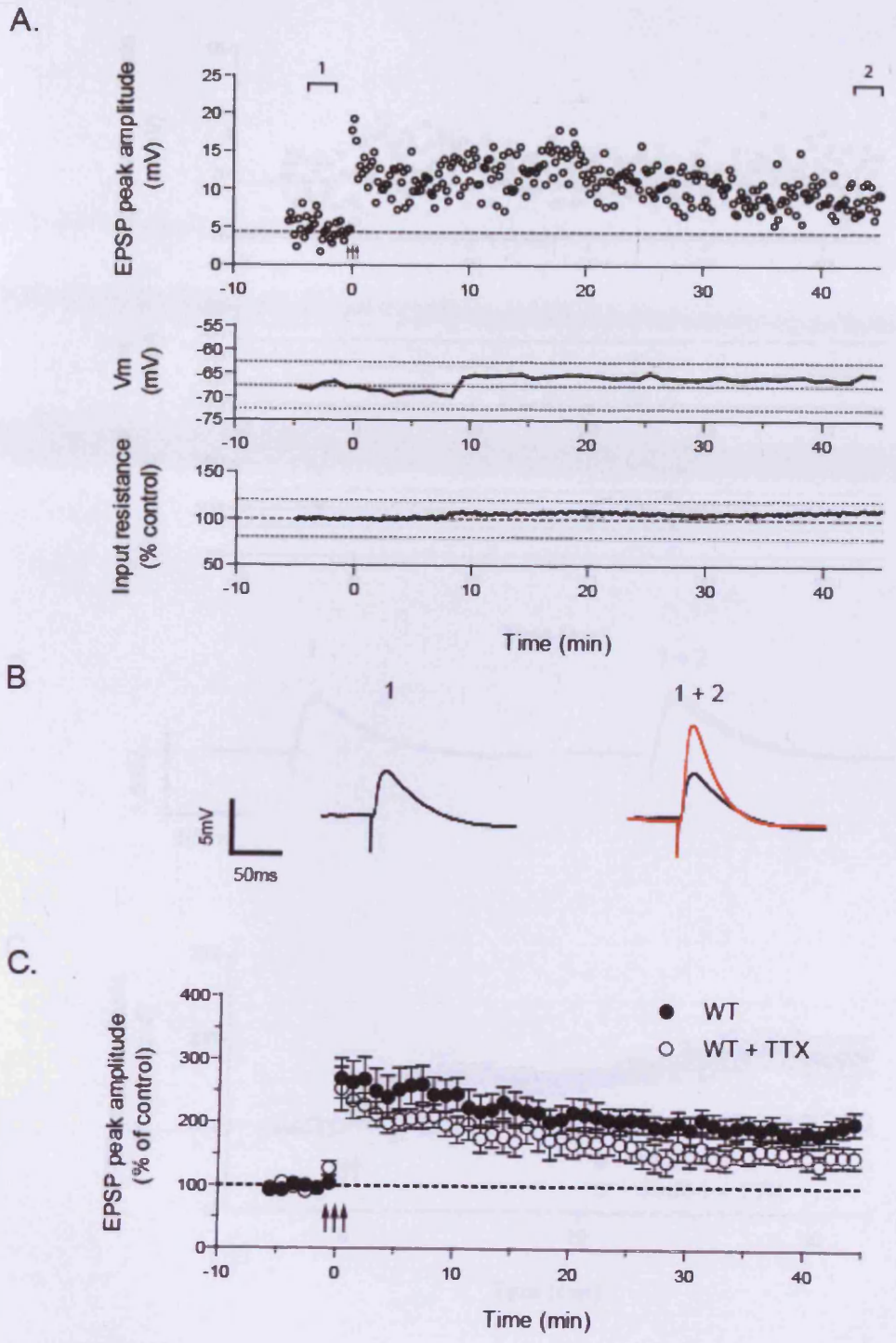


Figure 5.12 Blocking action potentials with TTX reduces but does not block the induction of LTP in wild-type.

A, Example experiment in wild-type with somatic spikes blocked during induction with a local TTX application. B, Example traces at time points indicated by the bars. C, Averaged data showing that LTP is reduced compared to control (•) (n=13) but can still be induced if somatic action potentials are blocked with TTX (○) (n = 12).

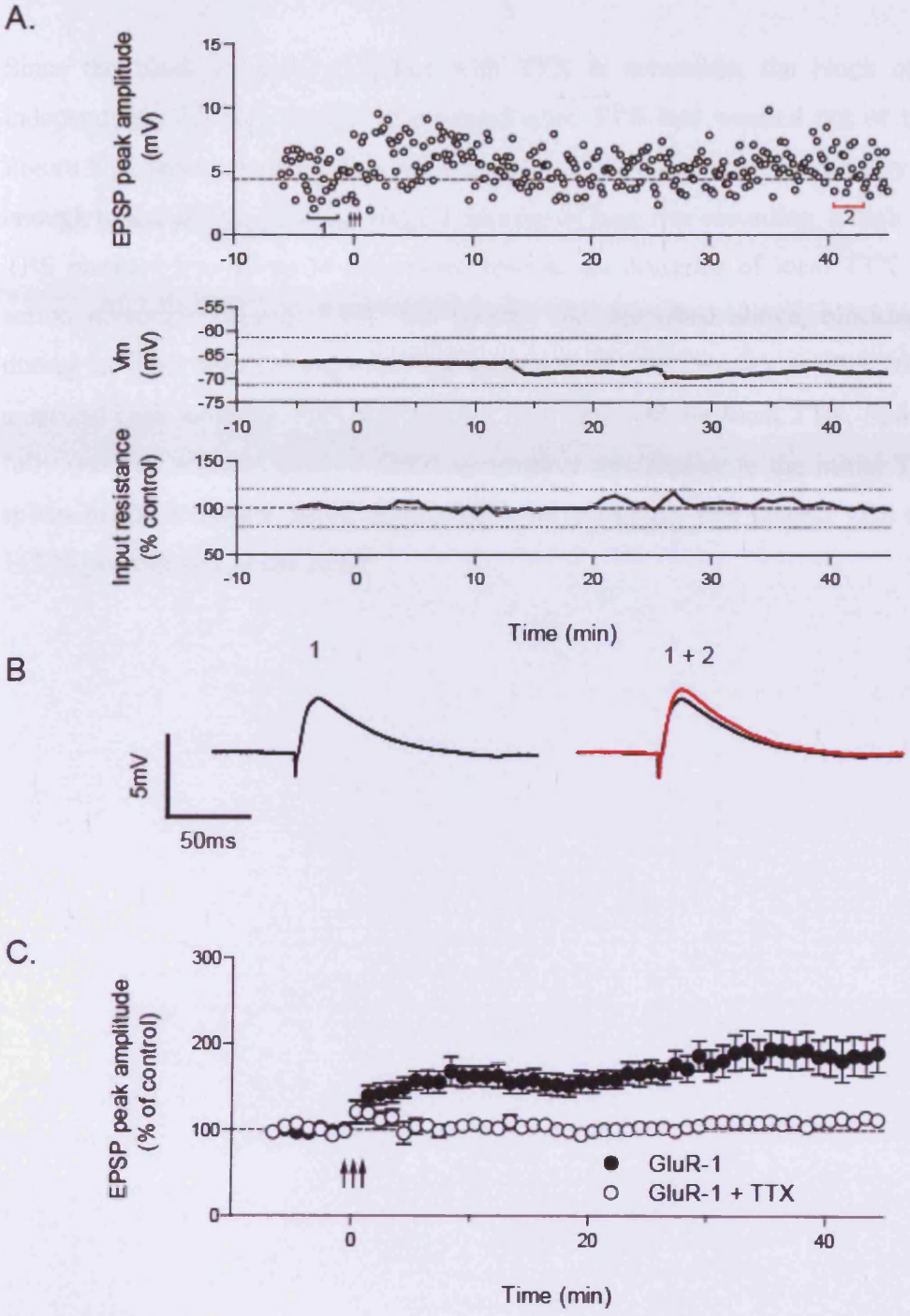


Figure 5.13 Somatic spikes are required for the plasticity in GluR-1 knockout mice.
A, Example experiment in GluR-1 knockout with somatic spikes blocked during induction with a local TTX application. B, Example traces at time points indicated by the bars. C, Averaged data showing that LTP is abolished in the GluR-1 knockouts if somatic action potentials are blocked with TTX (o) (n = 19).

Since the block of somatic spikes with TTX is reversible, the block of GluR1 independent LTP should also be reversed once TTX had washed out of the slice. Figure 5.14 shows an individual experiment in which a cell was kept healthy for long enough to test this prediction. After 5 minutes of base line recording, a high intensity TBS protocol was given at time point zero in the presence of local TTX to block action potentials (Figure 5.14C left panel). As described above, blocking spikes during the TBS protocol prevented the induction of LTP. However, after 20 minutes a second high intensity TBS was applied, this time without local TTX. Spiking was fully restored and the level of EPSP summation was similar to the initial TBS with spikes blocked (Figure 5.14C right panel). As predicted, this second TBS caused a 143 % potentiation of the EPSP.

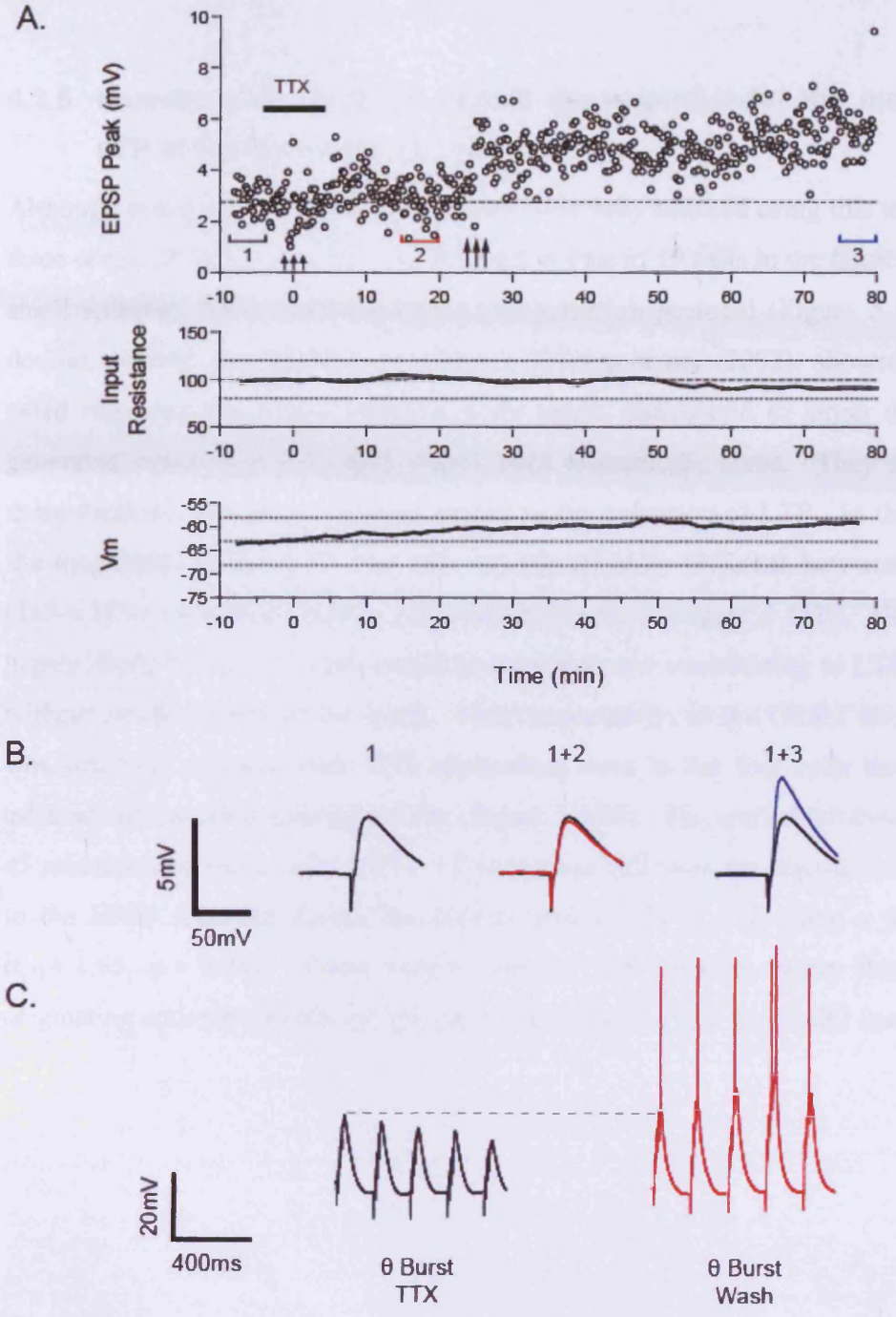


Figure 5.14 An example experiment showing that the TTX dependent block of GluR1 independent LTP is reversible within a slice.
A, EPSP amplitude before and after TBS with and without 10μM TTX. Arrows indicate the time at which the TBS was given, and the bar indicates when TTX was pressure applied. The first TBS was given in the presence of TTX and no change in the EPSP was observed. After approximately 25min a second TBS was applied after wash out of TTX and the EPSP shows potentiation. B, Representative traces taken at time points indicated by the bars. C, Example traces showing that somatic action potential generation during TBS was reversibly blocked during TTX application. There was no difference in the level of EPSP summation.

4.2.5 Somatic over dendritic spikes are essential for the induction of LTP in the GluR1 knock outs.

Although somatic back propagating spikes were fully blocked using this technique, in three of the 12 cells recorded in wild-type and four of 19 cells in the GluR1 knockout, small spikelets were observed during the induction protocol (Figure 5.15). Using double, somatic and dendritic recordings Golding et al., (2002), showed that these small regenerative events, recorded in the soma, correspond to larger dendritically generated events that propagate poorly back towards the soma. They suggest that these dendritic events are strongly related to the induction of LTP. In the wild-type the magnitude of the LTP was also not significantly different between cells with (165 ± 18%) or without (153 ± 12%) dendritic spikes (Figure 5.15B). However it is highly likely that these events could be occurring and contributing to LTP induction, without being evident at the soma. More importantly, in the GluR1 knockout LTP was still fully blocked with TTX application, even in the four cells that did show evidence of dendritic spiking activity (Figure 5.15B). The size of the evoked EPSPs, 45 minutes after the tetanus (105 ± 11%) in these cells was not significantly different to the EPSP recorded during the control period (101 ± 1%) using a paired t-test ($t_{(3)} = 1.65$, $p > 0.05$). These results indicate that somatic, rather than dendritic originating action potentials are essential in inducing LTP in the GluR1 knockout.

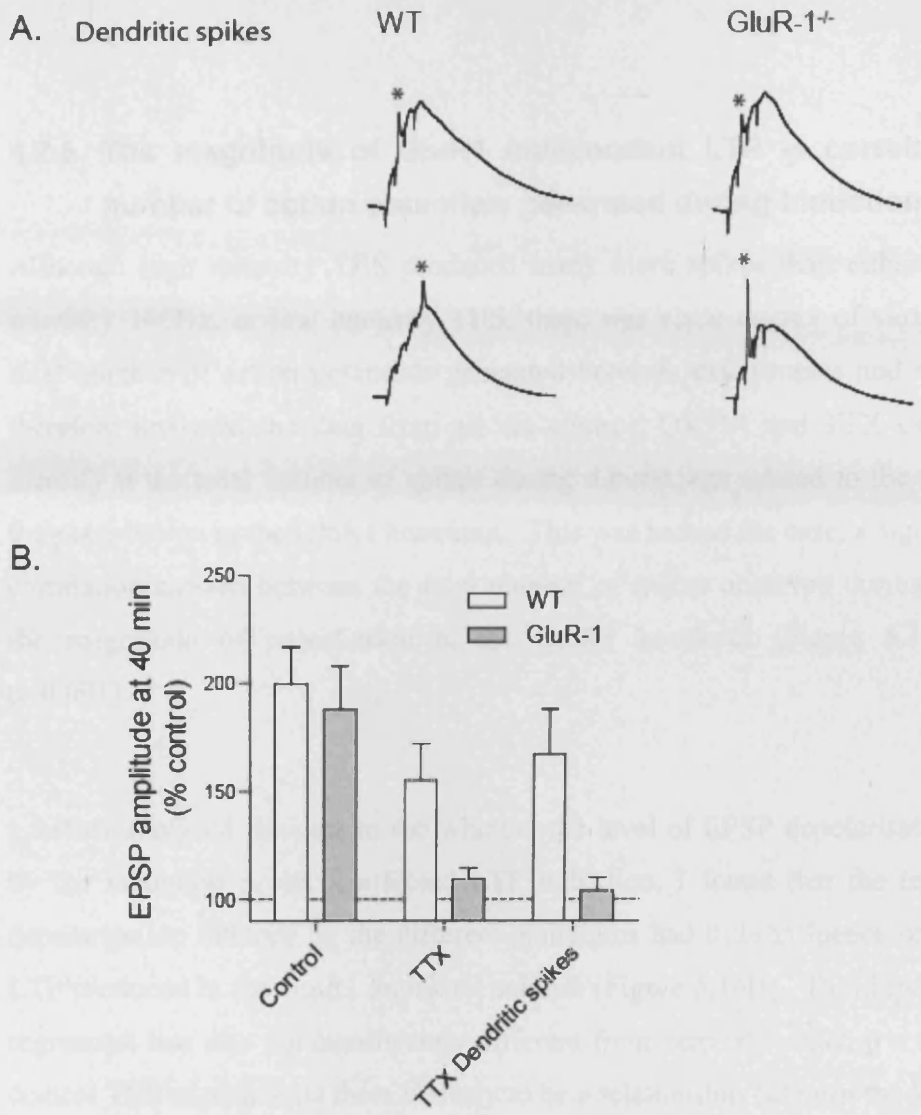


Figure 5.15 Somatic over dendritic spikes are essential for the induction of LTP in the GluR-1 knock outs.
A, Example traces showing the small spikelet's that were observed in a subset of experiments during the TTX applications, in both wild-type (Left) and GluR-1 knock-out (right). These are presumably due to the activation of regenerative events in the dendrites. B, Bars showing that LTP could not be induced in the GluR-1 knockout even if dendritic spikes were present during induction (n = 4).

4.2.6 The magnitude of GluR1 independent LTP is correlated to the number of action potentials generated during induction.

Although high intensity TBS produced many more spikes than either high or low intensity 100Hz, or low intensity TBS, there was some degree of variability in the total number of action potentials generated between experiments and conditions. I therefore analysed the data from all the control, QX314 and TTX experiments to identify if the total number of spikes during a burst was related to the magnitude of the potentiation in the GluR1 knockout. This was indeed the case, a significant linear correlation existed between the total number of spikes observed during a burst, and the magnitude of potentiation in the GluR1 knockout (Figure 5.16A, $r^2=0.81$, $p<0.001$) .

I further analysed the data to see whether the level of EPSP depolarisation produced by the induction protocol affected LTP induction. I found that the level of EPSP depolarisation induced by the different paradigms had little influence on the level of LTP produced in the GluR1 knockout animals (Figure 5.16B). The slope of the linear regression line was not significantly different from zero ($r^2 = 0.02$, $p > 0.05$). In the control TBS experiments there is likely to be a relationship between the level of EPSP depolarisation and the number of action potentials produced. It is valid to exclude these data from the correlation analysis, as spiking was not or at least less dependent on the level of EPSP depolarisation in the TTX or QX314 experiments. If this data is excluded, it becomes even more obvious that the level of depolarisation is not related to the magnitude of LTP in the GluR1 knockout (Figure 5.16C). Of course, the action potentials themselves produced a depolarisation, but this was not an important factor in controlling LTP induction in wild-type, which showed comparable levels of LTP both with and without spikes.

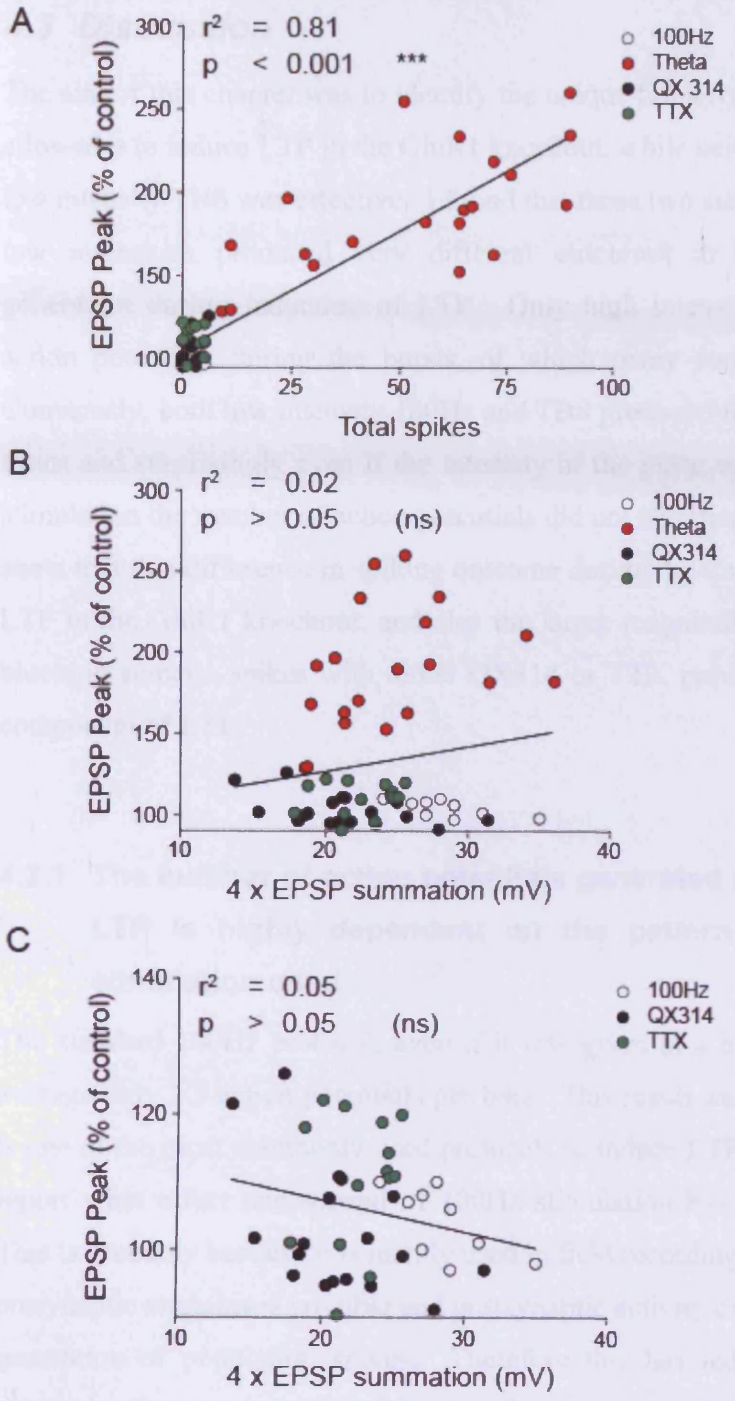


Figure 5.16 The level of potentiation in the GluR-1$^{-/-}$ mice is correlated to the number of spikes observed in the burst and not to the level of depolarization in the burst.
A, Magnitude of LTP in the GluR-1$^{-/-}$ depends on the total number of spikes during the burst. Experiments show a correlation between the number of spikes in a burst and the increase in EPSP observed with LTP. B, Magnitude of LTP in the GluR-1$^{-/-}$ is not correlated with the average amplitude of summated EPSPs during the TBS. C, Same data as shown in C with control TBS data excluded as spiking will be related to the level of depolarization. Data is pooled from LTP experiments generated by high intensity HFS (○), TBS (●) TBS with QX 314 0.2mM (●) and TBS with TTX 10μM (●).

4.3 Discussion

The aim of this chapter was to identify the unique property of high intensity TBS that allowed it to induce LTP in the GluR1 knockout, while neither 100Hz stimulation, nor low intensity TBS was effective. I found that these two standard protocols at high and low intensities produced very different outcomes in terms of action potential generation during induction of LTP. Only high intensity TBS robustly produced action potentials during the bursts, of which many consisted of complex spikes. Conversely, both low intensity 100Hz and TBS produced few, if any spikes during the trains and surprisingly even if the intensity of the pulse was increased during 100Hz stimulation the number of action potentials did not significantly increase. I went on to show that this difference in spiking outcome during the trains was critical in inducing LTP in the GluR1 knockout, and also the larger magnitude LTP in the wild-type, as blocking somatic spikes with either QX314 or TTX prevented the induction of this component of LTP.

4.3.1 The number of action potentials generated during the induction of LTP is highly dependent on the pattern and intensity of the stimulation used.

The standard 100Hz protocol, even if it was given at a high intensity, produced on average only 2.3 action potentials per burst. This result was surprising, as although it is one of the most commonly used protocols to induce LTP, very few studies actually report what effect one second of 100Hz stimulation has on the post synaptic cell. This is probably because it is mainly used in field recording, where only control of the presynaptic stimulus is possible and postsynaptic activity can only be estimated by the generation of population spikes. Therefore this has led to many field recording studies looking at what effect the rate and/or frequency of presynaptic stimulation has on LTP induction, without reporting the effect on the post synaptic firing rate. The importance of identifying postsynaptic activity during LTP induction was identified in a study by (Pike et al., 1999). They showed that increasing the frequency of presynaptic stimulation during a 5Hz train was not sufficient to induce LTP if only one action potential was paired during the induction, however if two or more action potentials were paired, LTP could be induced.

Another source of variability in the number of action potentials generated during induction is in the stimulus intensity that these induction protocols are given at. This is particularly important during TBS where doubling the intensity of the pulse caused a nine fold increase in the number of action potentials generated during induction. Surprisingly, doubling the pulse width during the 100Hz stimulation did not have a large effect on the number of action potentials generated. Unlike in the theta burst protocol, where only short bursts (40ms) of high frequency stimulation were applied, the prolonged one second stimulation associated with the 100Hz protocol caused the action potentials to rapidly accommodate and fail.

