A Study of Ion Channels Modulating Synaptic Transmission
Using a Cerebellar Purkinje Cell Nerve-Bouton Preparation

Alan Douglas Robertson

UCL

Submitted for the examination of a PhD
I, Alan Robertson confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been the case.
Abstract

In this thesis a nerve-bouton preparation of Purkinje cells has been characterised. Mechanically isolated Purkinje cells are shown to retain active afferent nerve terminals. This provides a simplified system where the effects of manipulating the ion channels in nerve boutons can be studied without the potentially confounding influences of the rest of the presynaptic cell or surrounding tissue. Isolated Purkinje cells were initially identified for whole cell patch-clamp recordings by their distinctive size and shape. Vesicular release of neurotransmitter was evident by spontaneous inward synaptic currents with a characteristic time course. Antagonist application established that isolated Purkinje cells receive a mixture of inhibitory GABAergic and excitatory glutamatergic inputs. Changes in the frequency, amplitude, and burst behaviour of these spontaneously occurring synaptic currents were used to infer properties of the afferent boutons. Because rat Purkinje cells can be distinguished by their lack of postsynaptic NMDA receptors the presynaptic effects of NMDA application could be readily investigated. NMDA caused an increase in the frequency of postsynaptic events. The NMDA-induced increase was found to be sensitive to external magnesium and TTX application. NMDA application was found to increase the frequency of both GABAergic events and glutamatergic events. Physiologically, NMDA receptors in afferent inhibitory terminals are thought to be activated by the retrograde release of glutamate. So experiments were performed to determine if retrograde release of glutamate could also increase the frequency of glutamatergic events, however it was found that this process has a much more pronounced influence on the GABAergic events. Properties of afferent boutons were also probed with a range of potassium channel blockers. The relevant topics covered are pharmacology, synaptic transmission, and the role of NMDA receptors in the cerebellum and the main technique used is whole-cell patch clamp recording.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>aCSF</td>
<td>artificial cerebral-spinal fluid</td>
</tr>
<tr>
<td>$A_f$</td>
<td>proportion of an interval distribution that consists of the peak with the faster time constant</td>
</tr>
<tr>
<td>AMPA receptor</td>
<td>2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid receptor</td>
</tr>
<tr>
<td>AP5</td>
<td>2-amino-5-phosphonopentanoic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis(2-aminophenoxy)ethane-$N,N,N',N'$-tetraacetic acid</td>
</tr>
<tr>
<td>BK channels</td>
<td>big conductance calcium-activated potassium channels</td>
</tr>
<tr>
<td>CA1</td>
<td><em>cornu ammonis</em> area 1</td>
</tr>
<tr>
<td>CACA</td>
<td><em>cis</em>-4-aminocrotonic acid</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CB$_1$ receptor</td>
<td>cannabinoid type 1 receptor</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CPA</td>
<td>cyclopiazonic acid</td>
</tr>
<tr>
<td>CPCCOEt</td>
<td>7-(hydroxyimino)-cyclopropa[b]chrome-n-1a-carboxylate ethyl ester</td>
</tr>
<tr>
<td>DISC</td>
<td>depolarisation-induced slow current</td>
</tr>
<tr>
<td>DNQX</td>
<td>6,7-dinitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>DPE</td>
<td>depolarisation-induced potentiation of excitation</td>
</tr>
<tr>
<td>DPI</td>
<td>depolarisation-induced potentiation of inhibition</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>DSE</td>
<td>depolarisation-induced suppression of excitation</td>
</tr>
<tr>
<td>DSI</td>
<td>depolarisation-induced suppression of inhibition</td>
</tr>
<tr>
<td>DTX</td>
<td>dendrotoxin</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>EA1</td>
<td>episodic ataxia type 1</td>
</tr>
<tr>
<td>EAATs</td>
<td>excitatory amino acid transporters</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>eIPSCs</td>
<td>evoked IPSCs</td>
</tr>
<tr>
<td>EPSC</td>
<td>excitatory postsynaptic current</td>
</tr>
<tr>
<td>FM 1-43</td>
<td>N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma aminobutyric acid</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor</td>
<td>GABA type A receptor</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;C&lt;/sub&gt; receptor</td>
<td>GABA type C receptor</td>
</tr>
<tr>
<td>GCL</td>
<td>granule cell layer</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid</td>
</tr>
<tr>
<td>IPSC</td>
<td>inhibitory postsynaptic current</td>
</tr>
<tr>
<td>K&lt;sub&gt;ATP&lt;/sub&gt; channel</td>
<td>ATP-sensitive potassium channel</td>
</tr>
<tr>
<td>LSE</td>
<td>large slow event</td>
</tr>
<tr>
<td>LTD</td>
<td>long term depression</td>
</tr>
<tr>
<td>M-current</td>
<td>muscarinic sensitive current</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
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mEPSC  miniature EPSC
mIPSC  miniature IPSC
ML    molecular layer
Mwk   moonwalker mutation
NEM   N-ethylmaleimide
NMDA  N-methyl-D-aspartate
NOS   nitric oxide synthase
NR1   NMDA receptor subunit 1
NR2   NMDA receptor subunit 2
NR2A  NMDA receptor subunit 2A
NR2B  NMDA receptor subunit 2B
NR2C  NMDA receptor subunit 2C
NR2D  NMDA receptor subunit 2D
NR3A  NMDA receptor subunit 3A
NVP-AAM077  (R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid
P     postnatal day
PCL   Purkinje cell layer
PPF   paired pulse facilitation
pDPE  putative depolarisation-induced potentiation of excitation
Pgu   pingu mutation
QX-314  N-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium chloride
<table>
<thead>
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<th>Description</th>
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<tr>
<td>Ro 8-4304</td>
<td>4-{3-[4-(4-fluoro-phenyl)-3,6-dihydro-2H-pyridin-1-yl]-2-hydroxy-propoxy}-benzamide</td>
</tr>
<tr>
<td>RP</td>
<td>rebound potentiation</td>
</tr>
<tr>
<td>sIPSC</td>
<td>spontaneous IPSC</td>
</tr>
<tr>
<td>SK channels</td>
<td>small conductance calcium-activated potassium channels</td>
</tr>
<tr>
<td>TEA</td>
<td>$N,N,N,N$-tetraethylammonium chloride</td>
</tr>
<tr>
<td>TPMPA</td>
<td>1,2,5,6-tetrahydropyridine-4-yl methylphosphinic acid</td>
</tr>
<tr>
<td>TRPC</td>
<td>transient receptor potential canonical</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>UCL 1848</td>
<td>8,14-diaza-1,7(1,4)-diquinolinacyclotetradecaphanedium di-trifluoroacetate</td>
</tr>
<tr>
<td>vGluT2</td>
<td>vesicular glutamate transporter 2</td>
</tr>
<tr>
<td>vGluT3</td>
<td>vesicular glutamate transporter 3</td>
</tr>
</tbody>
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Chapter 1: Introduction

This introductory chapter provides some relevant background information for the project.

1.1 Purkinje Cells

This thesis looks at presynaptic ion channels modulating neurotransmitter release. All of the work is done on cerebellar Purkinje cells. Purkinje cells are the only output from the cerebellar cortex, the cell and synapse rich outer layer of the cerebellar lobules. The Purkinje cells integrate many different inputs, both excitatory and inhibitory, which will affect the response of the Purkinje cell. The timing and strength of these inputs will influence the frequency, type, and duration of the spikes that form the output from the Purkinje cells (Hausser & Clark, 1997; Davie et al., 2008). The role of presynaptic ion channels in modulating the timing and strength of the inputs to cerebellar Purkinje cells may be critical to the function, normal or pathological, of the cerebellum in its role in motor learning, coordinating movement, and correcting errors (Apps & Garwicz, 2005)
1.2 Excitatory inputs

Excitatory inputs can be evoked from climbing fibres and parallel fibres. Parallel fibres are the axons from the granule cells, the somas of which are located further within the cerebellar lobule from the Purkinje cell layer. The axons project into the molecular layer, the outer layer of the lobule, where they bifurcate and the two lengths run parallel to the other granule cell axons. These parallel fibres synapse onto the perpendicular planar but extensive dendritic tree of the Purkinje cells in addition to some other cells such as molecular layer interneurons. Each Purkinje cell receives inputs from thousands of individual parallel fibres (Konnerth et al., 1990). Purkinje cells can also receive inputs directly from granule cells in the granule cell layer as their ascending axon synapses directly onto the soma of the Purkinje cell (Isope & Barbour, 2002). Parallel fibres tend to have a low probability of releasing neurotransmitter (Foster et al., 2005) but this probability can be increased with high external calcium and application of forskolin which promotes the production of cyclic adenosine monophosphate (cAMP) (Bender et al., 2009). Paired pulse facilitation also increases the probability of release at parallel fibres. This is where excitatory postsynaptic currents (EPSCs) are evoked twice in quick succession and the second EPSC is larger (Konnerth et al., 1990; Perkel et al., 1990). The EPSC derived from a stimulating a single parallel fibre tends to be quite small, with amplitudes of around 10s of picoamperes (Isope & Barbour, 2002). However when evoking EPSCs by stimulating parallel fibres in the molecular layer the amplitude increases with the size of the stimulation as more and more parallel fibres are recruited into coordinating glutamate release (Konnerth et al., 1990; Perkel et al., 1990).

Repetitive stimulation of cerebellar parallel fibres induces a slow EPSC in Purkinje cells (Hartmann et al., 2008). The slow EPSC is characterised by a durable
inward current accompanied by a rise in intracellular calcium that is critically mediated by channels expressing transient receptor potential canonical (TRPC) 3 subunits (Hartmann et al., 2008). There was evidence that the current is carried by TRPC1 based on the association of TRPC1 with the alpha subunits of a metabotropic glutamate receptor (mGluR) and producing a current with similar pharmacology in Chinese hamster ovary (CHO) cells expressing TRPC1 and mGluR1 (expression of TRPC3 failed to produce a current) (Kim et al., 2003). However, in TRPC3 knockout mice the slow EPSC is absent but the calcium rise remains intact. No changes in glutamate release at parallel fibres were seen in the knock out animals. Similarly TRPC3 knockout mice lack the slow EPSC when mGluR agonists are applied to the Purkinje cells, but not the calcium rise, supporting the idea that the slow EPSC is activated separately from some of the other actions of mGluRs. Knockout mice lacking TRPC1, TRPC4, and TRPC6 subunits maintain the slow EPSC (Hartmann et al., 2008). But the slow EPSC may be mediated by a heteromeric channel. Inducing the expression of the non-conducting TRPC1(F561A) subunit decreases the amplitude of the slow EPSC as does including anti-TRPC1 antibodies in the internal solution (Kim et al., 2003).

The slow EPSC is thought to play an important role in motor coordination. Unlike wild type, TRPC3 knockout mice have an abnormal gait where the hind paws are regularly placed outside the mice’s body width and also have a higher susceptibility to slipping when moving along a ladder with irregular spacing or an elevated narrow beam. Supporting the idea that TRPC3 is important in motor behaviour is that the TRPC1 knock out, the second most prominent transcript of TRPC in Purkinje cells, do not exhibit abnormal motor behaviour (Hartmann et al., 2008). The importance of TRPC3 to normal motor behaviour and Purkinje cell function is also apparent from investigating into the moonwalker (Mwk) mutation (Becker et al., 2009). Moonwalker is
a mutation found when mice that had been treated with a mutagen were screened for ataxic behaviour. It is a heterologous dominant mutation which encodes a point mutation in the TRPC3 channel that substitutes a threonine in position 635 for an alanine (T635A). The threonine residue is part of the intracellular linker between S4 and S5 which can be phosphorylated by phosphokinase C gamma. Phosphorylation becomes impaired in the T635A mutation which may underlie some of the changes in gating of TRPC3 such as the lower concentration of mGluR agonists needed to induce a current in Purkinje cells compared with wild type. The ataxia of moonwalker mice is characterised by a wider gait than wild type, not just the hind paws as with TRPC3 knockout mice, with a shuffling meandering, more irregular steps and a decrease in the time taken to fall from a stationary raised beam. Ataxia is apparent before the progressive loss of Purkinje cells occurs; the Purkinje cells themselves exhibit shorter dendrites that cover a smaller area (Becker et al., 2009).

The climbing fibres project into the cerebellar cortex from the inferior olive. Although just one input, climbing fibres can form extensive and convoluted synapses around the base of the dendritic spines that can comprise of several active sites of neurotransmitter release (Kakizawa et al., 2005). Once EPSCs are evoked from climbing fibres, their characteristically large amplitudes do not vary with further stimulation giving the inputs a distinctive invariable all-or-nothing response (Konnerth et al., 1990; Perkel et al., 1990). The large amplitude EPSCs evoked from climbing fibre inputs are caused by multiple vesicular release from its many active zones (Wadiche & Jahr, 2001). The characteristically large invariable amplitude of climbing fibre inputs is also found in early development even, though there may be several climbing fibres at early stages of development which will eventually be reduced to just one (Llano et al., 1991b). Unlike parallel fibres, EPSCs evoked from climbing fibres
exhibit paired pulse depression where subsequent EPSCs have a reduced amplitude if evoked soon after the first input (Perkel et al., 1990). The climbing fibre input can be evoked in a cerebellar slice by stimulating directly over the Purkinje cell, in the adjacent granule cell layer (Konnerth et al., 1990) or in the white matter in the cerebellar lobules (Perkel et al., 1990). EPSCs evoked from both parallel fibres and climbing fibres are blocked by 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA) receptor antagonists such as 6-cyano-7-nitroquinoxaline-2,3,-dione (CNQX) (Konnerth et al., 1990), but are unaffected by amino-5-phosphonopentanoate (AP5) application even if magnesium is removed to stop any voltage block (Perkel et al., 1990). Changing the membrane potential of the postsynaptic Purkinje cell perfused with a caesium containing internal solution when EPSCs are evoked shows a linear relationship for both EPSCs evoked from parallel and climbing fibres (Perkel et al., 1990). The lack of sensitivity of the EPSCs to N-methyl-D-aspartate (NMDA) receptor antagonists but not AMPA receptor antagonists, and the linear voltage-current relationship when magnesium is present suggests both types of EPSCs are mediated by AMPA receptors alone without any contribution from NMDA receptors (Perkel et al., 1990).
1.3 Retrograde transmission acting on excitatory inputs

Retrograde transmission is where a messenger is released from the postsynaptic cell and influences the release properties of the afferent cells. Retrograde transmission can be induced by afferent nerve terminals, but the afferent that induces retrograde transmission need not be that afferent that has altered release properties (Duguid & Smart, 2004). Excitatory afferents to Purkinje cells are subject to retrograde signalling. Both climbing fibre and parallel fibre inputs can be inhibited by the release of endogenous endocannabinoids for the Purkinje cell in a process called depolarisation-induced suppression of excitation (DSE). The endocannabinoids act on cannabinoid type 1 (CB₁) receptors in the excitatory terminals reducing the amplitude of EPSC and the size of the calcium transient that is evoked with the same stimulation (Kreitzer & Regehr, 2001). The release of endocannabinoids can be induced by an increase in intracellular calcium associated with depolarising the Purkinje cell (Llano et al., 1994) which can be replicated physiologically by inducing complex spikes which result from evoking the climbing fibres inputs (Duguid & Smart, 2004). In parallel fibres, activation of CB₁ receptors causes a reduction in the calcium transient by activating potassium channels in the terminals (Daniel & Crepel, 2001). There is variability in the effects retrograde transmission has on the many different afferents to the postsynaptic cell. For example endocannabinoids were more effective at reducing the action-potential evoked calcium transient in larger parallel fibres terminals than smaller terminals (Zhang & Linden, 2009). In addition to endocannabinoids another form of DSE has been reported to act on parallel fibres, which is mediated by kainate receptors activated by the retrograde release of glutamate (Crepel, 2007). However, this form of DSE can only occur during a narrow developmental window between postnatal days eighteen and twenty-two.
1.4 NMDA receptors at excitatory terminals

NMDA receptors are thought to be involved in some forms of retrograde transmission (Duguid & Smart, 2004). Activation of presynaptic NMDA receptors are not thought to modulate excitatory inputs to Purkinje cells directly. Presynaptic NMDA receptors are not thought to modulate climbing fibre inputs (Piochon et al., 2007), but presynaptic NMDA receptors are thought to play a role in LTD which requires climbing fibre inputs (Bidoret et al., 2009). The frequency or amplitude of miniature EPSCs (mEPSCs) derived from parallel fibres are not affected by NMDA application even under experimental conditions similar to those where NMDA increases the frequency of miniature inhibitory postsynaptic currents (mIPSCs) (Glitsch & Marty, 1999). (Miniature post synaptic currents refer to currents caused by the release of a single vesicle of neurotransmitter, which tend to have smaller amplitudes and frequencies than non-miniature events (Southan & Robertson, 1998a), in practice this often means post synaptic events that are not evoked by action potentials such as when voltage-gated sodium channels are blocked.) NMDA application also failed to change the frequency of mEPSCs in interneurons which, like Purkinje cells, also receive inputs from parallel fibres (Glitsch & Marty, 1999). However there have been reports where NMDA seems to be able to induce a large response in Purkinje cells by activating several synaptic inputs throughout the neuronal network (Farrant & Cull-Candy, 1991). The response is mostly abolished by a combination of tetrodotoxin (TTX) and bicuculline, but has a resistant component with amplitude of around 20 pA which is sensitive to CNQX, suggesting NMDA is occasionally able to stimulate glutamatergic inputs. Applying NMDA and glycine when EPSCs are being evoked by stimulating the parallel fibres has been reported to cause a reduction in the EPSC amplitude (Casado et al., 2000). This is thought to be solely a postsynaptic effect as the paired pulse facilitation (PPF) of two
closely evoked EPSCs remains in the same ratio despite a NMDA induced reduction in amplitude suggesting there is no change to the release probability. It is proposed that the reduction in amplitude is caused by the NMDA application stimulating nitric oxide synthase in parallel fibre terminals which crosses into the postsynaptic cell setting off a signalling cascade which results in a reduction in postsynaptic AMPA receptors (Casado et al., 2000). The NMDA receptors in parallel fibres terminals are thought to be important in inducing long term depression (LTD) at the parallel fibres. The NMDA receptors are proposed to act as detectors for a high frequency of glutamate release where early action potentials occurring in a single parallel fibre is able to relieve the NMDA receptors of magnesium block for a time where they can then detect glutamate overspill from the synapse (Casado et al., 2002; Bidoret et al., 2009). However it has been proposed that nitric oxide stimulated by NMDA receptors does not originate from parallel fibres (which in the same studies are thought to lack NMDA receptors) but in other nerve terminals that do express NMDA receptors (Shin & Linden, 2005; Qiu & Knopfel, 2007). Interestingly, whereas NMDA application can affect the frequency of mIPSCs without glycine (Glitsch & Marty, 1999), the NMDA induced reduction in the amplitudes of parallel fibre EPSCs is dependent on the presence of glycine (Casado et al., 2000).
1.5 Molecular layer interneuron inhibitory inputs

The molecular layer interneurons consist of basket and stellate cells. Both types of interneurons are able to form multiple synapses that release the inhibitory neurotransmitter gamma aminobutyric acid (GABA) onto Purkinje cells. The molecular layer interneurons are parvalbumin positive GABAergic interneuron (Caillard et al., 2000) and are sparsely distributed throughout the cerebellar molecular layer. A working method to differentiate basket cells from stellate cells is based on their position in the molecular layer. Molecular layer interneurons found in the first third of the molecular layer directly adjacent to the Purkinje cell layer are classified as basket cells whereas molecular layer interneurons found in the outer two thirds are classified as stellate cells (Vincent et al., 1992). The more distant stellate cells form functional synapses with Purkinje cells later during basket cells than the closer basket cells. At postnatal (P) 9-11 basket cells are functional whereas stellate cells are still differentiating. Stellate cells mature between P12-15 but at P9-11 stellate cells do form functional connections to Purkinje cells which produce evoked IPSCs (eIPSCs) that have smaller amplitudes and are less variable than eIPSCs from basket cells at the same age (Vincent et al., 1996).

Purkinje cells spontaneously display a high frequency of postsynaptic currents. In one study from P11-17 rat slices found the spontaneous postsynaptic currents reversed at potentials predicted by the reversal potential for chloride ions when the concentrations were varied (Farrant & Cull-Candy, 1991), suggesting the majority of postsynaptic currents are mediated by anion channels. In two studies on P11-17 the majority of spontaneously occurring IPSCs under control conditions were completely blocked by bicuculline suggesting they are mediated by vesicular release of GABA that activate GABA type A (GABA\textsubscript{A}) receptors (Konnerth et al., 1990; Perkel et al., 1990). Molecular layer interneurons are rarely coupled electronically as neighbouring
interneurons lack correlation in firing and internal application of a dye is mostly retained in that cell (Vincent et al., 1992). Molecular layer interneurons can form connections with multiple Purkinje cells close together; it is rare to find Purkinje cells innervated by the same molecular layer interneuron when the postsynaptic cells are further than 300 μm apart. The amplitudes of eIPSCs originating from the same molecular layer interneurons are not correlated which suggests variability in neurotransmitter release (Vincent et al., 1996). IPSCs that can be attributed to spontaneous action currents in the presynaptic cell exhibit a broad range of amplitudes from a couple of picoamperes up to 2 nA. There are no obvious peaks in the amplitude histogram which would also suggest there is great variability in the spontaneous release process (Vincent et al., 1996), assuming the expression of postsynaptic receptors remains consistent. Although variable there is no correlation between amplitude and the preceding interval since the previous IPSC suggesting the synapses are not influenced by prior neurotransmitter release (Vincent et al., 1992). The amplitudes of IPSCs evoked from molecular layer interneurons increases with the size of the stimulation. However the mean amplitudes do not increase linearly with the size of the stimulation as plateaus have been reported at around 50, 800, and 1200 pA (Vincent et al., 1992). Molecular layer interneurons are able to generate a burst of IPSCs which can be evoked from one interneuron (Auger & Marty, 1997) with a single stimulation although the threshold for inducing a burst is greater than the threshold to evoke a single IPSC (Vincent et al., 1992). Increases in the frequency of eIPSCs correlate with an increase in the frequency of smaller amplitude asynchronous IPSCs that occur in bursts of IPSCs (Vincent et al., 1996).
Perfusion of afferent molecular layer interneurons with a caesium based internal solution produces IPSCs that are greater in amplitude but with less variation. The internal caesium blocks potassium channels as demonstrated by the failure to evoke outward potassium currents. The greater uniformity of IPSCs is thought to come about by reducing the variation in propagation of action potentials along the branch points along the basket cell axon (Vincent & Marty, 1996). Immunohistological studies showed basket cell terminals expressed high levels of Kv1.1 and Kv1.2 (Wang et al., 1993; Chung et al., 2001; McNamara et al., 1993). Blocking members of the Kv1 potassium channel family with alpha-dendrotoxin (DTX) causes a marked increase in the frequency of spontaneous IPSCs (Southan & Robertson, 1998b). This was not done by altering the frequency of action potentials in the soma of the afferent cell or effecting potassium currents in the soma as they were unaltered by application of alpha-DTX or 4-aminopyridine (4-AP), but the soma did express potassium currents sensitive to tetraethylammonium (TEA). This means that alpha-DTX is having an effect somewhere in the axonal compartment. The same group that found that the somas of molecular layer interneurons expressed a TEA sensitive current also found TEA does not affect the frequency of IPSCs but does cause an increase in the amplitude (Southan & Robertson, 1998a). Another study done with similarly aged rats instead of mice did demonstrate TEA could increase the frequency of IPSCs (Tan & Llano, 1999). An increase in frequency was also seen with alpha-DTX, 4-AP, and charybdotoxin which blocks big conductance calcium-activated potassium (BK) channels. Unlike the other blockers that have an effect on frequency, applying 4-AP also causes an increase in the intracellular calcium in the boutons. Applying TEA and charybdotoxin with 4-AP causes an enhancement of the calcium transient but alpha-DTX does not have any
synergic effects with 4-AP. Several basket cell axons can wrap themselves around the initial segment of the Purkinje cell axon forming a dense structure of interneuron release sites called the pinceau (McNamara et al., 1996). It is possible to make whole cell recordings from the pinceau which could be quite informative especially if the properties of the basket cell release sites appear different from the properties of the soma (Southan & Robertson, 2000). Whole cell recordings from the pinceau reveal currents can be evoked that are sensitive to both 4-AP and TEA. Both blockers were unable to reduce the outward current uniformly but instead there were components of the evoked potassium current that was quite sensitive to each blocker and a second component which was less sensitive. This was thought to correspond to high-threshold and low-threshold voltage-gated potassium channels in the terminal. Certainly after blocking the outward current with alpha-DTX which blocks low-threshold voltage-gated potassium channels there does remain a large component that can be blocked by 4-AP.

Mice lacking Kv1.1 were generated (Smart et al., 1998) and showed an increase in spontaneous IPSC (sIPSC) frequency (IPSCs that were not evoked but occur spontaneously which may include events mediated by action potentials) but not sIPSC amplitude. The increase in frequency was action potential dependent as no modification in frequency was seen with mIPSCs (Zhang et al., 1999). However no changes in the frequency of action potentials could be seen from recording from the soma suggesting that Kv1.1 has a specialised role in propagating action potentials in the basket cell axon or terminal. Kv1.1 knockout mice had motor defects as well as demonstrated by the decreased latency for falling off a thin beam. Interestingly their ability to stay on a rotarod was not significantly different from wild type mice. The pharmacology of neurotransmitter release was also changed in Kv1.1 knockout mice. The frequency of
sIPSCs became more sensitive to 4-AP application in Kv1.1 null mice but lost a significant sensitivity to 300 µM TEA. Both the wild type and knockout retained sensitivity to DTX, which in this study was a mixture of alpha-DTX and I-DTX (Zhang et al., 1999).

Mutations in Kv1.1 can cause episodic ataxia type 1 (EA1) and it is thought the role of Kv1.1 in basket cell terminals play an important role in the phenotype of the condition. EA1 is characterised by impaired motor coordination brought about by the onset of stress. Kv1.1 V408A is one mutation that causes EA1 and it was introduced to a line of mice (Herson et al., 2003). This mutation occurs at the cytoplasmic end of the S6 domain. The mutation is homozygous lethal; killing the embryo between embryonic day (E)4 and E9. Heterozygous mice have an increased frequency of spontaneous IPSCs being derived from basket cells without any changes in the action potentials at the afferent cell soma or effecting the frequency of mIPSCs. This again suggests Kv1.1 plays no role in the soma but has a specialised role in modulating neurotransmitter release from the axonal compartment. The heterozygous mice only display significant impaired motor coordination when stressed. Stressing the mice was achieved by injecting them with 10 mg kg$^{-1}$ of the beta-adrenergic agonist isoproterenol before encouraging them to run on a sloped treadmill for between 20 and 30 minutes. When stressed the heterozygous mice showed a decreased latency to fall from an accelerating rotarod and increased number of missteps when travelling along a narrow raised beam. No difference was seen between heterozygous and wild type mice when either group was treated with isoproterenol or the exercise alone. Also no significant difference was seen between treatments when running was preceded by a higher dose of 20 mg kg$^{-1}$ isoproterenol (Herson et al., 2003). However it was reported that some of the characteristic of the EA1 type mutation is only found in female mice (Hayden et al.,
Mutation in Kv1.2 can induce similar effects suggesting the two channels have a similar location and function. However, unlike the EA1 mutation in Kv1.1 which replicates an inherited disease, the pingu (Pgu) mutation Kv1.2 was found during a screen for ataxic mutants (Xie et al., 2010). Like the EA1 heterozygous mice, Pgu homozygous mice display an increase in spontaneous IPSCs in Purkinje cells without changes in the frequency of mIPSCs or action potentials in the soma of the afferent cell. These changes are accompanied by a severe ataxic phenotype and reduced weight.

Action potentials in molecular layer interneurons ultimately exert their effect on sIPSCs by activating calcium channels (Llano et al., 1997). When calcium channels are blocked with cadmium applying TTX to block action potentials has no further effect on the IPSC properties. The voltage-gated calcium channels that were involved in neurotransmitter release from molecular layer interneurons were identified as the P/Q type (Ca,2.1) (Forti et al., 2000). Selectively blocking these presynaptic channels with omega-agatoxin IVA decreased the frequency and amplitude of the sIPSCs that could not be altered further by either TTX or cadmium. P/Q type voltage gated calcium channel immunoreactivity could be located around the soma of Purkinje cells and co-localise with markers for inhibitory terminals (Stephens et al., 2001). The vesicle release site is tightly coupled to their source of calcium as internal perfusion of 10 mM of the slow calcium chelator ethylene glycol tetraacetic acid (EGTA) does not disturb IPSC properties (Vincent & Marty, 1996). Internal calcium stores in the terminals of molecular layer interneurons play a role in synaptic transmission. Applying a lower concentration of ryanodine of 10 µM can increase the frequency of mIPSCs (Bardo et al., 2002) and induce bursting behaviour by enhancing calcium hot-spots (Conti et al., 2004). Calcium stores are important for the amplitude of IPSCs (Galante & Marty, 2003). When the stores are depleted large amplitude IPSCs that are a result of the
simultaneous release of multiple vesicles cannot be sustained from molecular layer interneuron boutons resulting in a reduction in amplitude (Llano et al., 2000).
1.7 NMDA receptors and retrograde transmission acting at inhibitory inputs

Presynaptic NMDA receptors play a role in modulating inhibitory neurotransmission in the cerebellar cortex. The subunits that comprise NMDA receptor have been located to terminals of molecular layer interneurons (Petralia et al., 1994; Duguid & Smart, 2004). Applying NMDA can increase the frequency of IPSCs in the postsynaptic Purkinje cell (Farrant & Cull-Candy, 1991; Glitsch & Marty, 1999; Fiszman et al., 2005). This is as result of activating NMDA receptors that can induce an increase in the frequency of mIPSCs from both basket and stellate cells (Glitsch & Marty, 1999). Also openings of single NMDA receptors can be recorded from cultured molecular layer interneurons (Fiszman et al., 2005). Activation of presynaptic NMDA receptors can induce a long-lasting increase in the frequency of IPSCs, although the increase is not as large as when NMDA is initially applied (Liu & Lachamp, 2006). The prolonged, but lesser, increase in IPSC frequency is caused by an a long-lasting increase in calcium concentration provided by calcium-induced calcium release initiated by the preceding activation of the calcium-permeable NMDA receptors which is evident from the reduction in the magnitude and duration of the NMDA response when the calcium stores are depleted by 100 µM ryanodine (Duguid & Smart, 2004). Although calcium induced calcium release greatly prolongs the response of activating NMDA receptors, calcium induced calcium release is not required to enhance the frequency of inhibitory inputs as activation of the receptors themselves is able to increase the calcium concentration in the nerve terminal. NMDA can enhance spontaneous IPSCs by activating voltage-gated calcium channels (Glitsch, 2008). Although if calcium-induced calcium release and voltage-gated calcium channels cannot be activated in inhibitory
terminals it is unlikely NMDA can affect the frequency of inhibitory events as 100 µM ryanodine can abolish the effect of NMDA on the frequency of mIPSCs (Huang & Bordey, 2004). However there is also evidence from a calcium imaging study that the boutons of basket and stellate cells lack functional NMDA receptors. This was inferred from a lack of a calcium rise when a NMDA agonist was applied to the nerve-boutons along the axon (Christie & Jahr, 2008). A calcium rise would be expected when presynaptic NMDA receptors are activated. The same study claims there is an effect of activating NMDA receptors in the afferent cells where a depolarisation is induced in the somatodendritic compartment of the cell and is propagated to the release sites along the axon.

Presynaptic NMDA receptors are thought to be involved in several physiological processes. Blocking presynaptic NMDA receptors with a selective antagonist, such as AP5, reduces the frequency of IPSCs in Purkinje cells showing that NMDA receptors in terminals are activated under basal conditions (Glitsch & Marty, 1999; Huang & Bordey, 2004). However presynaptic NMDA receptors on molecular layer interneurons are also involved in detecting the overspill of glutamate from parallel fibres, that either synapses on the same postsynaptic cell or the molecular layer interneuron, which results in an increase of GABA being released onto the postsynaptic Purkinje cells (Huang & Bordey, 2004; Liu & Lachamp, 2006). Presynaptic NMDA receptors in inhibitory terminals are also involved in a form of retrograde transmission called depolarisation-induced potentiation of inhibition (DPI) (Duguid & Smart, 2004; Duguid et al., 2007). This is where a depolarisation of the postsynaptic Purkinje cell causes a rise in intracellular calcium that result in the retrograde release of vesicular glutamate which activates presynaptic NMDA receptors located in inhibitory terminals thus increasing the frequency of IPSCs and potentiating the inhibition onto the Purkinje cell. Although
depolarising the postsynaptic Purkinje cell results in an increase in calcium (Llano *et al.*, 1994) this can replicated with multiple inputs evoked from the climbing fibre (Duguid & Smart, 2004). The calcium sensitive proteins that mediate the retrograde release of glutamate cannot be closely coupled to the source of the calcium influx as DPI can be abolished by an internal application of slower calcium chelator EGTA (Duguid *et al.*, 2007). A similar reduction in DPI can also be caused with the faster calcium chelator BAPTA and application of 100 µM ryanodine (Duguid & Smart, 2004), although calcium induced calcium release is important to the calcium rise in the postsynaptic cell (Llano *et al.*, 1994) some of the reduction in the effects of ryanodine on DPI will be presynaptic as ryanodine can interfere with activation of presynaptic NMDA receptors. Vesicular release of glutamate is thought to be involved in the retrograde signalling as DPI is abolished by the internal application of the light chain of botulinum toxin B and N-ethylmaleimide (NEM) (Duguid *et al.*, 2007), both of which interferes with vesicular release, as well as in mice lacking the vesicular glutamate transporter 3 (vGluT3) which is normally expressed in Purkinje cells and would be the most likely transporter to load glutamate into vesicles in that neuron (Crepel *et al.*, 2011). The involvement of NMDA receptors has been confirmed by DPI being abolished by applying selective antagonist such as AP5 (Duguid & Smart, 2004; Duguid *et al.*, 2007).

