INTRINSIC MEMBRANE PROPERTIES AND PLASTICITY IN MEDIAL VESTIBULAR NUCLEUS NEURONES

by

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DECLARATION

The work described in this thesis was carried out by myself, apart from the experiments in Chapter 3 which were completed in conjunction with Dr. A.R. Johnston. The work was conducted under the supervision of Dr. M.B. Dutia in the Department of Biomedical Sciences, at the University of Edinburgh between October 1998 and October 2001.

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Contents

ABSTRACT

This thesis describes *in vitro* electrophysiological experiments on horizontal brainstem slices containing medial vestibular nucleus (MVN) from young adult and aged rats. The whole-cell patch clamp recording technique was used in MVN slices of young adult rats to study intrinsic membrane properties and spike firing characteristics of rostral MVN neurones and their role in vestibular compensation, the behavioural recovery that follows damage to the vestibular receptors or nerve of one inner ear. Extracellular recordings were made in MVN slices of young and aged rats to study age-related changes in the intrinsic activity and GABA receptor sensitivity of MVN neurones.

MVN neurones were classified into Type A and Type B cells based on their action potential shapes. Type B cells were further grouped into Type B_{DP} cells, which showed depolarising plateau potentials, and Type B_{LTS} cells, which showed low threshold spikes, according to their firing behaviours in response to depolarising and hyperpolarising current pulses. The results showed that significant adaptive changes take place in intrinsic membrane properties and firing characteristics of specific subpopulations of vestibular neurones in the rostral region of the ipsilateral MVN during vestibular compensation following unilateral labyrinthectomy (UL). Type B cells had significantly higher resting discharges and more depolarised resting membrane potentials in post-UL slices, while Type A cells did not show any change in their *in vitro* firing rates and resting membrane potential. However, after UL, a greater number of Type A cells expressed spike frequency accommodation, and there was a significant increase in the gain of Type A cells. Significantly more Type B cells showed low threshold spikes in MVN slices of labyrinthectomised rats suggesting that T-type calcium currents were up-regulated in some MVN cells during vestibular compensation. Up-regulation of intrinsic excitability in Type B cells and changes in dynamic membrane properties of Type A cells could be important *in vivo* in recovery of neuronal activity of MVN cells which are silenced soon after UL.

The intrinsic spontaneous activity of MVN neurones in aged rats was similar to that in young animals suggesting that intrinsic properties of MVN neurones are maintained in the aged animals. The responsiveness of the aged MVN neurones to the $GABA_A$ receptor agonist muscimol was significantly greater than in young adults, while their responsiveness to the $GABA_B$ receptor agonist baclofen was not significantly different from those in young animals. $GABA_A$ receptor up-regulation in aged rats may serve to counteract a possible decrease in efficacy of the commissural and cerebellar inhibitory systems with aging, while at the same time, alterations in $GABA_A$ receptor function in aged rats could be involved in the impairment of vestibular compensation in older animals.

ABBREVIATIONS

MVN	medial vestibular nucleus
LVN	lateral vestibular nucleus
SVN	superior vestibular nucleus
IVN	inferior vestibular nucleus
VOR	vestibulo-ocular reflex
VCR	vestibulo-collic reflex
FTN	floccular target neurones
INC	interstitial nucleus of Cajal
IO	inferior olive
AHP	afterhyperpolarisation
UL	unilateral labyrinthectomy
SN	spontaneous nystagmus
CNS	central nervous system
aCSF	artificial cerebrospinal fluid
MΩ	megaohm
h	hour
ms	millisecond
mM	millimolar
μM	micromolar
nA	nanoampere
pA	picoampere
mV	millivolt
ISI	interspike interval
LTS	low threshold spikes
S.E.M.	Standard error of the mean

Chapter 1

An overview of the vestibular system and vestibular compensation

1.1 Vestibular system: Anatomy and physiology

The vestibular system consists essentially of the vestibular organs, namely the semicircular canals and the otoliths, in the periphery and the vestibular nuclei and associated motoneurones in the brainstem. The vestibular organs, which are situated in the bony labyrinth of the temporal bone, provide information about head orientation and motion in three-dimensional space. There are three semicircular canals that are located in the inner ear on each side of the head. The horizontal, anterior and posterior semicircular canals, each oriented approximately in each plane of three-dimensional space, detect angular movements of the head such as nodding up and down, tilting side to side and rotating side to side. The otolith organs, the utricle and the saccule, are located between the semicircular canals and the cochlea of the inner ear, and oriented perpendicular to each other. These sac-like organs signal linear acceleration and head orientation relative to the earth's gravity. A major role of the saccule and the utricle is to keep a person vertically oriented with respect to gravity. If the head and body start to tilt, the vestibular reflex system compensates to correct postural alignment. This arrangement of the semicircular canals and the otoliths permits the most efficient signal detection in three-dimensional space. The information about the position and velocity of head movement sensed by the hair cells in the semicircular canals and the otolith organs is transmitted through the eighth cranial nerve to the second order vestibular neurones located in the brainstem vestibular nuclei (Figure 1.1). The cell bodies of the eighth nerve are located in Scarpa's ganglion of the labyrinth. There are four main vestibular nuclei on each side of the brainstem: the superior, inferior, medial and lateral vestibular nuclei. All four nuclei receive at least some primary vestibular fibres, and they differ as to the source of the primary afferents in the labyrinth; for example, the information from the three canals is predominantly represented in the medial vestibular nucleus (MVN) and superior vestibular nucleus (SVN) while lateral vestibular nucleus (LVN) receives input mainly from the otolith organs (Stein and Carpenter, 1967; Gacek, 1969). No major subdivision of the vestibular nuclei is supplied by only one part of the labyrinth.

