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An investigation into the role of climbing fibres in cerebellar function.

By

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Summary

This thesis involves an experimental investigation into the role of climbing fibre afferents in cerebellar function. A new hypothesis is proposed to explain how these fibres operate and why they are essential for normal cerebellar function.

It is proposed that the ongoing or tonic simple spike activity displayed by the cerebellar Purkinje cells *in vivo* is generated by an intrinsic spike generating mechanism, and that this mechanism is regulated by the climbing fibre input to a Purkinje cell. This control is proposed to be via injections of Ca^{2+} into the Purkinje cells by the climbing fibre input. The Ca^{2+} then activates the Ca^{2+}-dependent K^+ channels that generate a hyperpolarizing influence that regulates the level of simple spike activity. The theory predicts that limited changes in climbing fibre frequency can control the whole range of tonic simple spike firing, and that loss of climbing fibre input will severely disturb Purkinje cell excitability and background firing patterns.

The experiments involved a combination of standard electrophysiological techniques and microlesions, immunohistochemistry to identify the Ca^{2+}-dependent K^+ channels in the Purkinje cells, and the application of pharmacological blockers or toxins to investigate the role of the channels in the proposed mechanism.

The removal of climbing fibres demonstrated their importance in normal Purkinje cell function. In the absence of a regular climbing fibre input, simple spikes rose to an exceptionally high level, with a subsequent oscillating firing pattern which consisted of alternating bursts of high frequency discharge followed by periods of quiescence.

Evidence was obtained by the application of a glutamate receptor antagonist which indicated that tonic simple spike activity was generated intrinsically, rather than by ongoing excitatory input. This intrinsic activity could be modulated up or down over a wide range by stimulation of the climbing fibres within a range of frequencies that has been shown to occur in normal conditions.

Immunohistochemistry revealed the presence of both large and small conductance Ca^{2+}-dependent K^+ channels in the Purkinje cells. The application of Funnel Web Spider toxin to specifically block most of the Ca^{2+} entry associated with the climbing fibre input

produced effects on simple spike firing that were similar to those of climbing fibre removal. Similar effects were obtained by blocking the small conductance Ca^{2+}-dependent K^+ channels, but blockage of the large conductance channels had little action on normal Purkinje cells.

It is suggested that the actions of the climbing fibres described in this thesis may have an important role in determining the level of cerebellar cortical output, in maintaining normal levels of Purkinje cell excitability and in motor learning.

Declaration

To the best of my knowledge and belief, this thesis does not contain material that has been accepted for the award of any other degree or diploma in any university or other institution, nor does it contain any material which has been previously published or written by another person, except when due reference is made in the text of the thesis.

Nadia L. Cerminara

Publications

Part of the work presented in this thesis has been published in the following abstracts:

Cerminara, N. L. and J. A. Rawson (1999) Evidence that climbing fibres control an intrinsic spike generator in cerebellar Purkinje cells. Abstracts-Society for Neuroscience. Vol 25, Part 2:566.7

Cerminara, N. L. and J. A. Rawson (2000) Climbing fibre activation within its physiological range modulates background simple spike activity of cerebellar Purkinje cells. Proceedings of the Australian Neuroscience Society. 11:1-99

Cerminara, N. L. and J. A. Rawson (2000) Distribution of calcium-activated potassium channels in the cerebellum of the rat. Abstracts-Society for Neuroscience. Vol 26, Part 1: 255.6

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Abbreviations

Below are a list of abbreviations and symbols appearing in the text and figures of this thesis.

AMPA: α-amino-3-hydroxy-5-methly-isoxazole-4-propionate

B: basket cell

BK: large conductance Ca^{2+}-dependent K^+ channel

Ca^{2+}: calcium ions

CCN: central cervical nucleus

CCT: cuneocerebellar tract

CF: climbing fibre

cm: centimetre

ChTX: charybdotoxin

CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt

CNS: central nervous system

Co^{2+}: cobalt ion

Cs^+: cesium ion

Da: daltons

DAO: dorsal accessory olive

DC: direct current

DCN: deep cerebellar nuclei

DF-SOCP: dorsal funiculus spino-olivocerebellar pathway

DLF-SOCP: dorsal lateral funiculus spino-olivocerebellar pathway

DSCT: dorsal spinocerebellar tract

EM: electron microscopy

EPSP: excitatory postsynaptic potential

FRA: flexor reflex afferents

FTX: Funnel Web Spider toxin-3.3 (tetrahydrochloride)

g: grams

GABA: γ-Aminobutyric acid

GFAP: glial fibrillary acidic protein

GL: granular layer

Go: Golgi cell

Gr: Granule cell

Hz: hertz

I_{AHP}: apamin-sensitive afterhyperpolarization

i.p.: intraperitoneal

IO: Inferior Olive

IbTX: iberiotoxin

ISI: interspike interval

K^+: potassium ion

kg: kilograms

LF-SOCP: lateral funiculus spino-olivocerebellar pathway

L7-PKC: PKC deficient Purkinje cells in transgenic mice

LRN: lateral reticular nucleus

LTD: long-term depression

LTP: long-term potentiation

M: molar

MAIT: Marr-Albus-Ito theory

MAO: medial accessory olive

MF: Mossy fibre

mg: milligrams

mGluR: metabotropic glutamate receptor

mGluR1: metabotropic glutamate receptor type 1

mGluδ2: glutamate receptor δ type 2

ml: millilitres

ML: molecular layer

mM: millimolar

mRNA: messenger ribonucleic acid

mV: millivolts

Na^+: sodium ion

NGS: normal goat serum

nl: nanolitre

nm: nanometre

PB: phosphate buffer

PBS: phosphate buffer saline

P: Purkinje cell

PF: parallel fibre

PKC: protein kinase C

PL: Purkinje cell layer

PO: principal olive

pS: picosiemens

PSTH: peristimulus time histogram

RSCT: rostal spinocerebellar tract

S: stellate cell

sAHP: slow afterhyperpolarization

sFTX: synthetic Funnel Web Spider toxin

$_sI_{AHP}$: apamin-insensitive slow afterhyperpolarization

SOCP: spino-olivocerebellar pathway

SK2: small conductance Ca^{2+}-dependent K^+ channel type 2

SK3: small conductance Ca^{2+}-dependent K^+ channel type 3

TEA: Tetramethylammonium

TTL: transitor to transistor logic pulses

TTX: Tetrodotoxin

µg: microgram

µm: micrometre

µM: micromolar

VF-SOCP: ventral funiculus spino-olivocerebellar pathway

VOCC: voltage-operated Ca^{2+} channel

VSCT: ventral spinocerebellar tract

WM: white matter

3-AP: 3-acetylpyridine

%: percentage

°C: degrees centigrade

Chapter 1

General Introduction

Cerebellar research has a long and rich history, with the earliest documented idea about cerebellar function being proposed in 1664 by Sir Thomas Willis. Upon examination of the gross cerebellar structure of a variety of mammals, Sir Thomas was remarkably impressed with the uniformity of its structure, especially in comparison to the marked differences in the cerebrum from species to species. From this, Sir Thomas concluded that the cerebellum was responsible for the maintenance of basic ongoing bodily functions such as respiration, heartbeat, and digestion, functions that are essential to all mammals. Willis' conclusions promoted the first scientific investigations into the function of the cerebellum, which have continued to this day, spanning nearly two centuries. Crude experiments by Du Verny (1697, cited by Dow, 1958) which involved ablation of the cerebellum indicated that the cerebellum was in fact, not essential for life. Further successful ablation studies, notably by Rolando 1809; Flourens, 1824; and Luciani, 1891 (cited by Dow, 1958), went on to confirm and extend these observations, with Flourens concluding that

"...all movements persist following ablation of the cerebellum: all that is missing is that they are not regular and coordinated".

(cited by Glickstein et al., 1986)

Through these early studies, we know that the cerebellum is important for the performance of smooth and accurate movements. One of the earliest studies clearly demonstrating this in humans was in the 1920s by Gordon Holmes, an army surgeon, who described the results of cerebellar lesions on World War I patients. These patients had one side of their cerebellum destroyed by a gunshot wound while the other side was available as a control. The subjects were asked to perform simple movement tasks. Holmes observed that on the side that was normal, movements were found to be smooth and accurate, while on the side with the cerebellar lesion, the subject gave a performance that was irregular, clumsy, and disjointed. Movements were slow to commence and terminate, with a limb often over or undershooting its target. However, in these clinical studies, it was often difficult to define the precise location of the lesion, which made it

difficult to correlate particular movement disorders with specific lesion sites. With the advent of highly refined experimental techniques and the use of stereotaxic apparatus, other studies have since confirmed that the cerebellum is essential for the coordination of eye and limb movements (Thach, 1978), and the maintenance of muscle tone (Thach et al., 1992), balance and posture (Thach, 1972; Brooks, 1975; MacKay, 1988), thus confirming that the cerebellum is intimately involved with the maintenance and performance of smooth and accurate movements. In addition to its prominent role in motor control, recent evidence suggests that the cerebellum may also be involved in nonmotor processes such as sensory, autonomic, and cognitive functions (see Schmahmann, 1997 for details). In view of the remarkable structural uniformity of the cerebellar circuitry, it is likely that the cerebellum performs some consistent computation on the information it receives and therefore, it has been suggested that the cerebellum exerts the same influence on these other functions (Ito, 1984) as it does on motor behaviour.

Over the past four decades, extensive research of the cerebellum using anatomical and electrophysiological techniques has provided details of its cytoarchitecture, afferent and efferent connections, and neuronal networks. Much of this work has been documented in major monographs by Eccles, Ito, and Szentogothai (1967), Palay and Chan-Palay, (1974), and Ito, (1984). Ironically, with the wealth of knowledge at hand, the precise details of how (or moreover, agreed upon) the cerebellum operates to control movement and other functions remains unknown.

Perhaps one of the most controversial issues in cerebellar function today is the contribution of climbing fibres to cerebellar operations. Practically all published ideas invoke an important role for the climbing fibre system in cerebellar function, however many of these ideas are opposing and controversial. One aspect of the climbing fibres function that is agreed upon, is that they are known to be essential for normal cerebellar function, as their removal causes movement deficits that resemble those of cerebellectomy (Llinas et al., 1975). An examination of the role of climbing fibres in cerebellar cortical control will be the focus of this thesis. Before presenting an encapsulation of a number of theories of climbing fibre function, and the aims behind the work presented in this thesis, a brief summary of cerebellar structure and function will be presented.

1.1 Anatomy of the cerebellum

1.1.1 Gross anatomy

The cerebellum constitutes 10-15 % of the whole brain weight and occupies most of the posterior cranial fossa. It consists of two hemispheres united in the midline by a ridge know as the vermis. A series of major folds of folia, separated by deep fissures, run transversely across the surface of the cerebellum. Two deep fissures divide the cerebellum into three lobes; the anterior and posterior lobes divided by the primary fissure, and the flocculonodular lobe, separated from the posterior lobe by the posterolateral fissure. The anterior and posterior lobes are further subdivided by smaller fissures into lobules. In turn, each lobule is divided into sublobules and folia.

The organisation of the cerebellum is remarkably simple. It is composed of a convoluted cortex surrounding an inner core of white matter. Deep within the white matter lie three pairs of deep cerebellar nuclei (DCN) arranged either side of the midline. These nuclei, known as the medial (fastigial), interpositus (anterior and posterior divisions), and lateral (dentate) nuclei, form the final output from the cerebellum to the rest of the central nervous system (CNS). It is through these efferent connections that the cerebellum is thought to exert its influence on downstream structures in the brainstem and thalamus to control and coordinate body movement.

1.1.2 The cerebellar cortex

Most of the information processing in the cerebellum takes place in the cortex, and it maintains the same basic neuronal structure from region to region. The cortex can be divided into three layers. From the pia inwards, they are the molecular layer, the Purkinje cell layer, and the granular layer. These layers are built from five major types of neurones. They are Purkinje cells, granule cells, Golgi cells, stellate cells, and basket cells.

The Purkinje cell somata constitute the Purkinje cell layer, and are arranged in a monolayer. Their cell bodies are quite large in comparison to other cerebellar neurones, ranging from 35-65 μm in diameter. They are spontaneously active in both anaesthetised and conscious animals, discharging at rates of 30-100 spikes/s. The Purkinje cells contain γ-Aminobutyric acid (GABA), and their axons form the sole output of the cerebellar cortex. The axons leave the cortex to form inhibitory synapses in the cerebellar nuclei (Ito et al., 1964; Ito et al., 1970). They possess an extensive fan-like dendritic tree that projects upwards to the molecular layer where they receive incoming afferent inputs. The initial

ramifications of the dendritic tree, made up of the primary and secondary dendrites are relatively smooth whereas the distal tertiary dendrites are covered with small spines. The plane of the dendritic arborisation lies in a single perpendicular plane to the longitudinal axis of the folium.

Granule cells are the most numerous type of cell in the cerebellum, and are located in the granular layer. They are small glutamatergic interneurones with cell bodies 5-6 μm in diameter and with little cytoplasm. Each granule cell has four or five small dendrites that end in claw-like expansions known as rosettes. Rosettes from up to 28 different granule cells cluster together to form a single glomerulus. It is at these sites that synapses are formed with incoming mossy fibres (See Section 1.3.1) and with the axons of Golgi cells. The granule cell axons, which are unmyelinated, ascend into the molecular layer where they bifurcate in a T-like fashion to form what are known as parallel fibres. The parallel fibres run parallel with the longitudinal axis of the folium for several millimetres, about 5 mm in the rat (Pichitpornchai et al., 1994). The parallel fibres intersect at right angles to the plane of the Purkinje cell dendritic trees, and varicose swellings on the parallel fibres terminate in an *en passant* manner with the spines on the tertiary Purkinje cell dendrites. Recently, Pichitpornchai et al., (1994) found that the mean intervaricosity interval and varicosity size varied along the proximal and distal lengths of the parallel fibres. As size and interval of the varicosities decreased with increasing parallel fibre distance, it was concluded that the weakest action of the parallel fibres occurs at the distal ends, whereas it is thought that the proximal regions of the parallel fibres is where they exert their most powerful influence. The parallel fibres also form excitatory contacts with the dendritic arborisations of the Golgi, stellate and basket cells, but to a lesser degree.

Also located in the granular layer, are the larger inhibitory interneurones, the Golgi cells. The Golgi cells, with a soma diameter of 15-20 μm, have their cell bodies located in the granular cell layer just beneath the Purkinje cells. The greater part of their dendritic tree is located in the molecular layer that radiates in all directions where they make synaptic contact with the parallel fibres. The parallel fibres provide excitatory inputs to the Golgi cells. Golgi cells differ from other inhibitory interneurones of the cerebellum, as not only are they GABAergic, they are colocalised with glycine as well. The axons of the Golgi cells terminate on granule cell dendrites and inhibit the many granule cells present within the range of its axonal arbor (Eccles et al., 1966e; Hamori & Szentagothai, 1966) to provide a feedback inhibition to the granule cells. In addition, Golgi cells are contacted by

Purkinje cell recurrent collaterals (Scheibel & Scheibel, 1954; Hamori & Szentagothai, 1966; Palay & Chan-Palay, 1974; Sugihara et al., 1999; Shinoda et al., 2000).

Golgi cells discharge at 3-35 Hz at rest (Edgley & Lidierth, 1987; Vos et al., 1999b), and are capable of responding to tactile peripheral stimulation with large receptive fields (Vos et al., 1999b). Moreover, multiunit recordings indicate that Golgi cells are organised into beams (Vos et al., 1999a). The function of Golgi cells at present remains unknown, but recent reports suggest that Golgi cells through their inhibitory action on granule cells may control the timing of granule cell spikes, while their spatial organisation and large receptive fields may organise the granule cells into functional assemblies along the parallel fibre axis (De Schutter et al., 2000).

The neuronal cells with cell bodies lying in the molecular layer are the smaller stellate cells (5-10 μm) and the larger basket cells (20 μm). These GABAergic inhibitory interneurones are similar to each other. Both have dendritic trees that are less extensive, but have the same orientation as the Purkinje cells, projecting perpendicular to the folium. The axons of the basket cells run transverse to the folium, and give rise to collaterals which distribute around the soma and axon hillock of Purkinje cell forming a peri-cellular basket-like complex (Bishop, 1993). The axons of the stellate cells make synaptic contacts identical to those of the basket cells, terminating on nearby Purkinje cell soma or around the axon initial segment (Paula-Barbosa et al., 1983). Both stellate and basket cells receive excitatory inputs from parallel fibres, and exert a feedforward postsynaptic inhibitory action on Purkinje cells.

Also found in the granular layer of the cerebellar cortex are Lugaro and unipolar brush cells, and glial cells. Lugaro cells are fusiform neurones that are located at the border of the granule and Purkinje cell layers. Their horizontal dendrites extend in the parasagittal plane and their axon projects to the molecular and granular layers (Laine & Axelrad, 1996). Purkinje cell collaterals also contact them. In view of their projections and afferentation, Lugaro cells are believed to act as a feedback interneurone on the corticocerebellar output. Unipolar brush cells are another cell type found in the granular layer. They are characterised by a stubby dendrite that resembles a paintbrush which interdigitates with the mossy fibre rosette to form an excitatory synaptic contact. A single thin axon emanates from the unipolar brush cells that terminate in mossy fibre-like rosettes in the granular layer (Dino et al., 2000), where they are contacted by granule cell dendrites. The network of unipolar brush cells and mossy fibres may thus contribute a powerful form of feedforward excitation within the cerebellar circuitry (Dino et al., 2000).

A summary illustrating the cerebellar neural circuitry is shown in Figure 1.1.

1.2 Cerebellar cortical organisation

Early comparative anatomical studies (Ito, 1984; Voogd & Glickstein, 1998) originally partitioned the cerebellum into transverse subdivisions that have evolved successively in phylogeny, with each having their own functional divisions and anatomical connections. Roughly speaking, these subdivisions reflect the patterns of afferent terminations in the cerebellum, with vestibular afferents terminating in the archi- or vestibulocerebellum, projections from the spinal cord terminating in the paleo- or spinocerebellum and pontine inputs terminating in the neo- or cerebrocerebellum. This anatomical transverse subdivision is misleading as the sites of termination overlap considerably. Consequently, an alternative longitudinal or zonal organisation was suggested which divided the cerebellum into three parasagittal bands. The tripartite division of the cerebellum was based upon the findings that Purkinje cells in each longitudinal division project topographically to a distinct DCN, with the efferent connections of each nucleus projecting to different descending pathways, controlling a different aspect of motor control (Jansen & Brodal, 1940; Chambers & Sprague, 1955). The three divisions, known as the medial (or vermis), intermediate (or paravermal) and the lateral zones project preferentially to the fastigial and vestibular nuclei, the interpositus nucleus, and the dentate nucleus respectively. In turn, the fastigial and vestibular nuclei project to the cortical and brain stem regions which give rise to the medial descending pathways that control proximal muscles and maintain posture, the interpositus nucleus projects to the cortical and brainstem regions that act on distal limbs via the lateral descending pathway which is responsible for the control of movement, and finally the dentate nucleus is thought to act on the premotor and motor cortex which is important for the planning of voluntary movement.

The aforementioned division of the cerebellum is a useful introduction into the functional organisation of the cerebellum, however, it is now recognised that these three bands can be further subdivided into a series of parasagittal zones. Based initially on the pioneering anatomical work of Voogd (1964, 1969) and in subsequent anatomical studies (Groenewegen & Voogd, 1977; Groenewegen et al., 1979), the compartmentalisation of the cerebellar cortex into a number of longitudinal bands was revealed, with each sagittally orientated zone defined by its climbing fibre input from a circumscribed region of the inferior olive, and its efferent projection to a circumscribed area of the vestibular and DCN. Consequently, the cerebellar cortex was subdivided into the 7 sagittal zones A, B,

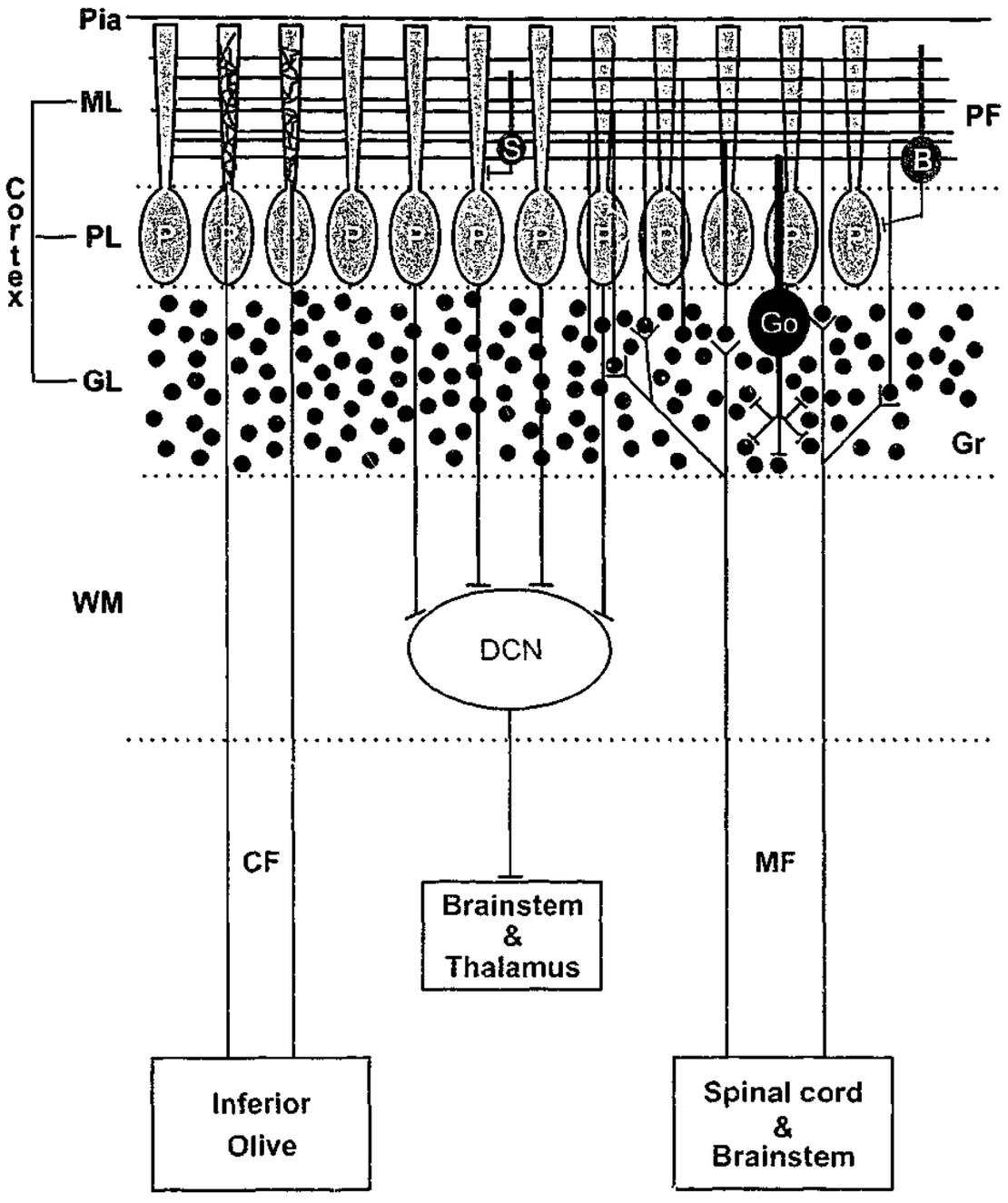


Figure 1.1

Diagrammatic representation of the principal features of the cerebellar circuitry and connections.. ML, molecular layer; PL, Purkinje cell layer; GL, granular layer; WM, white matter; DCN, deep cerebellar nucleus; CF, climbing fibre; MF, mossy fibre; PF, parallel fibre; P, Purkinje cell; Go, Golgi cell; Gr, granule cell; B, basket cell; S, stellate cell

C1, C2, C3, D1, and D2. Given that each region of the cerebellar or vestibular nucleus in turn innervates various ascending and descending pathways, each sagittal zone was suggested to constitute the basic functional unit of the cerebellum, with each controlling a different motor pathway and playing a distinct role in motor control (Ito, 1984).

In addition to the anatomical zonal arrangement of the cerebellum, electrophysiological studies also revealed a similar longitudinal zonal topography of the cerebellar cortex, with each zone displaying distinct climbing fibre response latencies and convergence patterns (Oscarsson, 1968; Larson et al., 1969a, b; Oscarsson, 1969; Ekerot & Larson, 1979a, b; Oscarsson, 1979; Ekerot & Larson, 1982). As input to the various zones was mediated via different spino-olivary pathways, it appeared that the function of the zones was related to its motor function (Oscarsson, 1980), as was suggested with the anatomical findings. The corresponding zones determined electrophysiologically were originally denoted by lower case letters: a, x, b, c1, c2, c3, d1, and d2 (note the addition of the x zone), as it was not known whether the physiologically defined zones were identical to the anatomical zones. It is now acknowledged that the division between the anatomically defined zones and the electrophysiological zones can be dismissed (Trott & Armstrong, 1987a, b; Armstrong, 1990; Trott & Apps, 1991), and therefore no distinction between the two is made.

From many years of electrophysiological and anatomical studies (for comprehensive reviews, refer to Brodal & Kawamura, 1980; Voogd & Bigare, 1980; Armstrong, 1990; Voogd & Ruigrok, 1997; Voogd & Glickstein, 1998) it is now generally agreed that the cerebellar cortex is aligned into narrow rostro-caudal zones (*ca* 1 mm), projecting to a circumscribed region of the cerebellar or vestibular nuclei and defined by their climbing fibre input from a circumscribed region of the inferior olive. As a result of the efferent and afferent projections to the cerebellar cortex, 9 main zones defined as A, X, B, C1, CX, C2, C3, D1, and D2/Y have been identified to date. There is now growing evidence that these well-established zones can be further subdivided into longitudinal microzones (for eg Andersson & Oscarsson, 1978a; Garwicz & Ekerot, 1994) which will be discussed further in Section 1.9.

1.3 Afferent projections to the cerebellum

The Purkinje cells with their extensive dendritic trees that extend across the molecular layer receive excitation from two main types of input, one direct and the other indirect. These two excitatory inputs, the mossy fibres and the climbing fibres determine the

output of the Purkinje cells, which in turn project to the cerebellar nuclei. The mossy fibres and the climbing fibres are distinct in their origins and terminations. The Purkinje cells also receive a third well less characterised type of input, the monoaminergic system. All three afferent systems will be discussed below.

1.3.1 Mossy fibre inputs

The mossy fibres are the most numerous source of afferent input to the cerebellum. They originate from a variety of sources, including brainstem nuclei, the basilar pontine nuclei, the cerebral cortex, vestibular nuclei, and spinal cord neurones that give rise to spinocerebellar tracts. Mossy fibres are thought to provide the cerebellum with information about the status of the body parts and local motor circuitry. Mossy fibres act on Purkinje cells indirectly by entering the cerebellum via all three cerebellar peduncles, and synapsing with granule cells, the excitatory interneurones of the granular layer. The granule cell axon, the parallel fibre, in turn contacts many Purkinje cells, and one Purkinje cell is contacted by many parallel fibres, around 175 000 in the rat (Napper & Harvey, 1988).

1.3.2 Monoaminergic inputs

As well as climbing fibres and mossy fibres, the monoaminergic system comprising noradrenergic, serotonergic and dopaminergic fibres, arises from several brainstem nuclei to also reach the cerebellum. Serotonergic inputs originate from various raphé nuclei (Shinnar et al., 1975; Chan-Palay, 1976; Bishop et al., 1985) and terminate in all three layers of the cerebellar cortex. At the ultrastructural level, serotonergic terminals have been shown to establish synaptic contacts with the dendrites of Purkinje cells (Chan-Palay, 1975). Noradrenergic fibres originate in the locus coeruleus and project to all areas of the cerebellar cortex in a patchy manner. Noradrenergic synapses contact the smooth dendrites and dendritic spines of Purkinje cells (Bloom et al., 1971) and the granule cell dendrites in the granular layer. Dopaminergic fibres arise from the ventral mesencephalic tegmentum and project to the Purkinje cell and granular cell layers. The precise roles of these three monoaminergic inputs are not fully understood, however it is believed that adrenergic and serotonergic afferents exert a diffuse modulatory influence on the activity of cerebellar neurones (Ito, 1984; Strahlendorf et al., 1984; Strahlendorf et al., 1986).

1.3.3 Climbing fibre inputs

Climbing fibres, so named because of the way in which they 'climb' up the dendritic tree of a single Purkinje cell, originate from cells of the inferior olive located at the base of the medulla, and are the sole provider of climbing fibres to the cerebellum (Armstrong, 1974; Desclin, 1974; Armstrong, 1978). In contrast to the mossy fibres, climbing fibres synapse with the Purkinje cells directly in a unique 1:1 relationship. As Purkinje cells are more numerous than olive cells (with a ratio of 7:1 in the rat, (Schild, 1970)), climbing fibres branch in both the medio-lateral and rostro-caudal planes to innervate different lobules of the cerebellar cortex. This has been demonstrated physiologically (Armstrong et al., 1969; Faber & Murphy, 1969; Armstrong et al., 1973a, b, c, 1974; Ekerot & Larson, 1982) and anatomically (Scheibel & Scheibel, 1954; Desclin, 1974; Brodal et al., 1980; Sugihara et al., 1999; Apps, 2000). Recently, studies examining the entire trajectories of single olivocerebellar axons in the cerebellar cortex with biotinylated dextran amine injected into the inferior olive demonstrated that on average, single inferior olive axons have seven climbing fibre axons (Sugihara et al., 2001). Thus, although each Purkinje cell is contacted by only one climbing fibre, each stem axon from an inferior olive cell can supply fibres to more than one Purkinje cell.

Climbing fibres project to the cerebellum via the inferior cerebellar peduncle and then ascend through the granular layer where at the level of the Purkinje cells, they become unmyelinated. Terminal arborisation of the climbing fibres is restricted to the inner two thirds of the molecular layer. Tendril fibres that arise from the primary branches of the climbing fibres form *en passant* and terminal swellings that synapse on the proximal smooth branches of the Purkinje cell dendritic tree. Besides terminal arborisations around the Purkinje cell dendritic tree, climbing fibre terminals also project to the Purkinje cell soma (Sugihara et al., 1999). Thin climbing fibre collaterals have also been observed to synapse with cells of the cerebellar nuclei and cerebellar interneurones (Scheibel & Scheibel, 1954; Sugihara et al., 1999).

1.4 Physiology of the Purkinje cell

Cerebellar Purkinje cells occupy a central position in cerebellar circuitry and have long been the focus of detailed electrophysiological studies. They integrate excitatory postsynaptic potentials (EPSPs) from climbing fibres and parallel fibres, and each afferent generates a distinct postsynaptic effect on the Purkinje cell. Furthermore, direct

stimulation of Purkinje cells in cerebellar slice preparations revealed that they possess unusual firing characteristics.

1.4.1 Effects of direct stimulation

In the landmark studies of Llinas and Sugimori (1980a, b) utilising guinea pig cerebellar slices, two types of spike activity were recorded from the dendrites and soma of the Purkinje cell upon injection of current pulses through an intracellular microelectrode. Initially, current injection caused the Purkinje cell to fire fast repetitive action potentials superimposed on a low threshold, slow-rising depolarizing plateau. With increasing stimulus intensity, a differing type of electroresponsiveness was observed. The onset of repetitive firing occurred earlier, and rather than continuing, rhythmic depolarizing spike bursts of low amplitude were observed. The burst of activity was quickly followed by a distinct membrane hyperpolarization that reactivated spike firing. The Purkinje cell could continue to oscillate in this way for the duration of the stimulus.

1.4.2 Ionic basis of Purkinje cell firing

In the same studies, Llinas and Sugimori (1980a, b) characterised the ionic species serving as the charge carriers for each of the two distinct types of firing. The replacement of extracellular sodium (Na^+) ions or the pharmacological block of Na^+ channels with tetrodotoxin (TTX) resulted in the abolition of the fast spikes, leaving the late slow-rising burst of spikes and the hypepolarization intact. This finding implied that the fast action potentials are carried by voltage-operated Na^+ currents, as occurs in other neurones. The depolarizing spike bursts were shown to be calcium (Ca^{2+}) dependent, as the blockade of Ca^{2+} currents with the addition of cobalt (Co^{2+}), whilst leaving the fast Na^+ action potentials unmodified, totally blocked the depolarizing spike bursts. In addition to the removal of the depolarizing spike bursts, the blockade of the Ca^{2+} conductance revealed a slow-rising response capped with the fast action potentials. The amplitude of the fast action potentials rapidly decreased as the slow-rising response stabilised at a plateau potential of about -30 mV. At this level, a total inactivation of the fast spike generating mechanism was observed. This plateau potential could be blocked by TTX or by the removal of Na^+ from the bath solution, thus indicating that slow or non-inactivating Na^+ channels are responsible for its generation. Furthermore, the plateau could be abolished or shortened by a strong outward current injection, and following the intracellular injection of tetraethylammonium (TEA), the level of the Na^+-dependent plateau shifted

towards a more positive value, indicating that the plateau represents an equilibrium between the non-inactivating Na^+ conductance and a non-inactivating K^+ conductance.

With intradendritic recordings, Llinas and Sugimori (1980b) demonstrated that the Na^+ dependent fast spikes did not invade the dendritic tree actively. Instead, the Ca^{2+}-dependent depolarizing spike bursts were observed to be most prominent at the dendritic level. Llinas and Sugimori (1980b) further showed that two types of Ca^{2+} conductances were responsible for the spike bursts. At low stimuli intensities, a plateau-generating Ca^{2+} conductance was produced, whereas further depolarizations produced large dendritic action potentials. Both classes of responses were abolished by the blockade of Ca^{2+} currents or by the removal of extracellular Ca^{2+}.

A compartmental model of Purkinje cells has thus been suggested, with Na^+ action potentials restricted to the soma, and Ca^{2+}-dependent action potentials constrained to the dendritic tree (Llinas & Sugimori, 1980a, b; see also Hounsgaard & Midtgaard, 1988). Dual patch recordings in the soma and dendrites of Purkinje cells further support the notion of a compartmental model (Stuart & Hausser, 1994), as do modelling studies (Rapp et al., 1994). This is also consistent with Na^+ imaging and TTX binding studies which demonstrated low Na^+ channel densities in the dendrites of Purkinje cells (Lasser-Ross & Ross, 1992), and a high density of Ca^{2+} channels in the dendrites (Llinas & Sugimori, 1980b; Llinas et al., 1992). The compartmentalisation of the soma and dendrites indicates that the Purkinje cells may act as relatively independent computational compartments.

1.4.3 Response of Purkinje cells to climbing fibre and parallel fibre activation

Activation of the parallel fibres generates a graded EPSP resulting in the generation of 'simple spikes' (Figure 1.2A) (Eccles et al., 1966a, d), consisting of fast Na^+ spikes that are now known to be initiated in the Purkinje cell initial segment which then spread passively into the dendritic tree (Stuart & Hausser, 1994). Climbing fibre activation produces a distinct powerful all-or-none response known as a 'complex spike' (Figure 1.2B) (Thach, 1968). When recorded intracellularly, the complex spike consists of an initial fast action potential of large amplitude followed by a long-lasting depolarization that lasts for many tens of milliseconds. Superimposed on this long lasting depolarization is a succession of 2-6 smaller wavelets. The large depolarization underlying the climbing fibre response has been identified as an EPSP (Eccles et al., 1966f). Modification of the extracellular ionic milieu and the use of ion channel blockers has demonstrated that the initial spike is

generated by voltage-dependent Na^+ conductances at the somato-axonic levels, whereas the prolonged depolarization is due to Ca^{2+}-dependent action potentials originating in the dendritic tree (Llinas & Sugimori, 1980a, b). When recorded extracellularly the complex spike consists of an initial spike similar to the simple spike followed by a slow wave lasting 10-15 ms upon which smaller secondary spikes occurring at a rate of approximately 500/s are superimposed. Due to its two distinct types of discharges, the Purkinje cell is one of the few places in the CNS where the morphology of the response indicates the mode of afferent excitation, indicating that the mossy and climbing fibre inputs each respond differently during motor and sensory stimuli.

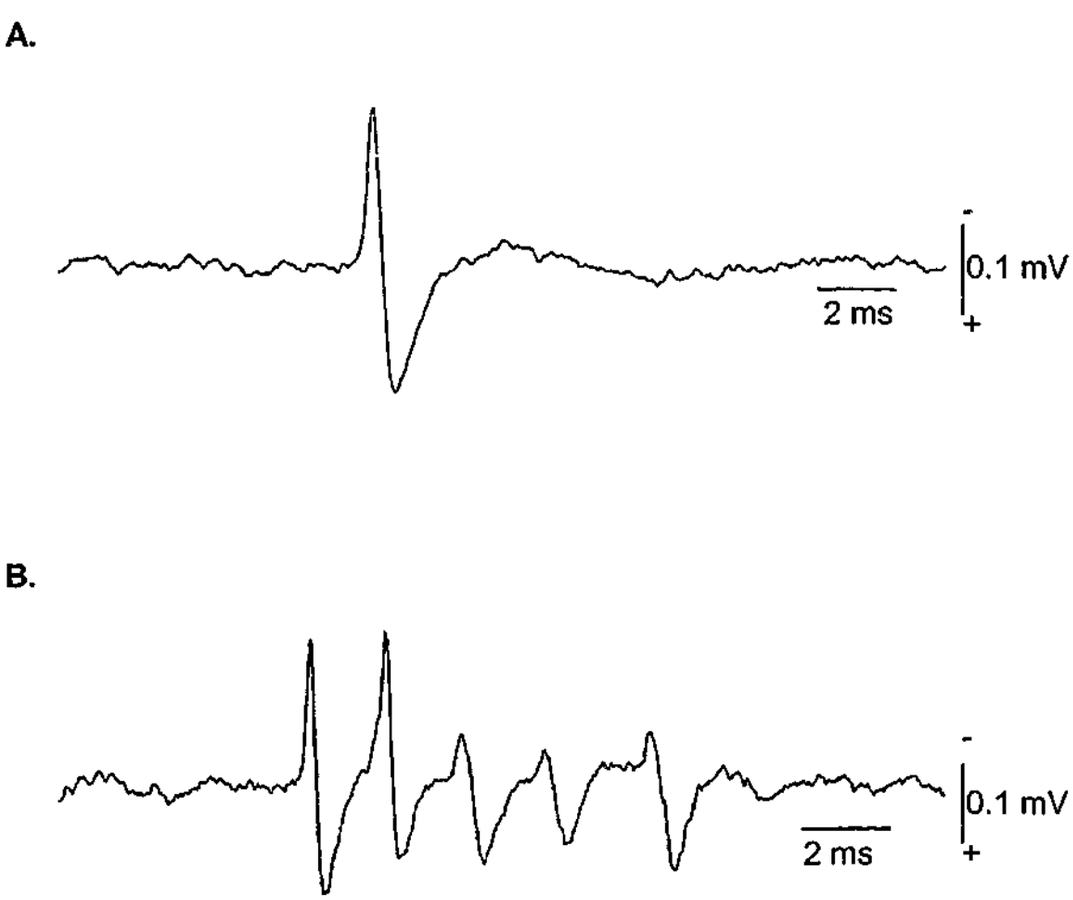


Figure 1.2

Purkinje cell response to parallel fibres and climbing fibres. **A.** Example of a simple spike response. **B.** Example of a complex spike response.

1.5 Organisation of mossy fibres

A large number of mossy fibres originate in the spinal cord and these are organised into spinocerebellar tracts. The cells of origin of these tracts are acted upon by primary

afferent dorsal root fibres, and transmit information from various proprioceptors and exteroreceptors. Five distinct somaesthetic spinocerebellar tracts have been identified; dorsal, ventral, rostral, cuneo, and central cervical spinocerebellar tracts. Other sources of mossy fibres include the lateral reticular nucleus and the pontine nuclei.

1.5.1 The spinocerebellar tracts

The dorsal spinocerebellar tract (DSCT) is known to convey information to the cerebellum from the lower body and hindlimb. Cells from this tract arise from Clarke's column, situated on the medial side of the dorsal horn between the lower thoracic and upper lumbar spinal cord segments, and are topographically organised (Xu & Grant, 1994). Projections from the DSCT enter the cerebellum via the inferior cerebellar peduncle, to terminate ipsilaterally in lobules I-V in the anterior lobe and in the posterior lobe vermis and paravermis (Oscarsson, 1969; Matsushita & Ikeda, 1980; Matsushita & Okado, 1981; Matsushita & Hosoya, 1982). The DSCT receives monosynaptic input from primary afferents and is divisible into proprioceptive and exteroceptive components. The proprioceptive neurones of the DSCT receive monosynaptic input from Golgi tendon organs and muscle spindles that are usually restricted to a single muscle. Exteroceptive neurones receive monosynaptic inputs from hair receptors and slowly adapting touch receptors that have receptive fields located within discrete areas of skin (Holmqvist et al., 1963b). DSCT neurones are thought to act as an efficient relay of sensory signals to the cerebellum, rather than as integrative neurones, as the response properties of the tract neurones closely resembles the primary afferents with which they are connected.

The ventral spinocerebellar tract (VSCT), the contralateral counterpart to the DSCT, originates from the ventrolateral grey matter of the lumbar spinal cord in the L3-L6 segments. These mossy fibres enter the cerebellum via the superior cerebellar peduncle to terminate bilaterally in the vermis and paravermis of the anterior lobe (Xu & Grant, 1994). VSCT neurones are excited by Group Ia and Ib muscle spindles and Golgi tendon organs, with the dominant excitation of the VSCT neurones being influenced by nerves to synergistic muscles that act around a particular joint. VSCT neurones also receive disynaptic inhibition from group I afferents, collaterals from the Renshaw cell pathway, and various descending pathways (Lundberg & Weight, 1970, 1971; Lindstrom & Schomburg, 1974). Based upon these neural connections, the VSCT is thought to provide internal feedback to the cerebellum by monitoring the internal state of lower motor centres rather than a direct transfer of sensory information as occurs in the DSCT (Lundberg & Weight, 1971; Ito, 1984). Recordings from VSCT neurones in cats during

locomotion and scratching support this idea. It was revealed that VSCT neurones were active during locomotion even after movement was abolished by paralysis (Arshavsky et al., 1972). Similar findings were seen in the decerebellated cats. VSCT neurones maintained their rhythmic discharge during the flexor phase of the scratch reflex cycle after the reflex movement was suppressed by decapitation (Arshavsky et al., 1978a).

The rostral spinocerebellar tract (RSCT) and the cuneocerebellar tract (CCT) are believed to be the forelimb homologues of the VSCT and the DSCT respectively. The CCT, like the DSCT is also divisible into proprioceptive and exteroceptive components. Unlike the DSCT, the CCT originates within the brainstem rather than the spinal cord, with the proprioceptive component originating in the external cuneate nucleus, and the exteroceptive component originating in the main cuneate nucleus (Cooke et al., 1971a; Cheek et al., 1975). The two components enter the inferior cerebellar peduncle to terminate in the ipsilateral vermis and paravermis of the anterior lobe and the paramedian lobule (Cooke et al., 1971b; Rinvik & Walberg, 1975; Somana & Walberg, 1980; Haring & Rowinski, 1982; Gerrits et al., 1985; Jasmin & Courville, 1987). Various electrophysiological studies have revealed that the CCT neurones faithfully transmit inputs from primary afferents innervating single muscles (Johnson et al., 1968; Rosen, 1969; Campbell et al., 1974), and cutaneous mechanoreceptors (Holmqvist et al., 1963a; Cooke et al., 1971a). There is some segregation of the proprioceptive and exteroceptive components of the CCT in the cerebellar cortex. Proprioceptive inputs were found to terminate in the depths of the cerebellar folia whereas the exteroceptive inputs terminated more superficially (Ekerot & Larson, 1972).

The RSCT with its cells of origin located at the base of the dorsal horn in C3-T1 spinal cord segments (MacKay & Murphy, 1974; Matsushita & Hosoya, 1979) has afferent projections and connections to the cerebellum comparable to the VSCT. It is therefore considered to transmit analogous information as the VSCT, but this time for the forelimb. The RSCT terminates bilaterally in the anterior lobe.

The projection of the spinocerebellar tract originating in the central cervical nucleus (CCN) is a direct spinal projection to the cerebellum. The CCN extends from C1-C4 spinal cord segments (Matsushita & Ikeda, 1975; Wiksten, 1979a). Axons of the CCN are crossed and enter the superior cerebellar peduncle to terminate bilaterally in lobules I-VIII (Wiksten, 1979a, b). Electrophysiological studies have demonstrated that the CCN is one of the major relay nuclei from neck muscles and the semicircular canals, indicating a role

in signalling head position and movement with regard to the rest of the body (Hirai, 1983; Hirai et al., 1984).

1.5.2 Reticular mossy fibres

The lateral reticular nucleus (LRN) which lies in the lower medulla lateral to the inferior olive, is another major source of mossy fibres to the cerebellum. Whereas the spinocerebellar tracts arise from peripheral sources and are relayed to the cerebellum via the spinal cord and the medullary relays, the LRN transmits information from peripheral receptors indirectly. Projections from the LRN terminate bilaterally in the anterior lobe and ipsilaterally in the paramedian lobule (Clendenin et al., 1974a, b). The LRN is responsive to cutaneous and high threshold muscle afferents, i.e. flexor reflex afferents (FRA) (Rosen & Scheid, 1973; Clendenin et al., 1974c). Cutaneous receptive fields are generally large, and may encompass all four limbs. The most dominant input to the LRN is from FRA that originate from all four limbs. In addition to the ascending inputs from peripheral receptors, the LRN is strongly influenced from descending inputs, such as the sensorimotor cortex, the red nucleus, and vestibular nuclei (Rosen & Scheid, 1973; Corvaja et al., 1977). The LRN is believed to integrate these ascending and descending inputs, and similar to the VSCT, conveys messages to the cerebellum regarding the activity of spinal cord motor mechanisms (Clendenin et al., 1974c; Arshavsky et al., 1978b).

1.5.3 Pontine mossy fibres

By far the greatest source of mossy fibres is from the pontine nuclei. Pontine nuclei project to the cerebellum through the middle cerebellar peduncle, to provide mossy fibres to nearly all regions of the cerebellum. The pons receives massive projections from the ipsilateral cerebral cortex, with a preponderance of origin from the motor, premotor, somatosensory, and visual cortices (Brodal, 1978; Wiesendanger & Wiesendanger, 1982; Glickstein et al., 1985; Mihailoff et al., 1985; Lee & Mihailoff, 1990). Recent anatomical and electrophysiological studies have demonstrated that projections from the cerebral cortex are more widespread, with connections from the frontal and prefrontal cortex (Middleton & Strick, 2001). There is little experimental evidence to indicate what sort of information is transmitted via the cerebropontocerebellar pathway, but as the primary and premotor cortex showed a strong projection to the pontine nuclei, it has been suggested that this pathway provides the cerebellum with a reference copy of descending motor instructions (Allen & Tsukahara, 1974). Presumably, these motor commands can then be compared with ascending sensory feedback from the spinal cord to detect errors between intended

and actual movements. More recently, as sensory areas of the cerebral cortex project strongly to the pons, it has been argued that the function of this pathway is for the sensory guidance of movement (Stein & Glickstein, 1992). Additionally, the generation of smooth-pursuit eye movement has also been shown to be dependent on the cerebropontocerebellar pathway (Suzuki et al., 1999).

The pontine nuclei are also known to receive inputs non-cortical in origin. These include the dorsal column nuclei (Swenson et al., 1984; Kosinski et al., 1988b), cerebellar nuclei (Brodal et al., 1972; Watt & Mihailoff, 1983), the tectum (Wells et al., 1989; Mihailoff, 1995), zona incerta (Mihailoff, 1995) and the spinal cord (Mihailoff et al., 1989). Some of these non-cortical projections have been found to converge in the pons with cortical projections, indicating that the pontine nuclei may actually integrate somatosensory signals with descending commands from the cortex at the level of the pons, rather than in the cerebellum as previously thought (Kosinski et al., 1986; Kosinski et al., 1988a; Lee & Mihailoff, 1990).

1.6 Climbing fibre organisation

In contrast to the mossy fibres, it is now well established that the climbing fibres originate from only one source, the inferior olive located in the base of the medulla (Szentagothai & Rajkovits, 1959; Desclin, 1974; Desclin & Escubi, 1974; Campbell & Armstrong, 1983). The inferior olive is divided into three main subdivisions: the medial accessory olive (MAO), the dorsal accessory olive (DAO) and the principal olive (PO). The inferior olive receives afferent input from many ascending and descending sources, with each subdivision of the olive receiving its own distinct inputs (Armstrong, 1974).

The descending inputs to the inferior olive include the primary somatosensory cortex, pre and supplementary motor cortices, and the frontal association cortex (Sousa-Pinto & Brodal, 1969; Bishop et al., 1976; Brown et al., 1977; Berkley & Worden, 1978; Swenson et al., 1989). There is also an extensive input from the red nucleus (Martin et al., 1975; Brown et al., 1977; Burman et al., 2000). Other sources include the thalamus, tegmental field of Forel, the nucleus of Darkschewitsch, the interstitial nucleus of Cajal, caudate nucleus, and the pretectum (Brown et al., 1977).

Many studies have demonstrated that information from skin, muscle, and joints, are relayed to the inferior olive from a number of somatosensory structures including the spinal cord, dorsal column nuclei, lateral nucleus, the medullary reticular formation, lateral cervical nucleus, trigeminal nucleus, and vestibular nuclei (Oscarsson, 1973;

Oscarsson & Sjolund, 1974; Berkley, 1975; Martin et al., 1975; Hand & Van Winkle, 1977; Saint-Cyr & Courville, 1979; Walberg, 1982; Molinari, 1984, 1985). These pathways ascend in different funiculi of the spinal cord, synapsing on different numbers of spinal and brain stem relay cells before terminating in discrete subdivisions of the three olivary subnuclei. In turn, the inferior olive provides axons to the cerebellum in the form of spino-olivecerebellar pathways (SOCPs) that terminate in the cerebellar sagittal zones. Each sagittal zone receives at least one (and usually more) SOCP, with each zone defined by its own latency and peripheral receptive fields.

The SOCPs have been studied extensively, and the most studied of these are the dorsal funiculus, dorsal lateral funiculus, ventral funiculus, and lateral funiculus-SOCPs. Although these pathways differ anatomically, they are functionally similar in that most of these pathways are activated by FRA. The dorsal funiculus (DF)-SOCP, as the name suggests, ascends through the spinal cord via the dorsal funiculus, relaying information from the cuneate and gracile nucleus to the contralateral medial and dorsal accessory olive. The DF-SOCP projects to the anterior lobe and paramedian lobule to terminate within the A, X, B, C1, C2, C3, D1, and D2 zones (Oscarsson, 1969; Armstrong et al., 1973d; Ekerot & Larson, 1979a, b, 1982). The response latencies evoked through these pathways upon forelimb and hindlimb stimulation are 10-15 ms and 15-20 ms respectively.

The ventral funiculus (VF)-SOCP ascends the ventral funiculus, contacting olive cells in the medial and dorsal accessory olive before terminating in the anterior and paramedian lobules of the cerebellar cortex in the A, B, C1 and C3 zones (Oscarsson & Sjolund, 1977a, b, c; Andersson & Eriksson, 1981). This pathway has been studied in great detail and it is possible to distinguish 5 separate pathways within the VF-SOCP on the basis of their peripheral receptive field, response latency and termination zone (Oscarsson & Sjolund, 1977a, c). All 5 pathways are activated by FRA (Armstrong & Harvey, 1968; Oscarsson, 1968; Oscarsson & Sjolund, 1977c) and have large receptive fields. Their response latencies range from 14-27 ms.

The pathway that ascends the spinal cord within the dorsal part of the lateral funiculus is known as the dorsal lateral funiculus (DLF)-SOCP. The DLF-SOCP targets olivary neurones that project to the anterior and paramedian lobules (Larson et al., 1969b; Oscarsson, 1969; Ekerot & Larson, 1973). Within the anterior lobe, two components of the DLF-SOCP activated by forelimb nerves at a latency of 18-24 ms, terminate in the caudal

C1 and C3 zone. A third component terminates rostrally in the D1 zone, and is activated by the ipsilateral hindlimb with a latency of 18-24 ms.

More ventral than the DLF-SOCP is the lateral funiculus (LF)-SOCP which projects to the C2 zone of the anterior lobe, lobulus simplex and the paramedian lobule via the medial accessory olive (Armstrong et al., 1973d; Ekerot & Larson, 1979a). The LF-SOCP is activated polysynaptically by FRA from all four limbs, with long latencies (Larson et al., 1969a; Armstrong et al., 1973d).

1.7 Efferent projections of the cerebellar cortex

The sole output of the cerebellar cortex is via the Purkinje cell axons, which project to the DCN and vestibular nuclei located within the cerebellar white matter and medulla respectively. Purkinje cell terminals contain GABA (Obata et al., 1967; Curtis et al., 1970; Ottersen & Storm-Mathisen, 1984) and exert an inhibitory action on DCN neurones (Ito et al., 1964). As well as receiving a major projection from Purkinje cells, degeneration studies and neural tracer studies have demonstrated that the DCN also receive axon collaterals of mossy fibres and climbing fibres (Matsushita & Ikeda, 1976; Groenewegen & Voogd, 1977; Andersson & Oscarsson, 1978b; Gerrits et al., 1985; Mihailoff, 1994; De Zeeuw et al., 1997b; Ruigrok, 1997; Shinoda et al., 2000). Activation of the climbing fibre and mossy fibre collaterals has been demonstrated to evoke an excitatory postsynaptic response on the nuclear cells (Llinas & Muhlethaler, 1988).

Electrophysiological studies have demonstrated that DCN neurones are endowed with the capability of discharging spontaneously, even in the absence of any obvious movements (Thach, 1970b; Armstrong & Rawson, 1979b; Harvey et al., 1979). DCN neurones in both *in vivo* and *in vitro* preparations display tonic activity ranging from 20-80 Hz (Armstrong & Rawson, 1979b; Jahnsen, 1986b; Llinas & Muhlethaler, 1988; Mouginot & Gahwiler, 1995). The spontaneous activity of DCN neurones is reportedly due to the presence of pacemaker-like Na^+ and Ca^{2+} currents (Jahnsen, 1986a, b). As the DCN neurones receive projections from Purkinje cells and axon collaterals from mossy and climbing fibres, the output of all nuclear cell neural computations are shaped by inhibition from the cerebellar cortex and excitation from the mossy and climbing fibres.