4.3.2 High intensity TBS induces complex spike bursting in CA1 pyramidal cells.

TBS applied at the higher intensity not only increased the number of action potentials, but also increased the number of complex spikes seen during the trains. Complex spikes are, by definition, bursts of two or three action potentials with an inter-spike interval of under 10ms and they are a defining electrophysiological signature of CA1 cell firing *in vivo* (Ranck, 1973, 1975). This observation, that the intensity of TBS not only changes the number but also the mode of CA1 cell firing, is interesting for a number of reasons. Firstly, complex spikes seem to be critical in inducing some forms of LTP. If sub threshold EPSPs are stimulated at 5Hz, LTP is only induced if this presynaptic stimulation is paired with complex spikes and not if paired with single action potentials (Pike et al., 1999). In this study the action potentials were generated by current injection at the soma, however this is also true if these complex spikes are generated naturally by long trains of low frequency stimulation (Thomas et al., 1998). These long trains of high intensity 5Hz stimulation produced a large LTP, while shorter trains or long trains at low stimulation intensities were not potentiated. Thomas et al, 1998 showed that these long trains slowly increased the occurrence of complex bursts in the postsynaptic cell and went on to show that these complex spikes were critical in inducing this plasticity, as low concentrations of bath applied TTX prevent both the occurrence of complex bursts and LTP induction. Secondly, the occurrence of these complex spikes in response to purely orthrodromic stimulation

provides additional evidence that TBS is a good model for what is occurring *in vivo* during periods of initiation and consolidation of past experiences. Complex spiking activity is known to occur *in vivo* during the active exploration of novel environments (O'Keefe, 1976; O'Keefe and Dostrovsky, 1971) and individual CA1 pyramidal cells also tend to fire these bursts at particular phases of the underlying theta rhythm (O'Keefe, 1993). These complex spikes, shaped by the theta rhythm, are thought to be important in helping to develop spatial maps of the novel environment and hence the development of place fields (Otto et al., 1991).

4.3.3 The number of action potentials increases in later trains of TBS.

The trains of TBS were repeated three times. This repetition rate was chosen for two reasons; firstly, three trains of TBS have been reported to induce a maximal LTP in the hippocampus (Hernandez et al., 2005), and, secondly; this is the repetition rate that many groups routinely use and to enable comparisons between studies it useful to keep at least some of the parameters constant. I found that the number of action potentials and the number of complex spikes was significantly higher in the second and third trains of TBS, while only a small increase was seen in later trains of 100Hz stimulation. This observation is in agreement with Thomas et al., (1998) who showed that during 5Hz stimulation the probability of action potential generation increased with increasing numbers of pulses, reaching a maximum at ~ 75 pulses.

As discussed in chapter 3, Hernandez et al., showed that increasing the number of pulses during induction was directly correlated to the magnitude of LTP, irrespective of the pattern that they were given at. The magnitude of the LTP at lower pulse numbers (40 – 100 pulses) was similar between theta burst and 100Hz. TBS did produce a larger magnitude LTP if higher numbers of pulses were given (< 200). The pattern of stimulation only accounted for about 10% of the difference in LTP magnitude, while pulse number accounted for 30% of the difference. They concluded from this that the number of pulses was therefore more important than the pattern of stimulation given. However I showed in chapter 3, that high intensity TBS which produces somatic action potentials postsynaptically, induces a larger magnitude LTP compared to low intensity TBS that does not induce so many somatic action

potentials, while the number of pulses given was exactly the same. This therefore implies that the number of somatic action potentials generated during induction is also an important factor in determining the magnitude of LTP. Since the pattern of stimulation has a large effect on the generation of these spikes, then the pattern of stimulation must also be critical in determining the magnitude of LTP. This idea fits with the observation that neither low nor high intensity 100Hz stimulation produces postsynaptic somatic spikes, and both protocols produce a smaller magnitude LTP compared to high intensity TBS.

The results from the Hernandez at al study can be interpreted differently, especially since I have shown that during theta burst stimulation postsynaptic spiking increases with increasing numbers of trains. Hernandez at al gave the lower numbers of pulses in one continuous train of stimulation, while the higher numbers were given in repeated trains of 100 pulses. At the lower pulse numbers, spikes were presumably not produced, and hence the levels of LTP were similar between both TBS and 100Hz. However, my data would suggest that after multiple trains, PTP from the preceding train would cause the generation of action potentials during TBS, but not after repeated trains of 100Hz. This would explain why they only saw a larger magnitude LTP in theta burst LTP at the high pulse numbers, suggesting that the number of pulses is the most important factor in determining the magnitude of LTP irrespective of the pattern of stimulation. This is probably an over simplification as my data would suggest that the number of action potentials induced is also an important factor in determining the magnitude of LTP. Admittedly, the number of pulses given would indirectly affect this, but so would the intensity the pulses were applied at, as would any other factor that changes the probability of post synaptic action potential firing, for example temperature and age. This study again highlights the importance of identifying what effect different stimulation protocols have on the postsynaptic response, rather than just concentrating on the presynaptic frequency and rate of stimulation.

4.3.4 Somatic spikes are essential for the induction of GluR1 independent LTP.

Of all the protocols used in my study, only high intensity TBS reliably induces somatic action potentials. This is also the only protocol that can induce LTP in the GluR1 knockout, suggesting a critical role for these spikes in the induction of the GluR1 independent component of LTP. However in isolation this does not prove that the two are directly related. I used the same high intensity TBS protocol and blocked the postsynaptic spikes, either with intracellular QX314 or with a local application of TTX to the soma. If the somatic spikes were blocked LTP was totally abolished in the knockout. The magnitude of the LTP in the GluR1 knockout was also highly correlated with the number of action potentials generated during the burst, while the level of depolarisation also seemed to be different. These results suggests that two components of LTP can be identified; one that does not require somatic action potentials and relies on the insertion of GluR1 containing receptors, and a second GluR1 independent component that appears to be expressed presynaptically and requires the generation of somatic spikes. 100Hz stimulation can only induce the GluR1 dependent component as it does not produce many somatic spikes during induction. While TBS, as long as it is given at a high enough intensity to allow the generation of postsynaptic spikes, can induce both the GluR1 dependent and independent component of LTP. As discussed in chapter 4, a number of studies have shown that TBS can induce two independent components of LTP which differ in their induction and, in some cases, expression mechanisms (Grover and Teyler, 1990; Morgan and Teyler, 2001). This LTP seems to be expressed both pre and post synaptically (Bayazitov et al., 2007; Zakharenko et al., 2003; Zakharenko et al., 2001). 100Hz stimulation on the other hand seems to only be able to induce a postsynaptically expressed LTP (Zakharenko et al., 2003; Zakharenko et al., 2001). Surprisingly none of these studies recorded postsynaptically during induction, and hence the relevance of postsynaptic spikes in the induction of this compound LTP has until now been unknown. Further evidence that post synaptic spiking activity can produce multiple forms of LTP was provided in a recent study which showed that the persistence of different forms of LTP was dependent on the occurrence of spiking activity during LTP induction (Raymond, 2008).

Although blocking somatic action potentials in the GluR1 knockout completely abolished the induction of LTP, blocking somatic spikes with either TTX or QX314 in the wild-type reduced, but did not prevent, the induction of LTP. This observation, confirms previous findings that LTP in the hippocampus can be induced in the absence of postsynaptic action potentials (Gustafsson et al., 1987; Harsanyi and Friedlander, 1997; Kelso and Brown, 1986; Krasteniakov et al., 2004; Lee, 1983).

4.3.5 Somatic over dendritic spikes are important in the GluR1 independent component of LTP.

Hoffman et al, has previously shown that pairing somatic action potentials with sub threshold EPSPs can produce LTP in the GluR1 knockout (Hoffman et al., 2002). However, the relevance of these back propagating somatic action potentials in wild-type LTP has been challenged (Lisman and Spruston, 2005). In wild-type, if the paired somatic action potential is blocked with TTX, then LTP can still be produced as long as complex spikes are produced in the dendrites (Golding et al., 2002). In fact a single burst of presynaptic activity is sufficient to induce LTP in wild-type as long as this single burst is large enough to induce a dendritic spike in the postsynaptic cell (Remy and Spruston, 2007). These result, along with the observation that somatic action potentials propagate poorly back into distal dendrites (Lisman and Spruston, 2005), casts doubt on the relevance of these back propagating spikes to the rules that govern LTP induction. My results tend to agree in principle with some of these observations. In wild-type, LTP can be produced in the absence of somatic action potentials as long as supra-threshold EPSPs are produced. Although dendritic recording were not performed, it is highly likely that the magnitude of the summated EPSPs during the burst gave rise to dendritic spikes. The occasional somatic spikelet observed during the TTX applications adds weight to this argument. However, the requirement for dendritic spikes during LTP induction is not supported by the observation that LTP can be induced in wild-type with QX314 included in the patch pipette, as QX314 would not only block the somatic but also the dendritic spikes. This inconsistency can possibly be explained by the low concentration of QX314 used in my experiments. QX314 must dialyse into the postsynaptic cell to block the voltage gated Na channels from the inside of the membrane. As a result a decreasing

concentration gradient is likely to exist with increasing distance from the soma. The concentration of QX314 was only just high enough to block spikes initiated by current injection at the soma and therefore spikes initiated at distal dendrites are likely to be affected less. These results support the hypothesis that back propagating somatic action potentials are not absolutely critical in the induction of LTP in wild-type. Where my study differs from the Golding study is in the conclusion that somatic spikes are therefore not important in the rules that govern synaptic plasticity *in vivo*. While I agree this may be the case for the GluR1 dependent component of LTP, the GluR1 independent component does require the generation of somatic action potentials, as both TTX and QX314 block the induction of LTP in the GluR1 knockout. Importantly, LTP was still blocked even if dendritic spikes were present at the soma, highlighting the importance of somatic over dendritic action potentials in the GluR1 knockout. Although I only observed these spikelets on a minority of occasions, it is known that these regenerative events propagate extremely poorly and are therefore likely to be present even if no sign of them is seen at the soma.

In summary the GluR1 independent component of LTP requires the activation of back propagating somatic action potentials, while the GluR1 dependent component is not dependent on somatic action potentials, but seems to require the activation of dendritic initiated spikes. Distinguishing between these two types of process is important when trying to understand how neurons integrate synaptic information. As dendritically spikes propagate poorly in dendrites, they can only produce local dendritic depolarisation and therefore only inputs in a spatially restricted manner will be potentiated. This would have the effect of making dendritic compartments computational units in there own right, greatly increasing the computational power of the neuron. Conversely, back propagating somatic action potentials would serve as a global signal to the neuron, allowing spatially restricted inputs to be potentiated if the timing and strength of the inputs were sufficient to induce a somatic action potential. It is likely that these two mechanisms work in parallel, with dendritic spikes helping to initiate somatic spikes, while also serving to boost the back propagating spike into distal dendrites. This would keep the link between pre and postsynaptic activity and therefore still allow dendritic spike induced LTP to obey the fundamental Hebbian learning rule.

5 The role of NO in the induction of LTP in wild-type and GluR1 knockout mice

5.1 Introduction.

"When a thing ceases to be a subject of controversy, it ceases to be a subject of interest" William Hazlitt 1778 - 1830

NO is well suited to serve as a retrograde messenger because of its capacity for rapid diffusion and also its short half life. A number of reports document a broad range of NO effects in the CNS, such as the modulation of neuronal development, nociception, apoptosis, synaptic plasticity, and complex behavioural responses (for review see Prast and Philippu 2001; Hofmann et al. 2003). 20 years ago, Garthwaite and co-workers (1988) recognized for the first time the potential of NO as a neuromodulator. They observed that activation of NMDA receptors in cultured cerebellar granule cells triggers the release of a messenger similar to endothelium-derived relaxing factor, which is identical to NO (Ignarro et al. 1987; Furchgott 1996). Based on these results, they put forward the hypothesis that NO may provide a universal link from postsynaptic activity to functional modifications of neighbouring presynaptic terminals and glial cells.

NO is produced by a complex reaction via oxidative release from L-arginine giving rise to L-citrulline. Three mammalian isoenzymes catalysing this reaction have been identified in various cell types, the constitutively expressed neuronal and endothelial NO synthase (nNOS/NOS1 and eNOS/NOS3), and the inducible NO synthase (iNOS /NOS2). The NOS1 isoform is abundantly expressed throughout the CNS and represents the principal source of NO in many neuronal populations (Bredt and Snyder 1990; Dawson and Dawson 1996; Prast and Philippu 2001). It is a Ca^{2+}/calmodulin-regulated enzyme, which can be activated by Ca^{2+} influx via N-methyl-D-aspartate (NMDA) receptors (Garthwaite et al. 1988). This functional relationship is thought to be especially effective, because the scaffolding molecule PSD-95 keeps the NOS I protein in close proximity to the NMDA receptors (Christopherson et al. 1999; Valtschanoff and Weinberg 2001). The NOS III isoform, another Ca^{2+}/calmodulin-dependent NOS which was initially detected in endothelial cells, has also been reported to be expressed in hippocampal pyramidal cells and

neurons of other brain regions (Dinerman et al. 1994; O'Dell et al. 1994). However, this observation has been challenged by others (Stanarius et al. 1997; Demas et al. 1999; Blackshaw et al. 2003). In contrast to the two constitutive forms of NOS, the NOS II isoform is normally not detectable in the CNS, but is unregulated following toxic or inflammatory stimuli.

The first direct evidence that NO was involved in the induction of LTP was provided in 1991 by four independent groups (Bohme et al., 1991; Haley et al., 1992; O'Dell et al., 1991a; Schuman and Madison, 1991). All four groups found that inhibitors of NOS blocked the induction of LTP and that the inhibition was reversed by giving an excess of the amino acid L-arginine, suggesting the inhibitors used were relatively specific. O'Dell et al., (1991a) and Schuman and Madison, (1991) also showed that it was postsynaptic NOS activity that was important, as inhibitors included in their patch pipette blocked the induction of LTP. They could also block the induction of LTP by applying the NO scavenging protein hemoglobin, indicating that NO must diffuse out of the post synaptic cell to produce its effect.

These results became controversial as in the early 1990s many papers were published that either confirmed that these NOS inhibitors could block LTP (Boulton et al., 1995; Doyle et al., 1996; Mizutani et al., 1993), while others could not see any effect on LTP (Bannerman et al., 1994; Cummings et al., 1994; Kato and Zorumski, 1993), or proposed that blocking of LTP was only possible under particular experimental conditions (Chetkovich et al., 1993; Gribkoff and Lum-Ragan, 1992; Haley et al., 1993; Haley et al., 1996; Malen and Chapman, 1997; O'Dell et al., 1994; Williams et al., 1993). These studies identified many different experimental conditions, including age, temperature, pathway, and the type and intensity of the induction protocol that could change the sensitivity of LTP induction to NOS blockade.

Another unresolved issue in the field is identifying which NOS isoform is responsible for generating the NO signal during LTP. Analysis of mice lacking NOS-I and/or NOS-III revealed the functional contribution of either NOS isoform to hippocampal LTP. Mice deficient in either NOS-I or NOS-III appear to be capable of normal LTP

in the CA1 region of the hippocampus (O'Dell et al. 1994; Son et al. 1996), while a strong impairment was observed in double NOS-I/NOS-III knockout mice (Son et al. 1996). Other groups have shown that the lack of NOS-III alone can result in reduced LTP in the Schaffer collateral pathway (Wilson et al. 1999) and other regions (Haul et al. 1999; Doreulee et al. 2001). A major role for NOS-III has also been suggested by Kantor et al. (1996), who reported that disrupting the localisation of NOS-III to the membrane causes impairment of LTP, and that this effect could be overcome by expressing a chimeric form of NOS-III constitutively targeted to the membrane. On the other hand, the importance of NOS-I has been demonstrated by a recent report which shows that selective application of NOS-I inhibitors reduced hippocampal LTP (Hopper and Garthwaite 2006). It was further suggested that phasic and tonic NO signals are needed for LTP, and that in hippocampal pyramidal cells these signals are derived from NOS-I in response to neural activity and NOS-III expressed in nearby vascular endothelial cells (Garthwaite et al. 2006; Hopper and Garthwaite 2006).

The findings discussed so far demonstrate the ability of NO to support LTP, but they do not discriminate between possible sites of its action. The observation that bath application of hemoglobin, a membrane-impermeable scavenger of NO, blocks LTP (O'Dell et al., 1991a; Schuman and Madison, 1991) suggests that NO travels through the synaptic cleft to the presynaptic terminal. Additional evidence for a presynaptic action of NO comes from studies of miniature excitatory postsynaptic currents (EPSC) (O'Dell et al. 1991) and NMDA receptor-dependent LTP in cultured hippocampal neurons (Arancio et al. 1995, 1996, 2001; Wang et al. 2005). NO increases the frequency of spontaneous miniature EPSCs and oxymyoglobin, another membrane-impermeable NO scavenger, is able to suppress LTP between pairs of cultured neurons following a tetanus, but its extracellular application has no effect on LTP induced by a weak tetanus in conjunction with photolytic uncaging of NO in the presynaptic cell. Studies using the styryl dye FM1-43 and two-photon microscopy to directly visualize transmitter release have also demonstrated that LTP is associated with a NO dependent increase in vesicular release in the hippocampus (Stanton et al., 2005).

NO signal transduction generally occurs through binding to guanylyl cyclase (GC)-coupled NO receptors, resulting in the formation of cGMP, with cGMP-dependent protein kinase being one of the downstream mechanisms leading to changes in synaptic strength (Feil et al., 2005) There are some reports that NO can support LTP through cGMP-independent mechanisms, e.g., by stimulating the ADP ribosylation of proteins regulating synaptic transmission (Schuman et al. 1994; Zhang et al. 1994; Sullivan et al. 1997).

However the bulk of pharmacological studies, as well as the phenotypes of transgenic mouse models, support the conclusion that NO signals via the sGC – cGMP pathway. LTP-inducing stimuli elicit an increase of cGMP in the hippocampus, which is sensitive to NOS inhibitors and NO scavengers (Chetkovich et al. 1993). Moreover, the membrane permeable cGMP analogue dibutyryl cGMP partially restores LTP blocked by a NOS inhibitor (Haley et al. 1992), and various sGC inhibitors suppress LTP (Zhuo et al. 1994; Arancio et al. 1995; Boulton et al. 1995). Evidence for the presynaptic localisation of sGC came from studies of LTP using pairs of cultured hippocampal neurons (Arancio et al. 1995). Importantly, cGMP produces activity dependent LTP when it is injected into the presynaptic neuron, but not when it is injected into the postsynaptic neuron. The observation that 8-Br-cGMP mimics the NO effect of increasing the frequency of spontaneous miniature EPSCs, together with results from quantal analysis support the conclusion that the cGMP-dependent potentiation of synaptic transmission is due to an increase in presynaptic transmitter release.

The role of NO in LTP is still controversial; probably due to the many contradictory findings described above. The confusion was possibly increased in the early 90s by the raging debate over the locus of LTP expression. Papers may have overstated results to promote one theory over others. LTP can be expressed simultaneously at both pre and postsynaptic sites. Therefore, to identify a NO dependent component in LTP, the experimental conditions must be optimised to produce a presynaptic rather than a postsynaptic change and controlling this is challenging in wild-type. Since the LTP in the GluR1 knockout seems to be expressed presynaptically and also requires

the postsynaptic activation of CaMKII (See chapter 4 and Hardingham and Fox, 2006), then it seems likely that a retrograde message is involved in the expression of this LTP. If NO is mediating this presynaptic component, then blockade of NOS should have a larger effect in the GluR1 knockout than in wild-type. Using this genotype would then help to tease out the mechanisms of NO signalling as postsynaptic effects would not confuse the results. In this chapter I have looked at the sensitivity of LTP to NOS inhibition in both wild-type and GluR1 knockout, I then used double GluR1 and NOS knockout to clarify which of the NOS isoforms mediates the observed NO effects.

5.2 Results

5.2.1 Choice of induction protocol is critical in identifying a NO dependent LTP in wild-type.

NG-Nitro-L-arginine (L-NNA) is a competitive inhibitor of NO synthase (NOS) with selectivity for the neuronal and endothelial isoforms of the enzyme. L-NNA (100μM) was bath applied to the slice 5 minutes preceding the induction of LTP. No effect was seen of 100μM L-NNA on base line transmission (pre drug fEPSP 102 ± 0.5%, post drug fEPSP 101 ± 0.6%). In agreement with Cummings et al., (1994) LTP induced using a high intensity 100Hz stimulation was unaffected if 100μM L-NNA was present during induction (Figure 6.1). The magnitude of the potentiation measured 120 minutes after the tetanus (141 ± 6%, n = 12) was not significantly different from control (147 ± 8%, n = 11) using a Bonferroni corrected t-test ($t_{(22)}$=1.75, $p > 0.05$,).

However, an affect of L-NNA could be seen on LTP induced using a high intensity theta burst stimulation (Figure 6.2). The LTP was not fully blocked, as the size of the fEPSP measured 120 minutes after the tetanus (128 ± 5%) was still significantly different from the S2 un-tetanised pathway (100 ± 8%, paired t-test $t_{(15)}$ = 6.76, $p<0.001$). However, the magnitude of the potentiation was significantly reduced in the presence of L-NNA (128 ± 5%) compared to control levels (160 ± 9%, Bonferroni corrected t-test, $t_{(26)}$= 4.74, $p< 0.001$). A 2-way ANOVA of the results revealed that there was no interaction between drug and stimulation type ($F_{(1,45)}$ = 2.6 , $p > 0.05$). A Bonferroni post hoc test identified that this was because the magnitude of the potentiation after theta burst stimulation in the presence of L-NNA (128 ± 5%) and the magnitude of 100Hz without L-NNA (147 ± 8%) were not significantly different ($t(25)$= 2.6, $p > 0.05$). This suggests that the fractionally larger magnitude LTP produced by high intensity theta burst stimulation compared to 100Hz, described in chapter 3, is due to a NO dependent mechanism.

The effect of NOS blockade on the level of potentiation seemed to increase with time, which is why I followed the LTP for over 120 minutes after the tetanus in these experiments. In fact, if the theta burst data was grouped into 10 minute bins and tested

in a repeated measures 2 way ANOVA, a significant interaction was seen between time after tetanus and drug ($F_{(275,11)} = 5.47$, $p < 0.05$). A Bonferroni post hoc test performed at each time point revealed that this interaction was because there was only a significant effect of drug in the last 120 minute period of the experiment ($t_{(25)} = 2.9$, $p < 0.05$).

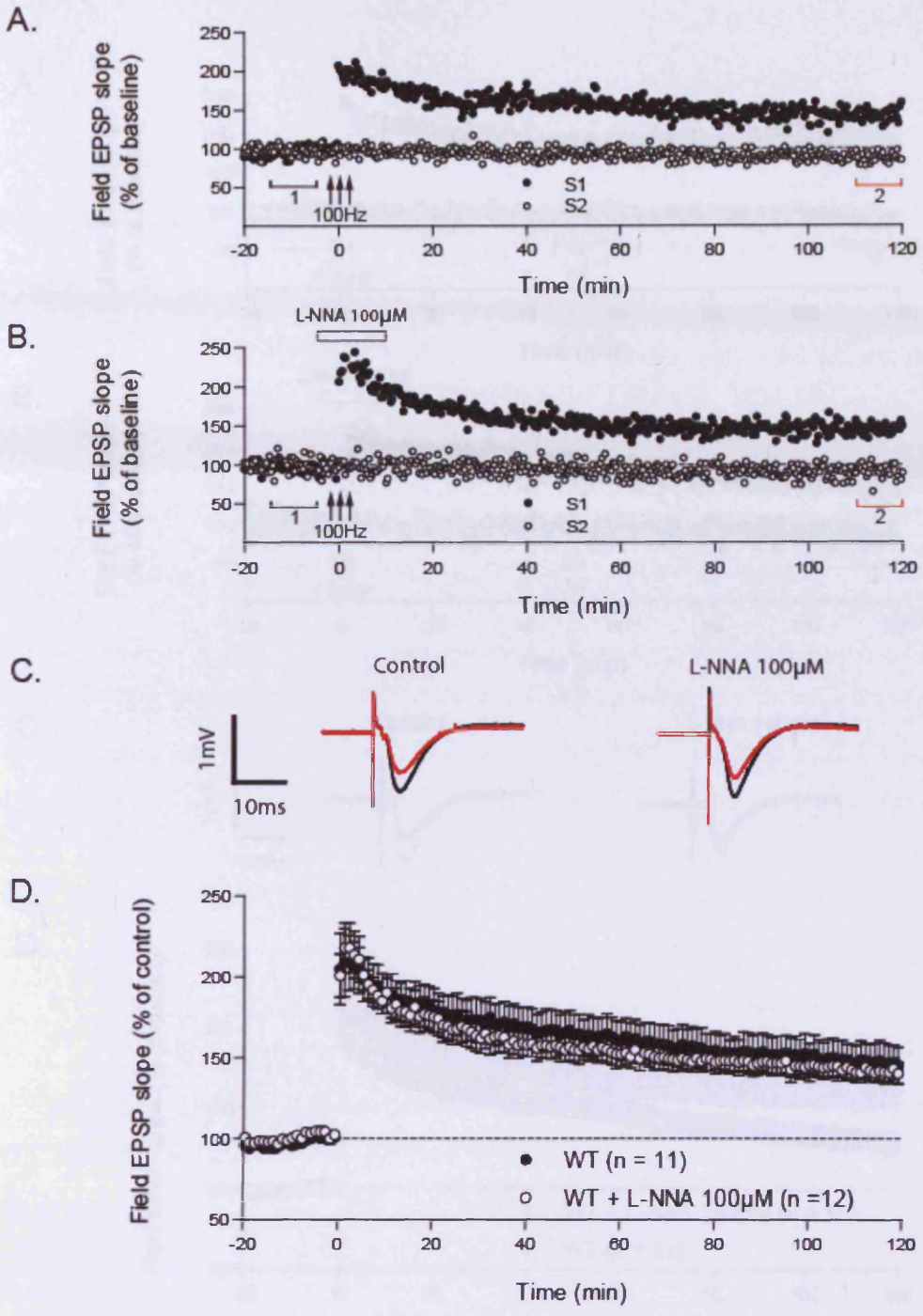


Figure 6.1 Inhibition of NOS did not affect the magnitude of LTP induced using 100Hz stimulation.

A, An example control experiment without NOS blockade, LTP was induced using a 100Hz stimulation at the time indicated by the arrow and then recorded for 120 minutes after the tetanus. B, Example experiment, the application of L-NNA (100µM) during the 100Hz protocol (time indicated by the bar) did not block the induction of LTP. C, Example traces taken during the control period (red line) and 120 minutes after the induction of LTP (black line). D, Average data, showing that the magnitude of LTP was not affected if NOS was blocked during induction (○). Control data (●).