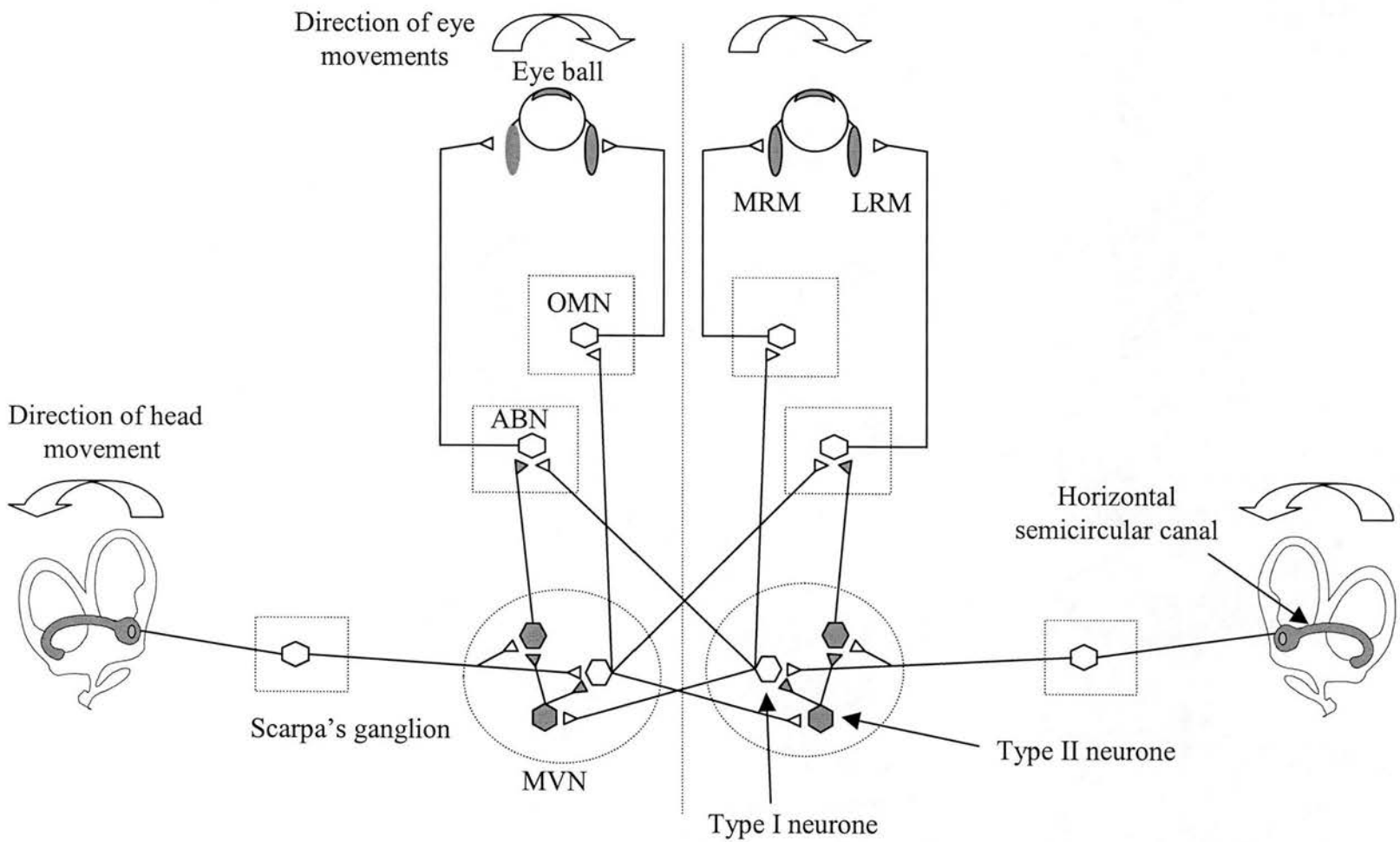

Figure 1.1. Simplified schematic diagram of the neuronal network mediating horizontal vestibuloocular reflex (VOR). LRM, lateral rectus muscle; MRM, medial rectus muscle; OMN, oculomotor nuclus; ABN, abducens nucleus; MVN, medial vestibular nucleus. (⬡ ;excitatory neurone, ⬢ ; inhibitory neurone)

The function of the vestibular system is to stabilise eye and body position in order to ensure precise, goal-directed movements and clear vision. The vestibular synergies that link the vestibular end organs to the target oculomotor and spinal motoneurones are employed for this purpose. The most direct neuronal network between the semicircular canals and eye muscles in the vertebrate is the three-neurone vestibulo-ocular reflex (VOR) arc, which can transmit information rapidly and provide a motor reaction within 14 ms after a vestibular stimulus (e.g. head movement) has occurred (Lisberger, 1984).

This three-neurone arc, as schematically described in figure 1.1, consists of;
(1) The primary vestibular neurones in Scarpa's ganglion located in the inner ear connecting the sensory cells in the crista ampullaris with the neurones in vestibular nuclei.
(2) The secondary vestibular neurones in brainstem vestibular nuclei sending their axons to the oculomotor nuclei via the medial longitudinal fasciculus.
(3) The motoneurones innervating the various extra-ocular muscles.

