Synaptic integration and dendritic excitability in cerebellar Purkinje neurons

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Abstract

Dendrites receive most of the synaptic input to the neuron, but their contribution to synaptic integration is not well understood. In this thesis I investigate synaptic integration in Purkinje neurons in cerebellar slices, focusing on the contribution of dendritic excitability using simultaneous somatic and dendritic patch-clamp recordings.

I first investigated the ionic conductances underlying intrinsic bistability in Purkinje cell action potential firing. The hyperpolarisation-activated mixed cation current I_h promotes the tonically firing state, with the firing rate being limited by the calcium-activated potassium current. Physiological modulation of I_h by serotonin is shown to enhance bistability triggered by inhibitory synaptic inputs.

I next explored the interaction between excitatory postsynaptic potentials (EPSPs) and voltage-gated channels. Near action potential threshold, parallel fibre (PF) EPSPs are non-linearly amplified. Application of the sodium channel blocker TTX abolishes this subthreshold EPSP "boosting". Strong depolarisation reveals an additional boosting mechanism which is mediated by calcium channel activation. Sodium channel-mediated boosting is perisomatic in origin, while calcium channel-mediated boosting is dendritic. The potassium channel blocker 4-aminopyridine abolishes the somato-dendritic polarity of calcium boosting and also hyperpolarises the voltage threshold of both sodium and calcium boosting. Blocking I_h increases the time constant of EPSP decay, thereby augmenting EPSP boosting. These results indicate that EPSPs are shaped by a complex interplay of several channel types depending on stimulus intensity and membrane potential.
I demonstrate that the very powerful synaptic input provided by the climbing fibre (CF) triggers an EPSP which depolarises the entire dendritic membrane to near zero mV. Pairing the CF with depolarisation or PF input triggered secondary calcium spikes which could be confined to dendritic branches and depended on the relative timing of CF and PF input. Inhibitory GABAergic synaptic input mimicked using dynamic clamp could completely suppress or delay CF-evoked dendritic calcium spikes in a temporally and spatially precise manner.
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Chapter 1: Introduction

It is a profound and necessary truth that the deep things in science are not found because they are useful; they are found because it was possible to find them.

Robert Oppenheimer

1.1 Dendritic research is a reborn field

Two types of processes extend from the soma of most neurons – a single axon and one or more dendritic arborisations. In the mammalian nervous system the axon is normally a long, thin (<1 µm diameter) process with relatively little branching. The purpose of the axon is to faithfully carry action potentials over distance and as such the axon is relatively uninteresting in terms of information processing, though the gating of action potential propagation has been proposed (Debanne et al. 1997).

Dendrites on the other hand are vastly more interesting in terms of the computational properties of the nerve cell. Their sheer density suggests a very important role in the functioning of the brain – an impressive 0.5 km/mm³ (Braitenberg & Schüz 1998) – and they receive the vast majority of the synaptic input made to the neuron. While dendrites certainly could increase the response repertoire and thus richness of computation of the individual neuron their contribution made to whole animal behaviour remains unclear. Only for a few mechanisms such as recurrent inhibition has the requirement been positively demonstrated (Koch 1999) through the need for spatial compartmentalisation.
through dendritic arborisation. I prefer to take the viewpoint that such elaborate and delicate structures do not arise without a purpose. The conservation of the dendritic structure of particular cell types across species further suggests a particular function of dendrites within each network.

In the groundbreaking work of Ramon y Cajal (1904), the neuronal structures of the central nervous system were described in detail for the first time. Simply observing the elaborate dendritic arbours on one side of the cell body and the long thin axon on the other side led Cajal to deduce the general direction of information flow in the nervous system. Cajal proposed that signals enter a neuron through a synapse at the level of the dendritic tree and then travel to the soma where signals arriving from everywhere else in the neuron are added up to form the output of the neuron. The output then travels along the axon, which in turn makes contacts with further neuronal dendritic trees along its course. In this view dendrites serve as “neuronal aerials” that pick up distant synaptic input and simply funnel to the soma. While that description covers some fundamental aspects of dendritic function, research over the past 50 years has shown that it is also woefully incomplete.
1.2 The cable equation

The biggest obstacle to progress in understanding dendritic properties has been technical. Dendrites are thin, delicate structures and thus hard to record from and manipulate. Only recently have experimental measurements on dendrites been made. As a consequence, most of our initial understanding of dendritic function came from theoretical considerations pioneered by Wilfred Rall (Segev et al. 1995). Rall applied cable theory, first developed for transatlantic telegraphic cables, to the dendritic tree. His characterisation of the electrical properties of a passive dendritic tree still underlies most present-day attempts at understanding dendritic function.

A stretch of dendrite can be considered as a cylinder of high resistance membrane surrounding a conducting core. The surrounding fluid has a comparatively low electrical resistance, which is usually assumed to be zero.

Figure 1.1
Diagram showing the electrical elements of a passive cable. From Johnston and Wu, 1995.
That leaves three parameters: the specific intracellular resistance, $r_i$, the specific membrane resistance, $r_m$, and the specific membrane capacitance, $c_m$, all of which are determined by the geometry of the dendrite and the properties of the neuronal membrane and intracellular milieu.

For a point $x$, some distance from the site of a current pulse, $i_i$, the voltage can be described as a decaying function of distance and time given by the resistance of the dendritic cable

$$\frac{\partial V_m(x,t)}{\partial x} = -r_i i_i$$  \hspace{1cm} (1.1)

However, $i_i$ is not constant but decreasing as current leaks out across the membrane as described by

$$\frac{\partial i_i}{\partial x} = -i_m = c_m \frac{\partial V_m}{\partial t} + V_m$$  \hspace{1cm} (1.2)

where $c_m$ is the membrane capacitance. Substituting from equation 1.2 into equation 1.1 and rearranging we obtain.

$$\lambda^2 \frac{\partial^2 V_m}{\partial x^2} = \tau_m \frac{\partial V_m}{\partial t} + V_m$$  \hspace{1cm} (1.3)
The two parameters in equation 1.3 $\lambda = \sqrt{\frac{r_m}{r_i}}$ and $\tau_m = r_m c_m$ are the space and time constants, respectively. Equation 1.3 is called the cable equation. The steady state solution for an infinite cable with a current injection at point $x = 0$ is plotted in figure 1.2. An adult rat Purkinje neuron is fairly electrically compact under steady state conditions with an electrotonic length of approximately $0.2 \lambda$ (Stuart & Häusser 1994; Roth & Hauser 2001).

**Figure. 1.2**

Diagram describing the steady state voltage decay to zero along an infinite cable in response to a current injection at point $x = 0$ (From Johnston and Wu, 1995). Under steady-state conditions the electrical length of Purkinje dendrites is $\sim 0.2 \lambda$.  

The two parameters in equation 1.3 $\lambda = \sqrt{\frac{r_m}{r_i}}$ and $\tau_m = r_m c_m$ are the space and time constants, respectively. Equation 1.3 is called the cable equation. The steady state solution for an infinite cable with a current injection at point $x = 0$ is plotted in figure 1.2. An adult rat Purkinje neuron is fairly electrically compact under steady state conditions with an electrotonic length of approximately $0.2 \lambda$ (Stuart & Häusser 1994; Roth & Hauser 2001).
1.3 Technological advances that allow the direct study of dendrites

As stated above, the main obstacle in studying dendrites is the experimental difficulties involved in making measurements from them. Two technological advances have each provided a quantum leap forward in our ability to make measurements from dendrites. First, the development of infrared differential interference contrast (IR-DIR) microscopy (Stuart et al. 1993) has meant that, using patch-clamp techniques, we can now make electrophysiological recordings from identified neurons in brain slices under direct visual control. The use of contrast enhancement combined with the reduced scattering of the longer infrared wavelengths of infrared light, means that dendrites become visible to the experimenter. Specifically, IR-DIC microscopy allows the experimenter to make several simultaneous electrophysiological recordings from the same neuron in order to directly assess spread of electrical signals within the neuron.

Second, the development of fluorescent calcium dyes and laser scanning microscopy has meant that we can now make detailed optical measurements, down to the level of the spines - \( \sim 1 \, \mu m \) - of the intracellular calcium concentration changes associated with most postsynaptic activity. Optical measurements are limited in time by the need to collect enough photons over a sufficient dendritic area for signals to be detected. It follows that the greater the area, the poorer the temporal resolution of optical calcium measurements. Presently the range of data acquisition rates is from \( \sim 50 \, Hz \) for two-dimensional line scan imaging up to \( \sim 2 \, kHz \) for one-dimensional line scans or
confocal spot detection. Particularly useful is two-photon laser microscopy, which permits very high spatial and temporal resolution in deep tissues, even in vivo (Denk et al. 1994).

1.4 Early biophysics

In the beginning of the 20th century Julius Bernstein proposed the membrane hypothesis. It suggests that at rest the neuronal membrane is selectively permeable to potassium ions (Bernstein 1902; Bernstein 1912). The intracellular concentration of potassium is higher than the extracellular meaning that potassium will tend to diffuse out of the cell leaving behind a negative charge; the membrane potential. The neuronal potential, as the action potential was called in those days, was thought to be due to a collapse in this permeability selectivity, essentially short-circuiting the neuron. Later the suggested increase in membrane permeability was demonstrated in the squid giant axon (Cole & Curtis 1939). However, with the first intracellular recordings of the action potential from the squid giant axon (Hodgkin & Huxley 1939; Curtis & Cole 1940) it was realised that collapse of selective permeability could not underlie the action potential. The simple reason is that during the action potential the membrane potential ‘overshoots’ zero mV to reverse the membrane polarity. A non-selective collapse in permeability can only bring the neuron to zero mV.

Following the development of the voltage clamp method, in four seminal papers (Hodgkin & Huxley 1952a; Hodgkin & Huxley 1952b; Hodgkin & Huxley 1952c; Hodgkin & Huxley 1952d), that would also win them a Nobel Prize, Hodgkin and Huxley described the ionic currents underlying the action potential.
They also introduced the, by now classical, Hodgkin-Huxley (HH) model of the action potential. Hodgkin and Huxley found the action potential to be composed of a transient inward sodium current followed by an outward potassium current which repolarises the membrane to reset the system.

**1.5 Ion channels of the neuronal membrane**

With only minor modifications, the HH-model of action potential generation has shown itself to be essentially correct. In fact, many of its predictions have later been confirmed experimentally (see Hille 1992 for review). Particularly, the discovery of gating currents and the demonstration (using the patch-clamp method) that ionic currents are passing through a number of different discrete channels in the neuron membrane has supported the HH-model.

In the HH-model of axonal action potential generation only two voltage-gated channels, a sodium and a potassium current, as well as a passive leak were necessary. That, though, does not explain the full picture of ionic conductances in neuronal membranes. Here a brief overview of our present state of knowledge will be given with the emphasis on three types of neurons that have received the closest study: layer 5 and hippocampal CA1 pyramidal and cerebellar Purkinje neurons. What we know about the distribution of ion channels across neuronal membranes comes from a variety of approaches. Cell attached voltage clamp experiments have provided direct measurements of ion channel activity (Magee 1999), antibody labelling has provided qualitative evidence for relative channel densities (Nusser 1999; Talley et al. 1999) and
sodium (Callaway & Ross 1997) and calcium sensitive dyes (Canepari & Mammano 1999) have been used to map the intracellular concentration changes of each ion.

1.5.1 Sodium channels

Sodium conductance has three major components, known as transient (Hodgkin & Huxley 1952a), persistent (Kay et al. 1998) and resurgent (Raman & Bean 1997). The persistent and the resurgent components probably arise as separate opening states of the transient channel (Kay et al. 1998; Raman & Bean 2001).

The properties of the transient sodium conductance are fairly uniform across cell types with a rapid activation and inactivation kinetics. Similarly the persistent component has been described in several cell types (Llinás & Sugimori 1980; Stafstrom et al. 1985; Andreasen & Lambert 1999). The resurgent component, which results from channels briefly exiting a blocked state during repolarisation, such as after an action potential, has so far only been described in Purkinje neurons (Raman & Bean 1997; Raman & Bean 2001), where it is thought to facilitate depolarisation in the interspike interval during continuous firing.

In layer 5 and CA1 pyramidal neurons the sodium channel density is relatively constant across the somato-dendritic axis (Stuart & Sakmann 1994; Magee & Johnston 1995). The properties of the channels also appear to be similar with the notable exception of an activity-dependent slow recovery from inactivation of the dendritic channels (Spruston et al. 1995). In Purkinje neurons
there are only few functional sodium channels present in the dendritic membrane (Llinás & Sugimori 1980; Stuart & Häusser 1994).

1.5.2 Calcium channels

Whereas a few excitable cell types do not possess sodium channels, such as crustacean muscle fibres (Hagiwara & Nakajima 1966), no excitable membrane has been found that does not express voltage-gated calcium channels. Calcium channels are found at lower densities than sodium channels in all cell types (Hille 1992).

A range of calcium channel subtypes exist that have been distinguished biophysically, pharmacologically, and more recently on a molecular level. The low threshold, low conductance transient calcium T-type channel is found in pyramidal cells as well as Purkinje cells, although at different densities (Takahashi et al. 1989; Markram & Sakmann 1994; Mouginot et al. 1997; Raman & Bean 1999; Talley et al. 1999). The high threshold, high conductance sustained L-type calcium is found in an appreciable densities in the CA1 (Takahashi et al. 1989) and layer 5 pyramidal (Markram et al. 1995; Schiller et al. 1997) neurons but less so in Purkinje neurons (Raman & Bean 1999). Finally, the P-type channel, named after Purkinje neurons where it was first discovered (Llinás et al. 1989; Usowicz et al. 1992), are also found in CA1 pyramidal neurons (Kavalali et al. 1997) and in layer 5 pyramidal neurons (Brown et al. 1993; Stewart & Foehring 2000). They are the predominant calcium channel type in Purkinje neurons (Usowicz et al. 1992; Raman & Bean 1999) with minor contributions from R and N type (Raman & Bean 1999).
The spatial distribution of the calcium channel types varies across the somato-dendritic axis. In CA1 pyramidal neurons there is a roughly constant overall density of calcium channels along the apical dendrite (Magee 1999); however, the density of L-type channels is higher proximally, while T-type channels are more even distributed (Christie et al. 1995; Magee & Johnston 1995). The distribution of P-type channels has, so far, not been studied in layer 5 neurons. The available evidence demonstrates the presence of calcium channels over the entire dendritic tree, including the distal-most branches in layer 5 pyramidal neurons (Schiller et al. 1995). The exact density and subtype distribution has not been studied, but calcium spikes can be generated in the distal apical dendrites (Schiller et al. 1997). A high dendritic density of calcium channels with P-type like properties, have been inferred from an early study on dendritic excitability in Purkinje neurons (Llinas & Sugimori 1980). The only direct measurements of P-type channel distribution (Usowicz et al. 1992), however, failed to find a somato-dendritic gradient. Interestingly, it has been suggested that P-type channels in Purkinje neurons may cluster around bifurcation points in the dendritic tree to form so-called ‘hot-spots’ of calcium channel activity (Hillman et al. 1991; Usowicz et al. 1992), although there is still no direct evidence to support this.

1.5.3 Potassium channels

Potassium channels are fundamental in dampening neuronal excitation. There is a wide range of potassium channel sub-types and a full description is outside the scope of the present introduction (see Jan & Jan 1997 for review),
here I will consider three categories: delayed rectifier, A-type and calcium-activated channels.

All three classes of K conductances are found in the neuron types under consideration here. Delayed rectifier, the type of potassium channel used in the HH-model (Hodgkin & Huxley 1952a), activates rapidly and inactivates on a scale of several seconds during membrane potential depolarisation, and upon repolarisation the channel quickly deactivates. These properties are ideal for the repolarisation of the membrane following during an action potential. The subcellular distribution of delayed rectifier is known in CA1 and layer 5 pyramidal cells, where the distribution is even across the somato-dendritic membrane (Hoffman et al. 1997; Bekkers 2000).

The A-type potassium current starts activating and inactivating at around \(-60\) mV (Hoffman et al. 1997), with the consequence that it is transient in nature. The activation is rapid (~1 ms) while the inactivation is slightly slower and voltage dependent (~2-8 ms over the range \(-30\) - \(+70\) mV) (Hirano & Hagiwara 1989; Hoffman et al. 1997; Bekkers 2000; Korngreen & Sakmann 2000). In the CA1 pyramidal neuron, A-type potassium conductance density increases five-fold as one moves along the somato-dendritic membrane (Hoffman et al. 1997). In layer 5 cells the density of A-type also increases along the somato-dendritic membrane, though not as dramatically as in CA1 cells (Bekkers 2000).

The distribution of calcium activated potassium channels \(K_{(Ca)}\) has not been measured directly but has been inferred as being low in the distal dendrites in CA1 and layer 5 pyramidal neurons. This is because there is little
evidence of an after-hyperpolarisation as expected if $K_{(Ca)}$ were prominent (Stuart et al. 1997; Poolos & Johnston 1999).

Of the above-mentioned three potassium channel types, only a high voltage activated transient potassium current, has so far been described in high densities in the dendrites of Purkinje cells (Martina et al. 2001). Delayed rectifier channels seem to be restricted to near the soma (Martina et al. 2001). It has been suggested that a high dendritic density of A-type current and $K_{(ca)}$ regulate the observed patterns of dendritic calcium spiking (see Magee 1999 for review). This issue will be addressed further in this thesis.

1.5.4 Hyperpolarisation activated current

Hyperpolarisation activated current ($I_h$), unlike other channels, is activated by hyperpolarisation (as the name indicates). The channel activates a mixed cation conductance with slow activation and inactivation kinetics; it starts activating at potentials hyperpolarised to ~ -60 mV and has a reversal potential of ~ -30 mV (see Pape 1996 for review).

The density of $I_h$ rises along the somato-dendritic membrane for both layer 5 (Williams & Stuart 2000) and CA1 pyramidal neurons (Magee 1998). The density along the somato-dendritic membrane in Purkinje neurons has not been studied in detail. However, current clamp measurements suggest a fairly even distribution of $I_h$ (Häusser & Clark 1997) (see also chapter 3).
1.6 Synaptic integration

1.6.1 Passive integration

Integration of synaptic input in a passive dendritic tree can generally be divided into two categories – summation of EPSPs and the influence of inhibitory post-synaptic potentials (IPSPs) on EPSPs.

When two inputs are activated simultaneously on the dendritic tree, the manner in which they summate will depend crucially on the location of the inputs relative to one another. If the inputs are in close proximity, the depolarisation at one site will be seen at the other site and reduce the driving force thereby reducing the size of the EPSP. Furthermore, since synaptic inputs activate membrane conductances, they will reduce the effective membrane resistance and thus the membrane space and time constant. Consequently EPSP originating in a background of other local EPSPs will decay faster and attenuate over a shorter distance than will a single EPSP on its own. Taken together this means that under passive conditions neighbouring EPSPs will summate sub-linearly.

