Metabotropic Glutamate Receptors and Synaptic Transmission in the Cerebellar Cortex

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Thesis submitted in fulfilment of the degree of Doctor of Philosophy, University College London (Wolfson Institute of Biomedical Research)

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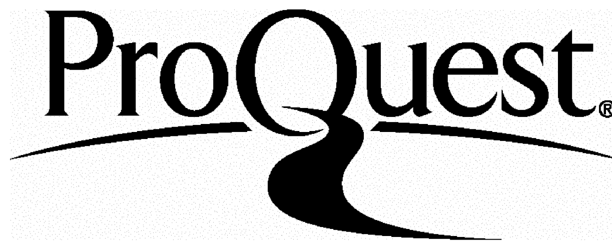

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Abstract

The metabotropic glutamate (mGlu) receptors are a family G-protein-coupled receptors; the 8 members of which are divided into 3 groups. In the rat cerebellum, pre- and postsynaptic mGlu receptors exist at the parallel fibre-Purkinje cell synapse, and exogenously-applied agonists can depress glutamatergic synaptic transmission both at this synapse, and at others in the CNS. There is, however, relatively little evidence that the synaptically released agonist has these actions. Using an *in vitro* slice preparation and single cell electrophysiological recording techniques, the principle aim of the research described in this thesis has been to identify conditions under which these receptors might be activated by synaptically released agonist.

Using agonists and antagonists of the mGlu receptors that act differentially on the 3 groups of mGlu receptors expressed in heterologous expression systems, it was found that activation of the group I and group III receptors caused a reversible reduction in the AMPA receptor-mediated EPSP amplitude, and appropriate antagonists inhibited these effects. Agonists of the group II receptors had no effect on transmission at this synapse.

Brief tetanic stimulation of the parallel fibres was found to result in a depression of synaptic transmission that lasted for several seconds after the end of the tetanus, a phenomenon we have termed post-tetanic depression. Through the use of selective antagonists, this novel form of synaptic depression was found to be mediated by $GABA_B$ receptors and group I mGlu receptors. These receptors are probably located pre- and postsynaptically respectively.

It is concluded that the group I and III mGlu receptors can depress excitatory synaptic transmission in the rat cerebellum. In addition, the results indicate that postsynaptic group I mGlu receptors have a previously undescribed role in short-term synaptic plasticity. These observations provide the first evidence that the group I mGlu receptors may be activated by synaptically released agonist to depress synaptic transmission.

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Chapter 1: Introduction

Glutamatergic Synaptic transmission

The transfer of information between neurones is, in the majority of cases, conducted via chemical neurotransmitters, which are released from the presynaptic terminals of one cell, and trigger a response by binding to postsynaptic receptors embedded in the plasma membrane of another cell. The main excitatory neurotransmitter in the mammalian CNS is the acidic amino acid L-glutamate, which plays a role in a large number of neuronal activities, and is also implicated in a number of pathological conditions, such as neurodegeneration (Meldrum & Garthwaite, 1990). Although glutamate is now universally accepted as a neurotransmitter, establishing this was initially difficult as the amino acid also plays important role in cellular metabolism. Glutamate is formed from glucose through the Krebs cycle and may also be formed directly from glutamine, which is synthesised in glial cells. In common with other transmitters, glutamate is stored in nerve terminals in vesicles, which release their contents in a Ca^{2+}-dependent manner. It is estimated that each vesicle releases some 4000-5000 glutamate molecules into the synaptic cleft (Riveros *et al.*, 1986; Burger *et al.*, 1989; Bruns & Jahn, 1995).

The glutamate concentration in the synaptic cleft is controlled by diffusion and reuptake by transporters (Bergles *et al.*, 1999). To date 5 different glutamate transporters have been cloned. Of these GLT-1/EAAT2 and EAAC1/EAAT3 are observed throughout the CNS in glial and neuronal cells respectively (Attwell, 2000). The other transporters

have a more restricted distribution: GLAST/EAAT1 is found in retinal Muller cells and cerebellar Bergmann glia; EAAT4 is in Purkinje cells and EAAT5 is in retinal photoreceptors and bipolar cells. Glutamate tends to be at a higher concentration inside the cell compared to the extracellular space and is negatively charged in solution, thus uptake is not a passive process. The energy for uptake is provided from the cotransport of ions down their electrochemical gradients. In addition to glutamate, Na^+ and H^+ ions enter the cell, and K^+ ions leave. This movement of charged species creates a net entry of positive charge and generates a detectable current (Takahashi *et al.*, 1996a). The presence of these transporters allows the basal glutamate concentration to be maintained at low levels, and at some synapses plays a role in terminating the synaptic response. For example, at the cerebellar parallel fibre-Purkinje cell synapse where the excitatory post-synaptic current (EPSC) decay is prolonged in the presence of blockers of glutamate uptake, such as L-trans-pyrrolidine-2,4-dicarboxylic acid (Barbour *et al.*, 1994).

Glutamate receptors

Ionotropic Glutamate Receptors

Glutamate acts on two broad classes of receptor: ionotropic and metabotropic. The ionotropic (iGlu) receptor family can be pharmacologically divided into three subtypes: AMPA, kainate and NMDA receptors. Glutamate binding to these receptors typically mediates fast synaptic transmission by the opening of an integral cation channel, allowing an influx of positive ions producing an EPSC. It should be noted that the classification of AMPA, kainate and NMDA receptors as ionotropic may be misleading

as recent evidence suggests that the AMPA and kainate receptors may exert some of their effects by coupling to G-proteins (Rodriguez-Moreno & Lerma, 1998; Wang *et al.*, 1999).

AMPA Receptors

The AMPA receptors are formed from 4 subunits, GluR1 to 4, each of approximately 900 amino acids with ~70% sequence homology (Hollmann & Heinemann, 1994). The subunits exist in 2 different forms, flip and flop, generated by alternative splicing. During development the expression of the variants alters, with the flip form dominating prior to birth, and flop expression increasing after. When stimulated by glutamate, heteromeric receptors containing only the flop variant produce peak and steady-state currents some five times smaller than those produced by receptors containing only the flip variant (Sommer *et al.*, 1990). This has lead to the proposal that a switch in expression from flop to flip might play a role in long-term potentiation (Sommer *et al.*, 1990). Each of the subunits can form functional homomeric channels, although homomeric GluR2 receptors produce only a small current and have a different current/voltage relationship compared to the other homomeric receptors. In addition, GluR2 containing receptors are impermeable to Ca^{2+} and the presence of this subunit is postulated to determine Ca^{2+} permeability of the AMPA receptors. Neurones express multiple AMPA receptor subunits, for example Purkinje cells express mRNA for GluR 1 and 2 flip and flop and GluR3 flip, with GluR2 being must abundant (Lambolez *et al.*, 1992; Bochet *et al.*, 1993). The exact subunit composition of native receptors is not known, although the existence of Ca^{2+}-permeable receptors in some neurones suggests that at least some AMPA receptors might not contain the GluR2 subunit (Jonas & Burnashev, 1995).

The main function of the AMPA receptors is to mediate fast synaptic transmission. Examples of presumed AMPA EPSCs and excitatory post-synaptic potentials (EPSP) at the cerebellar parallel fibre-Purkinje cell synapse are illustrated in Chapters 3 - 5 of this thesis. AMPA receptor-mediated synaptic currents typically have a rapid rise time (< 2 ms) and rapid decay. The dynamics of the AMPA current are dictated by a range of factors, and differ between synapses; for example, due to the prolonged presence of glutamate in the synaptic cleft, the parallel fibre-Purkinje cell synapse EPSC is relatively long-lasting, decaying with a time constant of 6.1 ms, compared to parallel fibre-interneurone EPSCs which decay with a more rapid time constant of 1.3 ms (Llano *et al.*, 1991; Barbour *et al.*, 1994). In addition to their postsynaptic role, there is evidence that AMPA receptors may exert a presynaptic modulation of transmitter release (Satake *et al.*, 2000).

Kainate Receptors

The kainate receptor family consists of 5 subunits GluR5, GluR6, GluR7, KA1 and KA2, and this number is expanded with a number of splice variants. GluR5-7 share ~75% sequence homology and show relatively low kainate affinity compared to KA1 and 2, which share 68% homology (Chittajallu *et al.*, 1999). In expression systems functional homomeric receptors are formed from GluR5, 6 and 7, although those formed from GluR7 exhibit low agonist potency. KA1 and 2 do not form functional homomeric receptors. The exact subunit composition of native receptors is unclear, although the development of pharmacological agents that can distinguish between

different homomeric and heteromeric receptors, in combination with molecular techniques, will hopefully improve this situation.

Identifying physiological roles for the kainate receptors has recently been facilitated by pharmacological developments that have allowed kainate and AMPA receptor activities to be discriminated. Postsynaptic kainate receptor-mediated synaptic currents have been identified following tetanic stimulation (Vignes & Collingridge, 1997; Castillo *et al.*, 1997) and, presynaptically, there is evidence that transmitter release at both excitatory and inhibitory synapses may be regulated by these receptors (Chittajallu *et al.*, 1996; Clarke *et al.*, 1997).

NMDA Receptors

The first cloned NMDA receptor subunit was NMDAR1 and this is capable of forming functional homomeric receptors. At least 8 splice variants of the NMDAR1 subunit have been identified. Receptors formed from these variants show differences in, for example, their response to zinc and polyamines, thus potentially increasing the functional diversity of the receptor (Hollmann & Heinemann, 1994). Subsequent to cloning NMDAR1, 4 other subunits (NMDAR2A-D) were identified. None of these subunits form functional homomeric receptors, however, when expressed with NMDAR1, functional receptors are formed, with different properties compared to homomeric NMDAR1 receptors. Compared to AMPA receptors (EC_{50} 500-1000 μM), NMDA receptors are activated by glutamate at much lower concentrations with an EC_{50} of less then 3 μM. The AMPA and NMDA receptors also differ in the kinetics of their synaptic currents. NMDA EPSCs are much slower to peak (10-20 ms) due to slow activation of the receptor, and produce a longer-lasting current (Edmonds *et al.*, 1995)

An important feature of the NMDA receptors is that they are permeable to Ca^{2+}, with a permeability ratio compared to Na^{+} ions of 10:1, and some 7% of the NMDA receptor current is carried by Ca^{2+} ions (Edmonds *et al.*, 1995). Ca^{2+} ions activate a number of cellular processes, and NMDA receptors are implicated in physiological activity, beyond direct synaptic transmission between neurones. One area that has attracted particular interest is the role of NMDA receptors in long-term potentiation (LTP). Donald Hebb (1949) developed the highly influential hypothesis that learning required a change in synaptic strength that would only occur when pre- and postsynaptic elements were simultaneously active. At resting membrane potentials NMDA receptors conduct relatively poorly due to voltage-dependent block of the receptor channel by physiological concentrations of extracellular Mg^{2+}. If the postsynaptic cell is depolarised the Mg^{2+} block is relieved and glutamate released from the presynaptic terminals can activate the receptor leading to Ca^{2+} influx and activation of messengers involved in LTP development. Thus, by responding to the simultaneous activity of pre- and postsynaptic cells (i.e. depolarisation and glutamate release respectively) the NMDA receptors may be the molecular coincident detector necessary for Hebbian learning to occur.

Like AMPA receptors, the NMDA receptors are typically viewed as mediating postsynaptic responses, but they have also been identified presynaptically where they act to regulate transmitter release. For example, in rat cerebellar slices, NMDA application results in an increase in the frequency of mini inhibitory postsynaptic currents recorded in Purkinje basket, and stellate cells, indicating a presynaptic action on GABA release (Glitsch & Marty, 1999).

Metabotropic Glutamate Receptors

The range of actions of glutamate are greatly expanded by the existence of a second class of receptors, the metabotropic glutamate (mGlu) receptors. These are typically coupled to G-proteins and induce longer-lasting modifications to neuronal activity compared to many of the ionotropic receptor-mediated events.

The first evidence of a mammalian mGlu receptor was provided by the observation that glutamate stimulated inositol phosphate production in cultured striatal neurones, hippocampal slices and cultured cerebellar granule cells (Sladeczek *et al.*, 1985; Nicoletti *et al.*, 1986a; Nicoletti *et al.*, 1986b). In a subsequent study using *Xenopus* oocytes injected with rat brain mRNA, it was demonstrated that this action of glutamate was mediated through an interaction with G-proteins (Sugiyama *et al.*, 1987). The first cloned mGlu receptor was reported independently by two groups using an assay involving *Xenopus* oocytes. RNA, synthesized *in vitro* from pools of rat cerebellar cDNA clones, was injected into the oocytes. The oocytes were then monitored for changes in an endogenous Ca^{2+} -activated chloride conductance induced by inositol 1,4,5-triphosphate (IP_3)-mediated release of Ca^{2+} from internal stores. By successively dividing the pools of cDNA that gave positive results a single cDNA clone, the mGlu1a receptor, was isolated (Houamed *et al.*, 1991; Masu *et al.*, 1991). By using the mGlu1a receptor sequence either as a probe to screen cDNA libraries, or to design degenerate primers for PCR, an additional 7 related receptor genes have been identified (Okamoto *et al.*, 1926; Abe *et al.*, 1992; Tanabe *et al.*, 1992; Nakajima *et al.*, 1993; Saugstad *et al.*, 1994). In addition to these 8 receptors a number of splice variants have been identified for receptors mGlu1, 4, 5, 7 and 8 (Schoepp *et al.*, 1999).

On the basis of sequence homology the receptors have been divided into three groups: group I contains receptors 1 and 5, group II receptors 2 and 3 and group III contains receptors 4, 6, 7 and 8. Within the groups, the receptors share about 70% sequence homology and across the groups about 45% (Pin & Duvoisin, 1995). The mGlu receptors are postulated to have seven transmembrane regions, but do not share sequence homology with previously identified seven transmembrane region G-protein linked receptors. These receptors are thus part of a new receptor family, also containing the calcium-sensing receptor originally isolated from a bovine cDNA (Brown *et al.*, 1993), $GABA_B$ receptors (Kaupmann *et al.*, 1997) and vomeronasal receptors (Bargmann, 1997).

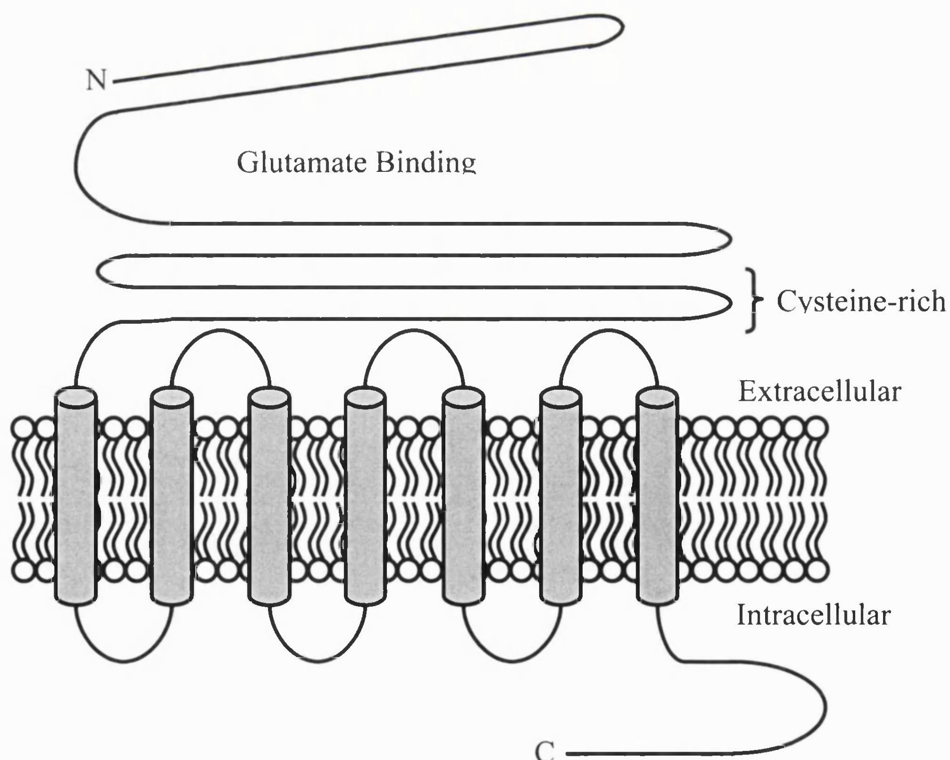


Figure 1.1. Basic structure of a metabotropic glutamate receptor

A receptor expressed in *Xenopus* oocytes from salmon cDNA has been found to have sequence homology with mGlu receptors. This receptor is activated by either glutamate or extracellular Ca^{2+} to produce an increase in IP_3, and represents the first example of a G-protein couple receptor having two distinct endogenous agonists (Kubokawa *et al.*, 1996). It has subsequently been found that mammalian group I mGlu receptors are also activated by Ca^{2+} at concentrations expected to occur in the synaptic cleft (Kubo *et al.*, 1998). The physiological significance of these findings is presently unclear, although it is suggested that the persistent activation of the receptor by Ca^{2+} might be important in the maintenance of long-term synaptic plasticity. The $GABA_B$ receptors, whilst not being activated by Ca^{2+}, do show increased sensitivity to GABA in the presence of Ca^{2+} (Wise *et al.*, 1999).

Functional mGlu receptors may exist as dimers as the mGlu receptors 1, 2 , 3 ,4 and 5 all migrate as apparent dimers on non-reducing gels. The group I receptor dimers are converted to monomers by reducing agents, and are postulated to be formed by disulphide bonds between cysteine residues located in the extracellular N-terminal domain (Romano *et al.*, 1996; Romano *et al.*, 1998). Dimers of group II and III receptors persist in the presence of reducing agents, and may therefore involve other linkages such as ester bonds (Romano *et al.*, 1998). The dimerisation may be necessary for receptor function as mGlu5-mediated increases in Ca^{2+} concentration were absent in the presence of reducing agent, whilst Ca^{2+} mobilisation by unrelated G-protein coupled receptors (GPCRs) was unaffected (Vignes *et al.*, 1992). The $GABA_B$ receptors may also exist as dimers as in expression systems fully functional $GABA_B$ receptors are only formed when the GBR1 and 2 subunits are coexpressed, and they are believed to form

heterodimers through an interaction at their intracellular carboxy-terminals (Jones *et al.*, 1998; White *et al.*, 1998; Kaupmann *et al.*, 1998a).

Agonist Binding and G-Protein Activation

For many G-protein coupled receptors (GPRCs), ligand binding occurs in a pocket formed by the ring of the seven transmembrane sections, but this does not appear to be the case for the mGlu receptors. All the cloned mGlu receptors have a large extracellular N-terminal domain which bears some sequence homology with bacterial periplasmic binding proteins, sharing the closest homology with the leucine, isoleucine, valine binding protein (LIVBP). Based on this similarity, O'Hara *et al* (1993) constructed a three-dimensional model for the predicted glutamate-binding site. The model predicts that the extracellular domain of mGlu1 receptor forms two globular regions joined by a hinge region where glutamate binds. It is suggested that glutamate binding results in the closing of the "jaws" of the extracellular domain. This conformational change in the protein may then be transmitted to the transmembrane regions via this cysteine-rich region, leading to G-protein activation (Pin & Bockaert, 1995). The iGlu receptors also exhibit homology with the bacterial proteins; thus, the metabotropic and ionotropic receptors may share common mechanisms for ligand binding (O'Hara *et al.*, 1993).

In other GPRCs the third intracellular loop appears to be involved in the specificity of G-protein coupling (Hulme, 1990). Across the mGlu receptor family this region is highly conserved so is unlikely to play such a role. The construction of chimeric receptors shows that in the mGlu receptors the predicted second intracellular loop plays

a key role in specificity of coupling to G-proteins (Pin *et al.*, 1995; Gomeza *et al.*, 1996a). The splice variants mGlu1a, b, and c differ in the length and sequence of their C-terminal, and these three receptors show differences in the kinetics of evoked release of Ca^{2+} from internal stores and in their sensitivity to PTX (Pin *et al.*, 1992; Pickering *et al.*, 1993). It is therefore suggested that the intracellular C-terminal also plays a role in transduction mechanisms of the mGlu receptors, and studies with chimeric receptors demonstrate that this region is involved with G-protein coupling (Pin *et al.*, 1994). The C-terminal domain also has numerous serine and threonine residues so provides a means of regulating receptor activity by kinases; for example, the phosphorylation of the threonine residue at position 840 of mGluR5a by PKC is responsible for the generation of Ca^{2+} oscillations in cells expressing mGluR5a (Kawabata *et al.*, 1996). These observations provide evidence that the splice variants may provide additional functional diversity, beyond that already provided by the eight cloned receptors.

Transduction Mechanisms

G-proteins are heterotrimers, consisting of an α, β and γ subunit. When inactive, the α subunit is bound to a molecule of guanosine 5'-diphosphate (GDP) and is associated with the other two subunits. Upon agonist binding to a receptor a conformational change occurs, resulting in the α subunit having a reduced affinity for GDP, and the GDP is therefore exchanged for guanosine 5'-triphosphate (GTP), which is at much higher concentrations in the cytosol. GTP binding to the subunit has two effects: firstly, the G-protein dissociates into 2 parts, GTP-α and $\beta\gamma$ subunits; secondly, the α subunit will dissociate from the receptor. The α and $\beta\gamma$ subunits can then activate a range of messenger processes through out the cell. The dissociation from the activated receptor

allows additional G-proteins to be activated, and in this way the signal produced by activation of a membrane receptor can be amplified in the cell.

The α subunit is a GTP-ase and hydrolysis of the GTP to GDP allow the 3 subunits of the G-protein to re-associate and the G-protein thus deactivates. The GTP hydrolysis by purified α subunits occurs slowly; for example, *in vitro* the G_α subunit of the G-protein transducin has a turnover number of 1-2 min^{-1}. This is far too slow to account for physiological responses of mammalian rods and cones which recover from light activation in less than 1 s (He *et al.*, 1998). A group of proteins, the regulators of G-protein signalling (RGS) proteins, have been identified which share a 120 amino acid domain and bind to activated G_α subunits (Hepler, 1999). RGS proteins have been identified that interact with a number of different G_α subunits, and this interaction results in an acceleration of the GTPase activity of the subunit (Hunt *et al.*, 1996; Watson *et al.*, 1996; He *et al.*, 1998). The existence of these proteins explains the reported discrepancies between the time course of physiological events and the rate of α subunit GTPase activity and they are likely to play an important role in regulating the duration of signals mediated by GPCRs.

A family of novel dendritic *Homer* proteins has been identified which contain a single PDZ-like domain that binds specifically to the C-terminus of group I mGlu receptors (Brakeman *et al.*, 1997). PDZ proteins have been identified which interact with iGlu receptors and ion channels and suggested roles for these proteins include: organising and anchoring the proteins in the membrane, and assisting in the assembly of signal transduction complexes at postsynaptic sites (Kornau *et al.*, 1995; Kim *et al.*, 1995; Niethammer *et al.*, 1996). The *Homer* proteins are suggested to play a role in trafficking

and locating the mGlu receptors in the membrane (Brakeman *et al.*, 1997; Roche *et al.*, 1999). It has also been found that group I mGlu receptors and IP_3 receptors from brain tissue coimmunoprecipitate with *Homer* (Tu *et al.*, 1998). Locating the mGlu and IP_3 receptors in close proximity by *Homer* allows for more efficient Ca^{2+} signalling as cultured Purkinje cells transfected with a variant of *Homer* lacking the region that interacts with the mGlu receptors exhibit quisqualate-evoked Ca^{2+} responses of reduced amplitude and increased latency to onset. In addition, there is evidence that the expression of *Homer* is modulated by physiological synaptic activity (Brakeman *et al.*, 1997); thus, activities involving mGlu receptors, such as synaptic plasticity may be subject to highly complex regulation involving *Homer*, RGS and other proteins (Xiao *et al.*, 1998; Tu *et al.*, 1999).

The classification of the mGlu receptors into three groups based on their sequence homology is supported by their transduction mechanisms. In common with many other GPCRs, group I mGlu receptors in expression systems, cultured neurones and brain slices stimulate phospholipase C (PLC) and phosphoinositide hydrolysis (Pin & Duvoisin, 1995). The enzyme PLCβ is stimulated via activation of G-proteins containing an α subunit from the pertussis (PTx) and cholera toxin (CTx)-insensitive α_q class (including α_q, α_{11}, α_{14}, α_{15} and α_{16} (Hepler & Gilman, 1992)) or the $\beta\gamma$ subunits from G-proteins formed with the PTx-sensitive α_i subunit (α_{i1}, α_{i2}, α_{i3}, α_{oA}, α_{oB}, α_g and α_z (Hepler & Gilman, 1992)). Activated PLC hydrolyses the membrane phospholipid phosphatidylinositol 4,5-biphosphate (PIP_2) to produce the hydrophobic diacylglycerol (DAG) and water-soluble IP_3. DAG activates protein kinase C (PKC) and IP_3 stimulates release of Ca^{2+} from internal stores via activation of the IP_3 receptors. IP_3 and PKC are important second messengers that have been implicated in numerous physiological

activities, such as synaptic plasticity (Linden & Connor, 1991), and Ca^{2+} signals, mediated by synaptically activated mGlu receptors and IP_3 production, have been demonstrated (Takechi *et al.*, 1998; Finch & Augustine, 1998).

Adenosine 3',5'-monophosphate (cAMP) is synthesised from ATP by the enzyme adenylate cyclase (AC) which was the first enzyme identified that is linked to G-protein stimulation. AC is stimulated by G_s and inhibited by G_i containing G-proteins (Simonds, 1999). The group II and III mGlu receptors are negatively coupled to adenosine 3',5'-monophosphate (cAMP) production. This is PTX sensitive and is therefore presumed to be via coupling to a Gi containing G-protein. In CHO and BHK cells the group III inhibition of cAMP formation is relatively weak, so this is perhaps not their main transduction mechanism (Conn & Pin, 1997).

In addition to the ability to stimulate PLC and decrease cAMP, a number of other transduction mechanisms have been reported for the mGlu receptors. Although typically regarded as reducing cAMP, it has been suggested that the mGlu receptors can potentiate the activity of adenylate cyclase stimulated by other receptors; thus increasing cAMP levels (Pin & Duvoisin, 1995; Conn & Pin, 1997). This action is possibly due to the $\beta\gamma$ subunits from G-proteins being able to potentiate the effects of the α subunit of the G-protein Gs on adenylate cyclase (Sternweis, 1994). In CHO cells expressed mGlu1a can stimulate adenylate cyclase and cAMP production (Aramori & Nakanishi, 1992), possibly by via Gs. In neuronal tissue there is little evidence that mGlu receptors can directly couple to Gs and cAMP formation; although in immortalized hypothalamic neurones (GT1-7 cells) a group II/III agonist increased basal and forskolin-stimulated cAMP accumulation, suggesting a novel mGlu receptor

subtype which apparently shares the same transduction pathway of other Gs-coupled receptors (Sortino *et al.*, 1996). The possibility that GPCRs can couple to more than one type of G-protein further increases the diversity of effects mediated by the receptor.

Release of glutamate from depolarised photoreceptors causes hyperpolarisation of the ON cells in the retina. This hyperpolarisation is due to activation of mGlu6 receptor (Nakajima *et al.*, 1993) which activates a cGMP phosphodiesterase leading to reduction of the cation current through the cGMP-gated channels (Nawy & Jahr, 1990; Shiells & Falk, 1990). Levels of cGMP can also be altered by mGlu receptors in other brain areas; for example, in cerebellar slices agonists increase the levels of cGMP in a NOS-dependent manner (Okada, 1992). In the rat nucleus tractus solitarii cGMP levels are also raised by mGlu receptors, in an apparently NOS-independent manner (Glaum & Miller, 1993).

The group I agonist 3,5-dihydroxyphenylglycine (DHPG) stimulates phospholipase D (PLD) in hippocampal slices (Klein *et al.*, 1997a; Klein *et al.*, 1997b). In slices taken from the hippocampus, and other brain regions of adult rats, mGlu receptor agonists can also stimulate PLD through a phospholipase C (PLC) and protein kinase C (PKC)-independent mechanism (Pellegrini-Giampietro *et al.*, 1996). In the hippocampus this response showed novel pharmacology: being activated by quisqualate and (1S,3R)-1-amino-1,3-cyclopentanedicarboxylic acid ((1S,3R)-ACPD), and antagonised by DHPG. The pharmacology indicates that a novel mGlu receptor may be involved. Also of interest is the fact that L-cysteine sulfinic acid, which has been implicated as a neurotransmitter, is a more potent agonist of this response than glutamate, leading to the suggestion that L-cysteine sulfinic acid may be an endogenous agonist at this receptor

(Boss *et al.*, 1994). L-cysteine sulfinic acid also acts at a metabotropic receptor to increase cAMP production in hippocampal slices (Boss & Boaten, 1995), a response that does not involve potentiation of the effect of other receptors linked to G_s-type G-proteins.

In cultured striatal neurones coactivation of AMPA and mGlu receptors causes arachidonic acid production via stimulation of PLA_2 (Dumuis *et al.*, 1993). In lateral septal neurones some actions of mGlu receptors may be mediated by PLA_2 as (1S,3R)-ACPD induces immediate early gene expression and this is reduced by PLA_2 inhibition (Kaatz & Albin, 1996). Arachidonic acid release can also be evoked from striatal astrocytes, but with a pharmacology that differs from known mGlu receptors (Stella *et al.*, 1994).

A recent report suggests that the group I mGlu receptors can sometimes act independently of the G-proteins (Heuss *et al.*, 1999). In hippocampal CA3 pyramidal cells a short train of stimuli (4 at 100 Hz) to the mossy fibres evoked a mGlu receptor-mediated EPSC. Although inhibition of G-proteins in the CA3 cells blocked the mGlu receptor-mediated inhibition of the afterhyperpolarisation current, the mGlu EPSC was unaffected, and appeared to be mediated via a src family tyrosine kinase.

Pharmacology

The sensitivity to glutamate (based on functional assays) of the mGlu receptors expressed in cell lines is in the micromolar range with quoted EC_{50}s of between 2.5 and 56 μM. The only exception to this appears to mGlu7, which has a much lower

sensitivity, with a quoted EC_{50} of 1000 μM (Pin & Duvoisin, 1995). Research into the physiological roles of individual mGlu receptors was initially hampered by a lack of selective ligands for each receptor. High affinity ligands are now becoming available which allow the responses of different groups of receptors to be characterised, although as yet the ability to discriminate between all the individual receptors is limited. A general overview of mGlu receptor pharmacology is presented below; the topic is discussed further in Chapter 3.

Group I Agonists

The pharmacological profile of group I agonists (on expressed mGlu1a receptors) is as follows: quisqualate > (S)-3,5-dihydroxyphenylglycine (DHPG) > glutamate > (1S,3R)-1-amino-1,3-cyclopentanedicarboxylic acid ((1S,3R)-ACPD) = ibotenate > (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-1)> 3-hydroxyphenlyglycine (3-HPG) > *trans*-azetidine-2,4-dicarboxylate (t-ADA). The initial studies of the PLC-linked group I receptors were performed using the agonist quisqualate. While this compound is highly active at these receptors, it is also active at iGlu receptors, making its use inappropriate for many studies, such as those into synaptic transmission.

The compounds DHPG, 3-HPG and t-ADA are all selective for group I receptors (Conn & Pin, 1997; Schoepp *et al.*, 1999). There is a report that DHPG can displace the group II antagonist LY 341495 from human mGlu3 receptors in a binding assay (Johnson *et al.*, 1999), but there do not appear to be any reports of this compound producing biochemical or physiological effects through group II receptors. More recently developed analogues of quisqualate can selectively activate mGlu5 over mGlu1; for

example (Z)- and (E)-1-amino-3-[2'-(3',5'-dioxo-1',2'4'-oxadiazolidinyl)]cyclobutane-1-carboxylic acid ((Z)- and (E)-CBQA; Fig 1.2) have no activity (1 mM) on mGlu1a expressed in BHK cells, but activate mGlu5a with EC_{50} of 11 and 49 μM respectively. These compounds were ineffective at expressed group II and III receptors (Littman *et al.*, 1999).

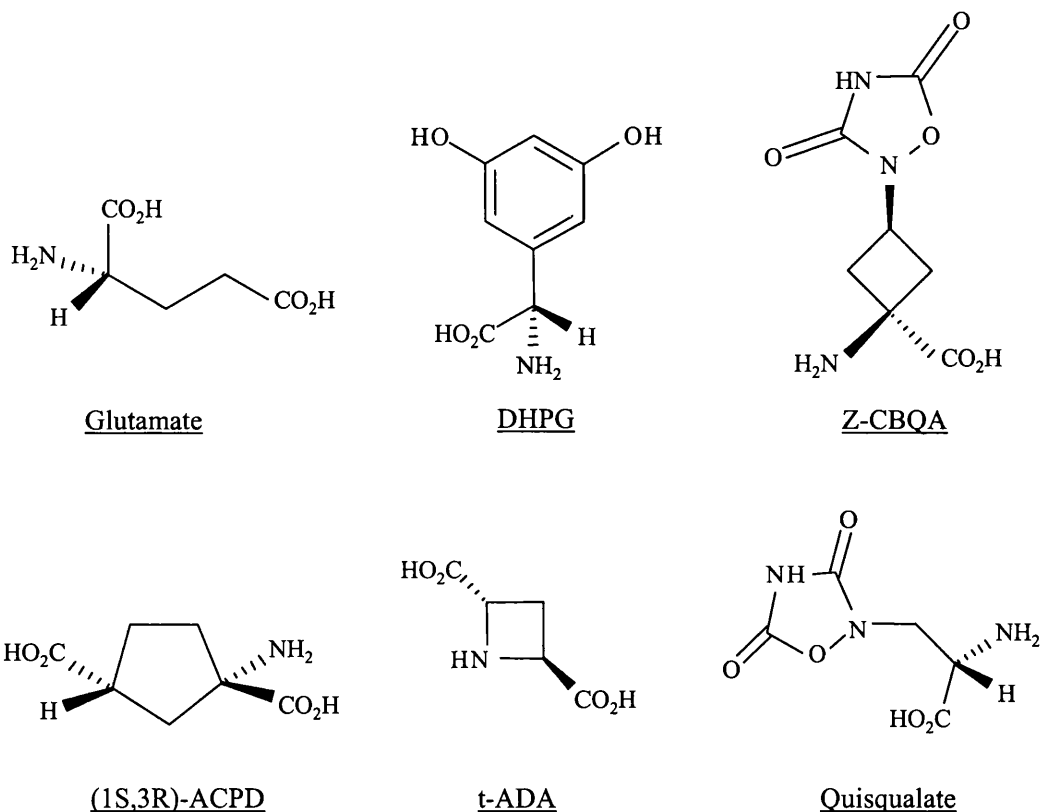


Figure 1.2 Chemical structures of group I mGlu receptor agonists

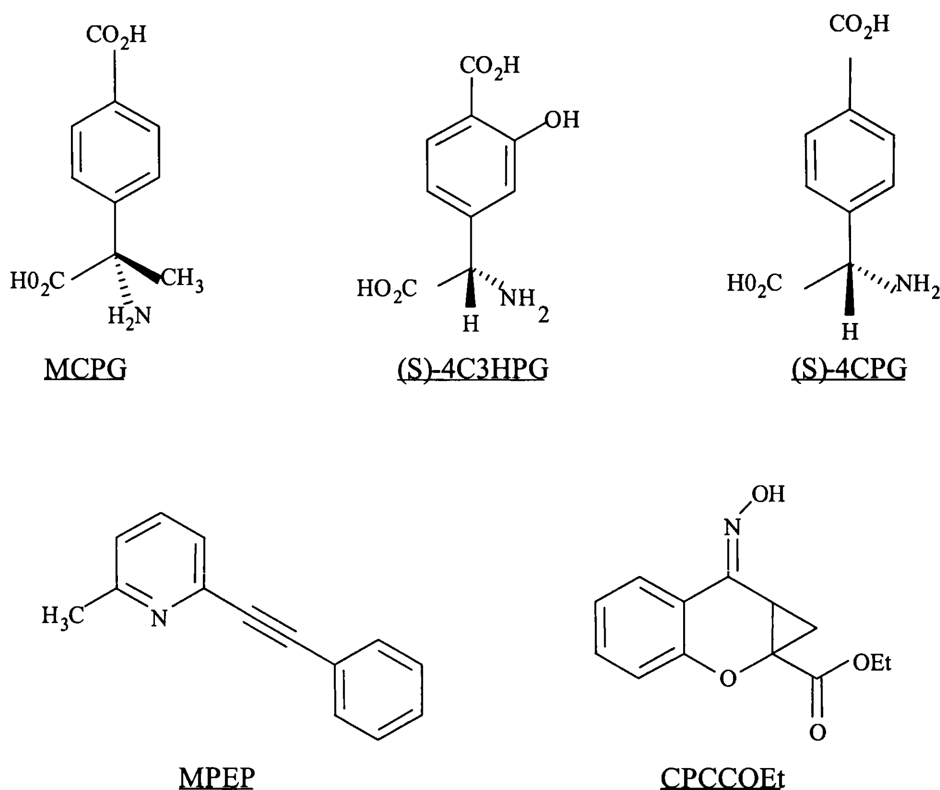


Figure 1.3. Chemical structure of group I mGlu receptor antagonists

Group I Antagonists

The first significant group I antagonists were carboxyphenyl glycine analogues. These could inhibit the actions of mGlu receptor agonist, without affecting iGlu receptors, although none of these compounds were totally selective for group I receptors over other mGlu receptors (Schoepp *et al.*, 1999). These antagonists include (S)-α-methyl-4-carboxyphenylglycine (MCPG), (S)-4-carboxyphenylglycine (S)-4CPG and (S)-4-carboxy-3-hydroxyphenylglycine((S)-4C3HPG). More recently, specific antagonists have been developed that can discriminate between the receptors mGlu1 and 5. Examples of these compounds include the mGlu1 antagonist

7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt; Annoura *et al.*, 1996) and the systemically active mGlu5 antagonist, 2-methyl-6-(phenylethynyl)pyridine (MPEP; Gasparini *et al.*, 1999a).

Group II Agonists

For the group II receptors the profile of agonists is as follows (measured against mGlu2): (1S,2S,5R,6S)-(+)-2-aminobicycol[3.1.0]hexane-2,6-dicarboxylic acid (LY354740)>(2S, 2'R, 3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG IV)=L-CCG-1 > 2R, 4R-4-aminopyrrolidine-2, 4-dicarboxylate (APDC) > glutamate > (1S,3R)-ACPD = (1S,3S)-ACPD > 4C3HPG > ibotenate. LY354740, DCG IV and APDC are selective for group II over other mGlu receptor groups, although DCG IV is also an NMDA receptor agonist (see Chapter 3). LY354740 is highly potent at rat and human group II mGlu receptors (EC_{50} 5-50 nM), but with no action at group I receptors. It is also inactive at mGlu4 and 7, although at micromolar concentrations it has agonist activity at mGlu6 and 8 (Monn *et al.*, 1997; Schoepp *et al.*, 1997; Monn *et al.*, 1999)

Group II Antagonists

The first antagonist identified with activity at the group II receptors was the non-selective MCPG. Subsequently, more potent competitive and selective antagonists have been identified, including 2*S*-2-amino-2-(1*S*,2*S*-2-carboxycyclopropyl-1-yl)-3-(xanth-9-yl)propanoic acid (LY341495), (2S,3S,4S)-2-methyl-2-(carboxycyclopropyl)glycine (MCCG), (2S,1'S,2'S,3'R)-2-(2'-carboxy-3'-phenylcyclopropyl)glycine (PCCG-IV)

and (2S)-α-ethylglutamic acid (EGLU).

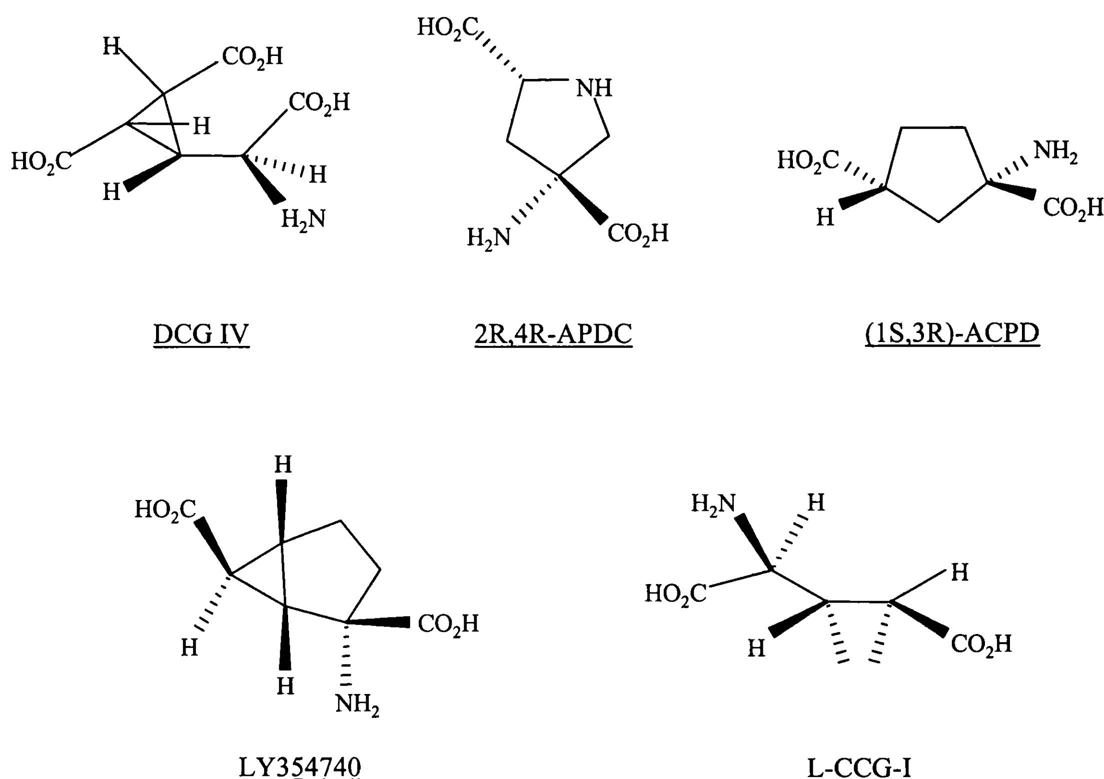


Figure 1.4. Chemical structure of group II mGlu receptor agonists

To date little progress has been made identifying compounds that can act selectively between the receptors mGlu 2 and 3. The pharmacology of mGlu3 agonists appears similar to mGlu2, although a difference has been identified in the sensitivity of the two receptors to quisqualate, which is a potent agonist at mGlu3, but is only weakly active at mGlu2 (Tanabe *et al.*, 1993). It may also be possible to discriminate between these two receptors based on antagonist potencies: MCPG is reported to be more potent at mGlu2 than 3 and MCCG is more potent at mGlu3, than 2 (McCool *et al.*, 1996).