The function of this VOR pathway is to counter-rotate the eyes in the orbits by an equal and opposite amount to the head movement, so that they remain fixed on the same point in space. Via this connectivity, for example, horizontal canal stimulation results in contraction of the contralateral lateral rectus muscle and the ipsilateral medial rectus muscle at the same time with the inhibition of antagonistic muscles, ipsilateral lateral rectus muscle and contralateral medial rectus muscle (Figure 1.1)

Many vestibular nucleus neurones also project monosynaptically to the neck muscle motoneurone pools in the cervical spinal cord (Bankoul and Neuhuber, 1992) and mediate the vestibulo-collic reflex, VCR. The VCR evokes reflex contractions of those neck muscles most appropriate to resist and counteract the head movement, thereby stabilising the head itself in relation to the neck. This integrated stabilisation of eyes and head gives the subject an essential base on which to execute co-ordinated eye and head movements. (For a review see Dutia, 1988).

1.1.1 Afferent connections of the MVN

The major input to the vestibular nuclei comes from the vestibular organs carried by the eighth cranial nerve. The second-order neurones in vestibular nuclei are monosynaptically and polysynaptically activated by the sensory afferents coming from the ipsilateral labyrinth (Precht and Shimazu, 1965; Markham 1968; Shinoda and Yoshida, 1974). The majority of primary vestibular projections to the MVN arise from the horizontal semicircular canals (Stein and Carpenter, 1967; Gacek, 1969; Korte, 1979; Carleton and Carpenter, 1984; Sato et al., 1989). The MVN cells respond to both natural stimulation, such as head turn, and electrical stimulation of the eighth nerve. The inputs to the MVN from the canals are functionally excitatory and mediated by excitatory amino acid neurotransmitters: glutamic acid and/or aspartic acid (Raymond et al., 1984; Touati et al., 1989; Yamanaka et al., 1997) acting on mainly non-NMDA receptors (Doi et al., 1990; de Waele et al., 1990; Carpenter and Hori, 1992; Capocchi et al., 1992). NMDA receptor mediated vestibular input to the MVN has also been described recently in the guinea pig (Babalian et al., 1997). In the monosynaptic vestibular input to the MVN is also a component that is resistant to both NMDA and non-NMDA receptor blockers (Babalian et al., 1997). Although horizontal canal afferents were shown to terminate mainly in the rostral part of the MVN, the rostral MVN neurones were also shown to be responsive to the other semicircular canals, anterior and posterior semicircular canals, (Shimazu and Precht, 1965; Markham, 1968; Jones and Milsum, 1970; Curthoys and Markham, 1971) and to the otoliths (Stein and Carpenter, 1967; Gacek, 1969, Schor et al., 1998).

Primary vestibular afferents constitute the major, but not the sole input to the vestibular nuclei. Various other sensory systems such as the visual and somatosensory apparatus and input from other areas of the brain combine with the vestibular organs in assuring a high sensitivity for the adjustment of the body in space.

Cerebellar and brainstem input to the MVN

A large afferent contribution to the vestibular nuclei comes from the vestibulocerebellum, which includes flocculus, nodulus, uvula and paraflocculus, and from the cerebellar nuclei. Immunohistochemical tracing studies showed that fibres from the flocculus and the paraflocculus terminate mostly in the rostral part of the ipsilateral MVN (Yamomoto et al., 1978; Sato et al., 1982; Langer et al., 1985; Umetani, 1992; Nagao et al., 1997; Balaban et al., 2000) whereas axons from the nodulus terminate in the caudal part (Angaut and Brodal, 1967; Langer et al., 1985; Matsushita and Wang 1986). The MVN also receives projections from the ipsilateral medial cerebellar nucleus and from the ipsilateral interstitial nucleus (Compoint et al., 1997; Teune et al., 2000). The electrophysiological data concerning the cerebellar connections is in agreement with the anatomical data; the rostral MVN neurones were shown to be inhibited by stimulation of the flocculus at monosynaptic latencies (Sato et al., 1988; Lisberger et al., 1994; Stahl and Simpson, 1995) and these cells were identified as floccular target neurones (FTN). Monosynaptic inhibitory postsynaptic potentials from the flocculus were bicuculline sensitive, suggesting a $GABA_A$ receptor mediated transmission from Purkinje cells to vestibular nucleus neurones (Babalian and Vidal, 2000). FTNs are involved in the three-neurone arc of the VOR and send glycinergic inhibitory projections to the ipsilateral abducens nucleus. In contrast to floccular Purkinje cells the fastigial nucleus has a tonic facilitatory influence on MVN neurones (Ito, 1970; Shimazu and Smith, 1971). Through these connections the floccular cortex, which receives convergent vestibular (Brodal and Hoivik, 1964; Barmack et al., 1993; Bäurle et al., 1998), visual (Maekawa and Simpson, 1973) and oculomotor information (Kimura and Maekawa, 1981), is involved in controlling vestibular related motor activities. Apart from its role in normal vestibular physiology, the cerebellum has been suggested to play an important role in vestibular plasticity; mediation of long-term plasticity of the VOR gain (Lisberger et al., 1994, du Lac et al., 1995) and lesion induced plasticity during vestibular compensation (Kitahara, 1997).