On the other hand, two simultaneous EPSPs which are distant from each other will not interact with each other and should consequently summate linearly at the soma. Furthermore, given that two distant EPSPs will have travelled a greater distance through the dendritic tree prior to summing, both EPSPs will be attenuated. This has the side effect that the time window over which inputs can summate is increased. Thus EPSPs of distal origin arriving at the site of action potential initiation will be smaller and broader than proximally originating EPSPs.
– in other words, the neurons will display location-dependent summation of EPSPs.

The synaptic efficacy of EPSPs can be modulated by IPSPs. Inhibitory synaptic conductances caused by GABA_\text{A} receptor activation are chloride-dependent (Hille 1992) and therefore have a reversal potential near the E_{Cl}, usually in the region –75 to -80 mV. The effect of IPSP is known as a shunting inhibition. At rest the driving force of an IPSC is small, thus the net current through the open channels is small and it results only in a small voltage deflection. However, given the occurrence of an EPSP, the driving force for the IPSC increases and so does the current through the channels. A fixed inhibition near the site of an EPSP can be overcome by a larger EPSP; one can think of this as the total sum of the local conductances. A greater excitatory conductance can negate the presence of a neighbouring inhibitory conductance.

If, on the other hand, the inhibitory conductance is placed somewhere in the path from the excitatory synapse to the soma, the IPSP will always inhibit the EPSP. This, like the case of distant EPSPs summing, is because the synaptic conductances do not interact – only the resulting post-synaptic potentials. This latter form of inhibition will depend linearly on the voltage deflection away from E_{rev} of the IPSC – thus making the inhibition divisive in nature. It is true for all conductance changes that the local effect at the site of the conductance is governed mainly by the size and time course of conductance itself, only after the conductance is over will the membrane properties determine the shape of a synaptic potential. This is very important
when one thinks about shunting inhibition, because it means that shunting inhibition is localised in time and space.

In conclusion, the somatic post-synaptic potential generated by synaptic input is critically dependent on the synaptic activity in the vicinity of the input, as well as on the path to the soma - i.e. the synaptic input will effectively be modified by its context.

1.6.2 Active dendritic integration

Active properties of dendrites were first investigated in spinal motor neurons in the 1950’s (Brock et al. 1952). However, due to the difficulty dendritic experiments, little was made of the active properties of dendrites until recently when direct experiments became feasible. For the purpose of this overview I shall class active dendritic integration into three categories: the integration of synaptic potentials with excitatory dendritic conductances; the interaction of EPSPs with inhibitory input; and the generation and spread of dendritic spikes.

1.6.2.1 Summation of EPSPs in an active dendritic tree

Dendrites are now known to express a plethora of voltage gated ion channels though the exact distribution across the somato-dendritic membrane is cell type specific (see above and Magee 1999 for review). It is therefore likely that dendrites do more than simple passive filtering to the synaptic input on its propagation to the soma.

Synaptic potentials are affected by the cable properties during propagation through the dendritic tree. A number of voltage gated ion channels could potentially influence this process. Hyperpolarisation activated channels
have been shown to decrease the membrane resistance (Magee 1998), thereby further reducing EPSP propagation by means of current leak. For the integration of trains of input, this has the effect of normalising temporal summation (Magee 1999). It follows from cable theory that EPSP propagation from a high input resistance area to an area with low input resistance, as is typically the case for propagation dendrites → soma, is less effective than spread in the opposite direction. However, several studies have found that EPSPs of dendritic origin are amplified by persistent sodium channels (Stafstrom et al. 1985; Sutor & Hablitz 1989; Andreasen & Lambert 1999) near or at the soma (Deisz et al. 1991; Stuart & Sakmann 1995). It is possible that A-type potassium channels along the dendritic membrane limit dendritic regenerative activation of voltage gated ion channels (Hoffman et al. 1997).

1.6.2.2 Interaction of EPSP with IPSPs

Considerations from the passive dendritic tree dictate that an inhibitory input more efficiently affect somatic activity when placed proximally to the any excitatory input (Qian & Sejnowski 1990; Koch 1999). Experiments confirm this prediction from theory. It is now clear that dendritic inhibition may strongly reduce dendritic regenerative events with little influence on the somatic output pattern (Callaway et al. 1995; Miles et al. 1996). Similarly, inhibition near or at the soma may reduced action potential generation, leaving the dendritic activity relatively unperturbed (Callaway et al. 1995; Miles et al. 1996).

1.6.2.3 Dendritic spikes

As described above, the dendritic membrane contains a range of voltage gated ion channels. In some cell types, e.g. layer 5 pyramidal neurons, these
allow, in combination with a relatively low capacitance load, for the propagation of the action potential from the soma into the dendritic tree (Stuart et al. 1997; Vetter et al. 2001), a phenomenon known as backpropagation. In the Purkinje neuron, the absence of dendritic sodium channels, combined with the high capacitance load of the dendritic tree, means that action potentials propagate only poorly into the dendritic tree (Stuart & Häusser 1994).

Spikes can also be initiated in the dendrites. However, in a branched structure, a significant portion of any local depolarising current will dissipate into the surrounding structure rather than depolarise locally (Rapp 1997). This makes the initiation of dendritic spikes critically dependent of surrounding cable structure (Schaefer et al. 2000) and the distribution of synaptic input. Furthermore, dendrites have lower ion channel densities than does the site of axonal spike initiation. The combined effect is that, while voltage-gated ion channels may be activated, the *regenerative* activation initiates only poorly. Once initiated the propagation of dendritic spike is again dependent on cable structure, in a highly branching environment, such as the Purkinje cell dendritic tree, dendritic spikes propagate poorly (Vetter et al. 2001). Intense stimulation may trigger dendritic spikes (Chen et al. 1997; Golding & Spruston 1998), as may the combined effect of pre- and postsynaptic activity (Stuart & Hausser 2001). This is believed to be a basic mechanism for induction of synaptic plasticity. In the case of Purkinje neurons, with its poor backpropagation of somatic spikes, the pairing of climbing fibre and parallel fibre activity may trigger dendritic spikes. The initiation and spread of these spikes is described in chapter 5.
1.7 The cerebellum

1.7.1 The cerebellar cortical molecular layer

This thesis concerns the properties of dendritic integration in Purkinje cells of the cerebellar cortex. The cerebellum is one of the most regular neuronal networks in the CNS. Moreover, the basic circuitry of the cerebellum is preserved across the vertebrates. The structural regularity and the possibility to make direct across-species comparisons have made the cerebellum a prime target for attempts to decipher some basic principles of CNS activity.

The molecular layer of the cerebellar cortex has a very regular crystalline-like structure (Figure 1.3) (Palay & Chan-Palay 1974). From the region bordering to the granule cell layer the flat dendritic arbours of the Purkinje neurons project into the molecular layer, in a style reminiscent of open handheld fans stacked on top of each other. Orthogonally to the plane of Purkinje dendritic arbours run the parallel fibres, the axons of the densely packed granule cells situated in the area below the Purkinje cell layer in the aptly named granule cell layer. The parallel fibres make as many as 200,000 excitatory connections per Purkinje neuron. The second excitatory innervation to the Purkinje neuron, the climbing fibre, rises from the inferior olive through the white matter. In adulthood, each Purkinje neuron is innervated by one and only one climbing fibre, though the same climbing fibre innervates more than one Purkinje neuron.

Whereas the excitatory synaptic input to the Purkinje neuron arises mainly outside the cerebellar molecular layer, the neurons providing inhibitory input are located within the molecular layer. There are two main groups of
inhibitory neurons, the basket and the stellate cells. The distinction between the two types is anatomical and sometimes disputed. The axons of the inhibitory neurons run in the sagittal plane. Interneurons thus make inhibitory synaptic contacts with adjacent Purkinje cells, as well as with Purkinje cells hundreds of microns distant in the sagittal plane (Palay & Chan-Palay 1974). The interneurons are activated in a feed-forward manner by parallel fibres making direct contact with the Purkinje cell being recorded from (known as “on-beam” inhibition) as well as by parallel fibres adjacent to the Purkinje cell (“off-beam” inhibition (Eccles et al. 1967)

The anatomical arrangement of the cerebellar cortex is particularly favourable for slice preparations. By making sagittal slices of the central vermis of the cerebellum (i.e. in the rostro-caudal direction), one obtains a preparation with the Purkinje dendritic tree in plane with the surface of the slice and at the same time preserving the micro-circuitry innervating the Purkinje neuron.
A striking feature of the cerebellar cortex is that the Purkinje neuron is the only cell type with an axon projecting out of the cortex. It follows that all computations taking place in the cerebellar cortex must be represented in the firing pattern of the Purkinje neurons. This simple fact makes the Purkinje neuron the final integrator of cerebellar cortical activity. Thus a complete understanding of Purkinje cell synaptic integration is critical for the proper understanding of cerebellar function. That the Purkinje neuron has the most branched dendritic tree in the central nervous system (Vetter et al., 2001) is a broad hint that this structure is important.
1.7.2 Role of the cerebellum in learning and motor control

Much of the work on the role of the cerebellum has focused on its involvement in motor coordination and learning. It was realised early on (Florens, 1824) that the cerebellum does not initiate movement on its own; rather the cerebellum plays a crucial role in the modulation of movement. However, an understanding of the specific role of the cerebellum in overall brain function has proven difficult to obtain, and only recently have we developed some initial insights.

An early model of cerebellar learning was provided by David Marr (Marr 1969). He proposed that the regular structure of the cerebellar cortex provides a means of storing patterns of activity that may be altered by changes in synaptic strength of the parallel fibre synapses. It is now well-established that pairing parallel fibre with climbing fibre activity causes long-term depression at the parallel fibre synapses (Ito 1982). This theory has led to attempts to identify plastic aspects of motor behaviour which may involve changes in the cerebellar cortex, in particular the vestibulo-ocular reflex and classical conditioning paradigms.

1.7.2.1 Motor control - the vestibulo-ocular reflex

As an example of motor coordination, the horizontal vestibulo-ocular reflex (VOR) is a relatively simple neuronal system. In response to head rotation the brain has an internal model for generating a motor output to rotate the eyes in the opposite direction in order to retain the point of visual fixation. Masao Ito has proposed a simple motor learning model, which explains the rapid
adaptation of this system to different conditions (Ito 1984). In this model the mossy fibres receive the neuronal signal arising from both vestibular organs in response to circular head acceleration. If the oppositely directed eye-movement does not hold the gaze the image the eye sees will move across the retina, so-called retinal slip. In the model of Ito this slip is signalled to the cerebellar cortex via the CFs. Thus the CF is the signal, which conveys the error signal if the eye motor system is ill-adjusted to accurately compensate for head movements. Any retinal slip will result in the coincident arrival at the Purkinje dendrites of PF and CF activity leading to long-term depression at the PF synapses. This synaptic plasticity then mediates a rectifying gain change in the VOR (see Ito 2001 for review) The location of plastic changes, however, has been the subject of controversy: it has been argued that the cerebellum is not necessarily the location of retention of the movement parameters; the cerebellum may only fulfil the role of error-correcting mechanism.

1.7.2.2 Classical conditioning – the eye blink response

The cerebellar cortex has been proposed to be the site of some forms of Pavlovian learning – as exemplified by eye-blink conditioning, where an air puff to the eyeball is paired with a tone. The neural mechanism for the conditioning is still not fully understood but the critical dependence on the cerebellar cortex is established (McCormick et al. 1982). A model of cerebellar cortical learning (Hesslow & Yeo 1998) envisages that mossy fibres carry the auditory (conditioned) stimulus with the CFs providing the unconditioned air-puff signal. If so, the two stimuli will meet in the cerebellum at the level of the Purkinje neurons. That MFs carry the auditory input to the cerebellum during the pairing
protocol has now been demonstrated (Hesslow et al. 1999). However, it is still a matter of debate whether or not the CFs carry the unconditioned air-puff stimulus (McCormick & Thompson 1982; Yeo et al. 1986).

1.7.3 Previous work on Purkinje cell synaptic integration

From the seminal work of Llinas, it has been realised that Purkinje neurons have an intricate range of possibilities for synaptic integration (see Llinas & Sugimori 1992 for fuller review). The distribution of voltage gated ion channels and synapses have led previous investigators to suggest a general scheme of electrophysiological properties of Purkinje cells. In this scheme the axonally initiated sodium spikes back-propagate poorly into the dendritic tree (Llinas 1975; Llinas & Sugimori 1980; Stuart & Häusser 1994) due to low dendritic sodium channel density combined with a high degree of branching (Vetter et al. 2001). The dendritic tree, however expresses prominent calcium electrogensis (Llinas & Sugimori 1980; Miyakawa et al. 1992).

The climbing fibre (CF) makes synaptic contacts over the proximal two thirds of the dendritic tree and is mediated by AMPA receptor activation. Near the soma and the CF elicits a burst of sodium action potentials. In the dendritic tree the CF response has been suggested to be due primarily to activation of dendritic calcium channels (Llinas & Sugimori 1980; Chan et al. 1989; Miyakawa et al. 1992), on the basis of the large dendritic calcium signals triggered by CF activation At depolarised membrane potential the dendritic CF response triggers a plateau potential, which is also thought to be calcium-mediated.
The other excitatory input to Purkinje neurons, the parallel fibres, appear to be mediated primarily by AMPA receptor activation, with metabotropic glutamate receptor activation occurring under conditions of intense or repetitive stimulation (Finch & Augustine 1998; Takechi et al. 1998). As each PF makes only one or two contacts with the Purkinje cell dendritic tree, the PF input is much weaker than the CF input (Barbour 1993). At depolarised potentials, however, powerful PF stimulation may elicit dendritic calcium spikes (Miyakawa et al. 1992; Midtgaard et al. 1993), though with more physiological stimulation intensities the PF only trigger somatic sodium action potential firing. But at the site of PF input, the EPSP is associated with postsynaptic intracellular calcium increases in the spine being innervated. This calcium increase occurs via both activation of voltage gated calcium channels (Denk et al. 1995) and through the metabotropic glutamate mediated pathway using inositol-tri-phosphate (IP$_3$) as the secondary intracellular mechanism (Finch & Augustine 1998; Takechi et al. 1998).

The pairing of these two excitatory input pathways induces calcium mediated LTD at the PF synapses (see Ito 2001 for a more in-depth review). Recently supralinear calcium mediated coincidence detection of the two inputs has been described at the level of the single PF spine (Wang et al. 2000) This coincidence detection appears to depend either on the mGluR pathway or on activation of voltage-gated calcium channels, depending on the level of synaptic activity.

As mentioned earlier, synaptic inhibition can be highly localised in both time and space, depending on its location relative to the excitatory synaptic input. Inhibition in the dendritic tree can depress the Purkinje cell response to
CF input (Llinas et al. 1968; Callaway et al. 1995) with the effect being much less pronounced at the soma (Callaway et al. 1995). To properly depress calcium influx, though, inhibition must be very precisely timed (Midtgaard 1992). The effect of inhibition on tonic firing in Purkinje neurons is to decrease the regularity of firing, suggestion an important role of inhibition in the integration of PF input as well as CF input (Häußer & Clark 1997; Jaeger & Bower 1999). Given the local effect of inhibition, however, the effect at the site of inhibition has not previously been investigated in detail.

1.8 Outline of the thesis

The present thesis is divided into three experimental chapters, each describing an aspect of synaptic integration and dendritic excitability in cerebellar Purkinje neurons, preceded by a chapter on materials and methods (chapter two)

In chapter three I describe Purkinje cell bistability, characterized by a quiescent and a continuously firing state. I will describe how two conductances, $K_{\text{Ca}}$ and $I_h$, serve to dampen the expression of bistability under physiological conditions.

The following two chapters are concerned with the post-synaptic integration of the two types of excitatory synaptic that Purkinje neurons receive. Chapter four describes how parallel fibre inputs to the Purkinje cell dendritic tree are shaped by the following four ion channels: voltage-gated sodium, calcium and potassium channels as well as $I_h$. I describe how near firing threshold somatic sodium channels amplify EPSPs, and that this is limited by potassium
channels and $I_h$ channels. Calcium channels activate only during continuous firing.

Chapter five describes local dendritic control of initiation and spread of secondary calcium spikes elicited by the climbing fibre input to Purkinje cells. I describe how these secondary spikes are voltage-dependent and may be elicited locally through focal depolarisation by PF input. I also describe how inhibition may locally control the spread of secondary spikes in response to CF input.

The thesis concludes with a general discussion.
Chapter 2: Materials and Methods

2.1 Introduction

The data presented in this thesis were obtained from Purkinje neurons maintained in the acute slice preparation. The electrophysiological recordings were carried out using the patch clamp technique originally developed by Erwin Neher and Bert Sakmann (Sakmann & Neher 1995) in the whole-cell current clamp mode.

2.2 Slice preparation

Acute sagittal slices were prepared from 18-25 day old Sprague-Dawley rats. The rats were decapitated in accordance with Animals Scientific Procedures Act 1996. The scalp was quickly removed to reveal the skull, which was cut open with scissors along the side in a caudo-rostral direction. The entire brain was then scooped out of the skull into a dish containing ice-cold extracellular solution (see section 2.4). For the remainder of the dissection procedure, the brain was kept in ice-cold extracellular solution. The blood from the dissection was washed off the brain, which was transferred to a petri dish. There the brain was pinned down through the neocortex and the pia mater covering the vermis of the cerebellum was removed with a pair of watchmaker’s forceps (Dumont No. 5). The vermis was then isolated by two rostro-caudal cuts between the vermis and either cerebellar hemisphere. The vermis was fixed onto one of its cut sides in the mount of a vibrating slicer (DTK-1000, D.S.K.,
Dosaka E.M. Co. Ltd., Japan) using cyanoacrylate glue and then covered in saline. During slicing the extracellular solution was bubbled with 95% O₂ / 5% CO₂. Slices of 250-300 µm thickness were cut and incubated in extracellular solution continuously bubbled with 95% O₂ / 5% CO₂ at 31-32 °C for one hour. Following incubation the slices were kept at room temperature until transfer to the recording set-up.

2.3 Electrophysiology

2.3.1 Whole cell current clamp recordings

Single, double and triple simultaneous whole cell current clamp recordings were performed (Miles & Poncer 1996) at near-physiological temperatures of 32-35 °C using identical AxoClamp 2B amplifiers (Axon Instruments, Foster City, USA). Recording electrodes were pulled from standard borosilicate capillaries (GC150F-7.5, Harvard Apparatus Ltd. Edenbridge, UK), using a two-stage vertical puller (PC-10, Narashige, Japan). Electrodes had a resistance of 5-8 MΩ when filled with internal solution (see section 2.4).