1.8 Efferent projections of the DCN and vestibular nuclei

1.8.1 Nucleofugal projections

Anatomic pathway tracing studies have clarified the connections and topographic organisation of the DCN. The bulk of cerebellar output exits the cerebellum via the superior and inferior cerebellar peduncles to project to a number of areas in the CNS. Efferents from the interpositus and dentate nuclei leave the cerebellum via the superior cerebellar peduncle crossing at the midline to the contralateral midbrain tegmentum, where at this point the tract divides into ascending and descending pathways. The descending pathways innervate the pontine nuclei, inferior olive and medullary reticular formation (Faull, 1978; Teune et al., 2000), whereas the ascending axons innervate the red nucleus (Faull & Carmen, 1978; Teune et al., 1995) and the ventrolateral thalamus or what has been termed the 'cerebellar thalamus' (Thach et al., 1992; Horne & Butler, 1995). Termination at both these sites is topographically organised (Stanton, 1980; Asanuma et al., 1983a). Fibres from the fastigial nucleus course through the inferior cerebellar peduncle, and like the superior cerebellar peduncle, it also has ascending and descending branches. The descending fibres of the fastigial nucleus terminate in the vestibular nuclei, pontomedullary reticular formation, and the inferior olive (Voogd, 1964; Sugimoto et al., 1980; Dietrichs & Walberg, 1981; Asanuma et al., 1983b). The ascending branch terminates in the superior colliculus (Angaut & Bowsher, 1970), and visual structures of the midbrain. The vestibular nuclei project to the spinal cord and extraocular muscles via the vestibulospinal tract and medial longitudinal fasiculus respectively.

Recordings from DCN targets have shown that the cerebellar nuclei generate large EPSPs, and thus it was presumed that the output of the cerebellar nuclei is excitatory in nature. In support of this, medium to large sized neurones of the DCN are known to use glutamate and/or aspartate as their neurotransmitter (Monaghan et al., 1986; Kumoi et al., 1988; Chen & Hillman, 1993).

1.8.2 Nucleocortical projections

As well as nucleofugal terminations, all three DCN have been shown to project to different areas of the cerebellar cortex. Axons of the DCN terminate in the granular layer of the cerebellar cortex, with a large degree of reciprocity displayed between a group of nuclear cells and the area of the cerebellar cortex from which they receive their Purkinje cell projection (Tolbert et al., 1976; Tolbert & Bantli, 1979; Dietrichs, 1981; Trott et al., 1990, 1998a, b). In addition, non-reciprocating pathways have been recognised, with some

DCN terminating in cortical areas outside of their Purkinje cell innervation area (Sastry et al., 1997). Taken together, these observations suggest that as well as possessing a cortico-nucleo-cortico feedback loop, communication between different cortical areas can also occur through the cerebellar nuclei (Ito, 1984; Sastry et al., 1997).

1.8.3 Nucleo-olivary projections

As well as comprising of medium to large sized neurones containing glutamate and/or aspartate, the cerebellar nuclei possess a population of small GABAergic neurones which are known to project to the inferior olive (Angaut & Sotelo, 1989; De Zeeuw et al., 1989; Ruigrok & Voogd, 1990), and are inhibitory in action (Andersson & Hesslow, 1987b; Andersson et al., 1988).

Olivocerebellar fibres that terminate in the cerebellar cortex as climbing fibres and are also known to send axon collaterals to the cerebellar nuclei. It has been established that the climbing fibre collaterals are organised as the reciprocal of the nucleo-olivary projection (Dietrichs & Walberg, 1981; Ruigrok & Voogd, 2000). Superimposed on this is the reciprocal projections between Purkinje cells and the DCN, as well as the normal climbing fibre projection from the inferior olive to Purkinje cells. Thus, it appears that a complex yet precise arrangement exists between the DCN, inferior olive and cerebellar cortex.

1.9 Zones and microzones

It is now generally agreed that at least major portions of the cerebellum are arranged into sagittally orientated strips or zones, with projections from functionally related inferior olive cells that target Purkinje cells arranged into narrow rostro-caudally orientated zones within the cerebellar cortex (See Section 1.2). In general, Purkinje cells within a given zone receive climbing fibres from a restricted region of the inferior olive characterised by its general receptive field and response latency to peripheral stimulation (Oscarsson & Sjolund, 1977a; Oscarsson, 1979, 1980; Armstrong, 1990). The Purkinje cells within each zone in turn project to a particular cerebellar or vestibular target nucleus, which receives collaterals from the climbing fibres that terminate in that zone (De Zeeuw et al., 1994).

With the advent of refined experimental techniques, a highly detailed analysis of the zones has been instrumental in revealing that some of the zones are further divisible into 'microzones' (Andersson & Oscarsson, 1978a; Ekerot & Larson, 1979b; Ekerot et al., 1991b). Microzones are narrow (*ca* 200 μm), longitudinal cortical strips, defined by climbing fibres with similar peripheral receptive fields, and innervating specific groups of

cells of the target efferent nucleus of that zone (Andersson & Oscarsson, 1978a; Garwicz et al., 1998; Jorntell et al., 2000). For example, the B zone which runs along the lateral edge of the vermis is divisible into 5 microzones on the basis of its climbing fibre properties, with each subgroup projecting to a differing group of cells within the lateral vestibular nucleus (Andersson & Oscarsson, 1978a, b). Moving along the 5 microzones, the most medial microzone is responsive to tail stimulation, followed by hindlimb, trunk, forelimb, and most laterally, the face (Andersson & Oscarsson, 1978a; Andersson & Eriksson, 1981). The C3 zone in the paravermis of the cat is also divisible into a series of microzones, numbering about 30 and responsive to both tactile and noxious limb stimulation (Ekerot & Larson, 1979b; Ekerot et al., 1991b; Garwicz et al., 1998). Subsequently, it has been proposed that the microzones represent the functional units of the cerebellum in motor control (Oscarsson, 1979; Ito, 1984; Garwicz et al., 1998).

In addition to the microzonal organisation, 'modules' consisting of microzones with similar climbing fibre receptive fields and converging on common groups of cerebellar nuclear cells (Garwicz & Ekerot, 1994) are also believed to represent functional units of the cerebellum in motor control (Garwicz & Ekerot, 1994; Garwicz et al., 1998; Apps, 2000).

1.10 Climbing fibre function

In both anaesthetised, and quiescent awake animals, Purkinje cell complex spike discharge occurs at very low rates, around 1.5 Hz on average (Thach, 1968; Armstrong & Rawson, 1979a; Armstrong & Edgley, 1984b; Andersson & Armstrong, 1987; Armstrong et al., 1988; Stone & Lisberger, 1990a; Keating & Thach, 1995). During motor performance, for example in monkeys slowly executing arm movements, complex spike activity of limb-related Purkinje cells seldom exceeded rates of more than 4 Hz (for eg Thach, 1968; Harvey et al., 1977). In comparison, simple spikes, which typically discharge in a tonic fashion at a rate of approximately 65 Hz, can attain rates of up to 200 Hz during motor performance. Similar findings have also been found during visually guided tasks (Ebner & Fu, 1997), locomotion (Armstrong & Edgley, 1984b; Armstrong et al., 1988) and in relation to eye movements (Lisberger & Fuchs, 1978; Stone & Lisberger, 1990a, b). As simple spike frequency modulation appears to dominate the Purkinje cell output during behavioural changes, the information-laden mossy fibre system is thought to be important for the moment to moment operation of the cerebellum.

Although the low rate of climbing fibre activity makes it unlikely that the information carried by these fibres is of importance in direct modulation of the frequency code of the Purkinje cells, the climbing fibres are essential for normal cerebellar operation. Selective destruction of the inferior olive with neurotoxins or by lesioning produces movement disorders in experimental animals closely resembling those of total cerebellectomies (Desclin, 1974; Llinas et al., 1975). Animals were ataxic and presented with a distinctive gait, and aggerated flexion of the limbs. Climbing fibre activity, although infrequent, must therefore have a vital role in cerebellar operation. Many theories have been proposed about the climbing fibres in an attempt to explain the meaning and consequences of their actions. While a brief review of some of these theories will be presented below, the reader is referred to Bloedel and Bracha (1998) and Simpson et al., (1996) for a more comprehensive discussion on climbing fibre theories.

1.10.1 Comparator, and event or error detector hypothesis

The convergence of cerebral inputs with spinal inputs at the level of the inferior olive led Oscarsson (1969) and Miller and Oscarrson (1970) to conclude that the climbing fibre afferents act as a 'comparator'. As Oscarsson (1969) originally found that climbing fibres transmitting information from the spinal cord to the cerebellum were readily activated by electrical stimulation, but were difficult to activate using natural stimulation, it was believed that the function of the inferior olive was not to signal peripheral events, but rather to 'compare' command signals from higher centres with the activity of commands evoked in the spinal cord. In this hypothesis, a disparity between the two types of inputs would result in the climbing fibres forwarding an error signal to the cerebellar cortex where a corrective signal would be generated to act on lower or higher motor centres. Oscarsson (1980) went on to modify his original hypothesis by proposing that the inferior olive in fact signals 'unexpected' errors by detecting either perturbations in the motor commands as a consequence of reflex activity or perturbations in the evolving movement as a result of unexpected changes in load or resistance.

In comparison to the original studies by Oscarsson, numerous studies have now demonstrated that olive cells and climbing fibres are in fact capable of signalling peripheral events evoked by natural mechanical stimulation and passive manipulation of the joints (Rushmer et al., 1976; Gellman et al., 1985; Ekerot et al., 1991a, b). Rushmer et al., (1976) demonstrated that small passive movements of the forepaw in the plantar and dorsal directions yielded climbing fibre responses, leading the authors to conclude that the climbing fibre system serves as an 'event detector', signalling footfall and footlift

during locomotion. Contradictory to this, further studies examining the occurrence of complex spikes from animals performing movement tasks and during periods of rest demonstrated that complex spike rates were not time locked to any particular phase of movement, and moreover, complex spikes discharged at the same rate during movement as during periods of rest (Thach, 1968, 1970a; Andersson & Armstrong, 1987; Armstrong et al., 1988). Furthermore, Gellman et al., (1985) demonstrated that olivary cells that were responsive to passive cutaneous stimuli failed to respond when a similar stimulus was produced by an active movement by the animal.

Although the original 'comparator' hypothesis did not receive experimental support, studies have supported the modified hypothesis of an 'unexpected event or error detector'. For example, in awake cats trained to walk on a horizontal ladder, complex spike discharge occurred when a rung the cat was stepping on unexpectedly gave way (Andersson & Armstrong, 1987). Similar findings of climbing fibres signalling unexpected events were made in other studies with differing experimental paradigms (Gilbert & Thach, 1977; Lou & Bloedel, 1992a, b).

1.10.2 LTD and motor learning

Since Hebb (1949, cited by Ito, 2001) first postulated that learning occurs when coactivation of pre- and postsynaptic synapses induces synaptic modification, synaptic plasticity has been thought to be essential for the formation of memory. Based on the convergence of a single climbing fibre and numerous parallel fibres onto a single Purkinje cell, Brindley (1969, cited by Ito, 2001) was the first to view the cerebellum as a site of Hebbian synaptic plasticity. Marr (1969) subsequently went on to propose a learning model of the cerebellum whereby memory traces are stored when climbing fibres, which were thought to signal correct performance, are activated in conjunction with the parallel fibres, causing a facilitation of the parallel fibre synapses. Albus (1971) modified this theory to propose that climbing fibres signal errors in performance and that rather than a facilitation, parallel fibre synapses are weakened. Experimental evidence that the parallel fibre synapses are capable of plastic change when activated in conjunction with the climbing fibre input to a Purkinje cell was first obtained by Ito, Sakurai, and Tongroach (1982) who demonstrated that a brief period of conjunctive stimulation of mossy and climbing fibre could result in a long lasting long-term depression (LTD) in transmission at the conditioned parallel fibre synapses. The Marr-Albus-Ito theory (MAIT) proposes that a subset of parallel activates a particular Purkinje cell. If an error in motor performance were to occur, the climbing fibres which would signal this error would become activated,

with the resulting conjunctive stimulation of the two inputs inducing LTD and leading to a weakening of the parallel fibre inputs.

The occurrence of LTD remained controversial until demonstrated in cerebellar slice preparations (Crepel & Jaillard, 1991; Konnerth et al., 1992) and Purkinje cell cultures (Linden et al., 1991). The phenomenon of LTD and the parameters required for its inducement has been extensively investigated and widely accepted as an effective functional mechanism, so much so that standard textbooks dogmatically describe LTD as the substrate for motor learning in the cerebellum. While there is no doubt that the climbing fibres can in fact modify parallel fibre transmission under experimental conditions, the assumption that the LTD plays a physiological role in motor learning has been strongly challenged by recent theoretical considerations and experimental findings.

A major disagreement for LTD by cerebellar researchers is the temporal sequence of stimulation of the climbing fibre and parallel fibre pairings required to induce LTD. Stimulation of parallel fibres simultaneously with or following climbing fibre activation is generally required for the induction of LTD, with maximal depression obtained when parallel fibres are stimulated 10-100 ms after climbing fibre stimulation (Ekerot & Kano, 1989; Schreurs & Alkon, 1993). The relative timing of the paired stimulation is in fact opposite to that predicted by the MAIT and of that used in classical conditioning, which suggests that the error signal, carried by the climbing fibres should come after the sensory input, the parallel fibres.

Another factor against LTD, is that it is very difficult to obtain substantial LTD *in vivo* with what would be considered normal physiological patterns of climbing fibre firing. In order to obtain a measurable degree of depression in parallel fibre transmission *in vivo*, it is generally necessary to activate 100-1000 pairings of climbing fibres and parallel fibres at stimulation rates of 1-4 Hz. In comparison, a stimulation rate of around 1 Hz is generally sufficient to cause LTD in slice preparations and cultured cells (Karachot et al., 1994). In the original investigations of Ito et al., (1982), they used a stimulation rate of 4 Hz for 25 seconds to obtain modified responses whereas Ekerot and Kano (1985) obtained only a modest depression with conjunctive stimulation at 2 Hz for 8 minutes. Ekerot and Kano (1989) went on to demonstrate that the amplitude of LTD depended on the climbing fibre stimulation frequency, with conjunctive stimulation at 4 Hz being more effective at inducing LTD than frequencies of 1 and 2 Hz. In awake animals undertaking a wide range of tasks, rates of climbing fibre activity rarely exceed more than 2.5-3 Hz, and even then such rates only persist for relatively brief periods and are not sustained over many

minutes (Thach, 1968, 1970a; Harvey et al., 1977; Armstrong & Rawson, 1979a; Andersson & Armstrong, 1987). Even under conditions of motor learning or when animals respond to an imposed perturbation, the Purkinje cells may display only one or two extra complex spikes on some trials associated with the learning task or disturbance (Gilbert & Thach, 1977; Harvey et al., 1977; Andersson & Armstrong, 1987). Furthermore, although one would expect during conditions of motor learning a decrease in simple spike activity induced by complex spike activity, in many cases an increase in simple spike discharge was often observed that is not consistent with the MAIT (Ebner et al., 1983; Sato et al., 1992). Thus, under natural conditions it is possible that the climbing fibres will not fire at a sufficiently high rate for a period of time sufficient to induce the synaptic plasticity observed experimentally.

The difference in stimulation rates required to induce LTD in *in vivo* and *in vitro* studies may be explained by the block of inhibitory transmission in cerebellar slices achieved either by pharmacological block (Crepel & Jaillard, 1991) or by severing the axons of the inhibitory interneurones (Konnerth et al., 1992), as it is very difficult to obtain cerebellar LTD when normal inhibition is present (Ekerot & Kano, 1985; Schreurs & Alkon, 1993). LTD failure with normal levels of inhibition occurs because strong stimulation of parallel fibres induces postsynaptic inhibition through the parallel fibre-stellate cell pathway (Ekerot and Kano 1985). A new hypothesis by De Schutter (1995) suggests that the interaction between LTD induction and inhibitory neurones acts as a local negative feedback safety mechanism to prevent the excessive depolarization of the Purkinje cell and accumulation of toxic amounts of Ca^{2+} by the parallel fibres, especially as each Purkinje cell receives more than 175 000 parallel fibre inputs (Napper & Harvey, 1988).

More recently, the actual requirement for climbing fibres inducing LTD has been questioned (De Schutter & Maex, 1996). LTD is known to be dependent on three factors: elevation of dendritic Ca^{2+} concentration, the activation of α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) receptors, and the activation of metabotropic glutamate receptors (mGluR). It is generally assumed that the climbing fibres are responsible for the increase in intracellular Ca^{2+} whereas parallel fibre stimulation activates the AMPA receptors and mGluR. Recent experimental evidence suggests that, as well as the classical form of climbing fibre-induced LTD, LTD can also be induced by other mechanisms that are capable of increasing intradendritic Ca^{2+}, such as parallel fibre stimulation alone (Hartell, 1996) or the application of depolarizing pulses (Crepel & Jaillard, 1991). In addition, experiments on gene knock-out animals with impaired cerebellar LTD have also

been instrumental in questioning the role of climbing fibres in synaptic plasticity. Mutant mice with targeted deletion of genes encoding metabotropic glutamate receptor type 1 (mGluR1), glial fibrillary acidic protein (GFAP), and the glutamate receptor δ type 2 (mGluδ2) all display impaired cerebellar LTD (Kashiwabuchi et al., 1995; Shibuki et al., 1996; Kano et al., 1997; Ichise et al., 2000). Moreover, mGluR1 and mGluδ2 knock-outs which display persistent multiple climbing fibre innervation of Purkinje cells, also show severe motor coordination deficits, whereas the GFAP mutants display Purkinje cells with monosynaptic climbing fibre innervation and normal motor coordination. These results suggest that the 1:1 relationship between climbing fibres and their Purkinje cells is more important than LTD for normal cerebellar function.

1.10.3 Gain change hypothesis

After the occurrence of a complex spike, a pause in simple activity for a duration of 10 to several hundred milliseconds has been noted by many investigators (Bloedel & Roberts, 1971; Latham & Paul, 1971; Murphy & Sabah, 1971; Burg & Rubia, 1972; Armstrong & Rawson, 1979a). This period of reduced simple spike activity is known as the 'climbing fibre pause'. After the pause, a change in Purkinje cell excitability was observed (McDevitt et al., 1982) that formed the basis of the gain change hypothesis. In a series of experiments by Ebner and Bloedel (Ebner & Bloedel, 1981; Ebner et al., 1983; Ebner & Bloedel, 1984) on decerebrate, unanaesthetised cats, it was concluded that when simple spike activity is preceded by climbing fibre activation, a short-lasting enhancement of the Purkinje cell to its mossy fibre input occurs. This period of facilitation could last for several hundred milliseconds and was manifested as an increase in both the excitatory and inhibitory simple spike responses.

More recently, Bloedel and colleagues have extended the gain change hypothesis in keeping with the modular organisation of the climbing system (Kelly et al., 1990; Lou & Bloedel, 1992a, b). The 'dynamic selection' hypothesis predicts that sagittally aligned Purkinje cells with a synchronous activation of their climbing fibre input would, through the enhancement of their mossy fibre responses, be modulated far more than Purkinje cells that were activated by comparable mossy fibre inputs alone. In this way, climbing fibres are thought to select mossy fibre inputs that produce the greatest modulation of Purkinje cell simple spike activity.

1.10.4 Timing device hypothesis

Inferior olive cells are electrotonically coupled between their dendrodendritic gap junctions (Llinas et al., 1974; Sotelo et al., 1974; De Zeeuw et al., 1997a). This dynamic coupling allows olivary cells to fire rhythmically and synchronously, and has led Llinas and colleagues to propose that the climbing fibres act as a 'timing device' for movement execution. The interaction of ionic conductances located differentially in the dendrites and soma of the olivary cells is thought to underlie their tendency to fire rhythmically at 10 Hz (Llinas & Yarom, 1981a, b). The synchronous activity of the olivocerebellar system translates at the level of the cerebellar cortex into Purkinje cells displaying complex spike activity arranged into parasagittally orientated strips (Sugihara et al., 1993; Fukuda et al., 2001) that is determined by the physiology, not the anatomy of the system. The banding pattern is dynamic, as GABAergic inhibitory input to the inferior olive from the DCN (Sotelo et al., 1974; De Zeeuw et al., 1989) can modulate the electrotonic coupling between the olive cells (Lang et al., 1996), as can blockade of the excitatory glutamatergic inputs (Lang, 2001). Blockade of GABAergic input disrupts the banding pattern (Lang et al., 1996), whereas blockade of glutamatergic input enhances it (Lang, 2001). Studies have demonstrated that strengthening the rhythm of the olivary neurones enhances the banding pattern of complex spike synchrony (Lang et al., 1999) by increasing the synchronization between coupled cells and decreasing the synchrony of poorly coupled cells (Lang et al., 1997).

The dynamic complex spike-banding pattern is thought to represent various functional states of the olivocerebellar system, with distinct patterns of synchronous complex spike activity generating distinct movements. Olivary neuronal clusters are thought to represent particular muscles, so that varying the coupling amongst different sets of olivary cells would recruit different sets of muscles at appropriate times during movement (Welsh et al., 1995). Evidence supporting the timing hypothesis was initially provided by studies correlating motor performance with synchronous olivary activity. Studies utilising the tremorgenic agent harmaline induced synchronous olivary activity and tremors in phase with this activity (de Montigny & Lamarre, 1973; Llinas & Volkind, 1973). More recently, (Welsh et al., 1995) found that rhythmic climbing fibre activity was time-locked with skilled tongue movements in the rat However, other studies examining compensatory eye movements in the awake rabbit to natural visual stimulation or in the monkey trained to perform wrist movements have not found any evidence that movement is timed by the oscillations of populations of inferior olivary cells (Keating &

Thach, 1995, 1997; De Zeeuw et al., 1998b). It remains to be determined whether the rhythmic olivary activity is important for generating non-rhythmic motor behaviour.

1.10.5 Internal models

It has been suggested that the cerebellum contains an internal model, which generates internal feedback signals that are used to adjust ongoing motor commands. Internal models are theoretical concepts that are believed to predict the consequences of motor commands in order to cancel the sensory effects of movement and to provide a mechanism to distinguish whether movements are self-generated or imposed by external sources (Wolpert & Flanagan, 2001). Thus, it has been proposed that discrepancies between actual and predicted sensory feedback would be utilised for motor control. It was also proposed that in order to learn the predictions and to ensure that they are accurate, they are compared with sensory feedback. In the case of the cerebellum, this would occur at or before the level of the inferior olive, with the climbing fibres signaling any discrepancies between the two (Miall et al., 1998). In the cerebellar circuitry, the parallel fibres (and thus, simple spikes) are thought to represent the predictive signal. Thus, it would be expected that a delay between an increase in simple spike activity and the resulting complex spike should be observed that is equal to the prediction interval. Indeed, Miall et al., (1998) observed in monkeys trained to track a visual target with a cursor, that a small increase in simple spike activity preceded complex spike activity by 150 ms. This 150 ms interval was believed to be due to the visuomotor feedback delay, and the subsequent output of the Purkinje cell represented a predictive signal. Miall et al., (1998) went on to suggest that the change in the Purkinje cell predictive signal would then translate to the DCN, and via their projection to the inferior olive, the DCN would then cause the activation of a complex spike. Complex spike activation would, through LTD, trigger a down regulation of the simple spike activity to counteract the excessive potentiation that could occur if the ongoing activity of the parallel fibre system was left unchecked. In this way, complex spike activity would correct for cerebellar errors that follow parallel fibre-Purkinje cell potentiation and keep the cerebellar cortex within its operational range.

1.11 The present study

It is evident from the above account of climbing fibre theories that there is no general consensus as to what contribution climbing fibres make to cerebellar function. As already indicated, it seems unlikely that climbing fibre activity is of major importance in directly

contributing to the Purkinje cell frequency code and it still seems likely that its main influence on cerebellar cortical output is an indirect one related to the dominant simple spike activity. Moreover, it seems clear that whatever function the climbing fibres perform, it is achieved over a very limited rage of firing frequencies. This thesis presents a new hypothesis for climbing fibres in cerebellar cortical function. It is proposed that an important role of the climbing fibres is to regulate the level of background simple spike activity of the Purkinje cells. It is suggested that this is achieved by control over an intrinsic spike generating mechanism.

For many years it had been thought that the resting discharge of cerebellar Purkinje cells is produced by the sustained postsynaptic depolarization of the cells by ongoing transmission along the mossy fibre-granule cell pathway or by spontaneous activity in the granule cells, as spontaneous simple spike activity was still present after sectioning of the cerebellar peduncles (Eccles et al., 1967a). Convincing evidence now exists that is not necessarily the case. For example, in agranular cerebella, the Purkinje cells still display high levels of ongoing simple spike discharge (Woodward et al., 1974; Siggins et al., 1976). Some spontaneous activity is also present in cerebellar slices (Hounsgaard, 1979; Llinas & Sugimori, 1980a; Crepel et al., 1984; Hounsgaard & Midtgaard, 1988; Hausser & Clark, 1997), in cultured Purkinje neurones (Gruol, 1983; Gruol & Franklin, 1987), and even in isolated Purkinje cell bodies (Nam & Hockberger, 1997; Raman & Bean, 1999b). Moreover, simple spike activity can be observed when synaptic input to the Purkinje cells is blocked *in vitro* or before the appearance of functional synaptic input (Gahwiler, 1975; Dupont et al., 1979; Miyashita & Nagao, 1984; Konnerth et al., 1990; Hausser & Clark, 1997) suggesting that the spontaneous discharge is driven by an endogenous pacemaker mechanism.

Various models that involve the interaction between Na^+, K^+, Ca^{2+} and Ca^{2+}-dependent K^+ conductances in the Purkinje cell membrane have subsequently been proposed to explain the origin of the endogenous activity (De Schutter & Bower, 1994a; Nam & Hockberger, 1997; Raman & Bean, 1999b; Pouille et al., 2000). The mechanism suggested here is relatively simple and is based on the observations of Llinas and Sugimori (1980a, b) on Purkinje cells in cerebellar slices. It is proposed that the non-inactivating Na^+ conductance provides the basic depolarization for generating tonic simple spike activity. This conductance was first described in the Purkinje cells by Llinas and Sugimori (1980a). It has a lower threshold for activation and generates a prolonged plateau of depolarization in the Purkinje cell. Llinas and Sugimori (1980a) suggested that the level of the depolarising plateau generated by this conductance was limited by a non-inactivating

outward K^+ conductance. They went on to show that with blockage of K^+ channels with TEA in Ca^{2+} free medium, the non-inactivating Na^+ conductance caused the Purkinje cell to depolarize intensely to the point at which the soma spike generating mechanism was inactivated. It is suggested that *in vivo*, the balance between the non-inactivating Na^+ and K^+ conductances determines the level of ongoing Purkinje cell depolarization and thus the level of intrinsic simple spike activity. The model then proposes that this basic level of depolarization is regulated by a hyperpolarizing Ca^{2+}-dependent K^+ conductance that is activated by Ca^{2+} entry via the climbing fibre input to the Purkinje cells. Each climbing fibre depolarizes the Purkinje cells sufficiently to activate the voltage-operated Ca^{2+} channels (VOCCs) with a consequent influx of Ca^{2+} into the Purkinje cell cytosol. This activates the Ca^{2+}-dependent K^+ conductance, which then exerts an additional hyperpolarizing influence on the Purkinje cell. It is proposed that the frequency of the climbing fibre input determines the prevailing concentration of Ca^{2+} in the cytosol and thus the magnitude of the Ca^{2+}-dependent K^+ current. It is proposed that control over the entire range of simple spike frequencies is achieved over the limited range of climbing fibre frequency *in vivo* i.e. approximately 0.5-3 Hz. It is predicted that an increasing frequency of input will generate proportionally higher levels of Ca^{2+} with a corresponding increased activation of the Ca^{2+}-dependent K^+ current, increased hyperpolarization of the Purkinje cell and a reduction or cessation of intrinsic simple spike activity. This model fits very well with the finding that an increase in the frequency of the climbing fibre input to a Purkinje cell causes a decrease or cessation in ongoing simple spike activity (Colin et al., 1980; Rawson & Tilokskulchai, 1981b; Demer et al., 1985; Andersson & Hesslow, 1987a).

It is also predicted that cytosolic levels of Ca^{2+} will be lower at low climbing fibre frequencies, with correspondingly less activation of the Ca^{2+}-dependent K^+ current and a subsequent increase in intrinsic simple spike activity. This proposed mechanism fits well with experimental findings that simple spike activity rises substantially following removal or inactivation of the climbing fibres (Colin et al., 1980; Montarolo et al., 1982; Demer et al., 1985; Savio & Tempia, 1985).

This model also proposes that the climbing fibre input is the major source of Ca^{2+} entry to the Purkinje cells, and is the major determinant of the concentration of Ca^{2+} entry in the cytosol. Given that the time taken to fully sequester Ca^{2+} from the cytosol is in the order of 10-15 seconds (Tank et al., 1988), cytosolic Ca^{2+} will remain at an elevated level *between* climbing fibre impulses. Thus, the theory predicts that Purkinje cells, unlike other neurones, normally operates with a fairly high level of cytosolic Ca^{2+}, which is maintained by their unique climbing fibre input. It is expected that Ca^{2+} in the Purkinje cell cytosol

will fall to a very low level if climbing fibre activity ceases. This will not only cause depolarization but will almost certainly affect the activity of Ca^{2+} dependent biochemical processes in the Purkinje cell with a consequent disruption of cell function. It is thus predicted that the ongoing input from the climbing fibre is also essential for the maintenance of normal Purkinje cell function.

The experiments in this thesis involve investigations into:

1. An examination of the short and longer-term effects of removal of the climbing fibre input to the Purkinje cell.

2. The effect of controlled, low frequency stimulation of climbing fibres on Purkinje cell activity.

3. The determination of the type and cellular distribution of Ca^{2+}-dependent K^+ channels in the Purkinje cells.

4. An examination of the effects of specific channel blockers for VOCCs and the relevant Ca^{2+}-dependent K^+ channels.

1.12 Organisation of thesis

The results will be subdivided into 4 experimental chapters and a brief introduction, a description of specific methods, and discussion of the data will accompany each chapter. Chapter 2 will describe the methods that are common across all experiments. The final chapters consists of a general discussion of the functional considerations of the present findings and suggestions for future experimental directions.

Chapter 2

General Methods

Some of the materials and methods were common for different experiments. These general methods will be presented below, and those specific to particular experiments will be described in the relevant chapters.

2.1 Animal Preparation and Surgery

The experiments were performed on adult Long-Evans male rats that weighed between 300 and 650 g. All animals were obtained from the Central Animal breeding facility of Monash University. All surgical and experimental procedures were approved and performed under the guidelines set by the local Monash University Animal Ethics Committee, and the National Health and Medical Research Council of Australia.

2.1.1 Anaesthesia

All animals were anaesthetised with an initial dose of sodium pentobarbitone (Nembutal, Rhône Mérieux, Vic., Australia) 60 mg/kg delivered intraperitoneally (i.p.). Anaesthesia was maintained by the inhalation of oxygen containing 0.5-2 % Halothane (Isoflurane, Rhône Mérieux, Vic., Australia). Anaesthesia was maintained at a level at which there was no flexor withdrawal reflex in response to vigorous pinching of the skin between the toes.

2.1.2 Preparation and Surgery

After induction of anaesthesia, a cannula was inserted in the trachea to provide a clear airway and for the delivery of gaseous anaesthesia. A dose of 6 µg Atropine (Astra Pharmaceuticals, N.S.W., Australia) (i.p.) was given to ensure a clear air passage. The back of the rats head was shaved, then placed securely in a Narashige stereotaxic frame and held in place by atraumatic ear bars in the external ear canals in preparation for the craniotomy to expose the cerebellum and brainstem. Body temperature was monitored by a rectal probe and maintained at 37 °C with the aid of a homoeothermic blanket.

2.1.3 Craniotomy

A midline incision was made in the scalp, and the skin removed from the underlying connective tissue. The overlying neck muscles were bluntly dissected and reflected to expose an area extending from the foramen magnum to the bregma suture. Bleeding was controlled by the use of gelatin foam soaked in a solution of Thrombin (Thrombostat, Parke Davis, N.S.W., Australia) in 0.9 % saline. The intraparietal bone was removed under a dissection microscope with a high-speed dental drill and fine rongeurs to expose the underlying cerebellum extending as far laterally on both sides to the paramedian lobule. Exposed tissue was kept moist with 0.9 % saline during surgery. The overlying dura of the cerebellum was carefully removed with fine forceps. Paraffin oil or silicon liquid (Dow Corning, U.S.A.) was used during surface recordings to prevent tissue from drying. During microelectrode recordings, exposed tissue was covered with a 2-3 mm layer of agarose gel (1.5 % in 0.9 % saline; Sigma, St Louis, U.S.A.) to prevent dehydration and to minimise brain pulsations. Agarose was used rather than a traditional agar gel as it had the advantage of providing a clearer and firmer gel and it remained transparent at the concentration used. This enabled the surface of the cerebellum to be seen clearly through the dissecting microscope and the electrodes could be positioned to avoid blood vessels. Clear visibility was also advantageous in positioning the parallel fibre stimulating electrodes on the cerebellar surface.

2.2 Electrodes

Glass-insulated tungsten electrodes were used for both extracellular recording and stimulating. These were constructed using a variant of the method of Merrill and Ainsworth (1972). Briefly, 8 cm lengths of 200 µm diameter tungsten wire (GTE 926117, Sylvania, PA, U.S.A.) were electrolytically etched to a long fine taper. A second, sharp taper was then made at the tip of the electrode where the diameter was approximately 5 µm. This produced a pencil-point like tip, which improved mechanical stability. The electrodes were then coated with a thin layer of borosilicate glass. The amount of exposed tip required was removed by inserting the electrode tip into a bead of molten, low melting point glass. The bead was allowed to cool, whereupon the borosilicate glass broke from the electrode at the junction of the bead.

The effort to be in face is further called 'face work', i.e. "to designate the actions taken by a person to make whatever he is doing consistent with face" which "often become habitual and standardised practices" (1969:9) and may further be established within a certain social group. Goffman further mentions two basic kinds of face work, i.e. the 'avoidance process' and the 'corrective process' (to be discussed in Section 2.2.3.1)

Brown and Levinson's (1987) study of 'face' is based on that of Goffman (1969[1967]). First, they extend the concept of face (to be discussed in this section); and second they extend the strategies of politeness (to be discussed in Section 2.2.3.1). They explain the notion of face through two dimensions of an individual's face. A person is claimed to have a 'double face', a negative and a positive one. The negative face includes "the basic claim to territories, personal preserves, rights to non-distraction – i.e. to freedom of action and freedom from imposition" (1987:61). This face derives from one's desire to be on one's own, unintruded upon by others. In contrast, the positive face consists of "the positive consistent self-image or 'personality' (crucially including the desire that this self-image be appreciated and approved of) claimed by interactants" (1987:61). This second face reflects the characteristic of people as social beings. In this case, there is a desire that one's presence, ability, and capacity are to be acknowledged and further accepted or appreciated by others. Furthermore, these negative and positive faces reflect the 'face wants' of an individual.

2.2.1.2.2 Braun (1988); Altmann & Riška (1966)

Braun (1988) looks at politeness from a different perspective. With special focus on the use of address forms, Braun identifies two bases on which politeness is defined, and thus two definitions of politeness. First, the meaning of politeness is based on the notions of appropriateness and adequacy of use. In this case, forms of address are basically "called and considered polite when they are adequate for the situation. Thus a form of address which is appropriate to the relationship of speaker and addressee, and which is in accord with the rules of the community, or at least those of the dyad, will always be regarded as adequately polite" (1988:49). A violation of this concern will potentially lead to impoliteness. For example, the V pronoun may be considered aggressive or insulting when used in a situation where the T pronoun is expected. This relates to the ideological nature

of politeness, in that it is the knowledge shared by a society that will determine the conventions of politeness to be applied to the people concerned.

While the first definition refers to context of situation (upon which appropriateness is defined) as its dominant factor, the second base for determining politeness is more 'pure' and internal, i.e. before context is taken into account. In this case, the degree of politeness of a form is measured according to its place within the hierarchical scale of politeness. "Within a system, different variants can be ascribed different degrees of politeness according to their use (to superiors or inferiors, to distant or intimate addressees)" (1988:50). Within this definition, T is less polite than V or any other more respectful forms of address.

Braun realises that politeness is a difficult concept to define. For instance, there are politeness forms that can be used impolitely. This is true for most languages – perhaps all. For example, the Portuguese *você* (V) or indirect address with *o senhor/a senhora* 'Mr/Mrs, sir/madam' used among people who normally use T may convey irony, scorn, or disdain. Second, 'non-polite' forms can also be used to convey politeness. For example, a German speaker would not be considered impolite when addressing parents, uncles, aunts, or parents-in-law with *du,* because this is normal today. Realising this dilemma in defining politeness, especially as far as contradiction and ambiguity are concerned, Braun suggests the restriction of the term politeness to the notions of appropriateness and adequacy of use (the first definition). She further suggests the invention of another term for the internal, non-contextual notion of politeness (the second definition above), although she did not suggest a term for the latter. Braun's account implies that definition of politeness based on appropriateness in relation to context is primary, and thus implies the primacy of context in distinguishing what is polite from what is not.

The concern for appropriateness or observance to rule is also raised by Altmann and Riška (1966), who explain this phenomenon in terms of observance of a 'courtesy relation'. In their formulation, "The courtesy relation R, i.e. one which is mediated by the elements of L, is mostly understood as an expression of the relation of S to H (or to an absent person A) where S evaluates H higher than himself" (*sic*, 1966:4), in which L is defined as "the set of means (signs) which are potentially able to express any relations of linguistic communication" (1966:2). This clearly relates to the relation of power as expressed by Brown and Gilman (1960). Altmann and Riška further mention that as an institution, courtesy has three basic members; these may be called 'grades', e.g.

'meliorative' (in which S evaluates H higher than himself, and thus expressing politeness), 'equative' (in which S evaluates H equal to himself, thus expressing equality), and 'pejorative' (in which S evaluates H lower than himself, thus expressing impoliteness). Furthermore, Altmann & Riška identify sub-gradings of the courtesy relation, from most to least polite: R2 (absolute meliorative), R1 (relative meliorative), R0 (equative), R-1 (relative pejorative), and R-2 (absolute pejorative).

2.2.1.2.3 Allan and Burridge (1991)

Allan and Burridge (1991; see also Allan 2001) discuss euphemism and politeness by relating euphemism to the notion of face: "a euphemism is used as an alternative to a dispreferred expression, in order to avoid possible loss of face: either one's own face or, through giving offence, that of the audience, or of some third party" (1991:11). In fact, euphemism can be said to be one strategy of politeness (hence will be dealt with further in Section 2.2.3). This section discusses only the general notion of euphemism.

In general, euphemism and its opposite, dysphemism, are alternative ways of referring to an entity. A euphemistic or dysphemistic label for an entity is referentially identical to the expression that has been previously assigned to the entity. On the one hand, a euphemistic label is chosen to present an entity nicely, so as to make it sound more positive, and thus facilitating the maintenance of the face of the associated party. On the other hand, a dysphemistic label is chosen to attach a negative connotation to the entity referred to, threatening the face of the associated party.

Basically euphemism is a reaction to taboo (Salomon 1966; Allan and Burridge 1991). Allan and Burridge's discussion indicates three levels of euphemism. The first is euphemism based on taboo associated with physical or metaphysical harm. It is based on the (usually traditional) belief that if the euphemistic expressions are ignored, something dangerous will happen. For example, in several communities certain wild animals are called with terms different from the common nouns originally assigned to them. This is done in fear of physical harm that might be caused by direct reference (which can be assumed as impolite behaviour) to them. In some Sundanese regions, for example, a *maung* 'tiger' may euphemistically be called *urang leuweung* 'jungle dweller' or *karuhun* 'ancestor' (Prawirasumantri 1969). The second type of euphemisms is related to 'social taboo', which is associated with efforts to preserve social harmony: to avoid loss of face.

This kind of taboo, in English-speaking societies, "stretch from those on body effluvia, reproductive processes, and the associated body parts ... to such matters of taste as (not) talking about personal income, which are rarer" (1991:12). For example, *faeces* is used instead of *shit*. The third type of euphemism is "euphemisms without real taboo counterparts" (Allan and Burridge 1991:12). This type of euphemism is used to create a positive connotation (thus avoid negative connotation) about a particular entity in a certain context. For example, *sanitation engineer* sounds more prestigious than *garbage collector*.

Finally, euphemism, especially the second type, can be placed within the framework of politeness. In this case, euphemism is used when the speaker wishes to avoid loss of face of one or more of the participants in or referents of the speech event. This second type of euphemism is one of the major concerns of the present study. On the other hand, dysphemism is beyond the concern of the present study because the investigation focuses on unmarked behaviour (i.e. what is normally performed daily in a neutral state of emotion, conforming to the existing rules shared by the participants in an interaction).

2.2.1.3 Summary: the connections

Although the scholars mentioned above look at the phenomenon of politeness from different points of view, there is a common thread that connects politeness and the phenomena of power and distance. Concerns for politeness – efforts to save face (Goffman, Brown & Levinson), to observe the social rules or preserve courtesy (Braun; Altmann & Riška), or to be euphemistic (Allan & Burridge) – can be seen as efforts to comply with what are assumed to be proper behaviours. Such behaviours seem to be a reaction to and recognition of the existing power relations and social distance among the participants. To put it another way, the power relation and social distance among participants serve as the base for polite behaviour.

The above notions of power, social distance, and politeness are applicable to the sociolinguistic notion of reference. Reference is a communicative phenomenon whose implementation is governed by the rule of politeness, which stems from the notions of power and social distance. To be polite in referring means to choose the right form of reference as an effort to observe the power relations and social distance assumed to exist among the participants. Some examples have been cited, especially from the work of

Brown and Gilman (1960) on the use of terms of address. The discussion of the use of reference to a third person is dealt with in Section 2.3.

In addition to behaviours that observe ideology, there is another set of behaviours that may be classified as 'non-compliant' behaviours. These are behaviours that violate the harmony prescribed by the existing power relations and social distance. The interpretation of such behaviours, then, will have to "be sought in some attitude or emotion of the speaker" (Brown and Gilman 1960:273) or "contemporaneous feeling or attitude ... in response to a mood" (Brown and Gilman 1960:274). For example, to address a superior with T when the context prescribes the opposite is to create something out of the ordinary, such as an insult, which is manifestly dysphemistic and marked. This kind of behaviour is beyond the concern of the present study, which focuses on the unmarked use of terms of reference. However, the awareness of this non-compliant dimension is necessary when analysing the data.

2.2.2 *Social factors*

As mentioned earlier, the sociolinguistic account of reference in the present study is traceable to the notions of politeness, power, and distance. First, politeness seems to be based on the notions of power and distance. Furthermore, a question arises regarding the factors on which power and distance are based. In Brown and Gilman's (1960:255) words, these social factors serve as the 'bases of power'.

Scholars differ slightly in identifying these social factors, depending on the contexts within which their research was carried out. For example, Brown and Gilman, in investigating terms of address in some Indo-European languages, mention some: "physical strength, wealth, age, sex, institutionalised role in the church, the state, the army, or within the family" (1960:255). Das, in investigating forms of address and terms of RTTP in Bengali, mentions such factors as "religious, social and economic status of the participants in a linguistic activity. They are also influenced by certain social restrictions and religious taboos" (1968:19). Bates and Benigni (1975) chose to include three aspects in their investigation: age, gender, and social class. The identification of the dominant social factors appears to be different from society to society. The factors that are considered dominant in the Sundanese society will be discussed in Chapter 3.

2.2.3 *Strategies*

People realise their observance of the notions of power, distance and politeness through a variety of strategies, which can be non-linguistic (e.g. bow, nod) or linguistic (see Altmann and Riška 1966; Noorduyn 1963). The present study focuses on the linguistic strategies. In general the linguistic strategies are realised through indirectness, which can take a variety of forms. This notion of indirectness is regarded by Braun (1988) as a major, psychological explanation of why politeness expressions are used at all. In this case, to be polite means to be less direct or to employ less direct strategies. Regarding terms of address, for example, "the plural may be interpreted as an avoidance of direct address in the singular" (1988:54), as in the case of the plural second person pronoun *vous* used to address a singular second person. Following are the works of some scholars that deal with the strategies of politeness. These works are not specifically dealing with politeness in reference to a third person; however, some relevance can be noted.

2.2.3.1 Goffman (1969[1967]); Brown and Levinson (1987)

Goffman's 'face work', as mentioned in Section 2.2.1.2.1, consists of two types of strategy for keeping social harmony. First, people can use an 'avoidance strategy' to prevent any contact that is potentially face-threatening. This can be done by avoiding dangerous topics or changing the topic of conversation to one less threatening. Second, when things have gone wrong, and there is a need to handle its effects in order to bring the unpleasant event back into line, the second type of face work is implemented, i.e. the 'corrective strategy'. Typical actions will be in the forms of showing that the incident is not a very serious or important matter, showing that the offence was unintentional, or excusing it as only a joke. Apology and compensation for the injured are other forms of actions taken when the incident is more serious and does not seem to be able to be repaired through less effort.

Brown and Levinson (1987) further develop Goffman's (1969[1967]) work by providing a more detailed discussion of 'face-threatening acts' (FTAs) and strategies people use when confronted with face-threatening situations. They mention that there are acts that intrinsically have the potential to threaten both the positive and negative faces (see Section 2.2.1.2.1) due to their tendency to conflict with the face wants of the speaker and/or of the hearer. Brown and Levinson further propose four categories of FTAs, i.e. (i) those that threaten the hearer's negative face, such as *ordering, threatening,* and *warning;*

(ii) those that threaten the hearer's positive face, such as *complaining, criticising,* and *disagreeing;* (iii) those that threaten the speaker's negative face, such as *accepting an offer, accepting thanks,* and *promising unwillingly;* and (iv) those that threaten the speaker's positive face, such as *apologising* and *confessing*. Furthermore, they develop a taxonomy of strategies for dealing with these FTAs. This consists of five strategies (ranging from strategies for low-risk to high-risk situations): the bald on record, the positive politeness, the negative politeness strategies (all of which are categorised as on-record), the off-record strategy, and the complete avoidance strategy. These strategies are essentially a development of Goffman's avoidance strategy.

Although the above strategies are discussed in relation to the theory of speech acts, there are at least two connections that can be drawn between these strategies and the strategies of reference use. First, the notion of face-threatening situations also applies to the use of reference. Failure to choose the appropriate form of reference may create a face-threatening situation. Second, the notion of avoidance is also a concern of politeness in the use of reference. In this case, avoidance can be interpreted as the avoidance of an inappropriate reference term or, when necessary, complete avoidance of making any reference at all, in order to avoid creating face-threatening situations.

2.2.3.2 Altmann & Riška (1966)

According to Altmann and Riška (1966), the notion of politeness (or courtesy) can be realised in a variety of ways, i.e. non-linguistically, linguistically, or both. Altmann and Riška concentrate on the linguistic aspects of courtesy, and classify them into five elements: (i) 'graphic', such as the use of upper case initials for pronouns indicating the hearer; (ii) 'phonic', which includes intonation, tone of voice, rate of speech, clearness, articulation, and pauses; (iii) 'lexical', i.e. the use/choice of words, (iv) 'grammar', which refers to the use/choice of certain grammatical structures, and (v) 'stylistic', which includes various periphrases, euphemisms, or special ways of expression.

Altmann and Riška further mention that the first two categories are not comparable to the last three. The graphics are not as widely used as the others, and the phonic element, although its use is widespread, belongs to "the suprasegmental constituents of language; it may be said that they are a necessary but not a sufficient condition for the creation of R relation" (1966:4). Furthermore, each of the last three categories (lexical, grammatical, and

stylistic) are broken down into three subcategories. Subcategory (a) consists of 'improper courtesy elements', in which one linguistic means (lexical, grammatical, or stylistic) has several meanings, one of which (perhaps with some change) is dedicated for courtesy purposes. An example would be the word *honour* in *Your Honour*, which is a courtesy synonym of *you*. Subcategory (b) consists of 'courtesy equivalents', which consists of synonyms that differ only with regard to courtesy. For example, the Sundanese *dahar, teda,* and *tuang* all mean 'eat', but occupy different levels in the courtesy scale. They are ordered from least to most courteous (see discussion on 'speech levels' in Chapter 3). Subcategory (c) consists of 'proper (or pure) courtesy elements', which consists of linguistic means solely dedicated for courtesy purposes, such as the English *Mister* and *Sir*.

Put into Altmann and Riška's system, politeness strategy through reference appears to fall primarily within the lexical and grammatical categories. The lexical level is exemplified in the previous examples, i.e. the use of *Your Honour, Mister,* and *Sir*. In Sundanese, terms such as *raden, enden, aom, agan* are used to refer to people of nobility; however, they are rarely used today. The terms that are more commonly used these days are mostly kinship-based, such as the title *Bapa* 'father', which is roughly equivalent to the English 'Mr' or 'Sir' (see Chapter 5). The grammatical level is exemplified in the use of T/V pronouns (see Section 2.2.1).

2.2.3.3 Other works

Some other scholars have mentioned other strategies of politeness. Emphasising the indirectness strategy of politeness, Braun (1988) mentions that avoidance of direct use of pronouns of address can be done by (i) using another pronoun which has been grammatically shifted, (ii) using a nominal term of address, or (iii) avoiding completely the mention of the reference/address. Allan and Burridge (1991) mention a list of indirectness strategies through euphemism, as summarised in Table 2.1. How these strategies relate to the use of reference to a third person in Sundanese will be a concern of the present study.

Type of euphemism	Examples
Figurative imagery	*the cavalry's come* for 'I've got my period'
Circumlocution	*little girl's room* for 'toilet'
Abbreviation	*SOB* for 'son-of-a-bitch'
Omission	the use of *mhm* as in 'This is a little –h'm—isn't it?'
Synecdoche	*I've got a cough*, which ignores other unpleasant symptoms such as stuffed up nose, postnasal drip, and running eyes
Metonymy	*nether regions* for 'genitals'
Hyperbole	*flight to glory* for 'death'
Understatement	*sleep* for 'die'
Use of a learned term	*feces/faeces* for 'shit'
Use of a colloquial term	*period* for 'menstruation'
Use of a term borrowed from another language	*anus* (from Latin 'ring') for 'arsehole'

Table 2.1 Types and examples of euphemisms (after Allan and Burridge 1991:14-25)

2.2.3.4 Summary

The above discussions indicate that linguistic indirectness appears to be the major politeness strategy. Indirectness is realised through avoidance of mentioning certain linguistic forms, either by way of using other forms, or through complete avoidance of their mention. These strategies are employed in order to realise polite behaviours. Furthermore, these polite behaviours are, once again, a recognition of and reaction to the existing power relations and social distance among the participants of an interaction.

2.2.4 Concluding remarks

The sociolinguistic account of reference discussed in this section is a major concern of the present study. The philosophical and linguistic accounts discussed earlier will help identify the various forms of reference to a third person that exist in the Sundanese language and the denotative, non-social meaning of these forms. As a further step, the sociolinguistic account will examine the social significance of those forms of reference based on relevant social concerns. This will lead to the examination of the speaker's choice of a term from among the referentially possible forms as well as the social considerations underlying the choice. A further discussion of sociolinguistic concerns can be found in Chapter 4. While this and the previous sections have dealt with the notion of reference in general, the next section presents a discussion of reference to a third person.

2.3 Reference to a third person (RTTP)

While Sections 2.1 and 2.2 discussed the general accounts of reference, the present section focuses on reference to a third person (RTTP). It discusses the study of RTTP in relation to other studies of reference. Section 2.3.1 clarifies the terminology involved, which includes 'reference', 'address', and 'RTTP'. Section 2.3.2 briefly discusses where the study of RTTP is located within the broader study of reference. Finally, Section 2.3.3 briefly examines the major concerns that arise in the studies of reference, and how they are applicable to the study of RTTP.

2.3.1 Terminology: reference, address, RTTP

In the literature on person reference, the term 'reference' appears to be a cover term, involving the first, second, and third persons; however, a further distinction has been made. The first and third persons are normally labelled with the original term, 'reference'. However, since the second person is labelled 'addressee', research investigating reference to a second person is commonly described as the study of terms or forms of address (e.g. Das 1968; Braun 1988).

The use of the term 'reference' to deal specifically with the third person is imprecise because conceptually the term can be associated with the first, second, or third persons. It is for this reason that the present study employs a more specific, limiting term: 'reference to a third person' (RTTP).

2.3.2 The studies of reference

This section briefly deals with the major areas of reference that have been sociolinguistically studied, and how they are interrelated. These studies include terms of address, reference to the first person (self-reference), and RTTP. The term of address is discussed first because it has been studied more widely than the other two.

2.3.2.1 Terms of address

It appears that interest in investigating the sociolinguistic aspect of reference has generally focused on the second person. Within this field, regarded as the basis for most (if not all)

discussions is the work of Brown and Gilman (1960), which investigated pronouns of address within the framework of power-solidarity semantics (see Section 2.2.1.1). Undoubtedly, terms of address have been widely researched, and among the most comprehensive studies is that of Braun (1988). Other research normally focuses on a specific aspect of address terms in a language, e.g. Wittermans (1967) on terms of address in Indonesian; Ervin-Tripp (1972) on American address forms; Tyler (1972) on kinship terms in Koya; Casson (1975) on kinship terms in Turkish; Kramer (1975) on the relation between gender and the use of address terms; Östör (1982) on terms of address in Hungarian; Fang and Heng (1983) on address norms in Chinese; Hook (1984) on American first names and titles; Kempf (1985) on pronouns and terms of address in German; Keshavarz (1988) on address forms in Persian; Oyetade (1995) on address forms in Yoruba; and Dickey (1997) on the address system in ancient Greek.

