The involvement of metabotropic glutamate receptors in the induction of long-term potentiation in the medial frontal cortex of the rat.

by

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Activity-dependent changes in the efficacy of synaptic transmission in the medial frontal cortex, namely long-term potentiation (LTP) and long-term depression (LTD), can persist for tens of minutes or hours and may be the neural basis of learning and memory. Glutamate is the principle excitatory neurotransmitter in the CNS. It acts on both ionotropic receptors that directly gate ion channels, and on metabotropic receptors (mGluRs) that exert their effects indirectly through second messenger cascades. I have investigated the involvement of mGluRs in the induction of LTP in the medial frontal cortex in vitro.

The medial frontal cortex receives inputs from hippocampal regions in which neurones fire at theta frequencies when rats move in or explore their environment. We first established that repetitive bursts of stimulation at theta frequencies were an effective conditioning paradigm for inducing LTP. Intracellular recordings were made from layer V cells. Test shocks were applied to the layer I/II border. The involvement of mGluRs was investigated using the broad-spectrum antagonist (R,S)-α-methyl-4-carboxyphenylglycine (MCPG). This drug reduced the incidence of LTP at layer V synapses indicating mGluR involvement in LTP induction.

We demonstrated that mGluRs were located on layer V cells in the medial frontal cortex by bath application of mGluR agonists. All produced excitatory effects in layer V cells, even when synaptic transmission was blocked using the GABAB agonist, baclofen.

We investigated the effect of MCPG on test and conditioned responses. The drug had no significant effect on either. It was seen, however, to reduce the amplitude of a NMDA receptor mediated EPSP isolated using 6-cyano-7-nitroquinoxaline-2,3-
dione (CNQX), low Mg\(^{2+}\) and higher stimulus intensities than those used in LTP induction. This suggests that the block of LTP induction by MCPG is unlikely to be the consequence of modulation of layer V electrophysiology.

Activation of group I mGluRs leads to the production of IP\(_3\), which facilitates the release of Ca\(^{2+}\) from intracellular stores. In Ca\(^{2+}\) imaging experiments, intracellular Ca\(^{2+}\) was monitored in both hippocampal and medial frontal cortical neurones in culture using the indicator Fura-2. Application of selective group I agonists raised intracellular Ca\(^{2+}\), even in zero Ca\(^{2+}\) medium. This rise was inhibited by both MCPG and the group I specific antagonist (S)-4-carboxyphenylglycine (S-4CPG). These observations suggest the effect of MCPG on the incidence of LTP is, at least in part, the result of its ability to dampen the IP\(_3\)-mediated rise in [Ca\(^{2+}\)] triggered by mGluR activation.

The effect of the group I specific agonist DHPG on the induction of LTP was then investigated. TBS alone produced no net change in the field response in layer V of medial frontal cortex in vitro. DHPG alone reversibly reduced the field response. TBS in conjunction with bath application of DHPG produced LTP of the field response. Activation of group I mGluRs therefore facilitates the induction of LTP in prelimbic cortex.
DEDICATION

To Mum and Dad

ACKNOWLEDGEMENTS

All other creatures look down towards the earth,

but man was given a face,

so that he might turn his eyes towards the stars

and his gaze upon the sky.

(Ovid - Metamorphoses)

My special thanks go to my supervisor Dr Lynn Bindman for support and inspiration given during my studies. My thanks also to Professor Mike Spyer and all at U.C.L. who have lent a helping hand over the last three years.

For financial support during my time at U.C.L., I thank the M.R.C, the Graduate School of U.C.L. and the Linden Gardens branch of the Bank of Ceylon.

To the Nats: Thanks for the all the fish. Couldn’t have done it without you. Also a huge thank you to all who have kept me sane during these last months. I’ll never forget the view behind closed eyelids.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABS</td>
<td>Artola, Brocher &amp; Singer</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetyl choline</td>
</tr>
<tr>
<td>ACPD</td>
<td>trans-1S,3R-aminocyclopentane dicarboxylate</td>
</tr>
<tr>
<td>ADP</td>
<td>Afterdepolarisation</td>
</tr>
<tr>
<td>AHP</td>
<td>Afterhyperpolarisation</td>
</tr>
<tr>
<td>AIDA</td>
<td>1-aminoindan-1,5-dicarboxylic acid</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate</td>
</tr>
<tr>
<td>AP5</td>
<td>D-2-amino-5-phosphonovalerate</td>
</tr>
<tr>
<td>BMI</td>
<td>Bicuculline methiodide</td>
</tr>
<tr>
<td>[Ca^{2+}]_i</td>
<td>Intracellular Ca^{2+} concentration</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca^{2+}/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CHPG</td>
<td>(RS)-2-Chloro-5-hydroxyphenylglycine</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-2,3-dihydroxy-7-nitroquinoxaline</td>
</tr>
<tr>
<td>CPA</td>
<td>Cyclopiazonic acid</td>
</tr>
<tr>
<td>4CPG</td>
<td>(S)-4-Carboxyphenylglycine</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacglycerol</td>
</tr>
<tr>
<td>DCG-IV</td>
<td>(2S,1'R,2'R,3'R)-2-(2',3'-Dicarboxycyclopropyl)-glycine</td>
</tr>
<tr>
<td>DHPG</td>
<td>(S)-3,5-Dihydroxyphenylglycine</td>
</tr>
<tr>
<td>EBS</td>
<td>Electrical brain stimulation</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(aminoethyl ether) N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory postsynaptic potential</td>
</tr>
<tr>
<td>fEPSP</td>
<td>Population excitatory postsynaptic potential</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-amino-butyric acid</td>
</tr>
<tr>
<td>GABA_A</td>
<td>γ-amino-butyric acid type A receptor</td>
</tr>
<tr>
<td>GABA_B</td>
<td>γ-amino-butyric acid type B receptors</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks Buffered Saline Solution</td>
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<tr>
<td>HFS</td>
<td>High frequency stimulation</td>
</tr>
<tr>
<td>IB</td>
<td>Intrinsic bursting</td>
</tr>
<tr>
<td>IL</td>
<td>Infrafacial</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>IP3</td>
<td>Inositol-(1,4,5)-trisphosphate</td>
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<tr>
<td>IPSP</td>
<td>Inhibitory postsynaptic potential</td>
</tr>
<tr>
<td>LFS</td>
<td>Low frequency stimulation</td>
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<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
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<tr>
<td>MCPG</td>
<td>RS-a-methyl-4-carboxyphenylglycine</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal Essential Medium</td>
</tr>
<tr>
<td>MGlur</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidyl inositolide</td>
</tr>
<tr>
<td>PL</td>
<td>Prelimbic</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PPF</td>
<td>Paired-pulse facilitation</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PSP</td>
<td>Postsynaptic potentials</td>
</tr>
<tr>
<td>RS</td>
<td>Regular spiking</td>
</tr>
<tr>
<td>STP</td>
<td>Short-term potentiation</td>
</tr>
<tr>
<td>TBS</td>
<td>Theta burst stimulation</td>
</tr>
<tr>
<td>VDCC</td>
<td>Voltage-dependent Ca(^{2+}) channels</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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</table>
1.1 Neural basis of learning and memory.

By the end of the last century, it was uniformly accepted that the majority of mature neurones have lost the ability to divide. This discovery ruled out the possibility that learning and memory involve the synthesis of new brain cells, and the search for an alternative theory commenced. Both Tanzi (1893) and Ramon y Cajal (1894) independently proposed what became known as the connectionist view of learning and memory. This stated that learning involved the strengthening of connections between neurones and that memory was encoded in the product of this modification. Anatomical studies by Konorski (1948) led him to propose that these changes in connection strength occurred at the synapse.

Perhaps the most famous development of the connectionist theory was proposed by the psychologist Donald Hebb (1949). He suggested that the changes in synaptic strength underlying learning required a coincidence of activity in the presynaptic and postsynaptic compartments. This proposal, now commonly known as Hebb's postulate, states:

"When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased"
A synaptic connection that acts in such a manner is referred to as a Hebbian synapse. It was decades later that Bliss & Lømo (1973) described a phenomenon that appeared to be an electrophysiological example of such a synaptic modification. This phenomenon, termed long-term potentiation or LTP, is defined as an activity-dependent form of synaptic plasticity that involves a rapid and sustained up-regulation of synaptic efficacy. It was first demonstrated following the conditioning of the hippocampal perforant pathway with high frequency stimulation (HFS). Both the amplitude of the population excitatory postsynaptic potential (fEPSP) and the amplitude and latency of the population spike were recorded in the dentate gyrus and were shown to be potentiated in a robust and persistent manner for the whole 90 minute recording period. This potentiation was observed to last for days, even weeks in the intact, unanaesthetized rabbit (Bliss & Gardner-Medwin, 1973).

LTP has been observed in all of the three major excitatory synaptic connections in the mature hippocampus (Schwartzkroin & Wester, 1975; Alger & Tyler, 1976; Lynch et al., 1977; Andersen et al., 1977; Yamamoto & Chujo, 1978). It has also, however, been observed in many other brain regions and since there is evidence for regional variation in both the experimental conditions necessary for LTP induction and the mechanisms underlying its induction and maintenance, it is pertinent to briefly mention some of the most important studies into neocortical LTP. It has been demonstrated in the visual, somatosensory and motor cortex. LTP has been elicited, in vivo, in the visual cortex of kittens following low frequency tetanic stimulation of the optic nerve (Tsumoto & Suda, 1979). This enhancement of cortical field potentials can only be demonstrated within a narrow window of postnatal development, in kittens aged between 21 and 34 days (Komatsu et al., 1988). It can also be produced in vitro, in layers II and III of the rat visual cortex, following HFS.
of the subcortical white matter. This LTP is dependent on the presence of the γ-amino-butyric acid type A receptor (GABA_A) antagonist bicuculline methiodide (BMI; Artola & Singer, 1987). Associative LTP (see section 1.3 for definition) can be elicited in an *in vitro* preparation of sensorimotor cortex, leading to the long lasting potentiation of excitatory postsynaptic potentials (EPSP) in layer V. In the same preparation, HFS elicits LTP in layers III and VI (Bindman et al., 1988). Associative LTP of a monosynaptic connection was produced in adult cat motor cortex for the first time under anaesthesia (Baranyi & Szente, 1987a). More recently this has been reproduced in awake cats (Baranyi et al., 1991). LTP has also been produced in non-primary cortical regions. In frontal cortex, HFS of layer IV elicits LTP of polysynaptic components of the EPSPs recorded in layers II and III (Sutor & Hablitz, 1989a). This LTP was seen to persist undiminished for 5 hours. In prefrontal cortex, LTP of the monosynaptic connection between layers II and V was produced following tetanic stimulation (Hirsch & Crépel, 1990). LTP thus appears to be a widespread phenomenon. LTP expressed in these different brain regions all represent a phenomenon of synaptic change. However the mechanisms underlying LTP induction and maintenance can show considerable variation.

**1.2 Properties of LTP.**

The term LTP should only be used to describe a potentiation that remains stable for over half an hour. It is widely accepted that measurements taken at 10 and 30 minutes after conditioning are sufficient indicators of short-term potentiation (STP) and LTP respectively (Clark & Collingridge, 1995). The mechanisms thought to underlie LTP can be divided into three stages: induction, maintenance and expression. The induction phase is rapid, the maximal potentiation of EPSP slope appearing as
early as 30 msec after conditioning. The maintenance phase involves a cascade of biochemical events occurring in both the preynaptic and postsynaptic elements which act to stabilise the potentiation at a level just below that maximally achieved. The expression phase involves the manifestation of the effects of these biochemical cascades and it accounts for the long-term nature of the phenomenon. LTP is commonly studied within the hippocampus, a brain structure that has been implicated in learning and memory both through the technique of electrical brain stimulation (EBS) and through clinical studies following hippocampal lesions. The simple circuitry of the hippocampus has facilitated the study of LTP and it is in the hippocampus that many of the mechanisms underlying this form of synaptic plasticity were first described.

1.3 The induction of LTP.

There are three conditioning paradigms that have proven to be effective at inducing LTP. The first is termed a pairing or associative conditioning paradigm and combines low frequency stimulation (LFS; e.g. 900 pulses at 1Hz) to the test afferent pathway with the excitation of the postsynaptic element by means independent of this test pathway either by the activation of distinct presynaptic fibres or the injection of depolarising current postsynaptically (Kelso et al., 1986; Wigstrom & Gustafsson, 1986). The second is HFS (100Hz for 1s) to the afferent pathway (Bliss & Lømo, 1973). The third is theta burst stimulation (TBS) to the afferent pathway. Theta rhythm is a natural pattern of neuronal firing in the hippocampus, which modifies the firing of the output neurones and has been shown to occur when rats move in and explore their environment (Vanderwolf, 1969; Otto et al., 1991; see O'Keefe & Recce, 1993 for references). Conditioning TBS consists of a train of 4 shocks at 100
Hz, repeated ten times at 5 Hz. Five TBS trains are applied at 1 minute intervals. This paradigm has been proven to be an effective method of inducing hippocampal LTP in both \textit{in vitro} (Larson & Lynch, 1986; Larson et al., 1986) and \textit{in vivo} preparations (Staubli & Lynch, 1987). The induction of LTP in the dentate gyrus preferentially occurs when the stimuli coincide with the positive peak of the dentate theta rhythm and there is some evidence that the timing of the activity of pre and post-synaptic cells determines the amount and possibly the direction of synaptic change (Pavlides et al., 1988). LTP induced by the latter two conditioning paradigms have been demonstrated to occlude one another, indicating common mechanisms (Diamond et al., 1988).

Both HFS and TBS lead to release of the excitatory transmitter, glutamate, and the subsequent activation of Na$^+$-permeable $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors that mediate the EPSP (Neuman et al., 1988; Hestrin et al., 1990). In addition, glutamate acts on $\gamma$-amino-butyric acid (GABA)-ergic interneurons that mediate the inhibitory postsynaptic potential (IPSP) and these provide an inhibitory drive onto the glutamatergic neurones, thus dampening neuronal excitability. When large quantities of glutamate are released, as in the case of repetitive conditioning paradigms, the release of GABA from the interneurone population is depressed through a negative feedback loop utilising $\gamma$-amino-butyric acid type B receptors (GABAB). This reduction in the inhibitory drive facilitates neuronal depolarisation and the activation of N-methyl-D-aspartate (NMDA) receptors and thus the induction of LTP (Davies et al., 1991; Mott & Lewis, 1991). The induction of LTP can be further facilitated by the presence of GABA$\text{A}$ receptor antagonists (Wigstrom & Gustafsson, 1983).

Three principle characteristics have been attributed to LTP induction, namely cooperativity, associativity and input specificity. The term cooperatively refers to the
existence of an induction threshold. Activation of small numbers of afferent inputs by weak intensity stimulation fails to overcome this threshold and therefore fails to induce LTP. Stronger tetanic stimulation, on the other hand, activates a larger number of inputs, and exceeds the threshold and produces LTP. There is therefore a strong correlation between the intensity of the conditioning and both the incidence and magnitude of LTP. Associativity is the principle underlying classical conditioning, whereby a weak input can become potentiated if paired with a stronger converging input on to the same cell. Input specificity refers to the fact that only those synapses active at the time of conditioning exhibit any change in synaptic strength.

LTP induction within both the hippocampus and the neocortex is dependent on postsynaptic membrane depolarisation. This is shown by four experimental observations: First, the addition of the selective GABA\textsubscript{A} antagonist, picrotoxin, to the bathing medium facilitates the induction of LTP (Wigstrom & Gustafsson, 1985). Second, hyperpolarisation of the post-synaptic membrane prevents the induction of LTP (Malinow & Miller, 1986). Third, LTP can be depressed by mimicking the activation of the inhibitory receptors on the post-synaptic cell by the application of the inhibitory transmitter GABA (Scharfman & Sarvey, 1985). Finally, the application of a weak stimulus, one which does not ordinarily meet the threshold requirement, paired with the injection of a depolarising current into the post-synaptic cell results in LTP induction (Wigstrom & Gustafsson, 1986). This finding correlates with the postulate of Hebb.

The dependence of LTP induction on simultaneous pre and postsynaptic activity highlights a need for a mechanism whereby this coincidence of activity can be detected. It has been postulated that one of the glutamate/aspartate receptors, the NMDA receptor, acts as this coincidence detector and that it therefore plays a crucial
role in LTP induction. Collingridge et al., showed that the addition of the selective NMDA receptor antagonist, D-2-amino-5-phosphonovalerate (AP5) to the bathing medium of a hippocampal slice irreversibly blocked the induction of LTP in the majority of slices tested (Collingridge et al, 1983). It has been suggested that the distribution of the NMDA receptor subclass throughout the CNS can be used as indicator of those regions that exhibit associative plasticity. The highest concentrations of these receptors are found in the strata oriens and radiatum of the CA1 hippocampal field, however, high levels are also found in specific cortical and neocortical regions (Cotman et al, 1987). The NMDA receptors are blocked in a voltage-dependent manner by a Mg$^{2+}$ ion wedged in the channel pore. Depolarisation of the postsynaptic membrane evoked by either tetanic stimulation of the afferent fibres or by post-synaptic current injection alleviates this Mg$^{2+}$ blockade and opens the channel pore, allowing Ca$^{2+}$ influx. This property of NMDA receptor activation explains all three of the basic properties of LTP induction. Cooperativity arises from the fact that a weak stimulus would cause insufficient postsynaptic levels of postsynaptic depolarisation. Associativity is a consequence of strong conditioning at an independent, but convergent pathway causing sufficient depolarisation to allow for NMDA receptor activation in the weaker pathway. Input specificity results from the need for presynaptic glutamate release and/or other presynaptic changes in addition to postsynaptic depolarisation to activate NMDA receptors.

Considerably less is known about the NMDA receptor involvement in LTP induction within the neocortex. In visual cortical slices, AP5 has been shown to reduce the probability of LTP induction following conventional tetanic stimulation (Artola & Singer, 1987). In an in vitro preparation of the prelimbic area of the prefrontal cortex, LTP induction can be blocked by AP5 (Hirsch & Crépel, 1990).
addition, LTP induced by electrical stimulation of the CA1-subicular region of the
hippocampus following tetanic stimulation can also be blocked by AP5 (Jay et al.,
1995). Several groups have reported that the block of LTP induction by the
competitive antagonist AP5 is not absolute. LFS paired with strong postsynaptic
depolarisation, a protocol thought sufficient to remove the Mg\(^{2+}\) blockade of the
NMDA receptor channels was shown to induce LTP in only 15% of synapses in the
sensory neocortex. This could be attributed to heterogeneity in both the afferent
pathways and the neocortical cells, a phenomenon not encountered in the hippocampus
(Bindman et al., 1988). The pairing of LFS with strong postsynaptic depolarisation
succefully induced LTP at 50 to 60% of synapses in the motor cortex of awake cats
(Baranyi & Szente, 1987a). In the kitten visual cortex, LTP is unaffected by the
application of AP5, being dependent instead on Ca\(^{2+}\) entry through T channels
(Komatsu, 1994). A similar series of experiments using older kittens and a different
induction protocol, reveal a dependency on NMDA receptor activation (Bear et al.,
1992). It is thus clear that there considerable variation in NMDA receptor-dependency
between different areas of the neocortex.

1.4 The importance of Ca\(^{2+}\) in the induction of LTP.

The activation of NMDA receptors is known to result in an influx of Ca\(^{2+}\) ions
into the neuronal interior. This signal is crucial for the induction of LTP. The
intracellular injection of the Ca\(^{2+}\) chelator ethylene glycol-bis(aminoethylether)
N,N,N',N'-tetraacetic acid (EGTA) blocks hippocampal LTP (Lynch et al., 1983). In
both the visual cortex of young rats (Tsumoto, 1990) and in prefrontal neurons
(Hirsch & Crépel, 1992) chelation of post-synaptic Ca\(^{2+}\) leads to a block of LTP
induction.
NMDA receptor-mediated $Ca^{2+}$ influx represents only one of four possible routes by which $Ca^{2+}$ can enter the postsynaptic compartment. Cytosolic $Ca^{2+}$ levels also rise as a consequence of entry through voltage-dependent $Ca^{2+}$ channels (VDCC) and metabotropic glutamate receptor (mGluR)-mediated release from inositol-(1,4,5)-trisphosphate (IP$_3$)-sensitive intracellular stores. In addition, $Ca^{2+}$ is released from intracellular stores in response to the $Ca^{2+}$ signal generated by one or more of the other possible routes, so called $Ca^{2+}$-mediated $Ca^{2+}$ release (Mayer & Miller, 1990). $Ca^{2+}$ entry through postsynaptic VDCCs alone has been demonstrated to transiently potentiate synaptic transmission in the hippocampus (Kullmann et al., 1992). The STP was converted into LTP by the addition of presynaptic activity during induction.

Thapsigargin, the tumor promotor, which depletes intracellular $Ca^{2+}$ stores via inhibition of ATP-dependent $Ca^{2+}$ uptake (Thastrup et al., 1990), prevents the induction of LTP (Harvey & Collingridge, 1992). Since thapsigargin depletes both ryanodine and IP$_3$-sensitive $Ca^{2+}$ stores, its effect on LTP induction could be the consequence of a block of $Ca^{2+}$ release from either of these sources, release that normally acts to magnify the NMDA receptor-mediated $Ca^{2+}$ signal. A recent study combining confocal laser scanning and field recordings in the hippocampus (Wilsch et al., 1998) provides evidence that suggests that $Ca^{2+}$ release from intracellular stores is crucial for the induction of LTP by weak tetanization protocols. When stronger tetanic paradigms are used there is sufficient $Ca^{2+}$ entry through VDCC to bypass the necessity of this mGluR-mediated $Ca^{2+}$ release.

A number of synaptic modification rules have been proposed that suggest that the magnitude of the postsynaptic rise in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$; Lisman, 1989; Artola & Singer, 1993) determines the production and direction of synaptic change. In its simplest form, it is proposed that a modest rise in $[Ca^{2+}]_i$ is
thought to lead to the induction of LTD, whereas a higher rise in \([Ca^{2+}]\) induces LTP (Sejnowski, 1977). This hypothesis is supported by the experimental observation that procedures that reduce Ca\(^{2+}\) entry through the NMDA receptor channel such as an elevation in extracellular \([Mg^{2+}]\) or a reduction in extracellular \([Ca^{2+}]\), both favour the production of LTD by stimulation paradigms that would otherwise elicit LTP (Calibresi et al., 1992; Mulkey & Malenka, 1992). This model of synaptic plasticity in which the change in synaptic strength is a linear function of postsynaptic activity, has, however, been modified considerably in recent years. It is now suggested that the change in synaptic strength is a non-linear function of postsynaptic activity, in which there is a shifting threshold for LTP induction, whereby prior synaptic activity can influence the outcome of afferent stimulation (Artola & Singer, 1993; Cohen et al., 1996). The so called Artola, Brocher & Singer (ABS) rule involves two thresholds, one for LTD induction and another, higher threshold, for LTP induction. A positive correlation between the amplitude of the postsynaptic Ca\(^{2+}\) signal and the incidence of LTP induction has been proposed in both the hippocampus (Melchers et al., 1988) and in the neocortex (Kirkwood & Bear, 1995), however evidence exists that suggests further modification to this model is required (Neveu & Zucker, 1996; Barry, 1997).

1.5 Consequences of raised postsynaptic Ca\(^{2+}\).

Elevated Ca\(^{2+}\) levels within the neuronal compartment results in the activation of Ca\(^{2+}\)-dependent protein kinases. These trigger reversible post-synaptic cellular events and it has been postulated that protein kinase C (PKC) and Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) are involved in a mechanism by which a transient surge in postsynaptic Ca\(^{2+}\) levels can be translated into long-term changes in synaptic efficacy.
The importance of PKC in the induction of LTP is indicated by five experimental observations: First, biochemical assays reveal that hippocampal LTP is accompanied by an increase in the extent of protein phosphorylation. One of these phosphorylation events is of a protein co-discovered by several research groups and thus given more than one name: F1, GAP-43, B-50 and P-57 (see Perkel & Nicoll, 1991 for references). Second, application of selective kinase inhibitors to the hippocampal slice preparation prevents the induction of LTP (Lovinger et al., 1987; Malinow et al., 1989). Third, injection of PKC into the postsynaptic compartment triggers a potentiated synaptic response that mimics LTP (Hu et al.; 1987). Fourth, the use of PKC activators, such as phorbol esters, facilitates the induction of hippocampal LTP and even transforms STP into LTP (Linden & Routtenberg, 1989). The intracellular injection of phorbol esters into motor cortical neurons of awake cats also triggers a long-term increase in cellular excitability (Baranyi et al., 1987b). Lastly, PKC gamma mutant mice exhibit impaired hippocampal LTP (Abeliovich et al., 1993). These observations support the involvement of PKC in the maintenance of LTP. However, when the distribution of PKC within the cat/kitten visual cortex was mapped using a tritiated phosbol ester, no difference in expression was seen between developmental stages exhibiting an abundance of or no plasticity (Needler et al., 1988). In addition, when the tetanus used to evoke LTP was preceded by low frequency stimulation, PKC gamma-deficient mutant mice displayed normal LTP (Abeliovich et al., 1993). This led to the conclusion that PKC is an important component in the mechanism that regulates induced LTP, but not in the induction process itself.

The involvement of CaMKII in hippocampal LTP is supported by three experimental observations: First, LTP is accompanied by both an increase in CaMKII
activity (Fukunaga et al., 1993) and an increase in the extent of phosphorylation seen in CaMKII and its substrates (Fukunaga et al., 1995). Second, intracellular injection of a specific CaMKII inhibitor, prevents LTP (Malinow et al., 1989; Hvalby et al., 1994). Third, CaMKII-deficient mutant mice show deficits in both hippocampal LTP and spatial learning (Silva et al., 1992).

Since CaMKII is capable of auto-phosphorylation it retains its kinase activity long after the postsynaptic Ca\(^{2+}\) levels return to their baseline levels. This property makes it a promising biochemical candidate as the molecule that is responsible for long term changes in synaptic strength triggered by transient postsynaptic events (for review see Lisman, 1994). Further experimental evidence demonstrates, however, that both PKC and CaMKII are only important for the induction of, but not the maintenance of LTP (Malinow et al., 1989; Otmakhov et al., 1997). Application of a peptide inhibitor for the catalytic domain of CaMKII blocked the induction of both STP and LTP by a pairing protocol. However, the introduction of the inhibitor after LTP had been successfully induced had no effect, indicating that CaMKII was not necessary for the continued expression of LTP. These findings suggest that either the maintenance of LTP is attributable to a postsynaptic kinase or kinases not affected by the inhibitors used to date, that the critical kinases are not located postsynaptically or that phosphorylated CaMKII plays an important role in the maintenance of LTP as a structural protein rather than an enzyme (Otmakhov et al., 1997).

Modulation of glutamate receptor channels through phosphorylation may, in part, explain the increased efficacy of synaptic transmission seen following LTP induction (Swope et al., 1992). All three of the ionotropic glutamate receptors: AMPA, NMDA and kainate receptors are modulated by phosphorylation. PKC potentiates NMDA receptor currents through a mechanism that involves reduction in
the voltage-dependent Mg$^{2+}$ block of the receptor channel. It triggers a four-fold decrease in the dissociation constant for the Mg$^{2+}$ block and as a consequence this becomes less efficient at negative membrane potentials. NMDA receptor currents are thus generated at much more negative membrane potentials and the EPSPs have a larger NMDA component (Ben-Ari et al., 1992). CaMKII enhances kainate receptor currents in cultured hippocampal neurons (McGlade-McCulloh et al., 1993) and transiently enhances AMPA receptor-mediated synaptic responses (Wyllie & Nicoll, 1994).

In addition to the aforementioned protein kinases, other Ca$^{2+}$-dependent enzymes have been proven to play a role in the induction of LTP. Tyrosine kinases are thought to regulate, through phosphorylation, the function of the NMDA receptor channel (Rosenblum et al., 1996). Evidence for the importance of this modulation for LTP induction lies in the observation that tyrosine kinase inhibitors, such as lavendustin A, are effective at blocking hippocampal LTP (O'Dell et al., 1991). Inhibitors of the Ca$^{2+}$-dependent protease, calpain, also effectively block hippocampal LTP (Denny et al., 1990; del-Cerro et al., 1990). It has been suggested that calpain acts to degrade spectrin, a structural protein implicated in the regulation of the both the surface chemistry of cells and cell morphology. Its degradation would therefore be expected to bring about dramatic changes in the morphology and receptor expression at the synapse (Lynch & Baudry, 1987). Inhibitors of the Ca$^{2+}$-dependent phosphatase, calcineurin, block the induction of LTP in rat visual cortex (Funauchi et al., 1994) and hippocampus (Wang & Stelzer, 1994). These observations are especially interesting since they contradict the model of bidirectional control of synaptic weight proposed by Lisman (1989). He suggested that the activity-dependent postsynaptic rise in Ca$^{2+}$ determines whether the synaptic weight will be increased or
decreased. Low levels of Ca\(^{2+}\) are thought to preferentially activate protein phosphatases and lead to LTD induction, whereas higher levels preferentially activate protein kinases and lead to LTP induction. Inhibitors of calcineurin would therefore be expected to have effects on LTD not LTP induction.