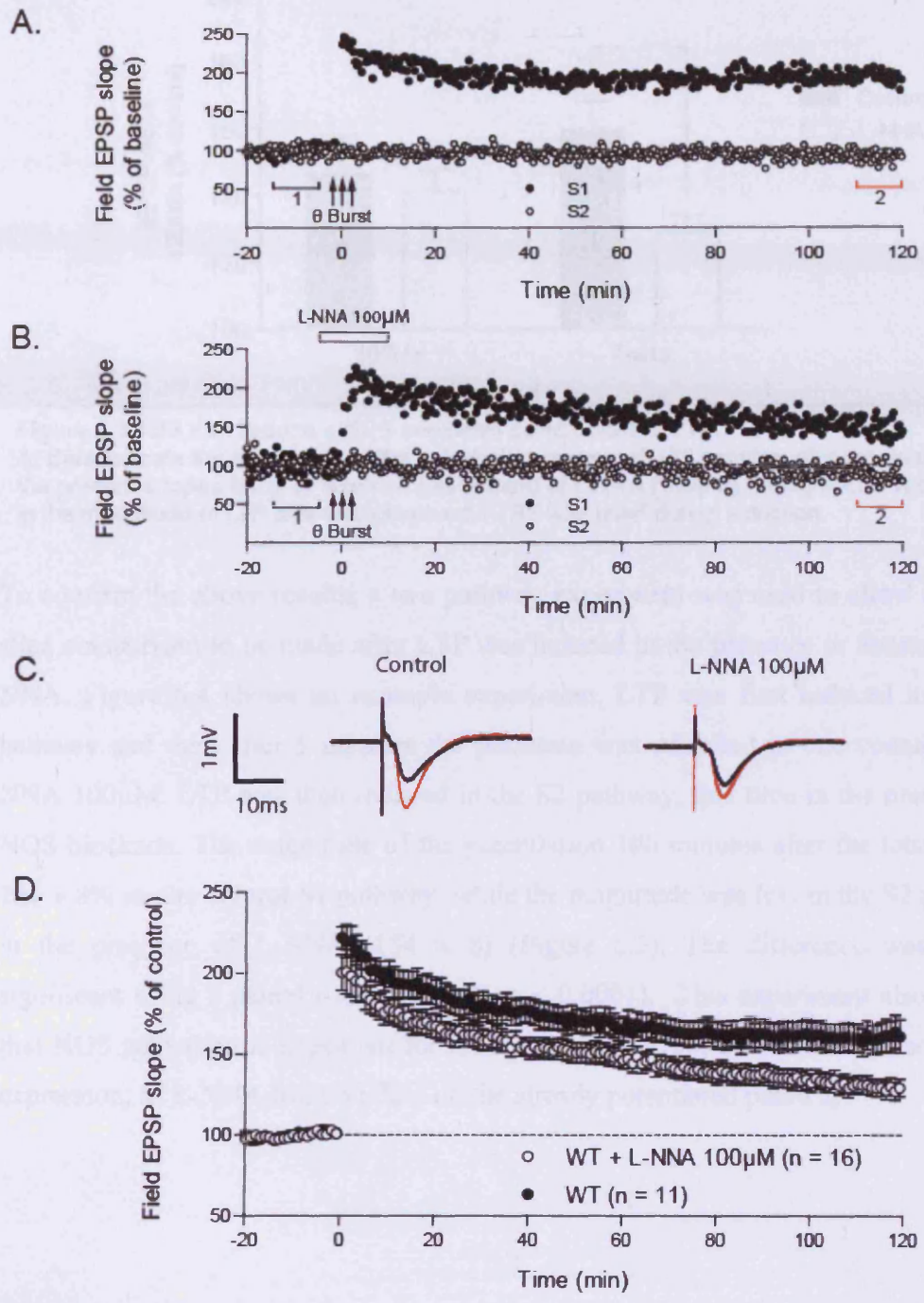


Figure 6.2 TBS produced a NOS sensitive component of LTP in wild-type.
A, An example control experiment without NOS blockade, LTP was induced using a TBS at the time indicated by the arrow and then recorded for 120 minutes after the tetanus. B, Example experiment, the application of L-NNA (100μM) during the TBS protocol (time indicated by the bar) reduced the magnitude of the LTP. C, Example traces taken during the control period (red line) and 120 minutes after the induction of LTP (black line). D, Average data, showing that if NOS was blocked during induction, the magnitude of LTP was reduced (○) compared to control (●).

A.

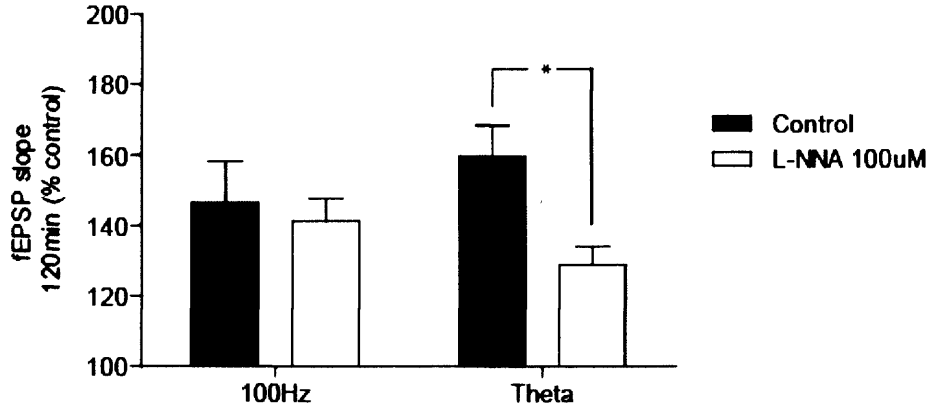


Figure 6.3 TBS can induce a NOS sensitive component of LTP.
A, Bars indicate the magnitude of the potentiation measured 120 minutes after the tetanus, in the presence (open bars) or absence (black bars) of L-NNA (100μM). A significant reduction in the magnitude of LTP was only observed if TBS was used during induction.

To confirm the above results, a two pathway experiment was used to allow a within slice comparison to be made after LTP was induced in the presence or absence of L-NNA. Figure 6.4 shows an example experiment, LTP was first induced in the S1 pathway and then after 5 minutes the perfusate was switched to one containing L-NNA 100μM. LTP was then induced in the S2 pathway, this time in the presence of NOS blockade. The magnitude of the potentiation 100 minutes after the tetanus was 181 ± 8% in the control S1 pathway, while the magnitude was less in the S2 pathway in the presence of L-NNA (154 ± 8) (Figure 6.5). The difference was highly significant using a paired t-test ($t_{(4)} = 22$, $p < 0.0001$). This experiment also reveals that NOS activation is important for the induction of LTP, but not for the continued expression, as L-NNA had no effect on the already potentiated pathway.

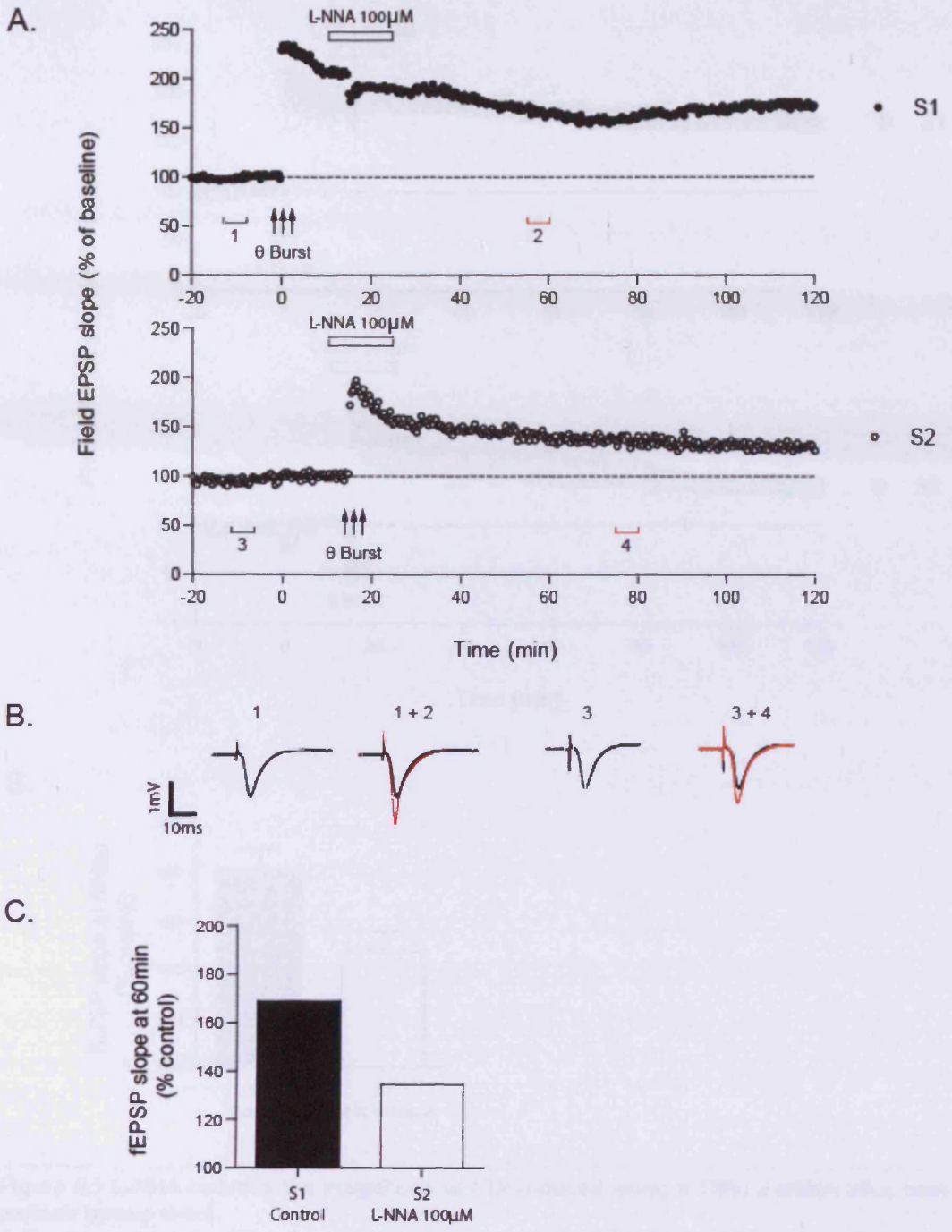
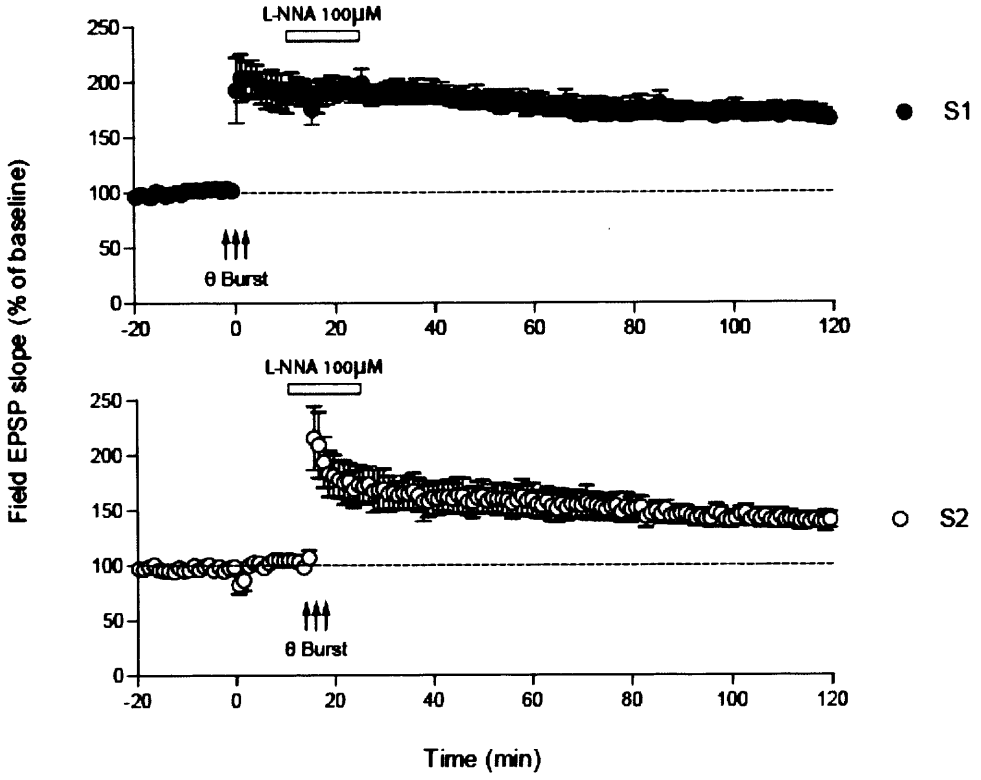


Figure 6.4 L-NNA reduces the magnitude of LTP induced using a TBS, a within slice comparison (example experiment).
A, Top trace shows the S1 pathway; bottom trace shows the S2 pathway. TBS was initially applied to the S1 pathway at t=0 (arrow), 10 minutes later L-NNA 100µM was bath applied and a second TBS was applied to the S2 pathway at t=15 (arrow). B, Example traces at time points indicated by the bars. C, LTP measured 60 minutes after the tetanus, was reduced in the S2 pathway.

A.



B.

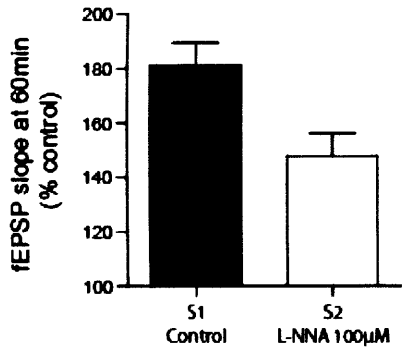


Figure 6.5 L-NNA reduces the magnitude of LTP induced using a TBS, a within slice comparison (group data).
A, Top trace shows the S1 pathway; bottom trace shows the S2 pathway. TBS was initially applied to the S1 pathway at t=0 (arrow), 10 minutes later L-NNA 100µM was bath applied and a second TBS was applied to the S2 pathway at t=15 (arrow). B, LTP measured 60 minutes after the tetanus, was significantly reduced in the S2 pathway (n = 5).

These data suggest that theta burst stimulation, a protocol that is known to induce somatic back-propagating spikes (chapter 4, and 5), is able to induce a NO dependent component of LTP in wild-type. However a protocol (100Hz), that is less effective in somatic spike generation cannot induce this NO sensitive component. Since theta burst stimulation is able to induce a slightly larger LTP than protocols that are less effective at generating somatic spikes, another possible conclusion from these data is that L-NNA actually affects the generation of somatic spikes during induction. This was tested by recording intracellularly during the induction protocols in the presence or absence of 100μM L-NNA. Application of L-NNA did not affect the number of spikes produced during high intensity theta burst stimulation, as the number of spikes generated during the burst was 17 ± 4 in control slices, compared to 19 ± 6 in slices treated with L-NNA. ($F_{(1,29)} = 1.16$, $p = 0.56$).

5.2.2 LTP in GluR1 knockout is more sensitive to NOS inhibition than in wild-type.

Since LTP in the GluR1 knockout seems to be expressed more presynaptically than in wild-type and requires the generation of postsynaptic somatic spikes, I hypothesised that this GluR1 independent component of the LTP might be fully dependent on NO. I tested this by perfusing either L-NNA, or L-NAME onto the slice during induction of LTP in slices taken from GluR1 knockout (Figure 6.6). L-NNA reduced the magnitude of LTP in the GluR1 knockout by ~ 80%. In untreated (control) slices LTP was reduced from 172 ± 16% (n = 26) to 115 ± 11% (n = 11) in slices perfused with L-NNA, which was highly significant using a Bonferroni corrected t-test ($t_{(36)} = 5.9$, $p < 0.001$). However, LTP was not fully blocked as the small residual LTP was still significantly different from the un-tetanised control pathway, using a paired t-test ($t_{(10)} = 3.29$, $p<0.05$,). These results were replicated with 100μM L-NAME, a non competitive inhibitor of NOS (Figure 6.6D). L-NAME reduced the magnitude of LTP by a similar amount to L-NNA (~82%), the magnitude of LTP in the presence of L-NAME was 112 ± 14 in comparison to 98 ± 4% in the S2 control (paired t-test, $t_{(12)} = 4.65$, $n = 13$, $p < 0.01$).

As for the wild-type, I checked that this was not due to blockade of somatic action potentials during induction. Application of L-NNA did not affect the number of spikes generated per train in slices taken from GluR1 knockout. Slices treated with 100μM L-NNA produced 25 ± 3.6 spikes per train during induction compared to 22 ± 2.4 in untreated slices ($F_{(1,29)} = 1.16$, $p = 0.56$).

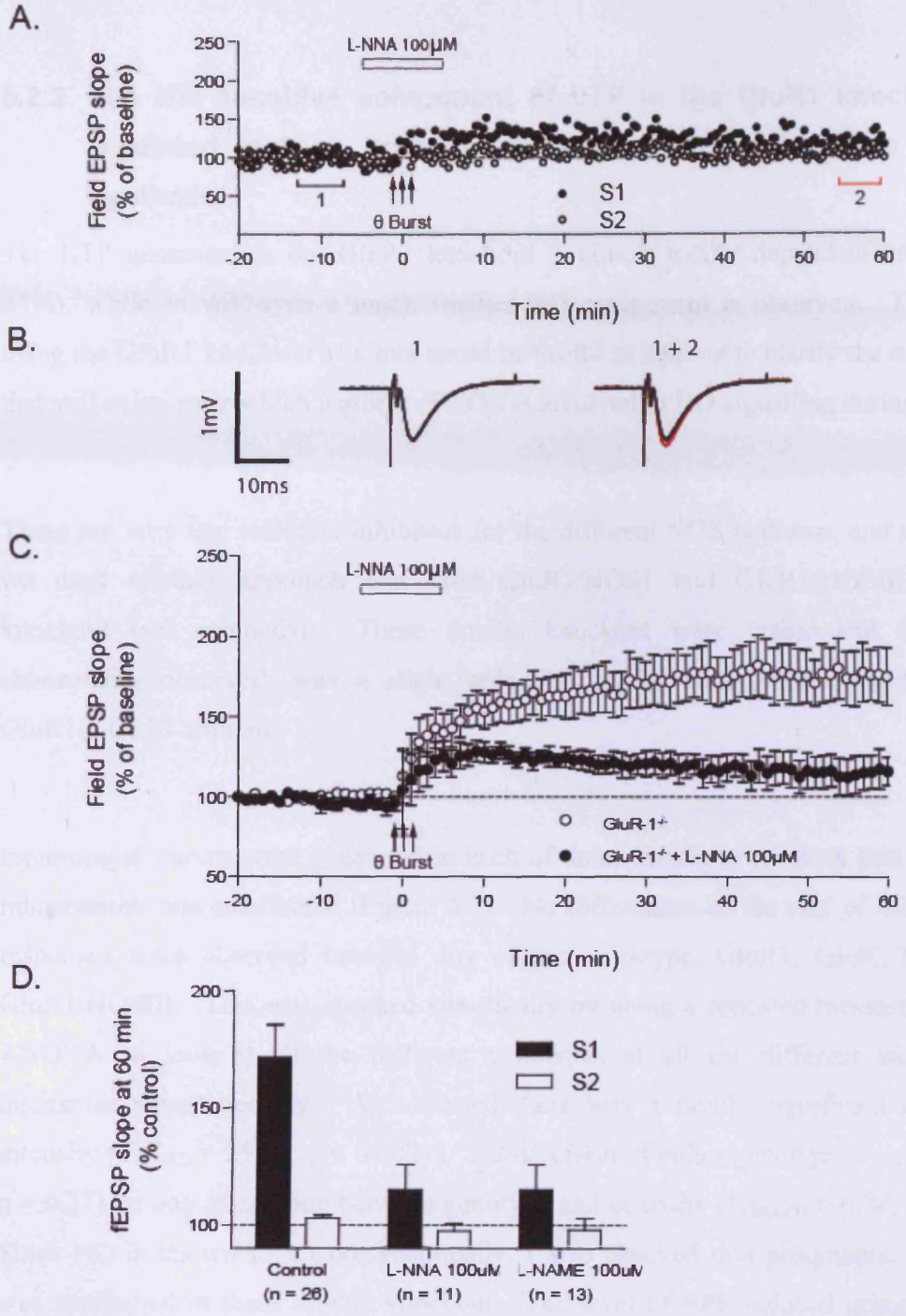


Figure 6.6 LTP in the GluR-1 knockouts is almost totally dependent on NOS activation.
A, Example experiment, application of L-NNA (100µM) during induction (time indicated by bar) almost completely blocked the LTP in the GluR-1 knockout. B, Example traces at time points indicated by the bars. C, Group data, LTP in the GluR-1 knockout (○) is largely reduced by a 5 min application of L-NNA (100µM) (●). D, Bars indicate the magnitude of the LTP measured 60 minutes after the tetanus. L-NNA (100µM) and L-NAME (100µM), significantly reduced the magnitude of the potentiation in the GluR-1 knockouts, although leaving a small residual LTP.

5.2.3 The NO sensitive component of LTP in the GluR1 knockout is mediated through both the NOS-1 and NOS-3 isoforms of NO synthase.

The LTP generated in the GluR1 knockout is almost totally dependent on NO (~ 85%), while in wild-type a much smaller NO component is observed. Therefore using the GluR1 knockout as a tool could be useful in helping to clarify the confusion that still exists over which isoform of NOS is involved in NO signalling during LTP.

There are very few selective inhibitors for the different NOS isoforms, and therefore we used another approach and bred GluR1/NOS1 and GluR1/NOSIII double knockout (see methods). These double knockout were viable and the only abnormality observed, was a slight reduction in the adult body weight of the GluR1/NOSIII animals.

Input-output curves were generated in each of these knockout to check that baseline transmission was unaffected (Figure 6.7). No differences in the size of the evoked responses were observed between any of the wild-type, GluR1, GluR1/NOSI or GluR1/NOSIII. This was checked statistically by using a repeated measures 2 way ANOVA to look at all the different genotypes at all the different stimulation intensities simultaneously. As expected there was a highly significant effect of intensity ($F_{(6,492)} = 257.8$, $p < 0.0001$), but no effect of either genotype ($F_{(3,492)} = 1.0$, $p = 0.37$), or any interaction between genotype and intensity ($F_{(18,492)} = 0.88$, $p = 0.6$). Since NO is known to act presynaptically, I also checked that presynaptic function was unaffected in these double knockout. The level of PPF induced using a 70ms inter-pulse interval was similar in both the GluR1/NOS-I mice (1.39 ± 0.13) and the GluR1/NOSIII (1.54 ± 0.23) double knockout compared to either the single GluR1 knockout (1.45 ± 0.1) or the wild-type (1.48 ± 0.1). This was confirmed using a one way ANOVA, which showed that there was no significant difference between the groups ($F_{(3,45)} = 1.2$, $p > 0.05$).

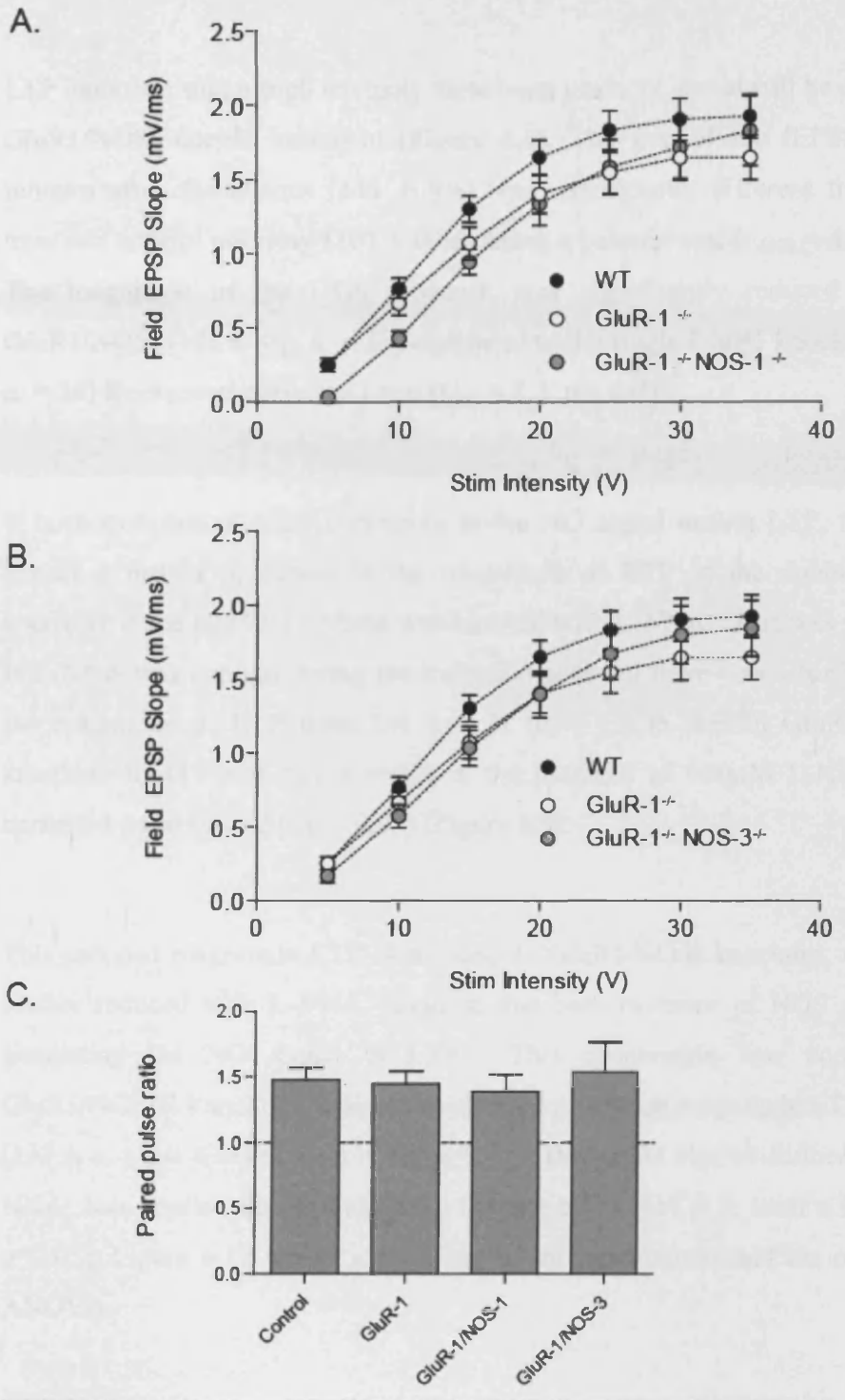


Figure 6.7 Base line transmission and presynaptic function are normal in the GluR-1 NOS double knockouts.

A, No difference is seen in the input/output response curves between wild-type (●), GluR-1 knockouts (○), GluR-1/NOS-I double knockouts (◍), and (B,) GluR-1/NOS-III double knockouts (◍). C, A 70ms paired pulse interval produced comparable levels of facilitation in all genotypes, indicating that presynaptic function is unaffected in the knockouts.

LTP induced using a high intensity theta burst protocol, could still be expressed in the GluR1/NOSI double knockout (Figure 6.8). The size of the fEPSP measured 60 minutes after the tetanus (141 ± 8%) was significantly different from the S2 un-tetanised control pathway (101 ± 0.65) using a paired t-test ($t_{(12)} = 4.94$, $p < 0.001$). The magnitude of the LTP, however, was significantly reduced in the double GluR1/NOSI (141 ± 8%, n = 13) compared to the single GluR1 knockout (171 ± 16% n = 26) Bonferroni corrected t-test ($t_{(35)} = 3.2$, $p < 0.01$).