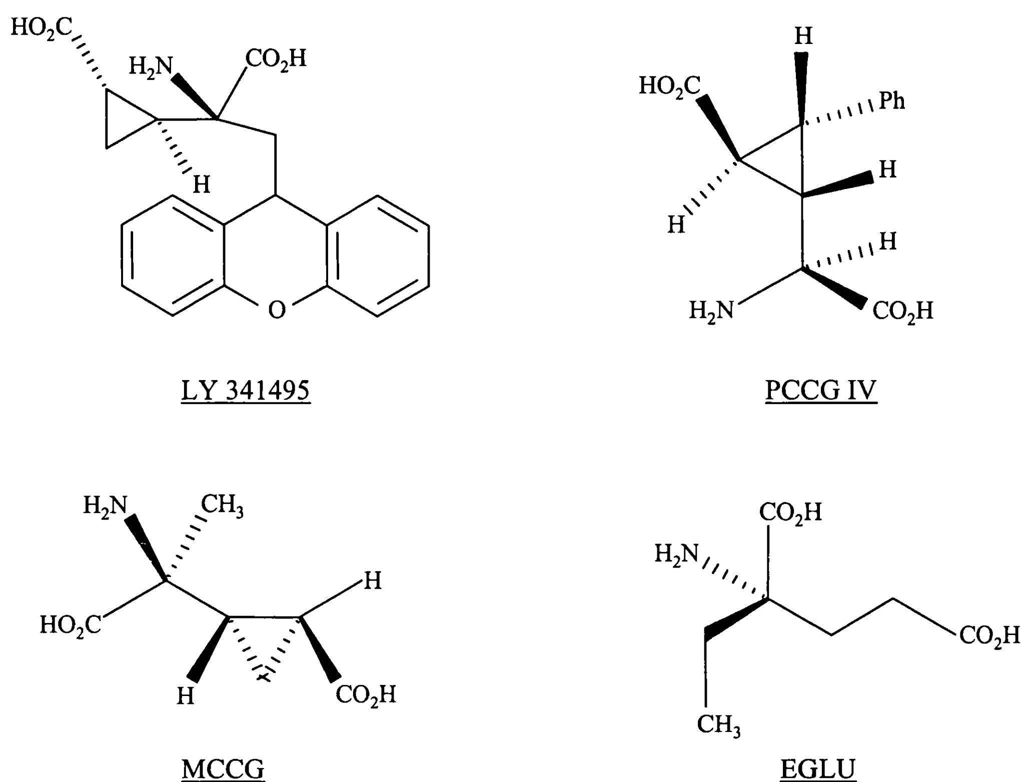


Figure 1.5. Chemical structure of group II mGlu receptor antagonists

Group III Agonists

The rank order of potency of group III agonists (measured on mGlu4) are as follows: L-2-amino-4-phosphonobutyric acid (L-AP4) > L-serine-O-phosphate (L-SOP) > glutamate > L-CCG-I > (1S,3S)-ACPD. Both L-SOP and L-AP4 are selective for the group III receptors. The mGlu7 receptor generally shows much lower potency for

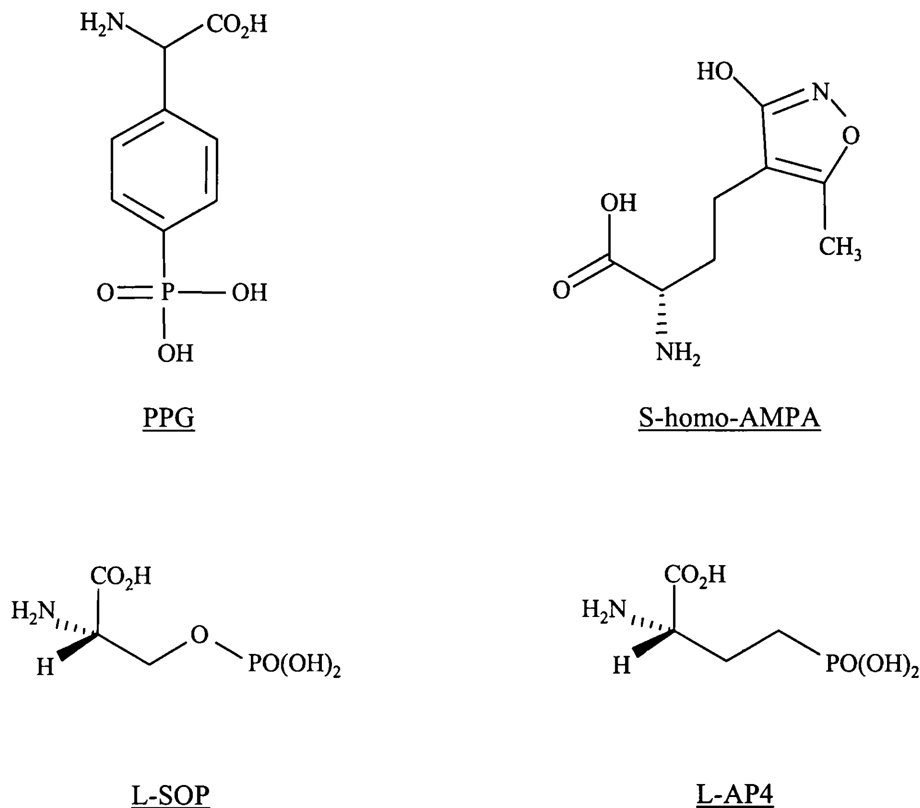


Figure 1.6. Chemical structure of group III mGlu receptor agonists

agonists compared to other group III receptors: glutamate showed a very low potency (EC_{50} > 1 mM) at mGlu7 expressed in BHK cells (Saugstad *et al.*, 1994). Similarly, other agonists, such as L-AP4 and L-SOP, are also only weak agonists at this receptor (Conn & Pin, 1997). The pharmacology of mGlu8 differs from the other mGlu receptors: (1S,3R)-ACPD is as potent an agonist at this receptor as it is at group I and II receptors, L-CCG-1 is equally potent at mGlu8 and 2, and is a more potent antagonist at this receptor that L-AP4, and MCPG acts as a mGlu8 antagonist, but is inactive at mGlu4 (Saugstad *et al.*, 1997; Schoepp *et al.*, 1999). Some progress has been made identifying agonists that selectively activate individual group III subtypes. (R,S)-4-phosphonophenylgycine (PPG) is an agonist at human group III receptors expressed in recombinant cell lines, but shows ~20 fold selectivity for mGlu8 over the other subtypes

(Gasparini *et al.*, 1999b).(S)-homoAMPA is an mGlu6 agonist with an EC_{50} of ~60 μM, but has no significant activity at the other mGlu receptors 1-7 expressed in CHO cells. The activity of the compound has not been tested on mGlu8 (Ahmadian *et al.*, 1997).

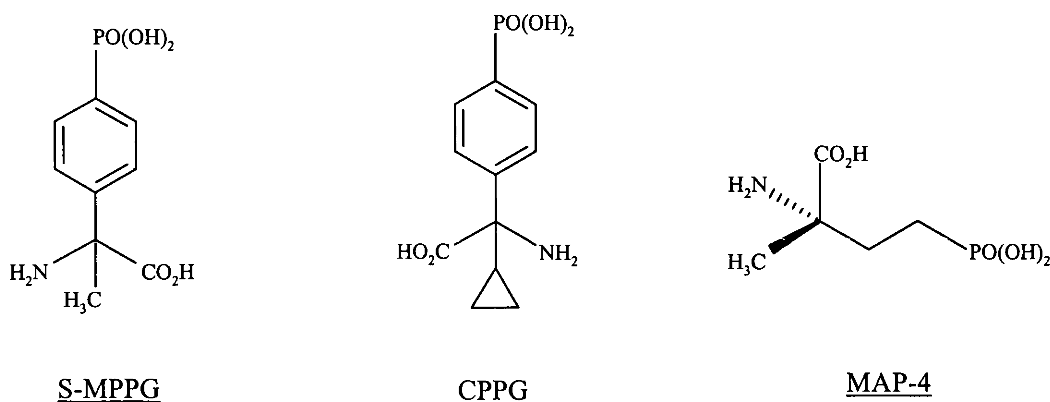


Figure 1.7. Chemical structure of group III mGlu receptor agonists

Group III Antagonists

The pharmacology of group III mGlu receptors is not as well developed as that for other groups and there is a paucity of potent group III selective antagonists. Compounds reported to be antagonists of group III include the MCPG analogue, (S)-α-methyl-4-phosphonophenylglycine (MPPG), (S)- α-methyl-2-amino-4-phosphonobutanoic acid (MAP-4) and (RS)-α-cyclopropyl-4-phosphonophenylglycine (CPPG), all of which have activity at other mGlu receptors (Schoepp *et al.*, 1999).

Actions of mGlu receptors in the CNS:

Regulation of Ionic Conductances

There is considerable evidence that activation of mGlu receptors changes neuronal membrane excitability by modulation of ionic conductances, including potassium, calcium and non-selective cationic conductances. Some of the effects of mGlu receptors are discussed below.

Voltage-sensitive Calcium Channels

The mGlu receptors can regulate cytosolic Ca^{2+} levels in two ways: either by triggering release from internal stores, or by modulating entry of Ca^{2+} into neurones via Ca^{2+}-permeable ions channels. Early evidence that that an mGlu receptor could depress these Ca^{2+} currents came from Lester and Jahr (1990). Working on cultured hippocampal CA1 pyramidal neurones, they demonstrated that a quisqualate-sensitive G-protein-linked receptor significantly reduced the high threshold Ca^{2+} currents. Subsequent work by Sahara and Westbrook (1993) showed that in cultured hippocampal neurones ACPD could inhibit both L- and N-type Ca^{2+} currents. There are now numerous reports that L-, N- and possibly P-type Ca^{2+} channels can be modulated by mGlu receptors in a variety of neuronal cells, as detailed below.

L-type Current

The L-type Ca^{2+} current is characterised by sensitivity to dihydropyridines, and high voltage threshold of activation. There is data suggesting that receptors from all three groups of mGlu receptors can modulate this current (Anwyl, 1999). In cultured mouse

cerebellar granule cells L-CCG-1 (presumed to be acting at a group II receptor) depressed the L-type current in a PTX-sensitive manner (Chavis *et al.*, 1994). Using the same preparation, Chavis *et al* (1996) demonstrated that group I mGlu receptors induce a functional coupling between ryanodine receptors and L-type Ca^{2+} channels, resulting in a large, oscillating increase in the L-type current. This coupling is blocked by GTP-β-S, but the mechanisms downstream of the G-protein are unclear, although it appears to be independent of IP_3 and kinase activity. In addition to this effect an increase in activity of the dihydropyridine-sensitive 7 pS Ca^{2+} channel present in cerebellar granule cells was also observed (Bossu *et al.*, 1994).

N-type Current

The N-type current is insensitive to dihydropyridines, is blocked by ω-conotoxin GVIA and has a lower threshold of activation than the N-type current. The group II mGlu receptor agonists inhibit the N-type current in a range of neuronal types (Stefani *et al.*, 1994; Ikeda *et al.*, 1995; Chavis *et al.*, 1995). In dissociated CA3 neurones mGlu receptor agonists inhibit a current pharmacologically identified as N-type. In the presence of GTP-γ-S the response became irreversible, and could be elicited once only, demonstrating involvement of G-proteins. Using cell-free outside-out patches and cell-attached patches the response did not appear to involve diffusible second messengers, leading to the suggestion that the G-protein are interacting directly with the Ca^{2+} channels (Swartz & Bean, 1992).

Other Ca^{2+} Currents

Although most experimental evidence points to mGlu receptors interacting with the N- and L-type currents, there is some evidence that other Ca^{2+} currents can be modulated.

In brain slices, some effects of mGlu receptor agonists on Ca^{2+} currents may be on the P-type current (Choi & Lovinger, 1996). All of the actions of mGlu receptors on Ca^{2+} channels discussed so far have been postsynaptic; however, recordings from the giant presynaptic terminal of the calyx of Held showed that agonists of mGlu receptors inhibited transmitter release by suppressing a high voltage-activated P/Q-type Ca^{2+} conductance in the presynaptic terminal (Takahashi *et al.*, 1996b).

Potassium Channels

Calcium-Activated Potassium Currents

Many neurones have Ca^{2+} -activated K^+ conductances. One of these, the after-hyperpolarisation current (I_{AHP}) is a conductance that plays a role in spike-frequency accommodation, and can be modulated by a variety of neurotransmitters including glutamate. Several groups have reported that mGlu receptor agonists, can alter the I_{AHP} in CA1, CA3 and dentate granule neurones of the hippocampus (Stratton *et al.*, 1989; Baskys *et al.*, 1990; Charpak *et al.*, 1990). In hippocampal slice culture Charpak *et al* (1990) showed that in CA3 pyramidal cells glutamate and quisqualate produced a marked increase in the number of action potentials elicited by depolarisation, presumably due to the observed block of the slow after-hyperpolarisation following spike discharge. This effect persisted in the presence of ionotropic glutamate receptor antagonists. The reduction of I_{AHP} was produced by concentrations of quisqualate that produced no measurable change to Ca^{2+} influx, suggesting that this is a more direct action on the K^+ influx.

In CA3 cells the reduction of I_{AHP} involves a PTX insensitive G-protein (Gerber *et al.*, 1992) and is produced by quisqualate, suggesting a group I receptor. The exact mechanism involved remains to be elucidated; in hippocampal CA1 neurones it may involve PKC as indicated by sensitivity to phorbol esters (Stratton *et al.*, 1990), but in CA3 cells the response does not appear to involve either PKC or PKA (Gerber *et al.*, 1992). In the dentate granule cells the block of I_{AHP} appears to involve G-protein mediated production of IP_3 and subsequent liberation of internal calcium stores (Abdul-Ghani *et al.*, 1996). The process also involves protein tyrosine kinase activity, possibly activated by calcium that was mobilised by IP_3 (Abdul Ghani *et al.*, 1996).

I_M

I_M is a voltage-dependent K^+ current, originally identified by its sensitivity to muscarine. This current plays a role in spike-frequency adaptation and may contribute to setting the resting membrane potential. Stratton *et al* (1989) and Charpak *et al* (1990) reported that a quisqualate-sensitive mGlu receptor produced a membrane depolarisation in hippocampal neurones that was accompanied by an increase in the membrane resistance; likely to be due to inhibition of I_M. Like the inhibition of I_{AHP}, this is thought to be caused by a mGlu receptor coupled to phosphoinositol hydrolysis. These effects of mGlu receptors on K^+ conductances are similar to those produced by muscarine. Both muscarinic and mGlu receptors are coupled to phosphoinositol hydrolysis and Charpak *et al* (1990) suggested that muscarinic and mGlu receptor inhibition of K^+ conductances, in hippocampal CA3 cells at least, may involve a common pathway. Blockade of I_M by mGlu receptor activation has also been observed in neurones from other brain regions such as the basolateral amygdala (Womble & Moises, 1994).

I_{LEAK}

In cells that show little I_{AHP} or I_M, or in cells studied under conditions where these conductances are inactive, mGlu receptor agonists can produce a decrease in the leak K^+ conductance (I_{LEAK}), thereby increasing excitability. In thalamic relay neurones, (1S,3R)-ACPD application results in a reduction of both input conductance and I_{LEAK} (McCormick & von Krosigk, 1992). Inhibition of this current is also seen in other brain areas, such as the hippocampal CA3 region (Guerineau *et al.*, 1994) and basolateral amygdala (Womble & Moises, 1994).

In addition to the effects on identified K^+ currents discussed above, Hu and Storm (1991) reported that mGlu receptor agonists (glutamate, quisqualate and ACPD) acted to slow the spike repolarisation in CA1 cells of the rat hippocampus. The mechanism of the spike broadening remains unclear, although the response is occluded by phorbol esters, suggesting that PKC may be involved. The authors suggest that mGlu receptor activation causes either inhibition of K^+ channels or may directly affect the Na^+ channels.

Non-Selective Cation Currents.

In CA1 pyramidal neurones (1S,3R)-ACPD, in addition to inhibiting K^+ currents, activates a Ca^{2+}-activated non-selective cation channel (CAN; Crépel *et al*, 1994). This current does not appear to be due to an electrogenic Na^+/Ca^{2+} exchanger as it is not reduced by substitution of the external Na^+ with lithium, nor is it temperature dependent

(see Crépel *et al*, 1994 for refs.). This current has subsequently been pharmacologically identified as being regulated by a group I receptor (Congar *et al.*, 1997).

In neocortical neurones mGlu receptor agonists cause the I_{AHP} seen after a train of spikes to be replaced by an afterdepolarisation (Greene *et al.*, 1992). Similar results are reported for neurones of the CA3 area of the hippocampus, of the olfactory cortex and the dorsolateral septal nucleus (Zheng & Gallagher, 1991; Constanti & Libri, 1992; Caeser *et al.*, 1993). In all these cells the afterdepolarisation is generated by mGlu receptor regulation of a Ca^{2+}-activated non-selective cation current.

In hippocampal CA3 neurones, when the extracellular K^+ concentration is raised above 5 mM, (1S,3R)-ACPD induces a biphasic current (Guerineau *et al.*, 1995). The initial phase is due to an increased Ca^{2+}-independent non-selective cationic current and the second due to reduction of I_{LEAK}. The enhancement of the cationic current appeared to be G-protein independent, being unaffected by GTP-γ-S and GTP-β-S and the authors postulate the involvement of a novel receptor, although the possibility that the GTP-γ-S and GTP-β-S failed to reach receptors in distal dendrites was not excluded. This cationic conductance is unlikely to be relevant to normal physiological activity as it requires unphysiological K^+ concentrations. However, during pathological situations, such as epilepsy and ischaemia, or periods of repetitive stimulation the K^+ concentration may be increased and this conductance may be active under such circumstances (see Guérineau *et al*, 1995 for refs.).

All the data discussed above describes the effects of exogenous mGlu receptor agonists on ion currents. There are, however, a growing number of reports show that synaptically

released glutamate can modulate ion conductances by activating mGlu receptors. In nucleus tractus solitarii, hippocampal, cerebellar and ventral tegmental neurones tetanic stimulation of glutamatergic fibres produces excitatory post-synaptic currents (Glaum & Miller, 1992; Batchelor & Garthwaite, 1993; Congar *et al.*, 1997; Shen & Johnson, 1997; Heuss *et al.*, 1999). These conductances all require tetanic stimulation of the presynaptic fibres, have a slow time course, peaking in ~1 s and lasting ~1-2 s. There is also a report that tetanic stimulation (10 stimuli; 66 Hz) of presynaptic fibres induces a mGlu receptor-mediated inhibitory postsynaptic potential (IPSP) in dopaminergic ventral tegmentum neurones. This IPSP was proposed to involve group I mGlu receptor-mediated liberation of Ca^{2+} from internal stores and activation of apamin-sensitive K^+ channels (Fiorillo & Williams, 1998).

Synaptic Plasticity

A discussion of the role of mGlu receptors in synaptic plasticity may be found in Chapter 4.

Pathology

A range of neurological disorders, including neurodegeneration, epilepsy and pain involve changes in neuronal excitability relating to increases in glutamate release. The iGlu receptors have been subjected to intensive research effort in the search for new drugs, but few compounds with therapeutic value have been developed. This is largely because inhibition of iGlu receptors will impinge on normal synaptic function. Rather

than simply block synaptic transmission the mGlu receptors modulate neuronal activity, and this makes these receptors attractive targets for drug research.

Neurodegeneration

Group II and III mGlu receptor agonists have been found to be neuroprotective in *in vitro* and *in vivo* models of neurodegeneration (Buisson & Choi, 1995; Buisson *et al.*, 1996; Bruno *et al.*, 1996; Kingston *et al.*, 1999). The role of group I receptors in neurodegeneration is more controversial. Both *in vitro* and *in vivo* studies have shown that group I agonists exacerbate the damage caused by NMDA application and oxygen-glucose deprivation (Buisson & Choi, 1995; Bruno *et al.*, 1995), and that group I antagonists are neuroprotective (Kingston *et al.*, 1999). However, in developing tissue activity of these receptors seems to be necessary for neuronal survival (Mount *et al.*, 1993), and there is data from *in vitro* models of neurodegeneration suggesting that group I activation may be protective (Pizzi *et al.*, 1993; Opitz *et al.*, 1995).

Pain

Several studies have suggested the mGlu receptors are involved in the transmission of painful stimuli. Intracerebral ventricular administration of the proposed mGlu1 receptor antagonist 1-aminoindan-1,5-dicarboxylic acid (AIDA) causes the pain threshold of mice to be raised (Moroni *et al.*, 1997). In the spinal cord the mGlu receptors may be involved in acute and chronic pain transmission (Meller *et al.*, 1993; Young *et al.*, 1995; Young *et al.*, 1997).

Epilepsy

In genetically epilepsy-prone rodents, and other *in vivo* models of epilepsy, mGlu receptor ligands have anticonvulsant properties. In genetically epilepsy-prone rats group II and III agonists potently raised the threshold to sound-induced seizures (Tang *et al.*, 1997). In electrically kindled rats the group III agonist L-AP4 inhibited seizure activity (Suzuki *et al.*, 1996; Abdul-Ghani *et al.*, 1997). Finally the group I agonist/group II antagonist (S)-4C3HPG attenuated audiogenic-induced seizures in DBA/2 mice (Thomsen *et al.*, 1994a).

Research for therapeutically active mGlu receptor ligands has been hampered by two factors: firstly, there have been difficulties in producing selective compounds for specific receptors; secondly, many of the currently available ligands share the alpha amino acid group of glutamate, limiting the ability of compounds to cross the blood brain barrier, a problem also encountered in the development of drugs acting at the iGlu receptors. Great steps have been made to overcome both of these obstacles: receptor-selective ligands are now available, such as the orally active anxiolytic and anticonvulsant group II agonist, LY354740 (Monn *et al.*, 1997). Although study of the mGlu receptors has focussed on their role in the CNS there is evidence of their existence in peripheral tissues, such as the liver and heart (Gill *et al.*, 1999; Storto *et al.*, 2000). The functional role of these receptors is presently unclear, but the presence of these receptors in peripheral tissues may prove significant in the search for therapeutic mGlu receptor ligands as a possible source of harmful side effects. Despite the potential difficulties, the mGlu receptors remain an attractive target for the development of new therapeutic agents.

The Cerebellar Cortex

The cerebellum is a significant proportion of the mammalian brain, occupying some 25% of the cranial capacity in man. Structurally it is divided into the medial cerebellar vermis and two lateral lobes, the cerebellar hemispheres, and consists of a superficial outer layer of grey matter (cerebellar cortex), internal white matter (medullary substance) and four pairs of nuclei embedded in the white matter. The cerebellar cortex exists as a highly convoluted sheet of cells and their processes, divided into a number of folia. The cerebellar cortex is divided into three layers: molecular, Purkinje cell and granule cell layer (see Chapter 2, Figure 2.2).

The outermost molecular layer contains the basket and stellate cells, the parallel fibres and Purkinje cell dendrites, and in the rat has a mean depth of 228 μm (Harvey & Napper, 1991). The basket and stellate cells are both excitated by parallel fibres activity and provide inhibitory input to the Purkinje cells. Frequently, in discussions of cerebellar anatomy and function these two cell types are grouped together as "inhibitory interneurones". It may be, however, that the two cell types serve differing roles as the basket cells synapse onto the Purkinje cell body whereas the stellate cells synapse onto the Purkinje cell dendritic tree (Palay & Chan-Palay, 1974). Thus, basket cell activity provides a "global" form of inhibition affecting all excitatory signals that reach the soma, and potentially altering action potential initiation. In contrast, the stellate cells might provide a local form of inhibition in the dendrites, affecting only a proportion of the excitatory inputs.

The Purkinje cell layer is formed from a single cell layer of flask-shaped Purkinje cell bodies. These GABAergic cells are among the largest neurones in the brain with a diameter of ~25 μm (Palay & Chan-Palay, 1974). In the rat the Purkinje cell layer, and therefore the cerebellar cortex, has a surface area of ~330 mm^2 (Harvey & Napper, 1991). A unique feature of the Purkinje cells is their extensive dendritic tree that lies in the molecular layer in a single plane, perpendicular to the parallel fibres. This arrangement allows the dendrites to contact with a large number of parallel fibres. Purkinje cells represent the sole output pathway from the cerebellar cortex, and project primarily to the deep cerebellar nuclei. These nuclei project to brain areas such as thalamic nuclei, which in turn project to the primary motor cortex. There is also evidence in humans that cerebellar nuclei project to non-motor areas, such as Broca's language area in the inferior prefrontal cortex (Leiner *et al.*, 1993).

The Purkinje cells receive excitatory inputs from the parallel fibres and the climbing fibres. The parallel fibres are the unmyelinated axons of granule cells that pass up through the Purkinje cell layer into the molecular layer where they bifurcate in a T-fashion and travel along the long axis of the folia, perpendicular to the Purkinje cell dendrites. The parallel fibres can have a span of some 5 mm along the folia (Harvey & Napper, 1991). Along the length of the parallel fibre, synapses are formed between varicosities in the fibres and the dendritic spines of the Purkinje cells. A parallel fibre will typically synapse once only with an individual Purkinje cell. It is estimated that in the rat each parallel fibre will synapse with a Purkinje cell on average every 5.8 μm (Pichitpornchai *et al.*, 1994) and each Purkinje cell forms synapses with ~175000 parallel fibres (Napper & Harvey, 1988). From the anatomical evidence that each parallel fibre synapses with Purkinje cells along its length one would expect granule cell

excitation to result in a beam of Purkinje cells being excited along the length of the parallel fibre. Using an intact cerebellum preparation and voltage sensitive dye, Cohen and Yarom (1998) have demonstrated that parallel fibre stimulation does indeed result in a beam of Purkinje cells being activated. However, this behaviour was not observed following excitation of the granule cells by stimulation of the white matter; instead, a circular patch of synchronised Purkinje cell activity was observed. These experimental observations may be explained by the facts that the ascending axon of the granule cell forms synaptic contacts with Purkinje cells prior to bifurcating (Pichitpornchai *et al.*, 1994). These synapses were originally proposed to be of functional significance by Llinás (1982), and it is estimated that some 20% of granule cell-Purkinje cell synapses occur on the ascending axon (Gundappa-Sular *et al.*, 1999). Interestingly, the synapses formed with the ascending axon were found to occur on the smallest diameter distal regions of Purkinje cell dendrites, whereas the parallel fibre synapses are located exclusively on intermediate and large diameter regions of the dendritic tree (Gundappa-Sular *et al.*, 1999). It has therefore been proposed that the ascending axon and parallel fibre synapses serve differing roles: the ascending axon synapses are postulated to provide the excitatory drive to the Purkinje cells, whereas the parallel fibre synapses may serve a modulatory role, by altering the dendritic membrane conductance, and thereby regulating the level of excitation in the Purkinje cells (Gundappa-Sular *et al.*, 1999).

The climbing fibres are non-myelinated axons of neurones in the inferior olive nucleus of the brain stem, which receives inputs from spinal and supraspinal areas relating to motor and sensory function. In adults, each Purkinje cell synapses with only a single climbing fibre that makes numerous synaptic contacts over the Purkinje cell dendritic

tree, and a single climbing fibre may synapse with 10-15 Purkinje cells. A significant feature of the climbing fibres is their powerful all-or-nothing excitation of the Purkinje cell, resulting in a complex spike (as opposed to the simple spike evoked by parallel fibre stimulation). This complex spike is due to activation of both Ca^{2+}- and Na^{+}-voltage-dependent ion channels. The complex spike *in vivo* can last for tens of milliseconds and can produce dramatic affects on subsequent simple spike firing, typically leading to a reduction, although in some cases an increase in simple spiking has been observed (Ito, 1984). *In vivo* the climbing fibre typically fire at frequencies of 1-4 Hz and a number of suggestions have been proposed for the role of these powerful yet relatively low frequency inputs. Probably the most influential proposal is that climbing fibres signal errors of performance which, should the Purkinje cell continue to be excited by further erroneous parallel fibre activity, will lead to induction of long term depression (Marr, 1969; Albus, 1971). Recently climbing fibre activity in monkeys trained perform a reaching movement to touch an area on a screen has been examined (Kitazawa *et al.*, 1998). By applying information theory (Shannon, 1948) the study suggested that the climbing fibres were not only encoding information regarding performance errors, but also encode information relating to the actual destination of the movement and its generation. The role of the climbing fibre may therefore be more complex than assumed in earlier theories.

The granule cell layer contains the granule cells which are small (soma 5-8 μm diameter), densely packed neurones and represent the most numerous cell type in the brain: in the rat there are estimated to be 9.2×10^7 granule cells, thus for every Purkinje cell there are 274 granule cells (Harvey & Napper, 1991). Each granule cell gives rise to approximately four short dendrites, which branch to form claw-like endings that enclose

a space occupied by the terminal of a mossy fibre, forming a structure known as a glomerulus (Palay & Chan-Palay, 1974). Together with the climbing fibres, the mossy fibres provide the main inputs of the cerebellar cortex and provide excitatory drive to the granule cells. The mossy fibres are myelinated axons which carry information to the cerebellar cortex from a variety of sources including: spinal cord, vestibular system, reticular nuclei, pontine nuclei and brain areas associated with autonomic function, such as nucleus of the solitary tract.

The other main cell type located in the granule cell layer is the inhibitory Golgi cell. In the rat there are reported to be two types of Golgi cells: large cells in the upper half of the granule cell layer, and smaller cells in the lower half (Palay & Chan-Palay, 1974); whether these two types are functionally distinct is unclear. Golgi cells may also be subdivided according to the receptors they express, for example the mGlu5 receptor is only expressed in a small percentage of these cells (Neki *et al.*, 1996). Synapses on these neurones are from a number of sources including the mossy fibres, climbing fibres, parallel fibres, basket cells and stellate cells (Palay & Chan-Palay, 1974). The axons of the Golgi cells branch repeatedly in the granule cell layer and each Golgi cell synapses with several thousand granule cells. It has been suggested that the Golgi cells, by sampling parallel fibre and mossy fibre activity, provide inhibition to the granule cells that will act to limit the number of parallel fibre active at any one time, thus preventing saturation of the Purkinje cell (Marr, 1969; Albus, 1971).

The cerebellum has long been recognised as serving an important role in the accurate control of movement and in the learning of some motor behaviour. Following damage to this area movements become imprecise, and frequently oscillate on the way to the

target (Ito, 1984). A significant proportion of the research into the contribution of the cerebellum in motor learning has focussed on the role it plays in simple reflex behaviours, such as the vestibulo-occular reflex (VOR). The VOR uses vestibular input to ensure that the eyes remain directed to a target during head movement. This reflex can adapt to reduce the retinal errors imposed by the distortion of vision induced by a lens. Lesions to the cerebellum impair the ability to modify the VOR, and the first evidence that synaptic plasticity in the cerebellar cortex might underlie some motor learning (Marr, 1969; Albus, 1971) was obtained studying this reflex (Ito, 1998).

Although the cerebellum is classically regarded as being involved in movement there is a growing body of evidence that in humans the cerebellum contributes to a whole range of other functions (Leiner *et al.*, 1993). For example, when human subjects were asked to compare sandpaper of varying degrees of coarseness which was rubbed against their fingers, without the subject moving, the dentate nucleus (one of the deep cerebellar nuclei receiving Purkinje cell axons) was found to be active (assessed using magnetic resonance imaging). In the same study subjects were asked to reach and pick up and object: this did not significantly activate the dentate nucleus. However, when the same task was repeated with the subjects being asked to compare the shape of the object to one held in the other hand, a significant activation of the dentate nucleus was observed (Gao *et al.*, 1996). Another area that has attracted interest is the role of the cerebellum in the non-motor aspects of language; for example, PET scans demonstrate cerebellar activation during processing of verbal information, distinct from activation relating purely to motor control (Fiez *et al.*, 1996).

Exactly how the cerebellum contributes to these various processes is unclear. As the cerebellum receives a range of sensory and motor information, it has been proposed that the cerebellum assimilates this information to ensure accurate performance. (Eccles *et al.*, 1967). Many movements occur too quickly for feedback control systems to operate; it has therefore been proposed that the cerebellum performs adaptive feedforward control, allowing accurate control of movements to be learned as a result of errors perceived from previous trials, with the cerebellar output adjusted by synaptic plasticity at the parallel fibre-Purkinje cell synapse (Marr, 1969; Albus, 1971). These hypotheses were developed before the idea that the cerebellum contributes to cognition had gained significant support. More recently, Masao Ito (1990) has suggested that the adaptive learning capabilities of the cerebellum, coupled with the inherent speed of performance that feedforward systems, such as the cerebellar cortex, possess when compared to feedback controllers, will be applicable to accurate performance in the other tasks that the cerebellum is implicated in.

The aims of the present work have been to investigate the role that mGlu receptors play in the regulation of synaptic function in the rat cerebellum. Regulation of the efficacy of synaptic transmission is an important aspect of CNS function and modulation of transmission in the cerebellum by the mGlu receptors could be of importance in a diverse range of physiological processes.

Chapter 2: Methods

Preparation of brain slices

All experiments were performed on sagittal slices of cerebellum from Wistar strain rats, aged between 12 and 17 days, obtained from Charles River, or from colonies maintained at either University College London or the Institute of Neurology.

Rats were killed by decapitation, using a Home Office approved technique, the surface of the skull was then removed by making lateral cuts in the skull and then lifting it away. Cold, oxygenated artificial cerebrospinal fluid (aCSF) was dripped on to the exposed surface of the brain, and the whole cerebellum was then dissected away from the rest of the brain and placed into cold (~8 °C) aCSF. For all experiments the aCSF contained (in mM): NaCl (120); KCl (2); $CaCl_2$ (2); $NaHCO_3$ (26); $MgSO_4$ (1.19); KH_2PO_4 (1.18); D-glucose (11), and was equilibrated with 5% CO_2 in O_2.

In initial experiments the slices were prepared using a Campden Instruments Vibraslice, but obtaining recordings from healthy neurones in these slices was relatively difficult, so for the vast majority of experiments slices were prepared using a Vibratome Series 1000 Sectioning System (TPI, Intracel). For slice preparation the cerebellum was carefully placed onto a piece of aCSF soaked filter paper, and then, by cutting in the sagittal plain just into the vermis with a disposable razor blade, the cerebellar hemispheres were removed. When removing the hemispheres care was taken to ensure that the cut was straight and perpendicular to the vermis. One of the cut sides of the

vermis was then glued to the cutting stage of the vibratome, and slices were cut from the centre portion of the vermis. Prior to gluing the tissue, a cube of agar was glued to the stage. The cerebellum was glued directly in front of the agar as this supported the tissue during the cutting. After cutting, slices were carefully transferred to a holding chamber containing aCSF at room temperature, and constantly bubbled with $95\%O_2/5\%CO_2$ (Fig 2.1).

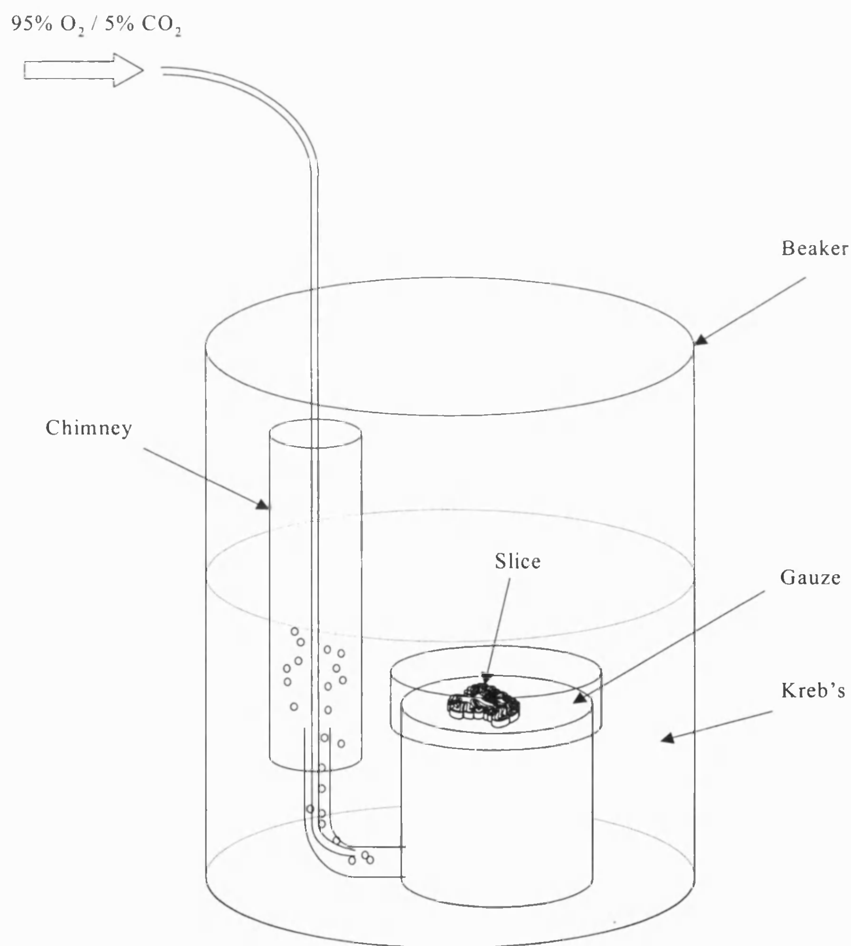


Figure 2.1. Holding Chamber for Brain Slices

The slices were left here for at least one hour before recording commenced. This period of recovery allows the cells in the slice to recover from the trauma associated with the cutting procedure (Alger *et al.*, 1984).

Brain slices provide a valuable preparation for the study of synaptic physiology: the synaptic connections that occur *in vivo* are maintained in the slice, neurones can easily be identified and accessed for recording, the external environment can easily be manipulated, and drugs can be applied and removed quickly (Crépel *et al.*, 1981). When preparing brain slices certain factors have to be considered. In particular, during the dissection and slice cutting the tissue will inevitably undergo periods of anoxia and physical trauma. The brief periods of anoxia associated with slice preparation are difficult to avoid. Neurones can, however, fully recover from brief anoxic episodes, apparently without any permanent damage, although during the anoxic period itself cells will swell, and changes in function are reported (Alger *et al.*, 1984). The damage associated with mechanical trauma is more difficult to avoid. Obviously, at all stages of the cutting process, the tissue is handled with great care, but loss of some cells, particularly those close to the cut edges of the slice is inevitable. One way to reduce the cell damage is to use tissue taken from younger animals, which seem to endure the process better than that from adults. This is possibly because in the younger tissue there are fewer myelinated fibres, thus allowing the blade to cut through easily. In adult rats cells are also larger with more extensive dendritic trees, thus increasing the likelihood of the cell being damaged by the blade (Alger *et al.*, 1984). The ability of tissue from younger rats to better endure slicing was one reason why they were used for the experiments described in this thesis.

The thickness of slices used in electrophysiological studies is determined by a number of factors. One key factor is the need to visualise the tissue to ensure correct electrode placement. For this a light source is placed under the tissue, and the thinner the slice, the clearer the cells can be seen. A second factor dictating thickness is the fact that slices do not have a blood supply to provide oxygen and glucose, and to remove waste products, and therefore have to rely on diffusion into and out of the slice. The thinner the slice is the better the oxygen supply will be to cells in the centre of the slice, and for this reason slices are usually less than 500 μm which should be thin enough not to have an anoxic core (Lipton & Whittingham, 1984). Unfortunately, the passage of the blade through the tissue leaves the outer section of the slice damaged, and this damage can penetrate in excess of 50 μm into the tissue. For the sharp electrode recording 400 μm thick slices were used. At this thickness slices were thin enough to allow visualisation of the tissue, and adequate oxygenation of the cells, but thick enough to have a central region that would hopefully be spared from the mechanical trauma associated with the cutting. To avoid the damaged tissue the electrode was routinely advanced ~40 μm into the slice before attempting to impale a cell. For the whole cell recording individual cells were selected under the microscope so the slices had to thinner (200 μm) to allow the light source to pass through the slice. For this technique the cells that are recorded from are located on the surface of the slice. This is the region damaged by the cutting process and, when viewed under the microscope, a proportion of the Purkinje cells are clearly damaged. This is a potential problem of the technique as, although recording is only performed on those cells that appear healthy, it is conceivable that they may have suffered subtle changes either as a direct result of the slicing, or from high concentrations of glutamate released from dying neighbouring cells. The synaptic circuitry is also potentially disrupted as cells lose their inputs from the dead neurones.

Such changes in the slice may have bearing on, for example, studies into synaptic plasticity where prolonged activation of glutamate receptors and depolarisation of neurones are linked to changes in synaptic strength, and could provide one explanation for different groups, apparently using similar techniques, preparations, and protocols, obtaining conflicting results.

Conventional Sharp Microelectrode Current Clamp Recording.

Recording Equipment

The recording set-up for current clamp recording was based around an Axoclamp 2A amplifier (Axon Instruments Inc). For data acquisition the amplifier was interfaced via a Digidata 1200 analogue-digital converter to a 486 personal computer running Axotape 2 (Axon Instruments Inc.). To allow real-time monitoring of the electrophysiological characteristics of the cell under investigation the signal from the amplifier was also sent to an oscilloscope. Parallel fibres were stimulated using pulses of a typical amplitude of about 30 V with a duration of 0.02 ms (Digitimer isolated stimulator model DS-2). The stimulation protocols were triggered via a Master 8 (A.M.P.I.), which also sent a signal, via the Digidata, to the computer to trigger data acquisition. The technique of sharp microelectrode recording is highly susceptible to disruption from mechanical vibration. To prevent loss of cells due to this the stimulating and recording electrodes and the recording chamber were mounted on an anti-vibration table. Recording quality can be impaired by radiative electrical pickup from a number of sources, including computer monitors and lights. To reduce these effects the headstage and bath were shielded with earthed aluminium foil.