1.6 The locus of LTP expression.

The increased postsynaptic response observed at synapses expressing LTP could be the result of both a presynaptic and/or postsynaptic change. There is evidence that LTP is accompanied by an increased postsynaptic responsiveness to quisqualate, a glutamate analogue (Davies et al., 1989). In addition, several groups have shown an increased presynaptic release of glutamate following LTP induction (Skrede & Malthe-Sorensen, 1981; Bliss et al., 1986; Kullmann et al., 1996). Alternatively, LTP could be the consequence of a morphological change in the synapse (Wallace et al., 1991; Bailey, 1993).

Much research has focused on the presynaptic versus postsynaptic debate, however, another possible explanation for changes observed following LTP induction is alterations within whole populations of neurones. Repetitive afferent stimulation has been shown to uncover previously latent excitatory pathways in the hippocampus (Miles & Wong, 1987b). These excitatory pathways emerge as a consequence of a depression of inhibitory circuits within the cortex and result in synchronous firing in populations of previously unconnected neurones. Yamamoto & Chujo (1978) proposed that alterations in the activity of excitatory local circuit neurones underlie the changes in synaptic efficacy observed at a monosynaptic connection. Tetanic stimulation was shown to elicit LTP at the synapse between the mossy fibre input and CA3 neurones within rat hippocampus. The long-lasting enhancement of the evoked response was accompanied
by the generation of afterdischarge trains. Test stimuli delivered to a distinct set of input fibres revealed that neurones not innervated by the tetanised input also exhibited afterdischarge trains. Therefore, LTP expressed in a population of neurones can effectively spread to neighbouring cells, the consequence of which is the formation of associated groups of previously unconnected neurones. This observation is of particular interest as learning and memory are thought to involve networks of neurones that form a matrix in which information is stored in a distributed format rather than as a change in a single connection.

1.7 Classification and structure of metabotropic glutamate receptors.

Glutamate acts on both ionotropic and metabotropic glutamate receptors (mGluRs). Presently, eight different mGluRs have been identified. These eight receptors can be classified into three groups according to the level of conservation of their amino acid sequences. This classification is further supported by their respective transduction mechanisms and pharmacology (Roberts, 1995). Group I receptors and their splice variants stimulate phospholipase C (PLC) as revealed by an increase in phosphoinositol turnover and Ca\(^{2+}\) release from internal stores. They comprise subtypes mGluR\(_1\) and mGluR\(_5\). Group II receptors are coupled to the inhibition of adenylyl cyclase and consist of the subtypes mGluR\(_2\) and mGluR\(_3\). Group III members are also negatively coupled to adenylate cyclase and comprise the subtypes mGluR\(_4\), mGluR\(_6\), mGluR\(_7\) and mGluR\(_8\). mGluRs have also been demonstrated to activate phospholipase D (PLD; Holler et al., 1993) on bath application of both \textit{trans}-1S,3R-aminocyclopentane dicarboxylate (ACPD) and (S)-3,5-Dihydroxyphenylglycine (DHPG). The subtype responsible for PLD activation has not yet been identified.
The general structure of the mGluRs has been determined and is postulated to comprise an extracellular domain, located at the mGluR amino terminus, seven closely located hydrophobic segments, predicted to form membrane-spanning structures, and an intracellular carboxy-terminal domain. The most conserved regions include an additional hydrophobic domain in the extracellular domain postulated to form the ligand binding domain and the first and third intracellular loops possibly involved in G-protein coupling. There is also evidence that the mGluRs possess an amino acid residue that confers innate sensitivity to extracellular Ca\(^{2+}\) (Kubo et al., 1998).

1.8 Distribution of mGluRs.

Immunostaining techniques show that mGluR\(_5\) mRNA and protein is abundant in cerebral cortex, including the frontal medial region of the adult female Sprague-Dawley rat (Romano et al., 1996). MGlur\(_5\) is present in all cortical layers, predominantly on postsynaptic spines and dendritic shafts. There is also some presynaptic axon staining, as revealed by light and electron microscopy, relating to the mGluR role of regulating the release of glutamate from the presynaptic terminal (Baskys & Malenka, 1991). In contrast, mGluR\(_1\) is never seen in presynaptic axons (Romano et al., 1995). MGlur\(_1\) is also seen in frontal cortex of adult rats, but the immunostaining is denser in layers II/III than in layer V, and the mGluR\(_1\) mRNA is confined to non-pyramidal neurones (Fotuhi et al., 1993). IP\(_3\), on the other hand, is concentrated within the pyramidal neurones.

Immunogold localisation reveals that, in both the hippocampus and cerebellum, there is subsynaptic segregation of metabotropic and ionotropic glutamate receptors (Nusser et al., 1994; Lujan et al., 1997). The ionotropic AMPA receptors
are found opposite the release site in the main body of the synaptic junction, whereas
the metabotropic receptors are located beyond the perimeter of the same synapses. A
possible functional implication of this arrangement is that mGluRs may be activated
only when repetitive synaptic activity causes sufficient glutamate release so that it
spills out of the synaptic region.

1.9 Effects of mGluR activation.

mGluRs have been shown to have various effects on membrane ion fluxes and
synaptic events including the blockade of the Ca\(^{2+}\)-dependent K\(^+\) conductance that
underlies the slow afterhyperpolarisation (AHP; Charpak et al., 1990), the
presynaptic inhibition of excitatory synaptic transmission (Baskys & Malenka, 1991),
membrane depolarisation (Stratton et al., 1990; Guerineau et al., 1994), the inhibition
of GABA\(_A\) receptor-mediated currents and potentiation of AMPA-mediated responses
(Glaum et al., 1992) and either the induction or augmentation of long-term
potentiation.

The neuronal response to the activation of mGluRs by bath application of
either a selective group I and group II mGluR agonist such as ACPD or the non-
selective agonists glutamate and quisqualate have been investigated in layer V
neocortical cells (Charpak et al., 1990). The principal postsynaptic effect of mGluR
stimulation in the presence of ionotrophic glutaminergic and muscarinic cholinergic
antagonists was the appearance of a slow afterdepolarisation (ADP), seen following a
train of evoked spikes. This ADP was accompanied by only small changes in input
resistance and is postulated to be the result of both a Ca\(^{2+}\)-mediated decrease in a
resting K\(^+\) current and by a Ca\(^{2+}\)-independent decrease in an inward cation current.
Conditioning-induced changes in a similar Ca\(^{2+}\)-dependent K\(^+\) current to that thought
to underlie the late AHP have been found in the B-photoreceptors of the mollusc Hermissenda (Coulter et al., 1989). The reduction in this current appears to be causal to the generation of a conditioned response and may be therefore a general mechanism underlying some learned behaviours. In rabbit hippocampal pyramidal cells, classical conditioning has been shown to reduce both the amplitude and duration of the Ca\(^{2+}\)-dependent AHP (Coulter et al., 1989).

The application of ACPD to both hippocampal CA1 pyramidal neurones and neocortical cells leads to depolarisation, an increase in input resistance and a reduction in spike frequency adaptation i.e accommodation (Desai & Conn, 1991; Davies et al., 1995), even in the presence of the GABA\(_B\) receptor agonist, baclofen, to block synaptic transmission. Membrane depolarisation is thought to be the consequence of the block of a leak K\(^+\) conductance (Stratton et al., 1990; Guerineau et al., 1994) and has been shown to be independent of changes in intracellular Ca\(^{2+}\) (Charpak et al., 1990). In addition to its direct effects on CA1 pyramidal cells, ACPD also indirectly enhanced excitatory synaptic responses in these cells by partially blocking synaptic inhibition. This was seen as an ACPD-induced decrease in paired-pulse inhibition and an ACPD-induced reduction in both the fast and slow components of evoked IPSPs (Desai & Conn, 1991).

The effects of mGluR activation of whole cell Ca\(^{2+}\) currents have been studied in pyramidal cells isolated from the dorsoparietal neocortex of rats (Sayer et al., 1992). ACPD was shown to suppress the high-threshold Ca\(^{2+}\) current, an effect not abolished by the application of 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX) and AP5. This finding suggests that mGluRs act to modulate L-type Ca\(^{2+}\) channels, through a mechanism shown to be partially Ca\(^{2+}\)-dependent. It is therefore proposed that one of the actions of mGluRs in neocortical cells is to suppress the component of
high-threshold Ca$^{2+}$ current conducted by these L-type channels. Also in cortical neurones, mGluRs have been shown to inhibit voltage-gated N-type Ca$^{2+}$ channels through a fast, voltage and calcium-independent pathway (Swartz & Bean, 1992; Choi & Lovinger, 1996). MGlurRs are also thought to facilitate NMDA receptor responses. This facilitation is seen as an ACPD concentration-dependent shift in the NMDA concentration-response curve and is seen at agonist concentration below the level necessary for the agonist to cause depolarisation on its own. In Xenopus oocytes this facilitation is mediated by PKC (Kelso et al., 1992). However, in the hippocampus, there is evidence both for (Aniksztejn et al., 1992) and against (Harvey & Collingridge, 1993) the involvement of PKC activation. This is not the only way in which the mechanism underlying the facilitation appears to differ between preparations. In the hippocampus, only the NMDA receptor mediated currents are facilitated (Aniksztejn et al., 1992), whereas in dorsal horn neurones, NMDA, AMPA and kainate responses are all seen to be facilitated (Bleakman et al., 1992). In neocortical neurones from dorsal sensorimotor cortex, mGluR agonists selectively facilitate NMDA receptor currents. This facilitation is dependent on intracellular levels of Ca$^{2+}$, but independent of PKC activation (Rahman & Neuman, 1996). A similar facilitation can be produced by the application of thapsigargin, suggesting that ACPD-induced facilitation is the consequence of IP$_3$ -mediated Ca$^{2+}$ release from internal stores. This is supported by the observation that, in the hippocampus, both ACPD and DHPG induce a dose-dependent and reversible enhancement of responses to NMDA (Fitzjohn et al., 1996).

It is well established that mGluRs are involved in the modulation of GABA transmission (Desai et al., 1994; Glaum et al., 1992). Group I mGluRs located on
hippocampal interneurones are known to enhance cell excitability and GABA release, whereas group II mGluRs act to reduce the probability of GABA release. It has therefore been proposed that mGluR-mediated regulation of inhibition represents an indirect role for mGluR$_1$ in synaptic plasticity (Poncer et al., 1995).

Activation of mGluRs also triggers the activation of PLD in the hippocampus, neocortex and striatum (Boss & Conn, 1992; Holler et al., 1993; Pellegrini-Giampietro et al., 1996; Klein et al., 1997). Both the broadspectrum mGluR agonist ACPD, the group I mGluR specific agonist DHPG and the group II mGluR specific agonist (2S,1'R,2'R,3'R)-2-(2',3'-Dicarboxycyclopentyl)-glycine (DCG-IV) all stimulate PLD activation. The broadspectrum mGluR antagonist RS-a-methyl-4-carboxyphenylglycine (MCPG) has been shown to inhibit the PLD response elicited by glutamate (Klein et al., 1997) and ACPD (Pelligrini-Giampietro et al., 1996). However, by itself MCPG induces a significant PLD response. PLD leads to the production of DAG and subsequent activation of PKC and therefore its activation has important implications for the induction of LTP.

The activation of group I mGluRs is linked with the activation of PLC and subsequent phosphatidyl inositide (PI) hydrolysis. This yields two important second messengers, IP$_3$ and diacylglycerol (DAG). The former leads to an increased release of [Ca$^{2+}$] from intracellular stores, whereas the latter triggers PKC activation. The involvement of mGluRs in the modulation of intracellular free Ca$^{2+}$ in the dendrites of CA1 pyramidal neurones has been demonstrated through patch-clamp recording combined with confocal microscopy. MCPG was shown to reduce the size of the Ca$^{2+}$ transient whilst either producing a small reduction or no effect on the synaptic currents evoked by the tetanus (Frenguelli et al., 1993). This demonstrates that mGluR activation contributes to postsynaptic increase in [Ca$^{2+}$] following trains of
action potentials. Conversely, ACPD greatly enhanced Ca^{2+} transients elicited by postsynaptic depolarization in dorsolateral septal neurones, another area rich in mGluR5.

In a preparation of acutely dissociated CA1 pyramidal neurons of the rat, group I mGluRs have been shown to activate three different types of Ca^{2+}-dependent K^{+} channels (Shirasaki et al., 1994). Through modulation of K^{+} channels, group I mGluRs are proposed to act as precise modulators of neuronal excitability.

There is evidence that group I mGluRs desensitize in response to prolonged or repeated agonist exposure. This desensitization was shown to be the result of a mechanism involving the activation of PKC and phosphorylation of the mGluRs (Gereau & Heinemann, 1998) through the use of selective PKC activators and inhibitors.

1.10 Involvement of mGluRs in the induction of LTP.

Group I mGluRs coupled with PLC that enhance NMDA receptor activity, depolarise neurones, and increase the intracellular [Ca^{2+}], would be expected to facilitate the induction of LTP, or even to induce LTP. Indeed, prior exposure to ACPD facilitated the induction of hippocampal LTP by weak (near-threshold) TBS (Cohen et al., 1995). As well as enhancing the magnitude of LTP, prior activation of mGluRs led to a more stable form of LTP. The priming effect of mGluR activation by ACPD can be mimicked by bath application of DHPG, and is proposed to be triggered by the activation of PLC (Cohen et al., 1998). Administration of ACPD combined with a subthreshold tetanic stimulation also induced LTP in hippocampal slices (Aniksztejn et al., 1992). Likewise, exposure to the group I specific agonist
DHPG given in combination with a STP-inducing tetanus induces LTP in the dentate gyrus (Manahan-Vaughan & Reymann, 1996).

It is also possible to induce LTP in the hippocampus by bath perfusion of ACPD alone (Bortolotto & Collingridge, 1992) provided that the CA3 hippocampal field remains attached to the slice (Collins et al., 1995). The extent of the potentiation shows a strong negative correlation with the age of the animal from which the slices were prepared, the ability of slices to exhibit potentiation declining with both age and body weight. This could be explained by alterations in the mGluR subtypes present during the early postnatal development. Both mGluR3 and mGluR5 are down-regulated at the stage in postnatal development during which the preparation losses the ability to express ACPD-induced potentiation (Catania et al., 1994). Further support for developmental changes in mGluR expression is derived from the observation of age-dependent changes in agonist efficacy (Schoepp et al., 1991). The ACPD-induced potentiation occludes the conventional NMDA-receptor dependent LTP indicating common mechanisms (Bortolotto & Collingridge, 1993). It can be blocked by AP5, suggesting a synergy between the mGluRs and NMDA receptors (see Otani et al., 1993) and it can be distinguished from conventional tetanus-induced potentiation by its slow onset. This suggests that it lacks a STP component (Bortolotto & Collingridge, 1993).

A number of different mechanisms have been proposed to underlie the observed ACPD-induced potentiation depending on either or both group I and group II mGluR activation. It could be the direct result increased Ca\(^{2+}\) influx consequent upon both the block of the AHP and of spike frequency adaptation (Desai & Conn, 1991). Alternatively, it could the result of an indirect effect on the excitatory drive from CA3 neurones to the CA1 hippocampal field, either by generating burst firing in the CA3
neurones, thus creating a "physiological tetanus" or by triggering an increased excitability of the CA3 neurones, the consequence of which is a larger excitatory drive to the CA1 field. There is experimental evidence for an increase in both recorded spontaneous burst firing and CA3 neuronal excitability (Chinestra et al., 1994). However, such changes would be expected to be accompanied by changes in the amplitude of the presynaptic fibre volley. This cannot be demonstrated experimentally (Bortolotto & Collingridge, 1995). Bortolotto & Collingridge (1995) also demonstrated that ACPD-induced potentiation is independent of changes in synaptic inhibition, membrane potential and input resistance, that it is insensitive to the L-type Ca$^{2+}$ channel blocker, nimodipine, that it is dependent on rise in postsynaptic Ca$^{2+}$, that it involves an increase in AMPA sensitivity and that it can be disrupted by LFS. Hence it was suggested that the activation of mGluRs triggers release of Ca$^{2+}$ from internal stores through an IP$_3$-dependent mechanism and this Ca$^{2+}$ signal substitutes for the transient activation of the NMDA receptors during a tetanus. Exposure to ACPD would result in a prolonged activation of mGluRs and thus a sufficiently large Ca$^{2+}$ signal to negate the need for NMDA receptor activation.

In the dentate gyrus, a fast onset LTP was induced by application of the group I mGluR specific agonist, DHPG. The agonist-induced potentiation occluded conventional tetanus-induced LTP, was independent of NMDA receptor activation and was antagonised by the non-specific mGluR antagonist MCPG (O'Leary & O'Connor, 1997). In the CA1 hippocampal field, however, both DHPG and the mGluR$_5$ specific agonist, (RS)-2-Chloro-5-hydroxyphenylglycine (CHPG), were found to induce LTD (Palmer et al., 1997). The LTD was dependent on NMDA receptor activation, was reversed by MCPG and did not occlude tetanically induced forms of LTD. It can be
enhanced by bath application of the GABA_A antagonist picrotoxin or use of Mg^{2+}
free ACSF, both of which increase the global level of excitability within the slice.

A more definitive method of studying the involvement of mGluRs in
hippocampal LTP was possible following the development of the specific mGluR
antagonist MCPG. In hippocampus, bath application of MCPG blocked tetanus-
induced LTP in the hands of some groups (Bashir et al., 1993; O'Connor et al., 1994,
Little et al., 1995) but not of anothers (Chinestra et al., 1993, Manzoni et al., 1994;
Thomas and O'Dell, 1995; Selig et al., 1995). This discrepancy is thought not to be
the consequence of insufficient concentrations of antagonist (Manzoni et al., 1994)
nor developmental changes in expression that would lead to a correlation between age
and effectiveness of mGluR antagonism (Thomas and O'Dell, 1995). MCPG had no
effect on hippocampal STP, suggesting that mGluRs are not involved in the induction
of STP, but solely in the induction of LTP. This supports the observation that ACPD-
induced potentiation appears not to contain a STP component.

In the dentate gyrus, MCPG reversibly blocked the induction of LTP in vivo
(Riedel et al., 1994) and disrupts the performance of rats in a spatial learning version
of the Morris water maze task (Richter-Levin et al., 1994). The controversy continues
in this brain region as well, however, MCPG being demonstrated not to block LTP by
other research groups in comparable studies (Bordi & Ugolini, 1995; Martin &
Morris, 1997). In visual cortex, MCPG failed to block the NMDA receptor-dependent
forms of LTP, LTD and depotentiation (Huber et al., 1998). In the dorsolateral septal
nucleus, mGluR antagonists were shown to block tetanus-induced NMDA receptor-
independent LTP (Zheng & Gallagher, 1992). The group I mGluR antagonist (S)-4-
Carboxyphenylglycine (4-CPG) also blocked the induction of LTP in the hippocampus
of freely moving rats (Manahan-Vaughan, 1997).
A number of different theories have been proposed to explain the conflicting consequences of mGluR antagonism on the induction of LTP. First, it has been suggested that the activation of MCPG-sensitive mGluRs may be only required for the induction of LTP by relatively weak stimulation protocols (Thomas & O'Dell, 1995). They therefore act to facilitate the induction of LTP by weak, near threshold patterns of synaptic stimulation either by their ability to enhance NMDA receptor-mediated responses (Aniksztejn et al., 1991) or inhibit GABAergic inhibitory synaptic transmission (Liu et al., 1993). This is supported by the observation that the efficacy of MCPG is determined by the tetanisation strength and the magnitude of the resulting Ca\(^{2+}\) signal (Wilsch et al., 1998). Second, it has been proposed that mGluRs are only necessary for the induction of LTP at naive synapses. MCPG would therefore not block the second of two attempts at inducing LTP (Bortolotto et al., 1994). This implies that the mGluRs trigger a 'molecular switch' which remains activated thus negating the need for mGluR activation on subsequent attempts at inducing LTP. LFS (1Hz, 10 min), a conditioning paradigm proven to induce depotentiation (Fujii et al., 1991), would be expected to reverse this molecular switch and therefore, when delivered between tetani, would result in a consistent sensitivity of LTP induction to MCPG. This cannot be demonstrated experimentally (Selig et al., 1995), throwing doubt on the molecular switch hypothesis.

The development of mGluR-deficient mice has proven a valuable tool for investigating the physiological roles of specific mGluR subtypes. MGluR\(_1\)-deficient mice exhibited a motor deficit and impaired mossy fibre LTP, but retained the ability to undergo LTP in the four remaining hippocampal pathways (Conquet et al., 1994). These findings are not supported, however, by a comparable study by Aiba et al. (1994) who found that mGluR\(_1\)-deficient mice exhibited substantial impairment of
both context-specific associative learning and hippocampal LTP. A further study by Hsia et al. (1995) also contradicts the findings of Conquet et al. MGluR1-deficient mice were demonstrated to exhibit normal mossy fibre LTP. In an attempt to resolve this issue, these in vitro experiments have been repeated in vivo (Bordi et al., 1997). In vivo, mGluR1-deficient mice exhibited reduced LTP in the dentate gyrus. The proposed explanation for this discrepancy is that mGluR1 is responsible for modulating inhibitory circuits present in vivo, but lost in the slice preparation.

MGluR5-deficient mice also exhibit significantly reduced NMDA receptor dependent LTP, whilst LTP at the mossy fibre synapse on the CA3 region, an NMDA receptor independent pathway, remains intact. The mutant mice expressed deficits in the acquisition and use of spatial information in both the Morris water maze and contextual information in the fear conditioning test (Lu et al., 1997). These studies suggest that mGluRs are not absolutely required for LTP, since the absence of the gene encoding one of the receptor subtypes merely reduces the magnitude of LTP. Instead it is proposed that they act to modulate the plastic process and can, therefore, be seen to be analogous to a volume control, regulating the magnitude of LTP. This is another example of the synergy that exists between the mGluRs and other elements of the cascades that bring about the induction of LTP.

1.11 Neocortical LTP

The cortex lacks many of the technical advantages for the study of LTP offered by the hippocampus. Apart from the fact that the cortical pyramidal neurones are somewhat smaller than their counterparts in the hippocampus, the neocortical histology and circuitry is considerably more complex making it extremely difficult to stimulate a single input system in isolation. LTP was almost exclusively studied in the
hippocampus following its discovery by Bliss & Lomo. However, even earlier in 1964, activity-dependent changes in the efficacy of synaptic transmission was observed in the somatosensory cortex of the anaesthetised rat (Bindman et al., 1964). Since then, many studies have demonstrated the existence of LTP in other cortical regions, including the prefrontal cortex (Hirsch & Crépel, 1990).

1.12 Circuitry of the prelimbic region of rat medial frontal cortex.

In 1948, Rose & Woolsey defined the prefrontal cortex (PFC) as being that region of the cortex that receives afferents from medial dorsal thalamus (Rose & Woolsey, 1948). In the rat, the prefrontal cortex can be divided into three principal areas termed prelimbic cortex (PL), infralimbic cortex (IL) and ventral anterior cingulate cortex (Groenewegen, 1988). There is a potential confusion in the literature in the naming of the PL cortex. The region of medial frontal cortex that we term the PL area is clearly identifiable as a restricted part of the prelimbic cortex according to the maps of Condé et al. (1990), which are based on tracing of afferents from medial dorsal thalamus. It corresponds to the ventral portion of the anterior cingulate area 3 and the dorsal portion of IL cortex according to the cytoarchitectonic maps of Zilles (1985).

The PL region of PFC, designated Brodman area 32, possesses a well-defined laminar cytoarchitecture characterised primarily by a homogenous layer V containing densely-staining cells and the lack of a layer IV (Krettek & Price, 1977). It receives inputs from the medial segment of the mediodorsal nucleus of the thalamus, a fact confirmed both by the anterograde degeneration technique (Leonard, 1969) and a retrograde fluorescent tracer technique (Condé et al., 1990). The thalamic inputs form part of a complex pattern of innervation within the PL area of medial PFC. The PL
region also receives afferents from the lateral areas of the CA1 and CA2 hippocampal fields, the subiculum, the amygdala and the insular, entorhinal and piriform cortices (Condé et al., 1995) and the description of these inputs has led to the suggestion of PL involvement in autonomic function (Neafsey, 1990) and in limbic function (Sesack et al., 1989). For each medial cortical area, however, the main sources of input are the other MFC areas rostrally and caudally adjacent (Condé et al., 1995). The physiological significance of these reciprocal connections between MFC areas remains to be determined.

Stimulation at the border between layers I and II in PL cortex can therefore activate numerous axons both antidromically and orthodromically. It could activate cortico-cortical fibres that make synaptic connections with the apical dendrites of the layer V neurones and also small layer II pyramidal neurones whose axons collateralise and form numerous synaptic contacts with the dendritic tree of the layer V cells. In addition, it might backfire afferent connections to the PL region from the association cortices, hippocampus and subiculum (Jay & Witter, 1991).

The major output neurones of the PL cortex are the layer V-VI pyramidal cells. The PFC sends fibres to almost every structure from which it receives them. The dendrites of the layer V cells receive a complex array of inputs. Generally speaking, the inputs to the apical dendrites originate in the association cortices, whereas inputs to the soma and/or basal dendrites arise from adjacent cells within the same deep layer or from the thalamic nuclei (Jones, 1984; Levitt et al., 1993).
1.13 Electrophysiological and morphological properties of layer V neurones in rat prefrontal cortex.

The physiological and morphological characteristics of the pyramidal neurones within rat prefrontal cortex have been examined using intracellular recording coupled with biocytin injection following stimulation of the input to this brain region from the nucleus accumbens (Yang et al., 1996). Three principal pyramidal cell types have been identified in layer V: regular spiking (RS; 19%), intrinsic bursting (IB; 64%) and intermediate (IM; 4%). RS cells exhibited the ability to generate a train of spike in response to the injection of a suprathreshold depolarising current pulse. Each spike yielded a well defined AHP thought responsible for the progressively longer interspike interval between subsequent spikes. IB cells exhibited a distinctive Ca$^{2+}$-dependent depolarising afterpotential (DAP), as well as an obligatory initial spike doublet, to the injection of depolarising current pulses. IM cells possess physiological characteristics that lie between those described for the RS and IB cells.

1.14 Synaptic plasticity in the medial frontal cortex.

Synaptic plasticity in the medial frontal cortex, and particularly the PL cortex, warrants investigation for many reasons.

First, the PL region of PFC functions as a component of the hippocampal-neocortical neural circuit involved in a short-term working memory system (Goldman-Rakic, 1987) which acts to hold sensory information for a short time until a behaviour is produced or decision reached. It therefore represents a transient neural record of sensory and motor events (Kesner & Holbrook, 1987). Divac (1971) was the first to demonstrate that lesions of rat PFC lead to a deficit in spatial learning. This finding is now supported by more elaborate lesion studies in which lesions of the dorsolateral
PFC in primates and medial PFC in rats disrupts short-term memory utilised in spatial delayed response, spatial delayed alternation, delayed oculomotor response and spatial response discrimination tasks (Kolb et al., 1974; Fuster, 1989; Funahashi et al., 1993; Kesner & DiMattia, 1987). In addition to lesion studies, recordings of single unit activity from cells within primate dorsolateral PLC reveal either a persistent increased or decreased rate of firing during the delay period of the delayed oculomotor task (Funahashi et al., 1990). These prefrontal neurones also respond to egocentric locations in the visual field, which has led to the hypothesis that the dorsolateral PFC is involved in the short-term representation of egocentric space. Different subregions of PFC are thought to mediate different aspects of working memory. The rat prelimbic and infralimbic cortices, for instance, represent visual object-attribute information (Kesner et al., 1996).

Second, anatomical studies show that the prelimbic and infralimbic areas receive direct inputs from the hippocampus (temporal fields of CA1-CA2) and the subiculum). The medial frontal cortex receives inputs from the CA1 hippocampal field. These hippocampal projections have been visualised using the anterograde tracer leucoagglutinin (Jay and Witter, 1991). The terminals of CA1 efferents are mainly, but not exclusively in deeper layers of prelimbic cortex, stemming from layer VI to more superficial layers, while the subicular efferents are distributed through the depth of the grey matter.