If both isoforms of NOS contribute to the NO signal during LTP, then you would expect a further reduction in the magnitude of LTP in the double GluR1/NOSI knockout if the NOS III isoform was blocked with L-NNA. This was indeed the case. If L-NNA was applied during the induction protocol there was a further reduction in the magnitude of LTP from 141 ± 8 % (n = 13) in control GluR1/NOSI double knockout to 117 ± 9 % (n = 12) in the presence of 100μM L-NNA (Bonferroni corrected t-test $t_{(37)} = 2.6$, $p < 0.05$) (Figure 6.9).

This reduced magnitude LTP in the double GluR1/NOSI knockout, which could be further reduced with L-NNA, suggests that both isoforms of NOS are involved in generating the NO signal in LTP. This observation was confirmed in the GluR1/NOSIII knockout, which also showed a reduced magnitude LTP (Figure 6.10) (137 ± 6, t-test $t(37)=3.5$, n = 12, $p<0.01$) that could also be further reduced if L-NNA was applied during induction (Figure 6.11) (117 ± 5, t-test $t(37)=2.6$, n = 12 $p<0.05$). Figure 6.12 shows a summary of the experiments and the results from the ANOVA.

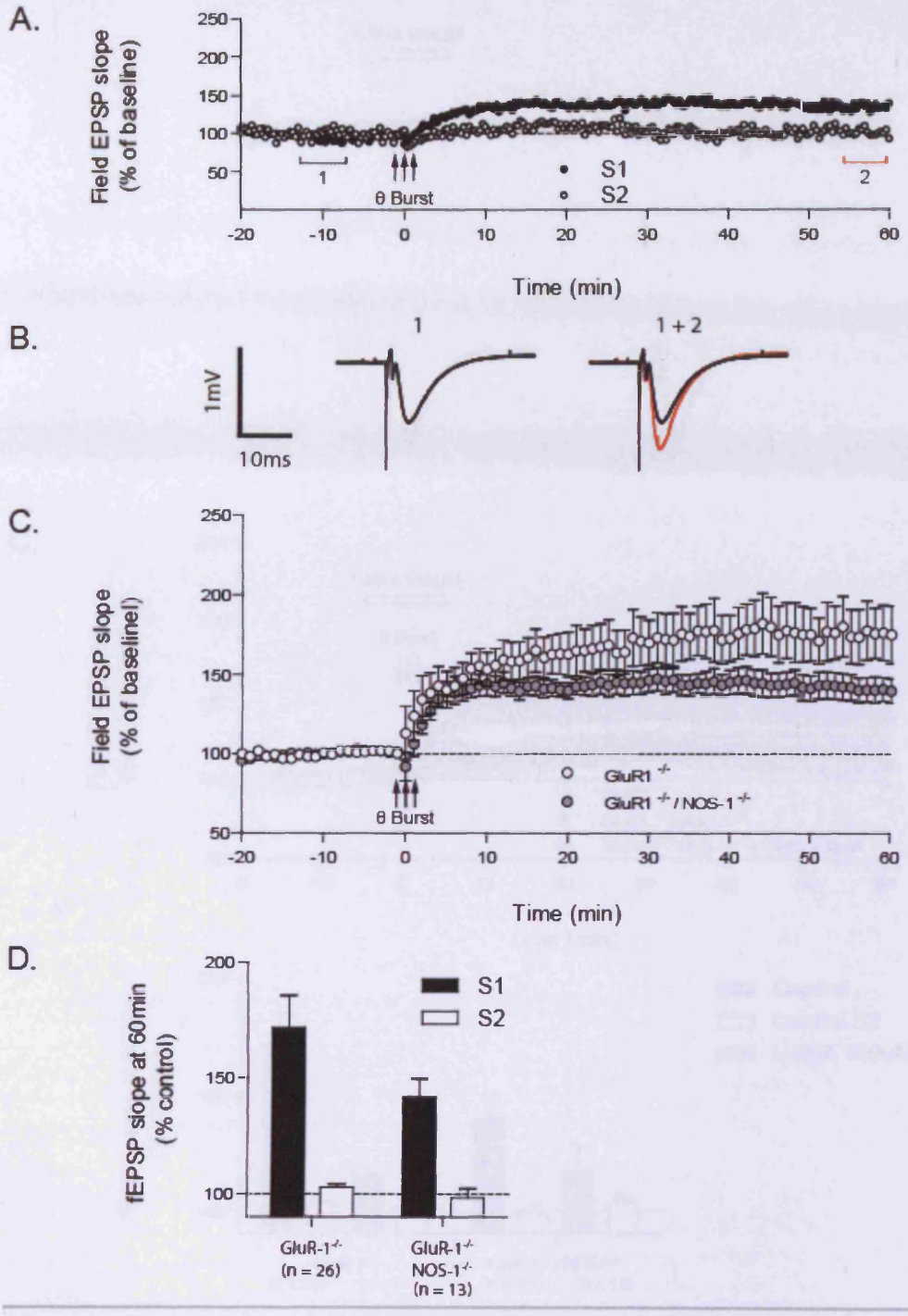


Figure 6.8 The magnitude of the LTP is reduced but not abolished in GluR-1/NOS-I double knockout

A, Example experiment, LTP can still be induced in the GluR-1/NOS-I double knockout. B, Example traces taken at time points indicated by the bars. C, Group data, LTP in the GluR-1/NOS-1 double mutant mice (●) is reduced when compared to the GluR-1 single mutant mice(○). D, Bars indicate the magnitude of the LTP measured 60 minutes after the tetanus.

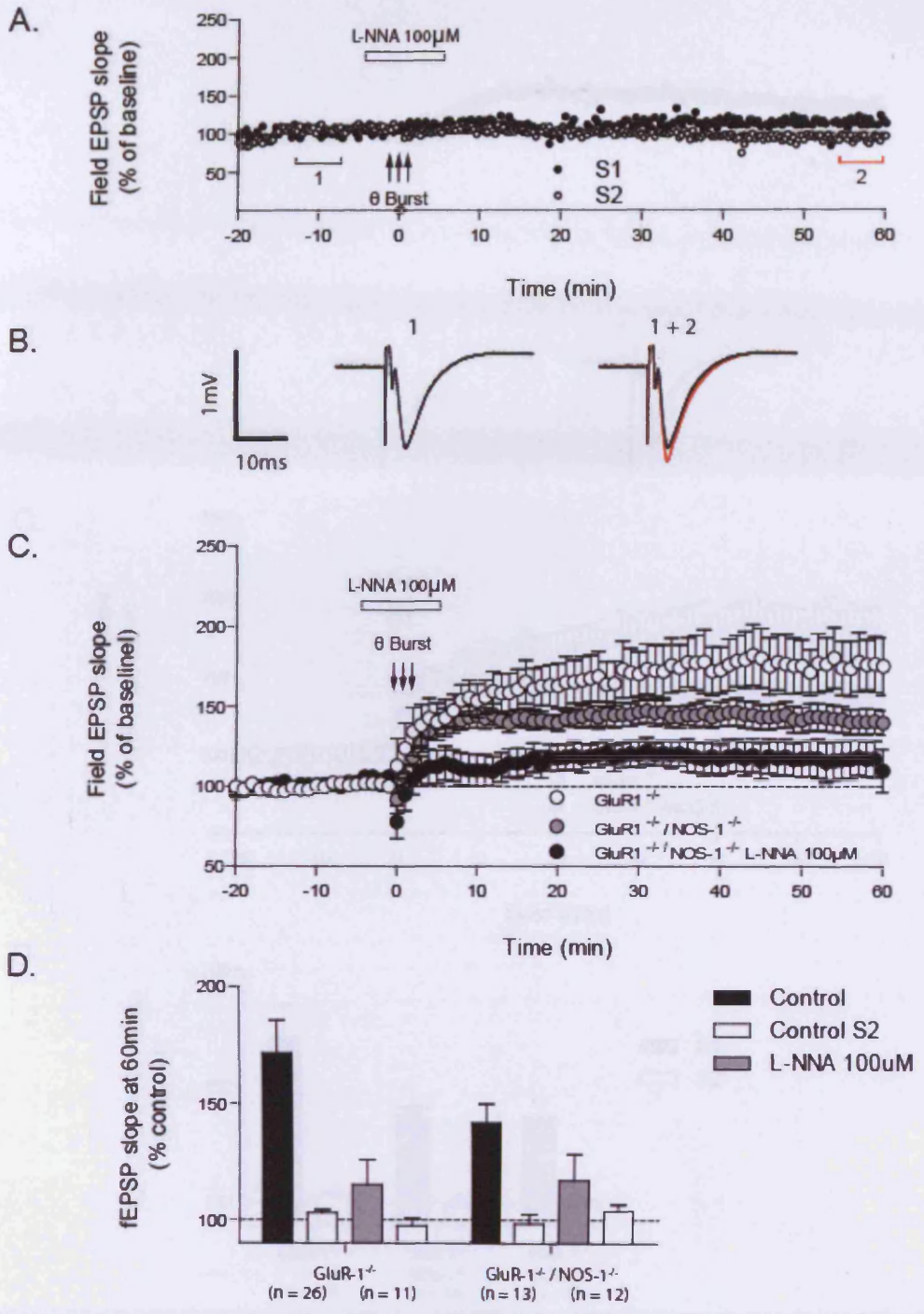


Figure 6.9 The magnitude of the LTP in the GluR-1/NOS-I double knockout can be further reduced with L-NNA.

A, Example experiment, application of L-NNA (100µM) during induction (time indicated by bar) further reduced and almost completely blocked the LTP in the GluR-1/NOS-1 double knockout. B, Example traces taken at time points indicated by the bars. C, Group data, LTP in the GluR-1/NOS-1 double knockout (●) is reduced when compared to the GluR-1 single knockout (○). The remaining LTP in the double GluR-1/NOS-1 knockout is further reduced by L-NNA (100µM (•) and is similar to LTP observed in single GluR-1 knockout with L-NNA (100µM) D, Bars indicate the magnitude of the LTP measured 60 minutes after the tetanus.

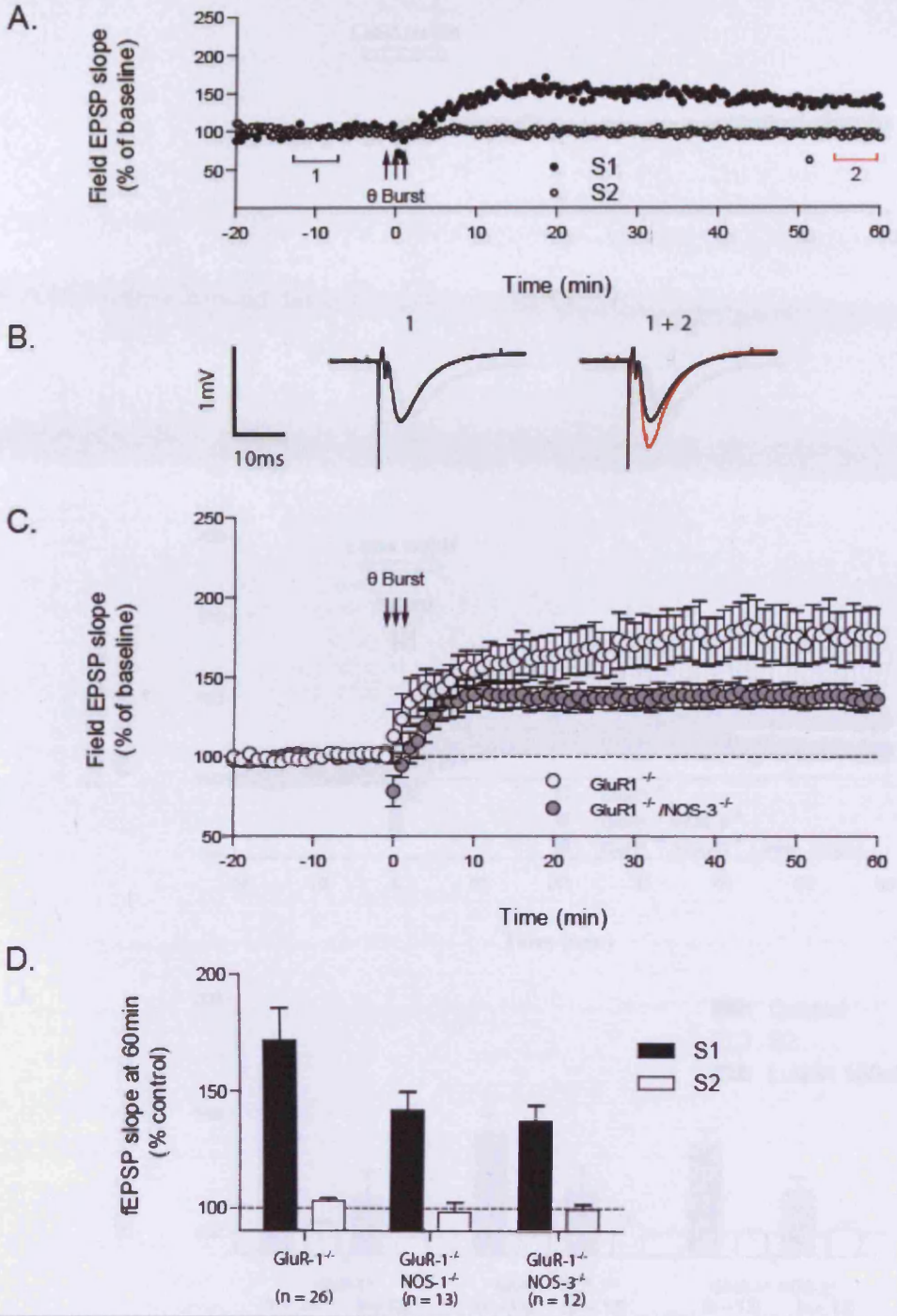


Figure 6.10 The magnitude of the LTP is also reduced but not abolished in GluR-1/NOS-III double knockout.

A, Example experiment, LTP can still be induced in the GluR-1/NOS-III double knockout.

B, Example traces taken at time points indicated by the bars. C, Group data, LTP in the GluR-1/NOS-III double knockout (●) is reduced when compared to the GluR-1 single knockout (○).

D, Bars indicate the magnitude of the LTP measured 60 minutes after the tetanus.

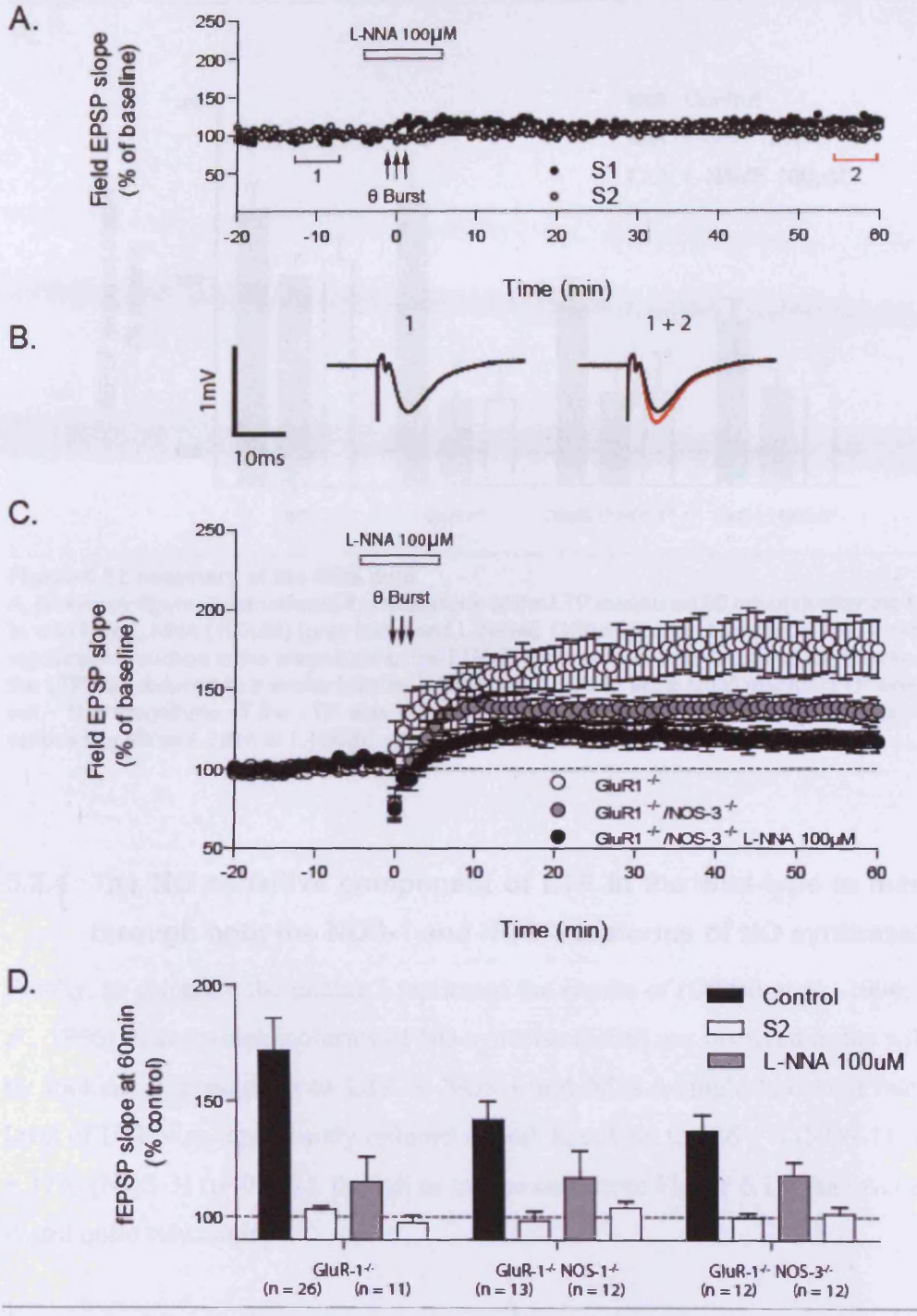


Figure 6.11 The magnitude of the LTP in the GluR-1/NOS-III double knockout is also further reduced by L-NNA.

A, Example experiment, application of L-NNA (100µM) during induction (time indicated by bar) further reduced and almost completely blocked the LTP in the GluR-1/NOS-III double knockout. B, Example traces taken at time points indicated by the bars. C, Group data, LTP in the GluR-1/NOS-III double knockout (●) is reduced when compared to the GluR-1 single knockout (○). The remaining LTP in the double GluR-1/NOS-III knockout is further reduced by L-NNA (100µM) (●) and is similar to LTP observed in single GluR-1 knockout with L-NNA 100µM. D, Bars indicate the magnitude of the LTP measured 60 minutes after the tetanus.

A.

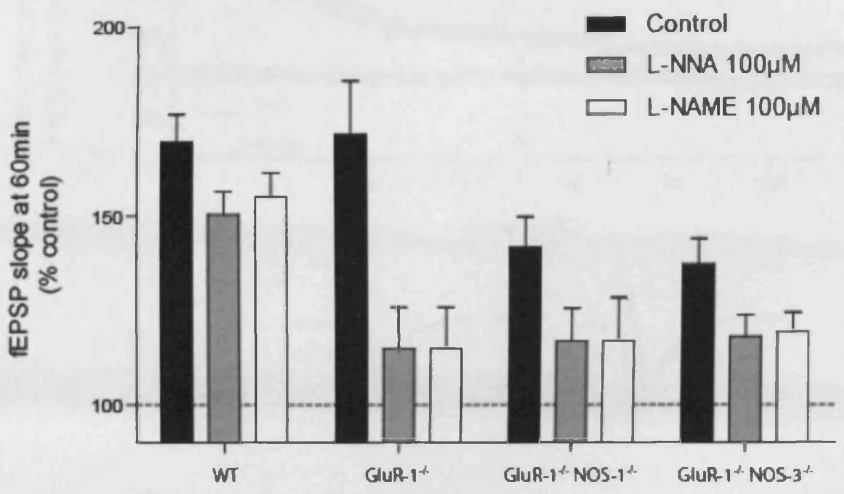


Figure 6.12 Summary of the NOS data.
A, Summary figure, bars indicate the magnitude of the LTP measured 60 minutes after the tetanus. In wild-type L-NNA (100μM) (grey bars) and L-NAME (100μM) (open bars) produced a small non significant reduction in the magnitude of the LTP 60 minutes post tetanus. In all the knockout mice the LTP was reduced to a similar level by L-NNA and L-NAME but a small residual LTP was apparent. The magnitude of the LTP was reduced in both double knockouts and both were further reduced by either L-NNA or L-NAME applications.

5.2.4 The NO sensitive component of LTP in the wild-type is mediated through both the NOS-1 and NOS-3 isoforms of NO synthase.

Finally, to complete the picture I replicated the results of (O'Dell et al., 1994; Son et al., 1996) to see which isoforms of NO synthase (NOS) are involved in the wild-type by looking at expression of LTP in NOS-1 and NOS-3 single knockout mice. The level of LTP was significantly reduced in both knockout to 136 $\pm$ 4 (NOS-1) and 137 $\pm$ 11% (NOS-3) ($p<0.001$), though as can be seen from Figure 6.13, the level of LTP is still quite substantial.

These studies show that approximately 50% of LTP is NOS dependent in wild-type while ~85% is NOS dependent in the GluR1 knockout. This NO dependence is only seen if a high intensity theta-burst stimulation is used to induce LTP, and it would appear that both NOS-1 and NOS-3 isoforms are involved.

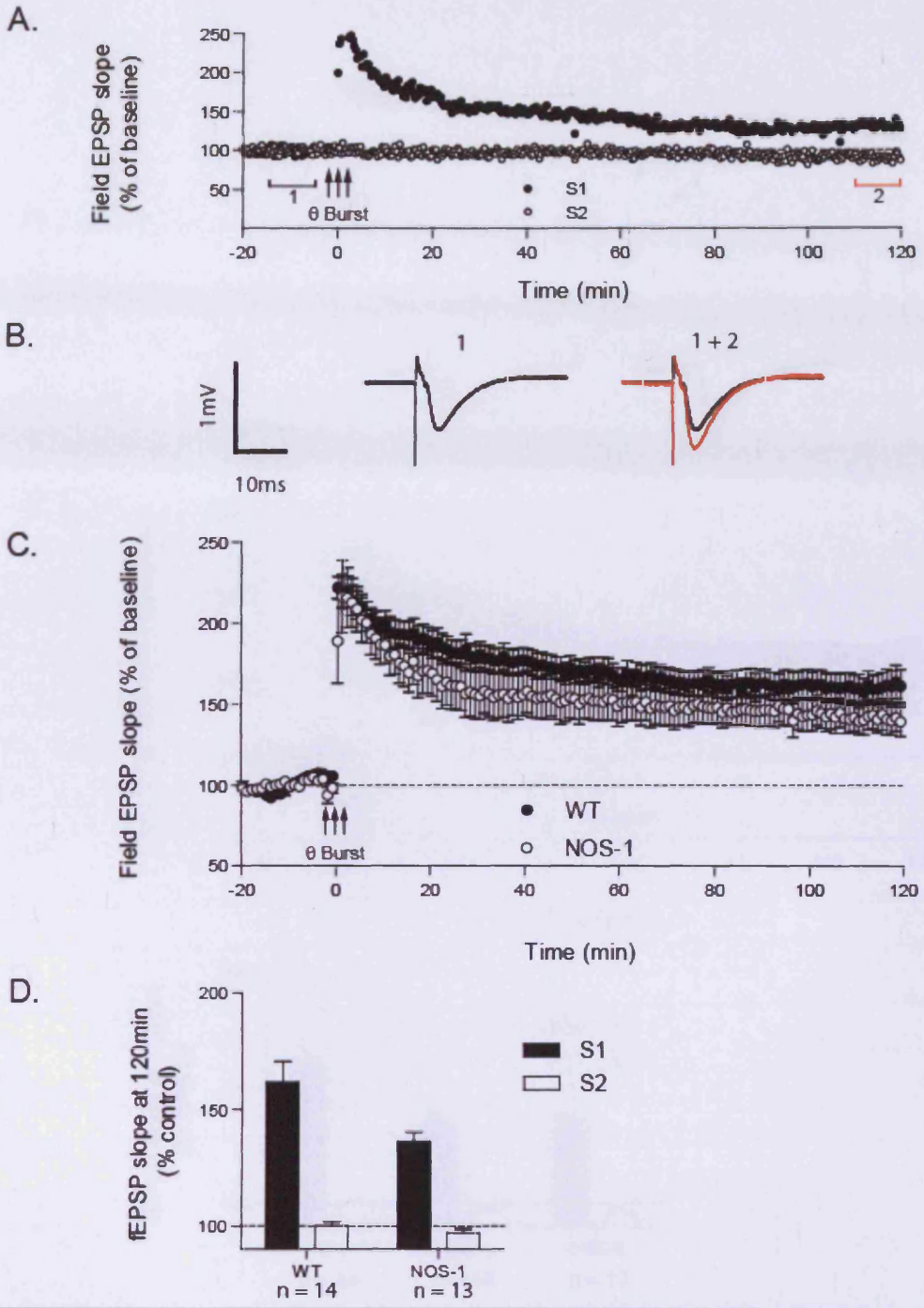


Figure 6.13 The magnitude of the LTP is reduced in the NOS-I single knockout.
A, Example experiment, LTP can be reliably induced in the NOS-I single knockout. B, Example traces taken at time points indicated by the bars. C, Group data, LTP in the NOS-I single knock-outs (○) is reduced when compared to wild-types (●) if a TBS is used during induction. D, Bars indicate the magnitude of the LTP measured 60 minutes after the tetanus.