Giga-seals were established in voltage-clamp mode using standard techniques. Positive pressure (~100 kPa) was applied through the pipette as it was lowered into the tissue. As the neuronal membrane was approached, the pressure was lowered to ~50 kPa and the pipette advanced until a clear ‘halo’ or “dimple” was seen around the pipette tip. At this moment, pressure was released, and gentle suction was applied until gigahm seal formation. The whole-cell recording configuration was established using a brief suction pulse
applied to the pipette to break the neuronal membrane and establish a continuum between the pipette and cell interior.

Once a low and stable access resistance had been established, I switched to current clamp mode. Purkinje neurons are normally spontaneously firing action potentials, thus a hyperpolarising current was applied to bring the membrane potential to \(-65\) mV for whole cell capacitance and access resistance compensation.

2.3.2 Whole cell capacitance and series resistance compensation

To empirically determine the properly adjusted whole cell compensation, simultaneous dual whole-cell recordings were performed from Purkinje neuron somata in whole cell current clamp configuration (Figure 2.2; \(n = 2\)). A 10 ms, \(-0.1\) nA step pulse was applied through one of the patch pipettes. Under the assumption that the soma is a single electrical compartment, one can perform compensation on the pipette used to inject current, with the aim of matching its electrical response to that of the uncompensated pipette, the passive follower. When the compensated recording is identical to the response of the passive recording the compensation is correct. This procedure could then be repeated “blindly” (i.e. without observing the response of the follower pipette) in order to “practise” the compensation procedure.

Somatic access resistances were in the range 7 – 20 M\(\Omega\), and dendritic access resistances were normally in the range 10 – 30 M\(\Omega\). Recordings were terminated if access resistance exceeded 50 M\(\Omega\). The junction potential between the extra- and intracellular solutions (~7 mV) was not corrected for.
Figure 2.1

Double somatic recording from a Purkinje neurone. A, top, voltage recording compensated for electrode capacitance and access resistance in response to a 10 ms, -0.1 nA step current through the pipette. Bottom, simultaneous voltage recording from the same Purkinje soma using an independent electrode. B, The recordings from A overlaid for visual comparison (averages of 375 recordings).
2.3.3 Dynamic clamp

For some experiments dynamic clamp (Robinson & Kawai 1993) was employed to mimic physiological GABA-A inhibitory conductances in the dendritic tree of the Purkinje neuron. The set-up for the dynamic clamp is demonstrated in figure 2.3. With dynamic clamp, the command voltage from the DAC-board represents a predetermined conductance rather than a fixed current, such that the injected current varies with the membrane potential. Using an analogue multiplication circuitry (Harsch & Robinson 2000) (SM-1 Cambridge Conductance, Cambridge, UK), the command voltage from the DAC-board is multiplied with the membrane potential to yield the voltage command signal for the current injection. The multiplication circuitry is designed according to Ohm’s Law (eqn. 2.1), where \( E_{\text{rev}} \) is the reversal potential of the mimicked conductance set by the experimenter. The other variables take their usual meaning. The modified circuitry introduces a loop, where the membrane potential feeds back into the voltage command signal. This feedback loop necessitates accurate access resistance and capacitance compensation to avoid oscillations.

\[
(V_m - E_{\text{rev}}) \ast g_{\text{synapse}} = I_{\text{inject}}
\]  

(2.1)
2.3.4 Extracellular stimulation

The major excitatory synaptic input pathways to Purkinje neurons were stimulated as follows. The climbing fibre was activated by placing an electrode in the granule layer immediately below the Purkinje neuron of interest. Parallel fibres were stimulated by burying an extracellular electrode in the molecular layer below the dendritic tree of the Purkinje neuron of interest. Inhibitory inputs were activated by placing an extracellular electrode in plane with, but adjacent to, the dendritic tree of the Purkinje neuron of interest.

**Figure 2.2**

The modified current clamp circuitry that allows injection of predetermined conductances by using an analogue voltage multiplier (SM-1 above). A feedback loop is introduced (dotted line).
2.4 Solutions

2.4.1 Internal

<table>
<thead>
<tr>
<th>Compound</th>
<th>Normal internal (mM)</th>
<th>High BAPTA internal (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-gluconate</td>
<td>125</td>
<td>85</td>
</tr>
<tr>
<td>KCl</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Na₂-ATP</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mg-ATP</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mg₂-GTP</td>
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<td>0.3</td>
</tr>
<tr>
<td>Na-phosphocreatine</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Internal solutions were calibrated to pH: 7.25 with KOH, and had an osmolarity in the range 285-295 mOsm. Note that the internal chloride concentration was set to produce an IPSP reversal potential of around –75 mV, which is close to the physiological value in Purkinje cells measured using the gramicidin perforated patch technique (Clark & Häusser 1995)
2.4.2 Extracellular solution

Extracellular solutions were continuously bubbled with 95% O₂ / 5% CO₂.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTX</td>
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</tr>
<tr>
<td>CdCl₂</td>
<td>100</td>
</tr>
<tr>
<td>ZD7288</td>
<td>40</td>
</tr>
<tr>
<td>4-AP</td>
<td>40 &amp; 100</td>
</tr>
<tr>
<td>QX-314</td>
<td>3000-4000*</td>
</tr>
</tbody>
</table>

* QX-314 was added to the intracellular solution.

2.4.3 Pharmacological agents

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
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</tr>
<tr>
<td>NaHCO₃</td>
<td>5</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>10</td>
</tr>
<tr>
<td>KCl</td>
<td>1</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2</td>
</tr>
<tr>
<td>Glucose</td>
<td>25</td>
</tr>
</tbody>
</table>

* QX-314 was added to the intracellular solution.
### 2.4.4 Abbreviations and purveyors:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-AP</td>
<td>4-Aminohydropyridine</td>
<td>Sigma</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrapotassium salt</td>
<td>Sigma</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>Cadmium chloride</td>
<td>Sigma</td>
</tr>
<tr>
<td>Glucose</td>
<td>D-Glucose</td>
<td>Sigma</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
<td>Sigma</td>
</tr>
<tr>
<td>K-Gluconate</td>
<td>Potassium gluconate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Mg-ATP</td>
<td>Magnesium adenosine triphosphate</td>
<td>Sigma</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
<td>Sigma</td>
</tr>
<tr>
<td>Mg₂-GTP</td>
<td>Magnesium guanosine triphosphate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Na₂-ATP</td>
<td>Disodium adenosinetriphosphate</td>
<td>Sigma</td>
</tr>
<tr>
<td>NaCl₂</td>
<td>Sodium chloride</td>
<td>Sigma</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Disodium hydrogen phosphate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Na-phosphocreatine</td>
<td>Sodium phosphocreatine</td>
<td>Sigma</td>
</tr>
<tr>
<td>QX-314</td>
<td>Lidocaine N-ethyl bromide</td>
<td>Sigma</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
<td>Tocris</td>
</tr>
<tr>
<td>ZD7288</td>
<td>4-Ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride</td>
<td>Tocris</td>
</tr>
</tbody>
</table>
2.5 Data acquisition and analysis

Data was acquired using Axograph software (courtesy of John Clements, Australian National University, Canberra, Australia). Data was digitised using an Instrutech ITC-18 DAC-board at 20-50 kHz after low-pass filtering at 2-5 kHz using a custom made 8-pole Bessel filter and stored on a Macintosh G3 computer.

Data was analysed off-line using Igor Pro Software (Wavemetrics, Lake Oswego, OR, USA) with custom written routines. Results are presented as mean ± SEM.
3.1 Introduction

Cerebellar Purkinje cells in vivo fire spontaneous action potentials. The mean interspike interval (ISI) is ~ 20 ms (~ 50 Hz), but the range of ISIs varies considerably, from just 2-3 milliseconds to several hundred milliseconds (Eccles et al., 1967). In the absence of synaptic input, Purkinje cells fire action potentials very regularly at a frequency of ~ 50 Hz (Häusser & Clark 1997). Since Purkinje cells release GABA at their synaptic terminals to inhibit their postsynaptic target neurons in the deep cerebellar nuclei, the duration of any pause in Purkinje cell firing presumably causes increased postsynaptic activity in the nuclear neurons. Modulating postsynaptic activity by changing the firing frequency of tonically active presynaptic neurons is an energetically costly way of neuronal communication. This raises the question of what functional role this method of communication plays in the computational abilities of the cerebellum. Furthermore, it focuses our attention on the intrinsic conductances which may underlie this type of behaviour. The present chapter is concerned with the latter of those two questions.

Purkinje cells are known to express a hyperpolarisation activated mixed cation current (Ih)(Crepel & Penit-Soria 1986). This conductance has a reversal potential of around ~ -30 mV and is activated from membrane potentials hyperpolarised to ~ -60 mV (Solomon & Nerbonne 1993; Roth & Hausser 2001), suggesting that it may be involved in prolonged action potential generation. Indeed, it has been found to participate in the control of tonic and
burst action potential generation in thalamic neurons (McCormick & Pape 1990), hippocampal interneurons (Maccaferri & McBain 1996) and supraoptic neurons (Ghamari-Langroudi & Bourque 2000). The specific role of \( I_h \) in the generation of tonic firing in Purkinje neurons has been investigated only in acutely dissociated somata (Nam & Hockberger 1997; Raman & Bean 1999), which retain spontaneous firing. However, in this reduced preparation only little activation of \( I_h \) is detectable around the membrane potentials of continuous firing. Consequently, and in contrast to the findings from other cell types, \( I_h \) was not found to significantly influence tonic firing (Raman & Bean 1999) (Nam & Hockberger 1997). However, the more intact neuronal structure of the slice preparation, with the entire dendritic tree and its associated synapses and ion channels present (Llinás & Sugimori 1980; Llinás & Sugimori 1980), may well cause the influence of \( I_h \) to be different from the situation seen in isolated somata, particularly as the soma-dendritic distribution of \( I_h \) is unknown in Purkinje neurons.

Purkinje neurons also express calcium activated potassium conductances \( (I_{K(Ca)} \) (Gruol et al. 1989; Muller et al. 1998), which are involved in the development of tonic firing during Purkinje cell maturation (Muller & Yool 1998). It is experimentally very difficult to quantify the contribution made by \( I_{K(Ca)} \) given it dependence on intracellular calcium concentration.

Here I explore the contribution of \( I_h \) and \( K_{(Ca)} \) to tonic action potential firing recorded from Purkinje neurons in the slice preparation. The results show that both \( I_h \) and \( K_{(Ca)} \) act as a “safety net” holding the Purkinje neuron near the voltage range for continuous action potential generation. Pharmacological block or down-regulation of \( I_h \), by ZD 7288 or serotonin respectively, causes the
spontaneous firing of the neuron to be interrupted by long (many hundred milliseconds) pauses in firing. Similarly, dialysing the Purkinje cells with a high concentration of BAPTA interrupts continuous firing for several seconds. This apparent bistability of the Purkinje neuron has been investigated in more detail in collaboration with the laboratory of Greg Stuart in the Australian National University, Canberra, Australia.

Whereas both Dr. Stuart’s group and I independently observed the basic phenomenon of apparent bistability caused by the block of \(I_h\), bistability has been explored in more detail by dividing the work between the group of Dr. Stuart and myself. In this chapter I describe my contribution to this analysis, and all figures and analysis presented here are my own. Where appropriate I also refer to relevant work carried out by Dr. Stuart’s group in the text.

3.2 Results

3.2.1 Purkinje neurons are intrinsically bistable

In the absence of synaptic input, Purkinje neurons fire action potentials continuously. I wished to ascertain whether this high level of activity is one of the equilibrium points of a bistable system. If continuous firing is an equilibrium of a bistable system, then the nature of the state, i.e. the rate of action potential generation, should be independent of the path taken to reach the equilibrium. In order to investigate this I recorded from somata of Purkinje neurons in the
Figure 3.1

Purkinje neurons are bistable. A. A Purkinje cell (top), a layer 2/3 pyramidal cell (middle) and a layer 5 pyramidal cell were injected with a current ramp (0.9, 1.2 & 2.0 nA ramp amplitude respectively). Prior to current ramp injection the Purkinje cell was hyperpolarised to –75 mV, the layer 2/3/ pyramidal neuron was resting at –77 mV and the layer 5 pyramidal neuron was resting at –65 mV. B. Plot of the instantaneous firing frequency during the ramp currents shown in A. Note that the onset frequency (indicated by an arrow) is very different for the Purkinje and pyramidal neurons. C. Relationship between slope of injected current and onset frequency for different ramps. Lines represent linear (top, middle) or polynomial (bottom) fits.
whole-cell current clamp configuration, while injecting positive current ramps (0.5 to 2 nA amplitude, 2.5 sec) from a hyperpolarised membrane potential (-70.9 ± 1.5 mV, n=7). The positive current ramps generated a gradual membrane depolarisation that, given a sufficient ramp amplitude, led to continuous action potential generation in the Purkinje neurons (Figure 3.1A, top). I then determined the relationship of the onset firing frequency with the slope of current ramp (Figure 3.1B & C). I found that onset frequency is only weakly dependent on the slope of the injected current ramp (correlation coefficient 0.32 ± 0.10; n=7). This indicates that Purkinje neurons, when initiating continuous firing, enter a state which is path independent, and is consistent with the properties of an intrinsically bistable system. It should also be noted that a considerable degree of hysteresis is observed when the Purkinje neuron goes back into quiescence during the downward slope, again indicative of tonic firing being a stable state.

To highlight these characteristics of Purkinje neurons I injected current ramps identical in time course to those injected in Purkinje neurons into layer 2/3 and 5 pyramidal neurons (Figure 3.1A, middle & bottom). In contrast to Purkinje neurons, the onset frequency of continuous firing in pyramidal neurons is a smooth rising function with increasing slope of the current ramp (Figure 3.1C, middle & bottom; n = 3 for layer 2/3, n= 4 for layer 5 neurons). In either type of pyramidal neuron there is only little hysteresis at the on → off transition.

Together these results suggest that Purkinje neurons display a form of bistability with equilibrium state being the tonic high frequency firing of action potentials.
Together these results suggest that Purkinje neurons display a form of bistability with equilibrium state being the tonic high frequency firing of action potentials.

3.2.2 Block of $I_h$ unmasks apparent bistability of spontaneous firing

During whole cell current clamp recording with no applied holding current, Purkinje cells fire action potentials at a high frequency while sitting at an average membrane potential of (-45.6 mV ± 2.0, n=5; Figure 3.2A). The selective $I_h$-blocker ZD7288 (40 µM) caused Purkinje neurons to adopt a pattern of irregular firing of sodium action potentials interspersed with calcium spikes. During this irregular firing there were pauses in firing that could last up to several seconds (Figure 3.2B, n = 7). During these pauses, the neurons became hyperpolarised by as much as much as 45 mV to –90 to -95 mV (Figure 3.3B). Thus Purkinje neurons can switch between states of continuous firing and quiescence following the block of $I_h$. This indicates that $I_h$ acts as a “safety net” to hold the Purkinje neurons within the range of membrane potentials for continuous firing. However, it is also clear from the results that $I_h$ is not the only factor involved, as the neuron is capable of sustained firing even in the absence of $I_h$. 
Figure 3.2

Blocking $I_h$ unmask bistability. Top, two concatenated 30 sec traces in whole cell current clamp of a Purkinje cell during regular tonic firing. Bottom, Same neuron after wash-in of 40 µM ZD7288. The tonic firing is irregular with interspersed calcium spikes. The neuron spontaneously fell silent for a period of tens of seconds. During quiescence the neuron hyperpolarised to a maximum of $-95$ mV.
3.2.3 $I_h$ lowers the input resistance of Purkinje neurons

In order to better understand the electrophysiological changes to the Purkinje neuron due to block of $I_h$, I injected long hyperpolarising current pulses (1 sec, 0.1 nA; $n = 7$) under control conditions and in the presence of ZD7288 from a membrane potential of $-65 \text{ mV}$.

Under control conditions the current pulse causes a negative voltage deflection that “sagged” back towards the holding potential (Figure 3.4). This “sag” is generally seen as confirmation of the presence of an $I_h$-current (Pape 1996). To test this prediction I repeated the protocol after having added ZD7288 to the perfusate. Under these conditions the sag disappeared, demonstrating its sole mediation by $I_h$. Furthermore there was a dramatic increase in the input resistance of the neuron (from $61.6 \pm 9.5 \text{ M}^*$ to $114.5 \pm 11.1 \text{ M}^*$; $n= 6$, $p < 0.01$).

These findings indicate that $I_h$ is a significant conductance in Purkinje cells that when activated can substantially lower the input resistance.
Figure 3.3

$I_h$ lowers Purkinje cell input resistance. **A.** Somatic and dendritic recordings of a Purkinje neuron held at –65 mV while a 1 sec., 0.1 nA hyperpolarising current is injected under control conditions and in the presence of 40 µM ZD7288. **B.** The same traces as in **A**, but with the control and ZD7288 conditions plotted separately.
3.2.4 Switching between states by simulated EPSPs.

By the injection of a steady hyperpolarising current Purkinje neurons can be silenced. One of the predicted characteristics of a bistable neuron is that, over a certain range of applied current, either of the two steady states will be stable. Thus, if $I_h$ helps to mask an inherently bistable system, then blocking $I_h$ should ease the transition from one equilibrium to the other.

In order to investigate this I hyperpolarised Purkinje neurons in whole cell current clamp mode, while injecting EPSC-like waveforms at a dendritic location and recording the resulting voltage change both at the soma and at the site of injection. When doing so, I found that a dendritic waveform injection of 1 nA, corresponding to a somatic EPSP of ~ 3 mV, made the neuron jump from quiescence at a more depolarised membrane potential in control as compared with ZD7288 in the perfusate (-56.0 ± 0.6 mV in control, -58.0 ± 1.2 mV, n= 4, p < 0.05; figure 3.4).

This result demonstrates the two stable states of the Purkinje neuron, and that single EPSPs can switch from the lower to the upper state. Furthermore, this switching is facilitated by the removal of $I_h$. 
Figure 3.4
Blocking $I_h$ eases the off → on transition. Somatic EPSPs from a Purkinje neuron double-patched at the soma and a dendritic location in the whole-cell current clamp mode. A double exponential waveform ($\tau_{\text{rise}}$: 0.3 ms, $\tau_{\text{decay}}$: 3.0 ms, amplitude: 1.0 nA) is injected through the dendritic pipette under control conditions and in the presence of 40 µM ZD7288. In the presence of ZD7288, the neuron makes the transition from quiescence to firing from a more hyperpolarised potential (-60 mV under control conditions and –57 mV in the presence of ZD7288).
3.2.5 Modulation by serotonin indicates a physiological role of $I_h$ in regulating firing

Activation of serotonin or adrenergic receptors negatively modulates $I_h$ in Purkinje neurons (Crepel & Penit-Soria 1986), (Li et al. 1993). This opens for the possibility that modulation of bistability in Purkinje neurons via a serotonergic pathway may be a physiological mechanism (Williams et al. 2001). To investigate this possibility I recorded from Purkinje neurons in whole cell current clamp while stimulating ‘off-beam’ inhibitory inputs in bursts of 10 stimuli @ 100 Hz once a second (Figure 3.5). Under control conditions from a membrane potential of –61 mV these bursts summated to an IPSP of 9.2 mV ± 1.9 mV ($n = 6$), while in the presence of serotonin the IPSPs summated to 11.9 ± 2.7 mV.