Like excitatory inputs to the Purkinje cell molecular layer interneurons are also the target of inhibition by retrograde transmission. A process called depolarisation-induced suppression of inhibition (DSI) exists which reduce the frequency of mIPSCs and the frequency and amplitude of spontaneous IPSCs (Diana & Marty, 2003). These changes are caused by a calcium dependent release of endocannabinoids that activate CB1 receptors in inhibitory terminals that result in a reduction in the calcium transients
in the boutons (Diana et al., 2002). Reductions in calcium transients are mediated by activation of presynaptic potassium channels and can be reduced by perfusing the afferent cell with a caesium based internal solution which will block some of the activated potassium channels (Diana & Marty, 2003). The decrease in amplitude of evoked events was observed when IPSCs were evoked from different parts of the molecular layer surrounding the afferent Purkinje suggesting the effect is not restricted to a single neuron (Vincent et al., 1992). However, DSI in Purkinje cells has also been thought to be mediated by the retrograde release of glutamate activating presynaptic mGluRs which result in a suppression of the inhibitory inputs (Glitsch et al., 1996).
1.8 Lugaro cell inhibitory inputs

Lugaro cells also provide inhibitory inputs to Purkinje cells. They are ovoid bipolar cells located just below the Purkinje cell layer are immunoreactive for calretinin unlike the parvalbumin positive molecular layer interneurons (Dumoulin et al., 2001). The soma of Lugaro cells that are afferent to a given Purkinje cell is normally located a couple of Purkinje cells widths along in the Purkinje cell layer, typically four cells along. In slices Lugaro cells normally have a very low activity or are silent, however their activity can be induced by directly stimulating the Lugaro cell or by applying serotonin (Dean et al., 2003). Recording from the Lugaro cell soma, serotonin is seen to increase the frequency of action potentials by inhibiting the slow afterhyperpolarisation from developing and increasing the afterdepolarisation. Inputs from Lugaro cells can be characterised by their relative insensitivity to bicuculline and they have been studied in isolation, albeit partially blocked, by recording inhibitory inputs in the presence of 20 μM bicuculline methiodide. Despite the inputs being sensitive to the GABA type C (GABA$_C$) receptor antagonist 1,2,5,6-tetrahydropyridine-4-yl methylphosphinic acid (TPMPA), GABA$_C$ receptors or receptors containing the rho GABA$_C$ receptor subunit were not thought to mediate the bicuculline resistant IPSCs. GABA$_C$ receptors were not thought to be involved because GABA$_A$ receptor antagonist, such as bicuculline and gabazine, would block the inputs eventually at high enough concentrations. The GABA$_C$ receptor rho subunit was not thought to be involved in GABA receptor heteromers as the inputs were sensitive to benzodiazepines and currents in the Purkinje cell could not be evoked with 200 μM of the GABA$_C$ receptor preferring agonist $cis$-4-aminocrotonic acid (CACA) (Dean et al., 2003). However CACA can evoke currents in mouse Purkinje cells by activating GABA receptors heteromers containing rho subunits (Harvey et al., 2006). At a concentration of 200 μM, CACA would not be able to induce
large currents under normal circumstances but this current would presumably be attenuated if recorded in the presence of 20 μM bicuculline methiodide possibly to the point it can no longer be observed.
1.9 Purkinje cell inhibitory inputs

Purkinje cells are themselves GABAergic neurons and can form inhibitory synapses onto other Purkinje cells within the cerebellum. The main axon from Purkinje cells descends to the deep cerebellar nuclei, but often has a branch or axonal collateral that loops back to the cerebellar cortex forming a highly uniform connection with nearby Purkinje cells (autaptic synapse were not observed). These axonal collaterals are thought to synchronise waves of activity in the cerebellar cortex of developing mice (Watt et al., 2009). Purkinje to Purkinje synapses are hard to find. In one study, after identifying potential pairs of connected Purkinje cells by perfusing the presynaptic cell with a fluorescent dye, only 10% of these pairs appeared to have functional synaptic connections (Orduz & Llano, 2007). The connections typically consist of 2-3 large varicosities from one axonal collateral, which is the most number axonal of collaterals a Purkinje cell is likely to receive. The axonal collateral has been reported to synapse mostly with Purkinje cells dendrites in mature mice (Orduz & Llano, 2007) or the somas in young developing mice (Watt et al., 2009). Currents evoked from Purkinje-Purkinje synapses were GABA<sub>A</sub> receptor mediated IPSCs as demonstrated by their sensitivity to bicuculline and gabazine, so are not mediated by the same receptors that respond to inputs from Lugaro cells and therefore cannot be isolated in similar fashion. Despite being able to coordinate activity in the cerebellar cortex, the axonal collateral to Purkinje cell synapses are thought to be a weaker connection when compared with inputs from molecular layer interneurons. The amplitude of evoked IPSCs appear to have a mean of around 55 pA and a narrow range with no event exceeding 200 pA which is smaller than evoked events from molecular layer interneurons. Also the calcium rise in axonal collateral nerve boutons in response to a train of depolarisations
is comparatively smaller than the calcium rises in other inhibitory inputs (Orduz & Llano, 2007).
1.10 Autocrine signalling

As the retrograde release of glutamate can reach the afferent synapses of molecular layer interneurons it was thought it was possible that the same glutamate could also be involved in autocrine signalling back on to the Purkinje cell that originally released it. After evoking the retrograde release of glutamate by causing a depolarisation of the Purkinje cell, a slow inward current can appear which is thought to be caused by activation of metabotropic glutamate receptors due to the sensitivity of the current to the antagonist 7-(hydroxyimino)-cyclopropa[b]chrome-n-1a-carboxylate ethyl ester (CPCCOEt) (Duguid et al., 2007). This slow current occurs separately from the appearance of another inward current thought to be mediated by calcium-activated chloride channels (Llano et al., 1991a). The current putatively mediated by autocrine glutamate signalling was dependent on vesicle release as it was blocked by internal application of the light chain of botulinum toxin B (Duguid et al., 2007) as well as bafilomycin A1 which disrupts the proton gradient necessary for loading vesicles by blocking a proton pump (Shin et al., 2008). This current and the proposed process was named depolarisation-induced slow current (DISC) (Shin et al., 2008). Autocrine glutamate signalling might suggest glutamate release is not directed at particular synapses but may affect many synapses in a diffuse area, or there is sufficient glutamate to spill over from a synapse and activate metabotropic receptors such as when the slow EPSC is evoked from parallel fibres (Hartmann et al., 2008). However further studies of DISCs shows it is not mediated by metabotropic glutamate receptors as DISC is not sensitive to other mGluR antagonists and is unchanged in mGluR1 knockout mice (Shin et al., 2009). Further evidence suggests DISC cannot be mediated by the retrograde release of glutamate as it is unaffected in mice lacking the vesicular glutamate transporter vGluT3 which is critical to expression of DPI (Crepel et al., 2011). DISC
was later identified as being caused by the autocrine release of dopamine acting via postsynaptic D₃ dopamine receptors (Kim et al., 2009). DISC can be blocked with internal application of blockers of transporters that load dopamine vesicles suggesting the dopamine signalling originates from the Purkinje cells meaning they are dopaminergic neurons in addition to being GABAergic and glutamatergic, although dopaminergic signalling is restricted to only some lobules of the cerebellum (Kim et al., 2009).
1.11 Lack of postsynaptic NMDA receptors

NMDA receptors are ligand-gated ion channels found in the central nervous system that are activated by the neurotransmitter glutamate (Petralia et al., 1994). NMDA receptors are composed of tetramers that form a calcium-permeable cation channel (Dingledine et al., 1999). In addition to being activated by glutamate, the obligate NMDA receptor subunit 1 (NR1) subunits require to have a ligand bound, usually glycine, as a precondition to receptor activation (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988). The pore of NMDA receptors can be blocked by magnesium which can be relieved if there is a sufficient depolarisation acting across the membrane (Nowak et al., 1984). Sensitivity to magnesium is influenced by the subunits that comprise of the NMDA receptor tetramer (Wrighton et al., 2008). A typical tetramer consists of two NMDA receptor subunit 2s (NR2), of which there are four types, in addition to two NR1 subunits (Dingledine et al., 1999). Tetramers containing the subunits NMDA receptor subunit 2A (NR2A) and NMDA receptor subunit 2B (NR2B) are more sensitive to extracellular magnesium than tetramers containing subunits NMDA receptor subunit 2C (NR2C) or NMDA receptor subunit 2D (NR2D) (Monyer et al., 1994; Wrighton et al., 2008). NR2 subunits also influences the duration of macroscopic currents, with tetramers composed on NR2A subunits giving comparatively briefer currents, than NR2B containing tetramers, which in turn are briefer than NR2C and then NR2D containing NMDA receptors (Monyer et al., 1994).

Unusually for central neurons, rat Purkinje cells lack postsynaptic NMDA receptors. Evidence for a lack of functional NMDA receptors include the insensitivity of any component of EPSCs to NMDA receptor antagonists and that bath applied NMDA agonists are unable to induce a inward current whereas AMPA receptor
agonists are able to do so in rats aged between P28 - 42 (Perkel et al., 1990). Outside-patches pulled from rat Purkinje cells, aged between P8 – 12, do not respond to NMDA but they do to AMPA agonists suggesting currents induced by NMDA are because of activation of receptors elsewhere in the neuronal network (Farrant & Cull-Candy, 1991). With in situ hybridisation there is a signal for NR1 transcripts in rat Purkinje cells but not for any NR2 subunits at any of the stages investigated, at P0, P7, and P12, suggesting a lack of functional receptors (Monyer et al., 1994). Similarly signals from immunohistochemistry are weak and variable (Petralia et al., 1994). However rat Purkinje cells do express functional NMDA receptors in early development but their expression declines after around postnatal day seven (Rosemund et al., 1992). It appears rat Purkinje cells transiently express NMDA receptors up to the first postnatal week (Rosemund et al., 1992) but rapidly lose them as indicated by in situ hybridisation (Monyer et al., 1994) and electrophysiological studies done on tissue from rats a couple of days older than a week (Farrant & Cull-Candy, 1991). After the first week of development there appears to be a lack of functional postsynaptic NMDA receptors in rat Purkinje cells. This is replicated in several studies covering such periods of development as P11 – 14 (Duguid & Smart, 2004), P12 – 14 (Glitsch & Marty, 1999), P18 – 26 (Casado et al., 2000), and P28 – 42 (Perkel et al., 1990). However, one study found in adult rats, P56 – 84, that EPSCs evoked from the climbing fibre did display a small component mediated by NMDA receptors that was absent from younger rats between P17 – 21 (Bidoret et al., 2009). The apparent lack of functional postsynaptic NMDA receptors is a species specific trait to rats. In mouse, Purkinje cells express NMDA receptors throughout development (Renzi et al., 2007) and are thought to play a specific role in contributing part of the input from the climbing fibre (Piochon et al., 2007).
1.12 The nerve-bouton preparation

This thesis uses the nerve-bouton preparation of Purkinje cells. The nerve-bouton preparation consists of cells and their adherent afferent nerve-terminals mechanically isolated from the surrounding tissue using a process called vibrodissociation. The nerve-bouton preparation was originally devised as a way to isolate neurons from a brain slice for electrophysiology without using proteases which could affect the function of surface proteins including some receptors and channels (Vorobjev, 1991). However it was later found that afferent terminals that have been retained by the preparation are spontaneously active (Akaike & Moorhouse, 2003). As only the nerve-terminals and the postsynaptic cell remain, changes in the behaviour of neurotransmitter release due to experimental treatments can be inferred to be caused by effects on one compartment or the other. If care is taken to keep the attributes of the postsynaptic cell constant and unchanging, changes in neurotransmitter release can be attributed to changes in the properties in the nerve-boutons with some confidence as has been done successfully in several previous studies with nerve-bouton preparations from a range of neurons with varied inputs (Ye et al., 2004; Shoudai et al., 2007). Nerve-bouton preparations allow for the study of nerve-bouton in situ with their native receptors being activating in their native postsynaptic cell isolated from the influence of surrounding neurons, glial cells, networks, and any non-bouton compartment of the afferent cell (Duguid et al., 2007).
1.13 Experimental rationale

Fast chemical signalling occurs between neurons at synapses when vesicles loaded with neurotransmitters are released from the presynaptic neuron activating the ligand-gated ion channels of the postsynaptic neuron at the other side of the synaptic cleft. Vesicles release their neurotransmitters into the synaptic cleft by fusing with the external membrane of the presynaptic terminal. Neurotransmitter release is controlled, for the most part, by proteins that rapidly fuse vesicles in response to an increase in calcium inside the nerve-terminal, although vesicles can also be released spontaneously at a much slower rate without a calcium increase (Lisman et al., 2007). The source of the calcium increase is often voltage-gated calcium channels in the nerve-terminal. Calcium channels are frequently located close to sites of vesicle fusion release where they are able to perform a specialised role in controlling neurotransmitter release. Neurotransmitter release can be coordinated with action potentials in synchronous release where vesicle fusion occurs due to activation of voltage-gated calcium channels caused by the depolarisation of an action potential (Neher & Sakaba, 2008). This means, in addition to voltage-gated calcium channels, channels that influence action potentials such as voltage-gated sodium and potassium channels also play a role in modulating neurotransmitter release.

Since neurotransmitter release is dependent on the actions of certain ion channels, postsynaptic events induced by neurotransmitters can be used to infer the behaviour of the presynaptic bouton. The behaviour of afferent nerve-terminals can be the product of many different neurons in a network (Fig 1.1) as well as being influenced by other nearby cells such as glial. By using the nerve-bouton preparation the postsynaptic cell is isolated whilst retaining some functional nerve-boutons (Akaike &
Moorhouse, 2003). This means the afferent terminals can be studied without influences from the rest of the presynaptic cell or surrounding cells. This can be useful both when the terminals of the cells are different from the soma, in this study in terms of what types of ion channels are expressed (Zhang et al., 1999), as well as isolating the influence of a channel at the nerve-terminal that may occur throughout the neuron (Christie & Jahr, 2008) (Fig 1.2). As the terminals are physically isolated there is no need to block the propagation of action potentials from the presynaptic cell as is often done in studies using brain slices (Glitsch & Marty, 1999).
**Figure 1.1.** Cerebellar circuit diagram. Purkinje cells are the focus of this study and they represent the sole output of the cerebellar cortex where there are acted on by several different types of neurons. Afferent inputs acting on the Purkinje cells include the excitatory parallel fibres from the granule cells and the climbing fibres as well as inhibitory inputs from the molecular layer interneurons: the basket and stellate cells. Not represented in the diagram are inhibitory inputs from Lugaro cells and Purkinje axonal collaterals. Figure courtesy of T.G. Smart (UCL) adapted from Haines & Ard, 1997.
**Figure 1.2.** A cartoon representation of the nerve-bouton preparation. After being isolated the Purkinje cells retain presynaptic nerve-boutons. These nerve-boutons are still functional and spontaneously release vesicles of neurotransmitter which can be detected as a postsynaptic event in the Purkinje cell. Treatments that change the properties of the postsynaptic events may be used to infer the properties of the nerve-boutons. In the Purkinje cell nerve-bouton preparations the nerve boutons express NMDA receptors in their terminals which are absent from the rat Purkinje cells at the ages used. The nerve-bouton also expresses potassium channels. There are potassium channels in the postsynaptic cell too, but they are unlikely to play much of a role due to the use of a caesium based TEA containing internal solution and the hyperpolarised holding potential.
1.14 Experimental aims

- To characterise the Purkinje cell nerve-bouton preparation, including which type of neurotransmitters are released and what controls the release under basal conditions.
- To investigate which potassium channels influence the nerve-boutons and therefore neurotransmitter release by applying potassium channel blockers.
- To investigate the presence and role of NMDA receptors in the nerve-boutons.
- To investigate the role of presynaptic ion channels in physiological relevant processes.
Chapter 2: Methods

In this chapter a brief description of the experimental procedures used will be given.

2.1 Preparing parasagittal Cerebellar slices

Purkinje cell nerve-bouton preparations were derived from 500 µm thick parasagittal cerebellar slices prepared from postnatal day 10 (P10) Sprague-Dawley rats (*rattus norvegicus*) of either sex (Charles Rivers International, Inc). Before being decapitated, the rats were killed by cervical dislocation in accordance to the procedures described in schedule I of the UK Home Office guidelines. The head was then placed in a Petri dish (Nunc) with iced-chilled cutting solution. (The compositions of all solution used in making the nerve-bouton preparations and the external solution used during electrophysiological recordings are given in Table 2.1.) The cutting solution is a high-magnesium carbogen-buffered artificial cerebral-spinal fluid (aCSF) consisting of 135 mM NaCl, 3 mM KCl, 20 mM NaHCO$_3$, 1 mM NaH$_2$PO$_4$, 15 mM glucose, 0.5 mM CaCl$_2$, and 3 mM MgCl$_2$ buffered with carbogen gas composed of 95 % O$_2$ and 5 % CO$_2$ (BOC). The skull was quickly uncovered by cutting the skin covering the dome of the skull from the foramen magnum towards the nose. The brain was exposed when the skull was opened by cutting around its circumference starting from the foramen magnum. The exposed cerebellum was separated from the rest of the brain by cutting downwards with a scalpel. The cerebellum was removed and placed on an ice-chilled platform then put into position under the arm of a McIlwain tissue chopper (Mickel Laboratory Engineering Co.) that has been fitted with a fresh double-sided razor blade.
(Wilkinson Sword). After cutting, the brain slices were removed to an incubation chamber where they were kept in incubation solution continuously bubbled with carbogen for at least an hour at room temperature before being vibrodissociated. The incubation solution is a low-magnesium carbogen-buffered aCSF consisting of 135 mM NaCl, 3 mM KCl, 20 mM NaHCO$_3$, 1 mM NaH$_2$PO$_4$, 15 mM glucose, 3 mM CaCl$_2$, and 0.5 mM MgCl$_2$ buffered with carbogen composed of 95 % O$_2$ and 5 % CO$_2$. 
<table>
<thead>
<tr>
<th>Substance</th>
<th>External solution concentration</th>
<th>Cutting solution concentration</th>
<th>Incubation solution concentration</th>
<th>Vibrodissociation solution concentration</th>
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<td>135 mM</td>
<td>135 mM</td>
<td>145 mM</td>
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<td>3 mM</td>
<td>3 mM</td>
</tr>
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<td>-</td>
<td>-</td>
<td>10 mM</td>
</tr>
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<td>20 mM</td>
<td>-</td>
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<td>1 mM</td>
<td>-</td>
</tr>
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<td>15 mM</td>
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<tr>
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<td>0.5 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
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<td>3 mM</td>
<td>0.5 mM</td>
<td>3 mM</td>
</tr>
<tr>
<td>pH</td>
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<td>buffered with 95% O₂ and 5% CO₂</td>
<td>buffered with 95% O₂ and 5% CO₂</td>
<td>7.35 with 1 M NaOH</td>
</tr>
</tbody>
</table>

**Table 2.1.** Compositions of the solutions used to produce the nerve-bouton preparation and the external solution used for electrophysiological recordings of spontaneous events.
2.2 Producing vibrodi ssociated cells

The nerve-bouton preparation, where mechanically-isolated neurons from brain slices retain functional afferent nerve-terminals, was produced by a process called vibrodissociation (Vorobjev, 1991). The apparatus used (Fig 2.1) consisted of a 2.5” 64 MΩ miniature loud speaker (Farnell) mounted on a course manipulator with the probe attached to loudspeaker cone. The probe is fashioned from a length of pipette glass (Harvard Apparatus) which has had one end pulled to a fine point which was fire polished to form a small glass bead. The movement of the glass bead was produced by supplying the loud speaker with a repeating rectangular pulse from a stimulator (S48, Grass). The voltage, frequency, and duration of the pulses produced by the stimulator were periodic adjusted to produce a good yield of viable Purkinje cells. The movement of the glass bead can be greatly affected by the parameters of the pulse generator as well as the length of the probe and the flexibility of the join to the loud speaker cone (most designs attempted featured a flexible section of tubing holding the probe at one end and a syringe plunger that had been glued to the loud speaker cone at the other but this is by no means a necessary feature). Estimates for the movement of the glass bead place the horizontal movement at 30-100 µm with a frequency of 80-130 Hz (Duguid et al., 2007). It has been advised that the best yield of cells is produced when the glass bead moves in a “figure-of-eight” (Yuri Pankratov (University of Warwick)) personal communication) although it is possible to get a good yield with an elliptical movement.
To vibrodissociate brain slices they are first transferred from the incubation chamber into a Petri dish containing ice-chilled vibrodissociation solution. The vibrodissociation solution is a magnesium-containing 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)-buffered aCSF consisting of 145 mM NaCl, 3 mM KCl, 10 mM HEPES, 15 mM glucose, 0.5 mM, CaCl₂, and 3 mM MgCl₂ with the pH adjusted to 7.35 with 1 M NaOH. Using a wide-bore Pasteur pipette the brain slice

**Figure 2.1.** An example of the set-up used for vibrodissociation. The set up consists of a dissecting microscope (foreground right) and a course manipulator (foreground left) with an attached 2.5” 64 MΩ miniature loud speaker connected to probe made from a glass tube drawn to a fine point with the tip fire polished to a small glass bead. The vibration that moves the probe comes from the loudspeaker supplied with a rectangular pulse generated from the S48 stimulator (background left). The dissecting microscope is illuminated with a light source (background right).
was transferred to a second Petri dish where it was carefully deposited so it is sitting in the centre of the dish in drop of chilled vibrodissociation solution. The brain slice is then held in place by being weighed down with a platinum “harp” which is a short cylinder of platinum formed into a U-shape with nylon threads attached. The vibrodissociation probe is placed just above the brain slice. When aiming to isolating Purkinje cells the probe is moved along cerebellar lobules at the junction of the molecular layer and the darker molecular layer. Viewing vibrodissociation under a microscope it is usual to see a fine material emanating from the brain slice which can appear like ripples of oil. After vibrodissociation the brain slice and platinum harp are removed and the isolated cells are left to settle for between 15 and 30 minutes.
2.3 Recording spontaneous events

After vibrodissociation, the cells were allowed to settle before they were perfused with external solution. External solution was perfused at a rate of ≈ 2 ml/min from a gravity driven perfusion system into a recording chamber with a volume of ≈ 1 ml. The external solution is a magnesium-free HEPES-buffered aCSF which consists of 145 mM NaCl, 3 mM KCl, 10 mM HEPES, 10 mM Glucose, and 2.5 mM CaCl\textsubscript{2} with the pH adjusted to 7.35 with 1 M NaOH. Spontaneous events were recorded from vibrodissociated Purkinje cells in the whole-cell configuration. Spontaneous events in this context refer to events which were spontaneously occurring and not induced by artificial electrical stimulation. This may include a mixture of events that are induced by action potentials as well as those that are not dependent on action potentials.

Vibrodissociated Purkinje cells were distinguished from the other cells on the basis of their relative large size and that they often retained a “stump” of their apical dendrite. Pipettes used to make recordings were pulled from thin-walled borosilicate glass (GC150TF, Harvard Apparatus) with a L/M-3P-A vertical pipette puller (List-Medical). The tips of the pipette were coated with Sylgard (Dow Corning) and were fire-polished at both ends in preparation for use in experiments. Pipettes had a resistance of between 2 to 5 M\(\Omega\) when filled with a CsCl based internal solution. The internal solution (Table 2.2.) consisted of 150 mM CsCl, 10 mM HEPES, frequently 5 mM \(N-(2,6\text{-dimethylphenylcarbamoylmethyl})\text{triethylammonium chloride (QX-314)},\) frequently 10 mM \(N,N,N,N\text{-Tetraethylammonium chloride (TEA)},\) 100 \(\mu\text{M 1,2-bis(2-aminophenoxy)ethane-}N,N,N',N'\text{-tetraacetic acid (BAPTA) or K}_4\text{BAPTA, 1.5 mM MgCl}_2, 2 \text{mM Na}_2\text{ATP, and 400 }\mu\text{M Na}_2\text{GTP with the pH adjusted to 7.3 with 1 M CsOH. Recordings were acquired with an EPC 7 amplifier (List-Medical), pClamp 8.}
software, and a 1320A Digidata digitiser (Axon Instruments, Molecular Devices, Inc) as well as an 8 pole low-pass Bessel filter that was built in-house. Events were recorded from the postsynaptic Purkinje cells with a gap-free protocol in the whole-cell configuration with cells voltage-clamped to a membrane potential of -80 mV. The records were sampled at 10 kHz and filtered at 2 kHz. Later recording were acquired with an additional filtering step at 10 kHz to reduce the noise of the baseline. This produced an effective filtering frequency of 1.96 kHz, where the reciprocal of the square of the effective filtering frequency equals the sum of the reciprocals of the squared individual filtering rates.
<table>
<thead>
<tr>
<th>Substance</th>
<th>Internal solution concentration</th>
</tr>
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<tbody>
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</tr>
<tr>
<td>HEPES</td>
<td>10 mM</td>
</tr>
<tr>
<td>QX-314</td>
<td>5 mM (when included)</td>
</tr>
<tr>
<td>TEA chloride</td>
<td>10 mM (when included)</td>
</tr>
<tr>
<td>BAPTA/K$_4$BAPTA</td>
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<td>MgCl$_2$</td>
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<tr>
<td>Na$_2$ATP</td>
<td>2 mM</td>
</tr>
<tr>
<td>Na$_2$GTP</td>
<td>0.4 mM</td>
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<td>pH</td>
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</tbody>
</table>

**Table 2.2.** Compositions of the internal solution used for electrophysiological recordings of spontaneous events.
2.4 Event detection

Events were initially detected from gap-free records using MiniAnalysis version 6.0.3 (Synaptosoft, Inc.). MiniAnalysis uses user defined parameters to detect events (Table 2.3). The program scans the current data for peaks according to a manually defined threshold. When the program finds the maximum extent of a peak it then goes backwards though the record to find when the peak started and from this calculates the time to peak. From the start of the peak an average of the baseline is taken before the peak started over the time specified to generate the amplitude of the peak. The time to decay is then calculated based the specified period to search for the decay and how long it takes to decay to the specified fraction of the peak amplitude. From the time to rise, time to decay, and the peak amplitude an area is calculated. Events are ultimately accepted or rejected based on if the area exceeds the user defined area threshold. Peaks can be detected during the decays of other events by using an extrapolated decay as the baseline. The records were checked twice by the user to ensure consistency and that no spurious events were included.

In addition to MiniAnalysis an in-house program was custom made to detect events (generously provided by G. W. J. Moss (UCL)). This program calculates the standard deviation of the baseline and will initially consider a change in the baseline a potential event if the height changes more than a specified multiple of the standard deviation of the baseline, typically anything in excess of five times the standard deviation, within a certain time frame. From these potential events the program detects the events peak and decay which are then checked manually.
<table>
<thead>
<tr>
<th>Detection Parameter</th>
<th>Formulae for suggested values</th>
<th>Suggested values based on sampling interval</th>
<th>Suggested values for detecting IPSCs</th>
<th>Values Used</th>
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<td>Period to reach local maximum (µs)</td>
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<tr>
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<tr>
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<td>0.5</td>
<td>0.37</td>
<td>0.5</td>
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<tr>
<td>Period to average baseline (µs)</td>
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<td>Area Threshold</td>
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<td>50</td>
<td>0.05</td>
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**Table 2.3.** Detection parameters used for MiniAnalysis version 6.0.3. Including the formulae for calculating the suggested values based on the sampling interval, the result of those calculations based on a sampling interval of 100 µs, values suggested by the program for the detection of IPSCs, and the values settled on to be used initially to detect events.
2.5 Data analysis

Once the events had been detected the times which they occurred were extracted and entered into a custom in-house program (courtesy of G. W. J. Moss (UCL)). From the times that the events occurred, the durations of the inter-event intervals were calculated and then the natural logarithm of the interval duration was taken. The logs of the interval durations were then automatically binned appropriately by the program and the square root of the count of each bin taken. In this form, the intervals can be plotted as a transformed interval distribution showing the time constants of different exponential components as time constants associated with different peaks whilst normalising the errors across the bins by taking the square root of the count (McManus et al., 1987; Sigworth & Sine, 1987). The binned data was then entered into OriginPro 8.1 (OriginLab Corporation) where they were attempted to be fitted with a double or a single exponential curve using the formula:

\[
\begin{align*}
    f_x(x) &= A_{fast} \tau_{fast}^{-1} \exp(x - \tau_{fast}^{-1} e^x) + A_{slow} \tau_{slow}^{-1} \exp(x - \tau_{slow}^{-1} e^x) \\
    \text{Where the tau values refer to the time constants derived from the peaks of the interval distribution and ‘A’ refers to the area associated with those peaks. Attempts to fit the distributions with double exponentials curves were always made first. If a double exponential curve was inappropriate the distributions were attempted to be fitted with a single exponential curve. When only one peak is present it tends to corresponds to the slower peak. Occasionally it was not possible to fit the data with a suitable curve. To compared a distribution with two time constants with a distribution with one or to get a time constant that corresponds to the overall frequency a weighted tau value was generated by combining the existing time constants proportionally:}
\end{align*}
\]
Equation 2.2

\[ \tau_{weighted} = \frac{A_{fast}\tau_{fast} + A_{slow}\tau_{slow}}{(A_{fast} + A_{slow})} \]

The area fraction \((A_f)\) of the total distribution that is represented by \(A_{fast}\) can also be an useful measurement and calculated thus:

Equation 2.3

\[ A_f = \frac{A_{fast}}{(A_{fast} + A_{slow})} \]

Significant differences in fitted time constants were taken to have occurred where the range of data that encompasses the time constant values plus and minus three times their standard deviations are distinct from each other.

No assumptions were made about the distributions of the amplitudes of the events. A non-parametric test for multiple groups was used to find significant differences among the populations of amplitudes. For this purpose the Kruskal-Wallis test was used followed by the Dunn-Holland-Wolfe test to find which groups are significantly different from each other if a significant difference was found. Occasionally the same non-parametric tests would be applied to the durations of intervals especially if there were a low number of events and curve fitting was difficult. Non-parametric tests were done with IgorPro 6.2.0.0. (WaveMetrics, Inc) and the threshold for significance is set at \(P < 0.05\).
2.6 Materials

NaCl, KCl, NaHCO₃, NaH₂PO₄, glucose, 1 molar CaCl₂ solution, MgCl₂.6H₂O, NaOH, and CdCl₂ were from BDH. HEPES was supplied by Calbiochem. CsCl was either from BDH or Calbiochem. N-methyl-D-aspartate (NMDA), glycine, QX-314, linopirdine, and tetrodotoxin (TTX) were from Tocris. TEA, 4-aminopyridine (4-AP), BAPTA, Na₂ATP, Na₂GTP, glibenclamide, 6,7-dinitroquinoxaline-2,3-dione (DNQX), and (+)-bicuculline were supplied from Sigma-Aldrich. CsOH was obtained from Aldrich. K₃BAPTA was provided from Invitrogen. 8,14-diaza-1,7(1,4)-diquinolinacyclotetradecaphanedium di-trifluoroacetate (UCL-1848) was synthesised in the Department of Chemistry at UCL.
Chapter 3: Characterisation of the nerve-bouton preparation

The experiments presented in this thesis makes extensive use of the nerve-bouton preparation of rat Purkinje cells. In this chapter some basic characteristics of the preparation are explored.

3.1 Introduction to the Purkinje cell nerve-bouton preparation

The Purkinje cell nerve-bouton preparation is produced by mechanically isolating Purkinje cells from a cerebellar brain slice (Vorobjev, 1991). Previous studies report attached and functional nerve-boutons are retained by the postsynaptic cell (Ye et al., 2004). This means bouton function can be inferred by the properties of events recorded in the postsynaptic cell. Thus, with care, the postsynaptic cell can acts like a passive antenna to monitor neurotransmitter release (a sort of cell-sized “sniffer” patch (Allen, 1997)) recording responses from multiple different types of afferent nerve-bouton. One advantages of studying synapses with the nerve-bouton preparation is that neurotransmitter release can be studied by investigating the effects on in situ ligand gated ion channels while the nerve terminals are isolated from the rest of the presynaptic cell or any influences from surrounding cells such as other neurons or glial cells.
3.2 Identification of Purkinje cells

Purkinje cells can be readily located in a cerebellar slice. Purkinje cells are present in all lobules of the cerebellar cortex, the cell and synapse-rich outer proportion of the cerebellum. However, they are restricted to the single-cell thick Purkinje cell layer that lies between the outer molecular cell layer and the inner granule cell layer. Purkinje cells in slices are characterised by an extensive dendritic tree which they project into the molecular layer (Fig 3.1a). Purkinje cells can also be easily distinguished from most other cells of the cerebellar cortex (such as the numerous and densely packed granule cells) as they have somas of around 20 to 25 µm in diameter, and are thus comparatively large.