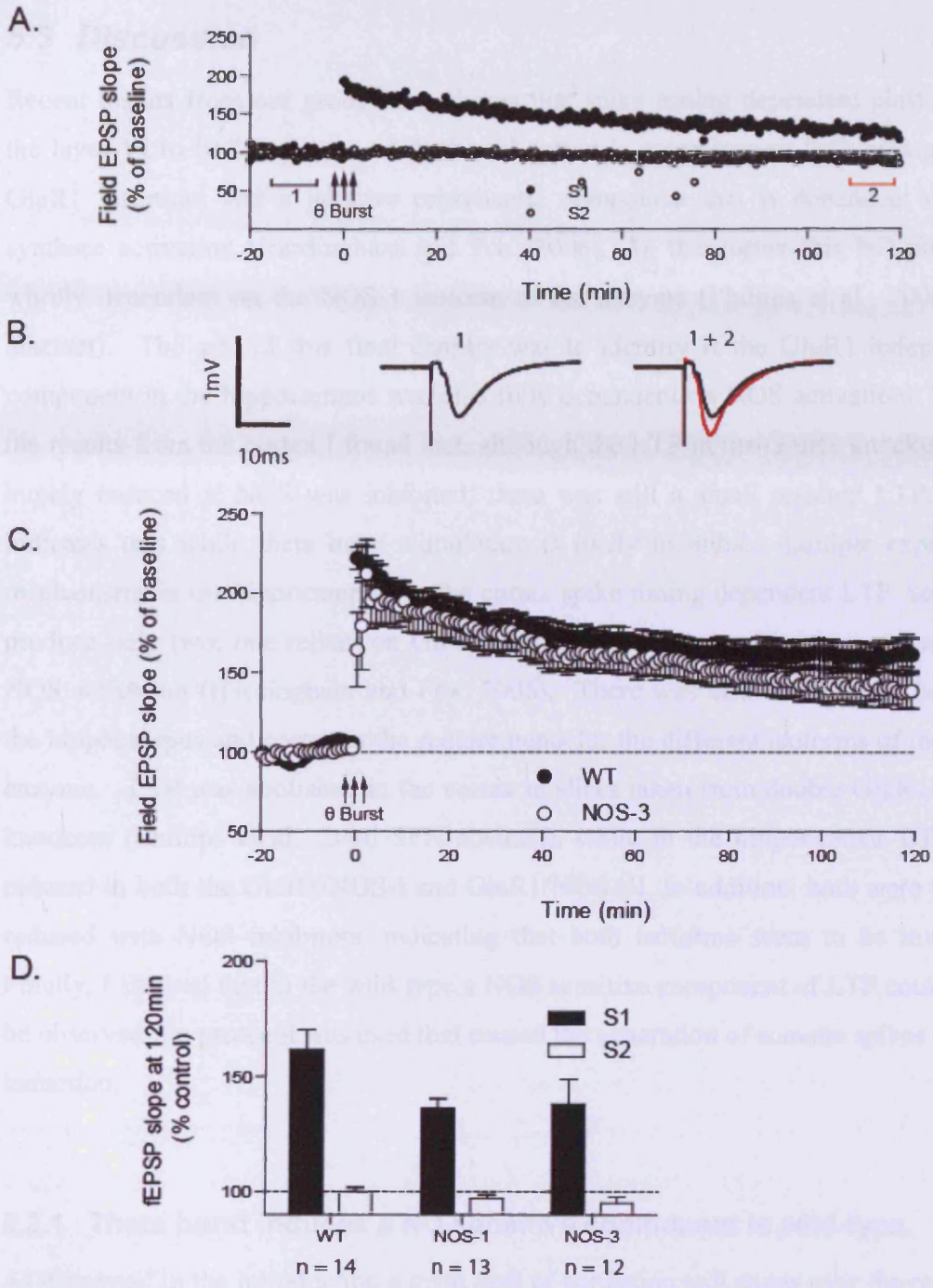


Figure 6.14 The magnitude of the LTP is reduced in the NOS-III single knockout.
A, Example experiment, LTP can be reliably induced in the NOS-III single knockout. B, Example traces taken at time points indicated by the bars. C, Group data, LTP in the NOS-III single knock-out (○) is reduced when compared to wild-type (●) if a TBS is used during induction. D, Bars indicate the magnitude of the LTP measured 60 minutes after the tetanus.

5.3 Discussion

Recent results from our group have shown that spike timing dependent plasticity in the layer VI to III/III synapse of the barrel cortex is dependent on both postsynaptic GluR1 insertion, and a putative presynaptic component that is dependent on NO synthase activation (Hardingham and Fox, 2006). In the cortex this NO signal is wholly dependent on the NOS-1 isoform of the enzyme (Phillips et al., 2006 SFN abstract). The aim of this final chapter was to identify if the GluR1 independent component in the hippocampus was also fully dependent on NOS activation. Unlike the results from the cortex I found that, although the LTP in the GluR1 knockout was hugely reduced if NOS was inhibited, there was still a small residual LTP. This indicates that while theta burst stimulation is likely to induce multiple expression mechanisms in the hippocampus, in the cortex spike timing dependent LTP seems to produce only two; one reliant on GluR1 and the other expressed presynaptically via NOS activation (Hardingham and Fox, 2006). There was also a difference between the hippocampus and cortex in the requirements for the different isoforms of the NOS enzyme. LTP was abolished in the cortex in slices taken from double GluR1/NOS1 knockout (Phillips et al., 2006 SFN abstract), while in the hippocampus LTP was reduced in both the GluR1/NOS-I and GluR1/NOS-III. In addition, both were further reduced with NOS inhibitors, indicating that both isoforms seem to be involved. Finally, I showed that in the wild-type a NOS sensitive component of LTP could only be observed if a protocol was used that caused the generation of somatic spikes during induction.

5.3.1 Theta burst induces a NO sensitive component in wild-type.

As discussed in the introduction a great deal of confusion still exists over the role that NO plays in the induction of LTP in the hippocampus. While some studies showed a complete block of LTP in the hippocampus after application of NOS inhibitors (1999; Bohme et al., 1991; Bon et al., 1992; Doyle et al., 1996; O'Dell et al., 1991a; Schuman and Madison, 1991) others found only a partial block of LTP (Bon and Garthwaite, 2003; Boulton et al., 1995; Chetkovich et al., 1993; Gribkoff and Lum-Ragan, 1992; Haley et al., 1993; Hopper and Garthwaite, 2006; Iga et al., 1993; Musleh et al., 1993; Southam and Garthwaite, 1996), or did not find any effects using

NOS inhibitors (Bannerman et al., 1994; Cummings et al., 1994; Williams et al., 1993). I found that blocking NOS with either L-NNA or L-NAME could only significantly reduce the magnitude of LTP if a high intensity TBS was used for induction, while no effect was seen using 100Hz stimulation. This observation fits with the data from a number of studies, which show a NOS sensitive component of LTP after TBS (Bon et al., 1992; Haley et al., 1993; Haley et al., 1992; Musleh et al., 1997; O'Dell et al., 1994; Williams et al., 1993) although (surprisingly) some studies have shown that LTP induced with 100Hz is also NO sensitive (Bohme et al., 1991; Bon and Garthwaite, 2003; Boulton et al., 1995; O'Dell et al., 1991a; Schuman and Madison, 1991). Thus, although there are conflicting reports as to whether 100Hz cannot induce a NO component (Cummings et al., 1994; Williams et al., 1993), to my knowledge no one has shown a lack of effect if a TBS is used. In all the studies mentioned above, the magnitude of LTP was measured 45 – 60 minutes after induction, while I did see a reduced component at these time points, the reduction was only significant at 120 minutes after the tetanus.

Everyone who studies LTP in the hippocampus knows there is a certain degree of variability in the magnitude of the potentiation between slices and experiments. This has the effect of making statistical analysis of subtle changes in the magnitude of LTP difficult, and is possibly the reason why I only saw a significant effect at 120 minutes after the tetanus, even though the magnitude was reduced from the value at ~20-30 minutes. To take account of this variability between slices I also looked at the effect of NOS inhibition using a two pathway experimental design. The advantage of this method is that it allows a within slice comparison to be made and therefore the use of paired statistics. A paired test calculates the difference between each set of pairs, and treats the errors based on the assumption that the differences in the entire population follow a Gaussian distribution. The whole point of using a paired experimental design and test is to control for experimental variability and is therefore useful in the LTP experiments. The variation in the magnitude of potentiation between slices cannot be controlled, however this should not affect the level of NO dependence. However, by analysing only the differences, a paired test corrects for the between slice scatter. I had previously shown in chapter 3, that if a TBS was given to one pathway, and then again 15 minutes later to the second pathway, no difference was observed in the

magnitude of potentiation. Using this method a significant reduction in the magnitude of LTP was observed after 60 minutes following the tetanus, which was more in line with the previously published results. This experiment also confirmed the finding that NOS activity is required for induction, but not the continued expression of LTP, as NOS inhibition did not affect the already potentiated pathway (Haley et al., 1992).

My results suggest that LTP induced using TBS is expressed via multiple mechanisms, one of which is likely to involve the activation of NOS. However in wild-type, LTP can also be expressed via the postsynaptic insertion of GluR1. Here lies one of the possible sources of confusion in the NO field. Blocking NOS will only block a single component of the LTP and not completely abolish it due to the engagement of other parallel expression mechanisms. This problem is enhanced by the observation that two standard induction protocols with differing outcomes in postsynaptic spike production will engage these multiple mechanisms differentially. One of the underlying aims of this thesis was to get around the problem of multiple expression mechanism by using the GluR1 knockout as tool to separate the postsynaptic mechanisms leaving only presynaptic ones. However in doing this, I identified a variable postsynaptic spike production that has previously not been considered in any of the field recording experiments which has a large effect in determining if a NOS sensitive component is induced in wild-type. A number of other variables have been identified that changed the sensitivity of LTP to NOS inhibition, including animal age, temperature (Williams et al., 1993) and stimulus intensity (Gribkoff and Lum-Ragan, 1992; Haley et al., 1993; O'Dell et al., 1994), these presumably change the balance of the multiple mechanisms to a more NOS sensitive one. How these variables might be related to somatic spike production will be discussed further in the next chapter.

5.3.2 LTP in the GluR1 knockout is more sensitive to NOS inhibition than that in wild-type.

In the GluR1 knockout almost all the LTP is NO sensitive (~90%), while in wild-type early stages of LTP are not NO sensitive. The residual component of LTP in the wild-type is likely to be GluR1 dependent, which would account for the large

difference in the NO dependent component between the two genotypes. Since the LTP in the GluR1 knockout seems to be expressed presynaptically (see chapter 4 and Hardingham and Fox, 2006) and requires post synaptic activation of CaMKII, it seems likely that the NO is acting as a retrograde messenger. NO is generated postsynaptically via NOS and then travels back to the presynaptic site, activating presynaptic sGC and thereby affecting transmitter release (Bon and Garthwaite, 2001; Burette et al., 2002; Haghikia et al., 2007). However, as I have no direct evidence for this and the evidence for a presynaptic change in the GluR1 knockout is based on two methods that have proved controversial in the past, it is still conceivable that NO is acting postsynaptically.

Recently a report has suggested that activation of a postsynaptic NO-sGC-cGMP pathway can cause the phosphorylation of S845 at GluR1 receptors in a cGMP dependent fashion and thereby increasing GluR1 levels in the plasma membrane (Serulle et al., 2007). In addition, the NO-cGMP pathway has been suggested to control the postsynaptic clustering of GluR1 in cultured hippocampal neurons (Wang et al., 2005). This is obviously unlikely to be mediating the NO mechanism in the GluR1 knockout as the GluR1 is absent. However it does raise the possibility that post synaptic AMPA trafficking can also be regulated via the NO-sGC-cGMP pathway. NO may also modulate cellular functions by S-nitrosylation of various proteins and it has recently emerged as a prevalent signalling mechanism. For example, S-nitrosylation of nuclear proteins associated with cAMP response element (CRE) binding protein is involved in regulation of its DNA binding, and thus CRE-mediated gene expression (Riccio et al. 2006). S-nitrosylation has also been shown to be involved in the regulation of GluR2 insertion during cerebellaa LTP (Kakegawa and Yuzaki, 2005). Possibly most relevant, was a recent study that showed if NMDA receptors were activated there was an increase in the un-clustering of dendritic PICK1, and therefore the release of GluR2-containing AMPA receptors to the membrane surface. This process was dependent on the activation of NO, resulting in the *S*-nitrosylation of ethylmaleimide *s*ensitive factor (NSF). They also found that NMDA receptor activation increased the NSF-GluR2 association and also the surface expression of AMPARs. NO donors mimicked the NMDA receptor activation induced decrease in dendritic PICK1 levels, and activation was also blocked by NO

scavengers. This fits with the model of LTP in wild-type discussed in chapter 4. Briefly, insertion of GluR1-containing receptors, which do not bind NSF, mediate the early phase of LTP (20-25 minutes) in a CaMKII-dependent manner (Hayashi et al., 2000; Plant et al., 2006). These receptors are then replaced by cycling GluR2/3 AMPARs (Plant et al., 2006). These data would then suggest that NO modulates the insertion of GluR2 containing receptors and hence blockade of NOS would block the later > 20 minutes of postsynaptic LTP in wild-type. This fits with the slow effect of NOS blockade, although it does not fully explain my results. In the GluR1 knockout the 100Hz protocol failed to induce LTP while in the wild-type it could induce a long lasting potentiation. Since GluR1 is thought to only be involved in the early phase of this potentiation and GluR2/3 in the later phase, then GluR2/3 must have required the initial delivery of GluR1. If it had not a slowly developing potentiation would also have been seen with 100Hz stimulation. Therefore it is unlikely that GluR2 insertion mediated the highly NOS sensitive, GluR1 independent, component of LTP. It is possible however that theta burst LTP activates a separate mechanism and thereby causes GluR2 insertion. Thus although my data suggests a presynaptic expression, it is possible that NO could be regulating the stabilization of GluR2 subunits and hence produce the slow, highly NO sensitive LTP that I observe in the GluR1 knockout.

5.3.3 The NO sensitive component of LTP in the GluR1 knockout is mediated through both the NOS-1 and NOS-3 isoforms of NO synthase.

Since the LTP in the GluR1 knockout was almost totally dependent on NOS activation, we hypothesised that the LTP in either the double NOS1/GluR1 or NOSIII/GluR1 knockout would be abolished. This would then help to resolve the confusion that still exists over which NOS isoform mediates that NO signal in LTP. In the past the results from single knockout of either NOSI or NOSIII have been confusing. It was first reported that LTP was preserved in NOSIII deficient mice (O'Dell et al., 1994), a result explained by NOSIII being located in pyramidal cells (now considered incorrect; see below). Subsequently, LTP was shown to be largely preserved in NOSIII knock-out mice, but lost when NOSI and NOSIII were both deleted (Son et al., 1996). Whereas others, using either a presumed dominant-negative

NOSIII construct (Kantor et al., 1996), or NOSIII knock-out mice (Wilson et al., 1997), reported a major loss of LTP. To our surprise, LTP was not fully abolished in either the NOS1/GluR1 or the NOSIII/GluR1 knockout. The magnitude of the LTP in both the knockout was reduced to a similar degree, and both could be further reduced with the application of NOS inhibitors. This result is in agreement with a recent report that suggested that both isoforms of the enzyme are important in the hippocampus (Hopper and Garthwaite, 2006). However it was surprising to us, as another member of our group has recently showed that in the barrel cortex LTP was completely abolished in the double NOS1/GluR1 knockout, while in the double NOSIII/GluR1 knockout the magnitude of LTP was similar to that seen in the single GluR1 knockout (Phillips et al., 2006). These results indicate that while in the cortex only NOS-I is involved in the production of NO during LTP, in the hippocampus both isoforms seem to be mediate the effect.

Although initially NOSIII was reported to be present in CA1 pyramidal cells (Dinerman et al., 1994), subsequent studies by the same laboratory (Blackshaw et al., 2003) and by others (Stanarius et al., 1997; Topel et al., 1998, Seidel et al., 1997) could not confirm this result, and instead, led to the conclusion that NOS-III mRNA and protein were found only in endothelial cells. As a result (Hopper and Garthwaite, 2006) concluded that NOSIII in endothelial cells generates a tonic level of NO, priming the presynaptic sites and keeping sGC in a state to be readily activate by a phasic NO signal mediated by postsynaptic NOSI. My results support this conclusion.

In summary, I have shown that in wild-type somatic spike generation during induction is essential in generating a NO mediated component of LTP. This NOS sensitive component did not affect the early phases of the potentiation, due to postsynaptic mechanisms dominating at this time. However after 2 hours the LTP was reduced by 50% and may have decreased even more if the experiment had been run for longer. In the GluR1 knockout, the LTP was almost fully dependent on NOS activation, although a small residual LTP did persist. Using the GluR1 knockout as a tool to separate the pre and postsynaptic mechanism, I have shown that both NOS isoforms

are responsible for mediating the NO signal. This study indicates that while NO is not wholly responsible for LTP expression, it is an important mechanism that mediates the prolonged expression of LTP and should not be ignored even if controversy surrounds it. Finally, I have also identified a unique residual LTP that requires the activation of NMDA receptors and CaMKII during induction, but is expressed through a GluR1 independent and NO insensitive expression mechanism. Given the small size of this LTP, what mediates this mechanism and its relevance to *in vivo* studies is an unanswered question.

6 Discussion

6.1 Summary

The original aim of this thesis was to clarify the role that NO plays in LTP in the hippocampus. To achieve this, the approach I took was to use GluR1 knockout as a tool to reduce the postsynaptic component of LTP leaving only a putative presynaptic component. The first step was to identify if LTP in the GluR1 knockout could be induced using a standard protocol in field recording experiments. This was important as, although GluR1 independent LTP has been reported previously, it was only possible to induce this component if a spike timing dependent protocol was used during induction. I found that while 100Hz was ineffective at inducing LTP in the GluR1 knockout, TBS produced a slowly developing plasticity that was almost completely dependent on NOS activation. The LTP in the GluR1 knockout was also expressed more presynaptically than in the wild-type, but required the activation of postsynaptic CaMKII, implying that NO was activating as a typical retrograde messenger. The surprising explanation that accounted for this large difference between protocols was that only TBS could reliably induce postsynaptic somatic potentials, many of which were complex spikes, while 100Hz (even if given at a high intensity) only produced action potentials during the start of the train, which then rapidly failed. I went on to show that it was these somatic action potentials that were essential in inducing this GluR1 independent component of LTP. In this study, the somatic action potentials were all induced naturally via orthrodromic stimulation and not by the standard method of current injection at the soma. This approach is important, as it has recently been suggested that local dendritic spikes are more important during induction of LTP over the back propagation of action potentials from the soma (Golding et al., 2002; Lisman and Spruston, 2005; Remy and Spruston, 2007). While this may be the case for the GluR1 dependent component, it is not true for the NO sensitive GluR1 independent component of LTP where back propagating action potentials are essential. Therefore, in identifying why TBS was more effective at inducing LTP in the GluR1 knockout, this also led to the realisation that somatic spikes were also maybe important for inducing the NO sensitive component of LTP in wild-type. Finally I went on to show that both the NOSI and NOSIII isoforms were responsible for this NO signal.

6.2 Can somatic spike production reconcile the confusion in the NO field?

Possibly the most disputed result in the NO field is whether NOS inhibitors can block or reduce the magnitude of LTP in the hippocampus. To date 19 studies have been published on this topic in the schaffer collateral to CA1 synapse alone, with many more studies published in other regions. Of these 19, ten used the same 100Hz protocol that I used in my study. Surprisingly, these results were almost evenly split between those showing an effect and those that did not. Three studies showed a full block, four showed a partial block, and in agreement with my results three showed no effect at all. However, from the five studies that used a TBS protocol, three saw a full block and two saw a partial block, while no one to my knowledge has reported a negative result. What could possibly explain these contradictory results? A number of studies have identified different experimental conditions that could account for these differences namely; the temperature that the experiment was performed at, the age of the animal, and the stimulus strength during induction. However, these studies do not explain or discuss the mechanism that could account for these differences. I have provided evidence that suggests that activation of NOS depends on post-synaptic spike production during induction, and since this is rarely monitored in extracellular field studies this is possible that this is the reason that different experimental conditions change the sensitivity of LTP to NO inhibition.

Williams et at showed that if LTP was induced with a 100Hz protocol then a NOS sensitive component was only apparent in experiments performed at low temperatures (24°C) while no effect was seen of NOS blockade at the more physiologically relevant temperature of 34°C (Williams et al., 1993). Reducing the temperature of the slice is known to make the slice hyper-excitable (Volgushev et al., 2000a). Volgushev et al., (2000a) showed that cooling the slice brings the cell closer to the threshold for spike generation, while the amplitude of excitatory postsynaptic responses were only slightly influenced by temperature changes in the range from about 21 to 36°C. They also showed that stimuli of constant strength often led to action potential generation at temperatures between 15°C and 25°C, but remained

sub-threshold at higher temperatures (34°C). The ionic mechanism of this hyper-excitability at room temperature is due to the strong dependence of potassium channel function on temperature (Volgushev et al., 2000b). Decreases in potassium conductance during cooling increase the input resistance and also lead to depolarisation of the cell membrane. The absolute value of the activation threshold of voltage-dependent sodium channels however does not change. As a result, less excitatory input would be sufficient to evoke spikes. Therefore, at lower temperatures the number of postsynaptic spikes during a 1 sec 100Hz pulse is likely to be increased and as at lower temperatures there is also an increase in the spike width (Volgushev et al., 2000a) the combined effect is likely to explain the increase in NO dependence at lower temperatures. At higher temperatures, stronger stimuli or primed burst stimuli are more effective in producing post-synaptic action potentials than a high frequency tetanus (Lum-Ragan and Gribkoff, 1993). Similarly, *in vivo* studies at body temperature have shown that the NO sensitive component of LTP is greater if TBS is used to induce it (Holscher, 1999), a protocol which would be likely to produce more spikes than a simple high frequency train.

Two studies have also suggested that the NO signalling mechanism in LTP is developmentally regulated. These showed that NO sensitivity was reduced (Boulton et al., 1994) or even absent in adult animals (Williams et al., 1993). Once again these studies used 100Hz to induce the LTP providing additional evidence in support of my observation that a 100Hz at high temperatures, in adult animals, does not produce a NO sensitive component of LTP. Although there is less evidence that cell excitability decreases with age, somatic spike production could also be involved in this process. It is known that GABAergic inhibition increases during development with an increase in the frequency of $GABA_A$ (Banks et al., 2002; Cohen et al., 2000) and $GABA_B$ (Nurse and Lacaille, 1999) mediated IPSCs up to approximately the 6^{th} post natal week. This developmental shift in the degree of inhibition was shown to be important in modulating the number of action potentials required to induce LTP during a theta burst pairing protocol (Meredith et al., 2003). In the adult hippocampus, LTP can only be induced during theta burst pairing if sub threshold EPSPs are paired with multiple bursts of complex postsynaptic action potentials (Pike et al., 1999). However in young animals single action potentials are sufficient to induce plasticity

(Meredith et al., 2003). This difference was due to an increase in the GABAergic tone in the adult animals, as single burst pairing was able to induce LTP in the adults if GABA transmission was blocked with bicuculline. This could explain why NO sensitivity is higher in young animals even after 100Hz stimulation While TBS produced many more complex spikes than the 100Hz protocol, 100Hz often produced 1-2 action potentials per burst. In young animals the reduced GABA inhibition could allow the recruitment of the NO pathway, despite the reduced number of spikes associated with the 100Hz protocol, and thus explain the increased NOS sensitivity.

Finally, the third variable that has been reported to change the sensitivity of LTP to NOS blockade is stimulus intensity. However this has also led to controversy as although Gribkoff and Lum-Ragan, (1992) showed that a "strong intensity" increased the level of NO dependence, Haley et al., (1993) reported the opposite finding. My results however suggest that it is not only the intensity that the stimulus is given at but also the pattern of stimulation that affects NO sensitivity. At high intensity 100Hz stimulation did not produce action potentials nor a NO dependent component of LTP, while if the same intensity was given during a TBS then postsynaptic spikes were produced and a significant NO component was observed. However care must be taken when interpreting what is meant by a strong or weak "intensity" protocol., While I define it to be the strength of the pulse given, other groups have used "intensity" as the number of pulses given during a train. I suggest that the number of somatic action potentials generated during the trains can explain these seemingly contradictory reports. (Gribkoff and Lum-Ragan, 1992) showed that the level of NO dependence was increased if multiple trains of 100Hz were given, while a single train of 100Hz stimulation did not induce a NO sensitive component. They also used young animals and therefore, as discussed above, fewer somatic spikes would be required to show a positive effect. I showed that even in adult animals, there was a small increase in the number of spikes produced after multiple trains of 100Hz. Therefore it is likely that in young animals repeated trains would produce the required spiking level to induce the NO mechanism while a single train would not be effective. Haley et al., (1993) however kept the number of trains constant, and changed the "intensity" of the protocol by increasing the number of pulses within a 100Hz train (25 pulses "weak", 50 pulses "strong"). As I showed that during 100Hz stimulation

spiking rapidly failed within the first 20 pulses of the train This is unlikely to change the number of spikes between the two protocols and explains their negative result.

One study has shown that 100Hz can induce a NO sensitive component of LTP in adult animals at 32°C (Hopper and Garthwaite, 2006). Although this result cannot be explained with my "somatic spike hypothesis", this study showed a greater sensitivity to NOS inhibition if bursts of 100Hz were given during the induction. This protocol is much more likely to produce somatic action potentials than a long train of stimuli and thus does not contradict my conclusions. To summarise Table 1 lists all the published studies on NOS inhibition of LTP, together with all the experimental conditions that were used and indicates whether the results fit with my somatic spike hypothesis. I believe that most of the results, positive or negative, can be explained by somatic spiking that affect the level of NO dependence. A number of questions still remain and further work is needed to prove this idea unequivocally, however I believe I have shown the importance of measuring the postsynaptic response during induction, rather than just concentrating on the presynaptic rate, frequency and intensity of the induction protocol used.

Table 1

Reference	Effect of NOS inhibition.	Induction Protocol Used	Species	Temp (°C)	Does the data fit with my "somatic spike hypothesis"
(O'Dell et al., 1991b)	Blocked completely	2 x 1 sec 100Hz	Guinea Pig	22	Yes, increased probability at low temperatures and young ages
(Bohme et al., 1991)	Blocked completely	2 x 1 sec 100Hz (high intensity)	Rat (immature)	32	Yes, increased probability at low temperatures and young ages
(Schuman and Madison, 1991)	Blocked completely	4 – 5 x 1 sec 100Hz	Rat (immature)	22	Yes, increased probability at low temperatures and young ages.
(Haley et al., 1992)	Blocked completely	TBS (high intensity)	Rats (immature)	22	Yes, TBS produces spikes, even more likely at young ages and low temperature.
(Bon et al., 1992)	Blocked completely	TBS	Rats (immature)	22	Yes, TBS produces spikes, even more likely at young ages and low temperature.
(Gribkoff and Lum-Ragan, 1992)	Low intensity no effect. High Intensity partial Block	*a.* 1 x 100Hz (low intensity) *b.* 2 x 100Hz (high intensity)	Rats (immature)	32	Yes, repeated 100Hz is more likely to induce more somatic spikes than a single train particularly at young ages.
(Williams et al., 1993)	Full block in young at low temp (TBS low intensity). Partial block in young at low temp (100Hz). No effect in adult.	*a.* TBS (low intensity) *b.* 100Hz low intensity	Rats 4 weeks (immature) 4mounths (adult)	24 30	Yes, At low temp spikes are more likely, hence the increased sensitivity at these temperatures. In adult animals at high temperatures, 100Hz will not produce as many spikes and hence NO sensitivity is less.
(Haley et al., 1993)	Full block at low intensity with short bursts No effect at high intensity long bursts	*a.* 2 x 250ms 100Hz (low intensity) *b.* 2 x 500ms 100Hz (low and high intensity)	Rats (immature)	31	Possibly. Train length will be less important as spikes rapidly fail with time. Block as young animals were used.
(Cummings et al., 1994)	No effect	4 x 1s, 100Hz	Rats (mature)	29	Yes, 100Hz at high temps and adult animals will not produce spikes and hence the NO component is not activated.
(O'Dell et al., 1994)	No effect of 100Hz Full block using 25 pulses of TBS Partial block using 10 pulses (high intensity)	*a.* TBS either 25 bursts (1train) at test intensity or 10 bursts (1train) at 50% max. *b.* 2 x 1s 100Hz low intensity.	Mice (adult)	30	Yes, TBS was more effective hence more somatic spikes.