Under control conditions Purkinje cell firing was abolished during the IPSP train, but quickly resumed following the termination of stimulation (mean pause: 195 ± 29 ms; $n = 6$). I then washed in 100 µM serotonin and repeated the stimulation protocol. Under these conditions the pause in tonic firing induced by the inhibitory burst was significantly prolonged (mean pause 279 ± 57 ms; $p < 0.05$). Furthermore the tonic firing would spontaneously be interrupted by periods of silence lasting from hundreds of milliseconds to a few seconds (figure 3.5). During these periods of silence the neuron would have a membrane potential of -52.2 ± 1.6 mV ($n = 6$). Thus the inclusion of serotonin in the perfusate would both lengthen the periods of silence introduced by inhibition and add intrinsic variability to the duration of tonic firing.
These results are consistent with the idea that negative modulation of $I_h$ provides a physiological mechanism for enhancing the intrinsic membrane bistability of Purkinje neurons.
Figure 3.5

Serotonin modulation of inhibition. **A.** Somatic recording from a Purkinje neuron under control conditions while stimulating inhibitory interneurons (10 stimuli @ 100 Hz every second) in the molecular layer ~ 300 µm from the recording pipette lateral to the sagittal plane of the slice. While activating inhibitory synapses the Purkinje neuron falls silent, but quickly resumes tonic firing after cessation of inhibition. **B.** In the presence of 100 µM serotonin, the inhibitory synaptic input causes the pausing in firing to increase beyond the end of stimulation. Also the continuity of tonic firing becomes variable in the presence of serotonin.
3.3.6 Bistability unmasked by high concentration of internal BAPTA

While in the upper firing state, calcium influx through voltage activated calcium channels will activate calcium-activated potassium channels. As these potassium channels are activated only while the neuron is continuously firing, it is possible that they play an important role in the maintenance of the upper firing state.

To test this I recorded spontaneous activity under control conditions and then introduced a high concentration (20 mM) of the fast calcium buffer BAPTA (figure 3.6) by including it in the recording pipette. Dialysis with BAPTA-containing intracellular solution increased the basal firing rate within seconds of break-in (figure 3.6). Over the first 15 seconds of rupturing the cell membrane the spontaneous firing frequency increased from 48.7 ± 12.3 Hz measured in the cell-attached configuration to 100.0 ± 14.2 Hz (n = 5).

Continued dialysis with BAPTA internal solution for 15-20 minutes led the cell to adopt a bistable activity pattern. For prolonged periods of time, the neuron would be stable and quiescent at a membrane potential of −61.3 ± 1.4 mV (figure 3.7 n = 10). The neuron spontaneously moved from quiescence to continuous firing. However, the initial firing frequency was considerably higher than seen in control with an average of 344.1 ± 45.8 Hz. During spontaneous firing action potentials decreased in amplitude, presumably due to sodium channel inactivation, and a second stable depolarised membrane potential was attained (−29.8 ± 1.0 mV). That sodium channel inactivation is incomplete at the upper stable membrane potential is suggested by the ‘spikelets’ that terminate the duration of the upper stable membrane potential (figure 3.6B2, right).
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Figure 3.6
Internal BAPTA rapidly increases spontaneous firing frequency in Purkinje neurons. **Aa**, A Purkinje neuron was recorded in the cell-attached mode using an internal solution containing 20 mM BAPTA (see Methods) and held in current-clamp mode without holding current (firing rate 55 Hz). The whole-cell configuration was achieved by break-in using applied suction at the arrowhead. **Ab**, Within 15 seconds of break-in, the spontaneous firing frequency had increased to 140 Hz. **B**, A continuous plot of the instantaneous firing frequency after break-in (at the arrow head).
Figure 3.7

Continued dialysis with BAPTA induces bistability. A. A somatic whole cell recording from Purkinje neuron under control conditions demonstrating tonic firing. The expanded view illustrates the regularity of firing. B. Continued dialysis with BAPTA (~20 min) following break-in led to a spontaneous bistable activity pattern. The top panel shows a continuous record of membrane potential in the absence of holding current, illustrating stereotyped bursts of firing interspersed with periods of quiescence lasting tens of seconds. Note the spontaneous transitions between quiescent and firing states and the rapid inactivation of sodium spikes following transition to the off → on transition (expanded view).
These results demonstrate that calcium activated potassium channels are involved in the regulation of the transitions between firing and non-firing states. Furthermore the results demonstrate the involvement of $K_{(Ca)}$ in setting the levels of the upper, by preventing runaway depolarisation, and the lower state.

3.3.7 Bistability in the presence of both ZD7288 and high internal BAPTA

To investigate the combined effect of blocking $I_h$ and $K_{(Ca)}$ I recorded spontaneous firing in the cell-attached configuration with a high BAPTA internal solution in the cell-attached patch pipette. Then I established the whole-cell configuration to dialyse the neuron with BAPTA. Finally I applied ZD7288 to the extracellular solution (figure 3.8).

With both $I_h$ and $K_{(Ca)}$ blocked the neurons adopted a pattern of activity reminiscent of that seen while blocking $K_{(Ca)}$ alone. However, there were some distinct differences. The quiescent periods lasted minutes rather than seconds and the hyperpolarised membrane potential seen between bursts of firing was very hyperpolarised (-93.8 ± 1.4, n=4). The upper state was reached following a slow spontaneous depolarisation and had an initial firing frequency of 118.7 ± 18.6 Hz. Following action potential inactivation a steady depolarised potential was reached (-29.6 ± 1.0 mV), which was not significantly different from that observed with high internal BAPTA alone.

Together with the results applying either ZD7288 or high internal BAPTA alone,
Figure 3.8
Continued application of BAPTA and ZD7288 slows oscillations. A somatic whole cell recording ~ 30 min following break-in with an internal solution containing 20 mM BAPTA and externally applied ZD7288 (40 µM) demonstrating very slow oscillations with stereotyped bursting and the silent periods lasting several minutes – longer than the quiescence observed with either BAPTA or ZD7288 alone. The expanded views demonstrate the stereotyped bursts with initial rapid inactivation of sodium spikes, followed by a relatively stable depolarised membrane potential and terminated by a series of sodium and calcium spike-bursts.
these results suggest that mainly calcium activated potassium channels determine the level of the upper state. The level of the lower quiescent state, on the other hand, is determined by a combination of both $I_h$ and $K_{Ca}$. 
3.3 Discussion

The results presented in this chapter demonstrate that Purkinje neurons are bistable. When brought to continuous firing, the initial firing frequency is only weakly dependent on the slope of the injected current used to depolarise the neuron. This path-independence strongly suggests a stable upper stable membrane potential equilibrium typical of a bistable system. Pharmacological block and physiological modulation of $I_h$ reveals that $I_h$ masks the inherent bistability of Purkinje neurons. Consequently, any down-regulation of $I_h$ will enhance this intrinsic bistability.

Furthermore, block of calcium activated potassium channels by the application of a high intracellular concentration of BAPTA also revealed a cellular pattern of activity with two distinct and stable membrane potentials, providing further evidence for a bistable system. The results suggest that $I_h$ and $K_{(Ca)}$ are important determinants of both upper and lower state,

3.3.1 Changes to activity patterns induced by block of $I_h$ and $K_{(Ca)}$

In the absence of synaptic input, Purkinje neurons fire action potentials at a high rate (Häusser & Clark 1997), (Nam & Hockberger 1997), (Raman & Bean 1999). In the dissociated Purkinje cell preparation, this high rate of activity has been ascribed to persistent sodium current, with $I_h$ playing only a minor role (Raman & Bean 1999). The work presented here is consistent with these findings, but it extends them by demonstrating that in the intact neuron, $I_h$ plays a critical role in maintaining Purkinje neurons in the voltage range where persistent sodium current operates. As such $I_h$ can be thought of as providing a
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‘safety net’ that holds the neuron in the voltage range for persistent sodium current to be activated.

The effect of \( I_h \) block appears to be related to the degree of block by ZD7288. In the experiments of Williams and Stuart, 10 \( \mu M \) ZD7288 (near the \( I_{C_{50}} \) for ZD7288 block of \( I_h \) (2-10 \( \mu M \)) (Harris & Constanti 1995; Gasparini & DiFrancesco 1997)), generates bistability exhibited by spontaneous firing interspersed by periods of silence of up to 1 second. When using 40 \( \mu M \) ZD7288 as in my experiments, where a more complete block of \( I_h \) can be expected, Purkinje cells fire both sodium and calcium spikes interspersed by periods of silence lasting several seconds.

The transition from quiescence to tonic firing is facilitated by removal of \( I_h \), again indicative of a function for \( I_h \) in masking inherent bistability.

### 3.3.2 Ionic mechanisms underlying bistability

Sodium currents in Purkinje neurons exhibit, in addition to a transient component, a resurgent and a persistent component (Gahwiler & Llano 1989; Raman & Bean 1997; Kay et al. 1998; Raman & Bean 1999). These two components have been proposed to play a critical role in sustaining tonic firing by providing inward current during the inter-spike intervals (Raman & Bean 1999).

The persistent component of the sodium current becomes available from membrane potentials depolarised to \( \sim -65 \) (chapter 4), about 15-20 mV depolarised to the action potential threshold in Purkinje neurons (-44.9 \( \pm \) 0.6 mV, \( n=47 \)). Williams and Stuart found that plateau potentials are diminished by
application of TTX before sodium action potential amplitude is affected. This points to an important role of persistent sodium current in maintaining the upper firing state of Purkinje neurons. The difference in effect of TTX on plateau and spike amplitude can probably be explained by considering that persistent channels represent a small fraction of channels - in a particular gating mode - out of the total sodium channel population of the cell (Raman & Bean 1999).

I have found that block of calcium activated potassium channels makes the membrane potential of the upper state more depolarised. This leads to inactivation of sodium channels. This suggests, in agreement with previous work (Raman & Bean 1999), that calcium currents are not directly involved in the maintenance of the upper firing state, rather calcium influx during tonic firing activate potassium channels, which then prevent run-away depolarisation. It is puzzling that with high internal BAPTA the membrane potential of the lower state is in the voltage range where persistent sodium channels are activated (chapter 4). Resolving this issue will require further experiments using cell attached voltage clamp in combination with whole cell current clamp recordings to establish the specific currents at the various membrane potentials. However, it is should be noted that only a small perturbation to the system is required for the off → on transition as it occurs with no applied external input. That $I_h$ is involved in keeping the membrane relatively depolarised during the lower state in the presence of high internal BAPTA is clear since the addition of ZD7288 hyperpolarises the lower state to almost $-100$ mV.
3.3.3 Physiological role of $I_h$ in Purkinje activity

Williams and Stuart find that serotonin down-regulates $I_h$ as has been observed before (Li et al. 1993). I find that application of serotonin lengthens the pausing in firing following a train of inhibitory input to the Purkinje neuron, though one should be careful not to ignore the possibility of a presynaptic effect of serotonin in addition to the postsynaptic effect described by Williams and Stuart. This points to an important role of $I_h$ in the \textit{in vivo} regulation of Purkinje cell excitability. My findings support the idea that the Purkinje neuron expresses bistability with $I_h$ helping to favour the upper firing state. Thus, since Purkinje neurons are innervated by serotonergic fibres, it is possible that serotonergic modulation of $I_h$ in Purkinje neurons can alter their spiking patterns \textit{in vivo}. Consistent with this idea, pauses in tonic firing lasting several seconds have been observed in sleeping, but not awake cats (McCarley & Hobson 1972), in concordance with higher serotonergic diurnal activity while awake (Partonen 1998). Similarly, stimulating the Raphe complex, which has a high density of serotonergic neurons, causes Purkinje neurons prolonged pauses in continuous firing (Strahlendorf et al. 1979; Weiss & Pellet 1982). In combination with the findings in the present chapter, the above findings suggest that, depending on arousal state, the Purkinje neurons may be subject to substantial output modulation by serotonergic neurons.
4.1 Introduction

The input-output relation of neurons is determined by the interaction between synaptic input and the active and passive properties of the postsynaptic neuron. Central to this transformation are the properties and distribution of voltage-gated channels, which can vary considerably across cell types, and which together with the passive electrotonic structure determines the electrophysiological “fingerprint” of each neuron (Mainen & Sejnowski 1996; Vetter et al. 2001). Recent experiments have demonstrated that the dendrites of neurons express voltage-gated channels at significant densities, and that consequently dendrites are electrically excitable (Usowicz et al. 1992; Stuart & Sakmann 1994). While there is strong evidence for activation of these dendritic channels by backpropagating action potentials (Spruston et al. 1995; Golding & Spruston 1998; Larkum et al. 1999), and during powerful synaptic activation (Eilers et al. 1995), (Midtgaard et al. 1993) there is considerable controversy, however, regarding the contribution of dendritic voltage-gated channels to synaptic integration under more physiological levels of synaptic input. For example, in neocortical and hippocampal pyramidal neurons, while there is evidence from imaging and patch-clamp recording studies for activation of dendritic Na and Ca channels, dual patch-clamp recordings have shown that under most conditions, any amplification of the EPSP by voltage-gated channels occurs near the soma (Stuart & Sakmann 1995) perhaps because dendritic activation of these conductances is balanced by a high dendritic
density of K and Ih channels (Hoffman et al. 1997), (Magee,1999). As the
distribution of both inward and outward voltage-gated currents depends on cell
type (Johnston et al. 1996); (Magee, 1999; Storm, 2000), the precise
contribution of voltage-gated channels will thus also depend on cell type.

Purkinje cells have one of the most elaborate dendritic trees of any cell in
the nervous system, and integrate their excitatory and inhibitory synaptic inputs
to form the sole output of the cerebellar cortex. Beginning with the pioneering
studies of Llinás and colleagues, a large body of evidence has accumulated to
indicate that a wide variety of voltage-gated channels are expressed in the
Purkinje cell dendritic membrane. Thus, patch clamp recordings have directly
revealed the presence of voltage-gated Ca channels (Usowicz et al. 1992;
Mouginot et al. 1997), Na channels (Stuart & Häusser 1994) and K channels
(Bossu et al. 1988; Gruol et al. 1991) in Purkinje cell dendritic membrane. There
is also strong evidence for the presence of other voltage-gated channels in the
somato dendritic membrane, including hyperpolarisation-activated (I_{\text{h}}) channels
(Crepel & Penit-Soria 1986), although their distribution has not been studied in
detail.

The contribution of these various channels to the excitability of Purkinje
cells has been the subject of intense study. The work of Llinas and Sugimori
(1980) demonstrated that the soma and dendrites exhibit different forms of
excitability in response to step current injections, with the soma primarily
showing Na spikes, and the dendrites Ca spikes and plateaus. These findings
have been supported by a number of more recent studies showing that voltage-
gated potassium channels can limit Ca spiking in dendrites in response to both
current injection and strong synaptic activation. Imaging experiments have also
shown that synchronous stimulation of parallel fibres can lead to local, voltage-dependent calcium increases in the spines and dendrites of Purkinje cells (Eilers et al. 1995); this has also received support from modelling studies (De Schutter & Bower 1994). However, these responses were observed in response to relatively powerful synaptic stimulation. The spatial distribution of activation of voltage-gated conductances in response to more physiologically relevant inputs remains unclear. Furthermore, the relative contribution of the various conductances, and their net effect at the soma, has been difficult to establish.

To address these questions, I have used simultaneous somatic and dendritic recordings from cerebellar Purkinje cells in slices in order to directly measure the spread of EPSPs from the site of synaptic activation to the soma. I have mimicked synaptic input using current waveforms, allowing us to precisely control input location and properties, and to apply specific pharmacological blockers which would normally interfere with synaptic transmission. Our findings demonstrate that subthreshold activation of voltage-gated channels plays a crucial role in shaping the relationship between synaptic input and output in Purkinje cells. The dominant effect at subthreshold potentials is that of perisomatic Na channels, which can provide a prominent voltage-dependent amplification that dramatically increases the window for temporal summation. When the Purkinje cell is firing spikes and is depolarised by dendritic synaptic input, then dendritic voltage-gated channels appear to be the dominant source of non-linearity. These findings have significant implications for our understanding of how Purkinje cells integrate their synaptic inputs.
4.2 Results

4.2.1 EPSP boosting of synaptic input

When eliciting parallel fibre EPSPs, the somatic EPSP shape changed as the holding potential was varied (fig. 4.1A) \((n = 7)\). While the shape of the somatic EPSP was independent of membrane voltage at potentials hyperpolarised to \(-65 \text{ mV}\), at potentials between \(-65 \text{ mV}\) and threshold for action potential generation, a progressive increase in size of the somatically recorded EPSP was observed. This amplification was expressed primarily as a prolongation of the EPSP rather than an amplification of the peak depolarisation. To quantify this amplification, I measured the amplitude of the somatic EPSP and estimated the EPSP integral by measuring the area under the curve from stimulus to return to baseline. These values were plotted against the holding potential (figure 4.1B). For the cell demonstrated in figure 1, the integral of the EPSP increased 9-fold (figure 4.1B, right) whereas the peak depolarisation increased by about a third (figure 4.1B, left). Hence, at potentials near spike threshold, there is a non-linear voltage-dependent amplification of synaptic EPSPs, which I refer to as ‘EPSP boosting’. A similar form of EPSP boosting has been observed previously in hippocampal and neocortical pyramidal neurons (Stafstrom et al. 1985; Deisz et al. 1991; Stuart & Sakmann 1995; Andreasen & Lambert 1999)
Figure 4.1

Subthreshold amplification of EPSPs A. Top, somatic Purkinje EPSPs recorded in response to distal synaptic input (stimulation pipette ~200 µm from the soma, 10 V) while varying the somatic holding potential. Stimulation at arrow. B. Left, the somatic peak depolarisation plotted as a function of holding potential. Right, integrals of the somatic EPSP plotted as a function of the holding potential of the neuron.
4.2.2 Dendritic current injections faithfully mimic EPSCs

To circumvent the effect of pharmacological agents on synaptic transmission (e.g. block of presynaptic voltage-gated channels), we simulated synaptic input by injecting current waveforms shaped like EPSCs via a dendritic recording pipette. Simultaneous whole-cell patch-clamp recordings were made from Purkinje neurons in at two locations (Miles & Poncer 1996), one in the dendritic tree and at the soma. Double exponential current waveforms ($\tau_{\text{rise}}$ 0.3 ms, $\tau_{\text{decay}}$ 3.0 ms) were injected via the dendritic recording electrode and the resulting EPSP was compared with EPSPs elicited by stimulating parallel fibre inputs with an extracellular electrode placed immediately below the dendritic pipette (Fig. 2; $n=4$). As shown in figure 4.2, the properties of the injected EPSP faithfully mimicked those of the stimulus-evoked EPSP.
Comparisons of EPSPs generated by extracellular stimulation and simulated EPSCs (189 µm from the soma). An extracellular stimulation pipette was placed in the distal molecular layer within 50 µm of the dendritic patch pipette and the parallel fibres stimulated repetitively (1 Hz, 9 V). The EPSC waveform was injected via the dendritic pipette ($\tau_{\text{Rise}}$ 0.3 ms, $\tau_{\text{Decay}}$ 3.0 ms, 0.5 nA peak).

