Regulation of calcium-permeable AMPA receptors at cerebellar interneuron synapses

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Abstract

Fast excitatory synaptic transmission in the central nervous system is mediated principally by glutamate, acting on AMPA receptors (AMPARs). The functional properties of these receptors reflect their subunit composition (GluR1-4) and dictate key features of the excitatory postsynaptic current, and thus the transmission process. Importantly, insertion or removal of AMPARs at the synapse underlies the expression of certain well-characterised forms of long-term synaptic plasticity. Recently, several additional forms of plasticity have been shown to involve the specific regulation of Ca^{2+}-permeable (GluR2-lacking) AMPARs. At parallel fibre synapses onto cerebellar stellate cells, Ca^{2+} influx through AMPARs triggers an autoregulatory change in their subunit composition. In this thesis I have investigated factors that may trigger or influence this type of subunit change. I discovered that a switch in AMPAR subtype (from Ca^{2+}-permeable to mainly Ca^{2+}-impermeable AMPARs) occurs during development of stellate cells. This change is accompanied by a decrease in synaptic channel conductance. Activation of either mGluRs or $GABA_B$Rs also results in switch in AMPAR subtype – a selective loss of synaptic Ca^{2+}-permeable AMPARs, triggered by a rise in intracellular Ca^{2+}. My experiments also reveal that both types of metabotropic receptor are tonically active, and therefore constitutively regulate subunit-specific synaptic targeting of AMPARs. My results identify a signalling mechanism likely to drive the dynamic switch in AMPAR Ca^{2+}-permeability, and demonstrate that AMPAR subunit composition can be modified by postsynaptic actions of GABA, as well as glutamate.

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1. Introduction

This thesis focuses on one aspect of synaptic transmission at one type of synapse. By studying fast excitatory transmission at the parallel fibre (PF) to stellate cell (SC) synapse in the cerebellum, it may be possible to gain insight into certain general principles of fast synaptic excitatory transmission, as well as the role it plays in SC function within the cerebellum. A special feature of the PF-SC synapse, which makes it especially worthy of study, is that SCs express calcium- permeable AMPARs (CP-AMPARs) (Liu and Cull-Candy, 2000a) which are both mediators of, and targets for plasticity (Cull-Candy et al., 2006). Recently, attention has focused on the regulation of these receptors and the special contribution they make to neurotransmission (Plant et al., 2006; Bellone and Luscher, 2006; Liu and Cull-Candy, 2005a). This thesis focuses on factors that control the calcium permeability of postsynaptic AMPARs in SCs; specifically the influence of development, activation of metabotropic glutamate and GABA receptors are investigated.

1.1 General principles of synaptic transmission

1.1.1 Background

The term synapse was first employed by the physiologist Sherrington (Foster and Sherrington, 1897) when he proposed that information must be passed between neurons *via* 'junctions', if, as Cajal had described, neurons were in fact 'independent elements' (Cajal et al., 1995). Studies from the peripheral nervous system implied that this information is conveyed chemically (Dale, 1953; Loewi, 1957). The development of intracellular recording techniques allowed Fatt and Katz to demonstrate that the large depolarization of the motor end plate (EPP) recorded in response to stimulation of the motor neuron, is indeed mediated by a chemical, acetylcholine (ACh) (Fatt and Katz 1951; Fatt and Katz, 1952b; Fatt and Katz, 1953;). In addition to the large EPP, they also recorded randomly spontaneous occurring 'blips' of very small amplitude (min EPPs), which were also shown to be mediated by ACh (Fatt and Katz, 1952a).

This fortuitous observation provided the basis of how we think about synaptic transmission today.

The small and consistent amplitude of these 'blips' suggested the all-or-nothing nature of these events, reflecting individual packets or 'quanta' of ACh molecules acting simultaneously on the end plate membrane. Further experiments showed that EPPs are dependent on external [Ca^{2+}] and always comprised of an integral number of quantal events. It was therefore concluded that the large depolarisation of the nerve impulse leads to an increased release of quanta which gives rise to a full-blown EPP. The coincident development of electron microscopy enabled the timely visualisation of vesicles clustered at presynaptic sites (De Robertis and Franchi, 1954) and thus, the quantal theory of vesicular chemical synaptic transmission was formed.

Action potentials (APs) trigger the release of neurotransmitter contained within vesicles in the presynaptic nerve terminal (Katz and Miledi, 1969a). The neurotransmitter then acts postynaptically on receptors mediating fast and/or slow changes in membrane conductance. The postsynaptic response can be predicted from the following parameters: the postsynaptic quantal size q, the number N of release sites, and the probability p that release of a quantum of transmitter occurs at a site (Del Castillo and Katz, 1954). Quantal analysis can be used determine these functional parameters at a given synapse and to correlate plasticity with a change in one or more of these three variables (Zucker, 1973; Bekkers and Stevens, 1995; Weis et al., 1999; Silver, 2003).

1.1.2 Transmitter release

The intricacies of the mechanisms of vesicular transmitter release and recycling, including the many stages and molecules involved are beyond the scope of this thesis. The purpose of this section is merely to provide a basic understanding and awareness of the presynaptic neuron as a gateway for the control and modulation of synaptic efficacy.

Presynaptic terminals are highly specialised secretory machines designed to cope with rapid and repeated rounds of release. Release is sustained by the existence of distinct synaptic vesicle pools and an exoendocytotic trafficking cycle comprising several steps (Sudhof, 2004; Schneggenburger et al., 2002; Becherer and Rettig, 2006). APs initiated in the presynaptic neuron, travel to nerve terminals to induce the opening of spatially concentrated voltage-gated Ca^{2+} channels (Schoch and Gundelfinger, 2006) which leads to a build up in intracellular Ca^{2+} concentration in local microdomains near the open channels (Schneggenburger and Neher, 2005; Yamada and Zucker, 1992). The binding of Ca^{2+} to the protein sensor synaptotagmin then initiates the merging of vesicle and plasma membrane – fusion (Fernandez-Chacon et al., 2001; Brose et al., 1992). Following release of transmitter *via* exocytosis (Breckenridge and Almers, 1987; Fernandez et al., 1984), vesicles are endocytosed and recycled *via* alternative routes (Sudhof, 2004; Rizzoli and Betz, 2005; Gandhi and Stevens, 2003).

1.1.3 The postsynaptic response

Once transmitter is released, it diffuses across the synaptic cleft to act on receptors in the postsynaptic membrane to mediate changes in membrane properties via the opening (or closing) of ion channels (Eccles and McGeer, 1979). Ions then diffuse through the channels down their electrochemical gradients. Depending on the ions to which the postsynaptic membrane becomes more permeable, their transmembrane distribution and the membrane voltage, the response will either lead to an excitatory postsynaptic potential (EPSP), or an inhibitory response (IPSP), and in the case of channels that conduct Ca^{2+}, the entry of a second messenger.

EPSPs depolarise the membrane towards AP threshold, whereas IPSPs hyperpolarize or hold the membrane potential away from threshold, reducing the likelihood that an AP will be generated. PSPs then summate either spatially or temporally to fire an AP if threshold reached. This simple point neuron hypothesis is adequate for considering the output of a neuron which is

dendritically relatively simple such as the SC (see below). However, for neurons with more extensive dendritic trees, integration is likely to be more complicated (Hausser and Mel, 2003; Magee and Johnston, 2005; Gulledge et al., 2005). The location of a synapse on the dendritic arbor in relation to the general morphology of the neuron, and the distribution of active conductances, as well as the presence of other active synapses, all have important roles in determining the postsynaptic response.

Postsynaptic responses vary according to the receptors activated. Ionotropic receptors gate ion channels directly to mediate fast synaptic transmission, whereas metabotropic receptors exert their effects over a relatively longer time period (usually hundreds of milliseconds) by using second messengers (Rodbell, 1980; Nicoll, 1988). These either trigger biochemical cascades that activate specific protein kinases which phosphorylate cellular proteins, thereby modulating their activity, or mobilise Ca^{2+} from intracellular stores which then initiates various cellular reactions. G proteins can also act directly on ion channels. However, not all of the effects of metabotropic receptor activation are G protein-dependent (Heuss and Gerber, 2000).

The actions of neurotransmitters are usually terminated by specific enzymes or by uptake mechanisms. The time course of transmitter in the synaptic cleft dictates (Clements, 1996) to a certain degree, the extent of the postsynaptic response by influencing receptor desensitisation, saturation and possibly the activation of extrasynaptic receptors.

1.1.4 Communication in the CNS

The principle excitatory and inhibitory neurotransmitters in the CNS are glutamate and GABA respectively and their action on ionotropic receptors mediates the majority of fast synaptic transmission. Glutamate binds to ligand-gated ion channels to elicit a fast depolarising inward current mediated mainly by Na^+ entering the cell (Mayer and Westbrook, 1987). GABA exerts inhibitory

effects by binding to ionotropic receptors, allowing Cl^- to flow into the cell, hyperpolarising the neuron. As well as being the currency for rapid communication, these transmitters can act upon metabotropic receptors, which like ionotropic receptors, can be both pre and postsynaptic.

An emerging theme in transmission is that different classes of receptors work to compliment each other. Ionotropic receptors are usually concentrated directly opposite release sites, have relatively fast agonist unbinding rates, detecting only local release of neurotransmitter. This allows for the rapid communication of specific information. Metabotropic receptors usually have a high affinity for their ligands and so can be activated relatively far from release sites allowing them to modulate transmission in response to dramatic increase in activity as well in response to more subtle changes in transmitter concentration involved in long-distance transmission and/or tonic activity.

1.2 Synaptic plasticity

Many features of a particular synapse affect how the postsynaptic neuron responds to the arrival of a presynaptic AP (AP) and these can be modified to enable appropriate transmission. Here I broadly describe certain factors which can determine synaptic strength *per se* and on another level, have the potential to be altered, providing scope for synaptic diversity, and the processing and storage of information.

Transmission at a synapse can be altered on both short and long time scales. Since the discovery of persistent enhancement of synaptic transmission by tetanic stimulation in the hippocampus (Bliss and Gardner-Medwin, 1973), a phenomenon now generally referred to as long-term potentiation (LTP), the study of activity-dependent synaptic plasticity has become a well-established field within neuroscience. Short-term changes are recognised as an important element of information processing in neuronal networks (Abbott et al., 1997; Tsodyks and Markram, 1997) and long term changes are thought to underlie mechanisms for development and learning (Collingridge et al., 2004).

Although LTP is persistent, it can be reversed by the process of depotentiation. Different patterns of activity can also lead to long-term depression (LTD) which can be reversed by de-depression. Bi-directional and reversible alterations in synaptic efficiency provide a possible dynamic mechanism for the brain to store large amounts of information (Collingridge et al., 2004; Jorntell and Hansel, 2006).

1.2.1 Transmitter release as a target for plasticity

Despite the presynaptic terminal being designed to cope with sustained transmitter release, the relationship between APs and release can be regulated by intracellular messengers, extracellular modulators and is dramatically altered by repetitive stimulation (Betz, 1970; von and Borst, 2002; Zucker and Regehr, 2002; Sakaba and Neher, 2001b). Release can be regulated both by the presynaptic neuron itself, through internal mechanisms or homosynaptic activation of autoreceptors (Davies et al., 1990; von and Matthews, 1997). It can also be modulated heterosynaptically by other neurons, *via* the release of retrograde neuromodulators acting on presynaptic receptors (Macdermott et al., 1999; Llano et al., 1991; Dittman and Regehr, 1997; Isokawa and Alger, 2005; Levenes et al., 2001; Zilberter et al., 1999; Hirasawa et al., 2004). Periods of elevated presynaptic activity can lead to an increase or a decrease in neurotransmitter release and boutons from the same axon can give rise to facilitating and depressing synapses depending on the target cell (Reyes et al., 1998; Scanziani et al., 1998; Delaney and Jahr, 2002).

Much effort has gone into analysis of the changes in statistics of transmitter release using the parameters *N* and *p.* (Zucker, 1973; Bekkers and Stevens, 1995). Variations in release probability (*p*) generally occurs either because of changes in peak Ca^{2+} concentration or changes in the probability of release for each Ca^{2+} influx. Peak Ca^{2+} concentration varies depending on the AP waveform (Sabatini and Regehr, 1997; Geiger and Jonas, 2000; Qian and Saggau, 1999 ;Borst and Sakmann, 1999), the Ca^{2+} concentration present at the point of Ca^{2+} channels opening (Wright et al., 1996;Kreitzer and Regehr,

2000;Zucker and Regehr, 2002) and the open probability of the Ca^{2+} channels (Borst and Sakmann, 1998; Cuttle et al., 1998; Forsythe et al., 1998; Borst and Sakmann, 1999) which can be inhibited by negative feedback and regulated via G-protein activation (Brody and Yue, 2000; Takahashi et al., 1998). Release of Ca^{2+} from intracellular stores can also affect transmitter release (Collin et al., 2005). Increases in $[Ca^{2+}]_i$ can also lead to a form of LTP, which is expressed presynaptically *via* an increase in cAMP, and in turn, activation of protein kinase (PKA) which facilitates transmitter release (Capogna et al., 1995;Regehr and Tank, 1991;Trudeau et al., 1996;Weisskopf et al., 1994).

Release probability as a function of peak Ca^{2+} concentration is highly variable and depends the number of release-ready vesicles (some could argue this is *N)*, and the Ca^{2+} responsiveness of these vesicles (Rettig and Neher, 2002). Variations in *N* usually arise from variability in vesicle pool dynamics (Neher, 1998; Wu and Borst, 1999; Wu and Betz, 1998; Zucker and Regehr, 2002; von and Matthews, 1997; Stevens and Wesseling, 1999). Probability of release can change as a result of depletion of the RRP (Betz, 1970; Rosenmund and Stevens, 1996), changes in recruitment of reserve vesicles or changes in the trafficking of vesicles between a releasable pool of cycling vesicles and a reserve pool of vesicles (Zucker and Regehr, 2002). Changes in pool size have been shown to be affected by protein kinase C and calmodulin, suggesting that pool size is under physiological regulation (Sakaba and Neher, 2001a; Smith et al., 1998; Junge et al., 2004; Burgoyne and Barclay, 2002).

It is generally thought that presynaptic regulation of neurotransmission occurs only through control of *p* and *N* (Auger and Marty, 2000) and that change in *q* is a postsynaptic phenomenon, reflecting a change in the availability and/or kinetics of postsynaptic receptors. However some argue that q can also be determined presynaptically (Liu, 2003; Bekkers et al., 1990; Harris and Sultan, 1995; Liu et al., 1999). Recent work has established that the mechanisms for presynaptic regulation of vesicular exocytosis might play more of a role than previously thought (Burgoyne and Barclay, 2002; Zakharenko et al., 2002 ;Krupa and Liu, 2004; Renger et al., 2001; Ishikawa et al., 2002). Intracellular $[Ca^{2+}]$ appears to have potential to modify fusion pore open time (Elhamdani et al., 2001), which could modulate the amount of transmitter being released into

the synaptic cleft. Studies show that quantal size can be varied by stimulation frequency, changes in second messenger levels, modification of the exocytotic machinery proteins and experience (Elmqvist and Quastel, 1965; Steinert et al., 2006; Machado et al., 2000; Machado et al., 2001; Naves and Van Der, 2001). These data raise controversial questions as to whether the same mechanisms could also regulate release from synaptic vesicles and contribute to synaptic plasticity.

1.2.2 Postsynaptic plasticity

The relation between input and output is also under postsynaptic control. Factors such as receptor number, affinity, occupancy and desensitisation (Katz and Thesleff, 1957; Jones and Westbrook, 1996; Trussell and Fischbach, 1989; Foster et al., 2002) can all shape the postsynaptic response and contribute to forms of plasticity (Chen et al., 2002;Wadiche and Jahr, 2001). Receptors can be upregulated and downregulated both in number (Harris et al., 1996; Corringer et al., 2006) and function (Benke et al., 1998; Derkach et al., 1999; Wang and Salter, 1994; Tan et al., 1994). Changes in the number and function of postsynaptic receptors have been extensively studied in associated with LTP and LTD. These will be described later.

1.3 Fast excitatory synaptic transmission

L-glutamate was unveiled as a neurotransmitter over half a century ago (Curtis et al., 1959) and its various receptors continue to be characterised pharmacologically, structurally and functionally with the development of specific agonists (Watkins, 1981) and antagonists, and through a combination of genetic (Seeburg, 1993; Hollmann and Heinemann, 1994) and electrophysiological techniques (Nowak et al., 1984; Cull-Candy and Ogden, 1985; Mayer et al., 1984).

1.3.1 Ionotropic glutamate receptors

The first characterised glutamate receptors were cation-selective ligand-gated ion channels. Based on electrophysiological studies, three distinct ionotropic glutamate receptor classes were proposed in the late 1970s and were initially named after the selective agonists kainate, quisqualate and *N*-methyl-D-aspartate (NMDA). It was later found that quisqualate also activates G-protein coupled metabotropic glutamate receptors and so 'quisqualate receptors' were renamed 'AMPA receptors', after the more selective agonist **(Figure 1.1A)** (Watkins, 1981). The advent of cloning allowed the molecular characterisation of receptor subunits (Hollmann and Heinemann, 1994) and revealed them to be structurally relatively similar. Each subunit consists of three transmembrane regions M1, M3, M4 with M2 (**Figure 1.1B**) being a re-entrant loop forming the channel pore. Multiple subunits exist for each receptor and these assemble to form channels with different properties.

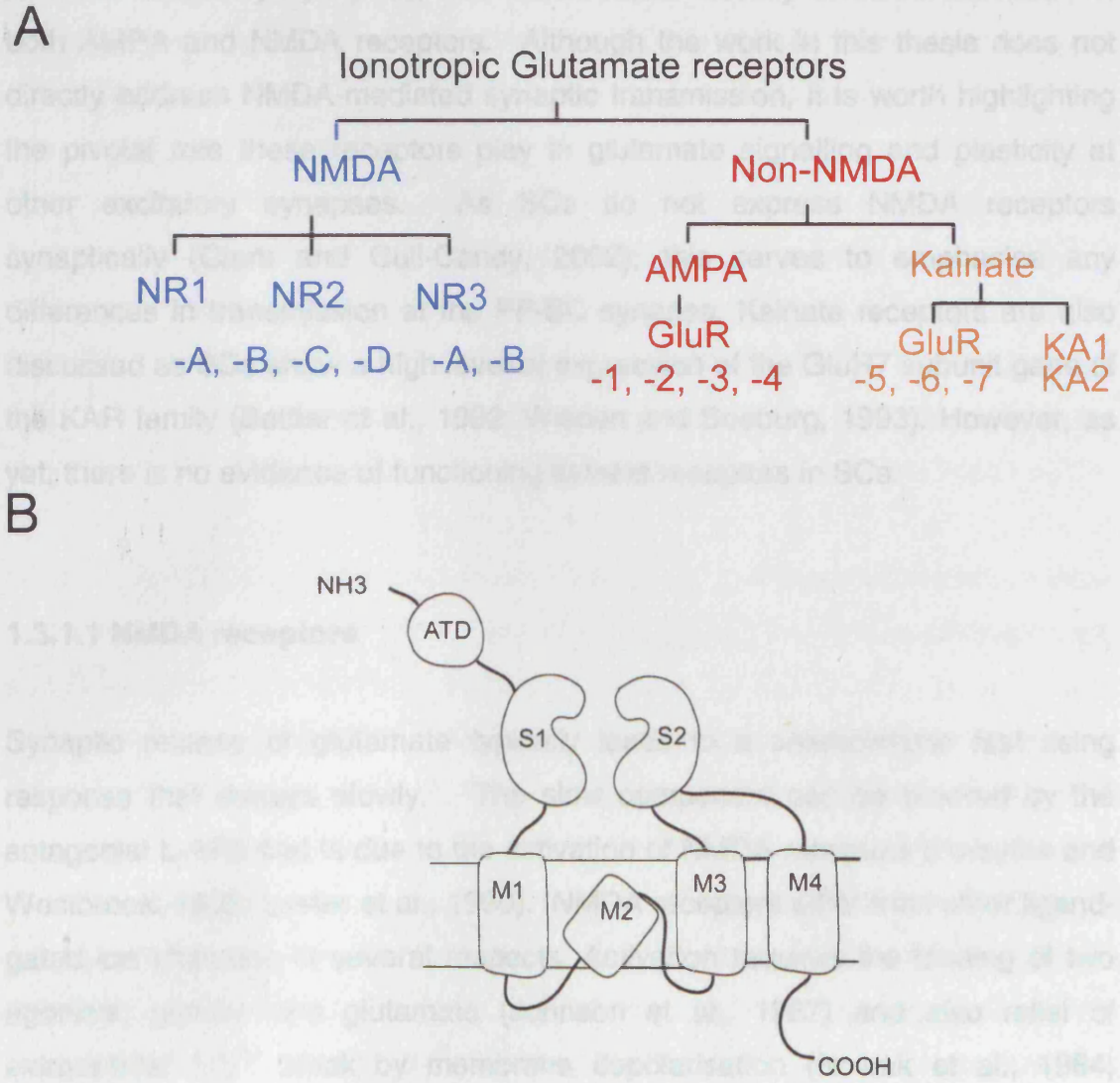


Figure 1.1 Classification and topology of ionotropic glutamate receptors. **A**. Classification of ionotropic glutamate receptors and their subunits. **B**. Each receptor subunit consists of 4 transmembrane domains (M1-4). S1 and S2 form the glutamate binding site. Amino-terminal domain (ATD) Adapted from (Biggin, 2002).

At most excitatory synapses, fast transmission usually involves activation of both AMPA and NMDA receptors. Although the work in this thesis does not directly address NMDA-mediated synaptic transmission, it is worth highlighting the pivotal role these receptors play in glutamate signalling and plasticity at other excitatory synapses. As SCs do not express NMDA receptors synaptically (Clark and Cull-Candy, 2002), this serves to emphasise any differences in transmission at the PF-SC synapse. Kainate receptors are also discussed as SCs show a high level of expression of the GluR7 subunit gene of the KAR family (Bettler et al., 1992; Wisden and Seeburg, 1993). However, as yet, there is no evidence of functioning kainate receptors in SCs.

1.3.1.1 NMDA receptors

Synaptic release of glutamate typically leads to a characteristic fast rising response that decays slowly. The slow component can be blocked by the antagonist L-AP5 and is due to the activation of NMDA receptors (Forsythe and Westbrook, 1988; Lester et al., 1990). NMDA receptors differ from other ligand-gated ion channels in several respects. Activation requires the binding of two agonists, glycine and glutamate (Johnson et al., 1987) and also relief of extracellular Mg^{2+} block by membrane depolarisation (Nowak et al., 1984; Mayer et al., 1984).

Although many NMDA subunits have been identified (NR1, which has eight splice variants, NR2 (A–D), and NR3 (A, B)), most native NMDARs are thought to function as heterodimers composed of two glycine-binding NR1 and two glutamate-binding NR2 subunits (Monyer et al., 1992; Chen et al., 2006; Cull-Candy and Leszkiewicz, 2004). Heterogeneity is still generated as one of the four NR2 subunits usually combines with a splice variant of the NR1 subunit, yielding receptors with distinct properties. The NR2 subunit critically determines a number of receptor properties, including sensitivity to Mg^{2+} block, open probability, deactivation time, single-channel conductance, and interactions with intracellular signalling molecules (Cull-Candy and Leszkiewicz, 2004; Dingledine et al., 1999). The type of NR1 splice variant in the receptor

assembly also influences certain key features, such as inhibition by protons, potentiation by polyamines, and inhibition by Zn^{2+} (Cull-Candy and Leszkiewicz, 2004; Traynelis et al., 1995). It is also important in the regulation of intracellular interactions and trafficking (Groc and Choquet, 2006; Carroll and Zukin, 2002; Mu et al., 2003; Prybylowski and Wenthold, 2004). Whether NR3 subunits can form part of the assembly with NR2-containing NMDARs is not known, but they have been shown to influence the surface expression and Ca^{2+} permeability of NR1/NR2 receptors (Perez-Otano et al., 2001) and NR1/NR3 assemblies can apparently function as glycine-gated cation-permeable ion channels, which are impermeable to Ca^{2+} and resistant to Mg^{2+} block (Chatterton et al., 2002).

Because NMDA receptors are blocked by Mg^{2+} in a voltage dependent manner and are permeable to Ca^{2+} (Macdermott et al., 1986), they are functionally well-suited to act as mediators of synaptic plasticity by acting both as coincident detectors of pre- and postsynaptic activity and by providing a means of Ca^{2+} influx to the postsynaptic cell, which can in turn initiate signal transduction cascades that modulate synaptic strength. The integration of chemical and electrical stimuli by NMDA receptors into a Ca^{2+} signal is crucial for forms of activity-dependent synaptic plasticity (Malenka and Bear, 2004) that are thought to underlie the basis for learning and memory (Bliss and Collingridge, 1993). Many immature neurons only express functional NMDA receptors and are therefore thought to be postsynaptically 'silent' because of the voltage-dependent block. During development, these synapses then acquire functional AMPARs, possibly *via* activity-dependent mechanisms similar to LTP (Molnar and Isaac, 2002). The flip side of having such a crucial role in both plasticity and development is that receptor dysfunction is linked with numerous pathologies including stroke, Parkinson's disease, Huntington's disease and schizophrenia (Cull-Candy et al., 2001).

NMDA subunits show distinct expression patterns in the central nervous system, which can be regionally or developmentally regulated (Monyer et al., 1994; Watanabe et al., 1994). In many neurons, NR2B (and NR2D) subunits are abundant at early developmental stages, with NR2A or NR2C subunits being progressively added during development. In the adult brain, NR2A is

ubiquitously expressed, NR2B is restricted to the forebrain, and NR2C is highly enriched in the cerebellum. NR1 occurs in eight splice variants, but whether two distinct variants exist within one NMDAR complex is unknown.

Subunits can also vary in their subcellular distribution. NR2A and NR2B subunits target preferentially synaptic and extrasynaptic locations respectively in some neurons (Massey et al., 2004; Stocca and Vicini, 1998). The subcellular localisation of NMDARs, and hence route of Ca^{2+} influx into the cell, is known to activate various signalling pathways that can differ between extrasynaptic and synaptic sites (Kohr, 2006).The kinetics and magnitude of the NMDAR-mediated Ca^{2+} signal can determine the polarity of the synaptic modification (Barria and Malinow, 2005; Liu et al., 2004a; Massey et al., 2004). Therefore, distinct NMDAR functions probably depend on a combination of subunit composition and spatial distribution. The evidence that NMDAR surface expression can be rapidly regulated, in a subunit specific manner, in response to activity (Williams et al., 2003; Barria and Malinow, 2002; Grosshans et al., 2002; Philpot et al., 2001) provides a powerful possible mechanism for metaplasticity (Collingridge et al., 2004; Perez-Otano and Ehlers, 2005).

1.3.1.2 Kainate receptors

Although structurally similar to AMPA and NMDA receptors, comparatively little is known about the role that kainate receptors play in synaptic transmission. Only recently, with the development of specific antagonists (Donevan and Rogawski, 1993) and the generation of knock-out animals (Mulle et al., 1998; Contractor et al., 2001; Ruiz et al., 2005), is their contribution being elucidated. Roles for kainate receptors have been revealed in synaptic transmission, neuronal development (Lauri et al., 2005; Marchal and Mulle, 2004), plasticity (Kidd and Isaac, 1999a) and disease (Mulle et al., 1998; Grigorenko et al., 1998; Kortenbruck et al., 2001).

Like the other ionotropic glutamate receptors, kainate receptors are also tetrameric assemblies that display various properties depending on their subunit

combination of a possible 5 cloned subunits - KA1, KA2, GluR5, GluR6 and GluR7. Patterns of mRNA and subunit specific antibody expression show that subunits are widely distributed throughout the CNS (Wisden and Seeburg, 1993). Subunits can also undergo alternative splicing (Bettler and Mulle, 1995) and RNA editing at various sites including the Q/R site in a manner similar to AMPARs (see below). Not all subunit combinations give rise to functional channels, however many are viable forming receptors which differ in their activation, desensitisation, sensitivity to antagonists and allosteric modulators, rectification and Ca^{2+} permeability (Sakimura et al., 1992; Howe, 1996;Schiffer et al., 1997; Cui and Mayer, 1999; Bettler and Mulle, 1995; Huettner, 2003; Lerma, 2003).

Kainate receptors can participate directly in postsynaptic excitatory currents (EPSCs) in many brain areas, both alongside AMPAR activation (Li and Rogawski, 1998; Eder et al., 2003; Kidd and Isaac, 1999a; Huang et al., 2004c; Vignes and Collingridge, 1997; Bureau et al., 2000; Castillo et al., 1997; Frerking et al., 1998) and also independently (Cossart et al., 2002). However kainate-mediated EPSCs display smaller amplitudes (10% of the total peak current) and have significantly slower rise time and decay than the AMPA-mediated component. These properties are unexpected given the results determined in culture and are thought not to be due to of receptor location but rather the intrinsic properties of the receptors (Bureau et al., 2000; Castillo et al., 1997; Cossart et al., 2002).

Presynaptically, kainate receptors can modulate transmitter release (Min et al., 1999; Huettner, 2003; Lerma, 2003 ; Lauri et al., 2003; Contractor et al., 2001), either by a direct influx of Ca^{2+} through the kainate-receptor channel, through depolarisation of the presynaptic membrane (Schmitz et al., 2001; Kamiya, 2002) or *via* a metabotropic action (Rodriguez-Moreno and Lerma, 1998; Cunha et al., 2000).

It appears, as for NMDARs, that the location and subtype of kainate receptor determines overall effect of receptor activation (Mulle et al., 2000). Targeting of kainate receptors to pre- versus postsynaptic sites is not clearly related to

specific subunits or splice variants but may involve the differential association of the various subunits and/or splice variants to interacting proteins (Jaskolski et al., 2005). Application of agonists suggests that kainate receptors are localised extrasynaptically (Ben-Ari and Cossart, 2000; Huettner, 2003; Lerma, 2003). However, the role of these receptors under physiological conditions is not clear. One source of kainate receptor activation may be glutamate released by astrocytes (Liu et al., 2004b).

As well as short term changes, kainate receptors participate in long term plasticity both pre- and postsynaptically. Activation of presynaptic kainate receptors in hippocampal mossy fibres contributes to the induction of a form of long term plasticity that is expressed presynaptically (Bortolotto et al., 1999; Contractor et al., 2001; Lauri et al., 2001; Schmitz et al., 2003). A kainate receptor-dependent enhancement of synaptic transmission has been observed in the basolateral amygdala (Li and Rogawski, 1998) Furthermore, in the thalamocortical pathway, pairing presynaptic stimulation and postsynaptic depolarization leads to an increase in AMPAR-mediated current but a decrease in kainite receptor-mediated response (Kidd and Isaac, 1999a). Recently kainate receptors have been shown to undergo LTD at synapses on layer II/III neurons in perirhinal cortex *via* a mechanism that involves interactions with mGluR5 receptors and PKC (Park et al., 2006).

1.3.1.3 AMPA receptors

The majority of fast excitatory transmission is mediated by AMPA receptors and changes in AMPAR expression are believed to underpin mechanisms responsible for development, learning and memory, ageing and disease. The properties of AMPARs depend critically on their subunit composition.

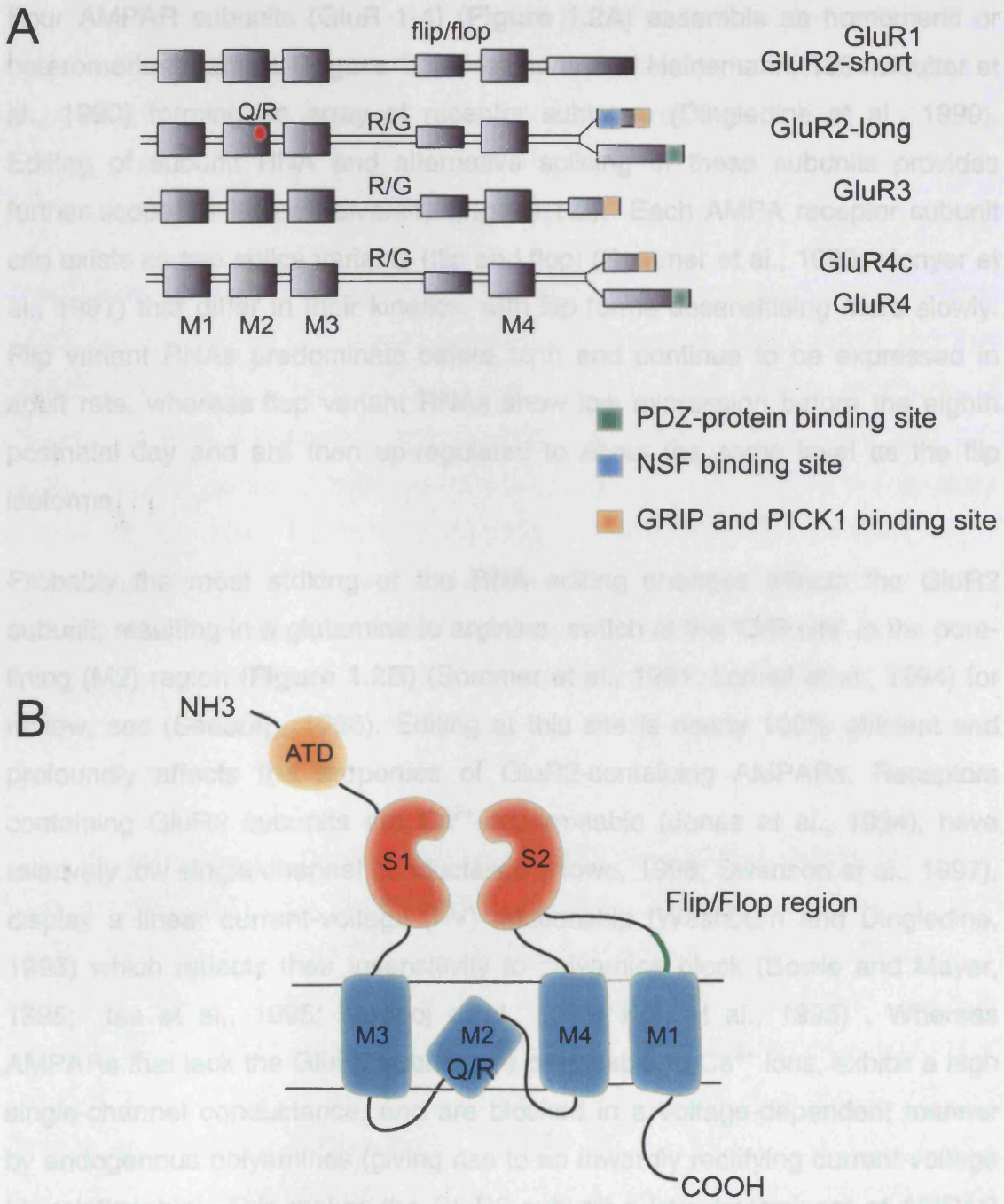


Figure 1.2 Alternative splicing and editing of AMPA receptor subunits. A. The flip/flop and C-terminal splice variants of the AMPA receptor subunits are depicted schematically. The regions buried in the membrane are shown as boxes M1 to M4. The Q/R- and R/G-editing sites are indicated. Different sites of interaction on both long and short C termini are shown (adapted from (Dingledine et al., 1999) and (Malinow and Malenka, 2002)). **B.** Same diagram as Figure 1.1 adapted to show location of AMPAR subunit post-translational modifications.

Four AMPAR subunits (GluR 1-4) (**Figure 1.2A**) assemble as homomeric or heteromeric tetramers (**Figure 1.3**) (Hollmann and Heinemann, 1994;Boulter et al., 1990) forming an array of receptor subtypes (Dingledine et al., 1999). Editing of subunit RNA and alternative splicing of these subunits provides further scope for isoform diversity (**Figure 1.2**). Each AMPA receptor subunit can exists as two splice variants (flip and flop) (Sommer et al., 1990; Monyer et al., 1991) that differ in their kinetics, with flip forms desensitising more slowly. Flip variant RNAs predominate before birth and continue to be expressed in adult rats, whereas flop variant RNAs show low expression before the eighth postnatal day and are then up-regulated to about the same level as the flip isoforms.

Probably the most striking of the RNA editing changes affects the GluR2 subunit, resulting in a glutamine to arginine switch at the 'Q/R site' in the pore-lining (M2) region (**Figure 1.2B**) (Sommer et al., 1991; Lomeli et al., 1994) for review, see (Seeburg, 1996). Editing at this site is nearly 100% efficient and profoundly affects the properties of GluR2-containing AMPARs. Receptors containing GluR2 subunits are Ca^{2+}-impermeable (Jonas et al., 1994), have relatively low single-channel conductance (Howe, 1996; Swanson et al., 1997), display a linear current-voltage (I-V) relationship (Washburn and Dingledine, 1996) which reflects their insensitivity to polyamine block (Bowie and Mayer, 1995; Isa et al., 1995; Kamboj et al., 1995; Koh et al., 1995) . Whereas AMPARs that lack the GluR2 subunit are permeable to Ca^{2+} ions, exhibit a high single-channel conductance, and are blocked in a voltage-dependent manner by endogenous polyamines (giving rise to an inwardly rectifying current-voltage I/V relationship). This makes the GluR2 subunit a key determinant of AMPAR function (**Figure 1.3**)

Although AMPAR subunits have similar extracellular and transmembrane regions, their intracellular cytoplasmic tails are distinct (for review see (Malinow and Malenka, 2002). GluR1, GluR4, and an alternative splice form of GluR2 (GluR2L) have longer cytoplasmic tails and are homologous. In contrast, the predominant splice form of GluR2, GluR3, and an alternative splice form of GluR4 that is primarily expressed in cerebellum (GluR4c) have short, homologous cytoplasmic tails. Through their C-terminal, each subunit interacts

with specific cytoplasmic proteins, most of which have single or multiple PDZ domains (**Figure 1.2**). Proteins containing PDZ domains have been suggested to play important roles in the targeting and clustering of membrane proteins to specific subcellular domains (Sheng, 2001). These will be described further in relation to AMPAR targeting and synaptic plasticity.

The different AMPAR subunits provide an array of functionally diverse receptors which are expressed differentially, both with respect to brain region and age (Takahashi, 2005). There is extensive evidence that AMPA receptors of different subunit composition can co-exist within the same neuron and that specific AMPA receptor subunits can be selectively targeted to the synapse (Wyllie et al., 1993 Rubio and Wenthold, 1997; Toth and McBain, 1998; Gardner et al., 1999). Expression and subcellular specific targeting of receptors can also be regulated in response to activity (Liu and Cull-Candy, 2000a; Collingridge et al., 2004). As with the other ionotropic glutamate receptors, the subtype and location of AMPARs has profound effects on synaptic transmission.

AMPARs are thought to be assembled as a dimer of heterodimers via a two-step process within the endoplasmic reticulum (ER), (**Figure 1.3**) although GluR1 and GluR2 subunits have been also shown to be synthesised in dendrites in an activity-dependent and activity-independent manner, respectively (Ju et al., 2004). The presence of a charged arginine residue at the Q/R site of GluR2 makes the juxtaposition of GluR2 subunits of different dimers energetically unfavourable (Greger et al., 2003). Although GluR2 subunits can form homodimers and assemble to produce functional homomeric GluR2 receptors in heterologous systems (Swanson et al., 1997), in neurons expressing a mixture of subunits the formation of homomeric GluR2 receptors is much less likely.

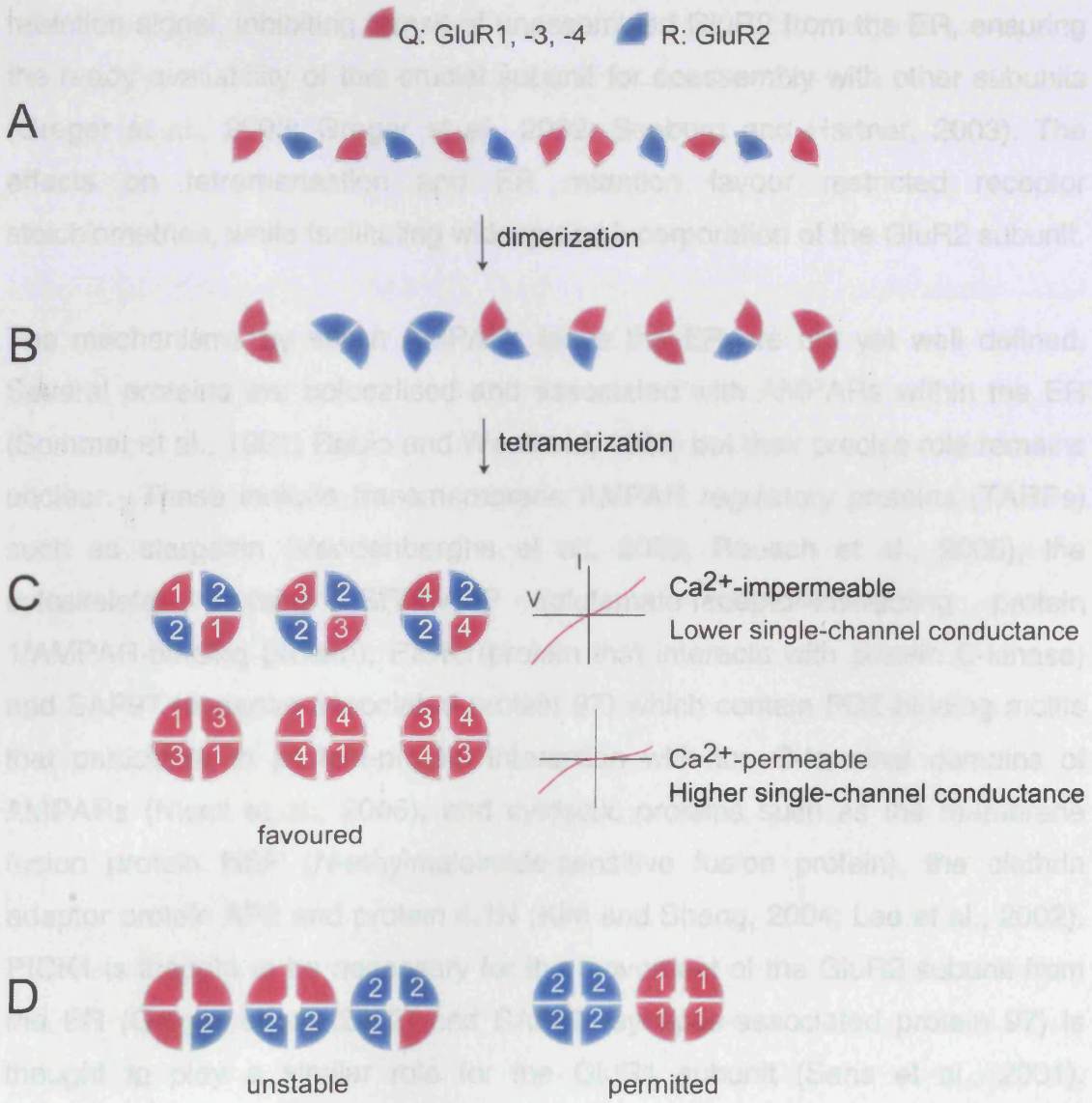


Figure1.3. Schematic representation of the subunit partnerships involved in formation of functional tetrameric AMPARs. GluR2 is shown in blue (R = arginine at Q/R site, Q = glutamine at Q/R site), other subunits (GluR1, 3 and 4) are in pink. Individual subunit types are identified by white numerals. **A**. Subunits are synthesised in the ER, then assembled in two stages: dimerisation followed by tetramerisation. **B**. Dimerisation; formation of heteromeric dimers is favoured, but homomeric dimers are also allowed. **C**. Tetramerisation: this results from assembly of a pair of dimers. Pairs of identical dimers preferentially co-assemble, producing a symmetrical stoichiometry. A range of expected subunit combinations is illustrated, assuming this rule applies to all unedited subunits. Corresponding I-V relationships shown on right. A number of Ca^{2+}-permeable combinations is permitted or favoured. **D**. (Left panel) A single copy of GluR2, or the presence of two copies of GluR2 in close juxtaposition, gives assemblies that are probably still Ca^{2+}-permeable but unstable. (Right panel)A number of Ca^{2+}-permeable combinations is permitted or favoured. Adapted from (Cull-Candy et al., 2006).