A number of other brainstem nuclei were also shown to send fibres to the MVN. Anatomical studies using HRP injection showed that the MVN receives afferent fibres from the interstitial nucleus of Cajal (INC) (Pompeiano and Walberg, 1957; Carpenter et al., 1970) and from the nucleus prepositus hypoglossi (Pompeiano et al., 1978). The direct projections from the INC to the MVN were also confirmed by electrophysiological studies by Markham et al. (1966). Because INC receives input from the cerebral cortex it may serve as a relay station for cerebro-vestibular connections. Pontine nucleus and medullary reticular formation, which receive afferents from the spinal cord, cerebral cortex and other higher levels of the brain, send descending fibres to the MVN (Grottel and Jakielska-Bukowska, 1993). Immunohistochemical studies have shown that MVN neurones receive a prominent innervation from the raphe nuclei (Steinbuch, 1981, 1991) and noradrenergic input from the locus coeruleus (Schuerger and Balaban, 1993). Anatomical and electrophysiological studies of the afferent projections from the inferior olive (IO) by Balaban (1984) showed that rostral part of the IO sends dense axonal projections to the caudal part of the contralateral MVN while the caudal dorsal cap of the IO and ventral outgrowth project to all areas of the contralateral MVN. These fibres are collaterals of climbing fibres that can control cerebellar Purkinje cell activity. Through the cerebellum the IO can also modulate vestibular neuronal activity.

Intrinsic connections

The intrinsic connections between the different subgroups of the vestibular nuclei on the same side were first demonstrated by Lapdli and Brodal (1968) using axonal degeneration techniques. Later studies using retrograde and anterograde axoplasmic transport techniques have confirmed the existence of the intrinsic connections in the vestibular nuclei (Rubertone et al., 1983; Carleton and Carpenter, 1983; Carpenter and Cowie, 1985; Ito et al., 1985; Epema et al., 1988). Reciprocal connections exist between the MVN and the inferior and the superior vestibular nuclei. There are also reciprocal connections between the rostral and caudal parts of the MVN (Pompeiano et al., 1978; Epema et al., 1988).

Spinovestibular projections

A relatively small number of spinal afferents ascending mainly with the dorsal spino-cerebellar tract have been described to project to the MVN and the other vestibular nuclei (Brodal and Angaut, 1967). In addition to this direct spino-vestibular pathway spino-reticulo-vestibular and spino-cerebello-vestibular pathways allow ample possibilities for spinal activity to reach the vestibular nuclei. Many cells located in the MVN were excited by spinal cord stimulation (Precht et al., 1967; Babalian et al., 1997) and proprioceptive influences arising from the joints of the neck can produce eye movements via these pathways (Koella et al., 1956).

Commissural connections

The vestibular nuclei on the two sides of the brainstem function in an integrated manner, even though all primary vestibular afferents are ipsilateral. The anatomical substrate for this integrative function is a massive commissural system interconnecting different parts of the vestibular nuclei. There are two types of commissural fibres: (1) those that interconnect corresponding regions of the same nuclei, and (2) those that interconnect parts of different vestibular nuclei. Using horseradish peroxidase retrograde labelling technique commissural connections between the MVN of the two sides and between the MVN and the contralateral superior vestibular nucleus (SVN) and inferior vestibular nucleus (IVN) have been demonstrated (Ruberteno et al., 1983; Ito et al, 1985; Epema et al., 1988). The most densely interconnected nuclei were the MVNs of the two sides. Electrophysiological experiments confirmed the dense commissural connections between the vestibular nuclei. Contralateral vestibular nerve stimulation was shown to produce field potentials in the MVN (Shimazu and Precht, 1966; Nakao et al., 1982; Furuya et al., 1992). This response was abolished following a midline incision, which interrupted the commissural fibres, confirming that the contralateral labyrinth influences the activity of the ipsilateral vestibular nuclei through the commissural fibres. Furuya et al. (1992) showed that the commissural inhibition induced by electrical stimulation of the contralateral labyrinth was abolished by bicuculline, indicating a $GABA_A$ receptor mediated commissural connection. Holstein et al. (1999) have also

presented immunocytochemical evidence that many vestibular commissural neurones are GABAergic. Recently Babalian et al. (1997) showed that in the guinea pig, contralateral vestibular stimulation produced inhibitory synaptic potentials with di- and trisynaptic latencies in 75% of the second order MVN neurones. In the remaining 25% of the neurones, stimulation of the contralateral nerve evoked excitatory synaptic potentials or had mixed excitatory and inhibitory effects on the second order MVN neurones. While the commissural connection is inhibitory in mammals, it is mainly excitatory in amphibians and mediated by glutamatergic receptors (Cochran et al., 1987; Knopfel, 1987; Dieringer, 1995).

1.1.2 Efferent connections of the MVN

Efferent projections from the vestibular nuclei pass to many other regions: the nuclei innervating the extra-ocular eye muscles, spinal cord, the cerebellum, and contralateral vestibular nuclei. Minor contingents have been traced to some other nuclei in the brain stem.