Reference	Effect of NOS inhibition.	Induction Protocol Used	Species	Temp (°C)	Does the data fit with my "somatic spike hypothesis"
(Boulton et al., 1995)	Partial block at 24°C and 30°C Age.	1 x 1s, 100Hz	Rats (immature)	24 30	Immature, therefore less spikes are required, and at low temperatures spikes are produced.
(Doyle et al., 1996)	Full block (*in vivo*)	20 stim at 200Hz repeated 10 times at 0.5Hz repeated 3 times at 0.003Hz	Rats Mature	37.4	Yes, Protocol very likely to produce somatic spikes.
(Son et al., 1996)	Partial block.	2 x 1s, 100Hz	Mice (adult)	29	Possibly, lower temperature could have produced more spikes.
(Malen and Chapman, 1997)	Full block	900 pulses at 50Hz	Rats (adult)	32	Possibly, Lower frequency stimulation could produce more spikes (would need to look at this directly).
(Musleh et al., 1997)	Full Block	TBS	Rats Organotypic culture (immature)	24	Yes, TBS produces many postsynaptic spikes. Possible full block seen due to culture and hence immature.
(Bon and Garthwaite, 2003)	Partial Block	3 x 100Hz	Rats (immature)	30	Yes, Immature and therefore fewer spikes are needed.
(Hopper and Garthwaite, 2006)	Almost full block	3 x 100ms 100Hz (high intensity)	Rats (mature)	30	No, however showed a greater sensitivity to NOS inhibition if bursts of 100Hz were given during the induction. This protocol is much more likely to produce somatic action potentials than a long train of stimuli and thus does not contradict my conclusions

6.3 Compensation in the GluR1 knockout.

The underlying approach that I took throughout the work reported in this thesis was to use the GluR1 knockout as a tool to study the NO sensitive mechanism without the confounding effects of postsynaptic processes. However, when using knockout one must always bear in mind that compensation of another mechanism might have occurred. Since the gene is missing throughout development, another gene, coding for a product in a different but related pathway, might take over the function of the

missing gene. One then might argue that the NO sensitive component in the GluR1 knockout, and hence the importance of somatic spikes, is not relevant to wild-type. To overcome this possibility I looked directly at the wild-type using a protocol that did not induce somatic spikes (100Hz) and also did not induce a NO sensitive component of LTP, and compared this to a TBS protocol which produced both somatic spikes and induced the NO sensitive component. Admittedly there was an increased sensitivity to NOS blockade in the GluR1 knockout, however this is to be expected if both mechanisms exist in the wild-type.

6.4 Mechanisms that link post synaptic somatic action potentials to NO release.

I have shown that somatic spikes are not required for the induction of the GluR1 dependent component of LTP and supra-threshold depolarisation alone is sufficient. However this is not the case for the NO sensitive component, where bursts of somatic action potentials seems to be critical in activating this mechanism. This result implies that a mechanism exists that requires somatic action potentials for the activation of NOS and thus NO release, which is not activated by EPSP induced depolarisation alone. Both the GluR1 and NO dependent mechanisms require the activation of NMDA receptors and CaMKII, implying that Ca^{2+} entry must be critical to the divergence of these mechanisms. How does a promiscuous second messenger like Ca^{2+} achieve this signalling specificity? There are two differing hypotheses that could explain these diverse effects. Firstly, there could be a spatial compartmentalisation of the Ca^{2+} signal whereby increase in Ca^{2+} in specific locations activates different effector mechanisms, (Raymond, 2007, 2008) or secondly, differences in the magnitude and timing of the Ca^{2+} influx at the synapse could preferentially activate the different pathways.

The first hypothesis would suggest that both TBS and 100Hz would induce localised Ca^{2+} signals in the synapse/spine, inducing the early GluR1 dependent mechanism. Somatic spikes on the other hand, would also lead to Ca^{2+} influx into other spatial compartments, namely the dendrites and the soma, leading to the activation of the

GluR1 independent component. This model however, would suggest that NO is acting postsynaptically, as it is highly unlikely that non synaptic NO production could modulate a synapse specific change presynaptically.

Experimental evidence for the second mechanism has recently been provided by Raymond, (2008) and Raymond and Redman, (2002, 2006). They showed that at high intensity TBS induced multiple components of LTP which were dependent on different Ca^{2+} sources in different spatially segregated regions of the cell. An early LTP (presumably GluR1 mediated) was dependent on Ca^{2+} induced Ca^{2+} release (CICR) via ryanodine receptors (RyRs) within the spine, and surprisingly this Ca^{2+} signal was not affected by action potentials. However, the intermediate LTP required somatic action potentials during induction which in turn induced a dendritic Ca^{2+} signal via IP_3 activation. Finally, they also showed that the late gene transcription dependent LTP also required action potentials, and was dependent on a Ca^{2+} signal at the soma via VDCC activation. This would fit with the idea proposed by Adams and Dudeh (2005) that calcium influx at the soma due to somatic action potential firing is the critical signal for the nucleus to commence transcription after the initiation of LTP. These data would imply that the NOS sensitive GluR1 independent component was activated via a non synaptic Ca^{2+} signal, either via IP3 or VDCC activation, and required gene transcription at the soma. While NO has no direct effect on gene expression *per se*, it is able to amplify the effect of calcium induced gene transcription (Peunova and Enikolopov, 1993). It has previously been suggested that NO and CREB may be involved in late-phase LTP (Lu et al., 1999). A view that is commensurate with the slow onset of NO dependent potentiation in the GluR1 knockout causing the gradually increasing differences between control and NOS inhibited wild-type LTP experiments beyond one hour post LTP induction. The factor linking gene expression with NO signalling might therefore be somatic action potentials initiating nuclear calcium influx.

The second hypothesis, that differences in the magnitude and timing of the Ca^{2+} influx at the synapse could preferentially activate the different pathways, would not exclude the possibility of a presynaptic change occurring. Different calcium levels at the synapse have for a long time been implicated in explaining how either LTP or LTD is triggered at a particular synapse. It is therefore possible that more subtle differences in the time course or magnitude of the Ca^{2+} signal could activate the different components of LTP that I identified. Prolonged depolarisations, associated with 100Hz stimulation, cause a gradual and prolonged increase in the magnitude of Ca^{2+} entry through NMDA receptors reaching a maximum at ~100ms after the end of the tetanus (Petrozzino et al., 1995). The size and time course of this Ca^{2+} transient is dependent on the duration and magnitude of the depolarisation given (Balaban et al., 2004; Perkel et al., 1993; Petrozzino et al., 1995). However, if somatic action potentials are paired with EPSP during a spike timing dependent protocol, then a much faster and larger Ca^{2+} signal is observed. This calcium signal is much larger than the sum of the Ca^{2+} signal from EPSP or action potentials delivered alone (ref). Patch electrode recordings from one dendritic location in CA1 pyramidal neurons showed that these Ca^{2+} transients evoked by EPSP-action potential pairing are correlated with supra-linear summation of electrical signals. This is termed "boosting" of the back propagating action potential (Watanabe et al., 2002) and was due to the inactivation of dendritic K channels. A similar boosting effect was found in the dendrites of both layer 5 (Williams and Stuart, 2000; Stuart and Hausser, 2001, Sjostrom and Hausser, 2006) and layer 2/3 pyramidal cells in the neocortex (Waters et al., 2003). In all cases the boosting of the dendritic action potentials were viewed as a mechanism potentially responsible for the depolarisation required to unblock the NMDA receptors and cause this maximal Ca^{2+} influx. Although the TBS that I used is not a true spike timing dependent protocol, the action potentials created during the bursts would all have occurred within 40ms of the first EPSP, and all spikes would have occurred within 10ms of the last EPSP of the burst and therefore within the critical time window for this boosting effect. TBS not only produced single action potentials, but also many complex spikes during the later trains which would further enhance this supralinear Ca^{2+} influx (Kampa et al., 2006; Waters et al., 2003). It is possible that NOS activation requires this large, fast Ca^{2+} transient, while the GluR1

dependent mechanism can be activated by the slower, longer Ca^{2+} influx associated with the depolarisation alone.

6.5 Conclusions

The results presented in this thesis show that LTP can be induced by conventional and physiologically relevant methods in adult GluR1 knockout. I have also shown that in wild-type somatic spike generation during induction could generate the NO mediated component of LTP which might explain some of the conflicting results reported in the literature. This NOS sensitive component did not affect the early phases of the potentiation. However, after 2 hours the LTP was reduced by 50% in the presence of NOS inhibition and may have decreased even more if the experiment had been run for longer. In the GluR1 knockout the LTP required NOS activation, although a small residual LTP did persist.

Using these knockout as a tool to separate the putative presynaptic and postsynaptic mechanism, I have shown that both NOS isoforms are responsible for mediating the NO signal. Previous studies have concluded that since these genetically modified mice showed normal spatial reference memory in the Morris water maze, the elevated plus maze and the Y-maze, hippocampal LTP is not necessary for spatial learning in this structure (Bannerman et al., 2003; Reisel et al., 2002; Schmitt et al., 2004; Zamanillo et al., 1999). While the present studies do not provide any evidence for hippocampal LTP being involved in spatial learning, they do argue against rejection of this theory based on a lack of hippocampal LTP in GluR1 knockout mice. Short term trial by trial working memory has been shown to be disrupted in these animals. This is consistent with the time course of the faster and more transient GluR1 component of potentiation which I have described and suggests that the rapid insertion of GluR1 is critical in this process. As a result, one would predict that the reference memory component that is persevered in the GluR1 knockout is dependent on a NO sensitive mechanism and that this would be lost if both NOS isoforms were blocked. Finally, I have also identified a unique residual LTP that requires the activation of NMDA receptors and CaMKII during induction, but is expressed through a GluR1 independent NO insensitive expression mechanism. Given the small

size of this LTP, what mediates this mechanism and its relevance to *in vivo* studies remains an unanswered question. This study indicates that while NO is not wholly responsible for LTP expression, its production is an extremely important mechanism that mediates its prolonged expression and should not be ignored, despite the controversy that still surrounds it.

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8 Publications

Phillips, K.G., Hardingham, N.R., and Fox, K.D. (2006). Comparing the role GluR1 and nitric oxide play in neocortical and hippocampal long-term potentiation. Atlanta, GA: Society for Neuroscience, *Abstract*, 38.37/D33.

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Development/Plasticity/Repair

Postsynaptic Action Potentials Are Required for Nitric-Oxide-Dependent Long-Term Potentiation in CA1 Neurons of Adult GluR1 Knock-Out and Wild-Type Mice

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Neocortical long-term potentiation (LTP) consists of both presynaptic and postsynaptic components that rely on nitric oxide (NO) and the GluR1 subunit of the AMPA receptor, respectively. In this study, we found that hippocampal LTP, induced by theta-burst stimulation in mature (>8-week-old) GluR1 knock-out mice was almost entirely NO dependent and involved both the α splice variant of NO synthase-1 and the NO synthase-3 isoforms of NO synthase. Theta-burst induced LTP was also partly NO-dependent in wild-type mice and made up ~50% of the potentiation 2 h after tetanus. Theta-burst stimulation reliably produced postsynaptic spikes, including a high probability of complex spikes. Inhibition of postsynaptic somatic spikes with intracellular QX314 or local TTX application prevented LTP in the GluR1 knock-out mice and also blocked the NO component of LTP in wild types. We conclude that theta-burst stimulation is particularly well suited to producing the postsynaptic somatic spikes required for NO-dependent LTP.

Key words: **plasticity; potentiation; nNOS; eNOS; working memory; long-term memory**

Introduction

The mechanisms underlying hippocampal long-term potentiation (LTP) have been studied extensively since its original discovery (Bliss and Lomo, 1973). Recently, efforts have concentrated on the postsynaptic mechanisms of LTP, which involve insertion of AMPA receptors into the postsynaptic membrane (Malinow and Malenka, 2002). However, presynaptic components of hippocampal LTP have also been documented (Malinow and Tsien, 1990), most recently by direct imaging (Stanton et al., 2005; Bayazitov et al., 2007) and less is known of the incipient mechanisms involved in this form of LTP. Because LTP can be induced postsynaptically (Malenka et al., 1989) and yet is partly expressed presynaptically (Stanton et al., 2005; Bayazitov et al., 2007), some retrograde factor must be involved in coordinating presynaptic and postsynaptic components of transmission strength (Lisman and Raghavachari, 2006). Nitric oxide (NO) became an early candidate for this retrograde factor (Haley et al., 1992; Kantor et al., 1996; Son et al., 1996), but research in this area has slowed partly due to the difficulty of reproducing findings in different laboratories on the role of NO in LTP and memory [for review, see Hölscher (1997)].

Recent evidence from the neocortex has shown that layer II/III cells exhibit LTP that can be separated into presynaptic and postsynaptic components by manipulating GluR1 and NOS (Hardingham and Fox, 2006). While GluR1 is responsible for the postsynaptic component of LTP in the neocortex, the presynaptic component is dependent on postsynaptic NO synthase (NOS) activation (Hardingham and Fox, 2006). In the neocortex, LTP cannot be abolished entirely by blocking either NOS or GluR1, but blocking both simultaneously eliminates LTP.

The first reports of GluR1-dependent LTP in the hippocampus indicated that LTP was completely absent in GluR1 knock-outs (Zamanillo et al., 1999), but it was later discovered that GluR1 dependent LTP was present in younger animals and required a spike-timing protocol to induce it (Hoffman et al., 2002; Jensen et al., 2003). This raises the possibility that this residual component of LTP is also NO dependent in the hippocampus, as it is in the neocortex.

Therefore, we looked at LTP in the hippocampus of GluR1 null mutants (Zamanillo et al., 1999) to test whether LTP is NO-dependent. We also studied the isoforms of NOS involved using the endothelial NOS (NOS-3) knock-out and the α neuronal NOS (αNOS-1) knock-out mice. The αNOS-1 knock-out shows a 94.5% reduction in catalytic activity (Huang et al., 1993) and lacks the major α splice variant but not the β and gamma splice variants of NOS-1 (Eliasson et al., 1997). The α splice variant contains a PDZ domain which links NOS-1 to PSD-95 and hence to the postsynaptic density, whereas the β and gamma isoforms do not and so are cytoplasmic (Eliasson et al., 1997). Deletion of the synaptically located αNOS-1 isoform therefore makes the αNOS-1 knock-out particularly well suited to studying synaptic deficits. Our studies reveal that both major NOS isoforms play a role in hippocampal LTP and that postsynaptic spikes are necessary for the induction of the NO component of LTP in both GluR1 knock-outs and wild-type mouse hippocampus.

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Materials and Methods

Animals. Subjects were mice aged P45–64 (6–9 weeks) for the intracellular experiments and P57–64 (8–9 weeks) for the extracellular experiments. AMPA receptor subunit 1 (GluR1) knock-out mice, αNO synthase isoform 1 (αNOS-1) knock-out mice, NO synthase isoform 3 (NOS-3) knock-out mice and wild-type littermates were bred into a C57BL/6 background and maintained in the colony as heterozygotes. Experimental null mutants and wild-type littermates were bred from heterozygote crosses (cousin mating). Double knock-out animals were created by breeding heterozygous single knock-outs until double heterozygous males and females were produced. Double knock-outs were produced by mating double heterozygous animals, or on a few occasions by mating GluR1$^{-/-}$ NOS$^{-/+}$ mice with double heterozygotes. The GluR1 knock-out mice were kindly supplied by Rolf Sprengel (Max Planck Institute for Medical Research, Heidelberg, Germany) via the Rawlins laboratory at Oxford University. The αNOS-1 and NOS-3 knock-outs were obtained from The Jackson Laboratory. We genotyped the animals used in this study by PCR using primers ordered from MWG. The following primer sequences were used, for αNOS-1: (oIMR13) 5′ CTT GGG TGG AGA GGC TAT TC 3′; oIMR14 5′AGG TGA GAT GAC AGG AGA TC 3′; (oIMR406) 5′ TCA GAT CTG ATC CGA GGA GG 3′; (oIMR407) 5′ TTC CAG AGC GCT GTC ATA GC 3′. For NOS-3: (oIMR94) 5′ TGG CTA CCC GTG ATA TTG CT 3′; (oIMR1823) 5′ ATT TCC TGT CCC CTG CCT TC 3′; (0IMR1824) 5′GGC CAG TCT CAG AGC CAT AC 3′. Jackson Laboratories supplied both αNOS-1 and NOS-3 primer sequences. For the GluR1 knock-outs we used (1005) 5′ AAT GCC TAG TAC TAT AGT GCA CG 3′; (MH60) 5′ CAC TCA CAG CAA TGA AGC AGG AC 3′; (3Int3) 5′ CTG CCT GGG TAA AGT GAC TTG G 3′. Rolf Sprengel supplied primer sequences for the GluR1 knock-outs.

Slice preparation. Mice were killed via cervical dislocation and decapitated. Brains were quickly removed and immersed into ice-cold artificial CSF (aCSF) [composition (in mM): 124 NaCl, 2.3 KCl, 2 $CaCl_2$, 1.3 $MgSO_4$, 1 NaH_2PO_4, 26 $NaHCO_3$, and 11 D-glucose] constantly bubbled with 95%O_2/5%CO_2 to maintain the pH at 7.4. Coronal sections (400 μm) were cut with a vibratome and incubated for at least 1 h in a submersion chamber kept at 32°C.

Extracellular field potentials. Slices were transferred to a submerged recording chamber perfused with aCSF at 32°C. Extracellular field potentials were recorded in the stratum radiatum of the CA1 region of hippocampus using carbon fiber electrodes. Responses were evoked in control and test pathways using a 20 μs square voltage step applied at 0.05 Hz through two monopolar electrodes located in stratum radiatum test (S1) and control (S2) pathways. The S1 electrode was placed approximately equidistant from the molecular and pyramidal layers on the CA3 side of the recorded cell. To ensure pathway independence, the stimulating electrodes were placed at slightly different depths in the stratum radiatum. The S2 electrode was placed either higher or lower than S1 (in alternate experiments) and was always located on the subiculum side of the recorded cell. If any effect on the S2 control pathway was observed after tetanus given to the S1 pathway, the recording was discarded. Input/output (I/O) curves were produced by gradual increases in stimulus strength at the beginning of each experiment, until a stable baseline of evoked response was reached. The test stimulus pulse was then adjusted to produce a field EPSP (fEPSP) whose slope and amplitude was 40% that of the maximum possible fEPSP and was kept constant throughout the experiment. The negative going slope of each fEPSP was measured over the 20–80% range of the peak amplitude. Responses were amplified (Axoclamp 2B), digitized [Cambridge Electronic Design (CED) 1401], and recorded using Signal (CED).

Dual extracellular and whole-cell patch-clamp recordings. Recordings were made in a submerged chamber perfused with aCSF at 32°C. To enable the results of the intracellular recordings to be compared directly to the extracellular recordings it was important to keep the stimulus strength constant between studies. This was achieved by recording an extracellular I/O curve before the intracellular recording was made. Intracellular recordings were then obtained from CA1 cells that were directly above the fEPSP recording electrode, perpendicular to the stratum radiatum. CA1 pyramidal cells were visualized using an Olympus Optical BH2 video microscope, using DIC optics. Patch electrodes with a resistance of 10–15 MΩ were pulled from borosilicate filamented glass capillaries using a horizontal puller (Sutter). Pipettes were filled with intracellular solution containing in mM: 110 K-gluconate, 10 KCl, 2 $MgCl_2$, 0.3 Na_2ATP, 0.03 Na_2GTP, and 10 HEPES corrected to pH 7.3 (290 mOsm). QX314 (20 μM) was included in the electrode filling solution for one set of experiments as described in the results. Whole-cell recordings were made at the post break-in potential (average E_m of −64 ± 3 mV for wild types, −68 ± 4 mV for GluR1 knock-outs) in the current-clamp mode but discarded if the input resistance changed by >20%. Responses were amplified (Axoclamp 2B), low-pass filtered below 4–5 kHz, digitized (CED 1401) and recorded using Signal (CED). When indicated in the text, 10 μM TTX dissolved in aCSF was pressure-applied through a patch pipette positioned near the soma under visual guidance. The flow of the solution was monitored by using 0.1% Fast Green in the TTX pipette and optimized to avoid TTX diffusion into the SR. Pressure application prevented postsynaptic action potential initiation and back-propagation while not affecting the EPSP amplitude.

Induction of synaptic plasticity. Extracellular fEPSPs were recorded from two independent pathways for a baseline period of 20 min. Intracellular recording only permitted a 4–5 min baseline period to prevent LTP washout. LTP was induced by either 100 Hz or theta-burst stimulation (indicated in figure legends). Stimulation (100 Hz) consisted of 100 pulses at a frequency of 100 Hz; this was then repeated three times at 0.05 Hz. In theta-burst stimulation, the bursts consisted of four pulses at 100 Hz, repeated 10 times at 5 Hz (theta frequency); these were also repeated three times at 0.05 Hz. These protocols were used either at a low stimulus intensity (defined as a control voltage of 40% of maximum and a pulse-width of 20 μs) or at a high stimulus intensity (defined as a control voltage of 40% maximum and a double pulse-width of 40 μs).

We measured the number of spikes produced by theta-burst and 100 Hz stimulation in the intracellular recording experiments. For the purposes of the analysis the number of "spikes per train" was considered as the number of spikes produced in a train of 10 theta-bursts (40 stimuli) and a train of 100 stimuli for 100 Hz stimulation. The number of "spikes per stimulus" was considered to be the total number of spikes produced divided by the number of stimuli during the stimulus train, which was 120 stimuli for theta-burst stimulation and 300 stimuli for 100 Hz stimulation.

Drugs. All drugs were applied in the aCSF unless otherwise stated. *N*-nitro-L-arginine (L-NNA), *N*-ω-nitro-L-arginine methyl ester (L-NAME), (D)-2-amiono-5-phosphopentanoate (D-AP5), *N*-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium chloride (QX314), and octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethan-o-10aH-[1,3]dioxocino[6,5-d]pyrimidine-4,7,10,11,12-pentol (TTX) were all obtained from Tocris Bioscience. Autocamtide-2-inhibitory peptide (AIP) (KKALRRQEAVDAL) was obtained from Calbiochem [described by Ishida et al. (1998)].

Data analysis. Responses were normalized with respect to the baseline and data groups expressed as mean ± SEM. For extracellular recordings, data comparisons were made between test and control pathways at the time points given in the text. For intracellular recordings, data were compared within pathway between baseline and 40–45 min after LTP induction. ANOVAs were then run to test for effects of genotype and drug application, together with any interaction terms using Graphpad software. *Post hoc* Bonferroni corrected *t* tests were then used to investigate the origin of effects further. For tests of statistical significance, *p* values are as reported in the text.

The mean amplitude and variance were measured for the whole control period plus stable periods of at least 50 (and usually 100) stimuli after the induction of LTP. At least two separate noise measurements were taken for each EPSP, from nonoverlapping parts of the baseline, to calculate the mean noise SD. This noise SD was then subtracted from the EPSP SD using the following equation: $(\text{EPSP SD})^2 = (\text{SD of combined EPSP + noise})^2 - (\text{noise SD})^2$.

Two periods were studied, the control period and the last 10 min of potentiation. We calculated the squared coefficient of variation (CV^2) from the variance and mean amplitude ($CV^{-2} = \text{mean}^2/\text{variance}$), nor-

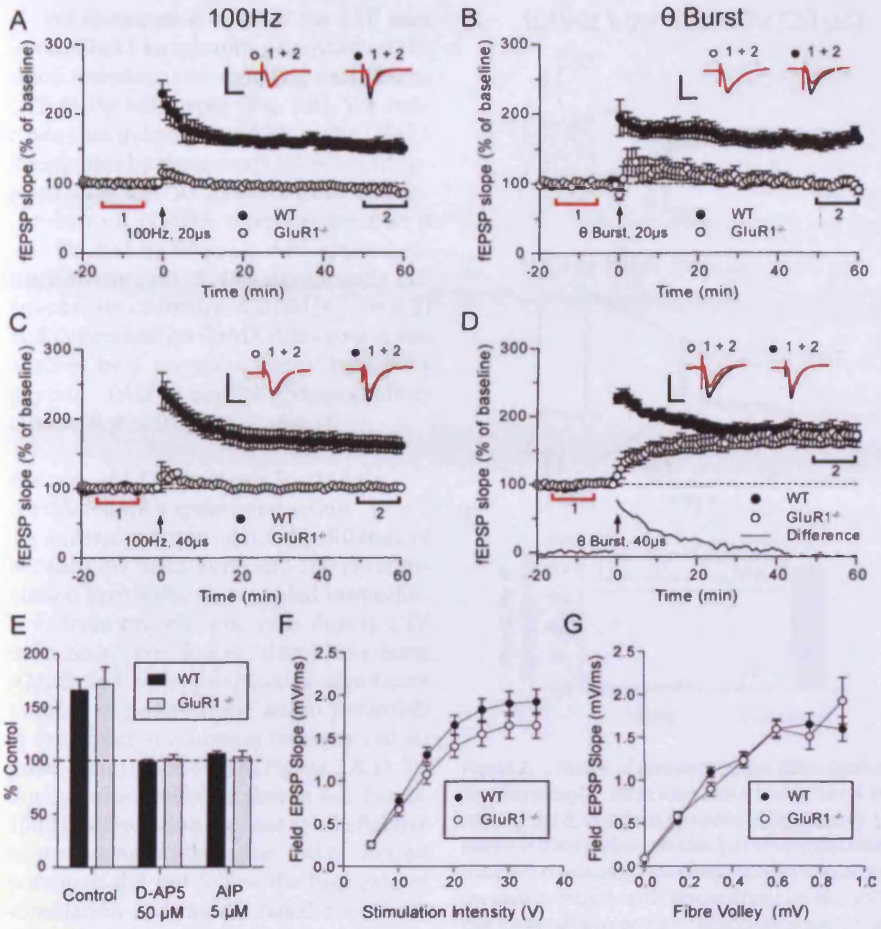

Figure 1. High-intensity theta-burst stimulation produces GluR1-independent LTP that depends on NMDAR and CaMKII. ***A***, ***C***, In GluR1 $^{-/-}$ mice (○), 100 Hz stimulation (100 pulses at 100 Hz, repeated three times at 0.05 Hz, delivered at arrow) at either low intensity (***A***) (test pulse-width 20 μs) or high intensity (***C***) (double test pulse-width 40 μs), produces no significant potentiation of the fEPSP, compared with a highly significant potentiation in wild-type mice (●). ***B***, Low-intensity theta-burst stimulation [four pulses at 100 Hz repeated 10 times at 5 Hz (theta) repeated three times at 0.05 Hz] also produces no fEPSP potentiation in the GluR1 $^{-/-}$ mice (○), whereas significant potentiation is seen in wild-type mice (●). ***D***, High-intensity theta-burst stimulation produces a slowly rising form of potentiation in GluR1 $^{-/-}$ mice (○) that is indistinguishable from the potentiation in wild-type mice (●) at 60 min. Each point plots the average amplitude of four successive fEPSPs normalized with respect to the baseline and expressed as mean ± SEM. Insets are representative traces taken at time points indicated by the bars (red, control period; black, 50–60 min) with the symbols identifying individual experimental conditions. Calibration: 1 mV, 10 ms. ***E***, 50 μM D-AP5 or 5 μM AIP completely block LTP in wild-type (filled bars) and GluR1 $^{-/-}$ mice (gray bars). ***F***, I/O curves for wild-type (●) and GluR1 $^{-/-}$ (○) mice show no difference in baseline transmission [$p > 0.05$, not significant (NS)]. ***G***, Fiber volley, I/O curves for wild-type (●) and GluR1 $^{-/-}$ (○) mice also show no difference in baseline transmission ($p > 0.05$, NS). WT, Wild type.

malized the mean amplitude and CV^{-2} values to the control period, and plotted values for the two time periods (Malinow and Tsien, 1990).