It has been found that terms of address are realised by a variety of means. Braun (1988) linguistically classifies them into (i) pronouns and (ii) nouns of address. 'Nouns' include proper names; kinship terms; titles; occupational terms such as the English *waiter*; words for certain types of relationship such as Turkish *arkadas* 'friend'; terms of endearment; and forms which define addressees as father, brother, wife, or daughter of someone else by expressing the addressee's relation to another person, such as the Arabic *Abu Ali* 'father of Ali'. It has also been claimed that the use of terms of address is influenced by social notions of power, social distance, and politeness, which are in turn affected by social factors such as age, gender, and social status.

2.3.2.2 Terms of self-reference

Reference to self has not been as widely explored as address terms. Indeed it seems to be even less explored than RTTP. Ide (1979, 1982) is a scholar who has investigated this area of reference. Ide (1979) compared the use of first-person and second-person references by children in Japanese and American English. She discovered that Japanese children used a greater variety of reference forms than American children, and that gender played a greater role in reference choice in Japanese. This is understandable because in terms of pronouns of self-reference alone English only has *I/me*, while Japanese has several forms that are used differently by males and females (see Table 2.2). In the other study, Ide (1982) discussed the use of first-person reference as part of the larger discussion of politeness and

electrophysiological observations that climbing fibres branch and terminate in sagittal zones (Groenewegen & Voogd, 1977; Ekerot & Larson, 1979a; Groenewegen et al., 1979; Oscarsson, 1979; Ekerot & Larson, 1982; Trott & Apps, 1993; Atkins & Apps, 1997; Voogd & Glickstein, 1998; Jorntell et al., 2000).

Responses produced in the cerebellar cortex by direct stimulation of climbing fibres are known to produce large negative-positive or predominantly positive-going potentials with a constant latency and constant amplitude for a given stimulus strength (Armstrong & Harvey, 1968; Armstrong et al., 1973a; Ekerot & Larson, 1982). The latencies ranged between 3 and 4 ms, and remained constant from trial to trial. A typical example is shown in Figure 3.2A, where single pulses of 12.5 μA evoked a positive-going surface potential that had a constant latency of 4 ms and amplitude of 0.3 mV. The constant latency and consistency of a response at a given stimulus strength was found to be an important criteria for localising the stimulating electrode close to the parent inferior olive cell or axon. Sometimes, climbing fibre responses were evoked by inferior olive stimulation with a fluctuating latency. In such cases, it was not possible to remove the climbing fibre input with a minimal amount of Xylocaine or the passage of 15 μA direct current for a few seconds. Presumably in such cases, the inferior olive cells were activated by stimulation of afferent fibres some distance away.

Extracellular recordings from individual Purkinje cells were then made in the area of cerebellar cortex determined to respond strongly to climbing fibre activation. Identification of Purkinje cells was based on their orthodromic activation on stimulation of the inferior olive to produce the characteristic all-or-nothing complex spike discharge. Figure 3.2B shows an all-or-nothing complex spike response at threshold strength for climbing fibre activation. The evoked complex spike had a constant latency of approximately 3 ms and consisted of 3-6 action potentials. Recordings were made from 24 Purkinje cells where complex spikes could be generated by weak stimulation of the inferior olive. Seventeen of the 24 cells were denervated over a period of minutes, while the remaining 7 cells were examined for periods ranging from ninety minutes, and up to five hours in one instance.

3.3.2 Temporary removal of climbing fibres

The effects of a single dose of Xylocaine lasted from 5-10 minutes and sustained suppression of the climbing fibre input could be achieved by repeat injection. Following removal of the climbing fibre input, simple spike activity started to rise above its resting

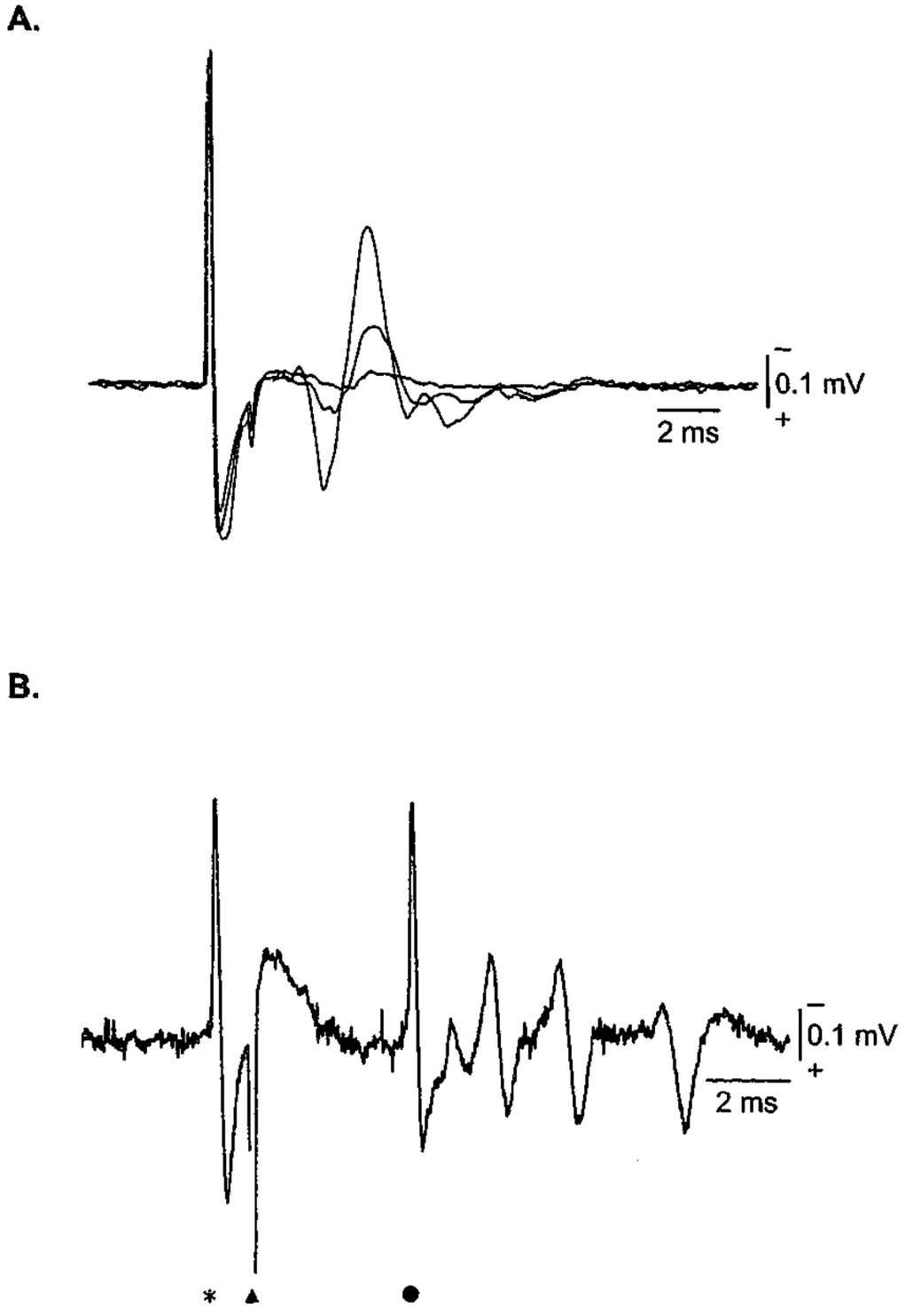


Figure 3.2

Identification of responses mediated by the stimulation of the climbing fibres via the inferior olive. A. Superimposed records of evoked responses recorded on the surface of the cerebellar cortex. Climbing fibre field was recorded below, just above, and at 2x threshold. Threshold was 12.5 mA. B. Example of an evoked complex spike generated by inferior olive stimulation. Complex spike is marked by a dot at the initial response. A spontaneously occurring simple spike can be seen prior to the complex spike. A spontaneously occurring simple spike is marked by the asterisk. Negativity in this and all following traces in this thesis is signalled by an upward deflection. The filled triangle indicates the stimulus artefact.

level within seconds of the disappearance of the spontaneous complex spikes (Figure 3.3A). The simple spike activity quickly rose to a steady level of discharge, where it remained for the duration of climbing fibre removal. This rapid rise in simple spike firing is especially evident when extracellular spike train recordings of Purkinje cell activity are examined before and after the removal of its climbing fibre input (Figure 3.4Ai-Aii. and Bi-iii.). Figure 3.4Ai-ii displays a period of control activity, where the complex spikes were found to have a firing rate of 1.3 Hz, and the resting firing rate of the simple spikes was 65 Hz. After the climbing fibre input was removed, simple spike rates progressively rose, becoming more regular and attaining a rate of approximately 100 Hz just one minute after climbing fibre denervation (Figure 3.4Bi-iii.). This remarkable increase in simple spike activity accompanying the disappearance of the complex spikes was found to occur in all 17 Purkinje cells. These cells were sampled from different areas of cortex and the response was constant among cells in different lobules or medial-lateral positions in the cerebellum. Climbing fibre removal significantly increased the mean simple spike activity of Purkinje cells from 27.59 ± 5.34 Hz to 55.75 ± 7.02 Hz ($n=17$, $p<0.05$) within one minute of climbing fibre denervation. Once climbing fibre activity resumed, as indicated by the reappearance of spontaneous complex spikes (Figure 3.3A and 3.4Ci.), simple spike activity started to decline to its initial control value. In contrast to the dramatic rise in simple spike firing rate that occurred upon climbing fibre denervation, the return to control levels after resumption of complex spike activity took somewhat longer, usually two to three minutes (Figure 3.3A and 3.4Ci-ii.). The return of complex spike firing rate to its control level mirrored that of the simple spikes, in that some time was required before firing rates were equal to pre-denervation levels. The form of the complex spike was also altered in the initial stages of its return. When compared to control (Figure 3.4Ai-ii. and 3.4Cii-iii.), complex spike form initially consisted of a large initial spike, with two or more wavelets or stunted spikes (Figure 3.4Ci.). Two to three minutes after its first reappearance, complex spike form was restored to its previous profile of a fairly large initial spike and distinct, well formed secondary spikes (Figure 3.4Cii-iii.).

3.3.3 Longer-term removal of climbing fibres

A further 7 cells were examined to investigate the effects of climbing fibre removal over longer periods. Long-term denervation was achieved by infusion of repeat doses of Xylocaine or by an electrolytic microlesion in the inferior olive. As with temporary denervation, mean simple spike firing rate increased significantly from control values after climbing fibre removal in the minutes after removal (control: 55.13 ± 9.23 Hz and

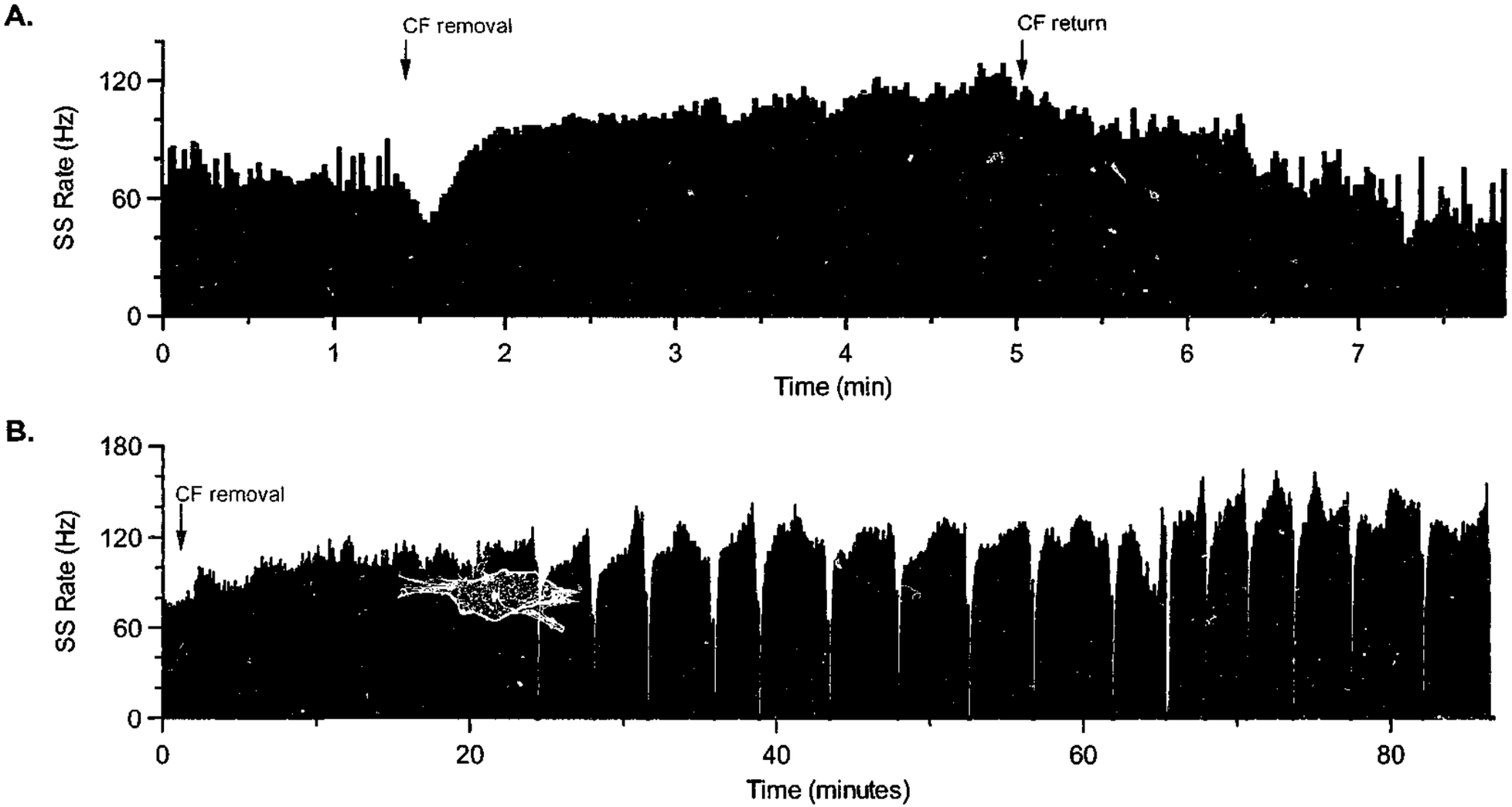


Figure 3.3

The effects of climbing fibre removal on Purkinje cell simple spike activity. A. Short-term climbing fibre removal. B. Long-term climbing fibre removal. Climbing fibre removal was achieved with 5 and 10 % Xylocaine respectively.

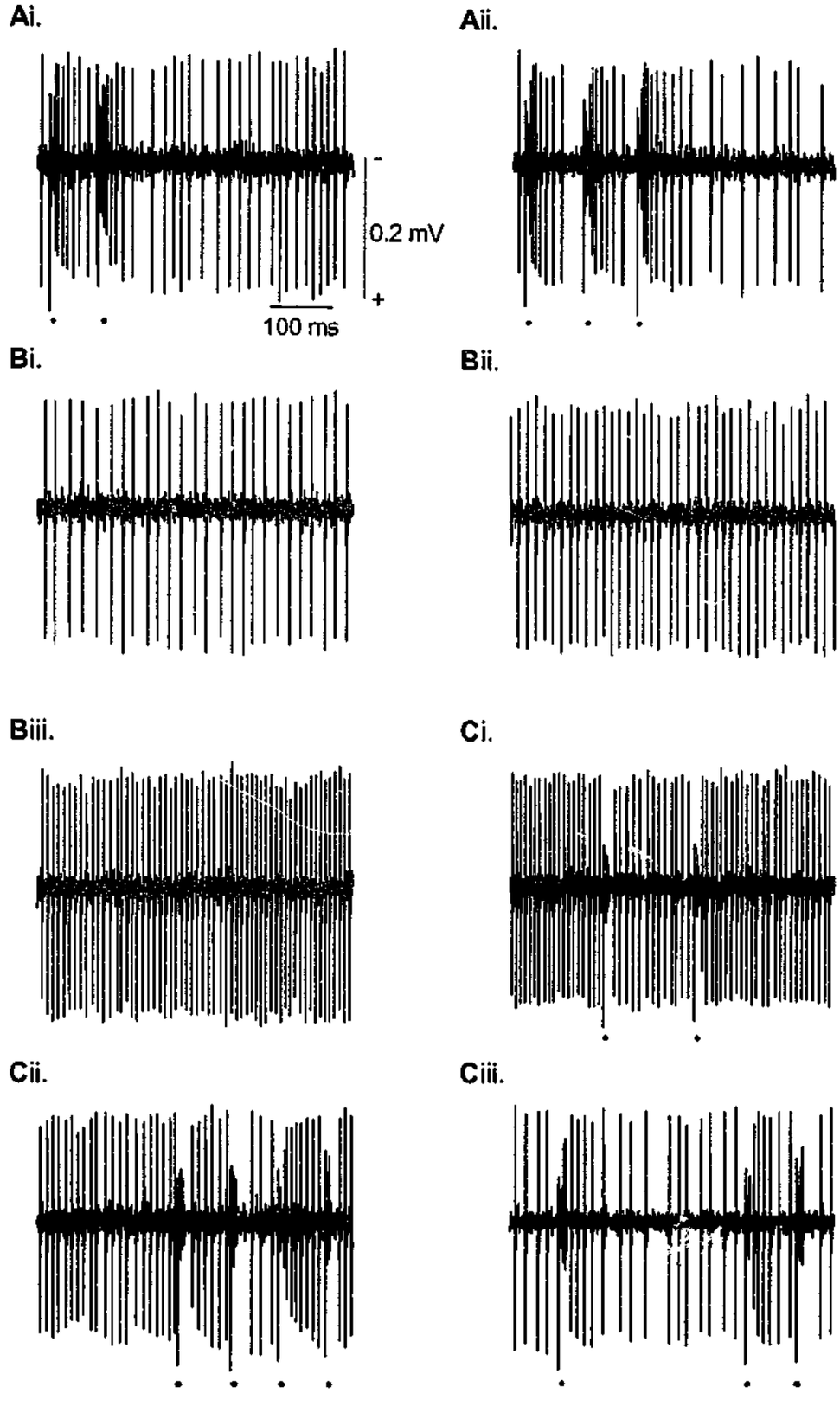


Figure 3.4

Recordings of Purkinje cell activity denervated with 5 % Xylocaine. Ai-ii. Examples of control activity, demonstrating the occurrence of both simple and complex spikes. Bi-iii. Purkinje cell activity 1, 2 and 3 minutes after its climbing fibre has been removed. Note the disappearance of complex spikes, and the increased firing frequency of the simple spikes. Ci-iii. Records of Purkinje cell activity post-climbing fibre denervation, at 1, 2 and 3 minutes after the first reappearance of the complex spikes. The simple spike rate progressively decreased with increasing complex spike discharge. The dots indicate the initial spike in the complex spike.

denervation 96.24 ± 12.71 Hz, $p<0.05$, $n=7$). However, Purkinje cell simple spike activity continued to progressively increase over a period of 25-75 minutes, attaining frequencies up to 150 Hz (Figure 3.3B). The cell then reached a point where its discharge pattern became unstable, and ceased firing for approximately 20 seconds, after which the cell commenced firing once more. The cell then started to oscillate between periods of total silence and bursts of rising discharge. After a period of 1.5-3 hours, the firing pattern of the Purkinje cell attained a strikingly regular period, exhibiting an oscillatory pattern of discharge of alternating periods of bursts of rapidly rising discharge with periods of quiescence lasting 15-20 seconds (Figure 3.5). This oscillatory discharge was seen to persist for up to five hours after climbing fibre removal.

3.3.4 ISI of denervated Purkinje cells

The firing patterns of Purkinje cells were irregular under normal control conditions, a fact reflected in the ISI histograms (Figure 3.6Ai, Bi.). The ISI distribution displayed a long tail for normal Purkinje cells. When the climbing fibre input to the cells was removed, the firing patterns of both short and long-term denervated cells became strikingly regular as demonstrated by the ISI histogram shift to a more narrow distribution (Figure 3.6Aii., Bii.), with cells denervated for long periods displaying an ISI distribution that was Gaussian in shape, with a single narrow peak. Presumably, the greater shift that occurred in the ISI distribution of cells denervated for long periods compared to those that were denervated temporarily, is a result of the longer time frame these Purkinje cells had to develop simple spike firing frequencies of greater regularity.

3.3.5 Restoration of long term denervated Purkinje cells

In one experiment where the climbing fibre input to a Purkinje cell was removed electrolytically with a DC lesion, it was possible to reactivate the climbing fibre after a long period of denervation (Figure 3.7).

Although lesioning the climbing fibre resulted in the removal of spontaneous complex activity, full degeneration of the climbing fibre requires some time (Sotelo et al., 1975; Desclin, 1976; Colin et al., 1980; Llinas & Sugimori, 1980a) and it is possible to stimulate the climbing fibre axons for up to 18 hours before the first sign of terminal degeneration.

As with all Purkinje cells, simple spike activity quickly increased from a control discharge rate of 70.23 ± 0.4 Hz to 130 Hz over a period of 40 minutes, after which the cell entered its

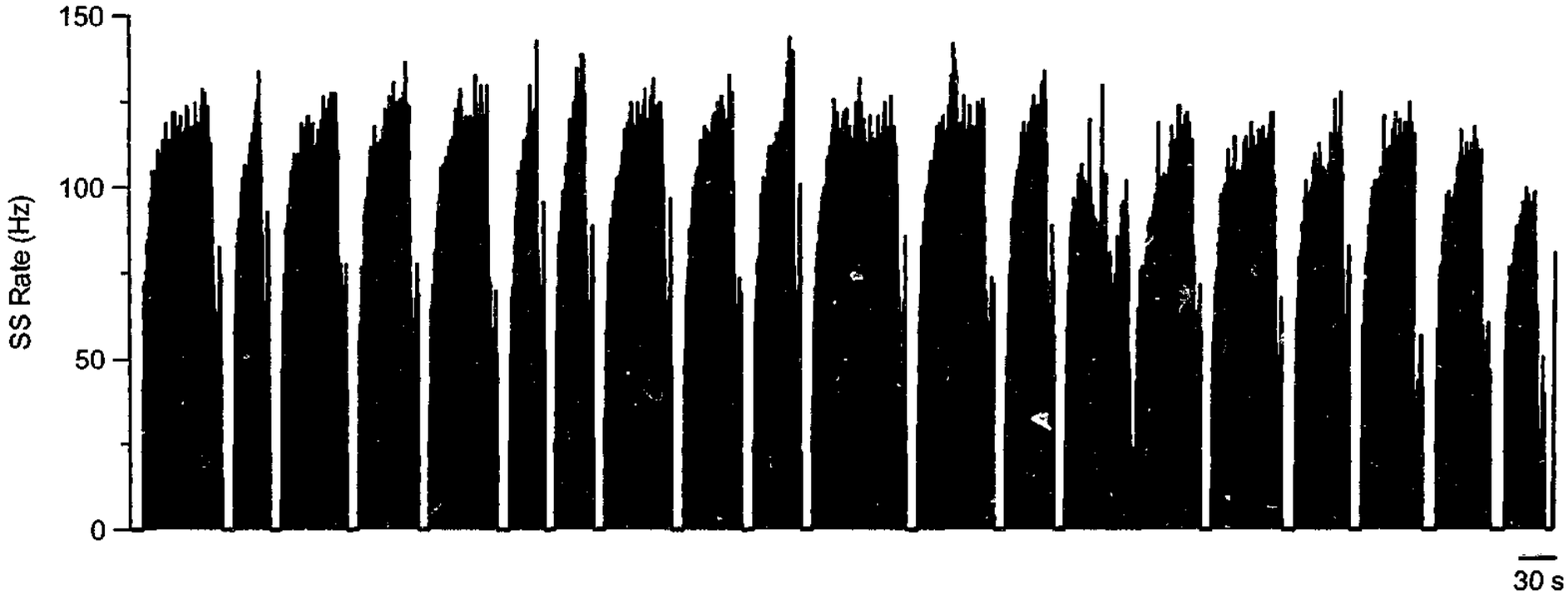


Figure 3.5

An example of an oscillating Purkinje cell which has been denervated for 2 hours. The cell has entered its oscillatory mode, with activity fluctuating between periods of intense firing and quiescence.

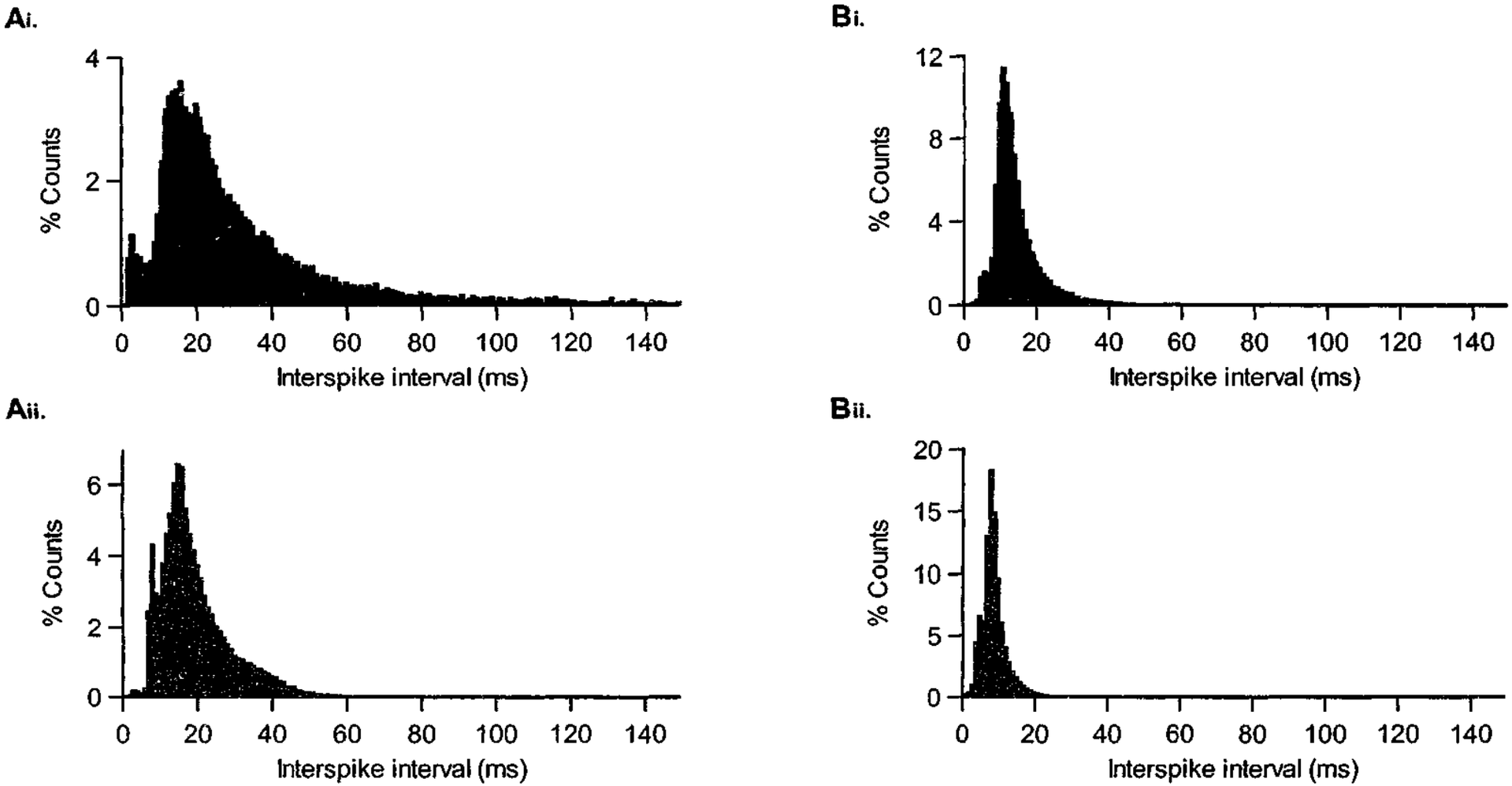


Figure 3.6

Interspike interval (ISI) histograms of short and long-term denervated Purkinje cells. **Ai.** & **Aii.** ISI histograms of all short-term dennervated Purkinje cells (n=17) **Ai.** ISI histogram of simple spike activity prior to removal of climbing fibre input. **Aii.** When the climbing fibre was removed, the firing pattern of the Purkinje cell became more regular, reflected in the narrow symmetric distribution of the ISI. **Bi.** & **Bii.** ISI histograms of all long-term denervated Purkinje cells (n=7) **Bi.** Pre-climbing fibre removal, and **Bii.** Post-climbing fibre removal. Note the shift in ISI distribution after climbing fibre removal.

oscillating mode, cycling between periods of discharge cessation and bursts of rising discharge attaining rates of up to 150 Hz (Figure 3.7A). This phenomenon was consistent with all long-term denervated Purkinje cells. After a denervation period of nearly 2 hours, climbing fibre activity was restored by electrical stimulation of the axons in the inferior olive. A climbing fibre stimulation rate of only 1 Hz resulted in an impressive return of simple spike firing rate to is pre-denervation level (Figure 3.7A and B). When the ISI histograms for this cell were examined, the distribution for normal control conditions and climbing fibre denervation resembled that of all other Purkinje cells, shifting from a histogram with long tailed appearance, to a narrower symmetrical distribution of ISIs during climbing fibre removal (Figure 3.8A and B). After climbing fibre input had been restored, producing a decrease in simple spike firing frequency to that of pre-denervation levels, the resulting ISI histogram also returned to a distribution matching that of control conditions (Figure 3.8C), demonstrating that spontaneous simple spike activity is very markedly under the control of climbing fibre activity, and that even after fairly long periods of climbing fibre removal, resumed climbing fibre activity is capable of restoring Purkinje cell simple spike activity to normal levels.

3.3.6 Confirming the intrinsic source of simple spikes- the effects of CNQX on tonic simple spike activity

The results above strongly support the view that the striking and sustained increase in Purkinje cell simple spike activity that follows the loss of climbing fibre input is due to loss of inhibition over an intrinsic spike generating current that is controlled by ongoing climbing fibre activity. Mossy fibres are also known to affect the simple spike rate of Purkinje cells along the mossy fibre-granule cell pathway (Eccles et al., 1966d). Fast excitatory synaptic transmission at parallel fibre-Purkinje cell synapses is via the AMPA class of glutamate receptors (Renard et al., 1995). Therefore, to confirm that Purkinje cells generate simple spikes intrinsically, the effects of 6-cyano-7-nitroquinoxaline-2, 3-dione disodium salt, or CNQX, a potent selective antagonist of the ionotropic glutamate AMPA/kainate receptor was infused around the Purkinje cell. This was achieved by replacing the recording microelectrode in the cerebellar cortex with a dual micropipette-microelectrode which contained CNQX (Tocris Cookson, U.K.). As mentioned in the General Methods section, a layer of 1.5 % agarose gel was placed over the exposed cerebellum to minimise pulsations. The dual electrode system however, could not be inserted through the agarose as this would have blocked the tip of the micropipette. Therefore, a selected patch of cortex was left free of agarose for insertion of the

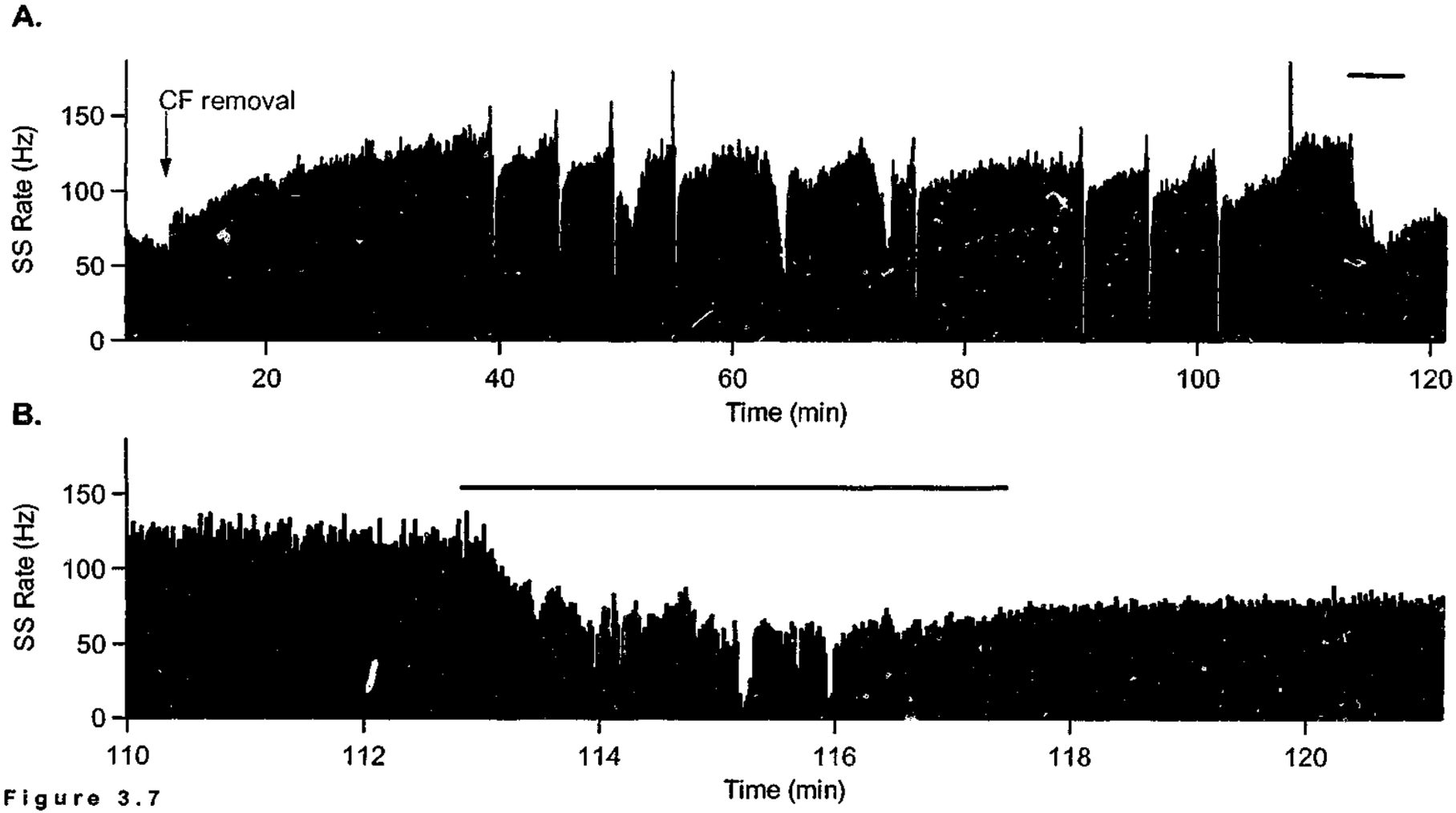


Figure 3.7

A. Effect of long-term removal on Purkinje cell simple spike activity, followed by a period of climbing fibre stimulation. Removal of climbing fibre was achieved with 10 % Xylocaine. After a 100 minute denervation period, the climbing was stimulated electrically at 1 Hz. Period of stimulation is indicated by the length of the blue bar. Stimulation of the climbing fibre resulted in a return of simple spike activity towards its pre-denervated rate. **B.** Expanded time base of the climbing fibre stimulation period.

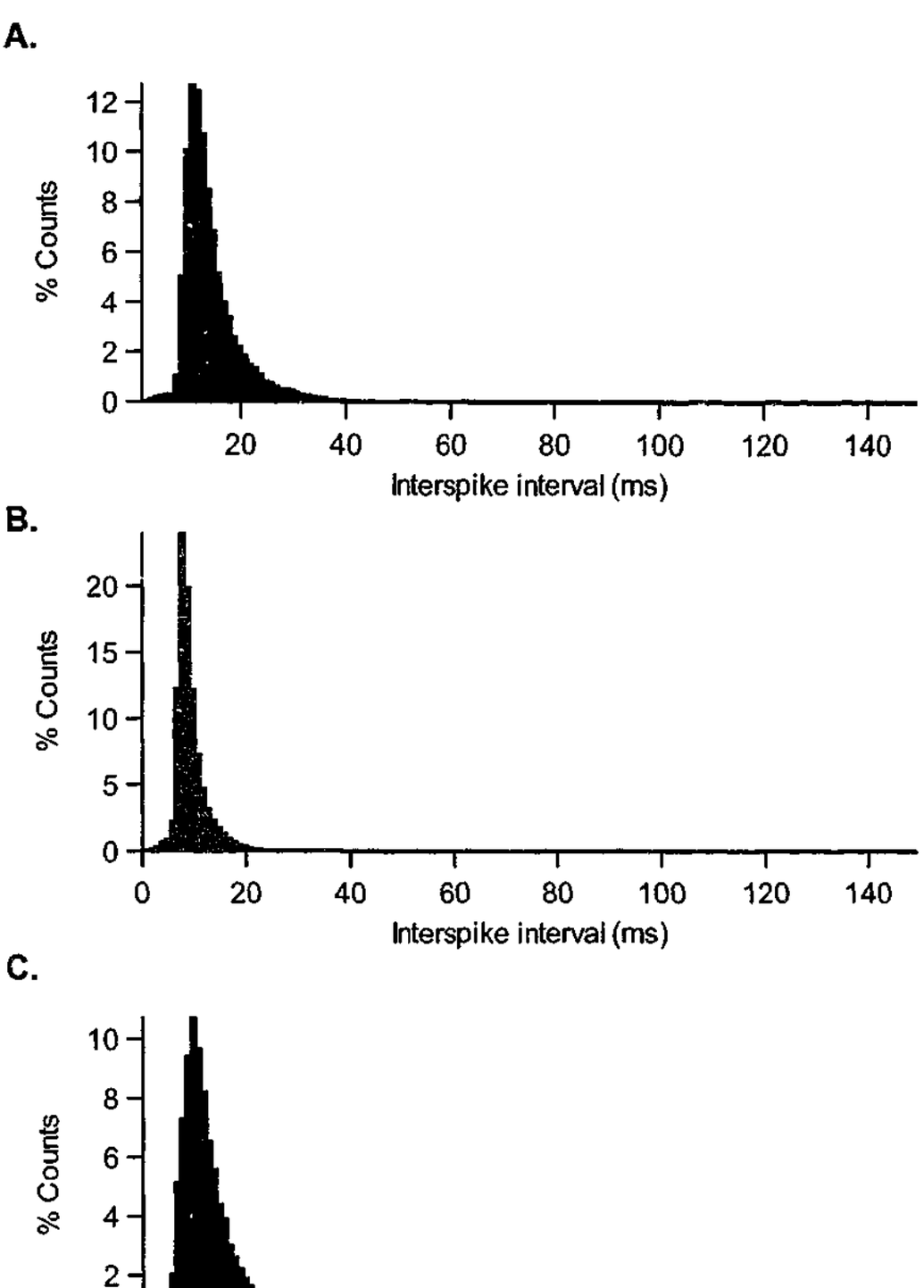


Figure 3.8

ISI histogram of a Purkinje cell whose climbing fibre has been removed, then stimulated after a 100 minute denervation period (See Figure 3.7). **A.** ISI histogram of the control period. **B.** ISI histogram of period of denervation **C.** ISI histogram after climbing fibre stimulation restored simple spike firing frequency to control levels.

micropipette and attached electrode. To do this, after removal of the dura, a small piece of gel foam around 0.5 mm in length and soaked in saline was placed over a selected area of cortex that was free of large blood vessels. A micromanipulator was then used to lower a glass rod of about 1 mm in diameter so that it just touched the gel foam. Agarose was then applied to the cortex and the glass rod and gel foam were removed after it had set. This produced an agarose free area of cortex through which penetrations were made, and the surrounding agarose still provided good overall mechanical stability. For repeat penetrations in subsequent areas, the entire layer of agarose was removed, and the above procedure was repeated.

Block of parallel fibre transmission at the parallel fibre-Purkinje cell synapse was achieved by infusing 50 μM CNQX around the Purkinje cell. CNQX was dissolved in distilled water and spun down with a centrifuge. The resulting supernatant was used in these experiments, as this did not crystallize and block the micropipettes. Additionally, the CNQX supernatant formed a dark brown colour, which made it easy to observe the extent of the CNQX infusion. This was ideal for ensuring that the amount of CNQX infused slowly over a period of 2-5 minutes, covered an area of around 1 mm, so that all the AMPA receptors on the entire dendritic tree of the Purkinje cell in question were blocked. CNQX was pressure-ejected around the Purkinje cell from the micropipette, which was attached to a Picospritzer. Parallel fibres were stimulated with a glass insulated tungsten microelectrode, placed in the cerebellar cortex 0.5-1.0 mm away and in the long axis of the folium of the Purkinje cell under study. Parallel fibre activation of a Purkinje cell was indicated by an evoked simple spike response. Thresholds were in the order of 3-5 μA. After ejection of CNQX, stimuli of up to 40 μA were tested to ensure that all parallel fibre input was blocked. Responses were continuously monitored with single stimuli delivered at 3.5 second intervals to check the effectiveness of the block on parallel fibre transmission. In order to make a reliable check on the effectiveness of the blockage of an evoked simple spike, the stimuli were applied at a fixed time after the occurrence of a spontaneous simple spike (See Figure 3.10) rather than in random relation to the background spikes. This eliminated the possibility that blockages of single spikes could be due to stimuli falling within the refractory period of the cell. In a final set of experiments, the combined effect of blocking parallel fibre transmission with climbing fibre denervation was investigated. The experimental setup for these series of experiments is shown in Figure 3.9.

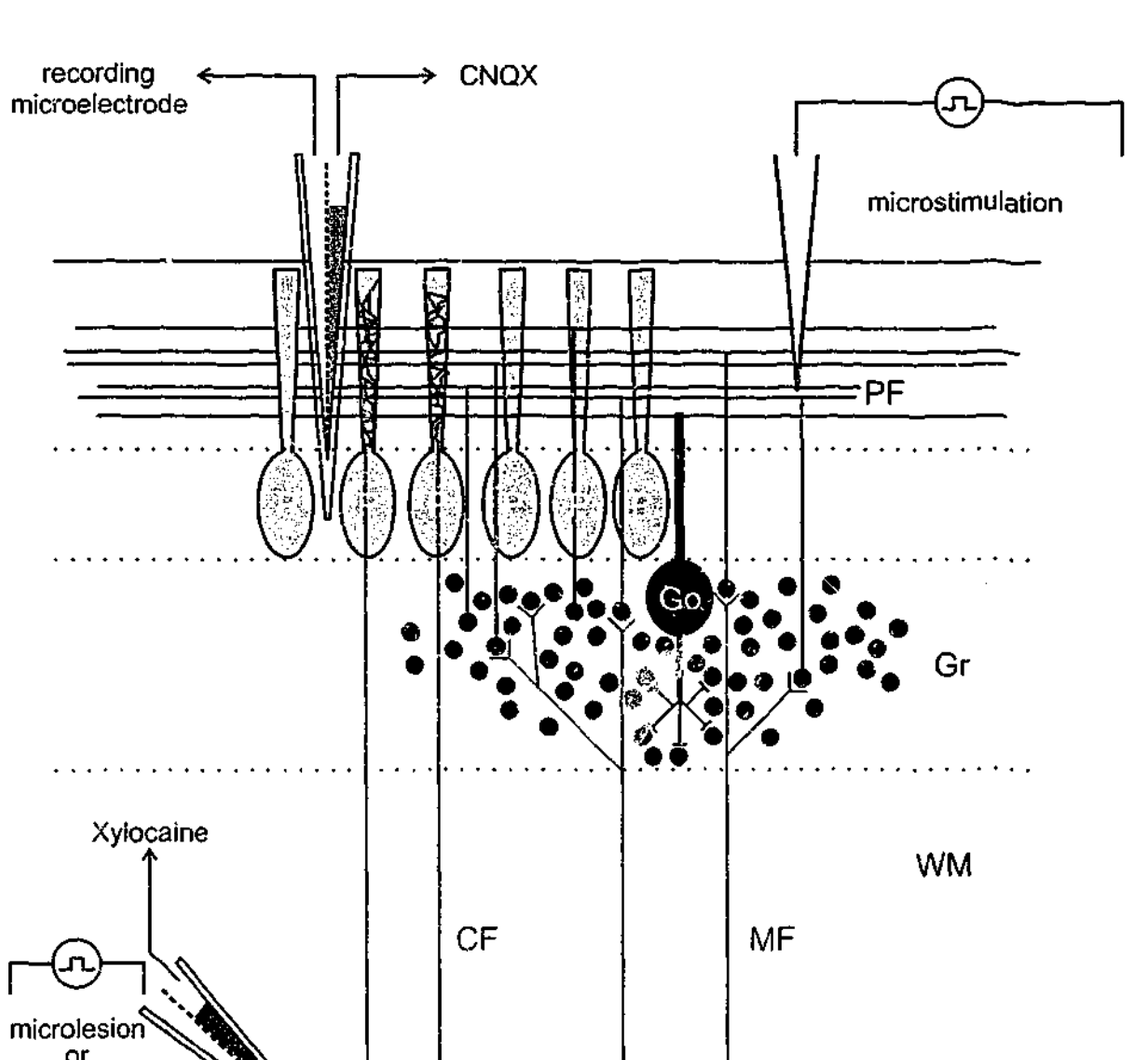


Figure 3.9

Diagram of experimental arrangement for the study of CNQX infusion on Purkinje cell simple spike activity, and the effect of CNQX infusion with climbing fibre removal. Two dual microelectrode-micropipettes were used, one inserted into the inferior olive for the stimulation and removal of the climbing fibres, and the other placed in the cerebellar cortex to record Purkinje cell responses to CNQX infusion, and climbing fibre removal. A glass-insulated tungsten microelectrode was used to activate parallel fibres in the molecular layer.

Application of CNQX totally blocked the simple spike responses to parallel fibre stimulation (Figure 3.10A-D), but had little or no effect on the resting simple spike rate of most of the Purkinje cells investigated (Figure 3.11A-B). Some Purkinje cells demonstrated a small decrease in simple spike rate upon infusion of CNQX (Figure 3.11C), and in a few other cells, simple spike discharge decreased substantially (Figure 3.11D). Tonic simple spike discharge however was not totally abolished in any of the Purkinje cells and all showed a rate of at least 20 Hz after CNQX application. Overall however, the simple spike resting firing rate of Purkinje cells remained unchanged (46.04 ± 6.2 Hz to 40.65 ± 6.26 Hz n=18, $p>0.05$, Figure 3.11E). The variability of simple spike rates after CNQX application may reflect the degree to which Purkinje cells receive input from ongoing activity in the granule cells. Clearly however, a very substantial portion of the activity in most cells was generated intrinsically and in most, any ongoing parallel fibre input did not contribute to the resting activity.

In the final series of experiments, it was examined whether CNQX could alter the responses of Purkinje cells which had their climbing fibre input removed. Infusion of CNQX did not alter the rise in simple spike discharge, nor did it alter discharge when the cell had entered oscillatory mode (data not shown, n=2). These findings demonstrate that Purkinje cells generate simple spikes intrinsically, and appear to confirm that the increase in simple spike rate that occurs after climbing fibre removal is due to a loss of inhibition over an intrinsic spike generator.

3.4 Discussion

The excitatory action of climbing fibres is extremely powerful and is fundamental for normal cerebellar function, as loss of climbing fibres is know to produce severe motor deficits. Many theories of climbing fibre function exist, including the hypothesis that climbing fibres control Purkinje cell excitability. Here, it has been demonstrated that in the absence of excitatory parallel fibre inputs, Purkinje cells can still generate simple spikes intrinsically. The possibility that climbing fibres can modulate the intrinsic spike generator was investigated by observing the effects of climbing fibre removal, accomplished either by microlesion or by applying lignocaine locally. It was found that following a microlesion or a reversible inactivation with lignocaine to the inferior olive, a suppression of complex spike activity occurred that was accompanied by an increase in simple spike frequency. Furthermore, climbing fibre denervation transformed the firing pattern of Purkinje cell discharge, causing Purkinje cells to discharge more regularly. In

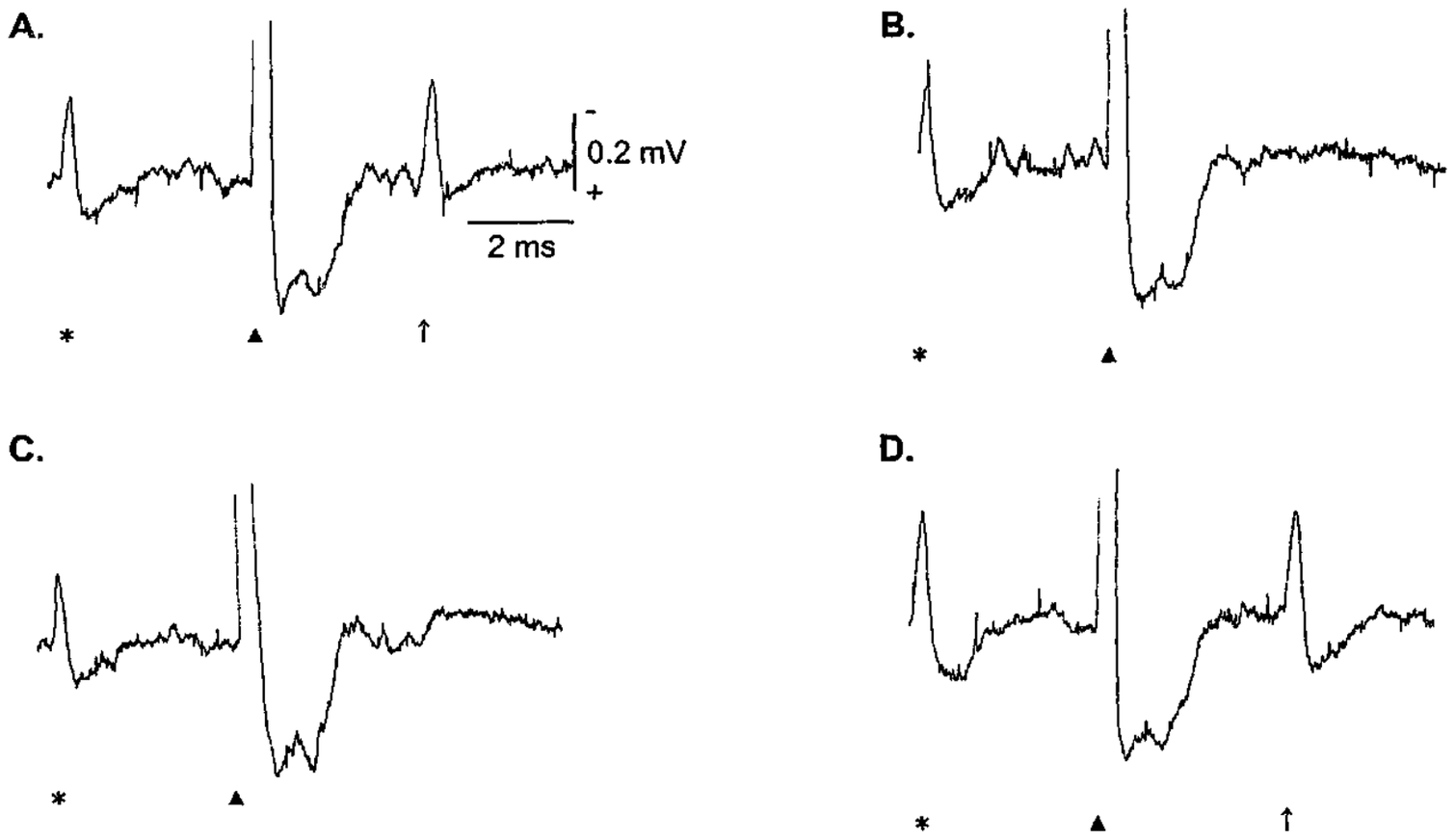


Figure 3.10
Simple spike activity evoked by parallel fibre stimulation and recorded in a Purkinje cell, before and after the infusion of 50 µm CNQX. **A.** Evoked simple spike before the infusion of CNQX. **B.** & **C.** Shortly after the addition of CNQX. Note the disappearance of the evoked s: ,ple spike. **D.** After the effects of CNQX has worn off, evoked simple spike activity returns. Filled triangles refer to the stimulus artefact. Spontaneously occurring simple spikes are indicated by the asterisk. Arrows indicates the evoked simple spike.