The vestibular nuclei provide the largest single source of fibres to the oculomotor nuclei. It is clear from physiological and anatomical studies that the main projections to the oculomotor nuclei originates from the MVN and the SVN, and are carried mostly in the medial longitudinal fasciculus (MLF) (Tarlov, 1970; Gacek, 1974, 1977; Baker and Highstein, 1978; Furuya and Markham, 1981; Langer et al., 1986; Babalian et al., 1997). In these studies it was shown that the MVN, chiefly the rostral part, projects to the abducens nuclei on both sides. Baker et al. (1969) electrically stimulated the MVN and recorded intracellularly from the abducens nucleus motoneurones. They observed monosynaptic IPSPs in ipsilateral abducens motoneurones and EPSPs in the contralateral side. Similar observations were made by Babalian et al. (1997) in the guinea pig isolated whole brain preparation showing the existence of strong disynaptic inhibitory and excitatory input to abducens motoneurones from the ipsilateral and contralateral vestibular afferents, respectively. The projections to the abducens nucleus on the ipsilateral side were demonstrated to

arise from the glycinergic MVN neurones (Spencer et al., 1989; de Zeeuw and Berrebi, 1995). The MVN was also shown to send fibres to the oculomotor nuclei in both sides, particularly to the medial rectus subdivision (Baker et al., 1969; Baker and Highstein, 1978; Kawaguchi, 1985; McCrea et al., 1987; Wentzel et al., 1995; Babalian et al., 1997). Projections to the contralateral trochlear nucleus were shown by tracing studies with HRP (Yamamoto et al., 1978; Gacek, 1979)

The lateral and medial vestibulospinal tracts (LVST and MVST respectively) are the major descending outflows from the vestibular nuclei to the spinal cord. The LVST originates from the LVN and IVN and projects to all segments of the spinal cord, terminating mainly in the extensor muscle motoneurone pools (Brodal et al, 1962). The MVN is the main source of the MVST (Nyberg-Hansen, 1964; Wilson et al., 1970) projecting predominantly to the ventral horn of the cervical spinal cord from C1 to C6 (Isu and Yokota, 1983; Bankoul and Neuhuber, 1992). Electrophysiological recordings in cats showed that about 70% of the second order vestibular nucleus neurones sent projections to the cervical spinal cord mainly on the contralateral side (Perlmutter et al., 1998). There are also fibres from the MVN innervating motoneurones at the thoracic level of the spinal cord (Wilson, 1972). These vestibulospinal projections constitute the short latency pathway for the vestibulocollic reflex, which together with the vestibuloocular reflex function enable the subject to pursue co-ordinated eye and head movements.

Vestibular related information is fed into the cerebellum through primary and secondary vestibulo-cerebellar projections. Primary vestibular projections consist of VIIIth nerve afferents that end as mossy fibres in the flocculus, nodulus and uvula, exclusively in the ipsilateral half of the cerebellum (Brodal and Hoivik, 1964; Carleton and Carpenter, 1984; Bäurle at al., 1998) and in the fastigial nucleus (Kotchabhakdi and Walberg, 1978). Secondary vestibular axons arising from the rostral part of the MVN project to the flocculi on both sides, and axons arising from the caudal part of the MVN project to the uvula and the nodulus (Rubertone, 1983; Barmack et al., 1992; Kitahara, 1997). The predominant fibre system in the cerebellum that uses acetylcholine as a transmitter is formed by mossy fibres

originating in the vestibular nuclei and innervates the nodulus and ventral uvula (Barmack et al., 1992, Jaarsma et al., 1997). Electrophysiological studies demonstrated that rostral MVN neurones send fibres to the vestibulocerebellum (Shinoda and Yoshida, 1975) with a strong contralateral preference (Cheron et al., 1996). These projections provide the vestibulocerebellum with information about head velocity, eye velocity and eye position. Thus the vestibulocerebellum has an important position as a site for convergence of visual, oculomotor and vestibular information (Lisberger and Fuchs, 1978; Noda, 1986).

The other efferent projections from the MVN include fibres to the contralateral MVN and to the other vestibular nuclei (Rubertone et al., 1983; Ito et al, 1985; Epema et al., 1988), to the contralateral inferior olive (Balaban and Beryozkin, 1994), to the pontine and medullary reticular formation (Carpenter and Nova, 1960; Ladpli and Brodal, 1968; Tarlov, 1969), and to the thalamus (Doi et al., 1997).

1.1.3 *In vivo* electrophysiological properties of MVN neurones

Both peripheral vestibular afferents and second order vestibular neurones have a spontaneous resting activity in the absence of any head movement (Shimazu and Precht, 1965; Precht and Shimazu, 1965; Goldberg and Fernandez, 1971). Spontaneously active vestibular neurones form a continuum between regularly and irregularly discharging cells with a mean discharge of about 30 spikes/s. On the other hand, some units in the nerve as well as in the vestibular nuclei are silent at rest and therefore show only a unidirectional response to rotation.

According to their response to horizontal canal stimulation four types of neurones in the MVN were described (Shimazu and Precht, 1965). Type I neurones increase their discharge frequency with ipsilateral horizontal canal stimulation and decrease with contralateral stimulation whereas type II neurones have the opposite response. Type III neurones increase their discharge frequency with rotation in either direction, and type IV neurones decrease firing with rotation in either direction. Shimazu and