The arginine residue acquired during RNA editing appears to function as a retention signal, inhibiting transit of unassembled GluR2 from the ER, ensuring the ready availability of this crucial subunit for coassembly with other subunits (Greger et al., 2003; Greger et al., 2002; Seeburg and Hartner, 2003). The effects on tetramerisation and ER retention favour restricted receptor stoichiometries, while facilitating widespread incorporation of the GluR2 subunit.

The mechanisms by which AMPARs leave the ER are not yet well defined. Several proteins are colocalised and associated with AMPARs within the ER (Sommer et al., 1991; Rubio and Wenthold, 1999) but their precise role remains unclear. These include transmembrane AMPAR regulatory proteins (TARPs) such as stargazin (Vandenberghe et al., 2005; Rouach et al., 2005), the cytoskeletal proteins GRIP/ABP (glutamate-receptor-interacting protein 1/AMPAR-binding protein), PICK1(protein that interacts with protein C-kinase) and SAP97 (synapse-associated protein 97) which contain PDZ-binding motifs that participate in protein-protein interaction with the C-terminal domains of AMPARs (Nicoll et al., 2006), and cytosolic proteins such as the membrane fusion protein NSF (*N*-ethylmaleimide-sensitive fusion protein), the clathrin adaptor protein AP2 and protein 4.1N (Kim and Sheng, 2004; Lee et al., 2002). PICK1 is thought to be necessary for the movement of the GluR2 subunit from the ER (Greger et al., 2002) and SAP97 (synapse-associated protein 97) is thought to play a similar role for the GluR1 subunit (Sans et al., 2001). Stargazin transports AMPARs early in the biosynthetic pathway (for a review, see (Nicoll et al., 2006)). These proteins participate in both the basal regulation of synaptic receptors and in processes underlying plasticity (see below) (Huang et al., 2005; Collingridge et al., 2004). Newly synthesised receptors then reach synapses either by vesicular transport or by lateral diffusion (Groc and Choquet, 2006).

1.3.2 Synaptic plasticity at excitatory synapses

A change in receptor number is recognised as a major way of altering synaptic strength (Bredt and Nicoll, 2003; Malenka and Bear, 2004; Malinow and

Malenka, 2002). Classically, attention has focused on the regulation of AMPARs at the synapse within the context of LTP and LTD. A general model is emerging in which synaptic plasticity involves regulated exocytosis and endocytosis of AMPARs at extrasynaptic sites and regulated lateral diffusion of AMPARs in and out of the synapse (Collingridge et al., 2004).

Long-term changes in AMPAR expression have been particularly well-characterised in the hippocampus. Knowledge gleaned from these studies is now being applied to the activity-dependent regulation of other forms of synaptic plasticity (e.g. (Bortolotto and Collingridge, 1993; Lauri et al., 2003)). It is becoming apparent that long term changes in synaptic efficacy can be induced in a number of different ways, for example activation via activation of mGluR (Watabe et al., 2002;Kemp and Bashir, 1999b), and that activity can alter the expression of other receptors aside from AMPA (Kidd and Isaac, 1999b). It is also well recognised LTP and LTD are certainly not the only means by which the activity generated by experience can modify neural circuit behaviour (Malenka and Bear, 2004; Abraham et al., 2001; Turrigiano and Nelson, 2004; Zhang and Linden, 2003) .

For simplicity, I shall outline the mechanisms involved in extensively-studied NMDA-dependent plasticity in the CA1 region of the hippocampus. Once again, this will serve to contrast with the expression of AMPAR-mediated plasticity observed at the PF-SC synapse.

1.3.2.1 Long-term potentiation

In the hippocampus, it is thought that basal levels of synaptic AMPARs are maintained by the constitutive cycling of GluR2/3 receptors *via* exo-and endocytosis, involving an interaction between GluR2 and the membrane fusion protein NSF (Shi et al., 2001; Kim and Lisman, 2001). During periods of high-frequency activity, synaptic NMDARs are relieved of Mg^{2+} block by membrane depolarisation, which leads to Ca^{2+}influx, a local rise in intracellular Ca^{2+} and ultimately the insertion of GluR1/2 receptors to the synapse (Malinow and

Malenka, 2002; Shi et al., 2001; Bliss and Collingridge, 1993; Malenka and Bear, 2004) An important requirement for this model is the existence of a pool of AMPARs near synapses, available for recruitment and indeed studies have shown that such a pool of AMPARs does exist (Baude et al., 1995; Kharazia et al., 1996; Martin et al., 1993; Petralia and Wenthold, 1992; Takumi et al., 1999).

The NMDAR-mediated increase in Ca^{2+} is presumed to trigger a number of intracellular pathways such as calcium/calmodulin-dependent protein kinase II (CaMKII), PKA, cAMP-dependent protein kinase, (Yasuda et al., 2003; Blitzer et al., 1998) protein kinase C (PKC) (Bliss and Collingridge, 1993; Hu et al., 1987;Linden and Routtenberg, 1989) and mitogen-activated protein kinase (MAPK), (Sweatt, 2004; Thomas and Huganir, 2004) amongst others. Conversely, protein phosphatases, protein phosphatase 1 (PP1) and protein phosphatase 2B (PP2B), have also been implicated in NMDAR-dependent LTD. Longer-lasting components of LTP require gene transcription and new protein synthesis (Abraham et al., 2001; Lynch, 2004 ; Pittenger and Kandel, 2003). Signalling molecules that are thought to link the activity that induces LTP to the nucleus include PKA, CaMKIV, and MAPK, which in turn activate the key transcription factor CREB as well as immediate early genes such as zif268 (Abraham et al., 2001; Pittenger and Kandel, 2003; Chen et al., 2000).

A possible mechanism for LTP involves GluR1 subunit binding to the PDZ-domain-containing protein SAP97 which, in turn, binds to motor proteins such as myosin-VI. By phosphorylating SAP97, CaMKII could drive the insertion of AMPARs at sites distant from the synaptic cleft, from which they would then diffuse laterally within the membrane to the synapse. In addition, the postsynaptic density protein 95 (PSD-95) interacts with AMPARs through stargazin (Chen et al., 2000). During plasticity, this interaction can traffic AMPARs to the plasma membrane, recruiting them from extra-synaptic to synaptic sites.

Receptors are then thought to be maintained at the synapse through interaction with PSD proteins. There is evidence that the concentration of PSD-95 at the synapse directly determines the number of synaptic AMPARs by interacting

with stargazin or other TARPs (Bredt and Nicoll, 2003). PSD-95 can be regulated by ubiquitination (Colledge et al., 2003) and palmitoylation (El-Husseini et al., 2002). The interaction of AMPARs with TARPs can also be dynamically regulated by ligand binding (Tomita et al., 2004). Together these provide additional mechanisms for regulating AMPAR number in synaptic plasticity.

It should be noted that LTP still occurs in some brain regions in mice lacking the GluR1 subunit (Zamanillo et al., 1999), suggesting that other AMPAR subunits can mediate activity-dependent synaptic plasticity. LTP can also involve the modification of the biophysical properties of AMPARs themselves, *via* their direct phosphorylation (Benke et al., 1998; Lee et al., 2003; Malenka and Nicoll, 1999; Derkach et al., 1999) and can also be associated with the structural remodelling of synapses (Abraham and Williams, 2003; Yuste and Bonhoeffer, 2001). Furthermore, the possible contribution the presynaptic terminal in forms of long term plasticity has yet to be fully determined (Malenka and Bear, 2004). Therefore, despite characterising many steps involved in LTP, the full array of mechanisms is still far from understood.

1.3.2.2 Long-term depression

LTD induced by low-frequency stimulation also requires activation of NMDARs (Dudek and Bear, 1992; Mulkey and Malenka, 1992) and a rise in postsynaptic Ca^{2+} concentration (Mulkey and Malenka, 1992). AMPARs are thought to be internalised before being released from the cytoskeleton. The interaction between NSF and GluR2, important for maintaining AMPARs at synapses, is disrupted by AP2, in response to an appropriate stimulus, initiating clathrin-dependent internalisation. NSF, through its ATPase activity, may also help to dissociate AMPARs from PDZ proteins ABP and GRIP (Collingridge et al., 2004). Both ABP and GRIP contain several PDZ domains and probably anchor AMPARs at both synaptic (Dong et al., 1997; Osten et al., 2000) and intracellular (Daw et al., 2000) locations. PICK1 is also involved in the internalisation of AMPARs (Perez et al., 2001; Kim et al., 2001), possibly by

phosphorylating GluR2 on serine 880. Once phosphorylated at this residue, GluR2 can bind PICK1 but not ABP/GRIP, providing a mechanism by which AMPARs can be freed from ABP/GRIP for internalisation. Endocytosis then occurs *via* clathrin-coated pits and requires the activity of dynamin. Internalised AMPARs may be recycled to the membrane, held in an intracellular pool or degraded (Collingridge et al., 2004).

LTD is thought to be maintained possibly by the ubiquitination and degradation of PSD-95 by the proteasome (Colledge et al., 2003). The ubiquitin/proteasome pathway is thought to be a critical regulator of the molecular architecture of glutamatergic synapses and therefore also of the long-term maintenance of LTD and LTP (Burbea et al., 2002; Cline, 2003; Ehlers, 2003). As with LTP, there is evidence that stable expression of LTD requires protein synthesis (Kauderer and Kandel, 2000; Manahan-Vaughan et al., 2000; Sajikumar and Frey, 2003).

1.4 Calcium-permeable AMPARs

Although the majority of AMPARs in the CNS are GluR2-containing, and hence Ca^{2+}-impermeable, there is significant expression of CP- AMPARs in neuronal and glial cells of various brain regions. It is now becoming apparent that the regulation of these CP- AMPARs - their expression, assembly, trafficking and turnover - is critical in synaptic plasticity, neuronal development and neurological disease(Cull-Candy et al., 2006).

1.4.1 Ca^{2+}-permeable AMPARs and development

CP-AMPARs can be developmentally up-regulated, down-regulated or expressed transiently, depending on location. The Ca^{2+} transients that CP-AMPARs mediate may represent important developmental signals which regulate cell growth and differentiation as activation of CP-AMPARs reduces

dendritic growth in embryonic chick retinal cells (Catsicas et al., 2001) and cultured motorneurons (Metzger et al., 1998).

Prenatal chick auditory neurons show a developmental increase in the expression of CP-AMPARs between E10 and E17/P2 (Sugden et al., 2002) and nucleus magnocellularis neurons (nMag) show developmental increase in EPSC rectification. This switch in receptor subtype can be prevented, but not reversed, by isolating nMag neurons in cell culture (Lawrence and Trussell, 2000) suggesting this change occurs in response to some external cues. CP-AMPARs are present at embryonic *Xenopus* excitatory spinal synapses, as well as extrasynaptically. Mature dorsolateral interneurons (DLi) also express CP-AMPARs, although relative P_{Ca} is decreased as compared with embryonic DLi, potentially reflecting a change in AMPAR subtype expression as synapses mature (Rohrbough and Spitzer, 1999).

Upregulation of CP-AMPARs occurs during the development of auditory brainstem neurons where Ca^{2+} permeability appears around the onset of hearing (P12) and is highest during the following 2 weeks. In the large fusiform neurons of the auditory brainstem, AMPA receptors composed of GluR1, GluR2 and GluR2/3 are gradually replaced by ones containing mainly GluR4 (Caicedo and Eybalin, 1999).

Motorneurons show decreased GluR2 labelling during postnatal weeks 3–5. There are also postnatal decreases in GluR2 in the striatum (Chan et al., 2003), basal ganglia (Jakowec et al., 1998) inner plexiform layer of the rat retina (Johansson et al., 2000) and rat trigeminal neurons (Turman, Jr. et al., 2000). Expression of CP-AMPARs may also be downregulated during development. In contrast with the auditory brainstem, the inner hair cell lacks the GluR2 subunit until the onset of hearing (P10) (Eybalin et al., 2004). A similar developmental change occurs in rat neocortical, layer V pyramidal neurons around P16 where synaptically evoked currents change from rectifying to linear, reflecting a switch in calcium permeability and subunit composition (Kumar et al., 2002).

Furthermore in hippocampal astrocytes possess inward rectifying receptors at P5 that are sensitive to extracellular JSTx - properties which are lost one week later (Seifert et al., 2003). GluR2 labelling increases developmentally in the outer plexiform layer and in bipolar cells in the developing retina (Johansson et al., 2000). Developmental increases in CI-AMPARs are also seen in the inferior olive (Chen et al., 2006), hippocampal pyramidal neurons (Sanchez et al., 2001a) and hippocampal interneurons (Lei and McBain, 2002).

Transient expression of CP-AMPARs can be observed during glial development. CP-AMPARS are detected only in oligodendroglial progenitors, but not pre-progenitors or mature oligodendrocytes, explaining a stage–specific susceptibility to excitotoxicity during differentiation (Itoh et al., 2002). Global hypoxia induces seizures and a decrease in hippocampal GluR2 in rat pups at P10- 12, but not in younger or older animals (Jensen et al., 1999)and this decrease in GluR2 is associated with an increase in AMPAR-mediated potentiation of hippocampal epileptiform activity *in vitro* (Sanchez et al., 2001a). Clearly, the presence or absence of GluR2 appears crucial in preserving neuronal survival and ensuring precise synaptic transmission.

1.4.2 Ca^{2+}-permeable AMPARs and synaptic plasticity

Short term plasticity: dynamic regulation by polyamine block

Intracellular polyamines are endogenous to all neurons (Tabor and Tabor, 1984), and cytoplasmic concentrations block GluR2-lacking AMPA receptors in a voltage-dependent manner (Bowie and Mayer, 1995; Kamboj et al., 1995; Koh et al., 1995; BarnesDavies and Forsythe, 1996). Initial evidence for the involvement of polyamines in synaptic plasticity came from the demonstration that repetitive stimulation of CP-AMPARs both in HEK cells and neurons leads to transient relief of the tonic polyamine block, resulting in facilitation of synaptic responses that would normally show depression (**Figure 1.4A**). The degree of facilitation depends on the frequency of stimulation and can last for several hundred milliseconds before channel re-block occurs (Rozov and Burnashev, 1999).

The recent finding that the basal concentration of polyamines, such as spermine, in neurons is non-saturating and can change, both during development and in response to activity (Aizenman et al., 2002; Shin et al., 2005), adds a further dimension to the influence of polyamines on plasticity. In pyramidal neurons in rat neocortex, there is a developmental decrease in the level of spermine and in the enzyme required for its synthesis (Shin et al., 2005). Concentration is highest in immature neurons, when the cells contain CP- AMPARs. As a result, immature cells display greater paired-pulse facilitation than mature cells (Shin et al., 2005).

Polyamine concentration also changes in response to activity, (Aizenman et al., 2002). After 4 hours of visual stimulation, CP-AMPARs expressed in immature optic tectal neurons display enhanced rectification due to increased polyamine synthesis (**Figure 1.4B**). Visually stimulated cells also display increased paired-pulse facilitation, indicating that this increase in polyamines impacts upon the use-dependent relief from tonic polyamine block. Taken together, these findings provide compelling evidence that control of intracellular polyamines can play a role both in short- and long-term plasticity.

Developmental and activity-dependent control of CP-AMPAR expression and internal polyamine concentration, modulated in combination with each other, provide a powerful mechanism for controlling the responsiveness of the immature neuron to repetitive stimuli and the kinetics of the AMPA response, especially at depolarised potentials (Bowie et al., 1998b).

CP-AMPARs and long-term plasticity

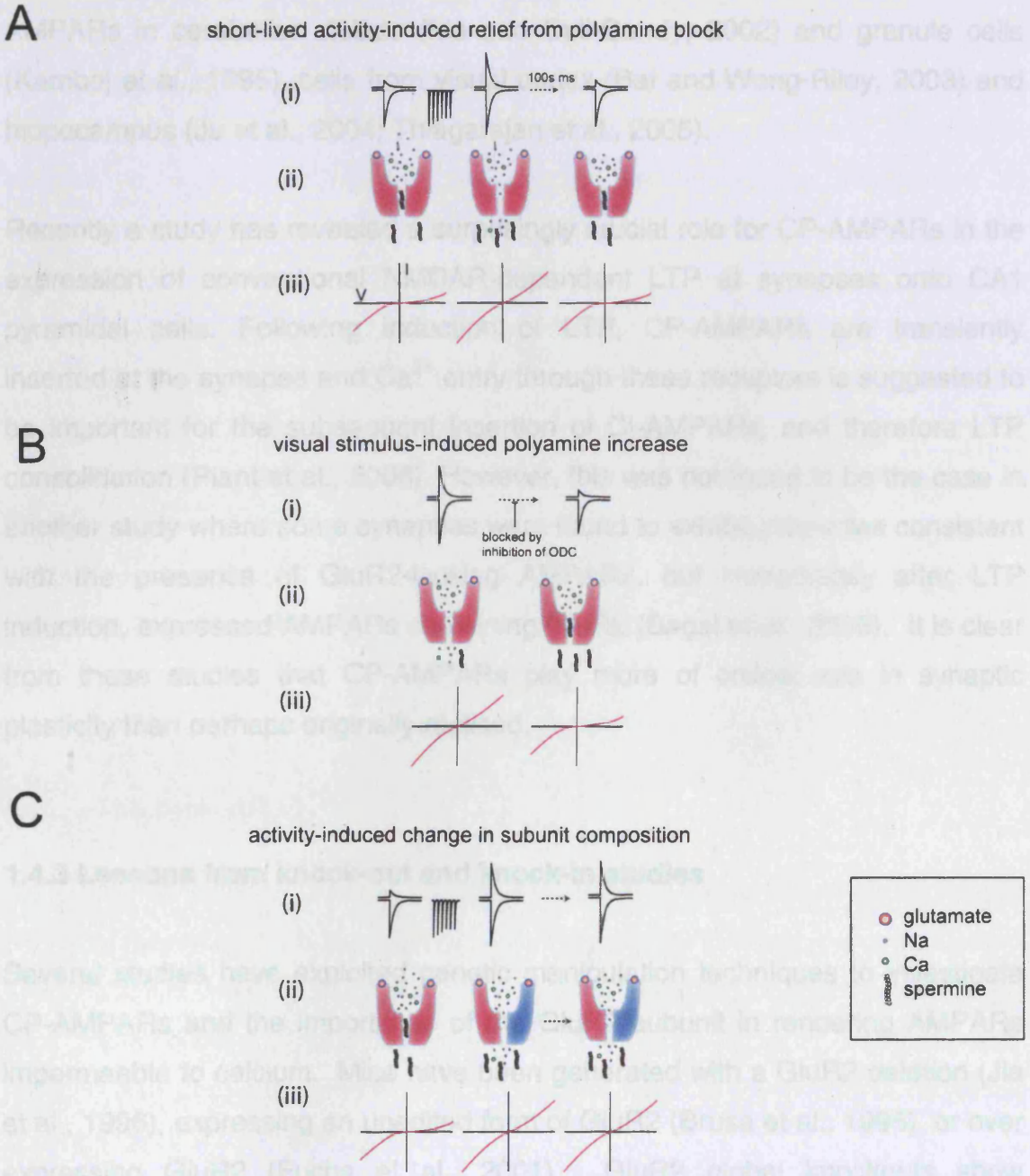
The Ca^{2+}-permeability of GluR2-lacking AMPARs makes them attractive candidates as mediators of synaptic plasticity - by offering a route for Ca^{2+} entry independent of NMDARs or voltage-gated Ca^{2+} channels. Indeed, stimulation of synapses containing CP-AMPARs can lead to LTP (Mahanty and Sah, 1998;Zalutsky and Nicoll, 1990), LTD (Soler-Llavina and Sabatini, 2006; Laezza

et al., 1999; Lei and McBain, 2004) and changes in subunit compostion (Liu and Cull-Candy, 2000a). CP-AMPAR mediated plasticity observed at the PF-SC synapse (Soler-Llavina and Sabatini, 2006; Liu and Cull-Candy, 2000a; Gardner et al., 2005b) is described in **section 1.5.3**.

Mossy fibre-interneuron synapses in the CA3 region of the hippocampus contain a continuum of AMPAR subtypes, ranging from predominantly GluR2-lacking, to predominantly GluR2-containing (Laezza et al., 1999; Lei and McBain, 2002). At synapses with a large proportion of CP-AMPARs, Ca^{2+} influx through these receptors is both necessary and sufficient to induce LTD (Laezza et al., 1999; Bischofberger and Jonas, 2002), which is expressed presynaptically (Laezza and Dingledine, 2004).

Depending on membrane potential, these synapses can also exhibit LTP (rather than LTD), if Ca^{2+} enters through both AMPARs *and* NMDARs (Laezza and Dingledine, 2004). This phenomenon is not inducible at GluR2-containing synapses, suggesting that CP-AMPARs can work both independently and in conjunction with NMDA receptors, allowing neurons to respond to high frequency inputs in a manner different from that seen in neurons lacking these receptors. The fact that NMDA receptors at these two synapses differ both in number and subunit composition (Lei and McBain, 2002) might be of some relevance, indicating subunit specific roles for interaction with CP-AMPARs.

1.4. Activity-dependent regulation of Ca^{2+}-permeable AMPAR-mediated currents. Schematic diagram showing (i) idealised EPSCs evoked at identical negative or positive membrane potentials, with (ii) underlying single-channel behaviour and (iii) corresponding whole-cell I/V relationship. **A** Short-term use-dependent relief of polyamine block. Stimulation reveals the presence of a rectifying response. Tetanic stimulation leads to temporary unblock of the channels, resulting in larger EPSCs at positive and negative potentials (indicating that a proportion of channels are blocked by polyamines at resting potential) and a more linear I/V plot. Channels re-block after several hundred milliseconds at rest. **B** Activity-induced increase in intracellular polyamine concentration. Unstimulated cells exhibit weakly rectifying EPSCs, indicating the presence of CP-AMPARs. Visual stimulation induces an increase in intracellular polyamines — unless the polyamine synthesising enzyme, ornithine decarboxylase (ODC), is inhibited — enhancing polyamine block of the channels, and increasing rectification. **C**. Activity-induced switch in subunit composition. Cells initially display inwardly rectifying EPSCs, reflecting polyamine block of GluR2-lacking receptors. Tetanic stimulation results in a switch in subunit composition, such that GluR2-lacking receptors are replaced by GluR2-containing AMPARs. This results in a linear I/V relationship, which remains linear for a prolonged period. Adapted from (Cull-Candy et al., 2006).



1.4. Activity-dependent regulation of Ca^{2+}-permeable AMPAR-mediated currents (see opposite).

Synaptic *inactivity* can also influence the Ca^{2+}-permeability and subunit composition of postsynaptic AMPARs. Inactivity induced by tetrodotoxin (TTX), high K^+ or AMPAR-blockers (in slice or culture) increases the expression of CP-AMPARs in cerebellar stellate (Liu and Cull-Candy, 2002) and granule cells (Kamboj et al., 1995), cells from visual cortex (Bai and Wong-Riley, 2003) and hippocampus (Ju et al., 2004; Thiagarajan et al., 2005).

Recently a study has revealed a surprisingly crucial role for CP-AMPARs in the expression of conventional NMDAR-dependent LTP at synapses onto CA1 pyramidal cells. Following induction of LTP, CP-AMPARs are transiently inserted at the synapse and Ca^{2+} entry through these receptors is suggested to be important for the subsequent insertion of CI-AMPARs, and therefore LTP consolidation (Plant et al., 2006). However, this was not found to be the case in another study where some synapses were found to exhibit properties consistent with the presence of GluR2-lacking AMPARs, but immediately after LTP induction, expressed AMPARs containing GluR2 (Bagal et al., 2005). It is clear from these studies that CP-AMPARs play more of critical role in synaptic plasticity than perhaps originally realised.

1.4.3 Lessons from knock-out and knock-in studies

Several studies have exploited genetic manipulation techniques to investigate CP-AMPARs and the importance of the GluR2 subunit in rendering AMPARs impermeable to calcium. Mice have been generated with a GluR2 deletion (Jia et al., 1996), expressing an unedited form of GluR2 (Brusa et al., 1995), or over expressing GluR2 (Fuchs et al., 2001). GluR2 global knockouts show enhanced LTP (Jia et al., 1996), unstable place-field recordings (Yan et al., 2002), increased mortality, reduced exploration, impaired motor coordination and increased sensitivity to pentobarbital-induced anaesthesia. Neurons which usually express GluR2-containing AMPARs show decreased expression of AMPARs while cells usually expressing GluR2-lacking AMPARs show an increase in AMPAR expression (Petralia et al., 2004). Mice globally expressing GluR2 in its unedited form undergo seizures and do not live past 3 weeks

(Brusa et al., 1995) and mice over expressing GluR2 in hippocampal interneurons show reduction of long-range synchrony (Fuchs et al., 2001), suggesting a role for CP-AMPARs in oscillatory precision.

Gross impairments resulting from GluR2 deletion or unediting can be minimised by restricting the deletion to specific brain areas. Mice lacking the GluR2 subunit in their hypothalamic and septal neurons show impairment in reproductive behaviour (Shimshek et al., 2006) When the deletion or expression of GluR2 (Q) is localised to the forebrain, mice show enhanced olfactory discrimination and learning but impaired olfactory memory. GluR2 expression in the piriform cortex and hippocampus is sufficient to rescue this impairment. However the enhanced olfactory discrimination and learning remains unaffected. Taken together, it is clear that CP-AMPARs play a special role in synaptic transmission: their expression, under both developmental and regional control, enables them to mediate exclusive forms of both short and long-term plasticity and allows them to perform specific roles in behaviour.

1.5 The cerebellum

Although this thesis addresses aspects of excitatory transmission at the PF-SC (PF-SC) synapse of the cerebellum, and the study of synaptic communication, at this level, is valuable *per se*, it is nevertheless tempting consider any findings in a wider context. Reciprocally, any broader knowledge of anatomy and function can help explain why certain characteristics of transmission might be appropriate at a particular synapse.

1.5.1 Cerebellar structure: cell types and connectivity

The cerebellum is attractive to study primarily because of its precise and geometric structure, as shown in **Figure 1.5A**, which has been well characterised since the work of Ramón y Cajal (Ramón y Cajal, 1888). It consists of the cerebellar cortex and three pairs of deep cerebellar nuclei.

Neuronal circuitry has a uniform structure over the entire cerebellar cortex where neurons are arranged into three layers: the granular, Purkinje cell and molecular layer (**Figure 1.5A**)(Eccles, 1967).

The cerebellum receives two major types of input: the climbing fibres and mossy fibres. Climbing fibres are the axons of inferior olive neurons while mossy fibres, which represent the vast majority of all afferent input to the cerebellum, originate from neurons of the brain stem, pons, and spinal cord. These both carry information to the deep nuclei and the cerebellar cortex. Climbing fibres enter the cerebellum and establish two branches, one to the deep nuclei and one to the Purkinje cells in Purkinje cell layer of the cerebellar cortex (**Figure 1.5B**), while mossy fibres branch to make contact with deep cerebellar nuclei and also granule and golgi cells within the granular layer. The cortex executes various computations to regulate the firing of Purkinje cells, which represent the sole output of the cerebellar cortex. Purkinje cells inhibit the deep cerebellar and vestibular nuclei and therefore regulate the extent to which the nuclei are activated by the mossy and climbing fibres (**Figure 1.5B**).

1.5.1.1 The granule cell layer

Upon entering the granular layer, mossy fibres branch extensively and make synaptic connections with dendrites of granule cells in specialised structures called glomeruli. Granule cells are the most abundant cell type in the cerebellar cortex. They have small spherical bodies of 5-8μM in diameter, an average of 4 short dendrites and are densely packed within the granular layer. In the centre of the glomerulus is an enlarged mossy fibre terminal (rosette) onto which up to 50 granule cell dendritic claws make contact (Hamori and Somogyi, 1983). Each dendritic claw originates from a different granule cell and makes on average three synaptic contacts upon a mossy fibre varicosity (Jakab and Hamori, 1988). Thus each granule cell receives on average four independent mossy fibre inputs. Conversely, there is large divergence in the mossy fibre–granule cell as one mossy fibre supplies excitatory synapses to 400–600 granule cells.

Golgi cells (Figure 1.5) are inhibitory interneurons and their axons also enter the glomerulus to make synaptic contacts upon granule cells, providing a means of feed-forward inhibition. Golgi cells are typically located in the top half of the granule cell layer, are sparsely distributed and easily distinguished due to their size (20-25µm diameter). Golgi cell–granule cell synapses produce significant cross talk with non-postsynaptic cells via spillover of GABA which may also play a role in regulating activity of granule cells (Rossi and Hamann, 1998; Chadderton et al., 2004).

Dendrites can also extend to the molecular layer (see below) where they receive PF input forming a negative feedback loop (Eccles et al., 1966b). Golgi cells also receive inhibitory synapses from Lugaro cells (see below) but not from Purkinje cells or other Golgi cells (Ito, 2006).

The granule cell layer also contains Lugaro (**Figure 1.5B**) cells (Sahin and Hockfield, 1990) and unipolar brush cells (Mugnaini and Floris, 1994). Comparatively little is known about these neurons, both in terms of their synaptic connections and their physiological activity. They can be distinguished by the shape and size of their cell bodies, their location within the granular layer and immunochemical markers.

Lugaro cells are fusiform shaped neurons, located at the top of the granular layer, just below the Purkinje cell bodies (Aoki et al., 1986) and (Sahin and Hockfield, 1990) Their relatively limited dendrites can extend either into the molecular, granular or Purkinje cell layer, making inhibitory contacts onto Golgi cells, SCs, basket cells but not Purkinje cells, and this varies from cell to cell. It is approximated that the cerebellar cortex contains one Lugaro cell and one Golgi cell for every 15 Purkinje cells. Also the axons of more than 10 Lugaro cells converge onto only one Golgi cell, while the axon of one Lugaro cell diverges onto 150 Golgi cells (Dieudonne and Dumoulin, 2000). Lugaro cells receive serotonergic input and discharge rhythmically at low frequencies (5-20 Hz) both *in vitro* and *in vivo*, providing oscillatory inhibitory input to their downstream targets, in particular the Golgi cells (Dieudonne and Dumoulin, 2000) and may play a role in synchronizing Golgi cell activity (Vos et al., 1999) .

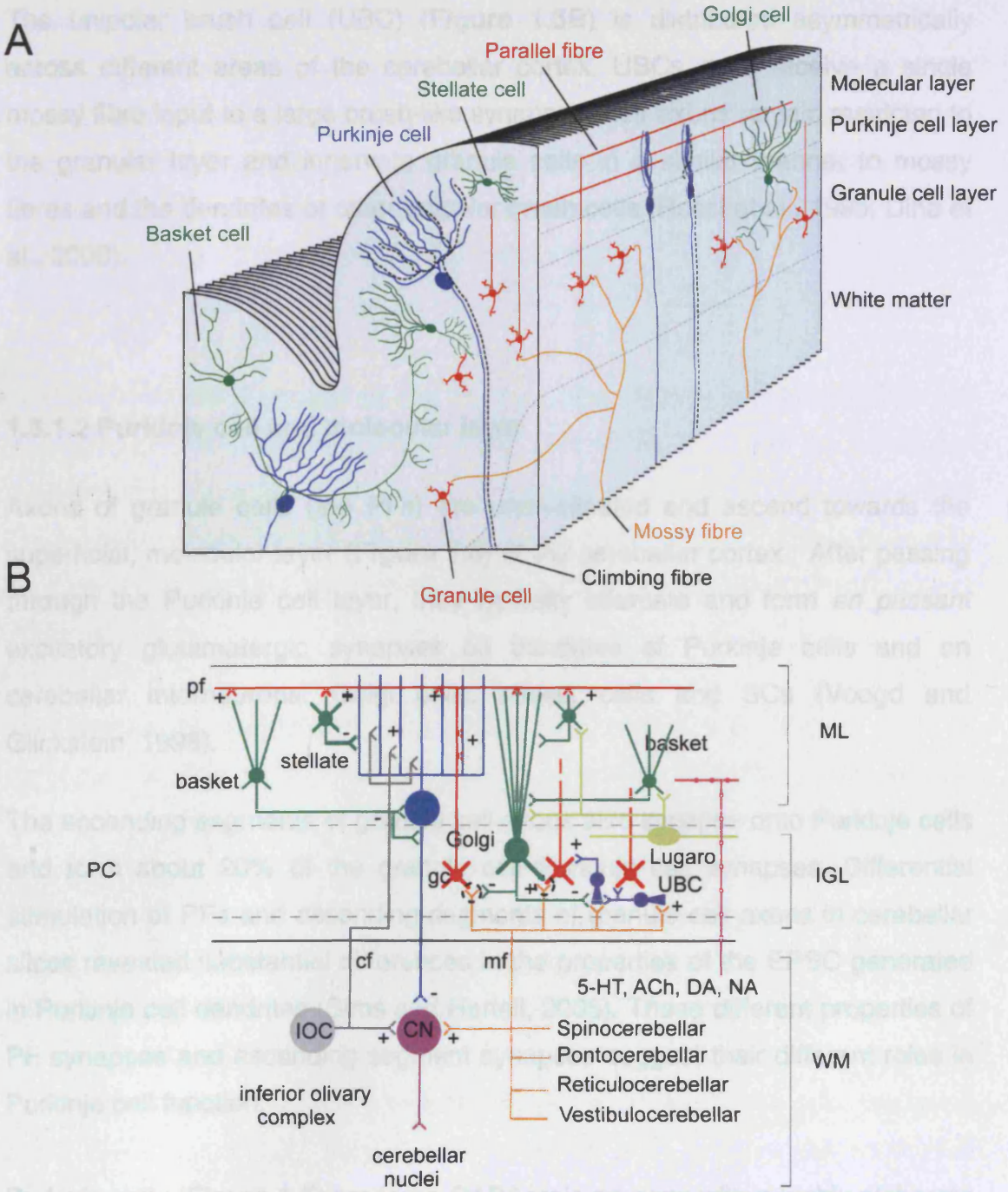


Figure 1.5 Circuitry of the cerebellum. **A**. Cross section of the cerebellar cortex showing the arrangement of different cell types within the various layers. **B**. Circuit diagram showing connectivity within the cerebellum. Molecular layer (ML), internal granular layer (IGL), white matter (WM), parallel fibre (pf), mossy fibre (mf), climbing fibre (cf), granule cell (gc), unipolar brush cell (UBC). Dashed circles represent glomeruli. 5-hydroxytryptamine (5-HT), Acetylcholine (Ach), dopamine (DA), noradrenaline (NA).

The unipolar brush cell (UBC) (**Figure 1.5B**) is distributed asymmetrically across different areas of the cerebellar cortex. UBCs each receive a single mossy fibre input to a large brush-like synapse. Their axons remain restricted to the granular layer and innervate granule cells in a similar manner to mossy fibres and the dendrites of other unipolar brush cells (Rossi et al., 1995; Dino et al., 2000).

1.5.1.2 Purkinje cell and molecular layer

Axons of granule cells (the PFs) are unmyelinated and ascend towards the superficial, molecular layer (**Figure 1.5**) of the cerebellar cortex. After passing through the Purkinje cell layer, they typically bifurcate and form *en passant* excitatory glutamatergic synapses on dendrites of Purkinje cells and on cerebellar interneurons: Golgi cells, basket cells and SCs (Voogd and Glickstein, 1998).

The ascending segments of granule cell axons also synapse onto Purkinje cells and form about 20% of the granule cell–Purkinje cell synapses. Differential stimulation of PFs and ascending segments of granule cell axons in cerebellar slices revealed substantial differences in the properties of the EPSC generated in Purkinje cell dendrites (Sims and Hartell, 2005). These different properties of PF synapses and ascending segment synapses suggest their different roles in Purkinje cell function.

Purkinje cells (**Figure 1.5**) are large GABAergic neurons with a highly elaborate dendritc tree which extends to the surface of the cortex. Neurons are arranged so that dendrites lie parallel, in the molecular layer, perpendicular to the PFs. Each Purkinje cell is innervated by a single climbing fibre, which entwines the main portion of dendritic tree (Llinas et al., 1975) - a consequence of the postnatal elimination of multiple innervation (Crepel et al., 1976; Hashimoto and Kano, 2003; Scelfo and Strata, 2005). Each climbing fibre forms numerous synaptic contacts with the dendrites of a single Purkinje cell (e.g., 26,000 in rat; (Nieto-Bona et al., 1997) which results in a particularly large EPSP

superimposed with Ca^{2+} spikes – a complex spike. In contrast, the excitatory convergence of PF/ascending limb synapses received by Purkinje cells is the largest known in the nervous system: up to 175,000 per Purkinje cell in rat (Napper and Harvey, 1988). Each PF is estimated to make one or two synaptic contacts upon every second or third Purkinje cell dendrite that it passes (Palay and Chan-Palay, 1976; Napper and Harvey, 1988).

Reciprocal inhibition occurs among Purkinje cells *via* their recurrent collaterals. The latter extend to neighbouring Purkinje cells within 300 µm of the parent cell (Hawkes and Leclerc, 1989). Axon collaterals of Purkinje cells also inhibit basket cells, which, in turn, inhibit Purkinje cells. Therefore, Purkinje cells may be involved in a mixed reciprocally inhibitory network containing Purkinje cells and basket cells.

Two classes of interneurons, the stellate and basket cells (**Figure 1.5**), lie amongst the PFs and Purkinje dendrites, and their dendritic processes are located exclusively within the molecular layer. Both cell types receive excitatory synaptic inputs *via* PFs and exert feed-forward inhibition of Purkinje cells (Mittmann et al., 2005). The two classes of neurons are closely related and are sometimes considered as a continuum (Sultan and Bower, 1998). The main difference between basket and stellate cells is their dendritic arborisation within the molecular layer (Palay SL & Chan-Palay V., 1974). Basket cells are mainly located in the deep third of the molecular layer, whereas stellate cells are found much more superficially. Basket cells innervate both Purkinje cell soma and axons though a basket shaped axonal plexus which surrounds the Purkinje cell body. Pinceux extensions of the plexus innervate the initial segment of the same Purkinje cell (Ramon Y Cajal, 1911). Stellate cell terminals usually end on Purkinje cell dendrites, however, sometimes they have descending collateral axons which end close to Purkinje cell bodies. It remains to be fully determined, however, as to whether basket and stellate cells are of distinct lineage or a result of external cues at the time of differentiation (Baader et al., 1999); (Yamanaka et al., 2004). Basket cells have been shown to preferentially express the hyperpolarization-activated cyclic nucleotide-gated cation channel

subunit HCN1 (Lujan et al., 2005). Also, differences between stellate and basket cells have been found in the decay kinetics of axonal Ca_i signals at early developmental stages (Collin et al., 2005). Recently it has been found that eliminating α1 subunit specifically reduced stellate cell synapses onto Purkinje cell dendrites by 75% whereas basket cell-Purkinje cell synapses were left unaffected (Fritschy et al., 2006) suggesting that there are further more subtle, non-morphological differences between these molecular layer interneurons.

Purkinje cells form, the pivotal elements around which all the cerebellar circuit is organised. They receive extracortical synaptic information which they process and channel towards cortical efferent pathways - the deep cerebellar nuclei and the brain stem nuclei, mainly the vestibular nuclei (Ito et al., 1964).