**Figure 4.2**
4.2.3 Boosting of simulated EPSPs

EPSPs simulated by dendritic injection of current waveforms were boosted similarly to evoked EPSCs. For the simulated synaptic input demonstrated in figure 3B the integrals for both the dendritic and somatic EPSP increased 7-fold over the voltage range of boosting (figure 4.3B). Over this range the somatic peak response was amplified by about one third from 3 to 4 mV, similar to the evoked EPSPs (figure 4.3B, somatic EPSP peak went from 2.9 ± 0.2 mV at –70 mV to 4.6 ± 0.4 mV at the most depolarised potential, n= 9, p < 0.01). The peak depolarisation of the dendritic EPSP does not change with holding potential (figure 4.3B). To quantify the onset of this non-linear amplification of the EPSP, I fitted a power function to the plot of EPSP integral versus membrane potential. I then differentiated and normalised the power function and determined at which membrane potential the differentiated function crossed the value of 0.3, which corresponds to the beginning of the upstroke of the magnitude of EPSP boosting. Using this measure the mean initial membrane potential for onset of EPSP amplification was –63.1± 0.5 mV (n=9).
Figure 4.3
Subthreshold EPSP amplification of injected EPSCs. A. Dendritic (dendritic pipette 135 µm from the soma) and somatic EPSPs generated by a dendritically injected EPSC waveform ($\tau_{\text{rise}}$ 0.3 ms, $\tau_{\text{decay}}$ 3.0 ms, 1.0 nA peak) while varying the holding potential at the two pipette locations. B. Left, somatic (blue circles) and dendritic (green circles) peak depolarisation plotted as a function of the holding potential of the neuron. Right, somatic and dendritic integrals of the EPSP plotted as a function of the holding potential of the neuron.
4.2.4 Contribution of sodium and calcium channels to EPSP boosting

To investigate the voltage-gated conductances underlying EPSP boosting I added pharmacological ion channel blockers to the perfusate while mimicking synaptic input using injected current waveforms.

I first investigated the contribution of voltage-gated sodium channels in Purkinje cells (Raman & Bean 1997; Kay et al. 1998) to EPSP boosting using the selective blocker tetrodotoxin (TTX). Adding TTX (0.5 µM) to the perfusate did not affect simulated EPSPs at hyperpolarised membrane potentials (figure 4.4A). However, the EPSP boosting observed under control conditions at depolarised potentials was abolished in the presence of TTX (figure 4.4A). Hence, these results indicate that the major source of EPSP boosting in Purkinje cells comes from TTX-sensitive sodium channels.

Next, I investigated the contribution of calcium channels to EPSP boosting, since Purkinje cells are known to express a variety of these channels (Mouginot et al. 1997), (Raman & Bean 1999), (Llinas et al. 1992). Over the range of subthreshold membrane potentials where EPSP boosting is observed under control conditions, CdCl₂ (100 µM) did not affect EPSP boosting (data not shown, but see figure 4.4A, middle).

The lack of influence of calcium channel blockers may be due to a lack of activation of calcium channels over the range of voltages investigated. To expand the range of holding potentials in the depolarised direction without generating confounding action potentials, I recorded EPSPs first in the
Figure 4.4
Pharmacology of EPSP boosting A. Somatic EPSP recorded in response to a dendritically injected waveform (τ_{Rise} 0.3 ms, τ_{Decay} 3.0 ms, 1.0 nA peak) for three different holding potentials (dendritic pipette located 96 µm from soma). Top, EPSPs recorded at a holding potential of -53 mV for two pharmacological conditions: 0.5 µM TTX and TTX + 100 µM Cd^{2+}. Middle, EPSPs at a holding potential of -60 mV for the two pharmacological conditions in top panel as well as in control conditions. Bottom, responses at a holding potential of -75 mV for the three pharmacological conditions in middle panel. B. Integrals of the EPSPs for the three pharmacological conditions in A (control, filled circles; TTX, open circles and TTX + Cd^{2+}, filled triangles) plotted as a function of holding potential.
presence of TTX and then in the presence of TTX + 100 µM cadmium (figure 4.4A; n = 3). The addition of cadmium to the TTX Ringer did not affect the EPSP at hyperpolarised potentials (figure 4.4A, bottom). Over the voltage range where boosting occurs in control, there was only a small difference between the responses in TTX and TTX + cadmium (figure 4.4A, middle), again confirming that under control conditions calcium channels make little contribution to EPSP boosting. However, depolarising the cell further activated another, TTX-insensitive EPSP boosting mechanism. This occurred over a voltage range where the Purkinje cell would tonically fire action potentials under control conditions (figure 4.4A, top). This TTX-insensitive boosting had a time course that lasted for several hundred milliseconds (figure 4.4A, top), however, with little to no peak amplification. Adding cadmium to the perfusate blocked this second form of EPSP boosting indicating that it is mediated by calcium channels.

To compare the relative contributions to EPSP boosting of calcium channels and sodium channels, I plotted the integral of EPSPs for the control and the two pharmacological conditions: TTX and TTX + cadmium (figure 4.4B). Relative to the control responses, EPSP boosting in the presence of TTX was shifted in the depolarised direction by ~10 mV (threshold −54.2 ± 1.0 mV, n= 6). Due to the longer duration of calcium channel mediated boosting, the integrals of the boosted EPSPs in the presence of TTX rose to values higher than those for EPSP boosting in control. In the presence of both TTX and cadmium there was no boosting. In fact, at depolarisation approaching zero mV, the EPSPs decreased in size (data not shown), presumably due to activation of voltage-activated potassium channels.
4.2.5 Spatial distribution of sodium and calcium channel boosting

It has been suggested (Llinás & Sugimori 1980) and more recently directly shown (Stuart & Häusser 1994) that Purkinje cells have higher somatic than dendritic sodium channel densities. Similarly, an opposite pattern of channel density has been suggested for calcium channels (Llinás & Sugimori 1980); however, dendritic cell-attached patch-recordings have thus far failed to confirm such a gradient (Usowicz et al. 1992). I therefore investigated the consequences any such sodium and calcium channel gradients might have on EPSP boosting. Specifically, I determined where in Purkinje cells the EPSP boosting originates.

To answer this question I subtracted the dendritic voltage response from the somatic voltage response in order to determine where the depolarisation was greatest during EPSP boosting. The resulting trace is a direct measure of any deviation from isopotentiality between the two recording pipettes. This is demonstrated in figure 4.5 for the same cell as in figure 4.4. In the left hand column of figure 4.5, averages of 3-4 somatic and dendritic traces are shown. In the right hand column the arithmetic difference between somatic and dendritic recording location is plotted such that points above the zero-line indicate that the soma is more depolarised than the dendrite. During the current injection itself, the dendritic voltage response was always bigger than the somatic.
**Figure 4.5**

Localization of EPSP boosting Somatic and dendritic EPSPs in response to a current waveform ($\tau_{\text{rise}}$ 0.3 ms, $\tau_{\text{decay}}$ 3.0 ms, 1.0 nA peak) injected through the dendritic pipette (96 µm form the soma, left column) and the arithmetic difference between them (right column). Same cell as in figure 3.4. For the traces in the right column, points above the zero line mean that the soma is more depolarised than the dendritic recording location. This is indicated by the arrowheads and letters $s$ (soma) and $d$ (dendrite). **A.** EPSPs (left) and the arithmetic difference between them (right) under control conditions at two holding potentials: -60 mV (top) and -75 mV (bottom). **B.** Somatic and dendritic EPSPs (left) in the presence of TTX and the arithmetic difference between them (right) at a holding potential of -53 mV. **C.** Somatic and dendritic EPSPs (left) in the presence of TTX + Cd$^{2+}$ and the arithmetic difference between them (right) at a holding potential of -53 mV.
response. However, at hyperpolarised potentials, the two pipettes reached isopotentiality very quickly after the actual current injection was over, indicating that at those potentials, EPSP decay following the termination of the EPSC is uniform across the cell (figure 4.5A). At membrane potentials where the EPSP is boosted under control conditions, the somatic voltage response became larger than the dendritic response by about 0.5 mV after cessation of the current injection. The duration of this voltage difference matched that of the boosting itself. This is good evidence that EPSP boosting is mediated by channels activated near or at the soma.

EPSP boosting mediated by activation of calcium channels is also non-uniform across the somato-dendritic axis. For this type of boosting, however, the polarity is reversed, i.e. the dendritic recording site is more depolarised than the soma (Figure 4.5B). The polarity of calcium channel mediated EPSP boosting is less pronounced than the polarity of sodium channel mediated boosting. In the presence of TTX + cadmium the cell was isopotential after termination of current injection at all holding potentials (figure 4.5C), confirming that any deviation from isopotentiality was due to activation of sodium and calcium channels.

4.2.6 EPSP boosting while varying EPSC amplitude

As well as the dependence of EPSP boosting on holding potential I investigated the dependence of EPSP boosting on EPSC amplitude. While
Figure 4.6

Amplitude-dependent EPSP boosting. **A.** Somatic and dendritic EPSPs recorded from a holding potential of –62 mV while varying the amplitude of the dendritically injected EPSC ($\tau_{\text{rise}}$ 0.3 ms, $\tau_{\text{decay}}$ 3.0, 35 µm from the soma). *Inset*, the same traces as in the main panel normalised to the peak depolarisation. **B.** EPSP amplitude plotted as a function of the EPSC amplitude for somatic (left) and dendritic recording pipette (right). **C.** EPSP integral plotted as a function of the amplitude of the dendritically injected EPSC, soma left, dendrite right. **D.** Difference plots as demonstrated in figure 4.5 for two EPSCs of different amplitude demonstrating perisomatic origin of the EPSP boosting.
holding the neuron at a constant membrane potential of –62 mV (within the range of membrane potentials where boosting is observed, see figure 1).

I varied the amplitude of the injected EPSC through the dendritic pipette (figure 4.5; n = 5). Under these conditions, the relationships between both peak depolarisation and EPSP integral with EPSC amplitude were linear for most of the range of subthreshold EPSC amplitudes (figure 4.6B). As the EPSC amplitude made the Purkinje cell approach the threshold for action potential generation, there was some amount of EPSP boosting (figure 4.6A, inset). As in the case of membrane potential-dependent EPSP boosting, the effect was expressed as a prolongation of the EPSP more than a peak amplification (figure 4.6A, inset and 4.6B).

By generating difference plots similar to those in figure 5, I was able to determine that EPSP boosting is, again, larger in amplitude at or near the soma (figure 4.6C). Amplitude-dependent EPSP boosting was much less pronounced but otherwise had similar properties to membrane potential-dependent EPSP boosting.

4.2.7 EPSP shaping by \( I_h \)

In addition to the inward currents mediated by sodium and calcium channels, Purkinje cells express a hyperpolarisation-activated current (\( I_h \)) (Crepel & Penit-Soria 1986; Roth & Hausser 2001). To investigate the influence of \( I_h \) on EPSP shaping, I injected dendritic waveforms while varying the membrane potential under control conditions and in the presence of 40 µM of the \( I_h \) blocker ZD7288 (n = 7) (Harris & Constanti 1995; Gasparini &
DiFrancesco 1997). At hyperpolarised potentials $I_h$ provides standing inward current with a reversal potential of $-30$ mV (Stuart & Spruston 1998). Blocking $I_h$ increased the input resistance at potentials where this current is normally activated. The input resistance in response to somatic hyperpolarising step currents (0.1 nA, 1 sec, figure 3.3) changed from $61.1 \pm 9.5 \text{ M}\Omega$ to $114.5 \pm 11.1 \text{ M}\Omega$ in ZD7288 ($p < 0.01$) and the dendritic resistance changed similarly from $51.6 \pm 10.0 \text{ M}\Omega$ to $104.1 \pm 12.1 \text{ M}\Omega$ ($p < 0.01$). There is a pronounced ‘sag’ at the onset and after the current injection (figure 3.3), which is completely blocked by the selective $I_h$ blocker ZD7288 (Roth & Häusser, 2001). The ratio between peak of the sag and the steady state membrane potential in response to the current step was $0.72 \pm 0.1$ at dendritic recording sites and $0.73 \pm 0.1$ at the soma. The similarity of the relative size of this sag is an indication of the distribution of $I_h$. That sag ratios and change in input resistance are equal at the dendritic and somatic locations indicates that $I_h$ is evenly distributed across the somato-dendritic membrane.

As a consequence of the increased input resistance in the presence of ZD7288 the decay of EPSPs at all potentials was slowed and the EPSP thereby widened (figure 3.3A). Fitting the somatic EPSP decay under control conditions with a single exponential gave a mean time constant of $41.9 \pm 11.1 \text{ ms}$, and in the presence of ZD7288, $93.4 \pm 5.4 \text{ ms}$.

The peak depolarisation at the soma, however, was not changed at hyperpolarised potentials. At depolarised potentials, boosting combined with the
Figure 4.7
EPSP Shaping by I_h. A. Somatic EPSPs generated by a dendritically injected waveform ($\tau_{\text{rise}}$ 0.3 ms, $\tau_{\text{decay}}$ 3.0 ms, 1.0 nA peak, 129 µm from the soma) under control conditions and in the presence of 40 µM ZD7288 at -75 mV (left) and -62 mV (right) holding potential. B. EPSP integrals plotted against holding potential for the two pharmacological conditions demonstrated in A.
slowed decay of the EPSP to dramatically increase the integral of the EPSP. This in turn shifted the threshold for action potential generation in the hyperpolarised direction (figure 3.7B) from $-62.8 \pm 1.5 \text{ mV}$ in control to $-74.2 \pm 3.1 \text{ mV}$ in ZD7288 ($n = 5$).

4.2.8 Temporal summation and $I_h$

The effect of $I_h$ on EPSP decay suggests that it may play an important role in temporal summation of EPSPs, as shown in hippocampal CA1 pyramidal neurons (Magee 1999). To investigate the contribution of $I_h$ towards summation of EPSPs in Purkinje cells, I used trains of 5 waveforms injected at 50 Hz (figure 4.8, $n = 6$). Similar to the results using single waveform injections, ZD7288 caused a widening of the EPSPs at hyperpolarised potentials and augmented boosting at depolarised potentials (figure 4.8A).

To determine which channels underlie the boosting observed in the presence of ZD7288, I added TTX in addition to ZD7288 (figure 4.8B) ($n = 4$). In the presence of both ZD7288 and TTX, boosting was abolished, which identified the boosting in the presence of ZD7288 as mediated by sodium channels. Subtraction plots like those shown in figure 4.4 confirmed that the boosting in ZD7288 is somatically mediated (not shown). In the presence of ZD7288, the after-hyperpolarisation, or 'sag', seen under control conditions (also see figure 4.8B) is not present, demonstrating its mediation by $I_h$.

In response to a train of EPSCs, overlapping somatic EPSPs do not summate indefinitely in amplitude, but rather reach a steady state peak depolarisation (Magee 1999). It has been argued that this normalisation of temporal summation in response to a train of EPSCs under control conditions is...
due to depolarisation-mediated inactivation of $I_h$ during the input train (Magee 1999). However, another possibility is that the difference in peak depolarisation seen when adding ZD7288 to the perfusate might be due to summation of voltage decays with different time constants. To distinguish between these hypotheses I did the following EPSP analysis, exemplified in figure 4.8C.

At hyperpolarised potentials for the either of the two experimental conditions, control and ZD7288, I compared the following: A single EPSP recorded at a membrane potential of $-72$ mV (figure 4.8C) was added onto itself four times with a shift in time of 20 ms. The result is a train of EPSPs simulating the response to a train of 5 EPSCs injected at 50 Hz. This simulated response was then overlaid with an actual response to a train of 5 EPSCs at 50 Hz injected at the same membrane potential. The resulting plot allows for direct visual comparison between the mimicked and actual response to a train of EPSCs. As figure 4.8 demonstrates, the simulated response to a train of inputs accurately replicates the observed response. This means that any degree of normalisation of the somatic EPSP peak during temporal summation with or without $I_h$ at hyperpolarised membrane potentials can be explained solely by summation of EPSPs with different decay time constants. It also suggests that there is only little change in the activation state of $I_h$ during input trains lasting up 100 ms.
Figure 4.8

Properties of Ih. 

A. Somatic EPSPs generated by a 100 ms long 50 Hz trains of dendritic current injections ($\tau_{\text{rise}}$ 0.3 ms, $\tau_{\text{decay}}$ 3.0 ms, 1.0 nA peak) at -65 mV (top) and -75 mV (bottom) holding potential under control conditions and in the presence of 40 µM ZD7288. 

B. Somatic EPSPs generated by a 100 ms long 50 Hz train of dendritically injected EPSCs ($\tau_{\text{rise}}$ 0.3 ms, $\tau_{\text{decay}}$ 3.0 ms, 1.0 nA peak) under control conditions, in the presence of 40 µM ZD7288 and in the presence of ZD7288 and TTX from a holding potential of -65 mV. 

C. EPSP in response to a 50 Hz train of 5 EPSCs ($\tau_{\text{rise}}$, 0.3 ms, $\tau_{\text{decay}}$ 3.0 ms, 1.0 nA peak, same cell as A) at -75 mV under control conditions (top) and in the presence of 40 µM ZD7288 (bottom). An EPSP in response to a single EPSC was added onto itself four times (baseline removed) with a 20 ms time shift in order to mimic the response to a 50 Hz train of 5 EPSCs. The calculated EPSP train and the single EPSP are overlaid with the actual response to allow direct comparison.
4.2.9 4-AP hyperpolarises the voltage range of EPSP boosting

It has been suggested that a high dendritic potassium channel density at synaptic locations limits activation of excitatory dendritic voltage-gated channels (Storm 2000) akin to a “shock absorber”. It is therefore possible that potassium channels inhibit regenerative activation of dendritic calcium channels thus allowing somatic sodium channels to drive EPSP boosting though a similar effect can be obtained with an uneven $r_m$ (London et al. 1999). The basis of the shock-absorber idea is a rising density of potassium channels as one moves out the dendritic tree away from the soma (Hoffman et al. 1997). However, there appears to be no universal principle describing the gradient of potassium channels along the somato-dendritic axis (Hoffman et al. 1997; Bekkers 2000; Korngreen & Sakmann 2000).