A third reason for investigating prelimbic LTP is that it can be induced at the synapses between the hippocampal input and neurones in prelimbic cortex in vivo (Laroche et al., 1990). In awake rats the LTP can persist for up to 3 days (Doyère et al., 1993). The LTP is NMDA-receptor dependent (Jay et al., 1995).
In addition, in isolated slices of prelimbic cortex, Hirsch & Crépel have uncovered several properties of LTP and LTD of monosynaptic EPSPs (Hirsch & Crépel, 1990). They showed that the EPSP elicited in layer V neurones by shocks applied to layer I had a substantial monosynaptic component, had both AMPA and NMDA receptor-mediated components, and could be modulated by exogenous noradrenaline or dopamine (see also Yang & Seaman, 1996; Gulledge & Jaffe, 1998).

Tetanic stimulation delivered to layer I elicited LTD of the EPSPs recorded intracellularly from layer V neurones in 50% of cases, LTP in 36% of cases and no effect in the remainder. LTP was elicited in a similar proportion of EPSPs by Nowicky & Bindman (1993) but LTD was elicited less often. Contrary to findings in the rat sensorimotor cortex, where the onset of the potentiated component of the layer V EPSP is often delayed by a few ms with respect to the onset of the EPSP (Bindman & Murphy, 1990), in the prefrontal cortex the potentiated EPSP is enhanced from its onset.

The PFC receives many modulatory inputs including all the major monoaminergic projections. Both dopamine (DA) and noradrenaline (NA) modulate activity in layer V/VI pyramidal cells. NA produces a long-lasting increase in the fEPSPs and population spikes recorded in both the dentate gyrus (Dahl & Sarvey, 1989) and CA3 hippocampal field (Hopkins & Johnston, 1988). The DA input to the PFC stems from the ventral tegmental area (VTA) and these modulatory synapses form on the dendritic tree of layer V-VI cells in close proximity to the major excitatory synapses (Descarries et al., 1987). DA decreases both spontaneous and evoked responses of PFC neurones (Ferron et al., 1984), but also potentiates excitatory responses of PFC neurones to subthreshold doses of NMDA (Cépeda et al., 1992) and acetyl choline (ACh; Yang & Mogenson, 1990). In a recent model it has
been proposed that the actions of DA on PFC neurones is neither inhibitory nor excitatory but depends instead on factors such as the timing and strength of the synaptic inputs and the resting membrane potential of the PFC neurone (Yang & Seaman, 1996).

1.15 Role of LTP and mGluRs in learning and mnemonic processing.

There is little direct evidence that a relationship exists between spatial learning and LTP. One approach used to clarify this issue is based on the prediction that experimental procedures that saturate LTP, such as repeated HFS, when delivered prior to the learning event, should prevent learning since the potential for further changes in synaptic efficacy has been abolished. Saturation preventing learning has been demonstrated experimentally by some research groups (e.g. Castro et al., 1989) but not others (e.g. Jeffery & Morris, 1993). It is, however, impossible to guarantee saturation has indeed been reached. Therefore, the reported negative results do not provide solid evidence against the involvement of LTP in learning and memory. Pharmaceutical manipulations of LTP, on the other hand, such as the intraventricular infusion of AP5 consistently impaired spatial learning in rats (Morris et al., 1986). In addition, both tyrosine kinase fyn mutant mice (Grant et al., 1992) and α-caMKII-deficient mutant mice (Silva et al., 1992) display learning deficits and a reduced ability for LTP. Furthermore the block of LTP in the dentate gyrus has been shown to prevent the establishment of a neural representation of newly acquired information. The infusion of AP5 into the dentate gyrus impairs the changes in dentate cell firing associated with tone-shock classical conditioning (Redini-Del Negro & Laroche, 1992). All these lines of experimental evidence support the hypothesis that LTP represents the neural basis of distinct forms of learning and memory.
In the majority of behavioural studies on mGluR involvement in spatial learning, agonists or antagonists are injected prior to training. It has been shown that i.c.v. infusion of MCPG disrupts memory formation in a spatial-alternation task performed in a newly developed shock-reinforced Y maze (Reidel et al., 1994). Similar retention deficits have been reported in water-maze learning in rats (Richter-Levin et al., 1994) and in passive avoidance learning in young chicks presented with a bead dipped in methylantranylate causing a clear disgust response (Holscher et al., 1994). The memory block has been shown to be dose-dependent and the injection of MCPG immediately before retention testing did not affect recall, indicating that the mGluRs are probably necessary during and for a short time after learning, but not during retention. In the water maze, the application of ACPD caused an impairment of acquisition suggesting that pre-training activation of mGluRs interferes with the learning process (Pettit et al., 1994). In young chicks, passive avoidance training is disrupted on the intracerebral injection of MCPG at 5 min, but not 15 min post-acquisition (Rickard et al., 1995).

The involvement of group I mGluRs in learning and memory has been tested directly through the use of the selective group I antagonist 1-aminoindan-1,5-dicarboxylic acid (AIDA). Exposure to AIDA prior to acquisition training blocks hippocampus-dependent contextual conditioning in rats (Nielsen et al., 1997). MGluR$_1$ deficient mice exhibit learning impairments during acquisition in the water maze (Conquet et al., 1994) and eyeblink conditioning (Aiba et al., 1994).

Three tentative conclusions can be drawn from the reports to date of the role of mGluRs in behaviour. First, it appears that not all forms of learning involve mGluR activation, for example brightness discrimination in rats is not MCPG-sensitive. Second, in other learning paradigms, such as passive avoidance, acquisition
of information in a spatial water maze or spatial alternation in the Y maze, paradigms that are believed to be hippocampus dependent, mGluR activation appears to be a prerequisite for memory formation. Third, the activation of mGluRs is limited to a short time window during and shortly after acquisition learning.

1.16 Aims of this study.

To investigate the involvement of group I mGluRs in the induction of LTP in prefrontal cortex.
CHAPTER 2

MATERIALS AND METHODS

2.1 The cortical slice preparation.

Male Sprague-Dawley rats (see 2.2 for details) were killed by cervical dislocation and then decapitated. The skull was exposed by a cut along the midline with a scalpel blade. The pelt was lifted aside, allowing access to the dorsal surface of the skull. Using a pair of dissecting scissors carefully inserted into the magnum foramen, a cut was made rostrally, along the dorsolateral edge of the skull, to the level of the olfactory bulbs, and then laterally across the frontal plate. The skull was then removed by the insertion of a pair of rongeurs under the bone, care being taken not to damage the underlying nervous tissue. In most cases, the dural membranes remained attached to the skull and were therefore removed at this stage. Any dura left on the surface of the brain was removed with a pair of fine forceps. Using the tip of a small spatula, the brain was lifted out of the cranial cavity and placed in a beaker containing ice cold artificial cerebrospinal fluid (ACSF) previously bubbled and saturated with 95% O₂ / 5% CO₂. The whole procedure so far described took less than a minute.

The brain was left intact for 2-3 minutes before being transferred to a petri dish, also containing ice cold ACSF. A razor blade was used to cut the brain coronally and thus to remove the caudal half of the brain. The frontal portion was then placed rostral surface down and the lateral and ventral surfaces trimmed. The remaining tissue was then lifted from the petri dish using filter paper, the rostral
Figure 2.1: Coronal section of rat medial frontal cortex.

Nissl stained, coronal section of rat brain including medial frontal cortex 2.7 mm rostral to bregma, taken from atlas of Paxinos & Watson (1982). Stimulating electrodes placed at border between layers I and II. Recording microelectrode placed halfway between medial surface and white matter.
surface dried and then stuck to the nylon stage of a Vibroslice tissue slicer (Campden Instruments Ltd.) with cyanoacrylate adhesive (RS components). The slicing chamber had been previously half filled with ACSF and stored in the freezer. Once the adhesive had dried (approx. 5 s), the brain was submerged in oxygenated ice-cold ACSF and the cutting chamber secured to the Vibroslice. The vibrating blade was then moved slowly toward the brain to cut slices 400 μM thick. Slices were discarded until the classic features of Bregma 2.2 to 3.2 (Paxinos & Watson, 1982; see figure 2.1) could be seen. These slices were transferred using either the tip of a spatula or a pipette with a tip diameter of 5 mm into petri dish resting on ice and containing filter paper moistened with ACSF. Between 6 and 8 slices were obtained per rat, the most rostral slice being that of Bregma 3.2 (Paxinos & Watson, 1982). The slices were placed in an incubation chamber. This perspex box (17.5 cm high, 16.5 x 16.5 cm width) contained a central pedestal onto which the petri dish was placed. It was filled to a depth of 3 cm with tap water continuously bubbled with 95% O₂/5% CO₂ through an airstone. A sheet of perspex was placed over the airstone to prevent drops of condensation from forming on the underside of the chamber lid and falling on the slices. The slices were left in this humidified, oxygenated atmosphere for at least one hour before any recording was attempted. Slices in the incubation chamber remained viable at room temperature for up to 12 hours.

2.2 Sprague-Dawley rats.

Male Sprague-Dawley rats between the ages of 26 to 35 days postpartum were used in all electrophysiological experiments. The majority of rats weighed between 80 and 100 grams, however no correlation between age and weight was found (figure
Figure 2.2: Relationship between age and weight of rat.

A Graph of age of rat in days plotted against weight of rat in grams for a total of 33 rats indicating lack of correlation between these two variables.
2.2, A).

2.3 Artificial cerebrospinal fluid.

ACSF was freshly prepared prior to every experiment and contained (in mM): 125 NaCl, 3.2 KCl, 26 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, 2 MgCl$_2$, 2 CaCl$_2$, and 10 D-glucose (all compounds supplied by BDH). Under the conditions of the experiments, the ACSF was continuously bubbled with 95% O$_2$/5% CO$_2$ and maintained at a temperature of between 31-33$^\circ$C (pH ca. 7.4).

2.4 Experimental recording chamber.

A pipette with a tip diameter of approximately 5mm was used to transfer a slice to a recording chamber for submerged slices (figure 2.4). A round glass coverslip (diameter 19 mm, BDH), the top surface of which was coated with Sylgard (10% w/v, Dow Corning), was attached to the base of the central well using silicon high vacuum grease (Dow Corning). A single D-shaped block of perspex (medical grade; dimensions: length 2.2 cm, width 7 mm, depth 5mm) was placed in the central well and once again stuck to the base using silicon grease. The presence of this perspex block reduced the total fluid capacity of the well to ca. 2 mls and improved the flow of ACSF across the slice. A "harp" (Edwards et al, 1989; see 2.6 for details) was used to firmly hold the slice down in the well. The slice was then continuously perfused with warmed oxygenated ACSF. A water bath, located immediately below the recording chamber, was used to heat the ACSF to a temperature of between 32 and 34$^\circ$C. Bath temperature was maintained within ± 0.5$^\circ$C during the course of an experiment using a thermistor-current feedback circuit (designed and built in the
Figure 2.4: The experimental recording chamber.

Diagram illustrating the top portion of the experimental recording chamber. Thick black arrows indicate the direction of flow of ACSF through the apparatus. ACSF enters the lower half of the chamber and is pumped through a water bath maintained at a temperature of between 32 and 34°C. The ACSF then ascends through a bubble trap, indicated by (a), in which is also placed the indifferent electrode, labelled (d). The ACSF flows over a Sylgard coverslip, indicated by (b) in the centre of the recording chamber, on to which the slice is placed. Excess ACSF is removed from this chamber by a bent hypodermic syringe needle, labelled (c), attached to a pump. Below the slice is a cool light source, indicated by (e), that illuminates the preparation (open arrow). Diagram taken from thesis submitted by Michael Francis Barry in March 1997.
departmental electronics workshop). The water in the bath was refreshed prior to every experiment and the coverslip replaced on a weekly basis. The well and perfusion tubes were washed with distilled water for ca. 20 min. after every experiment. Alcohol or Milton was used to sterilize the tubing on a weekly basis. The recording chamber was illuminated from below by direct light from a fibre-optic source (Micro Instruments, KL1500-T) and the slice viewed through a dissecting microscope (Prior Scientific Instruments Ltd, S2006).

2.5 ACSF perfusion system.

An 8 roller variable speed peristaltic pump (Watson-Marlow, 502S pump, 508 MC2 pump head) was used to deliver ACSF to the recording chamber. Fluid within the central well was then removed using suction supplied by a 3 roller peristaltic pump (Cronzet, 60 rpm) via a 21 gauge hypodermic needle attached to a 1ml syringe and Portex tubing. A 100 ml conical flask was used as an ACSF reservoir and this volume of fluid was continuously bubbled with the 95% O₂ / 5% CO₂ gas mixture. The fluid was recirculated at a flow rate of 2 to 3 ml/min. The dead space in the delivery tubing between the ACSF reservoir and slice was measured by syringe injection to be between 3 and 4 mls, including fluid contained in the well.

2.6 Harp construction.

The harp frame was formed from a 2.8 cm length of platinum wire (Goodfellows Metals; diameter 0.5 mm), bent into a U-shape (dimensions: 0.9 cms x 1.0 cms x 0.9 cms) and flattened in a vice. A fine nylon stocking was then laddered and the fibres stretched over the mouth of a glass bottle (diameter 2 cm). Individual nylon threads were dissected out under a microscope (Prior Scientific Instruments Ltd,
S2000) and arranged in a parallel array so as to be about 5mm apart. A thin layer of 
cyanoacrylate adhesive was applied to the side arms of the platinum frame. This was 
then placed over the threads so that the nylon spanned the gap between the two side 
arms. A £1 coin was placed on top of the harp ensuring contact between frame and 
stretched nylon threads. The adhesive was allowed to dry overnight, after which the 
£1 coin was removed and the nylon threads lying outside the frame trimmed. Before 
use, the harp was washed thoroughly with distilled water. A single harp was used for 
a maximum of 25 experiments, or until the nylon threads were no longer taut.

2.7 Recording electrodes.

Microelectrodes were pulled on a Flaming/Brown microelectrode puller (P-87, 
Sutter Instrument Company) from borosilicate glass (Clarke Electromedical 
Instruments GC120F-10).

Intracellular microelectrodes were filled with 4M potassium acetate. Only those 
microelectrodes with a series resistance of between 60 MΩ and 120 MΩ were used. 
Microelectrodes with series resistance less than 60 MΩ were shown experimentally to 
make poor cell penetrations, whereas those with series resistances greater than 120 
MΩ were often electrically noisy and resisted the passage of applied current.

Extracellular microelectrodes were pulled in the same manner, but filled with 
0.3M sodium chloride. Only those microelectrodes with series resistances of less than 
10 MΩ were used.

2.8 Voltage recording and current injection circuitry.

Intracellular microelectrodes were connected to the headstage (x0.1) of a 
Axoclamp 2A preamplifier (Axon Instruments) by way of a holder containing an
Ag/AgCl electrode. The reference electrode comprised another Ag/AgCl wire, inserted inside a 1.5 cm length of Portex tubing, previously filled with agar dissolved in physiological saline solution. This was then placed in the outer well of the bubble trap of the recording chamber, where it was in contact with the ACSF bathing the slice. The agar interface between the silver wire and the ACSF was used for two principal reasons: First, it minimised artefactual potential changes elicited by temperature fluctuations as a result of changing fluid level within the bubble trap. Second, it removed the small risk of noxious interaction between AgCl and the slice. The reference electrode was re-chlorided once a month by submersion in 0.3M NaCl solution whilst connected in circuit with a 9V battery. The Axoclamp pre-amplifier had both bridge balance (0 to 1000 M) and current injection (0 to 10 nA) facilities and amplified the voltage signal ten times. This output voltage was amplified a further ten times by a d.c. amplifier (built in the departmental electronic workshop). The signal was then acquired by a 486P/33 personal computer (Dell) via a T1-1 interface analogue-to-digital converter (range ± 10V) using pClamp 5.5. software (Axon Instruments). The resulting signal, digitized at 10 KHz, was then displayed on the computer screen. The Axoclamp preamplifier had both internal voltage calibration and input current measurement facilities.

Extracellular microelectrodes were connected to an Axoclamp (x1) headstage and pre-amplifier. The latter amplified the voltage signals by ten times and these were then treated in the same manner as described for the intracellular recordings.

2.9 Microelectrode support and drive systems.

The microelecrode holder was attached to a digital microelectrode drive (Robert Clark Engineering, Roslyn Precision Ltd.). This was supported by a mild
steel arm, the orientation of which could be crudely adjusted and which was in turn supported by a steel bar. This was firmly secured to a lathe-carriage and this allowed the calibrated movement of the microelectrode to any desired position within the recording chamber. Movement of the drive unit was initiated by remote control and this allowed the microelectrode to be lowered or withdrawn in controlled steps of either 2 \( \mu \text{m} \) or 10 \( \mu \text{m} \).

2.10 The stimulating electrodes.

The stimulating electrodes were constructed in the laboratory from nickel-chromium wire (termed "nichrome", a gift from G.L.Collingridge and Z.I.Bashir, University of Bristol). The tips of two 5 cm lengths of varnish-insulated nichrome wire (diameter 55 \( \mu \text{m} \)) were trimmed so as to remove the insulation. Each wire was then placed in a 5 mm length of stainless steel tubing, acquired from a disposable hypodermic needle (21 gauge, Sabre). At one end, the two wires were twisted together and thinly smeared with epoxy-resin (Araldite, 50% wt/wt, Ciba-Geigy). This acted to insulate the wires, whilst maintaining a distance of 100 to 150 \( \mu \text{m} \) between them. The wires were then placed through a glass pasteur pipette, shortened to 100 mm (from 230 mm, Bilbate) until 2 cm of the nichrome wire extended beyond the tip. Both ends of the pipette were then sealed with araldite. Once set, the tips of the wires were then trimmed to a length of between 1.5 and 2.0 cm. The other end of the nichrome wires were then attached to light-weight sleeved wire. All connections were insulated using heat-shrink tubing.

This bipolar stimulating electrode was connected to an isolated stimulator (Devices, MK IV), delivering an unipolar, constant voltage shock of duration 0.05-0.2 ms and 0-20 V strength. It was triggered by a -1.0 V pulse elicited by the pClamp.
software and subsequently amplified ten times by a d.c. amplifier. Movement of the electrode within the recording chamber was facilitated by a micromanipulator, the fine controls of which were operated via a hydraulic drive. Square wave pulses were delivered once every 15 or 20 s, so as to elicit a postsynaptic potential, just below action potential firing threshold.

2.11 Reduction of mechanical vibration and electrical interference.

The recording chamber and water bath were supported by four rubber bungs which rested on a mild steel base plate together with the recording and stimulation electrode assemblies and the dissecting microscope (Prior Scientific Instruments Ltd). This base plate was in turn supported by three inflated Mini car tyres acting as an anti-vibration device, reducing the incidence of lost cell penetrations due to incidental vibration.

The whole experimental assembly was surrounded on all but one side by an electrical shield of aluminium sheeting. Both this, the base plate and the hypodermic needle used for ACSF removal from the recording chamber were earthed through a common ground connection.

2.12 Positioning of stimulating and recording electrodes.

The stimulating electrode was lowered towards the slice using both the coarse and fine controls of a micromanipulator. It was placed lightly on the surface of the slice, at the border between layers I and II. When the light source is positioned correctly, layer I can be identified under the microscope as a translucent line.

Both intracellular and extracellular recordings were made from within a restricted portion of the prelimbic area of the medial frontal cortex as labelled by
Condé et al. (1995) based on the terminations of afferents from medial dorsal thalamus. The recording electrode was placed in a zone spanning 40 to 60% of the distance between the medial surface and the border of the white matter (Paxinos & Watson, 1982; figure 2.12), estimated to be layer V.

2.13 Intracellular microelectrode cell penetration.

The microelectrode was lowered towards the slice until it entered the ACSF and the recording circuit was completed. A current pulse of -0.1 nA, 60 ms duration was passed through the electrode. The tip of the microelectrode was cleared of any blockages through the application of maximum capacitance neutralisation without positive feedback (termed "ringing"). The bridge circuit on Axoclamp was balanced and the voltage offset adjusted to +40 mV. At this point, an estimate of microelectrode input resistance could be obtained and those failing to meet the criteria, discarded. Suitable microelectrodes were then advanced in 10 mm steps towards the slice until contact was made, indicated by a sudden increase in microelectrode input resistance. The microelectrode was advanced further, now in 2 mm steps, concurrent with brief applied depolarising current (~60 mV) evoked by quickly flicking the "clear" switch on the Axoclamp, a procedure shown to improve the likelihood of cell penetration. The microelectrode was advanced through the slice until a cell was penetrated or until a depth of ~350 mm was achieved, at which point the microelectrode was withdrawn and repositioned. The microelectrode tip often
Figure 2.12: Coronal sections of rat brain including medial frontal cortex showing recording electrode placement.

Three coronal sections of rat brain taken from atlas of Paxinos & Watson (1982) at various levels rostral to bregma (3.2, 2.7 and 2.2 mm from left to right) indicating sites of recording microelectrode in experiments using TBS as an induction paradigm. Solid triangles represent stable potentiations > 20 min after completion of TBS. Open circles represent those cells in which no effect was seen following TBS. The majority of points lie within prelimbic cortex, labelled according to Condé et al., (1995).

PL - Prelimbic cortex; IL - Infralimbic cortex
became blocked with tissue debris during its passage through the slice. This resulted in an increase in microelectrode input resistance. On many occasions it was possible to remove this blockage by triggering a brief oscillation of the Axoclamp capacitance neutralisation circuit. This was achieved by depressing the "buzz" button. Microelectrodes could be used for a maximum of five tracks or until the tip broke, indicated by a rapid drop in electrode resistance.

On penetration of a cell there was a sudden negative shift in potential, often accompanied by action potentials. Hyperpolarising current was immediately injected into the cell to prevent membrane depolarisation and action potential firing. This was maintained for at least 10 minutes following impalement. Occasional depolarising current pulses were applied to test cell excitability and this allowed non-excitable glial cells to be discarded. These glia typically exhibited membrane potentials of between -70 and -90 mV and did not show membrane charging following either a depolarising or hyperpolarising current pulse. Only neurones with membrane potentials more negative than -60 mV, action potentials greater than 60 mV in amplitude (threshold to peak) and apparent input resistance $> 20 \text{ M}\Omega$ were accepted for further experimentation. Cells failing to meet one or more of these criteria were abandoned.

2.14 Characterisation of membrane properties and synaptic responses.

Apparent input resistance ($R_{in}$), membrane potential ($V_m$) and spike firing threshold and amplitude were measured throughout the recording period by means a digital volt meter supplied with the Axoclamp pre-amplifier. This allowed any change in the cellular response to be confidently attributed to changes in synaptic strength rather than any overall change in the condition of the cell. Cells in which large ($>10 \text{ M}\Omega$) changes in $R_{in}$ were observed were discarded since such changes are indicative
of significant changes in $V_m$ and hence instability of recording. As changes in $V_m$
could be the consequence of drift in the potential measured at the Ag/AgCl reference
electrode, cell viability was confirmed by monitoring spike amplitude and threshold.
The former was measured on injection of depolarising current pulses. Action
potentials elicited on the EPSP are influenced by underlying conductance changes
within the cellular response and were therefore not used to obtain spike amplitude
measurements.

The I-V relationship of the membrane was examined in a number of cells
through the application of a series of depolarizing-hyperpolarizing current pulses in
the absence of evoked PSP, termed "passive pulse plot". The apparent input resistance
of the cell ($R_{in}$) was continuously monitored throughout the experiment by the
injection of hyperpolarizing current pulses (duration 60 ms, -0.1 nA) through the
recording electrode $> 100$ ms before the onset of the evoked response. The voltage
deflection elicited by the injected current pulse was measured at a point taken shortly
after the completion of membrane charging (judged by eye). Any error introduced by
bridge imbalance was corrected for. The threshold for action potential firing and the
spike amplitude were monitored during the experiment either by the occasional
injection of a depolarising current pulse, which occluded the measurement of $R_{in}$, or
by increasing the stimulus width and thus inducing spike firing synaptically.

At the start of some experiments, the membrane potential was altered through
the injection of a series of $200$ ms hyperpolarizing or depolarizing current pulses.
Synaptic stimulation was triggered between $30$ and $50$ ms after the onset of the current
pulse to ensure full charging of the membrane before the synaptic response. This
procedure, termed an "active pulse plot", was used to determine whether the observed
postsynaptic potential (PSP) was an excitatory postsynaptic potential (EPSP), a
reversed inhibitory postsynaptic potential (IPSP) or an EPSP-IPSP sequence. In the event that an EPSP could not be evoked or that the net synaptic response was an IPSP, the polarity of the stimulus was changed or an attempt was made to carefully lift up and reposition the stimulating electrodes without losing the cell. In some cases, the relative position of the stimulating and recording electrodes was such that the axon of the layer V cells was stimulated directly and an antidromic spike elicited. Once again, in this case, the stimulating electrodes were lifted up and repositioned.

2.15 Extracellular recordings.

The extracellular electrode was lowered towards the slice until it entered the ACSF and the recording circuit was completed. At this point, an estimate of microelectrode resistance was obtained and those failing to meet the criteria were discarded. Suitable microelectrodes were then advanced towards the slice and placed in layer V. Field potentials evoked by stimulation at the border between layers I and II could then be recorded. Much stronger stimulus intensities were necessary to evoke a fEPSP than needed to generate an intracellular response. The mean stimulation strength used in extracellular recordings was 2.7 mV (n = 10), compared to that of 0.97 mV (n = 10) in intracellular recording. Routinely, the relationship between input stimulation strength and output response was determined at the start of the experiment. The stimulus strength was then set at a level that elicited a response approximately 50% of the maximal response.
2.16 Induction of LTP.

The induction of LTP was attempted using a theta frequency induction (TBS) paradigm originally proposed by Larson et al. (1986). This comprised a train of 4 shocks at 100 Hz, repeated ten times at 5 Hz. Five trains were applied at 1 min intervals. Conditioning shocks were increased from 0.05 ms to 0.1 or 0.2 ms duration, and in some of the intracellular recordings, depolarizing current applied before each train to obtain firing in each theta burst within the train.

2.17 Pharmacology.

All drugs used in electrophysiological experiments were applied by bath perfusion. The GABA_A receptor antagonist bicuculline methiodide (Sigma) was present in all experiments at a concentration of 1µM with the exception of those experiments on the NMDA receptor-mediated component of the EPSP in which it was present at the higher concentration of 20 µM. MCPG (Tocris-Cookson) was used at 500 µM in ACSF containing 0.5 mM NaOH once the stock solution was diluted. ACPD (Tocris-Cookson) was used at 50 µM, S-3HPG (Tocris-Cookson) at 200 µM, DHPG (Tocris-Cookson) at 100 µM and CHPG (Tocris-Cookson) at 500 µM. Baclofen (Sigma) was bath applied at 200 µM. In those experiments in which the NMDA receptor-mediated component of the EPSP was isolated, 20 µM CNQX was used and the ACSF contained 1mM Mg^{2+}. DL-AP5 was used at 40 µM. In the Ca^{2+} imaging experiments, all aforementioned drugs were used at five times the stated concentration and were either bath applied or pressure ejected in puffs of 15s duration. 4CPG (Tocris-Cookson) was bath applied at 1 mM. KCl solution was used at 50 mM with isotonic replacement of NaCl. Glutamate was applied at 100 µM.
2.18 Analysis of experimental data.

Data collected during an experiment was analysed off line using the pClamp software analysis program Clampfit. Several consecutive points in each trace were averaged and imported into a spreadsheet (Microsoft Excel), in which measurements of Vm, Rin, EPSP slope, total spike amplitude and peak EPSP amplitude could be calculated. Spike threshold was measured manually from Clampfit. The experiments were then displayed in a graphical form using Excel and plotted on a Laserjet printer (Hewlett Packard). We classed the change for each experiment as LTP if the mean slope at 24 to 36 min post TBS was 120% of the baseline slope measured over the 4 min period before the start of TBS, and as LTD if the slope at 24-36 min was 80% of control, and as no change if the post-TBS slope fell between the two criteria.

A number of different parameters of the evoked field response were selected for analysis: (i) the peak-to-peak amplitude of the population spike, taken as the amplitude between the peak positivity and the peak negativity. (ii) the slope of the population spike usually measured over the first 1ms, but in a number of experiments measured over a shorter time period. Measurements were taken at an arbitrarily chosen latency, fixed for each experiment and not more than 1 ms after onset to prevent distortion by the subsequent population spike.