Once isolated by vibrodissociation it is possible to successfully identify individual Purkinje cell nerve-bouton preparations. In the nerve-bouton preparation, cells are mechanically isolated and have little or no dendritic tree remaining so Purkinje cells, like all other cells, can no longer be identified by either their relative positions or their dendritic structure. However, Purkinje cells have the advantage that they are still relatively easily identifiable on the basis of their size. Thus Purkinje cells in the nerve-bouton preparation appear to have a large isolated ovoid soma that lacks an axon and at most only a very short initial section of the dendritic tree; there is an absence of any observable dendritic branches (Fig 3.1b). Although there are no afferent terminals discernable when these cells are viewed using a bright field microscope. Purkinje cells do retain active nerve-terminals from formerly presynaptic cells. Furthermore, the continued activity of these nerve-boutons can be inferred by their ability to endocytose N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styril)pyridinium dibromide (FM 1-43 dye) which increases in fluorescence when retained by cell membranes (Fig 3.1c).
Figure 3.1. Locating and identifying Purkinje cells. a) A photograph of a parasagittal cerebellar slice from a P10 rat (typical of the type used throughout the reported experiments). Purkinje cells are the large ovoid cells in a single layer (Purkinje cell layer - PCL) between the darker (lower) cell-dense granule cell layer (GCL) and the lighter (upper) cell-sparse molecular layer (ML). This photograph was taken with the assistance of D. P. Bright (UCL) and the slice was viewed with differential interference contrast microscopy. b) Once isolated the Purkinje cells, seen here using a standard bright field microscope, can still be identified by their size relative to other cells. In this example a small section of retained dendrite is just visible, protruding from the cell towards the top of the image. c) Although isolated, the continued functionality of the nerve-boutons can be inferred by their ability to endocytose and retain the fluorescent dye FM 1-43. Cells prepared by the author. FM staining carried out and image taken by M. Caldwell (UCL).
3.3 The properties of synaptic events in the Purkinje cell nerve-bouton preparation

Voltage-clamped whole-cell recordings from the Purkinje cell nerve-bouton preparation revealed a relatively flat baseline punctuated by spontaneously occurring events of postsynaptic current. Under typical recording conditions when the Purkinje cells were voltage-clamped at -80 mV all of the spontaneously occurring events produce inward currents, albeit with variable amplitudes, with a caesium chloride based internal solution. Indeed the amplitude of these postsynaptic currents range widely from tens of picoamperes to several nanoamperes (Fig 3.2). The ranges of amplitudes were variable from cell to cell, although the smaller amplitude events tended to be more frequent.
The events have rapid onsets and are followed by a slower gradual decay to the baseline (Fig 3.3a). The time course was quite distinctive of the events. It aided detection of events, even if some of the smallest events were only a couple of times greater than the fluctuations in the baseline (Fig 3.3b). However, although distinctive, the duration of events was another variable characteristic. Some events decay markedly faster to the baseline (Fig 3.2c) than others although the decay always appears slower than the rise.

Some events showed complicated decay characteristics. The most striking were the large slow events (LSEs). These events frequently had amplitudes of 100s of pA,
which have a rapid onset and are followed by a decay that appears to have at least three distinct components (Fig 3.3d). The first component has a fast decay much like the ordinary faster postsynaptic current events. The second component appears as a near rectangular inward current. When the second component rapidly drops off, it reveals the third component which has smaller amplitude and returns slowly to the baseline over several seconds. Unfortunately, these events were too rare to study in detail (see discussion).
Figure 3.3. The decays of events are distinctive and variable. a) An example of an event. The amplitude was 275 pA and it took 10.6 ms to decay to half the amplitude. b) An example of a small event. Here the amplitude was 20 pA and it decayed to half its amplitude in 8.1 ms. c) An example of a fast event where the amplitude was 286 pA and decay to half its amplitude took a comparably fast 4.3 ms. d) An example of a large slow event (LSE). In this example the amplitude was 1.237 nA. As the decay had several components, a figure for the decay to reach half the amplitude would not meaningfully represent the longevity of LSEs. Here it takes 5.6 s for the current to return to baseline.
The frequency of events recorded from Purkinje cell nerve-bouton preparations varied widely. Some Purkinje cells displayed a high frequency of events (Fig 3.4a), while other cells, under the same conditions, showed much fewer events (Fig 3.4b). Occasionally recordings from Purkinje cells display very few or even no events. The low levels of spontaneous events meant they could not be used in the electrophysiological experiments and were often discarded, especially for experiments where the frequency of events is expected to be reduced (for example by blocking voltage-gated calcium channels). Cells that had a low frequency of spontaneous events were often cells that were unhealthy. Indicators of this included a low cell resistance and an unstable baseline.

The variability of the event frequency may vary with the number and health of boutons retained. This should not be surprising as a number of boutons that can be visualised with FM staining do vary among nerve-bouton preparations. The number and type of boutons retained in the nerve-bouton preparation may also vary further depending on how much of the dendritic tree remains. This last feature is likely to be especially important for excitatory inputs coming from parallel fibres which are predominantly located distally to the cell body.
Figure 3.4. The frequencies of events are subject to cell-to-cell variability. a) An example of a cell with a high frequency of events. In this example there are 2530 events occurring during a 5 minute recording under control conditions, which is the equivalent of a frequency of 7.83 Hz. The median interval is 51 ms and the interquartile length is 131.2 ms. A large number of small events contribute to the high frequency. b) An example of a cell with a low frequency of events. There are 95 events occurring during a 5 minute recording. This is the equivalent of a frequency of 0.32 Hz. The median interval is 1997.7 ms and the interquartile range is 4448.375 ms.
To study event frequency in greater depth the distributions of the inter event intervals were examined. Some studies report that the distribution of intervals between events can be described by an exponential decay and the time constant of the decay can be used as measure of event frequency (Auger et al., 1998; Llano et al., 2000). A single exponential decay distribution would be expected to be the case when each bouton has a constant release probability per unit time, even if release varies from synapse to synapse. Intervals between events recorded from the Purkinje cell nerve-bouton preparation grouped around the shorter interval lengths with a steady decline in the number of intervals as the duration increased. Indeed a curve described by a single exponential decay often produced with a $R^2$ value close to 1 (Fig 3.5a). However the curves often did not fit equally among the data points suggesting there was a deviation from a distribution described by a single exponential decay. This was caused by the bins representing the shorter intervals having a higher count than would be expected if the data did follow a single exponential decay. In turn, this suggested that the data would be better described by a bi-exponential decay (Fig 3.5b) and this seems to be the case based on the curve being better distributed above and below the data points as well as having smaller residual differences (Fig 3.5c).
Figure 3.5. The range of inter-event intervals can be described by an exponential function. a) The intervals from the same experiment as described in Fig 3.2a can be fitted with a single exponential decay but it is poorly distributed among the data. The time constant is 392.8 ms. b) A bi-exponential decay will produce a better fit. Here the time constants are 99 and 729 ms with the components respectively representing 83 % and 17 % of the curve area. c) The better fit by a bi-exponential decay compared to a single exponential decay can be seen by the reduced residuals that are distributed more evenly on either side of the curve.
When a curve is fitted to data best described by two exponential decays the time constants of the exponential decay can often be an order of magnitude different. This can create problems in displaying, binning and fitting the data. However this problem is frequently encountered when studying single ion channel kinetics, where approaches have been developed to transform the data to aid handling (McManus et al., 1987; Sigworth & Sine, 1987). To aid data handling the x-axis was transformed by taking the log of the interval durations, which allows multiple time constants possibly spanning several orders of magnitude to be handled with relative ease. Furthermore, the square root the count displayed on the y-axis was taken to keep errors approximately constant in all bins (Sigworth & Sine, 1987). Fitting several exponential decays to an untransformed distribution might have used the following equation:

\[ f(t) = \sum a_i \exp \left( -\frac{t}{\tau_i} \right) \]

In this equation the area of any component has a corresponding time constant. But if the distribution is transformed to aid fitting a probability function \( f_x(x) \) is generated, where \( x \) equals the natural log of time, as defined by this equation:

\[ f_x(x) = \sum a_i \tau_i^{-1} \exp \left( x - \tau_i^{-1} e^x \right) \]

In this form the peaks of the distribution correspond to values for time constants. This method of fitting worked quite well and often produced two clear peaks representing the different time constants (Fig 3.6a). One component appears to represent brief intervals while the other slower longer intervals. The time constants that correspond to these peaks were called \( \tau_{\text{fast}} \) and \( \tau_{\text{slow}} \) respectively.
However, adequately fitting the curves to the data requires enough intervals to reveal the underlying distribution. Occasionally there were too few intervals to fit any meaningful curve to the available data. At such times a curve described by a single exponential decay produced the best fit. This may have been because there were too few intervals to show both components, because the two time constants were very similar, or because a second component was absent. When there was only one time constant it was often most similar to the slower of the two time constants in a bi-exponential fit. The aid a comparison between distributions with one and two components a weighted time constant was calculated for the bi-exponential distributions (Fig 3.6b). Generating a tau weighted value may aid comparing different frequencies as it produces a single time constant that refers to the overall frequency.
Figure 3.6. The transformed interval distribution can be described by two exponential components. a) Transforming the interval data by taking the log of the intervals and the square root of the count produces a distribution where the peaks correspond to the time constants. The peaks correspond to time constants of 19.1 and 666.4 ms. These values are within 1 error of those time constants derived from fitting an exponential decay to the untransformed interval distribution, but themselves have smaller errors. The fraction of the faster peak of the entire distribution, \( A_f \), is 0.22. b) For reasons of comparison it is often necessary to combine the two time constants proportionally to get one time constant called tau weighted. Here the equivalent peak of tau weighted corresponding to a weighted time constant of 522.8 ms is imposed onto the distribution as indicated by the dashed green line.
The different time constants are unlikely to be the result of a consistent rate of neurotransmitter release. Even if multiple nerve-boutons had different but constant rates of neurotransmitter release an interval distribution would form over time that could be described by one time constant. The fast and slow time constants suggest there are different rates of neurotransmitter release. For a brief period after an event there appears to be an increased probability of another event occurring. This could result in a rapid succession or a burst of events. Bursts of events have been described in the literature (Auger & Marty, 1997; Auger et al., 1998) as well seen in experiments with the nerve-bouton preparation (Fig 3.7). These burst will terminate eventually. If the faster of the time constants represented bursting it could be quite interesting. Bursts of events are thought to occur from neurotransmitter release from one site (Auger et al., 1998) so it would be a way to infer the behaviour of individual terminals whereas tau slow would refer to the overall behaviour of all active inputs.
Figure 3.7. Events can occur in short bursts with brief intervals. a) Events frequently appear as just one isolated event. b) Occasionally there are bursts of events where an unusually high number of events occur in a short period of time. The faster time constant and its peak may correspond to the intervals between bursts of events and the prevalence of bursting behaviour.
3.4 Release properties of presynaptic boutons.

To investigate the release properties of the afferent nerve terminals the presynaptic voltage-gated calcium channels and the voltage-gated sodium channels were blocked. There was concern about the yield of viable cells per dish as well as the success rate of making good recordings from those cells. Also there was a high turnover of dishes as a fresh dish of cells was used after each record where drugs were applied so that prior drug application to the bath solution would not bias any future recordings made other cells, even if occasionally several different experiments and drug applications are carried out on the same record. For these reasons several drug applications were attempted on the same cell by blocking in turn voltage-gated calcium channels then allowing the cell to return to basal conditions before blocking the voltage-gated sodium channels. To block a range of voltage-gated calcium subtypes 100 µM cadmium was used and the block the voltage-gated sodium channels was induced by applying 500 nM tetrodotoxin (TTX) (Fig 3.8). Application of Cd\(^{2+}\) and TTX both appeared to reduce the frequency and amplitude of events.
Cadmium was bath applied for either one or two minutes to block the presynaptic voltage-gated calcium channels after a five minute control period. Blocking presynaptic voltage-gated calcium channels with cadmium tended to increase the value of the fitted time constants. Looking at the common means of the fitted data indicates that only during the washout of cadmium is there a significant increase in the tau weighted (Fig 3.9). In individual experiments, there was a trend for the tau weighted during cadmium application to be greater than during control conditions and for the tau weighted to then partially return to control levels when cadmium was washed out. This trend occurred in four experiments out of five, but only in two experiments were the increases significant. Although in both cases a significant increase in tau weighted, representing an overall decrease in event frequency, persisted during the wash-out of cadmium. Significance changes when cadmium is applied may be occluded by the large

Figure 3.8. Cadmium and TTX appears to reduce the frequency and amplitude of events. A trace from an experiment when voltage-gated calcium channels were blocked with 100 μM cadmium then had 500 nM TTX applied to block voltage-gated sodium channels.
errors associated with less accurate curve fitting that comes from fitting fewer intervals. Significant changes in tau weighted are underlined by changes in tau slow. Although there are examples where cadmium affects bursting where either there is a significant increase in tau fast or an absence of a faster peak in the interval distribution during either the brief application of cadmium or its wash-out.

Figure 3.9. Cadmium application causes increases in tau weighted. Applying 100 µM cadmium to block presynaptic voltage-gated calcium channels causes an increase in tau weighted indicating an overall decrease in event frequency. However, in the common mean (n = 5) there is only a significant increase when cadmium is washed out even though it is described by a lower time constant than during cadmium application.

As large errors associated with poorly fitted time constants may be occluding observation of a significant effect another analysis method was sought. During
cadmium application the intervals appeared to have increased and this is confirmed in four out of five experiments when looking for differences in the population intervals with a non-parametric test (Fig 3.10a). When cadmium was washed out, half of the records that had significantly longer intervals returned to being indistinguishable from the control. Cadmium application also caused a significant decrease in the amplitude of events that persisted beyond its application (Fig 3.10b). There was a significant decrease in the population of amplitudes in two experiments in five during cadmium application and three experiments it five when cadmium was being washed out. There were no incidences of cadmium significantly increasing amplitudes. Unlike the quickly reversible effects of cadmium on the intervals, the reduction in amplitude persists and can become more pronounced with time.
Figure 3.10. Adding cadmium increases the event intervals and decreases the amplitude persistently. a) Cadmium application is able to significantly increase intervals durations. b) There is also a reduction in amplitudes which persists after the removal of cadmium. The data for both plots are from one record and presented as a box plot where the edges of the box represent the interquartile range, the line through the box represents the median and the whiskers represent 10-90 % of the data range. An asterisk indicates a significant difference from control (P < 0.05).
The cadmium was allowed to washout for between two and three minutes, when the events were gauged to have returned to behaving as under control condition 500 nM TTX was bath applied to block the presynaptic voltage-gated sodium channels (Fig 3.8). TTX application appeared to reduce the frequency of events. In one experiment there was a reversible lack of events during TTX application. There is a trend for tau weighted to be larger during TTX application than during the preceding period, which was when cadmium was being washed out, however there are no incidences when the time constants are significantly different. Fitting two time constants to the distribution of intervals during TTX application was difficult. On only one occasion out of five experiments was it possible to fit two time constants during TTX application. As a comparison only once was it not possible to adequately fit two time constants during cadmium application. The absence of a fast time constant possibly indicates blocking voltage-gated sodium channels in nerve-boutons attenuates bursting behaviour.

Intervals are longer during TTX application and its washout (Fig 3.11a). Out of five experiments four cells have a population of intervals significantly longer when compared with when cadmium was being washed out, which was the preceding section of the record. This effect is persistent and continues in three out of five cells when TTX is washed out. There were no incidents of the duration of event intervals being significantly smaller duration application or its washout. During the experiments on TTX application, event amplitude increased as time proceeded. During TTX application there was one record that had a significant increase in amplitude compared to the cadmium wash-out period and when it became time to wash-out TTX there were two records with significantly increased amplitudes.

Although efforts were taken to wait until the cadmium had washed out and the cell returned to control behaviour, it was a concern that the previous cadmium treatment
might have biased the results. To explore this possibility the TTX results were compared with the true control period before cadmium was applied. Changes in the time constants were also not significant compared to the initial control period as with the cadmium washout. When comparing the intervals with a non-parametric test a similar proportion of cells display a significant lengthening of interval duration occurring during TTX application and TTX washout in four cells out of five (intervals from all treatment during the records were tested for significant and the significant differences were detected with the Dunn-Holland-Wolfe test which takes in to account multiple comparisons). Although similar proportions of cells have significantly longer interval durations there is one or two cases of different cells being significant depending which control period was used. By having two separate experiments during one recording some bias seems to have been introduced with regard to amplitudes. When compared with the initial control period no cells have significantly larger amplitudes. It appears there are cases that TTX application and TTX washout can display significantly larger amplitudes of events when compared with a period recovering from cadmium application but not when compared with the control. The most likely explanation is the cells were still recovering from the effects of cadmium reducing amplitude, although not the effects on intervals. This does not represent an action of TTX in itself, but the length of time the events take to recover from the effects of cadmium. Supporting this explanation that it is the length of time from when cadmium was added and not what drug were later applied is that the proportion of cells with significantly larger amplitudes increased with time as there is a higher proportion during TTX washout than TTX application. The persistent effects of cadmium on event amplitude but not frequency may be similar to the effect of severely reducing extracellular calcium on IPSCs as both treatments will impair the normal function of voltage-gated calcium
channels and the resulting intracellular calcium concentration of the nerve terminal (Llano et al., 2000).

Even though the amplitudes were not significantly different compared with the control, the relative frequency of the amplitudes during TTX application suggests there may have been an effect. If the relative frequencies of amplitudes during TTX application and during TTX washout are subtracted from the control it appears that during TTX application there is a reduction in the smaller events but an increased proportion of events in the adjacent bins (Fig 3.11b). This observation stands if comparing TTX application to the initial control or the period when cadmium was allowed to washout. Although when cadmium was being washed out there is an even greater proportion of small events, as indicated by the greater change in relative frequency towards the control, reflecting cadmium’s persistence effect decreasing the event amplitude. TTX application appears to increase the proportion of smalls event but decrease the proportion of the very smallest events. The increased propensity of small events would be expected as TTX treatment reduced spontaneous IPSCs to mini IPSCs (mIPSCs) which have smaller amplitudes (Southan & Robertson, 1998a). The smallest events are severely attenuated by TTX. These events may reflect events that occur as a burst or during the decay of another event and given there is less bursting behaviour this would suggest TTX application might abolish these types of events all together.
Figure 3.11. TTX application affects the frequency and amplitudes of events. a) Intervals are significantly longer during application of 500 nM TTX which persists during its washout. A box plot of intervals where the top and bottom of the box represent the interquartile range, the line through the box corresponds to the median, and the whiskers represent the 10 % to 90 % data range. Significant increases in interval duration compared to the control are indicated by an asterisk (P < 0.05). b) During application of TTX and its washout the relative frequency of events between 100 and 200 pA increases but there is a marked reduction in the relative frequency of events below 100 pA. The reduction of small events is more pronounced when compared with the preceding period of time when cadmium was being washed out as indicated by a larger positive change towards the control values.
3.5 The role of presynaptic potassium channels in neurotransmission

It is clear that a proportion of neurotransmitter release from the nerve-bouton in our Purkinje cell preparation depends on functional voltage-gated calcium channels and voltage-gated sodium channels. As voltage-gated processes play an important role in regulating neurotransmitter release from the nerve-boutons it was deemed likely there would be mechanism to repolarise and maintain an hyperpolarised membrane potential most likely regulated by potassium channels.

To investigate the role of presynaptic potassium channels, the preparations were treated with increasing concentration of 4-aminopyridine (4-AP) in the expectation they would be blocked. A 4-AP sensitive current has been reported in parallel fibres (Daniel & Crepel, 2001) and 4-AP has also been reported to block a current that can be evoked from basket cell terminals (Southan & Robertson, 1998b; Southan & Robertson, 2000) and increase the frequency of IPSCs (Tan & Llano, 1999). After a control period to establish the basal properties of the preparations they were treated with 200 µM, 500 µm, and 1 mM 4-AP each applied for one minute each. The lowest dose of 4-AP used, 200 µM, rapidly produced a clear increase in the frequency of events (Fig 3.12a).

Strangely for such a marked increase in event frequency the common mean for tau weighted exhibited no reductions that were considered significant, that is out with three times of their respective errors compared with the control at any concentration of 4-AP tested. The large errors are likely to be an artefact of combining weighted time constants representing a wide range of basal frequencies as when considering the individual records there are significant reductions in tau weighted occurring at least in four records out of five at every concentrations tested. These reductions in tau weighted
are because of significant reductions in tau slow, a trend that does show up in the common mean (Fig 3.12b) and is replicated in four out of five records at all the concentrations tested. No significant reductions were seen in the individual records for tau fast. The 4-AP induced increases in frequency, as described by significant reductions in tau weighted and tau slow, persisted during the wash out period. Occasionally, significant increases in the fraction of the interval distribution represented by the faster curve, A_f, occurs during 4-AP application. A_f is thought to correspond to the proportion of bursting behaviour in a record.

Although the effects of 4-AP are quite consistent the effects on event amplitude were more variable. When 4-AP causes a significant increase the event amplitudes, it increases the relative frequency of larger events at the expense of smaller events. In one example (Fig 3.12c) the smallest events are relatively unchanged whereas events between the smallest events and 300 pA decrease concurrently with an increase in events with amplitudes between 300 pA and 1 nA. It might be the case the 4-AP is able to affect the events that are slightly larger than the very smallest events and enhancing them into even larger amplitude events.
All concentrations tested causes significant increases in event amplitudes in at least two out of five preparations. However the first and lower concentration of 200 µM...
4-AP was able to cause a significant increase in the amplitude of events in four out of five cells, but only half of the preparations could sustain this significant increase during subsequent treatments with higher concentrations. This is unlikely to be a run down as the amplitudes do not become significantly smaller than the control with time, instead they appear to cease being significantly greater than control. There is possibly nothing special about the concentration of 200 µM 4-AP, it could just be the first treatment to cause a significant increase in event amplitudes which cannot be sustained for more than a couple of minutes. Looking at a preparation which has had 1 minute treatments with 50 and 100 µM 4-AP, before the tried and tested concentration of 200 µM and greater were added, shows a significant increase in amplitude can occurs at the earlier lower doses but is not sustained. Although being significantly greater than control also ceases at concentrations above 200 µM 4-AP it is clear the increases in the population of event amplitudes has lessened during the later treatments (Fig 3.13a).

The tried and tested concentrations of 200 µM 4-AP and greater all had an effect on increasing neurotransmitter release. To try a gauge how effective 4-AP is at enhancing neurotransmitter release the minimum concentration required to cause a significant increase in event frequency was sought. Examining the results from testing one preparation with 5, 10, 20, and 50 µM 4-AP shows that the lowest concentration a significant reduction in tau weighted occurs is when 50 µM 4-AP is applied (Fig 3.13b). In keeping with the idea that the earlier effective concentrations of 4-AP can cause an increase in event amplitude which cannot always be sustained during the later concentrations, 20 µM 4-AP causes a significant increase in event amplitude whereas the later treatment of 50 µM 4-AP does not.
Figure 3.13. Lower concentrations of 4-AP can also increase the frequency and amplitude of events. a) A significant increase in amplitudes occurs with effective doses of 4-AP but this often is not sustainable as time goes on. The data (n = 1) is presented as a box plot where the edges of the box represent the interquartile range, the line through the box represents the median and the whiskers represents the data range from 10-90 %. An asterisk indicates a significant difference from control (P < 0.05). b) From this example (n = 1) 50 µM 4-AP is the lowest concentration that causes a significant reduction in tau weighted. Significance is indicated with a star. If a time constant is out with three times the respective errors from the control the difference is considered significant.
The effects of tetraethylammonium (TEA), another potassium channel blocker, on neurotransmitter release was investigated. TEA application has been reported to increase the frequency of IPSCs from molecular layer interneurons afferent to Purkinje cells (Tan & Llano, 1999) and TEA sensitive currents can be evoked in cerebellar interneuron terminals (Southan & Robertson, 2000). Cells were recorded under control conditions for five minutes to establish the basal release properties. Three treatments of TEA, 200 µM, 500 µM, and 1 mM, were bath applied in order of increasing concentration to the preparations for one minute at each concentration.

TEA application up to 1 mM was found to have little effect on either the frequency or amplitude of postsynaptic events (Fig 3.14a). As it was expected for TEA to cause an increase in the frequency of events, tau weighted values were examined for significant reductions compared to control. Occasionally a significant decrease would be observed but they were rarely reproduced, sustained, or dependent on the concentration of TEA. Examining the common means for tau weighted (Fig 3.14b) as well as tau fast and tau slow shows no significant changes during the course of TEA application. TEA application also did not change the amplitudes of events.

Nerve-bouton preparations were also treated with a range of other potassium channel blockers. However there was no apparent effect when any of the blockers were applied individually. The other blockers applied were linopirdine to block the muscarinic-sensitive current (M-current) (Aiken et al., 1995), glibenclamide to block ATP-sensitive potassium (K<sub>ATP</sub>) channels (Inagaki et al., 1996;Gribble et al., 1997), and UCL-1848 to block small conductance calcium-activated potassium (SK) channels (Hosseini et al., 2001) (data not shown). This suggests these channels are not involved in neurotransmitter release in the Purkinje cell nerve-bouton preparation under control conditions.
Figure 3.14. TEA had no obvious effect on the frequency of events. a) An example of a trace where application of TEA had no obvious effect on the frequency or amplitude of events up to a concentration of 1 mM. b) Although significant differences in tau weighted were very occasionally observed there is no consistent effect of TEA. There are no significant differences in the common mean tau weighted when TEA is applied (n = 7 for control, 0.2 mM, and 0.5 mM, n = 5 for 1.0 mM, n = 2 for wash out).
3.6 Different types of event

Purkinje cells were expected to display currents caused by the opening of both anion channels, caused by GABA activating GABA\textsubscript{A} receptors, and cation channels opening in response to glutamate gating AMPA receptors. It has been determined experimentally that the cation conductances that mediate the excitatory inputs in Purkinje cells reverse at 0 mV when a caesium based internal solution is used (Perkel \textit{et al.}, 1990). The reversal potential for anion currents was calculated to be +2.24 mV based on the concentration of chloride ions. As the external solution was buffered by HEPES the concentration of bicarbonate ions did not need to be taken into account. Despite the expected reversal potentials being close together attempts were made to establish after an experiment if there was more than one type of conductance by holding the cell at around the expected reversal potentials to see if the events split into inward and outward currents. Holding the cell around the reversal potential of the conductances would diminish their driving force so any currents induced would be quite small. However, despite the reduced driving force, the possible differences in frequencies between inhibitory and excitatory events, and the small difference in reversal potentials it was possible to occasionally see the events split into inward and outward currents (Fig 3.15a). As all events reversed eventually when the cell was suitably depolarised the splitting of events was unlikely to be due to errors in voltage-clamping. Simultaneously displaying inward and outward currents at one holding potential suggests that Purkinje cell nerve-bouton preparations displays at least two types of spontaneous events. The identities of the two types of events were confirmed by being able to block all events with the appropriate antagonist. Applying 20 µM DNQX to block AMPA receptors and 50 µM (+)-bicuculline to block GABA\textsubscript{A} receptors repeatedly inhibited all the
spontaneously occurring events (Fig 3.15b) confirming the identity of the receptors involved.
Figure 3.15. Purkinje cell nerve-bouton preparations spontaneously displayed two types of events. a) Sufficient depolarisation beyond $\approx 0$ mV from the holding potential of -80 mV will cause all the events to become outwards currents. However at a narrow range of potentials the events occasionally split into inward and outward currents suggesting two populations of events. The holding potential in this example is +2 mV; events are indicated by an asterisk. b) All spontaneously occurring events are reliably blocked by a mixture of 20 µM DNQX and 50 µM (+)-bicuculline suggesting the events are mediated by AMPA and GABA$_A$ receptors. Frequently spontaneous GABAergic events dominate such as in this example where the AMPA antagonist, DNQX, does little to affect the apparent frequency of events.
The majority of events appeared to be GABAergic IPSCs. When trying to isolate the EPSCs by applying (+)-bicuculline often all or most of the spontaneously occurring events were blocked. This is to be expected as inhibitory inputs tend to somatically located and therefore better placed to be retained after vibrodissociation compared with the many of the more distally located excitatory inputs. Whereas IPSCs could be readily isolated, only about one preparation in two or three had a sufficiently high enough frequency of EPSCs to use in experiments. Looking at individual experiments where IPSCs and EPSCs were isolated by applying appropriate antagonists shows that they have different properties, which raises the possibility that the two different types of events could be distinguished by their properties when both are present. When EPSCs can be isolated they tend to have a slightly higher frequency than IPSCs as indicated by shorter interval duration (Fig 3.16a) although the ranges did over lap. Looking at the intervals of pharmacologically isolated IPSCs over 5 cells gives a mean interval duration +/- the standard error of 1315.8 +/- 91.02 ms (n = 1096) compared to pharmacologically isolated EPSCs from 4 cells with a mean interval of 612.9 +/- 28.88 ms (n = 1941). However the trend for a higher frequency of EPSCs could be due to a possible selection bias as experiments looking at EPSCs were selected for having a reasonable frequency before experiments began. The amplitudes (Fig 3.16b) and the decay times (Fig 3.16c) were strikingly different as the range of values for EPSCs were much smaller and had faster decays than the ranges of values for IPSCs. The mean amplitude for the isolated IPSCs from the same cells was 486.8 +/- 17.48 pA (n = 1101) whereas for the EPSCs the mean amplitude was smaller at 31.0 +/- 0.79 pA (n = 1945). The time to decay to half the amplitude of the event amplitude is a figure generated by MiniAnalysis which represents how long did it take for the event to reduce to half its own size and should not be mistaken for a fitted time constant. From the same cells the
mean +/- standard error of the time to decay to half the event amplitude was 7.70 +/- 0.166 ms (n = 1101) for IPSCs and 1.76 +/- 0.038 ms (n = 1945) for EPSCs. Although for both amplitudes and time to decay values the data range for EPSCs did overlap with the lower part of the ranges for IPSCs. It was not thought possible to unambiguously distinguish between an EPSC and an IPSC based on the properties alone during control conditions let along during future experimental treatments. Probably the easiest, quickest, and most robust way to distinguish between the two different types of events, whilst ensure both inputs produce inward currents at the holding potential, is to apply an appropriate antagonist to block the other type of input.
Figure 3.16. Inhibitory and excitatory events have different but not distinct properties. The data is presented are individual cells, when the inhibitory (n = 5) or excitatory (n = 4) events are isolated, as a box plot where the edges of the box represent the interquartile range, the line through the box represents the median and the whiskers represents 10-90 % of the data range. a) Excitatory events tended to occur at a higher frequency than inhibitory events as indicated by the shorter interval durations, but there were considerable overlap between the distributions of inhibitory and excitatory intervals. b) Excitatory events tended to have smaller event amplitudes than excitatory events under control conditions. c) The times taken for events to decay to half of their amplitude tended to be shorter for excitatory events than inhibitory events. Although the differences in amplitude and decay time between inhibitory and excitatory events were striking they are not distinct properties and thus cannot be used to distinguish the two types of events.
3.7 Conclusions

Nerve-bouton preparations of Purkinje cells have been found to be readily identifiable from their size and retain functional nerve-boutons despite the postsynaptic cell and the boutons being mechanically isolated from the surrounding tissue. The functionally of their remaining adherent nerve-boutons can be inferred by their ability to endocytose and retain FM dyes in addition to the brief events of inward currents that spontaneously occur in the Purkinje cells. These events often appear similar in form, frequency, and amplitude to ligand-gated ion channels being activated by synaptically released neurotransmitters. The events varied in event amplitude, decay times, and duration of inter-event intervals. The properties of events and how they changes with various treatments can be used to infer properties of the presynaptic boutons. When using nerve-bouton preparations there is no requirement to isolate the nerve terminals from being influenced by action potentials emanating from the somatodendritic compartment of the presynaptic cell as the terminals have already been mechanically isolated. Recording spontaneous events as opposed to mini events can mean recording larger more frequent events; certainly this is the case for IPSCs (Southan & Robertson, 1998a), but it can also mean bursting behaviour. The distribution of intervals can be described by a bi-exponential decay where the time constants of decays can be used as a measure of event frequency. However it was found data handling is improved by transforming the data so that two peaks were frequently seen corresponding to time constants (McManus et al., 1987; Sigworth & Sine, 1987). These time constants were within one error of the time constants fitted to untransformed data but were themselves were more precise. The faster of the two time constants are thought to represent bursting behaviour and are a feature of spontaneous events. Bursts of events suggest the spontaneously occurring events are more than just mini events as intervals between
minis can be adequately described by an exponential decay with one component unlike when bursting occurs were two components are often required (Llano et al., 2000).