Preparation of Stimulating and Recording Electrodes

Stimulating electrodes were prepared either as a bipolar electrode formed from twisting together two strands of insulated silver wire, with the insulation removed from the tips in contact with the slice, or from a patch pipette filled with 0.9% NaCl. The synaptic stimulation obtained with the monopolar electrode was more reliable, and was therefore used routinely. Recording electrodes were prepared from borosilicate glass capillaries with an internal filament to aid pipette filling (1.2 mm O.D. x 0.69 mm I.D.; Clark Capillaries, Clark Electromedical Instruments). Using a Flaming/Brown micropipette puller (Sutter Instrument Co., Model P-87) electrodes were produced which, when filled with 3 M KCl, had a resistance typically between 60 and 100 MΩ. Microelectrodes have very fine tips and, to keep the resistance to a minimum and achieve stable, low-noise recording, it is important that the tip of the electrode contains no air bubbles. To fill the electrode a small drop of filling solution was added just inside the open end of the pipette, in contact with the internal filament. This fluid would fill the tip by capillary action, and the tip was then back-filled so that about 1.5 cm of the tip contained the KCl. To connect the electrode to the recording circuit a silver wire was inserted into the microelectrode with the tip of the wire was in contact with the KCl. Prior to use the silver wire was electroplated by immersing it in 0.9% NaCl solution and connecting it to the positive pole of a 1.5 V battery. The circuit was completed with an electrode fashioned from aluminium foil, connected to the negative pole, and placed in the NaCl solution. Before the wire was coated it was cleaned with fine grade wet-dry paper. The wire was left in the NaCl until it achieved a reddish black colour, which took about 10-15 minutes.

Perfusion System

For all experiments the aCSF contained (in mM): NaCl (120); KCl (2); $CaCl_2$ (2); $NaHCO_3$ (26); $MgSO_4$ (1.19); KH_2PO_4 (1.18); D-glucose (11), and was equilibrated with 5% CO_2 in O_2. For recording the slice was held submerged in a bath by a slice weight produced from a flattened platinum wire with nylon strands glued across. The bath was perfused under gravity with aCSF (flow rate = ~ 1 ml min^{-1}). Immediately prior to entering the chamber the perfusate was heated to 30 °C (heater manufactured by The Technology group at the Wellcome Research Laboratories, Beckenham, Kent). During all recording the $GABA_A$ receptor antagonist bicuculline methochloride (30 μM) was added to the perfusate. To apply drugs the perfusing system was switched between the normal aCSF solution and tubes containing aCSF mixed with the drug. Drugs were either prepared on the day of the experiments, or were made from concentrated solutions, prepared in advance, and then frozen at –20°C until required. The perfusate was removed from the bath by suction from a vacuum pump. Care was taken to ensure that the level of the perfusate in the recording chamber was such that the fluid was maintained at a consistent level that only just covered the tissue. The aCSF-containing perfusion tubing can act as an antenna. To prevent this source of noise, a break was placed into the perfusion so that rather than a continuous flow, at one point it would instead drip.

Recording Technique

The Purkinje cells are located in a clearly defined cell layer (Fig. 2.2). For recording, a section of the cell layer that ran parallel with the direction of travel of the recording electrode was selected. Prior to attempting to impale a cell with the recording electrode, a stimulating electrode was positioned in the molecular layer. After impaling a cell with

the recording electrode, the stimulating electrode would often have to be moved to allow parallel fibre stimulation without directly stimulating the postsynaptic cell, or the climbing fibres. For this reason the stimulating electrode tip would be positioned on the surface of the molecular layer, rather than embedded in the tissue.

To record from a single Purkinje cell the electrode was positioned just above the cell layer, using a Narishige NM-3 hydraulic manipulator. Once in position the electrode was advanced into the slice in short steps using a Newport motion controller. As the tip advanced through the tissue it would occasionally penetrate a cell; these cells were usually unhealthy, and quickly died. A more satisfactory result was obtained by advancing the electrode so that it was in contact with a cell, and then rupturing the membrane using the buzz switch on the amplifier (this switch produces a brief increase in the capacitance compensation, causing the circuit to oscillate. Exactly how this leads to penetration is not known). That the electrode tip was in contact with a cell was assessed from the electrode resistance, which was monitored on the oscilloscope using short current steps (from Ohm's law: voltage = resistance x current). When the electrode tip contacted a cell an increase in resistance was observed. Once a cell was penetrated, it was quickly hyperpolarised to prevent the potentially harmful effects of voltage dependent Ca^{2+} entry and to suppress the spontaneous activity in the cell. For a few minutes after impalement the amplitude of current required to hyperpolarise the cell would decrease. Once this reached a stable level, recording could commence. During recording the cell was held at an apparent membrane potential of ~ -85 mV. Cells

requiring more than 1.5 nA to hyperpolarise the cell to –85 mV were rejected

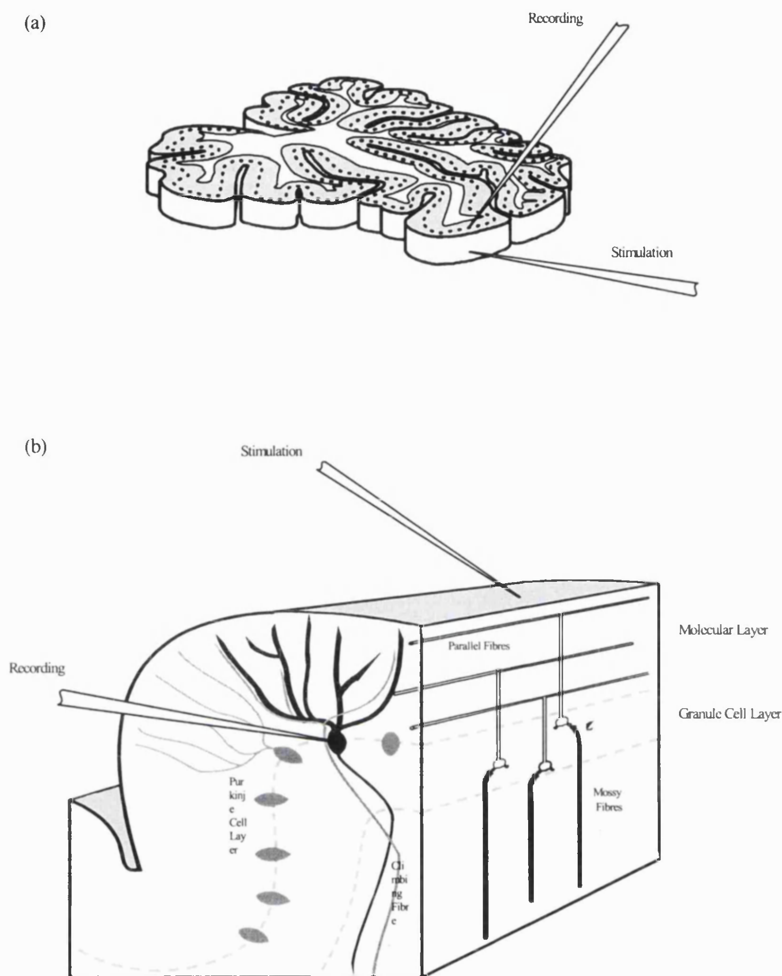


Figure 2.2. (a) Position of Electrodes in Saggital Slice of Cerebellum. (b) 3-D diagram of cerebellar cortex showing arrangement of parallel fibres and Purkinje cells.

Sources of error associated with sharp microelectrode recording

When using intracellular microelectrodes to measure the membrane potential of neurones there are a number of potential sources of error. Fortunately these are well documented, and steps can be taken to compensate for them.

When resistors (R) and capacitors (C) are placed in series or parallel in a circuit a filter can be formed. Whilst this characteristic of RC circuits is a useful tool there are situations when unwanted filtering of electrical signals occurs. One example of this comes from the electrode capacitance and the electrode resistance (together with stray capacitances from sources such as the rest of the microelectrode and grounded objects in close proximity) which form a low pass filter. Low pass filters, as the name suggests, allow low frequency signals, but eliminate high frequency signals, which results in an apparent slowing of a signal. Capacitance is proportional to area, and it is therefore necessary to minimise the area of the electrode in contact with the solution to reduce the capacitance across the glass wall (transmural capacitance). To do this the level of fluid in the bath was carefully controlled, so that it only just covered the slice. In addition checks were made that the bath solution was not travelling too far up the sides of the glass by surface tension. It is, however, not possible to completely remove this and other sources of capacitance by physical means; therefore, capacitance neutralisation circuitry of the amplifier was used. By passing short current steps, and monitoring the voltage, the capacative transients could be observed. By adjusting the capacitance neutralisation a current of equal magnitude to that lost to the total capacitance is added. In practise this was not totally effective and care had to be taken as over compensation produces oscillations of the circuitry which usually resulted in a lost cell.

Measuring cell membrane potential with a microelectrode, whilst simultaneously passing current through the same electrode results in erroneous measurement of membrane potential as a result of the voltage drop across the microelectrode resistance, which will add to the membrane potential. This can be compensated for by the bridge balance circuitry, which subtracts a signal that is proportional to that resulting from the

microelectrode current and resistance. With the electrode in the bath, but prior to attempting to penetrate a cell, a short current step was applied (0.2 nA for 35 ms; this ct step can be seen, for example, following the SS1 EPSP in Figs. 4.3 and 4.4). The change in voltage this produced was due to the microelectrode, and the bridge balance control was adjusted until this was eliminated. This function was originally performed using Wheatstone bridge circuitry, although in modern amplifiers operational amplifiers are used, but the name has been kept. The balance control on the Axoclamp-2A amplifier is calibrated so that the resistance of the electrode can be read. Once the cell was penetrated the bridge would often need to be adjusted as the electrode resistance would often increase. Accurate balancing of the bridge to an extent is subjective, as incomplete capacitance compensation can lead to difficulty in judging exactly when the bridge is balanced.

Another source of error in the measured voltage comes from junction potentials. These occur wherever two dissimilar conductors connect, such as the silver wire and the KCl solution, and between the KCl in the electrode, and the perfusate in the recording bath. Electrons flowing from the amplifier into the silver wire will convert the AgCl to Ag and the Cl^- ions become hydrated and enter the filling solution. When electrons flow in the opposite direction Ag atoms in the AgCl coated wire give up their electrons, and combine with Cl^- ions in the filling solution, producing insoluble AgCl. For this reaction to occur there must obviously be Cl^- ions present in the filling solution. The reaction is reversible and produces a predictable potential. It is because of junction potentials, and the fact that gas bubbles often form at metal/liquid interfaces that metal electrodes are not used for intracellular recording. The junction potentials are effectively removed when the electrode first enters the bath by using the input offset to introduce an

opposing potential to zero the summed junction potentials. Errors in measurement of the membrane potential can also result from changes in the tip potential (which often accompany changes in tip resistance). This is compensated for at the start of the recording with the DC offset control; it can, however, change during a recording due to the complex surface chemistry of the glass, and its interaction with the surrounding environment (Halliwell *et al.*, 1994). In practise, it was usually easy to tell if a large offset occurred during recording from the voltage dependent characteristics of the cell i.e. a change in the apparent firing threshold of the cell.

Tight-Seal Whole Cell Voltage Clamp Recording

Recording Equipment

For the whole cell voltage clamp recording an Axopatch 200B amplifier (Axon Instruments Inc.) was used. This was interfaced with a Pentium PC via a Digidata 1200 analogue-digital converter. The software used for acquisition was Clampex 7 (Axon Instruments Inc.). The slice was viewed with a Zeiss Axioskop upright microscope with a x40 magnification water-immersion lens. The holding chamber, manipulators and microscope were all mounted on an antivibration table. To allow real-time monitoring of the electrophysiological characteristics of the cell, the signal from the amplifier was also sent to an oscilloscope. Parallel fibres were stimulated using pulses of a typical amplitude of about 30 V with a duration of 0.02 ms (Digitimer isolated stimulator model DS-2).

Perfusion System

As with the sharp electrode recording the slice was held submerged in aCSF in the recording chamber by a slice weight. The aCSF was pumped in and out of the recording chamber (Ismatec Reglo pump) at a rate of ~1.5 $mlmin^{-1}$. Unless stated otherwise, the perfusate at room temperature (19-26°C).

Preparation of Recording and Stimulating Electrodes

For those experiments investigating excitatory synaptic transmission, a monopolar stimulating electrode (~1 MΩ patch pipette filled with 0.9% NaCl) was embedded in the molecular layer such that its tip was positioned beneath the dendrites of the selected Purkinje cells. The patch electrodes were produced using 1.5 mm diameter borosilicate glass capillaries (1.17 mm internal diameter; Clark Electromedical Instruments). Like the electrode glass used to fashion the sharp microelectrodes, the capillaries had an internal filament to aid filling of the electrode, although with the wide diameter pipettes filling of the electrode to the tip was considerably easier than with the microelectrodes. To produce the recording pipette a Narishige Model PB-7 electrode puller was used. This was set to pull electrodes with a resistance of approximately 1.5 MΩ. These were then heat-polished using Narishige microforge (model MF-9) to give a final resistance of between 2 and 2.4 MΩ and a diameter of about 1 μm at the tip. After polishing, the capillaries were coated with M-COAT D (Measurements group UK ltd.) so that about 2cm of the capillary was coated, starting from about 1 mm back from the tip. M-COAT is an air-drying acrylic (drying in about 10-15 mins), which was used to increase the wall thickness of glass wall, and thereby lower the transmural capacitance. The M-

COAT is also hydrophobic, and therefore stops the external solution travelling up the outside of the pipette by surface tension, again helping to lower the transmural capacitance. The constituents of the solution in the recording electrode are free to diffuse into the cell, and it is therefore important that the osmolarity, pH etc. of the solution are controlled, so that the health of the cell is maintained. Unless stated otherwise, the electrodes were filled with an internal solution containing 150 mM CsCl, 10 mM HEPES, 500 μM Cs-EGTA and 4 mM Mg-ATP. This solution had an initial pH of about 4, and CsOH was added to bring the pH up to a value of 7.4. The solution was initially prepared so that the osmolarity was slightly higher than desired, and distilled water was added to give a final osmolarity of between 295 and 298 mOsm. For some experiments Na-GTP (0.4 mM) was added to the solution. The prepared internal solution was stored at –20°C in 1 ml aliquots. On the day of an experiment an aliquot would be thawed, and then kept on ice. The frozen aliquots were stored for about 1 month and, once thawed, aliquots were not re-frozen.

As with sharp microelectrode recording a AgCl-coated silver wire connected the pipette to the recording circuitry. Junction potentials will exist between liquid-metal interfaces, and the summed potentials are compensated for when the electrode is in the bath.

Recording Technique

Healthy Purkinje cells could clearly be differentiated from the unhealthy neurones that would be large and swollen, or had a dull, shrivelled appearance. Once a Purkinje cell had been selected for recording, and the stimulating electrode had been positioned, the recording electrode was positioned above the cell, without entering the external

solution. Prior to lowering the electrode into the bath positive pressure (~0.6 PSI) was applied so that a steady stream of internal fluid would flow from the electrode tip. For a high resistance seal to form it is important that the tip of the electrode is clean, and this positive pressure provides a stream of internal solution which helps push debris away from the tip. As the electrode approached the cell, the stream of fluid produced a dimple in the cell membrane. The electrode was positioned such that on release of the positive pressure the membrane in the dimpled region would fall back into contact with the tip of the electrode. On release of the positive pressure, a seal would start to form between the glass of the pipette tip and the cell membrane. To aid formation of this seal, on release of the positive pressure, suction was applied (about 0.3 psi) and, as the seal began to form, negative voltage was applied. When applying the voltage, the holding current was kept less than 1 nA. The resistance of the seal was measured using seal test in Clampex. This applies a voltage step, and calculates the resistance. Using this technique it was possible to routinely achieve seals with a resistance of 2-3 GΩ. High resistance seals are necessary as any current lost through the seal will increase the noise in the recording (current noise, resulting from the random motion of charged particles in a conductor, termed Nyquist or Johnson noise, is inversely proportional to the resistance), and the lower the seal resistance the higher the fraction of current lost through the seal resistance and thus not measured. As with sharp microelectrode recording the pipette capacitance has undesirable effects on the recording. Keeping the bath level low, and increasing the pipette thickness with M-COAT, helped to physically reduce pipette capacitance and, once a seal was established, the pipette capacitance compensation controls were used to reduce the capacitive transients, seen at the start and end of the voltage step, into the noise. This is necessary as while the pipette capacitance is charging, the potential at the electrode tip will change only slowly, so high frequency

events may be distorted. Secondly, because the current flowing into the pipette capacitance is not in series with any resistance, the series resistance compensation circuitry (discussed below) will become unstable, and overload for this component of the current. Thirdly, increasing the pipette thickness with the insulator M-COAT will help reduce noise (Penner, 1995; Benndorf, 1995)

Before breaking the cell membrane under the patch the holding potential was set at –70 mV. To break the membrane, steadily increasing suction was applied and as soon as the membrane was ruptured the suction was ceased.. When the membrane patch ruptured large capacitive transients, resulting from the cell membrane capacitive current, could be seen at the onset and end of the voltage step. The amplifier has circuitry to compensate for these transients. This compensation is required, as during these events changing from one voltage to another would require excessively large currents and the system would saturate; resulting in the time taken for the membrane potential to change being significantly slowed. Once the compensation controls are set, it is possible to read off the values for the cell membrane capacitance, and the series resistance (resistance of the pipette and ruptured membrane patch).

The series resistance of the pipette and the ruptured membrane patch are large enough to give rise to significant voltage errors. In addition to reducing the control of the membrane potential, the series resistance, together with membrane capacitance dictates how quickly the membrane potential can be changed and can filter signals, potentially distorting high frequency signals (Rudy & Iversen, 1992). To compensate for series resistance a signal proportional to the measured current is added to the command potential. Total compensation of the series resistance is never possible as the circuitry is

degraded by feedback and will eventually oscillate. The series resistance was monitored throughout all experiments. At the start of recording this would typically be between 8 and 12 MΩ. If it fluctuated by more than ~20% the recording would be terminated. Compensation was typically set around 60%. The effects of series resistance compensation on parallel and climbing fibre EPSCs have been investigated in Purkinje cells (Llano *et al.*, 1991). For the climbing fibre EPSC the major result of compensation was to increase the amplitude of the EPSC. For the parallel fibre EPSC the effects were more variable, probably reflecting the proximity of the synaptic input to the soma. For presumed distal synapses the main action of the compensation was a decrease in the rise time of the current. Although this was not specifically investigated in the present work, clear changes in the time to peak of the parallel fibre EPSC could be observed by switching the compensation on and off.

In a small spherical cell with an electrode at the centre of the soma it is possible to voltage clamp the membrane at a single potential, and any membrane currents should be measured free from any distortion from voltage changes. In large irregular shaped cells, such as Purkinje cells, it is not possible for the membrane to be isopotential. The voltage drop across the internal resistance of the cell will attenuate the voltage signal from the electrode; this resistance will vary depending on the internal geometry of the cell. This lack of so called "space clamp" has a number of effects: firstly reversal potentials of distal synaptic inputs, measured in the soma, will not be equal to the synaptic reversal potential. Secondly, the conductance change at the soma will not equal that in the synapse. Thirdly, the low pass filtering properties of the dendrite will cause the time course of the synaptic current to be slower at the soma, than at the point of origin (Spruston *et al.*, 1993; Spruston *et al.*, 1994). This is one of the reasons that younger

animals were used: their Purkinje cells are more compact, with a less extensive dendritic tree, permitting better voltage clamp, compared to adult rats in which synaptic currents can arise from regions of the cell escaping voltage control (Llano *et al.*, 1991). To improve the space clamp steps are usually taken to increase the cell membrane resistance by blocking ion channels, and thereby make the cell more electrotonically compact. In the present experiments a CsCl-based solution was used to fill the recording electrode, as cesium blocks a number of K^+ channels. External application of ion blockers is also commonly performed, but for experiments studying synaptic transmission, use of these would not be appropriate as they could interfere with presynaptic mechanisms. For high frequency events the attenuation of the current comes primarily from the membrane capacitance and internal resistance, and therefore increasing membrane resistance is not expected to improve the voltage clamp (Spruston *et al.*, 1993). In fact for poorly clamped synapses increases in the membrane resistance may actually lead to a slowing of the measured current. This is because an increase in membrane resistance increases the membrane time constant, τ_m (the product of membrane resistance and capacitance), and, therefore, any change in voltage at the synapse will have a slower decay. The voltage clamp circuit will pass current for as long as this change in voltage is detected, and therefore the synaptic current will appear prolonged (Spruston *et al.*, 1993; Spruston *et al.*, 1994).

Advantages and Disadvantages of the Two Recording Techniques

The two recording techniques used both have their advantages and disadvantages. Obtaining useable cells with sharp microelectrodes was difficult. This was perhaps due to the Purkinje cells being in a single cell layer, making the probability of the electrode missing the cell body high, and the fact that the electrode has to pass through the

processes of Bergmann glial cells to access the Purkinje cell. However, once a healthy cell had been obtained the recording would often be long lasting, allowing data to be acquired over several hours. The high resistance of the microelectrode did create difficulties, in particular blocking of the electrode would occur. Occasionally the electrode could be cleared by carefully increasing the capacitance neutralisation so that the circuit started to oscillate, although cells were often lost doing this. Additionally, during prolonged recordings a DC offset would sometimes develop. Usually this was small (less than 10 mV), but occasionally was substantial enough for the measured membrane potential to be clearly inaccurate, and voltage dependent effects, such as firing threshold, to be active at apparently incorrect potentials.

It is possible that penetrating the cell membrane with the electrode reduces the health of the cell as cells recorded with sharp electrodes seem to have lower input resistance than those recorded with patch electrodes (Edwards *et al.*, 1989; Staley *et al.*, 1992; Spruston *et al.*, 1994). This may be due entirely to damage of the cell membrane during penetration, and additionally may be due to Ca^{2+} leaking into the cell around the edges of the electrode and activating Ca^{2+} -dependent conductances.

Using patch electrodes and whole cell recording, successful recordings were much easier to obtain, although compared to sharp electrode recording the duration of a stable recording was shorter, either because the cell would be lost, or series resistance would increase. The increased magnification used to view the cells also facilitated positioning of the stimulating electrode. This greatly reduced some of the problems found using the sharp electrode technique, where direct stimulation of the Purkinje cells, or activation of climbing fibres, was often a problem. A potentially significant problem associated with

the use of patch electrodes is that of washout. The soluble contents of the pipette and cell are free to diffuse, and this can lead to some responses becoming reduced during a recording, perhaps due to contents of the electrode solution disrupting enzymes and second messenger systems, or soluble contents of the cell diffusing into the pipette (Horn & Marty, 1988). Several methods are routinely used by investigators to overcome this problem, including supplementing the pipette solution with soluble constituents of cells that are likely to be diluted, thus impairing normal cell function, such as ATP and GTP, or by perforated patch recording. In this technique the patch of membrane under the patch pipette is not ruptured; instead, it is permeabilised by the addition of pore-forming agents, such as nystatin and similar agents, to the pipette solution (Horn & Marty, 1988). In the experiments described here, because of the potential complications of run down, individual cells were not used to perform multiple experiments. Run down is not a significant problem with conventional high resistance microelectrodes, where the narrow tip of the microelectrode restricts diffusion. Although disruption of second messengers can be a significant problem, diffusion between the cell and internal solution can be advantageous, allowing investigators to add known concentrations of agents intracellularly, such as inhibitors of G-proteins and other second messengers, to study the transduction pathways of second messenger-linked events. Diffusion from the pipette has also been exploited in experiments using ion and voltage sensitive dyes. Perhaps the most significant problem of whole-cell voltage clamp is that of space clamp, and it is important to remember that a current generated in the dendrites is likely to be distorted by the time it reaches the recording electrode (Armstrong & Gilly, 1992). The introduction of the tight-seal whole cell voltage clamp technique has, however, allowed investigators to study individual currents at a much greater resolution, and free from distortion by voltage-dependent effects, to a degree that was not previously

possible using conventional recording techniques, and is now the standard technique employed by neurophysiologists.

Chapter 3: Pharmacology of the depression of synaptic transmission at the parallel fibre-Purkinje cell pathway in the rat cerebellar cortex by selective mGlu receptor agonists

Introduction

A commonly observed action of G-protein coupled receptors is to regulate release of neurotransmitter, either acting to alter release of the receptor's own transmitter (autoreceptors), or that of others (heteroreceptors). Modulation of synaptic transmission has been shown to be mediated by, amongst others, receptors for adenosine (Dittman & Regehr, 1996), cannabinoids (Levenes *et al.*, 1998), dopamine (Harvey & Lacey, 1996; Nicola & Malenka, 1997), opioids (Cherubini *et al.*, 1985; Faber *et al.*, 1998) and GABA (Dutar & Nicoll, 1988a; Dittman & Regehr, 1997). The mGlu receptors are no exception, and receptors from each of the three groups of mGlu receptors regulate synaptic transmission, producing both facilitation and depression at different synapses. Co-application of the mGlu receptor agonist (1S,3R)-ACPD with arachidonic acid results in facilitation of glutamate release from cortical synaptosomes, (Herrero *et al.*, 1992), and potentiation of excitatory synaptic transmission at the Schaffer collateral-CA1 synapse (Collins & Davies, 1993). Subsequently, it was reported that application of the selective group I mGlu receptor agonist DHPG produced a PKC-dependent potentiation of glutamate release from cortical synaptosomes, without requiring the presence of arachidonic acid (Reid *et al.*, 1999). A more widely observed effect of mGlu receptor agonists is depression of fast excitatory transmission. In the early 1980s, the glutamate analogue L-AP4 was found to depress synaptic transmission at a number of synapses in the CNS (Koerner & Cotman, 1981; Davies & Watkins, 1982). At this

time the mGlu receptors had not been identified, although these early results were probably due to L-AP4 activating presynaptic group III mGlu receptors. Depression of synaptic transmission by mGlu receptor agonists, such as L-AP4 and (1S,3R)-ACPD, has been observed at synapses in a variety of brain areas such as the cerebellum (Crépel *et al.*, 1991; Glaum *et al.*, 1992), neocortex (Burke & Hablitz, 1994), olfactory bulb (Schoppa & Westbrook, 1997), hippocampus (Baskys & Malenka, 1991), nucleus tractus solitarius (Glaum & Miller, 1993), spinal cord (Jane *et al.*, 1994) and the striatum (Lovinger & McCool, 1995). The mGlu receptors also act to regulate the release of other transmitters such as GABA (Glitsch *et al.*, 1996; Morishita *et al.*, 1998) and dopamine (Ohno & Watanabe, 1995; Antonelli *et al.*, 1997).

The majority of studies into the action of mGlu receptors on synaptic transmission have involved the application of exogenous compounds, using ligands such as (1S,3R)-ACPD, which show poor selectivity between the different groups of mGlu receptors. For receptors expressed in cell lines, agonists and antagonists which act with a high degree of selectivity between the three groups of receptors are now commercially available (Conn & Pin, 1997; Schoepp *et al.*, 1999). With the exception of mGlu6, which appears to be restricted to the retina (Nakajima *et al.*, 1993; Laurie *et al.*, 1997), all of the cloned mGlu receptors are expressed in the rat cerebellar cortex, and mGlu receptors are known to exert both pre- and postsynaptic actions at the parallel fibre-Purkinje cell synapse (Pekhletski *et al.*, 1996; Batchelor & Garthwaite, 1997). Using group-selective mGlu receptor agonists, we have examined the actions of each of the 3 groups of mGlu receptors on fast synaptic transmission, which is presumed to be mediated through AMPA receptors (Batchelor & Garthwaite, 1997). In addition, the activity of antagonists, reported to act in a group-selective manner on receptors in cell

lines, has been tested against these agonists. The aim of these experiments was two-fold: firstly, to characterise the activity of agonists and antagonists, previously reported to be selective for receptor subtypes expressed in cell lines, on native receptors in neurones; secondly, having identified antagonists which can select between the mGlu receptors, to determine the physiological role of the different mGlu receptors.

Results

Group I

Figure 3.1a illustrates the effect of (S)-3,5-dihydroxyphenylglycine (DHPG; 30 μM) on the amplitude of EPSPs evoked by stimulation of the parallel fibres at a frequency of 0.067 Hz. In the presence of DHPG the EPSP amplitude was reversibly reduced. The depression of the EPSP was concentration-dependent (EC_{50}=18 μM, n = 3-8. Figure 3.1b), with the highest concentration (100 μM) depressing the EPSP to 17 ± 8 % of control levels ($P < 0.01$, n = 4).

To test whether this depression of the EPSP amplitude was mediated by group I receptors DHPG, at a concentration producing approximately 50% depression, was applied in the presence of one of two antagonists. When DHPG (30 μM) was re-applied in the presence of the group I antagonist 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt; 100 μM), the DHPG-induced depression was significantly inhibited from 40 ± 5% to 83 ± 6% of baseline ($P < 0.001$, n=5; Fig 3.2a,c). On washout of the CPCCOEt, DHPG was re-applied in the presence of the group II/III antagonist (RS)-α-methyl-4-phosphonophenylglycine ((RS)-MPPG; 300 μM), or its active enantiomer (S)-α-methyl-4-phosphonophenylglycine (MPPG 100

μM; Fig. 3.2b). This had no significant action on the DHPG-induced depression of the EPSP which was depressed to a mean level of 36 ± 11 % of baseline ($P > 0.05$, $n = 3$; Fig 3.2c).

At higher concentrations, DHPG (10-100 μM) application resulted in depolarisation of some Purkinje cells. This depolarisation was sufficient for the cells to reach firing threshold. To prevent a significant change in the membrane potential, the holding current was manually increased. The increase in holding current in the presence of DHPG was statistically significant compared to control conditions (see Table 1). DHPG (100 μM) produced no significant change to the measured Purkinje cell input impedance (calculated using by applying a 35 ms current pulse of 0.2 nA prior to the parallel fibre stimulation. see Table 1).

At 100 μM, DHPG produced a depolarising effect in all 3 cells tested (Table 1). At a lower concentration (30 μM), application of DHPG in 4 cells (of 8 tested) had a depolarising effect. As with the 100 μM concentration, the holding current was manually increased to counteract this. Under control conditions the mean membrane potential was held at -87 ± 3 mV with a mean holding current of -1.02 ± 0.08 nA. In the presence of 30 μM DHPG the membrane potential was manually clamped at a mean potential of -89 ± 4 mV, with the holding current increasing to -1.27 ± 0.12 nA ($P < 0.05$; $n = 4$). In 4 other cells, however, application of 30 μM DHPG resulted in no observable change to the membrane potential, and no change in the holding current was required during the drug application (membrane potential: control = -90 ± 4 mV; in DHPG = -91 ± 4 mV; holding current: control = -0.87 ± 0.20 nA; in DHPG = -0.88 ± 0.18 nA). Although in these four cells the compound did not appear to depolarise the

Purkinje cell, it did induce a significant depression in the amplitude of the parallel fibre-Purkinje cell EPSP recorded in these cells to 44 ± 8% of control values. ($P < 0.01$; $n = 4$). This degree of depression was not significantly different from that seen in the 4 cells that depolarised (40 ± 5%; $P > 0.05$) or from the whole population studied (42 ± 6%).

The antagonist CPCCOEt (100 μM) produced no significant changes to the EPSP amplitude (control = 10 ± 2 mV; CPCCOEt = 10 ± 1 mV; $P > 0.05$; $n = 5$), nor did it affect the membrane potential or input impedance (see Table 1).

	Membrane Potential (mV)		**Holding Current (nA)**		**Input Impedance (MΩ)**	
	Control	**Compound**	**Control**	**Compound**	**Control**	**Compound**
DHPG (n = 3-4)	-85 ± 1	-86 ± 2 (P > 0.05)	0.9 ± 0.1	-1.2 ± 0.2 (P < 0.05)	31 ± 2	27 ± 1 (P > 0.05)
DCG IV (n = 4)	-82 ± 2	-83 ± 3 (P > 0.05)	-0.9 ± 0.2	-0.9 ± 0.1 (P > 0.05)	27 ± 6	25 ± 6 (P > 0.05)
L-AP4 (n = 5)	-85 ± 2	-85 ± 2 (P > 0.05)	-0.6 ± 0.1	-0.6 ± 0.1 (P > 0.05)	35 ± 7	34 ± 7 (P > 0.05)
CPCCOEt (n = 5)	-90 ± 4	-90 ± 4 (P > 0.05)	-0.9 ± 0.1	-0.9 ± 0.1 (P > 0.05)	27 ± 3	26 ± 4 (P > 0.05)
MPPG (n = 5)	-88 ± 4	-89 ± 4 (P > 0.05)	-0.9 ± 0.1	-0.9 ± 0.1 (P > 0.05)	28 ± 4	28 ± 4 (P > 0.05)

Table 1. Effect of group-selective mGlu receptor agonists and antagonists on electrophysiological characteristics of Purkinje cells

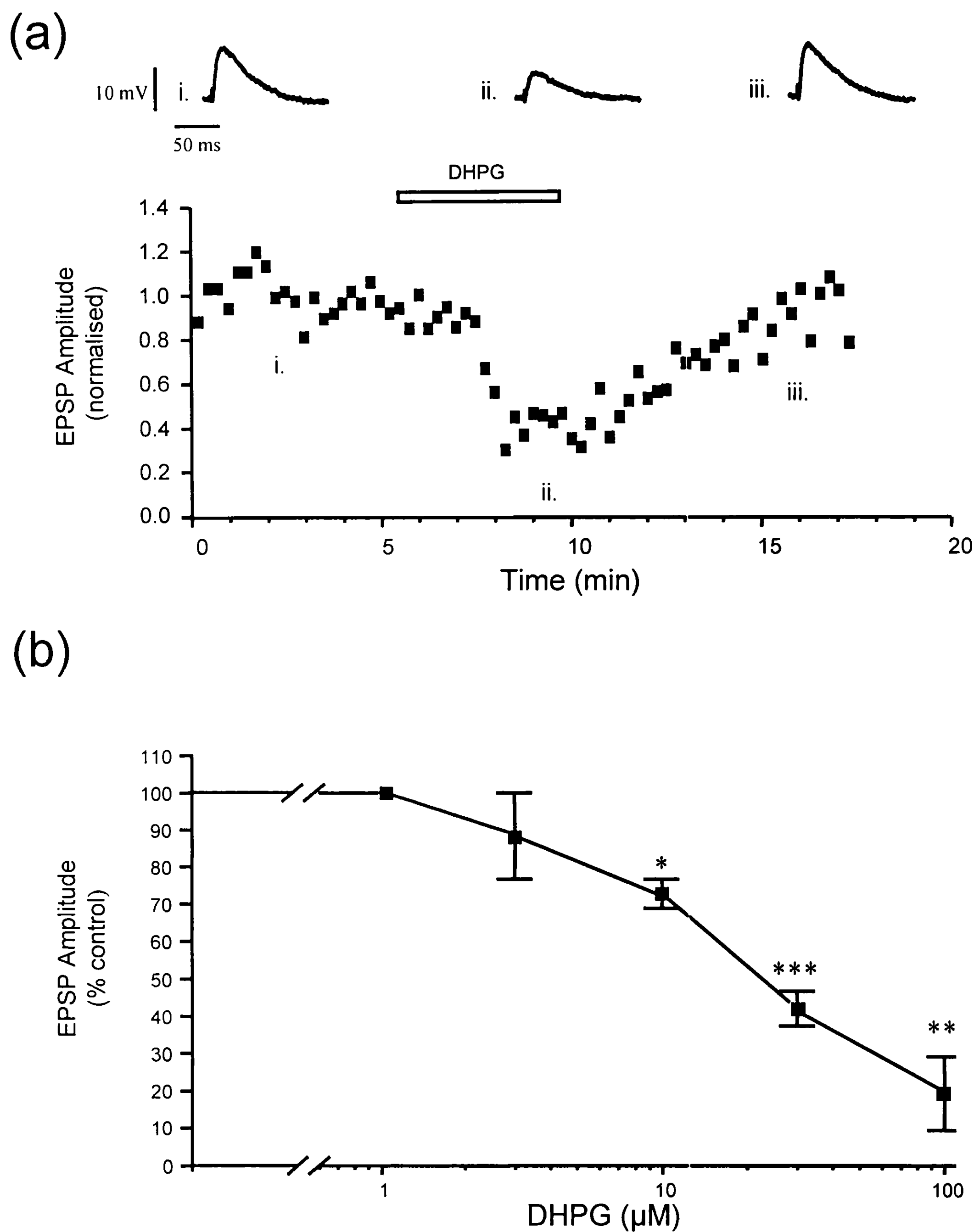


Figure 3.1. Depression of parallel fibre-Purkinje cell synaptic transmission by a group 1 mGlu receptor agonist. (a) Time course of the change in EPSP amplitude induced by DHPG (30 μM) perfusion, recorded in a single Purkinje cell. Each point is the amplitude of a single EPSP. The insets show averages of 5 EPSPs elicited at the times indicated (i, ii, iii). (b) Effect of DHPG (1 - 100 μM) on the amplitude of the parallel fibre EPSP (n = 3-7). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

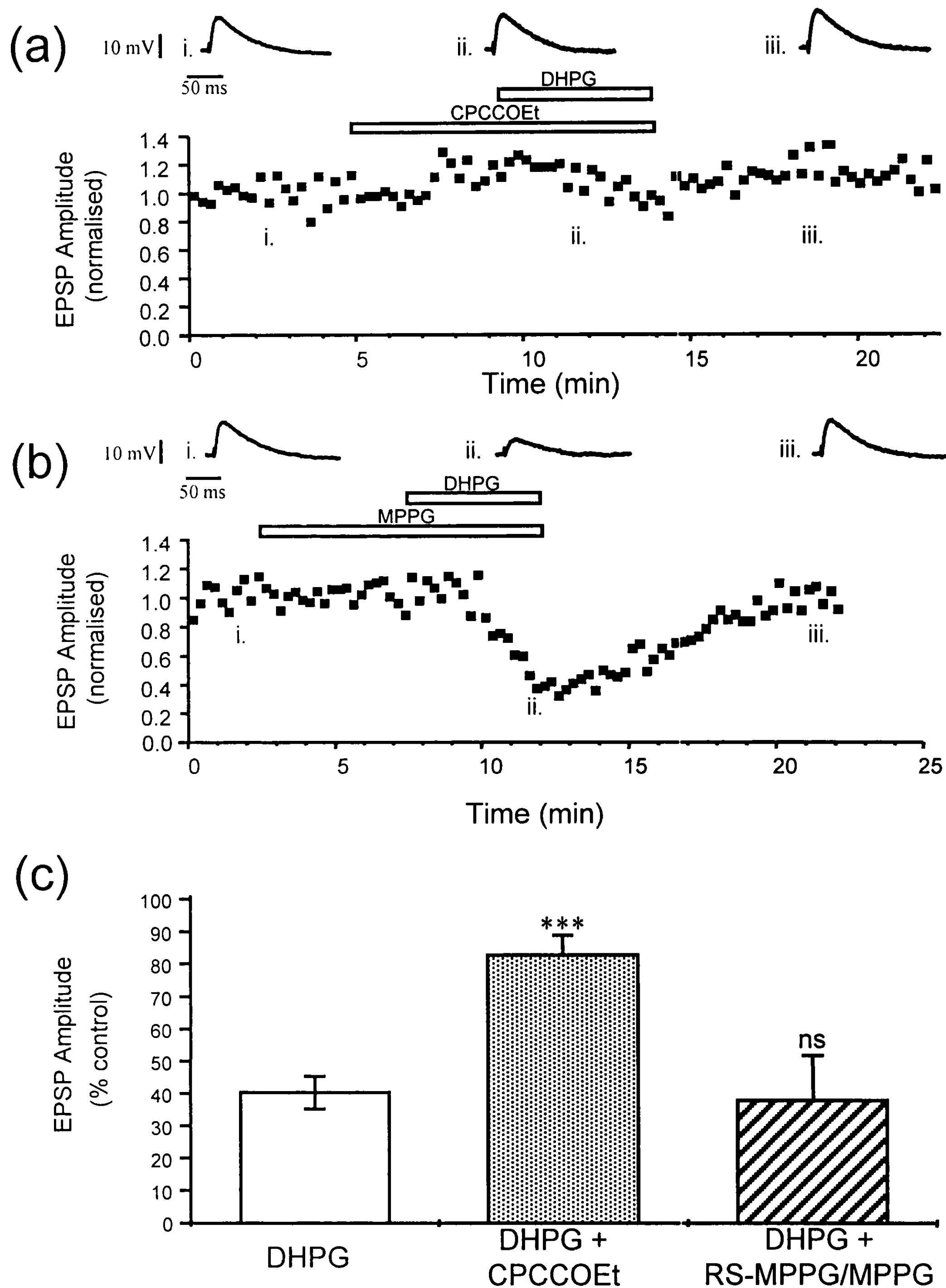


Figure 3.2. Pharmacology of the DHPG – induced synaptic depression. (a) In the same cell as Figure 1, perfusion of CPCCOEt (100 μM) inhibited the EPSP depression resulting from DHPG (30 μM) application. (b) Subsequent perfusion of the group II/III antagonist MPPG (100 μM) was ineffective. Each point is the amplitude of a single EPSP. The insets show averages of 5 EPSPs elicited at the times indicated (i, ii, iii). (c) Pooled data (n = 3-5) of the effects of CPCCOEt (100 μM) and MPPG, or RS-MPPG (100 or 300 μM) ***P < 0.001; ns, not significantly different from DHPG alone.

Group II

Application of (2S, 2'R, 3'R)-2-(2', 3'-dicarboxycyclopropyl)glycine (DCG IV, 1 μM), at a concentration reported to selectively activate the group II mGlu receptors (Ishida *et al.*, 1993; Lovinger & McCool, 1995; Kamiya *et al.*, 1996; Brabet *et al.*, 1998), produced no significant effect on the amplitude of the parallel fibre EPSP (103 ± 8% of control; n = 3; Fig. 3.3). Increasing the concentration of DCG IV (30 μM) did, however, result in a significant, reversible reduction in the amplitude of the EPSP to 33 ± 8% of baseline ($P < 0.001$; n=3; Fig 3.4 + 3.5). Some actions of DCG IV have been attributed to DCG IV being an NMDA receptor agonist (Wilsch *et al.*, 1994; Uyama *et al.*, 1997). To test whether such an effect could account for the depression of the parallel fibre EPSP, DCG IV was re-applied in the presence of the NMDA receptor antagonist D(-)-2-amino-5-phosphonopentanoic acid (AP5; 100 μM). This resulted in a significant inhibition of the DCG IV-induced depression (93 ± 4%. Significant difference from DCG IV alone: $P < 0.01$; n=3; Fig. 3.5b), with no significant difference from the baseline EPSP (i.e. mean = 100%; $P > 0.05$; n=3). On washout of the AP5, re-application of DCG IV depressed the EPSP amplitude (Fig. 3.4c).