1.5.2 Theories about cerebellar function

The predominating view of the cerebellum is that it acts as a coordination centre, using sensory inputs from periphery to improve movement and balance. It was known from early lesion studies (Holmes, 1939) that the cerebellum played a crucial role in motor control and its malfunction is associated with ataxia (Duenas et al., 2006; Schmahmann, 2004) specifically, difficulty initiating and concluding movements. Early theoretical proposals about the operation of the cerebellar function (Marr, 1969; Albus, 1971), which heavily influenced ideas about its function, suggested that patterns of motor activity are represented in the PFs and that Purkinje cells act as a pattern recognition device. Any error in motor output is thought to be conveyed to the cerebellum *via* the climbing fibres, resulting in complex spike activity in Purkinje cells. This results in an activity-dependent modification of the PF-PC synapse, which represents the memory element. This idea was confirmed experimentally when CF activation was shown to induce LTD of PF inputs (Ito and Kano, 1982). Extensive experimental evidence has since been, and continues to be gathered about the involvement of complex spike activity in support of climbing fibre-driven LTD at PF synapses (Ito, 2001; Hartell, 1996; Reynolds and Hartell, 2000; Hartell, 2002) especially within the context of experimental paradigms for motor learning such as the gain modulation of vestibular ocular reflex (De

Zeeuw and Yeo, 2005) and the classical conditioning of eyelid responses (Mauk et al., 1998) which are both disrupted by lesions of the cerebellum (Ito et al 1982, (McCormick and Thompson, 1984)).

Because of the simplicity of its architecture and the involvement of PF-PC LTD in certain forms of motor learning it is tempting to underestimate the complexity of the cerebellum both in terms of role and function. However, several issues need to be explored and resolved. Most synapses in the cerebellum have since been shown to undergo plasticity (Hansel et al., 2001), and long-term changes in PF–PC synaptic efficacy, both potentiation (Lev-Ram et al., 2002) and depression (Hartell, 1996), can occur in the absence of climbing fibre activation. Also mice with impaired or absent PF LTD can still form motor memories (De Zeeuw and Yeo, 2005).

Classical theories for the operation of the cerebellum (Marr, 1969; Albus, 1971) rely on the idea that information is spread along beams of PF activity. Although this can be achieved *via* direct stimulation of the molecular layer, in fact stimulation of the periphery or mossy fibre leads to spatially restricted patches of activity (Eccles et al., 1972; Bower and Woolston, 1983; Kolb et al., 1997; Cohen and Yarom, 1998). These observations are inconsistent with the idea that granule cell activity leads to the synchronised firing of PCs with shared input, but rather provides foundation for the idea that synapses formed by the ascending segment of the axon may carry greater synaptic weight than those formed by PFs (Sims and Hartell, 2005). Indeed ascending axon synapses have higher mean release probability and larger mean quantal amplitude than PF synapses. They are also selectively resistant to cerebellar LTD, implying an exclusive role within the cerebellum (Sims and Hartell, 2005).

Also, anatomical connections between the cerebellum and brain structures such as the hypothalamus (Haines et al., 1984) and prefrontal cortex (Kelly and Strick, 2003) suggest that the cerebellum may have a role in emotion and contribute to higher cognitive processing. Indeed, LTP has been found to occur between PFs and Purkinje following fear conditioning (Sacchetti et al., 2004) and cerebellar cognitive affective syndrome patients, with lesions confined to the

cerebellum display impairments in executive function, spatial cognition and linguistic processing which can occur in the absence of the cerebellar motor syndrome (Schmahmann and Sherman, 1998). The cerebrocerebellar communication loop found in the prefrontal cortex, supports the idea that the cerebellum provides a forward model for higher mental functions (Ito, 2006).

1.5.3 Development

Histogenesis of the cerebellum is largely postnatal (for reviews, see (Altman and Bayer, 1978;Wang and Zoghbi, 2001). In rodents postnatal development occurs in two main phases: the neonate and infantile phase which occurs from birth/postnatal day 0 (P0) to P21 where cell growth, migration and synapse formation occurs and the juvenile and young adult phase, which extends from P21-P60, during which more subtle refinement of circuitry takes place.

The cerebellum develops from the dorsal region of the posterior neural tube. Cells arise from two germinal matrices to radiate laterally and evolve into the deep cerebellar nuclei and Purkinje cells of the cerebellar cortex. The first cells form the deep cerebellar nuclei. The ventricular zone then produces cells that eventually form the Purkinje neurons. These proto-Purkinje cells send dendrites to the PFs of the granule neurons. The full number of Purkinje cells is present early on, but their mature monolayer forms postnatally. Purkinje cells continue their maturation after birth, projecting to the deep cerebellar nuclei and refining the input they receive from the climbing fibres of inferior olivary neurons.

Unlike most of the cell types of the cerebellum, which are born at the ventricular zone, cerebellar granule neurons come from a specialised germinal matrix called the rhombic lip. In the mouse embryo, granule cell precursors exit the rhombic lip and start their migration by E13. By E16 most of the cerebellar surface is covered by granule cell precursors, constituting the external granule cell layer (EGL). Granule cells migrate from the EGL, leaving their axons to form the molecular layer, passing through the Purkinje cell layer into the internal granule cell layer.

There is very little or no molecular layer during the first few days after birth. Between P1 and P14, the ventricular zone gives rise to stellate and basket interneurons (Miale and Sidmman, 1961). Stellate/basket cell progenitors proliferate in the white matter during the course of migration from the ventricular zone to the molecular layer, where they then differentiate into a variety of cortical interneurons to reach their final location between P7 and P21 (Altman and Bayer, 1978;Zhang and Goldman, 1996). In the rat cerebellum, cells in the inner half of the ML (presumed basket cells) are generated between P2/3 and P16/17 with a peak at P6/7, and cells in the outer half of the ML (presumed SCs) are produced between P4/5 and P18/19 with a peak at P8/11 (Yamanaka et al., 2004; Altman and Bayer, 1978)

1.5.4 The role of stellate cells within the cerebellum

1.5.4.1 General features of stellate cells

Cerebellar SCs are small, aspiny inhibitory interneurons which receive excitatory inputs from granule cell axons PFs, and inhibitory inputs from other SCs (Palay SL & Chan-Palay V., 1974; Midtgaard, 1992b; Kondo and Marty, 1998). SC axons are either confined to the proximal area of their cell bodies (30-40 µm) or they can extend up to 450 µm from the soma (Palay SL & Chan-Palay V., 1974; Sultan and Bower, 1998) forming a dense interconnected population which mediate inhibition to Purkinje cell dendrites (Eccles et al., 1966b)(see below). They are also interconnected through electrical synapses which allow them to synchronise their activity (Mann-Metzer and Yarom, 1999). SCs fire at high frequencies (>10 Hz) both *in vivo* and *in vitro*. Firing can be periodic (Midtgaard, 1992a; Hausser and Clark, 1997; Carter and Regehr, 2002), irregular (Jorntell and Ekerot, 2003) or bursting (Mann-Metzer and Yarom, 2002b).

SCs are small with high input resistance, therefore excitatory and inhibitory quantal events produce significant changes in membrane potential (Carter and Regehr, 2002). Only small numbers of coincident quanta are needed to trigger an AP (Carter and Regehr, 2002). SCs exhibit several intrinsic membrane

conductances at the resting membrane potential, including I_h (Crepel et al., 1976; Hashimoto and Kano, 2003; Midtgaard, 1992a), and a non-inactivating Na^+ current (Mann-Metzer and Yarom, 2002a) which contribute to determining the duration of the train of APs induced by an excitatory current.

In addition to synaptic AMPA and GABA receptors that mediate fast transmission, SCs express a variety of receptor types at different locations. Activation of extrasynaptic receptors usually leads to changes in SC firing while activation of receptors located on SC terminals results in altered release of GABA (see below). SC firing can be increased by activation of postsynaptic mGluR1α (Karakossian and Otis, 2004) and β-adrenergic (Kondo and Marty, 1998) receptors, whereas activation of postsynaptic $GABA_B$ receptors leads to a reduction in firing rate (Mann-Metzer and Yarom, 2002b). SC terminals express $GABA_B$ (Mann-Metzer and Yarom, 2002b), $GABA_A$ (Mejia-Gervacio and Marty, 2006), AMPA (Satake et al., 2004), and NMDA receptors (Harvey et al., 2004). Activation of these receptors, either homo- or heterosynaptically, leads to alterations in transmitter release. It is worth mentioning that SCs show a high level of expression of mRNA for the GluR7 subunit (Bettler et al., 1992; Wisden and Seeburg, 1993) although no functional kainate receptors have been found in these cells.

1.5.4.2 PF-SC synapse

Characteristics of synaptic transmission

Synaptic transmission at the PF-SC synapse is mediated solely by AMPARs. However, upon high frequency PF stimulation, extrasynaptic NMDA receptors and mGluR1α can also be recruited (Clark and Cull-Candy, 2002; Karakossian and Otis, 2004). SC evoked EPSCs are characterised by their large amplitude, high failure rate, variable latency and significant asynchrony (Rancillac and Barbara, 2005; Atluri and Regehr, 1998). Synaptic reliability is low in response to single stimuli, however short bursts of presynaptic activity are more effective and PF-SC synapses are reliable at frequencies above 60 Hz (Rancillac and Barbara, 2005), consistent with patterns of granule cell activity *in vivo*, which

can involve short high-frequency bursts (Eccles, 1967; Chadderton et al., 2004).

1.5.4.3 Plasticity at the PF-SC synapse

Short term plasticity

PF-SC synapses show paired-pulse facilitation (Atluri and Regehr, 1998). It is also likely that use-dependent relief of polyamine block of CP-AMPARS (Rozov and Burnashev, 1999) also mediates a form of short-term facilitation at this synapse, however this has yet to be determined. Low-frequency stimulation of PFs can result in short term facilitation caused by activation of presynaptic kainate receptors and facilitation switches to depression upon high–frequency stimulation (Delaney and Jahr, 2002). Interestingly, both high and low-frequency stimulation continues to facilitate PF-Purkinje cell synapses reflecting the synapse specificity of kainate receptor modulation of glutamate release from PFs. High-frequency trains of stimuli can also lead to a form of short term depression at the PF-SC synapse which is mediated by the release of endocannabinoids (from an unidentified source) and activation of presynaptic CB1 receptors (Rancillac and Barbara, 2005).

Long term plasticity

Although attention has previously been focused on cerebellar long term plasticity at the PF-PC synapse, recent investigations have shown the PF-SC synapse can also store information in the long term. It seems that PF-SC synapses can undergo both LTP and LTD, however results vary depending on the protocol used. Both high- (8Hz) and low-frequency (2Hz) stimulation of PFs can induce LTP as a result of different mechanisms (Rancillac and Crepel, 2004). Low-frequency stimulation induces a form of LTP which is thought to be presynaptic and NO-dependent. When low frequency stimulation is paired with SC depolarisation, the resulting LTP can be blocked by both AP5 and

intracellular BAPTA, suggesting some post-synaptic involvement. On the other hand, LTP induced by high frequency stimulation is dependent on cAMP and NO, suggesting some post-synaptic mechanism. However the site of this form of LTP remains inconclusive as one cannot exclude the involvement of retrograde messengers.

Two forms of LTD have also been described at this synapse. One is induced by low-frequency (2 Hz) stimulation and requires the activation of mGluRs. However, it does not require a rise in intracellular [Ca^{2+}], or CB1R activation (Rancillac and Crepel, 2004). While this form of LTD is suggested to be presynaptic, there is little strong evidence. The other form of LTD described at this synapse is induced by repetitive stimulation (4 x 25 stimuli at 30 Hz, delivered at 0.33 Hz) and also involves mGluR activation. This form *is* dependent on a rise in intracellular [Ca^{2+}] and CB1R activation. This form of plasticity is presynaptic and input specific, reflecting the ability of SCs to compartmentalise Ca^{2+} even in the absence of spines (Soler-Llavina and Sabatini, 2006). Clearly, these different stimulation protocols highlight the ability of the PF-SC synapse to undergo long-term changes by a variety of different mechanisms.

Long-term plasticity at this synapse has also been confirmed *in vivo* (Jorntell and Ekerot, 2002; Jorntell and Ekerot, 2003). While burst stimulation of a PF bundle, unpaired with climbing fibre activity, induces a long-lasting decrease in the size of receptive fields of inhibitory interneurons, it causes a large increase in the size of receptive fields in Purkinje cells. Conversely, PF stimulation paired with climbing fibre activity induces a long-lasting increase in the size of receptive field in interneurons, but a decrease in the size of receptive fields in Purkinje cells. Thus, it appears that PF- interneuron synapses undergo LTP complementary to LTD in Purkinje cells and vice versa.

Activity dependent change in AMPA subunit composition

Aspects of the work described in this thesis relate to previous observations showing that high frequency simulation at the PF-SC synapse induces a lasting

switch in subunit composition and Ca^{2+} permeability of postsynaptic AMPARs (**Figure 1.4B**)(Liu and Cull-Candy, 2000b). The rapid replacement of Ca^{2+}-permeable receptors by ones containing GluR2 results in an EPSC of reduced amplitude at negative potentials, reflecting the insertion of receptors with a lower mean channel conductance. In contrast the EPSC is increased at depolarised potentials reflecting the presence of receptors that are no longer blocked by intracellular polyamines. EPSC rectification correlates well with the intrinsic level of synaptic activity in the SC population (Liu and Cull-Candy, 2002), suggesting that similar plasticity changes can occur *in vivo*. It has been shown that Ca^{2+} influx through GluR2-lacking AMPARs drives the insertion of GluR2-containing AMPARs into the membrane, implying a self-regulating mechanism.

Recent experiments have examined the trafficking of CP-AMPARs with regards to this form of plasticity (Liu and Cull-Candy, 2005a; Gardner et al., 2005b). From the subunits known to be present (Petralia and Wenthold, 1992), it seems likely that the switch in receptor subtype involves the replacement of homomeric GluR3 assemblies with heteromeric GluR2/3 assemblies. Block of AMPAR interactions with the intracellular protein partners PICK1 or NSF (Collingridge et al., 2004; Bredt and Nicoll, 2003) has little effect on cerebellar SC mEPSCs, whereas inhibiting GRIP produces a clear rundown in mEPSC amplitude (Liu and Cull-Candy, 2005a). This reduction occurs only at negative potentials, when CP- AMPARs contribute to the current, implying that neither PICK nor NSF are required for basal delivery of AMPARs, but that GRIP is required for the anchoring of the CP-AMPARs at the synapse. By contrast, both NSF and PICK1 binding are required for the activity-dependent delivery of GluR2-containing AMPARs (Gardner et al., 2005a; Liu and Cull-Candy, 2005a). Block of GRIP during high-frequency stimulation increases the loss of CP-AMPARs suggesting that Ca^{2+} entry through AMPARs leads to their dissociation from GRIP and subsequent loss from the synapse (Liu and Cull-Candy, 2005b). Surprisingly, the interaction between GRIP and CI-AMPARs appears unaffected by activity.

It seems that the switch in AMPAR subtype in SCs, involves selective disruption of the interaction between GRIP and GluR2-lacking AMPARs, and

that PICK and NSF regulate the concomitant activity-dependent delivery of GluR2-containing receptors. Experiments on mutant mice (lacking the last 7 amino acids in the C-terminus of GluR2 or GluR3, and knockouts for PICK1, GRIP1 –2, and GluR3 and -4), support the view that GluR2 PDZ ligand, and GluR2-PICK interaction are required for the activity dependent delivery of GluR2-containing AMPARs, but that the C-terminus of GluR3 is not needed (Gardner et al., 2005b). These results, together with findings that extrasynapstic receptors in these cells are largely GluR2-containing (Liu and Cull-Candy, 2002), suggest that extrasynaptic CI-AMPARs may act as a reservoir for rapid synaptic delivery during activity.

1.5.4.4 SC impact on Purkinje cell output

Activation of basket/SCs induces powerful IPSPs in Purkinje cells (Eccles, 1967). Purkinje cells *in vivo* fire spontaneously at a highly irregular rate (Eccles, 1967). This irregular discharge is caused not by excitatory synaptic drive but by tonic influence from inhibitory interneurons (Hausser and Clark, 1997). As a consequence, both excitation and feed-forward inhibition are superimposed on a background of activity, and can act not only to initiate individual spikes, but also to accelerate or delay spontaneous spikes. The proximity of the membrane potential to threshold means that even small input signals can influence spike output (Hausser and Clark, 1997; Carter and Regehr, 2002).

SC firing can have important consequences for cerebellar function (Eccles et al., 1966a; Hausser and Clark, 1997; Midtgaard, 1992b). It shapes the magnitude and spatial extent of calcium signals in Purkinje cell dendrites and even single spikes in SCs have been shown to significantly delay Purkinje cell firing (Hausser and Clark, 1997). Because PCs and SCs share a joint source of excitation, the local axons of SCs can provide each Purkinje cell with inhibition, which is proportional to the amount of its excitation (De and Bower, 1994) and the temporal organisation of the responses of both neurons dictate their interrelated output (Mittmann et al., 2005). The width of Purkinje cell dendritic trees is in the order of 200 µm corresponding to one 'beam' of PFs. Purkinje cells which are excited by PF input are inhibited by interneurons receiving the

same PF input (Eccles 1967). The length and orientation of the longer axons of the molecular layer interneurons enable them to induce lateral or off-beam as well as feed-forward or on-beam inhibition (Eccles et al., 1967). However, in view of the fact that mossy fibre activation is unlikely to lead to beams of Purkinje cells activity (Cohen and Yarom, 1998), the role of the long-range axons of the molecular layer interneurons remains to be elucidated.

In the presence of a continuous baseline of inhibitory activity, Purkinje cell spiking is suppressed by synchronized inhibitory inputs, and precisely timed spikes are triggered by transient pauses in inhibitory input (Jaeger and Bower, 1999). The overall effect of SC interconnections could lead to an increase in granule cell triggered spike correlations, or a limitation in excitatory responses, or both. In turn, the resulting spike pauses in Purkinje cells could be to lead to well-timed spike responses in the deep cerebellar nuclei (Gauck and Jaeger, 2000).

1.3.4.5 Modulation of inhibition

Much of the work in this thesis is aimed at understanding transmission at the PF-SC synapse, which is a major determinant of SC output and therefore PC inhibition. However, SCs can influence inhibition of PCs through a number of other mechanisms which should not be overlooked. A number of forms of plasticity lead to altered inhibition of Purkinje cells. Changes may be short or long- lasting, and are usually mediated by a retrograde messenger or by spillover of neurotransmitter to SC terminals to altering release of GABA.

For example, endocannabinoids, released from Purkinje cells as a result of their depolarisation (Diana et al., 2002), act as retrograde messengers to activate presynaptic CB1 receptors on SC terminals resulting in inhibition of GABA release for tens of seconds. Group 1 mGluR activation on Purkinje cells also leads to cannabinoids release and reduced release of GABA from SCs.

Glutamate can also act as a retrograde messenger at the SC-Purkinje cell synapse. Activation of presynaptic NMDA receptors by glutamate released from PCs leads to presynaptic Ca^{2+}entry and release of Ca^{2+} from ryanodine-sensitive stores. This in turn increases the rate of vesicular GABA release for around 15 min. (Harvey et al., 2004). Activation of presynaptic NMDARs can also lead to a longer-lasting increase in transmitter release. This requires the presence of glycine but the source glutamate remains unknown (Liu 2006). Conversely, spillover of glutamate from climbing fibres activating presynaptic AMPARs, reduces the release of GABA. This is thought to be *via* a metabotropic-like action (Satake et al., 2006).

Inhibition from interneurons to PCs can also be increased via activation of adrenergic, 5HT and group 1 mGluRs receptors (Mitoma et al., 1994; Mitoma and Konishi, 1999; Kondo and Marty, 1998; Llano and Gerschenfeld, 1993a; Karakossian and Otis, 2004) Depending on the amount of NA released into the BC–PC synapse, the resulting presynaptic β-adrenoceptor-mediated modulation of GABA can be shifted from short-term to long-term potentiation (Saitow et al., 2005).

Inhibition may also be altered postynaptically. Co-activation of an inhibitory input together with the Purkinje cell results in input-specific suppression of a form of plasticity known a rebound potentiation (RP). RP, induced by PC depolarisation or activation of the CF (Kano et al., 1992) leads to enhanced $GABA_A$ receptor currents for a prolonged period as a result of postsynaptic receptor phosphorylation. However, co-activation of an inhibitory input and Purkinje cell specifically suppresses the active input but potentiates inactive inputs (Kawaguchi and Hirano, 2000), enabling an otherwise indiscriminate form of plasticity to show synapse specificity.

Interestingly, a role for interneurons in mediating cerebellar LTD at the PF-PC synapse has recently been found. It is suggested that SCs (rather than PFs) are the source of NMDA receptor-triggered NO production that is required for induction of cerebellar LTD (Shin and Linden, 2005). Coupled with the previously mentioned finding that a NO-dependent form of LTP may be

produced at PF-SC synapses (Rancillac and Crepel, 2004), cerebellar interneurons may ultimately serve to persistently attenuate Purkinje cell activity both as a result of LTD of PF-Purkinje cell synapses and by LTP of PF-interneuron synapses.

1.5.5 Summary

The combined properties of SCs and their synaptic inputs make these small interneurons well-suited to be coincidence detectors, performing rapid synaptic computations (Mittmann et al., 2005) to influence Purkinje cell firing. As quantal events shape SC firing (Carter and Regehr, 2002) and synaptic input even from single SCs can lead to PC spike suppression or a shift of Purkinje cell spike timing (Hausser and Clark, 1997), even modest changes at the PF-SC synapse could profoundly affect cerebellar output. Inhibition of Purkinje cells can also be modulated in response to changes in patterns of activity *via* plasticity at both PF-SC and SC-PC synapses thereby providing alternative sites for information storage. Although the full extent to which this plasticity and on/off beam inhibition may contribute to actual cerebellar function is not understood, it is clear that SCs play an important role by exerting powerful control over Purkinje cells, the sole output of the cerebellar cortex. Because Purkinje cells provide the sole inhibitory output from cerebellar cortex to the deep cerebellar nuclei, any increase in interneuron activity will ultimately disinhibit the deep nuclei and lead to an increase in cerebellar output.

1.6 Aim of thesis

The aim of this thesis was to examine a variety of hypothesis relating to the regulation of AMPAR subunit composition at the parallel fibre-stellate cell synapse, following the finding that at this synapse, high-frequency stimulation at the leads to a form of plasticity involving a change in AMPAR subtype (Liu and Cull-Candy, 2000a). Investigations were carried out using the patch-clamp technique by recording evoked, spontaneous and quantal AMPAR- mediated events from stellate cells as described in the Methods (**Chapter 2**).

Chapter 3 Investigates the possibility that there is a change in the contribution of GluR2 to the subunit composition of synaptic AMPARs during the maturation of the PF-SC synapse. This was determined by analysing the rectification properties of evoked EPSCs and estimating the conductance of single channels underlying the spontaneous EPSCs from animals during different stages of development.

Chapter 4 investigates whether group 1 mGluR activation can change the subunit composition of synaptic AMPARs at the PF-SC synapse, as has been observed in the ventral tegmental area (VTA)(Bellone and Luscher, 2005). In Purkinje cells it has been shown that $GABA_B$ receptor activation enhances the group 1 mGluR response (Hirono et al., 2001), therefore the possibility that $GABA_B$ receptor activation may also regulate the subunit composition of AMPARs at this synapse was also investigated. Given that in a number of brain regions, tonic activation of extrasynaptic receptors has been observed, experiments were carried out to test the possibility that mGluR and GABAB receptors at his synapse are tonically activated.

Chapter 5 Investigates the possibility that a previously undescribed type of synaptic current in stellate cells is mediated by AMPARs and is mediated by glutamate release from PFs.

The thesis concludes with a general discussion (**Chapter 6**) intended to highlight and unite the main findings.

2. Materials and Methods

2.1 Introduction

All data presented in this thesis were obtained from whole-cell patch-clamp recordings (Neher and Sakmann, 1992) of visually identified stellate cells in acute brain slices of the rat cerebellum.

2.2 Slice Preparation

All procedures were performed in accordance with UK Animals (Scientific Procedures) Act 1986. Coronal cerebellar slices (200μm) were made from the vermis of 18 day old (unless stated otherwise) Sprague-Dawley rats. Rats were deeply anaesthetized with isoflurane and decapitated. The head was immediately immersed in ice-cold (0-4ºC) 'slicing solution' in which Na^+ was partially replaced with sucrose (Table 2.3.1). The scalp and skull were cut with scissors and peeled back sequentially to reveal the brain. The brain was removed from the skull and placed in a beaker of fresh slicing solution. From this the brain was transferred to a Petri dish filled with ice-cold slicing solution where the cerebellum was dissected from the cerebrum with a razor blade. The cerebellum was then placed and fixed on the stage of a moving blade microtome (DTK-1000; Dosaka EM Company, Kyoto, Japan) for Chapters 3 and 5 and (650 V HM; Micron GmbH) for Chapter 4. Superficial pia matter and blood vessels were removed with fine forceps. Coronal cerebellar slices (200μm) were cut and transferred to a bathing chamber consisting of a glass beaker with a suspended sheet of surgical gauze, containing oxygenated slicing solution at room temperature. The slices were then kept at 32°C for 40mins. During the final 20mins of this period, the slicing solution was gradually exchanged with 'extracellular' solution with normal Na^+, also bubbled with 95% O_2 and 5%CO_2 (Table 2.3.2). Slices were then perfused with this solution at room temperature for a further 20min before recording.

2.3 Electrophysiology

2.3.1 Patch pipettes

Patch pipettes were made from thick walled borosilicate glass capillary tubing (GC-150F; Clark Electromedical, Pangbourne, UK, or Harvard Apparatus Ltd, Edenbridge, UK) on a two-stage puller (Narishige, Japan) on the day of use. The glass capillary tubing was lightly fire polished at both ends before pulling to minimize scraping of the AgCl coating on the connecting wire when mounting the patch pipette onto the pipette holder. In order to increase the thickness of the pipette walls and reduce their electrical capacitance, pipettes were coated with Sylgard (Dow Corning 184) which was cured by passing the tip into a heated coil. The resin increased the thickness of the pipette wall

2.3.2 Recording of SC EPSCs

Slices were viewed with a fixed stage upright microscope (Axioskop FS1; Zeiss, Welwyn Garden City, UK) using IR-DIC optics. Electrodes of 3-6 MΩ resistance were filled with intracellular solution (Table 2.3.3). Recordings were made with an Axopatch200A amplifier (Axon Instruments, Union City, CA). Whole-cell recordings were made at room temperature. Cells were chosen on the basis of their soma diameter (approximately 8-12μm) and location, which was in the superficial outer third of the molecular layer i.e. >60 μm from the Purkinje cell layer (Hausser and Clark, 1997);(Palay SL & Chan-Palay V., 1974) (**Figure 2.1.A**). In cell attached mode, cell attached spikes (Hausser and Clark, 1997) could often be observed (**Figure2.1B**). Whole-cell configuration (**Figure2.1C**) was achieved while clamping the cell at -60mV. Upon breaking into the cell numerous, large spontaneous IPSCs (Llano and Gerschenfeld, 1993) with a slow decay and more infrequent smaller EPSCs (Liu and Cull-Candy, 2000) could be observed (**Figure 2.1D**). The larger, more slowly decaying currents disappeared in the presence of (20μM) Bicuculline **(Figure 2.1E)**. Holding current was -20.70 ± 1.6pA at -60mV. Whole-cell capacitance and series

resistance were 5.67 ± 0.3pF and 12.8 ± 0.5 MΩ Series resistance was monitored repeatedly throughout each experiment and the experiment was discarded if it increased more than 30%. If the initial series resistance was >18 MΩ the cell was not used. Series resistance compensation was not used.

EPSCs were evoked by PF stimulation using a patch electrode (3-5 MΩ) filled with external solution placed in the molecular layer. Pulses of 60-99V were applied for 20-200 µsec at 0.5Hz. For the data presented in Chapter 3, synaptic currents were recorded at various holding potentials, for at least two minutes at each potential, which was changed at random. For the data presented in Chapter 4, EPSCs were evoked as above, then the cells were held at -60mV for a 10 min period without stimulation (during which 50µM DHPG was applied). Stimulation was then resumed as before. For experiments to assess the timecourse of DHPG action, EPSCs were continuously evoked at -60mV during application of the agonist. Antagonists (where used) were pre-applied for at least 20min before the start of the experiment (unless stated otherwise). For paired-pulse experiments, 2 stimuli with a 10ms interval were delivered at 0.3Hz. During experiments where baclofen (3µM) was applied, stimulation was continuous either at -60mV or at various potentials (depending on experiment). Events evoked in Sr^{2+} were all acquired at -60mV with a stimulus frequency of 0.5Hz.

To record spontaneous EPSCs whole-cell recordings were made at -60mV in the absence of stimulation. Miniature EPSCs were recorded similarly, but in the presence of 500nM TTX.

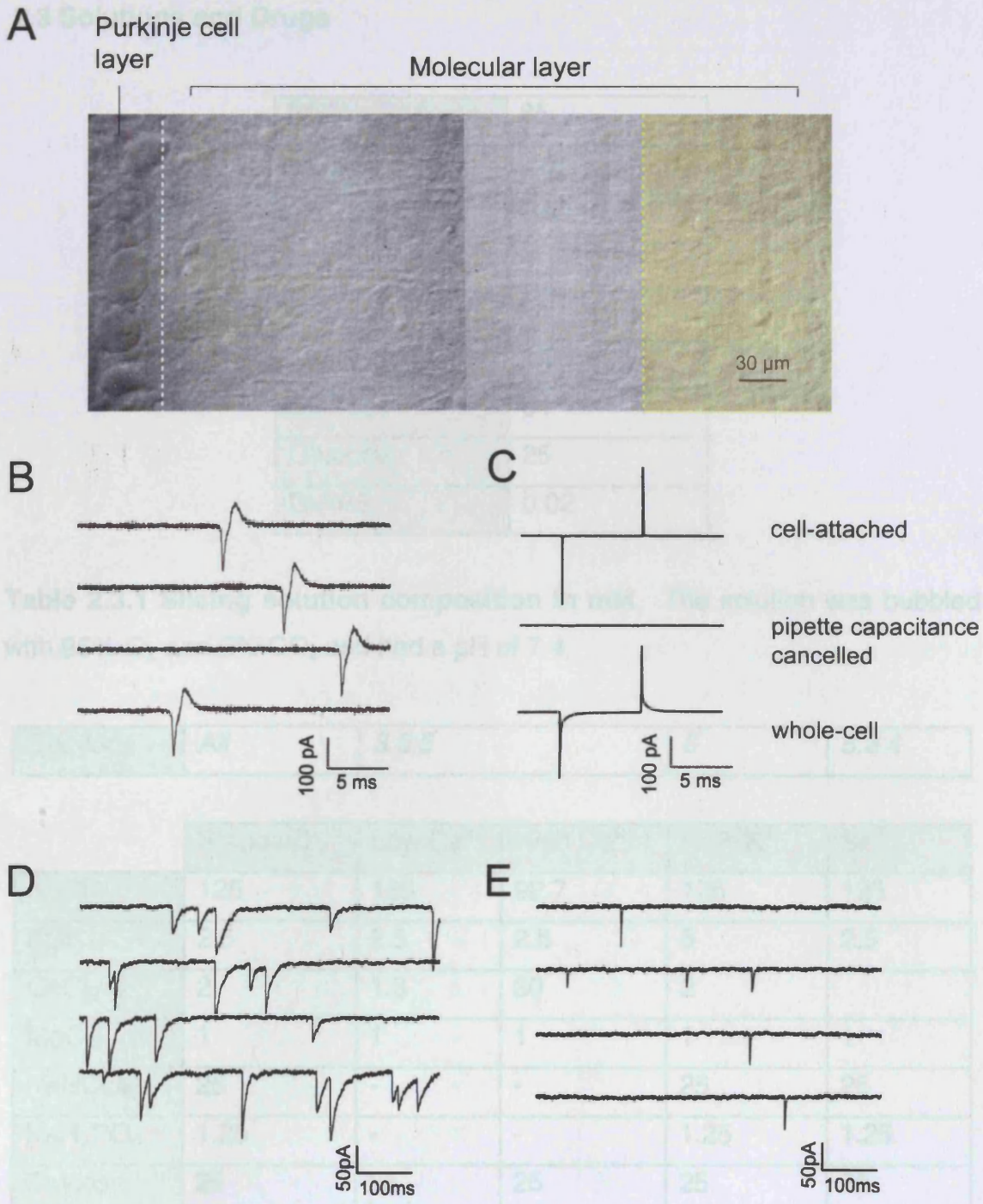


Figure 2.1. Identification of stellate cells. A. An example of the region of molecular layer selected for making recordings (highlighted in yellow). **B**. Cell-attached spikes observed in loose cell-attached mode. **C**. Mean of 100 sweeps in response to 5 mV step in different configurations. Once a seal has been achieved (cell-attached), the pipette capacitance is cancelled. Whole-cell configuration is achieved by light suction. **D**. Upon breaking into the cell, identification as a stellate cell is confirmed by the presence of frequent, large, spontaneous IPSCs. **E**. IPSCS are blocked in the presence of Bicuculline (20µM).

2.3 Solutions and Drugs

NaCl	85
KCl	2.5
$CaCl_2$	0.5
$MgCl_2$	4
$NaHCO_3$	25
NaH_2PO_4	1.25
Sucrose	64
Glucose	25
D-AP5	0.02

Table 2.3.1 Slicing solution composition in mM. The solution was bubbled with 95% O_2 and 5% CO_2 and had a pH of 7.4.

Sections	*All*	*3.3.5*	*5*	*5.3 4*

	Standard	Low Ca^{2+}	High Ca^{2+}	High K^+	Sr^{2+}
NaCl	125	135	92.7	125	125
KCl	2.5	2.5	2.5	5	2.5
$CaCl_2$	2	1.8	30	2	
$MgCl_2$	1	1	1	1	1
$NaHCO_3$	25	-	-	25	25
NaH_2PO_4	1.25	-	-	1.25	1.25
Glucose	25	25	25	25	25
HEPES	-	10	10	-	-
D-AP5	20	20	20	20	20
Bicuculine	20	20	20	20	20
Sr^{2+}	-	-	-	-	5

Table 2.3.2 Composition of recording solutions in mM. The solutions were bubbled with 95% O_2 and 5% CO_2 and had a pH of 7.4.

Section	*4*	*3, 5*	*4*

	Basic	Drugs	BAPTA
CsCl	140	128	140
HEPES	10	10	10
MgATP	2	2	2
$CaCl_2$	0.5	1	0.5
NaCl	4	2	4
EGTA (Cs)	5	10	-
BAPTA	-	-	10
TEA Cl	-	5	-
QX314	-	1	-

Table 2.3.3 Internal solution composition in mM. All solutions contained 100μM spermine unless stated otherwise (pH 7.4 with CsOH, 285 ± 5 mOsmol/l).

	Chapter	μM
DHPG	4	50
LY 367385	4	100
MPEP	4	10
AM251	4	1
Baclofen	4	3
CGP 52432	4	5
GYKI 53655	5	50

Table 2.3.4 Additional Drugs.

Salts were obtained from Sigma (Sigma-Aldrich Co Ltd., Poole, UK) and drugs were obtained from Tocris Bioscience (Bristol, UK), except GYKI 53655, which was a gift from David DiGregorio.

2.4 Analysis

2.4.1 I-V analysis

Evoked EPSCs were recorded directly to computer or onto digital audio tape (DAT120, Maxell Europe Ltd, Apley, U.K.) In both cases, for final analysis, signals were low pass filtered at 2 kHz and digitized at 20 kHz (pCLAMP8; Molecular Devices, Union City, CA). Waveforms were analysed using IGOR Pro V.5.0.3.0 (Wavemetrics, Lake Oswego, OR) and Neuromatic (http://www.physiol.ucl.ac.uk/research/silver_a/software). Events without a smooth rise were rejected. Averages at each holding potential were determined by aligning events at their 20% rise time. A minimum of 20 sweeps were used to derive each mean waveform, except for holding potentials between -10 and +10mV where events were rare with little variance. For Chapter 3, the mean EPSC amplitudes were plotted against membrane potential and fitted with a third order polynomial which was constrained to cross the X axis at the reversal potential. The rectification index (RI) was calculated by dividing the most positive current amplitude by the negative current value obtained at the membrane voltage equidistant from the reversal potential. Thus, for a linear I-V, RI=1, and for an inwardly rectifying response the RI is less than 1. In Chapter 4, mean EPSC amplitudes at +40 and -60mV were plotted against membrane potential and the I-V relationship fitted with a third order polynomial constrained to cross the abscissa at the mean reversal potential (-1.5 ± 0.3 mV; n = 33). The rectification index (RI) was calculated by dividing the amplitude of the EPSC obtained at the most positive voltage by the extrapolated amplitude of the EPSC at a membrane voltage equidistant from the reversal potential. As shown in Figure 2.1 the veracity of this approach was confirmed by comparing the RI values obtained by this method, with those obtained from fitting EPSC amplitudes obtained at multiple voltages (-60, -40, -20, +20, +40mV).

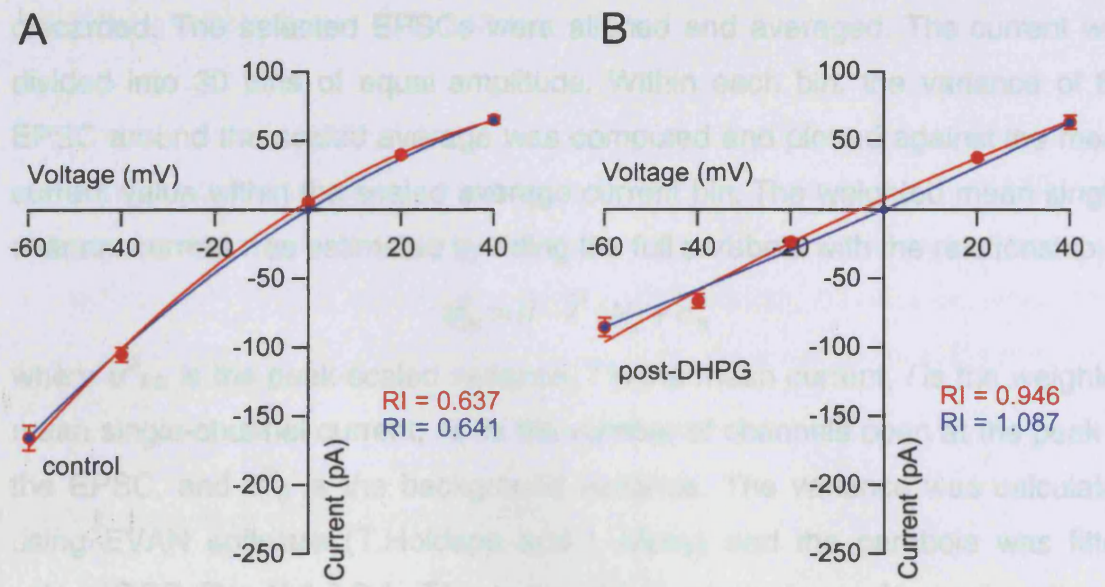


Figure 2.2 Method of determining RI. **A**. Representative current-voltage (I-V) relationship for evoked EPSCs obtained at membrane potentials between -60 and +40mV. Solid red circles show mean data and error bars show SEM. Red line shows an unconstrained third-order polynomial fit to data points (weighted according to the standard deviation). The RI for this I-V, calculated from the current at +40mV and the extrapolated current at a negative potential equidistant from the calculated reversal potential was 0.637. The blue line is a third-order polynomial fit using the values at +40mV and -60mV only, constrained to pass through the population reversal potential of +1.5mV. The RI calculated in this manner was 0.641. **B** Corresponding data from the same cell following 10 mins treatment with 50µM DHPG.

2.4.2 Noise analysis

Peak-scaled nonstationary fluctuation analysis was used to estimate the conductance of synaptic receptors (Traynelis et al., 1993). EPSCs recorded at -60mV were selected to exclude responses which were multi-quantal or contaminated with spontaneous synaptic activity. Only traces showing a fast monotonic rise time and uncontaminated decay were selected for analysis. The number of traces ranged from 11-105 (mean ± SEM = 44.6 ± 26.4). Selection was done initially by eye, then by examining the distributions of rise time and the time taken for the current to decay to 50% of its value (T_{50}). Restrictions on rise time (<0.27 ± 0.06ms) and T_{50} (<0.85 ± 0.26ms) were applied. If, after selection, data from a cell yielded insufficient waveforms, analysis was

discarded. The selected EPSCs were aligned and averaged. The current was divided into 30 bins of equal amplitude. Within each bin, the variance of the EPSC around the scaled average was computed and plotted against the mean current value within the scaled average current bin. The weighted mean single-channel current was estimated by fitting the full parabola with the relationship:

$$\sigma^2_{PS} = i\bar{I} - \bar{I}^2 / N_p + \sigma^2_B$$

where σ^2_{PS} is the peak-scaled variance, $\bar{I}$ is the mean current, i is the weighted mean single-channel current, N_P is the number of channels open at the peak of the EPSC, and σ^2_B is the background variance. The variance was calculated using EVAN software (T.Holdapp and I. Mody) and the parabola was fitted using IGOR Pro V.4.0.2.1. The mean chord conductance for each cell was calculated using Ohms Law and the calculated reversal potential for each cell. Spontaneous EPSCs were detected using a scaled template algorithm (Clements and Bekkers, 1997) implemented in IGOR pro/Neuromatic, and were analysed as for evoked EPSCs.

2.4.3 Predictions of shifts in E_{rev}

The predicted shift in reversal potential in 30mM Ca^{2+} was calculated using the equation:

$$P_{Ca}/P_{Na} = \frac{a_{Na}}{4a_{Ca}}\left(\exp\frac{(2V_{revCa} - V_{revNa})F}{RT} + \exp\frac{(V_{revCa} - V_{revNa})F}{RT}\right)$$

where a_{Na} and a_{Ca} represent the activities of Na^+ and Ca^{2+} in the extracellular solutions (0.88 and 064, respectively), and R, T, and F have their conventional meaning. V_{revNa} and V_{revCa} values were not corrected for liquid junction potentials of 3.9 mV and 6.6 mV, respectively (calculated using Clampex 7.0).