2.19 Measurement of intracellular [Ca$^{2+}$] using Fura-2 microfluorimetry.

Hippocampal mixed neuronal/glial primary cultures were prepared from rat pups age 2-4 days post-partum. Both hippocampi were dissected out and placed in ice-cold Gey's salt solution containing 20 mg/ml gentamycin. A pair of fine sissors was used to coarsely chop the tissue and this was then incubated in Ca$^{2+}$/Mg$^{2+}$-free Hanks Buffered Saline Solution (HBSS) containing 0.1% trypsin for 15 minutes at
36°C. After this period, the trypsin was inactivated by washing with normal HBSS and the cells were then dissociated by a sequence of three tituration steps using flame-narrowed Pasteur pipettes. In between each tituration step, cells were pelleted in HBSS and washed with fresh HBSS. Cells were pelleted and resuspended in Minimal Essential Medium (MEM) containing 10% horse serum. Dissociated cells were then plated on 25 mm round glass coverslips, previously coated with poly-D-lysine. The hippocampal cultures were grown at 36°C in a humidified atmosphere of 5% CO₂ and 95% air (pH 7.4) and fed bi-weekly with MEM containing 10% horse serum (Life Technologies, U.K.). Cultures were treated on day 3 with 1% cytosine arabinoside (Life Technologies, U.K.) for 24 hours to reduce glial growth, thus ensuring that the majority of cells in the hippocampal cultures were neuronal.

Medial frontal cortical mixed neuronal/glial primary cultures were prepared from male Sprague-Dawley rats between 12 and 14 days postnatum. The medial frontal region of cortex was isolated from slices of frontal cortex. The culture preparation was identical to that described above, with the exception that F12 was added to the culture medium. Cells were grown for a minimum of 7 days before using for experiments. Neurones were easily distinguishable from glia in these cultures by their phase-bright appearance, smooth rounded soma, and distinct processes.

Cytosolic changes in [Ca²⁺] within both somatic and dendritic compartments were measured using the ratiometric fluorescent Ca²⁺ indicator Fura-2 AM (Molecular probes, Eugene, OR.). The cultures were loaded for 30 minutes at room temperature with 5μM Fura-2 AM and 0.005% pluronic acid in standard recording medium (RM) containing (in mM): 156 NaCl, 3 KCl, 2 MgSO₄, 1.25 KH₂PO₄, 2 CaCl₂, 10 glucose and 10 HEPES, pH adjusted to 7.35 with NaOH. For experiments
in which zero Ca\(^{2+}\) buffer was used, the 2mM CaCl\(_2\) was omitted and 100 \(\mu\)M EGTA was added to the RM. Fluorescence measurements were obtained on an epifluorescence inverted microscope equipped with an oil-immersion x40 quartz objective. \([\text{Ca}^{2+}]_i\) was monitored using excitation light provided by a Xenon arc lamp, the beam passing through a 340nm and a 380nm filter housed in a computer controlled filter wheel. Emitted light was reflected by a 510nm dichroic mirror to a 12 bit CCD camera (Digital Pixel Ltd, U.K.). All imaging data were collected and analysed using Kinetic Imaging software (Liverpool, U.K.). A computer controlled shutter kept U.V. damage of the cells to a minimum by allowing exposure to excitation light only when required for imaging.

The responsiveness of the culture was tested by eliciting a Ca\(^{2+}\) response to the local application on ejection under pressure of either 50 mM KCl or 100 \(\mu\)M glutamate from a patch pipette (diameter 2-4 \(\mu\)m) placed near to the cell soma. Drugs were applied either in a similar manner or through bath application.

Analysis of the images obtained was conducted off-line following the completion of the experiment. A value of background fluorescence was obtained by measuring the fluorescent signal from a portion of the field containing no glial or neuronal elements. This background fluorescence was then subtracted from the images obtained during the experiment. A ratio of the 340 nm and 380 nm images was then taken and these measurements imported into a spreadsheet (Microsoft Excel). No calibration was done prior to the experiments and therefore the ratio of 340/380 was plotted against time. The changes in the Ca\(^{2+}\) fluorescence were therefore displayed, rather than quantitative measurements of the Ca\(^{2+}\) levels within the cell. When neurones were located on top of glia, the glial signal was subtracted as background to
yield a more accurate measurement of changes in \([\text{Ca}^{2+}]\) within the neuronal compartment.

Both neurones and glia were classed as having responded when the change in the 340 / 380 nm ratio observed exceeded 110% of control. Only those cell exhibiting changes in \([\text{Ca}^{2+}]\), within 20 sec of the start of agonist exposure were included in the pooled data. In addition, cells exhibiting spontaneous changes in \([\text{Ca}^{2+}]\), before agonist application were discarded.
CHAPTER 3

RESULTS: Electrophysiological experiments

3.1 Introduction.

Part I of this chapter describes the electrophysiological properties of layer V neurones within rat medial frontal cortex. Part II details the results of experiments in which the TBS protocol was used to induce LTP in this region of cortex. Part III describes the results of experiments in which the importance of mGluRs in the induction process was assessed by using the mGluR-specific, broad-spectrum antagonist MCPG, bathed applied prior to TBS. It includes data examining the effect of MCPG on normal synaptic transmission and membrane properties. Part IV shows the results of experiments designed to elucidate the location of the mGluRs and utilising mGluR-specific agonists both without and in the presence of the GABA_B agonist baclofen. Part V details the results of experiments in which a possible mode of action of MCPG was investigated. The ability of MCPG to antagonise the excitatory actions of the group I mGluR-selective agonist S-3HPG was tested, as was its action on an isolated NMDA-receptor mediated EPSP.
Figure 3.2i: Electrophysiological properties of layer V regular spiking (RS) neurone.

A Postsynaptic response of a RS neurone to an intracellually injected depolarising current pulse of +0.2 nA. Upper record, voltage; lower record, current plotted against time. Note that the current trace is drawn to represent the size and timing of the injected current pulses from the Axoclamp. Calibration bar: 20 mV, 40ms.

B Postsynaptic response of a RS neurone to an intracellually injected depolarising current pulse of +0.3 nA. Upper record, voltage; lower record, current plotted against time. Calibration bar: 20 mV, 40ms.

C Postsynaptic response of a RS neurone to an intracellually injected depolarising current pulse of +0.4 nA. Upper record, voltage; lower record, current plotted against time. Calibration bar: 20 mV, 40ms. The horizontal dotted line is drawn to clearly show the existence of an AHP following the train of spikes.

\[ V_m \text{ on exit } -78 \text{ mV.} \]
Part I: Electrophysiological and pharmacological properties of layer V neurones in rat medial frontal cortex.

3.2 Cell characteristics.

Intracellular recordings were obtained from a total of 66 frontal cortical slices from 60 rats. Out of a random population of 53 neurones, 79% of cells were found to be regular spiking (RS) neurones (McCormick et al. 1985; Yang et al., 1996) with resting membrane potentials more negative than -60 mV. All neurones were demonstrated to generate a train of spike in response to the injection of suprathreshold depolarising current pulses. Each spike yielded a well defined afterhyperpolarisation (AHP) thought responsible for the progressively longer interspike interval between subsequent spikes. An example of a RS neurone is illustrated in figure 3.2i. The remaining 19% of neurones were intrinsic bursting (IB) neurones. These exhibit a distinctive Ca\(^{2+}\)-dependent depolarising afterpotential (DAP) and initial spike doublet on injection of a depolarising current pulse. An example of an IB neurone is illustrated in figure 3.2ii. In all cells tested (ca. 40% of sample) paired pulse facilitation was seen at a 50 ms interval.

The electrophysiological characteristics of the layer V neurones are summarised in Table 3.2i.
Figure 3.2ii: Electrophysiological properties of layer V intrinsic bursting (IB) neurone.

A Postsynaptic response of an IB neurone to an intracellularly injected depolarising current pulse of +0.3 nA. Upper record, voltage; lower record, current plotted against time. Note that the current trace is drawn to represent the size and timing of the injected current pulses from the Axoclamp. Calibration bar: 20 mV, 40ms.

B Postsynaptic response of an IB neurone to an intracellularly injected depolarising current pulse of +0.4 nA. Upper record, voltage; lower record, current plotted against time. Calibration bar: 20 mV, 40ms.

C Postsynaptic response of an IB neurone to an intracellularly injected depolarising current pulse of +0.8 nA. Upper record, voltage; lower record, current plotted against time. Calibration bar: 20 mV, 40ms. The horizontal dotted line is drawn to clearly show the lack of an AHP following the train of spikes.

$V_m$ on exit -81 mV.
TABLE 3.2i: Layer V cortical cell characteristics: control value +/- SEM.

<table>
<thead>
<tr>
<th>Cell characteristic:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_m$ on exit (mV)</td>
<td>-76.9 +/- 1.1</td>
</tr>
<tr>
<td>Apparent $R_{in}$ (MΩ)</td>
<td>43.8 +/- 2.0</td>
</tr>
<tr>
<td>Firing threshold (mV)</td>
<td>23.5 +/- 0.5</td>
</tr>
<tr>
<td>Total spike height (mV)</td>
<td>91.6 +/- 1.1</td>
</tr>
<tr>
<td>EPSP slope at 1ms (mV/ms)</td>
<td>1.0 +/- 0.1</td>
</tr>
<tr>
<td>EPSP peak amplitude (mV)</td>
<td>4.8 +/- 0.4</td>
</tr>
</tbody>
</table>

$V_m$: membrane potential
Apparent $R_{in}$: apparent input resistance measured using a -0.1 nA current pulse.
Firing threshold: action potential threshold measured from $V_m$.
Total spike height: action potential measured from $V_m$ to peak.
EPSP slope: slope of evoked response taken from onset to 1ms.
EPSP peak amplitude: peak amplitude of evoked response.

3.3 Membrane properties of layer V cortical neurones.

The I-V relationship of the layer V cortical neurones was evaluated by measuring voltage deflections 40 ms after the onset of postsynaptically injected depolarising and hyperpolarising current pulses. At 40ms, the membrane was shown to be fully charged. An example of the current-voltage relationship of a RS neurone is illustrated in figure 3.3i. It is linear in nature, displaying only slight outward reticification at depolarised membrane potentials. The current-voltage relationship displayed by the IB class of layer V neurone was also found to be linear and an example is illustrated in figure 3.3ii.
Figure 3.3i: Current-voltage relationship of a RS medial frontal cortical neurone.

A  Superimposed postsynaptic responses of a medial frontal cortical neurone bathed in normal ACSF containing 1μM BMI to intracellular current injections. Upper records, voltage; lower records, current (-0.8 to +0.8 nA) plotted against time. Note that the current traces are drawn to represent the size and timing of the injected current pulses from the Axoclamp. Calibration bar: 20 mV and 0.6 nA, 40ms.

B  Graph of voltage (mV) measured 40 ms after onset of current pulse plotted against current (nA) for cell illustrated in A.

Apparent $R_{in}$ 27.5 MΩ (measured @ 40 ms; -0.2 nA current pulse); spike threshold 25.8 mV; spike amplitude 64.8 mV (threshold to peak). $V_m$ on exit - 83 mV.
Figure 3.3ii: Current-voltage relationship of an IB medial frontal cortical neurone.

A Superimposed postsynaptic responses of a medial frontal cortical neurone bathed in normal ACSF containing 1μM BMI to intracellular current injections. Upper records, voltage; lower records, current (-0.8 to +0.8 nA) plotted against time. Note that the current traces are drawn to represent the size and timing of the injected current pulses from the Axoclamp. Calibration bar: 20 mV and 0.6 nA, 40ms.

B Graph of voltage (mV) measured 40 ms after onset of current pulse plotted against current (nA) for cell illustrated in A.

Apparent $R_{in}$ 69.2 MΩ (measured @ 40 ms; -0.2 nA current pulse); spike threshold 23.4 mV; spike amplitude 65.7 mV (threshold to peak). $V_m$ on exit - 81 mV.
3.4 Postsynaptic potentials recorded in layer V cortical neurones.

Postsynaptic potentials (PSPs) were evoked in layer V neurones following the application of test shocks once every 15 or 20 s to the border between layers I and II. As illustrated in figure 3.4i, the size and shape of these PSPs exhibit considerable variation. The inhibitory component of the PSPs was routinely minimalised through the addition of 1µM of the GABA<sub>A</sub> antagonist bicuculline methiodide (BMI) to the ACSF. In a proportion of the cells, the predominantly excitatory nature of the PSP was confirmed by the delivery of a test shock superimposed on intracellularly injected depolarising and hyperpolarizing current pulses. An example of an active pulse plot is illustrated in figure 3.4ii. At more depolarised membrane potentials, the EPSP can be seen to reduce in amplitude until firing threshold is reached and a spike elicited.

The EPSP was shown to be composed of three known and one undefined component, as illustrated in figure 3.4iii. Bath application of 10µM CNQX reduced the EPSP peak amplitude to 51% of control. Application of 500 µM MCPG had no effect on the EPSP peak amplitude. Bath application of 20 µM AP5 reduced the EPSP peak amplitude by a further 16% to 36% of control. Application of 10 µM BMI reduced the EPSP peak amplitude a further 22% to 14% of control. The residual EPSP is possibly mediated by kainate receptors.

Evoked EPSPs were completely abolished by bath application of 200 µM baclofen, as illustrated in figure 3.4iv, A. Measurements of V<sub>m</sub> and R<sub>in</sub> were taken 0 to 4 min before and 8 to 12 min after drug application from a total of 10 slices, obtained from 10 rats. The mean value of V<sub>m</sub> in control was -73.7 +/- 2.5 mV. After bath application of baclofen, the mean value of Vm was -74.9 +/- 2.7 mV. This was not significantly different from control levels. The mean value of R<sub>in</sub> in control was 57.5 +/- 7.9 MΩ. This was reduced in the presence of baclofen to 77% of control to a mean value of 44.3 +/- 6.9 MΩ. This reduction in R<sub>in</sub> was found to be significantly different from control (P = 0.0162, two-tailed paired t-test, n=10).
Figure 3.4i: EPSPs exhibit variability.

Five superimposed, successive EPSPs evoked through stimulation at the border between layers I and II, illustrating the variability in both EPSP slope and amplitude. Calibration bar: 5 mV, 10 ms.
Figure 3.4ii: Postsynaptic response of a medial frontal cortical neurone to electrical stimulation superimposed on intracellular current injection.

Superimposed postsynaptic responses of a medial frontal cortical neurone to afferent stimulation (filled arrow) at 50 ms after the onset of varying levels of intracellularly injected steady current (-0.3 to +0.5 nA). The cell was bathed in normal ACSF containing 1 μM BMI. Upper records, voltage; lower records, current plotted against time. Note that the current traces are drawn to represent the size and timing of the injected current pulses from the Axoclamp. Calibration bar: 20 mV and 11.7 nA, 25 ms.

Apparent $R_{in} = 27.5 \, \text{MΩ}$, spike amplitude 68 mV (threshold to peak). $V_m$ on exit - 83 mV.
A

B

EPSP peak amplitude [% of control]

<table>
<thead>
<tr>
<th>Control</th>
<th>CNXQ</th>
<th>AP5</th>
<th>MCPG</th>
<th>BMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>60</td>
<td>40</td>
<td>40</td>
<td>20</td>
</tr>
</tbody>
</table>
Figure 3.4iii: Postsynaptic response of layer V cortical neurone has CNQX, AP5 and BMI-sensitive components.

A Superimposed postsynaptic responses to a paired-pulse protocol in control ACSF and in the presence of the following drugs: 10 μM CNQX; with additional AP5 at 20 μM; with additional MCPG at 500 μM and with additional BMI at 10 μM. Each traces represents an average of 12 consecutive responses. Calibration bar: 10ms; 5mV.

B Histogram of EPSP peak amplitude as a percentage of control plotted against time in control ACSF and in ACSF containing the drugs described in A.
Figure 3.4iv: Baclofen abolishes EPSPs evoked in layer V medial frontal cortical neurones.

A Postsynaptic responses to a paired-pulse protocol in control ACSF (upper trace) and ACSF containing 200 μM baclofen (lower trace). Each trace represents an average of 12 successive responses to electrical stimulation (indicated by vertical lines) at border between layers I and II. $V_m$ is plotted on the vertical axis and bath application of baclofen to the cell illustrated results in both the abolition of the evoked response and in membrane hyperpolarisation.

Control: Apparent $R_{in}$ 63.5 MΩ; spike threshold 12.9 mV; spike amplitude (threshold to peak) 67.4 mV. Thirty min. after baclofen application: Apparent $R_{in}$ 45 MΩ; spike threshold 19.3 mV; spike amplitude (threshold to peak) 68.8 mV. $V_m$ on exit -65 mV.

B Histogram illustrating pooled data from 10 cells showing $V_m$ measurements, as a % of control, taken 0 to 4 min before and 8 to 12 min after bath application of baclofen. Error bars are +/- SE.

C Histogram illustrating pooled data from the same 10 cells showing $R_{in}$ measurements, as a % of control, taken 0 to 4 min before and 8 to 12 min after bath application of baclofen. Error bars are +/- SE.
Part II: Induction of LTP in the medial frontal cortex.

3.5 Theta burst stimulation (TBS) as an induction paradigm.

The conventional high frequency tetanic LTP induction protocol has been demonstrated to produce mixed effects in the rat medial frontal cortex (Hirsch & Crépel, 1990; Nowicky & Bindman, 1993). Tetanic stimulation at 50-200 Hz in layer I/II elicited LTD in one half of cells, LTP in a third of cells and no effect in the remainder. It is not therefore a reliable method of inducing LTP in this region of cortex. TBS has been shown to be more effective at inducing hippocampal LTP in both *in vitro* (Larson & Lynch, 1986; Larson et al., 1986) and *in vivo* preparations (Staubli & Lynch, 1987). We first investigated whether TBS would prove to be a more effective method of inducing LTP in the prelimbic area of rat medial frontal cortex.

High frequency conditioning trains including theta frequencies were applied to 14 cells from 14 slices, obtained from 13 rats. All slices were bathed in 1 μM BMI, since this has been reported to facilitate the induction of hippocampal LTP (Wigstrom & Gustafsson, 1983). TBS elicited LTP of the initial slope of the EPSP, measured over the first 1ms and presumed to be the monosynaptic component, in 8 out of 14 cells (57%). These LTPs persisted 30 min after the completion of TBS. Four of the 8 LTPs were stable from 30 to 60 min, while the other half showed some decrease in the level of potentiation over this period. The level of potentiation seen following TBS in the 8 cells exhibiting LTP ranged from 129% to 350% of control, the mean potentiated EPSP slope in these 8 cells being 186%. An example of such a monosynaptic LTP elicited by TBS is illustrated in figure 3.5i, A and B. Interestingly, the LTP can be seen to slowly develop, only reaching a maximal potentiated level almost 30 min after the completion of TBS. This is similar to findings in the visual cortex in which tetanic stimulation triggers an initial depression of the response, followed by a gradual recovery to a potentiated level (Bear et al., 1992; Aroniadou & Teyler, 1992). However, this is not a consistent characteristic of LTP in the rat medial frontal cortex. LTD was produced in 1 of the 14 cells, and no persistent effect was seen in the remaining 5 cells. There was LTP of a presumed polysynaptic EPSP.
Figure 3.5i: Monosynaptic LTP is produced by TBS.

A Graph of EPSP slope plotted against time for a single experiment. Each point represents an average of sixteen successive responses over 4 min. Error bars are +/- SE. Vertical dashed lines indicate start and completion of TBS.

Control: Apparent $R_{in}$ 42 MΩ; spike threshold 27.1 mV; spike amplitude (threshold to peak) 73.9 mV. Thirty min. post-TBS: Apparent $R_{in}$ 40 MΩ; spike threshold 28.3 mV; spike amplitude (threshold to peak) 74.8 mV. $V_m$ on exit -72 mV.

B Superimposed averaged EPSPs taken at times shown by $a$ and $b$ in A. Calibration bar: 5mV; 5ms.

C Graph of monosynaptic EPSP slope plotted against time, normalised to the mean of 6 averages in control period. Pooled data for 13 cells, excluding the cell exhibiting LTD, are shown by the closed circles. Data from all 14 cells is shown by the open circles. Vertical dashed lines indicate start and completion of TBS.

D Graph of EPSP slope plotted against time for a single experiment. Each point represents an average of sixteen consecutive responses over 4 min. Error bars are +/- SE. Vertical dashed lines indicate start and completion of TBS.
component (the slope taken at 1 to 2ms and amplitude at 4ms) of two of the five neurones that showed no lasting monosynaptic plasticity. The pooled data from the 13 cells in which TBS produced either LTP or no effect are illustrated in figure 3.5i, C (closed circles). Excluding the cell that exhibited LTD, the mean change in EPSP slope in response to TBS was 150 +/- 19% (SE) at 28-32 min after the completion of TBS. The pooled data from all 14 cells is also shown in figure 3.5i, C (open circles). Even if the single LTD is included, the mean EPSP slope at 28-32 min is 145 +/- 19% (SE) and this is still significantly different from control ($P = 0.039$, two-tailed paired $t$-test, $n=14$). Another example of LTP is illustrated in figure 3.5i, D.

No correlation between the weight of the rat and the consequence of TBS was seen, as illustrated in figure 3.5ii, A. In addition, no correlation was seen between the size of the control EPSP slope taken at 1ms and the result of stimulation using the TBS protocol, as illustrated in figure 3.5ii, B.

**Part III: Involvement of mGluRs in the induction of LTP.**

3.6 Antagonism of mGluR decreases the likelihood of LTP induction.

An increase in Ca$^{2+}$ levels within the postsynaptic compartment has been demonstrated to be critical for the induction of LTP in the hippocampus (Lynch et al., 1983), visual cortex (Tsumoto et al., 1990) and prefrontal cortex (Hirsch & Crépel, 1992). In addition to Ca$^{2+}$ entry through NMDA-receptor mediated channels and through voltage-dependent Ca$^{2+}$ channels, intracellular Ca$^{2+}$ levels can be boosted by group I mGluR-facilitated release of Ca$^{2+}$ from IP$_3$-sensitive internal stores.

The involvement of mGluRs in the induction of LTP in the medial frontal cortex was assessed using the mGluR-specific broadspectrum antagonist MCPG. In the presence of MCPG, TBS produced monosynaptic LTP in only 1 of the 14 cells tested (14 slices from 14 rats). The effect of MCPG on reducing the incidence of LTP was found to be significant ($P < 0.02$, $X^2$ test). The LTP produced in the presence of mGluR antagonism is illustrated in figure 3.6i and reaches a potentiated level 290% of control. This falls within the range seen in ACSF alone. A more typical example of the effect of TBS in presence of MCPG is illustrated in figure 3.6ii, A, B. TBS
Figure 3.5ii: The outcome of TBS is not determined by either the weight of the rat nor the initial EPSP slope in control.

A  Graph of outcome of TBS plotted against weight of rat. Note only 10 of 14 points are displayed since no effect was seen in two rats of 88g and LTP was elicited in two rats of 95 g and 3 rats of 100g.

B  Graph of control EPSP slope (mV/ms) taken at 1ms plotted against cell number. Experiments resulting in LTP are shown by the filled black squares, LTD by the filled black circle and no effect by the filled black triangles.
produces STD that returns to baseline levels 8 to 12 min post-conditioning. Although no effect is seen on the presumed monosynaptic component, the slope at 1-2 ms, a presumed di-synaptic component, does show a slow-onset LTP. This is illustrated in figure 3.6ii, C, and is one of three examples of polysynaptic LTP seen in the presence of MCPG. The effect of TBS in MCPG and in ACSF alone was examined in a single cell, illustrated in figure 3.6iii. In the presence of MCPG, TBS elicited a STP that declines back to baseline levels. After 20 min of washout of MCPG, an identical TBS was delivered and this gave rise to LTP. This protocol was repeated in a further 3 cells. In 1 cell, MCPG blocked the induction of LTP and on washout of the drug an identical TBS elicited LTP of a presumed disynaptic component (Morris et al., 1996). In the remaining 2 cells, no effect of TBS was seen in or after washout of MCPG. TBS elicited STP in 3 out of the 10 cells in which no monosynaptic or polysynaptic LTP was observed. The pooled data from all 14 cells is shown in figure 3.6iv, A (open circles). The mean EPSP slope at 28-32 min. after the completion of TBS was not significantly different from control (n.s.d, two-tailed paired t-test, n=14). The pooled data from 13 cells, excluding the cell that exhibited LTP are shown on the same graph (filled circles). The effect of TBS in MCPG and in ACSF alone are shown on the same graph in figure 3.6iv, B. Repeated measures ANOVA confirms both that the initial EPSP slope in the control period does not differ between the two experimental groups \( P = 0.42; n = 14,14 \) and that, for the period from 4 min to 36 min after TBS ended, the initial EPSP slopes in MCPG were significantly different from those in ACSF alone \( P = 0.011; n = 14,14 \). This indicates that MCPG has a significant action in reducing the potentiation elicited by TBS.

3.7 MCPG has no effect on normal synaptic transmission or on cell membrane properties.

Bath application of MCPG had no effect by itself on the initial slope of the EPSP, as illustrated in figure 3.7, A. MCPG has been reported to have an effect on a NMDA receptor-mediated component (Collingridge et al., 1993). On the basis of this observation, the effect of MCPG on the level of excitation acheived during TBS was
Figure 3.6: TBS induces LTP in a single cell bathed in MCPG.

A  Graph of EPSP slope plotted against time for the single cell in which TBS elicited LTP. Each point represents an average of sixteen successive responses over 4 min. Error bars are +/- SE. Vertical dashed lines indicate start and completion of TBS.

Control: Apparent $R_{in}$ 28.5 MΩ; spike threshold 21.5 mV; spike amplitude (threshold to peak) 79.7 mV. Thirty min. post-TBS: Apparent $R_{in}$ 29 MΩ; spike threshold 22.7 mV; spike amplitude (threshold to peak) 79.5 mV. $V_m$ on exit -76 mV

B  Superimposed averaged EPSPs taken at times shown by $a$ and $b$ in A. The averaged spike in trace $b$ has been truncated. Calibration bar: 5 mV, 5 ms.
Figure 3.6ii: MGluR antagonism reduces incidence of LTP.

A  Graph of EPSP slope plotted against time for a single experiment. Each point represents an average of sixteen successive responses over 4 min. Error bars are +/- SE. Vertical dashed lines: start and finish of TBS.

Control: Apparent $R_{\text{in}}$ 27.8 MΩ; spike threshold 31.6 mV; spike amplitude (threshold to peak) 64 mV. Thirty min. post-TBS: Apparent $R_{\text{in}}$ 25.2 MΩ; spike threshold 30.9 mV; spike amplitude (threshold to peak) 65.9 mV. $V_m$ on exit -81 mV

B  Superimposed averaged EPSPs taken at times shown by $a$ and $b$ in A.

C  Graph of EPSP slope, measured between 1 and 2 ms, vs. time for a single experiment. Each point represents an average of sixteen successive responses over 4 min. Error bars are +/- SE. Vertical dashed lines: start and finish of TBS.
Figure 3.6iii: Effect of TBS in MCPG and in ACSF alone examined in a single cell.

A  Monosynaptic EPSP slope plotted against time for a single cell. Each point represents an average over a 4 min. period and is shown +/- SE. Vertical lines represent the start and completion of TBS. Duration of exposure to MCPG is indicated by the solid horizontal bar. Inserts (above dotted lines) are representative traces showing the depolarisation and spiking during the 5th burst of the first TBS train in MCPG and in ACSF alone. Calibration bar: 10 mV, 10 ms.

B  Superimposed averaged responses at times indicated in A. Top traces: Calibration bar: 5 mV, 10 ms. Bottom two traces are identical to those shown above, merely at a larger scale. Calibration bar: 2 mV, 1 ms. Horizontal black bars (below traces) indicate the times over which values were averaged to obtain EPSP slope.

Five values were averaged around 0 ms and this mean value subtracted from the mean of five values averaged around 1ms.
Figure 3.6iv: MCPG reduces potentiation seen following TBS.

A  Graph of monosynaptic EPSP slope plotted against time, normalised to the mean of 6 averages in control period. Pooled data for 14 cells bathed in MCPG throughout recording period (open circles). Pooled data for 13 cells, excluding cell displaying LTP is also shown (closed circles).

B  Graph of monosynaptic EPSP slope plotted against time, normalised to the mean of 6 averages in control period. Pooled data for 14 cells bathed in ACSF alone, including cell displaying LTD (closed squares); pooled data for 14 cells bathed in MCPG throughout recording period, including cell displaying LTP is also shown (closed circles). Vertical lines represent start and completion of TBS.
examined. The number of spikes elicited by each stimulus burst was taken as an indicator of the level of excitation achieved during TBS in the conditioning trains. There was no significant difference in the number of spikes elicited during TBS in cells bathed in MCPG or in ACSF alone, as illustrated in figure 3.7i, B. Table 3.7 summarises measurements taken of various cell parameters, 0 to 4 min before bath application of MCPG, 8 to 12 min after washin of the drug, and at least 8 to 12 min following washout. None of the cell characteristics measured differ significantly between the two experimental groups.