Blocking voltage-gated calcium channels with 100 µM cadmium results in a decrease in the frequency of events although did not abolish them completely. This suggests voltage-gated calcium channels actively facilitate the frequency of neurotransmitter release under control conditions. Whereas the effects of cadmium on frequency were partially reversed the effects on reducing amplitude were more persistent. The reason for the long lasting effect on amplitude may be blocking presynaptic voltage-gated calcium channels has similar actions to severally reducing extracellular calcium (Llano et al., 2000). When extracellular calcium is reduced there is a persistent decrease in the amplitude of IPSCs due to internal calcium stores being depleted and that are unable to be replenished. If a similar process is occurring in the nerve-bouton preparations it would suggest the process that initiates the release of a GABA filled vesicle under control conditions tends to rely on calcium from outside the nerve terminals, whereas the process that causes several vesicles to be released simultaneously resulting in large amplitude IPSCs tends to rely on calcium from internal stores.

TTX had a similar effect to cadmium by reducing the frequency, but not the amplitude, of events suggesting that the presynaptic boutons retain the ability to produce action potentials and they play a role in enhancing neurotransmitter release. It is worth remembering that TTX has only a presynaptic effect as the voltage-gated sodium channels in the hyperpolarised postsynaptic Purkinje cell would be blocked as they were perfused with an internal solution containing QX-314. In one cell out of five, TTX blocked all events suggesting in that preparation for a period all events that would have occurred would have been evoked by action-potentials. Although a reduction in
the frequency of events was often clear, it was hard to confirm with significant changes in fitted time constants. The difficulty of fitting time constants describing events during the application of TTX is likely to be due to the reduced number of events but there is also the difficulty of identifying the faster curve in the transformed interval distribution. If the lack of a faster time constant was a true effect of applying TTX, and previous studies (Llano et al., 2000) as well as abolishing the smallest events suggest it should be, this would suggest blocking action potentials is more effective at abolishing bursts than blocking voltage-gated calcium channels. This is unexpected as action potentials exert their influence on neurotransmitter release by activating voltage-gated calcium channels (Stephens et al., 2001). As the block of voltage-gated calcium channel currents by 100 µM cadmium is thought to be approximately complete (Shah & Haylett, 2000) this could reflect differences in how quickly the blockers of the different channels act.

As neurotransmitter release is partly mediated by the actions of depolarisation of the nerve-bouton, resulting in activation of voltage-gated sodium channels and voltage-gated calcium channels, it is not entirely surprising these processes would be countered by potassium channels. The potassium channels will exert influence on the nerve-boutons by maintaining a hyperpolarised membrane potential in addition to controlling the frequency and repolarisation of action potentials. The potassium channels that control neurotransmitter release under control conditions were quite sensitive to 4-AP at concentrations of 200 µM and greater but insensitive to TEA up to a concentration of 1 mM. Whereas the concentrations of 4-AP routinely used were in excess of the concentration required to have an effect, significant actions were first seen around 20 µM to 50 µM 4-AP, it is possible an effect with TEA might have been seen if higher concentrations had been used. TEA at a concentration of 2.5 mM has been reported
increase the frequency of IPSCs from molecular layer interneurons (Tan & Llano, 1999). A TEA sensitive current can be evoked from molecular layer interneurons (Southan & Robertson, 2000), but it is not necessarily active or plays a role in neurotransmitter release under control conditions. Application of 4-AP also causes a marked increase in the amplitude of events. When an increase in amplitude occurs there is a decrease in the relative frequency of moderately sized events and an increase in the relative frequency of larger events. It is possible a moderately sized event occurs it is enhanced by the actions of 4-AP to become a large event. The relative frequency of smallest events are unchanged, which fits with 4-AP not altering the tendency for bursting behaviour. However the increase in amplitude is often not sustained and thus appears more effective at earlier applications of lower concentrations of 4-AP. Since large IPSCs rely on internal calcium stores (Llano et al., 2000) perhaps some of the earlier applications of 4-AP depletes them. Certainly 4-AP can evoke a large calcium transient from within molecular layer interneuron terminals (Tan & Llano, 1999). Although if 4-AP application did deplete the calcium stores by inducing large calcium transient it would not be expected to stop replenishment of calcium stores, unlike when voltage-gated calcium channels are impaired, which may explain why amplitudes returned to control level and did not end up being reduced further.

Purkinje cell nerve-bouton preparations are able to spontaneously display both IPSCs and EPSCs that are caused by activation of postsynaptic GABA\textsubscript{A} or AMPA receptors respectively. These receptors are thought to mediate the vast majority if not all events. IPSCs are more ubiquitous so the majority of effects on postsynaptic events would relate to the behaviour of inhibitory inputs. Because there was interest in the behaviour of boutons rather than one type of input both types of events were recorded. However if the behaviour of inputs differed this would lead to misleading or inexact
results. Attempts were made to differentiate the inhibitory and excitatory events by their properties so they could be identified in a mixed population of events. Although different, overlapping ranges of values meant the properties were not distinct enough to unambiguously identify them. EPSCs tend to have a higher frequency based on reductions in interval duration, but this is misleading as it is taken from preparations that have a notable level of excitatory inputs. There appears to be a limited range of frequencies with EPSCs under control conditions, which suggests either EPSCs are present at a relatively high frequency or absent. EPSCs tended to have a narrower range of small amplitudes, but again this was not distinct. Small amplitudes, typically in the range of 10s of pA, would suggest EPSCs are derived from single parallel fibre inputs (Isope & Barbour, 2002). Using the unfitted decay times from Minianalysis shows EPSCs have faster decay times. In previous studies this has been used to distinguish IPSCs from EPSCs (Fiszman et al., 2005). Differences can be enhanced by the decay times of IPSCs mediated by GABA_A receptors which can increased by a high internal chloride concentration (Houston et al., 2009). However under control conditions the differences are not distinct enough and they may become less different during experimental treatments. A quick and robust way to study one input would be to block the other type of input with an antagonist. Although not as ubiquitous as IPSCs in the nerve-bouton preparation, it is unusual to be able to record spontaneous EPSCs. Regardless which excitatory synapse they are coming from the excitatory inputs normally require stimulation to be reliably recorded from Purkinje cells in slices (Konnerth et al., 1990b). Also parallel fibres, if that is where inputs are coming from, are considered to have a low probability of release (Bender et al., 2009). Although it is not unheard of that there may be bicuculline insensitive events spontaneously occurring in Purkinje cells in slices (Farrant & Cull-Candy, 1991), it would appear the excitatory
nerve-terminals once isolated from the rest of the presynaptic cell, are much more excitable and more prone to spontaneously release glutamate in the Purkinje cell nerve-bouton preparation.
3.8 Summary

- The nerve-bouton preparation of Purkinje cells retain spontaneously active nerve-boutons as inferred by the spontaneously occurring events that can be recorded in the postsynaptic cell and the ability of the preparation to endocytose FM dyes.
- Voltage-gated calcium channels in nerve-boutons contribute to the frequency and amplitude of postsynaptic events.
- The nerve-boutons retain the ability to fire action potentials and do so spontaneously which contributes to the frequency of postsynaptic events.
- Neurotransmitter release from nerve-boutons is controlled by potassium conductances which are quite sensitive to 200 µM 4-AP but insensitive TEA up to a concentration of 1 mM.
- The spontaneously occurring events consisted of GABA\textsubscript{A} receptor mediated IPSCs and AMPA receptor mediated EPSCs which can be isolated pharmacologically but are not unambiguously distinguishable by their properties.
Chapter 4: Presynaptic NMDA receptors

This chapter details the potential actions of activating presynaptic NMDA receptors on synaptic neurotransmitter release.

4.1 Introduction to presynaptic NMDA receptors

There is varied evidence about the role of NMDA receptors in afferent terminals modulating neurotransmitter release onto postsynaptic Purkinje cells. NMDA receptors have been detected in the terminals and en-passant boutons of cerebellar molecular layer interneurons (Petralia et al., 1994a; Duguid & Smart, 2004). Activating the presynaptic NMDA receptors have been found to increase the frequency of mIPSCs in postsynaptic Purkinje cells (Glitsch & Marty, 1999; Glitsch, 2008) and are thought to play a role in such physiological processes such as depolarisation-induced potentiation of inhibition (DPI) (Duguid & Smart, 2004; Duguid et al., 2007) as well as the overspill of glutamate from parallel fibres (Huang & Bordey, 2004). There have even been single ion channel openings of activated NMDA receptors recorded from artificially enlarged boutons in cultured molecular layer interneurons (Fiszman et al., 2005). However it has been reported in stellate cells NMDA receptors are not present near release sites (Christie & Jahr, 2008). This is inferred from calcium-imaging studies where the absence of NMDA receptor-mediated calcium influx in axon varicosities (thought to correspond to release sites) when an agonist is applied. NMDA receptor-mediated calcium rises do occur in the somatodendritic compartment of stellate cells and the resulting depolarisation spreads to axon varicosities where it can activate voltage-gated calcium channels. The Christie and Jahr study concludes that activation of NMDA receptors in the
somatodendritic component of the presynaptic cell could increase neurotransmitter release but not by NMDA receptors located in the afferent side of the synapse or indeed anywhere near release sites in the axon. The same study also reports the same results were found in basket cells.

Parallel fibre terminals are also thought to express NMDA receptors (Petralia et al., 1994a; Bidoret et al., 2009) but they have not been proposed affect neurotransmission by either increasing EPSC frequency or decreasing the threshold needed to evoke glutamate release. The NMDA receptors are thought to mediate a calcium influx that activates nitric oxide synthase located in the parallel fibre terminals (Casado et al., 2000). The newly made nitric oxide acts across the synapse that sets off a signalling cascade in the Purkinje cell that ultimately causes a decrease in the amplitude of EPSCs that can be mediated by the postsynaptic AMPA receptors. No changes in the parallel fibre behaviour are thought to occur. However this too has been called into question by a study where it is proposed the production of nitric oxide occurs in response to activation of NMDA receptors located at molecular layer interneuron terminals (Shin & Linden, 2005).

As the nerve-bouton preparation allows for the study of the behaviour of nerve terminals isolated from other cell types, unless they too form synapses with the postsynaptic cell, the preparation was used to look at potential roles of presynaptic NMDA receptors. The presence and role of presynaptic NMDA receptors in modulating neurotransmitter release was studied with immunohistochemistry and by activating the receptors with a bath applied selective agonist, in this case NMDA co-applied with glycine. The presynaptic NMDA receptors afferent to rat Purkinje cells was well suited to the bath application of selective agonists as, unlike most other central neurons, and indeed Purkinje cells from mice (Renzi et al., 2007), they do not express functional
postsynaptic NMDA receptors after a approximately the first week of postnatal development (Rosemund et al., 1992a). This means the effects of activating presynaptic NMDA receptors in afferent nerve-bouton can be achieved with an application of an agonist without the resulting effect being confounded or obscured by the activation of NMDA receptors in the postsynaptic cell. Although the lack of postsynaptic NMDA receptors are useful for studying their role in afferent terminals, it is not a requirement as it is possible to stop the activation of the postsynaptic receptors with an internal application of a NMDA receptor pore blocker (Berretta & Jones, 1996; Rodriguez-Moreno & Paulsen, 2008). If it can be established that activation of presynaptic NMDA receptors can effect neurotransmitter release, further characterisation of the response should be carried out such as the effects of extracellular magnesium, beginning to investigate how activation of presynaptic NMDA receptors can affect neurotransmitter release, and if NMDA application modulates IPSCs and EPSCs differently.
4.2 Immunohistochemistry

Immunohistochemistry was used to establish if presynaptic NMDA receptors were present in the Purkinje cell nerve-bouton preparation. If they were present in the afferent nerve-boutons they would be expected to co-localise with immunoreactive sites against synaptic markers. Therefore, Purkinje cells were prepared by vibrodissociation then treated with antibodies against the synaptic protein synaptophysin and the NMDA receptor subunit NR1 (Fig 4.1). Antibodies against the synaptic protein synaptophysin highlight several areas of immunoreactivity mostly around the cell’s circumference. The immunoreactivity picks out the afferent nerve-boutons and contributes to the evidence that synapses are retained in the nerve-bouton preparation. Purkinje-cell nerve bouton preparations are also immunoreactive for the NR1 subunit, which, as an obligate component of the NMDA receptor tetramer, suggests NMDA receptors are present. Additionally the immunoreactivity for the NR1 subunit co-localises closely with immunoreactive sites for the synaptic protein synaptophysin but little or none is seen elsewhere in the preparation. This suggests the NMDA receptors that are present in the Purkinje cell nerve-bouton preparation are mostly expressed in the vicinity of synapses, which fits with the expectation that NMDA receptors will be expressed solely in afferent side of the nerve-boutons and not in the postsynaptic cell. Co-localisation of the NR1 subunit and synaptophysin was successfully reproduced with several cells in three preparations. However, this evidence alone is not enough to support the proposed presynaptic location. The NMDA receptors could be located close to synaptic proteins but be expressed in the postsynaptic cell opposite the afferent nerve-bouton, as the resolution of confocal microscopy (~250 nm) is not sufficient to separate these possibilities. Also no controls for immunohistochemistry, such as absence of the primary antibody, or adding an excess of the epitope, were carried out.
Figure 4.1. Immunohistochemistry was used to infer the presence and location of NMDA receptors. a) A confocal image where green fluorescence indicates synaptic locations detected with an antibody against the synapse specific protein synaptophysin. b) Immunoreactivity against NR1 subunits, an obligate component of the NMDA receptor, is indicated by red fluorescence. c) Bright field image of the Purkinje cell nerve-bouton preparation. d) An overlay of the three previous images showing that the immunoreactivity for synaptophysin and NR1 co-localise (indicated by the yellower colour). NMDA receptors are likely to be located in the vicinity of synapses in the Purkinje cell nerve-bouton preparation; this was reproduced in several cells in three preparations. Cells prepared by the author, immunohistochemistry and confocal imaging were provided by M. Caldwell (UCL).
4.3 Activating presynaptic NMDA receptors

Changes in the frequency and amplitude of postsynaptic events were examined to study the effects of activating presynaptic NMDA receptors. To increase the likelihood of observing an effect from presynaptic NMDA receptors experimental conditions were made conducive to receptor activation. This consisted of applying NMDA with glycine, at the saturating concentration of 10 µM, to ensure receptor activity was not limited by a lack of activation at the NR1 site, where glycine can act as an agonist (Johnson & Ascher, 1987b). In addition magnesium containing salts were omitted from the external solution as Mg$^{2+}$ ions can limit receptor activity by blocking the pore of the NMDA receptor when the membrane is not sufficiently depolarised (Nowak et al., 1984a).

Bath applying NMDA to Purkinje cell nerve-bouton preparations isolated from P10 rats induced a pronounced increase in the frequency and amplitude of post-synaptic events in approximately half of all applications (Fig 4.2). Interestingly, even under these favourable conditions, the only currents induced during NMDA application were similar in form to the post-synaptic currents seen under control conditions. The lack of a NMDA induced current in this preparation distinct from the postsynaptic currents observed under control conditions strongly suggests a lack of functional NMDA receptors in the postsynaptic cell. This is an important conclusion. Firstly it suggests the NR1 immunoreactivity in the vicinity of synapses (Fig 4.1) is more likely to be in the presynaptic boutons than the postsynaptic cell. Secondly, without any postsynaptic NMDA receptors, any observed effects induced by applying NMDA to the nerve-bouton preparation can be attributed to activating NMDA receptors in the presynaptic boutons. Thirdly, the lack of a postsynaptic NMDA current also suggests that
identifying Purkinje cells after vibrodissociation has been successful as lack of functional NMDA receptors are indicative of a rat Purkinje cells at this stage of development (Rosemund et al., 1992a). The lack of postsynaptic NMDA currents can be attributed to recording from rat Purkinje cells with some confidence as under similar experimental conditions bath applied NMDA induces a prolonged inward current when recording from nerve-bouton neuron preparations derived from pyramidal neurons of the rat *cornu ammonis* area 1 (CA1) region of the hippocampus (J. Chen and D.C.H. Benton, unpublished results).

A range of NMDA concentrations were tested on the Purkinje cell nerve-bouton preparation (Fig 4.2c). The goal of these experiments was to find a dose that would produce a consistent but sub-maximal NMDA response. If a sub-maximal concentration could be found, additional manipulations would have the range to potentially increase or decrease the amplitude and frequency of the post-synaptic events induced by NMDA receptor activation. Concentrations of 10, 20, and 50 µM NMDA were tested (n = 7 for 10 and 20 µM, n = 6 for 50 µM), each co-applied with 10 µM glycine, applied in order of increasing concentration for one minute at a time. To support that the effects observed are the sole result of activating NMDA receptors a suitable blocker, such as the competitive antagonist 2-Amino-5-phosphonopentanoic acid (AP5) (Olverman et al., 1984a), could have been added to see if this eliminated the NMDA induced effect, but this was not done.
Figure 4.2. NMDA application increases the frequency and amplitude of events seen under control conditions without inducing a separate postsynaptic current. a) A recordings made from a Purkinje cell nerve-bouton preparation under control conditions displaying a low frequency of spontaneously occurring events. b) During application of 10 µM NMDA with 10 µM glycine there is a notable increase in the amplitude and frequency of events. c) NMDA was bath applied to Purkinje cell nerve-bouton preparations at concentrations of 10, 20, and 50 µM each co-applied with 10 µM glycine for one minute at a time. d) Applications of NMDA induce an increase in the number of events seen per minute. Data points represent mean of events per minute, the error bars represent +/- the standard error and the number next to the data point corresponds to the n value.
From visually inspecting the records it was apparent that applying NMDA can increase the frequency of events. To assess this more carefully, interval distributions were examined. As increases in event frequency were expected values of tau weighted, then the individual fitted time constants, were examined for being significantly smaller than the control values. The common mean for tau weighted (Fig 4.3a) is significantly smaller than the control values for all concentrations of NMDA tested indicating a significant increase in frequency. The decreases in tau weighted occurred due to significant decreases in tau slow (Fig 4.3b). In contrast, there were no significant differences in tau fast values (Fig 4.3c) or in the fractions of interval distribution representing the shorter intervals at any concentration tested (Fig 4.3d).
A NMDA induced significant increase in event frequency was poorly replicated in individual experiments. Roughly half of the records show a significant reduction in tau weighted at any of the NMDA concentrations tested. The highest concentration of NMDA tested at 50 µM was able to cause a significant reduction in tau weighted in three experiments out of seven. Changes in overall frequency were mostly described by significant reductions in tau slow (Fig 4.4) but these changes do not always result in an

Figure 4.3. The NMDA-induced increase in event frequency can be described by changes in parameters of the interval distributions. a) The common mean for tau weighted values decreased when 10, 20, and 50 µM NMDA is applied indicating an overall increase in event frequency. A star indicates a significant decrease compared to the control value (n = 7). b) Decreases in tau weighted are caused by decreases in tau slow (n = 7). c) However the common means for tau fast (n = 4 - 7) and d) $A_f$ does not change significantly at any concentration of NMDA tested (n = 4 - 7).
overall decrease in tau weighted. Most records did display a significant difference in tau slow at least one concentration tested.
Figure 4.4. Significant changes in tau weighted are often caused by significant changes in tau slow. Values are time constants +/- standard error a) The distribution of intervals during the control period. Often distributions of event intervals can be described by two curves with one peak representing a time constant faster than the other. The distribution consists of 210 events with a tau fast value of 20.2 +/- 6.20 ms and a tau slow of 1600.5 +/- 115.39 ms. The time constants can be combined to produce a tau weighted, 1409.7 +/- 103.96 ms, that describes the overall frequency. b) Adding 20 µM NMDA to the same cell as in a) causes a significant decrease in the value of tau weighted of 450.8 +/- 50.20 ms, which is caused by a significant decrease in tau slow, 511.4 +/- 58.49 ms, but not tau fast, which has a value of 18.1 +/- 11.4 ms and did not change significantly. The distribution consists of 136 events.
As a concentration of NMDA that produced a sub-maximal increase in frequency would be potentially useful, the effectiveness of the different concentrations was examined. All three concentrations of NMDA tested, 10, 20, and 30 µM, are able to significantly reduce tau weighted compared to the control in approximately half of the applications. However it was very rare to observe an application of NMDA causing a significant increase in event frequency over the frequency induced by one of the lower concentration of NMDA tested. This would suggest all three concentrations are able to induce a maximal response in this preparation. However a closer inspection of the tau slow values shows there is a trend for the values to decline with increasing concentrations of NMDA. Although the differences between applications of different concentrations of NMDA are almost never significant, it is interesting that out of twenty-two NMDA applications there are only three exceptions to the trend that every application produces a further reduction in the value of tau slow compared with the previous treatment (and two of these three exceptions are from the same record). This would not happen between applications of NMDA if all concentrations could induce a truly maximal response. Possibly there are differences among the abilities of different concentrations inducing an increase in event frequency, but the differences are either too small or methods used not sensitive enough to detect them. It seems none of the concentrations tested could induce a sub-maximal increase in frequency but hopefully the response has enough range so it could be significantly reduced and still be detectably greater than the control.

NMDA application also caused a significant increase in the amplitudes of postsynaptic events. The highest concentration tested, 50 µM, significantly increased the event amplitude in approximately half of applications which suggests the effect on amplitude is roughly as potent as the one on interval duration. All concentrations tested
seemed roughly as effective at increasing amplitude as the highest dose. Examining a record where 10, 20, and 50 µM NMDA produced a significant increase in the frequency and amplitudes of events shows the sizes of postsynaptic currents have broadly similar ranges regardless of concentration of NMDA applied (Fig 4.5a). Although, applying NMDA does evoke a handful of large events that have amplitudes greater than the largest events seen during the control period. Looking at the relative frequency of the different event amplitudes during NMDA application shows that there is a lower frequency of the smaller events, below 100 pA, but a higher proportion of events between 100 and 300 pA (Fig 4.5b). The increase in the proportion of events between 100 and 300 pA, in addition to a couple of large outliers, causes the significant difference in amplitude when NMDA is applied. However the increase in event amplitude may not just be an increase in the frequency of large events. The slight decrease in the relative frequency of smaller events may suggest that the increases in amplitude could be caused in part by NMDA inducing the nerve-boutons producing the smaller events to release more neurotransmitter resulting in more events with larger amplitudes whilst there is a corresponding decrease in the smaller events.
Figure 4.5. Applying NMDA can significantly increase the amplitude of events. a) Taking the amplitudes from a cell, that experienced a significant increase in amplitude with all concentrations of NMDA tested, shows the ranges of amplitude are similar with and without NMDA. Although there does seem to be some outlying large events evoked by NMDA application. b) The relative frequency of the amplitudes show that when NMDA is applied there might be a slight decrease of the smaller events and an increase in the relative frequency of events between 200 and 400 pA.
Glycine was consistently applied with NMDA at a concentration of 10 µM. It was applied with NMDA to ensure activation of the NR1 subunit which is essential for receptor activation (Johnson & Ascher, 1987b; Kleckner & Dingledine, 1988a). However glycine can act as an agonist in its own right at ionotropic glycine receptors, which can be located in presynaptic terminals and influence neurotransmitter release (Turecek & Trussell, 2001). To eliminate the possibility that glycine may itself be influencing neurotransmitter release one experiment had the NMDA omitted from the first drug application so that only the 10 µM glycine remained (Fig 4.6). Glycine on its own does not appear to alter neurotransmitter release when applied to the Purkinje cell nerve-bouton preparation at this stage of development. The tau weighted values during control and application of 10 µM glycine not significantly different and were actually quite similar. The weighted time constant +/- the standard error was 3888.7 +/- 512.88 ms for the control and 4134.6 +/- 1682.5 ms when 10 µM glycine was applied alone. However NMDA and glycine are not always able to produce a significant increase in neurotransmitter release. To confirm glycine lacks an affect and not that the preparation cannot be induced to increase the frequency of events, NMDA with glycine was applied. Both 20 and 50 µM NMDA were able to significantly reduce tau weighted compared to control (values +/- standard error were 761.7 +/- 148.84 ms for 20 µM NMDA and 641.2 +/- 130.10 for 50 µM NMDA), but not compared to when glycine was applied presumably because of the larger errors associated with fitting a curve to fewer event intervals.
Figure 4.6. Glycine alone has no effect on the frequency of events. NMDA is always co-applied with 10 µM glycine. To test that it does not have an independent effect on neurotransmitter release glycine was added to a cell without NMDA. During glycine application tau weighted is not significantly different from the control value, indeed they were quite similar. The cell was able to show changes in neurotransmitter release as when NMDA is applied there are significant decreases in tau weighted compared to the control (indicated by a star) but not to glycine application.
4.4 Magnesium sensitivity of presynaptic NMDA receptors

Experimental conditions were made conducive to NMDA receptor activation by including glycine and omitting magnesium salts from the external solution. Extracellular magnesium can block the pore of the NMDA receptors and seriously impede the current it can mediate if the potential acting across the ion channel is not sufficiently depolarised to repel the positively charge magnesium ions (Nowak et al., 1984a). However it is possible under these experimental conditions that the omission of external magnesium is an unnecessary precaution and that NMDA is able to increase neurotransmitter release when external magnesium ions are present, as has been reported in experiments elsewhere (Glitsch & Marty, 1999; Duguid & Smart, 2004). If magnesium was added during NMDA activation it could indicate if it was a necessary precaution or not. Also if magnesium does affect the increase in event frequency it could be informative about the nature of the presynaptic bouton and the NMDA receptors that mediate the increase in neurotransmitter release.

To test these possibilities 1 mM MgCl₂ was added during NMDA application. Recordings were made from three nerve-bouton preparations of Purkinje cells for five minutes to establish the basal properties of the events under control conditions. After the control period 10 µM NMDA with 10 µM glycine was bath applied for a minute which was followed by the solution being switched to a solution of 10 µM NMDA and 10 µM glycine supplemented with 1 mM MgCl₂ for one minute which was then switched back to the magnesium free NMDA and glycine solution for a further minute. The purpose of the second magnesium-free NMDA application was to ensure that any effect magnesium may have is reversible when application ceases.
A NMDA induced increase in event frequency was observed to be rapidly and reversible attenuated when the NMDA solution was switched to one supplemented with magnesium to the extent that the postsynaptic events appeared similar to events under control conditions (Fig 4.7). First the occurrence of a NMDA-induced effect needs to be established before considering if it has been attenuated or not. Examining the common mean to look for trends over several experiments shows that during both applications of NMDA without magnesium as well as its wash-out a significant decrease in tau weighted occurs (Fig 4.8a). However when NMDA is applied with magnesium there ceases to be a significant decrease in tau weighted compared with control suggesting the presence of magnesium reduces the potency of NMDA to increase neurotransmitter release. The increase in tau weighted when magnesium is present with NMDA is caused by the tau slow value increasing so it is more similar to the value describing the control period (Fig 4.8b). There were no significant changes in tau fast although when NMDA was applied with magnesium the common mean for tau fast was approximately double that of the values during the control and NMDA application (Fig 4.8c). It was not investigated if 1 mM Mg\(^{2+}\) could affect the frequency of events during control conditions. However adding magnesium with NMDA does not reduce the event frequency to levels lower than control suggesting magnesium at the concentrations used is acting by impeding the effects of NMDA.
Figure 4.7. The NMDA-induced increase in event frequency is sensitive external application of magnesium. a) Adding 10 µM NMDA can increase the frequency of events. If magnesium is added during NMDA application a sudden and reversible decrease in the event frequency can be seen. b) A five second segment during the control period showing the low frequency of events. c) Detail of the record during NMDA application showing the increase in frequency and amplitude. d) A close-up of the record when magnesium is applied with NMDA, detailing a reduction in event frequency to similar levels seen during the control period.
Figure 4.8. NMDA ceases to induce significant decreases in tau weighted when magnesium is present. a) The common mean for tau weighted from three cells shows a significant decrease when NMDA is applied but only without magnesium (significant reduction are indicated by a star). b) These changes are caused by changes in tau slow. c) The values for tau fast do not change significantly but do lengthen when NMDA is applied with magnesium.
As with the common mean, when changes in tau weighted are observed in individual experiments when NMDA is applied the changes are due to reductions in tau slow (Fig 4.9). Both applications of NMDA induced a significant decrease in tau weighted in two records out of three but never when NMDA was applied with magnesium. Although the ability of NMDA to increase the frequency of events was replicated in these experiments there was no significant increase in event amplitude when NMDA was applied. Therefore it is hard to say definitely if magnesium will block the increase in amplitude, but since both the increase in frequency and amplitude are thought to work through the common mechanism of activating NMDA receptors it seems quite likely. In one preparation there was no significant change in event frequency in response to NMDA. Including magnesium with NMDA also did not have an effect suggesting that magnesium at the concentrations used acts primarily via NMDA receptors but not inhibiting the neurotransmitter release process itself. The one record that did not have a significant NMDA response was made from a preparation that exhibited a high frequency of large bursting events (Fig 4.10). Some records display an apparent insensitivity to NMDA application. In records that already exhibits a high frequency of events this may be because a further increase in event frequency are unattainable as the afferent nerve-boutons are unable to be induced into releasing more neurotransmitter.
Figure 4.9. NMDA-induced changes in interval distributions are partially reversed when magnesium is included. Values are time constants +/- standard error. a) An interval distribution from a preparation under control conditions showing two peaks. There were 118 events, tau fast was 36.5 +/- 8.76 ms and tau slow was 3433.8 +/- 412.84 ms b) The distribution during NMDA application displays a leftward shift of the second peak. This can be described by a significant reduction in tau slow. There were 207 events, tau fast was 25.2 +/- 4.05 ms, and tau slow was 568.8 +/- 77.06 ms. c) Adding magnesium caused a tau slow to cease being significantly smaller than control. There were 49 events when magnesium was applied with NMDA, tau fast was 18.5 +/- 4.22 ms, and tau slow was 3271.4 +/- 1059.99 ms.
Figure 4.10. Some records do not respond to NMDA may do so because they cannot be induced to release more neurotransmitter. a) A record that does not appear to respond to concentrations of NMDA that induce responses in other cells. Note applying magnesium during NMDA application has at most a slight effect. b) There were no significant differences in the time constants that describe the frequency of events. However, there was an increase in the values when NMDA was applied with magnesium.
The concentration of NMDA used, 10 µM, was the lowest concentration tested. Throughout one experiment the NMDA concentration was doubled to 20 µM to see if this lessened magnesium block (Fig 4.11). As with 10 µM of NMDA, 20 µM NMDA produces a significant increase in the frequency of events according to a significant reductions in tau weighted. When magnesium was applied with 20 µM NMDA there was still a significant increase in event frequency over control, something that did not happen when using 10 µM. Although magnesium not stopping a higher concentration of NMDA causing a significant increase compared to the control does not necessary mean magnesium ceased having an effect. The first application of NMDA produced a significant increase in frequency over both the control period and when magnesium was present with NMDA, meaning the magnesium did attenuate the NMDA-induced response. The second application was not significantly more frequent than when magnesium was present as it was probably still partially under its effects. Since magnesium is applied during NMDA application the apparent intermediate and lesser effects may be because the prior stimulation with the higher concentration of NMDA causes the nerve-boutons to be more depolarised and therefore more resilient to block. When magnesium is added during an application of a higher NMDA concentration most of the events occurs during the start of the application as a continuation from when NMDA was applied without magnesium (Fig 4.11a). This observation supports the idea that higher concentrations of NMDA decrease magnesium block by keeping the boutons depolarised for longer. Although a decrease of events down to control levels appears to occur eventually within the one minute application. Using a higher concentration of NMDA did significantly increase the amplitude of events. The amplitudes were attenuated so they were not significantly different from control when magnesium was
applied suggesting this is another NMDA-induced process which is also magnesium sensitive.
Figure 4.11. Magnesium block seems lesser with a higher concentration of NMDA.

a) Magnesium does reduce the NMDA response down to near control levels eventually but the effect is less immediate than when 10 µM NMDA is used, possibly indicating the higher concentration keeps nerve-boutons depolarised for longer.  
b) The first application of 20 µM NMDA produces a tau weighted that is significantly smaller than control and NMDA with magnesium which, unlike with 10 µM, is itself significantly reduced compared to control. Significant reductions compared to control are indicated by a star.
4.5 Action potential dependence of the actions of presynaptic NMDA receptors

There is an accumulation of evidence supporting a role for presynaptic NMDA receptors located in afferent nerve-boutons. Increases in neurotransmitter release induced by applying NMDA with glycine, but not glycine alone, and that are sensitive to extracellular magnesium are good indicators that presynaptic NMDA receptors are likely to be involved in stimulating neurotransmitter release. However this evidence alone does not give any information about how activation of presynaptic NMDA receptors leads to an increase in neurotransmitter release. Neurotransmitter release from the afferent-boutons of the Purkinje cell nerve-bouton preparation has previously been seen to be partly dependent on action potentials. The depolarisation that is expected to accompany NMDA receptor activation could induce an increase in the frequency of action potentials which may be necessary to mediate some of the increased neurotransmitter release. Although action potentials are expected in the preparation and may play a role in the effects of activating presynaptic NMDA receptors, they are known not to be absolutely necessary as NMDA application can induce increases in mIPSCs frequency when the induction of action potentials is blocked (Glitsch & Marty, 1999; Duguid & Smart, 2004).