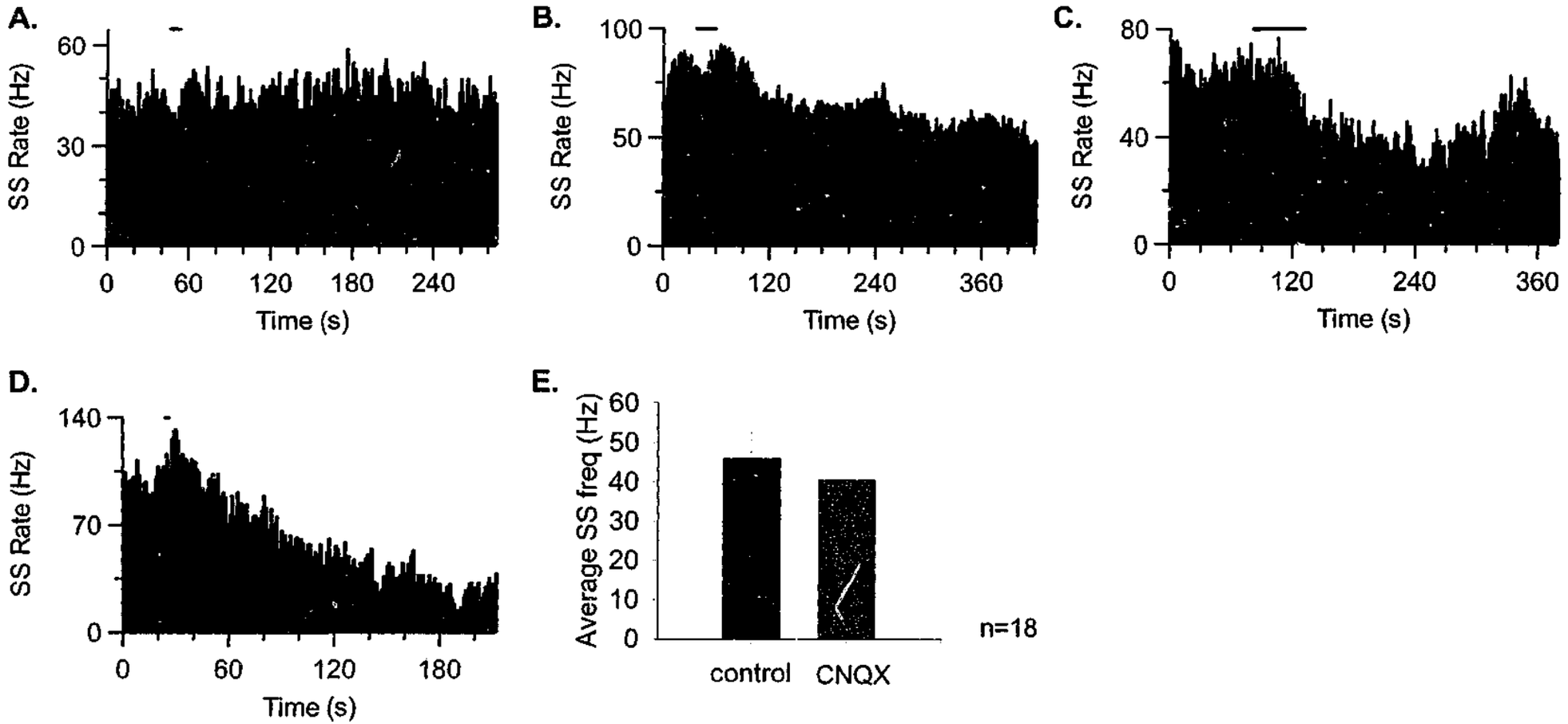


Figure 3.11

Effects of 50 μM CNQX on Purkinje cell simple spike rates. **A-D.** Various responses to infusion of CNQX. Period of CNQX infusion indicated by the red bar. **E.** Summary of effects of CNQX on all Purkinje cells studied. The difference between control and CNQX was not significant.

addition, the experiments presented in this study demonstrate that Purkinje cells denervated for long periods (up to five hours in one instance) start to fire in bursts where activity oscillates between periods of high frequency discharge of up to 150-200 Hz and 10-15 s periods of total quiescence. Thus, it appears that the intrinsic spike generator present in Purkinje cells is normally suppressed by climbing fibre activity. The increase in simple spike activity that followed climbing fibre denervation is thus due to the removal of an inhibitory action that climbing fibres exert on the intrinsic pacemaker.

3.4.1 Comparisons with previous studies- short-term denervation

The observation that an increase in Purkinje cell simple spike activity develops following the removal of its climbing fibre is in accordance with previous observations (Colin et al., 1980; Montarolo et al., 1982; Demer et al., 1985; Savio & Tempia, 1985). These studies produced climbing fibre suppression, either by chemical or electrolytic lesions, or by inactivation of the inferior olive by cooling or application of lignocaine hydrochloride. All reported a salient increase in simple spike activity that accompanied the abolition of complex spike discharge, demonstrating a reciprocal relationship between climbing fibre activation and tonic simple spike activities.

3.4.2 Comparisons with previous studies- long-term denervation

An observation not described in previous studies of climbing fibre denervation (Colin et al., 1980; Montarolo et al., 1982; Demer et al., 1985; Savio & Tempia, 1985) was the oscillatory bursting discharge of Purkinje cell simple spikes that occurred after the suppression of complex spike activity over long periods of time. Previous studies observed cells only for a matter of minutes, and as Purkinje cells did not start to oscillate until at least 25 minutes after climbing fibre denervation, it is likely that this is the reason that this phenomenon remained previously undetected. The mechanism underlying the cycles of intense, high frequency simple spike discharge and periods of silence will be discussed below in Section 3.4.4.

As well as not being reported from earlier acute studies, this oscillatory pattern of discharge by Purkinje cells after climbing fibre denervation has not been reported to occur in chronic experiments, where an irreversible lesion of the inferior olive was performed prior to the recording sessions. These chronic experiments investigated the electrophysiological, metabolic, and morphological changes of climbing fibre removal on the olivocerebellar system (Desclin & Colin, 1980; Bardin et al., 1983; Benedetti et al., 1984). One study in particular (Benedetti et al., 1984), followed Purkinje cell simple spike

activity for one month subsequent to lesions of the inferior olive by means of cryocoagulation, electrocoagulation, and the administration of 3-AP. In this study, an initial significant increase in simple spike activity occurred after the destruction of the inferior olive, however after five days, simple spike firing rates started to decrease, and gradually continued to do so, taking about 1 month to reach levels similar to controls. It therefore seems puzzling that the authors did not report any oscillating cells, at least prior to the observed decrease in simple spike rate. It is however possible that oscillating cells may have been mistaken for injured or dying Purkinje cells and discounted. In some ways, the oscillating activity is similar to the discharge of damaged or dying Purkinje cells that have been injured by the microelectrode. These often display brief bursts of high frequency action potentials and periods of silence. It is not thought that the present oscillatory behaviour was an artefact for several reasons. It could be observed in Purkinje cells where the discharge amplitude was relatively low (~0.5 mV) indicating that the electrode was not in close proximity to the cell membrane. Its appearance was strictly correlated with the removal of the climbing fibre input to the Purkinje cell. Lastly, and perhaps, most importantly, the oscillating activity could be removed by restimulating the climbing fibre input.

It may also be mentioned that the repeat injection of Xylocaine and microlesion of the inferior olive generally resulted in the removal of the climbing fibre input to a small patch or strip of cortex. Thus, microelectrode penetrations made several hours after and close to the location of the originally denervated Purkinje cell commonly encountered Purkinje cells with oscillating type firing (as described above). These displayed no complex spikes, but their depth below the cerebellar surface and the large-amplitude action potentials with characteristic simple spike wave forms almost certainly indicated they were Purkinje cells. Such concentrations of cells with oscillating firing were never encountered in normal cortex with its regular climbing fibre input.

3.4.3 Evidence of an intrinsic pacemaker

Spontaneous simple spike activity *in vivo* has previously been attributed to the continuous secretion of excitatory transmitter from parallel fibre synapses (Eccles et al., 1967a). The present results now indicate that the resting activity of the mossy fibre-granule cell pathway does not contribute substantially to the spontaneously generated activity of Purkinje cells. In the absence of excitatory parallel fibre inputs blocked by the antagonist CNQX, Purkinje cells continued to display spontaneous simple spike activity and there was generally little change in the discharge rate. This finding is consistent with earlier

studies *in vitro*, which demonstrated that Purkinje cell simple spike discharge is preserved when synaptic transmission is blocked (Hounsgaard & Yamamoto, 1979; Hausser & Clark, 1997). The persistent firing of simple spikes that remains in the absence of functional parallel fibre synapses suggests that the resting or tonic Purkinje cell activity is an inherent property of the Purkinje cell, and has been attributed to the underlying ionic mechanisms of the Purkinje cell membrane. Convincing evidence now exists suggesting that the spontaneous simple spike activity is controlled by an intrinsic pacemaker, which is comprised of the interaction of different classes of ion channels present in the Purkinje cell membrane, including Na^+, K^+, and Ca^{2+} (Llinas & Sugimori, 1980a, b; Hounsgaard & Midtgaard, 1988; Gruol et al., 1991; De Schutter & Bower, 1994a; Nam & Hockberger, 1997; Raman & Bean, 1999b; Pouille et al., 2000). The present study indicates that the climbing fibres are responsible for controlling the interaction of the ionic conductances that generate the simple spike activity.

3.4.4 Ionic conductances involved in the intrinsic pacemaker: A proposed model

It is proposed here that the tonic simple spike activity of the Purkinje cells is generated primarily by a non-inactivating Na^+ current, which is counterbalanced to a degree by a non-inactivating K^+ current. Both these currents have identified in Purkinje cells in several studies (Llinas & Sugimori, 1980a; Hounsgaard & Midtgaard, 1988; De Schutter & Bower, 1994a). While the present model is similar in some regards to previous models that explain the origin of endogenous activity (De Schutter & Bower, 1994a; Raman & Bean, 1999a, b), it incorporates the new feature of control over the intrinsic spike generating mechanism by the climbing fibre input to the Purkinje cell. It is proposed that the depolarization set by the balance between the non-inactivating Na^+ and K^+ currents is further regulated by an additional hyperpolarizing Ca^{2+}-dependent K^+ current generated as a result of Ca^{2+} entry associated with climbing fibre activation.

P-type VOCCs, first described in Purkinje cells (Llinas et al., 1989), are very slowly inactivating and underlie the high threshold Ca^{2+} action and plateau potentials in adult cerebellar Purkinje cells (Llinas et al., 1989; Llinas et al., 1992). They are prominent in Purkinje cell dendrites, extending through the secondary and tertiary bifurcations of the dendritic tree (Hillman et al., 1991). Most importantly for the present study, the P-type channels are also responsible for a large part of the substantial Ca^{2+} influx that occurs with climbing fibre depolarization of the Purkinje cell (Mintz et al., 1995). VOCCs are known to be closely coupled with Ca^{2+}-dependent K^+ channels (Blatz & Magleby, 1987; Lancaster

& Nicoll, 1987; Robitaille & Charlton, 1992; Gola & Crest, 1993). Ca^{2+}-dependent K^+ conductances are regulated by the concentration of free intracellular Ca^{2+} (Marty, 1989; Sah, 1996; Vergara et al., 1998). Thus, the increase in cytosolic Ca^{2+} that occurs as a result of climbing fibre activation of P-ype VOCCs, will activate Ca^{2+}-dependent K^+ channels with a consequent hyperpolarizing influence on the Purkinje cell that counteracts the depolarization set by the balance between the non-inactivating Na^+ and K^+ conductances. Thus, the climbing fibre system may represent a means by which the intrinsic pacemaker is regulated, and which ultimately controls Purkinje cell excitability.

When Ca^{2+} enters the Purkinje cell cytosol, it is sequestered into internal stores over a time course of about 10-15 seconds (Tank et al., 1988). Given that climbing fibres discharge at rate around 1 Hz, there will be insufficient time to fully sequester Ca^{2+} from the cytosol between successive climbing fibre impulses. The result will be a somewhat elevated level of free Ca^{2+} in the Purkinje cell, and this level will depend on the actual frequency of the climbing fibre input. The free Ca^{2+} in the cytosol will cause a hyperpolarizing current through the activation of the Ca^{2+}-dependent K^+ channels. This will counteract the inward Na^+ current and limit simple spike firing.

It is predicted that when climbing fibre activity ceases, the Purkinje cell will be deprived of its regular injection of Ca^{2+} through the P-type VOCCs, and cytosolic Ca^{2+} will fall to a very low level due to sequestration. Consequently, current flow through the Ca^{2+}-dependent K^+ channel will cease, and the Purkinje cell will depolarize to the level set by the balance between the non-inactivating Na^+ and K^+ conductances. Thus, simple spike rate should rise to the level determined by this depolarization. This proposed mechanism is clearly consistent with the rapid elevation in simple spike activity that occurred soon after the removal of the climbing fibre input.

The next question that arises, is what mechanism can account for the oscillatory behaviour that occurred after climbing fibre input had been blocked for a longer period of time?

As explained above, climbing fibre input is likely to maintain an elevated level of Ca^{2+} in the Purkinje cell cytosol and this may be the normal operating mode for the Purkinje cell given the ongoing nature of climbing fibre activity. Thus, when climbing fibre activity ceases, cytosolic Ca^{2+} will fall to a very low level and it will be maintained at this low level in the absence of regular influxes of Ca^{2+} via the VOCCs. This will almost certainly affect the activity of Ca^{2+}-dependent biochemical processes in the Purkinje cell, particularly Ca^{2+}-dependent protein kinases that phosphorylate and dephosphorylate proteins and activate and inactivate them. It is suggested that the Purkinje cell will continue to

depolarize after climbing fibre removal because of a progressive inactivation of non-inactivating K^+ channels, possibly via lack of phosphorylation. The reason for focussing on these channels is that Llinas and Sugimori (1980b) showed that with blockage of K^+ channels in Ca^{2+} free medium caused the Purkinje cell to depolarize dramatically and to the point at which the spike generating mechanism was inactivated. This suggests that an unopposed Na^+ current is capable of powerfully depolarizing the Purkinje cell. Thus, if current through the non-inactivating K^+ channels declines after climbing fibre removal, there will be progressively less opposition to the Na^+ current which will progressively depolarize the Purkinje cell with a corresponding increase in simple spike rate. The depolarization then reaches a level sufficient to activate VOCCs with an influx of Ca^{2+} into the Purkinje cell. The Ca^{2+} activates Ca^{2+}-dependent K^+ channels which generate a hperpolarization that shuts off Purkinje cell firing. The Ca^{2+} is subsequently sequestered and the hyperpolarizing current declines. The Na^+ current then proceeds to depolarize the Purkinje cell with a corresponding high frequency of simple spike discharge, until the depolarization is again sufficient to open VOCCs and cause Ca^{2+} entry, and the cycle is repeated. Thus, it is envisaged that the oscillating behaviour of the Purkinje cell results from a strong depolarization that is periodically interrupted by hyperpolarization generated by entry of Ca^{2+} and activation of Ca^{2+}-dependent K^+ channels.

It may be noted that the oscillating firing of the present Purkinje cells after removal of their climbing fibre input, is practically identical to the spontaneous firing pattern of Purkinje cells in cerebellar slice preparations (eg Llinas & Sugimori, 1980a, b), where the cells have been deprived of their climbing fibre input for some considerable time. The cell firing is characterised by periods of bursting and silence, with about the same period as described here. Moreover, in support of the present mechanism, Ca^{2+} imaging studies showed regular oscillations in cytosolic Ca^{2+} in Purkinje cell that displayed autorhythmic activity in slice preparations (Tank et al., 1988). Tank et al., (1988) tentatively ascribed the rhythmic increases in cytosolic Ca^{2+} to oscillations in the release from internal stores (though oscillations were not seen in Ca^{2+} free media). The present model suggests that such changes in cytosolic Ca^{2+} in climbing fibre deprived Purkinje cells represents periodic influx through VOCCs.

A further point to make, is that although the non-inactivating Na^+ conductance in the absence of climbing fibre activation would be expected to continually depolarize the Purkinje cell to the point of spike inactivation due to depolarization block, this does not appear to be the case (De Schutter & Bower, 1994a; Raman & Bean, 1999b). Pouille et al.,

(2000) demonstrated that the blockade of P-type VOCCs in cultured cerebellar slices, which would mimic climbing fibre denervation, causes an increase in the Purkinje cell depolarization to a value of -30 mV. P-type VOCCs present in Purkinje neurones are known to be activate at potentials of -50 to -30 mV (Regan, 1991). Thus, it is likely that as the Purkinje cell deprived of its climbing fibre becomes more depolarized as a consequence of the decline of current flow through the Ca^{2+}-dependent K^+ and non-inactivating K^+ channels, then the unopposed Na^+ current will progressively depolarize to the level sufficient to activate the P-type VOCCs.

3.4.5 Recovery of climbing fibre denervation

It does appear however from the chronic studies (Benedetti et al., 1984) that the intense increase in simple spike activity and oscillations which follow climbing fibre removal is not permanent, as simple spike discharge rates apparently return to control values around one month after initial inferior olive destruction. Behavioural studies have shown following inferior olive lesions, the animal presents with severe motor disorders which persists for a period of days, after which some recovery of function occurs (Desclin & Escubi, 1974; Llinas et al., 1975). Thus, initial and severe effects of climbing fibre loss could well be due to the abnormal oscillating behaviour of the Purkinje cells. Following lesions of the inferior olive, climbing fibre varicosities degenerate, and very few Purkinje cell spines remain opposed to the degenerating climbing fibre terminals. After some time, the number of Purkinje cell dendritic spines emerging from secondary and tertiary dendritic trunks increases, and some of the spines become innervated by neighbouring parallel fibres, which persist until these neurones become reinnervated by climbing fibre collaterals (Sotelo et al., 1975; Sotelo, 1978; Desclin & Colin, 1980). Morphological studies revealed that seven days after inferior olive lesions there was reinnervation of the Purkinje cells, with surviving climbing fibres beginning to sprout collaterals that form new climbing fibres around neighbouring Purkinje cell dendritic trees (Rossi et al., 1989; Rossi et al., 1991a; Rossi et al., 1991b). Within 1-2 months post-lesion, new terminal plexuses were evident which displayed structural features which were similar to normal climbing fibres (Rossi et al., 1991a; Rossi et al., 1991b). At the electron microscopy (EM) level, the presence of newly formed synaptic contacts formed by the sprouted climbing fibres and spines of the proximal Purkinje cell dendrites was confirmed (Rossi et al., 1989). The plasticity that occurs following lesions of the inferior olive appears to restore the climbing fibre-Purkinje cell synapse and may contribute to the partial recovery in motor function.

3.4.6 Are the cerebellar inhibitory interneurones involved?

The next question that arises is to what extent the effects of climbing fibre removal on simple spike activity may result from loss of excitation of inhibitory interneurones by way of climbing fibre collaterals? Montarolo et al., (1982) credited the increase in simple spike firing rates following cooling of the olive not only on the direct action of climbing fibres on the Purkinje cells, but also on the influence of cerebellar cortical interneurones. They based this conclusion on the results obtained from a series of experiments where a bilateral chemical lesion of the inferior olive using 3-AP was performed 217 days prior to the recording session. They then recorded from Purkinje cells that displayed complex spikes as a result of a few remaining olivary cells. In comparison to intact animals, the increase in rates attained by simple spikes after cooling of a prelesioned inferior olive was considerably less. The greater increase in intact rats was thought to be due to the suppression of the activity of many olivary neurones, which led to the conclusion that a given Purkinje cell is not only influenced by the direct action of its climbing fibre, but also, indirectly, via the inhibitory interneurones.

In order to account for the dramatic effects of climbing fibre removal in inhibitory interneurones, the synaptic contacts between climbing fibres and interneurones would need to be both extensive and numerous. Collaterals of climbing fibres were first reported to contact basket and stellate cells (Scheibel & Scheibel, 1954). Synaptic contact between collaterals and Golgi cells were also demonstrated in several studies (Scheibel & Scheibel, 1954; Hamori & Szentagothai, 1966; Palay & Chan-Palay, 1974). Ultrastructural examination of the cerebellar cortex after the degeneration of climbing fibres using 3-AP found no evidence of such a connection, and questioned the existence of climbing fibre collaterals to the inhibitory neurones (Desclin, 1976; Desclin & Colin, 1980). With the advent of anatomical tracers such as biotinylated dextran amine (Veenman et al., 1992), which have made it possible to trace the pathway and trajectories of single axons, the question of climbing fibre collaterals has recently been re-examined by Sugihara, Wu and Shinoda (1999). As well as forming a dense terminal arborisation around Purkinje cells. swellings from the terminal arborisations were demonstrated to contact interneurones located in the molecular layer of the cerebellar cortex. In addition, thin collaterals arising from the stem axon and thick branches of olivocerebellar axons before they formed climbing fibres were found to terminate in the granular layer. These collaterals displayed few ramifications and coursed through the granular layer for a relatively short distance. Sparsely distributed swellings on these collaterals appeared to contact presumed Golgi cells, supporting earlier studies (Scheibel & Scheibel, 1954; Hamori & Szentagothai, 1966;

Palay & Chan-Palay, 1974), and were found amongst aggregates of granule cells. Thus, it would appear that climbing fibre collaterals are in fact present, and appear to contact inhibitory interneurones. Despite this contact, EM studies by Hámori and Szentágothai (1980) excluded the formation of a synaptic contact between them. Electrophysiological studies also prove rather inconclusive, with reports of a weak excitatory effect, no effect, and a strong inhibitory effect on cerebellar interneurones upon climbing fibre stimulation (Eccles et al., 1966c; Bloedel et al., 1972; Schulman & Bloom, 1981; O'Donoghue et al., 1989). Therefore it appears that existence of climbing fibre collaterals may be infrequent. Furthermore, when a comparison of the number of swellings and trajectories of climbing fibre collaterals in the molecular and granular layer were compared to the number of swellings and the extent of branching of climbing fibres acting directly on Purkinje cells, they were also found to be rather infrequent and limited (Sugihara et al., 1999; Shinoda et al., 2000). Thus, it would appear likely that the actions seen with climbing fibre removal result predominantly from the direct action of climbing fibres.

The effects of the infusion of CNQX (which blocks ionotropic glutamate receptors, in particular AMPA receptors) also tend to support this idea. These infusions would almost certainly have blocked ongoing excitatory transmission in a fairly wide area of cortex around the Purkinje cell under study, including any ongoing excitatory transmission from granule cells and their parallel fibres to the inhibitory interneurones. If this system were exerting a major influence on the simple spike activity seen in the present Purkinje cells, then blockage should have resulted in an increase in simple spike activity, due to disinhibition. However, as reported above, there was no change in simple spike activity after application of CNQX.

Recent computer models of Purkinje cells propose that several intrinsic currents contribute to the pacemaker firing pattern of Purkinje cells, most notably the persistent Na^+ and K^+ currents, Ca^{2+} currents, and Ca^{2+}-dependent K^+ currents (De Schutter & Bower, 1994a). Complimentary studies then added excitatory and inhibitory inputs to this model in order to examine the model's response to synaptic input and to replicate background simple spike firing properties of Purkinje cells *in vivo* (De Schutter & Bower, 1994c; Jaeger et al., 1997). When random granule cell inputs were applied at varying frequencies the model generated high minimum firing rates of simple spikes of approximately 80 Hz, with little variability of the ISI. Moreover, as the input frequencies of granule cells were increased, the Purkinje cells started bursting, a type of behaviour that although seen in slice recordings, either spontaneously or during current injection (Llinas & Sugimori, 1980a) is never seen *in vivo*. When inhibitory inputs were added to

the model in addition to the excitatory inputs, the model showed a complete range of firing frequencies that emulates the firing range seen *in vivo*. Furthermore, with the addition of inhibitory inputs, ISI was highly variable. When the ISI histogram from a spontaneous Purkinje cell recorded *in vivo* was compared to the histogram obtained from the model, they were both very similar. The addition of excitatory and inhibitory inputs transformed the model, altering the spike pattern to behave more like Purkinje cells recorded *in vivo*. Thus, continuous excitatory and inhibitory synaptic inputs appear to be important for generating the irregular simple spike activity that Purkinje cells display. Further findings also using the realistic model of Purkinje cell firing found that inhibitory rather than excitatory inputs are more influential in controlling spike timing (Jaeger & Bower, 1999), as the duration of the interspike interval was correlated with the amplitude of the inhibitory conductance. Furthermore, it was shown that a tonic level of baseline inhibitory inputs to the Purkinje cells was required to preserve the *in vivo* discharge pattern of simple spikes. Without these tonic inputs, Purkinje cells were seen to burst, a phenomenon not seen *in vivo*, but present in tissue slice preparations.

The finding that inhibitory inputs are necessary in maintaining *in vivo* patterns of spike firing has also been substantiated by Hausser and Clark (1997) who assessed the contribution of inhibitory synapses to the generation of irregular firing patterns of Purkinje cells. This was examined not in computer modelling, but rather, in *in vitro* preparations by blocking synaptic inhibition using the GABAergic blockers gabazine, bicuculline, and picrotoxin. When inhibition was blocked, Purkinje cell firing rates increased by 41 %, a value comparable to the increase observed in the present study upon climbing fibre denervation. Identical to climbing fibre removal, blockade of the inhibitory synapses also altered the firing pattern of Purkinje cells, with simple spikes firing more regularly, a fact reflected in the narrower ISI distribution. The shift in ISI distribution was identical to that that occurred as a result of climbing fibre removal presented in this chapter. So the question remains: is the intrinsic Purkinje cell pacemaker under the control of climbing fibres or inhibitory interneurones? While the issue of the influence of inhibitory synaptic input has not been dealt with directly in this thesis, given the above observations, this issue needs to be addressed, and will be dealt with below.

Firstly, blockade of synaptic inhibition increased the rate and regularity of Purkinje cell simple spikes (Hausser and Clark, 1997). This increase would be assumed from knowledge of the inhibitory actions of cortical interneurones. Although both the current investigation and that by Hausser and Clark (1997) both reported an increase in simple spike frequency after the blockade of climbing fibre and inhibitory inputs respectively, an

obvious difference between them is that Hausser and Clark (1997) did not describe an oscillatory or bursting discharge mode for any of the Purkinje cells investigated. This is especially surprising as computer models found that a tonic baseline of synaptic inhibition was required, otherwise Purkinje cells began to discharge in a bursting mode (De Schutter & Bower, 1994c; Jaeger et al., 1997; Jaeger & Bower, 1999). It may be argued that Hausser and Clark (1997) may not have studied the Purkinje cells for a long enough period, as it must be recalled that the bursting mode appeared at least 25 minutes after climbing fibre removal. Similarly, an *in vitro* study where synaptic inhibition was blocked with 1 μM bicuculline produced an increase in Purkinje cell firing, but did not produce bursts (Aubry et al., 1991). Conversely, *in vivo* studies where 5 mM bicuculline was applied to the surface of the cerebellar cortex, altered Purkinje cell firing to cycle between a quiescent state and periods of bursting (Jaeger & Bower, 1994). The difference in responses between these three studies may be explained by the finding that the effects of bicuculline are concentration dependant (Gahwiler, 1975). More importantly, recent observations have found that bicuculline causes a significant blockade of apamin sensitive Ca^{2+}-dependent K^+ channels (Gahwiler, 1975; Johnson & Seutin, 1997; Debarbieux et al., 1998; Seutin et al., 1998; Aizenman & Linden, 1999). Ca^{2+}-dependent K^+ channels are predicted to have a significant role in the control of the pacemaker function of Purkinje cells (see Section 3.4.4). As it will be demonstrated later in this thesis (Chapter 5), apamin sensitive Ca^{2+}-activated K^+ channels are indeed present in the Purkinje cell, and it will be shown that the blockade of these channels with the highly specific blocker apamin, significantly increases the discharge frequency of simple spikes. More importantly however, it will be demonstrated that the blockade of these channels induces Purkinje cells to burst (Chapter 6). Thus, the interpretation of the effects of bicuculline is ambiguous, as the effects seen could be confused with their direct action on Purkinje cells rather than via the inhibitory interneurones. Of course, an obvious way in which this problem can be clarified is to consider the use of more specific compounds for GABAergic receptors. These include picrotoxin and gabazine. Some evidence suggests that the use of these blockers will not induce Purkinje cells to oscillate. As described earlier, Hausser and Clark (1997), as well as using bicuculline, also used gabazine and picrotoxin to study the effects of withdrawal of synaptic inhibition on Purkinje cell spontaneous discharge, and as mentioned earlier, they did not report any Purkinje cell oscillations. Further experiments infusing these GABAergic blockers around the Purkinje cell in a similar manner as employed in the CNQX studies would be useful in resolving this issue.

3.4.7 Effects of climbing fibre removal on DCN

The changes in background simple spike activity that are associated with climbing fibre removal would have a marked effect on the target cell of the Purkinje cells in the DCN. Nuclear cells normally display tonic discharge (Thach, 1968; Armstrong & Rawson, 1979b; Harvey et al., 1979; Armstrong & Edgley, 1984a) and it is well established that Purkinje cells exert a monosynaptic inhibitory action on the nuclear cells (Ito et al., 1970). Hence, an increase in Purkinje cell activity will decrease tonic nuclear cell firing by inhibition. Indeed temporary suppression of the olivocerebellar pathway induced a decrease in intracerebellar nuclear cell discharge that paralleled the increase in simple spike activity (Benedetti et al., 1983). These changes are accompanied by a marked increase in metabolic activity of Purkinje cell axon terminals (Bardin et al., 1983; Batini et al., 1984). The consequence of the depressed DCN activity will be marked changes in intracerebellar output and thus in cerebellar influence over the operations of its target cells in the brainstem and thalamus. Supporting this suggestion is the finding that inferior olive lesions decreased the metabolic activity of the red nucleus, one of the DCN targets (Bardin et al., 1983).

At present however, no electrophysiological studies have examined the long-term effects of climbing fibre removal on DCN. In the long term it could be predicted, on the basis of observation on Purkinje cells, that DCN activity may return to near normal levels. What happens in the shorter term (perhaps several days) where the Purkinje cells display oscillating activity is not known. However, the periodic burst-like firing and silent periods of the Purkinje cells may not necessarily produce corresponding patterns of firing in the DCN. For this to occur, the firing pattern of the Purkinje cells that project to a population of DCN cells would need to be in phase with one another. This seems unlikely as some preliminary multi-unit recordings from patches of climbing fibre denervated Purkinje cells did not indicate a tendency for the cells to oscillate in phase (Rawson, 2001, personal communication). It seems more likely that the net effect of oscillating Purkinje cell activity on the DCN will be a somewhat decreased level of activity, as the bursts in Purkinje cell firing were longer than the periods of silence.

3.4.8 Summary

The results of the present chapter suggest that the tonic simple spike activity of Purkinje cells *in vivo* is generated intrinsically. It was proposed that the mechanism for establishing

the level of intrinsic spike activity is a balance between opposing non-inactivating Na^+ and K^+ currents.

Evidence was obtained to suggest that this intrinsic spike generating mechanism is normally controlled by ongoing input in the climbing fibre projection to the Purkinje cells. It was suggested that the mechanism of this control was an outward Ca^{2+}-dependent K^+ current which is generated by Ca^{2+} influxes through P-type VOCCs activated by climbing fibre depolarization of the Purkinje cells.

A sustained input from the climbing fibre to its Purkinje cell seems essential for maintaining an inhibitory control over the spike generating mechanism. With removal of the climbing fibre input it was suggested that there would be a rapid loss of control via the Ca^{2+}-dependent K^+ current and then a more prolonged and progressive loss of control, leading to an oscillatory mode of firing. The proposed theory of climbing fibre control of the intrinsic pacemaker also predicts that limited changes in climbing fibre frequency will affect the level of Ca^{2+} in the Purkinje cell cytosol. Thus, the higher the climbing fibre frequency the higher the prevailing Ca^{2+} concentration and subsequently, the greater the Ca^{2+}-dependent K^+ conductance which will cause a proportionately lower simple spike rate. The opposite would apply to low climbing fibre rates where less accumulation of Ca^{2+} will occur, reducing the outward K^+ current and causing an increase in simple spike rate. This idea that climbing fibre frequency controls and determines the level of tonic activity will be examined the subsequent chapter.

Chapter 4

The effects of climbing fibre inputs within physiological rates on Purkinje cell simple spike activity.

4.1 Introduction and Aims

The contribution of climbing fibre input to cerebellar function is currently unresolved. The limited range of complex spike discharge that in resting conditions occurs at a rate of 1.5 Hz (Thach, 1968, 1970a; Gilbert & Thach, 1977; Armstrong & Rawson, 1979a; Andersson & Armstrong, 1987) is unlikely to make a major direct contribution to the conventional frequency coding of the Purkinje cell, as the addition of a few complex spikes would appear rather insignificant in comparison to the tonic simple spike rates of 50-100 Hz (Eccles et al., 1967b; Latham & Paul, 1971; Armstrong & Rawson, 1979a). Consideration has therefore focussed on their interactive influence on the Purkinje cell simple spike discharge, namely the pause in simple spike activity that follows the onset of a complex spike.

Many investigators have noted the inhibitory effect on Purkinje cell discharge exerted by climbing fibre inputs. First described by Granit and Phillips (1956) and Eccles et al., (1966b), complex spike discharge is known to be followed by a distinct pause or reduction in the ongoing simple spike discharge that has a duration of 10-20 ms to several hundred ms (Bell & Grimm, 1969; Bloedel & Roberts, 1971; Latham & Paul, 1971; Murphy & Sabah, 1971; Armstrong et al., 1979; Armstrong & Rawson, 1979a; McDevitt et al., 1982; Sato et al., 1992, 1993). This effect is known as the climbing fibre pause, and it is highly variable among individual Purkinje cells. Bloedel and Roberts (1971) noted that the duration of the climbing fibre pause could outlast the duration of the complex spike and suggested that climbing fibre pause could in fact be of greater importance than the complex spike for cerebellar function. Presumably, the pause in simple spike discharge will result in a corresponding reduction in the inhibitory action exerted by the Purkinje cells on cerebellar nuclei activity which would produce an increase in nuclear cell discharge. Indeed, conditions where climbing fibre discharge consistently evoked climbing fibre pauses of 400-500 ms, pronounced facilitation of cerebellar nuclei activity was observed to occur after climbing fibre activation (Armstrong et al., 1979; Armstrong & Rawson, 1979a).

Investigations of the mechanism contributing to the climbing fibre pause found that the inactivation of the Purkinje cell spike generator by the depolarizing action of the climbing fibre accounted for 10-20 ms of the post climbing fibre pause, as after this, it was possible to evoke a test antidromic spike in the Purkinje cell (Bloedel & Roberts, 1971; Martinez et al., 1971). Pauses longer than 20 ms has been attributed to the influence of climbing fibre collaterals on cerebellar cortical neurones that would directly inhibit the Purkinje cells (via the basket cells) or to decreased transmission of the mossy fibre-granule cell pathway via the Golgi cells (Bloedel & Roberts, 1971; Latham & Paul, 1971; Murphy & Sabah, 1971; de Montigny & Lamarre, 1973; Armstrong et al., 1979; Armstrong & Rawson, 1979a). This proposal was based on the observation that the duration of the pause could be graded by the strength of inferior olive stimulation. Furthermore, Murphy and Sabah (1971) demonstrated that a pause in Purkinje cell simple spike activity could occur without directly activating its climbing fibre input, suggesting that the pause was generated by the activation of climbing fibres supplying nearby Purkinje cells.

An extended reduction of the ongoing level of simple spike discharge can also be produced by increasing the level of inferior olive activity. The use of tremorgenic drugs such as harmaline induces a synchronous olivary discharge of alternating cycles of membrane hyperpolarization and rebound depolarization with a period of 8-10 Hz (Llinas & Yarom, 1986). The synchronous climbing fibre activity leads to a corresponding rhythmic complex discharge in the Purkinje cells and a significant suppression of the simple spike activty (Lamarre & Mercier, 1971; de Montigny & Lamarre, 1973; Lamarre & Weiss, 1973; Llinas & Volkind, 1973; Hawdon et al., 1988; Rawson et al., 1988). Likewise, repetitive electrical stimulation of the inferior olive at rates of 1-10 Hz reduced and eventually eliminated simple spike activity (Colin et al., 1980; Rawson & Tilokskulchai, 1981b; Demer et al., 1985). With both types of olivary stimulation, the decrease in simple spike activity was ascribed to a direct action of climbing fibres on the Purkinje cells, with little or modest contributions from the cerebellar cortical interneurones and climbing fibre pauses. In the case of electrical stimulation, the sustained inhibitory effect was observed only when the stimuli were above threshold for activating the climbing fibre innervating the Purkinje cell under study (Colin et al., 1980; Rawson & Tilokskulchai, 1981b). Conversely, the reverse effect, whereby a silencing of the inferior olive was achieved either by cooling, chemical lesion or by the application of lignocaine, caused an increase in the tonic simple spike activity (Colin et al., 1980; Montarolo et al., 1982; Demer et al., 1985). Only a small proportion, if any, of the increase in simple spike rates following

abolition of the complex spike activity could be attributed to the removal of the climbing fibre pause.

Results presented in the preceding chapter verified that the temporary and longer-term inactivation of the inferior olive produced a distinct increase in the background simple spike rates of cerebellar Purkinje cells, that are now recognised to be, at least in part, generated intrinsically (Woodward et al., 1974; Gahwiler, 1975; Dupont et al., 1979; Llinas & Sugimori, 1980a; Gruol, 1983; Crepel et al., 1984; Konnerth et al., 1990 Hounsgaard, 1988 #217; Hausser & Clark, 1997; Nam & Hockberger, 1997; Raman & Bean, 1999b). Thus, it was established that the tonic Purkinje cell activity produced by the endogenous pacemaker has a reciprocal relationship with its climbing fibre input, purportedly due to the release of an inhibitory action. The proposed mechanism for simple spike initiation presented in the previous chapter and supported by many other studies (Llinas & Sugimori, 1980a, b; De Schutter & Bower, 1994a; Raman & Bean, 1999b), implicated the non-inactivating Na^+ and K^+ conductances as generating simple spike discharge intrinsically. In turn, it was suggested that the climbing fibres control the intrinsic spike generator by modulating the Ca^{2+}-dependent K^+ current via the activation of the P-type VOCCs with each discharge. Therefore, it is proposed that the climbing fibre inputs can determine the level of Purkinje cell background activity over its entire physiological range of simple spike discharge. The idea that the climbing fibres regulate the ongoing simple spike activity is supported by the aforementioned studies, which demonstrated that an increase in climbing fibre stimulation caused a reduction in Purkinje cell activity whereas inactivation of the climbing fibres caused a rapid and substantial increase in background firing. This theory depends upon the ability of the climbing fibres being able to alter background activity within its limited range of normal operation. Thus, the aim of this study was to determine whether climbing fibre activation over its *in vivo* range could alter the level of background simple spike activity.

Under natural rest conditions, Purkinje cells discharge complex spikes at a frequency of approximately 1.5 Hz. In awake animals undertaking a wide range of behaviours, rates of complex spike discharge did not exceed more than 2.5-3 Hz (Thach, 1968; Harvey et al., 1977; Armstrong & Rawson, 1979a; Andersson & Armstrong, 1987). Additionally, inferior olive cells are known to discharge with either a single impulse or a burst of 2-6 impulses at about 500 Hz (Armstrong & Harvey, 1968; Armstrong & Rawson, 1979a). Previously it has been shown that the repetitive activation of climbing fibres at high frequencies had a suppressive effect on background simple spike rates (Rawson & Tilokskulchai, 1981b; Demer et al., 1985). Demer et al., (1985) found that the cut-off frequency, defined as the

complex spike stimulus frequency where simple spike activity was absent, was on average 5 Hz. In contrast, Rawson and Tiloskulchai (1981b) who examined the effects of climbing fibre stimulation in the cat, reported no changes in the ongoing simple spike rates during climbing fibre stimulation at rates of up to 4 Hz. Rather, stimulation at higher rates had an inhibitory effect that was dependent on the simulation frequency, with rates of 8-10 Hz completely suppressing simple spike activity. Both these cut-off frequencies probably exceed the normal physiological range of complex spike discharge, whereas the cut-off frequency found in the study conducted by Colin et al., (1980) was 2 Hz, which lies within the observed *in vivo* range of complex spike activity. However, Colin et al., (1980) used the toxin 3-AP to first destroy the olivary cells and then stimulate the climbing fibre axons which could continue to evoke complex spikes for some time after the inferior olivary lesions. A limitation of 3-AP is that it is highly toxic, producing a mortality rate of 61 % (Colin et al., 1980) and although it was thought to be specific to the inferior olive, it has been demonstrated that 3-AP can also effect other areas of the brain including the nucleus ambiguous and the nucleus of the solitary tract (Desclin & Escubi, 1974). Therefore, the Purkinje cell activity in the Colin et al., (1980) study may have been depressed, influencing the cut-off frequencies obtained.

Clearly, it is uncertain what range of climbing fibre frequency influences the Purkinje cell simple spikes produced intrinsically. Moreover, previous studies used stimulation frequencies outside the physiological range. Therefore, a need exists to assess what effects climbing fibres exert in the cerebellum over their observed and limited natural range of firing.

In addition, the influence of varying not only the rate but the number of impulses in the complex spike discharge also needs to be examined. Apart from the study by Rawson and Tiloskulchai (1981b), other studies have used only single pulse stimulation of the climbing fibres (Colin et al., 1980; Demer et al., 1985). Rawson and Tiloskulchai (1981b) reported that when climbing fibres were driven with bursts of stimuli, suppression or reduction of simple spike firing was proportional to the number of stimuli in the burst. Again however, the overall climbing fibre frequencies used were at the very upper limits of the physiological range.

Therefore, the experiments in this chapter aimed to examine in detail how repetitive climbing fibre activation directly influences the ongoing Purkinje cell simple spike discharge that is generated by the intrinsic pacemaker, and also to examine their effects on cortical inhibitory interneurones. Specifically, the aims for this chapter are:

- To determine how Purkinje cell simple spike activity is affected by climbing fibre stimulation at rates that fall within the reported *in vivo* range. The experiments were designed so that the climbing fibre input to a cell could be controlled precisely by electrical stimulation. Additionally, the effects on cerebellar cortical interneurones were also examined.

- Varying the number of impulses used to stimulate the climbing fibres were also used to determine how this affects tonic simple spike activity of Purkinje cells.

- In a final series of experiments, Purkinje cells will be infused with the AMPA-type glutamate receptor antagonist CNQX. Application of CNQX is known to block excitatory synaptic transmission at the parallel fibre-Purkinje cell synapse. In the previous chapter, application of CNQX blocked the simple spike responses to parallel fibre stimulation however, its tonic simple spike discharge was not abolished providing evidence that Purkinje cells can generate simple spikes intrinsically. The effects of climbing fibre stimulation on the intrinsic simple spike activity of Purkinje cells, once AMPA receptors were blocked with 50 μm CNQX, will also be investigated.

The results will show that climbing fibre activation within a very limited and presumably physiological range can exert a direct inhibitory action on the intrinsic Purkinje cell simple spike activity. The amount of simple spike activity regulated will be shown to be dependent on the frequency of stimulation as well as the number of impulses in the discharges of the climbing fibres.

4.2 Methods

The experiments were performed on 15 adult male Long-Evans rats. General aspects of the experimental techniques were described in Chapter 2. A schematic diagram of the specific experimental setup used in this chapter is shown in Figure 4.1. Microelectrodes were used to record from single Purkinje cells and an electrode (with about 50 μm tip exposure, see Section 2.5) in the inferior olive was used to lesion and then stimulate the climbing fibre axons. Methods for localising the climbing fibre input to the Purkinje cell were described in the previous chapter (Section 3.2.2).

Once a Purkinje cell with a low threshold evoked complex spike was located, an attempt was made to block normal orthodromic conduction up the climbing fibre by a

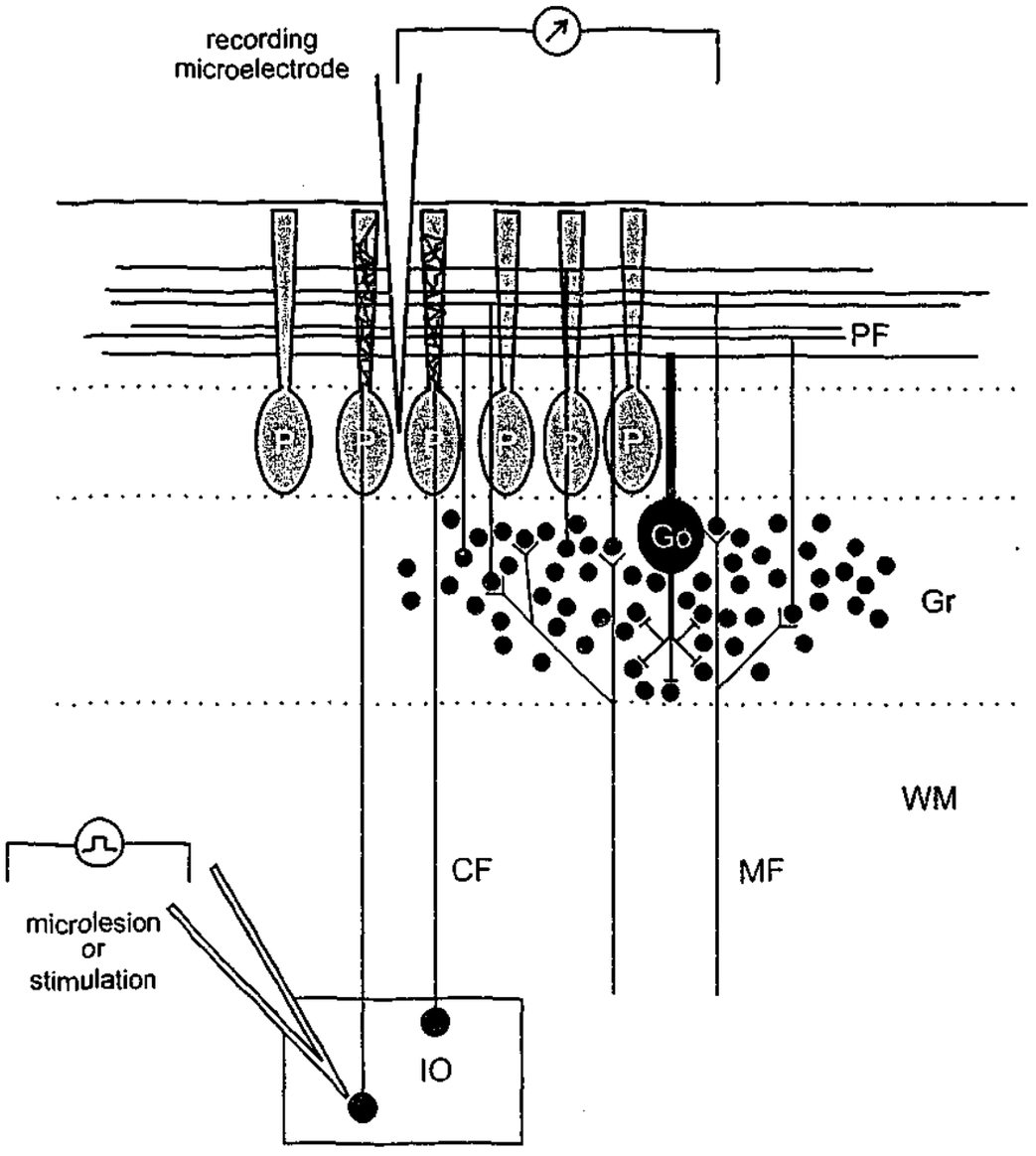


Figure 4.1

Schematic diagram of the experimental arrangement used for stimulating the climbing fibres. A dual microelectrode-micropipette was inserted into the inferior olive for the stimulation and removal of the climbing fibre input to the Purkinje cells. Extracellular recordings were made with a glass-insulated micropipette inserted into the region of cortex which received the most intense climbing fibre activation. Climbing fibre removal was accomplished by either a microlesion, or by applying 5-10 % Xylocaine to the parent cell or axon in the inferior olive.

IO, inferior olive; CF, climbing fibre MF, mossy fibre; P, Purkinje cell; Gr, granule cell; PF, parallel fibre; Go, Golgi cell; WM, white matter

microlesion. This was done by passing 15 μA of DC (electrode tip positive) through the electrode in the inferior olive to completely abolish the spontaneous complex spike activity. Usually only several seconds of constant delivery was sufficient to accomplish this with evoked responses of low threshold (<20 μA). The stem climbing fibre axon was then stimulated electrically with 0.1 ms duration pulses to evoke complex spikes in the Purkinje cell. Once the climbing fibre input had been removed by the microlesion, a 'holding' stimulus of 0.5 Hz was initially applied to prevent the dramatic rise in simple spike activity described in the previous chapter. Frequencies of climbing fibre stimulation above and below the holding frequency in the range of 0.1-3.5 Hz were then tested.

As already mentioned, it has been established that inferior olive cells discharge with either single or bursts of 2-6 impulses at approximately 500 Hz that are propagated up the climbing fibre axon (Armstrong & Harvey, 1968; Armstrong & Rawson, 1979a). On average, inferior olive cells generate a burst of 3 impulses in their discharge (Armstrong & Rawson, 1979a). Thus, the climbing fibre response or complex spikes of a Purkinje cell can be generated by a single pre-synaptic impulse in the climbing fibre or by a brief high frequency burst of impulses. In addition to using single pulses for climbing fibre activation, the effects of bursts of 2-4 pulses at 500 Hz (delivered at the same overall rates as the single pulses) were also tested. Bursts of climbing fibre impulses were used to assess how the natural pattern of climbing input from the parent inferior olive cells affect the Purkinje cell.

When stimulating with bursts of stimuli, a stimulus strength of twice the threshold for evoking a complex spike with a single pulse was used to ensure secure driving of the climbing fibre at 500 Hz. Responses were constantly monitored on an oscilloscope to ensure that complex spikes were generated with each stimulus.

4.3 Results

4.3.1 General comments

Surface recordings of the cerebellar cortex were made in each experiment prior to climbing fibre removal to determine the region of cortex that displayed climbing fibre responses to weak stimuli applied through the stimulating electrode located in the inferior olive. Figure 4.2A shows an example of a climbing fibre field response evoked on the surface of the cortex. The characteristics and latency of the response were identical to those described in the previous chapter (Section 3.3.1).

Detailed observations were made on the discharge patterns of 75 Purkinje cells whose particular climbing fibre could be activated by stimulating its axon in the inferior olive. Purkinje cell recordings were generally very stable with little fluctuation in spike amplitudes that ranged from 400 μV-2 mV. Observation periods ranged from 30 minutes up to 4 or 5 hours. None of the cells studied showed obvious signs of injury discharge, such as variations in spike amplitude or burst-like firing. Once a Purkinje cell was isolated with a good signal to noise ratio, no deliberate attempt was made to maximise the size of the action potential in order to minimise the risk of mechanical stimulation or damage by the microelectrode. Purkinje cell identification was based on the presence of spontaneous complex spikes and complex spikes evoked by inferior olive stimulation. Figure 4.2B shows an example of a Purkinje cell with an all-or-nothing complex spike response at threshold strength of climbing fibre activation. Such cells showed a constant latency complex spike that responded consistently to stimuli just above threshold. Occasionally, complex spikes were evoked inconsistently and with a variable latency. These responses could have been due to activation of the inferior olive cells by stimulation of afferents, and were not studied further. The firing rates of the Purkinje cells ranged from 5-148 Hz, with a mean rate of 62.7 ± 3.5 Hz.

4.3.2 Effects of climbing fibre frequency on Purkinje cell discharge.

Activation of climbing fibres at various frequencies evoked a complex spike for each stimulus in each Purkinje cell studied. The effect of this on Purkinje cell simple spike activity was examined in detail. It was found that increasing the frequency of climbing fibre stimulation had an inhibitory effect on tonic simple spike firing rates and decreasing the frequency of climbing fibre stimulation caused simple spike rates to rise. Low frequency stimulation of climbing fibres within the range of 0.1-3.5 Hz and with bursts of 1-4 pulses was found to decrease simple spike activity of Purkinje cells in a gradable manner. Simple spike activity was blocked invariably by stimulation at 4 Hz if used for a sufficient period of time. Climbing fibre stimulation at rates of 4 Hz and above always blocked simple spike activity quite rapidly, but such rates approach the end of the physiological range (Harvey et al., 1977; Rawson & Tilokskulchai, 1981b; Demer et al., 1985).

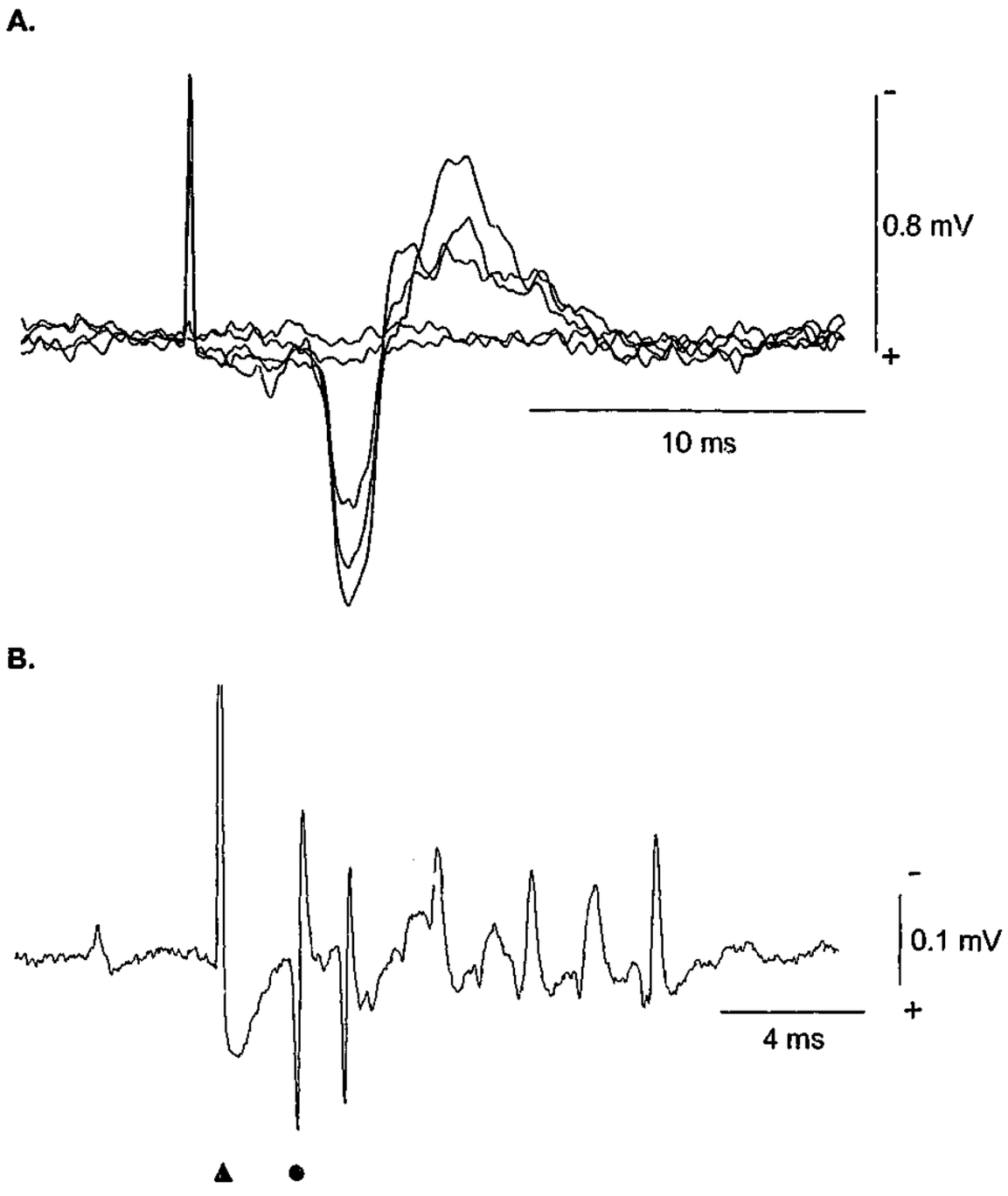


Figure 4.2

Identification of responses mediated by stimulation of the climbing fibres via the inferior olive. **A.** Superimposed records of evoked climbing fibre field responses recorded from the surface of the cerebellar cortex. **B.** An example of an evoked complex spike generated by inferior olive stimulation. The filled triangle indicates the stimulus artefact. Complex spike is marked by a dot at the initial response.