TABLE 3.7: Layer V cortical cell characteristics do not differ between two experimental groups: mean value +/- SEM.

<table>
<thead>
<tr>
<th>Cell characteristic</th>
<th>0 to 4 min. before washin of MCPG</th>
<th>8 to 12 min. after washin of MCPG</th>
<th>8 to 12 min. after washout of MCPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vm on exit (mV)</td>
<td>-78.2 +/- 1.8 (n = 17)</td>
<td>-77.2 +/- 1.6 (n = 17)</td>
<td>-77.1 +/- 1.9 (n = 9)</td>
</tr>
<tr>
<td>Apparent Rin (MW)</td>
<td>36.4 +/- 2.8 (n = 17)</td>
<td>34.7 +/- 3.2 (n = 17)</td>
<td>35.2 +/- 5.3 (n = 9)</td>
</tr>
<tr>
<td>Firing threshold (mV)</td>
<td>23.7 +/- 0.93 (n = 17)</td>
<td>23.6 +/- 0.97 (n = 17)</td>
<td>25.6 +/- 1.25 (n = 9)</td>
</tr>
<tr>
<td>Total spike height (mV)</td>
<td>89.3 +/- 2.2 (n = 17)</td>
<td>88.6 +/- 2.6 (n = 17)</td>
<td>87.7 +/- 3.8 (n = 9)</td>
</tr>
<tr>
<td>EPSP slope (mV/ms)</td>
<td>0.9 +/- 0.12 (n = 17)</td>
<td>0.89 +/- 0.13 (n = 17)</td>
<td>0.88 +/- 0.13 (n = 2)</td>
</tr>
<tr>
<td>EPSP peak amplitude (mV)</td>
<td>4.8 +/- 0.71 (n = 17)</td>
<td>4.5 +/- 0.69 (n = 17)</td>
<td>5.6 +/- 1.19 (n = 9)</td>
</tr>
<tr>
<td>AHP peak amplitude (mV)</td>
<td>1.7 +/- 0.2 (n = 14)</td>
<td>1.6 +/- 0.2 (n = 14)</td>
<td>1.5 +/- 0.3 (n = 6)</td>
</tr>
</tbody>
</table>

AHP peak amplitude: peak amplitude of the afterhyperpolarisation seen after a train of spikes. Number of cells from which each measurement was taken and averaged is shown in brackets.
Figure 3.7: Lack of effect of MCPG on electrophysiology of test and conditioned responses.

A  Graph of initial EPSP slope plotted against time, normalised to 6 points in the control. Pooled data from 17 cells. Slices were bathed in 500 μM MCPG for time indicated by black horizontal bar. Error bars are +/- SE.

B  Histogram illustrating the number of spikes elicited during TBS in MCPG (black bar; n = 5) and in ACSF alone (white bar; n = 5). Two cells of the 5 cells were analysed in both solutions.
Part IV: Location of group I mGluRs in medial frontal cortex.

3.8 Group I mGluRs mediate excitatory effects on layer V cells.

The activity of group I mGluRs has not previously been examined in prelimbic cortex. Twenty-five slices obtained from 23 rats were used to investigate the effects of the mGluR agonists ACPD, S-3,HPG, and CHPG on the peak afterhyperpolarising potential (AHP) after short trains of spikes (induced by 70-400 ms depolarising current pulses). In the majority of cells, the application of these agonists had a depolarising effect that caused an increase in the number of spikes by a current pulse of fixed strength. Therefore AHPs were measured while steady hyperpolarising current was applied intracellularly to restore the membrane potential to control levels or to elicit the same number of spikes per depolarising current pulse.

In preliminary experiments, ACPD was bath applied at 50 μM to four cells. It consistently produced membrane depolarisation, an increase in the number of spikes per current pulse, and either a reduction in the peak amplitude of the AHP or reversal to an afterdepolarising potential (ADP) as found by Greene et al. (1994) in sensorimotor cortex (Figure 3.8i, A). The $V_m$ differed by only 1 mV at times of the 3 traces, although by 10 min ACPD produced depolarisation from a control level of -78.9 to -74.7 mV (means of 8 successive measurements). Recovery was to -77.3 mV after 8 min of washout. The observed effects are excitatory and therefore likely to be mediated by group I mGluRs. We tested this hypothesis by the bath application of 200 μM S-3HPG to three cells. It produced a reduction in the amplitude of the AHP in all the cells and depolarisation of the membrane potential in two of the three cells (Figure 3.8i, B-D). The pooled data from all 3 cells indicates that the mean reduction in AHP amplitude on agonist application was to 45.5 +/- 13% (SE) of control. This does not reach levels of significance. Little recovery was seen on washout of the agonist. The increase in mean input resistance (from 52.7 +/- 9.4 MΩ to 53.4 +/- 10.6 MΩ) was not significant. The group I mGluR specific agonist DHPG was also shown to cause membrane depolarisation, increased spike firing on a current pulse of fixed strength and a reduction in the amplitude of the AHP. These findings are described in greater
**Figure 3.8:** MGluR agonists reduce peak amplitude of AHP in slices of medial frontal cortex.

A  Superimposed responses to intracellular depolarising current pulses in control (*black trace*), 50 μM ACPD (*thick black trace*) and after washout of the drug (*grey trace*). Calibration bars: 5 mV, 10 ms.

B  Histogram of AHP amplitude as a % of control mean at 0-4 min before and 8-12 min after bath application of S-3HPG, and at least 8-12 min after washout of drug. Vertical bars, +/− SE.

C  Superimposed responses to longer duration current pulses showing reduction in AHP amplitude in S-3HPG, although 10 spikes were elicited in presence of drug (cf. 9 spikes in control and washout). Calibration bars: 5 mV, 10 ms.

D  Graphs of (i) AHP amplitude (mV), (ii) V_m (mV) and (iii) spike height (mV) plotted against time for cell illustrated in C. In (i) each point represents an average of 8 consecutive measurements. In (ii) individual measures of V_m are displayed. In (iii) each point represents an average of 16 consecutive measurements. Black horizontal bar on top graph indicates duration of exposure to S-3HPG.
depth in relation to the effect of DHPG on the induction of LTP (see chapter 5). To establish whether these excitatory effects were mediated by the mGluR₁ or mGluR₅ subtype, we bath applied 400 μM CHPG to two cells. It produced a reduction in the AHP and membrane depolarisation in both cells (not illustrated). These preliminary experiments indicate that group I mGluRs mediate excitatory effects in slices of rat medial frontal cortex, however the existence of these receptors on the layer V cells still remained to be established.

To determine whether or not the effects of the group I agonists were mediated by postsynaptic receptors on the layer V cells from which our recordings were made, 200 μM baclofen was used to block the evoked release of transmitter and hence evoked EPSPs. In the presence of baclofen, 17 cells were exposed to either ACPD, S-3,HPG or CHPG. The AHP was then measured whilst using steady hyperpolarising current to ensure that the same number of spikes were elicited by a depolarising current pulse of fixed strength both with and without the mGluR agonist. Bath application of 50 μM ACPD to five cells in the presence of baclofen, significantly reduced the AHP to 56.6 +/- 11.3% (SE) of control (P=0.018, two-tailed paired t-test, n=5). Washout produced partial recovery within 20 min (Figure 3.8ii, A-C). Bath application of 200 μM S,3HPG to five cells in the presence of baclofen significantly reduced the AHP to 63.0 +/- 7.3% (SE) of control (P=0.007, two-tailed paired t-test, n=5). Once again, washout produced partial recovery within 20 min (Figure 3.8iii, A-C). Bath application of 400 mM CHPG to seven cells in the presence of baclofen, significantly reduced the AHP to 87.0 +/- 5.1% (SE) of control (P=0.0158, two-tailed paired t-test, n=7). Washout produce partial recovery, however, this did not reach levels of significance (Figure 3.8iv, A-C). In all three cases, no consistent change in input resistance was seen.
Figure 3.8ii: ACPD reduces peak amplitude of late AHP in slices of medial frontal cortex in which synaptic transmission was blocked with baclofen.

A  Superimposed responses to intracellular depolarising current pulses in control (black trace), 50 μM ACPD (thick black trace) and after washout of the drug (grey trace). Membrane potential held at constant level before current pulse by steady current injection. Calibration bars: 5 mV, 10 ms.

B  Graphs of (i) AHP amplitude (mV), (ii) $V_m$ (mV) and (iii) spike height (mV) plotted against time for cell illustrated in A. In (i) each point represents an average of 8 consecutive measurements. In (ii) and (iii) individual measures of $V_m$ are spike height respectively are displayed. Horizontal bar on top graph indicates duration of exposure to ACPD.

C  Histogram of AHP amplitude as a % of control mean at 0-4 min before and 8-12 min after bath application of ACPD, and at least 8-12 min after washout of drug. Vertical bars, +/- SE.
Figure 3.8iii: S-3HPG reduces peak amplitude of late AHP in slices of medial frontal cortex in which synaptic transmission was blocked with baclofen.

A Superimposed responses to intracellular depolarising current pulses in control (black trace), 200 μM S-3HPG (thick black trace) and after washout of the drug (grey trace). Membrane potential held at constant level before current pulse by steady current injection. Calibration bars: 5 mV, 10 ms.

B Graphs of (i) AHP amplitude (mV), (ii) $V_m$ (mV) and (iii) spike height (mV) plotted against time for cell illustrated in A. In (i) each point represents an average of 8 consecutive measurements. In (ii) individual measures of $V_m$ are displayed. In (iii) each point represents an average of 16 consecutive measurements. Horizontal bar on top graph indicates duration of exposure to S-3HPG.

C Histogram of AHP amplitude as a % of control mean at 0-4 min before and 8-12 min after bath application of S-3HPG, and at least 8-12 min after washout of drug. Vertical bars, +/- SE.
Figure 3.8iv: CHPG reduces peak amplitude of late AHP in slices of medial frontal cortex in which synaptic transmission was blocked with baclofen.

A  Superimposed responses to intracellular depolarising current pulses in control (black trace), 400 μM CHPG (thick black trace) and after washout of the drug (grey trace). Membrane potential held at constant level before current pulse by steady current injection. Note traces contaminated by 50Hz noise, but effect on AHP amplitude is clear. Calibration bars: 5 mV, 10 ms.

B  Graphs of (i) AHP amplitude (mV), (ii) $V_m$ (mV) and (iii) spike height (mV) plotted against time for cell illustrated in A. In (i) each point represents an average of 8 consecutive measurements. In (ii) individual measures of $V_m$ are displayed. In (iii) each point represents an average of 16 consecutive measurements. Horizontal bar on top graph indicates duration of exposure to CHPG.

C  Histogram of AHP amplitude as a % of control mean at 0-4 min before and 8-12 min after bath application of CHPG, and at least 8-12 min after washout of drug. Vertical bars, +/- SE.
Part V: Mode of action of MCPG.

3.9 MCPG does not consistently antagonise excitatory effects of S-3HPG.

200 μM S-3HPG was bath applied to a total of 3 slices, obtained from 3 rats, both in control ACSF and in ACSF containing 500 μM MCPG. In all 3 cells S-3HPG produced membrane depolarisation and an increase in the number of spikes elicited by a current pulse of fixed strength. The AHP amplitude was measured whilst using steady intracellularly injected current to ensure that the same number of spikes were elicited by a depolarising current pulse of fixed strength both in the presence of and without S-3HPG. In 2 of the 3 cells, S-3HPG had a less pronounced effect on the amplitude of the AHP in the presence of MCPG. One of these cells is illustrated in figure 3.9i, A and B. On the initial application of S-3HPG, the AHP amplitude was reduced to 44% of control. After 8 to 12 min of washout of the drug, the AHP amplitude recovered to 65% of control levels. On a second application of S-3HPG, in the presence of MCPG, the AHP amplitude is reduced to 49% of control. If values for this second agonist application are normalised not to the first, but the second control period, this can be seen to represent a reduction to 56% of control. After 8 to 12 min of washout of the drug, the AHP amplitude has recovered to 64% of control. In the remaining 1 cell, the agonist-induced reduction in AHP amplitude was more pronounced in the presence of MCPG than in control ACSF. When the data from all 3 cells is pooled, the mean agonist-induced reduction in AHP amplitude in control ACSF to 61% of control does not reach levels of significance. However, experiments detailed in section 3.8 have shown that S-3HPG is capable of significantly reducing the amplitude of the late AHP. In the presence of MCPG, the mean agonist-induced reduction in AHP amplitude is to 67.2% of control and once again this does not reach levels of significance. These preliminary experiments suggest that MCPG is capable of attenuating the effect of S-3HPG on the AHP amplitude.

There is evidence for Group I mGluR receptor desensitization (Gereau & Heinemann, 1998) and therefore it is possible that the above observations are the direct consequence of decreased receptor sensitivity on the second agonist application. This is further supported by the observation, in a single cell, that the initial reduction
Figure 3.9i: MCPG attenuates excitatory effects of S-3HPG on membrane conductance and cellular excitability.

A (i) Superimposed responses to intracellular depolarising current pulses in control (black trace), 200 μM S-3HPG (thick black trace) and after washout of the drug (grey trace). Membrane potential held at constant level before current pulse by steady current injection. Spikes on current pulse are truncated. Note that the traces are contaminated with 50 Hz noise, however this does not affect validity of AHP amplitude measurement. Calibration bars: 2 mV, 20 ms. (ii) Superimposed responses to intracellular depolarising current pulses in conditions described for (i) with exception that slices were bathed in 500 μM MCPG for duration of experiment.

In control ACSF: Control: Apparent $R_{in}^\circ$ 51.7 MΩ; total spike amplitude 94.4 mV. 10 min after application of drug: Apparent $R_{in}^\circ$ 55 MΩ; total spike amplitude 96.1 mV. 10 min after washout of drug: Apparent $R_{in}^\circ$ 52.7 MΩ; total spike amplitude 96.3 mV.

In 500 μM MCPG: Control: Apparent $R_{in}^\circ$ 52.3 MΩ; total spike amplitude 95.7 mV. 10 min after application of drug: Apparent $R_{in}^\circ$ 51.2 MΩ; total spike amplitude 95.3 mV. 10 min after washout of drug: Apparent $R_{in}^\circ$ 54.7 MΩ; total spike amplitude 95.9 mV. $V_m$ on exit -78 mV.

B Graphs of (i) AHP amplitude (mV) and (ii) spike height (mV) plotted against time for cell illustrated in A. In both graphs, each point represents an average of 8 consecutive responses. Horizontal bars on top graph indicate duration of exposure to S-3HPG. Vertical dashed lines indicate period over which slices were bathed in MCPG.

C Histogram of AHP amplitude as a % of control mean at 0-4 min before and 8-12 min after bath application of S-3HPG, and at least 8-12 min after washout of drug for slices bathed in control ACSF (first 3 bars) and in ACSF containing MCPG (last 3 bars). Duration of exposure to MCPG is indicated by horizontal black bar. Values are shown +/- SE.
in AHP amplitude on S-3HPG application is much larger than in subsequent applications (figure 3.9ii). On the initial bath application of S-3HPG, the AHP amplitude is reduced to 49% of control. After at least 8 to 12 min of washout of the drug, the AHP amplitude has recovered to 89% of control. In the presence of MCPG, the second application of S-3HPG elicits a reduction in AHP amplitude to 61% of control. After at least 8 to 12 min of washout of the drug, the AHP amplitude has recovered to 89% of control. After 20 min of washout of MCPG, the third and final application of S-3HPG reduces the AHP amplitude to 65% of control. After at least 8 to 12 min of washout of the drug, the AHP amplitude recovers to 82% of control. This is mirrored by the effect of S-3HPG on the peak amplitude of the EPSP, measured at 17.5 ms after the onset of the response, as illustrated in figure 3.9ii, B. Initial application of S-3HPG triggers a pronounced increase in the peak amplitude of the EPSP. The second application, in the presence of MCPG, produces a smaller increase in peak EPSP amplitude, however, the third application, after at least 20 min of washout of MCPG, produces no effect at all. These experimental observations suggest that, although we observe an attenuation of the S-3HPG-induced reduction in AHP amplitude in the presence of MCPG, this could well be the consequence of receptor desensitization rather than direct antagonism of the group I mGluR-mediated excitatory effects by MCPG.

We tested this hypothesis using a modified experimental protocol. In a total of 4 cells, obtained from 3 rats, the response to S-3HPG was first tested in the presence of MCPG and then, following at least 20 min washout, a second challenge given. In 3 out of 4 cells the application of S-3HPG in the presence of MCPG produced membrane depolarisation and an increased cellular excitability comparable to that seen in control ACSF. Once again the AHP amplitude was measured whilst using steady intracellularly injected current to ensure that the same number of spikes were elicited by a depolarising current pulse of fixed strength both with and without S-3HPG. In only a single cell was the reduction in the AHP amplitude on application of S-3HPG comparable to that seen in agonist alone. The remaining 3 cells exhibited only a small reduction in the AHP amplitude. An example is illustrated in figure 3.9iii. The
Figure 3.9: Response to S-3HPG declines on repeated exposure even under normal conditions.

A  Graph of AHP amplitude (mV) plotted against time for a single experiment. Vertical dashed lines indicate duration of exposure to 500 μM MCPG. Black horizontal bars indicate period over which S-3HPG was present in the bathing medium.

In control ACSF: Control: Apparent $R_{in}$: 38.5 MΩ; total spike amplitude 90 mV. 10 min after application of drug: Apparent $R_{in}$: 38.8 MΩ; total spike amplitude 90 mV. 10 min after washout of drug: Apparent $R_{in}$: 38.9 MΩ; total spike amplitude 89.6 mV.

In 500 μM MCPG: Control: Apparent $R_{in}$: 38.2 MΩ; total spike amplitude 90.9 mV. 10 min after application of drug: Apparent $R_{in}$: 38.4 MΩ; total spike amplitude 90.5 mV. 10 min after washout of drug: Apparent $R_{in}$: 38.4 MΩ; total spike amplitude 90.4 mV.

Second application in control ACSF: Control: Apparent $R_{in}$: 37.8 MΩ; total spike amplitude 91.9 mV. 10 min after application of drug: Apparent $R_{in}$: 38.1 MΩ; total spike amplitude: 90 mV. 10 min after washout of drug: Apparent $R_{in}$: 37.7 MΩ; total spike amplitude 92.1 mV. $V_{in}$ on exit -68 mV.

B  Graph of peak EPSP amplitude, measured at 17.5 ms after onset of the response and plotted against time. Vertical dashed lines indicate duration of exposure to MCPG. Black horizontal bars indicate period over which S-3HPG was present in the bathing medium.
reduction in AHP amplitude in response to S-3HPG was much more pronounced in the absence of MCPG antagonism in the bathing medium, despite the fact that it was the second of two agonist applications. When the data from all 4 cells is pooled, the mean agonist-induced reduction in AHP amplitude in ACSF containing MCPG was to 75 +/- 10% (SE) of control. This does not reach levels of significance and can be compared to a reduction to 45.5 +/- 13% (SE) of control seen in S-3HPG alone. Although clearly in individual experiments the effect of S-3HPG is more dramatic in control ACSF than in the presence of MCPG, when the data are pooled there is no significant difference between the reduction in AHP amplitude observed in the two experimental groups. On washout of S-3HPG in the presence of MCPG there is complete reversal of the observed excitatory effects. As described already, in S-3HPG alone there is almost no recovery on washout of the agonist and therefore reversibility is another factor that differs between those cells exposed to S-3HPG under control conditions and those exposed in the presence of mGluR antagonism. The recovery of AHP amplitude following washout of the agonist is significantly different under the two experimental conditions (P<0.01, two-tailed unpaired t-test assuming unequal variance, n=3,4).

In addition to changing the conductances underlying the AHP, S-3HPG was also seen to have an effect on the peak amplitude of the EPSP, as illustrated in figure 3.9iv. Application of S-3HPG was seen to reversibly increase the peak amplitude of the EPSP, this effect being more pronounced on the second EPSP evoked by a paired-pulse protocol. Agonist-induced membrane depolarisation was counterbalanced by the application of intracellularly injected hyperpolarising current, however changes in the EPSP were thought not to be wholly independent of changes in membrane potential.

3.10 Effect of MCPG on an isolated NMDA receptor-mediated EPSP.

In neocortical neurones, mGluR agonists have been demonstrated to selectively facilitate NMDA receptor currents (Rahman & Neuman, 1996). We therefore isolated the NMDA receptor-mediated component of the EPSP by bath application of 20 μM CNQX and 10 μM BMI in low Mg²⁺ (1mM) ACSF. The stimulus strengths used
Figure 3.9iii: MCPG attenuates response to S-3HPG.

A  (i) Superimposed responses to intracellularly injected depolarising current pulses in control ACSF containing 500 μM MCPG (black trace), 10 min after wash in of S-3HPG (thick black trace) and after 10 min of washout of both drugs (grey trace). (ii) Superimposed responses to intracellularly injected depolarising current pulses in control ACSF (black trace), 10 min after wash in of S-3HPG (thick black trace) and after 10 min of washout of the drug (grey trace). Number of spikes elicited by current pulse held at constant level by steady current injection. Spikes have been truncated. Calibration bars: 5 mV, 20 ms.

B  (i) Graph of AHP amplitude (mV) plotted against time for a single experiment. Vertical dashed lines indicate duration of exposure to MCPG. Black horizontal bars indicate period over which S-3HPG was present in the bathing medium. (ii) Graph of spike height (mV) plotted against time for a single experiment. Markers as above.

In 500 mM MCPG: Control: Apparent $R_{in}$ 52.3 MΩ; total spike amplitude 76.5 mV. 10 min after application of drug: Apparent $R_{in}$ 51.8 MΩ; total spike amplitude 77.5 mV. 10 min after washout of the drug: Apparent $R_{in}$ 52.5 MΩ; total spike amplitude 76.4 mV.

In control ACSF: Control: Apparent $R_{in}$ 52.3 MΩ; total spike amplitude 76.6 mV. 10 min after application of drug: Apparent $R_{in}$ 52.7 MΩ; total spike amplitude 75.4 mV. 10 min afterwashout of drug: Apparent $R_{in}$ 51.9 MΩ; total spike amplitude 76.1 mV. $V_{m}$ on exit -64 mV.
Figure 3.9iv: S-3HPG potentiates EPSP peak amplitude.

A Graph of peak amplitude (mV) of first EPSP plotted against time for a single experiment. Amplitude measurements taken at 7.2 ms after the onset of the response. Black horizontal bar indicates periods during which S-3HPG was present in the bathing medium.

B Graph of peak amplitude (mV) of second EPSP plotted against time for a single experiment. Amplitude measurements taken at 10 ms after the onset of the response. Black horizontal bar indicates periods during which S-3HPG was present in the bathing medium.

C Superimposed responses to paired pulse stimulation protocol in control (black trace), in 200 mM S-3HPG (thick black trace) and after washout of the drug (grey trace). Calibration bar: 1 mV, 10 ms.

In control ACSF: Control: Apparent $R_{in}$ 45.2 MΩ; total spike amplitude 84.8 mV. 10 min after application of drug: Apparent $R_{in}$ 45.3 MΩ; total spike amplitude 84.6 mV. 10 min after washout of drug: Apparent $R_{in}$ 45.1 MΩ; total spike amplitude 86.4 mV. $V_m$ on exit -81 mV.
were considerably larger than those used under normal experimental conditions, in an attempt to evoke a response of sufficient amplitude. Preliminary experiments investigating the effect of S-3HPG on this isolated NMDA-receptor mediated response proved inconclusive. In addition to any effects on the evoked response, bath application of S-3HPG elicited membrane depolarisation. Although an attempt was made to combat these changes in membrane potential by intracellular current injection, there was concern, however, that current injected at the soma would not efficiently clamp the membrane potential in the dendrites. It was therefore decided to test the effect of MCPG on the isolated NMDA receptor-mediated EPSP. We have already shown a lack of effect of MCPG on the peak amplitude of the mixed AMPA and NMDA receptor mediated EPSP, however MCPG has been demonstrated to modulate NMDA receptor-mediated currents in the hippocampus (Collingridge et al, 1993). Slices were bathed in low Mg²⁺ (1mM) ACSF containing 10 μM CNQX and 10 μM BMI. Once again, stronger stimulation strengths were used to evoke a response of sufficient amplitude. MCPG reduced the peak amplitude of the evoked response and an example of such an effect is illustrated in figure 3.10, A and B. Bath application of 500 μM MCPG reduced the NMDA receptor-mediated EPSP to 59% of control. After 20 min of washout of the drug, the EPSP peak amplitude recovered to 80% of control. MCPG was bath applied to a total of five slices, obtained from five rats and the pooled data is illustrated in figure v, C. MCPG reduced the peak amplitude to a mean value of 77% +/- 5% of control (P=0.005, two-tailed paired t-test, n=5). Following 8 - 12 min of washout, the EPSP peak amplitude was seen to recover to 90% +/- 3% of control, however this was not significant.
Figure 3.10: MCPG reduces peak amplitude of isolated NMDA receptor mediated EPSP.

A Graph of peak amplitude (mV) of first EPSP plotted against time for a single experiment. Amplitude measurements taken at 4 ms after the onset of the response. Black horizontal bar indicates period during which 500 μM MCPG was present in the bathing medium. Slice was perfused with 10 μM CNQX, 10 μM BMI and 1mM Mg\(^{2+}\) ACSF at time point A. 40 μM AP5 was bath applied at time point B. The slice was returned to control ACSF at time point C.

Control: Apparent R\(_{in}\) 55.5 MΩ; total spike amplitude 97.4 mV. 20 min after application of drug: Apparent R\(_{in}\) 52.8 MΩ; total spike amplitude 96.8 mV. 20 min after washout of drug: Apparent R\(_{in}\) 55.1 MΩ; total spike amplitude 96.7 mV. V\(_m\) on exit -73 mV.

B Superimposed postsynaptic responses taken at time shown by i, ii and iii in A. Each trace is an average of 12 consecutive responses. Calibration bar: 1 mV, 1 ms.

C Graph of peak EPSP amplitude (mV) plotted against time showing pooled data from fivecells, normalised to the mean of 3 average in control period. Slices were bathed in 10 μM CNQX, 10 μM BMI and 1mM Mg\(^{2+}\) ACSF for period indicated by black horizontal bar. Vertical dashed lines indicate duration of exposure to 500 μM MCPG.
CHAPTER 4

RESULTS: Effect of group I mGluR activation by DHPG on the induction of LTP.

4.1 Introduction.

In order to investigate the effect of the group I mGluR agonist DHPG on the induction of LTP in slices containing the prelimbic (PL) region of prefrontal cortex (PFC) we utilised field potential recordings. Part I of this chapter therefore describes and interprets field potentials recorded in this region of cortex. Part II describes experiments in which the TBS protocol was used to find a paradigm that was subthreshold for reliable induction of LTP of the prelimbic field response within slices of rat medial frontal cortex. Part III details experiments investigating the effect of the group I mGluR specific agonist DHPG on both the cortical field potentials and on the induction of LTP. Intracellular studies were also used to aid the interpretation of the field potentials and the action of DHPG on the field responses.

Stimulation at the border between layers I and II in PL cortex can activate numerous axons and circuits within the cortex. The existence of cortico-cortical connections contributes a substantial polysynaptic component to the field potential response. The organisation of the PL region is very different from other cortical regions, such as the hippocampus and visual cortex, in which extensive analysis of field potentials have already been conducted.

The work in the chapter was a continuation of preliminary experiments by S. Knevett and E. Lerner in our laboratory.
Part I: Interpretation of field potential recordings in prelimbic area of rat medial frontal cortex.