NMDA receptor agonists do not exert any pre- or postsynaptic actions at the parallel fibre-Purkinje cell synapse (Llano *et al.*, 1991; Glitsch & Marty, 1999 and refs. therein). We therefore tested the possibility that DCG IV depresses transmission indirectly, via excitation of inhibitory interneurones, and subsequent suppression of glutamate release by spill-over of GABA acting on $GABA_B$ receptors on the parallel fibre terminals. In a single experiment DCG IV (30 μM) was applied in the presence of the $GABA_B$ receptor

antagonist CGP 35348 (1mM). In the presence of this antagonist the effects of DCG IV on the parallel fibre EPSP appeared to be inhibited (Fig. 3.4d).

DCG IV (30 μM) produced no significant change to the membrane potential or input impedance (see Table 1).

Group III

Application of the group III selective agonist, L(+)-2-amino-4-phosphonobutyric acid (L-AP4), at concentrations of 3-100 μM, produced a concentration-dependent depression of EPSP amplitude (EC_{50}= 5 μM; n = 3-7; Fig 3.6b). This depression was reversible on washout of the agonist (Fig 3.6a). The depression produced by 10 μM L-AP4 (52 ± 8 % of control; n = 3) was inhibited when L-AP4 was re-applied in the presence of 100 μM of the group II/III antagonist MPPG (97 ± 3% of baseline; $P < 0.01$; n = 3); but was unaffected by 1mM of the group I/II antagonist (S)-α-methyl-4-carboxyphenylglycine (MCPG; 46 ± 11% of baseline; $P < 0.05$; n = 3; Fig 3.7b).

In the presence of L-AP4 (100 μM) no significant changes were observed to the cell's membrane potential and input impedance (see Table 1). The antagonist MPPG (100 μM) produced no significant changes to the EPSP amplitude (control = 11 ± 2 mV; MPPG = 11 ± 2 mV; $P > 0.05$; n = 5), nor did it affect the membrane potential, holding current or input impedance (see Table 1).

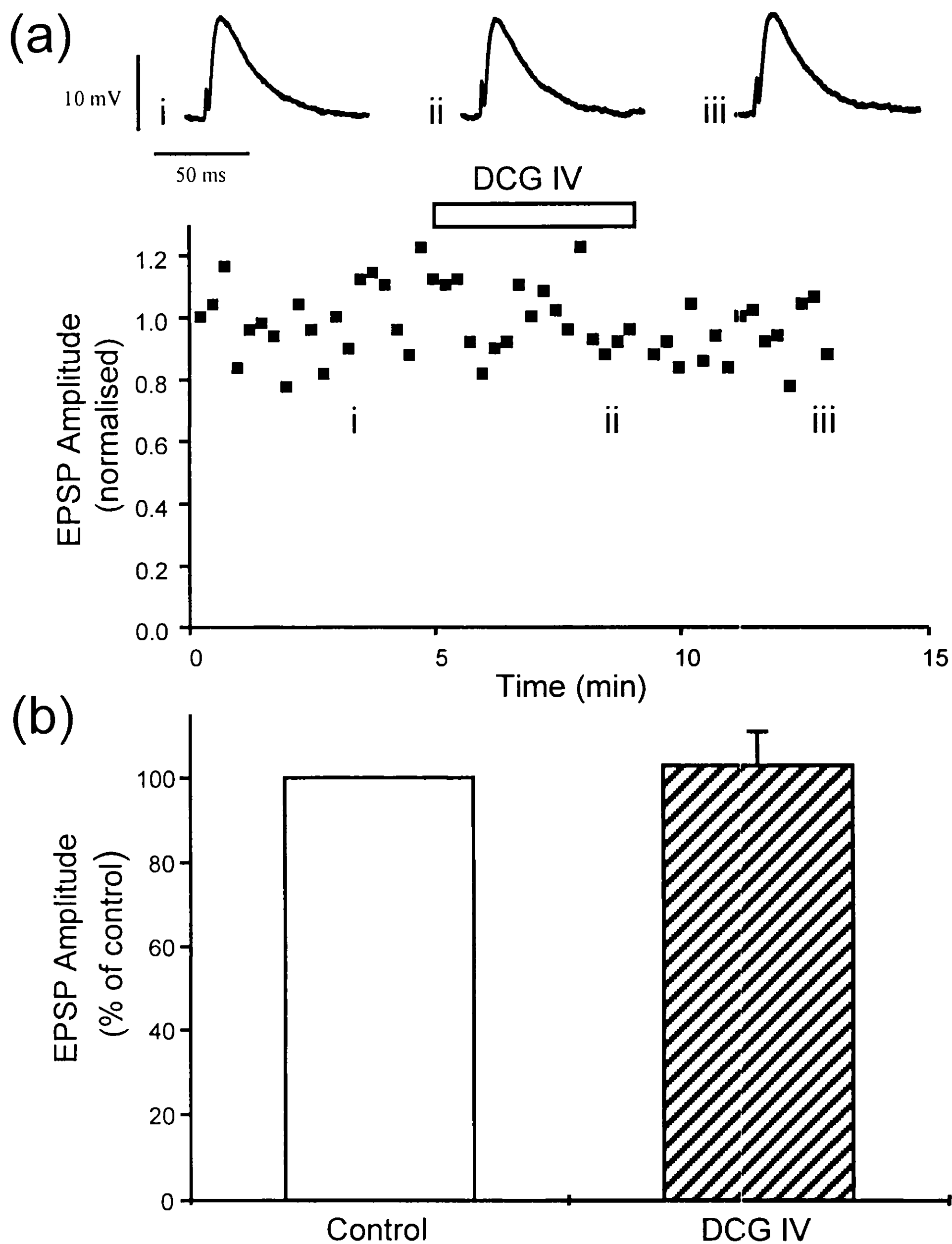


Figure 3.3. Lack of effect of a group II agonist. (a) EPSPs recorded in a single cell were unaffected by perfusion of DCG IV (1 μM), at a concentration known to activate group II mGlu receptors. Each point is the amplitude of a single EPSP. The insets show averages of 5 EPSPs elicited at the times indicated (i, ii, iii). (b) Pooled data from 3 cells.

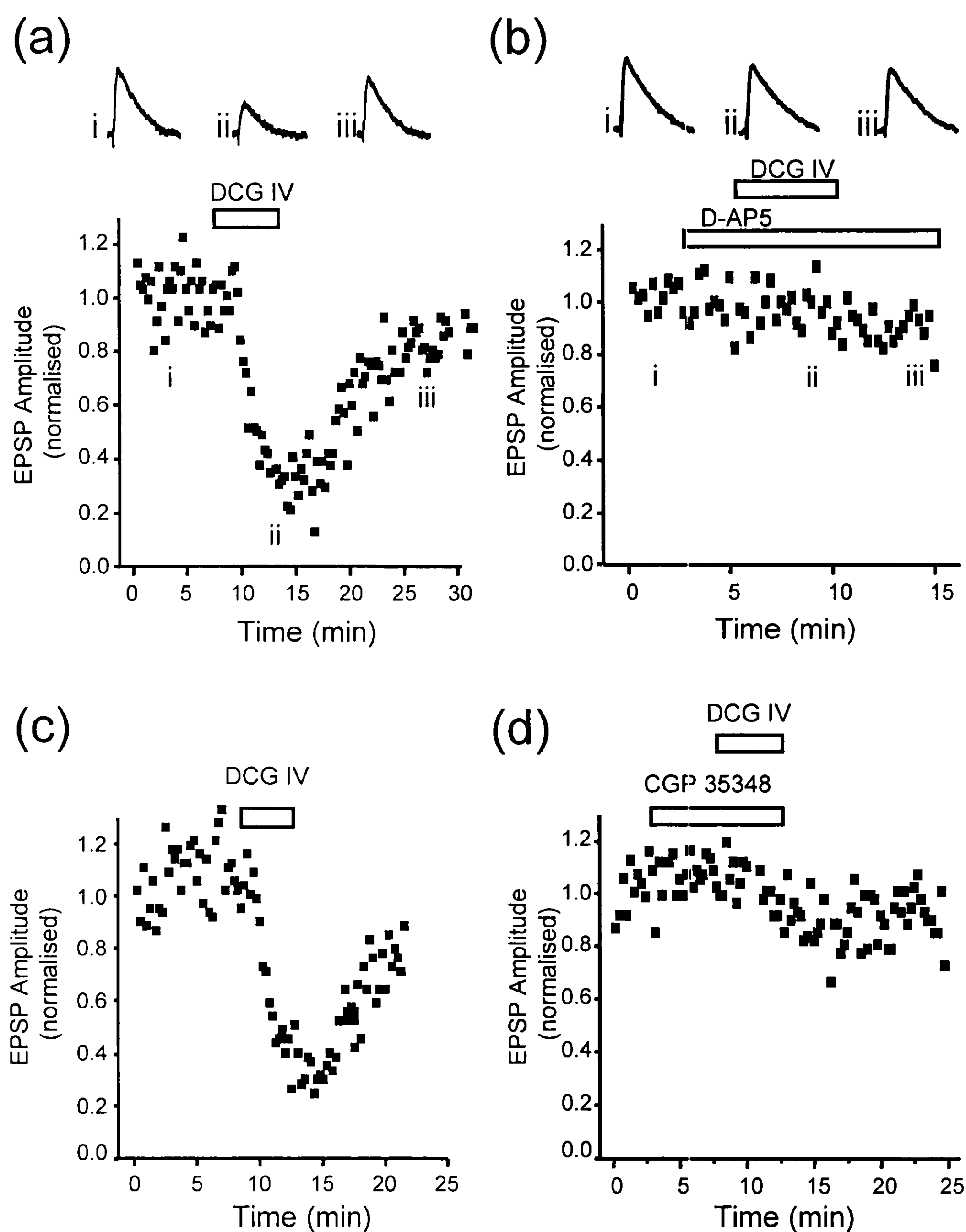
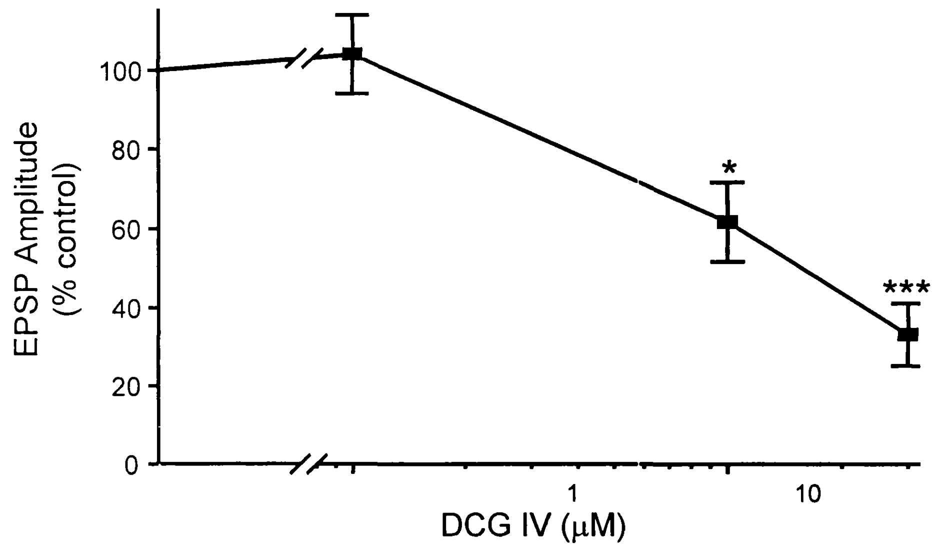


Figure 3.4. A high concentration of DCG IV depresses parallel fibre-Purkinje cell synaptic transmission, independent of mGlu receptors. (a-d) illustrate the effect of DCG IV (30 μM) on EPSPs when perfused (a) alone, (b) in the presence of D-AP5 (100 μM), (c) alone after D-AP5 washout and (d) in the presence of CGP 35348 (1 mM). Each point is the amplitude of a single EPSP. The insets show averages of 5 EPSPs elicited at the times indicated (i, ii, iii).

(a)



(b)

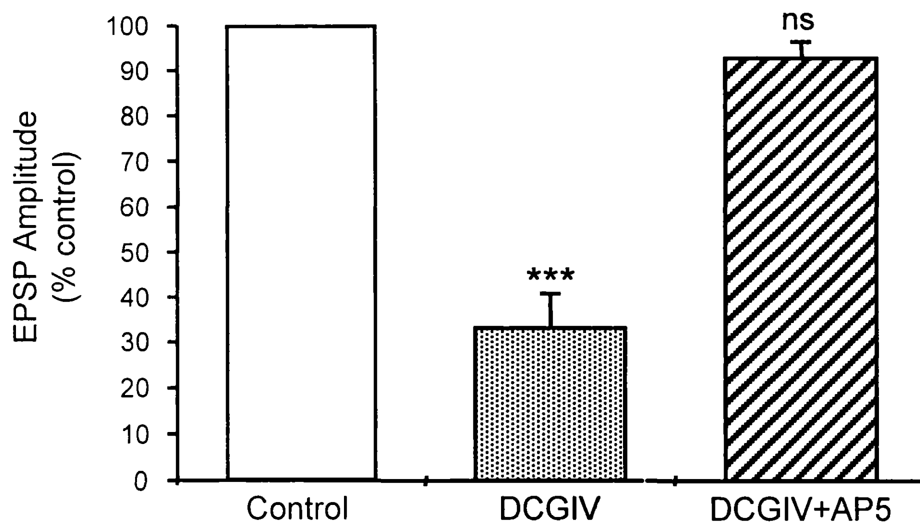


Figure 3.5. Higher concentrations of DCG IV depress parallel fibre-Purkinje cell neurotransmission via NMDA receptor activation. (a) Concentration-response curve for DCG IV (n = 3-4). (b) Pooled data (n=3) for DCG IV (30 μM) in the presence, and absence of D-AP5 (100 μM).
*** $P < 0.001$; ns, not significantly different from control.

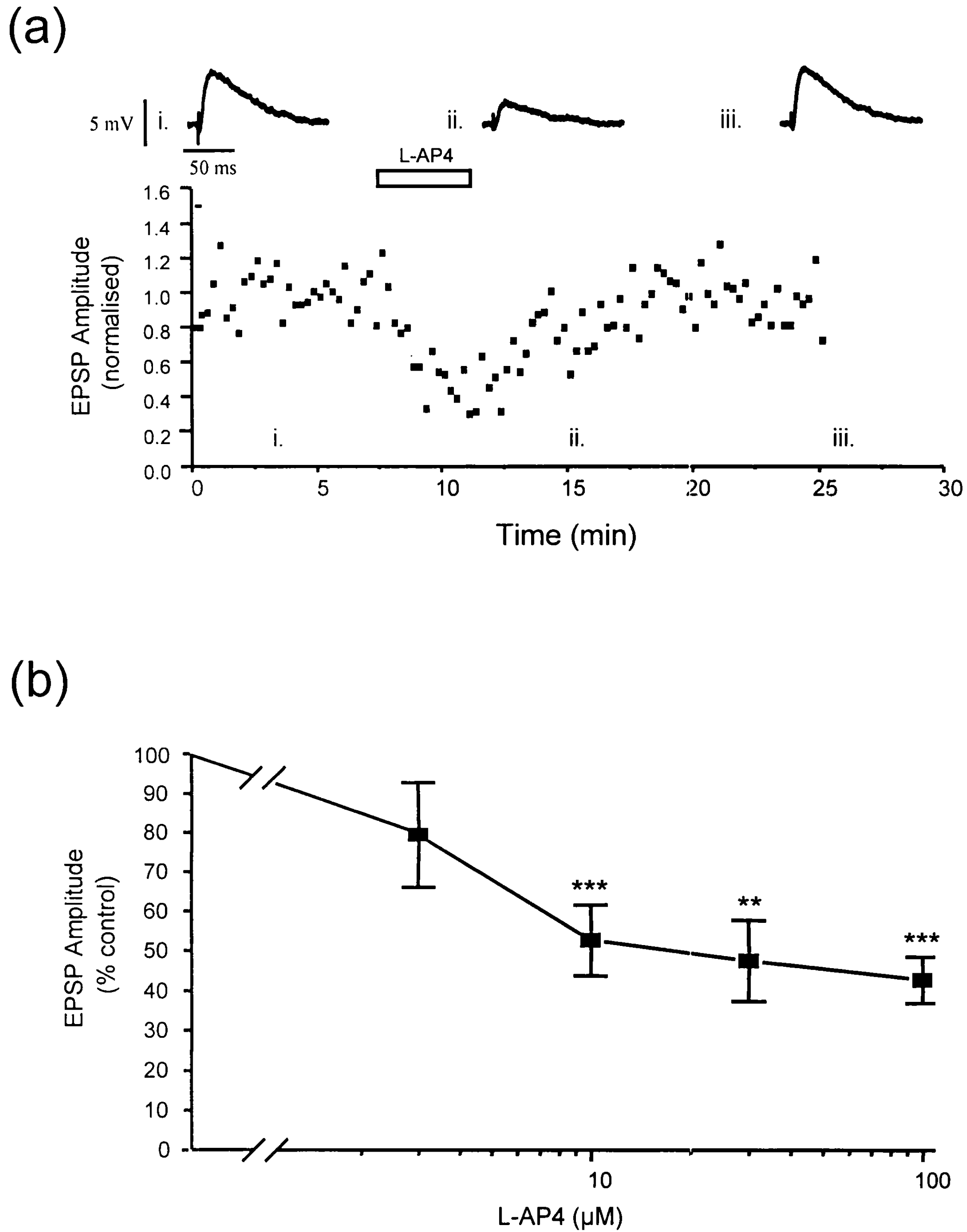


Figure 3.6. Reversible depression of parallel fibre-Purkinje cell synaptic transmission by a group III receptor agonist. (a) Effect of L-AP4 (10 μM) recorded in a single Purkinje cell. Each point is the amplitude of a single EPSP. The insets show averages of 5 EPSPs elicited at the times indicated (i, ii, iii). (b) Concentration response curve for L-AP4 (n = 4 – 7;. **P < 0.01; ***P < 0.001).

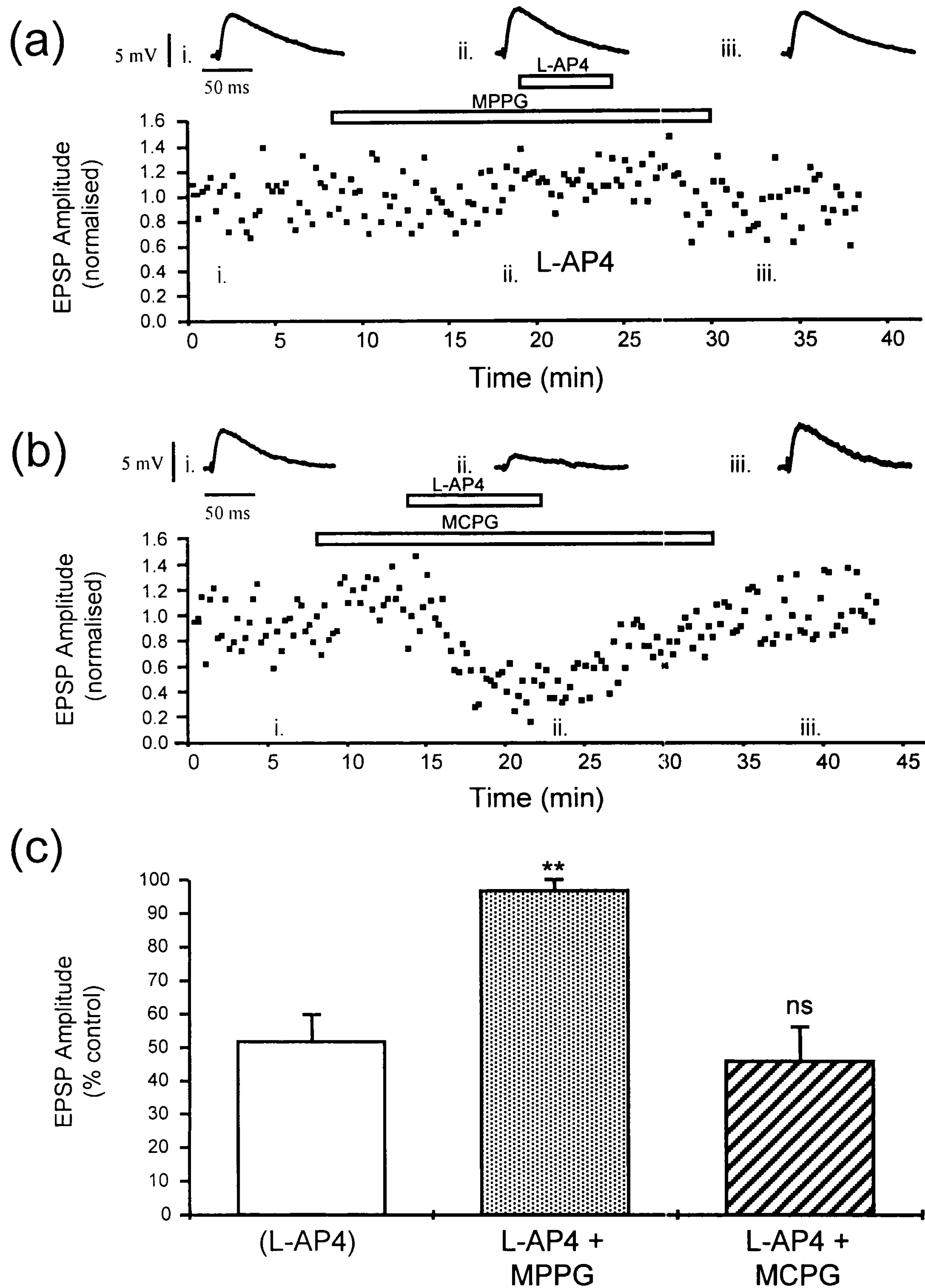


Figure 3.7. Pharmacology of the synaptic depression evoked by L-AP4. (a) In the same cell as Figure 3.6a, the L-AP4-induced depression was inhibited in the presence of the group II/III Antagonist MPPG (100 μM). In the same cell (b), the depression was unaffected by the antagonist MCPG (1 mM). Each point is the amplitude of a single EPSP. The insets show averages of 5 EPSPs elicited at the times indicated (i, ii, iii). (c) Pooled data of the antagonist effects. (n = 3; **P < 0.01; ns, not significantly different from control).

Discussion

Using the sharp microelectrode recording technique, in rat cerebellar slices, I have tested the effects of group-selective mGlu receptor ligands on synaptic transmission at the parallel fibre-Purkinje cell synapse.

Effects of Group I mGlu Receptor Activation

Application of the agonist DHPG caused a concentration-dependent depression of AMPA receptor-mediated synaptic transmission at the parallel fibre-Purkinje cell synapse, with 100 μM (the highest concentration used) depressing the EPSP by approximately 80%. From the evidence discussed below it is likely that this depression of synaptic transmission is due to the compound activating the group I mGlu receptor mGlu1.

It has previously been demonstrated that synaptic transmission at the parallel fibre-Purkinje cell synapse can be depressed by bath application of the agonist t-ACPD (Crépel *et al.*, 1991), or its active isomer (1S,3R)-ACPD (Glaum *et al.*, 1992). On receptors expressed in cell lines t-ACPD and (1S,3R)-ACPD, are non-selective agonists, acting at group I, and, more potently, at group II mGlu receptors (Pin & Duvoisin, 1995; Conn & Pin, 1997). (1S,3R)-ACPD is also an agonist at some of the group III mGlu receptors such as mGlu8 (Saugstad *et al.*, 1997). The effects of this agonist on synaptic transmission are, however, absent in mutant mice lacking the group I receptor mGlu1, suggesting that the depression is due to the activity of the compound at the group I receptors.

In *Xenopus* oocytes, injected with rat mGlu1 mRNA, (RS)-DHPG activates a Ca^{2+}-dependent chloride current, with an EC_{50} similar to that found in the present work (Ito *et al.*, 1992), and in adult hippocampal slices the compound stimulates phosphoinositide hydrolysis (Schoepp *et al.*, 1994) with a potency similar to (1S,3R)-ACPD. In the same preparation, DHPG produced no reduction in basal or forskolin-stimulated cAMP levels, consistent with the compound being a group I agonist, but having no activity at the group II or III mGlu receptors.

Consistent with the DHPG-mediated depression being due to the activation of a group I mGlu receptor, it was inhibited by the antagonist CPCCOEt. This compound reduces glutamate-evoked increases in intracellular Ca^{2+} in CHO cells expressing mGlu1a (Annoura *et al.*, 1996), and reduces quisqualate-evoked phosphoinositide hydrolysis in CHO cells expressing mGlu1b (Casabona *et al.*, 1997). Although CPCCOEt is a potent mGlu1 antagonist, concentrations of up to 100 μM have no effect on quisqualate-evoked phosphoinositide hydrolysis in cell lines expressing the human mGlu5a receptor (Casabona *et al.*, 1997). CPCCOEt may therefore be of value in studies elucidating the physiological roles of different group I receptors. In addition, CPCCOEt has no effect at either group II or III mGlu receptors, as assessed by the compound's lack of effect on either (1S,3R)-ACPD-mediated reduction in forskolin-evoked increases in cAMP in cell lines expressing the human subtype mGlu2, or L-AP4's effects in cells expressing mGlu4a, -7b or –8a. (Litschig *et al.*, 1999). This antagonist is relatively unusual among the mGlu receptor antagonists as it inhibits in a non-competitive manner, showing no effect on [^{3}H]glutamate binding, whereas most other mGlu receptor antagonists act competitively (Schoepp *et al.*, 1999; Litschig *et al.*, 1999). Studies with chimeric receptors and point mutations have demonstrated that CPCCOEt interacts with Thr815

and Ala818 on human mGlu1b. These two residues are located on the extracellular surface of the transmembrane region of the receptor where interaction with CPCCOEt is postulated to disrupt intramolecular signalling between the extracellular agonist-binding domain, and regions associated with activation of the transduction pathway. None of the mGlu receptors 2-8 have homologous residues in these positions, thus explaining the selectivity of CPCCOEt for mGlu1 (Litschig *et al.*, 1999).

In mouse Purkinje cells CPCCOEt inhibits a tonically active inward current, as evidence from a small (mean = 12 pA) reduction in the holding current (Yamakawa & Hirano, 1999). This does not occur in rat Purkinje cell neurones as, in the present study, CPCCOEt did not affect the Purkinje cell membrane potential or the EPSP amplitude.

The group II/III antagonist MPPG, which is inactive at group I receptors (Gomeza *et al.*, 1996b, Thomsen *et al.*, 1996) exerted no significant effect on the depression of synaptic transmission. Thus, DHPG did not appear to be exerting any effects on transmission via the group II or III mGlu receptors.

To date the only other reported action of DHPG is as an antagonist of a PLD-linked mGlu receptor (Pellegrini Giampietro *et al.*, 1996). The pharmacology of this possibly novel receptor is quite different from the response of DHPG described here in that (1S,3R)-ACPD acts as an agonist, but DHPG an antagonist (Pellegrini Giampietro *et al.*, 1996). It is therefore unlikely that the depression of transmission at the parallel fibre-Purkinje cell synapse is due to this receptor.

In addition to depressing synaptic transmission, DHPG also caused the Purkinje cell to depolarise. This effect has been previously reported both for this agonist (Gruol *et al.*, 1996), t-ACPD and (1S,3R)-ACPD (Crépel *et al.*, 1991; East & Garthwaite, 1992; Glaum *et al.*, 1992; Staub *et al.*, 1992). Like the depression of synaptic transmission, this is likely to be due to activation of the group I receptor mGlu1 as in the mGlu1 knockout mouse (1S,3R)-ACPD does not evoke the inward current seen in the wild type mouse (Conquet *et al.*, 1994). The mechanism for this depolarisation in Purkinje cell is at present unknown. In hippocampal neurones, mGlu receptor agonists produce membrane depolarisation (Stratton *et al.*, 1989; Litschig *et al.*, 1999), accompanied by an increase in membrane resistance, postulated to be due to inhibition of the voltage dependent, muscarinic-sensitive K^+ conductance, I_M (Litschig *et al.*, 1999). The characteristics of the inward current evoked by t-ACPD in Purkinje cells have been investigated in rat cerebellar slice cultures (Staub *et al.*, 1992). This current is unlikely to be due to an affect on K^+ channels: the K^+ channel blockers barium and TEA were ineffective; the reversal potential was calculated to be +18 mV, and the input conductance is increased (although this could be due to activation of voltage dependent conductances in the dendrites, where voltage clamp would be relatively ineffective).

The inward current was dependent on external Na^+ and internal Ca^{2+} as replacement of external Na^+ with choline or lithium, or loading the cell with the Ca^{2+} chelator BAPTA suppressed the current. This dependence of the current on external Na^+ and internal Ca^{2+} supports the hypothesis that the current is due to a Ca^{2+}-activated non-specific cation (CAN) channels (Partridge *et al.*, 1994). In hippocampal CA1 neurones (1S,3R)-ACPD activates a current (Crepel *et al.*, 1994), which has subsequently been shown to be due to group I mGlu receptors (Congar *et al.*, 1997). As with Purkinje cells, this action of

(1S,3R)-ACPD was reduced by internal BAPTA and reducing the external Na^+ concentration by substitution with choline. The current in the CA1 neurones was only minimally affected by temperature, which would be consistent with ion permeation through ionic channels, rather than a metabolic pump. This evidence is all consistent with this current being due to activation of a CAN current. Unlike the situation in the CA1 neurones, however, the current evoked in Purkinje cells was reduced when external Na^+ was substituted for lithium. As the CAN channels are typically permeable to monovalent cations, such as lithium (Yellen, 1982; Partridge *et al.*, 1994), and larger cations, such as TEA and choline (Lipton, 1986; Partridge & Swandulla, 1987; Partridge *et al.*, 1994) it is therefore unlikely that this current is due to a CAN channel. The other candidate, consistent with the evidence discussed above, is an electrogenic Na^+/Ca^{2+} exchanger (Reeves, 1990; Perry & McNaughton, 1991), which are affected by internal Ca^{2+} buffering, or by substitution of external Na^+ with ions such as choline (Blaustein, 1977). In neurones of the rat basolateral amygdala a Na^+/Ca^{2+} exchanger is postulated to underlie a (1S,3R)-ACPD-evoked inward current, similar to that observed in Purkinje cells, which is inhibited by the Na^+/Ca^{2+} exchanger inhibitory peptide XIP (Keele *et al.*, 1997). In mouse Purkinje cells, however, BAPTA or the Na^+/Ca^{2+} exchange inhibitor KB-R7943 both produced only small reductions in the amplitude of the current evoked by (1S,3R)-ACPD (Hirono *et al.*, 1998). It is however difficult to draw any firm comclusions from these results as the authors provided no positive control for the KB-R7943, and in cells loaded with 20 mM BAPTA greater than 25% of the (1S,3R)-ACPD-evoked Ca^{2+} signal remained. Further studies are therefore necessary before the mGlu receptor-mediated inward current in Purkinje cells may be identified with confidence.

The amplitude of the EPSP recorded in the soma reflects the amplitude of the underlying synaptic current. As this current flows from dendrites to the soma a proportion of the current will be lost across the membrane resistance; thus, if the DHPG-evoked inward current involves an increase in membrane conductance, it could explain how activation of group I mGlu receptors depress the amplitude of the parallel fibre EPSP. In the experiments described here application of DHPG, and t-ACPD in another study (Crépel *et al.*, 1991), produced no detectable change in input resistance, at concentrations that depress synaptic transmission. In both of these studies the recording conditions were perhaps not optimal for detecting such a change, and other studies have detected an increase in conductance (Staub *et al.*, 1992; Yamakawa & Hirano, 1999). It is, however, unlikely that the depression of EPSP amplitude is related to the depolarisation as at lower concentrations of DHPG (30 μM) half the cells tested showed no evidence of the depolarisation, but the EPSP amplitude was depressed to a similar degree in all the cells. The absence of a depolarisation in some cells is probably a developmental phenomenon, as mGlu receptor-mediated EPSPs (Batchelor *et al*, 1994) are always observed in Purkinje cells of adult rats. In accordance, the ACPD-induced population depolarisation of Purkinje cells is smaller in developing rats (East and Garthwaite, 1992), as is the mGluR-mediated population EPSP (Batchelor and Garthwaite, 1993).

Effects of Group II mGlu Receptor Activation

In HEK293 cells transfected with rat mGlu2 or mGlu3 DCG IV had an EC_{50} of 0.35 and 0.09 μM respectively (determined from inositol phosphate accumulation in cells co-expressing G-protein α-subunit G_{qi9}), but was ineffective in cells transfected with

mGlu1, 4, 5, 6, 7 or 8, even at a concentration of 1 mM (Brabet *et al.*, 1998). At a concentration of 1 μM application of the DCG IV resulted in no significant depression of the parallel fibre-Purkinje cell EPSP. At corticostriatal, mossy fibre-CA3 and spinal cord synapses, similar concentrations of this agonist depresses synaptic transmission (Ishida *et al.*, 1993; Lovinger & McCool, 1995; Kamiya *et al.*, 1996). From the EC_{50} for DCG IV on expressed receptors, and its reported efficacy elsewhere in the CNS, if the group II receptors play a role in the regulation of synaptic transmission between parallel fibre-Purkinje cell synapse 1 μM should have been sufficient to activate them. The lack of effect of 1 μM DCG IV suggests that the group II mGlu receptors do not play a role in the regulation of synaptic transmission at this synapse.

In the present studies, DCG IV, when applied at higher concentrations, did produce a significant depression of the parallel fibre EPSP. This result was surprising as histological studies have not located the group II receptors at this synapse (Ohishi *et al.*, 1994; Ohishi *et al.*, 1998), and the depression of transmission at this synapse by the non-selective agonist (1S,3R)-ACPD is absent in mutant mice lacking the mGlu1receptor (Conquet *et al.*, 1994). It is unlikely that this effect was due to DCG IV activating one of the other mGlu receptors as, at least for receptors expressed in cell lines, it has no agonist activity. In fact, for mGlu1, 4, 5, 6, 7 and 8 DCG IV is reported to be an antagonist (Brabet *et al.*, 1998). In the CA1 region of the rat hippocampus DCG IV (10 μM) causes depression of synaptic depression, which was unaffected by mGlu receptor antagonists, but was reversed by 50 μM of AP5 (Wilsch *et al.*, 1994), a NMDA receptor antagonist (Evans *et al.*, 1982; Davies & Watkins, 1982). In rat cortical slices DCG IV depolarisation of neurones is inhibited by NMDA receptor antagonists, but unaffected by mGlu receptor antagonists (Uyama *et al.*, 1997). At the parallel fibre-

Purkinje cell synapse the depression of the EPSP amplitude evoked by DCG IV was inhibited by AP5, suggesting that this depression is due to DCG IV activating NMDA receptors. This depression also appeared to be inhibited by the $GABA_B$ receptor antagonist CGP 35348. The Purkinje cell receives inputs from two inhibitory interneurones, the basket and stellate cells. These cells are excited by NMDA receptor agonists (Llano *et al.*, 1991; Farrant & Cull Candy, 1991), and excitation of these cells can lead to a depression of parallel fibre-Purkinje cell synaptic transmission, via $GABA_B$ receptor activation (Dittman & Regehr, 1997). The depression of synaptic transmission produced by DCG IV may be due to the compound exciting inhibitory cells via NMDA receptors, thereby increasing GABA release. Alternatively, there is evidence that NMDA receptors are expressed on the terminals of the inhibitory interneurones (Glitsch & Marty, 1999), and DCG IV could be increasing GABA release via these presynaptic receptors.

Effects of Group III mGlu Receptor Activation

Depression of synaptic transmission by L-AP4, apparently by a presynaptic mechanism, was reported several years before evidence demonstrating the existence of the mGlu receptors was obtained (Harris & Cotman, 1983), and the compound has been widely used to study the actions of mGlu receptors in neuronal cells. Although active at all of the cloned group III receptors, L-AP4 does not appear to have any activity at the group I or II mGlu receptors (Pin & Duvoisin, 1995; Conn & Pin, 1997). At the parallel fibre-Purkinje cell synapse L-AP4 produced a concentration-dependent reduction in EPSP amplitude. It is probable that this depression is due to activation of the group III receptor mGlu4 which is expressed presynaptically on the parallel fibre terminals

(Kinoshita *et al.*, 1996; Mateos *et al.*, 1998). In the mGlu4 knockout mouse the depression of synaptic transmission evoked by L-AP4 in the wild type mouse is absent in the mutant (Pekhletski *et al.*, 1996). In addition, the depression was inhibited by MPPG, a group III receptor antagonist, but not by MCPG, which has antagonist activity at group I and II mGlu receptors and some group III receptors (Saugstad *et al.*, 1997; Wu *et al.*, 1998), although not at mGlu4 (Hayashi *et al.*, 1994; Thomsen *et al.*, 1994b).

Conclusion

In conclusion, these experiments have confirmed that functional mGlu receptors are present at the parallel fibre-Purkinje cell synapse in the rat cerebellar cortex, and that activation of these receptors results in a depression of synaptic transmission. Using selective agonists and antagonists for these receptors, we have demonstrated that it is possible to pharmacologically distinguish between the different groups of receptors at this synapse. Activation of either the group I or III mGlu receptors results in a reversible depression of synaptic transmission. The group II receptors do not appear to have a role in the regulation of excitatory transmission at this synapse.

Bathing an entire slice with an exogenous agonist is not necessarily going to identify the response produced when receptors are activated by their endogenous agonist, released under physiological conditions. To fully understand the roles of the different mGlu receptors in synaptic transmission we need to identify the conditions necessary for the receptors to be activated by their endogenous agonist. These experiments have identified pharmacological tools that can distinguish between the actions of the group I

and III mGlu receptors on synaptic transmission at the parallel fibre-Purkinje cell synapse, thereby facilitating investigations of the activation of these different mGlu receptors to be studied under physiological conditions.

Chapter 4: Group I mGlu receptors and $GABA_B$ receptors mediate post-tetanic depression at the parallel fibre-Purkinje cell synapse

Introduction

The efficacy of synaptic transmission is not fixed, but can be modified subject to the activity of the individual synapse, or that of neighbouring synapses. Such use-dependent plasticity appears to be a ubiquitous feature of CNS synapses, and may result in an increase (Bliss & Collingridge, 1993), or decrease (Linden & Connor, 1995) in the strength of transmission, at both excitatory (Bliss & Lomo, 1973; Bliss & Collingridge, 1993) and inhibitory (Aizenman *et al.*, 1998; Oda *et al.*, 1998) synapses. The changes in synaptic strength range in duration from a few hundred milliseconds, as seen in paired-pulse facilitation (Zucker, 1999), to the apparently permanent forms of long-term potentiation ((Bliss & Collingridge, 1993) and depression ((Linden & Connor, 1995). The synapse was suggested as the site of learning and memory storage towards the end of the nineteenth century (see Brown *et al.*, 1990 for refs), and the hypothesis that long-term changes in synaptic strength underlie memory formation still represents the most widely supported explanation of the cellular mechanisms of memory. In light of the potential importance that these phenomena have for our understanding of the functioning of the nervous system, synaptic plasticity has become an area of intensive research. In particular, efforts have focused on the long-lasting forms of synaptic plasticity occurring at excitatory glutamatergic synapses. Long-term potentiation (LTP) was first reported at the synapse between the perforant path fibres and granule cells in the rabbit hippocampus (Bliss & Lomo, 1973). This area of the brain appears to be

important for some forms of memory, as damage to the hippocampus results in impaired memory in both humans and animals (Mishkin, 1978; Rolls, 1991; Squire, 1992). LTP has now been demonstrated at all the hippocampal excitatory synapses, and at numerous other synapses in both vertebrates and invertebrates, with probably the best studied example of LTP in vertebrates being that which occurs at the synapse between the hippocampal CA3 and CA1 pyramidal neurones (Bliss & Collingridge, 1993).

Long-term depression (LTD) of synaptic transmission has been observed at a number of synapses and, depending on the stimulation protocol, either LTP or LTD may be evoked at the same synapse (Sakurai, 1989). A well-documented form of LTD occurs at the parallel fibre-Purkinje cell synapse in the cerebellar cortex. It is well established that the cerebellum plays a role in the control of movement and learning of motor co-ordination (Ito, 1984), and several theories have been proposed as to how the cerebellum might achieve this (Braitenberg, 1961; Braitenberg & Onesto, 1962; Marr, 1969; Albus, 1971; Pellionisz & Llinás, 1982; Thompson, 1990). One of the more influential of these is that developed from the hypotheses published independently by Marr and Albus (Marr, 1969; Albus, 1971). The essence of this hypothesis is that Purkinje cells are activated by inputs from a number of parallel fibres. If this parallel fibre activity results in performance errors the climbing fibre synapsing onto the Purkinje cell will fire, and those parallel fibre synapses simultaneously active with the climbing fibre would be weakened. Experimental evidence for such plasticity was unavailable for more than ten years until Ito and colleagues (1982) demonstrated that pairing of either vestibular nerve stimulation, or iontophoretic application of glutamate, with stimulation of the inferior olivary nucleus produced a depression of the Purkinje cell excitation produced by the vestibular nerve stimulation. This depression was

specific to those inputs involved in the conjunctive stimulation. Subsequently, more direct evidence that LTD required activation of the parallel and climbing fibres was obtained *in vivo* (Ito & Kano, 1982) and *in vitro* (Sakurai, 1987) and has become an important area of research in the field of cerebellar physiology.