To discern if the NMDA induced increase in neurotransmitter release is wholly or partly dependent on action potentials in the nerve-bouton preparation the ability to initiate action potentials was blocked pharmacologically by applying TTX during application of NMDA and glycine. As with the previous experiment NMDA and glycine was applied under control conditions on either side of NMDA application that occurred with further experimental manipulations. The first application is to establish
what would happen under control conditions and the second application is to ensure that effects observed were due to the additional treatment and were not concurrent with a sudden change in the release properties of the afferent boutons. NMDA was applied to the cells twelve to sixteen minutes into the recording at a concentration of 20 µM, supplemented with 10 µM glycine, for between one and two minutes. Action potentials were then blocked during continuing application of NMDA and glycine supplemented with 500 nM TTX lasting between one and two minutes before being switched back to a solution of NMDA and glycine. The reason NMDA was applied relatively late into the recording was because these experiments were carried out on the five cells that were used to study the release properties in afferent boutons by blocking voltage-gated calcium channels and voltage-gated sodium channels. It was ambitious to obtain results from several treatments from one preparation. The length of the recording and the multiple drug applications should be taken into account when evaluating the changes in the release properties of the afferent-boutons. However there were some advantages to this approach. Firstly the lack of a long slow inward current when the NMDA is applied strongly infers a lack of postsynaptic NMDA receptors and supports that the cells were correctly identified as Purkinje cells. Secondary as the nerve-bouton preparations already had already been treated with TTX an informed comparison could be made between the actions of TTX during control conditions and during NMDA application within the same record. As the results of these experiments are derived from records that have received multiple drug applications there is a choice of possible controls with which that further drug applications can be compared. The seemingly most intuitive period to be the control is the drug-free period of the record that immediately precedes the first NMDA application, which is the one to three minutes that the preparation was allowed to recover from TTX application. But further drug applications could also be
compared with the true control period which consisted of the first five minutes of each recording, as the immediately preceding period is likely to experience some residual effects from the TTX application.

Applying NMDA appears to cause an increase in the frequency and amplitude of events compared to the events during the preceding TTX wash-out period. Adding 500 nM TTX during the NMDA-induced event frequency increase is seen to cause a reduction in frequency and amplitude of events towards the levels that occurred during the control. The effect of TTX seems to be reversible as when the solution is switched back to a solution of NMDA and glycine the frequency and amplitude of events again increase (Fig 4.12).

![Graph showing the effect of TTX on NMDA-induced increase in event frequency.](image)

**Figure 4.12.** Adding TTX attenuates the NMDA-induced increase in event frequency. As the sodium channels in the nerve-boutons will be blocked by the TTX application it can be inferred that at least part of NMDA-induced increase in frequency is dependent on action potentials.

The common means for the individual fitted time constants were examined for significant reductions, or lack thereof, compared to the control period which initially is
taken as the TTX wash-out period. The common mean for tau weighted (Fig 4.13a) during both applications of NMDA were significantly smaller than the values during TTX wash-out. However the first application was more potent as it was also significantly smaller than when NMDA was applied with TTX and the 2nd NMDA application, presumably as there are some lingering effects of TTX. The reductions in tau weighted are caused by significant reductions in tau slow (Fig 4.13b) whereas tau fast (Fig 4.13c) and A_/ (Fig 4.13d) remain relatively constant. The common mean derived from the values describing tau weighted when NMDA is applied with TTX is not significantly smaller than any other treatment. Since this treatment is flanked by two applications of NMDA, applying NMDA with TTX could be thought to as inducing NMDA application to cease being significantly different from control. The NMDA induced increase in event frequency is seriously attenuated by applying TTX and therefore is at least partly action-potential dependent.
The conclusions drawn from the common mean were the same irrespective of the section of the record that was used as the control period, be it either the TTX wash-out prior to the first NMDA application or the true control at the start of the record.

**Figure 4.13.** Applying TTX with NMDA attenuates the significant reduction in tau weighted. a) Changes in event frequency as described by tau weighted over the course of the experiment. An asterisk indicates when the common mean value is significantly smaller than the TTX wash-out which acts as the control period (n = 5). b) Changes in frequency as described by tau weighted are because of changes in tau slow (n = 5). c) The common mean for tau fast do not change significantly over the course of the experiment. Although when NMDA is applied with TTX, tau fast does lengthen to beyond levels seen under control conditions (n = 2 – 5). d) Changes are non significant for A_f but there is a slight increase when NMDA is first applied which will contribute to the tau weighted (n = 2 – 5).
However when examining individual experiments there is not only a disappointing
dearth of significant NMDA responses but also there are some apparent differences
depending on which control is used. When TTX wash-out is being taken as the control
only the first application of NMDA could only cause a significant reduction in tau
weighted twice out of five records. If NMDA treatments are compared to the true
control at the start of the record there is one additional reduction in tau weighted
occurring during the second application (Fig 4.14). The reason there were two controls
was because multiple treatments were applied to one preparation. It appears the two
periods are not equivalent and can change the conclusions drawn, albeit only slightly.
When NMDA was applied with TTX there was never a significant difference from
control regardless of which plausible control was used. As with the common mean, no
individual record had significant differences in tau fast regardless which control is used.
Significant increases in $A_f$ were quite rare, only occurring once when NMDA was
applied compared to either plausible control.
The proportion of records that did respond to NMDA was disappointingly low, although a trend was seen with the significant differences in the common mean. However, the point of the experiment was not to see if NMDA can increase neurotransmitter release, that has already been established, but to see if the increases in neurotransmitter release induced by activating presynaptic NMDA receptors are

**Figure 4.14.** Significance can depend on which plausible control is used. In experiments where TTX is added during NMDA application either the preceding section of the record (TTX wash-out) or the start of the record (the ‘true’ control) can be used (n = 5). In this example both applications of NMDA and the NMDA wash-out period have a significantly smaller tau slow (indicated by a red asterisk) compared to the ‘true’ control, but not compared with TTX wash-out despite having a larger value. This is because the errors associated with the TTX wash out period are larger as its value is derived from fewer events.

The proportion of records that did respond to NMDA was disappointingly low, although a trend was seen with the significant differences in the common mean. However, the point of the experiment was not to see if NMDA can increase neurotransmitter release, that has already been established, but to see if the increases in neurotransmitter release induced by activating presynaptic NMDA receptors are
dependent on action potentials. Obviously it becomes more difficult to see if TTX attenuates the NMDA-induced increase in neurotransmission if there are only a few incidences of NMDA actually increasing neurotransmission. But it seems at least part of the effect of activating presynaptic NMDA receptors is dependent on action potentials. To follow up this finding it might be worth investigating if all of the NMDA-induced increase requires action potentials. As the cells have already been treated with TTX under control conditions it is possible to make that comparison within the same preparation (Fig 4.15). The tau weighted values suggest out of five records there is one incidence of NMDA and TTX causing a significant increase in event frequency over TTX without NMDA. One of the five records had no events when TTX was applied and as there are enough events during application of NMDA and TTX to fit a time constant to the intervals this also represents a notable increase in event frequency. Out of four records, where both treatments could be described by fitted time constants, three records saw a reduction in tau weighted from the period when TTX was applied to when NMDA and TTX was applied. This would suggest that activating presynaptic NMDA receptors should be able to induce an increase in neurotransmitter release when action potentials are not present.
Drawing conclusions from fitted time constants based on a small number of events becomes difficult due to the size of the errors; a useful alternative is to test differences in the populations of inter-event intervals using a non-parametric test. Using the TTX wash-out period as a control, the first NMDA application, as well as

**Figure 4.15.** NMDA can increase the frequency of events without action potentials. Out of five records four have interval distributions that can be described by time constants during application of TTX with and without NMDA (a fifth record had all events abolished by TTX application so cannot be described by an interval distribution). Three of these four records have a smaller tau weighted values when TTX is applied with NMDA than TTX alone. One of the reductions is significant (significance is indicated by a star) as judged by the usual criteria that significant changes have happened if the three times the respective errors of the time constants are distinct from each other. Here the tau weighted value +/- the standard error was 1730.0 +/- 263.5 ms with TTX and 600.8 +/- 107.80 ms with TTX and NMDA.
NMDA and TTX are often able to significantly reduce the duration of event intervals. A significant reduction with the first application of NMDA occurs in four records out of five whereas when the NMDA is applied with TTX a significant reduction occurs three times out of five. This suggests NMDA is quite capable of increasing the frequency of events compared with the last drug-free section of record and so is NMDA with TTX. Comparing the interval durations between the first NMDA application and NMDA with TTX shows that TTX causes a significant increase in interval duration in two out of five records. This would suggest that NMDA induces a significant increase in event frequency which can be significantly reduced by adding 500 nM TTX during the NMDA application, but NMDA with TTX can induce frequencies of events that are themselves significantly more frequent than that during control conditions and presumably also when TTX is applied under control conditions. In three out of five records there was a significant reduction in inter-event intervals when NMDA was added in the presence of TTX compared with when TTX was applied under control condition. This should occur in approximately half of the records were NMDA can induce a significant increase in event frequency.

There were no incidences of significant increases in amplitudes compared to control during any of the treatments used in these experiments. There are however changes in the relative proportions of event amplitudes when NMDA is added with and without TTX (Fig 4.16). When NMDA is able to increase the amplitude of events there tends to be an increase in large events at the expense of small events. However without an increase in amplitude there are appears to be an increase in the relative frequency of the smallest events. Since TTX application under control conditions, and to some extent during the TTX wash-out, inhibits bursts perhaps NMDA is reintroducing the very smallest events that occur in bursts. As this occurs whether TTX is included with the
NMDA or not, activation of the presynaptic NMDA receptors can contribute to bursts as action potentials in the afferent bouton would normally.

**Figure 4.16.** NMDA with and without TTX increased the frequency of smallest events. Compared with the previous drug free section of the record which was when TTX was being washed out, NMDA application with or without TTX can increase the relative frequency of smaller events of up to 50 pA. The threshold of 50 pA may not be meaningful beyond that small events that occur within bursts are found within this range which may be caused by activation of presynaptic NMDA receptors.
4.6 Effects of Activation of Presynaptic NMDA Receptors on IPSC and EPSC Properties

Applying NMDA with glycine can evoke increased neurotransmitter release by stimulating the nerve-boutons afferent to Purkinje cells in a nerve-bouton preparation. There are at least six different inputs that can form synapses onto Purkinje cells (inhibitory inputs from basket cells, stellate cells, Lugaro cells and Purkinje cell axonal collaterals in addition to excitatory inputs from parallel and climbing fibres) and it is unknown which synapses are retained and active in the nerve-bouton preparation. It is therefore difficult to distinguish which synapses do reliably contribute to neurotransmitter release, either under control conditions or when stimulated with NMDA. So far the most robust way to begin characterise the inputs from different synapses is to differentiate between inhibitory and excitatory inputs. This is necessary as the Purkinje cell nerve-bouton preparation can receive both types of input. Separating inhibitory from excitatory inputs is done by applying appropriate antagonists that block the ligand-gated ion channels that mediate the IPSCs and EPSCs.

The majority of events evoked by NMDA are probably inhibitory as events recorded under control conditions are most likely to be inhibitory. As inhibitory synapses are numerous and located around the soma they are well placed to be retained in the nerve-bouton preparation. The apparent higher frequency of EPSCs in the nerve-bouton preparations as reported in the previous chapter is misleading as these frequencies are taken from approximately every second to third cell to find a sufficiently high enough frequency of excitatory events. It is likely that cells with a high frequency of EPSCs have a high frequency of IPSCs too. This would be expected if the frequency of events varied with the amount of dendrite retained, and therefore nerve-
boutons, by the preparation. The amplitudes of events during NMDA stimulation also suggest that they are likely to be mostly inhibitory as the larger amplitudes and larger range of amplitudes of IPSCs are more similar to events evoked during NMDA application than the EPSCs evoked from a single nerve-bouton. However it is not safe to assume the events evoked with NMDA application are just a higher frequency of the events that occur under control conditions. Activating presynaptic NMDA receptors may change neurotransmitter release properties which may be reflected in different event characteristics. Indeed it was seen that NMDA application causes changes in the proportion of events with different amplitudes. There is also the possibility that activating presynaptic NMDA receptors may induce neurotransmitter release from afferent boutons that were previously inactive under control conditions.

To investigate the effects of NMDA on inhibitory and excitatory inputs in isolation, NMDA was applied to cells recorded in the presence of appropriate antagonists. Antagonists were applied soon after the whole-cell configuration was successfully formed with the nerve-bouton preparation and were constantly applied throughout the record after being given sufficient time to act. During the NMDA application in the presence of one antagonist a second antagonist is applied specific to the type of events thought to be isolated. The second antagonist is to confirm that only one type of event was present during the NMDA application, and presumably during the control period. The solutions were switched back to just NMDA, glycine and one antagonist (the second application of NMDA) to ascertain that the release properties had remained relatively constant during the additional drug treatment. As the purpose of the second antagonists is to see if all inputs can be blocked, which requires definite answer, a 30 second wash-in period was included to allow the second antagonist to have a full effect. A corresponding wash-out period was not included as purpose of the second
application of NMDA is to establish that the preparation has not run down during application of the second antagonist.

To study the inhibitory inputs in isolation, one cell had all excitatory inputs blocked by applying 20 µM DNQX throughout the recording. After recording from the cell under control conditions for three minutes NMDA was applied for a minute and a half before the solution was switched to a NMDA solution supplemented with bicuculline for another minute and a half, incorporating 30 seconds of wash-in time, which was followed by switching back to the NMDA solution. Examining the trace shows that a clear increase in the frequency of inhibitory events can be induced by NMDA application (Fig 4.17). Allowing for the wash-in time all the events are blocked by the combination of the bicuculline and the DNQX present throughout the recording. The bicuculline application confirms that, when the excitatory events are blocked, the increases in events during NMDA application are solely IPSCs mediated by GABA_A receptors.
Changes in event frequency were examined by looking at the fitted values that describe the distribution of intervals. The tau weighted values during both applications of NMDA were significantly and very noticeably smaller than the values for control but

Figure 4.17. NMDA can increase the frequency of inhibitory events. a) NMDA can increase the frequency of events in a cell where all the excitatory events are blocked. Adding bicuculline confirms the events seen are inhibitory and mediated by GABA<sub>A</sub> receptors. b) Detail of the record under control conditions. Mean IPSC amplitude (values given with +/- standard error) was 381.4 +/- 61.36 pA (n = 108) and mean interval was 1664.5 +/- 209.35 ms. c) Detail of the record when NMDA is applied. Mean IPSC amplitude was 616.0 +/- 53.01 pA (n = 304) and the mean interval was 298.7 +/- 29.71 ms. d) Detail of the record when bicuculline blocks the increase in events caused by NMDA.
not from each other (Fig 4.18). This indicates a pronounced increase in IPSCs
frequency. The reductions in tau weighted are caused by decreases in tau slow, whereas
tau fast may have declined slightly the changes are not significant compared to control.
The proportion of the area of the distribution taken up by the peak representing the
faster time constant increases which will contribute to the overall change in tau
weighted but was not the main cause of it (Fig 4.18b). It was clear that activating
presynaptic NMDA receptors can increase neurotransmitter release from inhibitory
afferents and as it was subsequently duplicated later as part of a further set of
experiments it was not thought worth repeating in this form.
Figure 4.18. NMDA can significantly reduce the time constants that describe IPSC interval distribution. a) When 50 µM NMDA was applied when IPSCs were isolated the reduction in tau weighted and tau slow was significant compared with the control (indicated by an asterisk) but not tau fast. b) However tau fast will contribute slightly more to the overall tau weighted during 50 µM NMDA application as there are non-significant increases in $A_f$. 

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The amplitude of IPSCs increases with NMDA application. A significant difference was detected in event amplitude during NMDA treatment but only for the first application. This suggests when a significant increase in event amplitude occurs with a potentially mixed population of events, i.e. without isolating inhibitory inputs with DNQX, IPSCs could mediate the increase in amplitude. A likely reason the second application of NMDA did not produce a significant increase in event amplitude may be because this period included events that occurred when bicuculline was being washed-out. As bicuculline goes from producing a total block of IPSCs to no apparent block there will be an intervening period where partially blocked IPSCs may display reduced amplitudes. If this were the case it would be possible that amplitudes cease to be significantly larger without having a detrimental effect on the significant increase in frequency, which fits with what was observed. If a run-down had occurred during the block of all postsynaptic events a reduction in amplitude and frequency would likely coincide. The distribution of event amplitudes is remarkable in that it neatly divides between large and small events (Fig 4.19). It is more usual for the event amplitudes to have a continuous distribution. Taking all the events during the control period, the first NMDA application, and the second NMDA application, which even includes events recovering from the effects of bicuculline block, there are no events with amplitudes between 1.3 nA and 2.2 nA, but with events as large as around 3.2 nA. It would be interesting to know if the large and small events were mediated by different types of synapses or if one common type of synapse was able to produce events with such distinct amplitudes. However this seems unlikely as examining the inhibitory events during NMDA application (Fig 4.17c) shows large events can be followed by a burst of smaller events suggesting that a nerve-bouton that is able to produce a large IPSCs can also produce a burst of small IPSCs. Large and small events occur during both the
control period and during NMDA application. The relative proportions of large and small events during NMDA application are relatively similar to control suggesting NMDA is able to stimulate an increase in frequency of both types of event (Fig 4.19d). However the first application of NMDA tends to increase the relative frequency of larger events that occur within the distinct populations encompassing both the smaller and larger events.
An analogous study was carried where the effects of stimulating presynaptic NMDA receptors were studied on excitatory inputs in isolation. Inhibitory inputs were blocked by treating the cell with 50 µM (+)-bicuculline throughout the entire recording. To ensure the effects of NMDA application on excitatory inputs could be examined

**Figure 4.19.** NMDA application can stimulate both large and smaller IPSCs. In a record that had discrete populations of large and small events, the amplitude range remained similar during a) control, b) the first NMDA application, and c) the second NMDA application. d) The relative frequency of different amplitudes did not change much more than 10 %. The significant increase in the amplitude of IPSCs during the first application of NMDA may be caused by an increase in the relative proportion of the larger events within the discrete groups of smaller and larger event. Increases in relative frequency with a treatment compared to control are indicated by negative percentage changes.
cells were selected that displayed excitatory events under control conditions. This meant somewhere between only every second to third cell could be used in experiments as often cells did not display spontaneous excitatory events and were therefore likely to have either too few or too dysfunctional excitatory afferents. This was not a problem for inhibitory inputs. Results derived from these cells should come with the caveat that they are drawn from a sub-set of cells that already spontaneously display EPSCs.

The experiment on isolated inputs followed a similar protocol to the one looking at inhibitory inputs. A control period of three minutes was used to establish the basal properties of spontaneous EPSCs. The cell was then treated with bath application of 50 µM NMDA and 10 µM glycine for a minute and a half before switching to a solution that maintained the same concentrations of NMDA, glycine, and bicuculline in the preceding interval but supplemented with 20 µM DNQX for a further minute and a half. The duration of application included a 30 second wash-in period for the DNQX. The wash-in period was necessary to allow the drug to work fully and to confirm that all postsynaptic events can be blocked by a combination of DNQX and bicuculline. After DNQX application the solutions was switched back to a NMDA containing solution with just one blocker. The second period where NMDA was present without DNQX lasted six minutes. The reason for the long duration was that the record appeared to recover from DNQX block over several minutes.

Visually inspecting the resulting trace shows that there was a high frequency of spontaneous low amplitude events during the control period of the record (Fig 4.20a, b). Under control conditions the mean amplitude (values +/- standard error) was 34.0 +/- 1.1 pA (n = 803) and the mean interval was 224.1 +/- 8.2 ms. Despite the apparent high frequency of EPSCs, NMDA application seems to be able to increase both the frequency and amplitude of events (Fig 4.20c) where the mean amplitude was 134.0 +/- 1.6 pA (n = 160).
5.0 pA and the mean interval was 68.8 +/- 3.1 ms. All these events during NMDA application appears to be blocked by adding DNQX (Fig 4.20d) which would act in conjugation with the bicuculline that is present throughout the recording to block all postsynaptic events. Ceasing DNQX application allows the events to return to a frequency and amplitude similar to that seen before the antagonist was applied (Fig 4.20e), however the washout is very slow and it took several minutes for the events to appear to have recovered. The reason for the long time may be because the recording from this particular cell became unstable as suggested by the sudden drop in the baseline when DNQX was being washed out in the presence of NMDA.
Figure 4.20. NMDA can stimulate EPSCs. a) When excitatory events are isolated, by adding 50 µM (+)-bicuculline, activating presynaptic NMDA receptors can increase their frequency and amplitude. b) High frequency of small EPSCs under control conditions. c) NMDA can increase the amplitude and frequency of EPSCs. d) All events can be blocked by DNQX confirming their identity as EPSCs. e) The events were able to partially recover when DNQX was removed.
Unlike other interval distributions with a reasonable number of intervals, the distribution that represents the control period appears to have only one peak (Fig 4.21a). This could be a result of having two very similar time constants. There is a slight bulge on the right of the distribution emerging below half the height of the distribution curve suggesting a possible contribution from a second curve. But if there were a second component that could be adequately fitted, it would produce a curve with a comparatively minor area and a time constant very similar to the one derived from the main curve as no distinct second peak was seen. During the first NMDA application two peaks can be adequately fitted but they are so close together that a second peak cannot be seen (Fig 4.21b). The main peak is to the left of the smaller peak which by the nomenclature used means it represents tau fast. This is unusual as the main component is more frequently represents tau slow. Interestingly, the location of the proposed second peak in the distribution of control period intervals which could not be adequately fitted roughly corresponds to the position of the large tau fast fitted to intervals during the first NMDA application. The second application of NMDA included the wash-out period for DNQX so it is not surprising the distribution of intervals was shifted to the right as many events will be blocked by DNQX, reducing the apparent frequency (Fig 4.21c). During the second application of NMDA, the distribution of intervals could be adequately fitted with two peaks, but as with previous sections of record only one peak was prominent.
Figure 4.21. NMDA can change the interval distribution of intervals between EPSCs. Values are time constants +/- standard error. a) The distribution of intervals during the control period is fitted with one curve as two cannot be adequately fitted. There were 803 events and the time constant was 221.5 +/- 7.70 ms. b) NMDA significantly decreases the tau weighted but not individual time constants. There were 1308 events and the individual time constants were tau fast 36.6 +/- 6.03 ms and tau slow 170.1 +/- 44.26 ms. c) The record does not recover fully from DNQX block. When NMDA is applied for a second time tau weighted was significantly higher and there was a decrease in EPSC frequency. There were 695 events, tau fast was 67.2 +/- 24.64 ms and tau slow was 540.70 +/- 46.70 ms.
During the first application of NMDA there is a decrease in tau weighted indicating an increase in event frequency (Fig 4.22). However the time constants decrease differently from other records that show an increase in the frequency of events. Normally an increase in the frequency of events would be caused by a significant decrease in tau slow. There may occasionally be a trend for a decrease in tau fast but it is rare that the value changes significantly. Continuing the assumption that when there is only one time constant it is treated as the equivalent of tau weighted and corresponds to tau slow, tau slow decreases during the first application of NMDA but the decrease is not significant (Figure 4.22a). However when comparing the tau weighted values, which takes into account both time constants, the value during NMDA application was significantly smaller. This is because during the first NMDA application the smaller tau fast time constant contributes greatly to tau weighted. The value of tau fast during NMDA application is not itself notable; it is not significantly different from the value during the second application of NMDA or the best attempt at fitting a value to the distribution during the control period. The fraction of the total distribution that is taken up by the faster curve is notably large (Fig 4.22b). In this preparation A,f is the value that changes notably and which is likely to cause the significant change in tau weighted.

The second application of NMDA also produces an unexpected result. From previous results the second NMDA application either produces a frequency similar to the first application or at least similar to control. However in this record looking at EPSCs the frequency during the second application of NMDA is significantly lower than either the first application or the control period as reflected in tau weighted. The fitted values for tau fast and tau slow are both increased during the second application, but only the tau slow is significantly greater than control (Fig 4.22a). The fraction of the distribution area that represents tau fast is notably smaller than during the first
application of NMDA. However visually inspecting the record suggests that during the second application of NMDA a frequency of EPSCs similar to the first application of NMDA may be regained eventually. If the six minute second application is divided into two minute sections and had time constants fitted to the distribution of intervals the values for tau slow are still significantly larger than both the values during the control period and the first NMDA application indicating a persistent significantly lower frequency. But this does not matter too much as the actions of DNQX still indicates that the events are glutamatergic events mediated by AMPA receptors and the nerve-boutons are still active after block with both antagonists even if a full recovery was not made.
Figure 4.22. NMDA changes fitted time constants describing EPSC frequency. a) The first application of 50 µM NMDA causes a significant reduction in tau weighted (indicated by a star), although not the individual time constants. Values for tau slow and tau weighted describing the second 50 µM NMDA application are significantly larger than control values (as indicated by a plus sign). b) $A_f$ during the first NMDA application is noticeably large and may explain the difference in tau weighted.
As with inhibitory events, activating presynaptic NMDA receptors result in an increase in EPSC amplitude. There is a significant increase in event amplitude during both applications of NMDA. An increase during the second dose is unexpected as the frequency is significantly lower than both the first application of NMDA and the control period. This would suggest that the NMDA is still having an effect after blocking the inputs with DNQX even if there is not an increase in frequency. It might also hint at why there is a reduction in frequency. The excitatory events, either during control or NMDA application, have much smaller amplitudes than inhibitory events. The limited range of amplitudes would mean that partially blocked events are more likely to fall below the detection threshold, whereas an inhibitory event could be partially blocked by an antagonist washing out and still have comparatively large, and thus detectable, amplitudes. The action of the lingering DNQX during wash-out may effectively remove a proportion of events while the ones that are detected are, as a population, significantly larger than during the control period. The smallest events being blocked by a residual effect of DNQX may also explain why there is an apparent decline in bursts as indicated by a smaller $A_T$ (Fig 4.22b). EPSCs under control conditions and when stimulated with NMDA have a propensity for small amplitudes. However with NMDA application the EPSCs do not have the same range of amplitudes as under control conditions (Fig 4.23a). Under control conditions the maximum amplitude is a little more than 200 pA, but when stimulated with NMDA events can be several times larger. Examining the changes in the distribution of event amplitude, it is striking the similarity between the first and second doses of NMDA despite the difference in frequency (Fig 4.23b). Applying NMDA reduces the proportion of the smallest events while increasing the proportion in the next couple of adjacent bins. It is likely that the events during NMDA application are the same type of events and from the same source as the events during
the control period. It appears activating presynaptic NMDA receptors causes an increase in the efficiency of excitatory synapses which manifests as an increase in the frequency and amplitude of events.

As with the record where inhibitory events were isolated the increase in frequency during the first application of NMDA, at least, seems blatant enough not to need repeating. This is especially the case as the experiment will be replicated as part of another set of experiments. However unlike the experiment when inhibitory events were isolated, applying NMDA to a cell with isolated excitatory events was repeated (Figure 4.24). It was found that inducing an increase in EPSC frequency by applying NMDA to the nerve-bouton Purkinje cell preparation was reproducible.
Figure 4.23. Activation of presynaptic NMDA receptors increases EPSC amplitude.

a) EPSCs under control conditions and when NMDA is applied tend to have small events, but when NMDA is applied the EPSCs have a much greater range of amplitudes. b) Both applications of NMDA cause a decrease in the relative frequency of the smallest events but caused an increase in events with a range of larger amplitudes.
Figure 4.24. Activating presynaptic NMDA receptors in the presence of bicuculline caused a replicable increase in the frequency and amplitude of EPSCs. The finding is repeatable and will be examined more in a further set of experiments. In all records a combination of DNQX and bicuculline seems to block all events. Recovery from DNQX can be rapid.
4.7 Discussion

NMDA receptors are likely to be located in the terminals afferent to rat Purkinje cells. The obligate NMDA receptor subunit NR1 is detected in the vicinity of synapses of the Purkinje cell nerve-bouton preparation. Applying NMDA under favourable conditions results in an increase of postsynaptic events, similar to the ones seen under control conditions, suggesting they are located in a position to influence neurotransmitter release. NMDA application does not induce a separate long prolonged inward current in the postsynaptic cell of the preparation. The lack of a newly induced current by NMDA application is important as it suggests a lack of functional postsynaptic NMDA receptors which, along with the size of the soma, are identifying features of rat Purkinje cells after about postnatal day 7 (Rosemund et al., 1992a). From this finding, Purkinje cells can be said to be successfully located after vibrodissociation with more confidence.

Activating presynaptic NMDA receptors located in the afferent nerve-terminals of the Purkinje cell nerve-bouton preparation can increase the frequency and amplitude of postsynaptic events. This increase does not require the somatodendritic compartment of the afferent cell to occur. However inducing significant increases in event frequency is variable as a significant increase can be evoked by applying a NMDA agonist in roughly half of experiments, regardless if increases in frequency are investigated by reduction in fitted time constants or by non-parametric tests on interval duration. Certainly the responses to activation of presynaptic NMDA in single boutons have been reported to be a variable process (McGuinness et al., 2010a). Besides the variability of the boutons, presumably some preparations cannot be induced to release more neurotransmitter, for example in preparations that already displays a high frequency of
events as seen in Figure 4.10. When presynaptic NMDA receptors are activated bursting activity does not change notably, possibly because the process cannot be increased further by NMDA receptor activation. But there is a notable decrease in the intervals between events that are not within a burst which causes the increase in frequency. Activation of presynaptic NMDA receptors can also cause an increase in the amplitude of postsynaptic events.

The evidence that NMDA receptors are being activated includes that they can be activated with a combination of the selective agonist NMDA and glycine which acts as an agonist at the NR1 site, but not by glycine alone. Further evidence that NMDA receptors are being activated is the sensitivity to the increase in frequency and amplitude induced by NMDA to an extracellular application of 1 mM Mg$^{2+}$. Magnesium can affect both the increase in frequency and amplitude suggesting they are increased via a common mechanism. At the concentration of magnesium used, the effect on neurotransmitter release works by attenuating the NMDA receptors, magnesium application does not affect an increase in neurotransmitter release caused by applying 4-AP (D. Benton unpublished observations). Although when there is not a NMDA receptor response, there was a slight and non-significant lengthening of time constants describing the frequency of events suggesting there may be non-specific effects on decreasing neurotransmitter release perhaps that is more effective at higher concentrations of magnesium.

The attenuation of the effect of applying 10 µM NMDA by extracellular magnesium is so severe it suggests if 1 mM Mg$^{2+}$ were present throughout the experiment no NMDA effect would have been seen. However applications of NMDA have been reported to be able to increase neurotransmitter release in the presence of extracellular magnesium (Glitsch & Marty, 1999; Duguid & Smart, 2004). The apparent
potency of the block could be an effect of the concentration of NMDA used or that the
effect of a brief application of magnesium differs from the effect it would have if it were
present throughout the record. Also the presence of magnesium has been reported to
enhance NMDA receptor function (Paoletti et al., 1995) so its acute effects may differ
from if it were present though out a recording. Adding Mg\(^{2+}\) during application of 20
µM NMDA still results in a response which is lesser to the response to NMDA without
magnesium but greater than control. This could mean a NMDA response may be seen in
a record that has magnesium present throughout the experiment if a higher
concentration of NMDA is used. However in the record where magnesium was applied
during treatment with 20 µM NMDA the frequency appears to be returning to control
like levels suggesting the magnesium block will be as severe but it will take longer to
occur. As magnesium is applied during NMDA treatment the slowing of the magnesium
effect may suggest that 20 µM NMDA is better at depolarising the nerve-boutons and
therefore resisting magnesium block than 10 µM NMDA. For magnesium to have such
an effect it would suggest the afferent nerve-boutons are capable of maintaining
hypermolarised membrane potentials. It also suggests the NMDA receptors that can
influence neurotransmitter release are particularly sensitive to magnesium meaning the
functional tetramers are more likely to incorporate the more magnesium-sensitive
subunits such as NR2A and NR2B (Wrighton et al., 2008).

The effects of activating presynaptic NMDA receptors are partially dependent
on action potentials in the afferent boutons. The initiation of action potentials was
blocked by applying TTX to inhibited voltage-gated sodium channels. TTX had to have
a presynaptic effect as the postsynaptic voltage-gated sodium channels would already be
blocked by an internal application of QX-314. The ability for NMDA to induce a
significant increase in event frequency was disappointingly rare in these experiments,
possibly due to the length of the records and the multiple treatments applied, but an action dependent effect was apparent during NMDA application. Action potentials may be required to propagate a depolarisation from the sites of the NMDA receptors in the afferent boutons to where the voltage-gated calcium channels that control neurotransmitter release are located. However given the small size of the boutons this seems unlikely. It is possible the depolarisation induced by NMDA receptors is enhanced by the presence of action potentials which could enhance the activity of the voltage-gated calcium channels that control vesicular release. Blocking action potentials has been reported to decrease the proportion of NMDA induced increase in IPSCs mediated by voltage-gated calcium channels (Glitsch, 2008). Although action potentials enhance the effect of activating presynaptic NMDA receptors there are not necessary for it, which corresponds to findings reported for mini events recorded in the presence of TTX (Glitsch & Marty, 1999; Duguid & Smart, 2004). TTX is very good at abolishing the faster intervals and therefore bursting behaviour, but this is not the case when TTX is applied with NMDA. If bursting behaviour requires calcium (Auger & Marty, 1997) and this could not be supplied when action potentials were blocked, perhaps because action potentials are required to activate voltage-gated calcium channels under control conditions (Stephens et al., 2001), activated NMDA receptors in boutons could provide enough calcium to reinstate bursting behaviour.