4.2 Typical field potentials recorded in rat medial frontal cortex.

Field potentials were evoked by test shocks applied to the border between layers I and II and routinely monitored at a depth of between 50 and 60% of the total distance between the pial surface and the white matter. Such a depth is comparable with the location of the recording electrode in the intracellular recordings described in chapter 3. In the majority of experiments the relationship between input stimulation strength and output peak-to-peak amplitude (I-O curve) was first determined. An example is illustrated in figure 4.2i. As the stimulus strength was increased, a distinct waveform appeared that comprised three overlapping components (described respectively in i, ii and iii):

(i) In most preparations there was an initial negative wave that could not be abolished by the AMPA receptor antagonist CNQX and which is therefore non-synaptic. We termed this the axonal volley. In figure 4.2i, the axonal volley is labelled A. The size of the axonal volley showed considerable variation between preparations. An example in which the axonal volley is very prominent is illustrated in figure 4.2ii, A. The waveform clearly starts with a large negative wave, which is not abolished by bath application of 20 μM CNQX. It does not, however, interfere with those aspects of the waveform that were monitored during the course of the experiment. An example of a field response in which the axonal volley was very small is illustrated in figure 4.2ii, B. In addition, the axonal volley becomes less pronounced in deeper layers of cortex. This could be the consequence of there being a smaller number of activated axons in deeper cortical layers, presumably due to the
A

![Graph showing stimulus strength vs. peak to peak amplitude](#)

**Stimulus strength (mV)**

**Peak to peak ampl. (mV)**

**Bi**

![Graphs labeled Bi, ii, and iii with various peaks labeled A, B, C, D](#)

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Figure 4.2i: Relationship between input stimulation strength and output peak-to-peak amplitude of population spike examined in a single slice.

A Graph of peak-to-peak amplitude of population spike (mV) plotted against stimulus strength (mV) for a single experiment. Each point represents an average of 8 consecutive responses.

B (i) Response to a stimulus strength of 1.7 mV to the border between layers I and II. Recording electrode placed 50% of distance between pial surface and white matter. Trace represents an average of 8 consecutive responses. Artefact has been truncated. Axonal volley labelled A. Onset of positive wave labelled B. Peak positivity labelled C. Peak negativity labelled D.

(ii) Response to a stimulus strength of 2.5 mV to the border between layers I and II. Recording electrode placed 50% of distance between pial surface and white matter. Trace represents an average of 8 consecutive responses. Artefact has been truncated. Labels as in A.

(iii) Response to a stimulus strength of 4.0 mV to the border between layers I and II. Recording electrode placed 50% of distance between pial surface and white matter. Trace represents an average of 8 consecutive responses. Artefact has been truncated. Labels as in A. Calibration bar: 0.2 mV, 2ms.
fact that only a subset of the population of axonal fibres span the entire depth of cortex in the slice. Alternatively, it could reflect the differences in conduction velocities within the population of axons, a factor that appears more pronounced as the distance between the site of stimulation and the recording electrode increases.

(ii) The axonal volley is followed by a positive wave, the onset of which can be partially obscured by the preceding axonal volley, as revealed by bath application of CNQX (see figure 4.2ii, A for example). The onset of the positive wave is labelled B and the peak positivity as C.

(iii) At larger stimulus intensities a prominent negative wave cuts into the positive wave. We termed this the population spike. By analogy with field potential recordings in the hippocampus, the population spike reflects the summed synchronous discharge of a population of neurones. It is negative since the action potentials that underlie it occur in the cell bodies.

The waveforms are more defined following the second of two stimuli given at an interval of 50 ms (figure 4.2iii, B). The apparent paired-pulse facilitation (PPF) is seen in all slices tested. Comparison of the field response elicited by the first and the second stimuli reveals an enhancement of the axonal volley following the second stimulus. This observation suggests that the second stimulus activates a larger number of afferent fibres and that, therefore, the observed PPF is actually a reflection of activation of a larger population of cells.

To aid the interpretation of PL field responses, field potentials were recorded at different depths of cortex measured from the medial surface and expressed as a % of the total depth of the grey matter, namely 470 μm (33%), 620 μm (43%), 820 μm (57%) and 1020 μm (71%). By analogy with the hippocampus, stimulation at the border between layers I and II should result in extracellular current flow from the cell
Figure 4.2ii: Experiments showing CNQX-sensitive components of fEPSPs and variations in fEPSP between slices of medial frontal cortex.

A  Superimposed traces showing field responses to test shocks applied once every 15s to border between layers I and II in control ACSF (thick black trace) and in 20 μM CNQX (grey trace). Recording electrode placed 50% of the distance between the pial surface and the white matter. Stimulus strength set at level that elicits a response 50% of the maximal response. Each trace is an average of 8 consecutive responses. The axonal volley is indicated by A. The onset of the positive wave is indicated by B. The peak positivity is indicated by C. The peak negativity is indicated by D. Note that the synaptic components are preceded by a large axonal volley not abolished in presence of CNQX. Calibration bar: 0.1 mV, 2 ms.

B  Superimposed traces showing field responses to test shocks applied once every 15s to border between layers III in control ACSF (thick black trace) and in 20 μM CNQX (grey trace). Recording electrode placed 50% of the distance between the pial surface and the white matter. Stimulus strength set at level that elicits a response 60% of the maximal response. Each trace is an average of 8 consecutive responses. Labels as above. Note that the response in CNQX is very noisy, however it is clear that the axonal volley preceding the synaptic components is negligible. Calibration bar: 0.2 mV, 2 ms.
body towards the dendrites, resulting in a negative potential at the synaptic region and a positive potential at the cell body layer. The field potentials recorded at different depths of cortex correspond with current knowledge of the fine anatomy of the medial frontal cortex. In a superficial layer of cortex, at approximately 33% of the total distance from the pial surface to the white matter, the field response comprised a small arrival volley followed by a large initial negative wave. This depth corresponds to the current sink and hence is likely to be the site of the synaptic connections on the apical dendrites of large pyramidal cells. At a depth approximately 43% of the distance between the pial surface and white matter, the response had a small initial positive component followed by the negative wave. The negative wave had a longer onset latency, which is likely to be the result of the placement of the recording electrode midway between current source and current sink. Alternatively, it could be the consequence of the afferent fibres to this depth of cortex exhibiting slower conduction velocities. If this were the case, however, a change in the onset latency of the axonal volley should also be recorded. The latency of the axonal volley remains constant. At a depth approximately 57% of the distance between pial surface and white matter, the response reversed to comprise an initial positive wave followed by a negative wave. This can be seen to be a mirror image of the waveform seen at a depth of 33% of the grey matter, the onset of the positive wave, labelled (ii), matching that of the negative wave seen in more superficial layers of cortex. The peak positivity, labelled (iii), is followed by a negative wave. The peak negativity is labelled (iv). A depth of 57% of the distance between pial surface and white matter corresponds both to the current source and also to the depth at which intracellular recordings described in chapter 3 were routinely made, presumably from the somata of layer V pyramidal cells. At a depth approximately 71% of the distance between the pial surface and the
Figure 4.2iii: Profile of medial frontal cortical field recordings.

A Superimposed traces showing field response to test shocks applied every 15 s to the border between layers I and II. Each trace is an average of 8 consecutive responses. Traces are shown for four depths of cortex. Depths are expressed both as an absolute depth measurement and as a percentage of the distance between the pial surface and the white matter. Trace labelled A: 470 μm (33%). Trace labelled B: 620 μm (43%). Trace labelled C: 820 μm (57%). Trace labelled D: 1020 μm (71%). The axonal volley is labelled (i). The onset of the positive wave is labelled (ii). The peak positivity is labelled (iii). The peak negativity is labelled (iv). The stimulus artefact has been truncated. It can be seen in isolation in the bottom trace, labelled E, and is largely finished by the onset of the synaptic response. Calibration bar: 0.5mV, 2ms.

B Superimposed traces representing the averaged response to the second stimulus of a paired-pulse protocol. The stimulus artefact has been truncated and can be seen in isolation in the bottom trace. Labels as in A. Calibration bar: 0.5mV, 2ms.
white matter, the response still comprised an initial positivity, however this was seen to have a longer onset latency reflecting either an increased distance between the stimulating electrode and the soma or possibly a second synaptic connection.

4.3 Comparison of the intracellular and extracellular responses.

In order to interpret the recorded field potentials, we compared intracellular and extracellular responses in a total of 5 slices obtained from 5 rats. We measured latencies of the onset of a number of different components including the EPSPs and spike firing and compared these with the field response recorded immediately outside the cell. The pooled data show that the field EPSPs have a latency comparable to that of intracellularly recorded EPSPs, whereas the population spikes have a latency comparable to that of intracellularly recorded action potentials. An example is illustrated in figure 4.3i. The onset of the fEPSP can be seen to be closely matched with the onset of the intracellular EPSP. However, the fEPSP onset is partially obscured by the preceding axonal volley. The population spike has a latency comparable to that of the intracellularly recorded action potential. However, in cells where the membrane potential is close to threshold for spike firing, an action potential can be elicited at an earlier time point that usually corresponds to the layer V positive wave of the fEPSP. The pooled data from five experiments is shown in figure 4.3ii and it shows a strong overlap between the latencies of the onset of the intracellularly recorded EPSPs and the onset of the positive wave of the recorded fEPSPs. In addition, the latencies of the action potentials corresponds to the onset of the population spikes.
Figure 4.3i: Comparison of intracellular response with fEPSP recorded on stepping 10 μm outside cell.

i Intracellular recorded postsynaptic response to test shocks applied once every 15s to border between layers I and II. The trace represents a single response. Dashed line labelled A indicates the onset of the stimulus artefact. Dashed line labelled B indicates onset of the EPSP. Dashed line labelled C indicates onset of action potential. Calibration bar: 20 mV, 1ms.

ii Same response as that illustrated in (i) but recorded on stepping 10 μm out of cell. Note that the headstage used was not appropriate for extracellular recording and therefore the stimulus artefact is very long lasting. Dashed line labelled A indicates the onset of the stimulus artefact. Dashed line labelled B indicates peak of axonal volley. Dashed line labelled C indicates the fEPSP component that reflects spiking in a small population of cells. The stimulus artefact has been truncated. Calibration bar: 100 μV, 1ms
A

- Onset of positive wave of fEPSP recorded in layer V
- EPSP onset

Bi

- IC spike latency

i

- Pop. spike latency

Latency (ms)
Figure 4.3ii: Comparison of latencies of extracellular and intracellular responses recorded in layer V.

A  Graph displaying distribution of latencies of the onset of intracellularly recorded EPSPs (thick black line) and the onset of the deep positive wave of extracellularly recorded fEPSPs (thin black line). Data from five cells / slices.

B  (i) Graph displaying distribution of latencies of the onset of intracellularly recorded action potentials (thick black line) and (ii) the onset of extracellularly recorded population spikes (thin black line). Data from 5 cells / slices.
Part II: Induction of LTP in the medial frontal cortex.

4.4 Theta burst stimulation (TBS) as an induction paradigm.

TBS was applied to a total of 9 slices, obtained from 9 rats. In exploratory experiments, several different parameters of the field response were measured in an attempt to isolate those parameters that most reliably reflected changes induced by TBS.

By analogy with hippocampal field potential recordings, the slope of the initial positive wave should be measured to detect changes in the field response. However, in the majority of experiments, the onset of the positive wave was obscured by the axonal volley. It was the population spike that changed most markedly and therefore the parameters of the evoked field response that were selected for analysis were (i) the early slope of the population spike reflecting its onset latency and (ii) the peak-to-peak amplitude of the population spike, measured from the peak positivity to peak negativity.

The peak-to-peak amplitude of the population spike was measured in 6 slices. Two of these slices exhibited LTP and an example is illustrated in figure 4.4i. The peak-to-peak amplitude exhibited considerable variability both before and after TBS, but the mean population spike amplitude at 30-32 min after TBS is clearly potentiated at 129% of control. LTD was elicited in a single slice, whereas the remaining 2 slices exhibited no effect. An example of such an experiment is illustrated in figure 4.4ii, A and B. The mean peak-to-peak amplitude of the population spike at 30-32 min after the completion of TBS for this single experiment was 118% of control. The pooled data from these 6 slices is illustrated in figure 4.4ii, C. The mean peak-to-peak amplitude at 30-32 min after the completion of TBS was found to be 114 +/- 7%
Figure 4.4i: TBS produces LTP of the peak-to-peak amplitude of the population spike, recorded in layer V, in 2 out of 6 slices.

A  Graph of peak-to-peak of the population spike plotted against time for a single experiment. Each point represents an average of 8 consecutive responses over 2 min. Vertical lines indicate start and completion of TBS.

B  Superimposed responses to test shocks applied once every 15s to border between layers I and II in control (thick black line; time point labelled a in A) and 30 min post-TBS (thin black line; time point labelled b in A). Each trace represents an average of 8 consecutive responses. Calibration bars: 0.1 mV, 5ms.
This does not reach levels of significance (P = 0.60, two-tailed paired t-test, n=6).

In a further 3 slices, the slope of the population spike was measured. Two slices exhibited STD, whereas no effect was seen in the remaining cell. One of the slices in which STD was induced is illustrated in figure 4.4iii, A and B. At 30-32 after the completion of TBS, the mean slope of the population spike was 74% of control. Although this satisfies the established criterion for LTD, the slope is not stably depressed and starts to return towards baseline almost immediately following the completion of TBS. It is therefore classified as an STD. The pooled data from these 3 slices is illustrated in figure 4.4iii, C. The mean slope at 10-12 min of 61 +/- 10% (SE) is not significantly different from control (P = 0.15, two-tailed paired t-test, n=3). The mean slope at 30-32 min after the completion of TBS of 87 +/- 4% (SE) is also not significantly different from control (P = 0.29, two-tailed paired t-test, n=3).

With both the peak-to-peak amplitude of the population spike and the slope of the population spike expressed as normalised values it is possible to pool both categories of measurement, since it is changes in the field potential that are being examined. The pooled data from the 9 slices are illustrated in figure 4.4iv. The mean change in the field response 30-32 min after the completion of TBS was 105 +/- 4% (SE). This is not significantly different from control (P = 0.97, two-tailed paired t-test, n=9).
A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)
Figure 4.4ii: TBS produces variable effects on the peak-to-peak amplitude of the population spike recorded in layer V.

A  Graph of peak-to-peak of the population spike plotted against time for a single experiment. Each point represents an average of 8 consecutive responses over 2 min. Vertical lines indicate start and completion of TBS.

B  Superimposed responses to test shocks applied once every 15s to border between layers I and II in control (thick black line; time point labelled $a$ in A) and 30 min post-TBS (thick grey line; time point labelled $b$ in A). Traces represent an average of 8 consecutive responses. Calibration bar: 0.1 mV, 5ms.

C  Graph of pooled data from 6 cells showing peak-to-peak amplitude of population spike plotted against time, normalised to the mean of 10 averages in control period. Vertical lines represent start and completion of TBS.
Figure 4.4iii: TBS produces variable effects on the slope of the population spike recorded in layer V.

A  Graph of slope of population spike plotted against time for a single experiment. Each point represents an average of 8 consecutive responses over 2 min. Vertical lines indicate start and completion of TBS.

B  Superimposed responses to test shocks applied once every 15s to border between layers I and II in control (thick black line labelled a and taken at time point also labelled a in A), 10 min post-TBS (thick grey line labelled b and taken at time point also labelled b in A) and 30 min post-TBS (thin black line labelled c and taken at time point also labelled c in A). Each trace represents an average of 8 consecutive responses. Calibration bar: 0.1 mV, 5ms.

C  Graph of pooled data from 3 cells showing slope of population spike plotted against time, normalised to the mean of 10 averages in control period. Values displayed +/- SE. Vertical lines represent start and completion of TBS.
Figure 4.4iv: TBS fails to induce LTP of the pooled field responses recorded in layer V.

Graph of pooled data from 9 slices showing field response plotted against time, normalised to the mean of 10 averages in control. Graph includes 6 slices in which the peak-to-peak amplitude of the population spike was measured and 3 slices in which the slope of the population spike was measured. Values are shown +/- SE. Vertical dashed lines represent start and completion of TBS.
Part III: Group I mGluR involvement in induction of LTP in medial frontal cortex.

4.5 Effect of DHPG on field response.

In the hippocampus, bath application of 100 μM DHPG elicits a sustained depression of synaptic transmission, termed DHPG-induced LTD (Palmer et al., 1997). The effect of activation of group I mGluRs by bath application of 100 μM DHPG for 10 min was examined in a total of 6 slices, obtained from 6 rats. DHPG decreased the field response in all 6 slices. An example of such an agonist-induced depression of the field response is illustrated in figure 4.5, A and B. By 10-12 min after agonist application, the peak-to-peak amplitude of the population spike had decreased to 45% of control. After 10-12 min of washout, the field response had recovered to 69% of control, and at 20-22 min the recovery was to 75% of control.

The pooled data from 6 slices is shown in figure 4.5, C. There was a significant reduction in the field response to 73 +/- 8% (SE) of control at 10-12 min after DHPG application (P = 0.03, two-tailed paired t-test, n=6). After 10-12 min of washout, the field response has recovered to 86 +/- 9% (SE) of control. At 20-22 min after the start of washout of the drug, the field response has recovered to 90 +/- 6% (SE) of control, and is significantly greater than that observed in the presence of DHPG (P = 0.026, two-tailed paired t-test, n=6). Using the criterion established by Palmer et al. (1997) in which LTD is defined as a stable depression of 10% or greater at 30 min, 3 out of 6 slices exhibited DHPG-induced LTD, although the pooled data does not display LTD. In our intracellular experiments, we classified LTD as a stable depression of 20% or greater at 30 min. Using this criterion, only 2 out of 6 slices exhibited DHPG-induced LTD.
Figure 4.5: Bath application of DHPG induces reversible depression of field response.

A  Graph of peak-to-peak amplitude of population spike plotted against time for a single experiment. Each point represents an average of 8 consecutive responses over 2 min. Vertical lines indicate period during which 100 μM DHPG was present in the bathing medium.

B  Superimposed responses to test shocks applied once every 15s to border between layers I and II in control (thin black line; time point labelled a in A), 10 min after DHPG application (thick black line; time point labelled b in A) and 20 min after washout of the drug (thick grey line; time point labelled c in A). Each trace represents an average of 8 consecutive responses. Calibration bar: 0.2 mV, 2.5ms.

C  Graph of pooled data from 3 cells showing peak-to-peak amplitude of population spike plotted against time, normalised to the mean of 10 averages in control period. Values displayed +/- SE. Vertical lines indicate period during which 100 μM DHPG was present in the bathing medium.
4.6 Effect of DHPG on membrane electrophysiology.

The reduction in the field response could be the consequence of a number of different DHPG-induced effects on the neuronal population, such as depolarisation of the postsynaptic cell or a reduction in transmitter release. Intracellular recording was therefore employed to establish the effect of DHPG directly on the layer V neurones. DHPG was bath applied to 3 slices and elicited membrane depolarisation, an increase in the number of spikes evoked by a current pulse of fixed strength and reduction in the amplitude of the AHP seen following a train of spikes. An example is shown in figure 4.6, A and B. Exposure to 100 μM DHPG elicited membrane depolarisation and increased spike firing (not illustrated). The depolarisation was counteracted by the application of steady hyperpolarisation to evoke the same number of spikes on a current pulse of fixed strength. The amplitude of the AHP was reduced to 39% following 10 min bath application of DHPG. After 40 min of washout, the AHP amplitude was fully recovered, reaching 97% of control. The observed changes in the AHP amplitude were accompanied by a transient reduction in the EPSP slope. However, the current injected at the soma to counterbalance the agonist-induced depolarisation would not efficiently clamp the membrane potential in the dendrites and therefore the effects on the EPSP were not wholly independent of the changes in membrane potential. Pooled data from 3 cells is illustrated in figure 4.6, C. The mean reduction in AHP amplitude on agonist application was to 27.1 +/- 17% of control (SE). This does not quite reach levels of significance (P = 0.051, two-tailed paired t-test, n=3). Washout produced partial recovery to 87.7 +/- 10% of control (SE).
Figure 4.6: DHPG elicits excitatory effects in layer V cells.

A Superimposed responses to intracellularly injected depolarising current pulses in control (thin black line), 100 μM DHPG (thick black line labelled with filled arrow) and after 10 min of washout of drug (thin grey line). Note control and washout traces cannot be easily distinguished since they superimposed almost exactly. Calibration bar: 30 ms, 10 mV.

B Graph of membrane potential (mV) plotted against time for cell illustrated in A. Black horizontal bar indicates period during which 100 μM DHPG was present in bathing medium.

C Histogram of AHP amplitude as a % of control mean at 0-4 min before and 8-12 min after bath application of DHPG, and at least 8-12 min after washout of drug. Vertical +/- SE.
4.7 The group I specific agonist DHPG facilitates induction of LTP.

The involvement of group I mGluRs in the induction of LTP in the medial frontal cortex was investigated in a total of 9 slices, obtained from 5 rats. The bath application of 100 μM DHPG was paired with the TBS conditioning protocol and the effect on LTP incidence investigated. DHPG was bath applied for 10 min, starting 6 min prior to the delivery of TBS. DHPG reduced the size of the field response prior to TBS. LTP was elicited following TBS in the presence of DHPG in 7 slices. Of the remaining 2 slices, one showed STD, whereas the other exhibited no effect. An example of an experiment in which LTP of both the peak-to-peak amplitude and the slope of the population spike was elicited by TBS in the presence DHPG is illustrated in figure 4.7i, A and B. It is interesting to note that the first point following the completion of TBS is still at control levels. This is typical of LTP evoked by this induction protocol. All 7 slices exhibiting LTP show this slow-onset, the time between the completion of TBS and the appearance of the maximal effect ranging between 2 and 10 min. This is likely to be the consequence of the reversal of the DHPG depressant effect following washout of the drug. The response is almost completely abolished by the bath application of CNQX. The pooled data for all 9 slices are illustrated in figure 4.7i, C. Points are normalised to the average of the 7 points in the control preceding the bath application of DHPG. The mean change in the field response 30-32 min after the completion of TBS was 147 +/- 12% (SE). This is highly significantly different from control ($P=0.0043$, two-tailed paired $t$-test, $n=9$).

No correlation was found between the magnitude of the depressant effect of DHPG and the consequence of TBS applied in the presence of the group I mGluR agonist ($r^2 = 0.176$). In addition, the peak-to-peak amplitude of the population spike within the last two minutes of control was not significantly different between those
Figure 4.7i: Application of DHPG combined with TBS induces LTP in 7 out of 9 slices.

A  Graph of peak-to-peak amplitude of population spike plotted against time for a single experiment. Black horizontal bar indicates period during which 100 μM DHPG was present in the bathing medium. Vertical dashed lines indicate start and completion of TBS. Arrow indicates time point at which 20 μM CNQX was added to bathing medium.

B  Superimposed responses to test shocks applied once every 15s to border between layers I and II in control (black line; time point labelled a in A) and 30 min post-TBS (grey line; time point labelled b in A). Calibration bar: 0.2 mV, 2.5 ms.

C  Graph of the field response plotted against time, normalised to the mean of 10 averages in control period. Pooled data for 9 slices, including 4 slices in which peak-to-peak amplitude of the population was measured and 5 slices in which slope of the population spike was measured. Horizontal bar indicates period during which 100 μM DHPG was present in the bathing medium. Vertical lines indicate start and completion of TBS. Error bars are +/- SE.
slices exposed to TBS and those exposed to TBS given in conjunction with DHPG ($P = 0.102$, two-tailed unpaired $t$-test, $n=6, 6$). When both the peak-to-peak amplitude of the population spike and the slope of the population spike are pooled, the field response in the two experimental groups are still not significantly different from each other ($P = 0.254$, two-tailed unpaired $t$-test, $n=9, 9$).

The effect of TBS in ACSF alone and in the presence of DHPG was examined in a single slice and the findings are illustrated in figure 4.7ii. TBS alone elicited STP that declined to baseline 26 min after the completion of TBS. The slice was then exposed to an identical TBS in the presence of DHPG, and a robust LTP was induced. In a second slice, not illustrated, TBS in control ACSF had no effect, whereas in the presence of DHPG, STD was elicited.
Figure 4.7ii: Ability of DHPG to facilitate induction of LTP examined in a single slice.

A Graph of slope of the population spike plotted against time for a single experiment. Each point represents an average of 8 consecutive responses. Black horizontal bar indicates period of which 100 mM DHPG was present in bathing medium. Vertical dashed lines indicate start and completion of TBS.

B (i) Superimposed responses to test shocks applied once every 15s to border between layers I and II in control (black line; time point indicated by a in A) and 30 min post-TBS (grey line; time point indicated by b in A) in normal ACSF. Calibration bar: 0.2 mV, 2.5 ms. (ii) Superimposed responses to test shocks applied once every 15s to border between layers I and II in control (black line; time point indicated by c in A) and 30 min post-TBS (grey line; time point indicated by d in A) in presence of 100 µM DHPG. Calibration bar: 0.2 mV, 2.5 ms.
CHAPTER 5

RESULTS: Measurement of the changes in intracellular [$Ca^{2+}$] produced by group I mGluRs.

5.1 Introduction.

This chapter describes experiments in which the effect of mGluR agonists on intracellular [$Ca^{2+}$] was tested. Part I details experiments in which the effect of the broadspectrum mGluR agonist ACPD, the group I mGluR-specific agonist S-3HPG and glutamate on levels of [$Ca^{2+}$], were examined in mixed neuronal/glia hippocampal cultures. Part II describes experiments in which the ability of MCPG and the group I mGluR-specific antagonist, 4CPG, to block the agonist-induced change in [$Ca^{2+}$], was tested. Part III describes preliminary experiments repeated in mixed cultures of medial frontal cortical neurones and glia.

All of the above experiments were performed by me in collaboration with Dr Julie Keelan and Dr Lynn Bindman in the laboratory of Dr Michael Duchen.

Part I: Measurement of changes in [$Ca^{2+}$], on application of mGluR agonists in hippocampal neurones and glia.

5.2 Effect of ACPD on [$Ca^{2+}$].

The change in somatic [$Ca^{2+}$], on focal application of the broadspectrum mGluR agonist ACPD at 50 µM was observed in a total of 11 neurones. Seven of these neurones were taken from a 7 day culture and the remainder from a 15 day culture. Eight out of 11 neurones responded to ACPD with a transient elevation in [$Ca^{2+}$], of greater than 110% of control. An example of such an agonist-induced rise in
Figure 5.2: ACPD elicits a transient rise in [Ca^{2+}] in cultured hippocampal neurones.

A  Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for a single experiment. Cultured neurones bathed in standard recording medium containing 2 mM Ca^{2+}. Filled black horizontal bar indicates period during which 50 μM ACPD was applied to the field through a puffer pipette.

B  Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 50 μM ACPD at time of peak effect (white bar) and 97s after agonist application (black bar). Pooled data from 11 neurones. Error bars are +/- SE.
[Ca\textsuperscript{2+}]\textsubscript{i} is illustrated in figure 5.2, A. The peak rise in [Ca\textsuperscript{2+}]\textsubscript{i} was to 165% of control. The [Ca\textsuperscript{2+}]\textsubscript{i} remained elevated above baseline levels for 30 sec after the peak effect was observed. The 3 non-responsive cells were subsequently tested with KCl solution and they responded to this with a transient elevation in [Ca\textsuperscript{2+}]\textsubscript{i}. When data from all 11 neurones are pooled, the mean peak rise in [Ca\textsuperscript{2+}]\textsubscript{i} was found to be 122 +/- 7% (SE) of control, as illustrated in figure 5.2, B. This reaches levels of significance (P=0.01, two-tailed paired t-test, n=11).

5.3 Effect of S-3HPG on [Ca\textsuperscript{2+}]\textsubscript{i}.

Next the action of the group I mGluR-specific agonist S-3HPG on [Ca\textsuperscript{2+}]\textsubscript{i} was observed in a total of 20 neurones. This total included 16 neurones from a 7 day culture, 2 neurones from a 8 day culture and 2 neurones from a 9 day culture. S-3HPG elicited a transient rise in [Ca\textsuperscript{2+}]\textsubscript{i} in all 20 neurones. An example is illustrated in figure 5.3i, A. The peak rise in [Ca\textsuperscript{2+}]\textsubscript{i} was to 204 % of control. The [Ca\textsuperscript{2+}]\textsubscript{i} remained slightly elevated above baseline levels until approximately 45 sec after the end of the period of agonist application. When data from all 20 neurones are pooled, the mean rise in [Ca\textsuperscript{2+}]\textsubscript{i} was found to be 159 +/- 9% (SE) of control, as illustrated in figure 5.3i, B. This rise is highly significant (P < 0.001, two-tailed paired t-test, n=20).

The effect of S-3HPG on [Ca\textsuperscript{2+}]\textsubscript{i} was also tested in 15 glia from the same cultures. S-3HPG elicited a transient rise in [Ca\textsuperscript{2+}]\textsubscript{i} in 8 out of 15 glia. Two examples are illustrated in figure 5.3ii, A. The peak rise in [Ca\textsuperscript{2+}]\textsubscript{i} in the first glial cell was to 343% of control, whereas that seen in the second glial cell was to 416% of control. Of the remaining 7 glia, 3 responded at a longer latency and were therefore classed as not expressing an agonist-induced rise in [Ca\textsuperscript{2+}]\textsubscript{i}. When we looked at the position of
Figure 5.3i: S-3HPG elicits a transient rise in [Ca^{2+}]_{i} in cultured hippocampal neurones.