2.4.4 Paired-Pulse ratio calculation

For Chapter 4, failure rate was calculated by dividing the number of failures by the number of stimuli for a given time period. Paired-pulse ratio (PPR) was determined by dividing the amplitude of the second EPSC (A2) by the amplitude of the first (A1; PPR = meanA2/meanA1). In this case, amplitudes were taken from averages constructed using all events in a given condition without alignment.

2.4.4 Analysis of sEPSCs and mEPSCs

Events were detected using a scaled template algorithm (Clements and Bekkers, 1997) and aligned as for evoked EPSCs. For **Chapter 5**, a histogram of baseline noise was constructed and fitted with a Gaussian. Events of greater amplitude than 3X the calculated SD of the baseline noise were analysed. sEPSCs and mEPSCs and events evoked in the presence of Sr^{2+} were detected within a restricted or unrestricted time window (as stated). Histograms of 10-90% rise times were constructed and fitted with a Gaussian function. EPSCs were considered as slow-rising if their measured rise times were greater than the mean + 3 × SD of a Gaussian fit to the rise time distribution (0.01 ms bin width). All other EPSCs were grouped as fast-rising events. Once separated, these events were aligned and averaged.

2.4.5 Statistics

All average data are expressed as mean ± SEM. When data were distributed normally (Shapiro-Wilk test), statistical differences between groups were tested using the two-tailed Student's *t*-test and considered significant at $p < 0.05$. If data were not normally distributed the Mann-Whitney U test or Wilcoxon matched pairs test was used, depending on the data set being compared. All correlations were tested using Spearmann rank order correlation. All statistical tests were carried out using GraphPad PRISM 4.03 (GraphPad Software Inc, San Diego, CA).

Table 2.3.4 Drug names

Abbreviation	Full name
AM251	N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
Baclofen	(RS)-4-Amino-3-(4-chlorophenyl)butanoic acid
Bicuculline	[R-(R*,S*)]-5-(6,8-Dihydro-8-oxofuro[3,4-e]-1,3-benzodioxol-6-yl)-5,6,7,8-tetrahydro-6,6-dimethyl-1,3-dioxolo[4,5-g]isoquinolinium bromide
$CaCl_2$	Calcium Chloride
CGP 52432	3-[[(3,4-Dichlorophenyl)methyl]amino]propyl] diethoxymethyl)phosphinic acid
Cs4 BAPTA	1,2,-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetracesium salt (from Molecular Probes)
CsOH	Cesium Hydroxide
D-AP5	D-(-)-2-Amino-5-phosphonopentanoic acid
DHPG	3,5-Dihydroxyphenylglycine
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (from Fluka)
Glucose	D-glucose
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulphonic acid
KCl	Potassium Chloride
LY 367385	(S)-(+)-α-Amino-4-carboxy-2-methylbenzeneacetic acid
Mg ATP	Magnesium adenosine triphosphate
$MgCl_2$	Magnesium Chloride
MPEP	2-Methyl-6-(phenylethynyl)pyridine hydrochloride
NaCl	Sodium Chloride
$NaHCO_3$	Sodium bicarbonate
NaH_2PO_4	Sodium dihydrogen phosphate
QX-314	Lidocaine N-ethyl bromide
Spermine	N,N'-Bis(3-aminopropyl)-1,4-butanediamine tetrahydrochloride
Sr^{2+}	Strontium
TEA-Cl	Tetraethylammonium chloride
TTX	Tetrodotoxin; octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethano-10aH-[1,3]dioxocino[6,5-d]pyrimidine-4,7,10,11,12-pentol

Table 2.3.4 Frequently used abbreviations

AMPAR	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
CB1R	Cannabanoid receptor 1
CP-AMPAR	Calcium permeable AMPAR
CI-AMPAR	Calcium impermeable AMPAR
EPSC	Excitatory postsynaptic potential
E_{rev}	Reversal potential
GABA	γ-Aminobutyric acid
$GABA_BR$	Metabotropic γ-aminobutyric acid receptor
LTD	Long term depression
LTP	Long term potentiation
mEPSC	Miniature excitatory postsynaptic current
mGluR	Metabotropic glutamate receptor
PF	Parallel fibre
PPR	Paired-Pulse ratio
RI	Rectification Index
SC	Stellate cell
sEPSC	Spontaneous excitatory postsynaptic current

3. The developmental regulation of CP-AMPAR expression at the parallel fibre-stellate cell synapse

3.1 Summary

To investigate changes in subunit composition during development, three approaches were employed. Intracellular spermine was used as a 'diagnostic' tool to determine the contribution of GluR2 to native receptors. EPSCs were recorded from animals at different developmental stages (P8, P18 and P28). Evoked EPSCs became significantly less rectifying during development, suggesting a reduction in the relative proportion of CP-AMPARs

Peak-scaled nonstationary fluctuation analysis (PS-NFA) of EPSCs was carried out to estimate any associated change in single channel conductance. While analysis of evoked EPSCs showed no significant correlation between single channel conductance and development, analysis of spontaneous EPSCs, however, yielded estimates of single channel conductance that showed a significant decrease with development.

EPSCs were also recorded in conditions of low and high external calcium, to assess the calcium permeability of AMPARs at different stages of development by looking at shifts in reversal potential (E_{rev}).

3.2 Introduction

Throughout the CNS, CP-AMPAR expression is developmentally regulated both pre- (Lawrence and Trussell, 2000; Sugden et al., 2002; Batista et al., 2002; Furuta and Martin, 1999) and postnatally. CP-AMPARs can be developmentally upregulated (Caicedo and Eybalin, 1999; Chan et al., 2003; Jakowec et al., 1998;Johansson et al., 2000; Turman, Jr. et al., 2000) replaced by GluR-2containing receptors (Eybalin et al., 2004; Kumar et al., 2002; Seifert et al., 2000; Johansson et al., 2000; Sanchez et al., 2001a; Lei and McBain, 2002), or expressed transiently (Liu and Wong-Riley, 2005; Turman, Jr. et al., 2000). The precise developmental control of the expression of CP-AMPARs is thought to serve significant roles in cell growth and differentiation (Catsicas et al., 2001; Metzger et al., 1998), neuroprotection (Turman, Jr. et al., 2000; Friedman and Veliskova, 1998; Jensen et al., 1991) and ageing (Pellegrini-Giampietro et al., 1991;Pellegrini-Giampietro et al., 1992; Hof et al., 2002; Wang and Manis, 2005).

In SCs, high frequency stimulation leads to an activity-dependent replacement of CP-AMPARs by GluR2-containing receptors (Liu and Cull-Candy, 2002). The possibility that GluR2 containing receptors are incorporated into the PF-SC synapse with development was investigated. Three approaches were used to investigate the subunit composition of SC AMPARs at various developmental stages.

Intracellular spermine blocks CP-AMPARs lacking the GluR2 subunit at depolarized potentials, resulting in an inwardly rectifying I-V relationship (Bowie and Mayer, 1995; Kamboj et al., 1995; Koh et al., 1995; Burnashev, 2005). SCs express CP-AMPARS that show rectifying I-V relationships which become linear after high frequency stimulation (Liu and Cull-Candy, 2000), reflecting an activity dependent change in subunit composition and Ca^{2+} permeability. Spermine (100μM) was therefore used as a pharmacological marker to identify the contribution of the GluR2 subunit to synaptic AMPARs in animals of different ages.

Secondly, studies of recombinant AMPA receptors have shown that homomeric, as well as heteromeric, channels display multiple conductance levels depending on their subunit composition (Swanson et al., 1997; Rosenmund et al., 1998). Single-channel conductance has been found to be relatively high for CP-AMPARs compared with channels containing the GluR2 subunit (Swanson et al., 1997). The difference in conductance is thought to be attributable to editing at the Q/R site (Hollmann et al., 1991). In neocortical non-pyramidal neurons, RI is negatively correlated with mean single channel conductance (Angulo et al., 1997) suggesting that this difference holds true in native receptors (but see (Stern et al., 1999). Therefore any developmental change in subunit composition should also be reflected by a change in single channel conductance. Peak-scaled nonstationary variance analysis (Traynelis et al., 1993; Traynelis and Jaramillo, 1998) of EPSCS was used to investigate any developmental change in the weighted mean single channel conductance of synaptic AMPARs.

Thirdly, AMPARs lacking the GluR2 subunit show high Ca^{2+} permeability, whereas GluR2-containing receptors have low Ca^{2+} permeability (Hollmann et al., 1991). Previously the Ca^{2+} permeability of AMPARs has been assessed by making outside-out patches from the soma (Itazawa et al., 1997; Jonas et al., 1994; Otis et al., 1995). However, in SCs, somatic AMPARs differ in their subunit composition to those at the synapse (Liu and Cull-Candy, 2002). To directly investigate the Ca^{2+}-permeability of synaptic receptors, the shift in E_{rev} of synaptically evoked currents was examined during exchange of solutions with different calcium concentrations (Kumar et al., 2002; Otis et al., 1995).

3.3 Results

3.3.1 An age-dependent change in rectification

I-V relationships were constructed and RI calculated (see methods) using mean current amplitudes of evoked SC EPSCs from animals of different age groups. Whole cell recordings were made in the presence of NMDA and $GABA_A$

receptor blockers. **Figure 3.1A** shows examples of averaged evoked EPSCs and the corresponding I-V relationships from SCs of different ages. Note the presence of varying degrees of rectification. At P7-8 (n=23), evoked SC EPSCs typically showed a strongly rectifying I-V relationship signified by a low RI value (0.30±0.04). This suggests that in immature animals, the synaptic AMPA receptors lack GluR2 and are Ca^{2+}-permeable. **Figure 3.1.B** shows RI values for all cells In SCs of P18 animals (n =19) or older and P28 (n=8), evoked EPSCs typically had I-V relationships that were less rectifying, represented by higher RI values (0.55 ± 0.05 and 0.49 ± 0.06 respectively). Rectification values in P7-8 animals differed significantly from those at P17-18 and P28 (both P<0.01). There was a strong correlation between age and RI (P<0.05; Spearman Rank order correlation), suggesting that the GluR2 subunit is inserted as the animal develops. As expected, recordings made with no spermine in the patch pipette had linear I-V relationships (**Figure 3.2**).

3.3.2 Evoked EPSCs yield synaptic channel conductance estimates that do not correlate with age or RI

After determining that the relative proportion of CI-AMPARs increases with age, it was next examined whether the change in AMPAR subunit composition was accompanied by an expected decrease in channel conductance of the native receptors. Peak-scaled nonstationary variance analysis of the EPSCS was used to estimate the weighted mean single channel conductance of synaptic receptors. This method exploits the fact that there are random fluctuations in the number of channel openings associated with stochastic gating (Traynelis et al., 1993; Traynelis and Jaramillo, 1998). By scaling the peak of the mean EPSC waveform to the peak amplitude of individual EPSCs, one can calculate variance, due to channel closure, independently of variance arising from other factors.

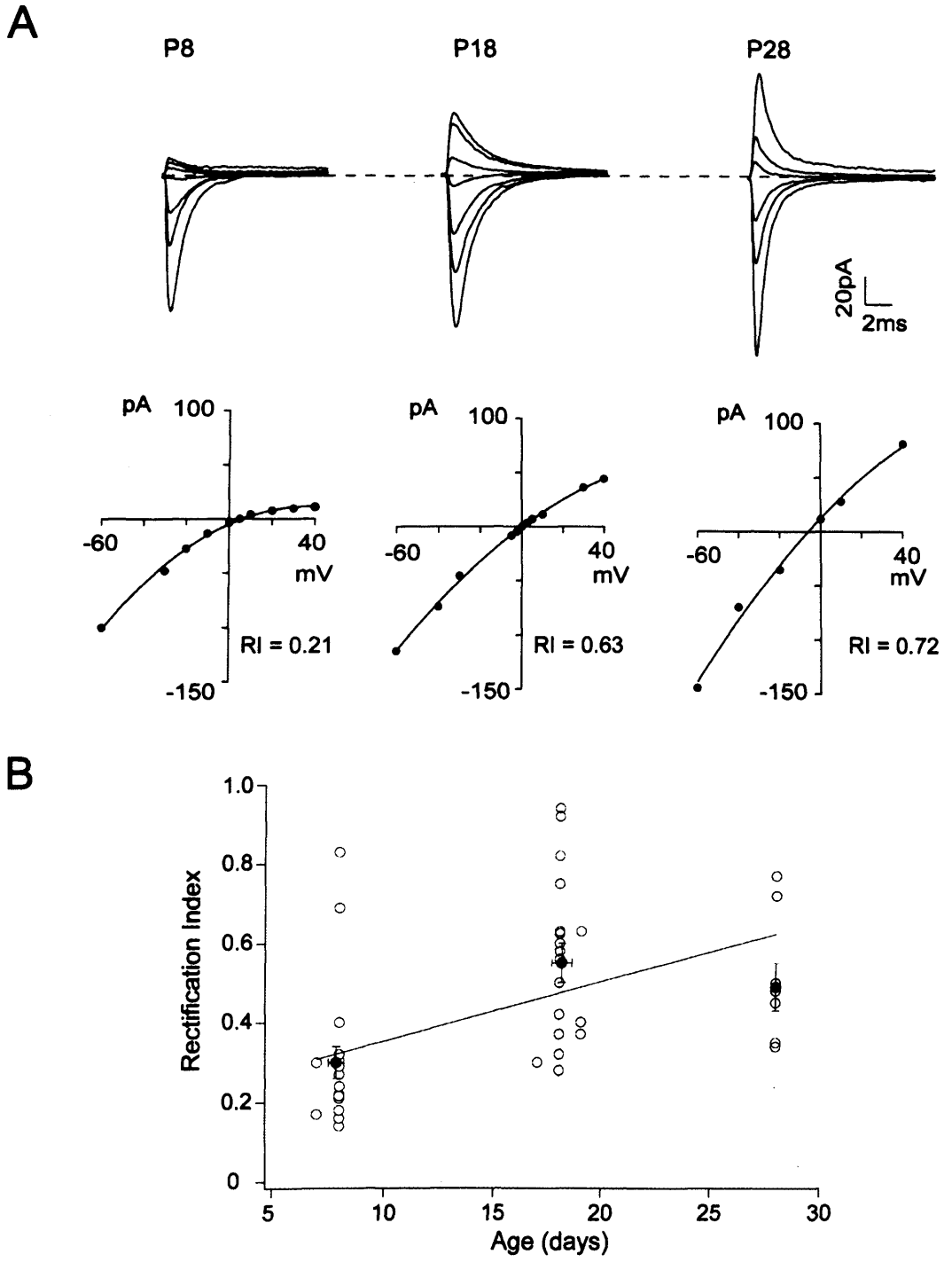


Figure 3.1 AMPAR rectification is greatest in young animals. A. Top panel shows evoked stellate cell EPSCs at various holding potentials from P8, P18 and P28 animals (upper panel) with corresponding I-V relationships (lower panel). I-V plots show varying degrees of rectification represented by different RI values. **B.** Mean RI values of evoked EPSCs from animals of various ages. Open symbols indicate individual values, solid symbols denote the mean x and y error bars showing SEM. Rectification at P8 was significantly greater (smaller RI) that at P18 or P28 (both $P<0.05$; Mann-Whitney U test). Overall there was a significant correlation with age ($P<0.05$;Spearman rank order correlation).

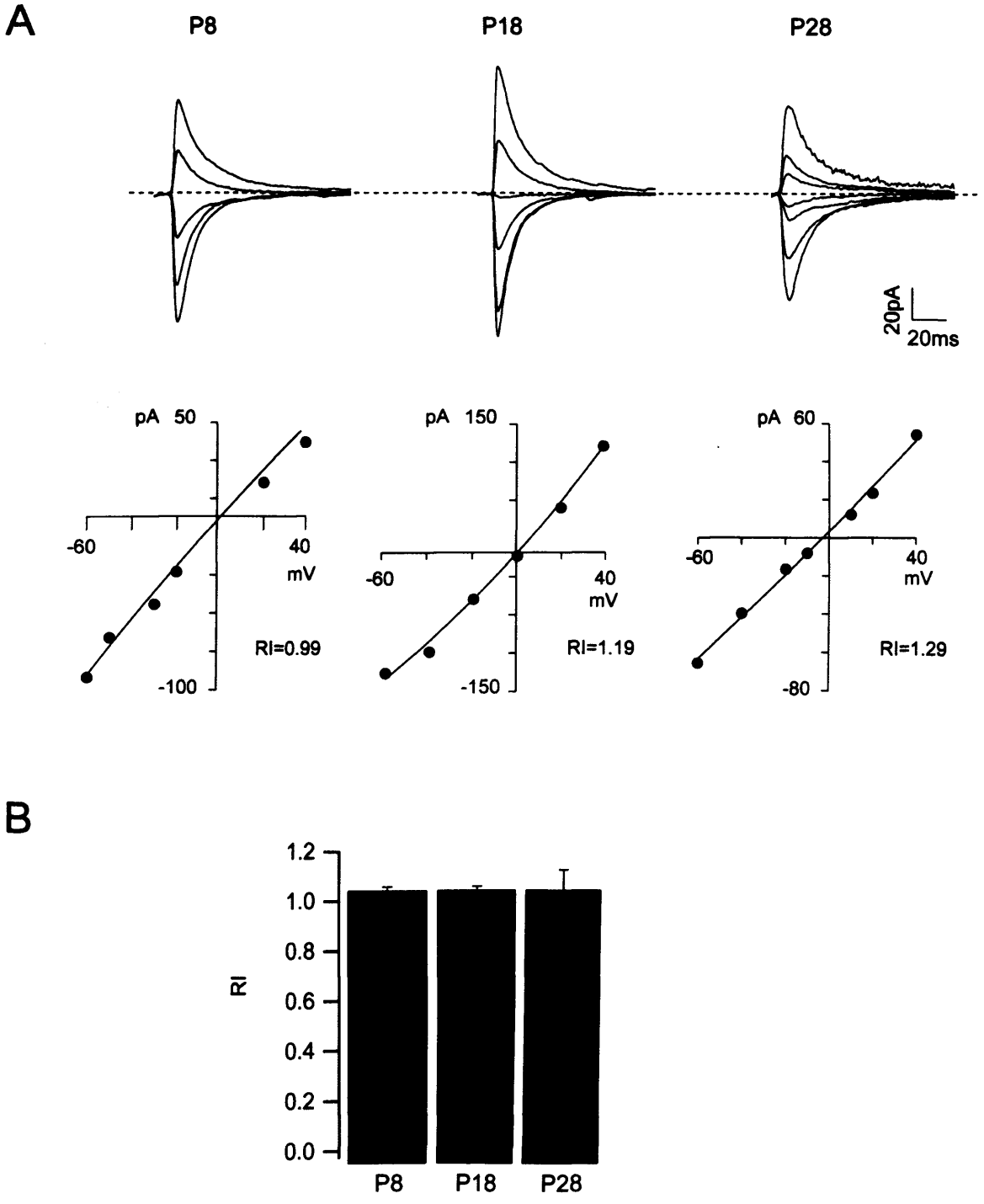


Figure 3.2 Non-rectifying I-V relationships of stellate cell EPSCs in the absence of spermine. A. Mean EPSCs evoked at various holding potentials in a stellate cells from a P8, P18 and P28 animals (upper panel) with corresponding I-V plots (lower panel). Spermine was not included in the pipette. **B**. Mean RI for control experiments. Rectification indices were consistent across all ages with mean values of 1.00 ± 0.01(*n*=6) at P7-8, 1.00 ± 0.01 (*n*=4) at P17-19 and 1.00 ± 0.07 (*n*=4) at P28.

When variance is plotted against current amplitude (example in **Figure 3.3B**), the parabolic relationship yields values for the weighted mean unitary current (*i*) from which single channel conductance can then be calculated.

Evoked EPSCs recorded at -60mV were analysed for all cells (*n* =36) and single channel conductance estimates calculated. **Figure 3.3A** shows evoked EPSCs from a SC selected for analysis and the relationship between scaled peak variance and response amplitude for the corresponding data (**Figure 3.3B**). To see if the decrease in rectification was accompanied by a change in the single channel conductance of native receptors, estimated conductance values was plotted against age (**Figure 3.3C**) and RI (**Figure 3.3D**). In both cases, no significant correlation was found ($P>0.5$).

3.3.3 Spontaneous EPSCs yield conductance estimates that decrease with age and RI

Stimulation of PFs can lead to asynchronous release of glutamate (Traynelis et al., 1993; Atluri and Regehr, 1998; Chen and Regehr, 1999; Barbara et al., 2003; Wong et al., 2003). In peak-scaled nonstationary variance analysis each peak is scaled to the peak of the mean. This assumes all channel are open before the peak, which is unlikely during EPSCs where release is occurring asynchronousy. I investigated this possibility by carrying out the same analysis on spontaneous EPSCs (**Figure 3.4**) which are thought to represent quantal events in SCs (Barbara et al., 2003). Analysis of spontaneous EPSCs showed a developmental decrease in single channel conductance (**Figure 3.4C**). Single channel conductance correlated significantly both with age and RI (**Figure 3.4.D**) ($P<0.05$ and $P<0.05$), supporting the idea that GluR2-containing receptors are inserted into the synapse during development.

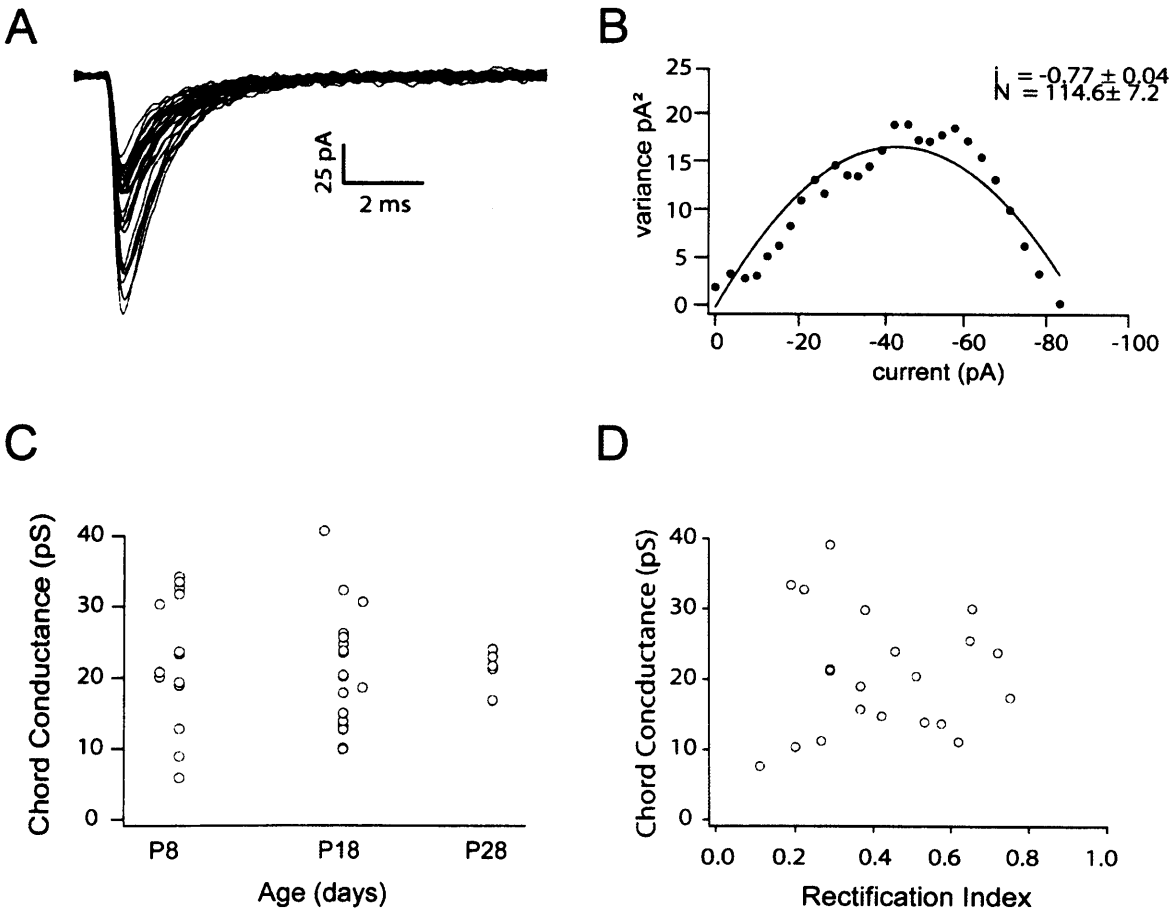


Figure 3.3 Peak-scaled nonstationary fluctuation analysis of evoked EPSCs. A. Evoked EPSCs recorded at -60mV from a stellate cell (P18). Events are aligned on 20% rise time. **B.** Current-variance plot for the data shown in A. The parabolic relationship yields values for the weighted mean unitary current (*i*) and channel number (*N*), from which single-channel conductance was calculated. **C.** Estimated single channel conductance of receptors underlying evoked EPSCs plotted against age. **D.** Estimated single-channel conductance of receptors underlying evoked EPSCs plotted against RI (n=22). There was no significant correlation in both cases ($P>0.5$; Spearman rank order correlation).

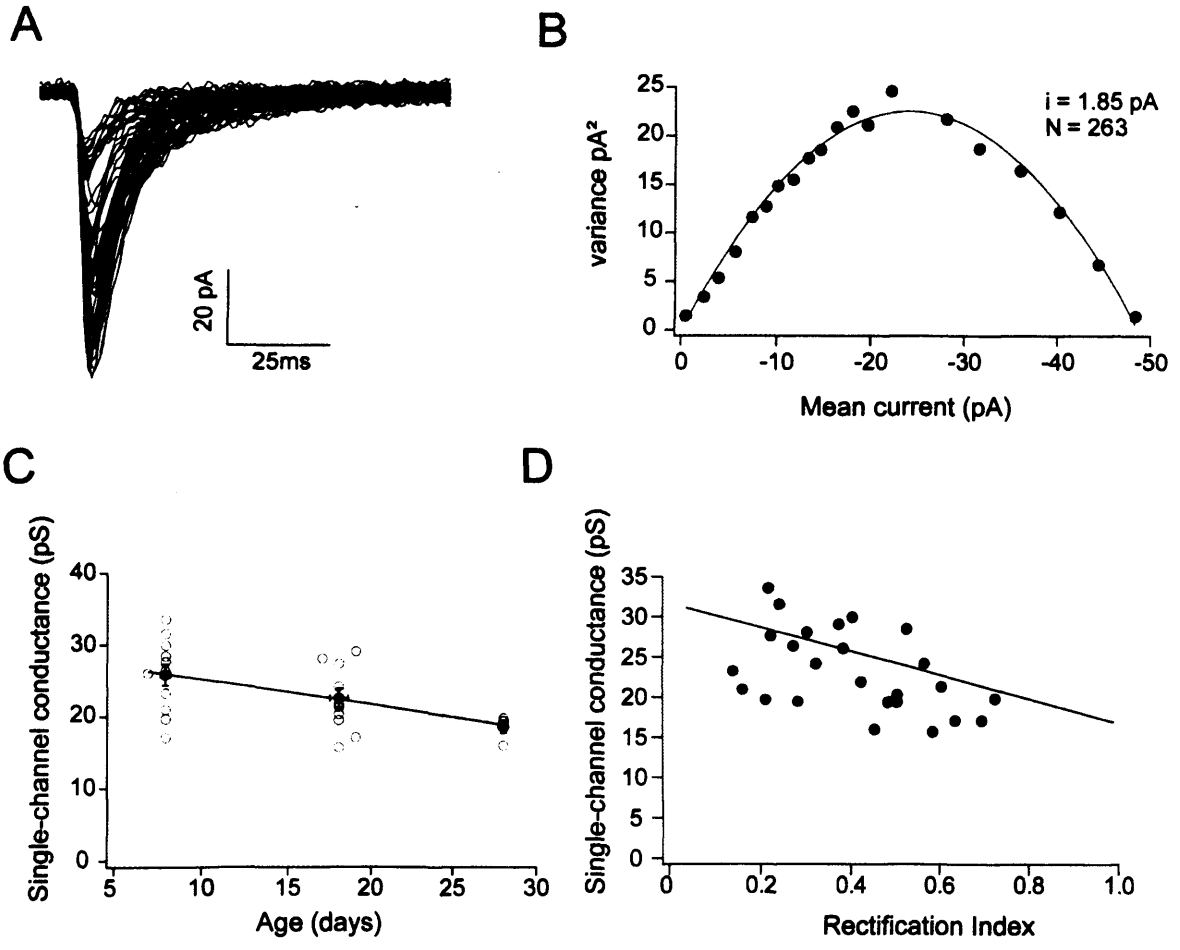


Figure 3.4 Peak-scaled nonstationary variance analysis of spontaneous EPSCs. A. Spontaneous EPSCs recorded at -60mV (P18). Events are aligned on 20% rise time. **B**. The current-variance relationship for the data shown in A. The parabolic relationship yields values for the weighted mean unitary current (*i*) and the number of channels open at the peak (*N*), from which single channel conductance can then be calculated. N = the channel number and I = mean weighted unitary current. **C**. Estimated single-channel conductance of receptors underlying spontaneous EPSCs from animals of various ages. Means are shown in black and error bars denote SEM. Age (n =34) was correlated with single-channel conductance (n =34) ($P<0.05$; Spearman rank order correlation). **D**. Conductance estimates plotted against RI. There is a significant correlation ($P<0.005$; Spearman rank order correlation).

3.3.4 Evoked and spontaneous EPSC kinetics

Kinetic analysis revealed no significant change in either 10-90% rise time, peak amplitude or weighted tau with either age or RI for the evoked EPSCS (P>0.1) (data not shown). For spontaneous currents (**Figure 3.5**) no significant change in either peak or rise time was found with development or RI, (**Figure 3.5A-D**) but spontaneous currents recorded from P18 animals decayed more quickly than currents recorded from P8 animals (**Figure 3.5F**). However, weighted tau was not significantly correlated with RI (P>0.1) (**Figure 3.5E**) or conductance (P>0.5) (data not shown) suggesting that the difference in decay is not associated with the developmental change in AMPAR subunit but may be a result of another aspect of synapse maturation, a change in geometry, for example.

3.3.5 Ion exchange experiments

To confirm that the developmental change in AMPAR subunit composition (as reflected by a change in RI and single channel conductance) is related to a change in AMPAR Ca^{2+} permeability, the effect of external [Ca^{2+}] elevation on the E_{rev} of synaptic currents was investigated. A lack of a shift, or a positive shift in E_{rev} with increasing Ca^{2+} concentration would reflect the presence of receptors permeable to Ca^{2+} whereas GluR2-containing receptors would be expected to cause a negative shift (Balazs et al., 1992; Otis et al., 1995).

Unlike studies performed on patches of AMPARs of known subunit composition, it was not possible to accurately predict the expected shift in reversal potential as a mixed population of AMPARs of various subunit composition were mediating the current. At a synapse containing purely GluR2- lacking AMPARs, raising the external Ca^{2+} from 1.8mM to 30mM would be expected to cause either no shift or a positive shift in reversal potential. For example, if it is assumed that CPAMPARS expressed in stellate cells have the same Ca^{2+} permeability as the CP-AMPARS in Bergman Glial cells (Geiger et al., 1995), then one would expect a maximum shift of +22mv (see **methods**). Where as at a synapse containing purely GluR2-containing AMPARs, with a Ca^{2+}-

permeability similar to CA3 pyramidal cells (Geiger et al., 1995) a negative shift of about -35mV would be expected.

The AMPARs expressed in hippocampal interneurons which are known also to express a mixed population of both CP- and CI-AMPARS express have been calculated to have a $P_{ap}a$ of 1.59 (Geiger et al., 1995). If the same were true of molecular layer interneurons of the cerebellum the predicted observed shift with the external solutions used in this study would be +13mV.

EPSCs were evoked in the presence of low (1.8mM) and elevated (30mM) $[Ca^{2+}]_o$. Elevation of Ca^{2+} lead to an increase in spontaneous activity, a reduction in EPSC amplitude and increased the stimulus threshold (Otis et al., 1995; Mathie et al., 1990). Initially, spermine was included in the pipette so that any correlation between E_{rev} shift and RI could be investigated. However after observing the dramatic effect of increased external $[Ca^{2+}]$ on EPSC amplitude, it was decided that the shift in E_{rev} could be more accurately determined if I-V relationships were non-rectifying.

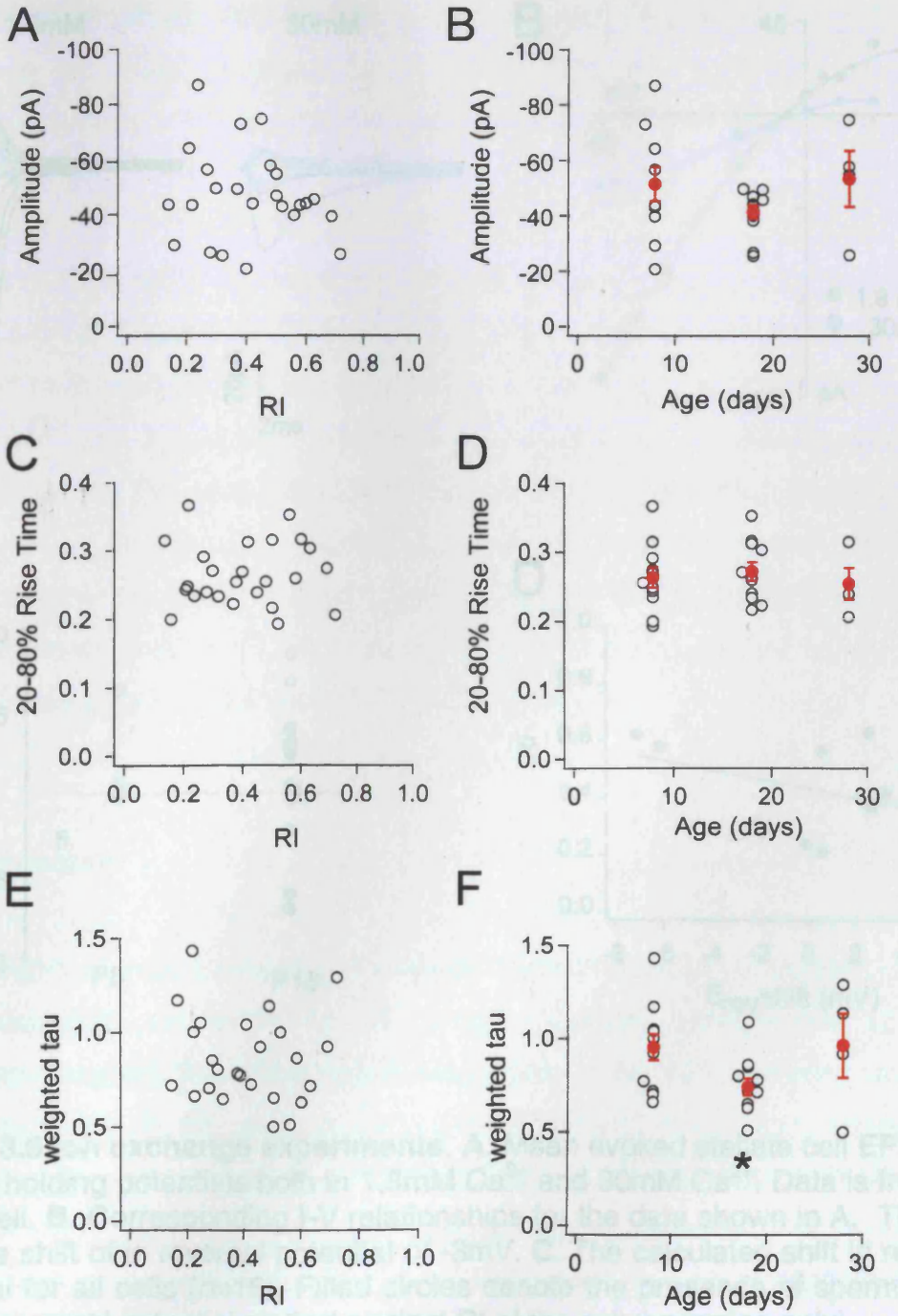


Figure 3.5 Spontaneous EPSC parameters. A. There was no correlation between peak current amplitude and RI (P>0.1; Spearman rank order correlation n=22). B. Peak current amplitude was consistent across all ages. Mean amplitude =-51.5 ± 6.2pA (P8), -41.51± 2.5 pA (P18) and -53.52 ± 1.6pA (P28). **C**. 20-80% rise time was not correlated with RI (P>0.05; Spearman rank order correlation n=22). D. 20-80% rise time was consistent across all ages (P>0.05; Spearman rank order correlation). **D**. Mean rise time = 0.26 ± 0.02 (P8), 0.27 ± 0.013 (P18) and 0.25 ± 0.02 ms (P28). **E**. τ_w was not correlated with RI (P>0.1; Spearman rank order correlation n=22). **F**. τ_w values = 0.9 5± 0.07(P8), 0.74 ± 0.08 (P18) and 0.96 ± 0.17 ms (P28). P8 values are significantly different to P18 (P<0.05 Mann-Whitney U test).

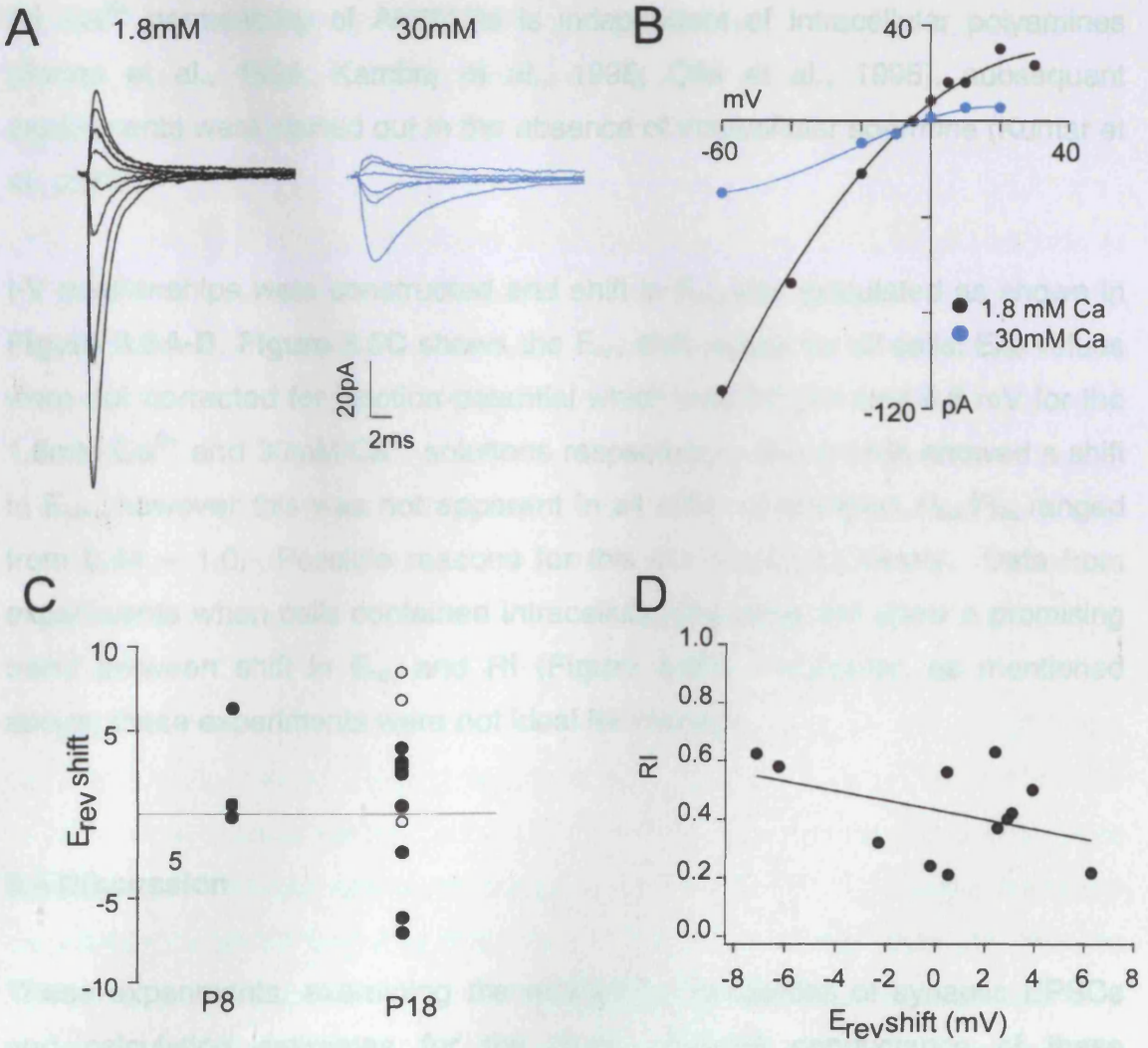


Figure 3.6 Ion exchange experiments. **A**. Mean evoked stellate cell EPSCs at various holding potentials both in 1.8mM Ca^{2+} and 30mM Ca^{2+}. Data is from the same cell. **B**. Corresponding I-V relationships for the data shown in A. This cell shows a shift of in reversal potential of -3mV. **C**. The calculated shift in reversal potential for all cells (n=16). Filled circles denote the presence of spermine. **D**. Shift in reversal potential plotted against RI of the cell (spermine only).

As Ca^{2+} permeability of AMPARs is independent of intracellular polyamines (Jonas et al., 1994; Kamboj et al., 1995; Otis et al., 1995), subsequent experiments were carried out in the absence of intracellular spermine (Kumar et al., 2002).