An example of climbing fibre activation within a limited range is shown in Figure 4.3, which illustrates the effects of varying climbing fibre frequency from 0.25-3.5 Hz with single pulses over a duration of two minutes. As the frequency of stimulation was increased, there was an increase in the depth of simple spike suppression that appeared to be gradable, depending on the frequency of climbing fibre stimulation (Figure 4.3A-H, Figure 4.5A). Increasing the frequency of climbing fibre activation also appeared to shorten the latency of onset of the inhibitory effect, and increased the time taken for simple spike activity to return to control levels upon cessation of stimulation (Figure 4.3E-H).

A similar pattern of response was seen when the number of pulses used in the stimulus paradigm was increased from single to double pulses. Figure 4.4A-H demonstrates an example of a Purkinje cell stimulated with 2 pulses and with varying frequencies of 0.25-3 Hz. As was seen with single pulses, increasing climbing fibre frequency increased the level of simple spike suppression (Figure 4.5B) while decreasing the latency of inhibition, and increasing the time taken for simple spike rates to return to control levels of discharge upon cessation of climbing fibre stimulation. The same effects were evident when the number of pulses were increased from 2 to 3 (Figure 4.5C). Thus, regardless of pulse number, a limited range of climbing fibre frequency was found to modulate Purkinje cell background simple spike activity over its entire range.

In most cells, stimuli were also delivered in 'steps', to examine the effects of sustained climbing fibre activation using physiological firing rates. Modest inhibitory effects were seen with low repetition rates, whereas increasing climbing fibre frequencies could modulate the depth of tonic simple spike to a greater degree. Illustrated in Figure 4.6 is a Purkinje cell that was stimulated in slow step increments from 0.5-2.5 Hz with 3 pulses at 500 Hz. With each change in climbing fibre frequency, tonic Purkinje cell simple spike activity increased or decreased in a stepwise fashion, with the level of activity dependant on the frequency of stimulation. It should also be noted that the time course needed to cause a change in simple spike discharge by climbing fibre stimulation at the lower end of the range was of the magnitude of a few seconds. This time course decreased with increasing rates of climbing fibre stimulation.

The directional change of simple spike frequency also appeared to be dependant on the previous history of climbing fibre stimulation with its corresponding simple spike discharge rate. Simple spike activity decreased when climbing fibre frequency was stepped from lower to higher repetition rates, and vice versa. Figure 4.7A illustrates

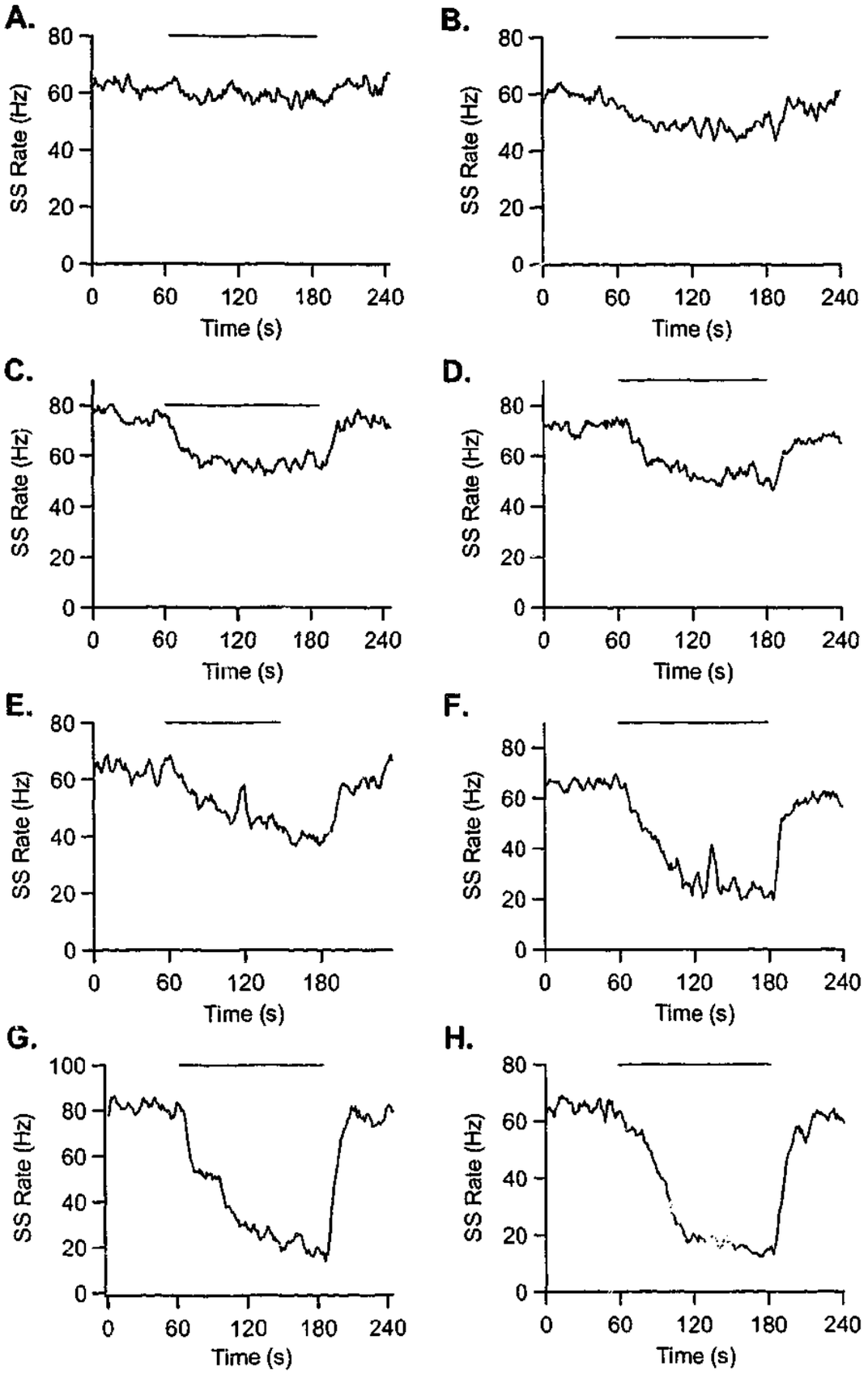


Figure 4.3

Effects of varying climbing fibre frequency on the simple spike rate of a Purkinje cell. In each trace, single pulses were employed. Period of stimulation is indicated by the length of the red bar. In each record, the duration of stimulation was 2 minutes. **A.** 0.25 Hz **B.** 0.5 Hz **C.** 0.75 Hz **D.** 1 Hz **E.** 1.5 Hz **F.** 2 Hz **G.** 3 Hz **H.** 3.5 Hz.

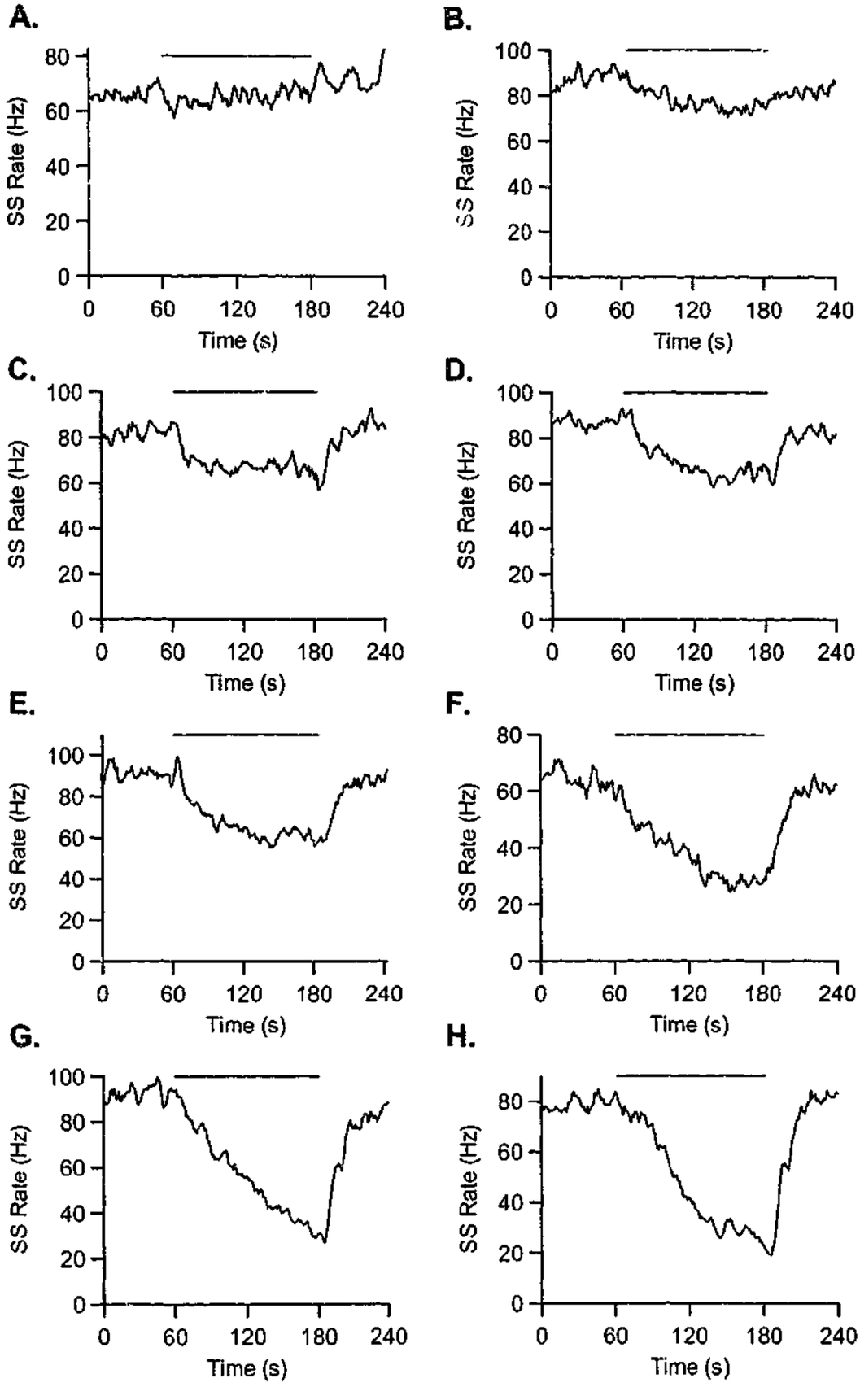


Figure 4.4

Effects of varying climbing fibre frequency on the simple spike rate of a Purkinje cell. This time, double pulses were employed. Period of stimulation is indicated by the length of the red bar. In each record, the duration of stimulation was 2 minutes. **A.** 0.25 Hz **B.** 0.5 Hz **C.** 0.75 Hz **D.** 1 Hz **E.** 1.5 Hz **F.** 2 Hz **G.** 2.5 Hz **H.** 3 Hz.

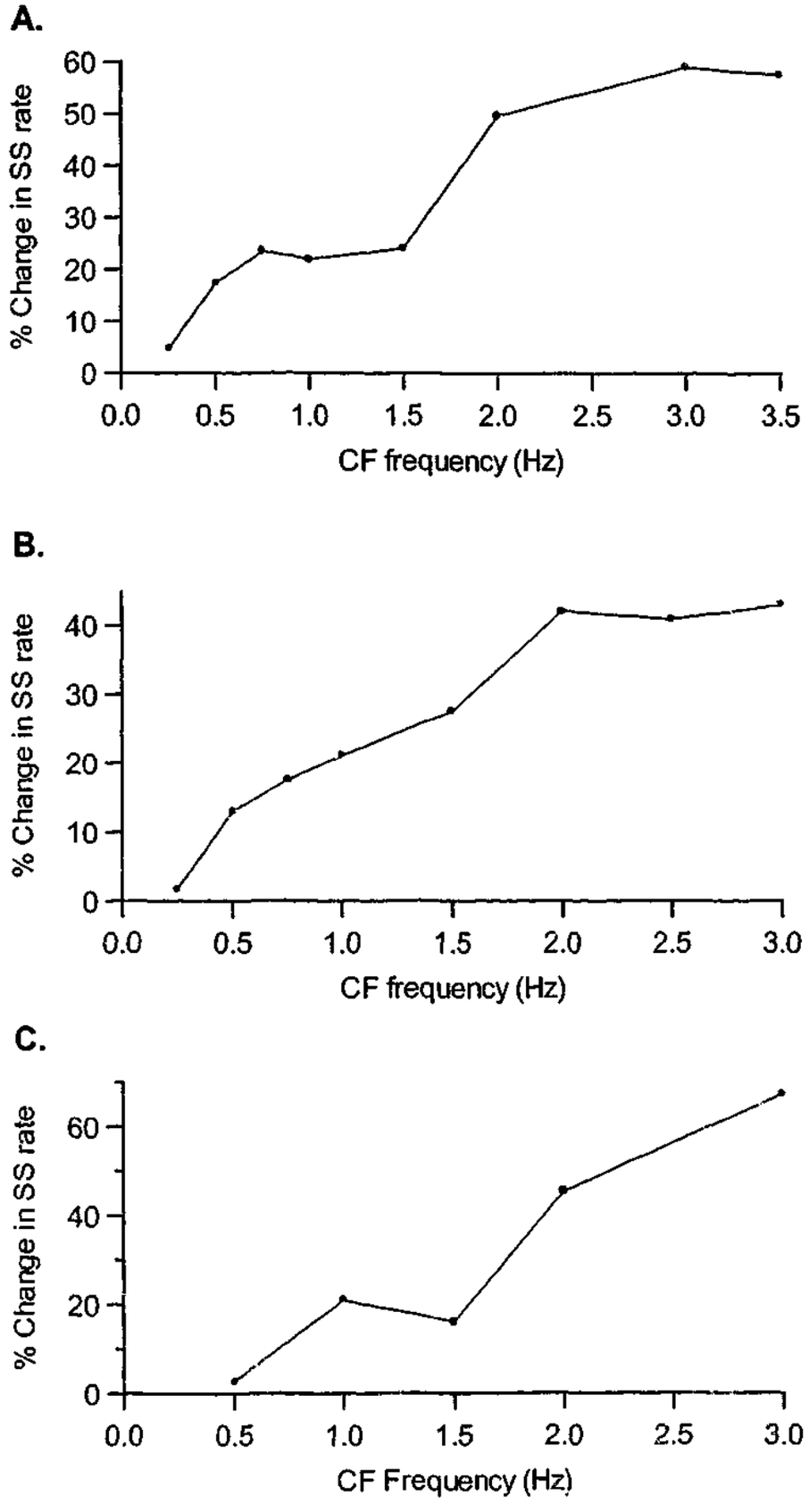


Figure 4.5

Effects of increasing the repetition rate of climbing fibre stimulation on Purkinje cell simple spike discharge. **A.** Stimulation with single pulses. **B.** Stimulation with bursts of 2 pulses. **C.** Stimulation with bursts of 3 pulses.

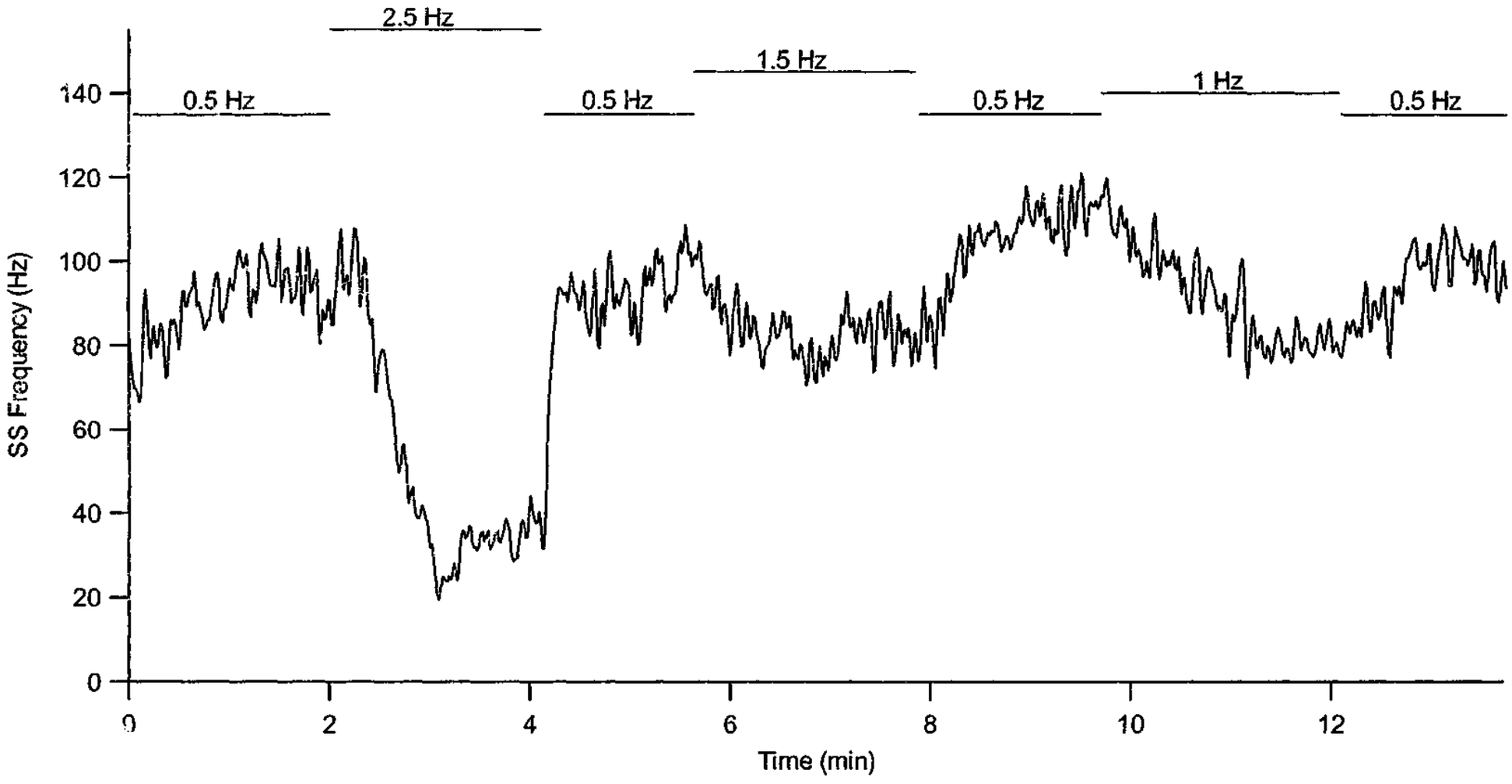


Figure 4.6

The effects of climbing fibre stimulation, delivered in steps, on Purkinje cell simple spike frequency. Climbing fibre frequency varied from 0.5 - 2.5 Hz, delivered in bursts of 3 pulses for the duration indicated by the red bar.

another example of a Purkinje cell with its climbing fibre stimuli delivered in steps. Climbing fibre stimulation consisted of single pulses at frequencies ranging from 0.1-2 Hz. Upon each change in climbing fibre frequency, simple spike rates were altered. Moreover, it was evident that each stimulus frequency was capable of sustaining a particular level of simple spike discharge that was not altered until the next change of stimulus delivery rate, even for frequencies as low as 0.25 Hz and 0.1 Hz. Even at very low rates of tonic simple spike discharge, small step-like changes in climbing fibre frequency could modulate background simple spike activity in an obvious manner (Figure 4.7B).

Another interesting feature that was observed in several of the Purkinje cells was that repetitive climbing fibre stimulation could increase the simple spike firing rate. This short unexplained facilitation in simple spike activity usually preceded the simple spike inhibition (Figure 4.7A, Figure 4.8). Figure 4.8 illustrates such an example. An increase in tonic simple spike discharge occurred at repetition rates of 2.5 and 3 Hz. At 2.5 Hz, the simple spike rate rose slowly over a period of a minute, after which, it declined, with a greater level of suppression developing as climbing fibre stimulation was continued. A similar effect was seen when the climbing fibre was stimulated at 3 Hz, although the increase appeared more rapidly with the increase in stimulus frequency, and persisted for a shorter period than at 2.5 Hz. This increase did not occur at stimulation rates of 1 Hz.

Whether the level of background activity determined if a Purkinje cells simple spike frequency would first increase before decreasing upon climbing fibre stimulaion was examined, as at first, it appeared that this phenomenon occurred when tonic activity was approximately 80-100 Hz. In a number of cells this appears to be true (Figure 4.7A, Figure 4.9C and D). In other cells (Figure 4.9A and B) with a background discharge of 50-70 Hz and stimulated at high repetition rates of 3 and 2 Hz, this effect did not occur. Therefore, this finding indicates that resting levels of simple spike discharge could be responsible for the facilitatory effect observed in some Purkinje cells. However, in a proportion of cells, the increase that preceded the suppression, occurred in cells that had a tonic discharge rate lower than 80 Hz (Figure 4.8). It should be noted however, that in these cases, the facilitatory effect was only seen when stimulus frequencies were greater than 2 Hz and with bursts of pulses. Therefore, it is possible that in some cells, climbing fibre frequency and pulse number influences the rise in simple spike rate via mechanisms yet to be determined.

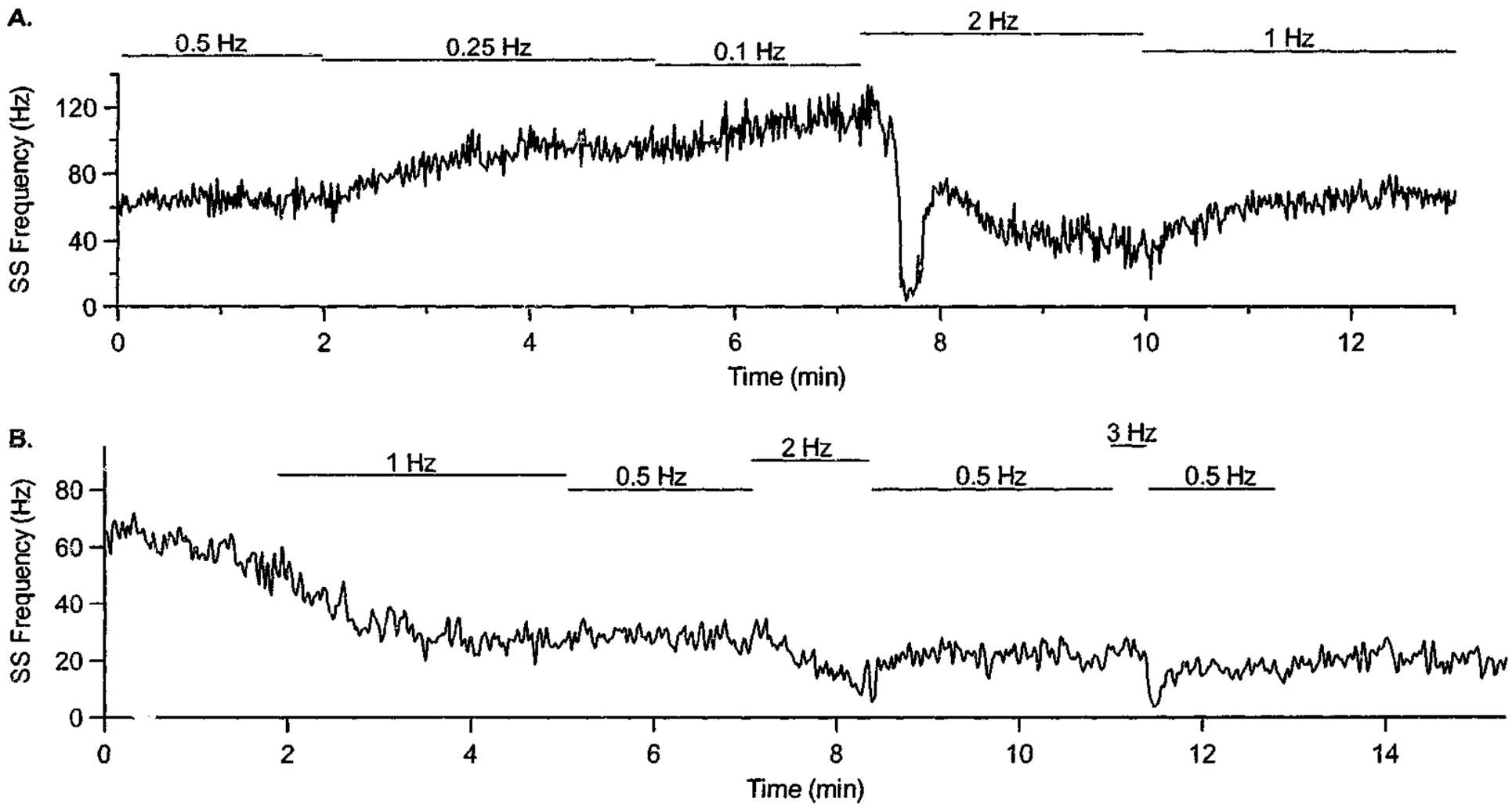


Figure 4.7

Effects of varying climbing fibre frequency delivered in steps. The duration of stimulation is indicated by the red bar. **A.** Single pulses. **B.** Bursts of 3 pulses.

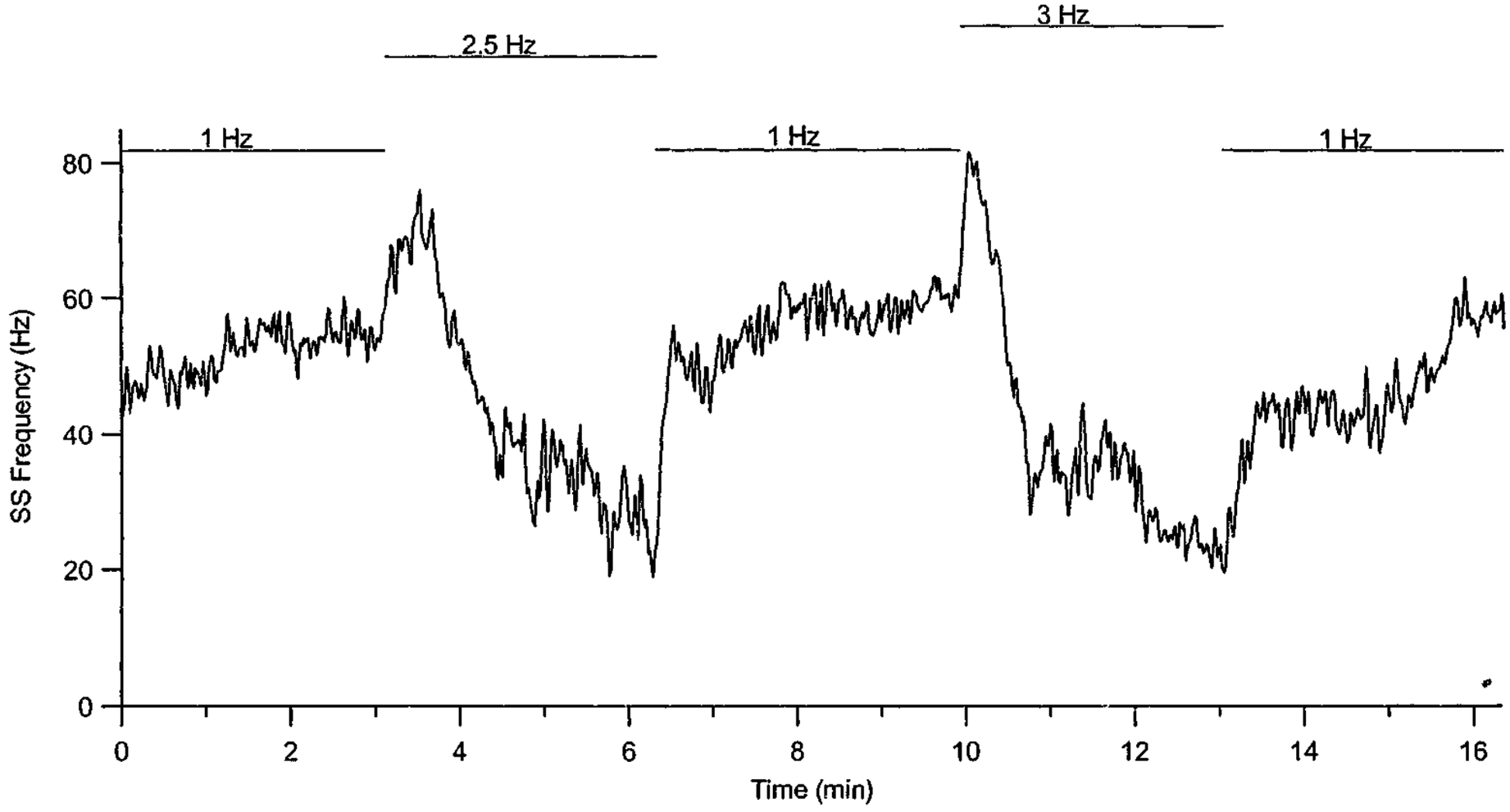


Figure 4.8

Effects of repetitive climbing fibre stimulation delivered in a step like manner. Stimulus consisted of bursts of 3 pulses, delivered for the duration indicated by the red bar.

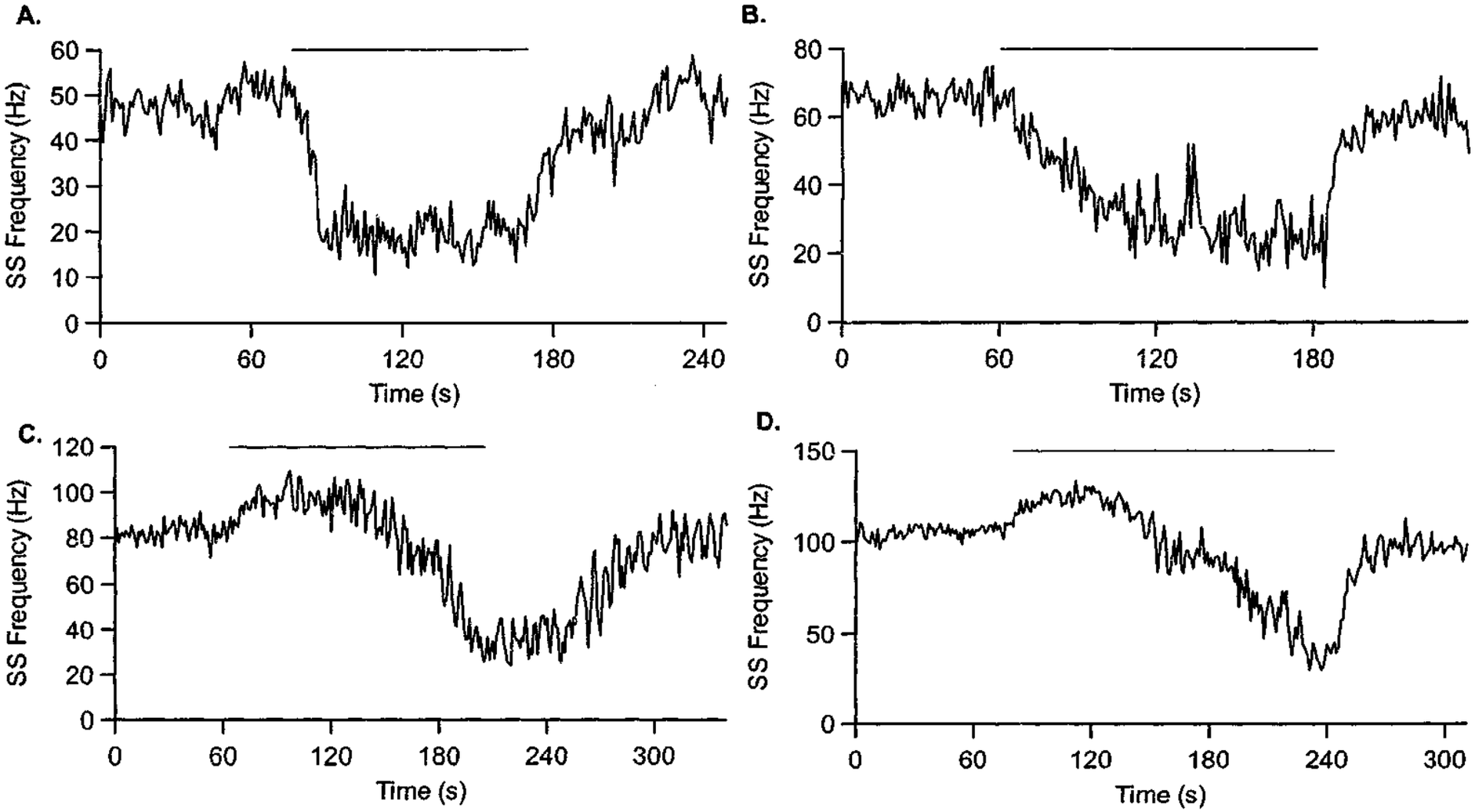


Figure 4.9

A comparison of whether the level of background simple spike activity affects Purkinje cell responses to climbing fibre stimulation. **A.** 3 Hz stimulation, double pulses **B.** 2 Hz stimulation single pulses **C.** 2 Hz stimulation, 2 pulses **D.** 2 Hz stimulation single pulses.

4.3.3 Effects of pulse number on simple spike discharge

Stimulation of climbing fibres with bursts of pulses was also found to play an important role in modifying tonic simple spike discharge. Repetitive activation of the climbing fibre input to a Purkinje cell with bursts of pulses produced a suppression of the simple spike activity as described above. Increasing the number of pulses enhanced the effectiveness and potency of simple spike suppression. Examples of increasing the number of pulses to activate the climbing fibres to a Purkinje cell are shown in Figure 4.10. Figure 4.10A demonstrates the effect produced by stimulating the climbing fibre at 0.5 Hz, with increasing pulse number from 1 to 2 pulses, delivered at 500 Hz. Clearly, the level of activity is influenced by the number of pulses used for climbing fibre activation, with 2 pulses having a greater inhibitory effect than 1 pulse. With increasing pulse number, there appeared to be a concomitant increase in level of inhibition. The same effect was evident when other climbing frequencies were investigated (Figure 4.10B and C). In the example shown in Figure 4.10B, climbing fibre inputs were generated at a rate of 2 Hz. Again, 2 pulses had a greater inhibitory effect on Purkinje cell simple spike rates than when single pulses were used. Moreover, the increase in climbing fibre frequency proved more effective than that of 0.5 Hz in Figure 4.10A. A comparison of Figure 4.10A with Figures 4.10B and C reveals that increasing the stimulus frequency also increases the degree of simple spike depression. In addition, it was noted that an increase in the number of pulses also led to a decrease in the onset of simple spike suppression. Typically, as shown in Figure 4.10C, 2 pulses had a greater effect in suppressing tonic simple spike activity than single pulses. When 3 pulses were applied, the difference in simple spike suppression did not differ markedly from that of 2 pulses, however the latency of onset of inhibition was considerably reduced.

4.3.4 Possible mechanisms of simple spike suppression by climbing fibre stimulation

The effects of climbing fibre stimulation that was subthreshold for the production of complex spikes did not produce a suppression or reduction in simple spike activity of the Purkinje cells under examination. Suppression of simple spikes was only observed when the stimulus intensity for climbing activation was at or above threshold. As can be seen in Figure 4.11A, stimuli that failed to evoke complex spikes in the Purkinje cell did not produce a reduction in simple spikes during stimulation. When the stimulus strength was increased to threshold, at the point indicated by the arrow, a reduction in simple spike activity was observed. This was also evident when the PSTHs were examined.

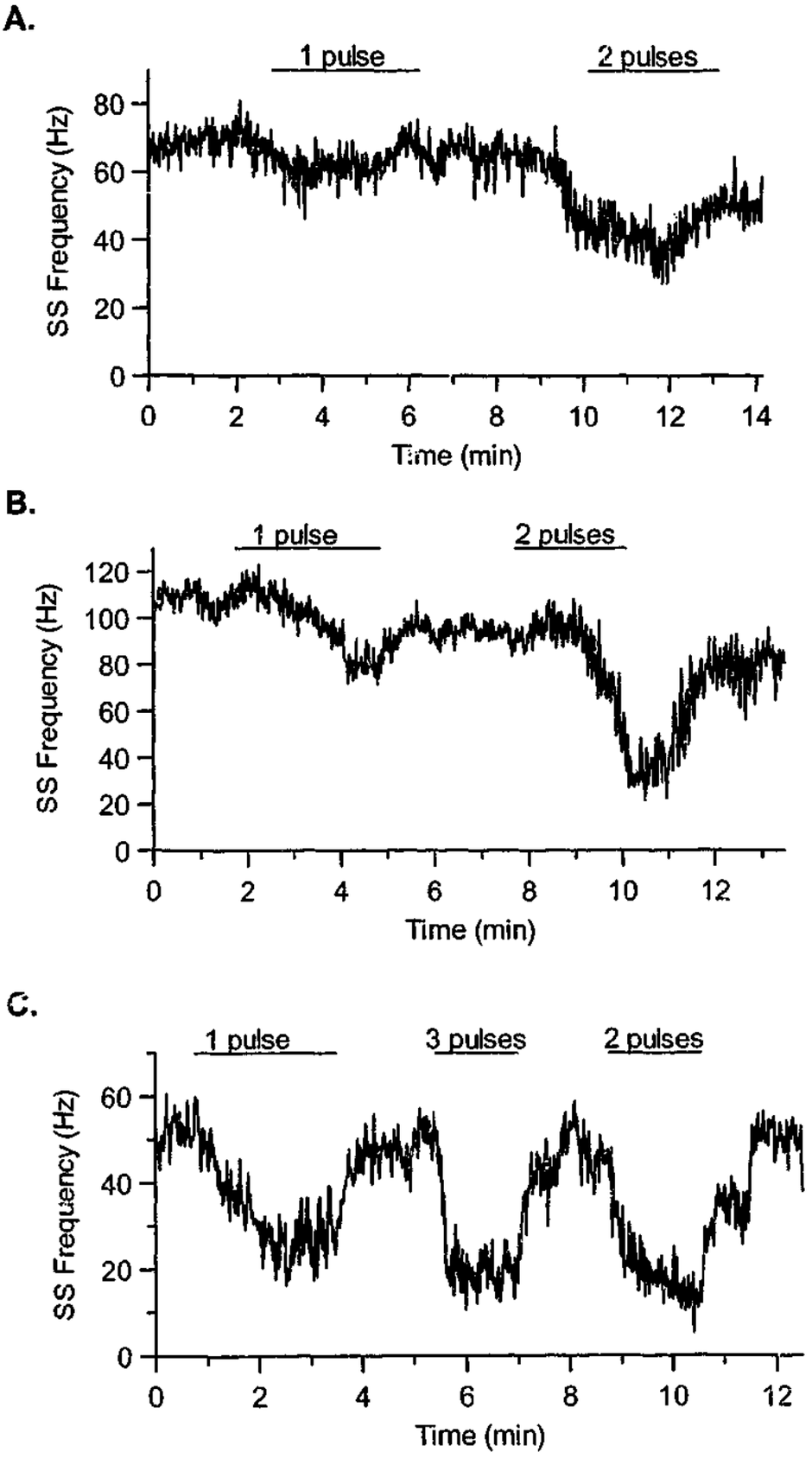


Figure 4.10

Depression of Purkinje cell simple spikes by trains of single and bursts of climbing fibre stimuli. The period of stimulation is indicated by the red bars. **A.** 0.5 Hz stimulation with single and double pulses. **B.** 2 Hz stimulation with single and double pulses. **C.** 3 Hz stimulation with 1, 2 and 3 pulses.

Little or no effect to simple spike discharge was seen during subthreshold stimulation of the climbing fibres (Figure 4.11Bi.), whereas a significant inhibitory effect was evident in the period of suprathreshold stimulation (Figure 4.11Bii.). A similar effect was seen with the reverse situation, first at suprathreshold strengths for climbing fibre activation followed by a period of subthreshold stimulation (Figure 4.12).

Since each Purkinje cell is innervated by only one climbing fibre in the adult, it seems reasonable to conclude that the powerful inhibitory effect on simple spike activity that was observed was predominantly and exclusively due to a direct action of the climbing fibre on the Purkinje cell it innervates. One question that arises is whether some of the inhibitory effect is attributable to the possible activation of inhibitory neurones by climbing fibre collaterals.

4.3.5 Effects of climbing fibre stimulation on cerebellar cortical interneurones

The effects of climbing fibre inputs on cortical inhibitory interneurones were examined to determine whether the decrease in tonic simple spike activity observed during climbing fibre stimulation might have resulted from the activation of inhibitory interneurones. Interneurones studied were located in regions of cortex where inferior olive stimulation evoked a large climbing fibre field potential and where Purkinje cell simple spike activity was depressed by climbing fibre inputs.

Neurones were categorised as cerebellar interneurones based on their depth in the cerebellar cortex, spike amplitude, and firing rate. The firing characteristics of cerebellar interneurones differed markedly from Purkinje cells, firing more regularly at rates of 4-30 Hz, compared to 30-50 Hz for Purkinje cells (Eccles et al., 1966c; Vos et al., 1999b). Neurones were assumed to be stellate interneurones when they were located in the superficial part of the cerebellar cortex, at depths of 100-300 μm below the surface, and with very small spike potential amplitudes (Eccles et al., 1966c). Basket cells were located deep in the molecular layer just above the Purkinje cell soma. Cells presumed to be Golgi cells generated large spike potentials, and were located in the granular layer, below the cell bodies of the Purkinje cells. Apart from depth below the surface, the most notable feature that indicated that the cells were cerebellar interneurones was the absence of spontaneous complex spikes and evoked complex spikes following stimulation of the inferior olive, even at strengths of up to 1 mA.

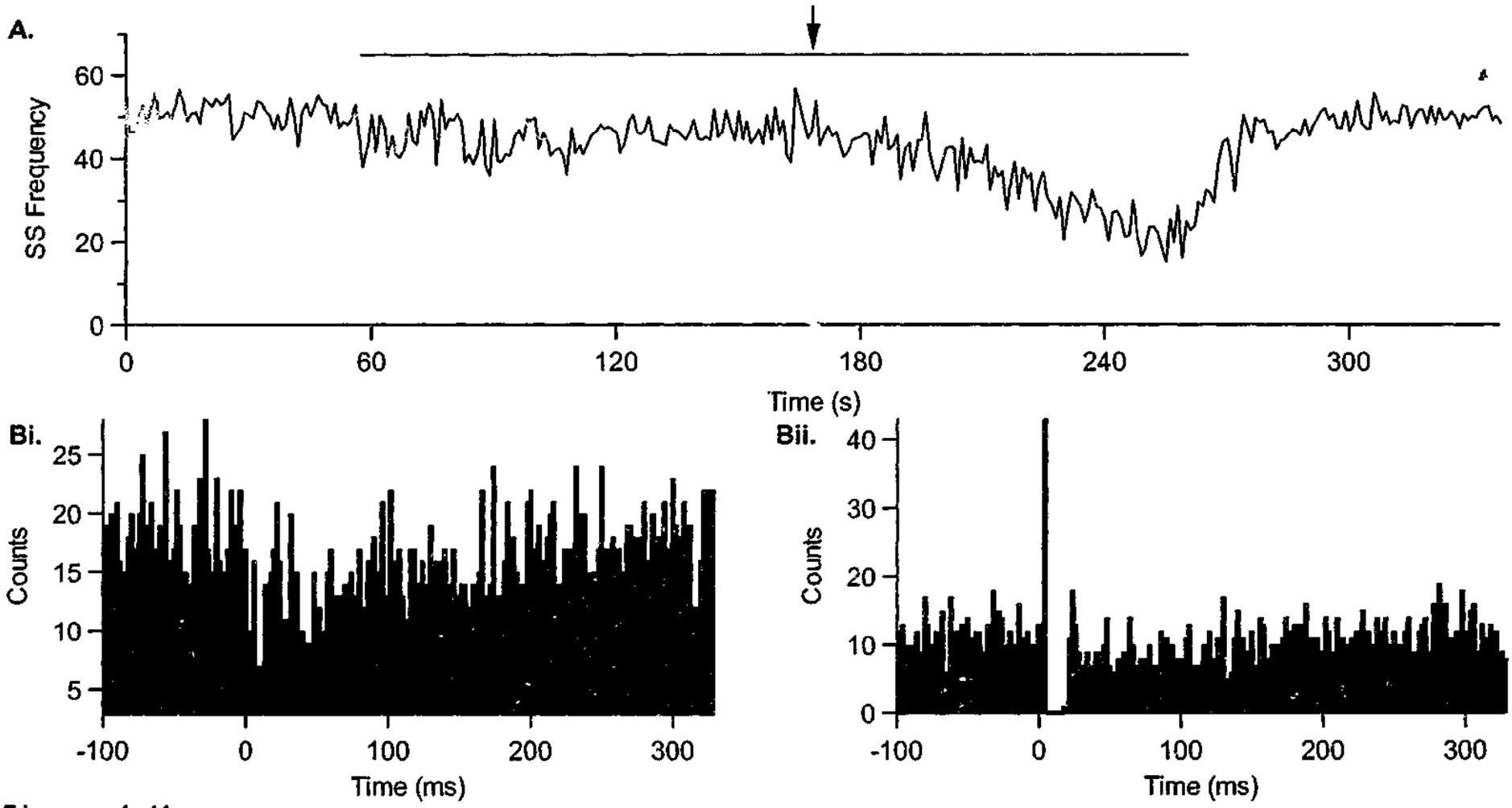


Figure 4.11

Possible mechanisms underlying inhibition of simple spike activity by climbing fibre stimulation. A. Train of 2 pulses at 3 Hz which were subthreshold for evoking complex spikes, failed to reduce simple spike rate. When stimulus strength was increased to above threshold, at the point indicated by the arrow, simple spike frequency was suppressed. The bar indicates the period of stimulation. (Threshold=23.5 μA). Bi. & Bii. PSTHs for subthreshold and suprathreshold stimulation. (n=169 and 140 respectively).

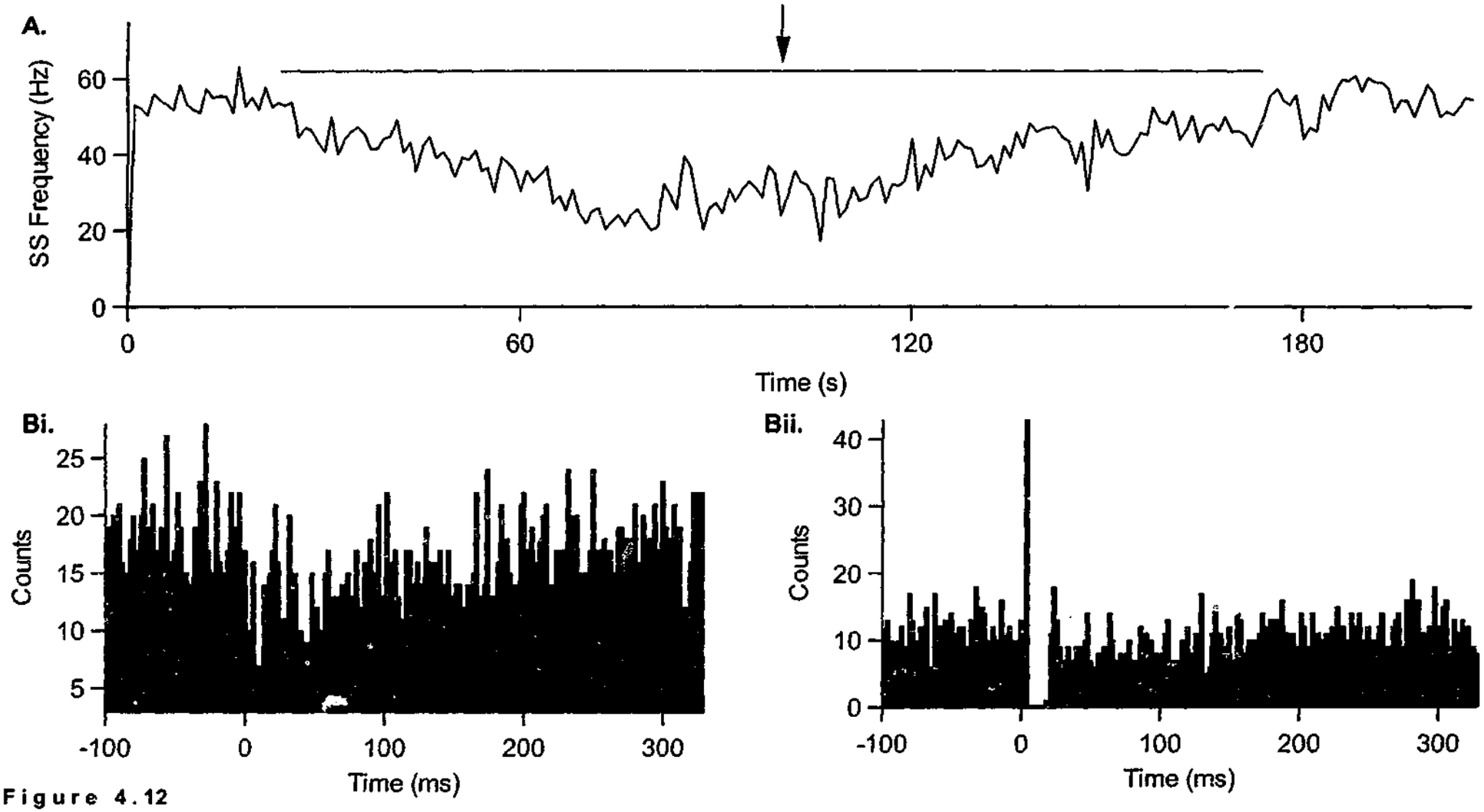


Figure 4.12

Possible mechanisms underlying inhibition of simple spike activity by climbing fibre stimulation. **A.** Train of 2 pulses at 3 Hz above threshold for evoking complex spikes suppressed simple spike activity. When stimulus intensity was decreased to subthreshold strengths, at the point indicated by the arrow, simple spike frequency returned to control levels. The bar indicates the period of stimulation. (Threshold=23.5 μA). **Bi.** & **Bii.** PSTHs for subthreshold and suprathreshold stimulation. (n= 170 & 120 respectively).

The effects of climbing fibre stimulation on the discharge properties of cerebellar interneurones was examined for 11 molecular layer interneurones (basket and stellate cells) that had a firing rates of 5.2-31.9 Hz. The activity of all 11 cells was unaltered by climbing fibre stimulation as shown in the PSTHs in Figure 4.13. Therefore, the inability of climbing fibres to induce facilitatory responses in molecular layer interneurones means that they are unlikely to be responsible for the suppression observed in Purkinje cell simple spike activity.

In addition, 7 neurones classified as Golgi cells, with discharge rates ranging from 9.1-27.7 Hz, were also found to be uninfluenced by climbing fibre inputs (Figure 4.14). A further 2 Golgi cells were substantially inhibited by stimulation of climbing fibres. As shown in Figure 4.15A, 1 Hz stimulation with 2 pulses exerted a inhibition of Golgi cell discharge during stimulation. The level of inhibition increased with increasing stimulation frequency (Figure 4.15B and C). This inhibitory response could be due to the excitatory effect of climbing fibres on Purkinje cells whose axon collaterals have contact with Golgi cells (Scheibel & Scheibel, 1954; Hamori & Szentagothai, 1966; Palay & Chan-Palay, 1974; Sugihara et al., 1999; Shinoda et al., 2000). It is clear however, that climbing fibre stimulation did not facilitate Golgi cell discharge. The inhibition observed in some of the Golgi cells examined would not be expected to account for the depression of simple spike discharge seen in Purkinje cells during stimulation of the climbing fibres, as the opposite effect might be expected by virtue of Golgi cell inhibition of granule cells.

4.3.6 Purkinje cells that did not follow previously described patterns of responses

While the majority of the Purkinje cells examined behaved in the manner described in the previous sections, 5 of the 75 sampled cells did not follow the reported pattern of simple spike discharge upon climbing fibre stimulation, as illustrated with 2 examples in Figure 4.16A and B. The manner in which these cells responded was unexpected and inconsistent with the behaviour Purkinje cells previously described in this study. Rather than inhibition of tonic simple spike activity during climbing fibre stimulation, these cells increased their tonic simple spike activity. Simple spike discharge remained elevated for the duration of climbing fibre activation, slowly returning to control levels once stimulation was suspended. The mechanism responsible for the increase in tonic simple spike activity is not known, but will be speculated on in the discussion.