A  Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for a single experiment. Cultured neurones bathed in standard recording medium containing 2 mM Ca^{2+}. Filled black horizontal bar indicates period during which 1 mM S-3HPG was applied to the field through a puffer pipette.

B  Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 1 mM S-3HPG at time of peak effect (white bar) and 97s after agonist application (black bar). Pooled data from 20 neurones. Error bars are +/- SE.
Figure 5.3ii: S-3HPG elicits a transient rise in [Ca$^{2+}$] in cultured hippocampal glia.

A Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for two individual glial cells in a single field. Cultured glia bathed in standard recording medium containing 2 mM Ca$^{2+}$. Filled black horizontal bar indicates period during which 1 mM S-3HPG was applied to the field through a puffer pipette.

B Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 1 mM S-3HPG at time of peak effect (white bar) and 97s after agonist application (black bar). Pooled data from 15 glia. Error bars are +/- SE
these three glia relative to both the puffer pipette and the other cells within the field, it appeared to be unlikely that the delayed response was the consequence of a larger distance between cell and puffer pipette than for the other cells in the field. Several glia within the field were observed to respond to S-3HPG and therefore the observed rise in $[\text{Ca}^{2+}]_i$ is not necessarily the direct result of agonist application, but could be an indirect consequence of agonist-induced changes in the other glia within the field. For this reason, only those glia that responded within a 20 sec time window, starting from the moment of agonist application, are classified as displaying an agonist-induced rise in $[\text{Ca}^{2+}]_i$. When data from all 15 glia are pooled, the mean peak rise in $[\text{Ca}^{2+}]_i$ was found to be 179 +/- 25% (SE) of control, as illustrated in figure 5.3ii, B. This is highly significant ($P = 0.007$, two-tailed paired $t$-test, $n=15$).

There is evidence that Group I mGluRs expressed in frog oocytes desensitize in response to prolonged or repeated agonist exposure (Gereau & Heinemann, 1998). A single cell was exposed to two consecutive applications of S-3HPG and the results are illustrated in figure 5.3iii. On the first exposure to agonist, the somatic $[\text{Ca}^{2+}]_i$ rose to 148% of control. S-3HPG was then applied for a second time, approximately 4 min after the initial application. The agonist-induced rise in $[\text{Ca}^{2+}]_i$ was still evident, but was reduced in magnitude in comparison with the initial application, the $[\text{Ca}^{2+}]_i$ only rising to 135% of control. In addition, on the second S-3HPG application, the cell was exposed to agonist for a longer period of time. The agonist was applied through the puffer pipette in a 60s pulse, compared with a 15s pulse in all other experiments. The $[\text{Ca}^{2+}]_i$ remained elevated for the duration of agonist exposure, the initial peak response being followed by a plateau phase in which the $[\text{Ca}^{2+}]_i$ remains elevated at 112% of control. An indication of receptor desensitization was also seen in another neurone.
Figure 5.3iii: Effect of S-3HPG on [Ca$^{2+}$], in hippocampal neurones declines on repeated exposure.

A Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for a single experiment. Cultured neurones bathed in standard recording medium containing 2 mM Ca$^{2+}$. Filled black horizontal bar indicates period during which 1 mM S-3HPG was applied, for the first time, to the field through a puffer pipette.

B Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for a single experiment. Cultured neurones bathed in standard recording medium containing 2 mM Ca$^{2+}$. Filled black horizontal bar indicates period during which 1 mM S-3HPG was applied, for the second time, to the field through a puffer pipette. Agonist applied for 60 sec.
Figure 5.3iv: Effect of prolonged exposure to S-3HPG on $[Ca^{2+}]$, in hippocampal glia.

A  Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for a single experiment. Cultured glia bathed in standard recording medium containing 2 mM $Ca^{2+}$. Filled black horizontal bar indicates period during which 1 mM S-3HPG was applied, for 60 sec, to the field through a puffer pipette.

B  Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 1 mM S-3HPG at time of peak effect (white bar) and 97s after agonist application (black bar). Pooled data from 3 glia. Error bars are +/- SE
The glial response to prolonged exposure of S-3HPG was tested in 3 cells. Two out of 3 glia responded to the agonist with a rise in \([\text{Ca}^{2+}]\), as illustrated in figure 5.3iv, A. When data from all 3 glia is pooled, the mean change in \([\text{Ca}^{2+}]\) was to 168 +/- 32% (SE) of control, as illustrated in figure 5.3iv, B. This did not reach levels of significance (P = 0.165, two-tailed paired \(t\)-test, \(n=3\)).

Group I mGluR agonists such as S-3HPG elicit membrane depolarisation as well as facilitating an IP\(_3\) -mediated release of \(\text{Ca}^{2+}\) from intracellular stores. This agonist-induced membrane depolarisation could trigger \(\text{Ca}^{2+}\) entry both through the NMDA receptor channel and through voltage-sensitive \(\text{Ca}^{2+}\) channels (VDCC). To determine whether S-3HPG is capable of eliciting a rise in \([\text{Ca}^{2+}]\), that is independent of membrane depolarisation, neurones were bathed in zero \(\text{Ca}^{2+}\) buffer containing 50 µM AP5, the NMDA receptor antagonist, and 100 µM EGTA, a potent \(\text{Ca}^{2+}\) chelator. Even under such conditions, application of 1mM S-3HPG was capable of eliciting a rise in \([\text{Ca}^{2+}]\), comparable to that seen in normal buffer medium in 8 out of 14 neurones tested. Two examples are illustrated in figure 5.3v, A. When data from all 14 neurones are pooled, the mean change in \([\text{Ca}^{2+}]\) was to 131 +/- 11% (SE) of control, as illustrated in figure 5.3v, B. This was significantly different from control (P = 0.015, two-tailed paired \(t\)-test, \(n=14\)). The agonist-induced rise in \([\text{Ca}^{2+}]\) is therefore independent of \(\text{Ca}^{2+}\) entry from the neuronal exterior and of membrane depolarisation elicited by the agonist.

The effect of S-3HPG on \([\text{Ca}^{2+}]\) was tested in 7 glia bathed in recording medium containing zero \(\text{Ca}^{2+}\), EGTA and AP5. The agonist elicited a rise in \([\text{Ca}^{2+}]\) in 3 out of 7 glia. Two examples are illustrated in figure 5.3vi, A. When data from all 7 glia are pooled, the mean change in \([\text{Ca}^{2+}]\) was to 165 +/- 31% (SE) of control, as illustrated in figure 5.3vi, B. This was not significantly different from control (P = 0.079, two-tailed paired \(t\)-test, \(n=7\)).
Figure 5.3v: S-3HPG elicits a transient rise in $[Ca^{2+}]_i$, in hippocampal neurones, even in buffer containing zero $Ca^{2+}$, EGTA and AP5.

A  Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for two neurones in a single field. Cultured neurones bathed in recording medium containing zero $Ca^{2+}$, EGTA and AP5. Filled black horizontal bar indicates period during which 1 mM S-3HPG was applied to the field through a puffer pipette.

B  Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 1 mM S-3HPG at time of peak effect (white bar) and 137s after agonist application (black bar). Pooled data from 14 neurones. Error bars are +/- SE
Figure 5.3vi: S-3HPG elicits a transient rise in \([\text{Ca}^{2+}]\), in hippocampal glia, even in buffer containing zero \(\text{Ca}^{2+}\), EGTA and AP5.

A Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for two glia in a single field. Cultured glia bathed in recording medium containing zero \(\text{Ca}^{2+}\), EGTA and AP5. Filled black horizontal bar indicates period during which 1 mM S-3HPG was applied to the field through a puffer pipette.

B Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 1 mM S-3HPG at time of peak effect (white bar) and 137s after agonist application (black bar). Pooled data from 7 glia. Error bars are +/- SE
Part II: Measurement of agonist-induced changes in $[Ca^{2+}]_i$ in presence of mGluR antagonists in hippocampal neurones and glia.

5.4 Effect of MCPG on ACPD-induced rise in $[Ca^{2+}]_i$.

The effect of the broadspectrum mGluR antagonist MCPG on the ACPD-induced rise in $[Ca^{2+}]_i$ was investigated in a total of 7 neurones. MCPG was seen to block the agonist-induced rise in $[Ca^{2+}]_i$ in all 7 neurones. An example is illustrated in figure 5.4i, A. The viability of the neurone was tested using 50 mM KCl solution and it responded to this with a transient rise in $[Ca^{2+}]_i$ (see figure 5.4i, B). Therefore the lack of effect of ACPD was the consequence of the mGluR antagonism rather than non-responsiveness of the culture. When the data from all 7 neurones are pooled, the mean change in $[Ca^{2+}]_i$ was to 101 +/- 1% (SE) of control, as illustrated in figure 5.4i, C. Although this is still significantly different from control ($P = 0.036$, two-tailed paired $t$-test, $n=7$), the agonist-induced rise in $[Ca^{2+}]_i$ is significantly reduced in the presence of MCPG ($P = 0.016$, unpaired $t$-test, $n=14,7$).

5.5 Effect of MCPG and 4-CPG on S-3HPG-induced rise in $[Ca^{2+}]_i$.

The effect of the broadspectrum mGluR antagonist MCPG and the Group I mGluR specific antagonist 4-CPG on the S-3HPG-induced rise in $[Ca^{2+}]_i$ was investigated in a total of 21 neurones.

The response to S-3HPG in the presence of MCPG was tested in 10 neurones. The ability of cells within the culture to respond to S-3HPG in the standard recording medium was first tested. Two examples of the agonist-induced rise in $[Ca^{2+}]_i$ are illustrated in figure 5.5i, A. The peak rise in $[Ca^{2+}]_i$ in these two neurones was to 178% and 204% of control respectively. MCPG was then added to the bathing medium. After 20 min of incubation, MCPG blocked the agonist-induced rise in $[Ca^{2+}]_i$ in 8 out of 10
Figure 5.4: MCPG reduces ACPD-induced rise in $[Ca^{2+}]_i$ in hippocampal neurones.

A  Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for a single experiment. Cultured neurones bathed in recording medium containing 1 mM MCPG. Filled black horizontal bar indicates period during which 50 µM ACPD was applied to the field through a puffer pipette.

B  Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for same cell as that illustrated in A. Cultured neurones bathed in recording medium containing 1 mM MCPG. Filled black horizontal bar indicates period during which 50 mM KCl was applied to the field through a puffer pipette.

C  Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 50 µM ACPD at time of peak effect (white bar) and 100s after agonist application (black bar). Pooled data from 7 neurones. Error bars are +/- SE
neurones tested. An example is illustrated in figure 5.5i, B. When the data from all 10 neurones are pooled, the mean change in $[\text{Ca}^{2+}]$ was to $105 +/-. 1\%$ (SE) of control. Although this was still significantly different from control ($P=0.001$, two-tailed paired $t$-test, $n=10$), MCPG reduced the peak effect on $[\text{Ca}^{2+}]$ from $159 +/-. 9\%$ (SE; $n=20$) of control (see section 5.3). This reduction is highly significant ($P < 0.001$, unpaired $t$-test, $n=20,10$) and therefore MCPG can block the S-3HPG-induced rise in $[\text{Ca}^{2+}]$.

The effect of the group I mGluR specific antagonist 4-CPG on the S-3HPG-induced rise in $[\text{Ca}^{2+}]$ was investigated in a total of 12 neurones. 4-CPG was seen to block the agonist-induced rise in $[\text{Ca}^{2+}]$ in 9 out of 12 neurones. An example is illustrated in figure 5.5ii, A. The neurones that failed to respond to S-3HPG were subsequently tested with $100 \mu\text{M}$ glutamate and responded with a transient rise in $[\text{Ca}^{2+}]$, as illustrated in figure 5.5ii, B. The lack of effect of S-3HPG on these 9 neurones was therefore either the result of a lack of expression of group I mGluRs or of the mGluR antagonism by 4CPG. When data from all 12 neurones are pooled, the mean change in $[\text{Ca}^{2+}]$ was to $108 +/-. 4\%$ (SE) of control, as illustrated in figure 5.5ii, C. Once again, although this is still significantly different from control ($P = 0.03$, two-tailed paired $t$-test, $n=12$), 4-CPG reduced the peak effect on $[\text{Ca}^{2+}]$ from $159 +/-. 9\%$ (SE; $n=20$) of control (see section 5.3). This reduction is highly significant ($P < 0.001$, unpaired $t$-test, $n=20,12$) and therefore 4-CPG is capable of blocking the S-3HPG induced rise in $[\text{Ca}^{2+}]$.

The reversibility of the effect of 4-CPG on the agonist-induced rise in $[\text{Ca}^{2+}]$ was examined in 4 neurones and an example is illustrated in figure 5.5iii, A and B. In the presence of 4-CPG, the agonist-induced change in $[\text{Ca}^{2+}]$ was to $101\%$ of control. After 20 min of washout of the antagonist, S-3HPG elicited a rise in $[\text{Ca}^{2+}]$ to $109\%$ of
Figure 5.5i: MCPG reduces S-3HPG-induced rise in $[Ca^{2+}]_{i}$ in hippocampal neurones.

A Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for two neurones in a single field. Cultured neurones bathed in standard recording medium. Filled black horizontal bar indicates period during which 1 mM S-3HPG was applied to the field through a puffer pipette.

B Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for a single neurone. Cultured neurones bathed in recording medium containing 1 mM MCPG. Filled black horizontal bar indicates period during which 1 mM S-3HPG was applied to the field through a puffer pipette.

C Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 1 mM S-3HPG at time of peak effect (white bar) and 100s after agonist application (black bar). Pooled data from 10 neurones bathed in 1 mM MCPG for duration of experiment. Error bars are +/- SE
A

Ratio 340/380

Time (sec)

B

Ratio 340/380

Time (sec)

C

Ratio 340/380 (% of control)

Control  S-3HPG & 4CPG  Recovery

n=12  n=12  n=12
Figure 5.5ii: 4-CPG reduces S-3HPG-induced rise in $[\text{Ca}^{2+}]_\text{i}$, in hippocampal neurones.

A Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for two neurones in a single field. Cultured neurones bathed in recording medium containing 1 mM 4-CPG. Filled black horizontal bar indicates period during which 1 mM S-3HPG was applied to the field through a puffer pipette.

B Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for same two neurones as illustrated in A. Cultured neurones bathed in recording medium containing 1 mM 4-CPG. Filled black horizontal bar indicates period during which 100 µM glutamate was applied to the field through a puffer pipette.

C Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 1 mM S-3HPG at time of peak effect (white bar) and 100s after agonist application (black bar). Pooled data from 12 neurones bathed in 1 mM 4-CPG for duration of experiment. Error bars are +/- SE
control. When data from 4 neurones are pooled, the mean change in \([\text{Ca}^{2+}]_i\) was to 106 +/- 1% (SE) of control, as illustrated in figure 5.5iii, C. Although this is significantly different from control \((P = 0.03, \text{two-tailed paired } t\text{-test, } n=4)\), it is clear that the agonist-induced rise in \([\text{Ca}^{2+}]_i\) has not fully recovered after 20 min of washout of 4CPG. Alternatively, the lack of recovery could reflect receptor desensitization on repeated exposure to agonist.

The effect of MCPG and 4-CPG on the S-3HPG-induced rise in \([\text{Ca}^{2+}]_i\) was investigated in a total of 13 glia.

The response to S-3HPG in the presence of MCPG was tested in 4 glia. After 20 min of incubation with MCPG, S-3HPG elicited a rise in \([\text{Ca}^{2+}]_i\) in 2 out of 4 glia tested. Two examples are illustrated in figure 5.5iv, A. When the data from all 4 glia are pooled, the mean change in \([\text{Ca}^{2+}]_i\) was to 110 +/- 6% (SE) of control. This is not significantly different from control \((P = 0.24, \text{two-tailed paired } t\text{-test, } n=4)\).

However, the mean change in \([\text{Ca}^{2+}]_i\) on application of S-3HPG in the presence of MCPG is not significantly different from that seen in S-3HPG alone \((P = 0.23, \text{unpaired } t\text{-test, } n=4,4)\).

The effect of 4-CPG on the S-3HPG-induced rise in \([\text{Ca}^{2+}]_i\) was investigated in a total of 9 glia. All 9 glia responded to S-3HPG with a transient rise in \([\text{Ca}^{2+}]_i\). An example is illustrated in figure 5.5iv, A. When data from all 9 glia are pooled, the mean change in \([\text{Ca}^{2+}]_i\) was to 139 +/- 15% (SE) of control, as illustrated in figure 5.5iv, B. This is still significantly different from control \((P = 0.042, \text{two-tailed paired } t\text{-test, } n=9)\).

Although 4CPG reduced the peak effect on \([\text{Ca}^{2+}]_i\) from 179 +/- 25% (SE) of control (see section 5.3), the observed reduction is not significant \((P = 0.4, \text{unpaired } t\text{-test, } n=9,9)\). 4CPG is therefore not capable of fully blocking the S-3HPG induced rise in \([\text{Ca}^{2+}]_i\).

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Figure 5.5iii: Effect of S-3HPG on $[Ca^{2+}]_{i}$ in 4CPG compared with that observed after 20 min washout of 4-CPG in hippocampal neurones.

A  Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for a single neurone. Cultured neurones bathed in recording medium containing 1 mM 4CPG. Filled black horizontal bar indicates period during which 1 mM S-3HPG was applied to the field through a puffer pipette.

B  Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for same neurone as illustrated in A. Cultured neurones bathed in standard recording medium. Filled black horizontal bar indicates period during which 1 mM S-3HPG was applied to the field through a puffer pipette.

C  Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 1 mM S-3HPG at time of peak effect (white bar) and 109s after agonist application (black bar). Pooled data from 4 neurones. Error bars are +/- SE.
Figure 5.5iv: MCPG fails to block effect of S-3HPG on $[Ca^{2+}]$, in hippocampal glia.

A  Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for two glia from a single field. Cultured glia bathed in recording medium containing 1 mM MCPG. Filled black horizontal bar indicates period during which 1 mM S-3HPG was applied to the field through a puffer pipette.

B  Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 1 mM S-3HPG at time of peak effect (white bar) and 109s after agonist application (black bar). Pooled data from 4 glia bathed in 1mM MCPG for duration of experiment. Error bars are +/- SE
Figure 5.5v: 4-CPG fails to block S-3HPG-induced rise in $[\text{Ca}^{2+}]_i$ in hippocampal glia.

A  Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for a single glial cell. Cultured glia bathed in recording medium containing 1 mM 4-CPG. Filled black horizontal bar indicates period during which 1 mM S-3HPG was applied to the field through a puffer pipette.

B  Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 1 mM S-3HPG at time of peak effect (white bar) and 109s after agonist application (black bar). Pooled data from 9 glia bathed in 1mM 4-CPG for duration of experiment. Error bars are +/- SE
5.6 Effect of MCPG on glutamate-induced rise in \([\text{Ca}^{2+}]_i\).

It is well established that glutamate elicits an increase in \([\text{Ca}^{2+}]_i\) (Murphy & Miller, 1988). The glutamate-induced changes in \([\text{Ca}^{2+}]_i\) are only partially inhibited by removal of extracellular \(\text{Ca}^{2+}\), presumably reflecting release of \(\text{Ca}^{2+}\) from intracellular stores. We tested whether MCPG could block the glutamate-induced rise in \([\text{Ca}^{2+}]_i\) in the presence of zero \(\text{Ca}^{2+}\) buffer containing 50 µM AP5 and 100 µM EGTA. Glutamate elicited a rise in \([\text{Ca}^{2+}]_i\) in 1 out of 5 neurones tested. An example is illustrated in figure 5.6. The peak rise in \([\text{Ca}^{2+}]_i\) was to 110% of control. When data from all 5 neurones are pooled, the mean rise in was 103 +/- 2% (SE) of control. This was not significantly different from control (\(P = 0.17\), two-tailed paired \(t\)-test, \(n=5\)). Our findings suggest that MCPG can block the glutamate-induced rise in \([\text{Ca}^{2+}]_i\) seen in zero \(\text{Ca}^{2+}\) buffer.

Part III: Measurement of changes in \([\text{Ca}^{2+}]_i\) on application of mGluR agonists in prelimbic neurones and glia.

5.7 Effect of S-3HPG on \([\text{Ca}^{2+}]_i\) in zero \(\text{Ca}^{2+}\) buffer.

The action of the group I mGluR-specific agonist S-3HPG on \([\text{Ca}^{2+}]_i\) in zero \(\text{Ca}^{2+}\) buffer containing 50 µM AP5 and 100 µM EGTA was observed in a total of 14 neurones. S-3HPG was bathed applied at a concentration of 1 mM. It elicited a transient rise in \([\text{Ca}^{2+}]_i\) in 13 out of 14 neurones. An example is illustrated in figure 5.7i, A. The peak rise in \([\text{Ca}^{2+}]_i\) observed was to 142% of control. When data from all 14 neurones are pooled, the mean rise in \([\text{Ca}^{2+}]_i\) was 158 +/- 11% (SE) of control, as illustrated in figure 5.7i, B. This rise is highly significant (\(P = 0.0002\), two-tailed paired \(t\)-test, \(n=14\)). S-3HPG therefore evokes a transient rise in \([\text{Ca}^{2+}]_i\).
Figure 5.6: MCPG blocks effect of glutamate on $[Ca^{2+}]_{i}$ in hippocampal neurones, even in buffer containing zero $Ca^{2+}$, EGTA and AP5.

A  Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for a single neurone. Cultured neurones bathed in recording medium containing 1 mM MCPG. Filled black horizontal bar indicates period during which 100 µM glutamate was applied to the field through a puffer pipette.

B  Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 100 µM glutamate at time of peak effect (white bar) and 147s after agonist application (black bar). Pooled data from 5 neurones bathed in 1 mM MCPG for duration of experiment. Error bars are +/- SE
that is independent of Ca\textsuperscript{2+} entry from the neuronal exterior in cultures of rat medial frontal cortical neurones.

The glial response to S-3HPG in zero Ca\textsuperscript{2+} buffer containing 100 \(\mu\)M EGTA and 50 \(\mu\)M AP5 was observed in 4 cells. Bath application of 1 mM S-3HPG elicited a rise in [Ca\textsuperscript{2+}]\textsubscript{i} in 4 out of 4 glia. An example is illustrated in figure 5.7ii, A. The peak change in [Ca\textsuperscript{2+}]\textsubscript{i} was to 135% of control. When data from all 4 glia are pooled, the mean rise in [Ca\textsuperscript{2+}]\textsubscript{i} was 143 +/- 8% (SE) of control. This reaches levels of significance (P = 0.017, two-tailed paired t-test, \(n=4\)). S-3HPG can therefore elicit a rise in [Ca\textsuperscript{2+}]\textsubscript{i} in cultures of rat medial frontal cortical glia, even in conditions of zero extracellular Ca\textsuperscript{2+}.

5.8 Effect of DHPG on [Ca\textsuperscript{2+}]\textsubscript{i} in zero Ca\textsuperscript{2+} buffer.

The effect of the group I mGluR-specific agonist DHPG on [Ca\textsuperscript{2+}]\textsubscript{i} in zero Ca\textsuperscript{2+} buffer containing 100 \(\mu\)M EGTA and 50 \(\mu\)M AP5 was observed in a total of 12 neurones. DHPG was bath applied at a concentration of 200 \(\mu\)M. It elicited a transient rise in [Ca\textsuperscript{2+}]\textsubscript{i} in 10 out of 12 neurones. An example is illustrated in figure 5.8i, A. The peak rise in [Ca\textsuperscript{2+}]\textsubscript{i} was to 218% control. When data from all 12 neurones are pooled, the mean rise in [Ca\textsuperscript{2+}]\textsubscript{i} was found to be 176 +/- 14% (SE) of control, as illustrated in figure 5.8i, B. This rise is highly significant (P = 0.0002, two-tailed paired t-test, \(n=12\)). DHPG therefore elicits a rise in [Ca\textsuperscript{2+}]\textsubscript{i} in cultured neurones from rat medial frontal cortex, even in zero extracellular Ca\textsuperscript{2+}.

The glial response to DHPG was observed in 9 cells. Bath application of 200 \(\mu\)M DHPG elicited a rise in [Ca\textsuperscript{2+}]\textsubscript{i} in all 9 glia. An example is illustrated in figure 5.8ii, A. The peak change in [Ca\textsuperscript{2+}]\textsubscript{i} was to 446% of control. Note that in the cell illustrated, DHPG elicits oscillations in [Ca\textsuperscript{2+}]\textsubscript{i} not seen before nor after the period
Figure 5.71: S-3HPG elicits a transient rise in [Ca\(^{2+}\)]\(_i\), in prelimbic neurones, even in buffer containing zero Ca\(^{2+}\), EGTA and AP5.

A  Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for two neurones in a single field. Cultured neurones bathed in recording medium containing zero Ca\(^{2+}\), EGTA and AP5. Filled black horizontal bar indicates period during which 1 mM S-3HPG was bath applied to the field.

B  Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 1 mM S-3HPG at time of peak effect (white bar) and 173s after agonist application (black bar). Pooled data from 15 neurones. Error bars are +/- SE.
Figure 5.7ii: S-3HPG elicits a transient rise in \([\text{Ca}^{2+}]\), in prelimbic glia, even in buffer containing zero \(\text{Ca}^{2+}\), EGTA and AP5.

**A** Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for a single glial cell. Cultured glia bathed in recording medium containing zero \(\text{Ca}^{2+}\), EGTA and AP5. Filled black horizontal bar indicates period during which 1 mM S-3HPG was bath applied to the field.

**B** Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 1 mM S-3HPG at time of peak effect (white bar) and 173s after agonist application (black bar). Pooled data from 4 glia. Error bars are +/- SE
Top panel: Control: Picture of ratio of fluorescent measurements taken at 340 and 380 nm from cultured medial frontal cortical neurones bathed in zero Ca\(^{2+}\) recording medium containing 100 μM EGTA.

Bottom panel: In DHPG: Picture of ratio of fluorescent measurements taken at 340 and 380 nm from cultured medial frontal cortical neurones bathed in zero Ca\(^{2+}\) recording medium containing 100 μM EGTA and 400 μM DHPG.
Figure 5.8i: DHPG elicits a transient rise in [Ca^{2+}], in prelimbic neurones, even in buffer containing zero Ca^{2+}, EGTA and AP5.

A  Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for a single neurone. Cultured neurones bathed in recording medium containing zero Ca^{2+}, EGTA and AP5. Filled black horizontal bar indicates period during which 200 μM DHPG was bath applied to the field.

B  Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 200 μM DHPG at time of peak effect (white bar) and 105s after agonist application (black bar). Pooled data from 12 neurones. Error bars are +/- SE
Figure 5.8ii: DHPG elicits a transient rise in \([\text{Ca}^{2+}]\), in prelimbic glia, even in buffer containing zero \(\text{Ca}^{2+}\), EGTA and AP5.

A Graph of ratio of fluorescent measurements taken at 340 and 380 nm plotted against time for a single glial cell. Cultured glia bathed in recording medium containing zero \(\text{Ca}^{2+}\), EGTA and AP5. Filled black horizontal bar indicates period during which 200 \(\mu\text{M DHPG}\) was bath applied to the field.

B Histogram displaying the 340/380 ratio as a % of control, in control bathing medium (hashed bar), in 200 \(\mu\text{M DHPG}\) at time of peak effect (white bar) and 105s after agonist application (black bar). Pooled data from 9 glia. Error bars are +/- SE
of agonist application. When data from all 9 glia are pooled, the mean rise in $[\text{Ca}^{2+}]_i$ was $393 \pm 107\%$ (SE) of control. This reaches levels of significance ($P = 0.028$, two-tailed paired $t$-test, $n=9$). DHPG is therefore capable of eliciting a rise in $[\text{Ca}^{2+}]_i$ in glia from cultures of rat medial frontal cortex, even in zero extracellular $\text{Ca}^{2+}$. 
6.1 Synaptic plasticity in the prelimbic region of rat medial frontal cortex.