As induction of LTP and LTD requires activation of second messengers pathways (Bliss & Collingridge, 1993; Linden & Connor, 1995) an involvement of metabotropic glutamate (mGlu) receptors is an attractive possibility, and a substantial body of evidence has now implicated a role for these receptors in long-term plasticity. For example, several lines of evidence suggest that mGlu1 is involved in LTD at the parallel fibre-Purkinje cell synapse: firstly, mGlu1a is strongly expressed in the Purkinje cells and immunohistochemical studies show that the receptor is located in both the dendritic and somatic membrane (Martin *et al*, 1992); secondly, Shigemoto *et al* (1994) blocked the induction of LTD with antibodies raised against two distinct extracellular sequences of the rat mGluR1 receptor and, thirdly, in mutant mice lacking the mGlu1 gene, two protocols, which produced LTD in normal mice failed to induce LTD (Conquet *et al*, 1994). What is still unclear is how the second messengers implicated in cerebellar LTD, including: PKC, nitric oxide and cGMP, and PLA_2 (Linden & Connor, 1991; Linden & Connor, 1995; Hartell, 1996; Boxall & Garthwaite, 1996), are connected with mGlu1 activation. Early studies into the role of mGlu receptors and LTP demonstrated that the proposed antagonist L-AP3 (Behnisch *et al.*, 1991; Izumi *et al.*, 1991) and later, the more selective compound MCPG (Bashir *et al.*, 1993; Riedel & Reymann, 1993), could disrupt the induction of hippocampal LTP and that mGlu receptor agonists could facilitate the development LTP in the hippocampus (McGuinness *et al.*, 1991). Additionally, in mice lacking the group 1 receptor mGlu5, CA1 region LTP is

significantly reduced (Lu *et al.*, 1997). In contrast, other groups have reported that (RS)-MCPG, despite inhibiting effects of t-ACPD, produces no effect on the development of LTP at the Schaffer collateral-CA1 synapse (Chinestra *et al.*, 1993; Manzoni *et al.*, 1998). The reasons for such conflicting data are unclear; Chinestra *et al* (1993) suggest several possibilities, including differences in temperature and species used. Alternatively, the discrepancy may be explained by the discovery that it is necessary for mGlu receptors to activate a molecular switch before LTP can be induced. Once this switch has been activated, mGlu receptors do not need to be activated during induction of LTP (Bortolotto *et al.*, 1994). If this molecular switch was activated, perhaps by high glutamate levels during tissue preparation, then LTP induced experimentally could appear to be mGlu receptor-independent.

Induction of LTD at the parallel fibre-Purkinje cell synapse typically involves pairing trains of low frequency stimulation of the parallel fibres (typically 1–4 Hz) with an increase in the intracellular Ca^{2+} concentration of the Purkinje cell, either by depolarisation or by climbing fibre stimulation. The physiological relevance of such stimulation protocols is, however, unclear. *In vivo* neurones do not appear to fire long trains of regularly spaced action potentials, but fire in a highly irregular fashion (Zador & Dobrunz, 1997), and often spikes are grouped into short, high frequency bursts (Mason & Rose, 1988; Connors & Gutnick, 1990). The mossy fibres, which provide the excitatory input to the cerebellar granule cells, when recorded *in vivo* fire in such short high frequency bursts (Kase *et al.*, 1980); and it is likely that the granule cells respond to this activity with a similar burst of action potentials. Certainly, when studied in the *in vitro* slice preparation, the granule cells have the capability to fire sustained, high frequency bursts of action potentials, and, under appropriate conditions, high frequency

electrical stimulation of the mossy fibres translates to high frequency firing in the granule cells (D'Angelo *et al.*, 1995). The small size and dense packing of the granule cells makes it difficult to reliably record the activity of single cells *in vivo*, but the available evidence suggests that, rather than prolonged, low-frequency firing, these cells do fire in short high frequency bursts (Eccles *et al.*, 1966).

In addition to long-term plasticity, synaptic transmission at the parallel fibre-Purkinje cell synapse undergoes other forms of activity dependent plasticity, which produce short-lived changes in synaptic strength, such as paired-pulse facilitation (Atluri & Regehr, 1996) and post-tetanic potentiation (Pekhletski *et al.*, 1996). As demonstrated in the previous chapter, activation of the group I or III mGlu receptors with exogenous agonists produces a reversible depression of the parallel fibre EPSP, which recovers on washout of the agonist. Similar effects of mGlu receptor agonists are seen throughout the CNS (Baskys & Malenka, 1991; Glaum & Miller, 1993; Burke & Hablitz, 1994; Jane *et al.*, 1994; Lovinger & McCool, 1995; Schoppa & Westbrook, 1997). Although there is strong evidence that mGlu receptors are involved in long-term synaptic plasticity, there are few examples of their involvement in the various forms of transient plasticity. This probably reflects a bias towards studying LTP and LTD over the other forms of plasticity, and the fact that the fields of mGlu receptor physiology and pharmacology are both relatively new, although rapidly expanding, areas of research. Previous studies have demonstrated that brief stimulation of the parallel fibres, designed to mimic the probable physiological activity of the granule cells, induces an EPSP, which lasts 1–2 seconds (Batchelor & Garthwaite, 1993). This has been pharmacologically identified as being mediated by group I mGlu receptors (Batchelor *et al.*, 1997). The studies described in the previous chapter identified antagonists that

inhibit the agonist-induced suppression of synaptic transmission in a group-selective manner. Using these selective antagonists, and the stimulation protocol already known to activate the postsynaptic group I mGlu receptors at the parallel fibre-Purkinje cell synapse (Batchelor & Garthwaite, 1993), we have conducted experiments to test whether the activation of mGlu receptors by synaptically released endogenous agonist can lead to a modification of synaptic strength.

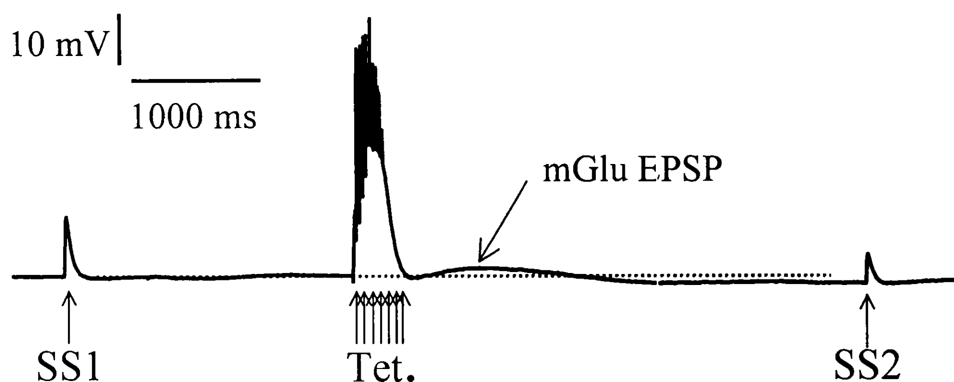
All of these experiments were conducted with sharp microelectrodes recording from Purkinje cells in current clamp at 30°C, as described in the methods chapter.

Results

Post-tetanic depression.

Figure 4.1 shows a sharp microelectrode current clamp recording from a single Purkinje cell. A single stimulus applied to the parallel fibres (SS1) evokes a fast EPSP, presumed to be mediated through AMPA receptors. Following the single stimulation, the parallel fibres receive a short tetanic stimulation (tet). This results in summated AMPA receptor EPSPs, followed by an mGlu receptor-mediated EPSP. In adult rats a clear mGlu receptor EPSP can be seen in all cells. In the younger animals used for this study (12-17 days) a clearly visible EPSP was seen in approximately 60% of cells. A few seconds after the tetanic stimulation the parallel fibre receive a single stimulus (SS2). This is of the same amplitude and duration as the SS1 stimulus, but the amplitude of the resulting EPSP is depressed compared to that evoked by SS1 (Fig. 4.1b).

(a)



(b)

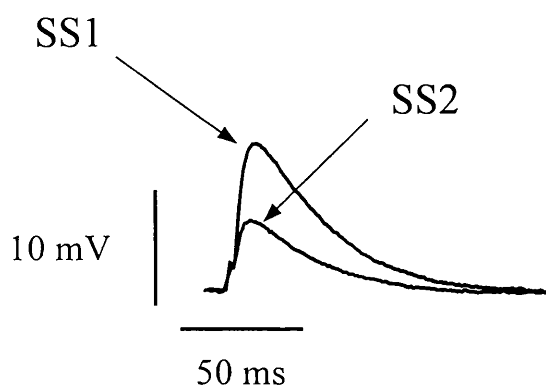


Figure 4.1. Post-tetanic depression of parallel fibre-Purkinje cell synaptic transmission. (a) Following tetanic stimulation (tet.) in a single Purkinje cell the SS2 EPSP is depressed compared to the SS1 EPSP evoked by single stimuli of identical strength. The dotted line highlights the slight hyperpolarisation of the membrane potential following the tetanic stimulation.
(b) The single EPSP SS1 and SS2 on larger scales

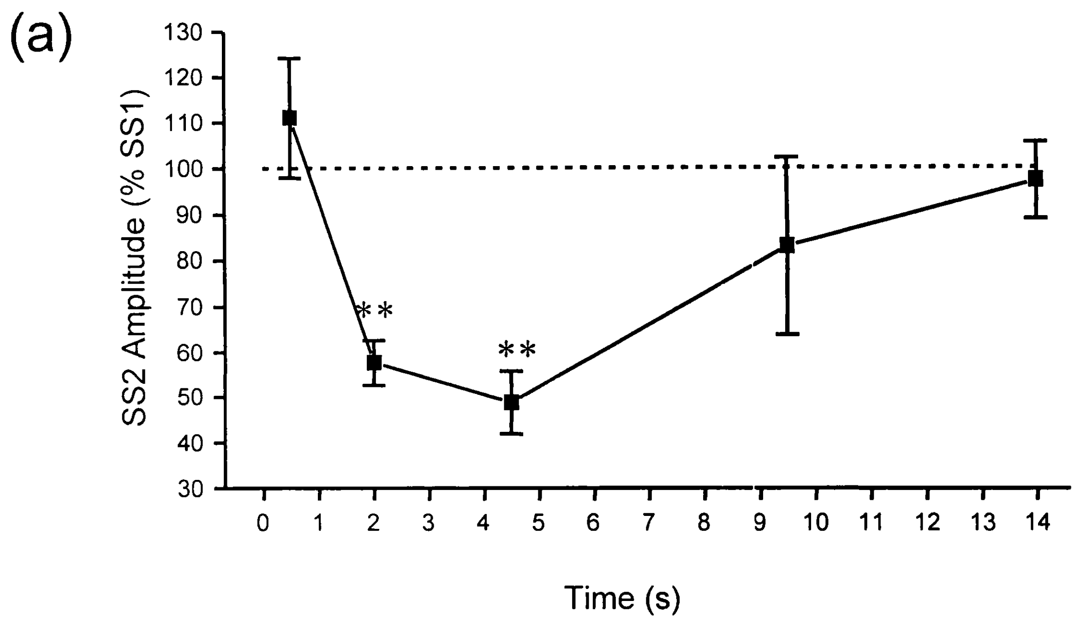
We have termed this depression post-tetanic depression (PTD). Following the tetanus a prolonged low-amplitude hyperpolarisation was observed (Fig. 4.1a); neither mGlu receptor nor $GABA_B$ antagonists noticeably affected this.

Time course of post-tetanic depression

The time course of PTD was tested following tetanic stimulation of the parallel fibres with 10 stimuli at a frequency of 100 Hz (Fig. 4.2a). At 0.5 s from the onset of the tetanus there was no significant depression of the SS2 EPSP amplitude compared to the SS1 control (111 ± 13%; n = 5; $P > 0.05$). At 2 s from the tetanus there was a significant depression of the SS2 EPSP compared to control (58 ± 5%; n = 5; $P = 0.001$). The PTD peaked at 4.5 s (49 ± 7%; n= 5; $P = 0.001$). By 14 s the amplitude of the SS2 EPSP had returned to baseline levels with no significant difference from control levels (97 ± 8%; n = 5; $P > 0.05$).

Frequency dependence of post-tetanic depression

Using a tetanus of 10 stimuli, the dependence of PTD on stimulus frequency was investigated for an EPSP evoked 4.5 s after the tetanus (Fig. 4.2b). At 20 Hz no significant depression was induced (95 ± 8%; n = 6; $P > 0.05$). Increasing the frequency to 50 Hz resulted in a significant depression of the SS2 EPSP, compared to the SS1 control (66 ± 11%; n = 6; $P < 0.001$). A further increase in the frequency to 100 Hz resulted in an increase in the degree of depression (56 ± 12%; n=6), which was significantly different from control levels ($P < 0.001$), but not significantly different from the depression seen at 50 Hz ($P > 0.05$).



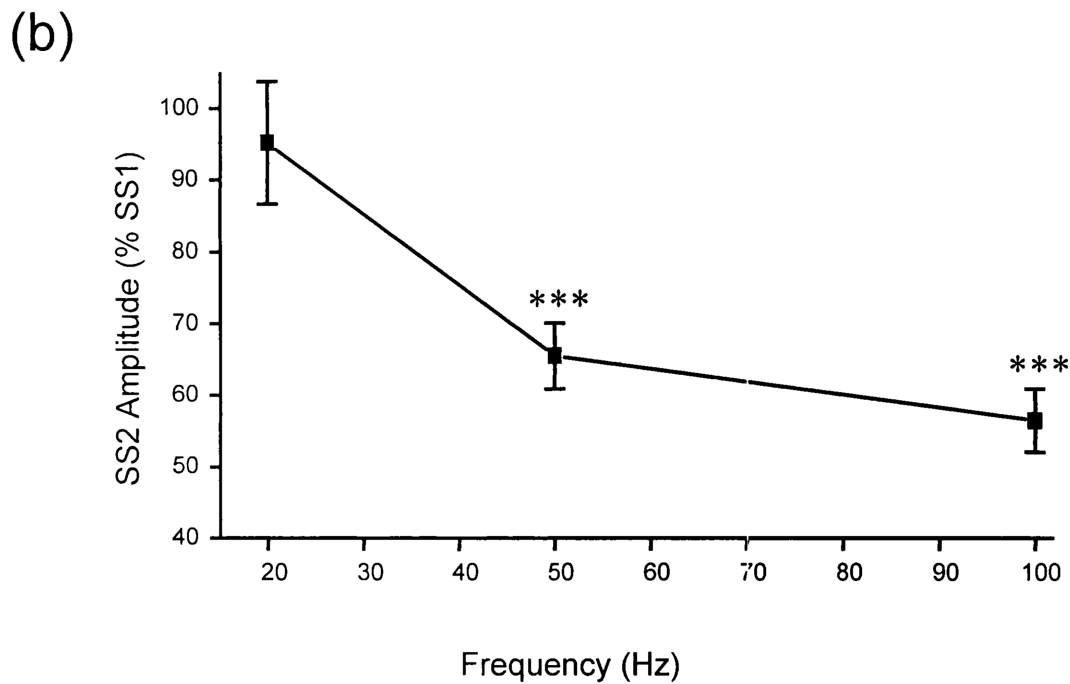


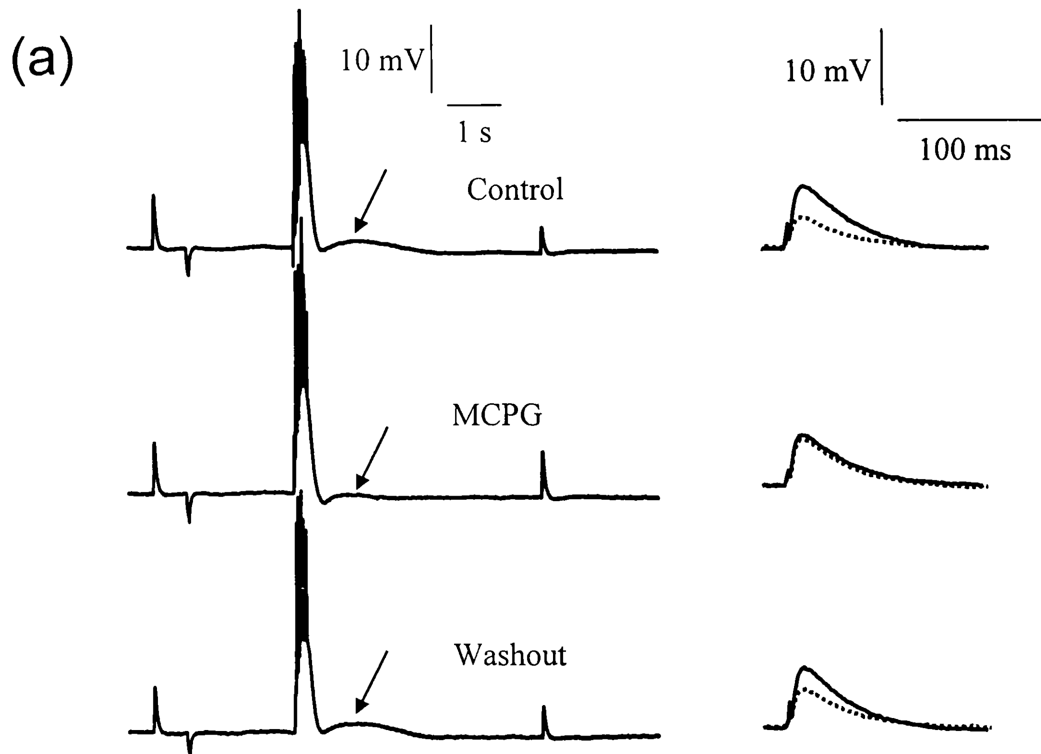
Figure 4.2. Time-course and frequency dependence of PTD.
(a)Time course of PTD following a tetanus of 10 stimuli at 100 Hz (n=5).
(b) Frequency dependence of PTD (n = 6-7). **P < 0.01; ***P<0.001.

Pharmacology of post-tetanic depression

In initial experiments the possibility that PTD was mediated through mGlu receptors was tested using the non-selective mGlu receptor antagonists MCPG and MPPG. Consistent with previous reports (Batchelor *et al.*, 1994), MCPG reduced the mGlu receptor EPSP evoked after stimulation the parallel fibres with a train of 10 stimuli at a frequency of 100 Hz. (Fig. 4.3a). In the presence MCPG (1mM) the PTD was also attenuated, with the mean amplitude of the SS2 EPSP in MCPG = 90.4 ± 10.2% compared to 57 ± 5.6% under control conditions ($n = 5$; $P = 0.01$; Fig. 4.3b). The effects of MCPG on both the PTD and the mGlu receptor EPSP were both reversible on washout of the antagonist (Fig 4.3a).

Application of the group II/III-selective mGlu receptor antagonist MPPG, at concentrations of up to 300 μM, produced no significant effect on the degree of PTD. The mean amplitude of SS2 was 67.6 ± 6.4% under control conditions, and 64 ± 9.2% in MPPG (300 μM; $n = 4$; $P > 0.05$; Fig. 4.4b). In addition, MPPG did not alter the amplitude of the mGlu receptor EPSP when present.

In the presence of MCPG, the PTD was not totally inhibited. Previous reports have suggested that $GABA_B$ receptors can be activated following tetanic stimulation of the parallel fibres (Dittman & Regehr, 1997). The possibility that the MCPG-insensitive component of PTD might be mediated through $GABA_B$ receptor activation was tested with the $GABA_B$ receptor-selective antagonist CGP 35348. A tetanus of 10 stimuli evoked a mean depression of SS2 to 52 ± 6% of SS1 ($n = 7$) when measured 4.5 s from the tetanus (Fig. 4.6a). Addition of CGP 35348 (1 mM) caused a significant



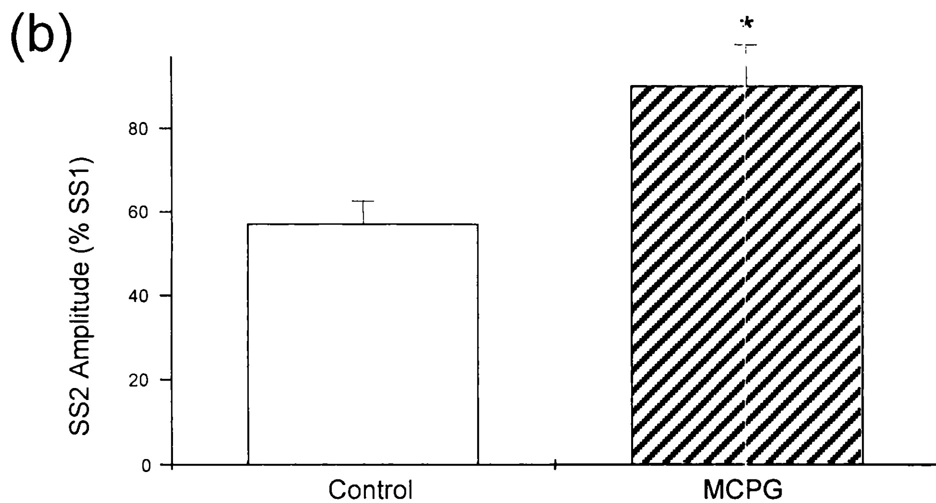
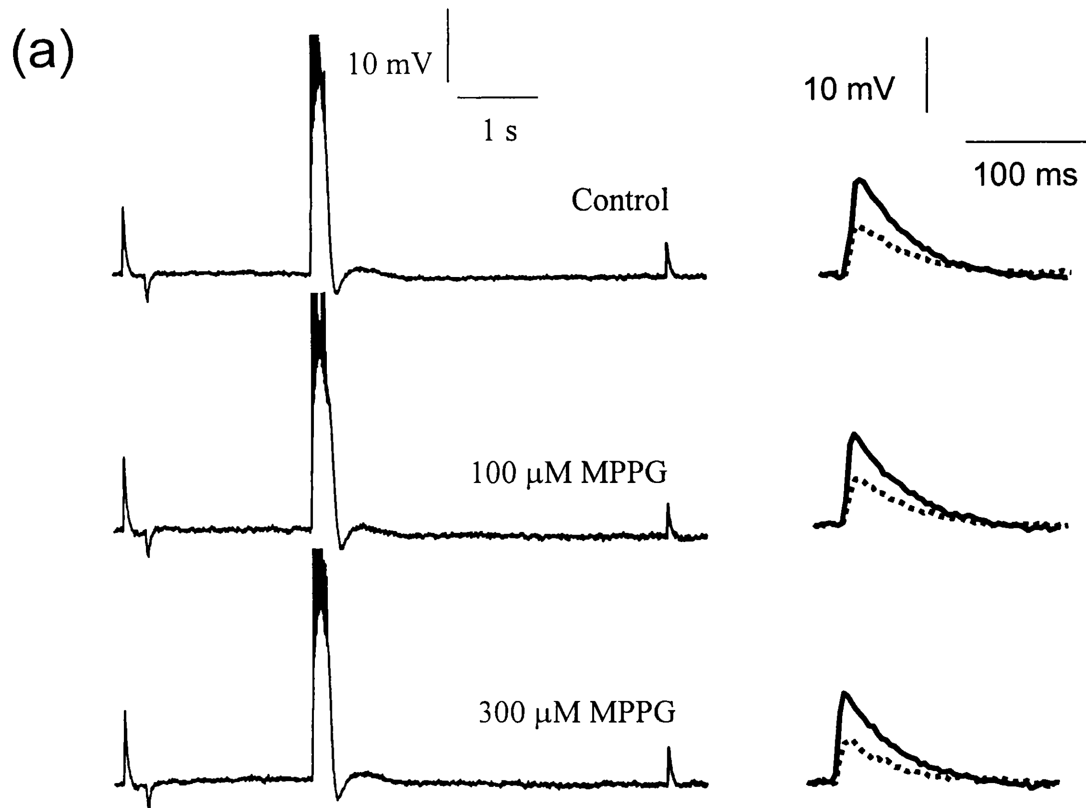


Figure 4.3. Effect of a non-selective mGlu receptor antagonist on PTD. (a) In a single experiment, MCPG (1 mM) reversibly reduced both PTD and the mGlu EPSP (indicated by arrows). (b) Pooled data (n=5) of MCPG (1 mM) on PTD. PTD measured on EPSP 4.5 s after tetanic stimulation. *$P < 0.05$



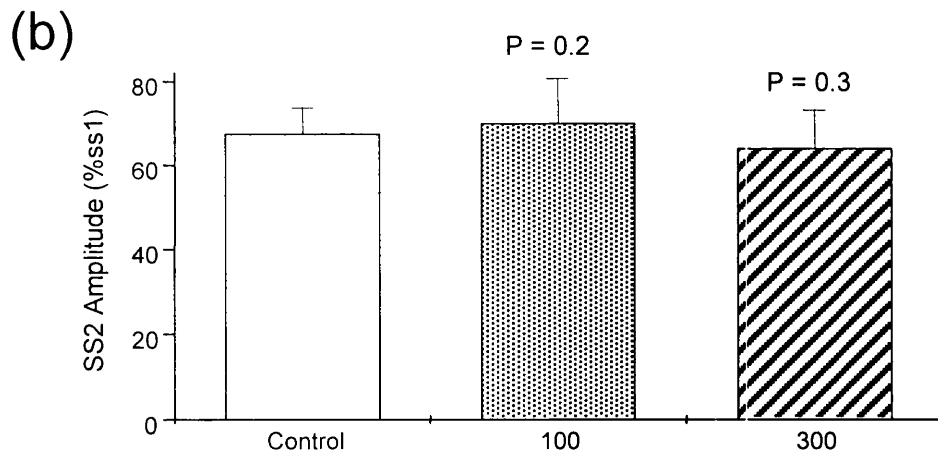


Figure 4.4. PTD is unaffected by group II/III mGlu receptor antagonist.
(a) Perfusion of MPPG (100 and 300 μM) has no effect on PTD recorded in a single Purkinje cell. The antagonist also appears not to affect the mGlu EPSP following the tetanic stimulation.

(b) Pooled data for MPPG (n = 4).

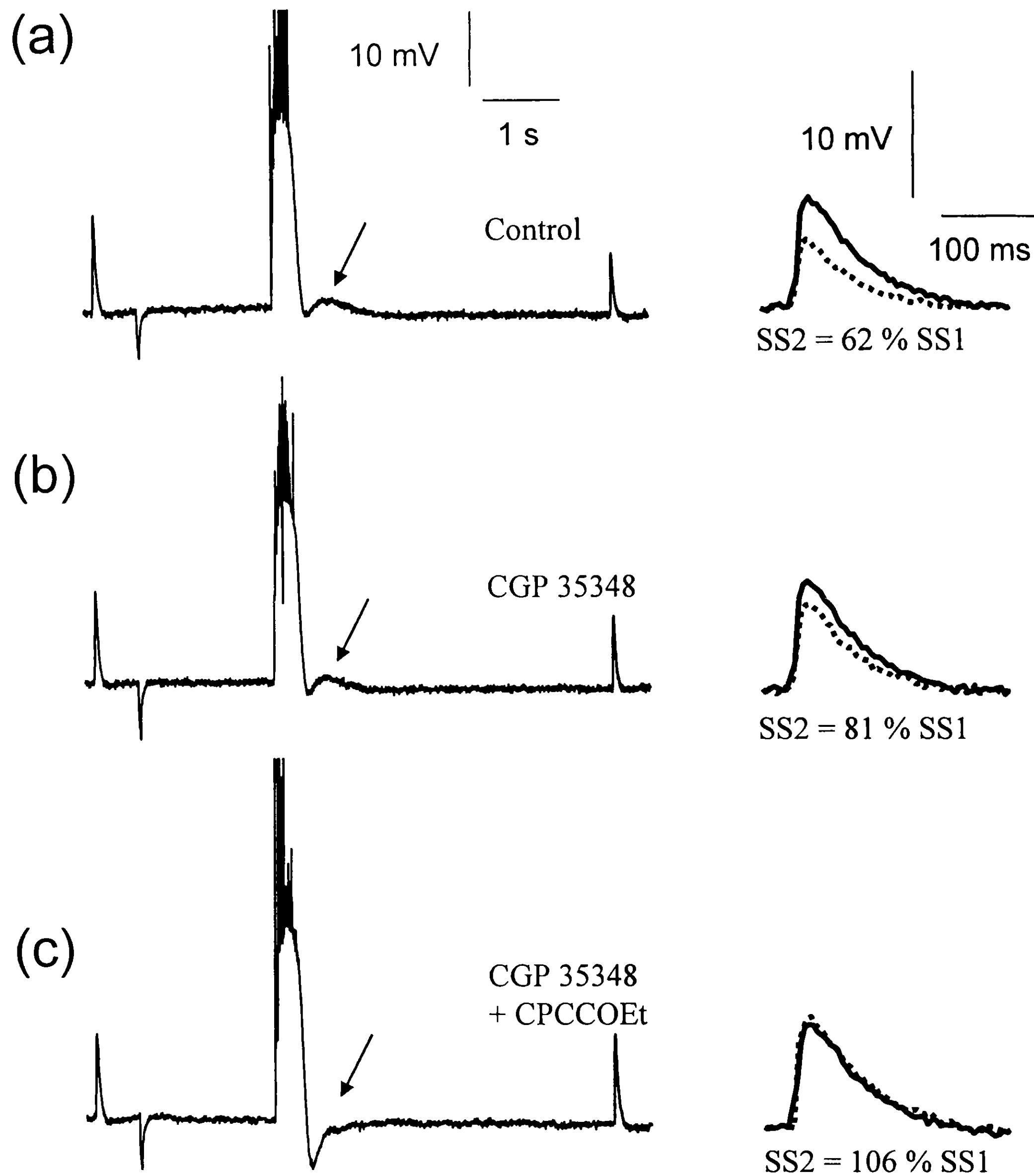
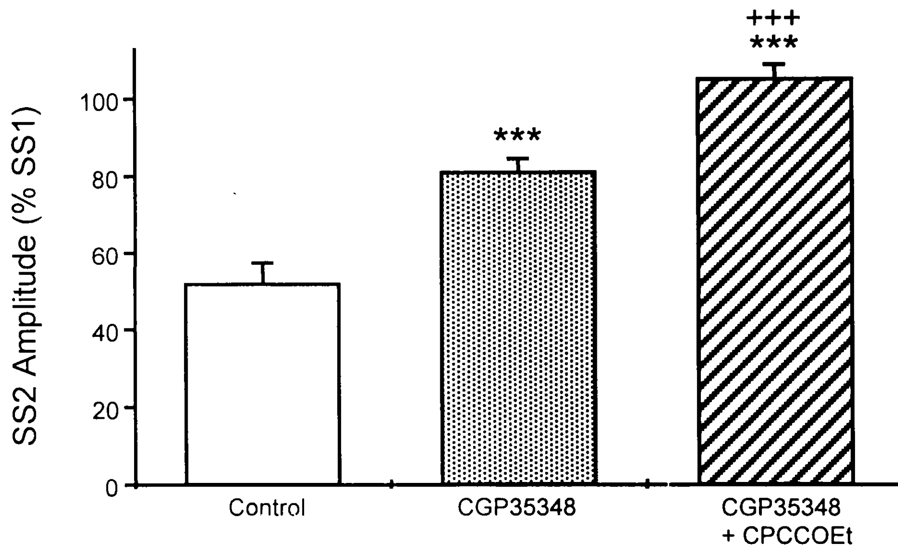


Figure 4.5. Pharmacology of PTD. In a single Purkinje cell the PTD observed under (a) control conditions is attenuated (b) in the presence of the $GABA_B$ receptor antagonist CGP 35348 (1mM), and is further reduced by the perfusion of CPCCOEt (300μM) in the presence of CGP 35348.
Additionally the mGlu EPSP (indicated by arrows) following tetanic stimulation is inhibited in the presence of CPCCOEt.

(a)



(b)

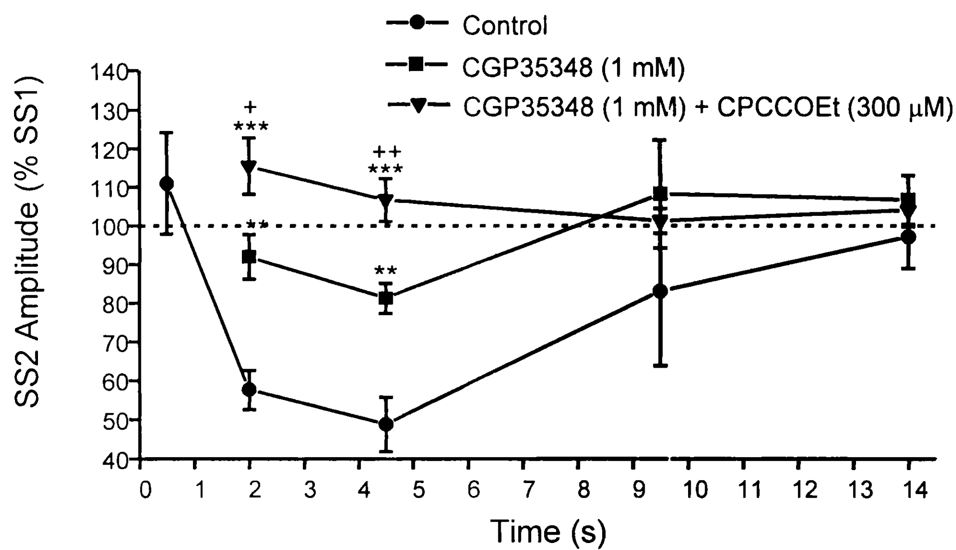


Figure 4.6. PTD is mediated through $GABA_B$ and group I mGlu receptors. (a) Pooled effects of the $GABA_B$ antagonist CGP 35348 (1 mM) alone, or in combination with the mGlu receptor antagonist CPCCOEt (300 μM) measured 4.5 s after tetanic stimulation. (b) The effect of the $GABA_B$ and mGlu receptor antagonists on PTD measures at different time points after the tetanic stimulation. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control; $+P < 0.05$, $++P < 0.01$ $+++P < 0.001$ compared to CGP 35348 alone.

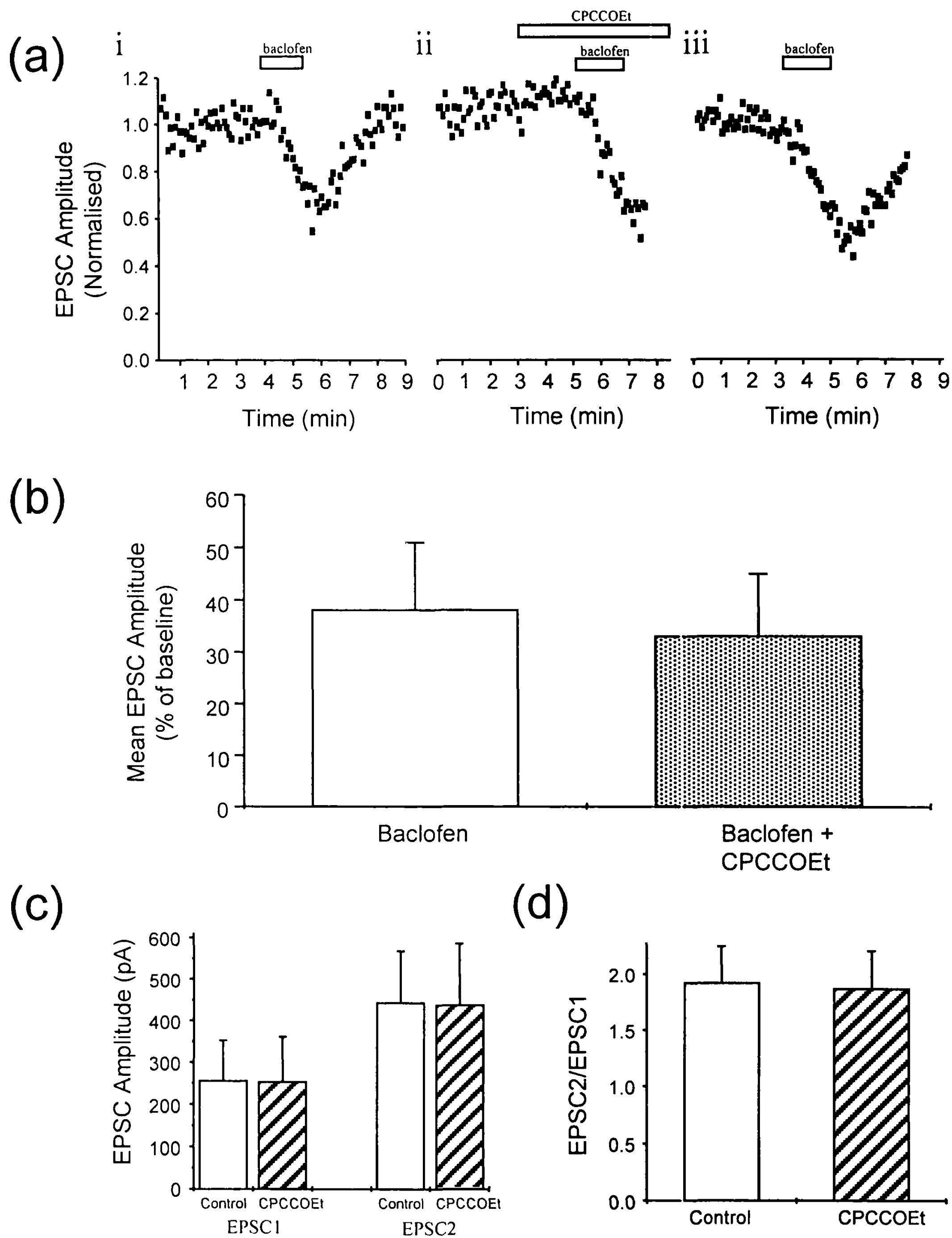


Fig 4.7. CPCCOEt has no effect on $GABA_B$ receptors, nor does it affect parallel Fibre-Purkinje cell synaptic transmission. (a) Single experiment demonstrating that the reversible EPSP depression evoked by baclofen (500 nM) is unaffected by CPCCOEt (300 μM) (b) Pooled data for baclofen (500 nM)-evoked depression in the presence and absence of 300 μM CPCCOEt (n = 3).
(c) Effect of CPCCOEt on EPSC amplitude and (d) paired-pulse facilitation.

reduction of the depression to 81 ± 4% of SS1 ($n = 7$; $P < 0.001$). CGP 35348 had no noticeable affect on the mGlu receptor EPSP (Fig. 4.5b). Application of the group I-selective mGlu receptor antagonist CPCCOEt (300 μM) resulted in a further reduction in the PTD to 105 ± 4% ($n = 7$) of control which was statistically significant compared to the effects of CGP 35348 alone ($P < 0.001$; Fig 4.6a). In the presence of CPCCOEt the mGlu receptor EPSP, when present, was reduced (Fig. 4.5c).

Figure 4.6b shows the time course of the CGP 35348- and CPCCOEt-sensitive components of PTD. When SS2 was elicited at a short time following the tetanic stimulation (0.5 s), it was not possible to measure the SS2 amplitude in the presence of antagonists as the EPSP was facilitated such that the cell fired action potentials. The EPSP was potentiated to such an extent that even further hyperpolarisation of the cell negative to –90 mV failed to prevent the cell reaching firing threshold during the EPSP. At subsequent time points after the tetanus the CGP 35348 produced a significant inhibition of the PTD (92 ± 6%; $n = 4$; $P < 0.01$) compared to vehicle alone (58 ± 5%, $n = 4$), when measured 2 seconds following the tetanic stimulation. In the continued presence of the $GABA_B$ antagonist application of CPCCOEt resulted in a significant inhibition of PTD compared to the CGP 35348 alone (116 ± 7%; $n= 4$; $P < 0.05$). By 14 s from the tetnus there was no significant depression, and addition of the CGP 35348 and CPCCOEt produced no significant effect on the EPSP amplitude.

As previous experiments suggested that a reportedly selective antagonist for $GABA_B$ receptors was a mGlu1 receptor antagonist (A. Batchelor, unpublished observations), the possibility that CPCCOEt may affect $GABA_B$ receptor activation was tested. Using the whole-cell voltage clamp technique and a paired pulse stimulation protocol (see

Chapter 5.) the $GABA_B$ receptor agonist R-baclofen (500 nM) produced a significant, reversible depression of EPSC amplitude to 38 ± 13 % of baseline levels. In the presence of CPCCOEt (300 μM) the depression induced by baclofen (33 ± 12 % of baseline levels) was not significantly different from baclofen alone ($P > 0.05$; n = 3; Fig. 4.7). CPCCOEt (300 μM) produced no significant effect on either the degree of paired-pulse facilitation (baseline = 1.92 ± 0.33; in CPCCOEt = 1.87 ± 0.34; n = 3; $P > 0.05$; Fig 4.7d.), nor on the EPSC amplitude (baseline = 254 ± 98 pA; in CPCCOEt = 253 ± 108 pA; n = 3; $P > 0.05$; Fig 4.7c.). In addition, the antagonist produced no visible changes to the train of summated EPSPs resulting from the tetanic stimulation.

Role of Group III mGlu Receptors in Post-Tetanic Potentiation

The characteristics of parallel fibre-Purkinje cell synaptic transmission have previously been investigated in mutant mice lacking the group III mGlu receptor mGlu4. A protocol (tetanus of 7 stimuli at a frequency of 40 Hz) which induced post-tetanic potentiation (PTP) of an EPSC elicited 200 ms after the tetanic stimulation in the wild type mouse, produced PTD in the mutant (Pekhletski *et al.*, 1996). In the present studies the same stimulus protocol also resulted in PTP of a single EPSP evoked 200 ms after the tetanus, compared to the amplitude of the first EPSP in the train. Application of the antagonist MPPG (300 μM), at a concentration that effectively blocks the effects of L-AP4, did not, however, result in any noticeable action on the PTP (Fig. 4.8).

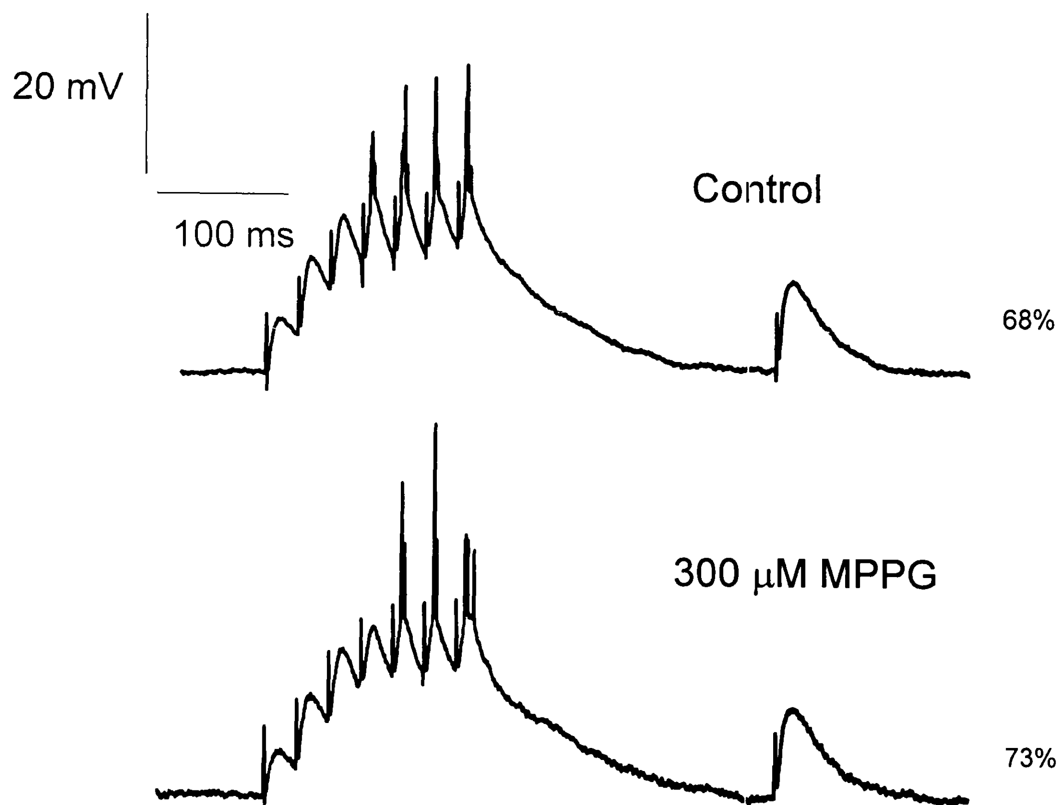


Figure 4.8. Lack of effect of MPPG on post-tetanic potentiation recorded in a single Purkinje cell.