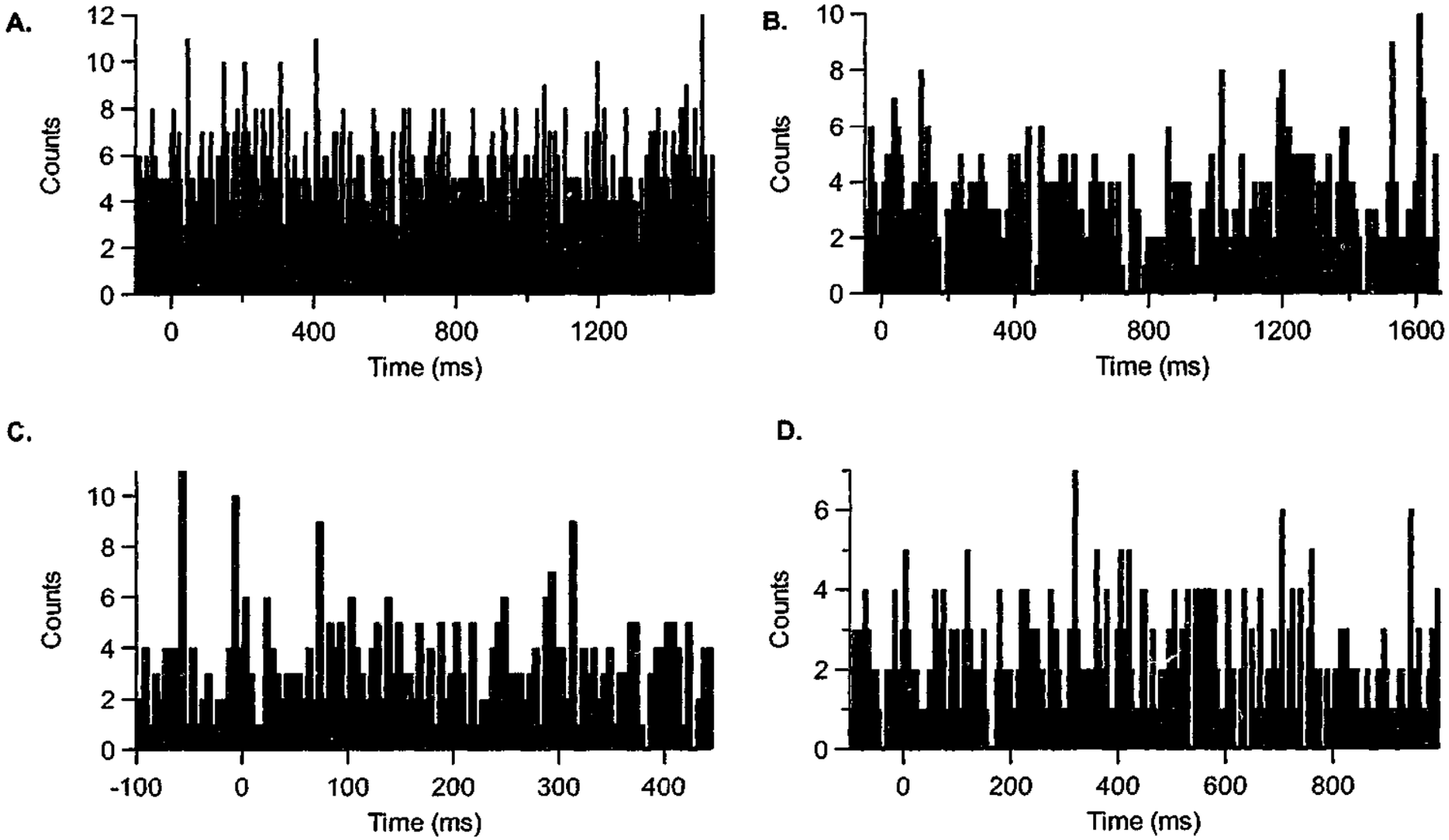


Figure 4.13

PSTH showing summed responses of cerebellar interneurones to climbing fibre stimulation. **A.** Interneurone stimulated at 1.5 Hz (n=50). **B.** Interneurone stimulated at 1 Hz (n=60). **C.** Interneurone stimulated at 2 Hz (n=60). **D.** Interneurone stimulated at 1 Hz (n=100).

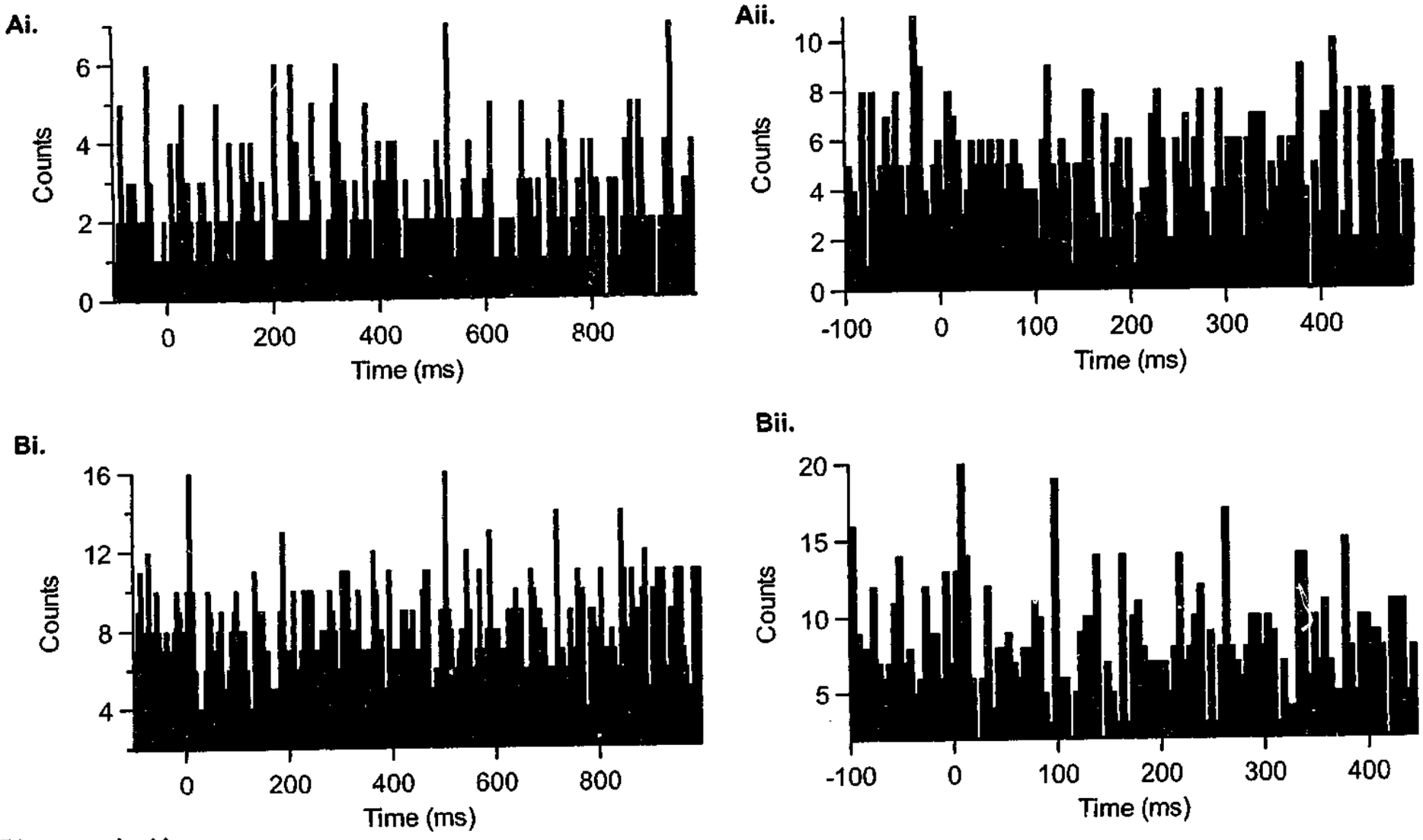


Figure 4.14

PSTHs of four Golgi cells showing a lack of responses to climbing fibre stimulation. Ai. & Aii. Single pulses at 1 and 2 Hz stimulation respectively. Bi. & Bii. Single pulses at 1 and 2 Hz stimulation respectively.

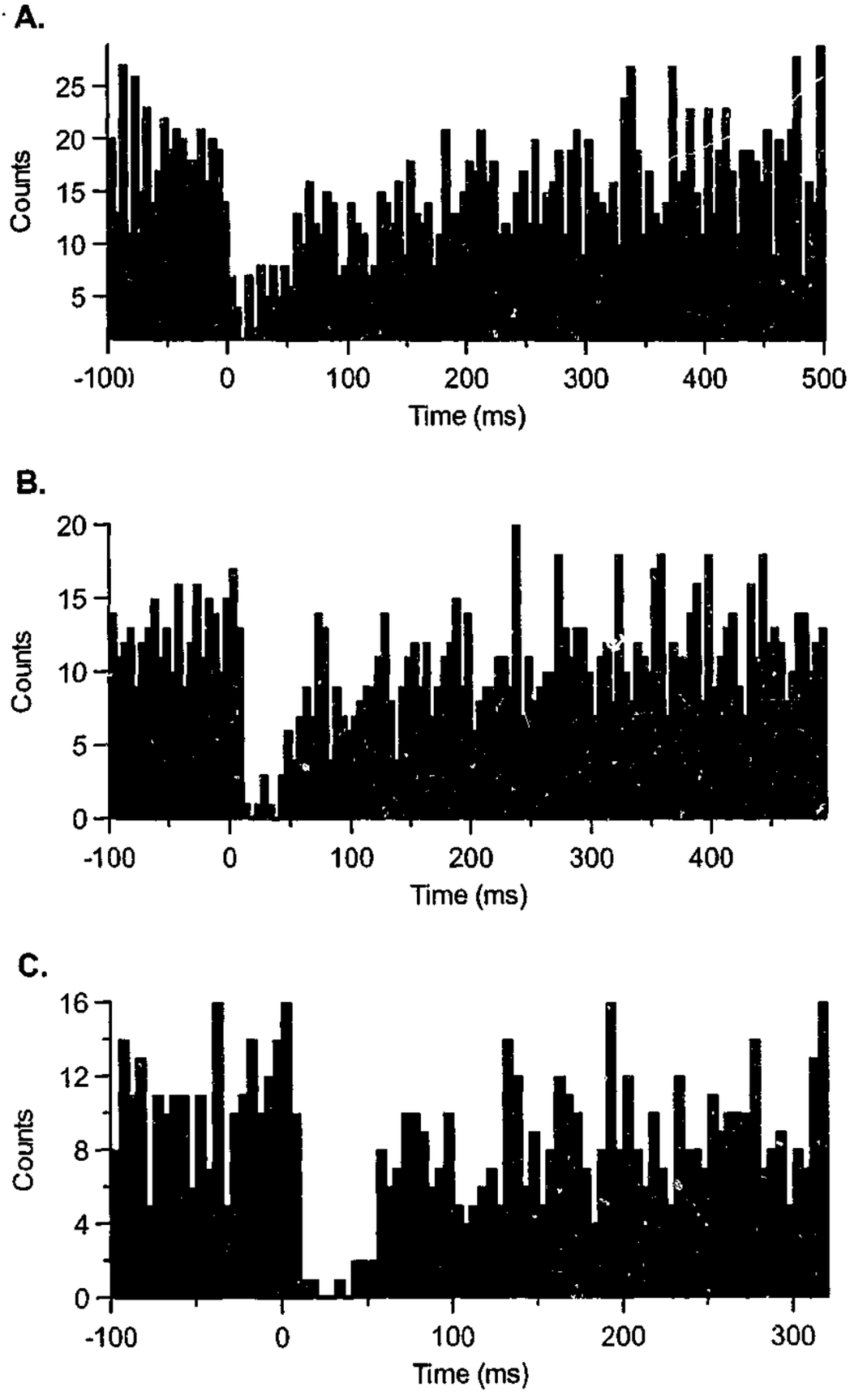


Figure 4.15

PSTHs of a Golgi cell to climbing fibre stimulation, consisting of single pulses delivered at A. 1 Hz (n=152) B. 2 Hz (n=105) and C. 3 Hz (n=127). Climbing fibre stimulation exerted a powerful inhibitory effect on Golgi cell discharge.

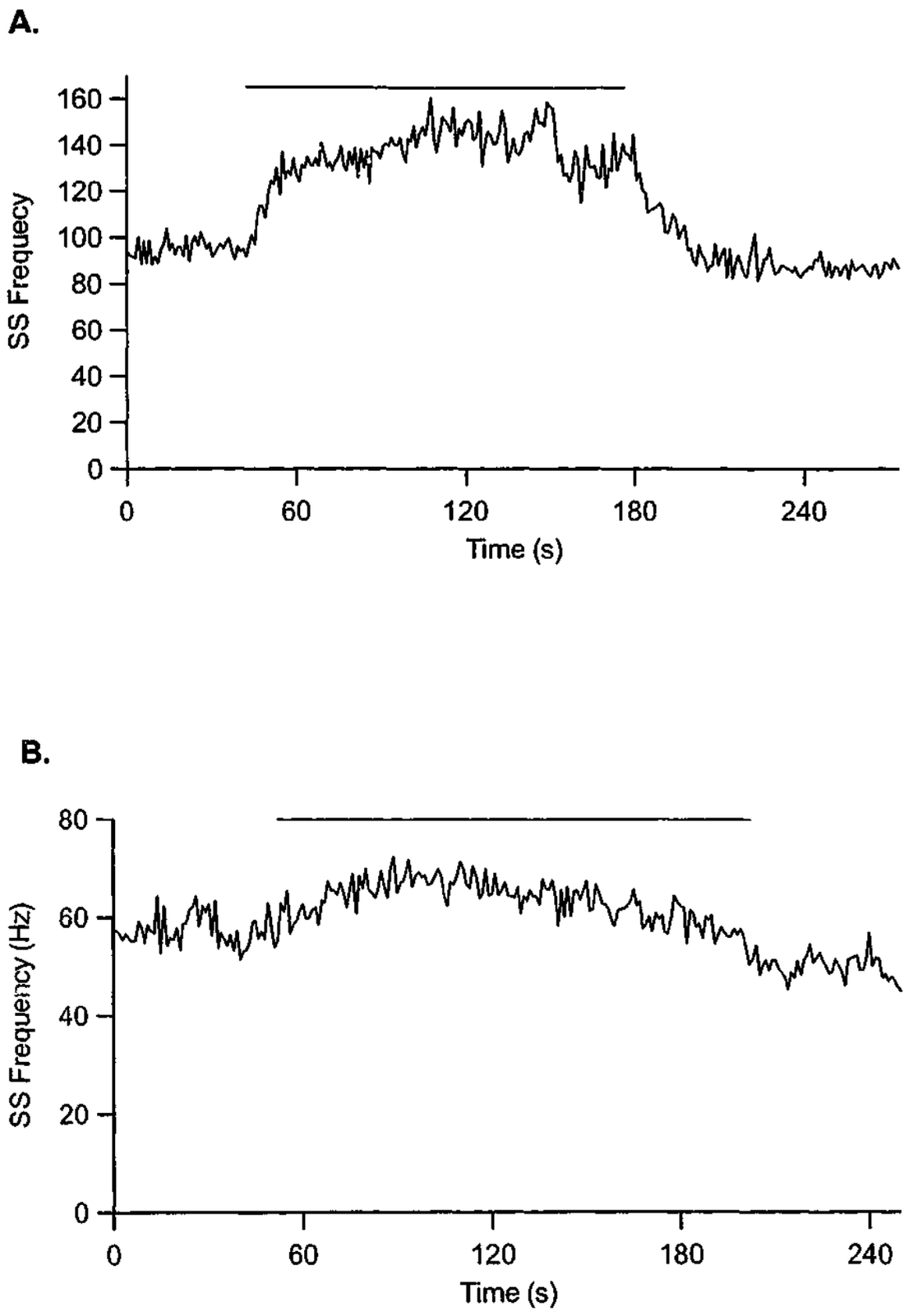


Figure 4.16

Effect of climbing fibre stimulation on Purkinje cell discharge displaying responses unlike those described previously. Five of the sampled 75 Purkinje cells behaved in this way. **A.** 0.5 Hz stimulation with single pulses. **B.** 3 Hz stimulation with single pulses.

4.3.7 Effects of climbing fibre stimulation on Purkinje cells infused with CNQX

The effects of climbing fibre stimulation on the intrinsic simple spike activity of Purkinje cells, once AMPA receptors were blocked with 50 μM CNQX was investigated in a further 2 Purkinje cells (data not shown). Parallel fibre transmission of these Purkinje cells was blocked with CNQX, leaving only simple spikes generated by the intrinsic spike generator. Climbing fibre stimulation depressed simple spike activity in these Purkinje cells in the same way described previously, both in terms of variation in climbing fibre frequency and the number of climbing fibre impulses. Therefore, it appears that the climbing fibres are capable of regulating the activity of the intrinsic spike generator to control the normal background level of Purkinje cell simple spike discharge.

4.4 Discussion

The present study was undertaken to examine the changes to Purkinje cell ongoing simple spike activity that occurs with controlled electrical stimulation of their climbing fibre inputs. The present study differs from previous studies in that the pattern of climbing fibre stimulation was designed to approximate the natural pattern of climbing fibre discharge. Previous studies used stimulation rates of up to 10 Hz, which, without the use of chemical excitants, does not occur in normal conditions. The results from the present study have shown that low frequency stimulation of the climbing fibres can modulate the level of tonic simple spike activity in Purkinje cells. Stimulation of climbing fibres over a limited range of frequencies was found to modulate the ongoing simple spike activity, with responses ranging from 20 to 120 Hz. The frequency of stimulation and the number of pulses used to stimulate climbing fibres influenced the level of simple spike activity. The modulation of simple spike discharge appears to occur from a direct action on the Purkinje cell, as inhibition was found to be tightly linked to the occurrence of complex spike activity that signalled the occurrence of climbing fibre stimulation. Moreover, substantial changes in simple spike rate were found to occur without the accompanying excitation of inhibitory neurones.

4.4.1 Comparison with previous studies

The results of the present study are consistent with earlier studies and provide direct support for the proposition that climbing fibres can reduce Purkinje cell simple spike discharge by a direct action on the Purkinje cell (Colin et al., 1980; Rawson & Tilokskulchai, 1981b; Demer et al., 1985). The results have further demonstrated that

climbing fibre activity within its known physiological range can precisely control the tonic activity of Purkinje cells produced by their intrinsic pacemaker. Furthermore, the modulation was dependent not only on the frequency of the climbing fibre stimulation, but also on the number of impulses delivered during stimulation. However, the effects described here differ somewhat to those described by early investigators. Rawson and Tiloskulchai (1981b) found that climbing fibre activation at rates of 1-2 Hz with single pulses did not cause a reduction in simple spike activity that was observed in the present study. Instead, stimulation rates of 2 Hz with bursts of impulses, or stimulation at rates greater than 4 Hz with single pulses were required to reduce simple spike activity. The disparity between the present study and that of Rawson and Tiloskulchai (1981b) could be due to a species difference (rat versus cat), or may have been influenced by the different type of anaesthesia used. The discrepancy may also stem from the differing manner in which the results were displayed. The current study presented the effects of climbing fibre stimulation on simple spike activity as an overall mean frequency whereas Rawson and Tiloskulchai (1981b) displayed the resultant Purkinje cell activity as a raw spike train, which may not have revealed the subtle changes in simple spike rates that occurred at low rates of climbing fibre stimulation. In addition, the changes in simple spike activity reported in the present study occurred more gradually at lower frequencies of climbing fibre stimulation, which may have been another reason why Rawson and Tiloskulchai (1981b) did not report a reduction at stimulation below 4 Hz.

Although part of this work corroborated the findings by Colin et al., (1980), a difference in the degree of modulation was found. Their study used a similar technique as that used in this thesis, whereby cells of the inferior olive were inactivated to eliminate their spontaneous firing so that activity of the climbing fibres could be controlled precisely. Colin et al., (1980) achieved this with the use of the neural toxin 3-AP to destroy the inferior olive. This removed the climbing fibre input to the Purkinje cell and, as full degeneration of the climbing fibre system does not occur until 18 hours later (Sotelo et al., 1975; Desclin, 1976), climbing fibre activity could still be produced by stimulating the fibres in the white matter. Using only single pulse stimulation, Colin et al., (1980) found a relationship between complex spike stimulation rates and simple spike discharge, with a gradual decrease in simple spike rates with increasing complex spike frequencies. However, they also found that a complete suppression of simple spike activity occurred when climbing fibres were stimulated at a rate of only 2 Hz, whereas in the current study, stimulation rates of 2 Hz with single pulses were shown to reduce but not silence Purkinje

cell discharge. A possible explanation for this could be their use of 3-AP, which may have affected Purkinje cell excitability.

The results presented in the current chapter appear contradictory to the findings of McDevitt, Ebner and Bloedel (1982), who found an increase in simple spike activity following the inactivation period evoked by spontaneous complex spikes. Although 5 of the 75 Purkinje cells sampled in the present study exhibited a maintained elevation in firing rates during climbing fibre stimulation, the great majority of the cells were in fact inhibited, or displayed an increase in firing rate prior to the observed decrease. However, several differences need to be considered when comparing this data to the findings of McDevitt, et al., (1982). The time course between the two studies is considerably different, as McDevitt, et al., (1982) examined these effects over a period of 500 ms, whereas the time course of the present study involved examining Purkinje cell responses over a period of minutes. Furthermore, McDevitt, et al., (1982) used spontaneously occurring complex spikes as the stimulus for investigating their effects on Purkinje cells while in the present study, activation of the climbing fibres was precisely controlled by blocking spontaneously occurring complex spikes and stimulating the climbing fibre axon electrically over a period of minutes.

4.4.2 Changes in simple spike activity by the climbing fibres is unlikely to occur as a consequence of cortical interneurones

Stimulation of the inferior olive caused a decrease in spontaneous simple spike discharge. Earlier studies suggested that this reduction in spontaneous simple spike activity was mediated by climbing fibre collaterals acting on cerebellar inhibitory interneurones. These can act either directly, by the excitation of stellate and basket cells, or indirectly, via the Golgi cells, which upon excitation, would in turn inhibit the granule cells and prevent transmission of the parallel fibre pathway (Eccles et al., 1966e). Thus, an examination of the effects of climbing stimulation on the inhibitory interneurones was performed to assess their possible contribution to simple spike modulation.

Molecular layer interneurones were unaffected by climbing fibre stimulation, indicating that they could not account for the profound changes that occurred to simple spike activity as a result of climbing fibre stimulation. This was not surprising given that few climbing fibre collaterals have been demonstrated to synapse with inhibitory interneurones (Hamori & Szentagothai, 1966, 1980; Sugihara et al., 1999; Shinoda et al., 2000). Electrophysiological studies examining the effect of climbing fibre stimulation on

cerebellar interneurones have also provided variable results, with effects ranging from no obvious change, to a weak excitatory or a strong inhibitory effect (Eccles et al., 1966c; Bloedel et al., 1972; Schulman & Bloom, 1981; O'Donoghue et al., 1989).

Approximately one fifth (22 %) of interneurones, putatively designated as Golgi cells were altered by climbing fibre discharge, and rather than an excitatory effect, the activity of these cells was strongly inhibited. Since Golgi cells are thought to inhibit the mossy fibre-granule cell pathway (Eccles et al., 1966e), inhibition of the Golgi cells would be expected to facilitate rather than suppress transmission of the parallel fibre pathway, leading to an increase in simple spike activity. Clearly, the observed decrease in endogenous simple spike discharge was not the result of Golgi cell inhibition.

The inhibition of Golgi cells by the activation of the inferior olive is in agreement with studies by Schulman and Bloom (1981), who also demonstrated Golgi cell inhibition by the olivocerebellar pathway in both rats and guinea pigs. As suggested by Schulman and Bloom (1981) the source of the inhibition could be due to axon collaterals from neighbouring Purkinje cells that were activated by incoming climbing fibre impulses. This suggestion is also supported by anatomical studies that have indicated that Golgi cells may receive Purkinje cell axon collaterals (Palay & Chan-Palay, 1974; Bishop, 1982; Bishop et al., 1987). One possible drawback to this explanation however, was the finding that most Golgi cells were uninfluenced by climbing fibre stimulation even though they were located in a region where inferior olive stimulation evoked large climbing fibre field potentials indicating activation of many Purkinje cells. Given that Purkinje cell axon collaterals have a limited spread of about 200 μm (Bishop & O'Donoghue, 1986; Bishop et al., 1987) one might also have expected Golgi cells to be inhibited if they received collateral inputs. It could be that Purkinje cell axon collaterals are restricted to a limited number of Golgi cells. Another possibility is a direct input to the Golgi cells from collaterals of climbing fibres (Scheibel & Scheibel, 1954; Hamori & Szentagothai, 1966; Palay & Chan-Palay, 1974; Sugihara et al., 1999; Shinoda et al., 2000). As climbing fibres release glutamate/aspartate and are excitatory to their known targets of Purkinje cells and DCN, one might expect Golgi cells to be excited by any direct climbing fibre input. However, it is interesting to note that a proportion of presumed Golgi cells were inhibited by direct application of glutamate (Yamamoto et al., 1976) suggesting that some Golgi cells may have receptors through which glutamate produces inhibition. Indeed, recent studies have demonstrated that some Golgi cells express group II mGluR which inhibit

adenyl cyclase production (Neki et al., 1996; Knoflach et al., 2001). Clearly, the possible action of climbing fibres on Golgi cells is an interesting topic for future study.

4.4.3 Proposed mechanism of climbing fibre action

It has previously been suggested that Purkinje cell activity is generated by an intrinsic mechanism that involves an interaction between Na^+, K^+ and Ca^{2+} conductances (Llinas & Sugimori, 1980a, b; De Schutter & Bower, 1994a; Raman & Bean, 1999b). The model proposed here is simpler in that the basic mechanism is a depolarization whose level is dependent on a balance between non-inactivating Na^+ and K^+ conductances. The climbing fibres are proposed to control the depolarization set by the currents via an additional Ca^{2+}-dependent K^+ current. The present results and those obtained in the previous chapter have clearly shown that climbing fibres can directly influence the pacemaker discharge of the Purkinje cell they innervate. How might the climbing fibres control and suppress the Purkinje cell intrinsic pacemaker?

The activation of the Ca^{2+}-dependent K^+ current is triggered by the rise in intracellular Ca^{2+} that occurs with climbing fibre activation, as the resultant depolarization opens P-type VOCCs which permit Ca^{2+} entry with every discharge (Tank et al., 1988; Gruol et al., 1992; Callaway et al., 1995; Eilers et al., 1995). As a consequence, Ca^{2+}-dependent K^+ currents will induce a hyperpolarization that counteracts the ongoing depolarization produced by the non-inactivating Na^+ current.

The theory of climbing fibre control proposed above can explain how varying rates of climbing fibre discharge can govern the rate of Purkinje cell discharge. It has been shown in cerebellar slices that once intracellular Ca^{2+} has peaked after the activation of P-type VOCCs, 10-15 seconds are required for the Ca^{2+} to be sequestered into internal stores, before cytoplasmic Ca^{2+} returns to its resting levels (Tank et al., 1988). Given this time course for sequestration, it is suggested that there will be an elevated level of Ca^{2+} in the cytosol of the Purkinje cell between each climbing fibre impulse, with the level of Ca^{2+} dependent on the frequency of climbing fibre activation. Thus, the higher the climbing fibre frequency the higher the prevailing Ca^{2+} concentration and subsequently, the greater the Ca^{2+}-dependent K^+ conductance, which will cause a proportionately lower simple spike rate. The reverse would apply to low climbing fibre rates where there will be more time for sequestration and less accumulation of Ca^{2+}, reducing the outward K^+ current and causing an increase in simple spike rate. Indeed, this does appear to be the case, with higher rates of climbing fibre activity resulting in a lower rate of simple spike activity

whereas lower rates of climbing fibre discharge caused simple spikes to discharge at a higher rate. In connection with these observations, Stockle and Bruggencate (1980) found that complex spikes are accompanied by an increase in K^+ and a decrease in Ca^{2+} ion concentration in the Purkinje cell extracellular space. The proposed mechanism that climbing fibres control the intrinsic Purkinje cell spike generator via variations in cytoplasmic Ca^{2+} concentration could be the subject of further investigations by performing Ca^{2+} imaging studies during repetitive climbing fibre. This would give a clearer indication of the validity of the present model.

An increase in simple spike discharge prior to their usual decrease was observed in a number of Purkinje cells during climbing fibre stimulation. This unexplained phenomenon has also been reported in other studies (Colin et al., 1980; Rawson & Tilokskulchai, 1981a; Andersson & Hesslow, 1987a). One possible explanation is that the increase may occur as a result of Golgi cell inhibition, releasing granule cells from tonic inhibition. Another mechanism could be through a dependency of Ca^{2+}-dependent K^+ channels on the level of intracellular Ca^{2+}. It is possible that as the amount of Ca^{2+} entry during initial climbing fibre stimulation may not have been sufficient to activate the Ca^{2+}-dependent K^+ channels. It could be that during repetitive climbing fibre activation, the accumulation of intracellular Ca^{2+} could continue to depolarize the Purkinje cell firstly generating the increase in simple spikes, until the threshold of intracellular Ca^{2+} is reached for activating the Ca^{2+}-dependent K^+ conductances and causing simple spike activity to become depressed.

Finally, the present hypothesis can also account for the irregular discharge that is normally seen in Purkinje cells. *In vivo*, climbing fibres do not fire at regular intervals (Armstrong & Rawson, 1979a), and this irregularity will almost certainly cause a temporal variation in intracellular Ca^{2+} levels. Also, variation in the number of impulses in the climbing fibre discharge would be expected to cause variation in Ca^{2+} entry.

4.4.4 Differing Purkinje cell responses: possible explanations

As already mentioned 5 of the 75 Purkinje cells studied in considerable detail displayed exceptions to the previously described inhibitory responses to climbing fibre stimulation. Rather than inhibiting the ongoing simple spike activity, a rise in simple spike discharge was observed throughout the duration of climbing fibre stimulation. It is unlikely that these Purkinje cells represent a different population of Purkinje cells, as they only depict less than 7 % of the total cells sampled. It has been demonstrated however that Purkinje

cells with similar resistances often respond with diverse firing frequencies to current pulses of similar amplitude and duration, indicating different integrative properties (Llinas & Sugimori, 1980a), a characteristic that could account for the differing response to climbing fibre stimulation observed in the present study.

It is also improbable that stimulating the Purkinje cell at higher frequencies causes the continuous increase in simple spike activity. As outlined previously, a low rate of climbing fibre stimulation would be expected to cause a lower level of intracellular Ca^{2+} concentration, resulting in a smaller Ca^{2+}-dependent K^+ current than expected during stimulation at higher frequencies. As the rise in Purkinje cell discharge was visible at both high and low climbing fibre stimulation frequencies, and with single and bursts of pulses, it would seem unlikely that this is the case. The increase in simple spike activity that occurs however, may be due to a higher threshold of intracellular Ca^{2+} required to trigger the activation of Ca^{2+}-dependent K^+ currents or possibly some cells have little expression of Ca^{2+}-dependent K^+ channels.

4.4.5 Summary

The current experiments have demonstrated an important inhibitory effect of climbing fibre stimulation on the intrinsic simple spike discharge of Purkinje cells. The results have shown that climbing fibre stimulation within a very limited and presumably physiological range can decrease and regulate tonic simple spike activity over a wide range of frequencies, from 20-120 Hz. The amount of activity regulated was found to be dependent not only on the frequency of climbing fibre stimulation, but also on the number of impulses in the climbing fibre discharge. Burst of impulses, which reflects the excitability of the inferior olive and hence the number of impulses ascending the climbing fibre, was found to cause a greater reduction in simple spike discharge than stimuli consisting of single impulses. Interestingly, the greater modulation of the Purkinje cell intrinsic spike generator with increasing number of impulses was seen to occur while the overall climbing fibre stimulation frequency remained constant, indicating that there are two possible independent mechanisms for controlling the intrinsic spike generator: via climbing fibre frequency and the number of impulses. The substantial changes in simple spike rate upon climbing fibre stimulation was found to occur without any accompanying excitation of inhibitory interneurones. While it has been demonstrated that climbing fibres can indeed control the Purkinje cell intrinsic spike generator, to confirm the proposed ionic mechanisms underlying climbing fibre pacemaker control, the following chapter, will use

immunohistochemical techniques to determine what Ca^{2+}-dependent K^+ channels are present in the Purkinje cell.

Chapter 5

Distribution of Ca^{2+}-dependent K^+ channels in the cerebellum of the rat.

5.1 Introduction and Aims

In the preceding two chapters, it was argued that Ca^{2+}-dependent K^+ currents that were activated by Ca^{2+} entry into the Purkinje cell through VOCCs may play a key role in the regulation of simple spike activity via the climbing fibres. The aims of this chapter were to determine the nature and distribution of Ca^{2+}-dependent K^+ channels in the Purkinje cells using immunohistological techniques. This knowledge would enable specific channel blockers to be used to determine the role of these channels in the proposed model of climbing fibre control. Before describing the methods and results, a brief review of the current information about the channels likely to be relevant to this study will be given.

Ca^{2+}-dependent K^+ currents generally play an important role in regulating neuronal activity. In particular, it has been demonstrated that Ca^{2+}-dependent K^+ channels contribute to action potential repolarization, the control of repetitive discharge, and underlying oscillatory membrane behaviour (Lancaster & Pennefather, 1987; Bourque, 1988). Ca^{2+}-dependent K^+ channels can be broadly divided into three subdivisions based on their electrophysiological, pharmacological and molecular profiles: large conductance (BK), small conductance (SK) and intermediate conductance (IK). Each subdivision plays a specific role in the particular physiological processes they influence. This study will focus on BK and SK channels, which are prevalent in neurones and mediate the fast action potential hyperpolarization (fAHP) and slow afterhyperpolarization (sAHP) respectively, following single or multiple action potentials.

BK channels were first cloned from *Drosophila*, and are composed of two membrane subunits, the pore-forming α-subunit, and a regulatory β-subunit (Toro et al., 1998). The β-subunit modulates the pharmacological and biophysical properties of the α-subunit (McManus et al., 1995; Kaczorowski et al., 1996; Hanner et al., 1997). BK channels are highly selective for K^+ and they exhibit a very high single channel conductance (100-200 pS). BK channels are voltage-sensitive and are blocked by a number of toxins including charybdotoxin (CbTX), iberiotoxin (IbTX), and tetraethlyammonium (TEA) at

submillimolar concentrations (Miller et al., 1985; Latorre et al., 1989; Reinhart et al., 1989; Galvez et al., 1990; Kaczorowski et al., 1996). The duration and frequency of BK channel opening increases with increasing Ca^{2+} concentration and membrane depolarization (Blatz & Magleby, 1987). In comparison to SK channels, BK channels are less sensitive to Ca^{2+} at negative membrane potentials and therefore may be less important in regulating K^+ conductance at resting membrane potentials (-50 to -70 mV). BK channels are often co-localised with VOCCs, indicating a putative role as feedback modulators of intracellular Ca^{2+}, as an increase in intracellular Ca^{2+} would lead to an efflux of K^+ and a subsequent hyperpolarization of the cell membrane (Lancaster & Nicoll, 1987; Robitaille & Charlton, 1992). This property may allow BK channels to play an important role in action potential shape and repolarization, and firing frequency and duration.

Three types of SK channels have been cloned: SK1, SK2, and SK3 (Kohler et al., 1996). In contrast to BK channels, all three SK channels are voltage-insensitive, TEA-insensitive, and display a smaller unit of conductance (~5-20 pS). SK channels are activated by intracellular Ca^{2+} at submicromolar concentrations (Lang & Ritchie, 1990; Lancaster et al., 1991; Park, 1994; Sah, 1995a), approximately 0.2-1 μM at negative membrane potentials. Therefore, SK channels are more sensitive to intracellular Ca^{2+} than BK, suggesting that they may be involved in controlling membrane excitability at resting membrane potentials.

SK channel activity is thought to be responsible for the sAHP that follows an action potential. Two types of sAHP can be distinguished: a sAHP that is sensitive to the bee venom toxin apamin (I_{AHP}), and a sAHP that is insensitive to apamin (sI_{AHP}) (for a review see Sah, 1996). SK1 channels are apamin-insensitive, whereas SK2 and SK3 are blocked with apamin, with SK3 having a lower sensitivity to this toxin than SK2 (Kohler et al., 1996). The apamin-sensitive current is also characterised by fast activation and decay times and is not regulated by neurotransmitters. Apamin-insensitive currents however, exhibit significantly slower kinetics and can be modulated by a number of neurotransmitters (Madison & Nicoll, 1982; Charpak et al., 1990). Both types of sAHPs are important in limiting the firing frequency of repetitive action potentials, a phenomenon known as spike frequency adaptation (Pennefather et al., 1985; Blatz & Magleby, 1986; Lancaster & Adams, 1986; Schwindt et al., 1988; Park, 1994; Sah, 1996).

Knowledge of the specific location and characteristics of the types of ion channels located in the neuronal membrane is fundamental in gaining an understanding of the ionic mechanisms involved in the intrinsic and integrative functions of Purkinje cells. Whereas

studies examining the distribution of Ca^{2+} channels in the cerebellum are quite extensive (for example, Hillman et al., 1991; Chung et al., 2000), few studies have examined the distribution of Ca^{2+}-dependent K^+ channels in any great detail. *In situ* hybridisation and immunocytochemical studies that have analysed the regional distribution of BK and SK Ca^{2+}-dependent K^+ channels have been directed to the rat brain in general (Knaus et al., 1996; Kohler et al., 1996; Bond et al., 2000; Stocker & Pedarzani, 2000), and none have examined solely on their distribution in the cerebellum.

Thus, the aims of this chapter were:

- To obtain a better understanding of the functional role of these channels
- Verify the existence of BK and SK Ca^{2+}-dependent K^+ channels in the rat cerebellum using immunohistochemical techniques
- To determine and compare the regional distribution and cellular location of each of these channels

The present study described the distribution of the Ca^{2+}-dependent K^+ channels located in the cerebellar cortex and the deep cerebellar nuclei (DCN). Immunostaining of BK channels and SK2 channels were observed in the cerebellar cortex and in neurones in the DCN, whereas SK3 immunoreactivity was undetectable.

5.2 Methods

5.2.1 Tissue preparation

Fourteen male adult Long-Evans rats (270-640 g) were deeply anaesthetised with sodium pentobarbitone (Nembutol, Rhône Mérieux, Vic., Australia, 150 mg/kg, i.p.), and perfused via the ascending aorta with 500 ml of warm heparinised saline, followed by 500 ml of cold 4 % paraformaldehyde in 0.1 ml phosphate buffer saline (PBS; pH 7.4), and finally with 500 ml of cold fixative with 10 % sucrose. The cerebellum and brain stem were removed and post fixed in 20 % sucrose and fixative overnight at 4 °C, or until the tissue had sunk. Serial sagittal and transverse sections of the entire cerebellum were cut on a freezing microtome at 40 µm, and collected as four separate sets of alternating sections into individual tissue wells containing PBS for free-floating immunohistochemistry.

5.2.2 Immunohistochemistry

Three sets of sections were processed using one of three primary rabbit polyclonal antibodies; anti-BK, anti-SK2, and anti-SK3 (Alomone Labs Ltd, Jerusalem, Israel). The remaining set was kept as a spare or used as a control. Sections were taken through the entire cerebellum to enable a detailed comparative study. Anti-BK channel is a polyclonal antibody raised in rabbit against a highly purified fusion protein and a C-terminal part (residues 1098-1196) of mouse *slo* α subunit. Anti-SK2 corresponds to residues 542-559 of rat SK2.1, and is a polyclonal antibody raised in rabbit. Anti-SK3 is a polyclonal antibody raised in rabbit corresponding to amino acid residues 2-21 of human SK3.1. An antibody to SK1 was not available at the time of this study (but see discussion).

All incubations were carried out using an orbital shaker and at room temperature, except for incubations with primary antibody, which were performed at 4 °C. Sections were first washed in PBS (3x10 minutes) and in between solution changes.

Sections were first incubated in 1 % sodium borohydride in PBS for 20 minutes, then in 50 % ethanol in 0.1 M phosphate buffer for 20 minutes to help break aldehyde bonds and permeabilise the tissue. Sections were then pre-incubated in a blocking solution containing 0.5 % Triton X-100, 5 % normal goat serum (NGS), and 1 µg of avidin/ml in PBS for one hour. This was followed by a 4 day incubation in either of the three primary antibodies, anti-BK, anti-SK2, or anti-SK3 used at a dilution of 1:100, plus 5 % NGS, 0.5 % Triton X-100, 1 µg of biotin/ml, and 1.25 % sodium azide. Following primary antibody incubation, the sections were incubated for 2 hours with biotinylated goat anti-rabbit IgG (H+L) (Vector, Burlingame, CA, U.S.A.) at a dilution of 1:300 in 0.1 M PBS with 5 % NGS. Visualisation was carried out with the fluorochrome strepavidin conjugated Cy3 (Jackson Immunoresearch Laboratories, PA, U.S.A.), diluted at 1:1000 with PBS and 5 % NGS for a period of 1 hour. Sections were mounted on gelatinised glass slides and allowed to air-dry overnight before coverslipping with Permafluor (Beckman Coulter, N.S.W., Australia) or with DAKO antifade mounting medium (DAKO Corporation, N.S.W., Australia). Microscope slides were stored at 4 °C when not in use.

Two methods were used to assess the specificity of immunostaining:

1. Sections were incubated without the primary antibodies.
2. Sections were incubated in solutions in which the primary antibody had been preabsorbed with the peptide antigen at a ratio of 2:1 (antigen supplied by Alomone Labs Ltd, along with the primary antibodies).

The sections were incubated with the secondary antibody and strepavidin Cy3 as described above.

5.2.3 Microscopy

Initially, sections were first viewed with a Nikon Eclipse E600 fluorescence microscope fitted with the Cy3 filter set (excitation 510-560 nm, barrier 590 nm), and photographed using Kodak Ektachrome P1600 slide film. Sections were also viewed using a Leica TCS-NT laser scanning confocal microscope equipped with a x4, x16, x40 (oil immersion), and x63 (oil immersion) lenses. Specimens were excited using the 568 nm line of the Kr/Ar laser. Emission was detected through a 665 nm longpass filter. Preparations were scanned in two planes and two-dimensional images were subsequently reconstructed using Voxblast software (Vaytek Inc, IA, USA). Some images were also visualised at a magnification of x10, x20 and x100 using a Fuji HC-2000 high-resolution digital camera.

5.3 Results

5.3.1 General comments

The cellular distribution of the Ca^{2+}-dependent K^+ channels, BK, SK2 and SK3 was determined in the adult rat cerebellum using specific antibodies and standard, indirect immunofluorescent techniques. The results are summarised in Table 5.1.

Table 5.1 A summary of the distribution of Ca^{2+}-dependent-K^+ channels BK, SK2 and SK3 immunoreactivites in the rat cerebellum.

Cell Type/Profile	BK	SK2	SK3
Parallel fibres	(-)	(++)	(-)
Purkinje cells			
–soma	(+++)	(+++)	(-)
–dendrites	(+++)	(-/+)	(-)
–axons	(++)	(-)	(-)
Granule cells	(-)	(++)	(-)
Golgi interneurones	(-)	(+)	(-)
Stellate/basket cells	(-)	(-)	(-)
DCN	(++/+++)	(+++)	(-)

(-) no detectable staining; (+) weak staining of profiles; (++) moderate staining; (+++) strong staining

The greatest immunopositive labelling of BK channels was evident in the Purkinje cell soma, dendrites and axons coursing through the white matter. Anti-SK2 immunoreactivity was present in all three layers of the cerebellar cortex, with strongest labelling occurring in the Purkinje cell soma. Within the granular layer, anti-SK2 immunoreactivity was associated with the cerebellar granule cells and a small number of presumed Golgi cells. SK2 immunopositive labelling was also evident in the parallel fibres of the molecular layer of the cerebellar cortex. Cells in the DCN were also SK2 immunopositive. No specific SK3 immunoreactivity was found in the cerebellum.

Controls in which the primary antibodies were preabsorbed with the respective antigens or in which the primary antibody was omitted did not show any specific immunostaining (Figure 5.1).

5.3.2 BK immunoreactivity

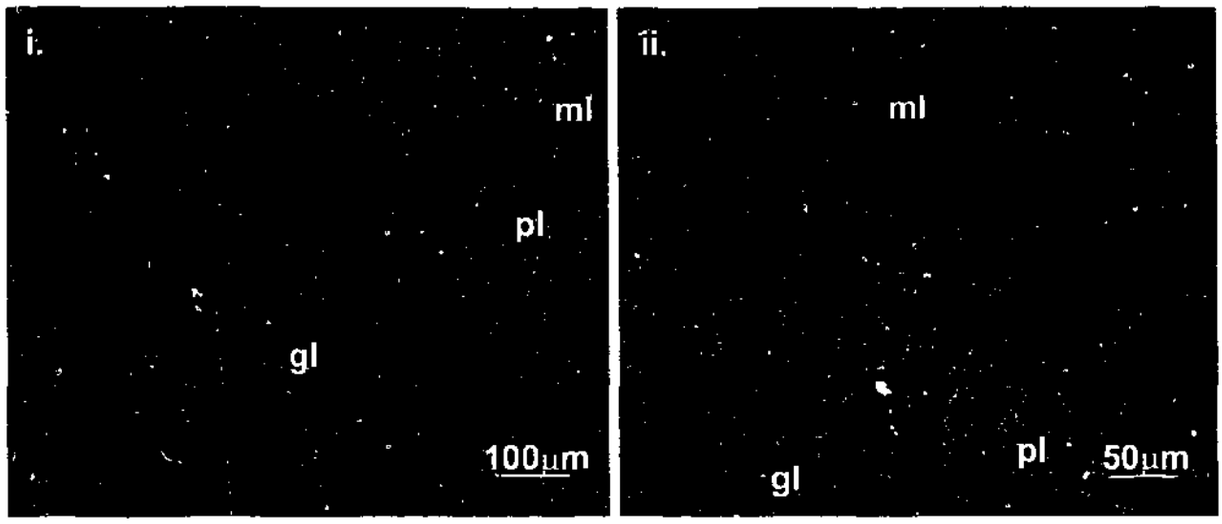
Examination of cerebellar sections showed that anti-BK immunoreactivity was expressed most prominently in the soma and dendrites of the Purkinje cells (Figure 5.2). Other neurones in the cerebellar cortex, including the granule cells of the granular cell layer and the interneurones, lacked any discernible BK label.

Purkinje cells were labelled across all folia of the entire cerebellar cortex. Anti-BK staining was strongly enriched in the soma and dendritic arbors of the Purkinje cell (Figure 5.2A-D). Intense staining was present in the axis of the main proximal dendrite of the Purkinje cell (Figure 5.2B, C, and D) and extended into the distal secondary and tertiary bifurcations of the dendritic tree, located in the molecular layer of the cerebellar cortex (Figure 5.2B). BK channels were sometimes localised in Purkinje cell axon collaterals, and their paths could be traced to their target Purkinje cells (Figure 5.2B). However, labelled axon collaterals were not abundant.

Anti-BK staining was moderate in Purkinje cell axons (Figure 5.2A, C, and E). The axon hillocks and proximal part of the initial axon segment were often densely labelled with BK (Figure 5.2B and C). In some instances, the positively stained axons of Purkinje cells were seen to terminate as Purkinje cell axon terminals in the cerebellar nuclei (Figure 5.3Ai. and ii.). In other cases, Purkinje cell axon terminals were not immunopositive (Figure 5.3Bii.).

Immunostaining was also present in populations of cerebellar nuclei cells, across all three DCN, where BK channels appeared to be localised in the soma and proximal dendrites (Figure 5.3). The number of anti-BK labelled cells was variable in different animals

A.



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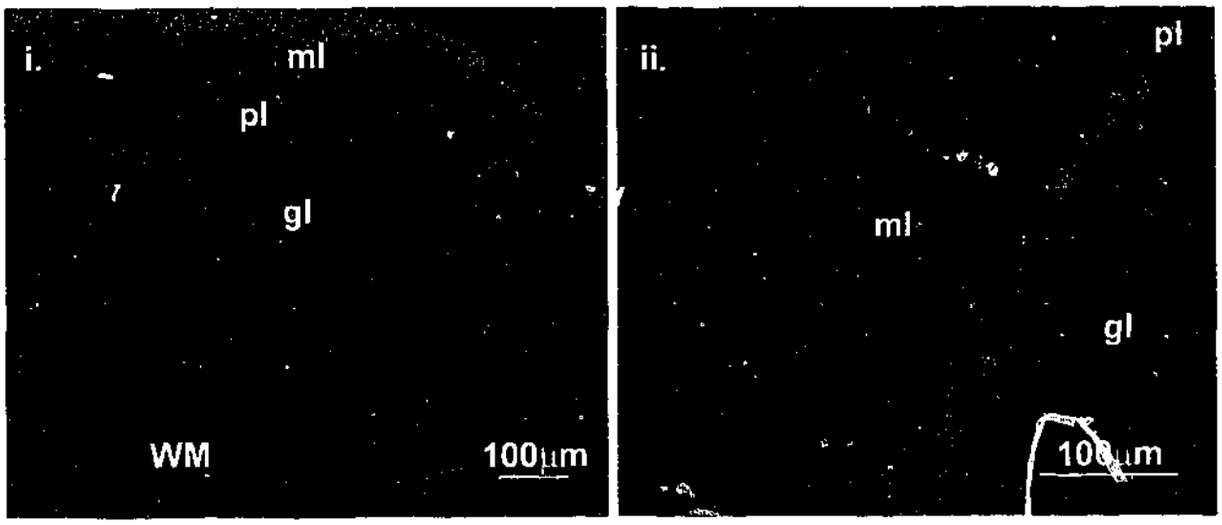


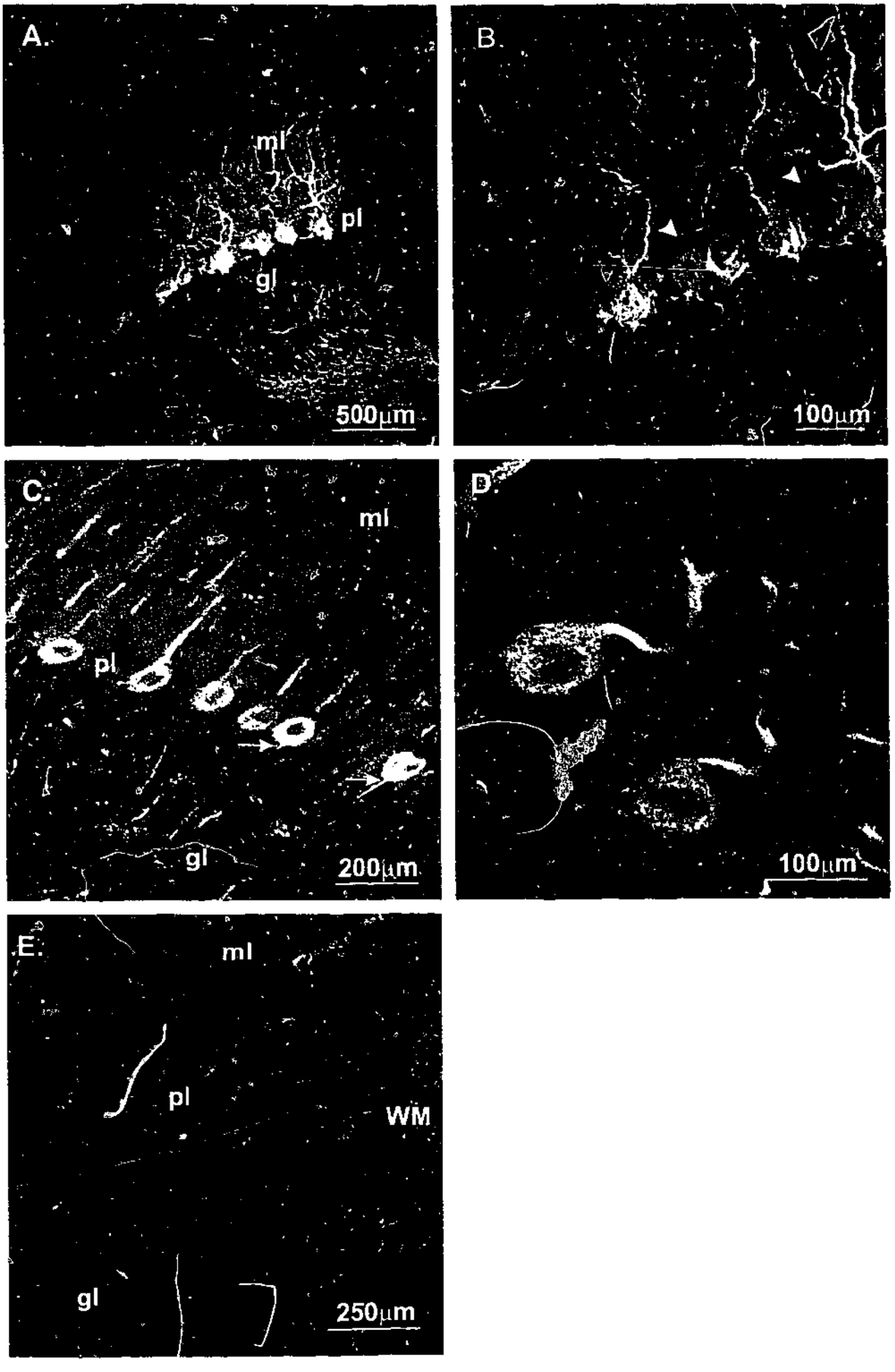
Figure 5.1

Original fluorescence photomicrographs showing specificity of staining with preabsorption controls. Tissue used in the controls were from the same animals as the labelled tissue.

Ai. Control BK with preabsorbed antibody. Aii. Normal BK antibody labelling in the Purkinje cell soma and dendrites. Bi. Control SK2 with preabsorbed antibody. Bii. Normal SK labelling in the Purkinje cell soma and granule cells.

Figure 5.2

Location of BK channels in the cerebellum. **A.** Low power confocal image of the distribution of BK channels in the Purkinje cells of the Purkinje layer and the nonreactive granular layer of the cerebellum. **B.** Confocal image of BK channel distribution in the cerebellar Purkinje cells, exhibiting immunoreactivity in the dendrites and in the cell soma. Note the immunoreactivity in the Purkinje cell collaterals (arrowheads) that lead to other Purkinje cells. **C.** Confocal image of Purkinje cells with BK channels located in the cell soma and dendritic tree. BK channels are also located in Purkinje cell axons and axon hillocks (arrows). **D.** High power confocal image of a Purkinje cell. **E.** Confocal image of BK channels present in the Purkinje cell axons.