Interaction between the hippocampus and the PFC may provide a crucial pathway whereby hippocampal structures direct aspects of cortical processing and thus act to stabilise the cortical representation of a learned association (Goldman-Rakic, 1987). LTP can be induced at the synapses between the hippocampal input and neurones in the prelimbic cortex in vivo (Laroche, 1990). In awake rats LTP at these synapses persisted for up to 3 days (Doyére et al, 1993), although there is some decline in the level of potentiation over this period. In the anaesthetised rat, LTP evoked in the hippocampal-prelimbic pathway lasts for at least several hours. We have demonstrated in our in vitro experiments that LTP can be induced at the monosynaptic connection between layers I/II and layer V in PFC. Four out of 8 neurones displaying LTP of the monosynaptic component exhibited potentiated synaptic transmission that remained undiminished even at 60 min after the completion of TBS. The LTPs observed in the remaining 4 neurones started to decay at times ranging between 36 and 56 min after TBS. Hirsch & Crépel (1990) also demonstrated, using in vitro recording, that some synapses in the PL region exhibited LTP of EPSP peak amplitude that persisted for up to 5 hours. This highlights an important issue, in that LTP in PL cortex appears to decay at a faster rate than most long-term memories. The decay would appear to rule out a LTP-like phenomenon as the neural basis of information storage on a long-term basis. In the hippocampus, this discrepancy is explained by the proposal that memories become reorganised over time, the hippocampal role in mnemonic processing diminishing and the role of other cortical
structures becoming more important (Zola-Morgan & Squire, 1990). The PL cortex is involved in a short-term working memory system utilised in spatial delayed response, spatial delayed alternation, delayed oculomotor response and spatial discrimination tasks (Kolb et al., 1974; Fuster, 1989; Funahashi et al., 1993; Kesner & DiMatteia, 1987). In addition, the PL region of the PFC forms part of a neural circuit through which spatial information is subsequently used to locate food on a radial arm maze after a 30 min delay (Floresco et al., 1997). The physiological basis of such a mnemonic mechanism would therefore only need to persist for 30 min. The observed LTP induced in the PL region therefore has a suitable duration for the performance of the described spatial tasks and this evidence implicates LTP in the PL cortex as a promising candidate for the neural basis of behavioural plasticity lasting over several hours rather than weeks.

6.2 Electrophysiological properties of layer V cells within PL cortex.

Although there is considerable variation in the microstructure of different regions of cortex, three principal types of pyramidal cells have been identified on the basis of their pattern of spike firing and other electrophysiological properties (Connors & Gutnick, 1990). The three classes are termed regular spiking (RS), intrinsic bursting (IB) and fast spiking (FS). RS neurones fire a single action potential in response to suprathreshold stimulation. At higher stimulus intensities, a train of spikes is elicited and each spike is followed by a prominent AHP that accounts for the observable spike frequency adaptation. The RS neurones have also been demonstrated on occasion to exhibit ADPs. IB neurones, on the other hand, fire a bursting pattern of spikes in response to stimulation and display a prominent DAP. FS neurones belong to a rare class of pyramidal cells that are characterised by the lack of either a prominent AHP, ADP or DAP. In the more recent study by Yang et al. in the PL region of PFC, the third of these
categories is referred to, not as FS neurones, but as intermediate (IM) neurones. Connor & Gutnick (1990) suggest that cells within the cortex actually express a continuous spectrum of properties and thus can fall between categories.

Our in vitro experiments confirm the findings of Connor & Gutnick (1990). We observed a single cell, initially classed as an IB neurone on the basis of the pattern of spike firing and the existence of a DAP, apparently switch to exhibit properties commonly displayed by RS neurones. The most abundant cell type in layer V of the rat PL region is the RS neurone (83%). The remaining neurones tested fell into the IB category (17%). Yang et al. (1996), who studied the properties of layer V neurones projecting exclusively to the nucleus accumbens, found that only 19% of neurones could be classed as RS cells, the remainder falling in either the IB (64%) or IM (4%) class. The difference between our findings and those of Yang et al. could be the result of the fact that stimulation at the border between layers I and II activates a distinct population of layer V cells from those that project to the nucleus accumbens. Alternatively, the angle at which their slices were cut was chosen to specifically preserve the axonal projections from the PFC to the nucleus accumbens and this could therefore alter the proportion of surviving cells.

6.3 Induction of LTP in prelimbic cortex.

Attempts at inducing LTP using theta burst stimulation (TBS) were successful in 57% of cells tested. TBS therefore represents a more effective induction protocol than the tetanic (50-200 Hz) stimulation used in previous studies (Hirsch & Crépel, 1990). Failure to induce a long-lasting enhancement of the field response following 5 trains of TBS is possibly the consequence of the level of depolarisation achieved in the population of layer V cells as a direct result of the excitatory driving during the TBS bursts. In
intracellular studies the level of excitation achieved during TBS can be both monitored and adjusted. In a small proportion of cells, depolarising current was applied through the intracellular electrode to ensure that the cell received sufficient excitation during TBS, thereby increasing the probability of inducing LTP. In extracellular studies it is not possible to monitor and regulate the level of depolarisation in the layer V cells in the same manner and therefore it is possible that the low incidence of LTP of the field response is related to the level of excitation achieved by the population of layer V cells as a consequence of TBS.

Alternatively, the stronger shocks needed to evoked field responses could also increase the level of inhibition within the slice and thus reduce the probability of inducing LTP. During low frequency stimulation, activation of the NMDA receptor system, essential for the induction of LTP, is modulated by GABAergic inhibition. Hyperpolarisation of the postsynaptic membrane enhances the Mg$^{2+}$ block of the NMDA receptor channel. When large amounts of glutamate are released, as in high frequency paradigms, the release of GABA from the interneurone population is depressed through a negative feedback mechanism utilising the GABA$_B$ autoreceptors (Davies et al., 1991; Mott & Lewis, 1991). A reduction in the level of inhibition, eleviates the Mg$^{2+}$ block and thereby permits the induction of LTP (Davies et al., 1991). The existence of such a regulatory mechanism makes it unlikely that increased levels of inhibition due to higher stimulus intensities account for the lack of LTP of field responses following TBS.

There are a number of possible explanations for the observed enhancement of the population spike in evoked field responses seen in LTP. It could reflect an increase in the number of presynaptic input fibres activated by the test shocks. If this were the case, however, one would expect to detect an increase in the presynaptic volley following TBS. This was not found to be the case in the hippocampus (Bliss & Lømo, 1973;
Andersen et al., 1977). Alternatively, it could reflect a decrease in the threshold for action potential firing. Once again, this cannot be detected experimentally (Andersen et al., 1977; Yamamoto & Chujo, 1978). Lastly, it could reflect either an increase in excitatory synaptic transmission or a depression of inhibitory synaptic transmission.

Yamamoto & Chujo (1978) showed that, following tetanic stimulation to the mossy fibre input to the CA3 hippocampal field, a population of CA3 neurones exhibited enhanced EPSPs, whilst a distinct population of cells exhibited depressed IPSPs. It is therefore likely that the observed changes in synaptic efficacy at the monosynaptic connection are the result of alterations in both excitatory and inhibitory synaptic transmission.

Repetitive afferent stimulation has been shown to uncover previously latent excitatory pathways in the hippocampus (Miles & Wong, 1987a, b). These excitatory pathways emerge as a consequence of a depression of inhibitory circuits within the cortex and result in synchronous firing in populations of previously unconnected neurones. Therefore, LTP expressed in a population of neurones can effectively spread to neighbouring cells, the consequence of which is the formation of associated groups of previously unconnected neurones.

In our intracellular in vitro experiments, LTP expressed in the monosynaptic component of the EPSP evoked in layer V cells is always also reflected in later components of the EPSP. In 2 cells in which no change in the monosynaptic component was seen, later, presumably polysynaptic, components of the EPSP exhibited LTP. The emergence of plasticity in a polysynaptic circuit without any enhancement of the monosynaptic connection suggests that the observed LTP is a property of activity in a population of interconnected neurones rather than in either the pre or postsynaptic compartments of a single connection. Alternatively, potentiation expressed in later components of the EPSP could reflect that the mechanisms underlying LTP expression
are voltage-sensitive e.g. NMDA receptor channels and/or VDCCs and that, therefore, a
certain level of depolarisation must be achieved before the channels expressing the
potentiation become activated. It is also possible that the population of fibres innervating
the cell, driving it to elicit an EPSP, are heterogeneous and therefore display a whole
spectrum of conduction velocities. If only the slower conducting fibres expressed LTP,
this would be manifested as a potentiation of solely the later components of the EPSP.

In our extracellular in vitro experiments, two parameters of the field response
were monitored: the slope and the peak-to-peak amplitude of the population spike.
Changes in the slope of the population spike were not always accompanied by changes in
the peak-to-peak amplitude of the population spike. On the other hand, changes in the
peak-to-peak amplitude were always also reflected in the slope measurement. A change
in the slope of the population spike can be equated with a change in the latency of the
population spike. An enhancement of the slope of the population spike could therefore
be either a manifestation of an increased level of cellular excitability or a greater
synchrony of firing within the neuronal population. Bliss & Lomb (1973) proposed that,
in the hippocampus, the population spike reflects the summed synchronous discharge of
a population of neurones and therefore an increase in its amplitude can represent a
number of possible changes in the slice. First, it could reflect an increased level of
excitability within the slice i.e. more neurones within the whole population of cells
overcoming the threshold for spike firing. Second, it could represent activity in the same
population of neurones, but with an enhanced strength of synaptic transmission. Third, it
could reflect a greater synchrony of neuronal firing. It is impossible based on the field
potential experiments to determine which of these factors accounts for the observed
increase in the population spike amplitude.
It is possible that the observed enhancement of the field response is E-S potentiation rather than a form of LTP. The currents generated at the excitatory synapse must propagate along the neuronal membrane to the axon hillock where an action potential is elicited if the threshold is reached. The efficiency with which the excitatory input at the level of the dendrites is relayed to the axon hillock is dependent on the space constant ($\lambda$) and hence the membrane resistance ($R_m$). Therefore if the $R_m$ increases due to, for example, the reduction of a membrane conductance, the current that reaches the axon hillock will be larger and thus more likely to exceed firing threshold and elicit a spike. The change in the field response would, therefore, be representative of a change in the coupling between EPSP and spike i.e. E-S coupling, which in turn is the direct consequence of a change in the membrane properties of the postsynaptic cell rather than a change in the strength of synaptic transmission. Unfortunately, in many of the field potential experiments, the layer V early positive wave is partially obscured by the preceding axonal volley. This makes it difficult to investigate the relationship between the field EPSP and the population spike. If the axonal volley were constant, it would be possible to measure the positive wave slope. However, the axonal volley exhibited a lot of variability within preparations and therefore no change in positive slope was detected.

6.4 Localisation of mGluRs in prelimbic cortex.

A major finding of our experiments is that group I specific mGluR agonists excite layer V cells in PL cortex. We tested for the presence of group I mGluRs in the medial frontal cortex of the male Sprague-Dawley rat by bath application of the broad-spectrum mGluR agonist ACPD, the group I mGluR-specific agonists S-3HPG and DHPG and the mGluR$_5$-specific agonist CHPG. All four agonists produced membrane depolarisation, an increased number of spikes elicited by a depolarising current pulse of fixed strength
and a reduction in the amplitude of the AHP seen following a train of spikes. These excitatory effects were not fully reversible on washout of the agonist. The AHP amplitude was still significantly smaller than control even after 20 min of washout of the drug. These experiments were then repeated in a preparation in which layer V cells were synaptically isolated using the GABAB receptor agonist baclofen. Once again, all mGluR agonists tested produced excitatory effects. These results indicate that group I mGluRs are present on layer V cells. The excitatory effects observed were considerably less dramatic in the presence of baclofen than in control conditions. This suggests that the mGluR present on layer V cells represent only a fraction of the total population of mGluRs mediating excitatory effects within the slice. Presumably in the absence of baclofen a large population of group I mGluRs become activated and this elicits widespread membrane depolarisation and an general increase in the level of excitability within the slice. When synaptic transmission is blocked in the presence of baclofen, the excitability of the layer V cells is no longer modulated by neural inputs from other cellular components of the cortex which may bring about a higher level of excitability nor by ionic changes or modulator-mediated changes resulting from widespread neural activity. The increased cellular excitability is therefore a direct consequence of group I mGluR activation on the layer V cells, rather than also an indirect effect of group I mGluR activation in neighbouring cells within the cortex.

An increase in input resistance on agonist application has been previously reported (Desai & Conn, 1991). We observed no consistent change in input resistance on application of any of the agonists tested. In cerebral cortex, mGluR activation leads to both a Ca\(^{2+}\) -mediated decrease in a resting K\(^+\) current and also a Ca\(^{2+}\) -dependent increase in an inward cation current (Greene et al., 1994). The opposing nature of these two mGluR-triggered conductance changes may explain our failure to see a consistent
change in input resistance, at a comparable membrane potentials, after the application of mGluR agonists.

There is evidence that group I mGluRs desensitise in response to prolonged or repeated agonist exposure (Gereau & Heinemann, 1998). We found evidence for receptor desensitisation in both our intracellular recordings and our imaging experiments. In 1 cell tested, the response to the group I mGluR specific agonist S-3HPG was shown to decrease on repeated exposure, despite the fact that the interval between the consecutive 10 min duration bath applications was as much as 40 min. Although it is not possible to draw any conclusions from the findings in a single cell, it is clear that the issue of desensitisation is of importance when designing experimental protocols using mGluR agonists.

6.5 MGlur involvement in synaptic plasticity.

The effect of MCPG on the induction of LTP in the hippocampus remains controversial. Our findings in the PL region of PFC support evidence previously reported in both the hippocampus (Bashir et al., 1993; O'Connor et al., 1994) and dentate gyrus (Riedel et al., 1994) and suggest a role for mGluRs in the induction of neocortical LTP. It is not surprising that different research groups produce conflicting results since mGluR activation represents only one of several pathways by which the production of LTP could be brought about. Subtle differences in experimental procedure or preparation used could well lessen or increase the importance of mGluRs in the induction process.

Our in vitro experiments showed that MCPG significantly reduced the incidence of LTP induction, as well as reducing the level of potentiation seen following TBS. LTP was induced in only one out of 14 neurones bathed in MCPG. The LTP had a magnitude and duration comparable to that seen in the absence of mGluR antagonism. The
emergence of LTP even under conditions of mGluR antagonism suggests that mGluRs are not absolutely required for the induction of LTP. It is plausible that some synapses may express a mGluR-independent form of LTP or that the involvement of mGluRs can be bypassed or substituted by other postsynaptic mechanisms.

Immunogold localisation reveals that, in both the hippocampus and the cerebellum, there is subsynaptic segregation of metabotropic and ionotropic glutamate receptors. The former are located beyond the perimeter of the synapse and the latter are found opposite the release sites in the main body of the synaptic junction (Nusser et al., 1996). A possible functional implication of this arrangement is that the mGluRs might only be activated when repetitive synaptic activity causes sufficient glutamate release so that it spills out of the synaptic region. In addition, as the distance from the synaptic site increases, the likelihood the receptor may be exposed to transmitter release from other terminals is also increased. The activation of mGluRs in response to synaptically released transmitter is often only detectable following repeated presynaptic activation (Charpak & Gahwiler, 1991; Batchelor & Garthwaite, 1997). This supports the view that in order for mGluR activation to be detectable, sufficient transmitter must be released from the presynaptic terminal so that it spills out of the synaptic region. In our in vitro experiments, preliminary studies investigating the effect of exposure to S-3HPG on the EPSP revealed that the enhancement of EPSP amplitude was considerably more dramatic on the response to the second of two shocks delivered in a paired-pulse stimulation protocol. This effect was not studied in a larger population of cells, however, since there was concern whether somatically injected current used to counterbalance the agonist-induced membrane depolarisation would efficiently clamp the membrane potential out at the dendrites. It is thus difficult to investigate the effect of S-3HPG on the EPSP, independent of the effects of S-3HPG on membrane conductances and cellular
excitability. However, these preliminary findings suggest, once again, that the involvement of mGluRs in modulating synaptic transmission only becomes critical when the stimulation parameters used release sufficient transmitter to spill out of the synaptic body.

We have demonstrated that group I mGluRs are involved in the induction of LTP in the PFC not only through the use of MCPG, but also through the use of the group I mGluR-specific agonist DHPG. We have shown that the activation of group I mGluRs by bath application of DHPG facilitates the induction of LTP. DHPG applied in conjunction with TBS elicits a long-lasting enhancement of the field response in 78% of slices tested. The probability of inducing LTP following TBS is therefore enhanced by simultaneous activation of group I mGluRs by DHPG.

6.6 Aspects of group I mGluR activity important for LTP induction.

There are numerous different consequences of group I mGluR activation that might prove important for the induction of LTP. First, their activation leads to the liberation of Ca\(^{2+}\) from IP\(_3\) -sensitive intracellular stores (see Pin & Duvoisin, 1994). Second, their activation triggers the activation of PKC via a DAG-dependent pathway. Third, they elicit excitatory effects on membrane conductances and cellular excitability producing membrane depolarisation, an increase in spike firing adaptation and a reduction in the amplitude of the AHP seen following a train of spikes (Charpak et al., 1990). Fourth, agonist-induced membrane depolarisation may increase Ca\(^{2+}\) influx into the neuronal interior via VDCCs and/or NMDA receptor channels. Furthermore, in pyramidal cells from the rat neocortex, mGluR activation leads to modulation of VDCC currents in a voltage-independent manner (e.g. Swartz & Bean, 1992). Fifth, their activation modulates NMDA receptor responses (e.g. Aniksztejn et al., 1992).
1992). Last, their activation has been demonstrated to modulate GABA-mediated transmission (Desai et al., 1994; Glaum et al., 1992).

It is well established that the induction of LTP requires both a transient elevation in dendritic \[\text{Ca}^{2+}\] and the activation of postsynaptic PKC. It is therefore very likely that the action of MCPG on LTP induction is a consequence of its ability to antagonise the excitatory effects of mGluR activation on increased intracellular \[\text{Ca}^{2+}\] via IP\(_3\) and PKC activation via DAG. For example, MCPG has been demonstrated to reduce the size of the \[\text{Ca}^{2+}\] transient evoked by tetanic stimulation in CA1 pyramidal cells clamped at -35 mV (Frenguelli et al., 1993) whilst either producing a small reduction or no effect on synaptic currents evoked by the tetanus. This demonstrates that mGluR activation contributes to a postsynaptic increase in \([\text{Ca}^{2+}]_i\) following trains of afferent action potentials. In our imaging experiment, we have shown, in hippocampal cultures, that MCPG reduces the \[\text{Ca}^{2+}\] rise elicited by mGluR activation on application of both ACPD and S-3HPG. MCPG was also demonstrated to reduce the rise in \([\text{Ca}^{2+}]_i\) induced by the application of glutamate in zero \[\text{Ca}^{2+}\] buffer containing both the \[\text{Ca}^{2+}\] chelator EGTA and the NMDA receptor antagonist AP5. Thus the innate excitatory transmitter in the cortex can also bring about a MCPG-sensitive release of \[\text{Ca}^{2+}\], presumably from intracellular stores. The ability of MCPG to reduce the \[\text{Ca}^{2+}\] signal evoked by mGluR activation may well therefore represent one possible mechanism through which it effects the induction of LTP.

A number of different synaptic modification rules have been proposed that suggest that the magnitude of the postsynaptic depolarisation (Bienenstock et al., 1982) and / or the postsynaptic rise in \([\text{Ca}^{2+}]_i\) (Lisman, 1989; Artola & Singer, 1993) determines the production and direction of synaptic change. These hypotheses propose that the greater the postsynaptic depolarisation and hence the greater the rise in postsynaptic \([\text{Ca}^{2+}]_i\), the
greater the likelihood of inducing LTP. Any experimental protocol that increases the 
$[Ca^{2+}]_i$ would therefore be expected to increase the probability of eliciting LTP.

There are two consequences of DHPG application that might be responsible for the 
observed effect of the agonist on LTP induction. We showed that DHPG elicits a 
transient rise in $[Ca^{2+}]_i$ in the absence of afferent stimulation and in zero $Ca^{2+}$ buffer. In 
addition, using intracellular recordings from layer V cells we have shown that DHPG 
evokes membrane depolarisation, a decrease in spike firing adaptation and a reduction in 
the amplitude of the AHP. It thus increases cellular excitability as well as triggering $Ca^{2+}$ 
entry via voltage-dependent pathways. Both of these consequence of group I mGluR 
activation influence the $[Ca^{2+}]_i$ and therefore could explain the increased incidence of 
LTP following TBS in the presence of DHPG.

Wilsch et al. (1998) propose that under conditions in which the $Ca^{2+}$ signal 
generated by $Ca^{2+}$ entry through NMDA receptor channels and VDCCs is subthreshold 
for LTP induction, mGluR activation can boost the postsynaptic $Ca^{2+}$ signal and thus 
facilitate the induction of LTP. We have demonstrated in cultured hippocampal neurones 
that activation of mGluRs leads to a transient elevation in $[Ca^{2+}]_i$. This rise is 
independent of $Ca^{2+}$ entry following agonist-induced membrane depolarisation as it can 
be produced even when the NMDA receptor channel is blocked by AP5 and $Ca^{2+}$ entry 
through VDCCs is eliminated by the removal of $Ca^{2+}$ from the bathing medium. A similar 
result was shown in cultures of medial frontal cortical neurones in zero $Ca^{2+}$ buffer. The 
contribution of the mGluR-induced $Ca^{2+}$ rise to the overall $Ca^{2+}$ signal induced by 
synaptic stimulation may be crucial only when stimulation paradigms close to threshold 
are used for LTP induction.

Postsynaptic group I mGluRs enhance cell excitability and hence $Ca^{2+}$ entry by way 
of membrane depolarisation (Guerineau et al., 1994), a decrease in spike firing
adaptation and a reduction in a Ca\(^{2+}\)-dependent K\(^+\) current underlying the AHP (Charpak et al., 1990). We have confirmed the action of group I mGluRs on layer V cells of PL cortex, even in the presence of baclofen. The ability of MCPG to block the excitatory effects of group I mGluR agonists was tested to elucidate another possible mechanism whereby it may reduce the incidence of LTP induction. Although some attenuation of the excitatory effects elicited by S-3HPG was seen in the presence of MCPG, the agonist was observed to still produce membrane depolarisation and increased cellular excitability. S-3HPG (200 \(\mu\)M) produced a substantial reduction in the AHP amplitude in only one out of 4 cells tested, which suggests that MCPG (500 \(\mu\)M) is capable of partially antagonising the excitatory effects of mGluR activation. However, in our small sample, the data does not reach levels of significance. The effect of MCPG on the induction of LTP is unlikely, however, to be the consequence of a dampening of the level of excitability within the slice. Using the number of spikes elicited during TBS as a measure of the level of excitation achieved as a consequence of TBS, no significant difference in the level of excitability within the slice was detected between those cells bathed in normal ACSF versus ACSF containing MCPG. However, the ability of MCPG to reduce excitatory group I mGluR-mediated effects is consistent with our experimental results on LTP induction in the presence of MCPG and indirectly supports, mGluR involvement in the induction of LTP.

Interestingly, the degree of recovery observed following washout of the agonist was significantly different between those cells exposed to S-3HPG in ACSF containing MCPG and those bathed in control ACSF. As in the presence of baclofen (see section 6.4), bath application of MCPG conferred the ability for a complete reversal of the excitatory effects on washout of the drug not seen in ACSF alone.
Activation of mGluRs has also been demonstrated to trigger the activation of PLD (Boss & Conn, 1992; Holler et al., 1993; Pellegrini-Giampietro et al., 1996; Klein et al., 1997), a compound linked to the production of DAG and the subsequent activation of PKC. MCPG has been shown to inhibit the PLD response elicited by glutamate (Klein et al., 1997) or ACPD (Pellegrini-Giampietro et al., 1996). However, even when applied by itself, MCPG (0.1-1 mM) induces a significant PLD response. MCPG should thereby act to increase, rather than decrease the likelihood of LTP induction. We can therefore rule out a role for PLD inhibition in our LTP experiments.

MCPG is a potent antagonist of mGluR$_1$, but has only minor antagonistic actions on mGluR$_5$. This is of particular concern since immunostaining of the frontal cortex of the adult rat reveals that mGluR$_1$ is mostly confined to non-pyramidal neurones in layer II/III. The obvious discrepancy may be the result of an action of MCPG on another, yet undescribed subtype of mGluR. Alternatively, MCPG might reduce the incidence of LTP through an network action, modulating activity via the mGluR$_1$ subtype, during TBS, within medial frontal cortical cells other than the layer V pyramidal cells.

6.7 Effects of mGluR antagonism on synaptic transmission.

MCPG has an antagonistic action on both group I and group II mGluRs (Pin & Duvoisin, 1994) and therefore might alter both excitatory and inhibitory activity in PL cortex. MCPG acting presynaptically might increase transmitter release (Baskys & Malenka, 1991), whereas acting postsynaptically it might reduce the NMDA receptor mediated component of the EPSP (Lu et al., 1997). In the hippocampus, MCPG has no effect on synaptic responses evoked by LFS that are mediated by AMPA, NMDA, GABA$_A$ or GABA$_B$ receptors (Bashir et al., 1993). In the medial frontal cortex, we saw no evidence for a significant depressant or facilitatory action of MCPG on single test
EPSPs. mGluRs have been shown to modulate NMDA (e.g., Fitzjohn et al., 1996) and GABA receptor-mediated (Desai et al., 1994) responses. Activation of mGluRs can therefore modulate the same currents as those that underlie the EPSP. However since these modulatory actions are possibly only of significance following repetitive synaptic stimulation when sufficient glutamate spills out to activate peripherally located mGluRs, one would not expect any detectable effect on single evoked responses. In accord with this expectation we found that the single EPSPs generated in layer V cells do not have a MCPG-sensitive component.

For the effect of an antagonist on NMDA responses to be demonstratable, both mGluR and NMDA receptors must be in an activated state to begin with. We demonstrated that MCPG had an effect on the peak amplitude of an isolated NMDA receptor mediated EPSP. Much stronger stimulation intensities were needed to evoke NMDA receptor mediated EPSP than for mixed AMPA/NMDA EPSP. The consequence of this is that glutamate released at the synapse is more likely to spill over to activate peripherally located mGluRs and therefore the depressant action of MCPG on the NMDA receptor can be revealed. In addition, a lower extracellular $[\text{Mg}^{2+}]$ was used and therefore a greater proportion of NMDA receptor channels are likely to have been in an open state. Both of these aspects of the experimental design increase the likelihood of simultaneous mGluR and NMDA receptor activation and therefore allowed us to investigate the modulatory action of mGluRs of NMDA receptor mediated currents. Our findings indicate that one of the possible ways in which MCPG reduces the incidence of LTP induction in the medial frontal cortex is through a reduction in the current mediated by NMDA receptors. TBS is a stimulation protocol that features high frequency repetitive firing. mGluR antagonism might be expected to dampen the level of excitability within the preparation through both an effect on spike accommodation.
leading to a reduced number of spikes per burst during TBS and an effect on the currents
mediated by the NMDA receptor channels. The action of MCPG on the level of
excitation within the slice during TBS was investigated by counting the number of spikes
resulting from each stimulus burst in the presence or absence of MCPG. There was no
evidence of a significant change of firing rate during TBS in MCPG compared with that
in ACSF. Presumably, the effects of MCPG on the NMDA receptor-mediated currents
are only observed in conditions of low extracellular [Mg$^{2+}$].

The lack of effect of MCPG on either test shocks or the global level of excitation
indicates that the action of MCPG in reducing the incidence of LTP in the PL cortex is
not likely to be a direct consequence of altered synaptic transmission or membrane
properties of the neurones. This is supported by the observation that MCPG itself has no
effect on membrane potential, input resistance or spike frequency adaptation (Davies et
al., 1995).

6.8 Further experiments.

The involvement of group I mGluRs in the induction of LTP could be further
examined by an investigation of the effect of DHPG application on the incidence of TBS-
induced LTP using intracellular techniques. This would support data already obtained
from extracellular field recordings. An investigation of both the effect of the mGluR$_5$-
specific agonist CHPG on the induction of LTP and the ability of MCPG to antagonise
mGluR$_5$-mediated excitatory effects would further elucidate the role of the mGluR$_5$
subtype in LTP induction.

The Ca$^{2+}$ imaging experiments could be extended to include a more
comprehensive study of the effects of mGluR agonists and antagonists on [Ca$^{2+}$]$_i$ in
mixed neuronal/glial cultures of prelimbic cortex. Further experiments using
cyclopiazonic acid (CPA), a compound capable of depleting intracellular Ca\(^{2+}\) stores, would confirm the source of Ca\(^{2+}\) that elicits the rise in \([\text{Ca}^{2+}]_i\), seen on application of mGluR agonists.

The role of PKC in the induction of LTP in the medial frontal cortex could be investigated. The ability, for example, of phorbol ester, a potent PKC activator, to facilitate the induction of LTP could be examined. Alternatively, the effect of one of a number of PKC inhibitors on the incidence of LTP in medial frontal cortex could be tested.
REFERENCES