Paired-pulse facilitation (PPF) was measured (interstimulus interval 75 ms) during the control period and 40 min after the induction of LTP. PPF was expressed as a ratio, i.e., the amplitude of the second EPSP was divided by the amplitude of the first. The average PPF during the control period was then compared with the PPF ratio 40 min after the tetanus. The change in the PPF ratio (ΔPPF) was then calculated by subtracting the post tetanus PPF ratio from the control PPF ratio in each individual experiment and then averaged (Hardingham and Fox, 2006).

Results

LTP can be induced by orthodromic theta-burst stimulation in adult mice

A tetanic stimulus applied at 100 Hz to the schaeffer collateral-CA1 pathway produced robust LTP in wild-type mice (mean ± SEM = 153 ± 9%) but not in GluR1 knock-out mice (96 ± 4%) (Fig. 1*A*). The stimulus intensity was routinely set at 40% of the maximal value measured from the I/O curves plotted at the start of the experiment. Theta-burst stimulation at the same pulse width (20 μs) or 100 Hz stimulation at an increased stimulus strength, (produced by doubling the width of the stimulus pulse from 20 to 40 μs) were also ineffective at inducing LTP in the GluR1 knock-outs (Fig. 1*B*,*C*). However, theta-burst stimulation in combination with the double pulse-width stimulus produced LTP of similar magnitude both in wild types and GluR1 knock-outs (173 ± 7% in wild types vs 171 ± 16 in GluR1 knock-outs measured at 60 min after tetanus) (Fig. 1*D*). LTP was significant in both cases here ($p < 0.001$, Bonferroni corrected *post hoc t* test).

Over the first 20 min, LTP in the GluR1 knock-outs increased more slowly than LTP in the wild types. Figure 1*D* (bottom, solid line) illustrates the difference in the time course of the potentiation by subtracting the potentiation seen in the GluR1 knock-outs from the potentiation seen in wild types; it is very similar to the LTP described by Hoffman et al. (2002) and Jensen et al. (2003). However, two factors were different in the present study; first LTP was produced purely by orthodromic stimuli and did not require postsynaptic current injection to ensure spike pairing. Second, the animals were at least 8 weeks of age and therefore the LTP was not restricted to immature synases.

We found that the induction of LTP in the GluR1 knock-outs depended not only on the intensity of stimulation but also on the parameters of the tetanus protocol. Neither theta-burst stimulation with a 20 μs stimulus pulse-width (Fig. 1*B*) nor 100 Hz stimulation with a 40 μs stimulus pulse-width (Fig. 1*C*) reliably induced LTP in our hands. The dependence of LTP on stimulus intensity in the GluR1 knock-outs could not be accounted for by lower levels of synaptic transmission (when compared with wild types), as the I/O curves were not significantly different between the two genotypes (Fig. 1*F*) (Bonferroni corrected *t* test, $p > 0.05$). To analyze this result further, the I/O function was also assessed relative to the size of the fiber volley. The fiber volley amplitude is proportional to the number of axons activated, allowing for an independent measurement of input strength and compensating for any small differences in stimulating and recording electrode placement between experiments. However, when the I/O response was plotted against the fiber volley we still found no difference between wild types and GluR1 knock-outs (Bonferroni corrected *t* test, $p > 0.05$) (Fig. 1*G*). As mentioned above, the stimulus intensity was routinely set at 40% of maximum response saturation corresponding to a mean value of ~10V (Fig. 1*F*). As can be seen from the I/O curve the response averages for the two genotypes at the 40% setting are very similar (Fig. 1*F*,*G*).

We investigated whether the LTP seen in the GluR1 knock-outs depended on the same receptors and signaling cascades as LTP in the wild types (Fig. 1*E*). We conclude that induction of LTP in the GluR1 knock-outs by theta-burst stimulation applied using a 40 μs stimulus pulse was dependent on NMDA receptors because it was blocked by 50 μM D-AP5 applied extracellularly (101 ± 4%; significantly different from control, $p < 0.001$; $t_{(27)} = 4.2$) and dependent on CaMKII because it was blocked by 5 μM autocamtide inhibitory peptide (AIP) applied intracellularly (103 ± 6, $p < 0.001$, $t_{(27)} = 4.1$).

Efficacy of LTP protocols is strongly correlated with spike production

To understand more about the differences between the theta-burst and 100 Hz stimulation protocols, we recorded intracellularly from postsynaptic cells during LTP induction. We found that theta-burst stimulation only produced a significant number of postsynaptic action potentials at the higher stimulation intensity (40 μs pulse-width) as shown in Figure 2*B*,*D*. To our surprise, we found that in our hands, 100 Hz stimulation was not at all effective in producing postsynaptic spikes. Action potentials did not follow the high rate of stimulation and rapidly failed over time, either due to depolarization block or perhaps due to spike accommodation (Fig. 2*A*,*C*).

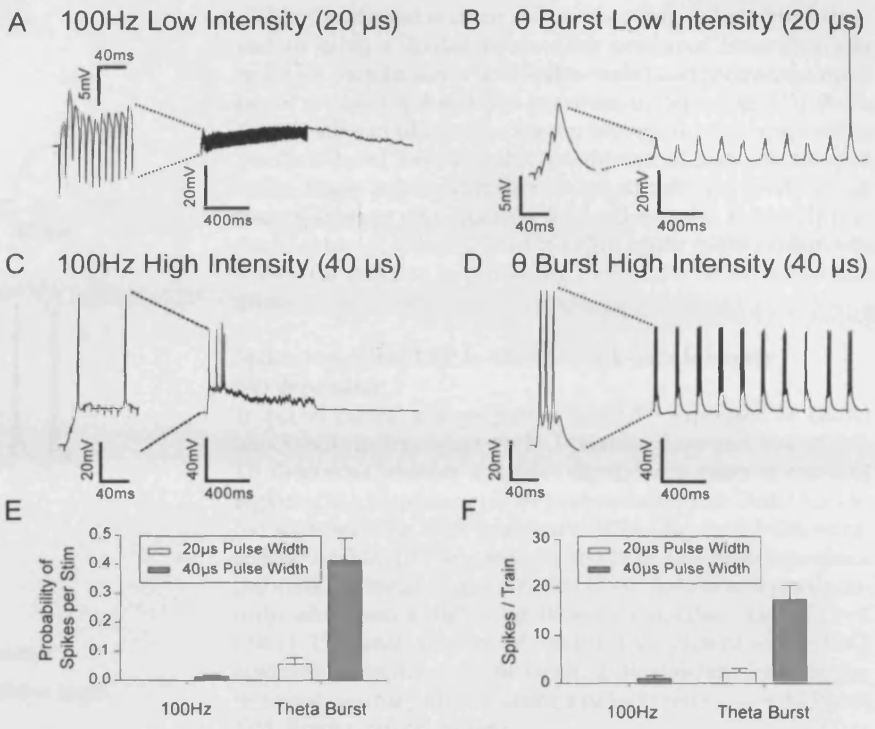

Figure 2. Number of postsynaptic spikes differs significantly between induction protocols. Example traces of intracellular recording during (***A***), 100 Hz stimulation at low intensity; ***B***, theta-burst stimulation at low intensity; ***C***, 100 Hz stimulation at high intensity; and ***D***, theta-burst stimulation at high intensity. Left (panels) are the first 100 ms of each burst. ***E***, Bars indicate the number of spikes produced per stimuli in the train (total number of spikes in train/total number of stimuli given in the train). The probability of generating a spike is significantly greater during high-intensity (gray bars) theta-burst stimulation compared with low-intensity (white bars) 100 Hz stimulation, high-intensity 100 Hz stimulation and low-intensity theta-burst stimulation. ***F***, The total number of spikes per train is significantly greater during high-intensity (gray bars) theta-burst stimulation compared with low-intensity (white bars) 100 Hz stimulation, high-intensity 100 Hz stimulation or low-intensity theta-burst stimulation.

We quantified these effects and found that the spike probability (per stimulus) was ~40-fold greater during theta-burst stimulation (44 ± 8% in wild-type, 53 ± 6% in GluR1 knock-outs) than for 100 Hz stimulation (1 ± 6% in wild-type, 2 ± 7% in GluR1 knock-outs) (Fig. 2*E*,*F*), using the same intensity of stimulation in each case (40% of maximum, 40 μs pulse-width). Consequently, applying more presynaptic stimuli during a 100 Hz protocol produced many fewer spikes than with a theta-burst protocol. Although it is possible that other experimenters produced action potentials using 100 Hz stimulation, we were not able to do so, and, as described below, this made 100 Hz stimulation a useful tool for some of the experiments in these studies.

Blocking somatic spikes prevents LTP in GluR1 knock-outs

To determine the importance of somatic spikes in LTP induction we recorded from CA1 pyramidal neurones using electrodes containing the sodium channel blocker QX314 and used the theta-burst LTP protocol (40 μs duration pulses). We found that after breaking into the cell QX314 rapidly abolished action potentials (Fig. 3*A*). Although spikes were eliminated during the theta-burst tetanus, the degree of EPSP summation with QX314 was almost identical to control levels (Control = 22.0 ± 0.7 mV; QX314 = 23.0 ± 1.1 mV). (Fig. 3*B*,*C*). We found that QX314 prevented induction of LTP in the GluR1 knock-out mice (Fig. 3*D*) but had no effect on wild-type LTP.

It was possible that QX314 acted by eliminating somatic or dendritic orthodromic spikes or by affecting targets other than sodium channels. Therefore, as a more specific test of the need for somatic action potentials in LTP induction in the GluR1 knock-outs, we applied the specific sodium channel blocker TTX extracellularly via a micropipette carefully positioned close to the soma of the cell being recorded from, under visual control (Fig. 4*A*). The bath aCSF flowed from dendrites to soma to further localize TTX to the soma. Using this technique it was possible to pressure eject TTX onto the soma and reversibly block action potentials (Fig. 4*B*). We found that blockade of somatic action potentials had little effect on the degree of EPSP summation caused by the stimulus (Control = 22.0 ± 0.7 mV, TTX = 19.0 ± 2.1 mV) (Fig. 4*C*,*D*).

Blocking somatic action potentials with TTX did not prevent LTP in wild types (mean = 155 ± 17%) (Fig. 4*E*), but it did prevent LTP in GluR1 knock-out mice (109 ± 5%) (Fig. 4*F*). Experiments with TTX were interleaved with control experiments. A two-way ANOVA showed an interaction between TTX treatment and genotype ($F_{(1,64)} = 5.08$, $p < 0.03$) and *post hoc* tests revealed that this was because the GluR1 knock-out only showed significant LTP without TTX ($t_{(35)} = 3.1$, $p < 0.01$), while wild types showed LTP with or without TTX ($t_{(23)} = 1.68$, $p > 0.05$). On several occasions we were able to hold the postsynaptic cell long enough to reverse the effects of TTX and recover normal action potentials (Fig. 4*B*) ($n = 3$). LTP was not induced when action potentials were blocked, but subsequently could be induced when action potential firing was restored (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

We further analyzed the data from the experiments described above to see if the depolarization level produced by the induction

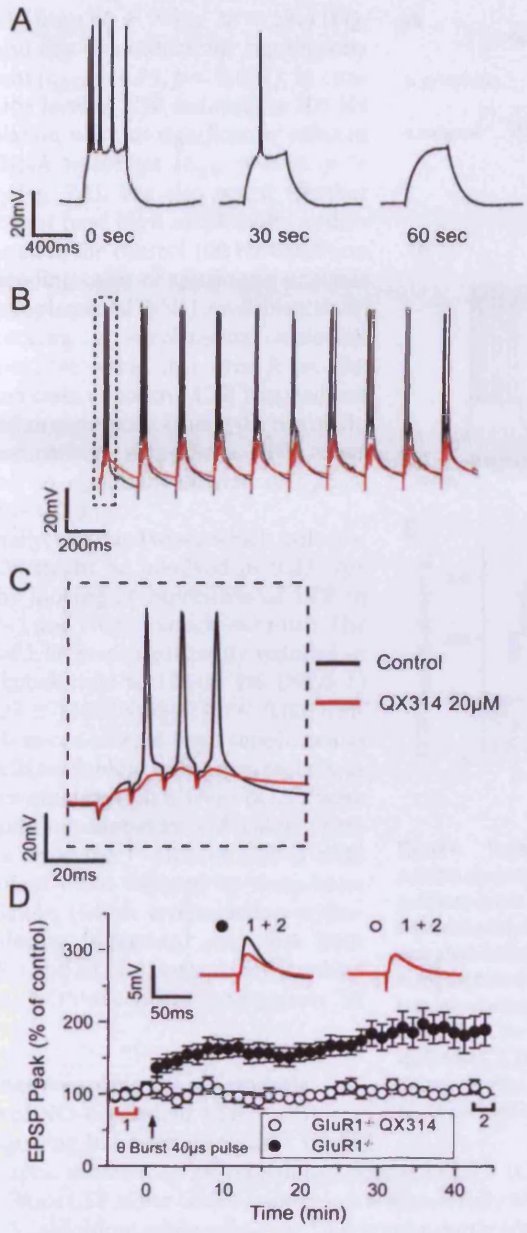

Figure 3. Intracellular QX314 blocks plasticity in the GluR1 $^{-/-}$ mice. ***A***, Spikes generated by depolarizing current injection (2.5 nA, 500 ms) are quickly blocked (<30 s) by QX314 (20 μM) as it dialyses into the cell (legends show time after gaining access to the cell). ***B***, Spiking during high-intensity theta-burst stimulation (black line) is blocked when QX314 is included in the patch electrode (red line). ***C***, EPSP summation is unaffected by the inclusion of QX314 in the electrode (control black line, QX314 red line) ***D***, LTP was induced after a 5 min control period by a high-intensity theta-burst stimulation at $t = 0$ (arrow). Potentiation in GluR1 $^{-/-}$ mice (●) was blocked by QX314 in the patch pipette (○). Each point plots the average amplitude of 9 successive EPSPs normalized with respect to the baseline and is expressed as the mean ± SEM. Insets are representative traces taken at time points indicated by the bars (red, control period; black, 42–45 min), with the symbols identifying the experimental conditions.

protocol affected whether LTP was induced successfully. We found that the level of depolarization produced by the different protocols had little influence on the level of LTP in the GluR1 knock-out animals (Fig. 5*B*,*C*) while the total number of spikes the protocol produced had a much bigger effect on the level of LTP (Fig. 5*A*). Of course, the action potentials themselves produced a substantial depolarization, but this was not an important factor in controlling LTP induction in wild types, which showed LTP both with and without spikes. On average, theta-burst stimulation using a double pulse-width produced more than one spike per train (mean = 1.75 spikes/train) and produced a number of complex spikes in the postsynaptic cells (Fig. 2*D*). From the 44 cells recorded, there was on average four complex spikes per theta-burst train and all cases showed at least one complex spike. Single pulse-width theta-burst stimulation produced far fewer spikes per train (mean = 0.22 spikes/train) and rarely produced complex spikes (2 from 16 cells), which might explain why it was less effective in producing LTP in the GluR1 knock-out mice.

Spike-dependent LTP in GluR1 knock-outs is largely NO dependent

In barrel cortex, a large part of the LTP expressed in GluR1 knock-outs is dependent on NO (Hardingham and Fox, 2006). To determine whether a similar dependency exists in the CA1 region of the hippocampus, we perfused alternate GluR1 knock-out slices with the NOS inhibitor L-NNA (Fig. 6*A*). With extracellular L-NNA, LTP was reduced to 115 ± 11% at 60 min after a theta-burst tetanus compared with 172 ± 16% in untreated controls, which was a highly significantly reduction (Fig. 6*D* $p < 0.001$). The small amount of residual LTP present with L-NNA application was however still significantly different from the untetanised control pathway [using a paired *t* test ($t_{(10)} = 3.29$, $p < 0.05$, Bonferroni corrected)].

Application of L-NNA did not decrease the probability of spike induction. Spike probability was 0.64 ± 0.09 in untreated GluR1 knock-outs and 0.56 ± 0.06 in L-NNA treated GluR1 knock-outs ($F_{(1,29)} = 1.16$, $p = 0.56$). This data therefore implies that NOS is significantly involved in hippocampal LTP. This conclusion was corroborated by evidence from double knock-out mice in which LTP was reduced in both GluR1/αNOS-1 and GluR1/NOS-3 double knock-out animals (Fig. 6*B*,*C*). In both cases, application of L-NNA further reduced LTP in the double knock-outs, indicating that both isoforms of NOS (endothelial (NOS-3) and neuronal (αNOS-1) are involved in LTP in the schaeffer collateral CA1 pathway. The residual component of LTP present in the GluR1/αNOS-1 knock-outs (142 ± 8%) was significantly different from the untetanised control pathway ($t_{(28)} = 3.92$, $p < 0.001$). Treatment of the double knock-outs with L-NNA (an unspecific NOS inhibitor) reduced but did not totally block LTP (mean = 117 ± 11%, $t_{(9)} = 1.7$, $p < 0.05$) (Fig. 6*D*, summary bars).

As was the case with L-NNA application to wild-type slices, reduction of LTP in the double knock-outs was not due to an inability to produce action potentials in the theta-burst tetanus, while the I/O curves were again indistinguishable from those of the single GluR1 knock-outs (Fig. 6*E*,*F*). These results therefore imply that NOS is involved in a substantial component of LTP in the GluR1 knock-outs.

Spike-dependent LTP in wild types is partly NO dependent

As the LTP in GluR1 knock-outs requires action potentials and is also largely NO dependent, we hypothesized that the same is true of a component of wild type LTP. We therefore again used two LTP induction protocols, one that caused consistent spike production (theta-burst) and one that in our hands only sparingly produced spikes (100 Hz). Both protocols produced LTP in the wild-type mice (Fig. 7). However, application of L-NNA reduced only the LTP produced by theta-burst stimulation and not that produced by 100 Hz stimulation. The level of LTP induced by theta-burst stimulation was almost halved by application of

L-NNA (from 60 + 9% to 28 + 5%) (Fig. 7*B*), and this was statistically significantly different ($t_{(25)} = 4.74$, $p < 0.001$). In contrast, the level of LTP induced by 100 Hz stimulation was not significantly affected by L-NNA treatment ($t_{(20)} = 0.49$, $p > 0.05$) (Fig. 7*A*). We also tested whether LTP might have been accidentally underestimated in the control 100 Hz condition by including cases of spuriously unstable LTP (supplemental Table 1, available at www.jneurosci.org as supplemental material). However, we found that even if we discounted cases of control LTP that had not reached an asymptotic value at the end of 2 h, the comparison with the L-NNA cases showed no significant difference ($t_{(17)} = 0.81$, $p > 0.05$).

Finally, we tested to see which isoforms of NOS might be involved in wild-type LTP by looking at expression of LTP in αNOS-1 and NOS-3 knock-out mice. The level of LTP was significantly reduced in both knock-outs to 136 ± 4% (NOS-1) and 137 ± 11% (NOS-3) ($p < 0.001$), although as can be seen from supplemental Figure 2 (available at www.jneurosci.org as supplemental material), levels of LTP were still quite substantial in both cases. These studies show that ~50% of LTP is NOS dependent when induced by theta-burst stimulation (which evokes action potentials during induction) and that both αNOS-1 and NOS-3 isoforms are involved in the NOS-dependent component of LTP.

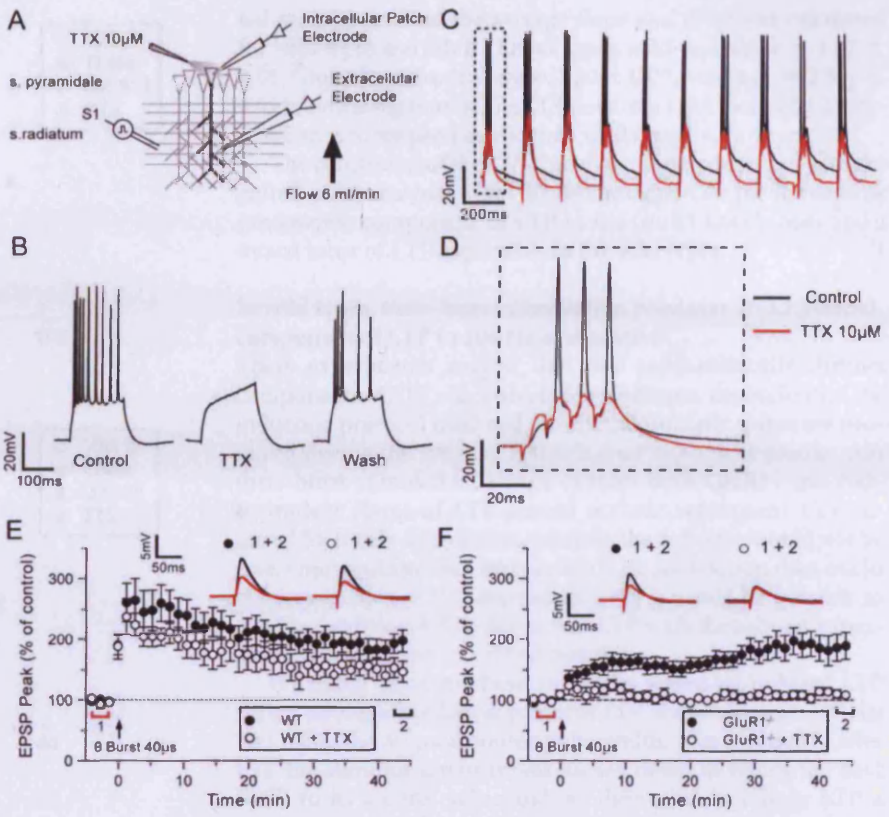

Figure 4. Somatic spikes are required for plasticity in GluR1 $^{-/-}$ mice. ***A***, Schematic diagram of the experimental setup. Action potential generation and propagation can be blocked by local pressure application of TTX (10 μM) to the soma. The slice is positioned so that TTX does not perfuse on the stratum (s.) radiatum. ***B***, Example trace illustrating how spikes generated by a depolarizing current injection (2.5 nA, 500 ms) can be reversibly blocked by the local somatic application of TTX (10 μM). ***C***, Spiking during high-intensity theta-burst stimulation (black line) is blocked if TTX is perfused on the soma (red line). ***D***, EPSP summation during the theta-burst stimulation is unaffected by somatic TTX application. ***E***, LTP was induced after a 5 min control period by a high-intensity theta-burst stimulation at $t = 0$ (arrow). Somatic TTX application (○) has a small effect on wild-type (WT) LTP at 45 min (●). ***F***, The LTP observed in GluR1 $^{-/-}$ mice (●) is completely abolished when somatic spikes are blocked with local TTX application (○). Each point plots the average amplitude of eight successive EPSPs normalized with respect to the baseline and expressed as mean ± SEM. Insets are representative traces taken at time points indicated by the bars (red, control period; black, 42–45 min) with the symbols identifying the experimental conditions.

Evidence regarding the presynaptic origin of NO-dependent LTP

NO signaling has been implicated in the presynaptic modulation of transmitter release in LTP (O'Dell, 1991). Since LTP in the GluR1 knock-outs is almost fully blocked by NOS inhibition, while wild-type LTP is only partly blocked, one might predict that the locus of expression of the LTP would also be almost entirely presynaptic in the GluR1 knock-outs and a mixture of presynaptic and postsynaptic in the wild types.

To test this, we monitored PPF before and 40 min after LTP induction. In the wild types, there was no overall change in PPF after LTP induction (ΔPPF = 0.02 ± 0.07, $t_{(19)} = 0.68$, $p = 0.5$) (Fig. 8*C*). There was a large variability in ΔPPF between individual recordings; six cells showed an increase, six cells show no change, and eight show a decrease in ΔPPF after LTP (Fig. 8*A*). However, in the GluR1 knock-outs there was far lower variability in the ΔPPF, with only one cell showing a substantial increase, six remaining unchanged while 14 cells showed a decrease in the PPF ratio after LTP (Fig. 8*B*). Using a paired *t* test and comparing each cell before and after LTP we found a significant decrease in ΔPPF for the GluR1 knock-outs following LTP (ΔPPF = 0.28 ± 0.04, t (20) = 2.9, $p < 0.01$). Consequently, ΔPPF was significantly different in wild types and GluR1 knock-outs ($t_{(32)} = 2.7$, $p < 0.05$) (Fig. 8*C*).