Precht (1965), demonstrated that 67% of neurones studied showed type I response, 29% type II response, 3% type III response and 1% type IV response to horizontal angular acceleration in both directions. The classification of MVN neuronal types by Shimazu and Precht is used throuout the thesis. The existence of two major types of neurones can be explained by the presence of functionally inhibitory commissural connections between the MVNs on the both sides. Physiological evidence for a crossed inhibitory pathway comes from evoked field potentials recorded in the vestibular nuclei as a result of vestibular nerve stimulation (Shimazu and Precht, 1966; Precht et al., 1973; Furuya et al., 1992). Studies using a combination of electrical stimulation and single cell recording have provided evidence that type II interneurones, which received excitation from type I neurones in the contralateral medial vestibular nucleus, inhibit ipsilateral type I neurones via the release of GABA acting on $GABA_A$ receptors (Precht et al., 1973; Nakao et al., 1982; Nakamura et al., 1989; Furuya et al., 1992; Furuya and Koizumi, 1998; Okada et al., 1998) (Figure 1.1). A direct $GABA_A$ receptor mediated inhibitory projection to the second order type I neurones from the contralateral MVN was also demonstrated (Furuya and Koizumi, 1998). Some type I neurones are excitatory and project to the motoneurones in the contralateral abducens nucleus and medial rectus subdivision of the ipsilateral ipsilateral oculomotor nucleus. Inhibitory type I neurones drive the motoneurones in the ipsilateral abducens nucleus and medial rectus subdivision of the contralateral oculomotor nucleus mediating the horizontal VOR (Baker et al., 1969; Steiger and Buttner-Ennever, 1979; McCrea et al., 1987; Sato et al, 1988; Wentzel et al., 1995; Babalian et al., 1997). Both types of type I neurones have projections to the spinal cord as well as their oculomotor projections (Nyberg-Hansen, 1964; Isu and Yokota, 1983; Bankoul and Neuhuber, 1992) and mediate vestibulocollic reflex (VCR), which acts synergistically with the VOR to provide the animal a stable base on which to execute compensatory eye movements for a stable gaze.

In addition to their response to horizontal canal stimulation some MVN neurones were shown to respond to stimulation in two vertical semicircular canal planes, and to linear acceleration (the otoliths) in the cat (Markham and Curthoys, 1972; Schor et

al., 1998). Through these connections, besides serving as a relay for horizontal canal signals, the medial vestibular nucleus may also be an important relay for information about orientation within the sagittal (pitch) plane. The MVN receives information from some extra-labyrinthine sources. The proprioceptive information from the neck muscle afferents arrives in the MVN through spinovestibular projections (Fredrickson et al., 1966). The MVN is also responsive to retinal slip stimulation (Azzena et al., 1980; Horn et al., 1983), to eye position and eye movements (McCrea et al., 1987; Scudder and Fuchs, 1992).

According to the signals they convey and their response to various stimuli, other classifications of MVN neurones were described *in vivo*: Floccular target neurones (FTNs), so called because they are the targets of floccular Purkinje cells (Lisberger and Pavelko, 1988; Lisberger et al., 1994) are true second order vestibular neurones identified in the rostral part of the MVN and constitute the part of the VOR pathway which is under cerebellar inhibitory control. They receive primary vestibular input at monosynaptic latencies (Broussard and Lisberger, 1992) and directly project abducens and oculomotor nucleus neurones (Scudder and Fuchs, 1992; Lisberger et al., 1994). FTNs exhibit large responses that consist of increases in their firing rate during the VOR evoked by head movements toward the side of recording (Lisberger and Pavelko, 1988; Lisberger et al., 1994). Sato et al. (1988) showed that 84% of the FTNs recorded in the MVN send inhibitory projections to the ipsilateral abducens nucleus. In a recent study Babalian et al. (2000) described physiological properties of the FTNs in the guinea pig isolated whole brain preparation. They reported that 15-20% of the second order MVN neurones were monosynaptically inhibited from the flocculus. Classified according to their action potential shapes these cells were all type B cells (see p.17 for definition). They receive both inhibitory and excitatory commissural inputs from the contralateral vestibular afferents with predominance of inhibitory ones. Eighteen percent of the FTNs were antidromically stimulated from the spinal cord suggesting involvement of the FTNs in the control of gaze and posture.

Scudder and Fuchs, (1992), divided rostral MVN neurones on the basis of their activity during horizontal head and eye movements and identified five major units: (1) Position-vestibular-pause (PVP) units discharge in relation to head velocity, eye velocity, eye position, and cease firing during saccades. (2) Eye and head velocity (EHV) units discharge in relation to eye velocity and head velocity in the same direction, so that the two signals partially cancel during the VOR. (3) Burst-position (BP) units have eye position and velocity sensitivity, a burst in the ON-direction, a pause in the OFF-direction and no head velocity sensitivity. (4) Eye-position (EP) units have eye position sensitivity, but no head velocity sensitivity. (5) Vestibular units are not modulated by eye position and eye velocity but by head velocity. The PVP neurones are the major conduit for head velocity information from horizontal canals to abducens nuclei and are the most abundant cell type in the rostral MVN. The nomenclature of Scudder and Fuchs is not used further in the thesis.

1.1.4 *In vitro* electrophysiological properties of MVN neurones

A prominent feature of MVN neurones is that they fire spontaneous action potentials in *in vitro* brain stem slices where all inputs to the nucleus are cut. Their *in vitro* discharge rate ranges from 0.5 - 60 spikes/s with an average firing frequency between 10 - 25 spikes/s (Gallagher et al., 1985; Lewis et al., 1987; Ujihara et al., 1988, 1989; Doi et al., 1990; Dutia et al., 1992; Johnston et al., 1993, 1994; Cameron and Dutia, 1997) which is comparable to their firing rates obtained *in vivo* (Shimazu and Precht, 1965; Precht and Shimazu, 1965; Goldberg and Fernandez, 1971; Hamann and Lannou, 1987; Ris et al. 1995).