I-V relationships were constructed and shift in E_{rev} was calculated as shown in **Figure 3.6A-B**. **Figure 3.6C** shows the E_{rev} shift values for all cells. E_{rev} values were not corrected for junction potential which was 3.9 mV and 6.6 mV for the 1.8mM Ca^{2+} and 30mM Ca^{2+} solutions respectively. Some cells showed a shift in E_{rev}, however this was not apparent in all cells. Calculated P_{Ca}/P_{Na} ranged from 0.44 – 1.0. Possible reasons for this are discussed below. Data from experiments when cells contained intracellular spermine, did show a promising trend between shift in E_{rev} and RI (**Figure 3.6D**). However, as mentioned above, these experiments were not ideal for analysis.

3.4 Discussion

These experiments, examining the rectification properties of synaptic EPSCs and calculating estimates for the single channel conductance of these receptors, suggest that the relative proportion of GluR2-containing receptors increases at the PF-SC synapse during development. Preliminary experiments increasing the concentration of extracellular [Ca^{2+}] suggest that AMPARs maintain some permeability to Ca^{2+} even in mature animals. The reasons for this, as well as the implications of a developmental change in AMPAR subunit composition will be considered.

3.4.1 I-V relationships of evoked EPSCs become less rectifying with age.

The degree of rectification caused by intracellular polyamine block is generally considered to reflect the relative contribution of GluR2 (Liu and Cull-Candy, 2000a; Plant et al., 2006; Washburn and Dingledine, 1996; Balazs et al., 1992)

to AMPAR subunit composition. The present findings therefore reflect the fact that the proportion of GluR2-containing receptors increases at the PF-SC synapse during the development.

Although rectification did decrease with age, there was still a large variation in RI amongst cells, even at the same developmental stage, consistent with previous observations at P18 (Liu and Cull-Candy, 2002). This variation likely reflects the dynamic process of GluR2-insertion and the fact that RI correlates with the level of spontaneous activity of individual SCs (Liu and Cull-Candy, 2002) and thus represents its synaptic history. It therefore follows, that SCs from mature animals would be expected to express more GluR2-containing AMPARs at synapses.

A similar decrease in rectification has been observed in neocortical pyramidal neurons (Kumar et al, 2002), where it was described as a 'developmental switch'. The change which occurs in neocortical pyramidal neurons is more dramatic as I-V relationships are completely linear by P18. I rarely observed a completely linear IV implying that, even in mature animals, SCs still express some receptors which lack the GluR2 subunit (see **general discussion**). The RI values between at P18 and P28 were not significantly different suggesting that a level of GluR2 is reached at adolescence and maintained throughout life. However, given the large variation in RI within age groups, this may be due to sampling.

3.4.2 Decrease in single channel conductance

The observed decrease in estimated single channel conductance underlying the spontaneous EPSCs with both age and RI fit with the prediction that an increased contribution of GluR2 to the synapse, as signified by a decrease in rectification, would be accompanied by a decrease in single channel conductance. Evidence from studies on recombinant AMPARs and knock-out animals suggests that single-channel conductance is relatively high for CP-

AMPARs compared with CI-AMPARs and this depends on the expression of edited GluR2 (Swanson et al., 1997; Feldmeyer et al., 1999). Single channel conductance is also inversely correlated with RI in neocortical non-pyramidal neurons (Angulo et al., 1997).

AMPARs displayed a variety of conductances which fall within previously described values. Neocortical nonpyramidal cell estimates range from 5.3 to 36.3 pS with an approximate mean of 20pS for regular-spiking nonpyramidal cells and 26 pS in fast spiking neurons (Angulo et al., 1997; Hestrin, 1993). Estimates in other neurons include 30pS for oxytocin neurons and 12pS for vasopressin neurons (Stern et al., 1999), 9-38pS for AII amacrine cells (Morkve et al., 2002), 1.5-22.3pS for CA1 synapses (Gebhardt and Cull-Candy, 2006; Luthi et al., 1999; Benke et al., 1998), 12pS for channels on cerebellar mossy fiber-granule cell synapse (Traynelis et al., 1993) and 7-8 pS at the climbing fibre-Purkinje cell synapse (Momiyama et al., 2003; Hausser and Roth, 1997). Cerebellar granule cell AMPA receptors have yielded estimates of multiple conductance levels of approximately 10, 20 and 30 pS (Wyllie et al., 1993) conductances of 27 pS in aspiny interneurons and 9 pS in pyramidal neurons (Hestrin 1993), 14pS for cultured hippocampal neurons (Lei and McBain, 2002; Lei and McBain, 2002)have been calculated.

As well as differences in subunit composition (Swanson et al., 1997), such variation could arise from the several observed conductance states of AMPARs (Rosenmund et al., 1998; Cull-Candy and Usowicz, 1987; Jahr and Stevens, 1987; Derkach et al., 1999; Dingledine et al., 1999; Smith et al., 2000) which can be agonist concentration (Rosenmund et al., 1998; Smith and Howe, 2000; Gebhardt and Cull-Candy, 2006) or phosphorylation-dependent (Derkach et al., 1999; Banker et al., 2000; Pincer et al., 2002) but see (Andrasfalvy and Magee, 2004).

It is difficult to directly study the channel properties of synaptic receptors given their location. Information about the conductance of synaptic channels is limited to estimates obtained from non stationary fluctuation analysis of synaptic

currents, where very small conductances cannot be resolved. This may explain why conductance estimates from single channel analysis (Swanson et al., 1997) were not observed at the synapse where these receptors reside (Traynelis et al., 1993). As single-channel conductance is estimated from a weighted mean, the presence of a mixed population of channels affects the estimate of channel conductance. For example the contribution of AMPARs with single channel conductances in the femtoseimen range (Swanson et al., 1997) could either heavily influence the final value, or go undetected. Analysis of synaptic receptors is further complicated by the variables discussed above such as agonist concentration and phosphorylation leading to altered conductance stated. All these factors should be taken into account when comparing single channel conductance values obtained from different neurons, using different methods of analysis.

3.4.3 Effects of calcium elevation on E_{rev}

The calculated shifts in Erev were less dramatic than would be expected if all AMPARs at the PF-SC synapse were Ca^{2+} impermeable (Kumar et al., 2002; Samoilova et al., 1999). This agrees with the observation that EPSC I-V relationships did not become completely linear and suggests that even in mature animals, SCs still express some receptors which lack the GluR2 subunit and are Ca^{2+} permeable. In addition, no dramatic positive shifts were observed either, suggesting that even in immature animals not all the AMPARs expressed are permeable to calcium. Intermediate values of AMPA receptor Ca^{2+} permeability, probably representing mixed populations of AMPA receptors (Burnashev et al., 1992), have also been observed in neurons of the hippocampus (Lerma et al., 1994; Isa et al., 1996), the dorsal spinal cord (Goldstein et al., 1995; Samoilova et al., 1999). Possible reasons for the retention of some Ca^{2+}- permeability are considered in the general discussion.

3.4.4 The developmental control of GluR2 expression

Changes in receptor subunits are often associated with synapse formation and developmental maturation of neural circuitry. Alterations in NMDA, GABA, and glycine receptor composition have been observed in various brain regions during early development (Carmignoto and Vicini, 1992; Takahashi et al., 1992; Farrant et al., 1994; Monyer et al., 1991; Gottmann et al., 1988; Gottmann et al., 1997; Singer et al., 1998). Throughout the CNS the expression of CP-AMPARs are developmentally regulated. I have found a developmental change in the proportion of GluR2-containing receptors with development at the PF-stellate cell synapse. A similar change has also been observed in the auditory brainstem, where the inner hair cells of the cochlear lack the GluR2 subunit until the onset of hearing (P10) (Eybalin et al., 2004). Increases in GluR2 during development also occur in rat, hippocampal astrocytes (Seifert et al., 2002), outer plexiform layer and bipolar cells in the developing retina (Johansson et al., 2000). GluR2 labelling shows a developmental increase in the inferior olive (Chen et al., 2006), hippocampal pyramidal neurons (Sanchez et al., 2001a) and interneurons (Lei and McBain, 2002). The functional relevance of this developmental control of CP-AMPAR expression is considered in the general discussion.

4. The involvement of mGluR- and $GABA_B$ receptors in mediating Ca^{2+}-permeable AMPAR plasticity at the parallel fibre-stellate cell synapse

4.1 Summary

The effect of group 1 mGluR activation on AMPAR-mediated transmission at the PF-SC synapse was investigated. Bath application of the agonist DHPG led to a significant and persistent decrease in evoked EPSC amplitude and rectification, accompanied by a significant increase in failure rate. Antagonists were employed to confirm activation of mGluRs and identify receptor subtypes involved.

The locus of DHPG action was examined using several approaches. Unchanged paired-pulse ratio, a decrease in mEPSC amplitude and block of DHPG action by 10mM intracellular BAPTA all implied a postsynaptic effect. Indeed, 10mM BAPTA itself induced an increase in EPSC rectification. The involvement of CB1 receptor activation was eliminated by using the antagonist AM251.

The role of $GABA_BR$ activation was also examined. Baclofen induced similar, but partially reversible changes in EPSC amplitude, rectification and failure rate. Baclofen increased paired-pulse facilitation and decreased sEPSC amplitude and frequency. Inclusion of 10mM BAPTA in the pipette abolished effects on RI and significantly reduced changes in failure rate and amplitude, reflecting the pre- and postsynaptic actions of the agonist.

Use of mGluR and $GABA_BR$ blockers revealed that mGluRs and $GABA_BR$ are tonically active in SCs. Treatment with group1 mGluRs antagonists increased evoked EPSC amplitude and rectification, and dramatically reduced failure rate. Similarly, a $GABA_BR$ blocker also enhanced evoked EPSC amplitude, in some cases increased rectification; failure rate was also decreased.

4.2 Introduction

It has previously been shown that high frequency stimulation of PFs onto SCs can lead to an activity-dependent switch in AMPAR subunit composition (Liu and Cull-Candy, 2000a); a change which requires calcium entry through GluR2-lacking AMPARs. Under physiological conditions however, high frequency activity will depolarise the cell (Carter and Regehr, 2002), therefore Ca^{2+} influx through CP-AMPARs will be limited by endogenous polyamine block and reduced electrochemical drive on calcium. SCs posses the intracellular trafficking molecules to enable this change in receptor subunit (Gardner et al., 2005b; Liu and Cull-Candy, 2005a). Furthermore, such changes occur during development (Chapter 3) suggesting that there may be other mechanisms that induce the switch in subunit composition under physiological conditions. These may be independent of, or in addition to, Ca^{2+} influx through CP- AMPARs.

It was recently demonstrated in dopaminergic neurons of the ventral tegmental area (VTA), that application of the group 1 mGluR agonist DHPG induces a long term depression of AMPAR-mediated EPSCS, accompanied by change in subunit composition of CP-AMPARs (Bellone and Luscher, 2006). SCs express group 1 mGluR receptors, which can be activated upon PF stimulation (Karakossian and Otis, 2004). Although group1 mGluRs are involved in plasticity at the PF-SC synapse (Rancillac and Crepel, 2004; Soler-Llavina and Sabatini, 2006), a role in the switching of postsynaptic AMPAR subtypes has not been observed.

There is currently interest surrounding the functional link between $GABA_BRs$ and mGluRs (Hirono et al., 2001; Tabata et al., 2004). SCs also receive strong inhibitory input from other SCs (Llano and Gerschenfeld, 1993b) which fire spontaneously (Hausser and Clark, 1997) as well as in response to PF stimulation, and have been shown to have functional $GABA_BRs$ (Mann-Metzer and Yarom, 2002b),

The experiments described in this chapter investigate the role of mGluR and $GABA_BR$ activation in the subunit specific targeting of postsynaptic AMPARs at

the PF-SC synapse. Once again, by recording whole-cell responses with intracellular spermine as a pharmacological indicator, the synaptic contribution of GluR2-containing AMPARs could be assessed.

4.3 Results

4.3.1 Effect of DHPG on evoked EPSCs

To test whether activation of mGluRs present in SCs (Rancilac & Crepel, 2003; Karakossian and Otis, 2004) could participate in the switch from GluR2-lacking to GluR2-containing AMPARs, EPSC properties were measured following application of the group 1 mGluR agonist DHPG (50μM), in the absence of stimulation. EPSCs were evoked at various holding potentials (**Figure 4.1A**) using PF stimulation for a minimum of 5 minutes before application of DHPG (50μM; 10mins). Recordings continued at various holding potentials. It was usually possible to hold the cell for 1hr. Evoked EPSC amplitude dramatically decreased at -60mV post DHPG application, as shown in **Figure 4.1C**. Mean evoked EPSC amplitude was reduced from 115.1 ± 8.7 pA in control solution, to 74.1 ± 7.7 pA (n = 22; **Figure 4.1F**). There was no obvious change in EPSC kinetics (**Figure 4.1E**). There was however a significant increase in failure rate (**Figure 4.1C**) from a mean value of 0.18 ± 0.03 before DHPG, to 0.35 ± 0.06 post DHPG (n = 20, P<0.05)(**Figure 4.1G**).

Figure 4.2A shows that at +40mV the EPSC amplitude remained virtually unchanged (or increased) following DHPG application. Mean EPSC amplitude at +40 mV was 30.9 ±3.8pA in control *vs* 33.2 ± 5.6 pA in DHPG, (n = 17). This resulted in an overall decrease in rectification of evoked EPSC I-V relationships (**Figure 4.2B**).

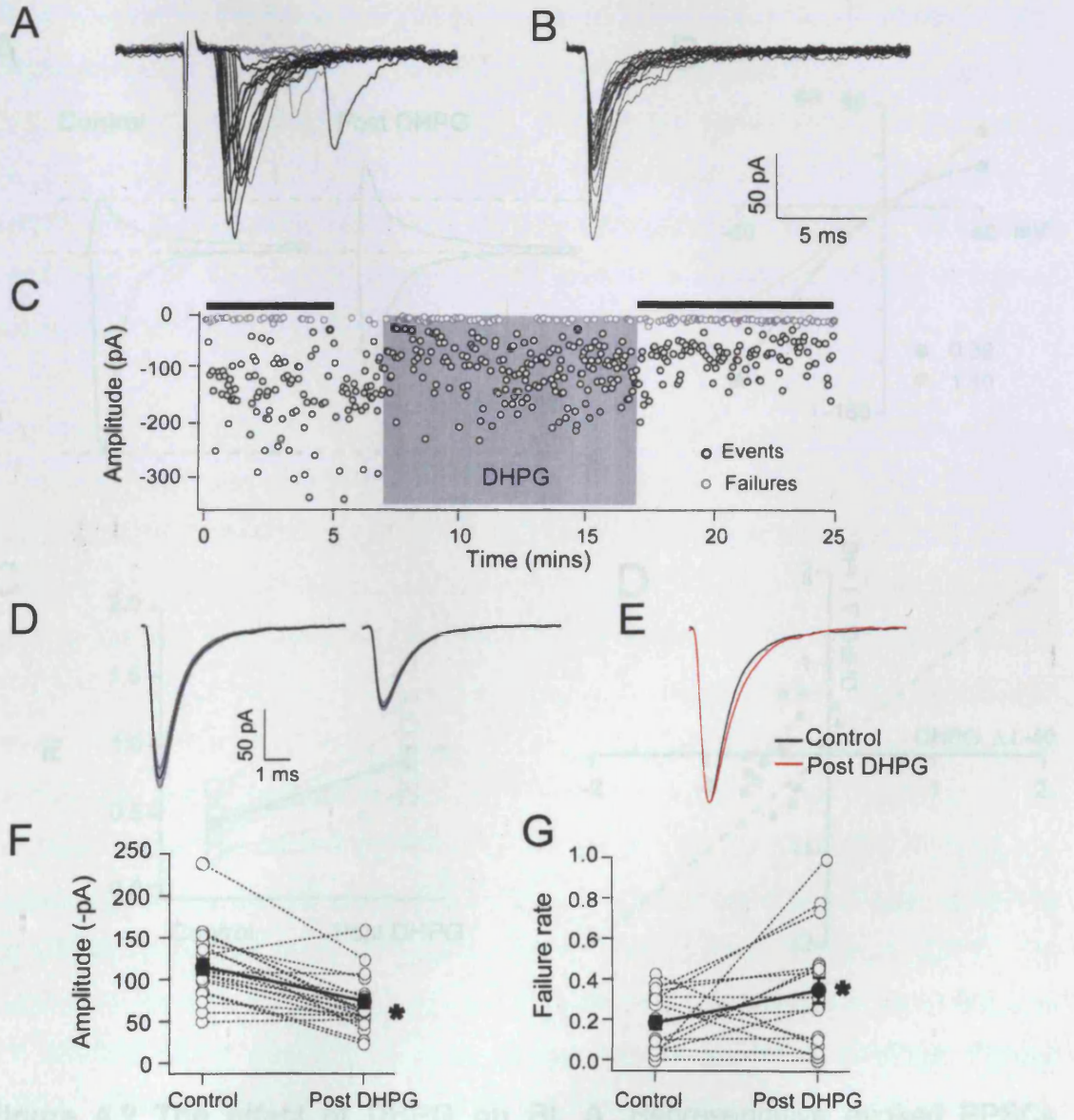


Figure 4.1 The effects of DHPG on evoked EPSCs in stellate cells.
A. Representative superimposed responses to parallel fibre stimulation. Traces are aligned on the stimulus artefact (truncated) and failures are shown in grey. **B**. Representative superimposed 'selected' (see methods) evoked EPSCs from the same cell as A. Only events with monotonic rise are included, and aligned on their 20% rise-time. **C**. Representative data from a complete experiment (different cell to A) showing the timecourse of the effect of DHPG (50 μM; grey box) on amplitude of selected EPSCs. Failures are shown in grey. Solid bars denote time periods from which mean amplitudes (pre- and post-DHPG) were calculated. **D**. Mean EPSC waveforms (from C) obtained in control conditions and following DHPG. Superimposed grey fill denotes the SEM. **E**. Normalised EPSCs showing the lack of effect of DHPG on EPSC kinetics. **F**. Pooled data (n =22) showing the reduction in EPSC amplitude produced by DHPG. Open circles show individual cells; mean amplitude from EPSCs measured in each condition. Filled circles mean data (error bars indicate SEM.). Asterisk indicates significant effect of DHPG (P<0.005 Wilcoxon matched pairs test). **G**. Pooled data showing increase in failure rate.

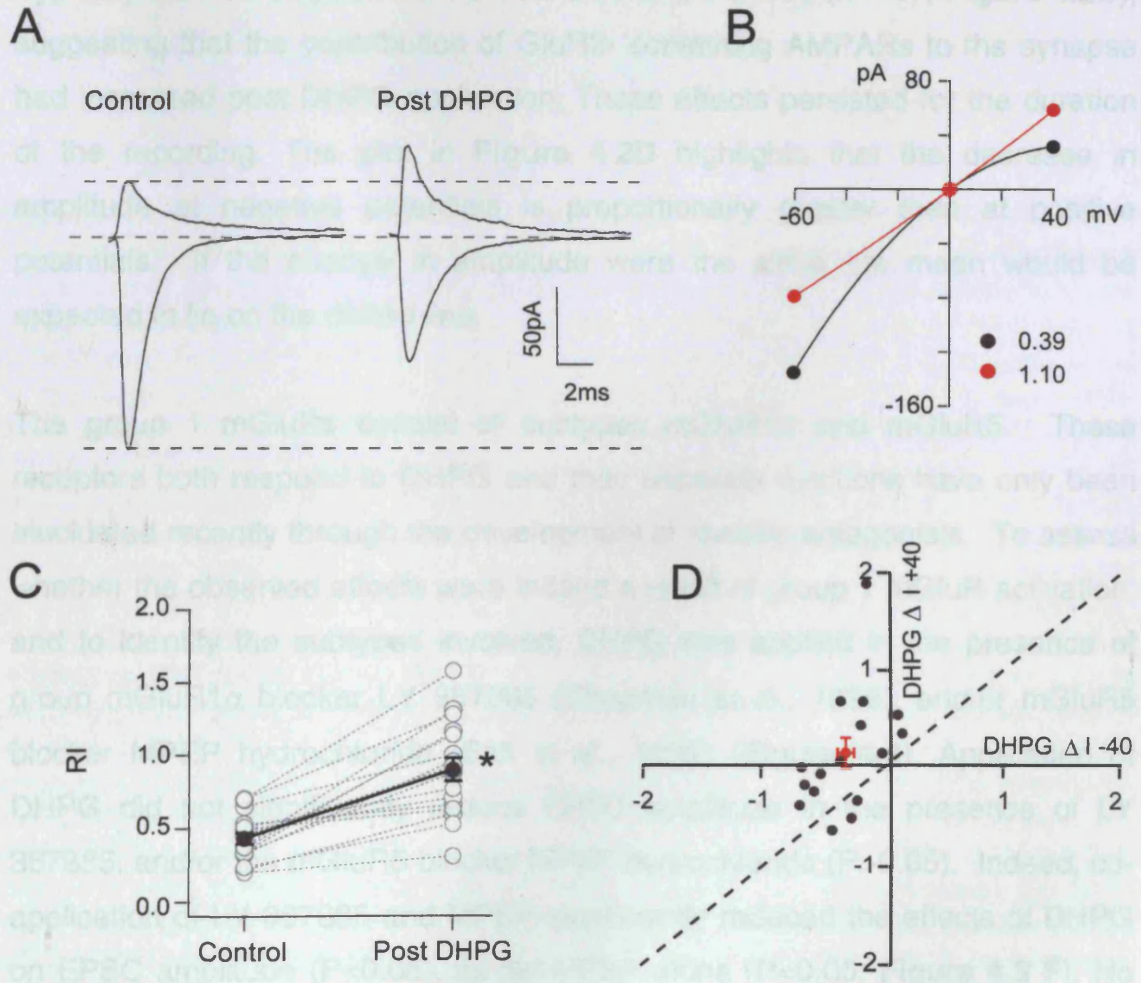


Figure 4.2 The effect of DHPG on RI. A. Representative evoked EPSCs recorded at -60mV and +40mV before and after application of 50µM DHPG (10 mins). Means were constructed from a minimum of 20 selected events (see methods). Dotted lines show the baseline and peak current for control EPSCs. **B.** I-V relationships constructed from data in A. Control EPSCs display an inwardly rectifying IV relationship. After DHPG application EPSCS display a linear I-V relationship. **C.** Effect of DHPG on rectification. On average RI was 0.44 ± 0.04 before and 0.90 ± 0.08 after DHPG application (n =17, P<0.05). Open symbols show individual cells, solid symbols show mean data ± SEM, and asterisks denote significant differences from control. **D.** Plot showing relative DHPG-induced changes in EPSC amplitude at -40 and +40mV. Each symbol is an individual cell; crossed bars show mean ± SEM. Voltage-independent changes in amplitude would fall on the dotted line.

Typically the RI increased from 0.44 ± 0.04 to 0.9 ± 0.08 (n = 17; **Figure 4.2C**), suggesting that the contribution of GluR2- containing AMPARs to the synapse had increased post DHPG application. These effects persisted for the duration of the recording. The plot in **Figure 4.2D** highlights that the decrease in amplitude at negative potentials is proportionally greater than at positive potentials. If the change in amplitude were the same the mean would be expected to lie on the dotted line.

The group 1 mGluRs consist of subtypes mGluR1α and mGluR5. These receptors both respond to DHPG and their separate functions have only been elucidated recently through the development of specific antagonists. To assess whether the observed effects were indeed a result of group 1 mGluR activation, and to identify the subtypes involved, DHPG was applied in the presence of group mGluR1α blocker LY 367385 (Chapman et al., 1999), and/or mGluR5 blocker MPEP hydrochloride (Salt et al., 1999) (**Figure 4.3**). Application of DHPG did not significantly reduce EPSC amplitude in the presence of LY 367385, and/or the mGluR5 blocker MPEP hydrochloride ($P>0.05$). Indeed, co-application of LY 367385 and MPEP significantly reduced the effects of DHPG on EPSC amplitude ($P<0.05$), as did MPEP alone ($P<0.05$; **Figure 4.3 F**). No significant change in RI was seen in the presence of antagonists ($P<0.05$) and LY 367385 alone significantly blocked the effects of DHPG ($P<0.05$; **Figure 4.3G**). There was also no significant change in failure rate in the presence LY 367385, and/or MPEP (**Figure 4.3H**).

Experiments were also carried out where EPSCs were evoked before and after a 10 minute break without DHPG application to ensure that any changes were not a result of time-dependent changes alone. As shown in **Figure 4.3 D-E**, EPSC amplitude, RI and failure rate remained unchanged in the absence of DHPG ($P>0.05$; **Figure 4.3D-H**).

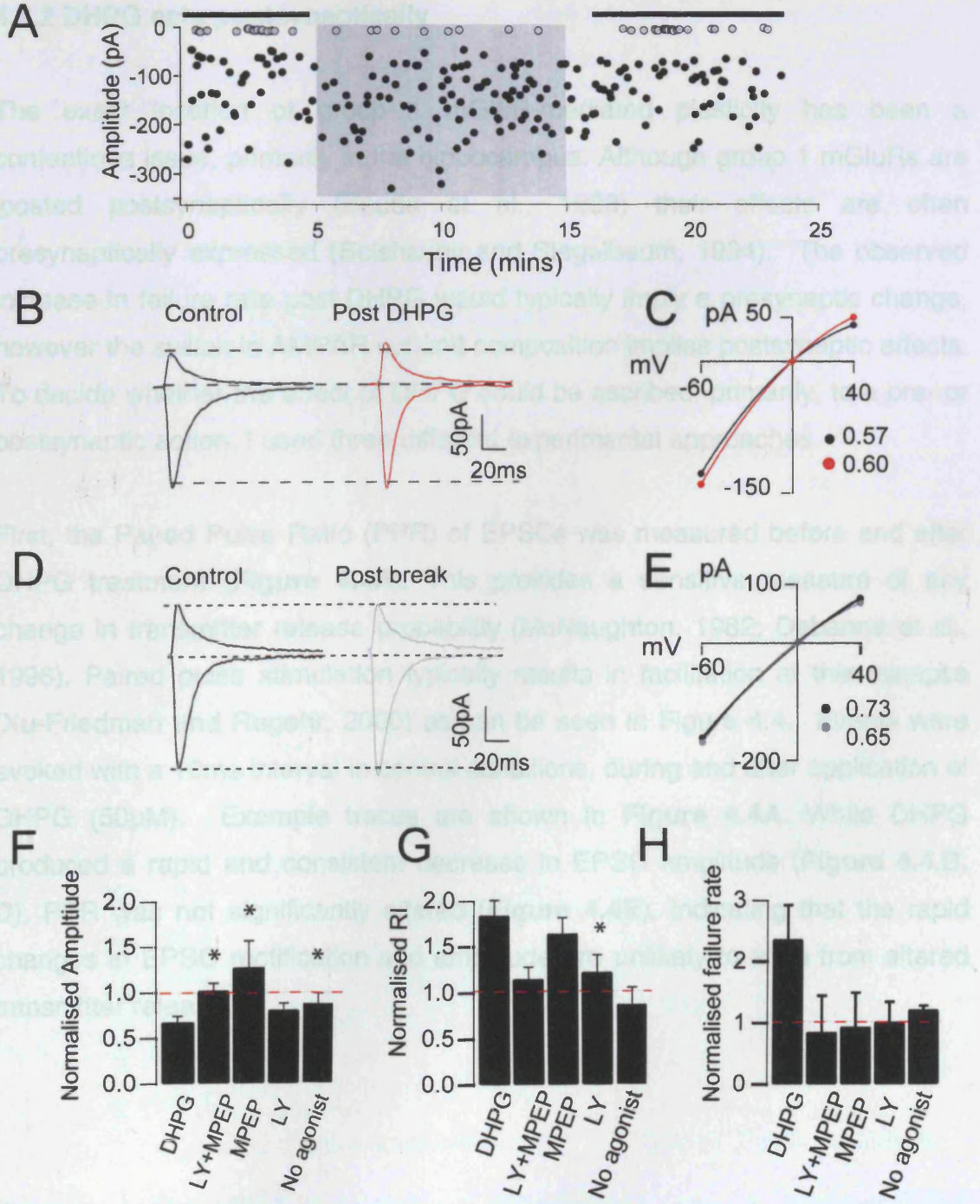


Figure 4.3 Group 1 mGluR activation mediates the change in RI and amplitude (see opposite).

4.3.2 DHPG acts postsynaptically

The exact location of group 1 mGluR-mediated plasticity has been a contentious issue, primarily in the hippocampus. Although group 1 mGluRs are located postsynaptically (Baude et al., 1993) their effects are often presynaptically expressed (Bolshakov and Siegelbaum, 1994). The observed increase in failure rate post DHPG would typically imply a presynaptic change, however the switch in AMPAR subunit composition implies postsynaptic effects. To decide whether the effect of DHPG could be ascribed, primarily, to a pre- or postsynaptic action, I used three different experimental approaches.

First, the Paired Pulse Ratio (PPR) of EPSCs was measured before and after DHPG treatment (**Figure 4.4A**). This provides a sensitive measure of any change in transmitter release probability (McNaughton, 1982; Debanne et al., 1996). Paired pulse stimulation typically results in facilitation at this synapse (Xu-Friedman and Regehr, 2000) as can be seen in Figure 4.4. Events were evoked with a 10ms interval in control conditions, during and after application of DHPG (50μM). Example traces are shown in **Figure 4.4A**. While DHPG produced a rapid and consistent decrease in EPSC amplitude (**Figure 4.4.B, D**), PPR was not significantly altered (**Figure 4.4E**), indicating that the rapid changes in EPSC rectification and amplitude are unlikely to arise from altered transmitter release.

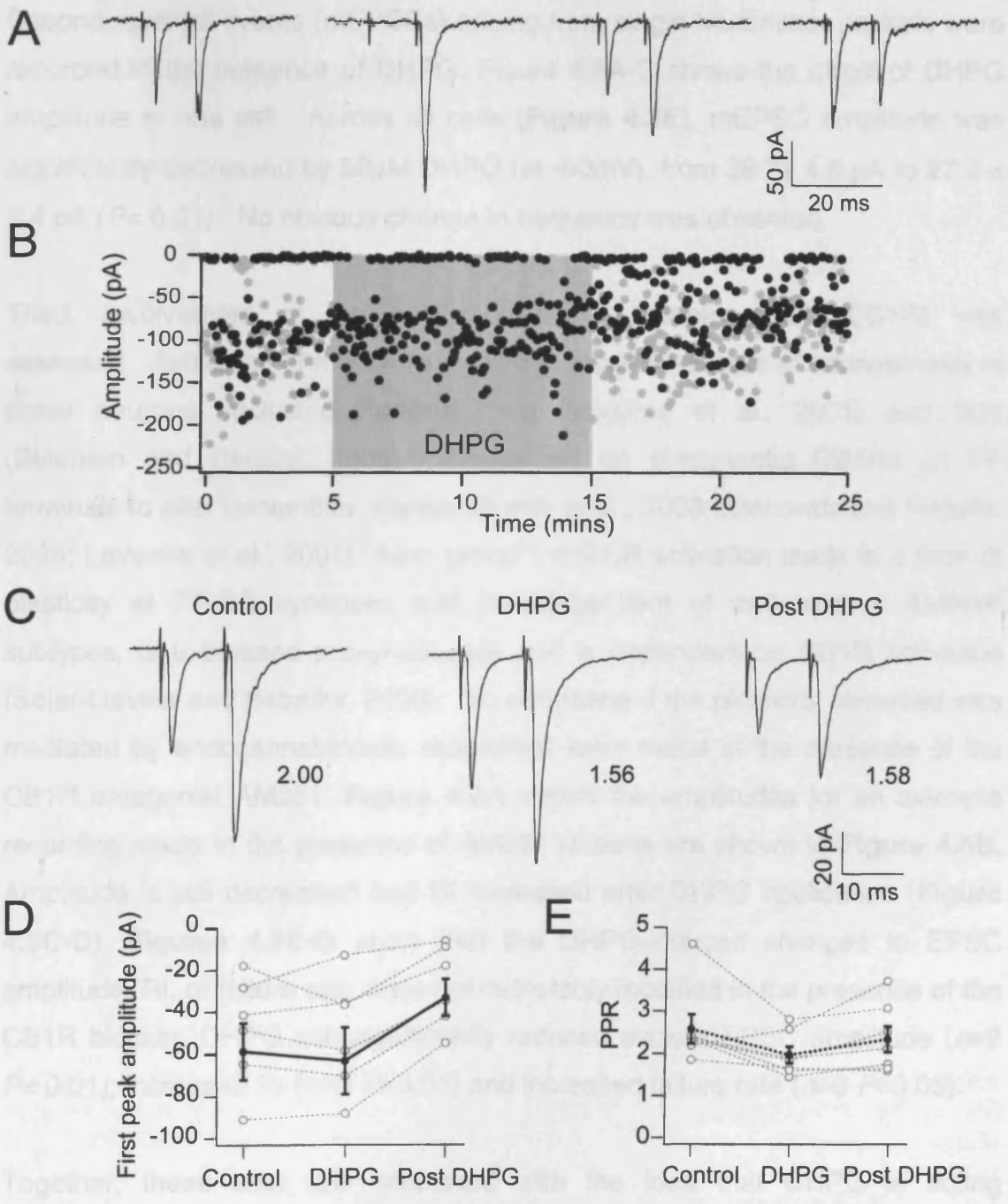


Figure 4.4 The effect of DHPG on paired-pulse ratio. A. Representative examples of individual paired-pulse traces. **B**. Representative data from a complete paired-pulse experiment during the application of DHPG (grey box). Amplitude of the first (black) and second peak (grey) are plotted. **C**. Mean paired pulse trace for control, DHPG and Post-DHPG conditions. Means were constructed by averaging all traces (including failures). Data is from the same cell as B. **D**. Amplitude of the first peak of the mean PPR trace in control, DHPG and Post-DHPG conditions (n=6). Mean and SEM. are shown in black. **E**. Paired Pulse ratios calculated from mean traces in control, DHPG and Post-DHPG conditions. Mean control PPR= 2.33 ± 0.08. Mean PPR in DHPG= 1.81 ± 0.16. Mean PPR Post-DHPG = 1.76 ± 0.14. Values are not significantly different (P>0.05.)

Second, quantal events (mEPSCs) arising from single transmitter packets were recorded in the presence of DHPG. Figure 4.5A-C shows the effect of DHPG amplitude in one cell. Across all cells (**Figure 4.5E**), mEPSC amplitude was significantly decreased by 50μM DHPG (at -60mV), from 38.7± 4.8 pA to 27.2 ± 2.4 pA ($P< 0.01$). No obvious change in frequency was observed.

Third, involvement of the endocannabinoid receptor CB1 (CB1R) was assessed. Activation of mGluR1α receptors leads to release of cannabinoids in some neurons, including Purkinje cells (Maejima et al., 2001) and SCs (Beierlein and Regehr, 2006). These act on presynaptic CB1Rs on PF terminals to alter transmitter release (Brown et al., 2003; Brenowitz and Regehr, 2005; Levenes et al., 2001). Also, group 1 mGluR activation leads to a form of plasticity at PF-SC synapses that is independent of switching of AMPAR subtypes, is expressed presynaptically and is dependent on CB1R activation (Soler-Llavina and Sabatini, 2006). To determine if the plasticity observed was mediated by endocannabinoids, recordings were made in the presence of the CB1R antagonist AM251. **Figure 4.6A** shows the amplitudes for an example recording made in the presence of AM251 (means are shown in **Figure 4.6B.** Amplitude is still decreased and RI increased after DHPG application (**Figure 4.6C-D**). **Figures 4.6E-G** show that the DHPG-induced changes in EPSC amplitude, RI, or failure rate were not detectably modified in the presence of the CB1R blocker. DHPG still significantly reduced evoked EPSC amplitude (n=9 $P< 0.01$), increased RI (n=8 $P<0.01$) and increased failure rate (n=9 $P<0.05$).

Together, these data are consistent with the idea that DHPG is acting postsynaptically, suggesting that group 1 mGluR activation triggers a robust decrease in postsynaptic CP-AMPARs. The increase in failure rate suggests that some synapses express a high proportion of GluR2-lacking AMPARs that can be rapidly removed by group 1 mGluR activation. This resembles the situation described for GluR2-containing AMPARs in hippocampal cells (Noel et al, 1999; Xiao, Zhou & Nicoll, 2001; Moult et al, 2006).

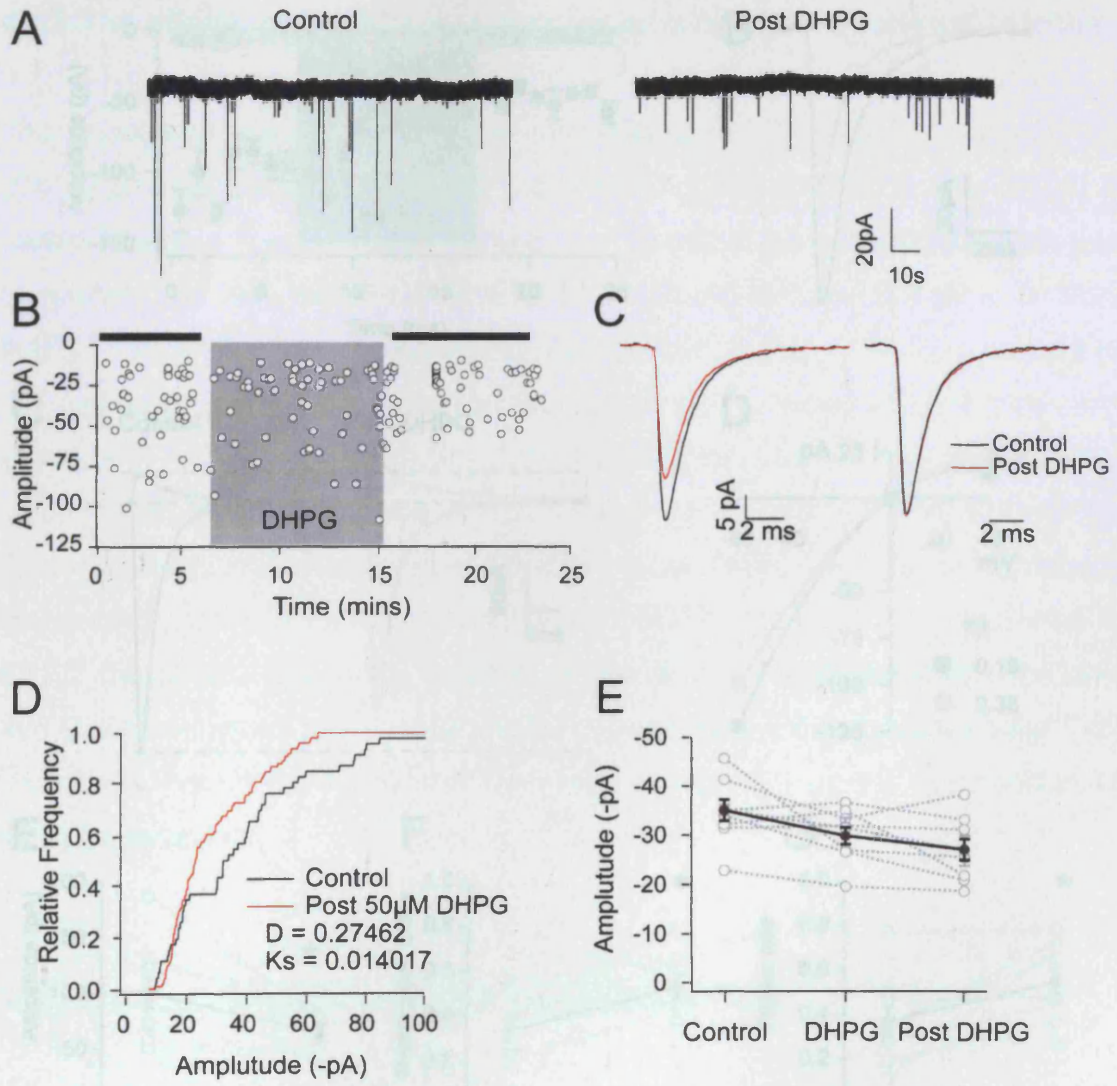


Figure 4.5 The effect of DHPG on mEPSCs. **A**. Example trace of a whole-cell stellate cell recording (-60mV) in the presence of TTX (500nM) pre (left panel) and post-DHPG application (right panel). **B**. Representative complete experiment showing the time course of the effect of DHPG (50μM) application (grey box) on mEPSC amplitude. **C**. Left panel shows mean mEPSC traces before (black) and after (red) DHPG application. Means were constructed from events detected (see methods) within the denoted time regions (black bars). Right panel shows normalised means showing the lack of effect of DHPG on EPSC timecourse. **D**. Cumulative plots showing that DHPG significantly reduces mEPSC amplitude for this cell. **E**. Average mEPSC amplitude in control, DHPG and Post-DHPG conditions for all cells (n=8). Means are shown in black and error bars denote SEM. Mean amplitude was 38.70 ± 4.84 in control, -33.77 ± 1.94 in DHPG and -27.18 ± 2.36 pA. Amplitude was significantly reduced Post DHPG (P< 0.01).