Discussion

Tetanic stimulation of the parallel fibres in the rat cerebellar cortex resulted in post-tetanic depression (PTD) of parallel fibre-Purkinje cell synaptic transmission. This depression was dependent on the stimulus frequency of the tetanus: low stimulus frequencies produced no evidence of synaptic depression, but higher frequencies resulted in a significant reduction of the EPSP amplitude. The depression lasted for several seconds following the tetanus, peaking at around 4 seconds after the train.

$GABA_B$ Receptor-Mediated Post-Tetanic Depression

A significant component of the PTD was found to result from the activation of $GABA_B$ receptors, as evidenced by the action of the competitive $GABA_B$-receptor antagonist CGP 35348 (Bittiger *et al.*, 1990). The $GABA_B$ and mGlu receptors share sequence homology (Kaupmann *et al.*, 1997), and, some $GABA_B$ receptor antagonists are active at group I mGlu receptors (SCH 50911 (100 μM) inhibits the Purkinje cell mGlu EPSC evoked by tetanic stimulation of the parallel fibres: A.Batchelor; unpublished observation). CGP35348 did not produce any observed effects on the mGlu receptor EPSP, and its actions on PTD are therefore unlikely to be due to interaction with the mGlu receptors. In addition, CGP 35348 shows negligible interaction with a range of other receptors, at concentrations of up to 1 mM (Bittiger *et al.*, 1990).

Depression of glutamatergic transmission by $GABA_B$ receptors, following tetanic stimulation of presynaptic fibres has previously been reported in both the cerebellum

and the hippocampus. In the guinea pig hippocampus tetanic stimulation in the dendritic region of CA1 neurones results in depression of excitatory transmission, via activation of $GABA_B$ receptors (Isaacson *et al.*, 1993). A similar result was observed at the parallel fibre-Purkinje cell synapse by Ditmann and Regehr (1997), who demonstrated that tetanic stimulation of the parallel fibres (10 stimuli at 100 Hz) resulted in a heterosynaptic depression of transmission. This depression was entirely blocked by the $GABA_B$ receptor antagonist CGP 35348 (100 μM). This heterosynaptic PTD lasted for less than 2 s, compared to the PTD reported here, which lasted about 10 s. The depression described by Dittmann and Regehr also had a quicker onset, with the peak effect at <0.5 s after tetanic stimulation. Dittmann and Regehr calculated that the decay of the $GABA_B$ effects had a Q_{10} of 3.3. The differences may therefore be explained, at least partly, by temperature differences (30°C vs. 32°C). In the Dittmann and Regehr study, the whole cell recording technique used necessitates recording from cells on the surface of the slice, where GABA concentration might attenuate more rapidly due to diffusion of the transmitter from the tissue into the bath. In the results described here, the recording technique allowed recording from cells deeper within the tissue where diffusion may be more restricted, resulting in prolonged exposure of the receptors to the agonist.

Previous studies have provided evidence that $GABA_B$ receptor-mediated depression of synaptic transmission, both at the parallel fibre-Purkinje cell synapse, and at other synapses, is induced by receptors located presynaptically (Dutar & Nicoll, 1988a; Dittman & Regehr, 1996). It is probable that the $GABA_B$ receptor-mediated component of PTD is also due to a presynaptic receptor (see Chapter 5). Two $GABA_B$ receptor subunits have been identified: GBR1 and GBR2 (Kaupmann *et al.*, 1997; Jones *et al.*,

1998; White *et al.*, 1998; Kaupmann *et al.*, 1998a). The GBR1 subunit exists in two isoforms, designated GBR1a and GBR1b. *In vivo,* the $GABA_B$ receptor is postulated to exist as a heterodimer formed by GBR1 and GBR2, as transfection of the GBR1 subunit in mammalian cells only results in fully functional receptors when co-expressed with the GBR2 subunit (Jones *et al.*, 1998; White *et al.*, 1998; Kaupmann *et al.*, 1998a)). In the cerebellar cortex the GBR1a isoform is located presynaptically at the parallel fibre-Purkinje cell synapse (Kaupmann *et al.*, 1998b; Billinton & Bowery, 1999). The GBR1b is expressed in Purkinje cells (Kaupmann *et al.*, 1998a; Billinton & Bowery, 1999), where it apparently co-localises with the GBR2 subunit, in an extrasynaptic location on the dendritic spines which form synapses with the parallel fibres (Kaupmann *et al.*, 1998a). It is therefore possible that there is a postsynaptic component of $GABA_B$ receptor-mediated PTD. Although the $GABA_B$ receptors do have a postsynaptic action at this synapse (an inhibitory postsynaptic potential; Batchelor & Garthwaite, 1992) there do not appear to be any examples in the literature of postsynaptic $GABA_B$ receptors exerting a direct effect on AMPA receptor-mediated synaptic transmission.

The GABA responsible for the activation of the $GABA_B$ receptors is probably released from the GABAergic basket and stellate cells (Gabbott *et al.*, 1986; Ottersen *et al.*, 1988). These inhibitory interneurones have synaptic contacts formed with the terminals of the parallel fibres (Palay & Chan-Palay, 1974), and are excited by ionotropic glutamate application (Llano *et al.*, 1991; Farrant & Cull Candy, 1991), or parallel fibre-stimulation (Midtgaard, 1992). It is difficult to predict the distances the GABA released from these cells would have to diffuse to affect transmitter release from parallel fibre terminals. The synapses formed between the Purkinje cell and the basket and

stellate cells are predominately located on the soma and dendritic shafts of the Purkinje cell respectively, although stellate cells are also suggested to form synaptic contacts with the dendritic spines of the Purkinje cell (Palay & Chan-Palay, 1974). As the GABA diffuses away from the release sites the concentration of the transmitter will decrease as the transmitter is dispersed over a wider area and, on the terminals of interneurones and in glia there are GABA transporters (Morara *et al.*, 1996; Itouji *et al.*, 1996), which will further reduce the extracellular GABA concentration. It is estimated that the concentration of GABA in the synaptic cleft rises to at least 500 μM during synaptic transmission (Maconochie *et al.*, 1994). Native and recombinant $GABA_B$ receptors have an EC_{50} for GABA of approximately 1 μM (Kaupmann *et al.*, 1997; Jones *et al.*, 1998), so presumably a similar concentration reaches the parallel fibre-Purkinje cell synapse.

The basal extracellular concentration of GABA, measured *in vivo* with a dialysis probe, is estimated to be ~0.8 μM (Lerma *et al.*, 1986; Tossman *et al.*, 1986). This concentration of GABA would be sufficient to activate the $GABA_B$ receptors, but application of CGP 35348 did not noticeably alter the amplitude of the single parallel fibre EPSP SS1. Isaacson *et al* (1993) reported a similar lack of effect of CGP 35348 on excitatory transmission in the hippocampus. Thus, assuming cerebellar $GABA_B$ receptors have a similar sensitivity to GABA as those reported elsewhere, the background level of GABA in the location of the $GABA_B$ receptors must be being maintained at much lower levels to prevent tonic $GABA_B$ receptor activation. In addition, low frequency stimulation of the parallel fibres does not appear to stimulate sufficient GABA release to depress synaptic transmission.

Group I mGlu Receptor-Mediated Post-Tetanic Depression

In the presence of the $GABA_B$ receptor antagonist, at a concentration expected to completely inhibit the receptor, the parallel fibre EPSP evoked following tetanic stimulation remained partially depressed. This was not observed in the heterosynaptic depression, which was completely blocked by CGP 35348 (100 μM; (Dittman & Regehr, 1997). We therefore tested whether the remaining component of the PTD could be due to activation of mGlu receptors.

Initial studies demonstrated that the mGlu receptor antagonist MCPG inhibited a component of the PTD, suggesting that at least a fraction of the depression was a result of mGlu receptor activation. The mGlu receptors are expressed on the inhibitory interneurones of the cerebellar cortex (Baude *et al.*, 1993; Grandes *et al.*, 1994). The MCPG-sensitive component of PTD could therefore be explained by excitation of the inhibitory cells following activation of the mGlu receptors after tetanic stimulation of the parallel fibres. To remove the possibility of such disynaptic effects all the additional experiments into the role of mGlu receptors in PTD were performed in the presence of a saturating concentration of CGP 35348.

MCPG is active at receptors from all three groups of mGlu receptors (Hayashi *et al.*, 1994; Thomsen *et al.*, 1994b; Saugstad *et al.*, 1997; Wu *et al.*, 1998). In the previous chapter it was shown that the antagonist CPCCOEt blocked the depression of the parallel fibre EPSP induced by the exogenous group I mGlu receptor agonist DHPG. In the presence of the $GABA_B$ antagonist, the remaining depression of the parallel fibre EPSP was abolished on application of the antagonist CPCCOEt. CPCCOEt is highly selective, apparently acting only at the mGlu1 receptor (Litschig *et al.*, 1999). In

previous studies in adult rats both CPCCOEt and MCPG inhibited the mGlu receptor-mediated EPSP elicited after tetanic stimulation of the parallel fibres (Batchelor *et al.*, 1997). In the younger rats used in this study the mGlu receptor EPSP, when present, was also inhibited by these antagonists.

The $GABA_B$ antagonist CGP 35348 is competitive, and there is a possibility that it was not at high enough concentration to completely inhibit the $GABA_B$ receptor, although from examples in the literature of this compound being used (e.g. Dittman & Regehr, 1997) 1 mM would be expected to provide total inhibition. As discussed above, the $GABA_B$ and mGlu receptors share sequence homology (Kaupmann *et al.*, 1997), and the $GABA_B$ antagonist SCH 50911 appeared to act as a mGlu receptor antagonist (A.Batchelor; unpublished observations). It was therefore important to confirm that the mGlu receptor antagonist was not acting at the $GABA_B$ receptor. As the results in Figure 4.7 illustrate, CPCCOEt, at the concentrations used to inhibit PTD, produced no effect on the depression of synaptic transmission induced by the $GABA_B$ receptor agonist R-baclofen (500nM). In addition, the basal extracellular concentration of glutamate is reportedly at concentrations sufficiently high enough to activate mGlu receptors (Lerma *et al.*, 1986; Tossman *et al.*, 1986); application of CPCCOEt, however, produced no evidence of constitutively active group I mGlu receptors at this synapse. Thus, the glutamate concentration is lower in the location of the receptors, or perhaps they are desensitised to lower concentrations of this agonist.

To our knowledge, this is the first report that transmission at this synapse can be depressed by synaptic activation of the mGlu1 receptors. Numerous studies have reported that exogenous mGlu receptor agonists can modulate the strength of synaptic

transmission, but there are very few examples indicating that synaptically released agonist can lead to the same effects. In the nucleus tractus solitarius a low frequency tetanus was reported to produce PTP in some cells and PTD in others (Glaum & Miller, 1993). The mGlu receptors were apparently depressing transmission because in the presence of MCPG all cells show PTP, and those cells originally producing PTP show an increase in the potentiation. In the hippocampus, raising glutamate concentration by high-frequency activity, or by blocking glutamate uptake, activated mGlu receptors, leading to an inhibition of transmitter release (Scanziani *et al.*, 1995).

The site of action of the group I mGlu receptor in PTD is unclear. We have considered three basic hypotheses: firstly, the receptor may be located presynaptically; secondly, it may be postsynaptic; thirdly, the receptor may be extrasynaptic (Fig. 4.9).

Regulation of synaptic transmission by G-protein coupled receptors is commonly due to receptors located presynaptically. The hypothesis that the mGlu receptor involved in PTD is located presynaptically is supported by functional studies. The agonist ACPD depressed synaptic transmission, but did not depress the postsynaptic potential produced by iontophoretic application of AMPA (in fact, the response was actually potentiated in the presence of the mGlu receptor agonist). This observation was interpreted as evidence that the depression of synaptic transmission is a presynaptic change in transmitter release, rather than a postsynaptc effect on the AMPA receptor (Glaum *et al.*, 1992). These studies were, however, potentially flawed: the AMPA was not applied in the presence of TTX, and the possibility that neuromodulators were released due to excitation of other cell types was not considered.

Significant evidence against the hypothesis that the mGlu receptor is located presynaptically comes from histological studies, which provide evidence of postsynaptic mGlu1 receptors (Martin *et al.*, 1992; Baude *et al.*, 1993), but, although granule cells do express mRNA for the group I receptors (Grandes *et al.*, 1994; Makoff *et al.*, 1997), provide no evidence that the receptors are located on parallel fibre terminals. The postsynaptic group I receptors are functional, as evidenced by the excitation of Purkinje cells by exogenous group I agonists (Crépel *et al.*, 1991; Glaum *et al.*, 1992 and previous chapter), and their activation by endogenous agonist following tetanic stimulation of the parallel fibres, producing an mGlu receptor-mediated EPSP (Batchelor *et al.*, 1994). Like the mGlu EPSP, PTD is only evident following high frequency tetanic stimulation, and the two phenomena appear to share the same pharmacology, although have different time courses. The need for repetitive stimulation may be due to the mGlu1 receptor being located on the periphery of the synapse (Baude *et al.*, 1993); therefore, trains of stimuli may be necessary to raise the glutamate concentration diffusing from cleft to sufficient levels to activate the mGlu receptor. Alternatively, second messengers such as cAMP and IP_3 are free to diffuse in the cytoplasm (Allbritton *et al.*, 1992; Kasai & Petersen, 1994), and the local concentration will reduce as they diffuse throughout the cell. A sustained activation of the receptor by tetanic stimulation may therefore be necessary to raise second messenger levels to a sufficient concentration to exert an observable effect.

The third hypothesis relies on glutamate diffusing out of the synapse to activate mGlu receptors located on another cell type, which may be either another neurone, or a glial cell. Apart from Purkinje cells, the group I mGlu receptors are expressed in granule cells, stellate cells, basket cells, Golgi cells, Lugaro cells and Bergmann glia (Grandes

et al., 1994; Grosche *et al.*, 1999). This hypothesis suggests that activation of the receptors of one of the other cell types leads to the release of a neuromodulator, which may then act either pre-, or postsynaptically to produce the PTD. For example tetanic stimulation of parallel fibres leads to Ca^{2+} signals in Bermann glia (Grosche *et al.*, 1999), which could conceivably result in release of a modulatory substance. In adult rats two of the more likely candidates for this, adenosine and nitric oxide, did not appear to play a role in PTD (A. Batchelor; unpublished results).

In the next chapter, experiments are described which were designed to help elucidate the site of action of the group I mGlu receptor responsible for depression of synaptic transmission at the parallel fibre-Purkinje cell synapse.

Synaptic Activation of the Group III mGlu Receptors?

The antagonist MPPG, at concentrations that inhibit the L-AP4-induced depression of parallel fibre–Purkinje cell synaptic transmission (see chapter 3), had no significant effect on PTD. In addition, consistent with MPPG being inactive at the group I mGlu receptors, this compound had no action on the mGlu receptor-mediated EPSP. These observations are in agreement with the hypothesis that both PTD and the mGlu receptor EPSP are mediated through the group I receptors, and that the inhibition of PTD produced by MCPG and CPCCOEt was entirely due to their activity at the group I receptors.

The only published evidence that group III mGlu receptors at the parallel fibre-Purkinje cell synapse may be activated by synaptically released agonist is from a study

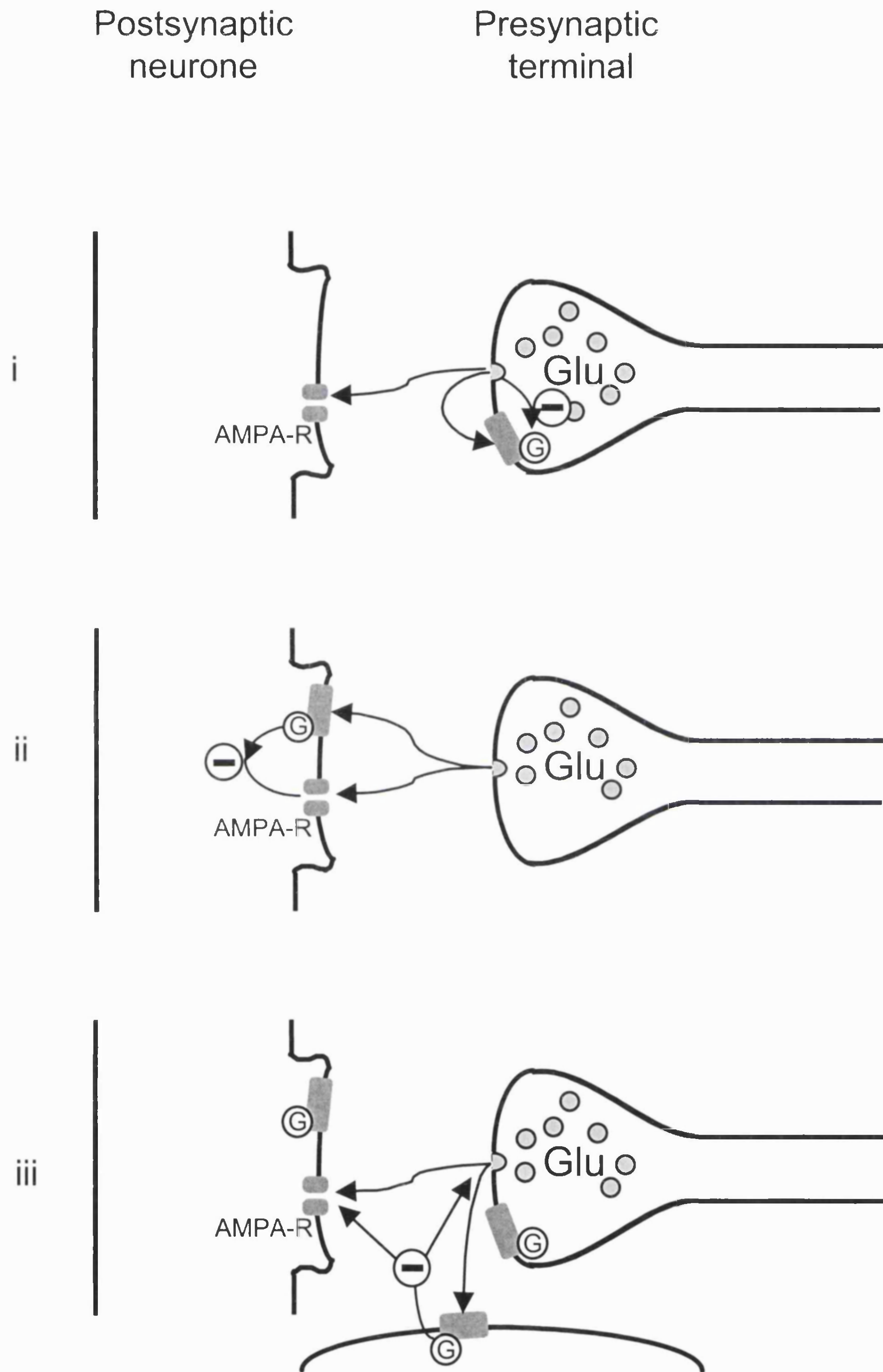


Figure 4.9. Hypotheses for the site of mGlu receptor-mediated PTD.
i. A presynaptic autoreceptor
ii. Postsynaptic receptor
iii. Extrasynaptic receptor
See text for further details.

examining mutant mice lacking the mGlu4 receptor (Pekhletski *et al.*, 1996). An EPSC elicited 200 ms after tetanic stimulation of the parallel fibres was potentiated in the normal mouse, but in the mutant was depressed compared to control EPSC amplitude. In the same study, however, application of the antagonist MAP4 to rat cerebellar slices did not result in the potentiation switching to depression. This negative result is difficult to interpret as MAP4 is an unselective compound, with reported agonist and antagonist activity at the group II and III receptors (Schoepp *et al.*, 1999). The lack of efficacy of MAP4 is, however, unlikely to be related to its poor selectivity. As described in this chapter, the stimulation protocol used to produce post-tetanic potentiation in the mouse also produced a potentiation of synaptic transmission in the rat, but this potentiation was unaffected by the more selective antagonist MPPG (Thomsen *et al.*, 1996; Gomeza *et al.*, 1996b). The negative results obtained with MPPG and MAP4 suggest that mGlu4 is not involved directly in the post-tetanic potentiation. The observations in the mutant mouse may therefore be evidence of abnormal development of synaptic machinery, rather than for a direct role of the receptor in synaptic transmission. When using the PTD stimulation protocol, EPSPs elicited at 500 ms after tetanic stimulation also showed evidence of potentiation. This was particularly evident in the presence of $GABA_B$ and mGlu receptor antagonists, when the EPSP would often be of sufficient amplitude to trigger action potentials. This type of potentiation is probably related to residual Ca^{2+} in the presynaptic terminals enhancing transmitter release (Zucker, 1999).

In the rat cerebellar cortex, mGlu4 receptors have been identified on the terminals of the parallel fibres (Mateos *et al.*, 1999), and are likely to mediate the depression of the parallel fibre EPSP caused by L-AP4 (Pekhletski *et al.*, 1996). One might therefore expect the mGlu4 receptor to act as a classic presynaptic autoreceptor, but the present

studies failed to find evidence that the group III mGlu receptors could be activated by synaptically released agonist. The concentration of glutamate in the synaptic cleft following a single stimulation is estimated to be in excess of 1 mM (Clements *et al.*, 1992), which is sufficient to activate both group I and III mGlu receptors (Pin & Duvoisin, 1995). Given the mGlu4 receptor's ideal location in the synapse to regulate glutamate release it was surprising that we found no evidence of it being activated by endogenous ligand. It is possible that to depress transmission the mGlu4 receptor needs to be activated for a sustained period, perhaps to allow the concentration of second messengers involved to reach levels sufficient to depress transmitter release. Such prolonged activation of the receptor would be expected in the studies using exogenous agonists, but would perhaps not be expected to occur during normal synaptic transmission, where glutamate is rapidly cleared from the synaptic cleft (Clements *et al.*, 1992; Clements, 1996). It is known that extracellular glutamate concentration can increase for prolonged periods during pathological conditions (Benveniste *et al.*, 1984), and the mGlu4 receptor may, therefore, have a role reducing transmitter release under these situations.

Role of Post-Tetanic Depression?

The present studies have demonstrated that PTD of synaptic transmission is produced by activation of $GABA_B$ receptors and group I mGlu receptors. In these experiments the two components of PTD seemed similar in terms of time course etc. *In vivo*, however, it is conceivable that activation of the $GABA_B$ and mGlu receptor components occurs under different circumstances. High frequency activation in this, and previous studies (Dittman & Regehr, 1997) has been shown to produce depression of fast synaptic

transmission, via presynaptic $GABA_B$ receptor activation. Reduction of glutamate release in this manner may provide an important mechanism for protecting cells from excitotoxicity. Although LTD is commonly proposed as a means of memory storage at the parallel fibre-Purkinje cell synapse, an alternative role has been proposed (De Schutter, 1995). *In vivo* Purkinje cells typically fire simple spikes, but with strong stimulation, such as from climbing fibres, or from parallel fibres in the absence of inhibition, fire complex spikes involving a mixture of Na^+ and dendritic Ca^{2+} spikes. It is known that strong activation of parallel fibres can induce LTD (Hartell, 1996), and De Schutter (1995) proposed that this LTD provides a mechanism of negative feedback to prevent overstimulation of the Purkinje cell. Such a permanent modification may be a drastic means of correcting a short period of increased synaptic activity. PTD may therefore serve a roll similar to that proposed by De Schutter for LTD, by reversibly depressing Purkinje cell excitation for several seconds following high frequency activity. The possible roles of short lasting forms of synaptic plasticity are discussed further in the next chapter.

Conclusion

Despite the large number of publications indicating a role for mGlu receptors in the regulation of excitatory synaptic transmission very few investigators have described the conditions necessary for these receptors to be activated by synaptically released agonist. We have discovered that group I mGlu receptors can be activated by stimulation protocols designed to mimic the presynaptic activity that occurs *in vivo*. This activation of the receptor has two effects: an EPSP, and post-tetanic depression. To our

knowledge, this is the first evidence that group I mGlu receptors can be activated in the rat CNS by endogenous agonist to depress synaptic transmission.

Chapter 5: Investigation into the site of action of G-protein linked receptors mediating depression of synaptic transmission in the rat cerebellar cortex

Introduction

Using conventional sharp microelectrode recording techniques, we have observed that the group I and III mGlu receptors can depress excitatory synaptic transmission in the rat cerebellar cortex (Chapter 3). In addition, conditions necessary for a group I receptor-mediated synaptic plasticity have been identified at the same synapse (Chapter 4). Synaptic transmission is a complex, highly regulated process and the resulting synaptic current may be attenuated as a result of either pre- or postsynaptic actions. In this chapter the action of mGlu receptors on neurotransmission at the parallel fibre-Purkinje cell synapse is further investigated. Using the whole cell voltage clamp technique, experiments were conducted to test whether the synaptic depression induced by mGlu receptors occurs pre- or postsynaptically.

Mechanisms of Neurotransmitter Release

Calcium Dependence of Release

Transmitter release is initiated by an action potential depolarising the cell membrane of the presynaptic terminal, facilitating opening of voltage dependent ion channels; in particular Ca^{2+} permeable channels. The resulting influx of Ca^{2+} via these channels triggers release of neurotransmitter. The fundamental importance of Ca^{2+} in release of

neurotransmitter release was recognised from experiments performed in the 1960s and 70s by Katz and colleagues (Katz & Miledi, 1968). Subsequently, direct recording of Ca^{2+} currents in squid nerve terminal demonstrated a clear correlation between influx of Ca^{2+} and release of transmitter (Augustine *et al.*, 1985). The relationship between release and Ca^{2+} is non-linear, and it was initially suggested that release was related to Ca^{2+} concentration raised to the power 4 (Dodge & Rahamimoff, 1967). However, the exact relationship has been found to vary between different synapses; for example, at the parallel fibre-Purkinje cell synapse transmitter release is related to the Ca^{2+} concentration raised to the power 2.5 (Mintz *et al.*, 1995). Pharmacological and biophysical studies have established the existence of several different classes of Ca^{2+} channel (L, N, P, Q, R, T) and several of these appear to be involved in the release of neurotransmitter at individual synapses. At the parallel fibre-Purkinje cell synapse release is triggered by at least three pharmacologically distinct Ca^{2+} channels (Mintz *et al.*, 1995; Cousin *et al.*, 1997).

The opening of voltage-dependent Ca^{2+} channels in synaptic terminals causes a large increase in the concentration of Ca^{2+}, and around the point of Ca^{2+} entry the concentration can reach 100-300 μM. Evidence for this comes from a number of sources including Ca^{2+}-sensitive dyes (Llinas *et al.*, 1992), the activation of Ca^{2+}-activated K^+ channels (Roberts *et al.*, 1990) and that release is only inhibited by high concentration of fast, high-affinity Ca^{2+} chelators (Adler *et al.*, 1991). The Ca^{2+} signal results in a rapid release of neurotransmitter, with postsynaptic responses being detected ~0.5 ms after depolarisation of the terminal. Taking into account other factors, such as time for Ca^{2+} channels to open, it is estimated that from channel opening to transmitter release takes about 200 μs (Llinas *et al.*, 1981). Such a time scale means the release

mechanism must be located close to the Ca^{2+} channels. Consistent with this freeze-fracture techniques have identified vesicles near to what are thought to be Ca^{2+} channels (Pumplin & Reese, 1978; Heuser & Reese, 1981). The rapidity of transmitter release makes the involvement of a complex biochemical cascade unlikely and suggests that release may involve faster events, such as Ca^{2+}-induced conformational changes.

Molecular Mechanisms of Transmitter Release

Several lines of evidence have lead to the development of the hypothesis that the synaptic vesicle protein synaptotagmin I (or P65) is the Ca^{2+}-sensor for triggering release of neurotransmitter (Südhof, 1995). This protein has two Ca^{2+} binding domains, and on Ca^{2+} binding undergoes conformational changes (Davletov & Sudhof, 1994). Cultured embryonic hippocampal neurones from mutant mice lacking synaptotagmin showed decreased Ca^{2+}-evoked transmitter release, although Ca^{2+}-independent release (mini EPSCs) was unimpaired (Geppert *et al.*, 1994), and injection of synaptotagmin peptides inhibits transmitter release in the squid giant synapse (Bommert *et al.*, 1993). Exactly how Ca^{2+} binding leads to release is not known, but it is suggested that Ca^{2+}-dependent multimerisation of synaptotagmin and binding to membrane phospholipids leads directly to creation of the fusion pore allowing transmitter to be released (Geppert & Sudhof, 1998).

Locating vesicles at the plasma membrane to be available for release involves a complex biochemical cascade. Within the terminal vesicles are clustered in two distinct pools: a store of vesicles located away from the terminal membrane, and the other consisting of vesicles docked at the membrane. The distal pool is postulated to be

regulated by synapsins, a family of four proteins (Ia, Ib, IIa, IIb) that are associated with the cytoplasmic side of the vesicle membrane. Synapsins are thought to anchor the vesicles to elements of the cytoskeleton such as actin (Greengard *et al.*, 1993; Pieribone *et al.*, 1995). A rise in Ca^{2+} concentration increases the phosphorylation of the synapsins, resulting in dissociation from the vesicles, thereby providing a mechanism to replenish supplies of vesicles released from the docked zone (Benfenati *et al.*, 1992; Greengard *et al.*, 1993). In support of this hypothesis injection into nerve terminals of the dephosphorylated form of synapsin I inhibits transmitter release, whereas Ca^{2+}/calmodulin-dependent protein kinase II increases release (Llinas *et al.*, 1991). Mutant mice lacking synapsin II, exhibit a small reduction in hippocampal post-tetanic potentiation and ~50% reduction of EPSP amplitude during repetitive stimulation. This is suggested to reflect an inability to replenish the supplies of vesicles docked at the membrane. Mice lacking synapsin I exhibited a different phenotype, with normal PTP and a ~20% increase in paired-pulse facilitation. Although this may suggest different roles for the synapsins, interpretation of the observations is complicated as the levels of other proteins implicated in transmission, such as synaptotagmin and the remaining synapsin were also reduced in both knockouts (Rosahl *et al.*, 1995). In addition, transmitter release is a necessary feature of synaptogenesis (Haydon & Drapeau, 1995); thus the phenotype of a mutant may be related to abnormal development of the synapse.

Vesicles are docked at the membrane by the interaction of SNAP receptor (SNARE) proteins, which form a stable, detergent resistant complex (Sollner *et al.*, 1993a). In the vesicle membrane the protein (or v-SNARE) is synaptobrevin (also known as vesicle-associated membrane protein or VAMP) and in the target cell membrane the t-SNAREs are syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa). The SNARE

proteins are also highly conserved among species with homologues to the three SNAREs existing in yeast (Sollner *et al.*, 1993b). Their importance in neurotransmission is demonstrated by the botulinium toxins which inhibit the release of transmitter by cleaving the presynaptic proteins synaptobrevin, SNAP-25 and syntaxin (Sudhof, 1995). For example, botulinium C1 toxin inhibits neurotransmission and causes an accumulation of vesicles in the terminal of the squid giant synapse (Marsal *et al.*, 1997). Thus, the SNARE proteins possibly function to fuse the two membranes together, rather than locate vesicles at the target membrane. Understanding of the formation of the complex of proteins associated with release is complicated by the involvment of numerous other proteins, including NSF, α-SNAP and munc18. Some of these assist formation of the complex and transmitter release, whereas others, such as syntaphilin, which competes with SNAP-25 to bind with syntaxin, can inhibit formation (Lao *et al.*, 2000).

<u>Mechanisms linking calcium channels to the vesicles</u>

SNARE proteins serve an additional role by interacting with subunits of Ca^{2+} channels, thus ensuring that the release mechanism is located close to the site of Ca^{2+} entry. Synataxin and synaptotagmin coimmunoprecipitate with N- and P/Q-type Ca^{2+} channels (Bennett *et al.*, 1992; Leveque *et al.*, 1994), and a SNAP-25 and syntaxin binding (synprint) site has been identified on the cytoplasmic loop between domains II and III of α_{1B} Ca^{2+} channel subunits (the pore forming component of N-type Ca^{2+} channels (Williams *et al.*, 1992; Sheng *et al.*, 1994)). The interaction of syntaxin and SNAP-25 is Ca^{2+}-dependent, with maximal binding occurring at a free Ca^{2+} concentration of 20 μM (Sheng *et al.*, 1996). Binding of syntaxin is competitively blocked by Ca^{2+}-independent

binding of synaptotagmin I to the synprint site (Sheng *et al.*, 1997). The α_{1A} subunits, which form P and Q-type channels (Mori *et al.*, 1991; Bourinet *et al.*, 1999), also have binding regions for syntaxin and SNAP-25 in the domain linking regions II and III, although syntaxin binding appears to be limited to the BI isoform, with the rbA isoform binding only SNAP-25 (Rettig *et al.*, 1996). The Ca^{2+}-dependence of SNARE protein interaction with the channels is also isoform specific. The rbA isoform binds SNAP-25 to the synprint region in a Ca^{2+} independent manner, but binds synaptotagmin in a Ca^{2+}-dependent manner. The BI isoform binds the SNARE proteins in a Ca^{2+}-independent fashion (Kim & Catterall, 1997). The α_{1B}, $\alpha_{1A(rbA)}$ and $\alpha_{1A(BI)}$ Ca^{2+} channel subunits are differently distributed in the CNS (Westenbroek *et al.*, 1992; Westenbroek *et al.*, 1995; Sakurai *et al.*, 1996); it is therefore possible that different synapses have subtle differences in the way they function, depending on the particular combination of Ca^{2+} channel that are present.

This interaction between SNARE proteins and the Ca^{2+} channels is of functional significance. Injection of synprint peptides into presynaptic cultured superior cervical ganglion neurones produced a depression of synaptic transmission to ~60% of control levels. This effect was maximal after about 10-15 minutes and reversed in about 40 minutes, probably due to the action of proteolytic enzymes (Mochida *et al.*, 1996). When syntaxin 1A is coexpressed with Ca^{2+} channels in *Xenopus* oocytes, channel availability at negative potentials (-60 and –80 mV) was reduced, and channels showed slowed recovery from inactivation compared to channels expressed alone. This effect was seen for both N- and Q-type channels, but not for L-type (Bezprozvanny *et al.*, 1995). Co-expression of SNAP-25, syntaxin and synaptotagmin with P/Q-type channels containing the rbA isoform resulted in normal inactivation compared to SNAP-25 alone

(-10 mV shift). This normal function was achieved without displacing SNAP-25 from the Ca^{2+} channel, and may, therefore, be a result of conformational changes resulting from the additional associations with the other proteins (Zhong *et al.*, 1999).

Importantly, the SNARE protein-Ca^{2+} channel interactions may provide a means for G-protein coupled receptors to modulate neurotransmission. G-protein coupled receptors commonly modulate transmitter release by depressing Ca^{2+} entry into the terminal via an interaction between G-protein $\beta\gamma$ subunits and the Ca^{2+} channel α subunit (Dolphin *et al.*, 1999). This effect may be facilitated by the molecular interaction between syntaxin 1A and Ca^{2+} channels as co-expression of syntaxin with N-type Ca^{2+} channels (consisting of $\alpha_1B + \alpha_2\text{-}\delta + \beta_{1b}$) resulted in channels tonically inhibited by G-proteins, and a negative shift in the voltage dependence of channel inactivation. The inhibition was diminished by the botulinium toxin C, suggesting that the syntaxin was responsible for the effect, and syntaxin is hypothesised to assist in directing the $G\beta\gamma$ subunits towards their binding site (Jarvis *et al.*, 2000). The interaction between the Ca^{2+} channels and the SNARE proteins may be regulated by second messenger systems as phosphorylation of a synprint peptide site by PKC or CaMKII prevented association with syntaxin and SNAP-25 (Yokoyama *et al.*, 1997). However, the physiological significance of this is unclear as blocking interaction with the synprint site inhibits synaptic transmission (Mochida *et al.*, 1996), but PKC and CaMKII typically enhance synaptic transmission (Malenka *et al.*, 1986; Shapira *et al.*, 1987; Llinas *et al.*, 1991; Capogna *et al.*, 1995). It does, however, raise the possibility that this is a potential target for second messenger-coupled receptors to modulate neurotransmission.

Postsynaptic Effects

Fusion of a vesicle at a glutamatergic synapse is estimated to release some 4000-5000 molecules into the synaptic cleft (Riveros *et al.*, 1986; Burger *et al.*, 1989; Bruns & Jahn, 1995). By analysing the displacement of the rapidly dissociating NMDA receptor antagonist D-aminoadipate the concentration of glutamate in the cleft is estimated to peak at 1.1 mM and decays with a time constant of 1.2 ms (Clements *et al.*, 1992). These estimates may, however, require revision as this concentration of glutamate would be expected to saturate the high affinity NMDA receptors but data obtained more recently suggests this is not the case: two photon imaging of NMDA-mediated Ca^{2+} signal in dendritic spines of CA1 neurones suggest that the NMDA receptor is not saturated as pairs of stimuli separated by 10 ms evoked Ca^{2+} signals that were larger than those evoked by single stimuli (Mainen *et al.*, 1999).

The glutamate released into the cleft binds to postsynaptic ionotropic receptors leading to opening of the integral cation channel and an influx of ions producing the excitatory postsynaptic current. The amount of charge entering a neurone through an ion channel is dictated by postsynaptic factors, such as membrane potential and receptor channel conductance and kinetics, all of which may be modified by the activity of second messenger systems. For example, AMPA receptors (GluR1) expressed in HEK293 cells with a constitutively active CaMKII mutant, show an increased occurrence of higher conductance states (21 and 28 pS) compared to AMPA receptors expressed alone where lower conductance (9 and 14 pS) states dominate (Derkach *et al.*, 1999). Such a change in conductance state of the channels may, at least in some instances, underlie hippocampal LTP. Benke *et al* (1998) observed (in 13 cells out of 21) that induction of

LTP was associated with more than 200% increase in the estimated single channel conductance.

Paired-Pulse Facilitation

An important question from the data in the previous chapter is where might the synaptically activated group I receptor be acting to depress parallel fibre-Purkinje cell transmission? A commonly used test of whether a modulation of synaptic strength has occurred pre-, or postsynaptically is paired-pulse facilitation. This technique involves giving two stimuli of the same amplitude to the presynaptic fibres, typically separated by tens of milliseconds. The second of the two resulting EPSCs is larger than the first (Fig.5.1a). Facilitation is universally regarded as a presynaptic action on release probability and, along with some other forms of plasticity, such as augmentation and potentiation, is dependent on the Ca^{2+} concentration in the presynaptic terminal (Kamiya & Zucker, 1994). Transmitter release has two components: the rapid phasic release evoked by high Ca^{2+} in the active zones, and a transient tail of release. The transient release persists for ~20 ms, and is thought to be due to residual Ca^{2+} in the terminal (Chen & Regehr, 1999). Originally it was believed that facilitation resulted from residual Ca^{2+} in the terminal, adding to Ca^{2+} from the second stimulus i.e. the small amount of Ca^{2+} persisting after an action potential invades the terminal will have relatively little effect, but, due to the non-linear relationship between Ca^{2+} concentration and release, combined with a second influx could exert a significant effect. Several lines of evidence suggest, however, that the Ca^{2+} involved in release and that associated with facilitation are acting at separate sites. Firstly, the level of residual Ca^{2+} can be less than 1 μM, whereas Ca^{2+} in the release zone following an action potential is in excess of 100

μM (Adler *et al.*, 1991; Zucker, 1999); addition of such a concentration of Ca^{2+} to the larger signal triggering exocytosis could not explain the large increase of transmitter release during facilitation. Secondly, the introduction of EGTA into synaptic terminals disrupts short-term plasticity, but does not affect transmitter release *per se* (Swandulla *et al.*, 1991). Thirdly, at invertebrate synapses where the relationship between Ca^{2+} and release is non-linear, facilitation appears to be linearly related to Ca^{2+} concentration (Wright *et al.*, 1996; Vyshedskiy & Lin, 1997). At the parallel fibre-Purkinje cell synapse Ca^{2+} plays an important role in paired-pulse facilitation as Ca^{2+} buffers accelerated both the decay of the residual Ca^{2+} and the facilitation. There is, however, a component of the facilitation that persisted after the Ca^{2+} had returned to baseline levels, suggesting that the duration of facilitation is related to residual Ca^{2+} and also the slower kinetics of either a Ca^{2+} binding or Ca^{2+}-activated processes (Atluri & Regehr, 1996).

It has been proposed that facilitation could result from an increase in the pool of vesicles available for release induced by Ca^{2+}/calmodulin kinase II phosphorylation of synapsin I. At some synapses this may be the case as, for example, in mutant mice lacking α-Ca^{2+}/calmodulin kinase II (αCaMKII) paired-pulse facilitation is reduced (Chapman *et al.*, 1995) and the CaMKII inhibitor KN62 (3.5 μM) reduced, although did not abolish, facilitation at mossy fibre-CA3 synapses (Salin *et al.*, 1996a). At other synapses, however, the enzyme does not appear to be involved and the molecular mechanism is unclear (Kamiya & Zucker, 1994; Salin *et al.*, 1996a).