The source and mechanisms of the spontaneous activity in vestibular neurones have been under study since it was first demonstrated *in vitro* by Gallagher et al. (1985). Available data since then has presented evidence that a major component of the spontaneous activity is generated by an intrinsic mechanism, as it persists after blockade of synaptic transmission with calcium channel blocking agents (Darlington et al., 1989; Doi et al., 1990; Serafin et al., 1991a; Dutia et al., 1992) and it is highly sensitive to changes in membrane potential (Serafin et al., 1990, 1991b; Gallagher et

al., 1992). Serafin et al., (1991b) demonstrated that a proportion of spontaneously active MVN cells have a sodium dependent plateau potential indicating that the voltage-gated non-inactivating sodium conductance may continuously drive the membrane potential to the firing threshold for fast sodium action potentials and therefore underlie their spontaneous activity. A small component of resting activity in vestibular neurones *in vitro* may come from the synaptic input from the neurones in the same nucleus, because blockade of synaptic input or application of major neurotransmitter antagonists modulates spontaneous activity of many neurones recorded in brain stem slices as will be discussed later in this chapter.

Two main cell types, type A and type B, were defined according to their action potential shapes and intrinsic membrane properties in intracellularly recorded MVN neurones in brain stem slices of guinea pigs, rats, mouse and chicks (Chapter 2, Figure 2.3.1.1)(Gallagher et al., 1985; Serafin et al., 1991a; Johnston et al., 1994; Dutia et al., 1995; du Lac and Lisberger, 1995). Type A MVN neurones were characterised by having broad action potentials with a single deep afterhyperpolarisation (AHP). Type B MVN neurones are in contrast characterised by narrower action potentials, and displayed a double component AHP consisting of first, fast AHP followed by a delayed, slower AHP. Serafin et al. (1991a), also described a third cell type, type C, which had intermediate properties and were not readily included in the other two groups. They argued that membrane properties of MVN neurones are actually distributed as a continuum in between the two stereotyped schemes corresponding to canonical type A and type B cells. However Johnston et al. (1994) were able to classify all recorded neurones into type A or type B using spike shape averaging, with about one third of the cells being type A and two third being type B.

1.2 Pharmacology of the MVN

In vivo and *in vitro* electrophysiological experiments demonstrated that the activity of the MVN neurones was modulated by major neurotransmitters and neuromodulators of the CNS. Glutamate, GABA and glycine mediate fast synaptic

actions mainly through postsynaptic ionotropic receptors. The monoamines (histamine, dopamine, serotonin, noradrenaline) usually have slower actions on neuronal activity mediated by metabotropic receptors. Vestibular neurones are also modulated by several neuroactive peptides (somatostatin, opioids, substance P, and ACTH). Recent immunocytochemical and autoradiographic studies identified several neuromodulators and their receptors within the vestibular nuclei.

Glutamate

Glutamate receptors mediate most of the excitatory neurotransmission in the mammalian central nervous system. The glutamate receptors are divided into two distinct groups, ionotropic and metabotropic receptors. The ionotropic receptors include alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptor channels. The metabotropic receptors (mGluRs) are coupled to GTP-binding proteins (G-proteins), and regulate the production of intracellular messengers (Ozawa et al. 1998). The presence of both ionotropic and metabotropic glutamate receptors has been identified in the vestibular nuclei neurones. There is a large body of biochemical studies showing that glutamate is involved in the excitatory synaptic transmission between the eighth nerve and the second order vestibular nuclei neurones. Vestibular ganglion neurones are labelled by antibodies raised against aminoacetyltransferase, an enzyme involved in the metabolism of glutamate (Raymond et al., 1984). The same study demonstrated that glutamate uptake by the presynaptic terminals of the vestibular nerve was reduced after vestibular nerve sectioning. Recent microdialysis experiments showed that glutamate is released from the eighth nerve (Yamanaka et al., 1995, 1997). Ligand binding studies have shown that there is high level of glutamate receptors in the vestibular nuclei, especially in the MVN (Touati et al. 1989; Walberg et al. 1990). All the four vestibular nuclei including the MVN are stained with antibodies made against AMPA-type and NMDA-type glutamate receptor subunits (Petralia and Wenthold, 1992; Petralia et al., 1994a,b). Glutamate immunoreactive vestibular afferents and cell bodies were shown in all subdivisions of the vestibular nuclei (Reichenberger and Dieringer, 1994; Reichenberger et al., 1997). Consistent with the evidence from the biochemical studies, many *in vivo* and *in vitro*

electrophysiological experiments have suggested glutamate as the neurotransmitter at the synapse between the vestibular nerve and the vestibular nuclei. Both NMDA and non-NMDA receptor agonists were shown to increase resting discharge of MVN cells recorded in brain stem slices (Smith et al., 1990; Smith and Darlington, 1992; Lin and Carpenter, 1993). NMDA receptor antagonists decreased the resting firing rate of the cells indicating that the tonic release of glutamate acting on NMDA receptors modulate the spontaneous activity of the MVN neurones *in vitro*. In intracellularly recorded MVN neurones the application of both NMDA and AMPA/kainate receptor agonists depolarised the membrane potential with a decrease in membrane input resistance (Lewis et al., 1987, 1989; Serafin et al., 1992). Although second order MVN neurones were shown to possess all types of the glutamate receptors, the vestibular nerve input to the MVN is mainly mediated by AMPA/kainate glutamatergic receptors as it was demonstrated that the monosynaptic EPSPs evoked by electrical stimulation of the eighth nerve were blocked by non-NMDA glutamate receptor antagonists (Cochran et al., 1987; Lewis et al., 1989; Doi et al., 1990; Carpenter and Hori, 1992). More recent patch-clamp studies have shown an NMDA receptor-mediated component in the vestibular nerve-MVN synaptic input (Kinney et al., 1994; Takahashi et al., 1994). Babalian et al. (1997) reported in the *in vitro* whole brain preparation of the guinea pig that about one third of the second order vestibular neurones had NMDA receptor-mediated transmission. In these cells the major part of the field potentials and monosynaptic EPSPs were blocked by CNQX, a specific antagonist of AMPA/kainate glutamatergic receptors, and the remaining small part being blocked by APV, a specific antagonist of NMDA glutamatergic receptors.