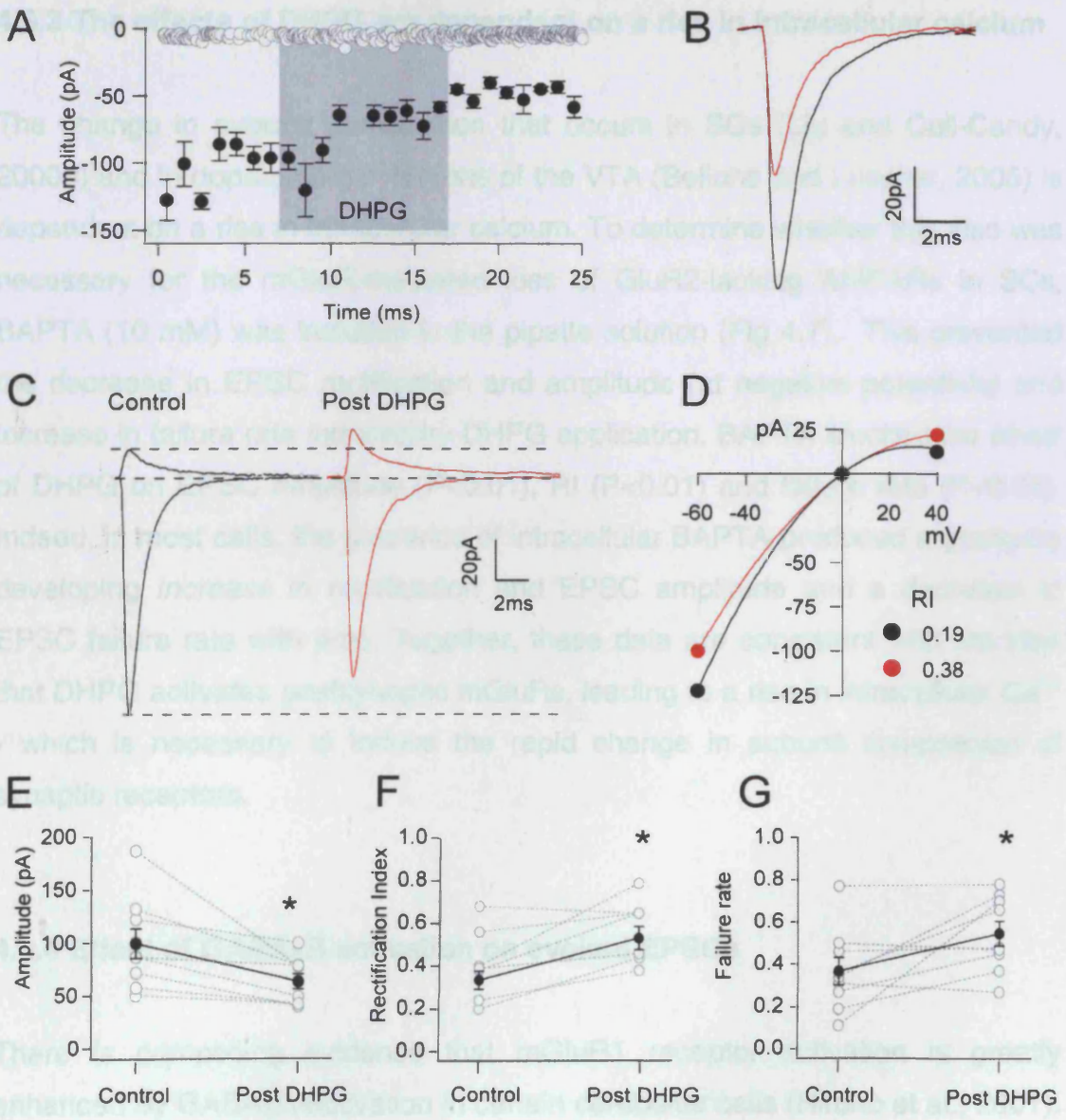


Figure 4.6 The cannabanoid antagonist AM251 does not block effects of DHPG on EPSC amplitude and rectification. **A**. Time course of the effect of DHPG in the presence of 1 μM AM251 for a single cell. Black circles represent an average of 20 sweeps. Error bars denote SEM. **B**. Mean EPSCs recorded before, and after DHPG application. **C**. Representative mean evoked EPSCs recorded in the presence of 1 μM AM251 at -60mV and +40mV before (black traces) and after 50μM DHPG (red traces). Dashed lines indicate baseline and peak current for control EPSCs. **D**. I-V relationships for traces in C showing DHPG significantly increases RI in the presence of AM251. **E**. Mean EPSC amplitude (-60mV), was significantly decreased by DHPG: control = -99.5 ± 14.2 pA, post DHPG = -63.43 ± 7.1 (n=9 p<001). **F**. Mean RI values are increased from 0.33± 0.04 in control to 0.53 ± 0.05 post DHPG (n=8 P<0.01). **G**. Failure rate was still significantly increased from 0.36 ± 0.06 to 0.54 ± 0.06 (n=9 P<0.05).

4.3.3 The effects of DHPG are dependent on a rise in intracellular calcium

The change in subunit composition that occurs in SCs (Liu and Cull-Candy, 2000b) and in dopaminergic neurons of the VTA (Bellone and Luscher, 2005) is dependent on a rise in intracellular calcium. To determine whether this also was necessary for the mGluR-mediated loss of GluR2-lacking AMPARs in SCs, BAPTA (10 mM) was included in the pipette solution (Fig 4.7). This prevented the decrease in EPSC rectification and amplitude (at negative potentials) and increase in failure rate induced by DHPG application. BAPTA blocked the effect of DHPG on EPSC amplitude ($P<0.01$), RI ($P<0.01$) and failure rate ($P<0.05$). Indeed, in most cells, the presence of intracellular BAPTA produced a gradually developing *increase* in rectification and EPSC amplitude and a decrease in EPSC failure rate with time. Together, these data are consistent with the idea that DHPG activates postsynaptic mGluRs, leading to a rise in intracellular Ca^{2+} - which is necessary to induce the rapid change in subunit composition of synaptic receptors.

4.3.4 Effect of $GABA_BR$ activation on evoked EPSCs

There is compelling evidence that mGluR1 receptor activation is greatly enhanced by $GABA_BR$-activation in certain cerebellar cells (Hirono et al., 2001). SCs receive a high level of spontaneous GABA-mediated synaptic activity (Nusser et al., 1997; Auger et al., 1998) arising from action potential firing in neighbouring SCs (Hausser and Clark, 1997). It was next considered whether activation of $GABA_B$ receptors in SCs may also be capable of influencing the expression of CP-AMPARs. Application of the $GABA_B$ agonist baclofen (3μM) produced a striking reduction in evoked EPSC amplitude. Figure 4.8A shows the effects of baclofen (3μM) on evoked EPSC amplitude on one cell. Baclofen also decreased rectification as shown in Figure 4.8B.

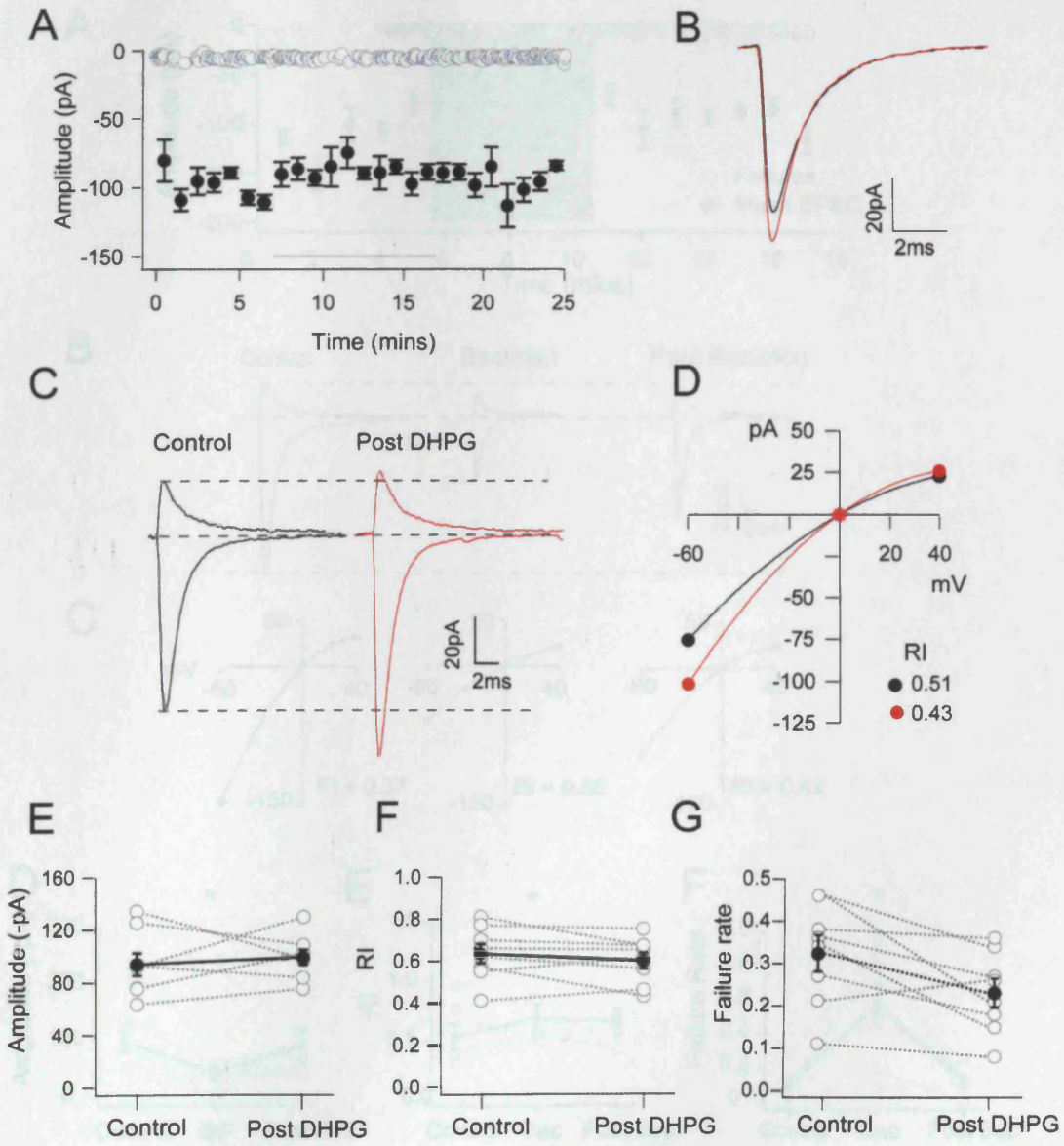


Figure 4.7 BAPTA blocks the effect of DHPG on EPSC amplitude and rectification. A. Time course of the effect of DHPG in the presence of BAPTA (10mM) for a single cell. **B**. Mean EPSCs recorded before (black trace), and after DHPG application (red trace). **C**. Representative evoked EPSCs recorded at -60mV and +40mV before, and after 50μM DHPG application with 10mM BAPTA in the patch-pipette. **D**. Corresponding I-V relationships for the data shown in C. **E**. Mean EPSC amplitude (-60mV) was not significantly changed by DHPG in these conditions; control = 93.8 ± 8.7 and post DHPG = 99.0 ± 5.8 pA (n=8) **F**. RI was not significantly changed in these conditions. Control RI = 0.64 ± 0.05, RI Post- DHPG=0.60 ± 0.04. **G**. There was no significant change in failure rate. Control failure rate = 0.32 ± 0.04 Post DHPG = 0.23 ± 0.03 (P>0.05).

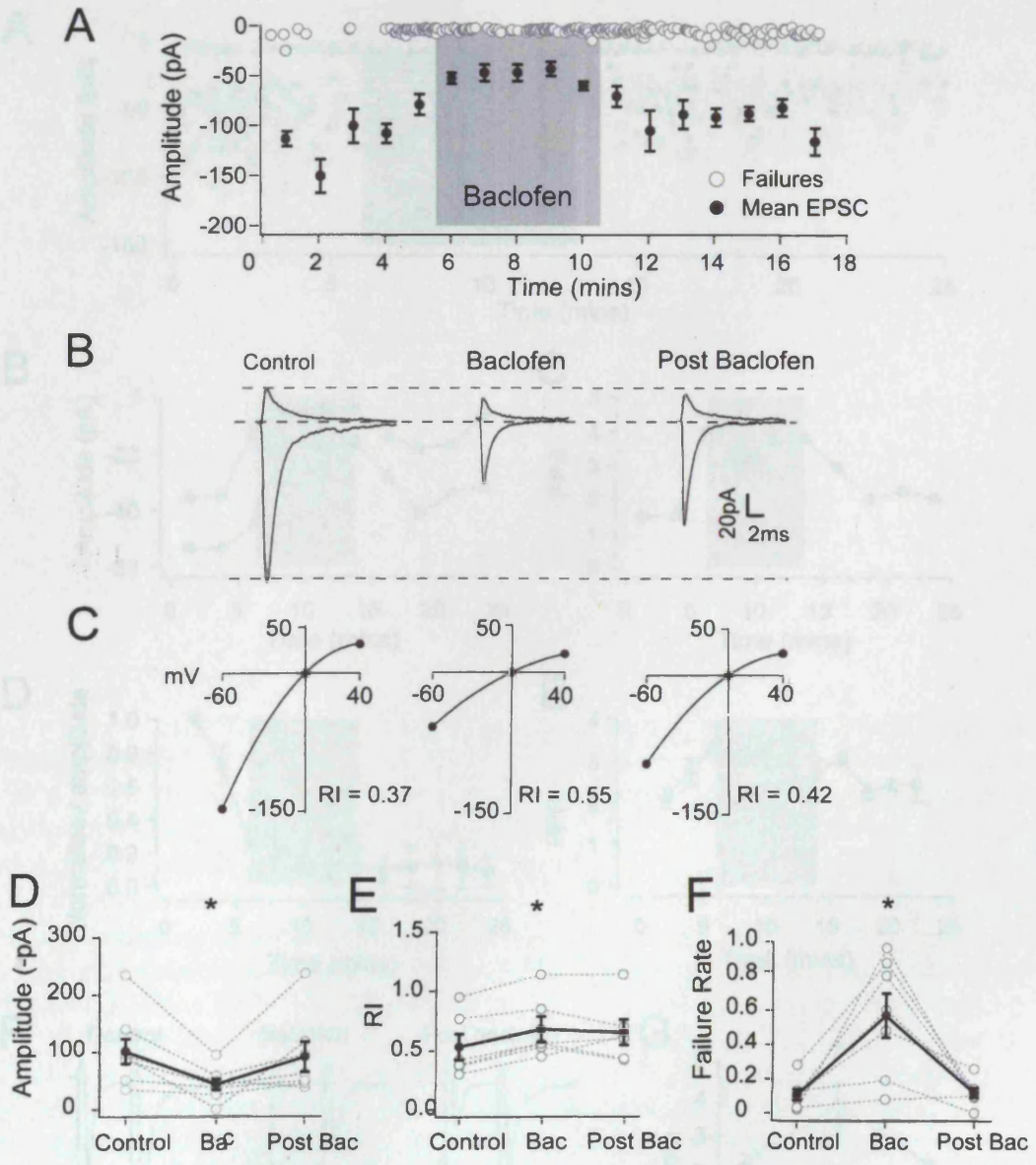


Figure 4.8 Effect of baclofen on evoked EPSCs. A. Representative data from a complete experiment showing the timecourse of the effect of baclofen (3 μM; grey box) on mean amplitude of selected EPSCs. Error bars denote SEM. Failures are shown in grey. **B**. Representative evoked EPSCs recorded at -60mV and +40mV before during and after application of 3μM baclofen (5 mins). Means were constructed from a minimum of 20 selected events (see Methods). Dotted lines show the baseline and peak current for control EPSCs. **C**. I-V relationships constructed from data in B. Control EPSCs display an inwardly rectifying I-V relationship. During application of baclofen EPSCs display a linear I-V relationship. Post-baclofen rectification partially recovers. **D**. Baclofen significantly reduces EPSC amplitude ($P<0.05$; Wilcoxon matched pairs test; asterisk). Mean amplitudes = -101.7± 21.9 (control) 43.3 ± 9.6 (baclofen) and -93.1 ± 23.1 post-baclofen. **E**. Baclofen increases RI ($P<0.05$; Wilcoxon matched pairs test; asterisk). Mean RI values = 0.53 ± 0.11 (control) 0.68 ± 0.12 and 0.64 ± 0.11(post-baclofen). **F**. Baclofen increases failure rate ($P<0.05$; Wilcoxon matched pairs test; asterisk). Mean failure rate values are 0.12 ± 0.03 (control) 0.57±0.13 (baclofen) and 0.12 ± 0.03 (post-baclofen).

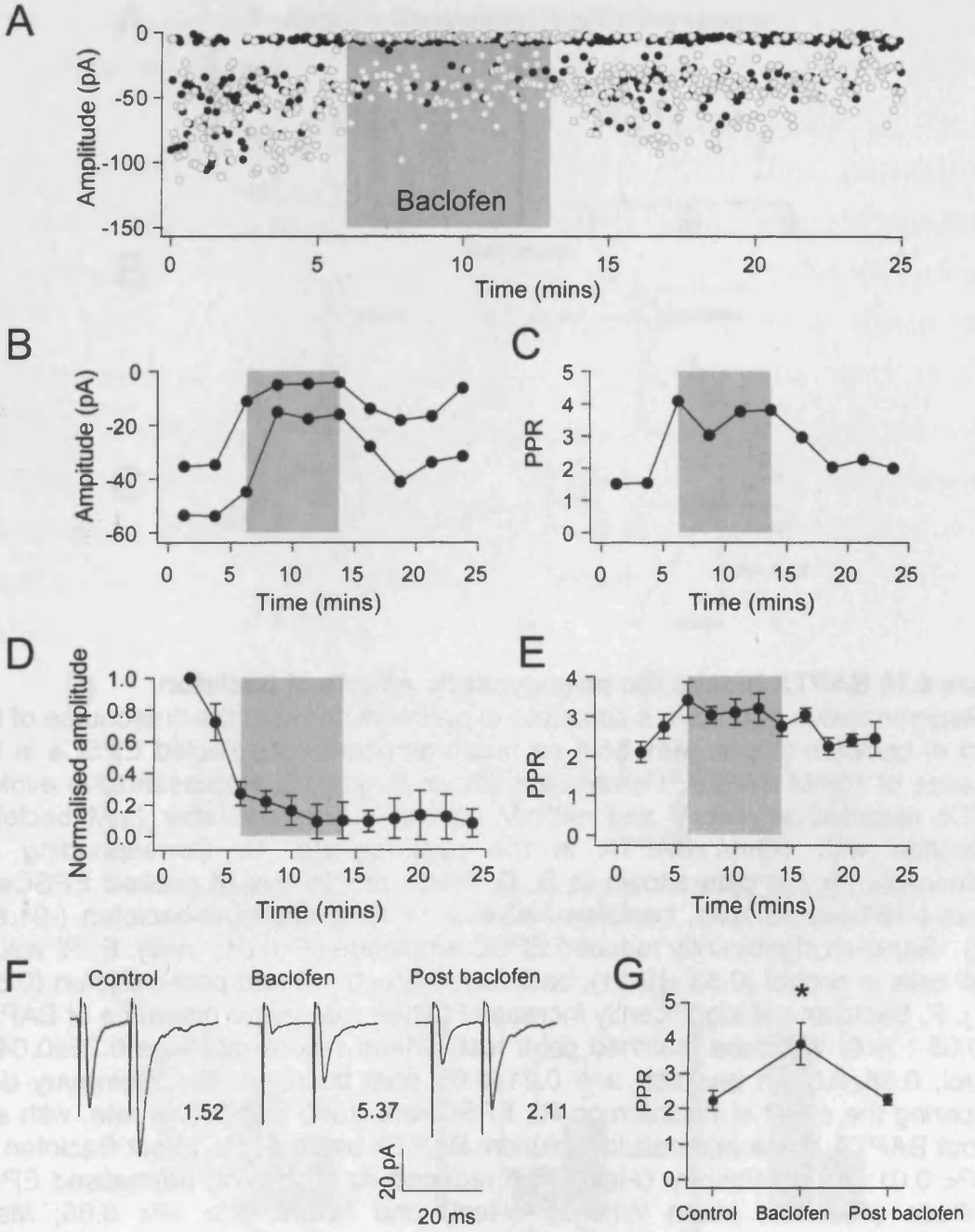


Figure 4.9 Effect of baclofen on paired-pulse ratio. A. Representative data from a complete paired pulse experiment during the application of baclofen (3μM) (grey box). Amplitude of the first (black) and second peak (grey) are plotted. **B**. Mean amplitude of the first and second peak for every 2 minute period. Data is from the same cell as A. Baclofen application shown in grey. **C**. Paired-pulse ratio during application of baclofen. Calculated from the data shown in B. **D**. Normalised change in amplitude of the peak of the first pulse across all cells (n=6). **E**.PPR calculated from data presented in C. **F**. Representative examples of mean traces calculated by averaging all events in control, baclofen and post-baclofen periods. **G**. Mean PPR values for all cells (n=6). Means calculated by averaging all events in control, baclofen and post-baclofen periods. PPR increased significantly from 2.19 ± 0.26 in control conditions, to 3.88 ± 0.61 in baclofen (n = 6, P<0.05; Wilcoxon matched pairs test; asterisk).

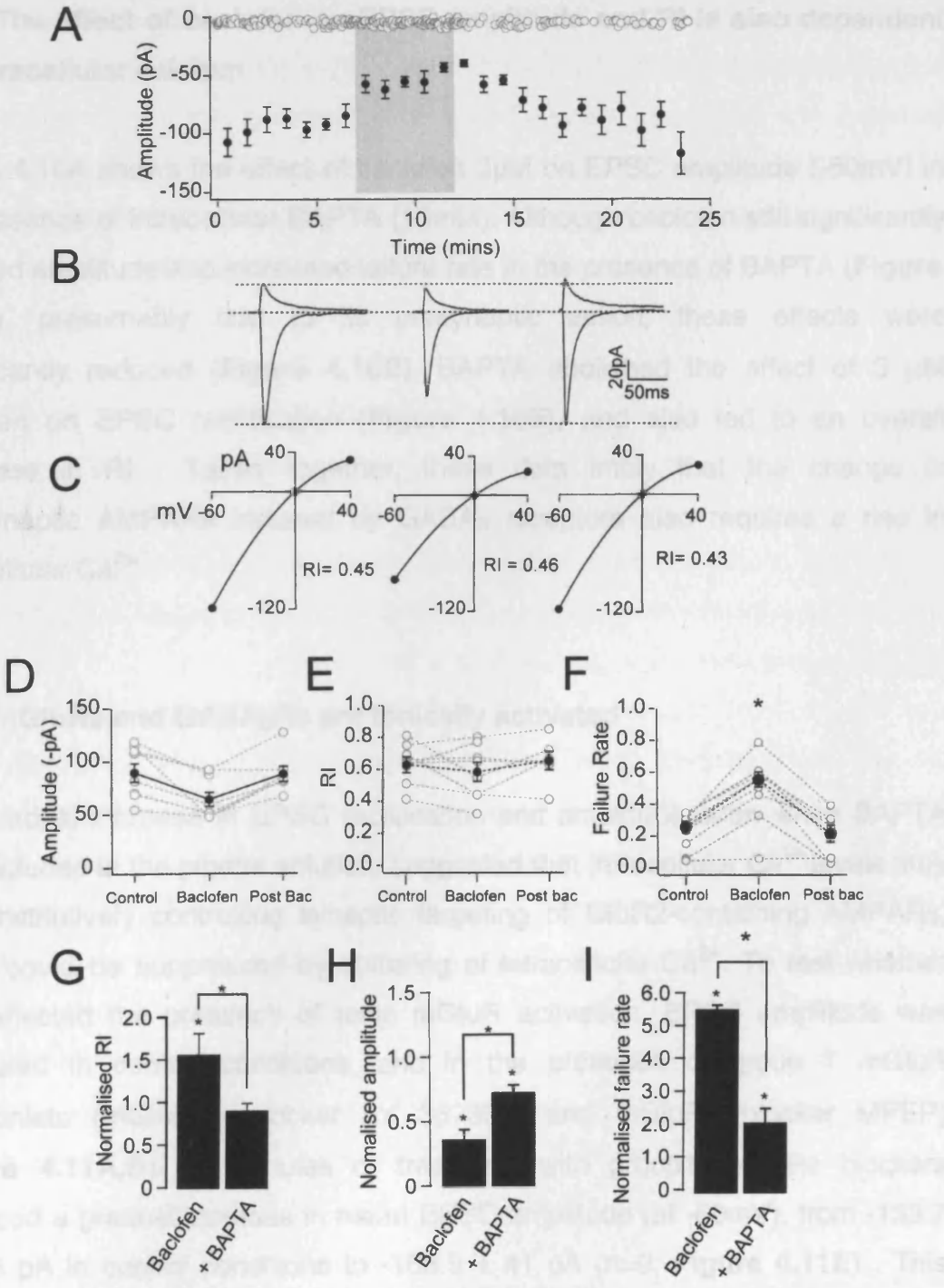


Figure 4.10 BAPTA blocks the post-synaptic effects of baclofen (see opposite).

Figure 4.10 BAPTA blocks the post-synaptic effects of baclofen.
A. Representative data from a complete experiment showing the timecourse of the effect of baclofen (3 μM; grey box) on mean amplitude of selected EPSCs in the presence of 10mM BAPTA. Failures are shown in grey. **B.** Representative evoked EPSCs recorded at -60mV and +40mV before, during and after 3μM baclofen application with 10mM BAPTA in the patch-pipette. **C.** Corresponding I-V relationships for the data shown in B. **D.** Mean amplitudes of evoked EPSCs in control (-104.4 ± 25.1pA), baclofen (-43.6 ± 11.1pA) and post-baclofen (-91.8 ± 26.5). Baclofen significantly reduced EPSC amplitude ($P<0.01$; $n=8$). **E.** RI values for all cells in control (0.53 ± 0.11), baclofen (0.68± 0.11) and post-baclofen (0.65± 0.11). **F.** Baclofen still significantly increased failure rate in the presence of BAPTA ($P<0.05$; $n=8$; Wilcoxon matched pairs test). Mean failure rate was 0.25±0.04 in control, 0.55±0.05 in baclofen and 0.21±0.05 post baclofen. **G-I.** Summary data comparing the effect of baclofen on RI, EPSC amplitude and failure rate, with and without BAPTA in the intracellular solution. BAPTA blocked the effect Baclofen on RI ($P< 0.01$; Mann Whitney *U*-test), and reduced its effects on normalised EPSC amplitude ($P<0.001$; Mann Whitney *U*-test) and failure rate ($P< 0.05$; Mann Whitney *U*-test).

4.3.6 The effect of baclofen on EPSC amplitude and RI is also dependent on intracellular calcium

Figure 4.10A shows the effect of baclofen 3μM on EPSC amplitude (-60mV) in the presence of intracellular BAPTA (10mM). Although baclofen still significantly reduced amplitude and increased failure rate in the presence of BAPTA (**Figure 4.10E**), presumably due to its presynaptic action, these effects were significantly reduced (**Figure 4.10E**). BAPTA abolished the effect of 3 μM baclofen on EPSC rectification (**Figure 4.10E**), and also led to an overall *decrease* in RI. Taken together, these data imply that the change in postsynaptic AMPARs induced by $GABA_B$ receptors also requires a rise in intracellular Ca^{2+}.

4.3.7 mGluRs and $GABA_B$Rs are tonically activated

The gradual increase in EPSC rectification and amplitude seen when BAPTA was included in the pipette solution, suggested that intracellular Ca^{2+} levels may be constitutively controlling synaptic targeting of GluR2-containing AMPARs, which could be suppressed by buffering of intracellular Ca^{2+}. To test whether this reflected the presence of tonic mGluR activation, EPSC amplitude was measured in control conditions, and in the presence of group 1 mGluR antagonists (mGluR1α blocker LY 367385, and mGluR5 blocker MPEP) (**Figure 4.11A,B**). 15 minutes of treatment with group1 mGluRs blockers produced a gradual increase in mean EPSC amplitude (at -60mV), from -133.7 ± 21.5 pA in control conditions to -188.9 ± 41 pA (n=9; **Figure 4.11E**) . This change was accompanied by a consistent increase in rectification (**Figure 4.11F**). As expected, EPSC amplitude was not significantly altered at +40mV, when the current is mediated entirely by GluR2-containing AMPARs. An example of a recording at +40mV before and during application of LY 367385, and MPEP is shown in **Figure 4.11C**.

Application of the $GABA_B$R blocker, CPG62349, also produced a striking enhancement of EPSC amplitude at negative potentials **Figure 4.12.A-B** and,

in some cases, rectification **Figure 4.12.C-D**. EPSC amplitude increased from -108.7 ± 10.8 pA in control conditions, to -146.0 ± 21.7 pA after 10 minutes in the presence of CGP (**Figure 4.12.F**). These findings are consistent with the view that, under resting conditions, group 1 mGluRs and $GABA_BRs$ are both tonically activate, and that suppressing their activation leads to an increase in the relative proportion of synaptic CP-AMPARs.

4.3.8 Effect of intracellular BAPTA (10mM) on evoked EPSCs in the absence of DHPG.

In order to establish whether the tonic activation of group 1 mGluRs and $GABA_B$ receptors, promoting the insertion of GluR2-containing receptors also requires intracellular Ca^{2+}, the effect of dialysing the cell with intracellular BAPTA (10mM) was investigated. **Figure 4.13.A** shows the amplitudes recorded at -60mV during dialysis of intracellular BAPTA (10mM). Overall there was no change in amplitude (-107.6 ± 13.8 at the start of recording and -107.4 6 ± 13.8 after 30 mins recording **Figure 4.13.D**). **Figure 4.13B** shows the effect of intracellular BAPTA on RI. In most cases RI was reduced (on average from 0.59 ± 0.04 to 0.45 ± 0.05, **Figure 4.13 E**). Dialysing the cell with BAPTA (10mM) had no effect on failure rate (**Figure 4.13.F**). Together, these results suggest that the tonic activation of group 1 mGluRs and $GABA_B$ receptors that drive the constitutive insertion of GluR-2 containing AMPARs, requires a certain concentration of intracellular calcium. Buffering calcium hinders the insertion of GluR2-containing receptors but rather promotes the insertion of GluR2-lacking AMPARs.

4.3.9 Effect of baclofen is reduced in the presence of group 1 mGluR antagonists

In order to establish whether the effects of baclofen on RI and amplitude were due to the enhancement of mGluR receptors through the activation of $GABA_B$

receptors, RI and EPSC amplitude were measured during application of baclofen in the presence of the group 1 mGluR antagonists LY 367385 (100μM) and MPEP (10 μM) (**Figure 4.14**). Although baclofen (3μM) still reduced EPSC amplitude (**Figure 4.14.A**) and increased failure rate (**Figure 4.14.C**), this was not significant and was smaller than compared with baclofen alone (**Figure 4.14D,F**). Baclofen also had little effect on RI (**Figure 4.14.B**). Together this suggests baclofen may indeed enhance the actions of mGluR activation, however one must also take into consideration that the mGluR antagonists will also block any tonic mGluR activity, which would result in an increased expression of CP-AMPARs and therefore counter the actions of baclofen.

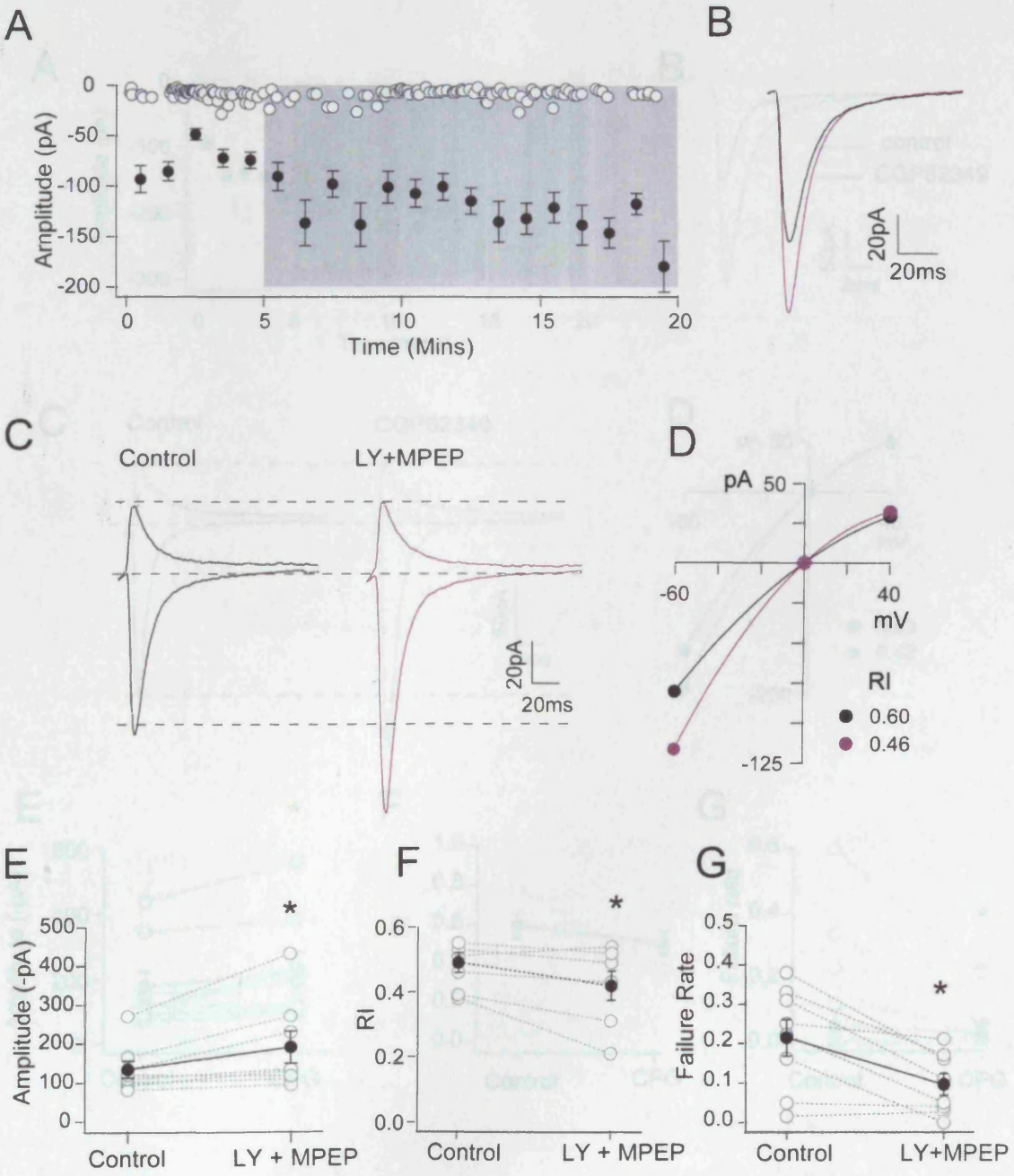


Figure 4.11 mGluR receptors are tonically activated. A. Timecourse of the effect of LY367385 (100μM) and MPEP (10μM) on EPSC amplitude at -60mV in a single cell. **B**. Mean EPSCs before (black) and after (purple) mGluR antagonist application. **C**. Evoked EPSCs at -60mV and +40mV, before (black traces) and after (purple traces) application of LY367385 and MPEP. **D**. Corresponding I-V plots for data shown in C. **E**. Mean EPSC amplitude increased significantly from 133.7 ± 21.52 pA in control, to 188.9 ± 41.0 pA in the presence of LY367385 and MPEP (n=8, P< 0.05; Wilcoxon matched pairs test). **F**. Control inwardly rectifying EPSCs (RI = 0.51 ± 0.03), show increased rectification in the presence of mGluR antagonists (RI = 0.44 ± 0.05; Wilcoxon matched pairs test) (n=6, P< 0.05; Wilcoxon matched pairs test).**G**. Failure rate was significantly reduced in the presence of mGluR antagonists from 0.21± 0.05 to 0.09 ± 0.03 (P < 0.01).

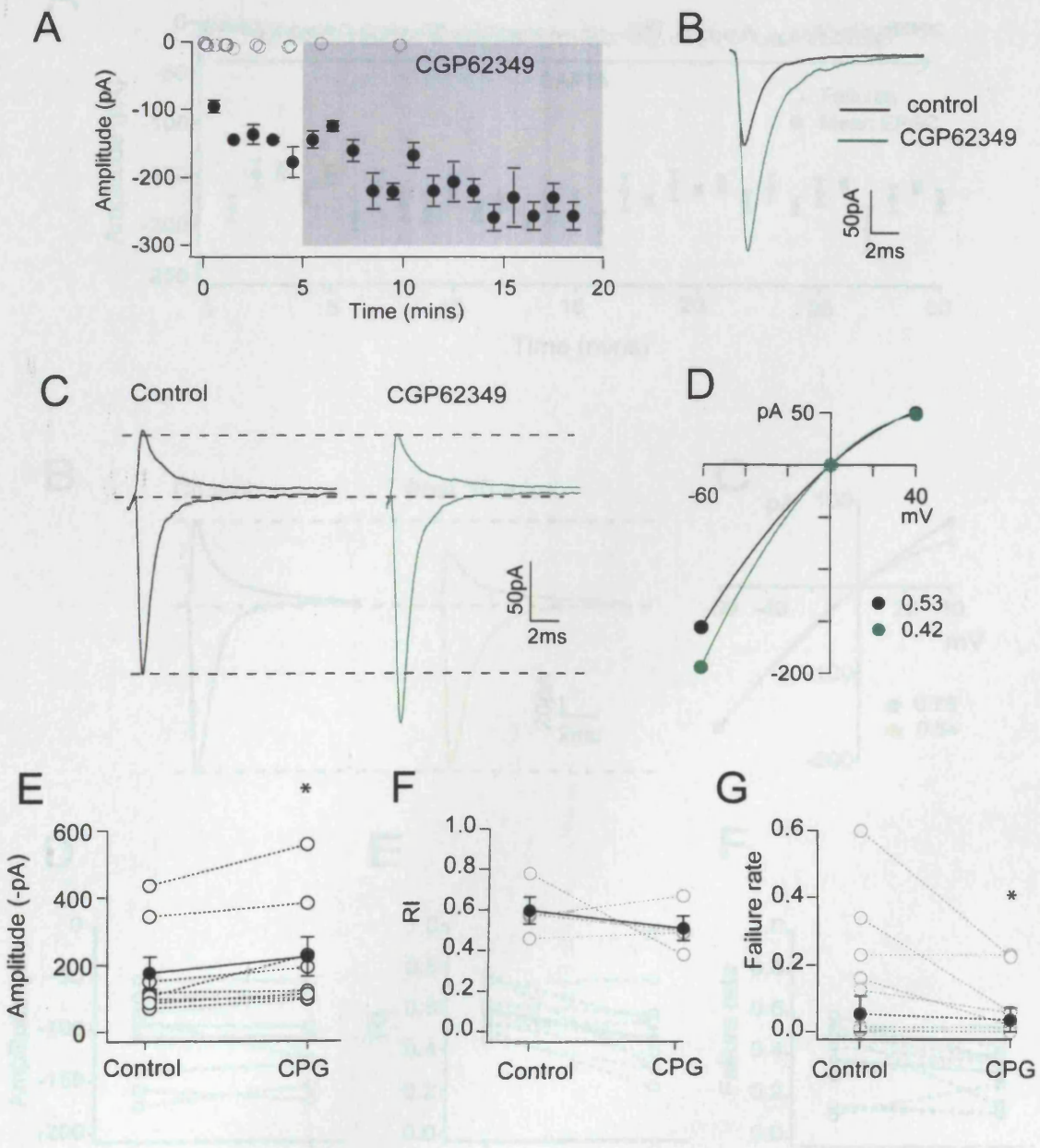


Figure 4.12 $GABA_B$ receptors are tonically activated. **A**. Timecourse of the effect of the $GABA_B$ antagonist CGP62349 (5μM) on EPSC amplitude at -60mV in a single cell. **B**. Mean EPSCs before (black) and after (green) $GABA_B$ antagonist application **C**. Evoked EPSCs at -60mV and +40mV, before (black traces) and after (red traces) application of CGP62349 (5μM). **D**. Corresponding I-V plots for data shown in C. **E**. Mean EPSC amplitude increased significantly from 108.7 ± 10.8pA in control conditions, to 146.0 ± 21.7pA in the presence of CGP62349. (n = 6, P<0.05; Wilcoxon matched pairs test). **F**. Control inwardly rectifying EPSCs (RI = 0.60 ± 0.07) show increased rectification in the presence of CGP62349 (0.51 ± 0.06; n = 4).**G**. Failure rate was significantly reduced in the presence of mGluR antagonists from 0.21± 0.046 to 0.09 ± 0.027 (P < 0.01).