In addition to facilitation, synapses show a use-dependent depression of synaptic transmission (Fig. 5.12). At the hippocampal CA3-CA1 synapse a single stimulus will trigger transmitter release with a mean probability of 0.3, although the variation

between synapses is high (range of 0.3 to 0.95; Allen & Stevens, 1994). The CA3-CA1 synapse has only a single release site, and by making paired recordings, or by minimal stimulation protocols, the nature of facilitation and depression at this synapse has been studied (Stevens & Wang, 1995). By using paired-pulse protocols it was found that the probability of release for the second stimulus is highly dependent on the outcome of the first i.e. if the first stimulus failed, then the second would have a high probability of release, whereas successful release for the first would result in lower probability of release for the second. This was especially true when the two stimuli were close together (5 ms) when the second stimulus would have almost zero probability of release following a successful first stimulus (Stevens & Wang, 1995). The depression of release is postulated to be due to a depletion of the releasable pool of vesicles, although other explanations, such as activity-dependent inactivation of the exocytotic machinery are plausible. When stimulating a number of presynaptic fibres, the degree of paired-pulse facilitation observed relates to the probability of release: a high release probability will result in a low degree of facilitation; a low probability will result in a high degree of facilitation. By altering the probability of transmitter release, as many neurotransmitters do, the degree of paired-pulse facilitation observed at a synapse will alter. Compounds acting postsynaptically, such as AMPA receptor antagonists, are not expected to alter the degree of facilitation, as they are not presumed to act in a use-dependent fashion. Thus, paired-pulse facilitation provides a means of testing whether a modulation of synaptic transmission is due to pre- or postsynaptic effects.

In addition to the PPF experiments, experiments were conducted to test whether PTD could be induced using the whole cell recording technique. When compared to sharp microelectrode recording, the whole cell technique can greatly facilitate studies into

second messenger-linked events. The contents of the postsynaptic cell may be manipulated by addition of compounds to the filling solution of the recording electrode thus allowing tests into whether PTD occurs pre- or postsynaptically to be conducted, and, if postsynaptic, the biochemical pathways involved in the depression can be investigated.

Results

Paired-pulse Facilitation

Except where mentioned, the paired-pulse facilitation experiments were performed at room temperature. The PTD experiments were all performed at 30 °C. In some of the PTD experiments, and those testing the effects of DHPG on paired-pulse facilitation, GTP was added to the electrode filling solution. No difference was observed between those recordings obtained with, and without GTP, and the data from these experiments is therefore grouped together. The actions of applied compounds on the average amplitude of 5 EPSCs evoked prior to commencement of washout were compared to the amplitude of the 5 EPSCs evoked prior to compound application. The only exception was DHPG; the effects of this agonist were measured at the point where the effect was maximal. Pairs of stimuli separated by 10s of milliseconds resulted in paired pulse facilitation (Fig. 5.1a). The degree of facilitation is expressed as the ratio of the amplitudes of the two EPSCs, and the second EPSC would typically be twice the amplitude of the second when seperated by 20 ms. As the example in figure 5.1b shows the facilitation lasts a few hundred milliseconds.

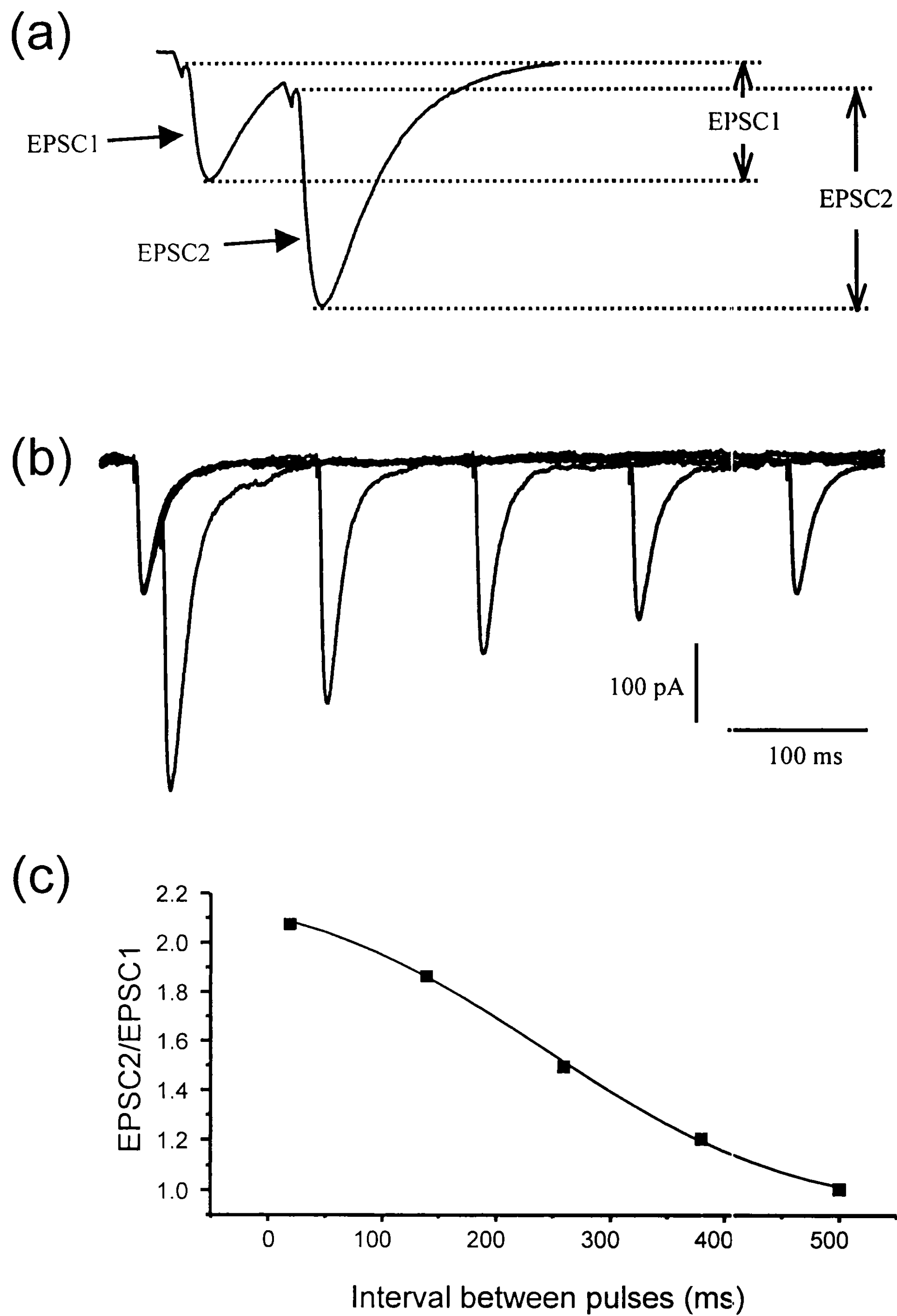


Figure 5.1. Paired-pulse facilitation of synaptic transmission at the parallel fibre-Purkinje cell synapse. (a) Illustration of paired pulse facilitation recorded in a single Purkinje cell. (b) and (c) Effect of interpulse-interval on the degree of facilitation in a single cell.

Effect of a $GABA_B$ receptor agonist on paired-pulse facilitation.

Bath application of R-baclofen (500 nM) resulted in a significant depression of EPSC1 from 312 ± 56 pA to 134 ± 26 pA ($P < 0.001$; n = 14; Fig. 5.2b), and EPSC2 from 522 ± 71 pA to 321 ± 35 pA ($P < 0.001$; n = 14; Fig.5.2b). In the presence of the R-baclofen there was a significant increase in the ratio of EPSC2 to EPSC1 from 1.8 ± 0.1 to 2.3 ± 0.2 ($P < 0.001$; n = 14; Fig. 5.2c). These effects all reversed on washout of the agonist (Fig. 5.2a). No effects were observed on the holding current of the postsynaptic cell in the presence of baclofen.

Effect of an A1 receptor agonist on paired-pulse facilitation.

The effect of A1 receptor activation on paired-pulse facilitation was tested using the A1 receptor agonist 2-chloroadenosine (3 μM). In the presence of 2-chloroadenosine EPSC1 amplitude reduced from 292 ± 58 pA to 133 ± 34 pA ($P < 0.01$; n = 6; Fig. 5.3b), and EPSC2 reduced from 518 ± 85 pA to 301 ± 69 pA ($P < 0.01$; n = 6; Fig. 5.3b). The EPSC2/EPSC1 ratio increased from 2.0 ± 0.2 to 2.5 ± 0.2 ($P < 0.01$; n = 6; Fig. 5.3c) in the presence of the 2-chloroadenosine. These effects of 2-chloroadenosine on synaptic transmission reversed on washout of the agonist (Fig. 5.3a).

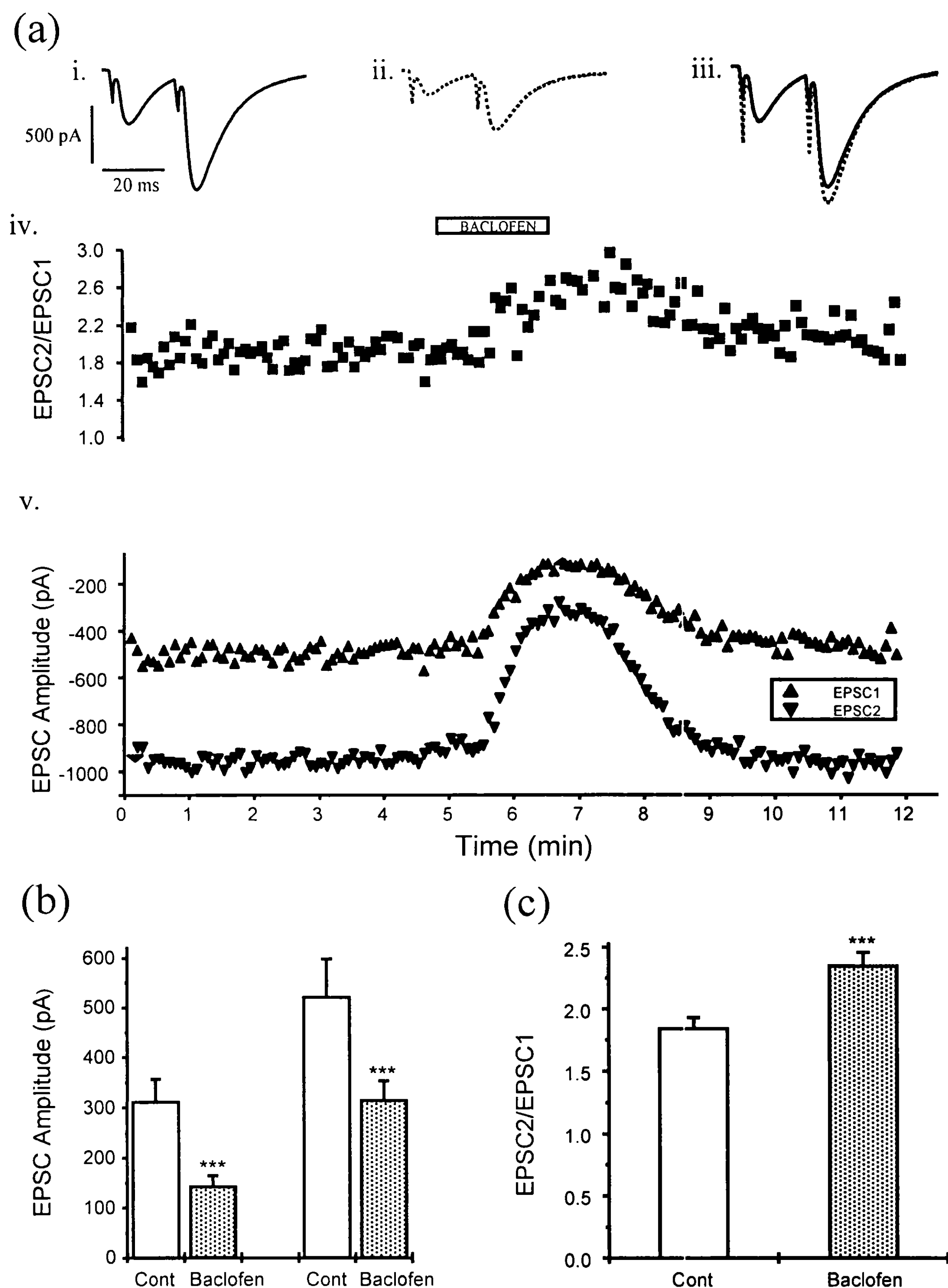


Fig 5.2. Effect of a $GABA_B$ receptor agonist on parallel fibre-Purkinje cell synaptic transmission and paired pulse facilitation. (a) Effect of baclofen (500 nM) on EPSC amplitude (v) and paired pulse facilitation (iv). Each point represents a single stimulation. (i) average of 5 EPSCs prior to drug application, (ii) prior to drug washout, and (iii) EPSC1 from (ii) scaled to EPSC1 from (i). Pooled data (n=14) for effects on (b) EPSC amplitude and (c) paired pulse facilitation (n = 14). * * * = $P < 0.01$

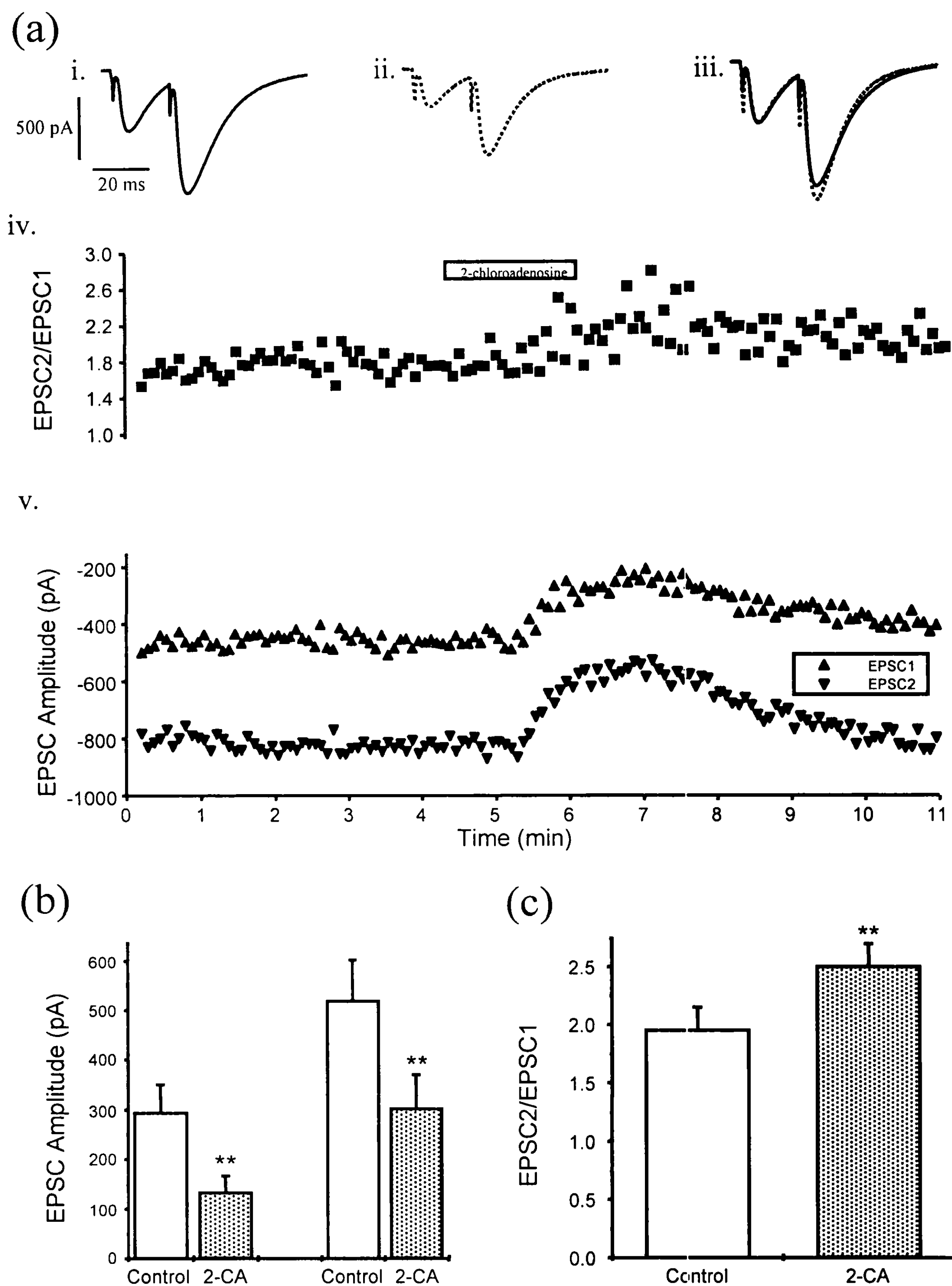


Fig 5.3. Perfusion of an A_1 receptor agonist reversibly depressed parallel fibre-Purkinje cell synaptic transmission and increased paired-pulse facilitation. (a) Effects of 2-chloroadenosine (3 μM) on EPSC amplitude (v) and paired pulse facilitation (iv) in a single experiment. Each point represents a single stimulation. (i) average of 5 EPSCs prior to drug application, (ii) prior to drug washout, and (iii) EPSC1 from (ii) scaled to EPSC1 from (i). (b) Pooled data (n=6) for the effects on (b) EPSC amplitude and (c) paired pulse facilitation. **P < 0.01

Effect of a Group III mGlu receptor agonist on paired-pulse facilitation.

The effects of group III mGlu receptors on paired-pulse facilitation were tested using the agonist L-AP4 (10 μM). In the presence of L-AP4 the amplitude of EPSC1 reduced from 290 ± 41 pA to 123 ± 28 pA ($P < 0.01$; $n = 6$; Fig. 5.4b), and EPSC2 reduced from 582 ± 77 pA to 298 pA ± 59 pA ($P < 0.01$; $n = 6$; Fig. 5.4b). The ratio of EPSC2/EPSC1 increased from 1.9 ± 0.1 to 2.5 ± 0.2 ($P < 0.05$; $n = 6$; Fig. 5.4c) in the presence of L-AP4. All effects of the drug returned to baseline levels on washout (Fig. 5.4a).

Effect of AMPA receptor antagonists on paired-pulse facilitation.

To test the effects that a postsynaptic depression of the parallel fibre EPSC has on paired-pulse facilitation the AMPA receptor antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulphonamide (NBQX; 10 nM) was applied. In the presence of the agonist EPSC1 was depressed from a mean of 574 ± 190 pA to 241 ± 83 pA ($P < 0.05$; $n = 6$; Fig. 5.5b), and EPSC2 depressed from 902 ± 277 pA to 389 ± 132 pA ($P < 0.05$; $n= 6$; Fig. 5.5b). There was no significant difference in the EPSC2/EPSC1 ratio in the presence of NBQX (1.6 ± 0.06) compared to control levels (1.6 ± 0.04; $P > 0.05$; $n = 6$; Fig. 5.5c). The EPSC amplitude was slow to recover on washout of the antagonist; recordings were therefore ceased before full recovery was obtained.

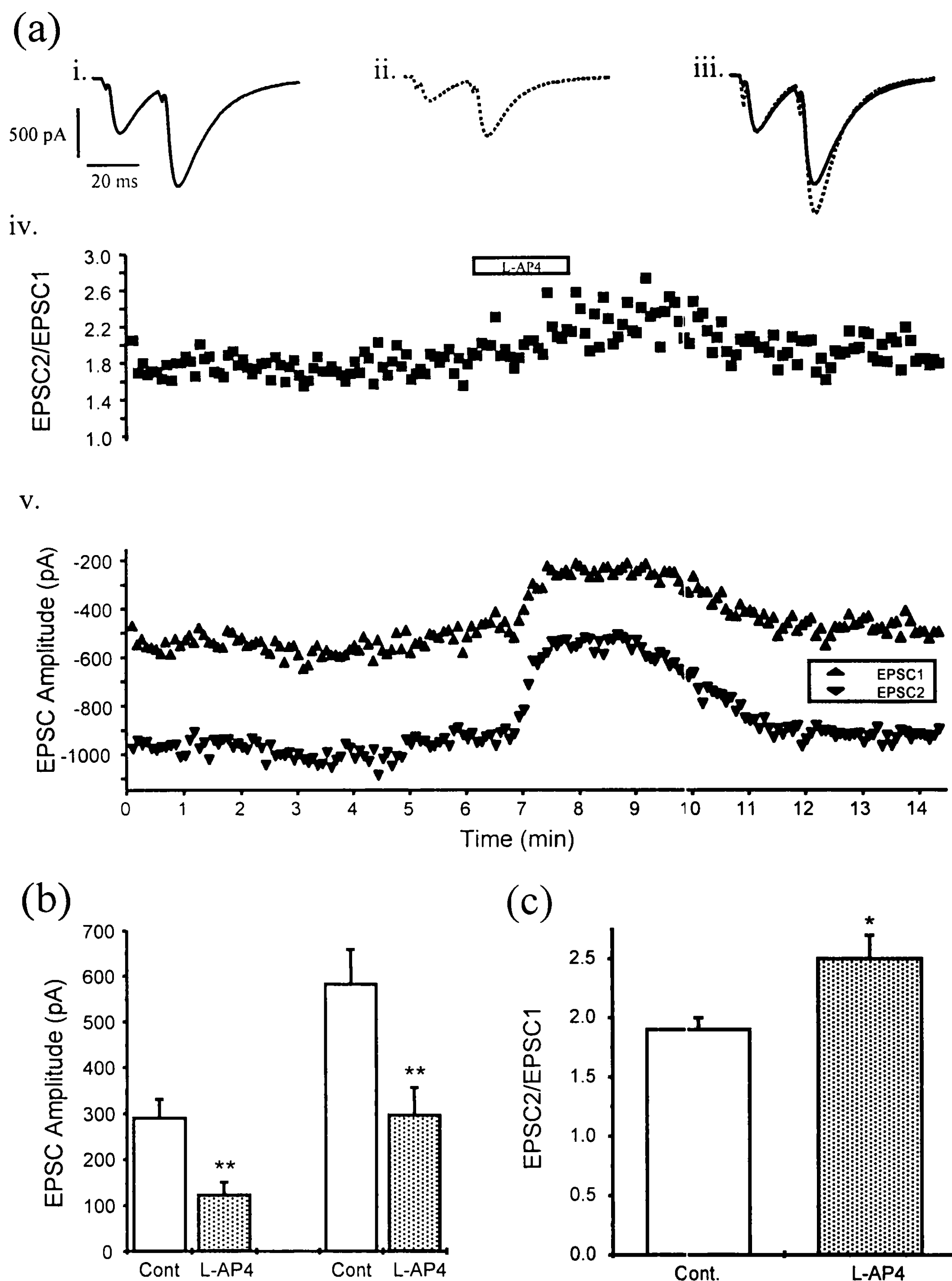


Fig 5.4. Group III mGlu receptors depress neurotransmission and increase paired-pulse facilitation at the parallel fibre-Purkinje cell synapse. (a) Effect of L-AP4 (100 μM) on EPSC amplitude (v) and paired pulse facilitation (iv). Each point represents a single stimulation. (i) average of 5 EPSCs prior to drug application, (ii) prior to drug washout, and (iii) EPSC1 from (ii) scaled to EPSC from (i). Pooled data (n=6) for effects on (b) on EPSC1 amplitude and (c) paired pulse facilitation. *P < 0.05; * *P < 0.01

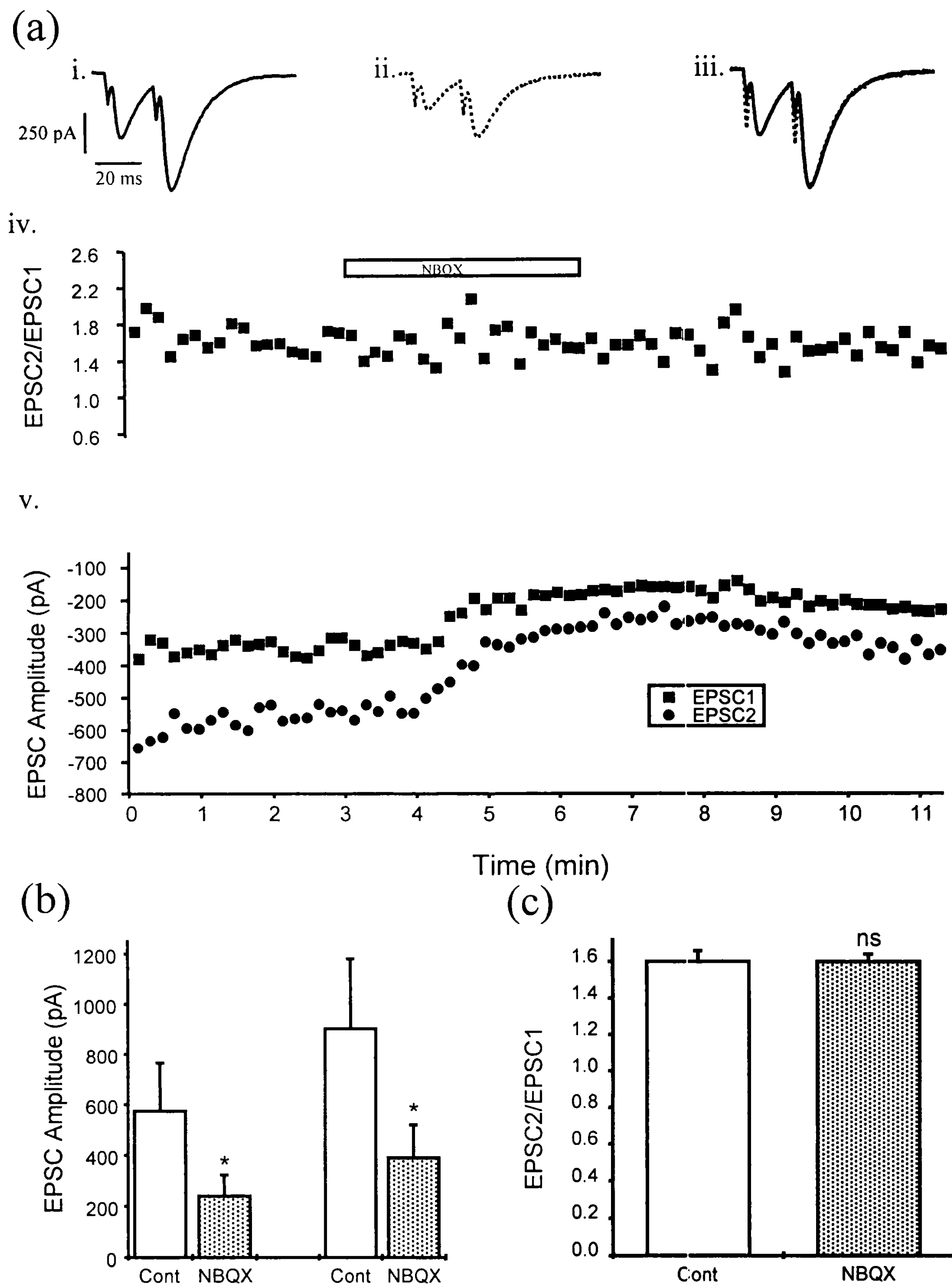


Fig 5.5. An AMPA receptor antagonist depresses parallel fibre-Purkinje cell transmission without affecting paired-pulse facilitation. (a) Effect of NBQX (10 nM) on EPSC amplitude (v) and paired pulse facilitation (iv). Each point represents a single stimulation. (i) average of 5 EPSCs prior to drug application, (ii) prior to drug washout, and (iii) EPSC1 from (ii) scaled to EPSC1 from (i). Pooled data (n=6) for effects on (b) EPSC amplitude and (c) paired pulse facilitation. *$P < 0.05$

Effect of Group I mGlu receptor agonists on paired-pulse facilitation.

The group I mGlu receptor agonist DHPG (100 μM) was applied to 25 cells from separate slices. The effects of the agonist on parallel fibre EPSCs can be divided into 2 categories: (1) DHPG only depressed synaptic depression; (2) DHPG induced a potentiation of synaptic transmission.

1. Reduction of the parallel fibre-Purkinje cell EPSC

In 21 neurones DHPG application resulted in a reversible depression of EPSC amplitude. EPSC1 decreased from 297 ± 31 pA to 182 ± 27pA ($P < 0.001$; n = 21; Fig. 5.6b), and EPSC2 from 594 ± 52 pA to 389 ± 48 pA ($P < 0.001$; n = 21; Fig. 5.6b). During the depression the degree of paired-pulse facilitation did not significantly change compared to baseline levels prior to drug application (baseline = 2.2 ± 0.1; DHPG = 2.3 ± 0.1; $P > 0.05$; Fig. 5.6c).

2. Enhancement of the parallel fibre-Purkinje cell EPSC

In 8 cells DHPG application resulted in a significant increase in EPSC amplitude: EPSC1 increased from 323 ± 60 pA to 474 ± 78 pA ($P < 0.001$ Fig. 5.7); EPSC2 from 671 ± 101 pA to 798 ± 100 pA. Of these cells 4 showed only enhancement whereas in the other 4 cells the DHPG evoked a brief depression that was followed by an increase in EPSC amplitude (Fig. 5.7a). Data from the 4 cells showing initial depression is included with the other 17 cells exhibiting depression only. The increase in EPSC amplitude reversed to baseline levels on washout of the agonist but, in contrast to the

DHPG-evoked EPSC depression, the enhancement was slower to reach maximal effect, often peaking after washout had commenced. This increase in EPSC amplitude coincided with a decrease in the EPSC2/EPSC1 ratio from 2.4 ± 0.3 to 1.9 ± 0.2 ($P < 0.001$; $n = 8$; Fig. 5.7c).

To test whether the potentiation was specific to DHPG the effects of the agonist (1S,3R)-ACPD on paired-pulse facilitation were tested. In the example illustrated in figure 5.8 the compound does not depress the EPSC, but does induce a potentiation in EPSC amplitude, accompanied by a decrease in the degree of paired-pulse facilitation.

It has been reported that at room temperature DHPG evokes a depression of synaptic transmission in the hippocampus, but at physiological temperatures a potentiation can be observed (Rodriguez-Moreno *et al.*, 1998). To test whether the recording temperature may account for the variability of the DHPG response the perfusate was heated to 35 °C. The slices were held in the chamber for 15 minutes at this temperature before attempts were made at seal formation. As the data from a single experiment illustrated in figure 5.8 shows, DHPG was still able to induce a potentiation of synaptic transmission at this temperature.

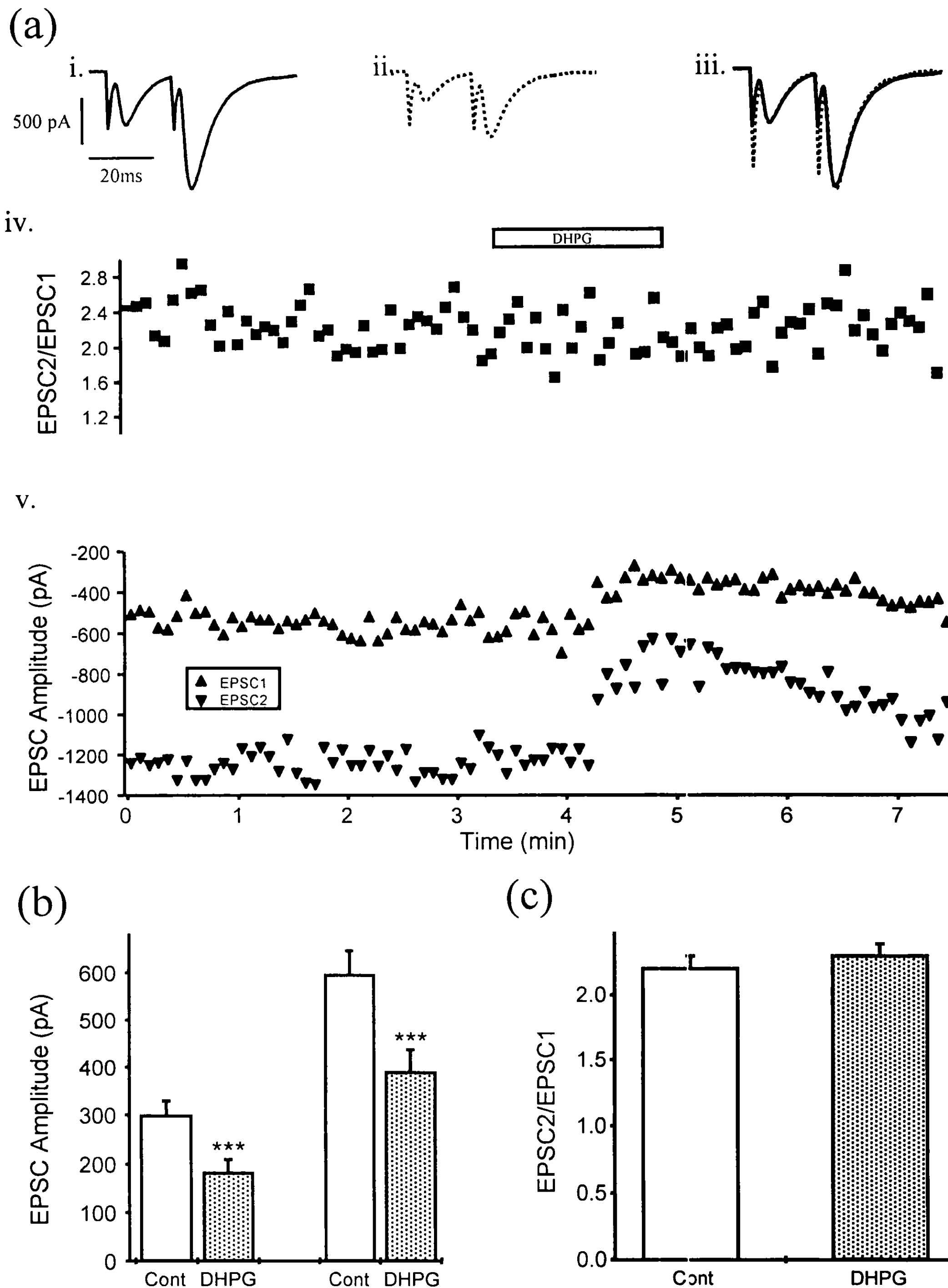


Fig 5.6. Depression of transmission at the parallel fibre-Purkinje cell synapse by DHPG (100 μM). (a) Effect on EPSC amplitude (v) and paired pulse facilitation (iv) observed in a single cell. Each point represents a single stimulation. (i) average of 5 EPSCs prior to drug application, (ii) prior to drug washout, and (iii) EPSC1 from (ii) scaled to EPSC1 from (i). Pooled data (n=21) for effects on (b) EPSC amplitude and (c) paired pulse facilitation. * * *P < 0.01

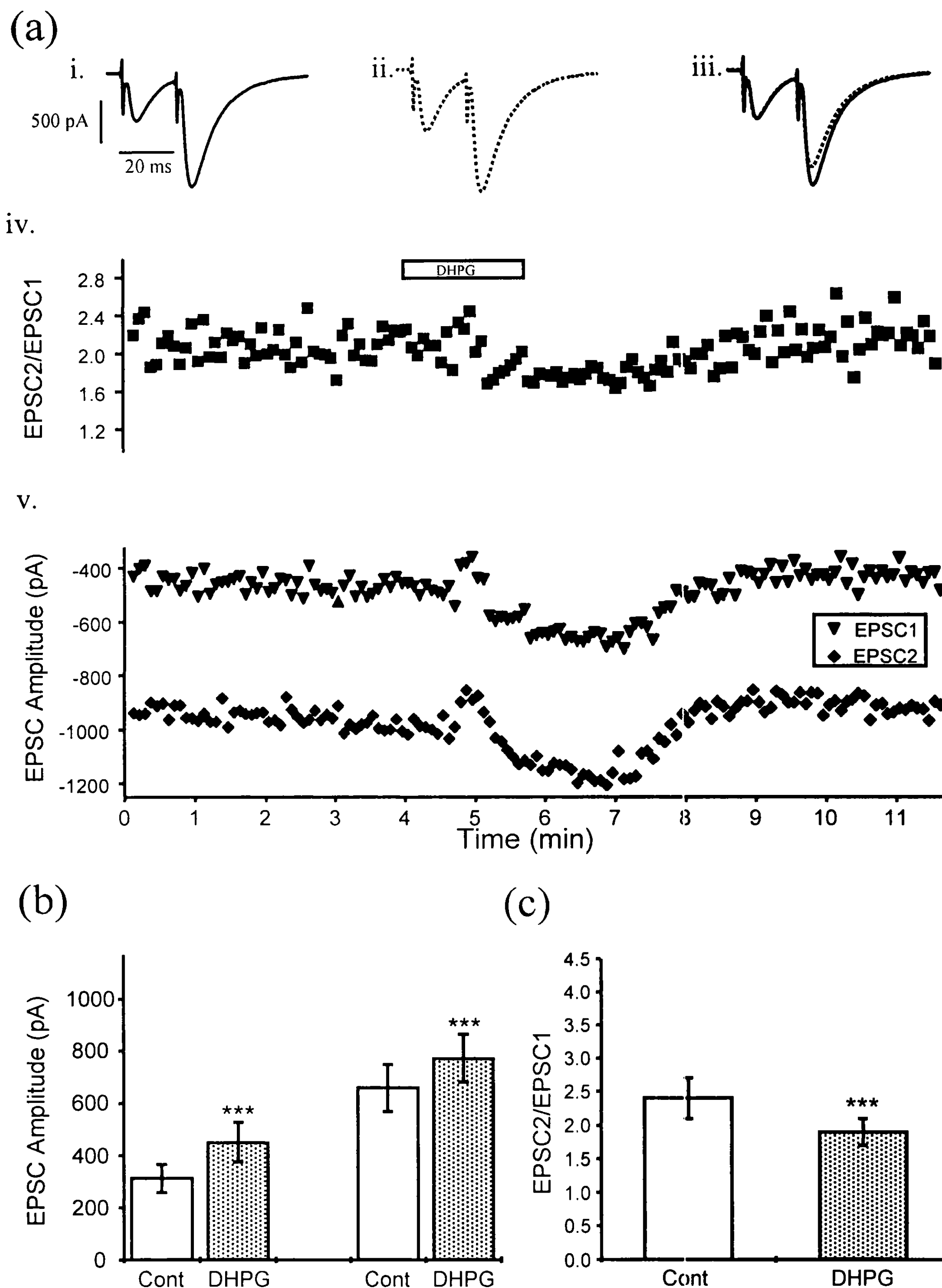


Fig 5.7. Enhancement of transmission at the parallel fibre-Purkinje cell synapse by DHPG (100 μM). (a) Effect on EPSC amplitude (v) and paired pulse facilitation (iv) observed in a single cell. Each point represents a single parallel fibre stimulation. (i) average of 5 EPSCs prior to drug application, (ii) prior to drug washout, and (iii) EPSC1 from (ii) scaled to EPSC1 from (i). (b) Mean effect of DHPG (100 μM) on EPSC amplitude (n = 8). (c) Mean effect of DHPG (100 μM) on paired pulse facilitation (n = 8). * * * = P < 0.01

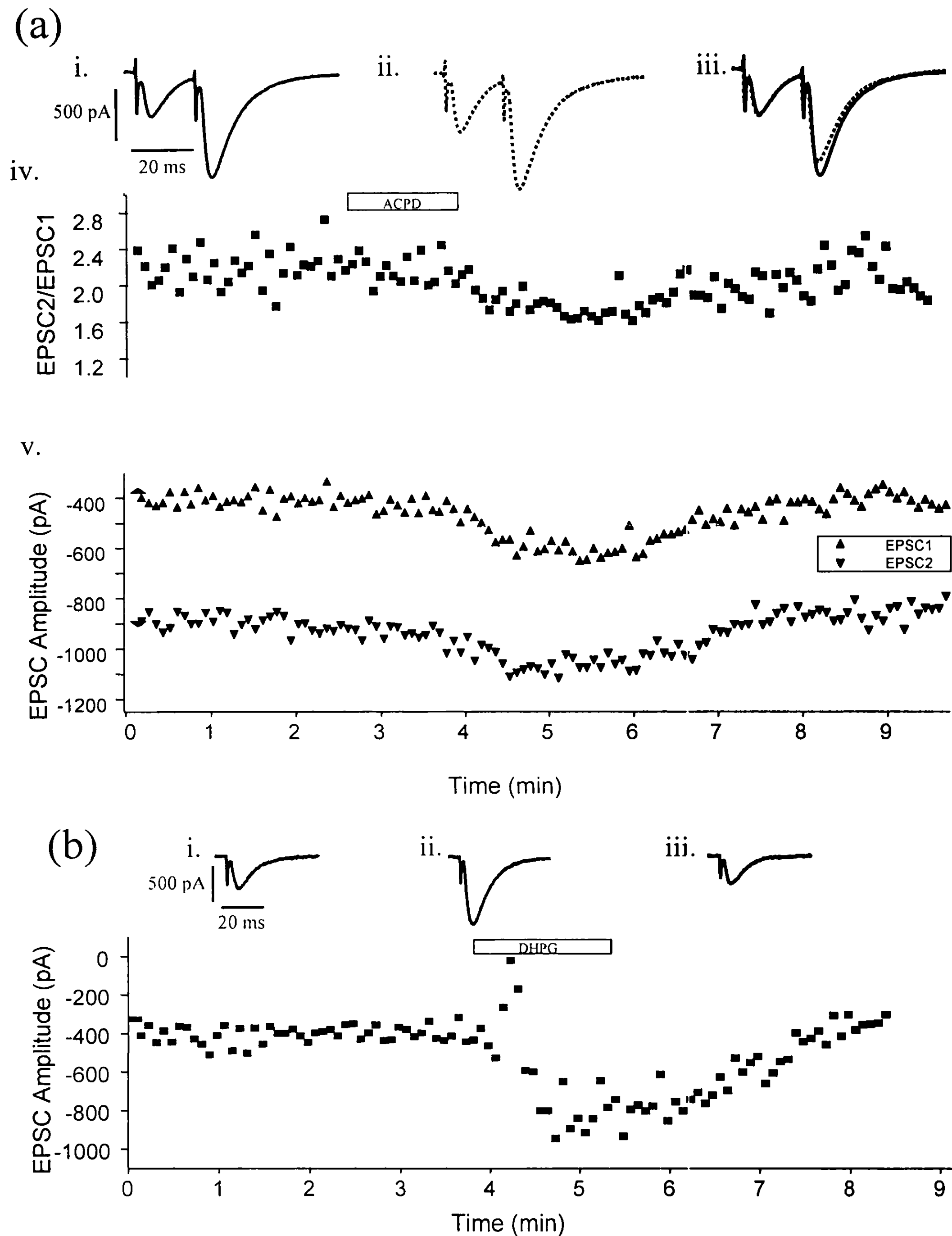


Fig 5.8. Effect of (1S-3R)-ACPD and DHPG on EPSC amplitude and paired-pulse facilitation at the parallel fibre-Purkinje cell synapse (a) Effect of 1S,3R-ACPD (100 μM) on EPSC amplitude (v) and paired pulse facilitation (iv) at the parallel fibre-Purkinje cell synapse stimulation. (i) average of 5 EPSCs prior to drug application, (ii) at peak of the potentiation, and (iii) EPSC1 from (ii) scaled to EPSC1 from (i). (b) Effect of DHPG (100 μM) on EPSC amplitude, with perfusate heated to 35°C. (i) average of 5 EPSCs prior to drug application, (ii) prior to drug washout, (iii) after drug washout

Post-tetanic Depression

The stimulation protocol described in the previous chapter (tetanus of 10 stimuli at 100 Hz followed 4.5 s later by a single stimulus) to evoke post-tetanic depression (PTD) of synaptic transmission was repeated using the whole-cell recording technique (Fig. 5.9). In contrast to the results obtained using conventional sharp microelectrodes that protocol did not result in depression of the SS2 EPSC (SS2 = 116 ± 11 % of SS1; n = 3; Fig. 5.9 a(i) + b). By increasing the strength of synaptic stimulation it was possible to make the cell fire during the train. Cells that showed no PTD following tetanic stimulation did show a significant depression of the SS2 EPSC when the cell fired during the tetanic stimulation (SS2 = 55.6 ± 4.3% SS1; $P < 0.01$; n = 3; Fig. 5.9b). The time course of this PTD was similar to that obtained using sharp electrodes, peaking at 4.5 s after the tetanus (SS2 = 70 ± 9 %; $P < 0.05$; n= 3; Fig. 5.9d), and returning to baseline levels after about 10 s. In ~90% of cells, firing during tetanic stimulation was followed by an inward current lasting a few seconds (Fig 5.9b). A component of this current was reversibly reduced by CPCCOEt (300 μM), and is therefore at least partially due to activation of group I mGlu receptors (Fig 5.9b).