The initial PPF ratio has been shown to be inversely related to the magnitude and sign of ΔPPF following LTP in the hippocampus, for instance by Schulz et al. (1994). In agreement with these data, we also saw a negative correlation between the initial PPF ratio and the ΔPPF after LTP both in wild types ($r = 0.47$, $n = 19$, $p < 0.05$) and GluR1 knock-outs ($r = 0.55$, $n = 21$, $p < 0.01$) (supplemental Fig. 3*A*, available at www.jneurosci.org as supplemental material). In the GluR1 knock-outs there was also a negative correlation between the ΔPPF ratio and the magnitude of LTP at 45 min ($r = 0.52$, $n = 21$, $p = 0.03$) and a positive correlation between the control PPF ratio and the magnitude of LTP ($r = 0.47$, $n = 21$, $p = 0.01$) (supplemental Fig. 3*B*,*C*, available at www.jneurosci.org as supplemental material); however, neither of these correlations were apparent in the wild types. The initial PPF and ΔPPF failed to predict the magnitude of LTP in wild types, presumably due to additional postsynaptic mechanisms involving GluR1, while in the GluR1 knock-outs these postsynaptic mechanisms are not available so presynaptic mechanisms dominate. Increased dependence of LTP on presynaptic mechanisms in GluR1 knock-outs has also been reported in the barrel cortex (Hardingham and Fox, 2006)

The presynaptic locus of LTP in GluR1 knock-outs was further corroborated by normalized mean CV^{-2} analysis (Malinow and Tsien, 1990). Purely postsynaptic changes would produce a plot with a horizontal trajectory (Fig. 8*D*), whereas changes in *N*

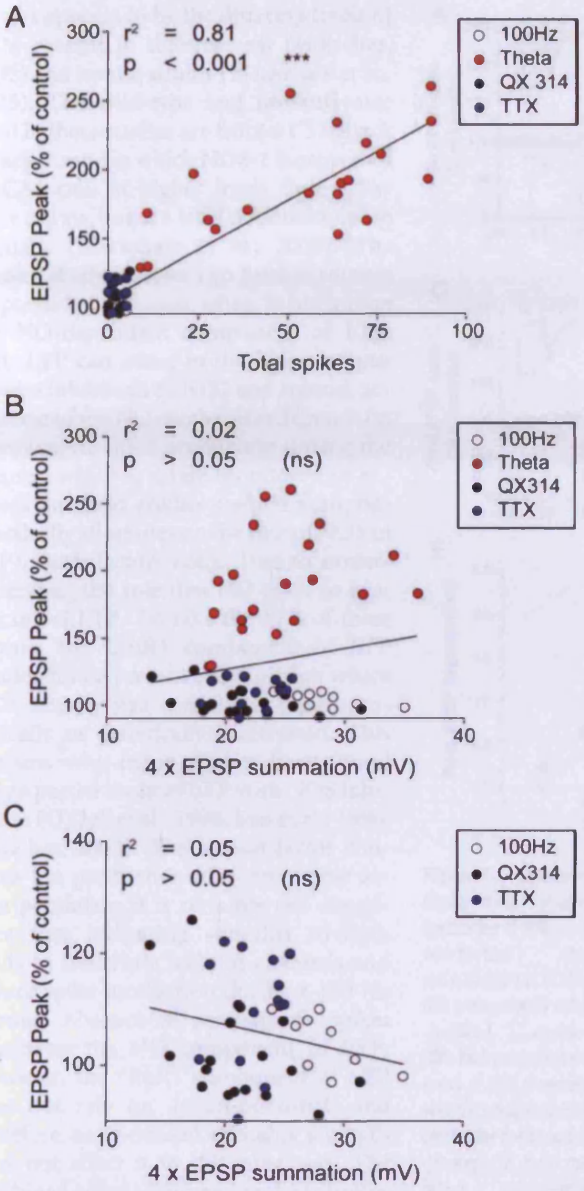

Figure 5. The level of potentiation in the GluR1 $^{-/-}$ mice is correlated to the number of spikes observed in the burst and not to the level of depolarization in the burst. ***A***, Magnitude of LTP in the GluR1 $^{-/-}$ depends on the total number of spikes during the burst. Individual experiments show a correlation between the number of spikes in a burst and the increase in EPSP observed with LTP. ***B***, Magnitude of LTP in the GluR1 $^{-/-}$ is not correlated to the average amplitude of summated EPSPs during the theta-burst stimulation. ***C***, Same data as shown in ***B*** with control theta-burst stimulation data excluded as spiking will be related to the level of depolarization. Data are pooled from LTP experiments generated by high-intensity 100 Hz stimulation (white circle), theta-burst stimulation (red circle), theta-burst stimulation with 0.2 mM QX 314 (black circle), and theta-burst stimulation with 10 μM TTX (blue circle).

or P_r would cause more vertical trajectories. This is because CV^{-2} is proportional to $NP_r(1 - P_r)^{-1}$ and is therefore not dependent on Q, whereas the mean amplitude is proportional to NP_rQ and is therefore proportional to Q (in which N is the number of release site, P_r is the probability of release, and Q is the quantal size). In wild types, the trajectory of the CV^{-2} plot was approximately diagonal, indicative of a mixed locus of potentiation [consistent with the work of Hardingham and Fox (2006)] (Fig. 8*D*). In GluR1 knock-outs, the trajectory of the CV–2 plot was significantly steeper than in wild types (linear fits were made to individual experiments and the average slope and error was calculated for wild types and GluR1 knock-outs, wild-type slope = 1.07 ± 0.05, GluR1 knock-out slope = 1.30 ± 0.07, t test $t_{(34)} = 2.5$, $p < 0.05$), indicating that in GluR1 knock-outs the locus of LTP expression is more presynaptic than wild types.

The conclusion of the CV^{-2} analysis seems consistent with the paired-pulse analysis in that they both suggested a predominantly presynaptic component of LTP in the GluR1 knock-outs and a mixed locus of LTP expression in the wild types.

In wild types, theta-burst stimulation produces an additional component of LTP to 100 Hz stimulation

These experiments suggest that two mechanistically distinct components of LTP are generated in wild types, dependent on the induction protocol used and on whether somatic spikes are produced during the tetanus. If this is true one might predict that theta-burst stimulation, which induces both GluR1- and NO-dependent forms of LTP should occlude subsequent LTP induced by 100Hz stimulation, whereas the converse would not be true. One would predict that since 100 Hz stimulation does not in our hands, induce NO-dependent LTP, it would be possible to produce additional NO-dependent LTP with theta-burst stimulation following the 100 Hz stimulation.

We tested this hypothesis in studies where we induced LTP with a strong stimulation protocol (3× theta-burst or 3× 100 Hz) using the 40 μs stimulus pulse width. Thirty minutes after LTP the stimulus intensity was turned down to return the field EPSP to its control value and we then tried to induce LTP a second time. We found that theta-burst stimulation produced LTP that occluded further LTP induced by 100 Hz stimulation (Fig. 9*A*). Transient potentiation (STP) was produced by the second tetanus but it fell back to baseline within 30 min (101 ± 2%) (Fig. 9*C*). However, if we swapped the order of the stimulus protocols so that the 100 Hz tetanus occurred before the theta-burst tetanus, a small LTP was observed (Fig. 9*B*). There was no clear post-tetanic potentiation episode and the potentiation rose then remained at a steady state level of (117 ± 7%) for the 50 min we followed it (Fig. 9*C*). Statistical analysis showed that the theta-burst LTP was significantly different from baseline ($p < 0.05$). This experiment supports the hypothesis that postsynaptic action potentials (recruited using theta-burst stimulation) activate a mechanistically different and additional component of LTP to that induced by depolarization without postsynaptic action potentials (produced, in our hands, using 100 Hz stimulation). It also suggests that the LTP component produced only by theta-burst is smaller than the LTP component common to both protocols.

Discussion

The main findings of this study are that postsynaptic action potentials are necessary for the NO-dependent component of hippocampal LTP in both GluR1 knock-out and wild-type mice. In GluR1 knock-out mice, almost all the LTP is NO sensitive, while in wild types the later stages of LTP are NO sensitive. In wild types, NO-dependent LTP accounts for ~50% of the potentiation 2 h post-tetanus. Presumably, the remaining component of LTP in wild types is GluR1 dependent, which would account for the large difference in the size of the NO-dependent component between the two genotypes.

Comparison with previous studies on the role of NO in LTP

Previous studies on the role of NO in LTP have investigated the source of discrepant results in different labs. One of the primary

factors appears to be the differing levels of NOS present in different rat (Hölscher, 2002) and mouse strains (Blackshaw et al., 2003). The wild-type and mutant mice used in these studies are from a C57/Black 6 background in which NOS-1 is expressed in CA1 cells at higher levels than 129sv mice or rats, but at a level not dissimilar to humans (Blackshaw et al., 2003). The present study exposes two further sources of possible confusion when investigating the NO-dependent component of LTP; first, LTP can occur in the hippocampus despite inhibition of NOS and second, activation of the NO mechanism depends on postsynaptic spike production during the tetanus, which is rarely monitored in extracellular field studies (which comprise practically all studies on the role of NOS in LTP). Both factors could lead to underestimating the role that NO plays in hippocampal LTP. To take the first of these factors; the GluR1 component of LTP would still be present even in cases where NOS activity was completely pharmacologically or genetically inactivated. This explains why many studies have found only a partial block of LTP with NOS inhibition (O'Dell et al., 1994; Son et al., 1996; Hölscher, 2002). The second factor concerns the production of postsynaptic action potentials; it is certainly our experience that increasing stimulus strength tends to inactivate sodium channels and reduce spike production during a 100 Hz tetanus. Absence of postsynaptic spikes eliminates the NO component of LTP. However, the GluR1 component of LTP does not rely on action-potentials and therefore an increased stimulus strength does not affect it in the same way. The combined effect of increasing the stimulus strength is therefore to decrease the NO-dependent component of LTP relative to the GluR1-dependent component. This explains several reports in the literature that increasing stimulus intensity reduces the NO-dependent component of LTP (Gribkoff and Lum-Ragan, 1992; Chetkovich et al., 1993; Haley et al., 1993; O'Dell et al., 1994).

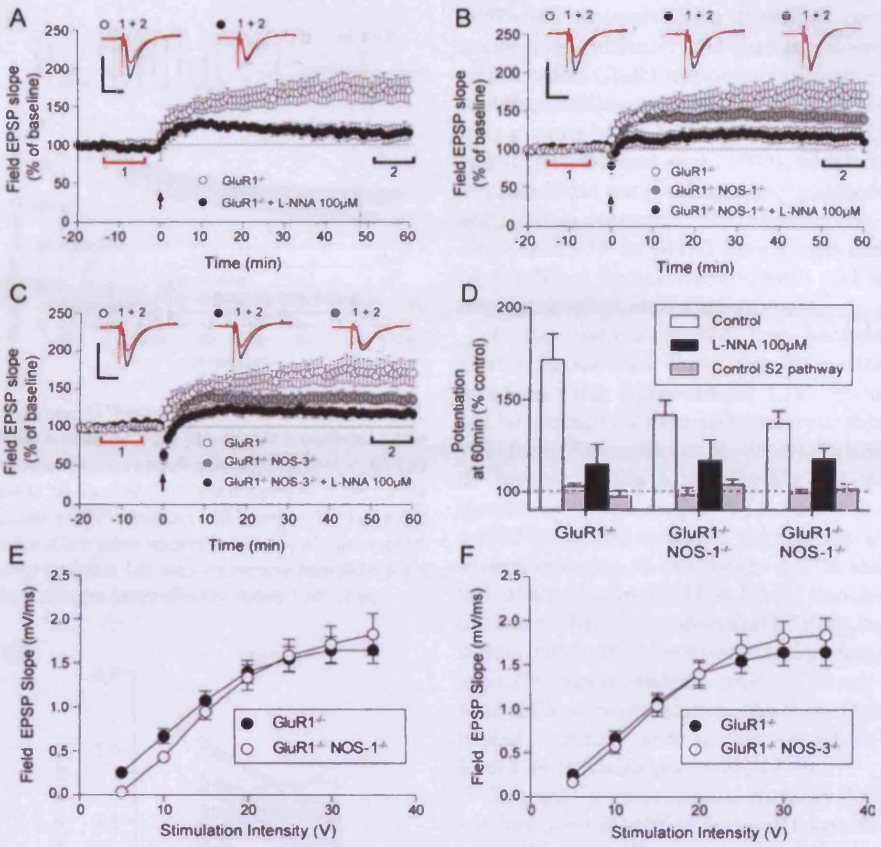

Figure 6. Comparison of LTP in GluR1 $^{-/-}$ single-knock-out and GluR1 $^{-/-}$ αNOS-1 $^{-/-}$ and GluR1 $^{-/-}$ NOS-3 $^{-/-}$ double- mutant mice with or without NOS inhibitor. ***A***, LTP in the GluR1 $^{-/-}$ (○) mice is significantly reduced by a 5 min application of 100 μM L-NNA (●). ***B***, LTP in the GluR1 $^{-/-}$ αNOS-1 $^{-/-}$ double-mutant mice (◌) is reduced when compared with the GluR1 $^{-/-}$ single-mutant mice (○). The remaining LTP in double-mutant mice is further reduced by 100 μM L-NNA (●) and is similar to LTP observed in single GluR1 $^{-/-}$ with 100 μM L-NNA. ***C***, LTP in the GluR1 $^{-/-}$ NOS-3 $^{-/-}$ double-mutant mice (◌) is also slightly reduced compared with the GluR1 $^{-/-}$ single-mutant mice (○) and is comparable with the LTP in GluR1 $^{-/-}$ −αNOS-1 $^{-/-}$ double-mutant mice. The remaining LTP in the double-mutant mice can be further reduced by 100 μM L-NNA (●). Each point plots the average amplitude of three successive fEPSPs normalized with respect to the baseline and expressed as mean ± SEM. Insets are representative traces taken at time points indicated by the bars (red, control period; black, 50–60 min) with the symbols identifying the experimental conditions. Calibration: 1 mV, 10 ms. ***D***, The average levels of LTP at 50–60 min are plotted for the S1 and S2 pathways in the three genotypes. Note the similarity of the LTP in the presence of L-NNA for all three genotypes. ***E***, ***F***, I/O curves for GluR1 $^{-/-}$ single-mutant (●) and GluR1 $^{-/-}$ NOS-1 $^{-/-}$ double-mutant (***E***) and GluR1 $^{-/-}$ NOS-3 $^{-/-}$ double-mutant (***F***) (○) mice show no differences in baseline transmission ($p > 0.05$, NS).

In our hands we found that theta-burst stimulation produced spikes more readily than 100 Hz stimulation. However, this is not to say that it is impossible to produce NO-dependent LTP with 100 Hz stimulation. In fact, some of the pioneering studies on the role of NO in LTP found that 100 Hz stimulation produced NO-dependent LTP, provided that the stimulus used was of a weak intensity (O'Dell et al., 1991,1994). We assume that in these cases the stimulus was weaker than we used in our studies and that it more successfully produced postsynaptic spikes during the tetanus.

Comparison with previous studies on GluR1 knock-outs

We found no differences in baseline levels of synaptic efficacy between GluR1 knock-out animals and wild types, consistent with previous studies (Zamanillo et al., 1999). It has also been shown that GluR1 knock-outs have normal levels of whisker evoked responses in layers II/III, IV and V of the barrel cortex and normal levels of synaptic response in the layer IV to II/III and II/III to V pathway (Wright et al., 2008). However, synaptic scaling is known to require GluR1 containing AMPA receptors in the hippocampus, which might predict a reduction in distal synaptic currents in the GluR1 knock-outs (Andrásfalvy et al., 2003). It may be that presynaptic plasticity mechanisms are able to compensate for the lack of postsynaptic scaling.

Earlier studies on GluR1 knock-outs concluded that LTP relied on spike pairing protocols that were effective in younger but not older animals (Jensen et al., 2003) or that it required pairing a burst of postsynaptic spikes with presynaptic stimulation (Hoffman et al., 2002). Here we show that spikes are essential for induction of LTP in the GluR1 knock-outs but that it is sufficient that they are produced naturally from orthodromic stimulation. Previous studies have shown that somatic action potentials are usually generated by prior dendritic spikes during theta-burst stimulation (Golding et al., 2002). Here we found that theta-burst stimulation reliably evoked postsynaptic action potentials

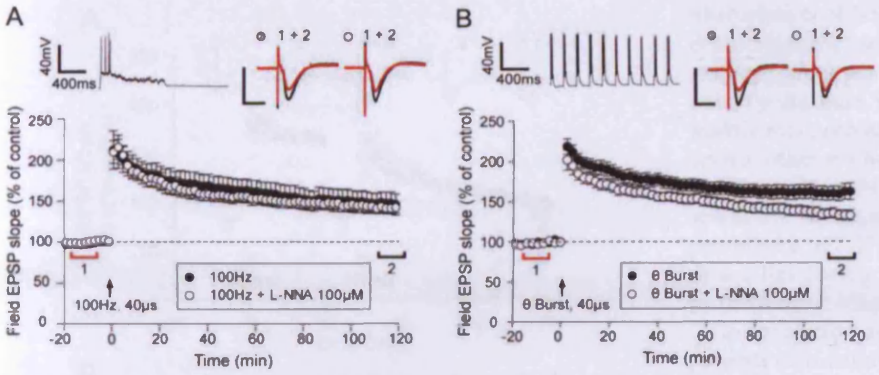

Figure 7. An NO sensitive component to LTP is only observed in wild types if LTP induction involves action potential firing. ***A***, Stimulation (100 Hz) at high-intensity stimulation produces LTP that is stable for >2 h (●). This LTP is unaffected by the application of 100 μM L-NNA (○). ***B***, High-intensity theta-burst stimulation produces a significantly larger potentiation (●) than the potentiation induced by 100 Hz stimulation and is more sensitive to 100 μM L-NNA (○). L-NNA is applied for 10 min starting at $t = -5$ min. Each point plots the average amplitude of six successive fEPSPs normalized with respect to the baseline and expressed as mean ± SEM. Right insets are representative traces taken at time points indicated by the bars (red, control period; black, 110–120 min) with the symbols identifying the experimental conditions. Left insets are example intracellular traces recorded to illustrate the type of spiking that occurs during induction. Calibration (unless otherwise stated): 1 mV, 10 ms.

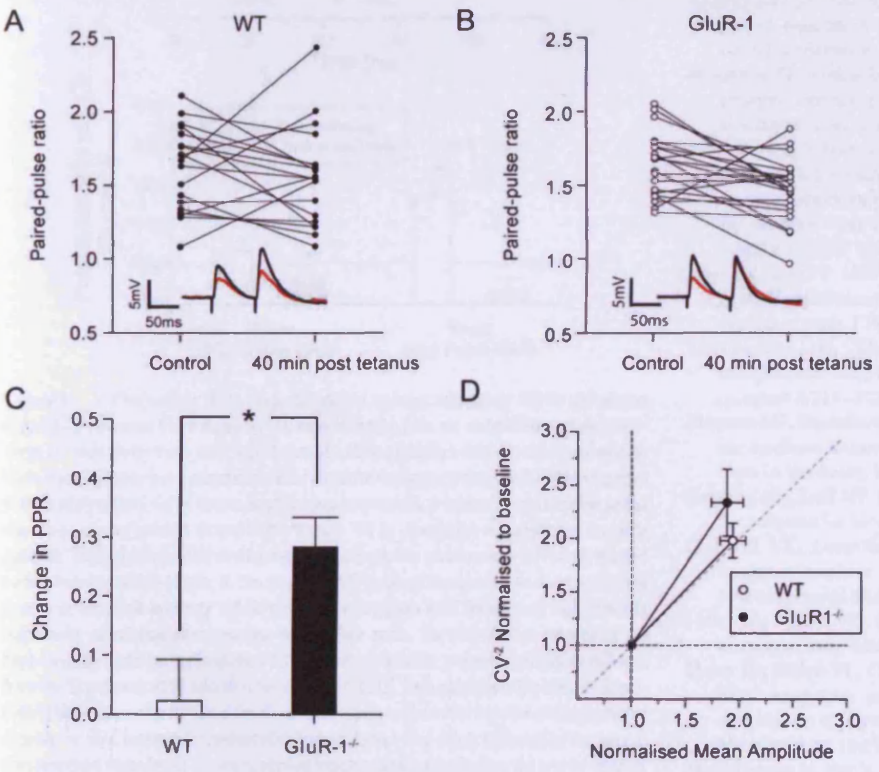

Figure 8. The locus of plasticity in wild types and GluR1 knock-outs. ***A***, Changes in PPF (75 ms interpulse interval) 40 min after LTP induction are highly variable in wild types (6 substantially increased, 6 do not change, and 8 decreased). ***B***, Less variability in ΔPPF is observed in the GluR1 knock-outs (1 substantially increased, 6 do not change, and 14 decreased), and there is a general decrease in the PPF 40 min after LTP induction. Insets are representative traces taken during the control period (red line) and 40 min after LTP induction (black line). ***C***, Bars represent the average ΔPPF for the wild types (open bars) and GluR1 knock-outs (black bars) (see Materials and Methods for definition of ΔPPF). PPR, Paired-pulse ratio. ***D***, The normalized mean response versus CV^{-2} trajectory is more vertical for GluR1 knock-outs than wild types. The origin (1,1) represents the baseline condition, whereas the points to the right are taken at 40 min after LTP induction. Both CV^{-2} and mean amplitude are normalized. WT, Wild type.

for approximately half the presynaptic stimuli and induced a significant number of complex spikes when a higher presynaptic stimulus strength was used. Our studies were all performed on animals older than 6 weeks of age (P45–P64) and the extracellular field experiments were on animals older than 8 weeks of age (P57–64); animals were therefore certainly not immature. The inability to see LTP in adult GluR1 knock-out animals in previous studies could be due to the stimulus protocols used, for example 100 Hz stimulation (Jensen et al., 2003), which in our hands did not produce many postsynaptic action potentials. We therefore conclude that LTP in GluR1 knock-outs can be produced by orthodox stimuli and is not restricted to immature animals.

It has been suggested that because GluR1 knock-outs show normal water maze learning, hippocampal LTP might not be necessary for spatial memory in this structure (Zamanillo et al., 1999). While the present studies do not provide any evidence for hippocampus LTP being involved in spatial memory, they do argue against rejection of this theory due to the lack of hippocampal LTP in GluR1 knock-out mice. This study shows that LTP can be induced by both conventional and physiologically relevant stimuli in GluR1 knock-outs while previous studies also show that spatial memory still occurs in GluR1 knock-outs (Zamanillo et al., 1999).

Recently, a more specific memory deficit has been identified in GluR1 knock-outs. While GluR1 knock-out mice are able to perform reference memory tasks such as the Morris water maze, where information needs to be recalled from previous trials (Zamanillo et al., 1999), they are impaired in working memory tasks that require within trial recall of recently acquired information (Sanderson et al., 2008). For example, GluR1 knock-out mice trained to retrieve food rewards from a radial arm maze made many more working memory errors (reentering arms which had previously been visited) when the food rewards were not replaced during the task, thereby requiring the animal to remember where it had just been (Schmitt et al., 2003). The working memory deficit in the GluR1 knock-outs is restored by forebrain expression of transgenic GluR1 (Schmitt et al., 2005) as is LTP in the hippocampus (Mack et al., 2001). It is conceivable that the GluR1 dependence of working memory is related to the early GluR1-dependent phase of LTP shown in this and previous studies (Hoffman et al., 2002; Hardingham and Fox, 2006). It is intriguing to think that the reference memory component that remains in the GluR1 knock-out animals might be NO-dependent, given that LTP in GluR1 knock-outs is largely NO-dependent. If so, it may rely on the slowly developing presynaptic mechanisms of plasticity recently described in the neocortex and hippocampus (Hardingham and Fox, 2006; Bayazitov et al., 2007).

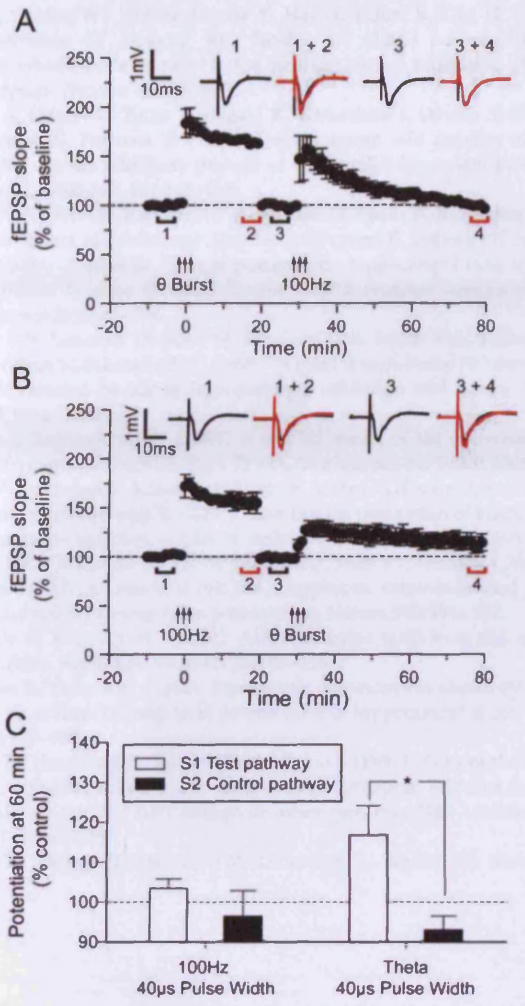

Figure 9. LTP induced by theta-burst stimulation occludes subsequent 100 Hz stimulation induced LTP, whereas LTP induced by 100 Hz stimulation does not completely occlude subsequent LTP induced by theta-burst stimulation. ***A***, LTP was initially induced in the S1 pathway by high-intensity theta-burst stimulation; after 20 min of further recording, the baseline was reset to the initial control level by decreasing the stimulus intensity. A further 10 min baseline period was then recorded before a second high-intensity 100 Hz stimulation was applied to the same pathway. The potentiation induced by the 100 Hz stimulation was transient (STP) and returned back to baseline within 45 min. ***B***, The same dual LTP protocol treatment as in ***A*** but performed in reverse: first, high-intensity 100 Hz stimulation was given to S1 followed by high-intensity theta-burst stimulation after resetting the baseline again. The potentiation induced by the high-intensity theta-burst stimulation did not return to baseline and was significantly different from the S2 pathway at 60 min after tetanus ($p < 0.05$). Each point plots the average amplitude of three successive fEPSPs normalized with respect to the initial baseline and is expressed as mean ± SEM. Insets are representative traces taken at time points indicated by the bars. ***C***, Bars represent mean levels of potentiation as indicated taken 60 min after the second tetanus.

A compound LTP with an early postsynaptic component and later presynaptic component has recently been described in the hippocampus (Bayazitov et al., 2007). Presynaptic function was monitored directly using a transgenic mouse strain expressing a pH sensitive fluorescent VAMP2 marker in neurons. The presynaptic component of LTP was only induced by theta-burst stimulation and not by 100 Hz stimulation (Bayazitov et al., 2007). Our study suggests that only theta-burst activity evokes presynaptic LTP in the Bayazitov study because it produces postsynaptic spikes more effectively than 100 Hz stimulation and hence enables the NO-dependent component of LTP. There is evidence that NO can play a postsynaptic role in LTP by producing direct nitrosylation of NSF and hence affecting insertion of AMPA receptors into the membrane (Huang et al., 2005). While we cannot rule out some postsynaptic action of NO in these studies, the paired pulse and CV^{-2} analysis in the present data suggest that NO mainly acts presynaptically, in common with the conclusions of several other studies (O'Dell et al., 1991; Hawkins et al., 1998). Finally, one further study has also reported that postsynaptic spikes are necessary during theta-burst LTP induction for the persistence of LTP (Raymond, 2008) again suggesting that the slower developing, NO-dependent component of LTP induced by theta-burst stimulation requires postsynaptic spikes.

In conclusion, the recent discovery of different temporal components of memory formation is paralleled by the discovery of different temporal components of LTP. This study and previous studies suggest that the early and late components have different presynaptic and postsynaptic loci. In this study, we further show that the later component relies strongly on NO, which in turn relies on postsynaptic spike production and may provide a means for dissecting different components of hippocampus-dependent memory in the future.

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