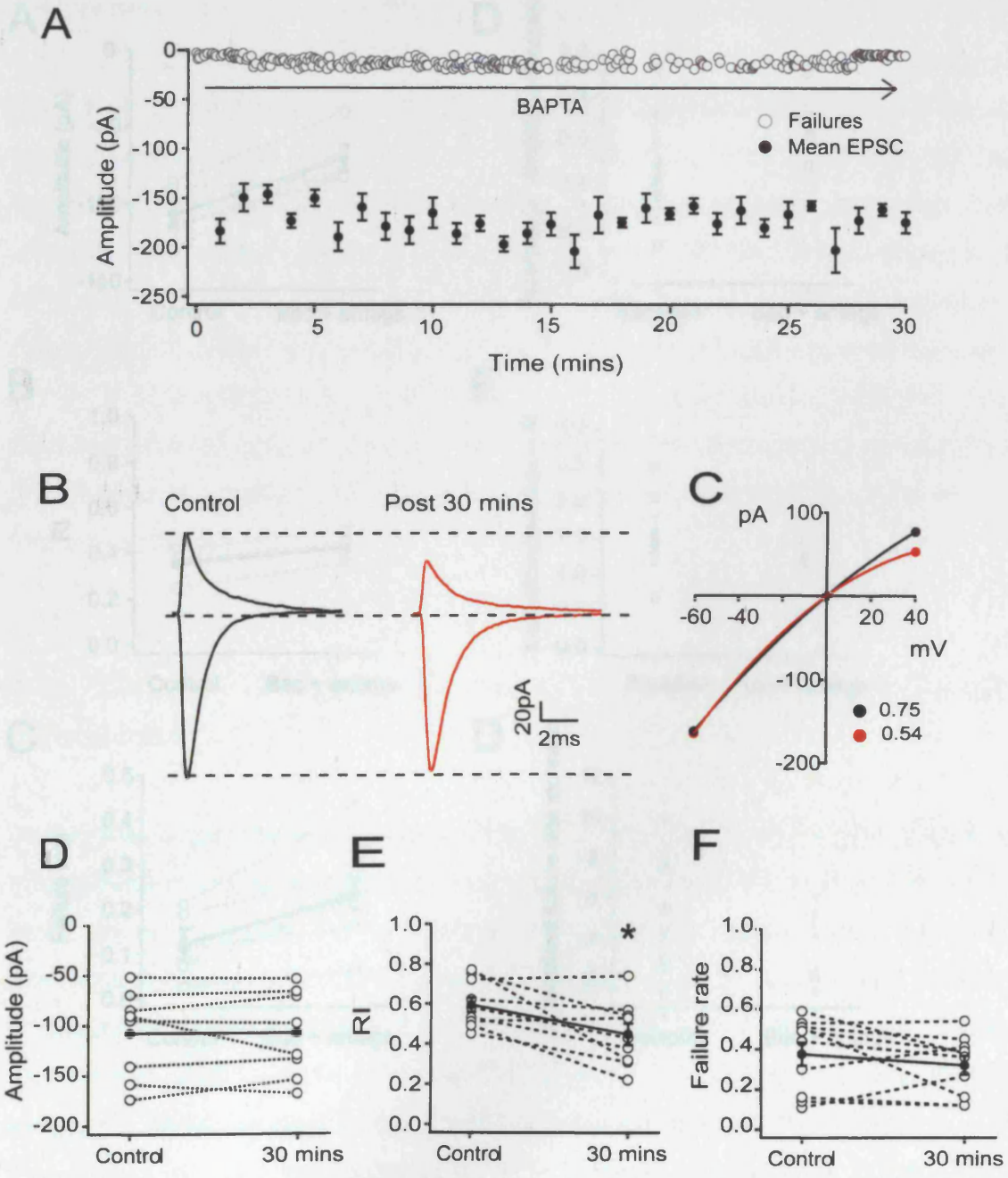


Figure 4.13 Effect of intracellular BAPTA (10mM) on evoked EPSCs
A. Timecourse of the effect of intracellular BAPTA (10mM) on EPSC amplitude at -60mV in a single cell. **B.** Mean EPSCs at the start of recording (black) and after 30 mins dialysis with intracellular BAPTA (10mM) (red) at -60mV and +40mV. **C.** Corresponding IVs for data in B. **D.** Corresponding I-V plots for data shown in **C**. **E.** Mean EPSC amplitude did not change significantly (-107.6 ± 13.8 at the start of recording and -107.4 6 ± 13.8 after 30 mins recording n = 10). **F.** Initially rectifying EPSCs (RI = 0.59 ± 0.04) did show increased rectification after 30 mins dialysis with intracellular BAPTA (10mM) (0.45 ± 0.05; n = 9).**G.** Failure rate was not significantly changed in the presence intracellular BAPTA (10mM). Mean failure rate was 0.38 ± 0.06 at the start of recording and 0.32 ± 0.05 after 30 mins recording.

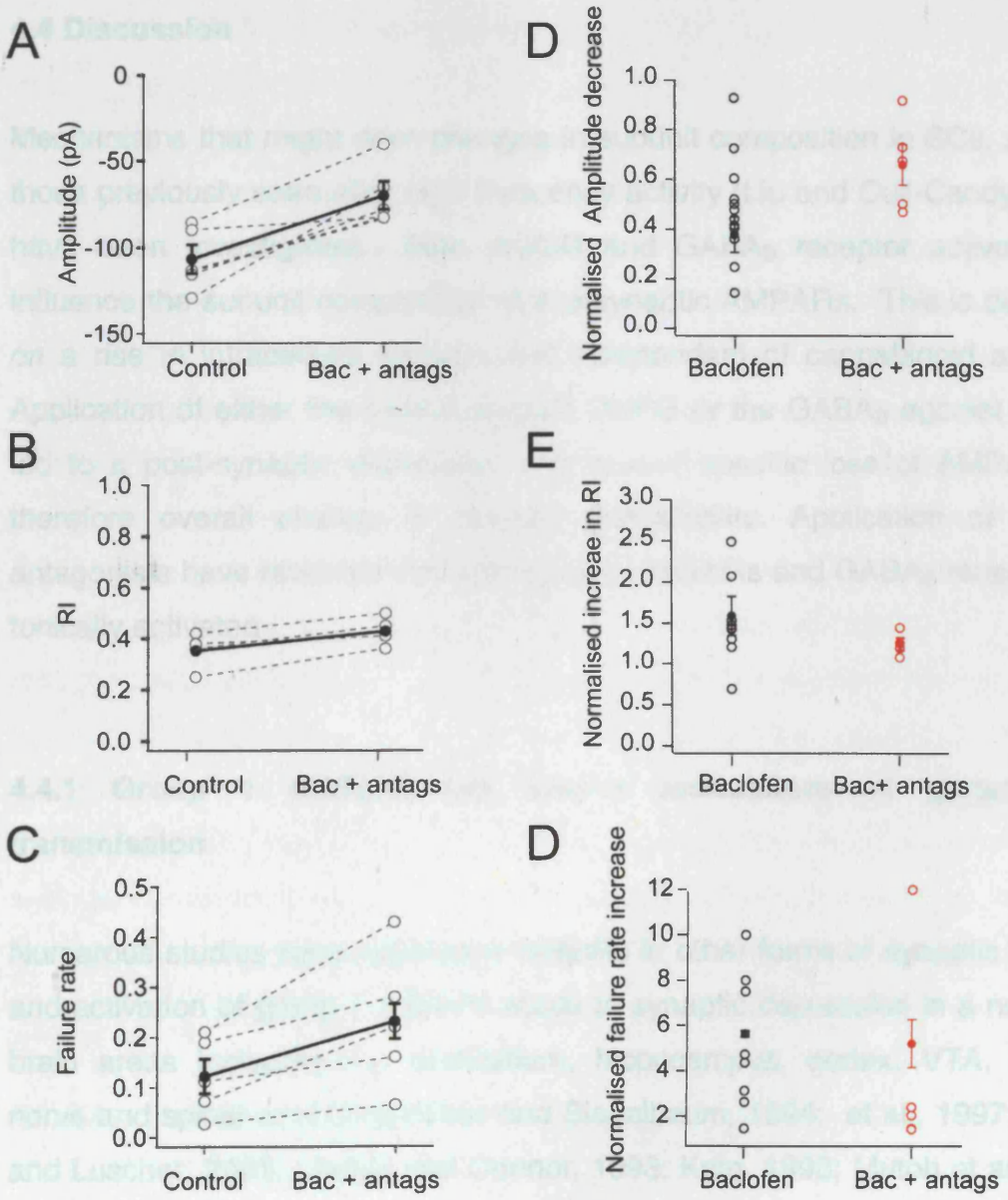


Figure 4.14. Effects of baclofen on EPSC amplitude and RI in the presence of group 1 mGluR antagonists. **A**. Baclofen did not significantly reduce EPSC amplitude in the presence of LY367385 (100μM) and MPEP (10 μM) (*n*=5 *P*>0.05; Wilcoxon matched pairs test). Mean amplitude in control was -106.7± 8.3 and in baclofen was -69.65 ± 8.0. **B**. There was no significant difference in RI in the presence of baclofen (*n*=4 *P*>0.1; Wilcoxon matched pairs test). Mean RI in control was 0.35 ± 0.04 and in baclofen was 0.43 ± 0.03. **C**. Baclofen did not significantly increase failure rate (*n*= 5 *P*>0.05; Wicoxon matched pairs test). Mean failure rate in control was 0.12 ± 0.03 and in baclofen was 0.23 ± 0.06. **D-F** Comparing the effects of baclofen alone and in the presence of group 1 mGluR antagonists.

4.4 Discussion

Mechanisms that might drive changes in subunit composition in SCs, similar to those previously seen after high frequency activity (Liu and Cull-Candy, 2000a) have been investigated. Both mGluR and $GABA_B$ receptor activation can influence the subunit composition of postsynaptic AMPARs. This is dependent on a rise in intracellular calcium and independent of cannabinoid activation. Application of either the mGluR agonist DHPG *or* the $GABA_B$ agonist baclofen led to a post-synaptic depression – a subunit-specific loss of AMPARs and therefore overall change in calcium permeability. Application of receptor antagonists have revealed that both group 1 mGluRs and $GABA_B$ receptors are tonically activated

4.4.1 Group 1 mGluRs are known modulators of glutamatergic transmission

Numerous studies have implicated mGluRs in other forms of synaptic plasticity and activation of group 1 mGluRs leads to synaptic depression in a number of brain areas including the cerebellum, hippocampus, cortex, VTA, olfactory nerve and spinal cord (Bolshakov and Siegelbaum, 1994; et al., 1997; Bellone and Luscher, 2005; Linden and Connor, 1993; Kato, 1993; Mutoh et al., 2005). Accordingly, DHPG application also led to synaptic depression at the PF-SC synapse. More unusual is the DHPG-induced change in RI, which has only also been described previously in the VTA (Bellone and Luscher, 2005). It will be interesting to see if group 1 mGluR activation exerts a similar effect at other synapses expressing CP-AMPARs.

4.4.2 The mGluR subtypes involved

The group 1 mGluRs consist of subtypes mGluR1α and mGluR5 both of which are expressed in the molecular layer (Negyessy et al., 1997; Hamori et al., 1996).These can have temporally and mechanistically distinct, or even opposing effects in the same neuron (Valenti et al., 2002) and with regards plasticity (Grassi et al., 2002; Volk et al., 2006; Grueter et al., 2006). Previously observed effects of group 1 mGluR activation in cerebellar molecular layer interneurons have been found to be blocked by both a specific mGluR1α antagonist (Karakossian and Otis, 2004; Soler-Llavina and Sabatini, 2006), and a global group 1 mGluR antagonist (Rancillac and Crepel, 2004). In the present experiments, it is clear that co-application of both mGluR1α and mGluR5 antagonists block the effect of DHPG on RI. Further experiments with specific antagonists are required to convincingly define the actions of individual subtypes. The results of these experiments would have to take into account the effect of blocking tonically active mGluRs. It would be interesting to see if one subtype is mediating the change in RI, and the other the change in amplitude, for example.

4.4.3 Are the actions of DHPG pre or postsynaptic?

The exact locus and mechanisms underlying the expression of mGluR-mediated LTD has been highly controversial, especially in the hippocampus and experiments argue for both pre- and postsynaptic effects. Evidence for presynaptic action includes a change in paired pulse facilitation (PPF), coefficient of variation (CV) and unchanged sensitivity to uncaged l-glutamate (Bolshakov and Siegelbaum, 1994; Oliet et al., 1997; Fitzjohn et al., 2001; Faas et al., 2002; Watabe et al., 2002; Rammes et al., 2003). However DHPG-LTD is also prevented by postsynaptic application of inhibitors of endocytosis or protein synthesis and involves changes in the surface distribution of GluR1 subunits in both slices and cultured neurons (Snyder et al., 2001; Huang et al., 2004a; Moult et al., 2006; Banko et al., 2006). Mechanisms are also thought to change during development (Nosyreva and Huber, 2006) and recently it was shown that

DHPG-LTD still occurs in the absence of protein synthesis in *Fmr1* knockout mice suggesting that other mechanisms can be recruited (Nosyreva and Huber, 2006).

The present results are consistent with the idea that at PF-SC synapses DHPG exerts its effects postsynaptically. Despite an increase in failure rate, there was no change in PPR post DHPG application (particularly noticeable when compared to baclofen). The reduction in mEPSC amplitude and lack of block by AM251 (see below) also provide strong evidence for a post synaptic mechanism. The increase in failure rate, could be explained by a rapid redistribution of AMPARs as described in the hippocampus (Noel et al., 1999; Xiao et al., 2001; Moult et al., 2006).

These findings differ from another form of mGluR-mediated plasticity at the PF-SC synapse (Soler-Llavina and Sabatini, 2006) which does include a change in PPR, suggesting a presynaptic mechanism. This form of plasticity is induced by low frequency stimulation, does not involve a switch in receptor subtype, is blocked by an mGluR1α antagonist and is dependent on cannabanoid activation (see below). Different forms of group 1 mGluR–mediated plasticity have been found to be expressed at the same synapse (Bellone and Luscher, 2005) and it should be noted that although DHPG is used as an effective way to study synaptically induced mGluR-LTD, several differences between chemically and electrically induced LTD have been found in the hippocampus. For example, DHPG-LTD expression requires sustained activation of mGluRs (Watabe et al., 2002; Rouach and Nicoll, 2003; Huang and Hsu, 2006) and the induction of DHPG-LTD is independent of postsynaptic Ca^{2+} entry (Fitzjohn et al., 2001).

4.4.4 CB1 activation is not required for DHPG mediated plasticity at the PF-SC synapse

It was important to investigate the possible involvement of cannabinoids in the plasticity described for two reasons. Firstly, activation of group 1 mGluRs is known to lead to release of endocannabinoids in a number of brain areas, leading to short and long term depression (Sung et al., 2001; Robbe et al., 2003; Azad et al., 2004; Maejima et al., 2001; Melis et al., 2004; Kushmerick et al., 2004; Narushima et al., 2006; Varma et al., 2001; Rouach and Nicoll, 2003; Bellone and Luscher, 2005; Brown et al., 2004; Kettunen et al., 2005; Chevaleyre and Castillo, 2003; Ohno-Shosaku et al., 2002) usually by acting on presynaptic receptors to alter transmitter release (Chevaleyre and Castillo, 2003; Gerdeman et al., 2002; Marsicano and Lutz, 2006; Robbe et al., 2002; Sjostrom et al., 2003) but see (Safo and Regehr, 2005). Therefore, establishing a role, or lack there of for cannabinoids would help confirm the location and mechanism of expression of this plasticity. The DHPG-induced increase in failure rate in the presence of AM251 argues against the idea that any presynaptic effect is being masked by a strong postsynaptic effect.

Secondly, bath application of DHPG, would inevitably lead to activation of group 1 mGluRs on other neurons in the slice. Activation of mGluR1αs on Purkinje cells is known to lead to release of endocannabanoids (Maejima et al., 2001) which can act on receptors on SCs (Galante and Diana, 2004; Rancillac and Barbara, 2005) and PFs (Brenowitz and Regehr, 2003; Brown et al., 2003; Levenes et al., 2001). Therefore, it was important to determine whether this plasticity was a result of activation of mGluRs on SCs rather than the repercussions of mGluR activation on Purkinje cells leading to CB1R activation on SCs themselves or on PFs which would affect glutamate release onto SCs (Rancillac and Barbara, 2005). The argument that it is the mGluRs on SCs that indeed mediate this plasticity, is further supported by the fact that it is abolished by inclusion of BAPTA in the pipette.

The difference between the type of plasticity described here and the presynaptic plasticity described by Soler-Llavina and Sabatini, (2006) is

emphasised by the fact that the presynaptic form of plasticity is blocked in the presence of AM251. Other cannabinoid-mediated plasticity reported at the PF-SC synapse (Rancillac and Barbara, 2005) is thought to arise from release of cannabinoids from neighbouring Purkinje cells. Soler-Llavina and Sabatini (2006) do not explicitly comment on the source of cannabinoids, however the fact that BAPTA blocks this form of plasticity suggests that either endocannabinoids are being released from SCs in a calcium-dependent manner, or that additional, calcium-dependent mechanisms are required for plasticity to take place. Indeed, it has recently been demonstrated that SCs can release cannabinoids, (Beierlein and Regehr, 2006), however this is dependent on NMDA activation and both the experiments in the present study, and those described by Soler-Llavina and Sabatini (2006) were performed in the presence of NMDA receptor antagonists.

If endocannabinoids *are* being released from SCs, it could be that cannabinoid release requires a certain level of highly localised Ca^{2+} elevation, as the plasticity described by Soler-Llavina and Sabatini (2006) is dependent on both CP-AMPAR and mGluRs activation and is synapse specific. Because I did not stimulate during the application of DHPG, calcium entry through CP-AMPARS is limited to purely spontaneous events and so may be below the threshold required for cannabinoid release. It should be noted that Soler-Llavina and Sabatini (2006) used 20mM BAPTA to block plasticity, where as I used only 10mM. Differences may also reflect the fact that I bath applied DHPG and possibly activated somatic receptors, including mGluR5 receptors, which are not required for the SCLTD they describe.

Both cannabinoid-dependent and -independent forms of mGluR-mediated plasticity can coexist at the same synapse. However, at these synapses, cannabinoids only meditate a transient depression and are not required for expression of LTD (Rouach and Nicoll, 2003; Bellone and Luscher, 2005). It is clear that plasticity at this synapse is a relatively new subject of enquiry, so further experiments will be needed to characterise the different forms present.

4.4.5 Decrease in RI and amplitude is dependent on a rise in intracellular Ca^{2+}

Many forms of mGluR-LTD are dependent on a rise in intracellular Ca^{2+}, and this can occur in a variety of ways. Firstly, group I mGluRs classically couple via G_q to phospholipase C (PLC) with two downstream pathways, leading to activation of PKC or IP_3 which leads to release of Ca^{2+} from intracellular stores (Hermans and Challiss, 2001; Nakamura et al., 1999; Conn and Pin, 1997; Fagni et al., 2000). Group 1 mGluR mediated Ca^{2+} signalling can also involve release from ryanodine-sensitive stores (Woodhall et al., 1999) Release from stores is involved in mGluR-LTD, in the hippocampus (Reyes and Stanton, 1996; Wang et al., 1997) and cerebellum (Safo and Regehr, 2005).

Secondly, Ca^{2+} elevation could arise from influx through voltage-dependent calcium channels (VDCCs) (Oliet et al., 1997; Bianchi et al., 1999; Mao and Wang, 2003; Endoh, 2004; Kettunen et al., 2002) and this is required for several forms of mGluR-LTD (Kreitzer and Malenka, 2005; Heinke and Sandkuhler, 2005). Membrane depolarization by DHPG alone can be strong enough to open L-type VDCCs (Bianchi et al., 1999; Mao and Wang, 2003). Furthermore, glutamate-induced Ca^{2+} influx through VDCCs may initiate Ca^{2+} release from intracellular stores to the cytoplasm (Usachev and Thayer, 1997). Reciprocally, release of Ca^{2+} from reticular Ca^{2+} stores leads to facilitation of L-type VDCCs (Perrier et al., 2002), via Ca^{2+} binding to calmodulin (Zuhlke et al., 1999).

Thirdly, transient receptor potential (TRP)1/5 channels are activated by G_q - protein coupled receptors (Strubing et al., 2001; Clapham et al., 2001) and mGluR1 activation leads to TRP-mediated responses in Purkinje neurons (Kim et al., 2003), midbrain dopamine neurons (Tozzi et al., 2003) hippocampal neurons (Gee et al., 2003;Topolnik et al., 2005) As the current experiments were carried out under voltage clamp and SCs are electrically compact, it is unlikely that VGCCs are being activated. However, the holding potential was changed at various points during the experiment to assess rectification, therefore the activation of VGCCs can not be completely ruled out. Elevation of

calcium in SCs is most likely to be a combination of release from intracellular stores and entry through the CP-AMPARS. Removing external calcium prevents the subunit change seen with high frequency activity (Liu and Cull-Candy, 2000a), suggesting that calcium entry is required in addition to other mechanisms. Alternatively external calcium may be required for optimal activation of mGluRs (Tabata et al., 2002; Tabata and Kano, 2004; Tabata et al., 2004; Kubo et al., 1998; Miyashita and Kubo, 2000).

4.4.6 What is the likely mechanism of DHPG-induced plasticity at this synapse?

The data presented points to a postsynaptic expression. In addition, amplitudes at negative potentials are dramatically reduced, whereas remain relatively unchanged at positive potentials, where the current is mediated only by CI-AMPARs. An attractive explanation therefore, is that group 1 mGluR activation in SCs leads to a subunit specific removal of CP-AMPARs from the synaptic density. This could be by internalisation (Snyder et al., 2001), or lateral diffusion (Moult et al., 2006).

DHPG-LTD in the hippocampus, which is thought to arise from a lasting decrease in postsynaptic receptors (Snyder et al., 2001; Xiao et al., 2001; Huang et al., 2004a), involves activation of G-proteins (Huang et al., 2004a; Kleppisch et al., 2001; Watabe et al., 2002), protein tyrosine phosphatases (Berman et al., 2006), mitogen-activated protein kinase (MAPK) cascades (Gallagher et al., 2004; Huang et al., 2004b) and local translation of dendritic mRNA to produce proteins required for the AMPAR internalisation (Huber et al., 2001; Kemp and Bashir, 1999a). It is thought that a MAPK cascade pathway *and* activation of a PTP to inhibit a PTK, resulting in a dephosphorylation of AMPARs, is required for LTD (Berman et al., 2006). It should be noted that these changes are independent of intracellular Ca^{2+} concentration (Fitzjohn et al., 2001), unlike the situation in SCs.

In Purkinje cells of the cerebellum, mGluR-induced AMPAR endocytosis is the result of a rise in postsynaptic Ca^{2+} and liberation of diacylglycerol which activates PKCα, to phosphorylate GluR2 Ser880. This suppresses the interaction between GluR2 and glutamate receptor interacting protein (GRIP), resulting in release of the receptor. GluR2 then binds to protein interacting with C kinase 1 (PICK1) and is removed via clathrin-mediated endocytosis (Matsuda et al., 1999; Chung et al., 2003; Xia et al., 2000; Wang and Linden, 2000; Leitges et al., 2004). In SCs, CP-AMPARS are thought to be homomeric GluR3 (Petralia and Wenthold, 1992), and little is known about phosphorylation of this receptor subtype. However, the site for phosphorylation and interactions with PICK are highly conserved between the two subunits (Malinow and Malenka, 2002) suggesting that phoshorylation of GluR3 subunit is possible.

4.4.7 $GABA_B$ receptor activation controls CP-AMPAR plasticity in stellate cells

The finding that $GABA_B$ activation can drive changes in AMPAR subunit composition similar to those seen with group 1 mGluR activation may at first appear surprising. How could a receptor classically known to act presynaptically on VDCCs to alter transmitter release (Mintz and Bean, 1993; Poncer et al., 1997; Sakaba and Neher, 2003) and postsynaptically induces slow inhibitory potentials by gating Kir3-type K^+ channels (Luscher et al., 1997), affect AMPAR subunit composition? One possibility, as other recent studies have suggested, is that $GABA_B$ activation enhances the actions of mGluRs (Tabata et al., 2004; Hirono et al., 2001; Patenaude et al., 2003; Hirono et al., 2001). As we have shown that both mGluRs and $GABA_B$ receptors are tonically active, one could propose that baclofen acts by potentiating the already active group 1 mGluRs. This would fit with the observation that blocking tonic $GABA_B$ activation alone is not sufficient to induce the opposite change in calcium permeability. My preliminary experiments show that the effect of baclofen seems to be reduced in the presence of mGluR antagonists, suggesting some cooperative activity between the receptors.

4.4.8 mGluR and $GABA_B$ receptors are tonically activated

Inclusion of BAPTA in the patch pipette and application of group 1 mGluR antagonists led to an increase in EPSC amplitude and rectification implying that mGluRs are tonically active and control the constitutive targeting of synaptic AMPARs. Potentiation of EPSCs with the inclusion of BAPTA in the pipette has been observed in SCs previously (Rancillac and Crepel, 2004) and also in the VTA (Robbe et al., 2003). It occurs in the hippocampus when endocytosis is blocked (Xiao et al., 2001) suggesting that receptors are constitutively removed from the synapse. Tonic activation of mGluRs has been detected in neocortex (Chen and Roper, 2004) calyx of Held (Renden et al., 2005) and medial vestibular nuclei (Grassi et al., 2002) (presynaptic) and second–order baroreceptor neurons (Sekizawa and Bonham, 2006), and hippocampal cells (Losonczy et al., 2003) (postsynaptic), and is presumed to be a result of ambient glutamate.

Similarly the application of $GABA_BR$ antagonists led to an increase in EPSC amplitude at all potentials, which is probably due to the relief of tonic activation of presynaptic receptors. SCs receive a constant barrage of GABAergic inputs arising from other SCs, which generally show a high level of spontaneous action potential activity, (Hausser and Clark, 1997) and this presumably results in continuous $GABA_BR$ activation. Persistent $GABA_BRs$ activation in SCs has been described previously (Mann-Metzer and Yarom, 2002b).

The structural features of the *en passant* PF-SC synapse facilitate tonic activation of metabotropic receptors. These synapses are not surrounded by glial processes, implying that the diffusion pathway to extrasynaptic sites is relatively clear; the distance to glial transporters is long and the density of GLT1 and GLAST transporters is low - compared with PF-Purkinje cells synapses (Chaudhry et al., 1995). Other factors, however, may contribute to tonic activation of metabotropic receptors. For example, Ca^{2+} has been shown to may be acting as an agonist either directly (Kubo and Tateyama, 2005; Miyashita and Kubo, 2000) or indirectly, through activation of $GABA_B$ receptors (Tabata et al., 2004; Wise et al., 1999). It has also been suggested that group1

mGluRs may be intrinsically active in the absence of agonists (Goudet et al., 2004; Kubo and Tateyama, 2005).

The functional implications of constitutive- and activity-dependent regulation of AMPAR subunit composition at the PF-SC synapse is considered in the General Discussion (Chapter 6). Recent work has shown that a single exposure to cocaine induces the expression of CP-AMPARs at synapses in the ventral tegmental area, and that this form of CP-AMPAR plasticity is also rapidly lost following activation of metabotropic glutamate receptors (Bellone and Luscher, 2005). My results suggest a similar mechanism could underlie a switch in AMPAR subunit composition, and Ca^{2+}-permeability in a variety of forms of plasticity.

5. Two kinetically distinct populations of currents underlie quantal AMPA-mediated events in stellate cells

5.1 Summary

Kinetic analysis of spontaneous EPSCs in SCs revealed that events were composed of two distinct populations of currents that differed in their rise time, decay time and amplitude. These were termed 'fast' and 'slow' sEPSCs ($sEPSC_{fast}$ and $sEPSC_{slow}$).

$sEPSC_{slow}$ events still occurred at the same frequency in the absence of presence of TTX and were blocked in the presence of by the AMPAR antagonist GYKI.

To determine whether $sEPSC_{slow}$ events could be evoked during PF stimulation, extracellular strontium was used to desynchronise transmitter release. Events detected within a 150ms time window after the synchronous EPSC displayed fast kinetics. This suggests that $EPSC_{slow}$ do not contribute to the evoked synaptic event.

5.2 Introduction

In previous chapters spontaneous EPSCs and quantal events have been examined in SCs to give information about the postsynaptic AMPARs. Indeed, analysis of spontaneous and quantal events at synapses has classically been employed to give information about both pre and postsynaptic properties of synaptic transmission (Katz and Miledi, 1969b). Factors altering transmitter release or postysynaptic receptor properties can be investigated by measuring changes in the frequency, amplitude and kinetics of miniature events (Silver, 2003; Bekkers and Stevens, 1994). Despite extensive use of mEPSC recordings in studies investigating neurotransmission, the role they might play in the CNS is often overlooked and has relatively recently been re-recognised to be potentially important (Zucker, 2005). The influence of mEPSCs or mIPSCs on SC activity may be of special significance given that single excitatory quanta can generate an action potential in these cells while single inhibitory quanta can suppress or delay postsynaptic firing (Carter and Regehr, 2002).

At central glutamatergic synapses, the rapid rise time of AMPAR-mediated synaptic currents (Forti et al., 1997; Geiger et al., 1997; Silver et al., 1992) and time course of displacement of competitive antagonists (Clements, 1996; Diamond and Jahr, 1997) suggests that during glutamate release 90% of vesicular content enters the synaptic cleft in less than 100μs. Glutamate released in this way would be expected to give rise to currents that rise and decay rapidly. However, AMPA-mediated conductances with slow rise times have also been recorded from central synapses, both at room temperature (Renger et al., 2001) and near physiological temperature (Carter and Regehr, 2000; DiGregorio et al., 2002; Schoppa and Westbrook, 2001).

The presence of slow rising events can be explained in a numbers of ways. Differences in the kinetics of quantal responses recorded at a single synapse could arise from a presynaptic mechanism (Sulzer and Pothos, 2000) such as the existence of different vesicle pools with varying fusion pore kinetics (Pothos, 2002). Alternatively, postsynaptic receptors may differ in their kinetics (Cossart et al., 2002). Slow rise times can be due to prolonged low concentrations of

neurotransmitter in the cleft. The kinetics of the glutamate transient depends on the density and location of transporters (Diamond and Jahr, 1997). Such prolonged low concentrations of neurotransmitter could arise from a narrow fusion pore, or the diffusion of neurotransmitter from distant sites – spillover.

Diffusion or 'spillover' of glutamate has been characterised at many synapses and can lead to activation of high-affinity mGluRs on neighbouring glutamatergic and GABAergic axon terminals (Scanziani et al., 1997; Mitchell and Silver, 2000) and high-affinity NMDA receptors located either extrasynaptically or in neighbouring synapses (Asztely et al., 1997; Isaacson, 1999; Carter and Regehr, 2000; Diamond, 2001; Clark and Cull-Candy, 2002). Although AMPA receptors have a lower affinity for glutamate and desensitise rapidly, studies have also reported the spillover-mediated activation of AMPA receptors (Carter and Regehr, 2000; DiGregorio et al., 2002). At the PF-SC synapse glutamate spillover leads to activation of both NMDARs and AMPARs (Clark and Cull-Candy, 2002; Carter and Regehr, 2000).

Spillover is not the only source of transmitter that is able to activate receptors extrasynaptically. Release of neurotransmitter vesicles at extrasynaptic sites (ectopic release) has been suggested by the presence of morphologically docked vesicles distant from PSDs in electron micrographs from tissues (Zenisek et al., 2000), including the ribbon synapses of bipolar neurons and saccular hair cells (Lenzi et al., 1999). Such release of glutamate has been shown to occur from climbing fibres in the cerebellum (Matsui and Jahr, 2004) which leads to activation of AMPARs on closely apposed Bergmann glia (Matsui et al., 2005). Modelling also predicts that ectopic release is likely to occur at cholinergic synapses in the chick ciliary ganglion (Coggan et al., 2005).

In this chapter I describe a type of spontaneous event recorded in SCs that differs from previously characterised spontaneous currents described both in kinetics and amplitude. Possible sources of this type of event are considered. It should be noted that for the analysis of spontaneous and quantal events in previous chapters, events with a slow rise time were rejected.

5.3 Results

5.3.1 Spontaneous EPSCs are composed of two distinct populations

Whole-cell spontaneous activity was recorded from 13 SCs in the presence of NMDA and $GABA_A$ antagonists. **Figure 5.1A** shows examples of individual spontaneous EPSCs varying in their time course. Histograms for 10-90% rise time, 37% decay and amplitude were constructed (**Figure 5.1 C-E**) and events were separated into two populations according to 10-90% rise time (see Methods). Separated events were then averaged. Mean traces of the two populations are shown in **Figure 5.1B**. The predominant type of event had a fast rise and decay, similar to the kinetics of an evoked EPSC. Events falling into this category were termed $sEPSC_{fast.}$ The other type of event had a much slower rise and decay time and smaller amplitude. These were termed $sEPSC_{slow.}$ Both types of event were consistently observed in all cells and displayed similar waveforms.

Across all cells (n=13), the mean $sEPSC_{fast + slow}$ (the average of all events) had a 10-90% rise time of 0.34 ± 0.02 ms, a 37% decay of 1.48 ± 0.07 ms and mean amplitude of -37 ± 5 pA. The mean $sEPSC_{fast}$ displayed a mean 10-90% rise time of 0.31 ± 0.02 ms, a 37% decay of 1.18 ± 0.04 ms, mean amplitude of -46 ± 5 pA. Fast events occurred at a mean frequency of 0.29 ± 0.09 Hz. The mean $sEPSC_{slow}$ had a 10-90% rise time of 0.70 ± 0.06ms a 37% decay of 2.5 ± 0.31 ms, and a mean amplitude of -19.6 ± 3.0 pA. Slow events occurred at a mean frequency of 0.10 ± 0.04 Hz.

Figure 5.1F shows the 10-90% rise time of the sEPSCs recorded from a single cell plotted against peak amplitude. This gave rise to an L-shaped distribution suggesting that the differences in kinetics and amplitude of events were not caused by dendritic filtering. If filtering of events was the primary origin of the kinetic differences, a more linear distribution would be expected. The L-shaped distribution also highlights the fact that while events with a fast rise time can still be of a small amplitude, events that rise and decay slowly are always of a modest amplitude.

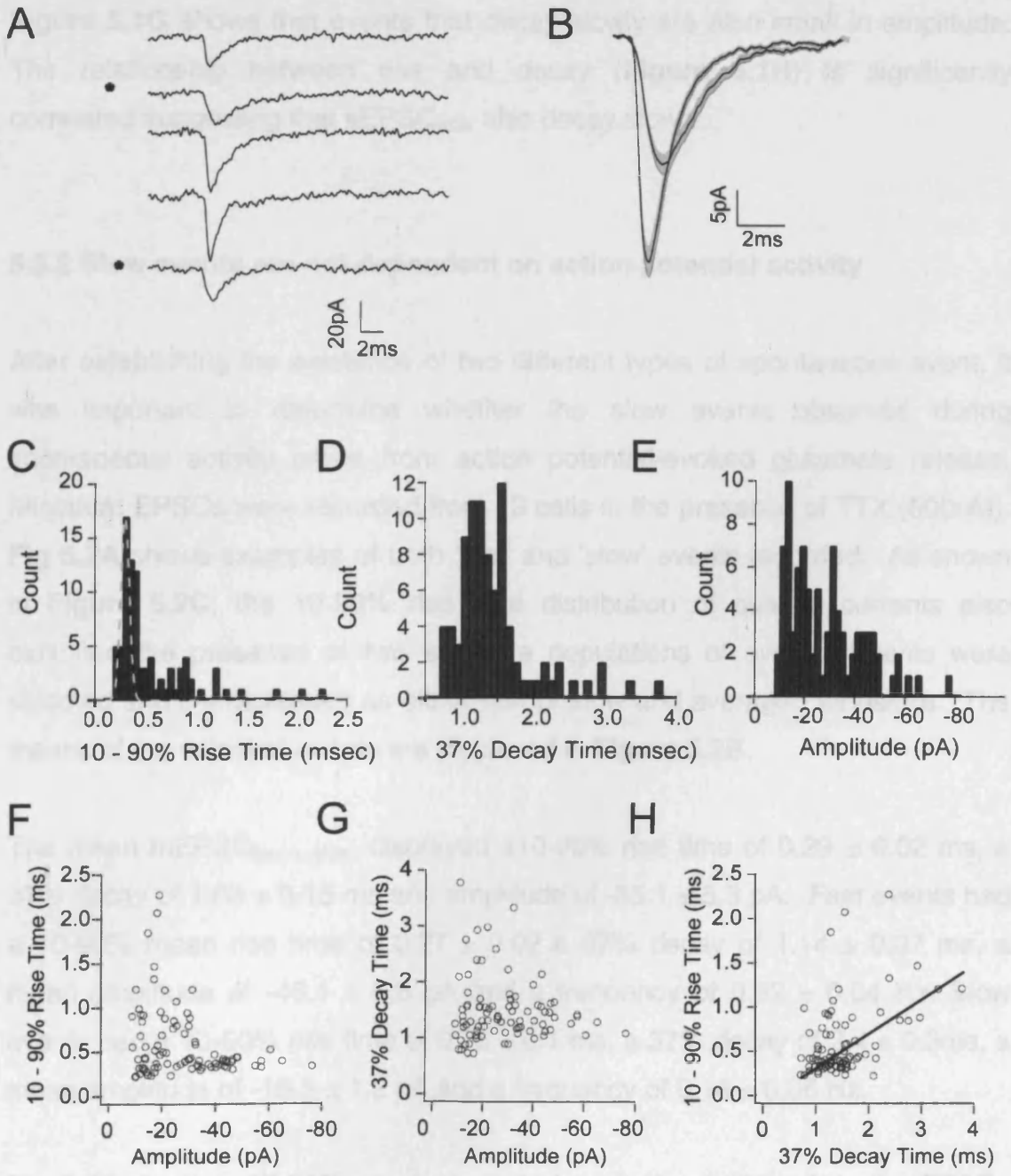


Figure 5.1 Identification of slow-rising sEPSCs. A. Representative stellate cell sEPSCs recorded from a single cell. Events with a slow rise (determined as in methods) are highlighted with an asterisk. All subsequent data presented is from the same cell. **B**. Average of all sEPSC $_{fast}$ (n= 57) and sEPSC$_{slow}$ (n=25) events after selection and alignment on 10% rise time SEM is shown in grey. **C**. Histogram of 10-90% rise time of events fitted with a Gaussian function (dashed line). **D**. Distribution of 37% decay. **E**. Distribution of amplitudes. **F**. 10-90% rise time plotted against amplitude gave rise to an L-shaped distribution and measures are not significantly correlated (P>0.1; Spearman rank order correlation). **G**. 37% decay plotted against amplitude give rise to an L-shaped distribution and are not significantly correlated (P>0.1; Spearman rank order correlation). **H**. Relationship between 10-90 rise time and 37% decay was significantly correlated (P<0.01; Spearman rank order correlation).

Figure 5.1G shows that events that decay slowly are also small in amplitude. The relationship between rise and decay (**Figure 5.1H**) is significantly correlated suggesting that $sEPSC_{slow}$ also decay slowly.

5.3.2 Slow events are not dependent on action potential activity

After establishing the existence of two different types of spontaneous event, it was important to determine whether the slow events observed during spontaneous activity arose from action potential-evoked glutamate release. Miniature EPSCs were recorded from 13 cells in the presence of TTX (500nM). **Fig 5.2A** shows examples of both 'fast' and 'slow' events recorded. As shown in **Figure 5.2C**, the 10-90% rise time distribution of quantal currents also exhibited the presence of two separate populations of events. Events were selected and characterised as either fast or slow and averaged as before. The means of the selected events are displayed in **Figure 5.2B**.

The mean $mEPSC_{fast + slow}$ displayed a10-90% rise time of 0.29 ± 0.02 ms, a 37% decay of 1.63 ± 0.15 ms and amplitude of -35.1 ± 5.3 pA. Fast events had a 10-90% mean rise time of 0.27 ± 0.02 a 37% decay of 1.14 ± 0.07 ms, a mean amplitude of -46.1 ± 5.6 pA and a frequency of 0.22 ± 0.04 Hz. Slow events had a 10-90% rise time of 0.75 ± 0.1 ms, a 37% decay of 3.4 ± 0.3ms, a mean amplitude of -16.3 ± 1.2 pA and a frequency of 0.16 ± 0.06 Hz.

Fig 5.2F displays 10-90% rise time plotted against amplitude for all mEPSCs detected in 1 cell. This distribution is also clearly L-shaped, suggesting that the differences in kinetics of quantal events observed was also not a result of filtering. **Figures 5.2 G** highlights the fact that miniature events that decay slowly are also small in amplitude. There is a significant correlation between rise and decay time as shown in **Figure 5.2H** suggesting that $mEPSC_{slow}$ also decay slowly. There were no significant differences between sEPSCs and mEPSCs in any of their parameters (**Fig 5.3**).

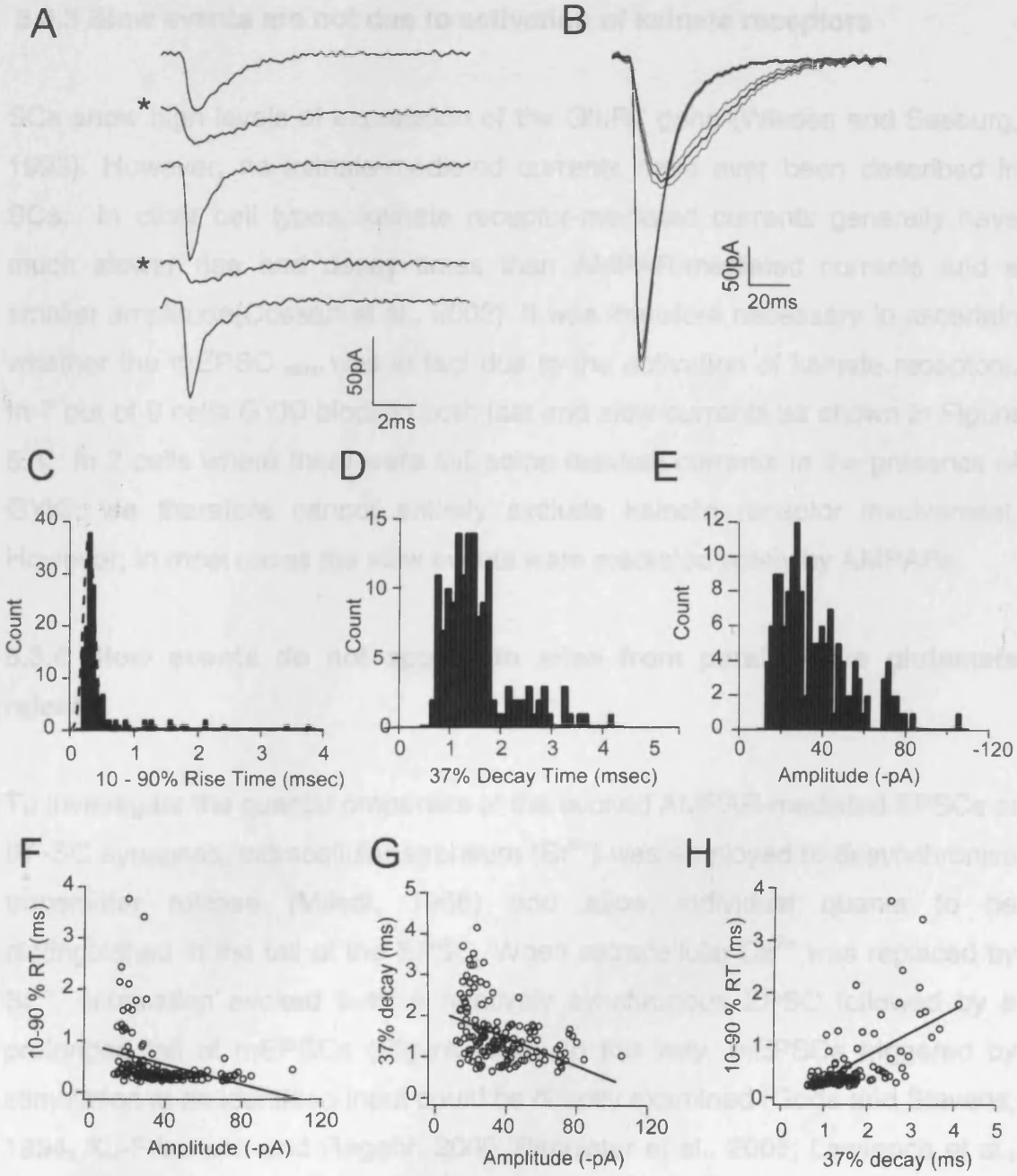


Figure 5.2 Identification of slow-rising EPSCs in the presence of TTX.
A. Individual stellate cells mEPSCs. Events with a slow rise (determined as in methods) are highlighted (all data is from the same cell as A). **B**. Average of all mEPSC $_{fast}$ (n= 113) and mEPSC$_{slow}$ (n=29) events after selection SEM. is shown in grey (all data is from the same cell as A). **C**. Histogram of 10-90% rise time fitted with a Gaussian function (dashed line). **D**. Distribution of 37% decay. **E**. Distribution of amplitudes. **F**. 10-90% rise time plotted against amplitude gave rise to an L-shaped distribution and measures were significantly correlated (P<0.01; Spearman rank order correlation). **G**. 37% decay plotted against amplitude give rise to an L-shaped distribution and was significantly correlated (P<0.01; Spearman rank order correlation). **H**. Relationship between 10-90 rise time and 37% decay was significantly correlated (P<0.01; Spearman rank order correlation).