I tested the effect of blocking 4-AP sensitive potassium channels on EPSP propagation in Purkinje cells. I found that Purkinje cells are extremely sensitive to bath-applied 4-AP. At concentrations above 200 µM the cells became highly excitable generating spontaneous large voltage deflection and action potentials even at hyperpolarised membrane potentials. Furthermore, 4-AP concentrations above 200 µM caused significant physical drift in the slice preparation, making dendritic patch recordings difficult if not impossible. To mitigate these problems I lowered the 4-AP concentration to 100 µM ($IC_{50} = 273$ µM reported for peak and steady state potassium currents for Purkinje cells in somatic outside-out patches (Southan & Robertson 2000).
4-AP hyperpolarises the voltage range of EPSP boosting **A**. EPSPs generated by a dendritically injected current waveform (τ\_rise 0.3 ms, τ\_decay 3.0 ms, 1.0 nA peak, 135 μm from the soma) under control conditions and in the presence of 100 μM 4-AP at three different holding potentials: -58 mV, top, -61 mV, middle and -82 mV, bottom. Left column, somatic EPSPs in response to the dendritic current injections at the three holding potentials. Right column, difference plots demonstrating peri-somatic origin of EPSP boosting. Notice the contribution of noise in the presence of 4-AP. **B**. Integrals of the EPSPs under control conditions (filled circles) and in the presence of 4-AP (open circles). Notice that the EPSP in the presence of 4-AP, the integral of the EPSP is variable due to noise.
Bath-applied 4-AP (100 µM) had no effect on somatic or dendritic EPSPs at hyperpolarized potentials (figure 4.9A). However, the voltage threshold for EPSP boosting was shifted in the hyperpolarized direction in the presence of 4-AP (threshold -63.5 ± 0.4 mV in control vs. -66.2 ± 0.4 mV in 4-AP; n= 4, p < 0.01). In order to investigate whether potassium currents could underlie the cell polarity of EPSP boosting, I subtracted the dendritic voltage recording from the somatic recording during EPSP boosting in control and in the presence of 4-AP. EPSP boosting was somatically mediated in both control solution and in the presence of 4-AP (figure 4.9B). Injecting dendritic EPSC waveforms with a peak of up to 8 nA did not alter the polarity of EPSP boosting (data not shown, but see figure 4.6).

4.2.10 4-AP abolishes polarity of calcium channel mediated EPSP boosting

At very depolarised potentials, Purkinje neurons exhibit calcium channel mediated EPSP boosting (figure 4.5A and figure 4.10A). The voltage range over which this calcium EPSP boosting occurs is shifted in the hyperpolarised direction in the presence of 4-AP on top of TTX (threshold in TTX –52.0 ± 0.3 mV, TTX + 4-AP –57.6 ± 0.5, n = 3 p < 0.01; figure 4.10A). By generating difference traces, I found, as before (figure 4.10, right column), that EPSP boosting in the presence of TTX in more prominent at the dendritic recording location. But after adding 4-AP to the perfusate, the spatial gradient of calcium channel mediated EPSP boosting is abolished (figure 4.10, bottom right).
4-AP abolishes polarity of EPSP boosting in the presence of TTX

A. EPSPs generated by a dendritically injected current waveform ($\tau_{\text{Rise}}$ 0.3 ms, $\tau_{\text{Decay}}$ 3.0 ms, 1.0 nA peak, 71 µm from the soma) in the presence of 0.5 µM TTX and TTX + 100 µM at three different holding potentials: -47 mV (TTX only), top, -53 mV, middle and –83 mV, bottom. Right column. Somatic EPSPs in response to the dendritic current injections at the three holding potentials. Left column, difference plots demonstrating dendritic origin of EPSP boosting in the presence of TTX and absence of voltage gradient in the presence of TTX & 4-AP. Notice the contribution of noise seen in the presence of 4-AP alone (figure 3.9) is absent in the presence of TTX.

B. Integrals of the EPSPs in the presence of TTX (black circles) and in the presence of TTX + 4-AP (red circles).
4.2.11 Temporal integration mediated by EPSP boosting

It has been suggested that transformation of transient synaptic input into a prolonged response neuron provides a mechanism for temporal integration and short-term memory (Abbott 2000; Seung et al. 2000). I therefore investigated the relationship between EPSP boosting and Purkinje cell output. Current waveforms were injected into a Purkinje cell dendrite while the neuron was continuously firing action potentials and at different levels of hyperpolarisation (figure 4.11A, top; n = 3), and post-stimulus time histograms (PSTH) were computed.

When the neuron was continuously firing (in the absence of holding current), the PSTH showed that a transient synaptic input only increases the firing frequency of the Purkinje cell briefly (figure 4.11A). However, when hyperpolarizing the cell to just below threshold for continuous firing (-60 mV, figure 4.11B), the same amplitude current waveform the neuron reliably displayed plateau potentials of up to several hundred milliseconds. These potentials caused a variable number of action potentials (figure 4.11B). The resulting PSTH showed an initially high probability of action potential generation following the input. The probability then falls over the following second (figure 4.11B). At more hyperpolarised potentials (-70 mV, figure 4.11C) a larger amplitude current waveform was needed to generate spikes, which however were tightly clustered and were not associated with a plateau potential. When
Figure 4.11

Temporal integration mediated by EPSP boosting. A Somatic EPSPs generated by a dendritically (97 µm from the soma) injected current waveform. Top, no holding current; peak waveform current 2.5 nA. B. Same cell held at -60 mV with the same waveform. C. same cell as above held at -70 mV and a current peak injection of 4.5 nA. Left column. Post stimulus time histograms of traces as demonstrated in the right hand column. Inset, PSTH for no holding current and a current injection peak of 4.5 nA.
the cell was continuously firing, increasing the amplitude of the current waveform did not affect the shape of the PSTH (Fig. 9). Together these results demonstrate that integration of transient input to the Purkinje cell is crucially dependent on the state of the cell at the time of the synaptic input. Under favourable conditions, with the membrane potential sitting just below firing threshold, a single input can be transformed into a long-lasting response, thus vastly expanding the window for temporal integration in Purkinje cells.

4.2.12 EPSP boosting by dendritic calcium channels

In sections 4.2.2 and 4.2.3 I described a mechanism for EPSP boosting mediated by calcium channels. Under control conditions (i.e. without block of potassium channels), this form of EPSP boosting has a relatively high voltage threshold and is more prominent in the dendritic tree than in the soma (Llinás & Sugimori 1980).

Given the high threshold for calcium channel mediated boosting I found little to no contribution at subthreshold membrane potentials, but I wished to ascertain the influence of dendritic calcium channels at membrane potentials close to where the Purkinje cell is spontaneously firing action potentials. In order to this I used the same data as described in section 4.2.11, where a single dendritic EPSC is converted into prolonged action potential firing (figure 4.12, n=3) I overlaid the dendritic and somatic voltage traces to determine where the depolarisation is the greatest. As can be seen in figure 4.12A, the voltage response was greater at the somatic location. This indicates that plateau generation from a single input is due mainly activation of somatic sodium channels (Llinás & Sugimori 1980).
It is possible that in order to overcome the voltage-sink effect of branching in the surrounding dendritic tree, a localised input will have to be prolonged for *regenerative* activation of dendritic calcium channels to occur (Rapp et al. 1994; Koch 1999). To test this I recorded simultaneously from two dendritic locations while activating the parallel fibres with 7-11 stimuli delivered at 100 Hz, such that the Purkinje dendrites innervated were in the area around one of the recording pipettes (figure 4.12B, n= 6).

In most cases (4 cells out of 6) parallel fibre activation lead to increased spike firing at the soma which was associated with a plateau potential outlasting the train of PF fibre input (figure 4.12B, *top*). This plateau was consistently larger at the distal dendritic recording electrode (i.e. the electrode closest to the synaptic stimulus), even long after the end of the stimulation, indicating that it was generated by activation of dendritic voltage-gated channels.

In a few cases (2 cells of 6), the input train of PF activity resulted in full dendritic spike generation at the site of PF activation. These spikes were large in amplitude (peak potential ~-10 mV; width ~ 3 ms) and comparable to calcium spikes triggered by strong dendritic depolarisation. Similarly to the plateau potentials, these spikes propagated only very poorly in the dendritic tree of the Purkinje cell, as they were almost undetectable at the site of the second dendritic pipette a few tens of microns away.

This suggest that dendritic calcium channels are activated *regeneratively* under conditions of intense excitatory stimulation, particularly when the Purkinje cell is firing sodium action potentials.
Figure 4.12

Location of EPSP boosting with action potential firing. A. Same cell as in figure 4.11A, middle, but here with the dendritic and somatic traces overlaid for comparison. The amplitude of the somatic action potentials have been clipped for clarity. Notice that throughout the action potential generating plateau, the soma is overall more depolarised than the dendritic recording location. B. Proximal (red traces) and distal (green traces) simultaneous double dendritic recordings (58, 137 µm, top and 58, 100 µm, bottom, from soma, respectively). In the cell demonstrated in the top panel, the activation at the distal recording pipette of 11 parallel fibre input @ 100 Hz generates a plateau which is attenuated strongly over the distance to the second pipette. For the cell in the lower panel, activation of 7 parallel fibre inputs @ 100 Hz locally caused a dendritic spike burst, which is almost undetectable at the more proximal pipette.
4.3 Discussion

These results demonstrate that amplification of EPSPs (‘EPSP boosting’) by voltage-gated channels is a prominent feature of the response repertoire of cerebellar Purkinje cells to synaptic input for both sub- and suprathreshold responses. This EPSP boosting acts primarily to prolong the duration of the EPSP and can lead to depolarising plateaus. At subthreshold membrane potentials sodium channels located near or at the soma mediate EPSP boosting, with little contribution of voltage activated calcium channels. However, in the presence of TTX, at membrane potentials corresponding to tonic firing, calcium channel mediated plateaus are observed. This calcium-channel mediated boosting was larger at the dendritic recording site. Indeed, when dendritic synaptic input strongly depolarised the dendritic membrane during action potential firing, dendritic plateau potentials presumably corresponding to calcium channel-mediated boosting were observed.

EPSP amplification by sodium and calcium channels was strongly regulated by the activity of other voltage-gated channels. Low concentrations of 4-AP abolished the somato-dendritic differences in boosting at depolarised potentials. This suggests that local somatic K channel activation, rather than a gradient of calcium channels, is responsible for limiting boosting at the soma at depolarised potentials, in agreement with direct measurements (Usowicz et al. 1992), showing a relatively even somato-dendritic density of calcium channels.

Hyperpolarisation activated channels also play an important role in regulating the interplay of these channels. Activation of Ih shortens membrane time constant, thus narrowing the window of temporal integration. As a
consequence of a more rapid effective membrane time constant, activation of persistent sodium channels is reduced at subthreshold potentials under control conditions.

Together these findings suggest that during silent periods in spontaneous firing (Eccles et al., 1967), (Jaeger & Bower 1999). Purkinje cells can transform a brief synaptic input into a prolonged output response. This feature is important for the temporal integration of synaptic inputs and may provide a form of state-dependent short-term memory in the Purkinje cell (Abbott 2000; Seung et al. 2000).

4.3.1 Subthreshold EPSP boosting is mediated by perisomatic sodium channels

Subthreshold boosting of EPSPs in cerebellar Purkinje cells is mediated by activation of sodium channels located near or at the soma. As EPSP boosting was a prolongation of the EPSP more than peak amplification, suggesting it is mediated by persistent sodium channels (I_{NaP}). The involvement of I_{NaP} in EPSP boosting has previously been suggested in pyramidal cells (Stafstrom et al. 1985), (Sutor & Hablitz 1989), (Stuart & Sakmann 1995), (Andreasen & Lambert 1999). Unfortunately, no selective blocker or I_{NaP} currently exists, making it difficult to test this hypothesis directly.

Under control conditions somatic sodium channel activation is limited by 4-AP sensitive potassium channels. Firstly, when applying a partial potassium channel block with 100 µM 4-AP, boosting started from a more hyperpolarised membrane potential relative to control (Hoffman et al. 1997). Secondly, in the presence of 4-AP there was a considerable amount of electrical noise in the voltage recordings. These noise fluctuations are abolished by the application of
TTX demonstrating that they are due to sodium channel activation. Together these findings suggest a role for potassium channels in keeping subthreshold EPSP boosting under control and to limit the influence of intrinsic noise on synaptic integration (Fricker & Miles 2000).

The polarity of subthreshold EPSP boosting under control conditions was not altered by 4-AP. This suggests that over the subthreshold range of membrane voltages, potassium conductances do not limit dendritic activation of inward ion currents, especially calcium channels. However the relatively low concentrations of 4-AP used means that further experiments are required to more thoroughly investigate this point.

4.2.2 Suprathreshold EPSP boosting is mediated by dendritic calcium channels

There is little to no contribution of calcium channels to subthreshold EPSP boosting under control conditions in cerebellar Purkinje cells. Only at membrane potentials where the cell fires action potentials continuously did the Purkinje cell exhibit boosting of EPSPs mediated by calcium channels. Calcium EPSP boosting started at membrane potentials close to –50 mV and had a time course of up to several hundred milliseconds. The depolarised threshold and long time course suggests mediation by P-type rather than T-type channels, the two major calcium channels found in Purkinje cells (Regan 1991; Llinas et al. 1992). At the steady state membrane potentials where calcium mediated EPSP boosting occurs, T-type calcium channels are inactivated, and (from a hyperpolarised pre-potential) have an inactivation time constant of ~ 30 ms (Regan 1991). EPSP boosting in the presence of TTX was larger in the dendritic tree than at the soma. This could indicate that the dendritic density of
calcium channels is higher than that at the soma, as has been suggested elsewhere (Llinás & Sugimori 1980). However, in the presence of 4-AP as well as TTX the spatial gradient of calcium channel EPSP boosting was abolished. The gradient of calcium channel mediated EPSP boosting is less pronounced than the gradient associated with sodium channel mediated boosting.

Together these observations are consistent with a uniform somato-dendritic distribution of P-type channels (Usowicz et al. 1992) and a high-somatic/low-dendritic density of potassium channels. Various labelling studies have found a high potassium channel density at the soma of Purkinje neurons (Rhodes et al. 1996; Serodio & Rudy 1998; Chung et al. 2001). Direct measurements of potassium current density using cell-attached patch-clamp recordings are still in progress (Martina et al. 2001)

The depolarised potentials required to elicit calcium EPSP boosting suggest that calcium EPSP boosting is triggered only under conditions of intense excitatory synaptic activity such as by the climbing fibre or during synchronous activation of many nearby parallel fibres. My results suggest that the conditions for regenerative activation of dendritic calcium channels exist but may be un-physiological for PF activity. The exact conditions under which parallel fibre input alone activates regenerative dendritic events need further clarification. However, several studies have described a plateau potential during the Purkinje cell response to climbing fibre stimulation both in vitro (Miyakawa et al. 1992; Midtgaard 1995) and in vivo (Campbell & Hesslow 1984). Calcium channel EPSP boosting may facilitate the dendritic plateau triggered by climbing fibre activity.
4.3.3 Hyperpolarisation activated cation current limits EPSP boosting

The experiments using ZD-7288 to block the hyperpolarisation-activated cation current \( I_h \) show that this current narrows the window of temporal integration and limits somatic sodium mediated boosting of EPSPs.

At hyperpolarised membrane potentials (where \( I_h \) is the dominant current) the decay time course but not the peak of single EPSPs is increased. For trains of EPSPs lasting up to 100 ms, the time course of EPSP decay determines attenuation of somatic peak summation. For longer time courses of EPSP decay the somatic EPSPs will summate to a higher steady state peak depolarisation. When summation of EPSPs at the soma is mimicked by adding a single EPSP onto itself with a time shift, this reproduces the experimental data, indicating that the time course of EPSP decay determines the level of EPSP summation. In other words, it is not necessary to invoke the inactivation of a standing inward current mediated by \( I_h \), as has previously been suggested (Magee 1999). However, it should be noted that in TTX & ZD7288 the EPSP after-hyperpolarisation seen at hyperpolarised potentials is abolished. The after-hyperpolarisation under control conditions indicates a small amount of \( I_h \) inactivation by the EPSP.

The distribution of \( I_h \) channels in Purkinje cells is not known. However, equal somatic and dendritic sag ratios in control and similar increases in somatic and dendritic input resistance following to block of \( I_h \) suggest a fairly even distribution across the cell, in contrast to pyramidal cells, where there is a steeply increasing gradient of \( I_h \) channels into the distal dendrites (Magee 1998; Williams & Stuart 2000).
4.3.4 EPSP boosting alters the input-output relationship

The pattern of action potential generation in Purkinje cells varies with membrane potential. While the cell is spontaneously firing or is hyperpolarised, EPSP boosting is not prominent, and action potentials triggered by EPSPs tend to be precisely locked to the input, with the width of the resulting peak in the PSTH being < 5 ms. However, when close to threshold the Purkinje cell can convert a single brief input into a plateau potential thereby significantly increasing the temporal integrative properties of the cell. While continuously firing the Purkinje cell tends to respond only briefly to synaptic input, unless the input is strong enough to trigger dendritic plateau potentials, which substantially outlast the period of synaptic stimulation.

These results demonstrate that Purkinje cells may integrate synaptic input over periods lasting from tens of milliseconds up to almost a second solely depending on the membrane potential of the neuron and the level of synaptic activity. Integrating input over time is a well-described feature of the control of eye movements, particularly when eye position must be maintained in space (Robinson 1989; Carpenter 2000; Seung et al. 2000), in which the cerebellum plays a key role as a gain integrator (Carpenter, 1991). It has been proposed that reverberating activity in local networks provides the integrator of eye-position over time (Seung et al. 2000). My results suggest an alternative possibility where the intrinsic properties of the Purkinje neuron provide a mechanism of integration over time.
5.1 Introduction

The climbing fibre (CF) innervation of Purkinje neurons is unique. In the mature animal each Purkinje neuron is contacted by one and only one CF, even though the same fibre may innervate ~10 Purkinje neurons. With a high probability of release and about 500 release sites in the rat cerebellum (Silver et al. 1998) this synaptic connection is one of the most powerful in the central nervous system. Activation of the climbing fibre input to Purkinje neurons triggers a stereotyped 'complex' spike (Eccles et al. 1966), normally consisting of 3-5 somatic spikes and one to three dendritic spikes (Eccles et al. 1966; Campbell & Hesslow 1986).