A.
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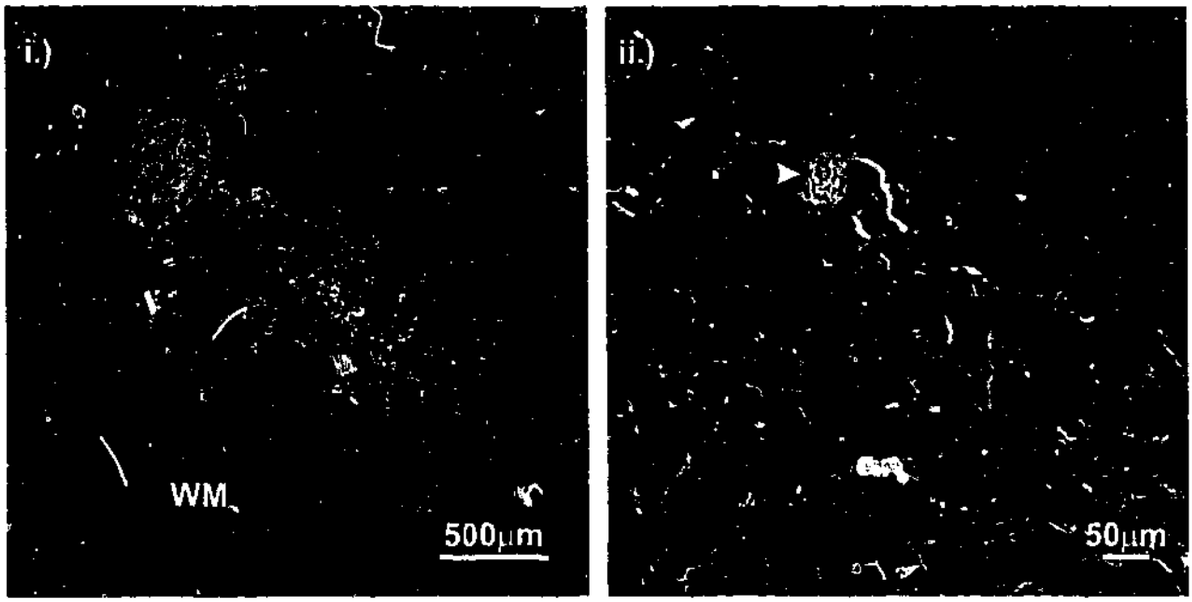

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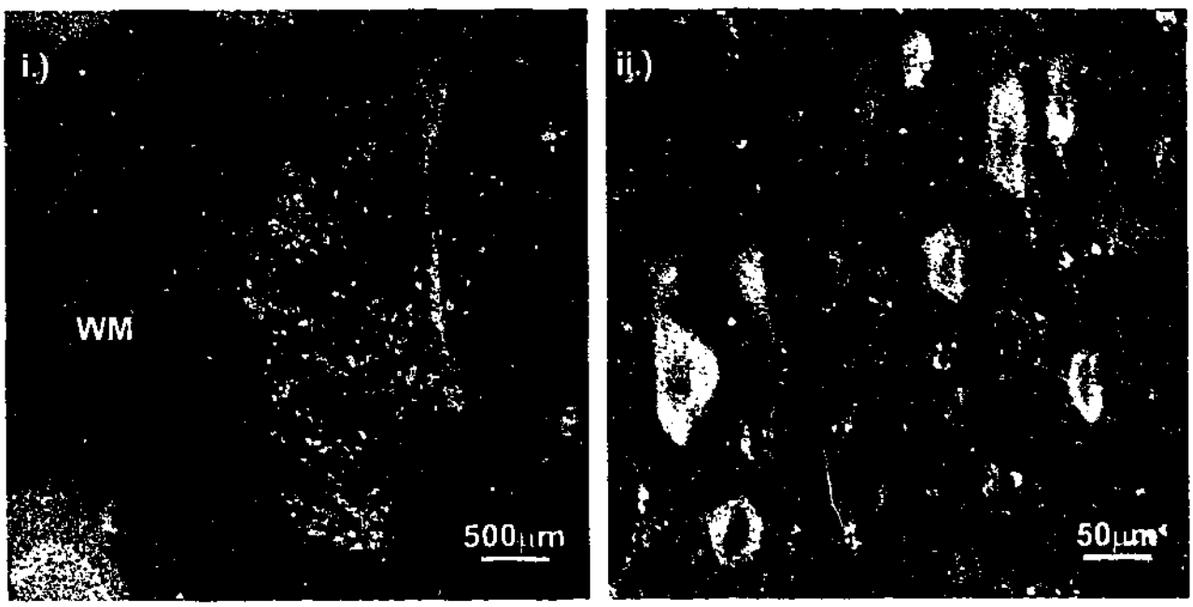

Figure 5.3

Confocal micrographs showing BK channel labelling in the cerebellar nuclei. **Ai.** BK channel labelling in the fastigial nucleus. **Aii.** Fastigial nucleus at higher magnification, displaying labelled nucleus cell (arrowhead) and Purkinje cell axons and terminals. **Bi.** BK channel labelling in the interpositus nucleus. **Bii.** Interpositus nucleus at higher magnification, displaying labelled nuclei cells, but with the absence of labelled Purkinje cell terminals.

(compare the number of cells labelled in Figure 5.3Aii. with that of 5.3Bii.). Whether this depicts an up or down regulation of the BK channels, or represents a subpopulation of cells that selectively express these channels, would need to be determined.

5.3.3 SK2 immunoreactivity

Anti-SK2 immunoreactivity was localised predominantly in the Purkinje cell and the granular cell layers. Moderate SK2 staining was detected in the granule cells in the granular layer (Figure 5.4A-C). SK2 was localised to the somata of the granule cells (Figure 5.4B, D and E), and extended into their parallel fibre projections in the molecular layer (Figure 5.5). However, it appears that only proportions of granule cells are immunopositvie (compare the number of labelled granule cells in Figure 5.4B with 5.4C). It would seem that, like BK labelling in the DCN, the proportion of SK2 labelled granule cells either represents a subpopulation of cells that exclusively express the SK2 channel, or is a reflection of induced expression.

SK2 immunostaining was also present in a number of Golgi interneurones (Figure 5.4B and E). Labelling was restricted to the Golgi cell soma and proximal dendrites. Basket and stellate interneurones were immunonegative.

Transverse examination of cerebellar sections revealed that SK2 labelling extended from the granule cells into their parallel fibre projections in the molecular layer (Figure 5.5A and B). The parallel fibres could be traced along the folium (Figure 5.5C and D), where they extended a considerable distance across the molecular layer and it was evident that SK2 immunoreactivity was concentrated in the parallel fibre varicosities (Figure 5.5E and F).

In contrast to BK, SK2 labelling was restricted to the Purkinje cell soma (Figure 5.4D and Figure 5.6A). Dense immunostaining of SK2 was evident in the Purkinje cell soma and, on some occasions, labelling was evident in the lower portions of the main proximal dendrite (Figure 5.6B). Strong SK2 staining was present in the nuclear membrane of some Purkinje cells (Figure 5.6C). However, the axons and the secondary and tertiary dendritic bifurcations of the Purkinje cells were anti-SK2 immunonegative.

Intense SK2 staining was present in many cells of the three DCN (Figure 5.7A). Labelling was localised to the soma and proximal dendrites of the nuclei cells (Figure 5.7B). Strong immunoreactivity was also seen in the nuclear membrane of some of these cerebellar nuclei cells (Figure 5.7C). No SK2 labelling was evident in the Purkinje cell axons or their terminals in the cerebellar nuclei.

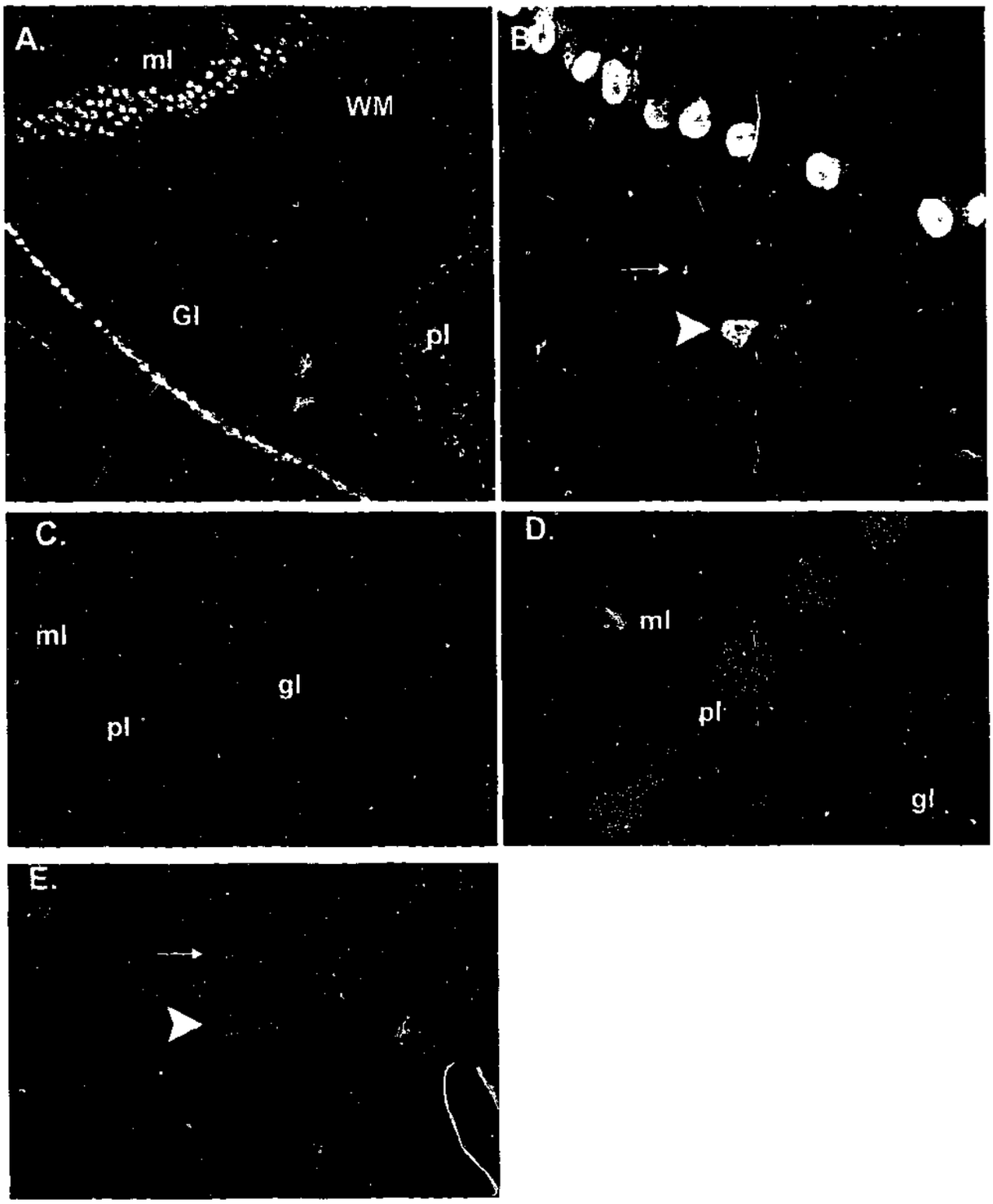

Figure 5.4

Examples of SK2 immunoreactivity in the cerebellar cortex. **A.** Low-power confocal image showing SK2 labelled Purkinje and granule cells. **B.** High-power confocal image displaying SK2 immunopositive Purkinje cells, a labelled Golgi cell (arrowhead) and granule cells (arrow). **C.** Low-power fluorescence photomicrograph of SK2 labelled Purkinje and granule cells. **D.** High-power fluorescence photomicrograph of SK2 labelled Purkinje and granule cells. **E.** High-power photomicrograph of granular layer showing labelled Golgi cell (arrowhead) and surrounding granule cells (arrow).

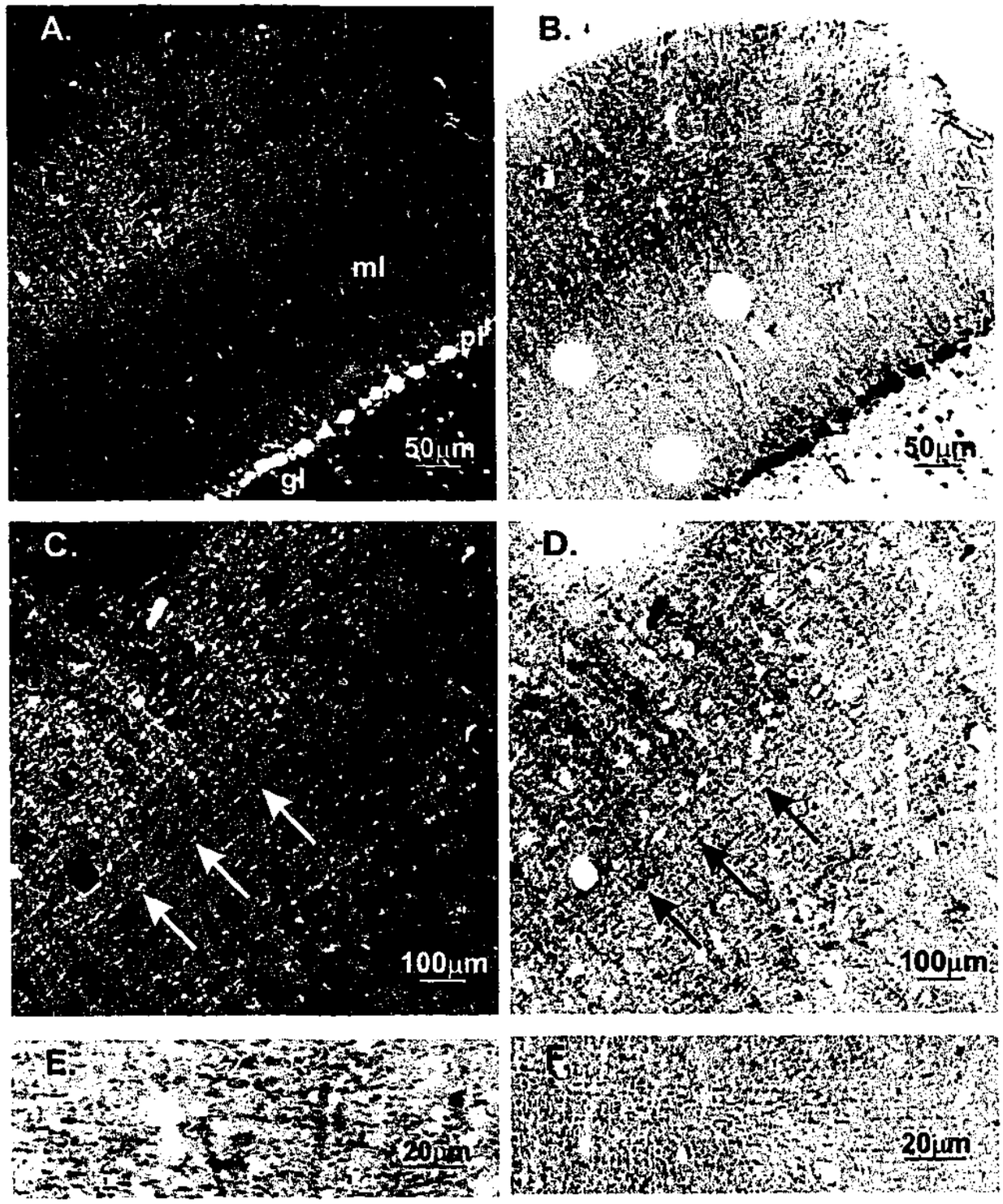

Figure 5.5

Distribution of SK2 channels in the parallel fibres. **A.** Confocal image of SK2 channel labelling in transverse cerebellar sections in the Purkinje cell soma and granule cells. SK2 immunoreactivity can be seen in the superficial parallel fibres of the folium. **B.** Negative image of **A. C.** High power confocal image of SK2 channels in the parallel fibres, arrows depict a single parallel fibre. **D.** Negative image of **C. E & F.** Negative images demonstrating SK2 immunoreactivity concentrated in the parallel fibre varicosities.

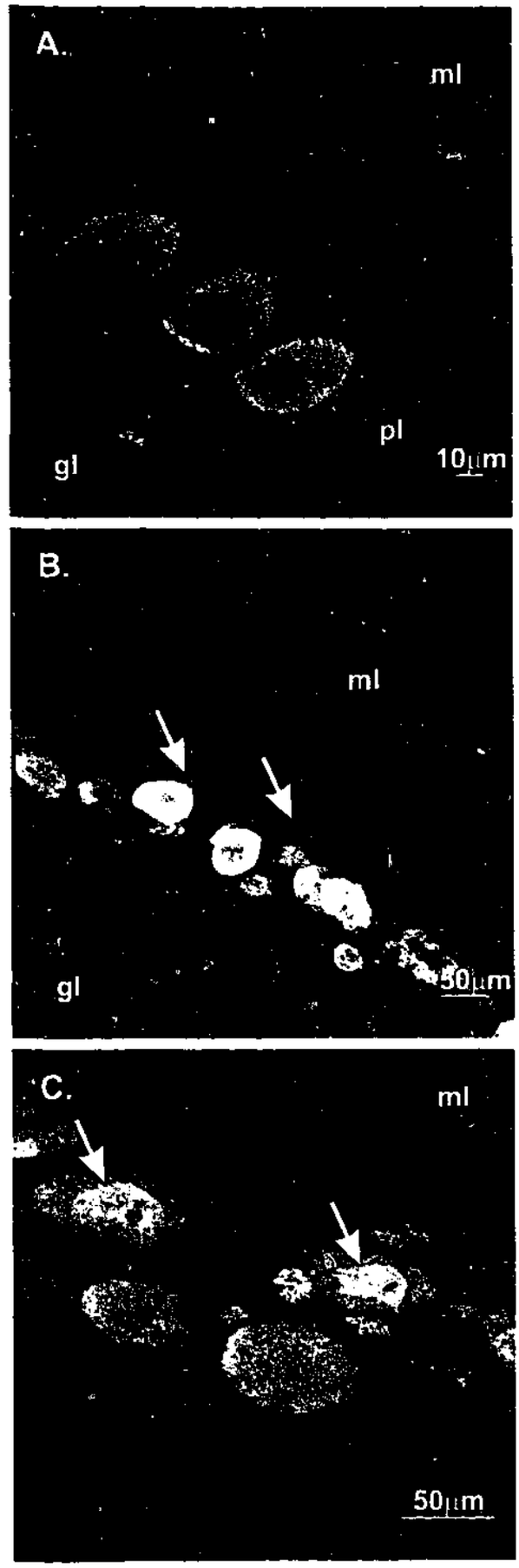

Figure 5.6

High power confocal images of SK2 channel labelling in the Purkinje cells. **A.** Immunopositive Purkinje cells. **B.** SK2 channels labelled Purkinje cells and granule cells. Note the immunostaining of proximal dendrites of some Purkinje cells (arrows). **C.** Staining of Purkinje cell nuclear membranes (arrows).

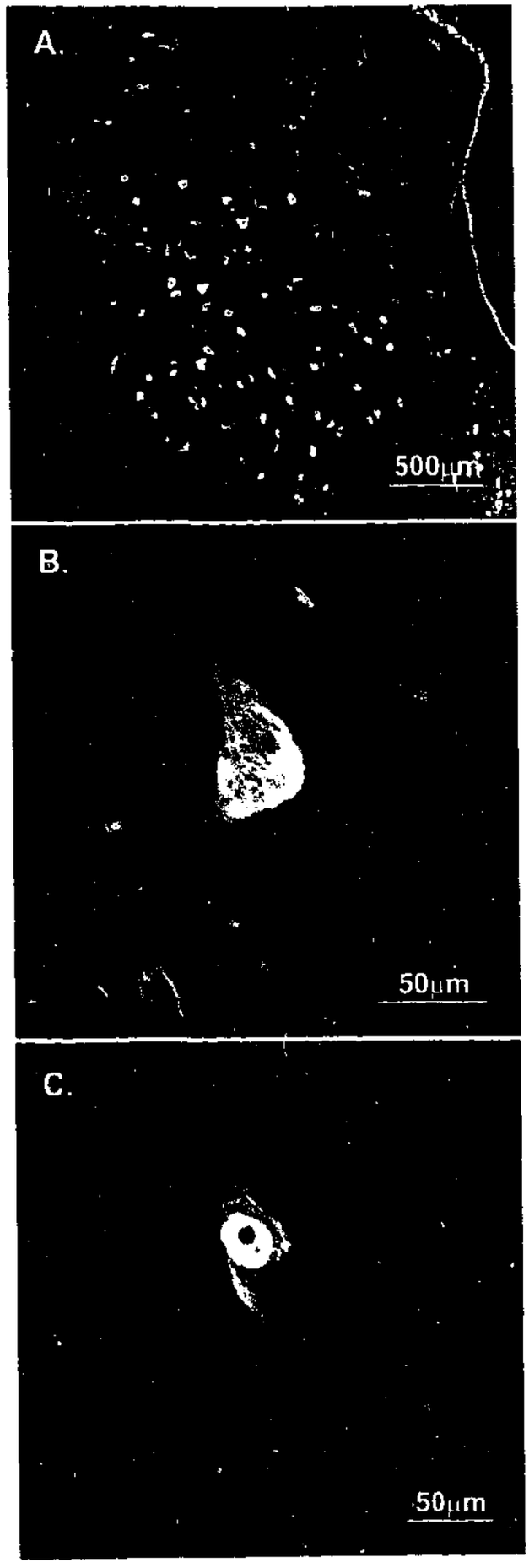

Figure 5.7

Confocal images of SK2 labelled cells in the cerebellar nuclei. **A.** Low power image of SK2 channel labelling in the lateral nucleus. **B.** High power image of a SK2 labelled nuclei cell. **C.** SK2 labelling of the nuclear membrane of a cerebellar nucleus cell.

5.4 Discussion

The distribution of Ca^{2+}-dependent K^+ channels in the cerebellum of the rat was determined by immunohistochemical techniques. While previous reports have examined the localisation of Ca^{2+}-dependent K^+ channels in the rat brain in general, here a detailed examination of the location of BK, SK2 and SK3 channels in the rat cerebellum has been made.

5.4.1 General comments and comparisons with other studies

Results from the current study demonstrate that BK channels are located predominantly in both the somatic and dendritic regions of Purkinje cells and also in a population of neurones of the DCN. Notably, BK immunoreactivity was also prominent in the axons and terminals of the Purkinje cells. These results are consistent with *in situ* hybridization and immunocytochemical studies by Knaus et al., (1996). In contrast to BK labelling, SK2 labelling was restricted to the Purkinje cell soma, and in some instances, the initial portion of the proximal dendrite. Strong labelling was also present in the DCN, and SK2 was present in the parallel fibres of the immunopositive granule cells, in which only a limited proportion of cells were labelled. The distribution of SK2 labelling in the Purkinje cell found here is not consistent with *in situ* hybridization and immunohistochemical studies. Stocker and Perdarzani (2000) detected only low levels of SK2 mRNA in Purkinje cells. Conversely, binding studies utilising radiolabelled apamin, which selectively binds to SK channels to map their distribution, was prominent in the granular layer, and restricted to the Purkinje cell soma (Mourre et al., 1986; Gehlert & Gackenheimer, 1993), a finding that closely correlates with the distribution of SK2 antibody labelling determined in this study. Interestingly, this study did not detect SK3 labelling anywhere in the cerebellum of the rat, a finding that is in contrast to *in situ* hybridization studies that describe very high levels of SK3 transcript located in Golgi interneurones in the granular layer of the cerebellar cortex (Xia et al., 1998; Stocker & Pedarzani, 2000). Whether these discrepancies represent post-transcriptional regulation of SK channel expression or whether subpopulations of SK2 and SK3 channels exist would need to be determined. The existence of the SK1 channel was not examined in this study, however the study by Stockler and Perdazani (2000) demonstrated that SK1 channels are present in the DCN but not the cortex.

Single-channel recording techniques have demonstrated that cultured Purkinje cells express several types of voltage-sensitive K^+ channels (Gruol et al., 1991). One such

voltage-sensitive channel, referred to by Gruol et al., (1991) as K1, was shown to exhibit increased sensitivity to increases in intracellular Ca^{2+} concentration, in addition to a large single unit conductance, properties similar to those described for BK channels located in other cell types. The location of K1 channels, as revealed by single-channel recordings, were found in both the somatic and dendritic regions of the Purkinje cells (Gruol et al., 1991), areas in which BK immunoreactivity in this study was extremely prominent. Moreover, application of TEA at concentrations known to block BK channels, was found to block K1 channel activity, preventing spike repolarization which in turn prolonged simple spike duration (Yool et al., 1988). Therefore, it appears that BK channels could play a prominent function in controlling Purkinje cell spike repolarization and firing, a role that BK channels display in other preparations (Wang et al., 1998; Lara et al., 1999; Shao et al., 1999).

5.4.2 Ca^{2+}-dependent K^+ channels and Purkinje cells

As well as membrane depolarization, BK channels require a high level of intracellular Ca^{2+} concentration for their activation (Tseng-Crank et al., 1994). In contrast, SK channels are voltage-insensitive and are activated by nanomolar concentrations of Ca^{2+} (Blatz & Magleby, 1987). In response to increases in intracellular Ca^{2+}, the probability of Ca^{2+}-dependent K^+ channel opening increases, leading to hyperpolarization of the membrane and, consequentially, the regulation of action potential repolarization and duration. The coupling of membrane potential to intracellular Ca^{2+} explains their proposed role as feedback modulators of Ca^{2+} influx through voltage-dependent pathways.

High levels of intracellular Ca^{2+} in Purkinje cells are attained by the activation of climbing fibres, which produce an increase in voltage-operated Ca^{2+} conductance mediated by P-type VOCCs. In order to optimise the concentration of intracellular Ca^{2+}, it has been proposed that Ca^{2+}-dependent K^+ channels are located close to Ca^{2+} channels (Blatz & Magleby, 1987; Lancaster & Nicoll, 1987; Robitaille & Charlton, 1992; Gola & Crest, 1993). Indeed, experimental evidence has demonstrated BK and SK2 channels are located in close proximity to VOCCs (Hounsgaard & Midtgaard, 1988; Gola & Crest, 1993; Robitaille et al., 1993; Viana et al., 1993; Sah, 1995b). Various studies have examined the distribution of P-type channels in the cerebellum of the rat using polyclonal antibodies generated from the protein Funnel Web Spider Toxin, a P-type channel antagonist (Hillman et al., 1991) or specific antibodies raised against the α_{1A} subunit of the P-type channel (Ludwig et al., 1997; Chung et al., 2000; Pouille et al., 2000). Hillman et al., (1991) found that P-type channels were located in the secondary and tertiary dendritic bifurcations of the Purkinje

cells. Conversely, Chung et al., (2000) and Ludwig, et al., (1997) demonstrated that P-type channel α_{1A} subunits were expressed in the Purkinje cell soma. In contrast, Pouille et al., (2000) detected α_{1A} subunits expression in both the Purkinje cell's soma and dendrites. Although the differential localisation of P-type VOCCs in these studies may reflect differences in tissue preparation or may represent subtypes of P-type VOCCs, overall, P-type VOCCs appear to be colocalised with BK and SK2 channels. Thus, it appears that Ca^{2+} entry via the P-type VOCCs could activate BK and SK2 channels located nearby.

Of course, the possibility exists that Ca^{2+} influx from the P-type VOCCs preferentially supplies either BK or SK2 channels. In several cell types, it has been found that Ca^{2+} influx via specific VOCCs activated a specific Ca^{2+}-dependent K^+ channel, with different types of VOCCs coupled to other Ca^{2+} dependent channels (Viana et al., 1993; Sah, 1995b). It should also be noted however that other classes of VOCCs have been demonstrated to exist in cerebellar Purkinje cells. VOCCs have been categorised as L, N, P, Q, R and T on the basis of differences in molecular structure, electrophysiological properties and their various sensitivities to pharmacological agents (Bean, 1989). The cloning of the pore-forming α_1 subunits has enabled antibodies to be produced to recognise each member of the VOCCs. As well as P-types channels, L, R, and T-type channels have also been demonstrated to exist in Purkinje cell soma and dendrites (Ludwig et al., 1997; Chung et al., 2000; Pouille et al., 2000). It may be conceivable that SK2 and BK channels are functionally linked to distinct classes of VOCCs. Double labelling studies demonstrating what class of VOCCs is colocalised with each Ca^{2+}-dependent K^+ channel would help in determining this.

One of the most striking findings in this study was the differential distribution of SK2 and BK channels in the Purkinje cells. SK2 channels were restricted to the Purkinje cell soma whereas BK channels had a somatic and dendritic distribution. The overlapping expression of more than one type of Ca^{2+}-dependent K^+ channel is a phenomenon seen in a variety of neurones (Lang et al., 1997; Wang et al., 1998; Aizenman & Linden, 1999). Given the spatial difference and the dissimilarity in concentration of intracellular Ca^{2+} required for their activation, it is likely that these two classes of Ca^{2+}-dependent K^+ channels subserve different roles in the overall regulation of Purkinje cell excitability.

Recordings from the Purkinje cell soma have that revealed Na^+ action potentials are initiated in the Purkinje cell soma and spread passively into the dendritic tree (Llinas & Sugimori, 1980a, b; Stuart & Hausser, 1994). Furthermore, recordings from Purkinje cell dendrites demonstrated that dendrites generate Ca^{2+} action potentials. A compartmental

model of Purkinje cells has been suggested, with Na^+ action potentials restricted to the soma and Ca^{2+} action potentials to the dendrites (Llinas & Sugimori, 1980a, b; Stuart & Hausser, 1994; Pouille et al., 2000). Thus a difference in Ca^{2+}-dependent K^+ channels in the soma and dendrites of the Purkinje cell as was demonstrated in this study, would influence action potential repolarization in each compartment without interruption to the other. This is in contrast to hippocampal and pyramidal cells, where somatic action potentials are actively propagated into the dendrites (Stuart & Sakmann, 1994). The active propagation of action potentials is believed to play an important role in synaptic integration and information processing (Stuart et al., 1997), and in the case of Purkinje cells, where propagation is passive, synaptic integration could occur without interruption to somatic firing (Stuart & Hausser, 1994).

5.4.3 Ca^{2+}-dependent K^+ channels and the DCN

DCN are the primary target of Purkinje cells, where they exert an inhibitory influence. DCN are spontaneously active in resting animals and in vitro preparations (Thach, 1968; Armstrong & Rawson, 1979b; Harvey et al., 1979; Jahnsen, 1986b; Aizenman & Linden, 1999; Raman et al., 2000), exhibiting high firing rates and bursting discharge ranging from 10-80 Hz. The block of Ca^{2+} currents in isolated nuclei cells terminated their spontaneous discharge by an apparent loss of hyperpolarizing drive, indicating that that Ca^{2+}-dependent K^+ channels play a significant role in membrane repolarization. Ca^{2+}-dependent K^+ currents have been observed in the DCN (Gardette et al., 1985; Jahnsen, 1986a; Llinas & Muhlethaler, 1988). Studies using cerebellar tissue slices and isolated DCN neurones suggest that Ca^{2+}-dependent K^+ channels contribute to the firing patterns and membrane repolarization of DCN cells (Aizenman & Linden, 1999; Raman et al., 2000). Consistent with these previous studies, experiments in this chapter have verified the existence of Ca^{2+}-dependent K^+ channels in the DCN, as revealed by the strong immunopositivity of BK and SK2 channels, albeit in a limited proportion of cells. Whether this restricted expression of BK and SK2 labelling in the DCN reflects a difference in cell type or population of neurones is unknown. Anatomical data has suggested the existence of three populations of neurones in the DCN: small GABAergic neurones that project to the inferior olive, GABAergic local interneurones, and large glutamatergic neurones that project to premotor areas (Voogd et al., 1996). However at present, no differences in electrophysiological properties amongst the three cell types has been demonstrated. Therefore, it is unlikely that BK and SK2 channels are preferentially located in a particular type of DCN cell. The circumscribed population of DCN exhibiting

BK and SK2 channels may be due to altered Purkinje cell inhibitory drive or by the influence of different neurotransmitters types such as serotonin and noradrenaline, which are know to modulate Ca^{2+}-dependent K^+ channels (Nicoll, 1988; Muller et al., 1992; Pedarzani & Storm, 1993), thus causing an up or down regulation of channels.

As well as expressing Ca^{2+}-dependent K^+ channels, *in situ* hybridization and electrophysiological studies have revealed that various VOCCs are also located in the DCN. Distinct punctate immunoreactivity of α_{1A}, α_{1B}, and α_{1D} were present in a population of DCN cells (Chung et al., 2000), which correspond to P, N, and L-type channels respectively. In addition, various Ca^{2+} currents have been described in DCN cells that resemble P, N, L, and T-type Ca^{2+} currents (Gardette et al., 1985; Llinas & Muhlethaler, 1988; Mintz et al., 1992a; Takahashi & Momiyama, 1993; Momiyama & Takahashi, 1994). As Ca^{2+}-dependent K^+ channels require Ca^{2+} for their activation, the population of DCN cells expressing BK and SK2 channels may depend on the particular class of VOCC that is coexpressed in the cell. This may also explain why only a limited proportion of DCN cells display immunopositive Ca^{2+}-dependent K^+ channels.

5.4.4 Ca^{2+}-dependent K^+ channels, nerve terminals and projection pathways

The moderate immunoreactivity of BK channels in Purkinje cell terminal areas and projection pathways is a phenomenon well documented in other brain pathways (Robitaille & Charlton, 1992; Knaus et al., 1996). This suggests a functional role for these channels in regulating presynaptic activity. Likewise, the presence of SK2 channels in the parallel fibres suggests that these channels may also have a role in regulating presynaptic activity at parallel fibre synapses. It is well established that localised Ca^{2+} entry through VOCCs participates in synaptic transmission (Dunlap et al., 1995; Fossier et al., 1999). These channels, located in presynaptic terminals, trigger the release of transmitters in response to Ca^{2+} influx. Pharmacological and localisation studies have revealed that neurotransmission in parallel fibre and climbing fibre synapses were mediated by N and P-type VOCCs (Hillman et al., 1991; Takahashi & Momiyama, 1993; Regehr & Mintz, 1994; Mintz et al., 1995; Westenbroek et al., 1995; Doroshenko et al., 1997). The coupling of Ca^{2+}-dependent K^+ channels with VOCCs, both functionally and structurally, in presynaptic terminals and projection pathways may serve to regulate the duration and interval of action potentials. This in turn would prevent the over-excitation of parallel fibres and climbing fibres by keeping Ca^{2+} concentration at an acceptable level, which would regulate the amount of transmitter being released. Moreover, this would

normalise the total amount of excitatory input to the Purkinje cells, which are targeted by climbing fibre and parallel fibre afferents, to prevent the over-excitation of Purkinje cells (De Schutter, 1995; Mourre et al., 1997).

5.4.5 Summary

In summary, this study demonstrates the expression of BK and SK2 channels in Purkinje cells and suggests that these K^+ channels are likely candidates for controlling the pacemaker or intrinsic spike generator. The results show that both BK and SK2 are present in the Purkinje cells, with immunopositive labelling of BK found in both the soma and dendrites, whereas SK2 channels were limited to the Purkinje cell soma. A proportion of DCN neurones were also BK and SK2 immunopositive. Notably BK channels were present in the axons and terminals of the Purkinje cells. SK2 was also present in the parallel fibres of the immunopositive granule cells. Interestingly, SK2 was evident in only a limited proportion of granule cells. Ultimately, these channels would play an important role in controlling Purkinje cell excitability under conditions of increased intracellular Ca^{2+}, as would occur during complex spike discharge.

Previous studies of Purkinje cells in slices, in culture and in computer models have found that spontaneous activity is generated intrinsically, and is likely to be the result of a balance between the ionic conductances of Na^+, K^+ and Ca^{2+}. In the proposed model of Purkinje cell pacemaker activity, it is likely that the spontaneous or intrinsic firing of Purkinje cells depends mainly on the non-inactivating Na^+ current, with the level of depolarization attained by the Na^+ conductance determined by the opposing, non-inactivating K^+ conductance. The intrinsic or baseline firing would then be modulated in part by a Ca^{2+}-dependent K^+ current that is determined by the activation of the climbing fibres which open the P-type VOCC. In the absence of Ca^{2+}, as would occur when climbing fibre inputs are removed, thereby preventing Ca^{2+} entry through the P-type VOCCs, intracellular Ca^{2+} concentration will decline, causing a concurrent decrease in Ca^{2+}-dependent K^+ current, as BK and SK2 channels will no longer be activated. Consequently, Purkinje cell membrane potential will depolarize and firing will increase to the intrinsic rate set by the balance between the Na^+ and the K^+ conductances. Indeed, this appears to be the case (Montarolo et al., 1982; Chapter 3 of this thesis). Therefore the contribution of currents passing through the BK and SK2 channels appear to be determined by the activation of climbing fibres, which in turn will regulate the Purkinje cell pacemaker. The role of these channels and their contribution in controlling the

Purkinje cell intrinsic pacemaker will be examined further in the following chapter, by applying pharmacological antagonists.

Chapter 6

Effects of Ca^{2+}-dependent K^+ and P-type Ca^{2+} channel blockers on spontaneous simple spike activity of cerebellar Purkinje cells.

6.1 Introduction and Aims

The present theory describing climbing fibre control over Purkinje cell firing suggests that Ca^{2+} entry into the Purkinje cell, as a result of climbing fibre activation of VOCCs, activates Ca^{2+}-dependent K^+ currents that exert a regulatory control over an intrinsic spike generating mechanism. The results of both the climbing fibre removal and stimulation experiments presented earlier are consistent with the hypothesis, and the immunohistochemistry results described in the preceding chapter indicated that Purkinje cells do in fact contain both BK and SK2 Ca^{2+}-dependent K^+ channels. There is considerable evidence that the predominant VOCCs in the Purkinje cell membrane belongs to a class of VOCCs known as P-type channels. These high-threshold Ca^{2+} channels, originally described in cerebellar Purkinje cells (hence the name 'P') by Llinas et al., (1989), have been estimated to account for about 90 % of the total Ca^{2+} current that is mediated by the climbing fibres (Mintz et al., 1992b; Norris et al., 1996). Their distribution in the cerebellum has been localised with the aid of antibodies directed against a fraction of the Funnel Web Spider toxin that specifically binds to and blocks these channels. In the cerebellar cortex, these P-type channels are associated exclusively and postsynaptically with the Purkinje cells (Cherksey et al., 1991), exhibiting intense immunoreactivity in the Purkinje cell dendrites and moderate staining in the soma (Hillman et al., 1991).

As well as identifying the types of ion channels present in the membranes of the neurones responsible for the generation and maintenance of the Purkinje cell pacemaker, the functional role played by these channels is fundamental in understanding how neuronal activity is generated and maintained. If the present theory is at least partly correct, then blocking the P-type VOCCs would have similar effects to climbing fibre denervation, in that simple spike activity would be expected to increase as a result of the loss of inhibitory control over the intrinsic spike generating mechanism. Likewise, simple spike activity would be expected to increase after blockage of Ca^{2+}-dependent K^+ channels due to a loss of a hyperpolarizing current. Therefore, the aim of this part of the study was to examine

how blockage of P-type VOCCs and Ca^{2+}-dependent K^+ channels effects Purkinje cell simple spike activity. Three types of channel blockers were used in this study; the FTX-3.3 fraction of the Funnel Web Spider toxin which is a specific channel blocker of P-type VOCCs (Dupere et al., 1996); apamin, which specifically blocks SK2 channels (Hugues et al., 1982); and iberiotoxin (IbTX), a specific blocker of BK channels (Galvez et al., 1990). The results demonstrated that P-type VOCCs, SK2, and BK channels all have distinct roles in the generation of intrinsic spike activity.

6.2 Methods

6.2.1 Experimental set-up

The experiments were performed on 14 adult Long-Evans male rats. Details of the general methods used were outlined in Chapter 2. A schematic diagram of the experimental set-up is depicted in Figure 6.1. Briefly, an area of the cerebellar cortex extending from the vermis to Crus I was exposed to allow for the insertion of a dual microelectrode-micropipette. As described earlier for the CNQX experiments (Section 3.3.6), a selected patch of cortex was left free of agarose to prevent blockage of the micropipette.

6.2.2 Preparation of channel blockers

The following drugs were used in the experiments described in this chapter: apamin (Auspep, Vic., Australia), an 18 amino acid polypeptide that was isolated from the venom of the honey bee *Apis mellifera* and targets SK Ca^{2+}-dependent K^+ channels; FTX-3.3 (tetrahydrochloride) (FTX), purified from the funnel web spider venom *Agelenopsis aperta* (Alomone Labs LTD, Jerusalem, Israel) that targets P-type VOCCs; and rIberiotoxin (IbTX) (Alomone Labs LTD, Jerusalem, Israel) which blocks BK Ca^{2+}-dependent K^+ channels. FTX and IbTX were dissolved in filtered distilled water to their final concentration (1 mM and 1 μM respectively), whereas apamin was dissolved in filtered water to form a stock solution (1 mM) and then diluted with physiological saline to its final concentration (60 μM) on the day of the experiment. As described for the CNQX studies (Section 3.3.6), a volume of toxin (50-150 nl) was infused over a period of 2-5 minutes to ensure that it encompassed the entire Purkinje cell under study. Pressure injection of the channel blockers occurred after a control period of approximately 2-5 minutes was obtained. Since the effects of the channel blockers could not be 'washed' out of the preparation, it was not possible to remeasure control activity after the injection of

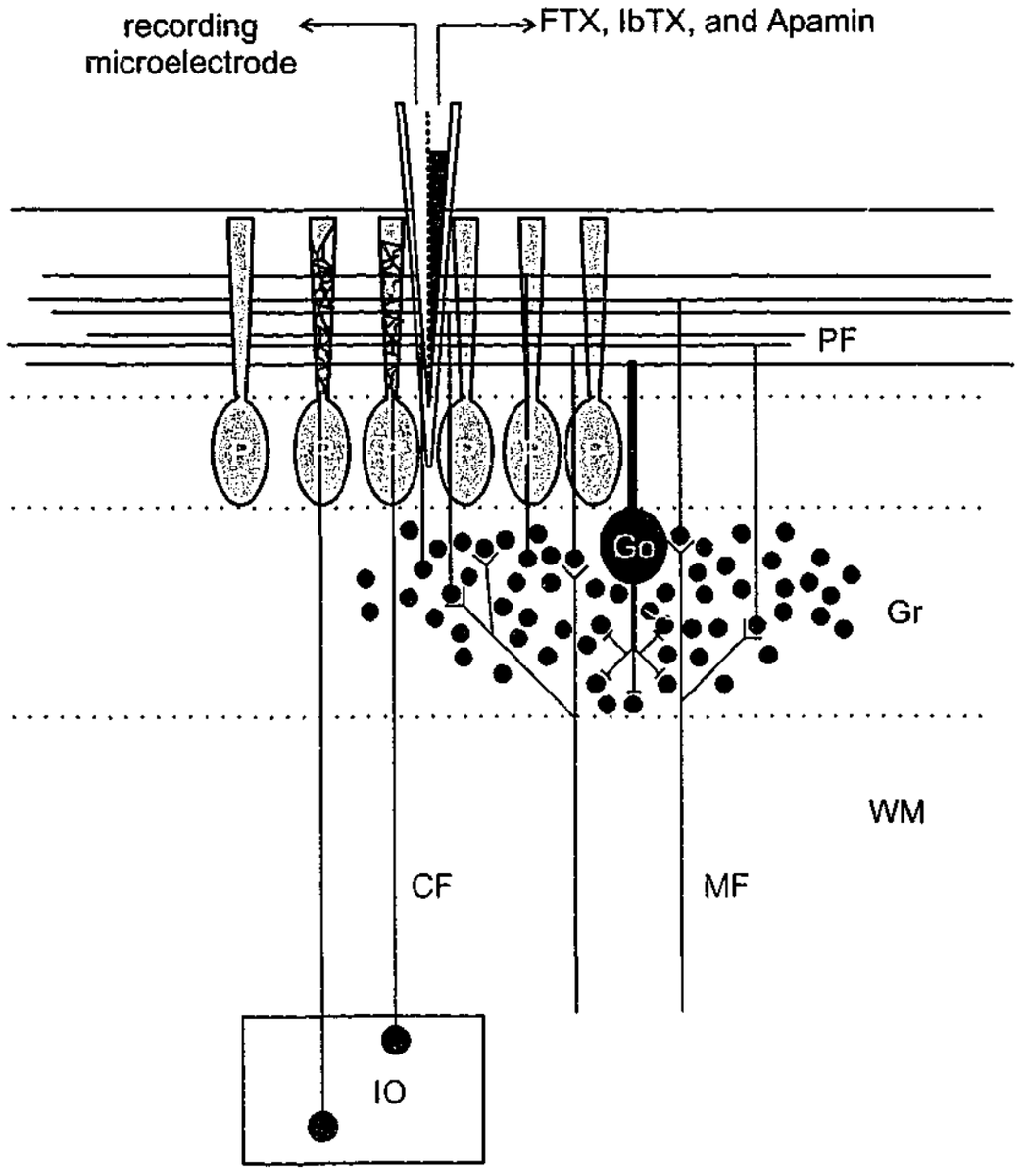

Figure 6.1

Schematic diagram of the experimental setup. A dual microelectrode-micropipette was used to record the effects of various ion channel blockers (FTX, IbTX or apamin) on the response properties of cerebellar Purkinje cells.

IO, inferior olive; CF, climbing fibre MF, mossy fibre; P, Purkinje cell; Gr, granule cell; PF, parallel fibre; Go, Golgi cell; WM, white matter

the toxin. Therefore, comparisons were only made between control and treatment groups.

6.2.3 Measurements and statistical analysis

To quantify the effects of the various drugs on the Purkinje cell simple spike activity the amplitude (peak-to-peak) and the frequency of simple spike discharge were measured before and after application of each channel blocker. In each experiment, control measurements of simple spike activity were averaged over a 2 minute period and compared with the activity measured after 2-10 minutes of exposure to each channel blocker. The results of a number of experiments were then averaged as indicated.

6.3 Results

6.3.1 General comments

Extracellular recordings using dual microelectode-micropipettes were obtained from 22 spontaneously active Purkinje cells. Of these 22 cells examined, 6 were recorded before and after the infusion of FTX, 7 were recorded before and after the infusion of apamin, while the remaining 9 were examined for their effects to the infusion of IbTX. Another 3 Purkinje cells served as controls whereby a saline vehicle was infused to examine any non-specific effects attributable to the infusion. The results from these control experiments (data not shown) showed that the Purkinje cell firing rate and spike amplitude was not affected by the saline injection. Therefore, it was concluded that the influences of the channels blockers resulted from a specific effect on their target channel and not from general effects relating to drug infusion.

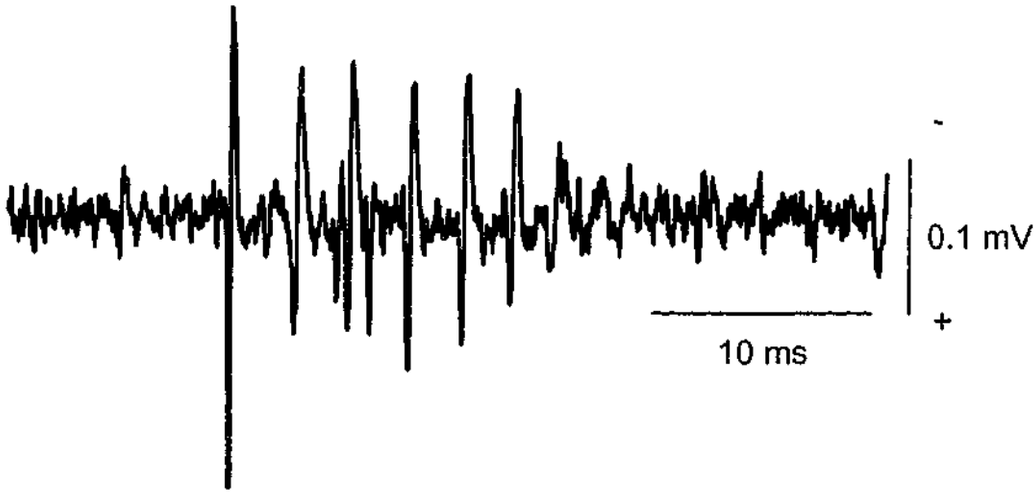

Figure 6.2

Example of a spontaneously occurring complex spike in a cerebellar Purkinje cell.

Purkinje cells were identified by their characteristic discharge, namely, by the presence of an all-or-nothing complex spike that consisted of a large, mainly positive-going initial spike that was followed by smaller wavelets (Figure 6.2). The firing rates of spontaneously active Purkinje cells prior to the infusion of the toxins ranged from 15-101 Hz, with a mean firing rate of 44.2 ± 4.7 Hz.

6.3.2 *Effects of FTX on Purkinje cell activity.*

The infusion of the P-Type VOCC blocker, FTX (1 mM) localised to a spontaneously active Purkinje cell caused a significant increase in average simple spike firing rate compared to its control values (control: 35.18 ± 5.76 Hz and FTX: 76.98 ± 15.5 Hz, $p<0.05$, n=6) (Figure 6.3). There was no significant effect of FTX infusion on spike amplitude ($p>0.05$, n=6). FTX took 2-10 minutes to take effect, with the maximum increase in discharge usually occurring 10-25 minutes after FTX application. The occurrence of complex spike activity in Purkinje cells was not affected by FTX, as these were shown to occur at the same frequency before and after FTX infusion. Presumably, these complex spikes were generated as a result of Na^+ entry into the cell via the glutamate/aspartate receptor. The changes observed with the application of FTX on Purkinje cell simple spike activity appeared to be similar to those observed with climbing fibre removal (Section 3.3.2). However, unlike climbing fibre removal, Purkinje cell simple spike activity never entered the oscillatory cycle of alternating periods of high frequency discharge with periods of quiescence when infused with FTX, even when the recording period was extended to 90 minutes.

To further quantify the effects of FTX on Purkinje cell simple spike activity, ISI histograms were examined before and after the application of the channel blocker. Figure 6.4A shows an example of a Purkinje cell that was infused with FTX, causing an increase in tonic simple spike activity. The distinct increase in simple spike firing rate was accompanied by a shift in the ISI distribution. Under control conditions, the firing pattern of the Purkinje cell was irregular as illustrated in the ISI distribution (Figure 6.4Bi). The firing rate of the Purkinje cell increased and became more regular in response to FTX infusion (Figure 6.4A), reflected in the ISI histogram, which shifted to a narrower distribution (Figure 6.4Bii.). This shift in ISI distribution was also observed in Purkinje cells whose climbing input was removed (Section 3.3.4). The similarity of the changes in Purkinje cell simple spike activity observed with the removal of climbing fibre influence and the infusion of FTX suggests that one mechanism by which climbing fibres act on Purkinje cells is mediated by the P-Type VOCC.

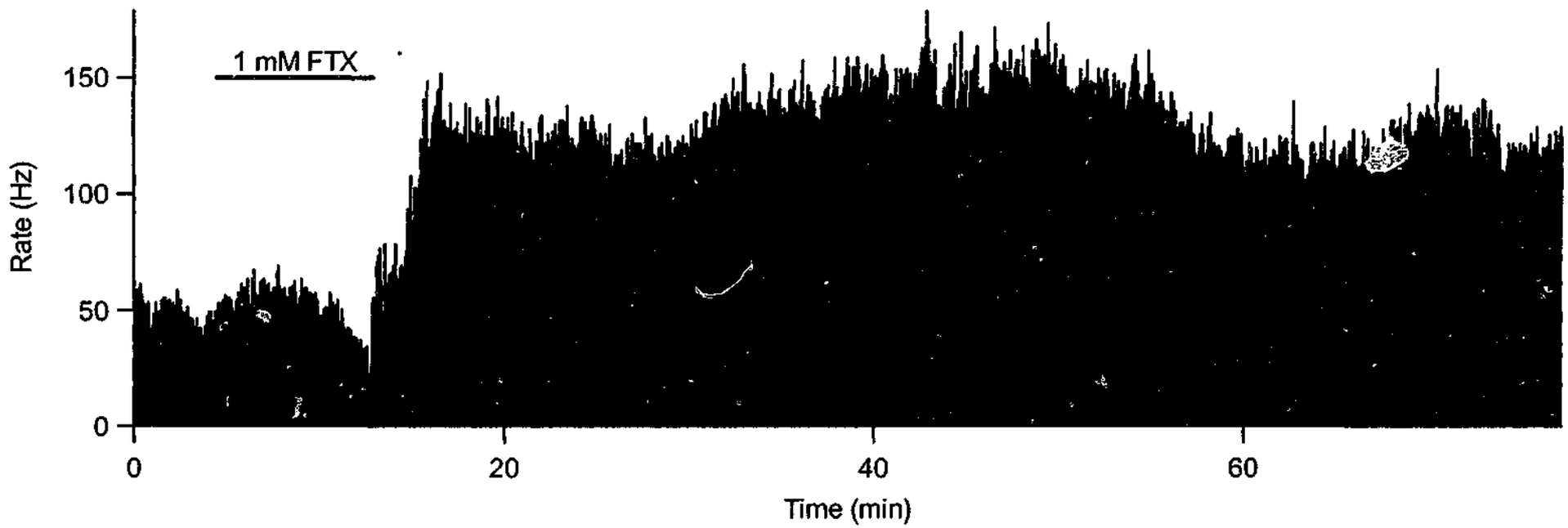

Figure 6.3

The addition of FTX (1 mM) caused a significant increase in the average simple spike firing rate of the Purkinje cell. In this example and those shown in subsequent figures, the period of infusion is indicated by the length of the red bar.