5.3.3 Slow events are not due to activation of kainate receptors

SCs show high levels of expression of the GluR7 gene (Wisden and Seeburg, 1993). However, no kainate-mediated currents have ever been described in SCs. In other cell types, kainate receptor-mediated currents generally have much slower rise and decay times than AMPAR-mediated currents and a smaller amplitude(Cossart et al., 2002). It was therefore necessary to ascertain whether the mEPSC $_{slow}$ was in fact due to the activation of kainate receptors. In 7 out of 9 cells GYKI blocked both fast and slow currents as shown in Figure 5.4. In 2 cells where there were still some residual currents in the presence of GYKI; we therefore cannot entirely exclude kainate receptor involvement. However, in most cases the slow events were mediated solely by AMPARs.

5.3.4 Slow events do not appear to arise from parallel-fibre glutamate release

To investigate the quantal properties of the evoked AMPAR-mediated EPSCs at PF-SC synapses, extracellular strontium (Sr^{2+}) was employed to desynchronise transmitter release (Miledi, 1966) and allow individual quanta to be distinguished in the tail of the EPSC. When extracellular Ca^{2+} was replaced by Sr^{2+}, stimulation evoked both a relatively synchronous EPSC followed by a prolonged tail of mEPSCs (Figure 5.5A). In this way, mEPSCs triggered by stimulation at an identified input could be directly examined (Goda and Stevens, 1994; Xu-Friedman and Regehr, 2000; Bannister et al., 2005; Lawrence et al., 2004).

EPSCs were evoked by PF stimulation in the presence of 5 mM Sr^{2+}. Examples of evoked events are shown in **Figure 5.5A**. **Figure 5.5B** shows the quantal events that were detected (as described in Methods) within a restricted time period of 150 ms after the initial large EPSC.

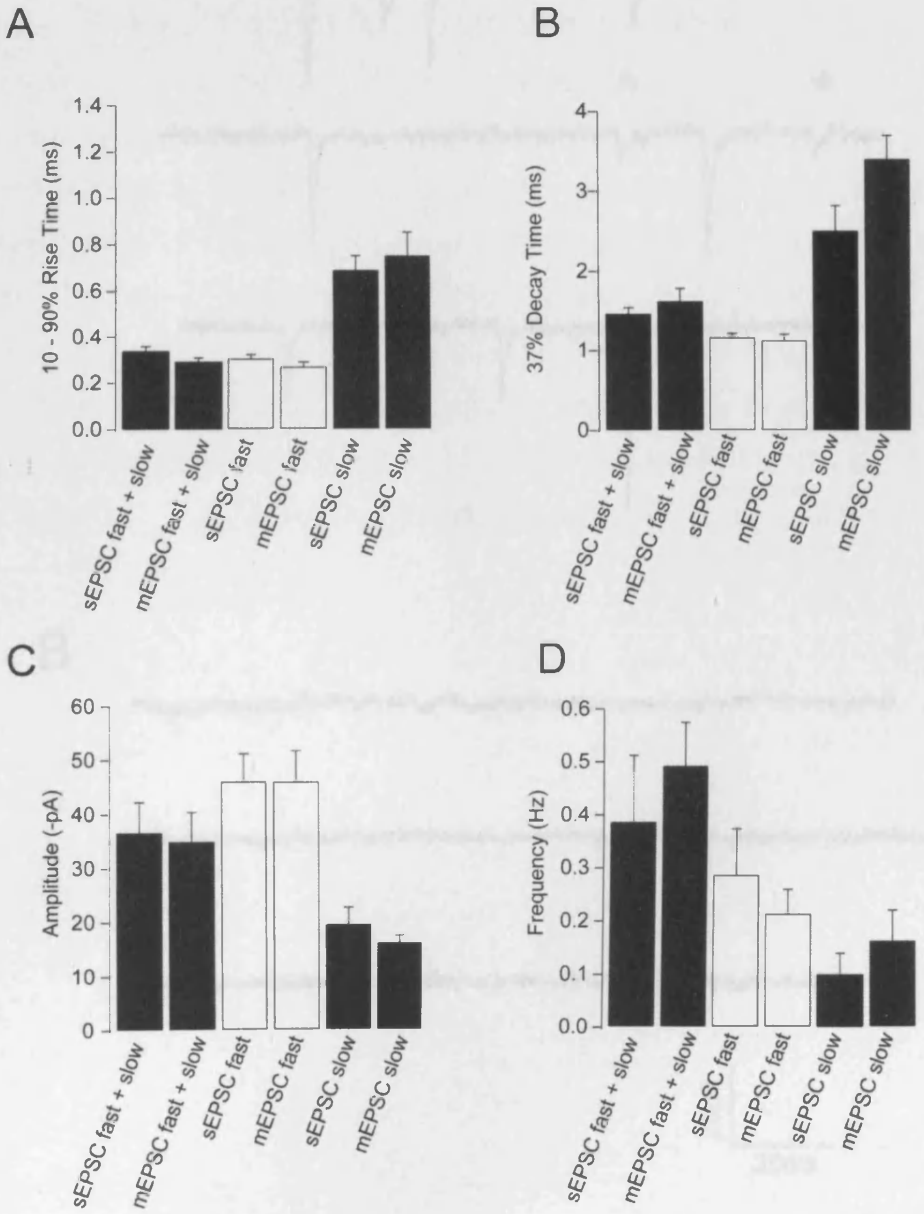


Figure 5.3 Comparison between spontaneous and miniature 'fast' and 'slow' events. A-D. For rise time, 37% decay, amplitude and frequency (non-selected, fast or slow), there was no significant difference between mEPSCs and sEPSCs (P>0.05; Mann Whitney *U*-test).

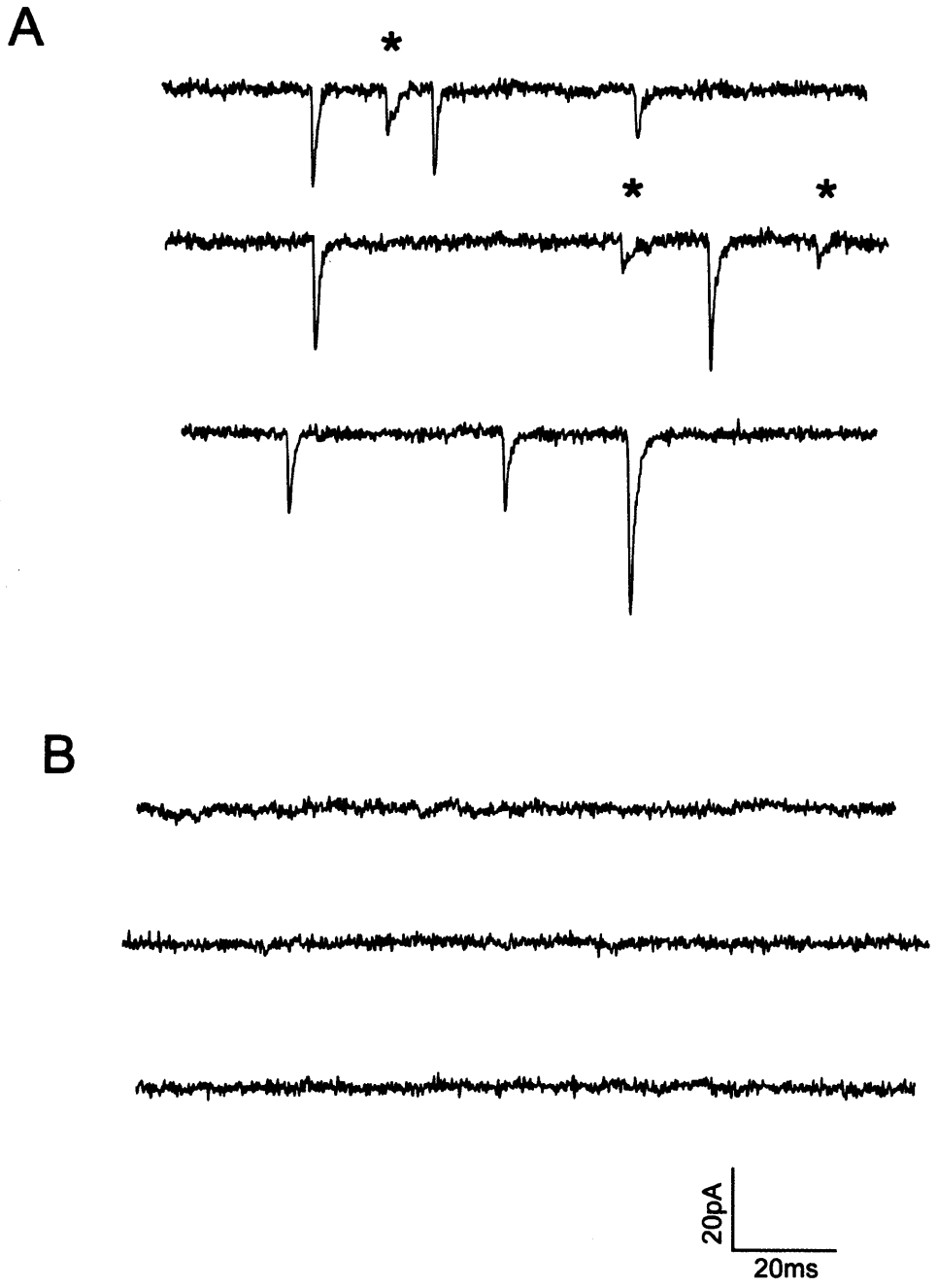


Figure 5.4 Fast and slow currents are mediated by AMPARs. A. Selected traces of whole-cell recording from a stellate cell in the presence of TTX (500nM) in the absence of GYKI. **B.** Selected traces from the same cell as A in the presence of GYKI (50μM). Asterisks denote slow rising events.

The distribution of 10-90% rise times (**Figure 5.5C**) and 37% decay (**Figure 5.5D**) showed that events were composed solely of currents with a fast rise time and decay. This was the case for all cells (n=10). However, in 4 cells, when all detected events were included in the analysis (not just those within 150ms of the evoked event), as shown in **Figure 5.6A**, EPSCs displayed a 10-90% distribution similar to that of spontaneous and miniatures currents (**Figure 5.6C**) being composed of two distinct populations. **Figure 5.6.B** shows the average traces for the fast and slow events. This suggests that Sr^{2+} could still support the type of neurotransmitter release required for the $mEPSC_{slow}$. Importantly, only the fast events appear to be impulse linked since slow events did not contribute to the EPSCs in the tail of the evoked event. As before, both $mEPSC_{fast}$ and $mEPSC_{slow}$ were blocked in the presence of GYKI (data not shown).

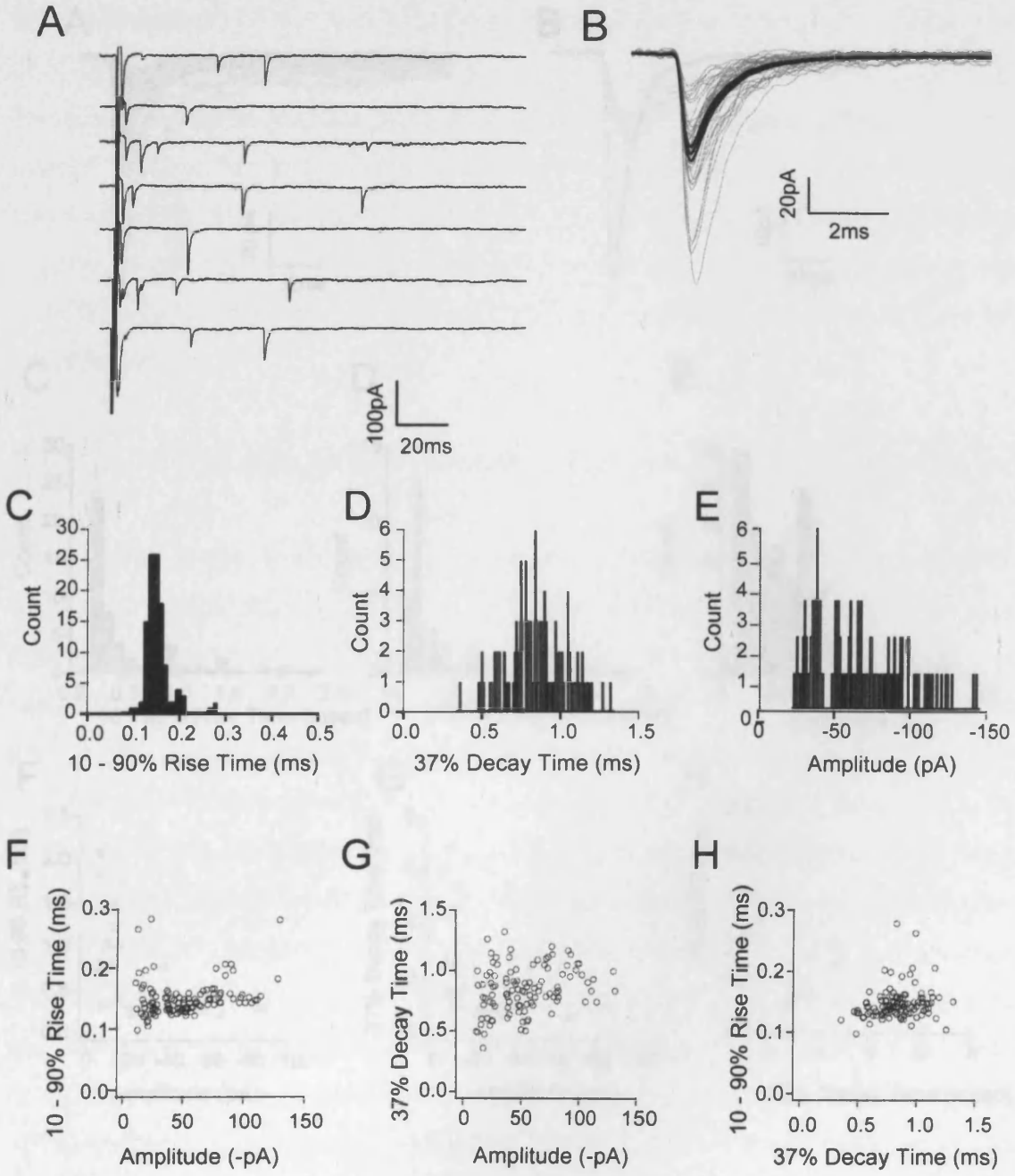


Figure 5.5. PF stimulation in the presence of Sr^{2+} does not give rise to slow events. **A**. Examples of EPSCs evoked by parallel fibre stimulation in the presence of Sr^{2+} (5mM). **B**. Events detected (see methods) within a 150ms time window after the end of the synchronous EPSC (grey). Events are from the same cell as A. Events are aligned on 20% rise time. Mean and SEM. are shown in black. **C**. Distribution of 10-90% rise times. Events are composed of a single population with a fast rise time. **D**. Histogram of 37% decay also displays a unitary population. **E**. Distribution of amplitudes. **F**. 10-90 rise time plotted against amplitude revealed no correlation ($P>0.1$; Spearman rank order correlation). **G**. 37% decay time was not correlated with amplitude ($P>0.1$; Spearman rank order correlation). **H**. 10-90% rise time was significantly correlated with 37%decay ($P<0.01$; Spearman rank order correlation).

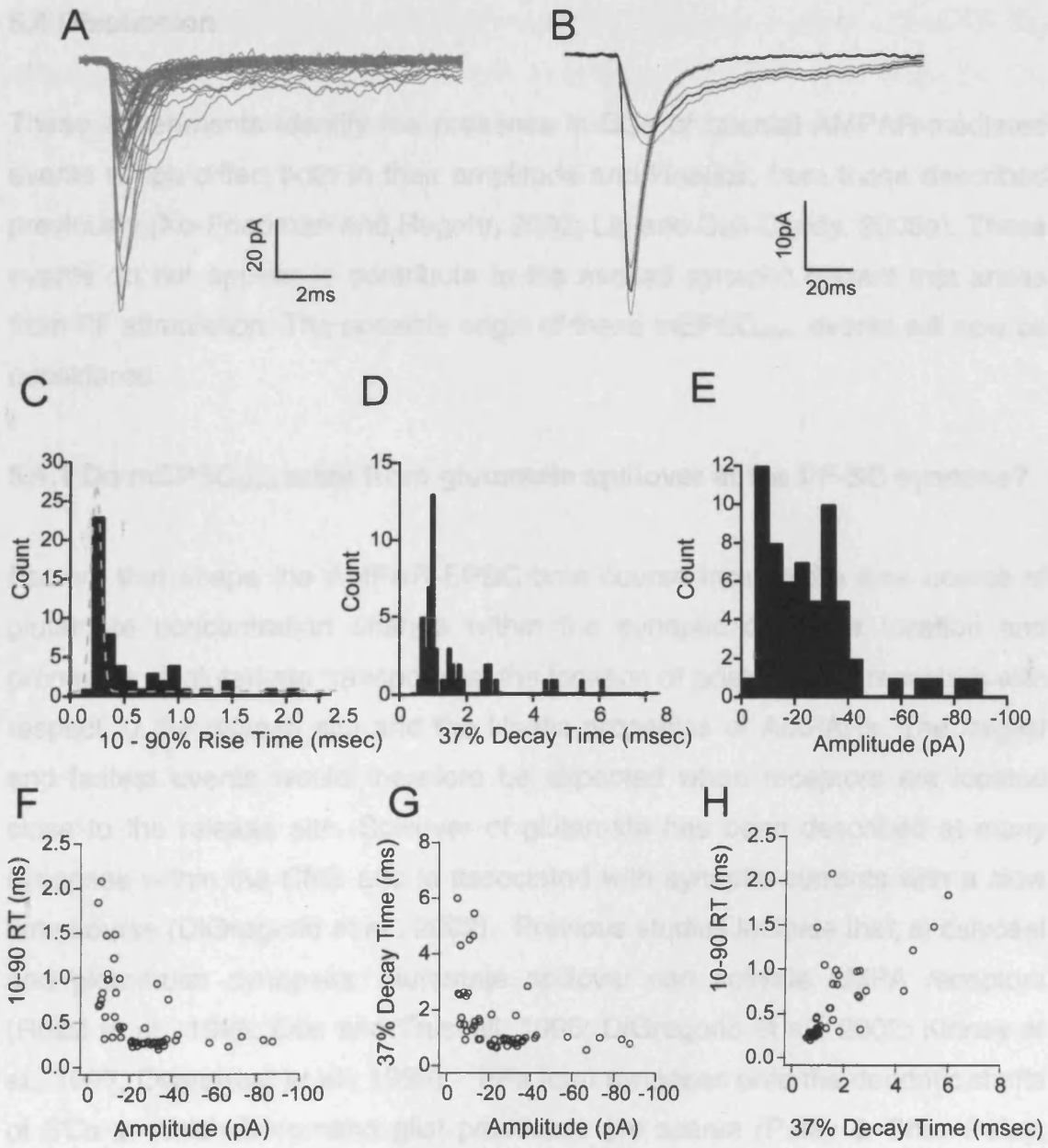


Figure 5.6 Slow events still occur in the presence of strontium. A. All events detected during a recording in Sr^{2+} (5mM) excluding initial evoked large EPSCs. **B**. Mean traces of fast and slow events. **C**. 10-90% rise time distributions of all events reveal the presence of slow events. **D**. The distribution of 37% decay times highlights the presence of slowly decaying events. **E**. Histogram of amplitudes. **F**. 10-90 rise time plotted against amplitude revealed no correlation ($P>0.1$; Spearman rank order correlation). **G**. 37% decay time was not correlated with amplitude ($P>0.1$; Spearman rank order correlation). **H**. 10-90% rise time was significantly correlated with 37% decay ($P<0.01$; Spearman rank order correlation).

5.4 Discussion

These experiments identify the presence in SCs of quantal AMPAR-mediated events which differ, both in their amplitude and kinetics, from those described previously (Xu-Friedman and Regehr, 2000; Liu and Cull-Candy, 2005a). These events do not appear to contribute to the evoked synaptic current that arises from PF stimulation. The possible origin of these $mEPSC_{slow}$ events will now be considered.

5.4.1 Do $mEPSC_{slow}$ arise from glutamate spillover at the PF-SC synapse?

Factors that shape the AMPAR-EPSC time course include the time course of glutamate concentration change within the synaptic cleft, the location and properties of glutamate transporters, the location of postsynaptic receptors with respect to the release site and the kinetic properties of AMPARs. The largest and fastest events would therefore be expected when receptors are located close to the release site. Spillover of glutamate has been described at many synapses within the CNS and is associated with synaptic currents with a slow time course (DiGregorio et al., 2002). Previous studies indicate that, at calyceal and glomerular synapses, glutamate spillover can activate AMPA receptors (Rossi et al., 1995; Otis and Trussell, 1996; DiGregorio et al., 2002; Kinney et al., 1997; Overstreet et al., 1999). PFs form synapses onto the dendritic shafts of SCs at which Bergmann glial processes are sparse (Palay & Chan-Palay, 1974) and glutamate transporter density is low (Chaudhry et al., 1995). Indeed a stimulus train is known to activate an AMPA-mediated spillover current at the PF-SC synapse (Carter and Regehr, 2000). However, it remains to be determined whether an individual quantum of glutamate is sufficient to activate extrasynaptic receptors. Furthermore, if the same source of glutamate release gave rise to both $mEPSC_{slow}$ and $mEPSC_{fast}$ one would expect to observe events comprised of both fast and slow waveforms. Yet biphasic quantal events composed of a fast and slow component were rarely, if ever observed (Figure 5.2H).

In their study describing an AMPAR-mediated spillover current at the PF-SC synapse Carter and Regehr (Carter and Regehr, 2000) also describe SC AMPAR activation by an indirect pathway i.e. glutamate release from PFs that do not make synapses directly onto SCs. The 'indirect' pathway gave rise to a small AMPA-mediated EPSC that developed gradually and decayed slowly. It may be that $mEPSC_{slow}$ arise from quantal release of glutamate from the 'indirect' pathway that Carter and Regehr describe.

5.4.2 Putative climbing fibre synapse?

Some studies have suggested that climbing fibres make synapses onto interneurons in the cerebellum (Ekerot and Jorntell, 2001). However this has yet to be demonstrated directly by the recording of climbing fibre evoked EPSCs in SCs. This may be relevant to the possible origin of $mEPSC_{slow}$ since, in addition to conventional release of glutamate, climbing fibres are known to support ectopic release of glutamate. CF varicosities contain synaptic vesicles, most of which are not associated with the presynaptic active zone facing the postsynaptic density (Palay SL & Chan-Palay V., 1974; Spacek, 1985; Xu-Friedman et al., 2001; Grosche et al., 2002). Furthermore, ectopic release of glutamate from climbing fibres can lead to activation of AMPARs on glia (Matsui and Jahr, 2004). Therefore, this may also provide an alternative source of glutamate for AMPAR activation on SCs.

If the source of glutamate producing $mEPSC_{slow}$ is indeed the climbing fibre, either through conventional or ectopic release, this would still not directly explain the slow kinetics of the $mEPSC_{slow}$. The AMPA-mediated currents that are generated in glia as a result of ectopic release of glutamate from climbing fibres display fast kinetics (200–300μs rise time, 1–2 ms to decay time; (Matsui and Jahr, 2004)). Therefore, it is more likely that rather than forming direct synapses with SCs, the climbing fibre glutamate release site, be it ectopic or conventional, is distant from the postsynaptic AMPARs on SCs. It is known that evoked glutamate released from climbing fibres can diffuse to activate AMPARs on SC terminals (Satake et al., 2000), however it is unknown whether individual

transmitter packets of glutamate from climbing fibres can also activate these receptors.

5.4.2 Do $mEPSC_{slow}$ arise from a distinct AMPAR subtype with slow kinetics?

Thus far, the possible explanations for the origin of $mEPSC_{slow}$ have assumed that glutamate release is remote from AMPARs and this then dictates the kinetics of the $mEPSC_{slow}$. The time course of the $mEPSC_{slow}$ could equally be explained by the existence of a population of AMPA receptors which differ in their receptor properties, from those previously described. In neurons, AMPARs are known to be associated with intracellular proteins e.g.: TARPs which can affect receptor properties (Tomita et al., 2006; Nicoll et al., 2006; Banke et al., 2000) and AMPAR properties can also be modified by phosphorylation (Banke et al., 2000). It may be possible, therefore, that a population of AMPARs, associated with particular intracellular proteins that affect receptor properties, exists in SCs to give rise to the $mEPSC_{slow}$. AMPARs with such properties have not been able to be characterised in recombinant systems because any such associated proteins have not been identified.

5.4.3 An alternative vesicle pool?

Although no $mEPSC_{slow}$ were detected within the tail of the currents evoked in the presence of Sr^{2+}, there still remains a possibility that $mEPSC_{slow}$ arise from a population of vesicles with fusion pores that open at different rates, (Choi et al., 2000) or that are triggered by something other than Ca^{2+} entry through voltage-gated Ca^{2+} channels (Zenisek et al., 2003; Matsui and Jahr, 2004).

5.4.4 Possible functional implications of $mEPSC_{slow}$

The time course of synaptic conductances determines basic properties such as temporal precision and reliability (Cathala et al., 2003; Galarreta and Hestrin, 2001; Harsch and Robinson, 2000) and the gain of rate-coded signals (Mitchell and Silver, 2003). Whether glutamatergic synapses operate independently or whether intersynaptic crosstalk occurs, has profound consequences on neuronal network functions such as its information storage capacity (Abbott and Regehr, 2004). To operate independently from their neighbours, it would be efficient for synapses if glutamate release was confined only to the area covering the directly opposing PSD. Such confinement could maximize the information capacities of a neural circuit. However, it may be necessary to allow for spillover, for example, in certain high-intensity activity conditions, to activate extrasynaptic receptors, some of which are required for the induction of synaptic plasticity. Spillover, even to neighbouring synapses, may also prove beneficial if such spillover causes a low level of activation of receptors at these synapses that could trigger depression. Depression at the neighbouring synapses could result in an arrangement to enhance synaptic specificity (Diamond, 2002).

Part of the uncertainty over whether spillover occurs or not stems from the difficulty of measurements of diffusion in the extracellular space (Thorne and Nicholson, 2006) and from ignorance of the two-dimensional distribution and density of receptors and transporters on the plasma membrane (Tanaka et al., 2005). Similar distributions of rise times and amplitudes of miniature events have been described for $GABA_AR$-mediated currents in SCs (Kondo and Marty, 1998), suggesting that the presence of $mEPSC_{slow}$ may be a feature of SC geometry.

6. General Discussion

This thesis has examined aspects of AMPAR-mediated transmission in SCs, with particular focus on factors affecting the subunit composition of AMPARs expressed at the PF-SC synapse. The data presented in Chapters 2 and 3 show that, in addition to high frequency stimulation (Liu and Cull-Candy, 2000a), the proportion of GluR2-containing receptors at the PF-SC synapse can increase both during development, and as a result of metabotropic glutamate and GABA receptor activation. Blocking the tonic activation of both group 1 mGluR and $GABA_B$ receptors leads to an increase in the proportion of GluR2-lacking receptors at the synapse, suggesting that the control of AMPAR subunit composition at this synapse can be dynamically regulated. Functional influences and consequences of such dynamic regulation of AMPAR subunit composition are discussed below.

6.1 Possible roles and functional consequences of the dynamic regulation of AMPAR subunit composition

6.1.1 The proportion of GluR2-containing AMPARs as a reflection of activity

The previous finding that, at the PF–SC synapse, high frequency stimulation induces a change in AMPAR subunit composition (Liu and Cull-Candy, 2000a) and that EPSC rectification is negatively correlated with spontaneous SC firing (Liu and Cull-Candy, 2002), is consistent with the idea that a switch in Ca^{2+} permeability may occur as a result of activity. If the change in RI that occurs during development relied on molecular or genetic cues, one might expect the developmental change in RI to be relatively uniform and values to be of a limited range within ages. As the observed change in rectification was not dramatic and the GluR2 contribution, signified by RI, varied from synapse to synapse (Figure 3.1), this might suggest the activity experienced during development is the trigger for GluR2 insertion.

PF activity evokes glutamate release at PF-SC synapses and release of GABA *via* feedforward inhibition at SC-SC synapses (Llano and Gerschenfeld, 1993b). While a single stimulus causes release of glutamate with limited diffusion, there is considerable spill over to adjacent sites in response to stimulus trains (Carter and Regehr, 2000; Clark and Cull-Candy, 2002; Karakossian and Otis, 2004). Postsynaptic mGluRs are generally distributed at the periphery of the synaptic density (Baude et al., 1993; Nusser et al., 1994). Therefore, one would predict that high frequency stimulation of PFs leads to coincident activation of both mGluRs and $GABA_BRs$ in these cells which would also lead to an increased proportion of GluR2-containing AMPARs at the synapse. During periods of inactivity however, tonic activation of metabotropic receptors would be expected to be reduced, allowing the expression of CP-AMPARs. Together this suggests that the extent of GluR2 contribution to AMPARs expressed at the synapse may reflect the previous activity experienced by the cell. However, it remains to be determined whether the level of neuronal activity can activate mGluRs and $GABA_BRs$ in such a way to dynamically control the expression of GluR2-containing AMPARs.

6.1.2 Increasing the proportion of GluR2-containing AMPARs as a neuroprotective response.

The increased contribution of GluR2 at the PF-SC synapse observed during development may be serving a role in neuroprotection against excitotoxicity, as seen in other brain areas. CP-AMPARs are detected in hippocampal oligodendroglial progenitors, but not pre-progenitors or mature oligodendrocytes, accounting for a stage-specific susceptibility to excitotoxicity (Itoh et al., 2002). The GluR2 subunit also offers protection against neuronal degeneration in response to hippocampal seizures but only during a critical developmental time window (Friedman and Veliskova, 1998; Friedman et al., 2003; Friedman and Veliskova, 1998; Jensen et al., 2001;Jensen, 2002). Global hypoxia induces seizures and a decrease in hippocampal GluR2 in rat pups at P10- P12 but not in younger or older rat pups (Jensen et al., 1998). Furthermore, this decrease in GluR2 is associated with an increase in AMPAR-

mediated potentiation of hippocampal epileptiform activity *in vitro* (Sanchez et al., 2001b).

It has been previously been suggested that the activity-dependent switch in SCs is a neuroprotective mechanism (Malinow and Malenka, 2002). The fact that a rise in intracellular calcium is required for the plasticity described in this study, and that calcium entry through CP-AMPARs the trigger for the switch in AMPAR subtype in response to activity (Liu and Cull-Candy, 2000a) suggests that this may be an autoregulatory, mechanism for the cell to limit calcium entry.

6.1.3 Polyamine block

Although, intracellular polyamine block was used, primarily as a 'diagnostic tool' in the present study the voltage dependence of polyamine block of CP-AMPARs is also of physiological significance. The unblock of AMPA receptors by polyamines accounts for the facilitation of peak responses observed following a brief train of agonist pulses, which may represent a mechanism of short-term plasticity for CP-AMPARs (Rozov and Burnashev, 1999). Any increase in GluR2 would reduce this type of plasticity and vice versa. It is interesting to note that the developmental loss of GluR2-lacking AMPARs seen in the neocortex (Kumar et al., 2002) is accompanied by a reduction in internal polyamine concentration (Shin et al., 2005), suggesting that its presence is serving some physiological purpose.

Regulation of CP-AMPAR expression and internal polyamine concentration, if modulated in unison, would provide a powerful mechanism for controlling the responsiveness of the SC to repetitive stimuli and the kinetics of the AMPA response, especially at depolarised potentials (Bowie et al., 1998a). This would allow neurons to cope with elevated levels of synaptic activity and filter out single, potentially unrelated events during periods of intense activity. Because of use-dependent relief of polyamine block, a neuron will be more likely to spike when it receives a train of inputs rather than a single one, ensuring that bursting stimuli will undergo plasticity and become consolidated while events would not.

6.1.4 Ca^{2+}-permeability

A linear IV relationship for PF-SC EPSCs was rarely observed in mature animals (Figure 3.1) and was not always observed after treatment with DHPG (Figure 4.2). Furthermore, the proportion of CP-AMPARs increased when tonic activation of mGluRs was blocked (Fig. 4.11) Together these findings suggest that the retention of some Ca^{2+}-permeability and the ability to for it to increase, may be of functional importance, given the diverse intracellular effects of Ca^{2+} as a ubiquitous second messenger in development (Aamodt and Constantine-Paton, 1999), synaptic plasticity (Malenka et al., 1989; Mahanty and Sah, 1998; Tian and Feig, 2006; Laezza et al., 1999) and excitotoxicity (Van Den and Robberecht, 2000; Kawahara and Kwak, 2005; Van et al., 2002).

Ca^{2+} influx, through AMPARs in SCs is especially significant given the absence of synaptic NMDA receptors in these cells (Clark and Cull-Candy, 2002). Recent imaging studies have provided insight into the functional relevance of Ca^{2+} signals mediated by CP-AMPARs especially in aspiny neurons (Goldberg et al., 2003). These Ca^{2+} signals are spatially and functionally distinct from Ca^{2+} signals mediated by other glutamate receptors (Soler-Llavina and Sabatini, 2006; Topolnik et al., 2005). Ca^{2+} entry through AMPARs is required for forms of plasticity at this synapse (Liu and Cull-Candy, 2002; Soler-Llavina and Sabatini, 2006).Therefore continuous expression of GluR2-lacking receptors in mature animals and the ability of the synapse to increase its Ca^{2+}-permeability through expression of CP-AMPARS, may be to allow the synapse to retain the potential for future modification, or to reset the baseline for synaptic plasticity once initial changes have been made.

6.1.5 Gating

The maintenance of CP-AMPARs at the synapse may also be important with regards channel kinetics. Mutant mice over-expressing GluR2 in interneurons exhibit severely disrupted oscillation synchrony between spatially separated sites in association with changes in interneuron firing patterns. This suggests that the expression of GluR2 needs to be tightly regulated to ensure precise

function. of interneurons. CP-AMPARS also generally have rapid kinetics, helping to maintain a faster time course of AMPA receptor-mediated EPSPs in interneurons. This might be necessary to allow gamma oscillations to synchronise over distance (Fuchs et al., 2001) and may contribute to the burst-firing patterns of interneuron populations and feed-forward and feedback inhibition (Buzsaki and Chrobak, 1995) within the neuronal network.

6.1.6 Consequences for stellate cell function within the cerebellum

Stellate cells receive excitatory input from PFs and provide feed-forward inhibition to Purkinje cells (Mittmann et al., 2005), the sole output of the cerebellum. Periods of high parallel fibre activity, for example during sensory stimulation (Chadderton et al., 2004) would likely lead to activation of extrasynaptic metabotrobic receptors at the PF-SC synapse. This in turn would result in a reduced synaptic current mediated mainly by GluR2-containing CI-AMPARS in response to glutamate release a form PFs. Stellate cells are small with high input resistance, therefore individual quanta can produce large changes in membrane potential and strongly influence firing (Carter and Regehr, 2002). Any change in AMPAR number and/or subunit composition at the PF-SC synapse would be expected to affect stellate cell firing, and therefore the inhibition of Purkinje cells (Hausser and Clark, 1997). Modelling would be required to accurately predict how such changes in synaptic currents would affect stellate cell firing (Carter and Regehr, 2002).

Modulation of Purkinje cell firing is the basis of most theories of cerebellar function, therefore any alteration in stellate cell firing is likely to affect cerebellar function. It must noted that EPSC properties in this study have been measured in isolation and that activation of both mGluRs and $GABA_BRs$ have other effects on stellate cells which will contribute to the overall and eventual consequence of metabotrobic receptor activation at this synapse. These additional effects would need to be taken into consideration when predicting the net effect of increased PF activity on Purkinje cell firing (via inhibition by stellate cells). It also remains to be determined whether increases in PF activity as observed in vivo (Chadderton et al., 2004) leads to activation of metabotropic receptors.

6.2 Conclusions

The results described in this study suggest that the role of CP-AMPARS at the PF-SC synapse are under dynamic regulation and have identified a novel mechanism by which this may occur. During development there is an increase in the contribution of GluR2-containing receptors. Furthermore the subunit composition of synaptic AMPARs can also change in response to mGluR and $GABA_BR$ activation. The results presented here identify, for the first time, a synapse where basic properties of postsynaptic glutamate receptors are directly modified by the action of GABA, as well as glutamate. Furthermore, targeting of GluR2-containing receptors is affected by tonic activation of group 1 mGluRs, suggesting these metabotropic receptors play a key role in both constitutive- and activity-dependent regulation of the synaptic AMPAR subtype at these synapses. Because the contribution GluR-2 containing receptors determines important functional properties such as Ca^{2+}-permeability and block by polyamines, such dynamic regulation affords the synapse a number of ways in which to respond to changes in activity.

6.3 Future directions

6.3.1 Developmental increase in the contribution of GluR2-containing receptors

The findings in this study, obtained with electrophysiological recordings could be supported by antibody staining showing an increase in GluR2 immunoreactivity with age. It would also be interesting to see whether there is a complimentary decrease in internal polyamine concentration with age as observed in neocortical neurons (Shin et al., 2005).

6.3.2 Does the group1 mGluR-mediated decrease in EPSC amplitude at negative potentials represent a decrease in receptor number of merely a change in subunit composition?

The present study has shown that activation of group 1 mGluRs leads to a decrease in EPSC amplitude at negative potentials and rectification. It remains to be determined whether the decrease in EPSC amplitude is a reflection of the change in AMPAR subunit composition or is in fact a result of a decrease in the total number of AMPARs at the synapse. This could be investigated using a variety of techniques including immunostaining (Kumar et al., 2002), peak-scaled non stationary noise analysis (Traynelis and Jaramillo, 1998), and fluorescence recovery after photobleaching (FRAP) (Bats et al., 2007).

6.3.3 Does $GABA_BR$ activation enhance the group 1 mGluR response?

The effects of $GABA_BR$ activation on the AMPAR-mediated response in stellate cells may be being mediated by mGluR receptors. $GABA_BR$ activation is known to enhance the group 1 mGluR response in Purkinje cells (Hirono et al., 2001). This possibility could be investigated by activating $GABA_BRs$ in the presence of group 1 mGluR antagonists.

6.3.4 Are metabotropic receptors tonically activated or tonically active?

In several brain regions the tonic activation has been attributed to ambient concentrations of neurotransmitter (Cavelier et al., 2005);(Chen and Roper, 2004). However the structure of these receptors id such that they can remain in a conformation that allows the receptor to be active, even in the absence of transmitter(Tabata and Kano, 2004). The similarity in structure to calcium receptors also means that the presence of extracellular Ca^{2+} may be enhancing activating these receptors (Tabata et al., 2004). This could be investigated pharmacologically by using various agonists, antagonists, inverse agonists and competitive antagonists.

6.3.5 Does physiological stimuli lead to activation of metabotropic receptors?

All the experiments carried out in the present study involved the bath application of agonists. The idea that activation of metabotropic receptors actually occurs in vivo would be strengthened by looking at whether they are activated in response to high-frequency stimulation of PFs.

6.3.6 Which protein partners are required for mGluR-mediated plasticity at the PF-SC synapse?

Protein partners such as PICK, GRIP and NSF are known to be required for change in AMPAR subunit composition seen in response to high-frequency activity (Liu and Cull-Candy, 2005);(Gardner et al., 2005). Peptides inhibitors such as pep-2SVKI and pep2m that interfere with the interaction between these proteins and AMPARs can be included in the pipette to identify if these protein partners are involved in the mGluR- and $GABA_B$-mediated change in AMPAR subunit composition.

6.3.7 Are $EPSC_{slow}$ mediated by glutamate spillover?

Further experiments could be carried out to determine the source of $EPSC_{slow}$. For example competitive glutamate antagonists with low affinity such as kyurenic acid could be used to see if the timecourse of the EPSCslow changes or if they disappeared altogether. External Ca^{2+} concentrations could be manipulated to alter transmitter release and to see how changing release affects the time course of the EPSCslow. Experiments could be performed at physiological temperature to see if EPSCslow are still present in these conditions. Also a glutamate re-uptake blocker such as TBOA could be used to assess the influence of increasing cleft glutamate concentration on EPSCslow.

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