The one-to-one nature and sheer magnitude of the CF response in Purkinje neurons suggests that it has a special function in the operation of the cerebellar cortex. While it is unclear what element of the CF response is transmitted down the axon (Campbell & Hesslow 1986), it is now well-established that pairing CF input with a train of parallel fibre inputs leads to long-term depression (LTD) at the parallel fibre synapses (Ito 2001). It follows that a neuronal mechanism for detecting the coincident CF + PF input to the Purkinje neuron must exist in one form or other. In layer 5 pyramidal neurons, backpropagating sodium spikes can function as coincidence detectors of pre- and postsynaptic activity (Stuart & Hausser 2001), and, given the poor backpropagation in Purkinje neurons (Stuart & Hausser 1994), it has been
speculated that the CF in Purkinje neurons fulfils a functionally similar role to the backpropagating action potential in layer 5 pyramidal cells (Häußer et al. 2000).

For the expression of LTD at the parallel fibre to Purkinje neuron synapse the increase in intracellular calcium is both necessary (Sakurai 1990; Konnerth et al. 1992; Freeman et al. 1998) and sufficient (Kasuno & Hirano 1994; Khodakhah & Armstrong 1997). This indicates that intracellular calcium changes may be the detection signal triggering LTD. Purkinje neurons do not express the calcium permeable ionotropic NMDA-receptor. However, it has been known for decades that dendrites of Purkinje neurons express prominent voltage activated calcium currents (Llinas & Sugimori 1980).

Early calcium imaging of Purkinje neuron dendritic trees revealed that a large global intracellular calcium concentration increase follows CF activation (Ross & Werman 1987; Miyakawa et al. 1992), indicating that the CF input activates voltage-gated calcium channels in the dendritic tree. Later studies (Finch & Augustine 1998; Suzuki et al. 2001) have confirmed and extended the original studies, demonstrating that PF activation can also cause local voltage-dependent increases in intracellular concentration (Eilers et al. 1995; Hartell 1996). Recently local supralinear calcium transient summation in dendritic spines has been observed in Purkinje neurons in response to paired activation of PF and CF input (Wang et al. 2000). A likely candidate for the mediation of supralinear calcium signals is the activation of regenerative calcium spikes (Midtgaard 1995).

In this chapter I investigate the electrical events triggered in the dendritic tree by the CF input using direct dendritic patch-clamp recordings. I investigate
the modulation in time and space of regenerative calcium channel activation in the dendritic tree of Purkinje neurons consistent with the idea of dendritic calcium spikes mediating the coincidence detection necessary for the induction of synaptic plasticity.

5.2 Results

5.2.1 Spread of calcium and sodium spikes in Purkinje cell dendrites

Electrogenesis is mediated by different mechanisms in the soma and the dendrites of Purkinje cells, with sodium spikes restricted to the soma. These different somato-dendritic features were highlighted in a famous figure by Llinas and Sugimori (figure 4, (Llinas & Sugimori 1980)), showing sodium spikes restricted to the soma and calcium spikes restricted to the dendrites. However, the figure of Llinas and Sugimori is a composite of recordings from different cells, due to the difficulty of making simultaneous recordings from multiple sites on the same neuron using the microelectrode techniques available at the time. This makes it difficult to understand the temporal relationships between electrical events at different locations, and in particular the precise site of initiation of individual events. Furthermore, the precise location of microelectrode recordings in the dendritic tree is difficult to determine, rendering the results qualitative in nature. I therefore used simultaneous triple-patch recording from Purkinje neurons in the whole–cell current clamp configuration to examine the initiation and spread of sodium and calcium spikes. Identical current pulses were injected simultaneously through all three pipettes to cause calcium spike activation (0.3-0.5 nA through each pipette, n= 2).
Sodium spikes were largest at the soma and then rapidly decremented in the dendritic tree. By examining the temporal relationship of the sodium spikes at the three locations (see inset), the sodium spike clearly occurred first at the somatic site, consistent with results showing axonal initiation of these events (Stuart & Hausser, 1994).

In contrast, dendritic calcium spikes under these recording conditions were always largest at the most distal dendritic recording site (located between 120 and 150 µm from the soma), and decremented rapidly as they propagated towards the soma. Remarkably, the calcium spike was almost undetectable as a distinct event at the soma. The amplitude of the calcium spikes at the dendritic sites was variable from spike to spike, and was not a stereotyped event like the somatic sodium spikes. Nevertheless, the relative amplitude of calcium spikes was usually consistent within a given cell for this method of stimulation, suggesting that the initiation site and properties of spread are relatively constant. Calcium spikes were generally preceded by a series of somatic sodium spikes, which were associated with a ramp of dendritic membrane potential. The overlay of single calcium spikes revealed that the onset of the calcium spike was less clearly defined than for the sodium spike at the soma, due to its smooth sigmoidal shape. Nevertheless, it was usually possible to distinguish from the onset that the Ca spike was initiated at the site at which the calcium spike was biggest.
Figure 5.1

Spread of sodium and calcium spike in Purkinje cell dendrites. Simultaneous voltage recordings at three different locations – at the soma, 96 µm from the soma and 138 µm from the soma. At all three recording locations a 1 sec, 0.3 nA depolarising current was injected to cause calcium spikes as well as sodium spikes, bottom inset. Sodium action potentials are initiated and back-propagate in a rapidly decrementing manner into the dendritic tree. Calcium spikes, top inset, however are initiated and are most prominent at the more distal recording site and do not propagate all the distance to the soma.
5.2.2 CF causes a stereotyped response with voltage dependent secondary dendritic spikes

At the soma the CF elicits a so-called “complex spike” consisting of the EPSP from which a variable number of sodium spikes and ‘spikelets’ are generated (figure 5.2, blue traces, n = 35). (Eccles et al. 1967). At dendritic recording locations more than ~ 50 µm from the soma, the CF response consisted of a big EPSP depolarising the neuron to near its synaptic reversal potential of 0 mV (absolute peak -1.6 mV ± 1.4, from a membrane potential of – 49.6 ± 1.2 mV, n=16) and sometimes above. When the Purkinje cell is continuously firing, one or more secondary calcium spikes (figure 5.2, top) were initiated at the dendritic location.
Figure 5.2

Climbing fibre activation causes a stereotyped and voltage-dependent response. Somatic and dendritic recording (96 µm from soma), while stimulating the climbing fibre in the granule cell layer. A. The somatic (blue) and dendritic (green) response while the neuron is firing spontaneous action potentials from a membrane potential of ~49 mV. Note that the multiple fast spikes initiated by the CF input at the soma, and the large EPSP followed by secondary calcium spikes at the dendritic recording location. B. Applying holding current through both recording pipettes polarises the neuron to –57 mV, and at that membrane potential the CF input does not cause regenerative spiking at the dendritic recording site, while at the soma multiple fast spikes are still triggered. Note the depolarising plateau following the CF response, probably caused by activation of persistent sodium channels as described in chapter 4. C. Hyperpolarising further to –72 mV hardly changes the nature of the CF input when compared to the response at –57 mV.
5.2.3 CF responses depolarise the entire dendritic tree

The CF innervates the proximal two thirds of the Purkinje cell dendritic tree over which it makes several hundred synaptic connections (Palay & Chan-Palay 1974; Silver et al. 1998). The distributed nature of the connection suggests that the whole dendritic tree becomes depolarised during CF activity (figure 5.3) (Llinas & Sugimori 1980). Also, prominent calcium transients have been recorded throughout the dendritic tree during CF activity in vitro (Miyakawa et al. 1992) and in vivo (Häusser & Svoboda 2000).

In order to determine the effective spread of the CF response through the Purkinje cell I plotted the ratio of EPSP amplitude/membrane potential for all the cells recorded from under two conditions; during tonic firing and from a membrane potential of ~ -65 mV. As figure 5.3 demonstrates, the CF response does not diminish in amplitude at either membrane potential while propagating through the dendritic tree. At all recording locations, the ratio of the CF response is near one, again demonstrating the powerful nature of the CF input.

At recording locations close to the soma (less than ~ 70 µm) the somatic spikes are evident, but not so at more distal sites in accordance with poor back-propagation of sodium spikes in Purkinje neurons (Stuart & Häusser 1994; Vetter et al. 2001).
Figure 5.3

The climbing fibre response depolarises the entire dendritic tree. A. A proximal (30 µm from the soma) and a more distal (138 µm from the soma) recording of the CF response during spontaneous firing and while applying holding current to –65 mV. Note for the proximal recording site that the backpropagating sodium spikes feature prominently in the recording while at the distal site only a very minor voltage deflection is left. B. Plots of the ratio of CF EPSP amplitude over membrane potential at two different membrane potentials – during continuous firing and around –65 mV. Notice over the dendritic tree reachable by patch pipettes the amplitude of the CF response is uniform.
5.2.4 Development of secondary dendritic spikes is history dependent

Even though the dendritic tree is depolarised to a similar extent during the CF EPSP independent of holding potential, only at depolarised potentials were secondary dendritic spikes generated. Previous work (Midtgaard et al. 1993) suggests that the difference lies in the inactivation of an A-type like potassium current at depolarised potentials. In order to investigate why secondary dendritic spikes were elicited at depolarised potentials, it was important to determine the timing relationship for depolarisation and the CF input triggering secondary dendritic spikes. In order to do this I applied simultaneous somatic and dendritic patch-clamp recordings (n=20) while applying a holding current to hyperpolarise the membrane to ~ -75 mV. To trigger secondary spikes, the holding current was released at various times relative to the CF stimulation (in 25 ms intervals, figure 5.5).

Following release of holding current, no change is observed in the complex spike waveform at the soma with increasing separation between release of holding current and CF stimulation (figure 5.5, *top*). However at the dendritic location there was a clear timing dependence of the development and initiation of secondary spikes (figure 5.5, *middle*). For the shortest time periods from release of holding current to CF stimulation no secondary spike was observed, then from periods of ~ 75 ms a graded regenerative event emerges, which transforms into a full secondary spike over a further ~ 200 ms. In order to quantify the time course of development of these secondary dendritic spikes I measured their amplitude and plotted them against time of relief of holding current (figure 5.5, *bottom*, half activation, $147.3 \pm 14.1$ ms, n=9).
Figure 5.4

History dependence of development of secondary dendritic spike. **A.** A somatic whole cell recording hyperpolarised to \(-72\) mV. In increasing steps of 25 ms up to 400 ms holding current was released before the climbing fibre was stimulated (arrow). In the expanded view on the right, one can see that the somatic CF response is unaffected by the varying time spent at depolarised potentials with spontaneous firing. **B.** A simultaneous dendritic recording from the same neuron as in **A.** In the expanded view on the right on the right the development with time of a dendritic secondary spike is demonstrated. **C.** The amplitude of the secondary dendritic spike plotted against the time of no holding current (half activation: \(140.1\) ms)
5.2.5 Pairing CF with PF input triggers local dendritic secondary spikes

In response to pairing of CF and PF inputs, Purkinje neurons express long term depression (LTD) at the PF synapses (Ito 2001). This synaptic plasticity is spatially restricted (Linden & Connor 1995; Reynolds & Hartell 2000).

I wished to directly measure the interaction of PF and CF input to the Purkinje neuron at the dendritic site where pairing is actually occurring. In order to do this I recorded simultaneously from two dendritic locations while stimulating the CF as well as the parallel fibres innervating the area around one of the recording pipettes (figure 5.5 & 5.6, inset). I did this under two different paradigms, either with the two recording pipettes on the same dendritic branch (figure 5.5, n=3) or on different branches (figure 5.6, n=3).

When pairing a single CF stimulus with a preceding train of PF inputs, it was possible to elicit additional dendritic calcium spikes (figure 5.5 & 5.6, top). At a frequency of 100 Hz the number of PF inputs necessary to produce this effect varied between 7 and 11, locally depolarised the membrane at the time of CF onset to -31.9 ± 1.0 mV (n= 5). These secondary calcium spikes were not produced by either of the PF or CF alone (figure 5.5 & 5.6, bottom). When the two recording pipettes were placed on either side of a branch point (inter-pipette distance 103 - 165 µm, figure 5.6) the additional dendritic calcium spikes elicited by pairing at one pipette location, were not detectable at the location of the other pipette (figure 5.6). However, when the two recording pipettes were placed along the same dendritic branch with pairing taking place at the distal-most pipette, some of the regenerative event elicited distally was always
Figure 5.5

Pairing of PF and CF input on the same dendritic branch. Voltage recordings from two locations along the same dendritic branch (58 and 137 µm from the soma). A stimulation pipette was placed in the PF fibre tract near one of the recording electrodes. Another stimulation pipette was placed in the granule cell layer to activate the CF. The recording setup is demonstrated in the top inset. A. A train of 11 PF stimuli were delivered at 100 Hz, followed after 10 ms by activation of the CF. The local pairing of PF and CF input to the dendritic caused the CF input to elicit additional secondary spikes. However, in opposition to observing separate dendritic branches, it is possible to detect the pairing induced secondary spikes further along the same dendritic branch B. Either one of the two synaptic inputs activated individually did not cause additional dendritic spiking.
Figure 5.6

Pairing of PF and CF input on separate dendritic branches. Recordings from two dendritic branches (135 and 112 µm from the soma, branch point 39 µm from soma). A stimulation pipette was placed in the PF fibre tract innervating the area around one of the recording electrodes. Another stimulation pipette was placed in the granule cell layer to activate the CF. The recording set-up is demonstrated in the top inset. A. A train of 11 PF stimuli were delivered at 100 Hz. followed after 10 ms by activation of the CF. The sequential activation of PF and CF input caused a steady depolarisation during the PF activity and a CF response with additional secondary dendritic spikes in the dendritic branch where the synaptic pairing is taking place. B. Either one of the two synaptic input activated individually did not cause additional dendritic spiking.
observed at the more proximal location (inter-pipette distance 35 – 79 µm, figure 5.5). These results indicate that in response to the pairing of a train of PF input and a CF input, local secondary calcium spikes are elicited in the dendritic tree of the Purkinje cell. This suggests that these local secondary spikes may be branch-specific.

5.2.6 Inhibition locally suppresses the CF response

A previous study found a profound effect of inhibition on the dendritic CF induced calcium signal (Callaway et al. 1995). I wished to investigate the electrical correlate of inhibiting the dendritic calcium signal. Simultaneous recordings were made from two nearby dendritic locations (n=3, distances were 29, 40 & 42 µm) along the same dendritic branch. While the Purkinje neuron was continuously firing I stimulated the CF and injected inhibitory waveforms (0.5-3 nS, $\tau_{\text{rise}}$: 0.3 , $\tau_{\text{decay}}$: 3.0 ms, $E_{\text{rev}}$: -75 mV (Southan & Robertson 1998)) using an analogue dynamic clamp circuitry (see Materials and Methods) (Harsch & Robinson 2000). The inhibitory waveforms were injected at 1 ms intervals over 7 ms following CF stimulation (figure 5.7).

For all neurons studied it was possible to substantially reduced the amplitude of the primary peak (75.8 ± 0.7%) or the secondary peak (65.0 ± 0.5%) by using a 3 nS GABA-like inhibitory conductances at the site of inhibition. However, no effect of the inhibitory conductance was detectable at the non-inhibited recording pipette nearby. To effectively reduce the dendritic CF response it was necessary to inject the inhibitory conductance coincidentally
Figure 5.7

Inhibition locally reduces the dendritic CF response. A. Simultaneous triple voltage recordings from the soma and at two sites along the same dendritic branch (96 and 138 µm from the soma). A stimulation pipette was situated in the granule cell layer to activate the CF. All three pipettes recorded membrane voltage, while an inhibitory synaptic conductance was injected via the more distal recording pipette using dynamic clamp (see Materials and Methods). The recording set-up is demonstrated in the top inset. B. At times 1 and 3 ms after CF activation, an inhibitory conductance ($\tau_{\text{rise}}$: 0.3, $\tau_{\text{decay}}$: 3.0 ms, $E_{\text{rev}}$: -75 mV, amplitude: 3 nS) was applied through the more distal pipette. When temporally coinciding with a peak in the CF response, the inhibitory conductance was able to ablate the dendritic depolarisation, A bottom traces. This ablation was very local as it was not detectable 40 µm along the same dendritic branch.
with the occurrence of the peak to be reduced, thus suggesting a very tight temporal localisation of the inhibitory effect.

It was not possible to inject conductances larger than 3 nS, due to ringing in the electrical feedback circuitry introduced by the dynamic clamp method (see section 2.3.3).

5.2.7 Secondary spikes are calcium mediated

Calcium imaging of climbing fibre responses demonstrate (Miyakawa et al. 1992) large calcium transients and Purkinje neuron dendrites support regenerative calcium spikes (Llinás & Sugimori 1980). It thus seemed likely that secondary dendritic spike in the CF response are calcium mediated. To test this I recorded simultaneously from three locations on the same Purkinje neuron while repeating the timing protocol demonstrated in the previous section (figure 5.4). The protocol was repeated this time substituting the CF stimulation with a 0.3 nA current waveform ($\tau_{\text{rise}}$: 0.7 ms, $\tau_{\text{decay}}$: 7 ms) simultaneously injected through each of the three recording pipettes. The relatively slow current waveform was chosen to compensate for the less distributed input the current injection represents in comparison to the CF. As shown in figure 5.7, distributed current injections are able to mimic the generation of secondary dendritic spikes under control conditions (figure 5.7, B). Particularly are they able to reproduce the time course of development of secondary calcium spikes after release of holding current (figure 5.7). The generation of secondary dendritic spikes is blocked by the application of cadmium (100 µM), thus demonstrating their mediation by voltage-gated calcium channels (figure 5.7, C, n = 1).
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Figure 5.8

Secondary dendritic spikes are calcium mediated. A Purkinje neuron was simultaneously patched in three different locations, at the soma and 69 and 127 µm from the soma along the same dendritic branch while applying holding current through all three pipettes to hyperpolarise the neuron to –72 mV. For clarity only the more distal recording is demonstrated here. **A.** At increasing times before stimulating the CF holding current is released and the neuron goes into continuous firing. As the time of no holding current increases CF activation starts to cause secondary dendritic spikes. **B.** Using the same protocol for the steady holding current as in **A,** three simultaneous current waveform injections ($\tau_{\text{rise}}$: 0.7, $\tau_{\text{decay}}$: 7.0 ms, 0.3 nA each) were injected at the same time as the CF was previously stimulated. Secondary dendritic spikes are generated over the same time course as with the CF input. **C.** After bath application of 100 µM CdCl$_2$ the same protocol as in **B** was repeated. In the presence of cadmium there was no development of secondary dendritic spikes.
5.2.8 Sodium channel contribution to the CF response

I wished to study the effect of sodium spikes on the CF response. The CF response at the soma is dominated by a number of sodium action potentials. It is known that there are calcium channels in the somatic membrane, so I wish to investigate whether shunting by sodium spike associated conductances prevent the regenerative activation of somatic calcium spikes. Distributed synaptic inputs can only with great difficulty be substituted for distributed current injections (figure 5.8), thereby allowing one to apply extracellular blockers. An easier approach is to apply intracellular blockers.