Most of the effects found in a mixed population of events are likely also to occur to inhibitory events given the ubiquity of IPSCs in the Purkinje cell nerve-bouton preparation. Activating presynaptic NMDA receptors can significantly increase the frequency and amplitude of IPSCs which corresponds to previous reports (Glitsch & Marty, 1999; Duguid & Smart, 2004). An increase in the frequency and amplitude of IPSCs can occur without the need for the somatodendritic compartment of the afferent
inhibitory neuron. However this seems to contradict the findings of a calcium imaging study that concluded that in stellate cells any effect NMDA has at the release sites is from the propagation of a depolarisation from the somatodendritic component (Christie & Jahr, 2008). There might not necessarily be a contradiction as it is unclear which synapse provides the increased frequency of inhibitory inputs when NMDA is added. Although since basket cells are also reported to not express NMDA receptors in their terminals there is a lack of synapses that could produce IPSCs with the range of frequencies and amplitudes recorded. If activation of presynaptic NMDA receptors increased the calcium concentration in the boutons the increase in the amplitude of inhibitory inputs could be explained by more frequent large IPSCs that can be produced from molecular layer interneurons (Llano et al., 2000).

Activation of presynaptic NMDA receptors can also significantly increase the frequency and amplitude of EPSCs. It should be noted at this point the receptors that induce the increases are presynaptic, as indicated by the lack of a postsynaptic current, but they are not necessarily located to the terminals that cause an increase in neurotransmitter release. This is a surprising result as the only reported function of NMDA receptors located to excitatory terminals afferent to Purkinje cells is to induce a signalling cascade that results in a decrease of EPSC amplitude (Casado et al., 2000). This could mean the signalling cascade is not preserved in the nerve-bouton preparation or the observed increase in amplitude is being partially occluded. The Purkinje cells receive two quite different excitatory inputs and it would be quite interesting to know which one can be enhanced by activating presynaptic NMDA receptors. The size of the EPSCs under control conditions suggests they are mediated by inputs from single parallel fibres (Isope & Barbour, 2002). When the amplitude of events is increased with NMDA application, the larger EPSCs are too variable and still too small to be likely to
be mediated by an input from an intact climbing fibre (Konnerth et al., 1990a). However the climbing fibre forms an intricate synapse around the bases of several dendritic spines encompassing several active zones (Kakizawa et al., 2005) which is unlikely to survive vibrodissociation intact. If retained section of climbing fibre were still active in the nerve-bouton preparation their inputs would be hard to identify if they lost their characteristic large invariable amplitude. The increase in EPSC amplitude during NMDA application is accompanied by a decrease the relative frequency of the smaller events that occur during the control period suggesting they are likely to be mediated by the same inputs. Parallel fibres can induce large EPSCs by the simultaneous activation of several inputs (Konnerth et al., 1990a), but since the inputs are reduced to single isolated boutons it would be hard to coordinate activity. If multiple parallel fibres and the climbing fibre inputs are unlikely to produce the large EPSCs that must mean individual inputs are responsible for most of them. The large amplitude EPSCs could be evoked by NMDA from a single parallel fibre that releases several EPSCs in quick succession (Isope et al., 2004) or is stimulated into releasing multiple glutamate vesicles at once (Bender et al., 2009).
4.8 Summary

- Purkinje-cell nerve-bouton preparations show immunoreactivity for NMDA receptor subunits that colocalised with synaptic proteins.
- Applying NMDA does not result in an inward current suggesting Purkinje cell can be successfully identified after vibrodissociation.
- Activating presynaptic NMDA receptors result in an increase in the amplitude and frequency of events.
- The NMDA effect is sensitive to extracellular magnesium.
- NMDA increases event frequency partly, but not entirely, though action potentials.
- Activating presynaptic NMDA receptors can increase the amplitude and frequency of both IPSCs and EPSCs.
Chapter 5: Presynaptic NMDA receptors in retrograde transmission

This chapter looks into the retrograde transmission by glutamate, which is a physiological process that is thought to involve presynaptic NMDA receptors.

5.1 Introduction to retrograde transmission

NMDA receptors in nerve terminals afferent to Purkinje cells are involved in several physiological processes including glutamate overspill from parallel fibres (Huang & Bordey, 2004), parallel fibre LTD (Casado et al., 2002), and depolarised-induced potentiation of inhibition (DPI) (Duguid & Smart, 2004). DPI is characterised by an increase in the frequency of IPSCs. It is caused by the activation of NMDA receptors in afferent inhibitory nerve terminals by the retrograde release of glutamate, or a glutamate like substance, from Purkinje cells which is typically evoked by depolarising the cell. Furthermore the ability to display DPI is persevered in the Purkinje cell nerve-bouton preparation (Duguid et al., 2007). Indeed by preserving the source of the retrograde signalling molecule, the postsynaptic cell, and the site of action, the inhibitory nerve terminals, confirms no surrounding cells or the rest of the presynaptic neuron is required for DPI.

It is plausible that an analogous system to DPI exists for excitatory inputs. As applying NMDA to Purkinje cell nerve-bouton preparations also causes an increase in EPSCs it is likely that the retrograde release of glutamate could activate the same presynaptic NMDA receptors that mediate the increase in the frequency of excitatory events. In keeping with the naming convention this process, if it existed, would be
called depolarisation-induced potentiation of excitation (DPE). Investigating the existence of this putative depolarisation-induced potentiation of excitation (pDPE) is a suitable starting point to search for a physiological role of presynaptic NMDA receptors modulating excitatory neurotransmission. The Purkinje-cell nerve bouton preparation should be suitable for searching for pDPE as the analogous mechanism DPI is preserved and the preparation often exhibited excitatory events that can be modulated by activation of presynaptic NMDA receptors.
5.2 Putative depolarisation-induced potentiation of excitation

To investigate the existence of pDPE Purkinje cell nerve-bouton preparations were first selected for having a notable frequency of excitatory events when the inhibitory events were blocked. After the whole-cell configuration was achieved 50 µM bicuculline was bath applied and if a substantial frequency of events remained a recording was made. This means not every cell with a sufficient frequency of events can be used for these experiments. Selecting cells with a sufficient level of excitatory inputs resulted in using between every second to third cell. Any result derived from these experiments must come with the caveat that the cells were selected for have a high frequency of excitatory events. Initially a five minute control period was recorded to establish the basal release properties of excitatory events. The amplitude, inter-event interval, and time to decay of spontaneously occurring EPSCs under control conditions, (such as have already been presented in Chapter 3) were measured to see if it were possible to distinguish between the two types of events that occur under control conditions from their properties alone. After the control period, the retrograde release of glutamate was induced by depolarising the postsynaptic Purkinje cell. The cell was depolarised from the holding potential of -80 mV to 0 mV for 1 second five times with a 10 ms interlude between depolarising steps (Fig 5.1). Repeated depolarisations can induced a larger calcium transient in Purkinje cells than one prolonged depolarisation (Llano et al., 1994). This stimulus was the equivalent to the longest and most depolarising that had been previously reported to be used to induce DPI and has a longer duration that the stimulus previously used to induce DPI in a Purkinje cell nerve-bouton preparation (Duguid et al., 2007). A large depolarisation was used to maximise the likelihood that pDPE would be seen if it existed. After applying the stimulus the cell was recorded for a further fifteen minutes as this is the duration of DPI. To confirm that
the cell was a Purkinje cell by the lack of a prolonged NMDA induced inward current distinct from the postsynaptic events and to test if the cell could respond to activation of presynaptic NMDA receptors, NMDA was applied. The cell was then applied with 50 µM NMDA with 10 µM glycine for up to five minutes. During the NMDA application 20 µM DNQX was applied. This would be expected to block all the events and confirm the events isolated with bicuculline were AMPA receptor-mediated EPSCs and not other bicuculline insensitive events. As effects of DNQX were judged on the abolition of all events or not, DNQX was given a thirty second wash-in period when it was first applied. DNQX application ceased and the second NMDA application with just one blocker was to demonstrate the events were recoverable from DNQX block.
Examining the traces, the frequency of EPSCs after stimulation appears unchanged compared with the frequency during the control period (Fig 5.2). In records where NMDA was applied (one record out of four did not have this additional treatment) there was an absence of prolonged inward currents but an increase in the

Figure 5.1. A large depolarisation was used to maximise the likelihood of seeing an effect from inducing the retrograde release of glutamate. a) The depolarisation consisted of 5 steps from the holding potential of -80 mV to 0 mV each lasting 1000 ms with 10 ms intervals. b) An example of the changes in current during the depolarising steps.
frequency and amplitude of post synaptic events were often seen. Also after an appropriate wash in period, DNQX blocked all remaining events during the NMDA-induced increase of events indicating that all events during NMDA application, and presumably the events before NMDA was applied, were AMPA receptor-mediated EPSCs. As DNQX was applied with bicuculline it indicates all events seen during NMDA application were either mediated by AMPA receptors or GABA<sub>A</sub> receptors and it was assumed all the excitatory events that were present before NMDA application persisted during its application as well as further applications of blockers.
**Figure 5.2.** An example of a trace used to investigate the existence of putative depolarisation-induced potentiation of excitation. a) An example of the control period before stimulation. Cells were treated with 50 µM bicuculline throughout the recording and selected for having a high frequency of EPSCs. b) The same cell after stimulation. The cell was recorded from for a further fifteen minutes to see if the stimulation had an effect. NMDA was then applied to confirm that the cell was likely to be a Purkinje cell and that it can respond to activation of NMDA receptors. DNQX was included during NMDA application to confirm the events were AMPA receptor-mediated EPSCs and not just insensitive to bicuculline. Enlarged sections of the record sections detailing a) the control period, b) after the stimulus, and c) during application of 50 µM NMDA.
When DPI is induced in a Purkinje cell the depolarisation can also induce other forms of retrograde transmission (Duguid et al., 2007). One such example is the retrograde release of endocannabinoids which can decrease the frequency of mIPSCs for a couple of minutes in a process called depolarisation-induced suppression of inhibition (DSI) (Diana et al., 2002). An analogous mechanism also lasting the first couple of minutes after depolarisation can decrease the release properties of the excitatory inputs to the Purkinje cell, called depolarisation-induced suppression of excitation (DSE) (Kreitzer & Regehr, 2001), which is also caused by retrograde release of endocannabinoids and also, at some developmental stages, glutamate (Crepel, 2007).

Because there can be more than one retrograde transmission mechanism happening simultaneously but often with different time courses, the fifteen minute record after the stimulus was divided up in to three five minute sections. Dividing the record up after stimulation may aid separating out the different retrograde signalling mechanisms if they had different time courses of action. If pDPE is analogous to DPI no significant increase in the frequency of EPSCs might be seen until any retrograde signalling mechanism that decrease excitatory inputs has ceased, for example in the first five minute section. DPI does persist for several minutes, if pDPE also persists for several minutes seeing a consistent increase in EPSC frequency over several sections would eliminate the possibility that the increase was due to a sudden burst of events. An advantage of dividing the response this way is interval distributions are constructed from a five minutes section of record, compared with as few as one minute sections during some drug treatments, which means more intervals and therefore better curve fitting to describe the distribution.

As DPI causes an increase in the frequency of IPSCs, a corresponding pDPE effect would also be expected to increase the frequency of EPSCs in preparations where
EPSCs occur spontaneously. Significant reductions in time constants that describe the distribution of intervals were not observed after the stimulation. Examining the common mean shows that a significant reduction in the tau weighted compared to control does not occur in any of the five minute sections after the stimulus, but a significant reduction in tau weighted was seen with both applications of NMDA (Fig 5.3). Far from an increase in frequency as indicated by a significant decrease in time constants, the common mean is significantly larger compared to the control after stimulation for the sections incorporating the first five minutes and from the tenth until the fifteenth minute. The decrease in frequency during the first five minutes is replicated in two records out of four and may be related to DSE. The decrease in frequency during ten to fifteen minutes was only replicated once out of four experiments. There was also an individual record out of four that exhibited a significant increase in tau weighted between the fifth and tenth minutes after stimulation, but again this was not replicated. The lack of significant decrease in tau weighted until NMDA is applied after the stimulation is well replicated in the individual experiments. No record had a significant decrease in tau weighted after the stimulation. This would suggest the retrograde release of glutamate from a Purkinje cell is unable to increase the frequency of EPSCs in a manner analogous to DPI increasing the frequency of IPSCs. The first application of NMDA caused an increase in EPSC frequency compared to the control as described by the significant reduction in tau weighted in two out of three records. The one record where there was not a significant reduction compared to control there was a significant reduction compared to the section of record immediately prior to NMDA application, this was in the record that had a significant decrease in events between ten and fifteen minutes after the stimulus. If the decrease in spontaneous EPSC frequency is related to DSE the effect seems particularly long lasting in the nerve-bouton preparation. This
confirms that the cells were able to display increases in EPSCs frequency with exogenous NMDA application but not with endogenous retrograde release of glutamate. Indeed there appeared to be occasions of a decrease in the frequency of EPSCs after a depolarising stimulus is applied.

**Figure 5.3.** There was no reduction in tau weighted describing the intervals between EPSCs after a depolarising stimulus. After a depolarising stimulus was applied there was no increase in frequency of EPSCs as would be expected if there was an analogous mechanism to DPI acting on presynaptic NMDA receptors. There were actually significant increases in tau weighted in the common mean between 0 to 5 minutes and 10 to 15 minutes after the stimulus (indicated by a plus sign). The cells can respond to activation of NMDA receptors as indicated by the significant reduction in the tau weighted during both applications of NMDA (significant reduction compared to control indicated by a star).
There were no significant reductions for either tau fast or tau slow compared to the control after the stimulus (Fig 5.4a) even if there were significant changes in tau weighted which may be caused by changes in $A_f$ (Fig 5.4b). Examining the distributions of intervals before and after the depolarising stimulus shows it is quite clear that there are two peaks and the values do not alter much (Fig 5.5). This differs from the example of NMDA on excitatory events in the previous chapter where the representative record had interval distribution where it was difficult to resolve two distinct peaks and therefore two different time constants. This supports the idea that excitatory events have a rate that can describe neurotransmitter release as well as a rate for bursts of neurotransmitter, as for a period after an event there is an increased likelihood of a subsequent event.
Figure 5.4. The individual time constants tau fast and tau slow rarely change significantly compared to control after a depolarising stimulus. a) Changes in tau fast, slow, and weighted from one record. There were no significant reduction compared to control but there was a significant increase in tau fast between 5 to 10 minutes (indicated by a plus sign), but this was only seen in this record. There were significant increases in the combined tau weighted during 0 to 5 minutes and 5 to 10 minutes. b) The increases in tau weighted are likely to be due to the significant reductions in $A_f$ compared to control (denoted by a star).
The cells were able to respond to activation of presynaptic NMDA receptors as indicated by the increase in frequency in the cells where exogenous NMDA was applied. When NMDA is added to a cell and there is an increase in the frequency of

**Figure 5.5.** The distribution of intervals between consecutive EPSCs can form two clear peaks. The positions of the peaks, which correspond to time constants, change little after the depolarising stimulus. Values are time constants +/- standard error. a) The control period before the depolarising stimulus consists of 267 events with a tau fast of 16.5 +/- 1.87 ms and tau slow of 2619.6 +/- 313.74 ms. b) The period from 0 to 5 minutes after the stimulus had 149 events with tau fast 19.5 +/- 5.50 ms and tau slow 2398.3 +/- 182.82 ms. c) From 5 to 10 minutes there were 100 events with tau fast 98.2 +/- 24.18 ms and tau slow 3615.8 +/- 299.84 ms. d) 10 to 15 minutes after the depolarising stimulus had 154 events with tau fast 31.0 +/- 9.18 and tau slow of 3517.9 +/- 770.52 ms.
events it can often be described by a significant reduction in tau weighted which is caused by a significant decrease in tau slow. In an experiment described in the previous chapter investigating what happens to the frequency of EPSC when presynaptic NMDA receptors are activated this expected changes in time constants did not occur, probably because the two peaks in the interval distribution were so similar. Taking a cell where the peaks of the interval distribution that describe the time constants are better separated shows when EPSCs are isolated activating presynaptic NMDA receptors causes a decrease in the value of the peak representing tau slow but not tau fast (Fig 5.6). This alteration in the interval distribution persists when during the second NMDA application after DNQX had been applied. This means the NMDA-induced increase in EPSCs frequency can occur in a similar manner to increases in the frequency of mixed event or IPSCs.
Figure 5.6. Activating presynaptic NMDA receptors causes an increase in the frequency of EPSCs. Increases in frequency can be described by a decrease in tau slow. Values are presented as time constants +/- standard error. a) Distribution of intervals between EPSCs during the control period of an experiment. The distribution is made up of 335 events, with a tau fast of 42.9 +/- 19.20 ms and a tau slow of 1004.6 +/- 81.66 ms. b) After attempting to evoke DPE 50 µM NMDA was applied with 10 µM glycine which causes an increase in event frequency. This increase in event frequency can be described as a decrease of the value of tau slow resulting in an overall decrease in tau weighted. There were 369 events, tau slow was 34.3 +/- 16.46 ms and tau slow was 291.6 +/- 29.94 ms. c) These changes persist during the 2nd NMDA application after DNQX had been removed. During the 2nd NMDA application there were 323 events, tau fast was 25.8 +/- 5.68 ms and tau slow was 381.1 +/- 26.98 ms.
Activation of presynaptic NMDA receptors can also significantly increase the amplitude of EPSCs. All three records that had NMDA applied to the cell towards the end of the pDPE experiment had a significant increase in the amplitude of EPSCs (Fig 5.7a). As seen before there is a decrease compared to control of the relative frequency of the smallest events but an increase in a range of slightly larger events (Fig 5.7b). During NMDA application the amplitude of the larger events evoked are still too small and too variable to likely to have originated from an intact climbing fibre input. After the depolarising stimulus there are no significant changes in the amplitude of EPSCs nor are there large changes in the relative frequency of amplitudes (Fig 5.7c).
Figure 5.7. NMDA application but not a response to a stimulus can increase the amplitude of EPSCs. a) Applying NMDA causes a significant increase in EPSC amplitude (significance denoted by an asterisk). Edges of the box plot represent the 1st and 3rd quartile whereas the whiskers represent the range of 10 to 90% of the data. b) Applying NMDA causes a decrease in the relative frequency of the smallest events but an increase in slightly larger events as indicated by the positive percentage change. c) After the depolarising stimulus intended to evoke DPE, the amplitude of EPSCs does not experience big changes in relative frequency of amplitudes.
Retrograde transmission, such as DPI and presumably pDPE, require adequate handling of intracellular calcium. These forms of retrograde transmission require for there to be a rise in intracellular calcium (Duguid & Smart, 2004) which can be supplied by the activation of voltage-gated calcium channels when the Purkinje cell is depolarised (Llano et al., 1994). Although DPI can be preserved in a nerve-bouton preparation of Purkinje cells, if the cells ability to handle calcium has been adversely affected it may explain why pDPE was not seen. Evidence was sought to suggest calcium dynamics was not impaired inside the cells used. Increases the intracellular calcium of a Purkinje cell can induce an inwards current. Examining the increase in the inward current when the Purkinje cell is held at -80 mV before and after the depolarising stimulus (Fig 5.1b) suggests the appearance of an inward current. This inward current can persist for a couple of milliseconds after the stimulus (Fig 5.8a) and has been attributed to a calcium-activated chloride current (Llano et al., 1991a). Often the inward current after a depolarising stimulus has two components: the first mediated by the calcium-activated chloride current and the second mediated by autocrine release dopamine, formerly thought to be mediated by glutamate, in a process called depolarisation-induced slow current (DISC) (Kim et al., 2009). DISC was not seen after the depolarising stimulus. This could be because the process is not a simply autocrine one but requires surrounding neurons and glial cells to work and thus cannot be induced in the nerve-bouton preparation. However a more likely explanation is that as expression of DISC varies throughout the cerebellum the small number of experiments could be have been made from cells from areas of the cerebellum that did not robustly express DISC. Another calcium-dependent process that can be investigated to ascertain the cell can handle calcium correctly is the induction of DSE. DSE is where depolarising Purkinje cells results in the retrograde release of endocannabinoids.
(Kreitzer & Regehr, 2001) and, at the right developmental stage, glutamate (Crepel, 2007) that decrease the release properties in evoked EPSCs. Changes in release properties of evoked EPSCs are characterised by reduction in EPSC amplitude and changes in paired-pulse facilitation. It is not known how DSE would affect an experiment that relied on spontaneously occurring EPSCs instead of evoked EPSCs. A reduction in EPSC amplitude may be expected. But out of four records there was only one significant reduction in EPSC amplitude compared with the control. This did happen in the first five minutes after a depolarising stimulus was applied but there was also one incidence of a significant increase in EPSC amplitude in the same period in a different record and so was not a convincing trend. DSE may act on spontaneous EPSCs by decreasing the frequency of events. Out of four records, two had decreases in the frequency of events compared to the control period as described by significant increases in tau weighted. The increases in tau weighted that describe the increases in frequency in the first five minutes are not accompanied by significant changes in tau fast or tau slow. But there are accompanied by significant reductions in $A_f$, where $A_f$ is the fraction of the interval distributions that is taken up by the faster curve. In every record there is a decrease in $A_f$ from control period to the first five minutes after the stimulus, in two out of four records this change is significant, yet there was no problem in fitting two peaks. The common mean describing the transition of $A_f$ from control to the first five minutes after the depolarising stimulus shows a significant reduction (Fig 5.8b). A decrease in $A_f$ would suggest a decrease in bursting events which could be the actions of retrograde released endocannabinoids, or another messenger, on spontaneous EPSCs occurring in a DSE-like mechanism. If a DSE-like mechanism has occurred it would suggest the cell’s ability to handle calcium or send retrograde messengers are not impaired and there must be others reasons that explain the lack of pDPE.
Figure 5.8. Calcium handling did not seem impaired in the nerve-bouton preparation. a) After the depolarising stimulus was applied there was often a brief inward current that could been attributed to a calcium-activated chloride current. b) The common mean for changes in $A_f$ (fraction of the distribution that is taken up by the fast curve) from control to the first five minutes after depolarisation significantly decreases (indicated by a star). There are also significant reductions in $A_f$ in two out of four individual records. As this could be a possible effect of a DSE-like mechanism on spontaneous EPSCs the calcium dynamics of the Purkinje nerve-bouton preparation do not seem to be impaired.
5.3 Depolarisation-induced potentiation of inhibition

Depolarisation-induced potentiation of inhibition was evoked to strengthen the finding that the retrograde release of glutamate cannot cause an increase of EPSC frequency similar to activating presynaptic NMDA receptors with a bath applied agonist. DPI has previously been demonstrated in a Purkinje cell nerve-bouton preparation (Duguid et al., 2007) suggesting DPI should be able to be elicited. The appearance of a possible calcium-activated chloride current as well a DSE-like mechanism suggests calcium handling has not been severely impaired in the nerve-bouton preparations. However if DPI can be evoked under present experimental conditions it will support that mechanisms involved in retrograde transmission of glutamate remain likewise intact and the absence of pDPE will be due to other reasons.

To study DPI the inhibitory inputs were isolated. After a whole cell recording was successfully made 20 µM DNQX was applied and maintained throughout the experiment to block any spontaneous EPSCs that may occur. If a sufficiently high frequency of events after DNQX treatment remained the cell was used to study DPI. Cells were hardly ever rejected because DNQX reduced the frequency of events to below usable levels. Unlike when trying to isolate EPSCs where cells could have a high frequency of events but a low frequency of excitatory events, cells with a sufficient frequency of mixed events generally also had a sufficient frequency of IPSCs. Spontaneous IPSCs were recorded for five minutes to establish the basal release properties in each experiment. The properties of IPSCs under control conditions, such as amplitude, inter-event interval, and time to decay have already been detailed in Chapter 3. After the control period a stimulus was applied to evoke the retrograde
release of glutamate from the Purkinje cells. The stimulus was the same one used to investigate pDPE (Fig 5.1) consisting of 5 depolarising pulses from the holding potential of -80 mV to 0 mV for 1000 ms with a 10 ms interval between the pulses. A large stimulus was used to maximise the likelihood of seeing DPI and to keep the experimental set up consistent with the investigation into pDPE. The response to the stimulus was recorded for 15 minutes which is the length of time DPI is reported to last (Duguid & Smart, 2004). After allowing a period of time to observe any response resulting from the stimulus 50 µM NMDA and 10 µM was applied for several minutes. The purpose of the NMDA application was to establish if the preparation did respond to activation of presynaptic NMDA receptors as well as confirming that the cell was likely to be a Purkinje cell by the absence of a prolonged NMDA-induced inward current. Applying NMDA to cells with isolated IPSCs also provides further opportunity to repeat the finding that activation of presynaptic NMDA receptors can increase the frequency of inhibitory events. As with previous experiments aiming to isolate either IPSCs or EPSCs appropriate antagonists were applied during the NMDA application. To confirm only IPSCs were being recorded 50 µM bicuculline was applied during the application of NMDA at the end of the experiment. As the expected result was a total block of all events the bicuculline was given 30 seconds to wash-in but not a corresponding period to wash-out as the continued NMDA application was to establish the effects of the antagonist were reversible.

Apart from the type of events isolated the experiments proceeded similarly to the experiments looking for pDPE. However there was one alteration in some later records. After the antagonists were given enough time to act, the perfusion system was switched off during the initial recording of the cell under control conditions, applying a depolarising stimulus, and then recording the response. The record was stopped and the
perfusion system switched back on for bath application of NMDA and further drugs. This change in procedure was made as it was thought to improve the likelihood of seeing the effects of the retrograde release of glutamate. Unlike in brain slices, where retrograde glutamate release should be shielded from the perfusion system by the surrounding tissue, the retrograde glutamate release from nerve-bouton preparations of Purkinje cells may be more exposed and dispersed quicker by the actions of the flowing solution. This could be a possibility if the retrograde glutamate release does not occur at tightly regulated structures like synapses but had a generalised release from the soma to several nearby nerve terminals.

Traces under control conditions often featured a high frequency of IPSCs with variable amplitudes (Fig 5.9a). Immediately after the depolarising stimulus there appears to be little change in the frequency of IPSCs, possibly even a reduction in event frequency, but the first five minutes after the stimulus there often appears to be a DPI like effect characterised by a persistent increase in the IPSC frequency (Fig 5.9b). NMDA application can induce a pronounced increase in the frequency of events which appear to be reversibly blocked by bicuculline (Fig 5.9b). Together this suggests that in cells that display an increased frequency of IPSCs in response to NMDA application can also display DPI, which appears less pronounced than the increase in IPSCs frequency when NMDA is applied but are thought to act through the same mechanism.

DPI is expected to cause an increase in IPSC frequency. To investigate increases in the frequency of events significant reductions in time constants that describe the distribution of event intervals were sought. As with the search for pDPE the 15 minutes used to record any response to the depolarising stimulus was divided into three 5 minute sections. This could help separate out different retrograde processes if they have different time courses as well aid fitting time constants with the increased number of
intervals that define the distribution. Taking a common mean of the derived time constants can be useful in highlighting trends that occur among the individual experiments.
Figure 5.9. An example trace of an individual DPI experiments. a) Example of IPSCs during a five minute control period. b) The same cell immediately after a depolarising stimulus was applied. Notice the apparent persistent increase in frequency a couple of minutes after the stimulus and before NMDA was applied. The NMDA application suggests that the preparation can respond to activation of presynaptic NMDA receptors and it lacks postsynaptic NMDA receptors whereas the block of all events suggests IPSCs were studied in isolation. Details of different sections of the record are given including c) during the control period, d) during the apparent increase in events after stimulus, and e) during application of 50 µM NMDA and 10 µM glycine.
Looking at the common mean for the response to a depolarising stimulus shows that from 10 minutes to 15 minutes after the stimulus there is a significant reduction in tau weighted compared to the control (Fig 5.10). There are also significant reductions in tau weighted with both applications of NMDA. Significant reductions in tau weighted after the stimulus occurred twice out of four records between ten minutes and fifteen after the stimulus and once from between five minutes and ten minutes after the stimulus. No significant reductions in tau weighted were observed in any individual record in the first five minutes after the stimulus, but unlike with pDPE there were no significant increases either. These significant reductions in tau weighted are likely to be caused by DPI which appears to be successfully evoked in half of the cells tested and is most pronounced between ten and fifteen minutes of a fifteen minute response. The NMDA responses in the individual experiments were more consistent. All records display significant decreases in tau weighted during both application of 50 µM NMDA, suggesting the observation that NMDA application can increase the frequency of IPSCs is reproducible as well as that these preparations respond to activation of presynaptic NMDA receptors. In all four records, that had NMDA added, at least one application was not only able to significantly reduce tau weighted when compared to the control period but also when compared with the sections used to record the response to the stimulus. As some periods during the response were themselves significantly smaller from control, this further significant decrease indicates that activating presynaptic NMDA receptors with 50 µM NMDA and 10 µM glycine is much more potent at increasing the frequency of IPSCs than activating them with the endogenous retrograde release of glutamate induced with a sustained depolarisation. Since all preparations tested respond well to activation of presynaptic NMDA receptors with application of an exogenous agonist it is disappointing that a higher proportion of cells did not display a
significant increase in IPSC frequency as a result of attempting to evoke DPI. One cell may not have displayed DPI due to the high frequency of events meant increasing the frequency further would have been difficult, although the preparation did respond to NMDA. Changes in tau weighted caused by DPI might not be big enough to be significantly different. It is notable in every record the tau weighted describing the section of record between 10 and 15 minutes after the stimulus was smaller than the control value even if these changes were not always significant.
Among the occasions when DPI causes a significant decrease in tau weighted there are two different ways a significant difference are achieved (Fig 5.11). There can the usual changes in the individual time constants where there is a significant reduction in tau slow which resulting in an overall decrease in tau weighted. This would appear as an increase in the frequency of the events that are not within a burst of events (although they may be at the start of a burst). Significant reduction in tau weighted caused by DPI

**Figure 5.10.** There tended to be an increase in IPSC frequency after a depolarising stimulus and when 50 µM NMDA was applied (n = 4, except the control and first period after the stimulus where n = 5). The common mean of tau weighted looking for DPI was plotting with the tau weighted values from individual experiments. There is a significant reduction in tau weighted compared to the control between 10 and 15 minutes after the depolarising stimulus (indicated by a star). There were also significant reductions in the common mean for tau weighted during both applications of NMDA.

Among the occasions when DPI causes a significant decrease in tau weighted there are two different ways a significant differences are achieved (Fig 5.11). There can the usual changes in the individual time constants where there is a significant reduction in tau slow which resulting in an overall decrease in tau weighted. This would appear as an increase in the frequency of the events that are not within a burst of events (although they may be at the start of a burst). Significant reduction in tau weighted caused by DPI
can also lack significant changes in the individual time constants $\tau_{fast}$ and $\tau_{slow}$.