The PTD could have been a result of factors associated with an increase in stimulus strength other than the cell firing, such as an increase in the amount of glutamate released due to an increase in the number of stimulated parallel fibres. To test whether the depression was related to the cell firing the effect of membrane potential on PTD was tested. At a holding potential of –70 mV the cell fired during the tetanic stimulation and the SS2 EPSC was depressed to 64 ± 6 % (Fig 5.10b) of SS1 levels. In the same cells the holding potential was increased to –100 mV, whilst maintaining the strength of

stimulation at the same level. Under these conditions the Purkinje cell did not fire and PTD was not apparent (SS2 = 105.5 ± 6.5% of SS1; n = 4; Fig. 5.10b).

The loss of voltage clamp during the tetanic stimulation could lead to activation of voltage and Ca^{2+} dependent conductances. Such an increase in membrane conductance would lead to an increased attenuation of the synaptic current as it flows through the dendrite to be measured in the soma. To test whether the observed EPSC depression was related to a decrease in membrane resistance a 5 mV voltage step was placed before SS1 and SS2, allowing the input impedance of the cell to be calculated. No significant difference was observed in the resistance prior to the SS1 and SS2 EPSCs (pre-SS1 = 148 ± 16 MΩ; pre-SS2 = 151 ± 17 MΩ; $P > 0.05$; n = 4; Fig. 5.10c)

The involvement of $GABA_B$ and group I mGlu receptors in the PTD induced in Purkinje cells that fired during the tetanus was tested using the antagonists CGP 35348 (1 mM) and CPCCOEt (300 μM). In the presence of CPCCOEt the PTD was significantly reduced compared to baseline levels (baseline = 44 ± 4 %; CPCCOEt = 72 ± 8 %; $P < 0.01$; n= 4; Fig. 5.11b). In the continued presence of CPCCOEt application of CGP35348 reduced the remaining depression (115 ± 17 %; $P < 0.05$, compared to CPCCOEt alone; n=4; Fig. 5.9b). The application of the two antagonists revealed an underlying potentiation of the EPSC. A similar effect was observed when studying PTD with sharp electrodes in current clamp (Chapter 4, Fig. 4.5 and 4.6). Although this was not examined, I suggest that it is a form of short-term plasticity related to residual Ca^{2+} in the parallel fibre terminals (Zucker, 1999). The CPCCOEt vehicle, DMSO (diluted 1:1000), exerted no significant effect on the PTD (control = 46 ± 14 %; DMSO = 42 ± 14 %; n = 3; Fig. 5.11c).

The strength of synaptic transmission shows a number of activity-dependent modifications such as paired-pulse facilitation, post-tetanic facilitation and PTD. The tetanic stimulation (10 stimuli at 100 Hz) used to evoke PTD was in the frequency range reported for granule cells *in vivo* (see Chapter 4). The effects of this tetanus on EPSC amplitude were therefore examined to see what activity-dependent plasticity was present during the train. Compared to the first EPSC of the tetanus the amplitude of subsequent EPSCs showed significant modifications. As expected from the paired-pulse facilitation experiments the second EPSC was facilitated (EPSC2/EPSC1 = 1.8 ± 0.3; $n = 6$; $P < 0.05$). The amplitude of later EPSCs in the train were depressed compared to the first EPSC (EPSC10/EPSC1 = 0.4 ± 0.07; $n = 6$; $P < 0.001$).

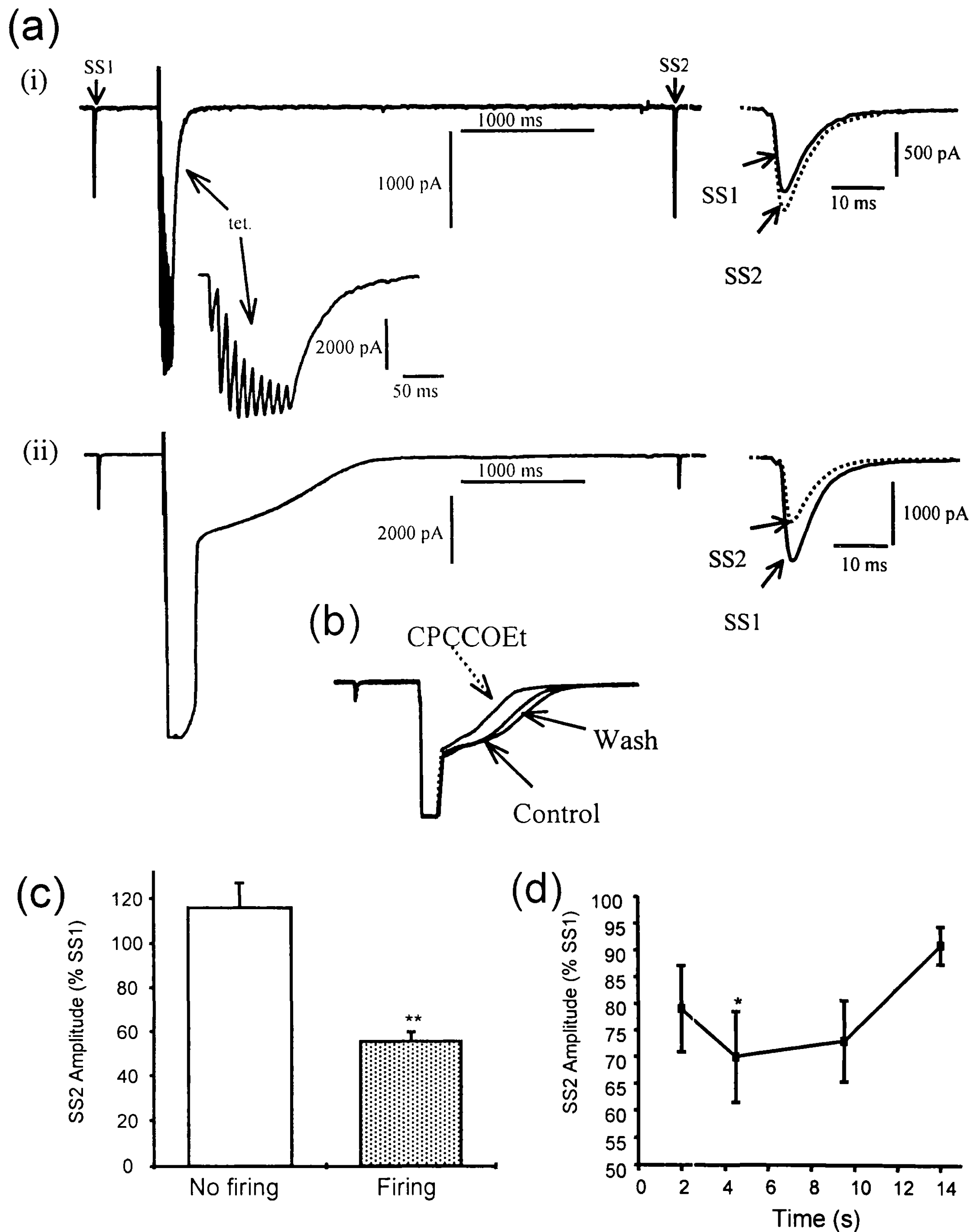


Fig. 5.9 PTD requires cell firing during the tetanus. (a) In a single cell SS2 is only depressed when the stimulus strength is increased such that there is firing during the tetanus. (b) The tail current following tetanic stimulation is reversibly depressed in the presence of CPCCOEt.(c) Pooled data (n=3) for effect of increasing the stimulus strength to induce firing on the amplitude of EPSC SS2 recorded 4.5 sec after tetanic stimulation (d) Time course of PTD (n = 3). *P < 0.05; **P < 0.01.

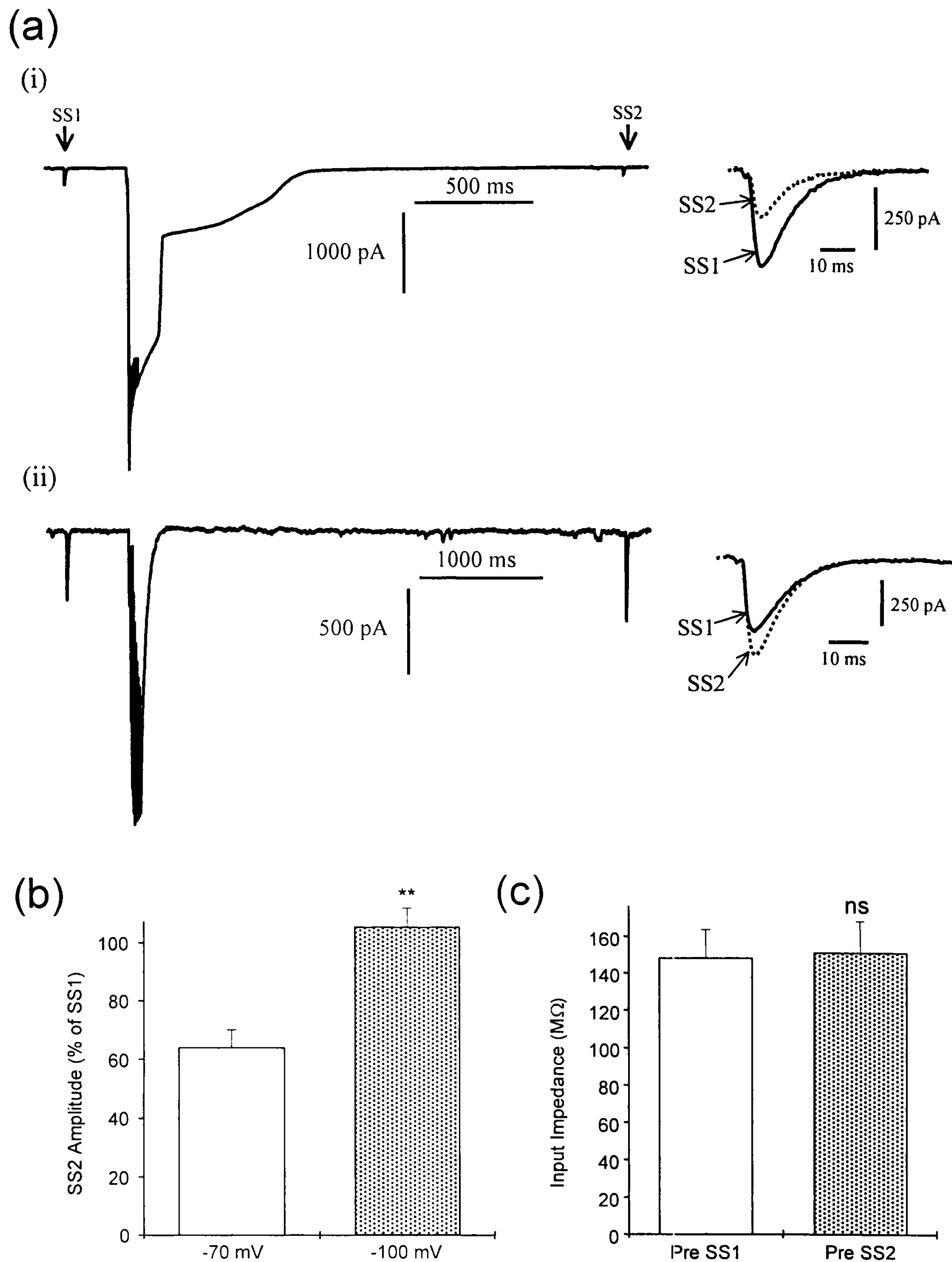
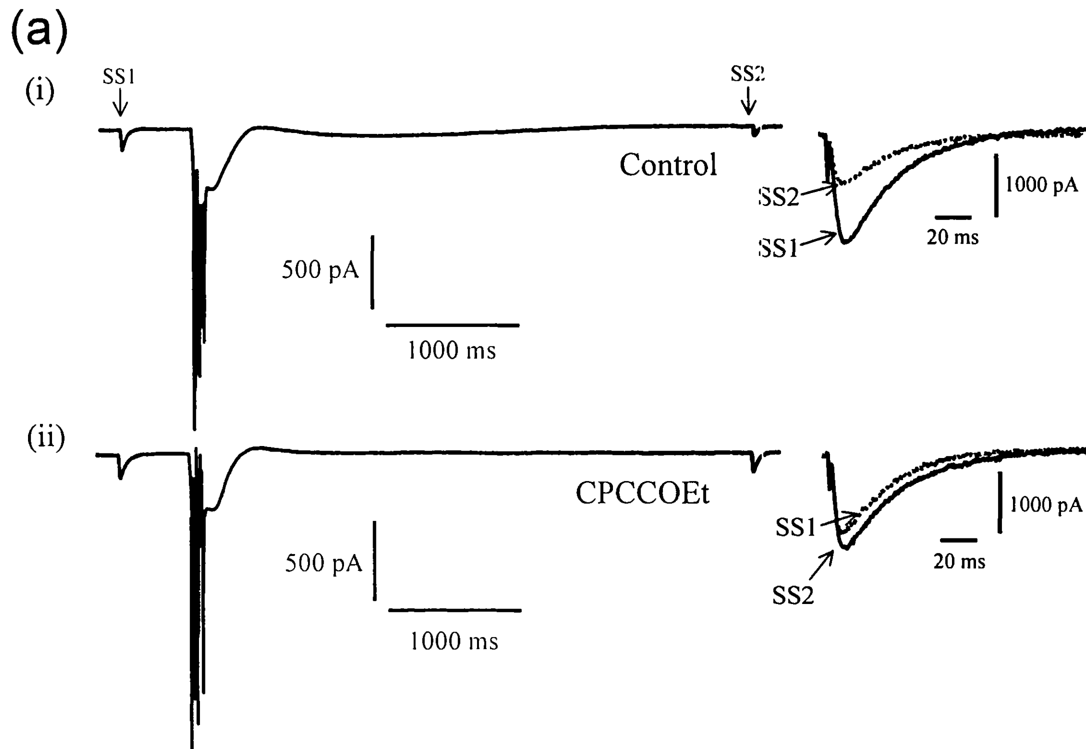


Fig 5.10. Effect of holding potential on PTD. (a) tetanic stimulation protocol results in synaptic depression when membrane is clamped at -70 mV (I), but not when at 100 mV (ii). (b) Mean data showing effect of membrane potential. (c) Input impedance measured prior to SS1 and SS2 (Vm = -70). n = 4; ns, not significantly different; **P < 0.01.



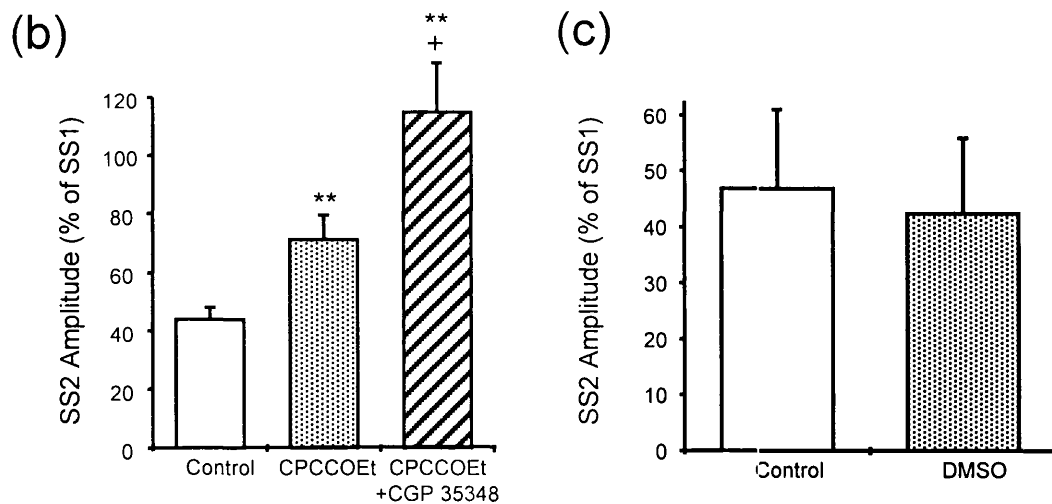
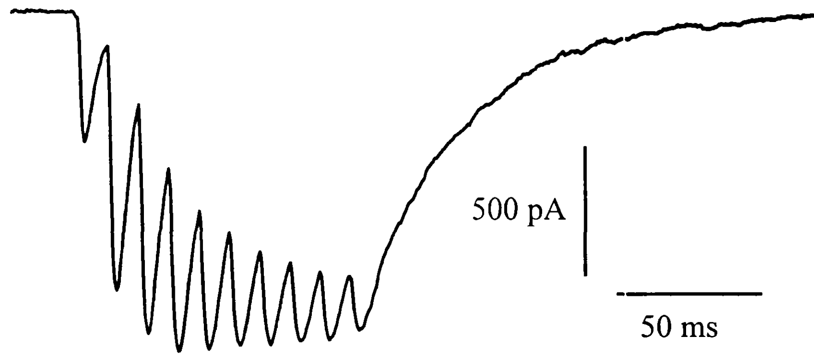


Fig 5.11. Pharmacology of PTD. (a) Tetanic stimulation results in depression of SS2 (i) which is attenuated in the presence of CPCCOEt (300 μM; ii) (b) Mean effect of CPCCOEt alone (n=10) and combined with CGP 35348 (1 mM; n=4) on PTD (c) Lack of effect of the vehicle (DMSO; 1:1000) on PTD (n=3). **P < 0.001 compared to control; +P < 0.05 compared to CPCCOEt alone

(a)



(b)

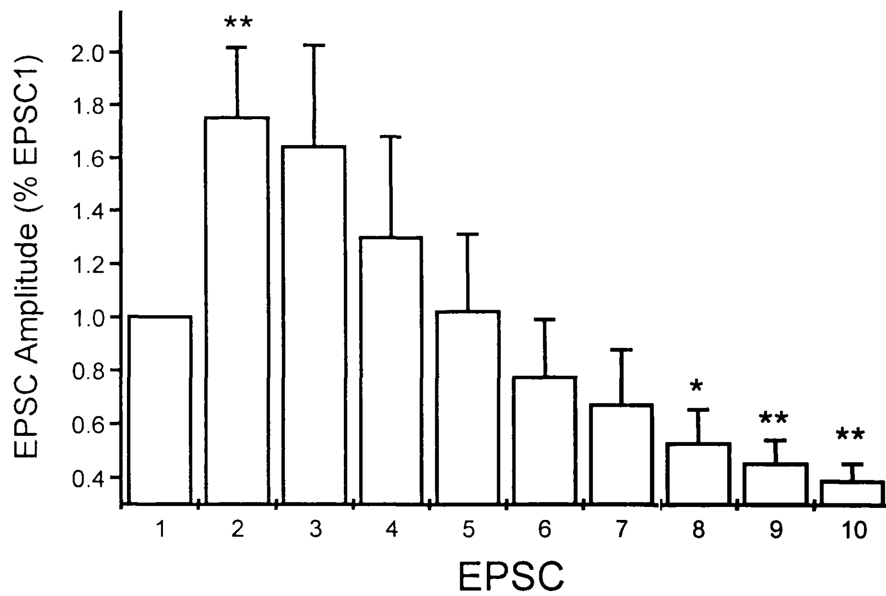


Fig. 5.12.Change in EPSC amplitude elicited by a train of 10 stimuli to the parallel fibres at a frequency of 100 Hz in (a) a single Purkinje cell; and (b) mean data from 6 cells. *P < 0.05; **P < 0.001

Discussion

Paired-Pulse Facilitation

Consistent with the numerous reports in the literature, pairs of stimuli applied to parallel fibres evoked EPSCs in Purkinje cells that exhibited paired-pulse facilitation. This facilitation lasted approximately 500 ms, a similar duration to that reported in a previous study of facilitation at this synapse (Atluri & Regehr, 1996).

Adenosine and $GABA_B$ Receptors

Depression of synaptic transmission by presynaptic $GABA_B$ and A_1 receptors is well documented (Dutar & Nicoll, 1988b; Thompson *et al.*, 1993; Dittman & Regehr, 1996). In agreement with previous data, depression of the parallel fibre EPSC by both baclofen and 2-chloroadenosine was accompanied with an increase in the degree of paired-pulse facilitation, consistent with the depression being due to presynaptic effects.

The type 1a $GABA_B$ receptor subunit (GBR1a) has been identified on the terminals of the parallel fibres (Kaupmann *et al.*, 1998b; Billinton & Bowery, 1999). *In vivo* these receptors are probably activated by GABA spilling over from the inhibitory interneurones in the molecular layer (see chapter 4). Alternatively, there is evidence that Purkinje cells may release a retrograde messenger, possibly glutamate, that depresses transmitter release from the inhibitory interneurones (Glitsch *et al.*, 1996). It is possible that under similar conditions Purkinje cells, or other cells could release GABA. Many G-protein coupled receptors, including the $GABA_B$ receptors, can depress Ca^{2+} currents

(Hille, 1994; Huston *et al.*, 1995; Chen & van den Pol, 1998). By imaging Ca^{2+} levels in the terminals, attempts have been made to elucidate the mechanism of depression mediated by $GABA_B$ receptors (Dittman & Regehr, 1996). Although baclofen exerted no effect on resting Ca^{2+} level, it did depress the Ca^{2+} influx associated with transmitter release. This depression was sufficient to explain the depression in synaptic transmission. The mechanism of G-protein-mediated inhibition of Ca^{2+} channels has not specifically been studied at the parallel fibre-Purkinje cell synapse but elsewhere, such as at the rat superior cervical ganglion, it seems to be membrane delimited, that is diffusible cytosolic second messengers are not involved (Shapiro & Hille, 1993). Unlike many G-protein mediated, events this is not regulated by the alpha subunit, but is apparently mediated by the beta gamma subunits of the G-protein, which seem to able to directly suppress Ca^{2+} channel activity. This inhibition is characteristically reversed by strong depolarising prepulses and shows slowing of, and a positive shift in, the voltage dependence of channel activation (Ikeda, 1996; Herlitze *et al.*, 1996).

$GABA_B$ receptor agonists are reported to induce a postsynaptic hyperpolarisation in the cerebellar Purkinje cells, and in other brain areas (Dutar & Nicoll, 1988b; Batchelor & Garthwaite, 1992). From the reversal potential this hyperpolarisation is likely to be due to an increase in K^+ conductance, and more recent studies suggest it is due to activation of a prominently inwardly rectifying K^+ channel (Sodickson & Bean, 1996; Luscher *et al.*, 1997; Jarolimek *et al.*, 1998; Kaupmann *et al.*, 1998b). In the present work no evidence of a postsynaptic effect of baclofen was observed. This is probably because the electrode filling solution contained cesium ions, which blocks a variety of K^+ channels.

The adenosine A1 receptor is a member of the 7 transmembrane region G-protein coupled receptors. The receptor is expressed in the cerebellum and is located in, amongst others, granule cells (Mahan *et al.*, 1991; Rivkees *et al.*, 1995). Unlike neurotransmitters, such as glutamate and GABA, adenosine does not appear to be stored and released from vesicles. Ecto-enzymes on cells may increase extracellular adenosine via the breakdown of ATP, which appears to function as a neurotransmitter mediating fast synaptic transmission (Evans *et al.*, 1992; Bardoni *et al.*, 1997). Adenosine may also be released from neurones in response to electrical stimulation, application of glutamate receptor agonists and ischaemic/hypoxic episodes (Hoehn & White, 1990; Pedata *et al.*, 1991; Manzoni *et al.*, 1994). In general, adenosine release appears to be linked to processes that increase the metabolic demand on the neurone and, by reducing neuronal activity, may have a neuroprotective function.

The AMPA Receptors

The AMPA receptor antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulphonamide (NBQX) depressed synaptic transmission, but did not significantly affect paired-pulse facilitation. Such a result is expected for compounds acting postsynaptically. If, however, the voltage clamp in the Purkinje cell at the location of the synapse were inadequate, it would be possible for the degree of paired-pulse facilitation to be altered by EPSC1 depolarising the cell, and thereby reducing the driving force on EPSC2. As NBQX did not affect the paired-pulse facilitation, changes produced in the presence of the other agonists are unlikely to be postsynaptic voltage effects. Although paired-pulse facilitation is classically regarded as being a presynaptic phenomenon, other postsynaptic explanations have to be

considered, such as the recently described polyamine-dependent facilitation of AMPA currents (Rozov *et al.*, 1998; Rozov & Burnashev, 1999).

The Group III mGlu Receptors

The depression of synaptic transmission by L-AP4 is absent in the mGlu4 receptor-deficient mouse (Pekhletski *et al.*, 1996); therefore the L-AP4-mediated depression is likely to be via this receptor. This receptor is located on the terminals of the parallel fibres, where the receptor forms into clusters (Mateos *et al.*, 1998; Mateos *et al.*, 1999). That the depression of transmission is due to a receptor on the presynaptic terminal is supported by the present observations that L-AP4 application altered the paired-pulse facilitation ratio. The mechanism of depression at the parallel fibre-Purkinje cell synapse is at present unclear. Simultaneous recordings from the giant presynaptic terminal, the calyx of Held, and its postsynaptic target in the medial nucleus of the trapezoid body have demonstrated that mGlu receptors inhibit transmitter release at this synapse by suppressing a Ca^{2+} conductance in the presynaptic terminal (Takahashi *et al.*, 1996b). A similar mechanism may account for the depression by L-AP4 described here, although mGlu receptors can depress synaptic transmission independently of an affect on voltage dependent Ca^{2+} channels; for example, in hippocampal slices mGlu receptor agonists reduce the frequency of miniature EPSCs, an effect that does not involve Ca^{2+} channels (Scanziani *et al.*, 1995). As discussed in the previous chapter, the mGlu4 receptor might be expected to act as an autoreceptor, providing negative feedback on the release of its agonist. As yet, however, the conditions necessary for this receptor to be activated by synaptically released endogenous agonist have not been identified.

The Group I mGlu Receptors

In the majority of cells, application of DHPG resulted in a depression of synaptic transmission that was not associated with a significant change in the degree of paired-pulse facilitation. This suggests that the depression of transmission is occurring postsynaptically. Such a conclusion is consistent with histological studies that have found the mGlu1 receptor postsynaptically (Martin *et al.*, 1992; Baude *et al.*, 1993). The degree of depression was less than that seen when using sharp microelectrodes (EPSC reduced to 62% of control compared to 17% of control with sharp electrodes). This may be due to washout of second messengers, a problem often encountered when using patch electrodes and whole cell recording (see Chapter 2).

In approximately a third of recordings, DHPG potentiated parallel fibre-Purkinje cell transmission, an observation that has not been previously reported. This action was not specific to DHPG as (1S,3R)-ACPD had similar effects. With sharp electrodes application of group I agonists produced only depression of the EPSP. The mGlu receptors have a potentiating effect on iontophoretically applied AMPA (Glaum *et al.*, 1992), but this is presumably a postsynaptic effect, whereas the potentiation described here was accompanied by a reduction of facilitation, suggesting a presynaptic origin. At room temperature DHPG (100 μM) is reported to depress excitatory transmission recorded in CA1 neurones (Rodriguez-Moreno *et al.*, 1998). However, in the same study, application of DHPG with the perfusate at a temperature of 35°C, resulted in an increase in EPSC amplitude followed by a depression (Rodriguez-Moreno *et al.*, 1998). Both the increase and depression of transmission were apparently due to a presynaptic receptor, and the authors suggest that basal levels of glutamate are sufficient to cause a

desensitisation of the receptor and, in the desensitised state, the receptor couples to alternative pathways. Raising the temperature of the perfusate increases the efficiency of glutamate uptake, thus the receptors do not desensitise. To test whether such an effect could explain DHPG-induced potentiation of EPSC amplitude described here, DHPG was applied to a slice perfused with aCSF at 35°C and a potentiation of transmission was observed. In addition, the dual effects described here differ in that depression appears to be occurring postsynaptically whereas potentiation is presynaptic, as it is associated with a decrease in the degree of paired-pulse facilitation. As this phenomenon has not been previously reported, occurred in only a proportion of cells and did not appear to be activated by synaptically released agonist following tetanic stimulation, the physiological significance is unclear. If this potentiation is indeed due to a presynaptic receptor, an important question is why histological studies have not identified the receptor presynaptically? The potentiation could result from excitation of other cell types by DHPG leading to release of transmitters or neuromodulators from other cell types in the slice (either increasing release of modulators that enhance, or by suppressing tonic release of agents which depress glutamate release.) that may spillover to the parallel fibre synapse to produce the observed effect.

Post-Tetanic Depression

When using sharp microelectrodes and recording in current clamp mode, mGlu receptor-mediated PTD was observed (Chapter 4). In initial experiments using whole-cell recording under voltage clamp conditions no evidence could be obtained for PTD. However, it was possible to induce CPCCOEt-sensitive PTD when the parallel fibre stimulation was increased to the point that the Purkinje cell fired during the train. This

depression was related to the cell firing as hyperpolarising the Purkinje cell, so that the cell no longer fired, prevented the PTD. The PTD was not simply due to voltage and/or Ca^{2+} dependent channels increasing the membrane conductance, as the apparent input conductance did not change. The need for Purkinje cell firing contrasts with the exogenous group I agonist that depressed transmission without such a requirement (Chapter 3). This difference may reflect the fact that DHPG will activate all group I receptors on the cell, not just those located at the synapse, and the prolonged presence of this agonist may allow an accumulation of second messengers, such as IP_3, to the extent that the spiking is unnecessary. The dependence of PTD on postsynaptic spiking, the lack of significant effect of DHPG on paired-pulse facilitation and the histological evidence for a postsynaptic location of mGlu1 all suggest that the mGlu receptor-mediated PTD is due to a mGlu receptor acting postsynaptically.

Based on results obtained so far and using evidence in the literature, it is possible to develop a hypothesis for the mechanism of mGlu receptor-mediated PTD. The dependence of PTD on firing of the Purkinje cell suggests that PTD induction might require a rise in the Ca^{2+} concentration of the cell. The dendrites of the Purkinje cell contain voltage-dependent Ca^{2+} channels which, when depolarised, produce Ca^{2+} spikes (Llinás & Sugimori, 1980). It is quite likely that the firing witnessed during tetanic stimulation involved areas of dendritic membrane escaping the voltage clamp leading to Ca^{2+} influx. The suggestion that mGlu receptor-mediated effects might be enhanced by a rise in intracellular Ca^{2+} is not without precedent. It has been established that increasing the intracellular Ca^{2+} concentration of a Purkinje cell, by depolarisation through current injection or climbing fibre stimulation, leads to a potentiation of the synaptically-evoked mGlu1-mediated EPSP (Batchelor & Garthwaite, 1997). Similarly,

in hippocampal CA1 pyramidal neurones, pairing repetitive synaptic activation of mGlu receptors with back-propagating action potentials resulted in a dendritic Ca^{2+} signal much larger than that evoked by either manipulation alone (Nakamura *et al.*, 1999). Synaptic activation of Purkinje cell group I mGlu receptors is known to lead to Ca^{2+} release from internal stores via activation of the IP_3 receptors, of which the IP_3R1 is the dominant form in Purkinje cells (Takechi *et al.*, 1998; Finch & Augustine, 1998). The release of Ca^{2+} via IP_3 receptor activation is most effective when Ca^{2+} and IP_3 are presented together (Berridge, 1998), and the IP_3 receptor has therefore been proposed to function as a coincidence detector, integrating the voltage-dependent Ca^{2+} entry and mGlu receptor-mediated IP_3 production. Such a role has been suggested for IP_3 receptors in cerebellar LTD (Berridge, 1993). I therefore suggest that PTD is a Ca^{2+}-dependent process, requiring mGlu1-mediated IP_3 production and a voltage-dependent increase in Ca^{2+}. The resulting Ca^{2+} signal might then activate a kinase, as desensitisation of many receptors appears to involve phosphorylation of the receptor protein (Huganir & Greengard, 1990). This hypothesis testable; for example the Ca^{2+}-dependence may be tested by loading the Purkinje cell with a Ca^{2+} chelator such as BAPTA, and the involvement of IP_3 can be tested for with agents such as heparin, which has been previously used to test for IP_3 involvement in mGlu receptor-mediated Ca^{2+} signalling in Purkinje cells (Takechi *et al.*, 1998; Finch & Augustine, 1998). In Purkinje cells, AMPA receptors can undergo temporary phosphorylation after exposure to AMPA and, when AMPA exposure is preceded by exposure to the cyclic GMP analogues 8-bromo-cGMP and dibutyryl-cGMP, or the phosphatase inhibitor calculin A, this phosphorylation becomes persistent (Nakazawa *et al.*, 1995). Such a persistent AMPA receptor phosphorylation parallels the AMPA receptor desensitisation, and it is postulated that this phosphorylation underlies LTD (Nakazawa *et al.*, 1997).

Incidentally, this phosphorylation occurs on Ser-696 on the GluR2 subunit and corresponding sites on the other subunits, compared to the CaM-KII-dependent phosphorylation of Ser-831 of GluR1 postulated to play a role in some forms of LTP (Derkach *et al.*, 1999). This raises the possibility that PTD and LTD may be related: both phenomena are postulated to require parallel fibre stimulation, mGlu receptor activation, a rise in intracellular Ca^{2+} and both result in desensitisation of AMPA receptors. Repeated application of a short tetanus to the parallel fibres can lead to LTD induction (Eilers *et al.*, 1997), although how the transient depression in PTD might switch to a more permanent form is presently unclear. If both phenomena require AMPA receptor phosphorylation, an additional messenger pathway may need to be activated to switch the phosphorylation to a more permanent form, perhaps involving a more sustained or higher amplitude Ca^{2+} signal.

If PTD requires a rise in intracellular Ca^{2+}, then the hypothesis described in the previous chapter that PTD provides a protective mechanism against overexcitation still stands. Perhaps PTD can protect the neurone from brief periods of overstimulation, but if the threat is sustained PTD switches to LTD.

Conclusion

Synaptic transmission is a complex process which may be modulated both pre- and postsynaptically by G-protein coupled receptors. The aim of these experiments was to determine whether mGlu receptor-mediated PTD occurs pre- or postsynaptically. The results are consistant with the hypothesis that the depression of synaptic transmission mediated by group I mGlu receptors occurs postsynaptically, and that by group III

occurs presynaptically. Additionally, it was found that the synaptic activation of mGlu1 receptors to induce PTD requires depolarisation of the Purkinje cell, leading to the hypothesis that PTD is a Ca^{2+}-dependent process involving activation of IP_3 receptors.

Chapter 6: Concluding remarks

Since the first mGlu receptor was cloned in 1991, this family of receptors has attracted huge interest and the field has advanced rapidly. The receptors are ubiquitous in the CNS, with each receptor showing a distinct distribution pattern; moreover, they have been implicated in a diverse range of physiological and pathophysiological activities.

The effects of mGlu receptors on synaptic transmission at the cerebellar parallel fibre-Purkinje cell synapse have been investigated previously using non-selective agonists and knockout mice, leading to the hypothesis that group I and III mGlu receptors depress neurotransmission at this synapse. However, this data has to be interpreted with caution as, for example, the wide range of abnormalities in the mutant could result in, or be a result of, altered expression of other receptors sensitive to the non-selective agonists used. Subsequent to these studies, mGlu receptor ligands have become available that are reported to select between the three groups of mGlu receptors in expression systems. In this thesis the effects of some of these compounds on parallel fibre-Purkinje cell synaptic transmission have been examined. The results indicate that the selectivity exhibited by these pharmacological agents towards mGlu receptor subtypes expressed heterologously in cell lines extends to native mGlu receptors at the parallel fibre-Purkinje synapse. Results using these agents compliment those from the studies on mutant mice, leading to the conclusion that group I (mGlu1) and III (probably mGlu4), but not group II, receptors, are associated with a reversible depression of synaptic transmission. In the case of mGlu1, this action is distinct from

membrane depolarisation, suggesting that this single receptor subtype can engage divergent pathways to generate either postsynaptic excitation or inhibition of glutamatergic synaptic transmission. The mechanisms giving rise to these actions remain to be determined. That antagonists acting at either group I or III mGlu receptors had no potentiating effect on the EPSPs implies that the receptors mediating synaptic depression are not activated during low frequency synaptic transmission.

Whilst numerous studies have demonstrated that bath applied exogenous mGlu receptor agonists can modulate neuronal function, there is a significant lack of data describing conditions necessary for the receptors to be activated by synaptically released endogenous agonist. The most important finding described in this thesis is the discovery that group I mGlu receptors can be activated synaptically following tetanic stimulation of the parallel fibres to produce a post-tetanic depression (PTD) of parallel fibre-Purkinje cell synaptic transmission. This is the first evidence that excitatory transmission at this synapse is modulated by synaptically released glutamate, and the first description of synaptically activated group I mGlu receptors causing a reversible suppression of synaptic transmission anywhere in the CNS. The PTD was evoked using a short tetanus at frequencies reported to occur *in vivo*, and the phenomenon might therefore be expected to be relevant to CNS function *in vivo*.

Following from the discovery of mGlu receptor-mediated PTD there are two key points that I feel require further exploration:

- Previous histological studies, and data described here, suggest that the receptor responsible for PTD is located postsynaptically. A hypothesis as to how

activation of this receptor may lead to synaptic depression was presented and this needs to be tested using inhibitors of the proposed transduction pathway.

- PTD and LTD have some similar characteristics, but the exact relationship between these two phenomena is unclear. In particular, it is hypothesised that under appropriate conditions PTD might be switched to LTD. A previous study demonstrated that LTD could be induced by repeated application of a short tetanic stimulation to the parallel fibres. What additional factor(s) might be required to make the switch needs to be investigated.

Although the principal aim of these studies has been to investigate the role of mGlu receptors, several forms of short-term plasticity of synaptic transmission at the cerebellar parallel fibre-Purkinje cell synapse have been demonstrated. The cerebellum is associated with control of motion and with cognitive functions, such as language, which require intense processing of temporal information. The natural firing frequencies of neurones during these tasks are likely to engage the short-term plasticities, and it is therefore probable that they have a role to play in the computation underlying these operations. Some progress is now being made in understanding the contribution these various forms of plasticity make to neuronal computation. For example, experimental studies of cortical neurones show that during high frequency activity the EPSC amplitude depresses to a steady state (similar to the type of depression illustrated in Chapter 5, Fig. 5.12). By modelling a neurone receiving inputs at 100 and 10 Hz it was found that synaptic depression endowed the cell with the ability to detect changes in the frequency of either signal, whereas without depression, the high frequency input saturated the cell, masking any changes in the 10 Hz input (Abbott *et al.*, 1997). By

simulating a network of randomly connected inhibitory and excitatory cortical neurones with the strength of synapses varying from synapse to synapse, it has been found that due to the time-dependent effects of short-term plasticity the network would be in a different state for each stimulus of a pair. It was therefore possible for the network to discriminate signals over a wide range of intervals (Buonomano & Merzenich, 1995; Buonomano, 2000). This is of importance as short-term plasticity may endow neuronal networks with the ability to process temporal information without the need for specialised structural features, such as parallel fibre delay lines proposed to operate in the cerebellar cortex (Braitenberg & Onesto, 1962).

Although synapses exhibit both long- and short-term forms of plasticity, little consideration has been given to how these processes might impinge on each other. There is evidence that LTP in the cerebellum, and some other brain areas, is due to a presynaptic effect on transmitter release (Salin *et al.*, 1996a; Salin *et al.*, 1996b). This long lasting change to the properties of transmitter release is likely to affect paired-pulse facilitation, and perhaps other forms of transient plasticity. This has been shown, for example at the hippocampal mossy fibre-CA3 synapse, where LTP induction results in a reduction in the degree of paired-pulse facilitation by about 60% (Salin *et al.*, 1996a). Beyond examining effects on paired-pulse facilitation there has, however, been little investigation into possible interactions of short- and long-term plasticity. By making paired recordings of layer 5 cortical neurones, Markram and Tsodyks (1996) demonstrated that LTP induction (induced by pairing pre- and postsynaptic depolarisation) induced a change in the postsynaptic response to trains of presynaptic stimuli. In essence, they observed that LTP resulted in a redistribution of the postsynaptic response such that the initial response to the train of stimuli was amplified

at the expense of later EPSCs, and that during high frequency stimulation steady state depression was reached sooner. The effects of LTP on the amplitude of the EPSC during steady state depression varied according to stimulus frequency: at 40 Hz the EPSC was unchanged, but at 5 Hz the steady state EPSC was increased following LTP. It has been recognised that the tetanus used to induce LTP may not be relevant to neuronal firing patterns *in vivo*, and efforts have been made to induce LTP with protocols which are perhaps more physiological, such as theta burst stimulation (Larson *et al.*, 1986; Greenstein *et al.*, 1988). It has been, however, largely overlooked that the low frequency single test stimulus used to evaluate the degree of LTP is also not physiological. The observations described above suggest that the effects of LTP and LTD could be far more complex than a simple change in the strength of low frequency synaptic transmission, and the effects of long-term synaptic plasticity on more natural firing patterns should be investigated

In conclusion, a large body of data has accumulated describing possible roles of mGlu receptors in the CNS. Much of this information has been acquired using bath applied exogenous agonists. In the future we need to acquire further evidence as to how these receptors are activated under more physiological conditions. The results described in this thesis have contributed to this aim.

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