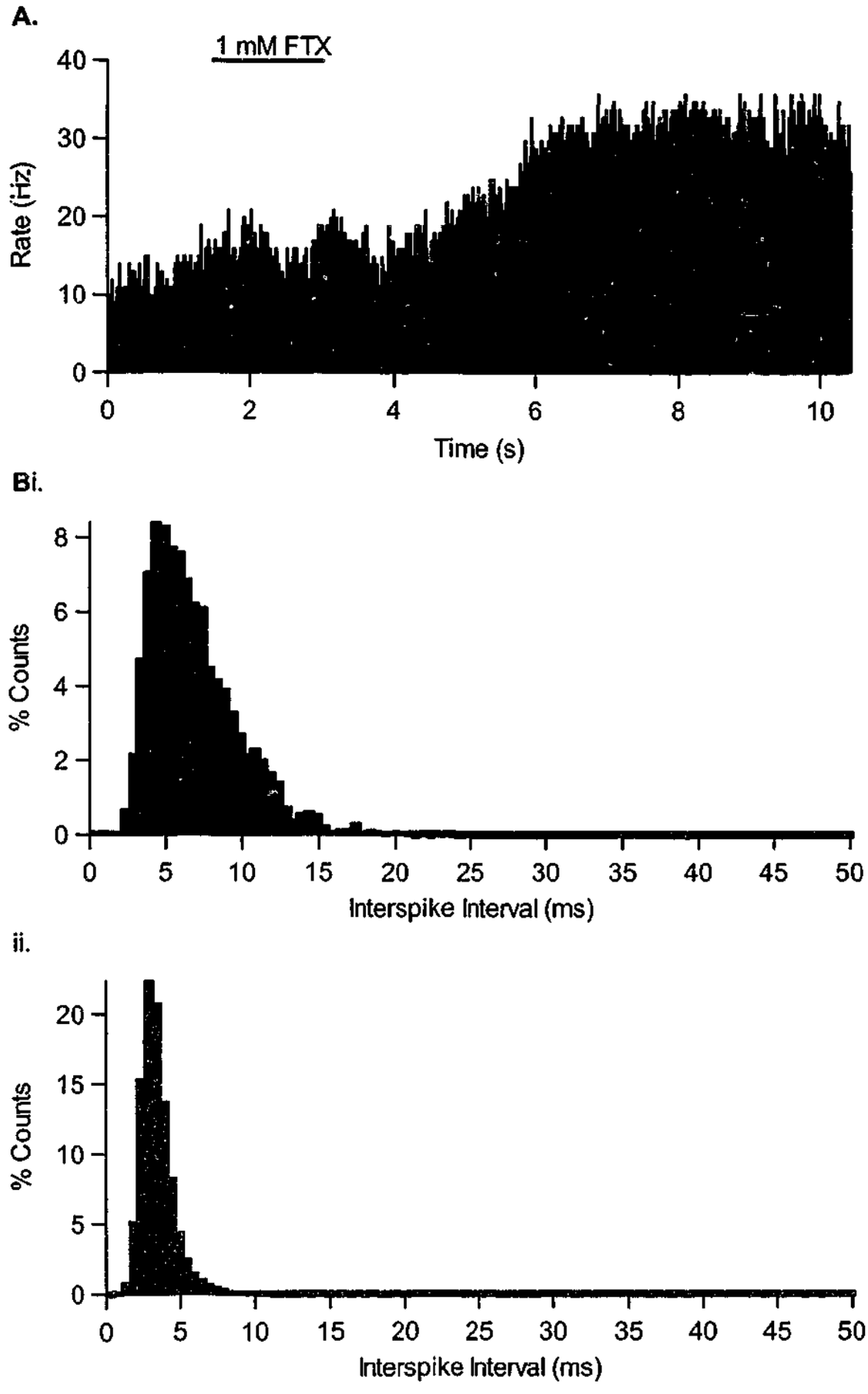

Figure 6.4

The effects of FTX (1 mM) on Purkinje cell activity. **A.** Example of a Purkinje cell infused with FTX. **Bi.** ISI histogram of control period of the same cell in **A.** **Bii.** ISI histogram after the addition of FTX. Note the leftward shift and narrower ISI distribution after the addition of FTX.

Interestingly, while the application of FTX to the Purkinje cell did not alter the rate or completely abolish complex spike discharge, it does appear however, that the infusion of FTX increased the number of secondary spikes appearing in the complex spike discharge. Figure 6.5 illustrates one such example. Before the infusion of FTX, complex spike discharge consisted of a large initial spike, followed by 6 secondary spikes (Figure 6.5A). After the application of FTX (Figure 6.5B), the initial spike remained unchanged, however the number of secondary spikes increased two-fold, from 6 to 12.

6.3.3 Effects of IbTX on Purkinje cell activity

The role of BK channels on Purkinje cell activity was investigated using IbTX (1 μM). Figure 6.6 displays 3 representative Purkinje cells in the presence of IbTX. Only 1 cell of the 7 cells sampled (Figure 6.6C) displayed an increase in Purkinje cell simple spike firing frequency in the presence of IbTX, whereas the remaining 6 cells (Figure 6.6A and B) showed little or no change in response to IbTX infusion. There was no significant change in firing frequency of simple spikes when IbTX was applied to the Purkinje cells (control: 52 ± 7.49 Hz and IbTX: 57.54 ± 9.28 Hz, $p>0.05$, n=7). Spike amplitude was also unaffected by IbTX infusion (control: 0.33 ± 0.052 mV and IbTX: 0.33 ± 0.035 mV, $p>0.05$, n=7).

To explore the role of BK channels in Purkinje cells that had entered the oscillatory mode, IbTX was applied to a further two Purkinje cells that exhibited oscillatory discharge after climbing fibre denervation. Figure 6.7 displays an example of one such cell. The Purkinje cell simple spike discharge before the infusion of IbTX, displayed a typical oscillatory firing pattern, with continuously alternating periods of silence and bursts of high frequency discharge. With the addition of IbTX, the oscillations ceased and simple spike activity returned to a steady rate. These results suggest that, while IbTX had no effect on spontaneously active Purkinje cells with normal simple spike discharge, the BK Ca^{2+}-dependent K^+ conductance may play an important role when Purkinje cells display oscillatory discharge.

6.3.4 Effects of apamin on Purkinje cell activity

To assess the contribution of SK channels to the spontaneous simple spike activity of Purkinje cells, apamin, a blocker of SK channels, was applied to a total of 7 Purkinje cells. The infusion of apamin did not significantly affect spike amplitude ($p>0.05$, n=7) whereas a significant increase in the average firing rate of simple spike activity compared to control values was observed (control: 44.13 ± 10.05 Hz and apamin: 79.2 ± 18.38 Hz,

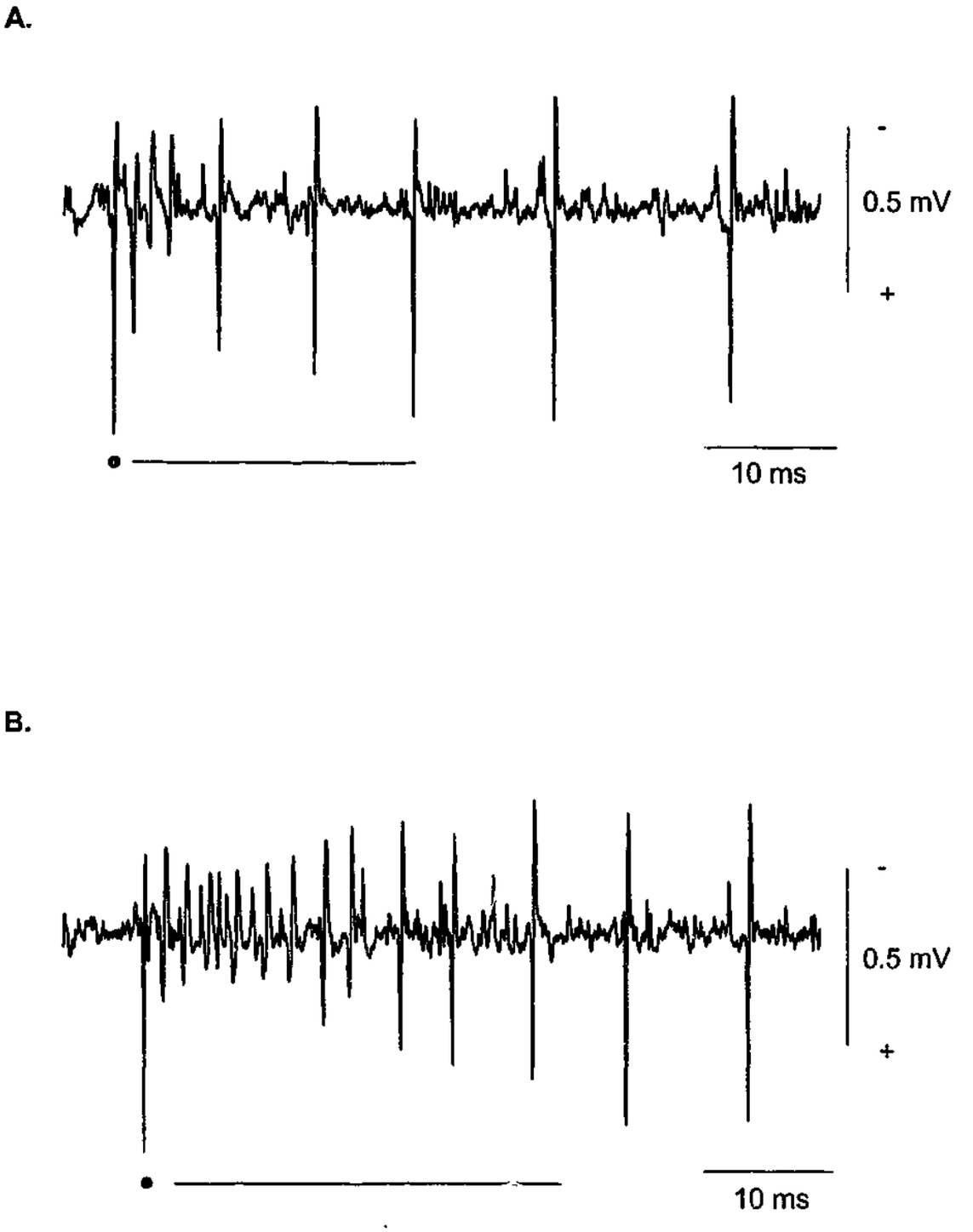

Figure 6.5

The effects of FTX (1 mM) on Purkinje cell complex spike discharge. **A.** Example of a typical complex spike recorded during the control period, prior to FTX infusion. The complex spike consisted of a mainly positive-going initial spike, followed by 6 secondary wavelets. **B.** Complex spike discharge that was recorded 10 minutes after the addition of FTX. The initial spike form was unchanged but the number of secondary spikes increased. For both **A** and **B** the red bar indicates the period where secondary spikes occurred. The dots indicate the initial spike in the complex spike.

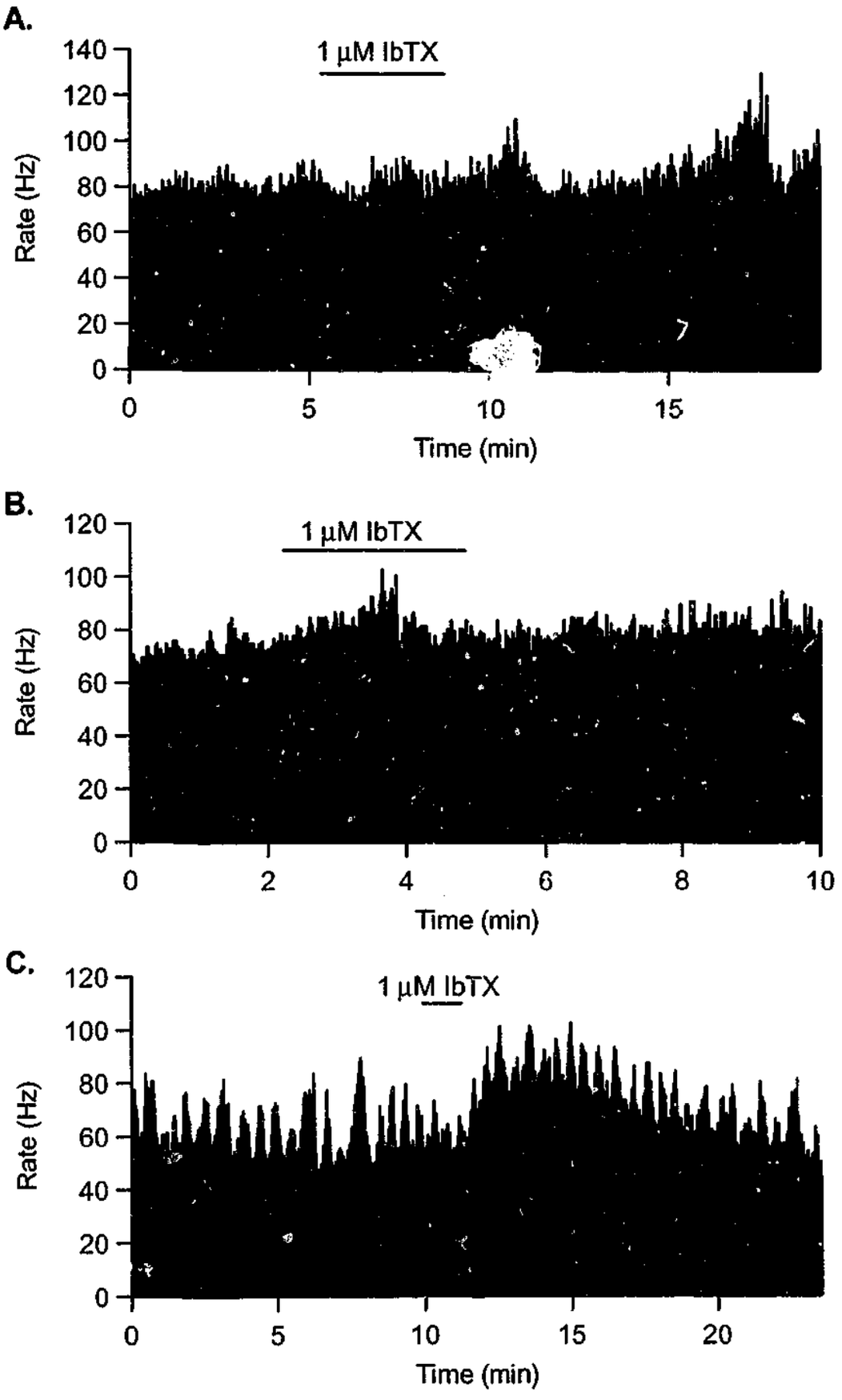

Figure 6.6

Examples of the firing behaviour of 3 Purkinje cells during IbTX (1 µM) infusion. Only one cell (C.) displayed an increase in firing rate, while the remaining cells (A and B) showed little change in firing rate to the infusion of IbTX.

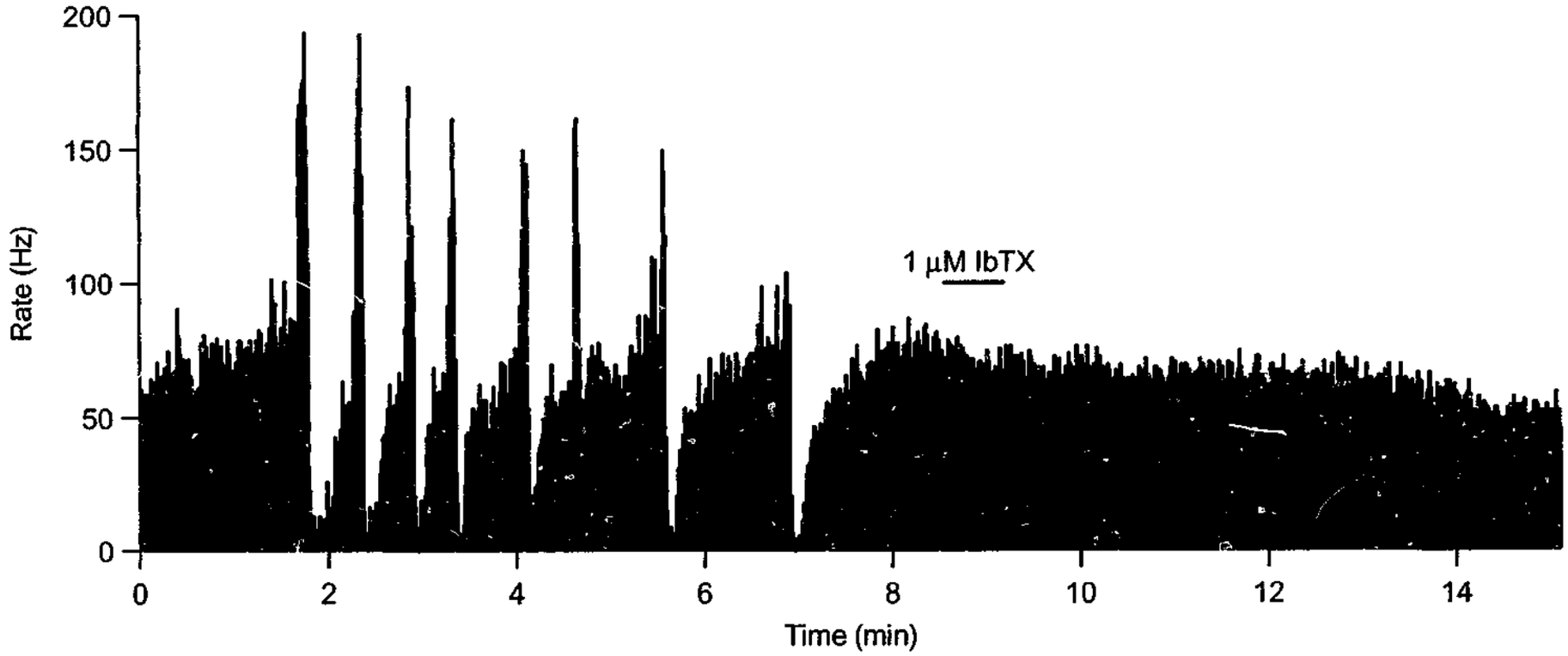

Figure 6.7
Effects of IbTX (1 µM) infusion on an oscillating Purkinje cell. After the infusion of IbTX the cell stopped oscillating and discharged continuously.

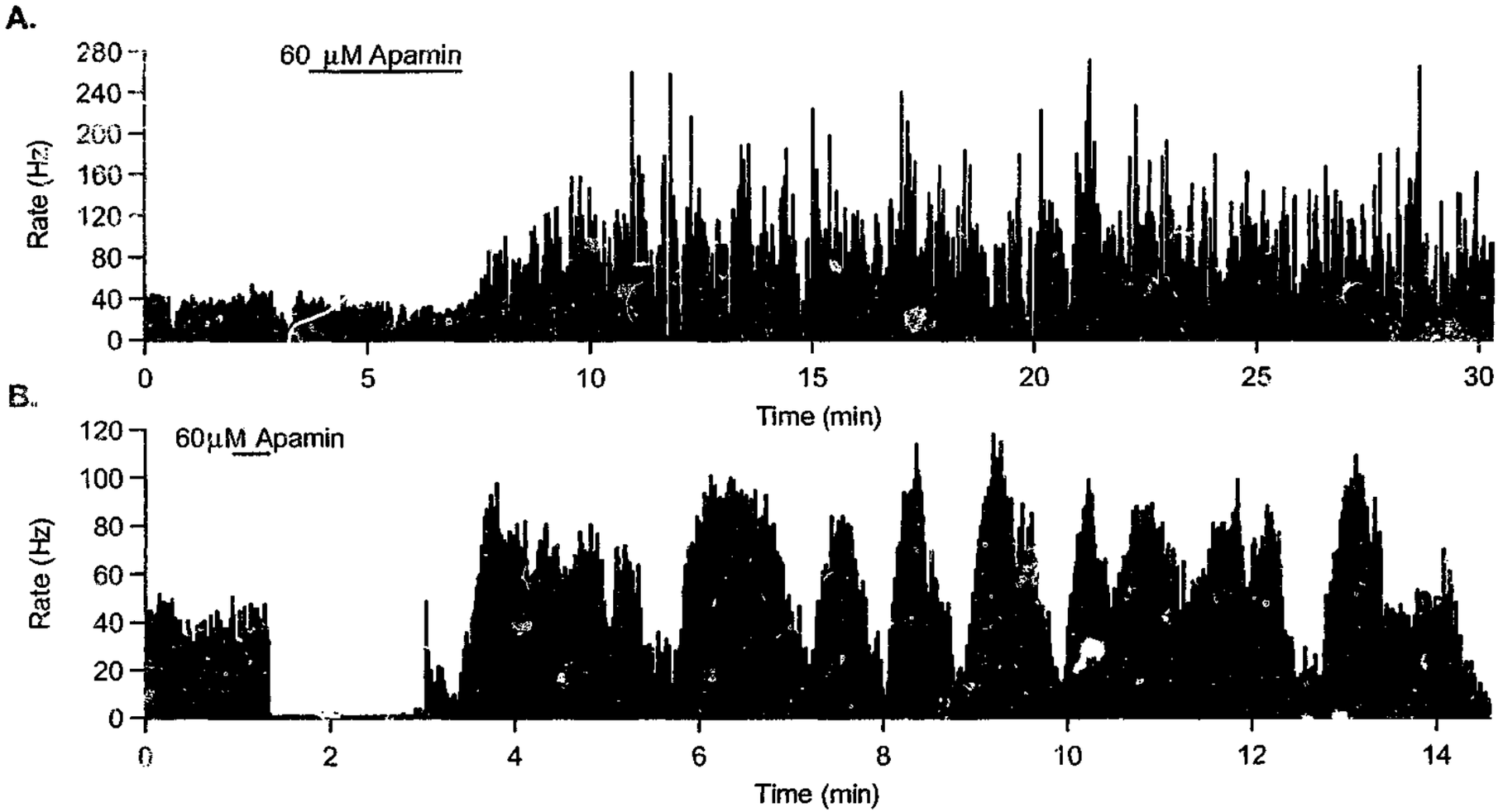

Figure 6.3

Examples of Purkinje cell firing rate during infusion of apamin (60 µM). Apamin significantly increased average Purkinje cell firing rate for the Purkinje cells shown in A. and B. In addition to an increase in firing rate, Purkinje cell firing pattern also resembled the oscillatory behavior of denervated Purkinje cells.

$p<0.05$, n=7). The infusion of apamin also produced bursts of high frequency discharge that were interspersed with periods of silence (Figure 6.8A and B). This bursting activity appeared to resemble the oscillatory behaviour of a Purkinje cell that was deprived of its climbing fibre. The increase in simple spike activity induced by apamin was also reflected in the ISI histograms. For example, in the two Purkinje cells displayed in Figure 6.8, with the addition of apamin, a leftwards shift and a narrowing of the ISI distribution (Figure 6.9Aii. and Bii.) was evident when compared to the ISI histograms measured during the control periods (Figure 6.9Ai. and Bi.).

6.4 Discussion

In the previous chapter, it was demonstrated that the Purkinje cells in the rat cerebellum express two types of Ca^{2+}-dependent K^+ channels, BK and SK2 channels, with the BK channels located in the soma and dendrites, and the SK2 channels restricted to the cell soma. P-type VOCCs are also reported to have a somato-dendritic location in Purkinje cells. The current experiments examined the effects of the channel blockers apamin, FTX, and IbTX on the tonic simple spike activity of Purkinje cells in the anaesthetised rat. In this chapter, the results indicated that the application of specific blockers of these channels had a marked effect on the spontaneous discharge rates of cerebellar Purkinje cells.

6.4.1 Direct or indirect influence of the channel blockers?

In the present investigation, recordings were made from cerebellar Purkinje cells in the *in vivo* anaesthetised rat. The channel blockers were infused around a Purkinje cell that was identified by its distinct firing rate and the presence of all-or-none complex spikes, and although the blockers were assumed to act locally, the precise location, or site of action, of the various channel blockers could not be verified. As described above, the existence of Ca^{2+}-dependent K^+ and P-type VOCCs in cerebellar Purkinje cells is well documented. Thus, it is likely that the changes in Purkinje cell discharge observed with the addition of FTX, apamin and IbTX were a result of direct influences of these drugs. However, the possibility does exist that the effect of the channel blockers is an indirect one, acting on cerebellar cortical inhibitory interneurones and granule cells.

Pharmacological, electrophysiological and expression studies have revealed that various types of VOCCs, including the P-type channel, are present in cerebellar interneurones and granule cells (Randall & Tsien, 1995; Volsen et al., 1995; Westenbroek et al., 1995; Ludwig et al., 1997; Forti et al., 2000). It is unlikely however, that the effects of FTX on cerebellar Purkinje cells observed in this study is attributable to the block of P-type Ca^{2+} channels

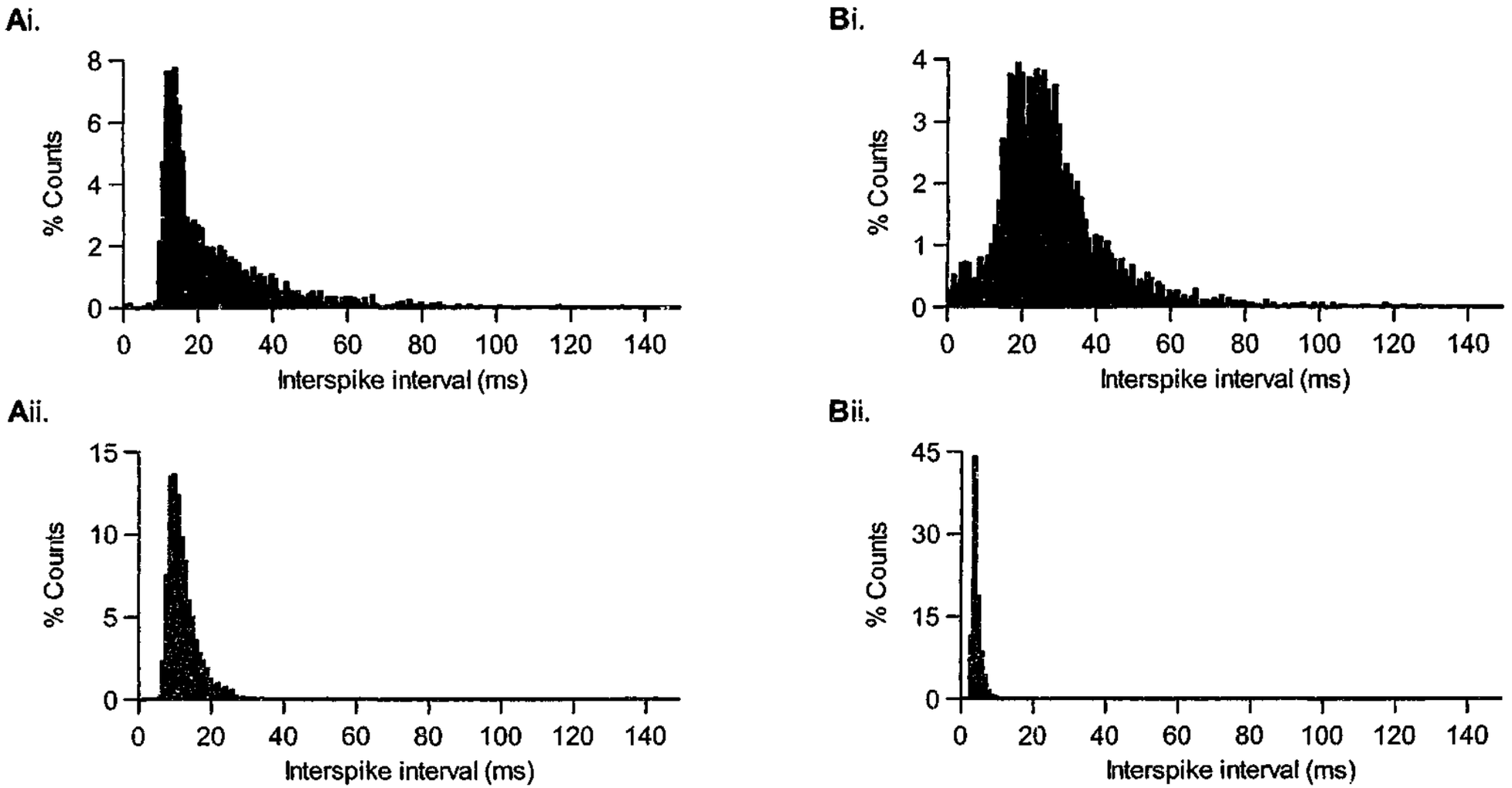

Figure 6.9

ISI histograms of Purkinje cells before and after the addition of apamin (60 μM). Ai. & Aii. ISI histograms of cells shown in Figure 6.8 before the infusion of apamin. Bi. & Bii. ISI histograms of cells shown in Figure 6.8 after the infusion of apamin. Note the leftward shift to a narrower ISI distribution.

located in granule cells, or as a result of disinhibiton of inhibitory interneurones. In support of this, Doroshenko et al., (1997) found that Ca^{2+} channels mediating synaptic transmission at inhibitory synapses are not suppressed by the P-type channel blocker ω-AgaIVA. *In-situ* hybridisation and immunohistochemical studies have detected the presence of N, P/Q, L, and R-type channels in granule cells (Rossi et al., 1994; Randall & Tsien, 1995; Westenbroek et al., 1995; Tottene et al., 1996; Ludwig et al., 1997; Chung et al., 2000). Pharmacological studies examining the contribution of each channel type to the total Ca^{2+} current revealed that P-type channels contribute approximately 11 % of the total Ca^{2+} current in granule cells (Randall & Tsien, 1995). Furthermore, studies by Tottene et al., (1996) found that the properties of P-type Ca^{2+} channels located in granule cells differ from those found in Purkinje cells.

Likewise, the change in Purkinje cell discharge upon the application of apamin and IbTX is unlikely to be attributable to SK and BK channels located in granule cells and interneurones. Firstly, *in situ* hybridisation studies indicate that BK channels are absent in molecular layer interneurones (Knaus et al., 1996), a finding confirmed by the immunohistochemistry results presented in the preceding chapter. Although SK channels are known to be expressed in granule cells and interneurones (Mourre et al., 1986; Gehlert & Gackenheimer, 1993; Stocker & Pedarzani, 2000; Chapter 5 of this thesis), patch-clamp recordings of granule cells has shown that granule cell spike discharge was unaltered by the application of apamin (D'Angelo et al., 1998), whereas in this study, apamin caused Purkinje cells to fire in high frequency bursts. BK channels, while not evident in granule cells in immunohistochemical studies performed in the previous chapter, were reported to be present in granule cells in both culture and in slice preparations (Fagni et al., 1994; D'Angelo et al., 1998). Studies examining the function of these channels in granule cells found that the application of charybdotoxin (ChTX) and submillimolar TEA resulted in an increase in granular cell firing frequency and reduced spike afterhyperpolarization (D'Angelo et al., 1998). Presumably, this would lead to an increase in the excitatory drive to the Purkinje cells via the parallel fibres. However, ChTX is not selective for BK channels alone, with many studies demonstrating that ChTX cross-reacts with several other types of K^+ channels (Hermann & Erxleben, 1987; Schneider et al., 1989; Schweitz et al., 1989; Sorensen & Blaustein, 1989; Tan & Llano, 1999). IbTX, which is highly selective for BK channels, was used in the present study to examine the effects of BK channel blockage on Purkinje cell activity. Moreover, the infusion of IbTX did not alter Purkinje cell firing rate when the climbing fibre input was intact. The effect of blocking BK

channels was only visible in oscillating Purkinje cells. Therefore, it is concluded that IbTX had a direct effect on Purkinje cell discharge.

6.4.2 Effects of FTX

P-type calcium channels are the predominant type of VOCC found in cerebellar Purkinje cells (Llinas et al., 1989; Usowicz et al., 1992; Stea et al., 1994). FTX, a blocker of P-type VOCCs (Llinas et al., 1989; Llinas et al., 1992; Usowicz et al., 1992), has been demonstrated to abolish Ca^{2+} action and plateau potentials, and to inhibit Ca^{2+} currents in Purkinje cells recorded from whole-cell patches or intracellularly (Llinas et al., 1989; Llinas et al., 1992; Usowicz et al., 1992; Dupere et al., 1996; Norris et al., 1996). In this chapter, the blocking of P-type channels with FTX resulted in a significant increase in Purkinje cell simple spike activity, a finding resembling the increase that occurred upon climbing fibre denervation described in Chapter 3.

However, unlike climbing fibre removal, Purkinje cells did not oscillate between periods of high frequency discharge and quiescence. Several possibilities could explain the lack of oscillatory activity. A small component of the total Purkinje cell Ca^{2+} current is carried by VOCCs other than P-type, such as N-, T- and L-type channels (Regan, 1991; Mintz et al., 1992a; Mouginot et al., 1997; Pouille et al., 2000) which would not have been blocked by FTX. It is possible that Ca^{2+} entry through non P-type channels was sufficient to activate Ca^{2+}-dependent K^+ channels and prevent extreme membrane depolarization and thereby the generation of oscillatory firing.

Another possibility as to why P-type channel block did not cause Purkinje cells to oscillate is that subpopulations of P-type channels may exist. A study by Dupere et al., (1996) compared the effects of a synthetic form of FTX (sFTX) that contains an amide carbonyl oxygen that is absent from the synthesised native form of FTX. Both compounds inhibited P-type channels, however differential inhibition of the two forms of FTX revealed that three subpopulations of P-type channels exist. Both types of FTX could block one subpopulation of P-type channels that mediated approximately 66 % of the Ca^{2+} current. The second subpopulation, which constituted 5-25 % of the Ca^{2+} current, was inhibited by sFTX but not FTX, while the third subpopulation representing 10-30 % of the Ca^{2+} current, could be blocked by FTX but not sFTX. Future experiments utilising other blockers that act on all subtypes of P-type channels could be used to examine this further. For instance ω-Aga-IVA, a polypeptide blocker also purified from the Funnel Web spider

Agelenopsis aperta, which binds to the α_{1A} subunit of P-type channels is one such candidate (Uchitel, 1997).

6.4.3 Effects of K^+ channel blockers

Ca^{2+}-dependent K^+ channels have an important role in regulating the afterhyperpolarization and thus the frequency of action potential discharge in neurones in which the ordinary somatic action potentials are mediated by a substantial Ca^{2+} current. In Purkinje cells, the situation is likely to be different as the ordinary somatic action potentials (the simple spikes) are generated purely via Na^+ currents, and these do not propagate to the dendrites (Llinas & Sugimori, 1980a, b; Stuart & Hausser, 1994) where the Ca^{2+} channels are concentrated. It is proposed that Ca^{2+}-dependent K^+ channels have an important role in regulating the overall level of depolarization of the Purkinje cell soma during climbing fibre activity. The actions of apamin, a well characterised SK2 channel blocker, which altered Purkinje cell spontaneous activity from relatively sustained discharge rates to an overall higher frequency burst-like pattern of firing, were consistent with this idea. A decrease in mean ISI was also observed. This resembled the Purkinje cell firing patterns observed in climbing fibre denervation studies. Taken together, these observations imply some functional equivalence between climbing fibre activation and Purkinje cells subjected to pharmacological blockade of SK channels, and that SK channels have an important role in regulating spontaneous Purkinje cell discharge.

In contrast, the addition of IbTX had no consistent effect on Purkinje cell simple spike frequency or interspike interval, except in instances when the climbing input to the Purkinje cell had been removed. The absence of any changes to Purkinje cell discharge with the infusion of IbTX was unexpected, because of the prominent expression of BK channels in the dendritic tree and soma described in the previous chapter. Seemingly, BK currents do not contribute to the regulation of normal Purkinje cell spontaneous discharge. The insensitivity of BK currents to IbTX cannot be explained by inadequate dispersal of the blocker to the Purkinje cells as the method of application of IbTX was identical to apamin, and BK and SK2 channels have been demonstrated to coexist in Purkinje cells. More importantly, an effect on Purkinje cell discharge was observed when climbing fibre denervation had occurred, providing evidence that IbTX was able to diffuse to the Purkinje cells.

BK channels are both voltage and Ca^{2+}-sensitive, opening rapidly in response to rises in intracellular Ca^{2+} within the range of 1-10 μM (Vergara et al., 1998). BK channels, as well as being gated by increases in intracellular calcium, are also modulated by membrane depolarization. An increase in the number and time in the open state of BK channels occurs when the membrane containing the channels is depolarized (Marty, 1981; Barrett et al., 1982; Blatz & Magleby, 1987). As IbTX could alter Purkinje cell discharge in cases where climbing fibre inputs were removed, this could indicate that the activation of BK currents depends on altered ion channel currents and changes in membrane potential that occurs as a result of climbing fibre denervation. Therefore, the increase in membrane depolarization (due to the lack of Ca^{2+} entry activating SK channels that is proposed to occur with climbing fibre denervation could activate BK channels to alter the intrinsic spike generator and aid in the control of Purkinje cell firing and membrane repolarization.

Alternatively, different subpopulations of BK channels may exist in cerebellar Purkinje cells. A diversity of BK channels with variations in Ca^{2+} and voltage dependence, kinetic and pharmacological properties have been described (Solaro et al., 1995; Lingle et al., 1996; Vergara et al., 1998). BK channels are composed of a large pore-forming α-subunit and a smaller modulatory β-subunit. The β-subunit alters the biophysical and pharmacological properties of the channel, including the Ca^{2+}/voltage sensitivity of the α-subunit. In particular, ChTX and IbTX resistant BK channels have been described (Reinhart et al., 1989; Dworetzky et al., 1996; Wallner et al., 1999; Xia et al., 1999; Meera et al., 2000). Recently, studies of BK channels associated with the β4-subunit found that these channels are much less sensitive to the blocking effects of IbTX and CbTX, and are highly expressed in the brain (Meera et al., 2000). Furthermore, *slo* (the BK channel gene), has been found to express numerous isoforms generated by alternative RNA splicing that differ significantly in their Ca^{2+} sensitivity(Tseng-Crank et al., 1994). Together, these observations suggest that different forms of BK channels, having broad functional roles could exist in cerebellar Purkinje cells that could account for the differences observed when IbTX was applied to intact and denervated Purkinje cells.

6.4.4 A compartmental model of SK2 and BK channels

Studies have demonstrated that Purkinje cells can be divided into somato-dendritic divisions with each generating their own activity (Llinas & Sugimori, 1980b; Stuart & Hausser, 1994). Intracellular recordings from Purkinje cells have revealed the presence of a voltage-dependant Na^+ conductance that was restricted to the soma and axon, while voltage-dependant Ca^{2+} conductances, capable of generating dendritic spikes, were

located exclusively in the dendritic tree (Llinas & Sugimori, 1980b; Stuart & Hausser, 1994). Given that BK and SK2 channels have diverse distributions, and that apamin and IbTX had such divergent effects on Purkinje cell discharge, it is likely that these two types of Ca^{2+}-dependent K^+ channels play distinct roles in the control of endogenous Purkinje cell activity, a fact observed in other neuronal populations (Lang et al., 1997; Wang et al., 1998).

SK channels differ from BK channels in two ways. First, SK channels are much more sensitive to intracellular Ca^{2+} than BK channels (Kohler et al., 1996). These channels are activated by nanomolar concentrations of intracellular Ca^{2+}, whereas BK channels require intracellular Ca^{2+} concentrations of 1-10 μM for their activation (Blatz & Magleby, 1987; McManus, 1991; Kohler et al., 1996; Sah, 1996; Vergara et al., 1998). Secondly, SK channel activity is independent of membrane potential, thus they are fully active at negative resting membrane potentials. In contrast, BK channels are voltage-dependent and become active once the membrane becomes depolarized. It is conceivable, that SK channels are important in controlling the overall level of Purkinje cell excitability in response to Ca^{2+} entry whereas BK channels may act to regulate the excitability of the dendritic tree where Ca^{2+} concentration reaches their highest levels during climbing fibre activation (Tank et al., 1988). With their predominant location in Purkinje cell dendrites, BK channels may contribute to the dendritic compartmental model first proposed by Llinas and Sugimori (1980a, b) and since supported by many other studies (De Schutter & Bower, 1994b; Stuart & Hausser, 1994; Pouille et al., 2000).

6.4.5 Summary

The experiments in this chapter examined the effects of various channel blockers specific to BK, SK2, and P-type VOCCs on the tonic Purkinje cell simple spike activity.

Blockage of P-type VOCCs with FTX caused a marked increase in simple spike frequency. This result is consistent with the proposed model that describes the contribution of Ca^{2+} entry, via climbing fibres, in the regulation of the Purkinje cell intrinsic spike generator. The addition of FTX was also shown to alter the firing pattern of Purkinje cell discharge, causing Purkinje cells to discharge more regularly. However, application of FTX did not completely mimic the effects of climbing fibre denervation, as the cell did not enter the oscillatory mode of firing. This could be explained by Ca^{2+} entry via non P-type VOCCs.

Similarly, apamin, which specifically blocks SK2 channel, caused a significant increase in tonic spontaneous Purkinje cell activity. Moreover, the addition of apamin resulted in the

appearance of bursts of high frequency discharge, which were interspersed with periods of quiescence, resembling Purkinje cell firing patterns observed in climbing fibre denervation. It appears that SK2 channels have an important role in regulating the overall level of depolarization of the Purkinje cell soma during climbing fibre activity. However, a surprising finding was that IbTX, a channel blocker specific to BK type channels, surprisingly did not affect the spontaneous activity of the Purkinje cell. BK channels may normally regulate the excitability of the dendritic tree where Ca^{2+} reach their highest levels during climbing fibre activation.

Chapter 7

Functional Considerations

The present findings have shown that ongoing activity in the climbing fibre input to the Purkinje cells is essential for maintaining normal Purkinje cell function. The effects described here can well explain why climbing fibre lesions so profoundly disrupt cerebellar function, especially in the short-term. Here, it is likely that the climbing fibre denervated Purkinje cells are discharging in an oscillatory manner, alternating between periods of high frequency discharge and periods of silence which appear to reflect bouts of intense depolarization and hyperpolarization. This will undoubtedly interfere with the ability of the Purkinje cell to process information arriving via the mossy fibre-granule cell-parallel fibre pathways. It can be predicted that parallel fibre inputs arriving during the depolarizing phase will contribute to spike inactivation, whereas all input is likely to be blocked during the hyperpolarizing phase. This would render the cerebellar cortex functionally inoperative, with no ability to effectively modulate nuclear cell firing.

In the longer-term, it does appear that background Purkinje cell firing returns to a more normal level and this is associated with some recovery of cerebellar function. However, it seems very unlikely that normal function and the ability to regulate baseline Purkinje cell activity could be regained in the absence of climbing fibres.

This action of the climbing fibres in regulating background simple spike discharge would intuitively seem to be of considerable importance in cerebellar operation, and this could subserve a number of functions under different conditions.

7.1 Regulation of cerebellar cortical output

First, this mechanism would permit the climbing fibres to determine a significant proportion of cerebellar cortical output. Thus, the output (at any one time) from Purkinje cells in a zone of cortex would be largely determined by the frequency of the climbing fibre input to the Purkinje cells. The climbing fibres could set the overall operational output from the cortex and the level of DCN activity and the amplitude of cerebellar influence on target neurones in the brainstem and the thalamus. Clearly however, factors other than the climbing fibres would help to determine the baseline level of Purkinje cell activity, particularly those that the establish the long-term setting of the levels of the

intrinsic conductances. It was evident here that the climbing fibres operate around different baselines of activity in different Purkinje cells, in that the simple spike activity did not jump to a common frequency after climbing fibre removal. Cells could also display different levels of simple spike activity in response to climbing fibre stimulation at the same frequency. It would be of interest to determine how the baseline level of simple spike activity is set and it would be of particular interest to establish whether the background is set individually in the Purkinje cells, or whether there is a climbing fibre-linked mechanism that provides a common adjustment for the Purkinje cells in a cortical zone.

7.2 Regulation of Purkinje cell excitability and neuroprotection

De Schutter (1995, 1997) has proposed that climbing fibre-induced LTD at parallel fibre synapses is part of a mechanism that operates to normalise Purkinje cell excitability and prevent excessive and potentially damaging depolarization. Clearly, the action of the climbing fibres described here in reducing Purkinje cell firing could also have a role in regulating Purkinje cell excitability. In fact, it appears that the presence of even a low level of climbing fibre input is essential in preventing excessive depolarization. In the shorter-term, an increase in climbing fibre input would have the effect of reducing overall Purkinje cell excitability. This effect has the advantage over LTD in that it is rapidly acting. Indeed, the indications from the present study are that climbing fibres are capable of dealing quite effectively and rapidly with even high levels of depolarization, as indicated by the observation that the higher levels of simple spike activity that occurred after climbing fibre denervation could be restored towards normal with climbing fibre stimulation rates of only about 1 Hz. It could therefore be that an increase in climbing fibre activity is the first line of defence to help prevent excessive depolarization by rapidly reducing overall excitability via activation of SK2 channels and hyperpolarization. The LTD mechanism proposed by De Schutter might then subsequently act in a more targeted fashion to reduce transmission through sets of excessively activate parallel fibres.

A striking indication of the importance of hyperpolarization mediated through SK2 channel activity in regulating Purkinje cell excitability comes from a study by Mouree et al., (1997) in which they chronically infused apamin into rats. These animals presented with a loss of postural control, paroxysystic jerking, and alternating periods of agitation with clonic-tonic convulsions and periods of total prostration, symptoms very much akin to those of acute climbing fibre loss. Moreover, they found bilateral degeneration of the

Purkinje cells in the apamin treated animals. From this, it was concluded that apamin had produced excessive depolarization of the Purkinje cells with the accumulation of toxic levels of Ca^{2+} which lead to the induction of cell death. It would indeed be interesting to know whether a substantial loss of climbing fibre innervation leads to Purkinje cell death.

Miall et al., (1998) have proposed that the ongoing activity of parallel fibres would, if left unchecked, lead to an ever increasing level of Purkinje cell simple spike firing. They suggested that complex spike activity in the absence of movement may have an 'autocorrective' effect, in that the increasing simple spike activity will reduce the cerebellar nuclear firing rate, in turn, disinhibiting cells of the inferior olive and causing an increase in climbing fibre activity which would then reduce parallel fibre transmission by LTD. In support of this hypothesis, a recent study by Bengtsson et al., (1999) has shown that the feedback loop from nucleus interpositus to the inferior olive is potentially quite potent in increasing climbing fibre activity. They demonstrated that injection of lignocaine into the output fibres from nucleus interpositus anterior caused a substantial increase in climbing fibre frequency to about 4 Hz, which led to a cessation of simple spike activity in the Purkinje cell (as would be predicted from the present discharge). If this feedback loop from the Purkinje cells and DCN to the inferior olive is indeed involved with normalising Purkinje cell excitability in the face of high levels of simple spike firing, then again, the mechanism of climbing fibre action described here is suggested as the prime candidate to perform this function (but see below for an alternative explanation).

7.3 Motor learning and classical conditioning

While it is very probable that the cerebellum is involved in motor learning, the experimental approach taken in this study cannot provide any direct evidence for or against the role of LTD. The idea that climbing fibres are obligatory in the process of LTD and motor learning is however questionable, as the MAIT predicts that sudden changes in climbing fibre activity should not alter Purkinje cell activity, as it is thought that climbing fibre activity should be able to modify simple spike activity gradually while learning takes place. However the results presented in this thesis and supported by others (Colin et al., 1980; Demer et al., 1985), show that changes in climbing fibre activity can rapidly alter Purkinje cell excitability.

Further evidence questioning the involvement of the climbing fibres in LTD and motor learning comes from the recent study by Goossens et al., (2001) who compared LTD,

motor learning and Purkinje cell properties in transgenic mice where protein kinase C (PKC) was specifically blocked in Purkinje cells (L7-PKC1) as compared with their wild-type litter mates. No difference in Purkinje cell simple and complex spike discharge properties (such as mean firing rate and interspike interval) were found between the L7-PKC1 and wild-type groups, however L7-PKC1 mutants showed impaired adaptation of the vestibular-ocular reflex, whereas their eye movement performance was unaffected. Earlier experiments indicated that L7-PKC1 transgenics up to 35d of age displayed multiple climbing fibre innervation (De Zeeuw et al., 1998a), however Goossens et al., (2001) who used animals 3-12 months in age found that in their L7-PKC1 mutants, multiple climbing fibre innervation no longer persisted, with Purkinje cells receiving inputs from a single climbing fibre. These experiments thus indicate that PKC is important for motor learning, but it appears that single climbing fibre innervation is more important for maintaining Purkinje cell firing properties. It would be interesting to examine the Purkinje cell firing properties activity prior to 3 months, in order to examine the difference between Purkinje cells innervated with mono and multiple climbing fibre inputs. A comparison could then be made with the current proposed theory of climbing fibre function, as it would of interest to contrast Purkinje cell discharge when its climbing fibre was removed with Purkinje cells innervated with multiple climbing fibres. Based on the observations from the climbing fibre denervation and stimulation studies, it would be expected that the increase in the number of climbing fibre inputs to the Purkinje cell will surely alter their firing properties.

Recent evidence from behavioural and physiological studies has indicated that the cerebellar nuclei may in fact be the site for plasticity and learning in the cerebellum (Garcia & Mauk, 1998; Aizenman & Linden, 2000). However, the only situation to date in which a motor learning trace has clearly been localised to the cerebellum is in classical conditioning (eg Thompson & Kim, 1996) where the paired presentation of a tone (the conditioned stimulus, transmitted via the mossy fibres) with a reinforcing unconditioned stimulus such as a puff of air on the cornea (transmitted via the climbing fibres) promotes the acquisition of a conditioned motor response (closing of the eyelid in response to the tone). Lesion experiments have conclusively shown that the conditioned trace is established in the nucleus interpositus or the overlying cortex (Thompson & Kim, 1996). Very recently Medina et al., (2001) have shown that inactivation of nucleus interpositus during the conditioning process prevents the acquisition of the conditioned response, indicating that the trace is normally established in the nucleus rather than the cortex. They then showed that even after the behavioural response was apparently extinguished,

there was still an enhanced trace to the conditioned input in nucleus interpositus, but this was only apparent after Purkinje cell inhibition had been blocked by picrotoxin. They proposed that the conditioned mossy fibre trace was established by long-term potentiation (LTP) at collateral synapses in the nucleus, and that a reduction in Purkinje cell firing and a removal of inhibition was essential to enable LTP to occur. Quite clearly, the action of the climbing fibres in reducing Purkinje cell firing as described here could provide the condition for learning or memory traces to be established in the cerebellar nuclei without the need to involve LTD in the cortex. This mechanism would not require precise coincidence between climbing and mossy fibre inputs and overcomes the timing constraints that are inherent in evoking LTD. According to the MAIT, in order to establish LTD, the mossy fibre-parallel fibre input needs to arrive conjunctively with, or very shortly after the climbing fibre input. This does not occur in classical conditioning in the cerebellum, where the mossy fibre input actually precedes the climbing fibre input. In light of this potential role of climbing fibres in setting the conditions for motor learning, it seems worthwhile to reconsider the possible function of the feedback pathway from the DCN to the inferior olive. As mentioned above it has been suggested that a decrease in the inhibitory output from the DCN to the inferior olive serves to normalise Purkinje cell excitability in the face of increased simple spike activity. However, it could be that a repetitive strong mossy fibre input that causes an increase in simple spike firing provides the signal for motor learning (at least in classical conditioning). Such that the periods of increased simple spike activity cause disinhibition of the inferior olive neurones and the increased climbing fibre input then suppresses Purkinje cell firing, which relieves the DCN from inhibition and provides the condition for establishing LTP at the mossy fibre collateral synapses with the DCN.

7.4 Regulation of mossy fibre responses

Most other theories of climbing fibre function take into account their interaction with the mossy fibre system, and there is a need to determine how the modulatory role of the climbing fibres on the Purkinje cell intrinsic pacemaker influences mossy fibre responses. As climbing fibre stimulation and removal was found to alter the ongoing simple spike activity generated by the intrinsic pacemaker, this suggests that the climbing fibres would determine the level of simple spike activity against which evoked mossy fibre responses will be superimposed. It is notable that Rawson and Tiloskulchai (1982) found that all Purkinje cell background activity disappeared with repetitive stimulation of the climbing fibres before the simple spike response evoked by parallel fibre stimulation. This suggests

that simple spike activity generated intrinsically is more sensitive to suppression than activity generated synaptically. Thus, it is possible that the climbing fibre input could reduce background discharge and cause an evoked response to be accentuated in terms of signal noise ratio. In simple terms, a mossy fibre will stand out against a low or absent background Purkinje cell discharge, but will be indistinct or submerged against a background that is high and irregular. Thus, it is possible that the climbing fibres could optimise the signal to noise ratio of the mossy fibre response by controlling the level of ongoing activity.

Of course, further experiments examining the effects of climbing fibre activity on mossy fibre responses are required to fully test this proposition. This could be achieved by evoking mossy fibre responses in Purkinje cells using controlled, reproducible, and natural stimuli, and determining how these responses are affected by changes in the frequency of climbing fibre input to the Purkinje cell.

Chapter 8

Future directions

The experiments described in the preceding chapters have largely verified the basic hypothesis that climbing fibres control intrinsic simple spike activity. Thus, it was demonstrated that very limited changes in the frequency of climbing fibre input could modulate the level of intrinsic activity up or down and over a wide range. The effects of climbing fibre removal also demonstrate that ongoing input from the climbing fibres is essential for maintaining normal Purkinje cell function. The effects of ion channel blockers were also largely consistent with the proposed model and indicated that Ca^{2+} entry via P-type VOCCs and modulation of excitability via SK channels are important components in climbing fibre control of the Purkinje cells.

Some aspects of the proposed mechanism could not be directly verified, and clearly, there are limits to which the details of ionic mechanisms can be elucidated in the present *in vivo* type of preparation, in which it is not possible to manipulate the extracellular ionic environment or to obtain long-term stable intracellular recordings from Purkinje cells.

In future, the present model would need to be studied further using an *in vitro* cerebellar slice preparation. Although the Purkinje cells would have lost their climbing fibre, and would display oscillating behaviour, it may be possible to 'normalise' such cells by repetitive stimulation of climbing fibre axons in the white matter. It would then indeed be of interest to monitor Ca^{2+} levels with different frequencies of climbing fibre stimulation and correlate them with simple spike activity. This could be achieved with imaging studies using Ca^{2+} sensitive dyes or with specialised ion selective microelectrodes to measure the changes in Ca^{2+} ion concentration.

The regulation of Ca^{2+}-dependent K^+ SK2 channels could also be the subject of further experiments, as these channels appear to be of considerable importance in mediating the action of the climbing fibres and for maintaining normal levels of Purkinje cell excitability. It would be interesting to determine how expression of these channels is regulated by climbing fibre loss and climbing fibre activity. In particular, it would be of interest to determine whether channel expression is upregulated after chronic climbing fibre loss to compensate for the reduced Ca^{2+} entry.

It has been proposed that the progressive depolarization of the Purkinje cell that occurs with climbing fibre denervation is due to a progressive inactivation of the non-inactivating K^+ channels through a lack of phosphorylation of Purkinje cell Ca^{2+} dependent biochemical processes. Further *in vitro* experiments involving protein kinase inhibitors would be useful in examining whether inhibition of protein phosphorylation affects tonic Purkinje cell activity and current flow through the K^+ channels.

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