This is often accompanied by an increase in $A_f$. A significant difference caused only by an increase in $A_f$ would mean bursts will tend to have more events but the interval duration between events within a burst would remain relatively constant. There is no reason to suppose that this process cannot also happen in addition to a significant reduction in $\tau_{slow}$. Applying 50 $\mu$M NMDA caused a significant reduction in $\tau$ weighted mostly through significant reductions in the value for $\tau_{slow}$. The increase in the frequency of IPSCs would appear as a greater number of events that either are not part of a burst of events or appear at the start of a burst, but not within a burst. The bursts that follow the events will be relatively similar to the bursts under control conditions. This repeats and confirms the finding that applying NMDA to a Purkinje cell nerve-bouton preparation can increase the frequency of IPSCs as was observed in the previous chapter.
Figure 5.11. DPI can cause increases in IPSC frequency described by significant reductions in tau weighted. Values are time constants +/- standard error. a) Changes in fitted time constants from fits of interval distributions in response to evoking DPI. Significant reductions compared to controls are indicated by a star. Note there can be a significant decrease in tau weighed without a significant decrease in either individual time constant. b) Interval distribution of IPSCs under control conditions. There were 55 events, tau fast was 37.1 +/- 8.22 ms, and tau slow was 8299.9 +/- 1152.2 ms. c) Interval distribution between 5 and 10 minutes after the stimulus had 198 events, tau fast was 17.0 +/- 2.71 ms, and tau slow was 2963.8 +/- 379.44 ms. The value of the peak of the slower curve has significantly decreased compared to control resulting in entire distribution being described by a significant decrease in tau weighted. d) Distribution of intervals between 10 and 15 minutes after the stimulus, where 146 events form a distribution with a tau fast of 13.3 +/- 1.46 ms and a tau slow of 4195.1 +/- 424.4 ms. A significant reduction occurs in tau weighted which is not replicated by tau slow.
Activation of presynaptic NMDA receptors often results in an increase in the amplitude of the postsynaptic events whether they are IPSCs, EPSCs, or a mixed population. If DPI is considered in isolation an increase in event amplitude would be expected as DPI is mediated by NMDA receptors in the nerve terminals, presumably the same ones that can cause an increase in event amplitude in the nerve-bouton preparation. However there were no incidences of significant increases in IPSC amplitude compared to the control after attempting to evoke DPI (Fig 5.12a). The lack of amplitude increases after the stimulus is not only surprising because DPI works through NMDA receptors which are able to increase the amplitude of events when activated by an exogenous agonist, but also because the depolarising stimulus would be expected to induce rebound potentiation (RP) (Kano et al., 1992). RP is where a calcium influx to the Purkinje cells causes an increase in the sensitivity of the postsynaptic GABA\(_A\) receptors to GABA resulting in the same vesicular GABA release inducing IPSCs with larger amplitudes. However studies into RP looked at the changes in amplitudes of mIPSCs, but in the current experiments looking at DPI recorded spontaneous IPSCs. Perhaps the larger amplitude of spontaneous IPSCs occludes any increase in the sensitivity to GABA by the postsynaptic receptors. There are only small changes in the relative frequency of amplitudes after attempting to evoke DPI (Fig 5.12b). Examining relative changes in a record that exhibits DPI shows a slight increase in the proportion of the smallest events and a tendency for an increase in medium sized events. The slight increase in the smallest events in the later sections of the response recording DPI could be due to a slight increase in the bursts of IPSCs. An increase in small bursting events may also contribute to the slight reduction in the median amplitude in later sections of the response (Fig 5.12a). Applying NMDA did not often result in an increase in IPSCs amplitude in the cells used to study DPI. Out of eight
applications of NMDA (four cells each treated twice) there were only two incidences of a significant increase in amplitude which appeared in two separate records. This may mean that the lack of significant increases in amplitude when attempting to evoke DPI may be because of the cells and not because of DPI or RP is unable to increase the amplitude of IPSCs. However, applying NMDA did occasionally result in a significant increase in amplitude (Figure 5.13a). In the previous chapter the effects of NMDA was studied in a cell that was unusual in that it exhibited discrete large and small IPSCs. In records that had a more continuous range of IPSCs a significant increase in amplitude caused a noticeable decrease in the relative frequency of the smallest events and an increase in the relative frequency of slightly larger events (Figure 5.13b). The decrease in the relative frequency of the smallest events can be seen even when applying NMDA does not cause a significant increase in amplitude.
Figure 5.12. There was not an increase in amplitude in response to a depolarising stimulus. a) There were no significant increases in amplitude compared to the control after DPI was evoked. Amplitude represented by box plots were edges represent the 1st and 3rd quartile and the whiskers represent the range from 5% to 95%. b) There were few notable changes in the relative frequency of amplitudes compared. The later sections of the response show a slight increase in smaller event, possibly part of IPSC bursts. The increase in smaller events may explain the slight decrease in median amplitude in events between 10 and 15 minutes post stimulus.
Figure 5.13. In DPI experiments NMDA could increase the amplitude of IPSCs. a) Applying NMDA at the end of an experiment can occasionally result in a significant increase in IPSC amplitude (significance denoted by an asterisk). b) When there is a significant increase in amplitude there is a decrease in the relative frequency of the smallest events relative to the control (as indicated up a positive percentage change). This also appears when there is not a significant increase in amplitude. Also when there is a significant increase in amplitude there is a noticeable increase in events with amplitudes slightly greater than the smallest events.
5.4 Discussion

In the Purkinje cell nerve-bouton preparation a phenomenon fitting the description of depolarised-induced potentiation of excitation was not found. It was thought since activation of presynaptic NMDA receptors causes an increase in the frequency and amplitude of spontaneous EPSCs, those same presynaptic NMDA receptors could be activated by the endogenous retrograde release of glutamate in a process analogous to DPI (Duguid & Smart, 2004). DPE was not seen because the preparations were unable to respond to activation of presynaptic NMDA receptors. When NMDA was added fifteen minutes after the depolarising stimulus was evoked it was capable of inducing an increase in the frequency and amplitude of events. This confirms a similar finding in the previous chapter.

Previous studies report that being able to evoke the retrograde release of glutamate from Purkinje cell nerve-bouton preparations remains intact (Duguid et al., 2007) so presumably the lack of DPE cannot be attributed to impaired release mechanisms or calcium dynamics. The presence of an inward current after depolarisation that could be attributed a calcium-activated chloride current, as it also occurs immediately after depolarisation, (Llano et al., 1991a) would also suggest calcium dynamics inside the Purkinje cell are not impaired. However there was a lack of two other calcium dependent processes. One process is DISC but this might not have been seen because expression of the current shows some variability depending on which lobules of the cerebellum the Purkinje cell is from which would be difficult to determine with vibrodissociated cells (Kim et al., 2009). Another calcium dependent process that seemed absent is RP where there is an increase in amplitude of mIPSCs (Kano et al., 1992). However in these experiments, spontaneous IPSCs were recorded
which tend to have greater amplitudes than mIPSCs (Southan & Robertson, 1998a) so the increase in IPSC amplitude by RP might be obscured by the presence of larger events.

DSE is another calcium dependent process that would be expected to be seen when Purkinje cells are depolarised. If DSE could be established to have occurred it would support the proposed health of the Purkinje cell nerve-bouton preparations. However, the effects of DSE has been described on evoked EPSCs where it reduces the amplitude of events, increases the threshold required to evoke an event, and changes the paired-pulse facilitation (Kreitzer & Regehr, 2001), so it is not known how DSE would affect spontaneously occurring EPSCs. After the depolarising stimulus there was often a decrease in EPSC frequency. The underlying change was a decrease in bursting behaviour from the excitatory inputs, since bursts are calcium dependent (Auger & Marty, 1997) the calcium influx, and therefore bursts, could be inhibited by activation of a 4-AP sensitive potassium current by endocannabinoids (Daniel & Crepel, 2001). This could be a DSE-like mechanism and since it appears long lasting it could be occluding observable effects of DPE.

The clearest test to see if the retrograde release of glutamate was intact in the nerve-bouton preparation was to induce DPI. A significant increase in IPSC frequency occurred in half of the records tested suggesting DPI could be induced and that while the retrograde release of glutamate did exist it could not have the same effect on the frequency and amplitude of EPSCs as activating presynaptic NMDA receptors. As some significant changes can take place because of changes in \( A_f \) and later sections of the record express more of the smaller events it is possible DPI sometimes increases the frequency of events by increasing bursting behaviour. The values for \( \tau_{fast} \) do not change so the intervals within bursts do not change but the length of bursts should
increase as indicated by increases in $A_I$. Perhaps this reflects how the endogenous retrograde release of glutamate can act which is not replicated by a prolonged bath application of a NMDA receptor agonist. If DPI was observed it appears the endogenous glutamate that normally activates the presynaptic NMDA receptors are less potent than 50 µM NMDA co-applied with 10 µM glycine. Adding NMDA replicates and confirms the earlier finding that activating the presynaptic NMDA receptors can increase the frequency of IPSCs without the need for the somatodendritic compartment of the afferent inhibitory neuron.
5.5 Summary

- DPI, a form of plasticity with inhibitory inputs that requires retrograde transmission of glutamate and presynaptic NMDA receptors, has been demonstrated to be maintained in Purkinje nerve-bouton preparations.

- However despite the continuance of retrograde glutamate transmission and having inputs that can be enhanced by activation of presynaptic NMDA receptors an analogous process was not seen with excitatory inputs.

- After applying a stimulus that should evoke retrograde glutamate release, a decrease in the frequency of excitatory events is occasionally seen.
Chapter 6: Discussion

In this chapter the main findings will be summarised, considered for validity and how they relate to the wider body of knowledge in the literature. Further experiments will be suggested to either clarify apparent disagreements with the literature or investigate any intriguing possible implications of the findings.

6.1 Validity of the nerve-bouton preparation

The nerve-bouton preparation is a little used preparation that has been around since the early Nineteen Nineties (Vorobjev, 1991). Designed to provide isolated neurons for electrophysiology from brain slices that did not have to be treated with proteases, the nerve-bouton preparation does provide for some interesting experimental procedures. The usefulness of the nerve-bouton preparation comes from the nerve terminals remaining attached to the postsynaptic cell. The neurons are isolated from the surrounding cells that would have been in close proximity in a brain slice, such as glial cells and other neurons, as well as being separating the neurons from their neuronal networks (Akaike & Moorhouse, 2003). Apart from the terminals, no other compartments of the formerly afferent cell remains as presumably if the somatodendritic compartment remained it would be seen with a bright-field microscope and it is unlikely that much would remain of the slender axons after vibrodissociation. It is thought that the isolated preparations are mostly free from fragments of the surrounding cells; Purkinje cells have been isolated lacking immunoreactivity for markers specific to Bergmann glial cells (Duguid et al., 2007). A proportion of these nerve-boutons derived from formerly presynaptic cells remain spontaneously active.
without the need to be stimulated into releasing neurotransmitter vesicles. It is quite possible other non-spontaneously active nerve-boutons are retained that are still able to release neurotransmitters but are require to be induced into doing so.

As the preparation consists of just the postsynaptic cell and the nerve terminals any alterations in the properties of neurotransmitter-induced events in response to experimental treatments must be acting on one of the two compartments. When making whole-cell recordings the two compartments can be controlled differently with the intracellular solution selectively acting on the postsynaptic cell but not the nerve-boutons. If changes in the postsynaptic cell can be controlled for or minimised any responses to experimental manipulations can be attributed to actions on the nerve terminals.

The nerve-boutons do have some disadvantages compared to brain slices. The cells cannot be identified by their relative position to other cells and gross brain structures. Also it is harder to stimulate one particular input as afferent connections are reduced to just the terminals, although neurotransmitter release can be still be evoked by a stimulating electrode (Akaike & Moorhouse, 2003). The frequency, type, and amplitude of events do vary in the nerve-bouton preparations, sometimes widely. This can be a particularly limiting factor if the experiment relies on spontaneously occurring events and a particular type or minimum frequency of events is required. The events that occur spontaneously may not always be the same sort of events that occur spontaneously in slices, presumable some nerve terminals behave differently when not under the influence of the rest of the presynaptic cell, and not all types of events can occur spontaneously.
Despite some disadvantages it is though Purkinje cells are an appropriate cell to study with a nerve-bouton preparation. After vibrodissociation isolated Purkinje cell nerve-bouton preparations can be successfully identified by being comparatively larger than other cells. Their ovoid shape and remains of the initial section of the dendritic tree can also aid identification. Rat Purkinje cells differ from most other central neurons as they do not express functional NMDA receptors (Perkel et al., 1990; Monyer et al., 1994) after the first week of development (Rosemund et al., 1992b). The lack of functional NMDA receptors can help confirm the identification of nerve-preparations derived from rat Purkinje cell of sufficient maturity. If a NMDA receptor agonist is added to a nerve-bouton preparation under conditions favourable to NMDA receptor activation during or after a whole-cell recording experiment and no slow prolonged inward current is not induced the cell is likely to lack functional postsynaptic NMDA receptors, and therefore a Purkinje cell. However there are other cell types that do not express functional NMDA receptors yet display events, for example oligodendrocyte precursor cells located in the hippocampus (Bergles et al., 2000), but these cells are comparatively smaller than Purkinje cells and found in a different brain region. In all the whole-cell recordings of putative Purkinje cells that were applied with NMDA (not all cells were tested in this way) no type of inward current was ever induced that was not observed during control conditions. This suggests the visual identification of Purkinje cell nerve-bouton preparations has been entirely successful. As visual identification works, incidences where cells were not or could not be tested with NMDA application, for example in some imaging experiments, are also likely to be Purkinje cells.

Purkinje cell nerve-bouton preparations were derived from cerebellar slices from P10 rats. The age was used as it is a compromise between being old enough to have
developed connections and not expressing postsynaptic NMDA receptors (Rosemund et al., 1992b) whilst being young enough to be frequently viable after the rough process of vibrodissociation. Given that recordings from the cell can be successfully made and that synaptic events can be observed while inward NMDA induced currents cannot, it seems the compromise in the age used was successful.

Throughout this study conclusions have been made about nerve-boutons afferent to Purkinje cells without ever having directly recorded their properties. The properties of the afferent nerve-boutons are inferred from changes in neurotransmitter release which are observed as events of inward current recorded from the postsynaptic cell. The inward currents are likely to be the result of neurotransmitters activating ligand-gated ion channels in the postsynaptic cell membrane. The events have similar amplitudes and time course to those induced by synaptically released neurotransmitters (Southan & Robertson, 1998b; Isope & Barbour, 2002). Also they can be blocked with appropriate antagonists against ligand-gated ion channels and have their polarity reversed when held around potentials where the ligand-gated ion channel currents are expected to reverse.

The spontaneously occurring events are either GABA<sub>A</sub> receptor mediated IPSCs or AMPA receptor mediated EPSCs. The identity of the two types of events has been repeatedly confirmed by the complete absence of events when blockers against both types of receptors have been applied. The two different types of events have often been studied in isolation. It might be a mistake to assume the total postsynaptic events is equivalent to the isolated IPSCs in addition to the isolated EPSCs. When an antagonist against either receptor is applied it not only blocks the postsynaptic receptor but also those receptors in presynaptic terminals which may change the event frequency during the course of an experiment. It has been reported that activation of GABA<sub>A</sub> receptors in parallel fibre terminals enhances the release of glutamate (Pugh & Jahr, 2011) which
may occur when there are mixed events but not when EPSCs are isolated with bicuculline application. Also when DNQX is applied glutamate will not be able to potentiate GABA release from molecular layer interneurons by activating AMPA receptors reported to be in their nerve terminals (Rossi et al., 2008). Although GABA and glutamate mediate most of currents seen in the postsynaptic cell the possibility that other mediators are released from nerve terminals should be considered. Even when the postsynaptic receptors of a synapse are blocked, it is possible that terminal may still continue to release mediators that can influence postsynaptic currents such as nitric oxide (Shin & Linden, 2005) or adenosine (Atterbury & Wall, 2009) particularly under conditions where those synapses are also thought to be stimulated.

Although GABA_A and AMPA receptors appear to mediate all the postsynaptic currents induced by the synaptic release of neurotransmitters, some records displayed events that seemed unlikely to be caused by either receptor. These were the long slow events (LSEs) which had typical amplitudes in excess of a nanoampere and had a decay consisting of several components which lasts for several seconds. The LSEs appeared only rarely in earlier records and no opportunity arose where their sensitivity to bicuculline or CNQX could be tested. Since earlier nerve-bouton preparations were thought less refined and consistent than the later preparations, this might suggests the vibrodissociation process may itself influence what types of events are seen. As only some records displayed LSEs there would have been too few opportunities to try and manipulate the spontaneously occurring currents, for example by investigating their sensitivity to a range of antagonists. If LSEs were induced by the synaptic release of neurotransmitters it might be difficult to replicate the time course closely enough with an application of agonist so it is recognisable as the same process. Also using a selective agonist might not be able to replicate LSEs if they were caused by multiple processes
occurring closely together. A likely candidate for at least a component of LSEs would be the slow EPSC. This is when a slowly rising and slowly decaying inward current can be induced by activation of postsynaptic metabotropic glutamate receptors which open channels that contain the TRPC3 subunit (Hartmann et al., 2008). However this does not account for the large amplitude component with the rapid decay that is always seen at the start of LSEs. If glutamate is responsible for the LSEs the large amplitude component with the rapid decay could be accounted for by a possible climbing fibre input (Konnerth et al., 1990b) or activation of glutamate transporters in the Purkinje cell (Canepari et al., 2001). Were the LSEs at least partly mediated by the slow EPSC, it may indicate that a sustained release of glutamate can occur from parallel fibres as this is the mechanism used to induce them in slices (Hartmann et al., 2008). Although the mechanisms that cause the LSEs may not be positively identified it was assumed that they were induced by neurotransmitter release and thus contributed to the overall frequency of events. This assumption has some support from an early experiment where NMDA application appears to increase the frequency of LSEs along with the increase of postsynaptic events thought to be induced by neurotransmitters.

Event frequency was described by fitted time constants describing the distribution of intervals. Deriving two different time constants from peaks in the interval distribution when the distribution is manipulated in a manner normally used for the analysis of the openings of single ion channels (McManus, et al., 1987; Sigworth & Sine, 1987) proved to be quite informative when deducing what alteration were behind changes in event frequency. It is unlikely that two time constants could have been as accurately fitted without transforming the distribution as the range of intervals can be orders of magnitude different. The technique was however often limited if there were too few events as this decreased the accuracy of fitting time constants. As many drug
applications lasted for around a minute, time constants were often poorly fitted unless there was an obvious increase in the frequency of events and made worse when the drug application caused a decrease in the frequency. To get around this problem, experiments in the future should have fewer longer-lasting drug applications so the effects can be observed on a larger population of event intervals.

The appearance of two peaks in the transformed interval distribution was not restricted to one type of input as it was seen when both IPSCs and EPSCs were recorded in isolation. The two peaks represent a minimum of two processes happening during neurotransmitter release. The two peaks indicate that instead having a fixed probability of release there is a short period of time after an event occurs where there is an increased likelihood of another event occurring. As there is in turn an increased likelihood that another event will occur after a previous event this could cause a series of events occurring separated by short intervals resulting in a burst. When bursts occur the first event may be quite large but the one that follow tend to be a similar amplitude if they are from the same source (Auger & Marty, 1997). It is this tendency to observed bursts that the faster time constant is thought to correspond to; with the time constant value corresponding to the duration of the intervals within bursts and the relative area that the faster peak contributes to the interval distribution, $A_f$, corresponding to the level of bursting activity within an interval distribution. When observing events in a postsynaptic cell, bursting behaviour is a different process than a higher frequency of events, as bursts are thought to originate from one input whereas the overall frequency depends on every afferent input (Auger et al., 1998).

When there is an increase in the frequency of events the fast tau rarely changes significantly. If the value was really unchangeable, this might suggest bursting is an intrinsic property of spontaneously occurring events. However when there is an increase
in event frequency no changes might also be seen if the system has reached a maximum. This is more likely as there are occasions where tau fast can change. For example when there was a DSE-like phenomenon affecting spontaneous EPSCs the $A_f$ decreases indicating less bursting occurs. The endocannabinoids that mediate DSE with evoked EPSCs decrease neurotransmitter release by activating potassium channels in the parallel fibres which in turn affect the presynaptic voltage-gated calcium channels that control release (Daniel & Crepel, 2001). A similar mechanism may work with a DSE-like effect on spontaneous EPSCs.

TTX and cadmium were applied to test if presynaptic voltage-gated sodium and voltage-gated calcium channels played a role in inducing synaptic events. Both treatments reduced the frequency of events. Cadmium had a longer lasting effect on the amplitude of events than the frequency; presumably blocking presynaptic voltage-gated calcium channels had the same effect as removing extracellular calcium in depleting intracellular stores which the larger events require (Llano et al., 2000). Besides an overall decrease in frequency, the two treatments often affected $A_f$ to the extent that a fast peak could not be detected. The lack of the faster peak may have been because during the brief applications of the drugs there were not enough events to construct a representative interval distribution. To eliminate this possibility and confirm the lack of the faster peak the experiments could be repeated but with longer durations of either TTX or cadmium, indeed initial results suggests prolonged applications of cadmium do lack burst of events and a corresponding faster peak in the interval distribution (D.C.H. Benton (UCL) unpublished results). Further experiments could be carried out to see if the same effect occurs with more selective voltage-gated calcium channels blockers and establish if the same voltage-gated calcium channels control bursting from inhibitory and excitatory inputs.
If a reduction in bursts of events occurs with TTX and cadmium application by blocking presynaptic channels there may be a common mechanism. This is likely to be the calcium influx mediated by the voltage-gated calcium channels which will either be blocked directly by the cadmium or not activated when there is an absence of action potentials caused by TTX application. Certainly a non-additive effect of blocking presynaptic voltage-gated calcium channels when action potentials are also inhibited has been reported with IPSCs from basket cells (Stephens et al., 2001). Presumably a similar mechanism also exists with excitatory inputs as the experiments were done with mixed spontaneous events. If bursts, represented by the faster of two peaks, were induced by action potentials activating voltage-gated calcium channels, it would mean the intervals between mini events during TTX application could be adequately described by a single curve, which has found experimentally and been reported (Llano et al., 2000).

If the bursts of events do depend on calcium rises in the nerve terminals, the calcium may not need to be provided by presynaptic voltage-gated calcium channels. Sustained bursts were seen from basket cells when treated with low concentrations of alpha-latrotoxin, a spider toxin that causes calcium permeable holes to form in the cell membrane (Auger & Marty, 1997). It was seen experimentally that activation of presynaptic NMDA receptors can not only increase the frequency of events during TTX application, it can reintroduce bursts of events where they were absent in the presence of TTX alone. The NMDA receptors could conceivable provide the calcium for this process but it is likely a proportion of it will be provided by activating voltage-gated calcium channels (Glitsch, 2008). Bursts can occur even when action potentials are inhibited without the need for an influx of external calcium. Basket cell terminals are thought to have spontaneous transients in calcium occurring at sites of neurotransmitter
release which are enhanced by a low concentration of 10 µM ryanodine (Conti et al., 2004) which can also increase the frequency of IPSCs (Bardo et al., 2002) which require two exponentials to describe the interval distributions unlike when there was only TTX (Llano et al., 2000). The rise in calcium in response to the ryanodine is approximately the same as an action potential (Conti et al., 2004) suggesting the ability to induce bursts from the inhibitory terminals is robust. However as parallel fibre terminals do not have calcium stores that can be induced to release calcium (Carter et al., 2002) so presumably will depend on action potentials to produce bursts of EPSCs possibly through trains of action potentials (Isope et al., 2004).
6.2 Presynaptic potassium channels in nerve-boutons

The higher concentrations (200 µM, 500 µ, and 1 mM) of 4-AP were the most effective potassium channel blocker tested at inducing an increase in neurotransmitter release. The sensitivity of afferent nerve-terminals to 4-AP agrees with findings in the literature the same drug can increase the frequency of spontaneous IPSCs from basket cell terminals (Tan & Llano, 1999). Part of this effect is likely to be caused by the inhibition of voltage-gated potassium channels located in the terminals. The terminals of several basket cell axons form a dense layer of connections around the initial segment of the Purkinje cell’s axon called a pinceau (McNamara, et al., 1996) from where it is possible to make whole-cell recordings (Southan & Robertson, 2000). Recording from the pinceau reveals there are at least two distinct populations of voltage-gated potassium channels, one population has an IC₅₀ for 4-AP of around 8 µM where as the other component has an IC₅₀ of approximately 8 mM (Southan et al., 2000). To get an effect on event frequency by applying 4-AP, it appears the component of the pinceau current with an IC₅₀ of 8 µM for 4-AP has to be blocked. This may correspond to an effect first being seen in the nerve-bouton preparation between 20 and 50 µM 4-AP; although this does suggest more than half of that 4-AP sensitive component need to be blocked before a significant change in neurotransmitter release is seen. Interestingly these voltage-gated 4-AP sensitive potassium channels are specifically located to the terminals as no 4-AP sensitive potassium current could be evoked from the soma of the basket cells (Southan & Robertson, 1998b). This raises a possible situation where the nerve-bouton preparation could be of use to study neurotransmitter when channels or receptors are not expressed in presynaptic cells but are restricted to the nerve-terminals. The range of potassium channels blocked by 4-AP is broad so it is hard to discern which channels 4-AP blocks and how those channels affect neurotransmitter release. More specific
blockers or enhancers could be used, but as it was seen linopirdine, glibenclamide, and UCL 1848 has no effect during control conditions suggesting against a role for M-current, $K_{ATP}$ and SK potassium channels. Since some voltage-gated potassium channels are blocked by 4-AP (Southan & Robertson, 2000) the block is likely, but not necessary, to be involved in increasing the frequency of action potentials. However, voltage-gated potassium channels can also influence the resting membrane potential (Zhang et al., 2010). To eliminate the possibility that channels that maintain the membrane potential may also be blocked and influencing neurotransmitter release, 4-AP could be applied to nerve-bouton preparations that have the initiation of action potentials blocked with TTX to see if an effect still takes place.

Applying TEA up to a concentration of 1 mM had no effect on the frequency or amplitude of events. However in almost mature mice a lower concentration of 200 µM was able to significantly increase the amplitude of spontaneous IPSCs in a Purkinje cell (Southan & Robertson, 1998a). Even more puzzling is using only slightly more mature rats, P12-15 compared to P10, the slightly higher concentration of 2.5 mM TEA caused an increase in both the frequency and amplitude of IPSCs from basket cells (Tan & Llano, 1999). A concentration of 2.5 mM TEA or higher could be added to a Purkinje-cell nerve bouton preparation to establish if the reason no response was seen was because the concentrations used just fell short of an effective dose. Also to investigate if a TEA sensitive current is present, it could be investigated if a TEA-sensitive current can be evoked in whole-cell recordings of the pinceau as has been reported in mouse (Southan & Robertson, 2000), although this will only show what currents can be evoked in the pinceau which are not necessarily the ones that influence neurotransmitter release under control conditions. If this experiment were to be attempted it would need to be done in brain slices as the pinceau does not appear to be left entirely intact after
vibrodissociation from viewing the cells with a bright field microscope, but the results found should be applicable to the remaining inputs retained from basket cells.

Regardless of whether an effective concentration was applied or not, the lack of response does indicate that potassium channels that are sensitive to 1 mM TEA are either absent in the nerve-terminals or are not active in playing a role in regulating neurotransmitter release. For example homomeric Kv1.1 channels are perhaps not likely to be involved in neurotransmitter release as they have an IC$_{50}$ for TEA of around 0.5 mM and would have been largely blocked at the concentrations applied (Al-Sabi et al., 2010). Species differences are not likely to be behind the differences as the results from rats were significant for both an increase in amplitude and frequency as well as being closer in developmental age (Tan & Llano, 1999). However, small alterations can change the sensitivity to blockers like TEA. It was found that in Kv1.1 Kv1.2 heteromers different arrangements of the subunits in the tetrameric structure affect the channel’s sensitivity to TEA (Al-Sabi et al., 2010). By expressing different arrangements of concatemers, where subunits are modified so that several subunits are expressed as single protein, it was found that when the different subunits are expressed next to each other they are less sensitive to TEA than if the different subunits were expressed diagonally from each other. The IC$_{50}$ for Kv1.1 Kv1.2 concatemers where the different subunits are expressed diagonally from each other is approximate 1 mM. The lack of effect in the nerve-bouton preparation with 1 mM TEA could be because the hetromeric Kv1.1 Kv1.2 channels are not arranged diagonally, assuming that blocking around half the current would have an effect. Heteromeric potassium channels with Kv1.2 and Kv1.2 subunits are likely to be involved in neurotransmitter release as they are located at basket nerve terminals (Wang et al., 1993; Mcnamara et al., 1993). In mouse at least some of the TEA sensitivity is caused by the presence of the Kv1.1
subunit. Kv1.1 knockout mice lack increases in the frequency or amplitude of postsynaptic IPSCs in Purkinje cells when TEA is applied (Zhang et al., 1999). Although it is also thought some of the TEA sensitivity comes from blocking high-threshold voltage-gated potassium channels made up of Kv3 subunits (Southan & Robertson, 2000) which are expressed in the pinceau and presumably other basket cell terminals (Bobik et al., 2004). If Kv3 subunits were responsible for TEA sensitivity seen in the literature but not the nerve-bouton preparation it could be because the terminals in the nerve-bouton preparation are insufficiently developed to express Kv3 subunits at P10. Taking the large glutamatergic calyx of Held synapse in the auditory system as an example, as development progresses the duration of the presynaptic action potential decreases as the expression of presynaptic Kv3 increases (Ishikawa et al., 2003; Dodson et al., 2003). Interestingly both Kv3 and Kv1 subunits were detected in the calyx of Held (Dodson et al., 2003). However, whereas Kv3 subunits were detected in the terminal, Kv1 subunits were found in a transitional zone between the end of the axon and the beginning of the terminal. This observation may be relevant to afferent terminals to the Purkinje cell but it might not matter too much as the vibrodissociation process is unlikely to be precise enough to sever the axon right up to the nerve terminal.

Application of 4-AP was seen to cause a notable increase in the amplitude of events. Although observed with a mixed population of events it is possible these events relate to the large IPSCs that come about from large multivesicular release induced by calcium transients (Llano et al., 2000) given that 4-AP application can increase the calcium in the terminals of molecular layer interneurons (Tan & Llano, 1999). Some records showed that large amplitude events run down back to control levels over multiple application of 4-AP. The amplitudes did not run down to values smaller than the amplitudes during the control period, so were probably related to the 4-AP
application and not because of the cell. It is possible that the multiple applications of 4-AP were able to exhaust internal calcium stores which would result in a decrease in amplitude (Galante & Marty, 2003). If this supposition is correct depleting internal calcium stores before 4-AP was added may retain the increase in frequency but limit the increase in amplitude, although perhaps not abolish it altogether as addition calcium could be sourced from a 4-AP induced depolarisation of nerve-terminals that can activate voltage-gated calcium channels (Shoudai et al., 2007).

The depolarisation and increased calcium in a nerve-terminal that occurs as a result of 4-AP application, but not the application of 4-AP itself, may activate further potassium channels such as the calcium- and voltage-sensitive BK channel. The narrowing of the presynaptic action potential and the increased action potential-evoked calcium rise which is induced by 4-AP can be augmented by application of TEA or charybdotoxin (Tan & Llano, 1999). Both these drugs block BK channels, but TEA application up to 1 mM had little or no effect on its own as it was seen from the Purkinje cell nerve-bouton preparation. It is likely that applying 4-AP increased the calcium rises enough to activate BK channels which in turn will narrow the action potential, which can be reversed and increase the calcium rise if the BK channels are themselves blocked. It would be interesting to see if BK channels can modulate neurotransmitter release from nerve-boutons. BK channels have been reported at the pinceau formed from several basket cell axons (Misonou et al., 2006). It should be relatively simple to infer a role for BK channels by blocking them once they are activated. This has been done in a hippocampal slice where BK channels were inferred to play a role in neurotransmission by blocking them with TEA, but after the application of 4-AP which presumably was needed to cause a calcium rise and depolarisation sufficient to activate the BK channels (Hu et al., 2001). However in hippocampal
organotypic cultures BK channels can modulate release under control conditions and do not need to be activated before being blocked with more selective drugs (Raffaelli et al., 2004). It is possible that BK channels do mediate neurotransmission in the cerebellum, although not necessarily from terminals afferent to the Purkinje cells, as a BK channel knockout mouse does display ataxia which is often associated with problems in the cerebellum (Sausbier et al., 2004).

Experiments with potassium channel blockers were done with a mixed population of events which could potentially contain both IPSCs and EPSCs, although IPSCs occur more reliably. Experiments could be repeated with only one type of input present. But it is likely that release from parallel fibres are controlled by a similar repertoire of presynaptic potassium channels as the parallel fibres do express a 4-AP sensitive potassium channel (Daniel & Crepel, 2001) and are sensitive to blockers of Kv1.1 and Kv1.2 potassium channels (Chen et al., 2005). There is also expression of Kv3 subunits as indicated by that the successful use of a promoter that normally drives Kv3 subunit expression can be harnessed to successfully express a calcium sensitive fluorescent protein in the parallel fibres of a transgenic mouse (Qiu & Knopfel, 2007). In a knockout study it was found that the lack of Kv3.1 and Kv3.3 slowed action potential repolarisation which caused broadening of action potentials in parallel fibres (Matsukawa et al., 2003). The same knockout mice also exhibited the lowering of a threshold to induce postsynaptic events from parallel fibres, changes in the ability to respond to certain frequencies of stimulation, and a reduction in the parallel fibre’s sensitivity to TEA application (Matsukawa et al., 2003).
6.3 Actions of presynaptic NMDA receptors

Activating NMDA receptors in nerve-terminals increases the frequency and amplitude of both inhibitory and excitatory events. The conclusions made about the NMDA induced increases in event properties were mostly done with cells displaying mixed events which potentially consisted of both inhibitory and excitatory events; but the increase in isolated inhibitory and excitatory events has been repeated and confirmed in the experiments described. At the concentrations tested (up to and including 50 µM) NMDA caused a significant increase in the frequency of mixed events roughly half the time. The results of activating presynaptic NMDA receptors have been found to be quite variable; at least in the hippocampus (McGuinness et al., 2010b). However in later experiments, such as the end of the DPI experiment, NMDA is more reliable at increasing the frequency of events. This is not an effect of using higher concentrations of NMDA as in earlier experiments all concentrations tested were roughly equally good at inducing an increase in event frequency. It may be because as more experience was gained, healthier, more consistent nerve-bouton preparations were being produced and better recordings were being made from them. Although there will be some preparations that have an event frequency that are not increased by the activation of presynaptic NMDA receptors. These preparations tend to be at the extremes of the range of observed frequencies. Preparations that cannot be influenced by NMDA often have either a very high frequency of events that cannot be increased further, such as was seen in one of the experiments where magnesium sensitivity of the NMDA effect was tested, or an exceptionally low frequency of events presumably as they have very few nerve-boutons that can be stimulated.
The NMDA receptors have to be in the nerve terminals as the postsynaptic Purkinje cell does not express the receptor (Perkel et al., 1990; Monyer et al., 1994). This has been repeatedly confirmed by the inability of NMDA application to induce a postsynaptic current that is distinct from the transient postsynaptic events mediated by ligand-gated ion channels. If functional postsynaptic NMDA receptors were present a prolonged inward current would be expected to be seen upon application of NMDA, as indeed it has in other cell types under similar conditions. Since there is no other components of the afferent cells remaining it means the NMDA receptors must be located in the nerve-boutons where they would be ideally positioned to influence neurotransmitter release. Although NMDA receptors must be located in the nerve-boutons there is no reason to suppose at the moment that they have to be in the terminals where the increased release of neurotransmitter is actually coming from. For example if NMDA receptors were located in inhibitory terminals but not excitatory terminals, activation of the NMDA receptors may cause release of a modulator which then influences release of EPSCs (Shin & Linden, 2005). Although this could be the case, the response to NMDA might not be so rapid. Presumably if an intermediate messenger was required this could be confirmed by applying appropriate blockers or by activating the system downstream of where NMDA has an effect.