To investigate the contribution of sodium spikes I made simultaneous somatic and dendritic double recordings initially with normal intracellular solution, then I carefully withdrew the somatic pipette and re-patched the soma with an internal solution containing 3-4 mM of the intracellular sodium channel blocker QX-314 (Butterworth & Strichartz 1990)(figure 5.8, n=5).

With QX 314 in the pipette, the somatic complex spike response was transformed from a burst of sodium spikes into a composite EPSP with a large broad initial EPSP followed by a secondary spike (figure 5.8, top left). I overlaid the somatic and the dendritic response to directly compare between sites (figure 5.8, bottom right), and I found that when the cell was dialysed with QX-314, the somatic and dendritic responses became qualitatively similar.

At membrane potentials where the neuron is continuously firing under control conditions, the dendritic CF response was qualitatively similar in QX-314 relative to response under control conditions. There was an
Figure 5.9

Sodium spikes shunt the somatic CF response. Somatic and dendritic (125 μm form the soma) voltage recordings under control conditions and with 3-4 mM QX-314 in the internal solution. The somatic recording pipette with control internal solution was retracted and replaced with a pipette containing 3-4 mM of the sodium channel blocker QX-314 as well as normal intracellular solution. A. When applying QX-314 the somatic response becomes similar to the dendritic CF response, which itself does not change significantly. B. Plot of the integrals of the CF responses at the soma and dendrites (n=5) demonstrating that under control conditions the somatic response is shunted relative to the case with sodium channel block, presumably via activation of voltage-gated sodium and potassium channels.
initial EPSP followed by a secondary spike, though the secondary spike was larger in amplitude in the presence of QX-314.

To quantify these differences I measured the integral of the CF response from stimulation until the responses in control and in QX314 became identical and started decaying together (see figure 5.8, insets) and plotted them in a bar chart (figure 5.8, B). Under control conditions the somatic CF response is significantly shunted, presumably secondary to activation of potassium channels (integral in control: $178.1 \pm 20.8$ mV*ms, in QX-314: $258.3 \pm 27.0$ mV*ms, $p < 0.01$) while the dendritic CF is hardly affected (integral in control: $258.2 \pm 19.3$ mV*ms, in QX-314: $272.1 \pm 25.9$ mV*ms). With the inclusion of QX 314 in the intracellular solution the secondary spikes would consistently occur first in the soma and only later in the dendrites (propagation speed, peak-to-peak: $0.46 \pm 2.0 \mu$m/ms, $n = 5$).

These results demonstrate that the conductances underlying action potential generations, and which are blocked by QX-314, prevent the generation of secondary calcium spike at the soma akin to those generated in the dendritic tree.

To study the contribution of sodium channels towards the generation of secondary dendritic spikes following CF stimulation depolarisations at various times relative to CF activation (section 5.2.4) were repeated under control conditions and then with a 3-4 mM QX-314 in the internal solution (figure 5.10, n=3)

The inclusion of QX-314 altered the amplitude of the secondary dendritic spikes (figure 5.9, B), but it did not change the time course of the development
Figure 5.10

QX-314 does not alter the time course of secondary spike development. Same neuron as in figure 5.9. **A** & **B**. Under both control conditions and with 3-4 mM QX-314 included in the internal solution a hyperpolarising current was released at increasing times relative to CF activation. Under both control and experimental conditions did CF activation elicit secondary dendritic spikes as the time of no holding current was increased **C**. A plot of the normalised amplitude of the secondary dendritic spikes plotted against time of release of holding current.
of secondary spikes after release of holding current (figure 5.10, C). To quantify the time course of development of secondary spikes I determined the half-activation time which was not significantly different between control conditions and with QX-314 in the internal solution (control; 161.4 ± 8.0 ms, QX-314; 175.0 ± 27.4 ms, n=3).

5.2.9 Calcium activated potassium channels do not regulate secondary dendritic spikes

Having observed large calcium mediated dendritic regenerative potentials Llinas and Sugimori suggested that dendritic excitability might be limited by calcium activated potassium currents ($K_{Ca}$) (Llinas & Sugimori 1980). It is conceivable that calcium influx at the depolarised membrane potentials of tonic firing might change dendritic excitability. I wished to investigate whether $K_{Ca}$ plays a role in the regulation of secondary dendritic CF spikes and their time course of development. I simultaneously patched Purkinje neurons in the whole-cell current clamp configuration at the soma and at a dendritic location (figure 5.11, n=3). Under control condition I repeated the timing protocol described in section 5.2.4. I then removed the somatic patch pipette and re-patched with a pipette containing 20 mM BAPTA, a fast calcium chelator, to block activation of $K_{Ca}$ channels. I waited 20 minutes and then repeated the protocol of releasing holding current at increasing times prior to CF stimulation (figure 5.11). Calcium activated potassium channels are important in setting the resting membrane potential of the neuron (chapter 3), and high BAPTA concentration in the internal solution the membrane potential of the neuron became irregular (see figure 3.7). Thus it was necessary to apply holding
Figure 5.11

Calcium activated potassium channels do not influence secondary spike generation. Somatic and dendritic (106 µm from the soma). A. Under control conditions a hyperpolarising holding current to –78 mV was released at increasing times before stimulating the CF. Then the somatic recording pipette was removed and replaced with another containing 20 mM BAPTA and the protocol of releasing holding current was repeated. Under both conditions does the time spend at depolarised potentials cause secondary dendritic spikes to an equal extend. B. Simultaneous somatic recordings to the dendritic recordings demonstrated in section A. The inclusion of high internal BAPTA has no discernable effect on the somatic CF response.
current to make the membrane potential under control and high internal BAPTA conditions similar. I then compared the development of secondary dendritic spikes for recordings where the membrane potentials were the same with and without BAPTA (figure 5.11, top). Under these conditions the CF responses in high internal BAPTA were virtually indistinguishable from the responses recorded under control conditions (n=3). I also compared the somatic responses (figure 5.10, bottom). Like the dendritic responses, the somatic CF responses for a given membrane potential were unaffected by the inclusion of high BAPTA in the intracellular solution.

5.3 Discussion

In this chapter I have described the conditions for initiation and local spread of secondary dendritic calcium spikes in response to a CF input to Purkinje neurons. These secondary spikes are initiated when CF and PF inputs are synchronously active, and thus represent a dendritic coincidence detection mechanism.

Generation of secondary dendritic spikes is a voltage dependent process with a half activation time of about 150 hundred milliseconds. Due to the shunting effect of sodium spiking, secondary spike generation is specific to the dendritic tree and does not have a significant impact on the somatic CF response. Local depolarisation by pairing preceding PF input with CF activation caused dendritic spikes. Some electrical correlate of these spikes was observed in more proximal recordings relative to the site of the pairing. However, when recording from a different dendritic branch, i.e. across a branch point away from the soma, there were often no detectable remains of the additional spikes.
resulting from the local pairing. This suggests that PF + CF pairing triggers branch-specific dendritic calcium spikes.

Blocking sodium channels, which are found almost exclusively at the soma in Purkinje neurons (Stuart & Häusser 1994), does not alter the time course of secondary spike development. This further underlines the primarily dendritic nature of the CF response modulation. Neither does blocking of $K_{\text{ca}}$ channels by a high concentration of internal BAPTA alter the development of secondary spikes in the CF response as suggested (Llinas & Sugimori 1980).

Taken together, the spatio-temporal regulation of secondary spike described is consistent with the properties expected of a trigger for LTD in the Purkinje cell dendritic tree.

5.3.1 Spatial regulation of secondary spike triggered by PF-CF pairing

Pairing a train of PF inputs to a Purkinje neuron with CF input causes LTD at the PF synapses (Ito 1984). Postsynaptic increases in calcium concentration are both necessary (Sakurai 1990; Konnerth et al. 1992; Freeman et al. 1998) and sufficient (Kasono & Hirano 1994; Khodakhah & Armstrong 1997) to cause LTD induction. I have demonstrated that pairing CF input with preceding trains of PF input may locally enhance secondary dendritic calcium spikes in a branch-specific manner. Current thinking suggests that LTD, and its functional opposite long-term potentiation (LTP), form the basis of learning and memory. For that to be true, LTD should be specific to the synapses at which the pairing of inputs occurs. In fact, experimental observations suggest that the spatial spread of LTD from the site of pairing is somewhat broader – on the scale of tens of microns (Ekerot & Kano 1985; Hartell 1996; Reynolds & Hartell 2000).
My finding that additional secondary calcium spikes are apparently localised to individual dendritic branches is consistent with the observations of spatial spread of LTD. It is thus conceivable that secondary dendritic spikes produced by local pairing of CF and PF activity represents the coincidence detection mechanism that is responsible for triggering LTD observed following conjunctive PF and CF activation. It is also consistent with the idea that calcium spikes triggered by sufficiently strong PF activation on its own can activate LTD (Hartell 1996; Reynolds & Hartell 2000). That observation indicates that the CF input per se is not necessary; it is the associated calcium signal, which is important. Rather, the more plausible trigger of dendritic plasticity is the large depolarisation elicited by the climbing fibre, which allows the regenerative activation of dendritic voltage gated calcium channels.

5.3.2 Spatial regulation of secondary spikes by inhibition

Previous work demonstrated a strong effect of inhibition on the dendritic calcium signal associated with the CF response (Callaway et al. 1995). The effect at the soma was much less pronounced. I have applied local GABAergic synaptic conductances to the dendritic tree of Purkinje neurons and found that small inhibitory conductances of 3 nS were sufficient to ablate either primary or secondary peaks in the dendritic CF response. The effect of inhibition was very local and was not observable 30-40 µm away from the site of inhibition. The local effect of inhibition is most likely explained by the drastic decrease in membrane resistance during the CF response and the consequent electrotonic lengthening of the Purkinje neuron during the CF response. That only a few nano-siemens were necessary to ablate a dendritic primary or secondary spike suggests a powerful role of inhibition on the regulation of calcium
electrogenesis (Miles et al. 1996). However, inhibition has only a very local effect and must occur with millisecond precision relative to the CF response to have an effect. It is possible that feed-forward inhibition (Llinas et al. 1968) via the molecular interneurons may provide the required spatio-temporal precision. But whether such input precision occurs *in vivo* is a question that remains to be answered. It is conceivable that the CF activates interneurons, which then inhibit Purkinje cells with the required degree of precision.

5.3.3 Ionic conductances underlying generation of secondary spikes

The major calcium channel types in Purkinje neurons appear to be P/Q and T-type channels (Regan 1991; Mougnot et al. 1997; Raman & Bean 1999; Ito 2001). At the time delay after depolarisation and over the range of membrane potentials where secondary spikes are generated T-type channels are almost completely inactivated (Crunelli et al. 1989; Kaneda et al. 1990) (Mougnot et al. 1997) suggesting that these channels do not play a significant role in secondary spike generation. P/Q-type channels, on the other hand have a higher activation threshold and much slower time course of inactivation consistent with the properties expected of channels underlying secondary spikes (Regan 1991). While the density distribution of T-type calcium channels across the somato-dendritic membrane is unknown, P-type channels appear to be evenly distributed (Usowicz et al. 1992) but limited in their somatic functional expression by activation of potassium channels (figure 4.9). Purkinje neuron sodium channels are located almost exclusively around the soma (Stuart & Häusser 1994; Callaway & Ross 1997). This highly polar density pattern across the somato-dendritic membrane leads to the question of how these channels contribute to the shaping of the CF response in Purkinje
neurons. At the soma, blocking the sodium channels with intracellular QX314 leads to an increase in the total depolarisation following CF stimulation. Blocking sodium channels also modulates the shape of the CF response such that it becomes similar to the dendritic response with fewer and broader depolarisations. Thus I conclude that sodium spikes primarily shunt the somatic CF response, presumably through the conductances associated with activation of voltage activated sodium and potassium channels (Hausser et al. 2001). As a corollary, shunting allows for the generation of multiple sodium action potentials presumably by preventing sodium channel inactivation. At the level of the dendrites, blocking sodium channels has little effect. The lack of influence following somatic sodium channel block on dendritic activity during the CF response is consistent with the considerable electrotonic lengthening of the neuron during the CF response. Activation of calcium channels in the dendrites of Purkinje neurons has led to the suggestion that subsequent activation of $K_{ca}$ channels regulates the termination of dendritic calcium spikes. My finding that dendritic calcium spiking is not altered by inclusion of 20 mM BAPTA in the internal solution strongly suggests that $K_{ca}$ channels are not directly involved in the regulation and/or termination of dendritic regenerative events. A high voltage-activated potassium channel is present at high densities in Purkinje neuron dendrites (Martina et al. 2001). It is likely that this channel is involved in repolarisation of dendritic calcium spikes. As shown in chapter three, blocking $K_{ca}$ with high internal BAPTA dramatically affects the resting membrane potential of Purkinje neurons. Thus one must take care to compare dendritic electrogenesis at equal membrane potentials under control conditions and with high internal BAPTA to achieve consistent results.
Previous studies have led to the suggestion that inactivation of an A-type like potassium conductance underlies the time delay in development of secondary calcium spikes when jumping from hyperpolarised to depolarised membrane potentials (Midtgaard et al. 1993; Midtgaard 1995). The idea being that potassium channel inactivation with time will unmask excitatory calcium conductances. I find it unlikely that a potassium channel inactivation is the correct explanation for several reasons. When stepping from hyperpolarised to depolarised potentials the primary EPSP during the CF response remains constant as the time at depolarised membrane potentials is increased (figure 5.4). If a potassium conductance inactivates over that same time period of depolarisation, it would be expected that, one, the amplitude of the primary EPSP would grow, and secondly, that the rate of the EPSP down-stroke would decrease. Neither of these effects is observed. Similarly, if the potassium conductance of the neuron decreases with time after a depolarising step, it would be expected that amplitude and rate of down-stroke of normal action potentials are likewise increased and decreased, respectively. Neither are any of these effects observed, while the application of low concentration of 4-AP (40 µM) demonstrates both of these latter two effects (data not shown). Furthermore, the published time-constants of A-type channels inactivation in Purkinje neurons (Hirano & Hagiwara 1989; Martina et al. 2001) are 1-2 orders of magnitude faster than the timescale of secondary spike development – on the order 2-5 ms at –50 mV. Together, these considerations make it highly unlikely that potassium channel inactivation is the factor responsible for the time-course of secondary spike generation.
A more plausible explanation is facilitation of P/Q-type channels as has recently been described (Lee et al. 2000; DeMaria et al. 2001). Previous activity causing calcium influx will facilitate subsequent calcium channel activation through a calmodulin-mediated pathway (Lee et al. 1999; DeMaria et al. 2001). P/Q-type calcium channel facilitation has a half-maximum-time constant of ~150 ms similar to the time-course of development of secondary dendritic spikes (DeMaria et al. 2001) consistent with a role in secondary spike development. However, if calmodulin-mediated facilitation of P/Q-type channels underlies development of secondary spikes, then the inclusion of 20 mM BAPTA in the intracellular solution should abolish the effect. This is not observed. Interestingly, a very recent study using fluorescence resonance energy transfer (FRET) labelling of calmodulin and the $\alpha_{1A}$ subunit of the P/Q-type channel has demonstrated calcium independent association of calmodulin with P/Q-type channel. This has the functional implication that the site of calcium binding to trigger facilitation is within ~10 nm of the ion-pore, thus increasing the possibility that calmodulin binds calcium before it is chelated by BAPTA.

5.3.4 Physiological significance of the CF response

It is not known what components of the CF input to the Purkinje neuron is transmitted down the axon to the deep cerebellar nuclei. Purkinje neurons are tonically firing, thus it is questionable whether the multiple action potential generated by the CF are distinguishable from tonic firing (Campbell & Hesslow 1986).
It has been suggested, consistent with Purkinje neurons being output neurons and thus the final integrator of the cerebellar cortex, that CF responses primarily provide signals for the induction of appropriate synaptic plasticity in the dendritic tree of Purkinje cells (Marr 1969; Albus 1971). My findings of dramatic dendritic modulation of the CF response by coincident parallel fibre input, often with little effect at the soma, is consistent with the idea that CF activity provides a LTD inducing signal for the Purkinje neuron. These experiments do not rule out, however, that the precise timing of the complex spike triggered by the CF input may play an additional role in processing within the cerebellar network (Welsh et al. 1995).
Chapter 6: General discussion

At the end of a long story, I feel it appropriate to round off with a little summary of the main conclusion and possible future direction contained.

6.1 Chapter 3 – Influence of \( I_h \) and \( K_{(Ca)} \) on bistability

Purkinje neurons are bistable with one state being associated with continuous action potential firing. Under physiological conditions this bistability is masked by both \( I_h \) and \( I_{(Ca)} \). It is possible that bistability \textit{in vivo} may be unmasked via serotonin down-modulating of \( I_h \). Bistability is a very persistent property of Purkinje neurons which we do not fully understand the ionic composition of. Future experiments should include cell attached recordings to dissect out the contributions made by the various voltage-gated ion channels in the Purkinje cell.

6.2 Chapter 4 – Shaping of parallel fibre EPSPs

At subthreshold potentials near firing threshold somatic persistent sodium channels amplify EPSPs. This mechanism allows brief synaptic input to be converted into trains of action potentials, thus dramatically expanding the time window of summation. Potassium channels, probably with a high somatic density, limit this effect as well as \( I_h \) channels. Calcium channels activate only over a voltage range, where the Purkinje neuron would be continuously firing action potentials and even at those membrane potentials, calcium channels are activated regeneratively only locally by strong excitatory input.
The functional distribution of potassium channels across the somato-dendritic membrane has thus far not been studied in detail. It to fully appreciate the importance of these channels, it is necessary to directly measure the potassium current component across the cell membrane using cell-attached recordings.

6.3 Chapter 5 – Regulation of secondary dendritic climbing fibre triggered calcium spikes

Climbing fibre activation produces a large EPSP that spreads throughout the dendritic tree of the Purkinje neuron. At depolarised membrane potentials secondary dendritic calcium spikes follow the EPSP. Secondary dendritic calcium spikes develop with a time course consistent with its mediation by facilitation of calcium channels. When elicited locally by pairing with depolarisation from parallel fibre input, the secondary spikes appear to be specific the branch where they are being elicited. Local inhibition may further localise the localisation of secondary spikes. This provides a secondary messenger system with properties similar to those expected of a trigger for synaptic plasticity.

Future directions would be to quantitatively map out the spread of secondary spikes elicited by the pairing of PF and CF input. It should be established whether or not the spatial spread of LTD is matched by the spread of dendritic secondary spikes. Both these questions should probably await the development of good voltage sensitive dyes before being attempted. Lastly, it should be established, perhaps with a mixture of cell attached recordings and
molecular biology, whether or not facilitation of calcium channels underlies the development of secondary spikes in time.
Bibliography


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