The evidence for the involvement of NMDA receptors mostly comes from the results of adding micromolar concentrations of NMDA with 10 µM glycine. Glycine application alone does not appear to influence neurotransmitter release, so inclusion of NMDA is critical to the effect. Glycine was not included in the control solutions. The observation that the effects of NMDA application are sensitive to external magnesium supports the proposition that NMDA receptors are involved in the increase in neurotransmitter release (Nowak et al., 1984b). However to further support that NMDA
receptors are involved and that the effect was not due to some nonspecific effect or contamination of the bath applied NMDA it should determined if a sufficient concentration of a competitive NMDA receptor antagonist such as AP5 (Olverman et al., 1984b) could block the increase in neurotransmitter release.

Activating presynaptic NMDA receptors in Purkinje cell nerve-bouton preparations causes an increased frequency under control conditions when both excitatory and inhibitory events are present. The NMDA induced increase in inhibitory events corresponds well to previous results from the literature done in slices (Glitsch & Marty, 1999; Duguid & Smart, 2004) however the results are not entirely compatible. Previous studies in slices differed from the experiments using the nerve-bouton preparation as they looked at only mIPSCs. Mini IPSCs are thought to be caused by release of a single vesicle of GABA and therefore they are considered to correspond to the smallest size of event that can be produced by vesicular neurotransmitter release. The mIPSCs are isolated by applying TTX which results in blocks action potential propagation into the nerve terminals from the soma which halts the incidences of action potential induced multivesicular release. Adding TTX isolates the terminal from action potentials propagating from the presynaptic cell meaning it is more isolated from the rest of the afferent cell than it would be without TTX. This was not necessary in the nerve-bouton preparation as nerve terminals were mechanically isolated so spontaneously occurring IPSCs could be studied without being influenced by the rest of the afferent cell (a mixture of mini IPSCs and IPSCs induced by action potentials that occur without being evoked). Also previous studies in slices were done with the presence of extracellular magnesium whereas, to improve the likelihood of seeing a NMDA effect, extracellular magnesium was removed in experiments using nerve-bouton preparations. If conditions corresponding to experiments in slices were applied
to the Purkinje cell nerve-bouton preparations it is possible that NMDA would not cause a significant difference in event frequency. Applying TTX to the nerve-bouton preparation so that only minis are present may result in NMDA causing an increase in frequency less often, if the effects of brief applications of TTX on mixed events are applicable to mIPSCs. But having magnesium present throughout the record is likely to occlude the NMDA effect entirely if the results on a mixture of excitatory and inhibitory events are indicative. However in the experiments looking at the magnesium effect on the NMDA-induced increase in frequency, magnesium was applied for only a short period of time during ongoing NMDA application which may have a more severe effect than if magnesium was present throughout the record. Indeed it has been reported that the presence of extracellular magnesium can potentiate NMDA receptors responses (Paoletti et al., 1995). Further experiments could be done to see if adding TTX and external magnesium throughout an experiment would always occlude an increase in frequency caused by NMDA as interpreted from previous results. If NMDA could increase the frequency of mIPSCs when TTX and extracellular magnesium is present, it would suggest the results from brain slices and the nerve-bouton preparation are entirely compatible.

Most experiments involving the NMDA induced increase of neurotransmission were done with preparations displaying a potential mixture of inhibitory and excitatory events. But, as there is some uncertainty and variability about which types of events are present in preparations, more definite conclusions could be made in the future when either only inhibitory or excitatory events are present. Since the properties of the inhibitory and excitatory events are not distinct from each other even under control conditions the most robust way to distinguish between the different types is to block one sort with an appropriate antagonist. Distinguishing events pharmacologically was
preferred to differentiate them based on their time courses as the time courses were not distinct. Blockers were also preferred to changing the internal solution so that outward and inwards currents are produced depending if an anionic or cationic conductance was activated as it was thought the analysis programs may have trouble with detecting potentially overlapping events with different polarities. Although inhibitory and excitatory events can be successfully isolated the type of synapse they come from is not always certain.

The molecular layer interneurons produce a high frequency of spontaneous IPSCs in slices (Konnerth et al., 1990b) so are likely to mediate a major proportion of the inhibitory events displayed in the Purkinje cell nerve-bouton preparation under both spontaneously under control conditions and when release was stimulated with NMDA. Inhibitory inputs are also made from Lugaro cells and other Purkinje cells. Inputs from Lugaro cells can be distinguished by being less sensitive to being blocked by bicuculline (Dean et al., 2003). However with the excitatory events blocked, 50 µM bicuculline was able to block all the remaining events. In the DPI experiments there were rare occasions when some events remained after bicuculline was applied during application of NMDA even after allowing 30 seconds for the antagonist to wash-in. But on closer inspection the remaining events are all blocked eventually during the rest of the bicuculline application. This may relate to the relative positions of the cell to the perfusion system and not due to insensitivity to bicuculline. This suggests inputs from Lugaro cells do not play a major role in either spontaneous or NMDA-induced neurotransmission. Nerve-boutons that were formerly from recurrent axon collaterals from adjacent Purkinje cells should be able to induce IPSCs in the postsynaptic cell. However Purkinje cells do not tend to receive many inputs from recurrent axon collaterals, possibly no more than one (Watt et al., 2009), the resulting IPSCs have a
small amplitude of around 50 pA, and they need to be stimulated in slices (Orduz & Llano, 2007) so they are poor candidates for mediating spontaneous IPSCs and if they did the IPSCs would probably have at most a minor contribution to the overall frequency of IPSCs. It is likely the majority of inhibitory inputs either under control conditions or being stimulated by NMDA application originate from molecular layer interneurons. Certainly the observed amplitudes of IPSCs are similar in size and frequency to those reported from molecular layer interneurons (Vincent & Marty, 1996).

It has been reported that calcium transients can be induced in axonal varicosities of stellate cells by depolarising the cell by either directly stimulating it or by applying a NMDA agonist to the somatodendritic component of the cell (Christie & Jahr, 2008). However when a NMDA agonist was applied to the axonal varicosities, which are swellings in the axon thought to correspond to sites of neurotransmitter release, no calcium transients were seen. This finding is reported to be repeatable with axon varicosities from basket cells (Christie & Jahr, 2008). The study inferred that there are no NMDA receptors in nerve terminals and any effect caused by applying NMDA receptor agonists are caused by depolarisation induced in the somatodendritic compartment of the presynaptic cell which propagates to the nerve terminals of the stellate cell presumably resulting in increased neurotransmitter release. This is at odds with the findings with the nerve-bouton preparation as presumably when NMDA does induce an increase in IPSC frequency there should be a calcium increase in the afferent nerve-boutons, be it either from the NMDA receptors themselves or subsequent activation of voltage-gated calcium channels (Glitsch, 2008), as well as calcium induced calcium release (Duguid et al., 2007). It would have been noticed if the somatodendritic compartment had been retained in the nerve-bouton preparation and the IPSC frequency
and amplitude are most likely to be from molecular layer interneuron inputs. It should be noted the study made very thorough use of imaging calcium transients and the same group could observe calcium rises in axonal varicosities in response to activation of other ligand-gated ion channels in cortical neurons (Christie & Jahr, 2009), but there was no record of what happened to the IPSC frequency when a NMDA agonist was applied to the axon. To reconcile these different findings it would be an interesting future experiment to perform calcium imaging with the nerve-bouton preparation to see if applying NMDA can cause a calcium rise in the afferent terminals. It is possible to monitor calcium transients in a nerve-bouton preparation by treating the cells with calcium sensitive fluorescent dyes that can permeate the membrane and then diluting out the dye in the post synaptic cell when a whole cell recording is formed so that the dye is only retained at useful concentrations in the nerve-boutons but not the postsynaptic cell (Ye et al., 2004). To ensure any calcium rise seen occurred in inhibitory nerve-boutons the study could be carried out in conjunction with a strain of mice that express green fluorescent protein (GFP) in their interneurons (Lopez-Bendito et al., 2004). Nerve-bouton preparations from Purkinje cells in these mice do retain fluorescent green boutons presumably from molecular layer interneurons (M. Caldwell (UCL) unpublished observations). If calcium transients were seen in the same nerve-bouton as green fluorescence when a NMDA agonist is applied that would be a good indicator that inhibitory terminals do express NMDA receptors that mediate calcium rises when activated. The proposed experiment could be repeated with 20 µM cyclopiazonic acid (CPA) to make it more similar to the calcium imaging study (Christie & Jahr, 2008). CPA had been added to deplete internal calcium stores to aid localising where the calcium transients originate. However in doing so the authors of the study may have made it harder to observe a calcium rises that should accompany
activation of NMDA receptors in nerve terminals as the calcium rise is partly mediated and prolonged by calcium-induced calcium release (Duguid & Smart, 2004) which would normally free calcium from internal calcium stores provided they are not depleted.

Activating presynaptic NMDA receptors also increases the frequency of EPSCs. Purkinje cells receive two very different excitatory inputs: a variable multitude of small inputs from parallel fibres or a single large and invariable input from the climbing fibre (Konnerth et al., 1990b). It would be quite informative to find out if it is possible to get inputs from both types of excitatory synapse. Under control conditions most of the EPSCs have quite small amplitudes which suggest they are from parallel fibres. Inputs from parallel fibres can have quite large amplitudes if evoked with a stimulus that is able to activate several parallel fibres at the same time. Presumably in Purkinje cell nerve-bouton preparations relying on spontaneous release there is no easy way to recruit several separated inputs from parallel fibre boutons so the majority of events will be individual inputs from just one bouton at a time. Certainly the amplitude of the vast majority of EPSCs under control conditions seems approximately within the same amplitude range of unitary EPSC induced by stimulating a single parallel fibre, which can be up to 30 pA (Isope & Barbour, 2002). There are a few rare EPSCs with higher amplitudes under control conditions; but given that there tends to be a high frequency of EPSCs when cells do display EPSCs it is possible that the larger events are two events that occur so close together that they could not be distinguished as individual events and appear to have a greater amplitude.

When NMDA is applied there is an increase in the EPSC amplitude beyond the range of amplitudes seen under control conditions. As these events are likely too big to be single inputs from parallel fibres (Isope & Barbour, 2002) it is worth considering if
the climbing fibre has a role. An initial approach would be to investigate if the climbing fibre has been retained in the nerve-bouton preparation. A possible approach is to infer that climbing fibres have been retained by looking for immunoreactivity against the vesicular glutamate transporter 2 (vGluT2) which among terminals afferent to Purkinje cells is specific for climbing fibres (Zander et al., 2010). If immunoreactivity is found attempts could be made to evoke release from the climbing fibre nerve-bouton.

Assuming the input from the climbing fibre can still be identified by its invariable large amplitude, it is apparent that applying NMDA will not evoke release from the terminal. If the climbing fibre could be stimulated and still be recognisable as such, applying an elevated concentration of potassium or adding 4-AP when EPSCs are isolated should be able to evoke release. It is also possible to stimulate nerve-boutons directly with a stimulating electrode (Akaike & Moorhouse, 2003). Stimulating around the apex of a Purkinje cell should be able to evoke an EPSC from the climbing fibre. It is possible that during vibrodissociation the climbing fibre is damaged to the extent that either it is incapable of releasing glutamate or the release properties have changed so the EPSCs are no longer identifiable as originating from climbing fibres.

If the climbing fibre is not responsible for producing EPSCs with increased amplitudes during NMDA application must come from parallel fibres. The variability in the size of the larger EPSCs suggests the climbing fibre is not involved. Also the increase in the larger events corresponds to a decrease in the relative frequency of the smaller events so they are likely to have the same source. However the increased amplitude is difficult to attribute to single inputs from parallel fibres. It is possible fragments of the large and convoluted climbing fibre terminal (Kakizawa et al., 2005) are viable and are able to induce larger EPSCs but not large and invariable enough to be characterised as such. As the frequency increases with NMDA application the chances
of parallel fibre EPSCs occurring simultaneously increases which could appear as events with greater amplitudes. Cells that display EPSCs tend to have a narrow range of high frequencies of excitatory events, so instead of waiting to record from a preparation with a lower frequency, the event frequency could be lowered. One way to lower the event frequency is to decrease the extracellular calcium concentration (Llano et al., 2000). If a cell with lowered frequencies of EPSCs could still have increased amplitudes when NMDA is applied then it is not likely to be caused by simultaneously occurring events. But this mechanism is unlikely as it is possible to observe an increase in EPSC amplitude when NMDA is applied whilst a reduction in frequency occurs. Nerve-boutons retain the ability to generate action potentials which contribute to neurotransmitter release and presumably the excitatory nerve-boutons retain this ability too. A parallel fibre can respond to a single stimulus by firing several action potentials which can result in EPSCs consisting of several close together peaks (Isope et al., 2004). Although this effect is not seen in brain slices before the age of P15, it may be relevant at P10 if the nerve-boutons derived from parallel fibres are more excitable than they would be in a slice. The presence of polyphasic EPSCs could be deduced if the NMDA induced increase in excitatory events ceased in the presence of TTX which will block the initiation of action potentials. Parallel fibres are able to release multiple vesicles simultaneously (Foster et al., 2005). Multiple vesicle release can be enhanced by increasing extracellular calcium (Bender et al., 2009). An increase in extracellular calcium might have an effect by increasing the amount of calcium entering the nerve boutons, something activated NMDA receptors located in excitatory nerve-terminals could reproduce or potentiate and explain the increase in amplitude. However an increase in extracellular calcium does not necessary result in an increase in intracellular calcium in a bouton meaning the effect cannot be replicated by activating NMDA
receptors. For example it has recently been reported in the hippocampus that increasing extracellular calcium causes an increase in the frequency of mEPSCs not by affecting voltage-gated calcium channels but by activating the calcium sensing receptor (Vyleta & Smith, 2011). If this multiple vesicular release could be enhanced by activating presynaptic NMDA receptors it could explain the increase in EPSC amplitudes. For this to be a likely mechanism it would mean the glutamate that causes EPSCs does not saturate the postsynaptic receptors under control conditions. Attempts were made to keep the state of the postsynaptic cell constant; however it is possible although unlikely that the increase in EPSC amplitude is caused by changes in the postsynaptic cell. Treatments that could be tried to prevent changes in postsynaptic cell include nitric oxide scavengers (Casado et al., 2000) and internal applications of a calcium chelator (Casado et al., 2002). Postsynaptic changes in receptors have been reported in Purkinje cells as a result of activating presynaptic NMDA receptors (Casado et al., 2000). However this nitric oxide dependent mechanism can reduce the amplitude of EPSCs to around 50% of the control value so when an increase in amplitude is observed it means this mechanism was either not seen or the increase in EPSC amplitude is partially occluded. Nitric oxide should be able to be produced in the Purkinje-cell nerve bouton preparations as these have a punctuate immunoreactivity for nitric oxide synthase (NOS), although the identity of the boutons are not known (S. L. B. Gordon-Smith (UCL) unpublished results, anti-NOS antibodies a generous gift from K. Bartus (UCL)).

An unresolved issue is does NMDA activate separate groups of presynaptic NMDA receptors located respectively on the inhibitory and excitatory terminals or are they only located on one type of synapse and influence the other type via some intermediate messenger. There are reports of presynaptic NMDA receptors influencing neurotransmitter release at one synapse but are located elsewhere (Qiu & Knopfel,
2007; Shin & Linden, 2005). If differences could be found in the subunits that comprise the NMDA receptors that mediate the increase in inhibitory and excitatory events it would confirm they are from separate populations and support the idea that different synapses have separate populations of NMDA receptors in their respective terminals. One approach is to use immunohistochemistry to identify if different combinations of NMDA subunits are present at different synapses with the identity of the synapses confirmed with appropriate markers. Immunoreactivity has been seen for NR2A, B, C, and D at terminals of cultured molecular layer interneurons (Duguid & Smart, 2004) and the presence of NR2A and to a lesser extent NR2B has been detected at the terminals of parallel fibres (Bidoret et al., 2009). However this confirms which subunits are present and not which subunits are needed for the increase in neurotransmitter release. To start to distinguish which subunits are involved in the increases in neurotransmitter release different pharmacological manipulations could be applied to detect if the NMDA receptors are sensitive to treatments that affect particular subunits. Many treatments that distinguish between different subunits are allosteric modulators and not competitive antagonists. Presynaptic NMDA receptors in the cortex have been reported to contain NMDA receptor subunit 3A (NR3A) forming receptors that presumably retain a NR2 subunit by their sensitivity to AP5 (Larsen et al., 2011). However given the lack of sensitivity to external magnesium that NR3A subunits exhibit it is unlikely that they contribute to the presynaptic NMDA receptors that causes increases in neurotransmitter release. The potency of magnesium block on the NMDA effect is so pronounced it suggests that the subunits that are less sensitive to magnesium, NR2C and NR2D, are also not strongly expressed in the relevant NMDA receptors. However there are antagonists that show some selectivity for NR2C and NR2D over other subunits which may be used to eliminate the possibility that these subunits are in
the NMDA receptors (Feng et al., 2004). 4-{3-[4-(4-fluoro-phenyl)-3,6-dihydro-2H-pyridin-1-yl]-2-hydroxy-propoxy}-benzamide (Ro 8-4304) is an allosteric modulator that works in a similar fashion to ifenprodil but is more selective in inhibiting NMDA receptors containing the NR2B subunit and would be a good place to start to infer which subunits are in the NMDA receptors that mediate the increases in neurotransmitter release (Kew et al., 1998). Receptors containing NR2A subunits are distinctive in that they can be partially inhibited by nanomolar concentrations of extracellular zinc including when they are present in heteromers (Paoletti et al., 1997). There is another drug, (R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl]-methyl]-phosphonic acid (NVP-AAM077), which is used to selectively block NR2A containing NMDA receptors. Although prior treatment with NVP-AAM077 shows some selectivity during steady-state agonists applications, the selectivity decreases when it is used to block NMDA receptors responding to synaptic inputs (Frizelle et al., 2006) so it might be an alternative to blocking a response to NMDA application but less suitable for testing on DPI which presumably is more synaptic like in its actions.

It would be interesting if the NMDA receptors that cause an increase in EPSCs were sensitivity to blockers selective for NR2A. Not only is there some dispute if the effects of activating presynaptic NMDA receptors are located to parallel fibres (Qiu & Knopfel, 2007; Shin & Linden, 2005) but when they are thought to be present they are not thought to influence the release of glutamate but mediate production of nitric oxide (Casado et al., 2000). If they did contain NR2A subunits it would suggest these NMDA receptor might be the same ones that activate nitric oxide synthase in parallel fibres (Bidoret et al., 2009). If the NMDA receptors that are located on parallel fibres and that produce nitric oxide in slices affected glutamate release in the nerve-bouton preparation
it might mean the nerve-boutons derived from the parallel fibres are more excitable when separated from the rest of the parallel fibre and granule cell. This could just be an artefact of using nerve-bouton preparations or there may be physiological situations where the excitability of the parallel fibre changes enough, such as switching off of a potassium conductance for example, so that presynaptic NMDA receptors become able to influence neurotransmitter release in slices.
6.4 Retrograde release of glutamate and the lack of DPE

It is possible that a process fitting the description of depolarised-induced potentiation of excitation does exist. The term has been used before to describe phenomena in other brain regions (Haj-Dahmane & Shen, 2009; Carta et al., 2010). In both cases the involvement of NMDA receptors had been ruled out by the lack of effect caused by applying the NMDA receptor antagonist AP5. The approach into investigating DPE differed from the one present in this thesis. When the term DPE has been applied to a phenomenon, it was first observed, described as DPE then attempts were made at understanding how it was mediated. However here DPE and the mechanism that induces it were proposed to exist first which was then followed attempts to confirm the hypothesis.

There are good reasons to suppose DPE might exist in Purkinje cells as an analogous mechanism to DPI. Given that applying NMDA to nerve-bouton preparations caused an increase in the frequency of EPSCs it seemed likely the retrograde release of glutamate, which can mediate DPI, could activate the same presynaptic NMDA receptors that can increase glutamatergic events. DPI had previously been reported in nerve-bouton preparations and had been confirmed in the experiments presented here. Inducing DPI was necessary to confirm the continuance of the ability to induce retrograde glutamate release to be sure DPE did not occur for other reasons. DPI was seen in the common mean and in half of the individual experiments which confirms the expected result of retrograde glutamate release. However there was one notable difference between the induction of DPI reported here and in the literature. When DPI was induced with a depolarising stimulus there was an increase in the amplitude of the mIPSCs caused by rebound potentiation which was also induced by the depolarisation
and the resulting calcium increase (Duguid & Smart, 2004). Rebound potentiation is where in response to a rise in intracellular calcium the postsynaptic GABA<sub>A</sub> receptors that mediate the IPSCs in the Purkinje cells increase their sensitivity to GABA, synthetically evoked or otherwise, resulting in an increase in the IPSC amplitude (Kano et al., 1992). The lack of rebound potentiation could be because the increase in amplitude is occluded by the larger spontaneous IPSCs that can be induced by action potentials in the nerve-boutons of the preparation. However in these experiments there were only the occasional increase in amplitude when NMDA was applied which is also known to cause an increase in IPSC amplitude, so perhaps the cells were unable to have increases in amplitude.

It is possible DPE does not exist in the proposed form in Purkinje cells. This would be the simplest explanation why DPE was not observed. This would be likely if there were just a few release sites for retrograde glutamate release and they were restricted to sites near inhibitory inputs so that the retrograde glutamate could not reach the NMDA receptors that mediate excitatory inputs under any physiological circumstances. Certainly having a low number of vesicles for retrograde glutamate release might explain why FM dye did not appear to be taken up in the soma when a depolarising stimulus was applied to Purkinje cells nerve-bouton preparations (Y. Pankratov unpublished observations). However if DPE was possible via the proposed mechanism there are many possible reasons why it was not observed. It would be informative to investigate the reasons why DPE was not observed as it may reveal further information about the nature of the excitatory nerve-boutons and the retrograde release of glutamate.

One parameter that was changed in the later DPI experiments and that could have affected DPE was the perfusion system. If the glutamate was required to travel a
distance greater than the width of a synaptic cleft it would be liable to be washed away by the actions of the perfusion system thus reducing its effective concentration. This proved an unnecessary precaution as DPI can be induced whilst the perfusion system running and repeating the pDPE experiment with the perfusion system off does not change the result that there are no increases in the frequency of EPSCs (D. C. H. Benton unpublished results). This suggests the retrograde release of glutamate is not affected by the perfusion system flowing at the rate used in these experiments.

DPE may exist but is not observable as its effects could be occluded by other actions of retrograde messengers induced by the same depolarisation. If this were the case DPE could be unmasked by using appropriate antagonists. For example in a previous instance when DPE was described, it was only observed after being unmasked by blocking CB₁ receptors (Haj-Dahmane & Shen, 2009). DSE has been previously studied on evoked EPSCs so it is not sure what effects it would have on spontaneous EPSCs. With evoked EPSCs from both parallel and climbing fibres there were reductions in amplitude and changes in paired-pulse facilitation after a depolarisation-induced retrograde release of endocannabinoids (Kreitzer & Regehr, 2001). After the depolarisation in experiments investigating pDPE there appears to be a DSE like effect where there are reductions in the bursts of EPSCs. It would be interesting to see if this was a DSE effect that is mediated by the retrograde release of endocannabinoids and can be blocked by CB₁ receptor blockers.

As glutamate is thought to be the messenger in retrograde release, the glutamate that is likely to be released by the depolarising stimulus should be able to act on several different type of glutamate receptor in nerve terminals, the actions of which may be able to occlude DPE. DSE has been reported to be mediated by the retrograde release of glutamate acting on kainate receptors in parallel fibre terminals in addition to
endocannabinoids (Crepel, 2007). This is one such potential process that could mask DPE however it is reported to occur in a narrow window of development between days P18 to 22. Also activation of kainate receptors in parallel fibres, while not always facilitating neurotransmission at every cell type, has been reported to enhance EPSCs in Purkinje cells at ages P13 to 17 which is closer in age to the P10 animals used in this study (Delaney & Jahr, 2002). The retrograde glutamate release could also activate metabotropic glutamate receptors in excitatory nerve terminals. Activation of mGluR4 in parallel fibre nerve terminals results in a depression of EPSCs (Abitol et al., 2008) which potentially could occlude DPE and contribute to DSE.

Excitatory inputs in general and the parallel fibres in particular, which are likely to be the main source of EPSCs, are located more distally than several of the inhibitory connections. Perhaps the lack of DPE, but presence of DPI, may be due to the increased distance from the presumed release sites of the retrograde glutamate located at the Purkinje cell soma and the excitatory terminals. Unfortunately it is difficult to reduce this intervening distance experimentally. Prolonging the retrograde release of glutamate could be a potentially useful manipulation to help understand the process regardless if distance is trying to be overcome or not. Increasing the depolarisation used to induce retrograde release of glutamate may prolong the glutamate transient. However the magnitude of the depolarisation already used is equivalent to the largest one used to evoke DPI in the literature and it might not be beneficial to prolong the depolarisation much more (Duguid et al., 2007). Transient increases in extracellular glutamate can be limited by the neurotransmitter being taking up by excitatory amino acid transporters (EAATs) (Wadiche & Jahr, 2005). The retrograde release of glutamate could be enhanced by applying EAAT blockers to prevent the up-take of glutamate (Duguid & Smart, 2004). Distance may not actually be an issue as it has been reported that at
certain developmental stages the retrograde release of glutamate can reach kainate receptors in parallel fibre terminals resulting in a decrease of excitatory transmission (Crepel, 2007). However the fact this is only an effect at certain developmental stages may actually mean distance is a problem if the reason the effect ceasing is caused by the development of the dendritic tree puts the kainate receptors on the parallel fibres out of range of retrograde glutamate release.

DPI relies on the release of additional calcium from internal stores to have an effect. Applying 100 µM ryanodine occludes DPI and limits the duration of an increase in mIPSC frequency when NMDA is applied (Duguid & Smart, 2004). This suggests calcium induced calcium release is required to see the full effect of activating presynaptic NMDA receptors in DPI and possibly the induction of DPI itself (although the calcium and retrograde signalling dependent DSI did not seem affected by the ryanodine). From experiments imaging calcium released from internal stores by applying caffeine it appears parallel fibres are not able to display calcium induced calcium release (Carter et al., 2002). Without calcium induced calcium release any DPE effect would be briefer compared to DPI meaning it is less likely to persist beyond any effects of DSE and more likely to be smaller in magnitude which might explain why DPE is not seen.

DPI has been shown to work through NMDA receptors. The NMDA receptors are activated by the retrograde release of a glutamate or glutamate like substance (Duguid & Smart, 2004). But for NMDA receptors to be activated they also require their NR1 site to be gated by an agonist (Johnson & Ascher, 1987a). Presumably when DPI is induced in a slice there is a sufficiently high enough concentration of small molecules, such as glycine or D-serine which can activate the NR1 site to permit NMDA receptor activation. If it were glycine activating the NR1 site as little as 10 nM
would be needed to see an effect (Johnson & Ascher, 1987a) and a concentration of just over 1 μM would be enough for the EC_{50} for heterologously expressed NMDA receptors containing any NR2 subunit (Chen et al., 2008). Apparently there is also a sufficiently high enough concentration of small molecules in the nerve-bouton preparation to also activate the NR1 site even though the preparation is isolated from surrounding cells and exposed to the solution (Duguid et al., 2007). At central synapses NMDA receptors can be potentiated by saturating the NR1 sites (Berger et al., 1998).

Perhaps the NR1 sites in presynaptic NMDA receptors are similarly unsaturated and act as a limiting factor to retrograde glutamate transmission. Limited activation of the NR1 site might explain the lack of DPE and the mixed results when investigating DPI in the nerve-bouton preparation. To investigate if a lack of NR1 activation is a limiting factor experiments investigating retrograde transmission could be repeated in a saturating concentration of glycine. Glycine of course has been seen to have no effect on its own.

A better option would be to use D-serine as even if it is less potent than glycine (Chen et al., 2008) its actions are prolonged as it has the advantage of being a poor substrate to be taken up by transporters (Berger et al., 1998). If this were the limiting factor an increased proportion of cells should exhibit DPI would be expected as well as observing DPE presuming insufficient activation of the NR1 was all that was lacking.
6.5 Future experiments

There are many directions future experiments could take to add to findings presented in this thesis. A general useful development would be to get more reliable stable cells. Experiments rely on there being a sufficiently high frequency of spontaneous events. To have more control of the frequency of events a stimulating electrode could be used to evoke events on demand. If it turned out, with a mixture of positioning and suitable thresholds, a particular type of input could be selectively activated it would open new experimental possibilities for the nerve-bouton preparation where one type of synapse could be studied in isolated from the rest of its presynaptic cell, any surrounding cells, and indeed any other types of nerve-boutons that happen to be on the same postsynaptic cell. It would be useful to know if the climbing fibre remains, whose presence could be determined by immunohistochemistry, and if it can be activated. If the climbing fibre were present it would be useful to know if it can be induced to mediate EPSCs, either by evoking it directly with an electrode or inducing neurotransmitter release pharmacologically such as with 4-AP when excitatory inputs are isolated. It would be interesting to determine if the apparent lack of climbing fibre inputs were the inputs were not recognisable as such or because they were absent from the nerve-bouton preparation.

The role of potassium channels in controlling neurotransmitter release could be developed further. It would be interesting to see if the apparent insensitivity to 1 mM TEA was because an insufficient concentration was used. Which potassium channels are involved could be elucidated further by using a wider range of blockers. As suggested it could be seen if the actions of 4-AP result in subsequent activation of potassium channels, such as BK channels, that are not involved in neurotransmitter release under
control conditions. Any interesting findings could be narrowed down to see if they are only applicable to excitatory or inhibitory inputs; which could be further supported with immunohistochemistry. Also these blockers could be combined with TTX to see if effective blockers act by enhancing action-potentials or if they act by depolarised the membrane potential in the bouton. As a side note, it could be investigated if the 4-AP induced increase in event amplitude was restricted to inhibitory inputs and requires calcium induced calcium release as was supposed.

The results that involve activation of presynaptic NMDA receptors should be ideally confirmed by abolishing the NMDA effect with at least one suitable blocker. Also to further the NMDA results it would be quite interesting to combine bath application of NMDA with calcium imaging, to confirm that activation of NMDA receptor in nerve-bouton do lead to rises in intracellular calcium. Experiments could be done to see how NMDA application on the nerve-bouton preparation compared to the literature. When considering the NMDA induced increase in IPSC frequency it would be interesting to see if an increase in event frequency still occurred under conditions similar to those used in slices, such as when magnesium and TTX is present. In regards to excitatory inputs it could be investigated if activation of presynaptic NMDA still caused a reduction in EPSC amplitude by stimulating NOS as was found in slices but appears to be absent or occluded in experiment done with the nerve-bouton preparation. It would be informative to know if the same types of NMDA receptors cause the increases in both inhibitory and excitatory events. It may be that appropriate treatments, such as NMDA subunit selective blockers or drugs that interfere with signalling cascades initiated by the activation of NMDA receptors, block the increase in one type of postsynaptic events which can be selectively ‘uncoupled’ from the activation of presynaptic NMDA receptors.
Understand why DPE was not seen but DPI was observed would be a rich source of potential experiments. Since Purkinje cell nerve-bouton preparations retain the ability to release glutamate as a retrograde messenger and have presynaptic NMDA receptors that enhance excitatory neurotransmission it is possible some sort of DPE analogous to DPI could occur, but it is not seen. If DPE could occur it could be occluded and attempts could be made to unmask DPE by blocking the actions of other retrograde messengers such as endocannabinoids or having the retrograde glutamate activating other glutamate receptors. If DPE is not occluded it could be investigated if the process is limited by a lack of ligands for the NR1 subunit or by the uptake of the retrograde glutamate by glutamate transporters.
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