GABA and Glycine

GABA and glycine are the main inhibitory neurotransmitters in the central nervous system. Two types of GABA receptors were described in the vestibular nuclei. $GABA_A$ receptors are ionotropic receptors and are directly coupled to chloride ion channels. They are assembled from a repertoire of at least 18 subunits (alpha1-6, beta1-3, gamma1-3, delta, epsilon, theta, rho1-3) (Rudolph et al., 2001). $GABA_B$ receptors are coupled to G-proteins and induces the inhibition of adenylyl cyclase

activity modulating various K^+ and Ca^{2+} channels (Misgeld et al. 1995). Glycine receptors are ionotrophic receptors similar to $GABA_A$ receptors (Betz et al. 1994). Although a third type of GABA receptor, the $GABA_C$ receptor, which is directly coupled to a chloride ion channel, exists in the brain (Zhang et al., 2001), it has not yet been identified in the vestibular nuclei.

Many immunohistochemical studies using antibodies against GABA or its synthesising enzyme, glutamic acid decarboxylase (GAD), and glycine have demonstrated that GABA and glycine are localized within the vestibular nuclei (Houser et al. 1984; Nomura et al. 1984; Raymond et al. 1988; Kumoi et al. 1987; Walberg et al. 1990; Holstein et al. 1996; de Waele et al. 1994; Rampon et al. 1996; Reichenberger et al. 1997). The release of GABA as a neurotransmitter within the vestibular nuclei was shown by high-performance liquid chromatography (HPLC) experiments (Li et al. 1994). The presence of $GABA_A$ and $GABA_B$ receptors in the vestibular nuclei was further supported by studies using radioligand binding and immunohistochemical methods (Hironaka et al., 1990; Calza et al., 1992; Holstein et al., 1992; Zanni et al., 1995). $GABA_A$ receptors are widely distributed post-synaptically on vestibular neurones and $GABA_B$ receptors have been shown to be distributed both pre- and post-synaptically (Holstein et al. 1992). *In vitro* electrophysiological experiments confirmed the neurotransmitter actions of GABA and glycine in the vestibular nuclei. The resting activity of extracellularly recorded MVN neurones is inhibited by bath application of GABA and GABA receptor agonists, and this inhibition can be blocked by selective $GABA_A$ and $GABA_B$ receptor antagonists (Smith et al. 1991; Dutia et al. 1992; Hutchinson et al., 1995). Intracellular recordings (Vibert et al. 1995a,b) showed that muscimol, a $GABA_A$ receptor agonist and baclofen, a $GABA_B$ receptor agonist, hyperpolarized all cells in the MVN in the presence of tetradotoxin (TTX) while some cells were depolarized in normal medium. This suggests that local inhibitory interneurones are spontaneously active and exert a tonic inhibition on the recorded neurones. This observation is supported by a recent morphological study (de Zeeuw and Berrebi, 1996), which demonstrated that GABAergic Purkinje cell axons terminate on both inhibitory and excitatory neurones in the vestibular nuclei.

The presence of post-synaptic glycine receptors in the MVN was shown by Lapeyre and de Waele (1995). Bath application of glycine produced a dose dependant decrease in the resting discharge of the cells, which persist in a high Mg^{2+}/low Ca^{2+} containing solution. This inhibition was suppressed by strychnine, a glycine receptor antagonist.

Available evidence suggests that glycine is involved in the inhibitory synaptic transmission from the vestibular nuclei to the abducens nuclei (Spencer et al. 1989; Babalian et al. 1997) and spinal cord (Felpel, 1972) while GABA is the neurotransmitter in the commissural system (Furuya et al. 1992; Babalian et al. 1997; Holstein et al. 1999), and in the cerebello-vestibular pathway (de Zeeuw and Berrebi, 1996; Babalian and Vidal, 2000).

Acetylcholine

Acetylcholine acts through two types of receptors: nicotinic and muscarinic receptors. The nicotinic receptors are directly coupled to a cation channel while muscarinic receptors act through G proteins and second messengers (for review, see Clarke, 1995). Both types of cholinergic receptors have been distinguished in the vestibular nuclei by biochemical and radioligand binding studies (Schwartz, 1986; Swanson et al. 1987; Wamsley et al. 1981; Calza et al. 1992). Vestibular nuclei neurones were labeled by monoclonal antibodies raised against specific subunits of the nicotinic acetylcholine receptors (Dominguez del Toro, 1994). Moreover all vestibular nuclei have been shown to display choline acetyl-transferase (ChAT), acetylcholine synthesizing enzyme, immunoreactivity, the highest acivity being recorded in the MVN (Burke and Fahn, 1985; Carpenter et al. 1987; Barmack et al. 1992).

In *in vitro* brainstem slices, acetylcholine was shown to predominantly depolarise the membrane potential of the MVN neurones, which was reversibly suppressed by muscarinic and nicotinic cholinergic receptor antagonists (Ujihara et al. 1988, 1989; Dutia et al., 1990; Phelan and Gallagher, 1992). These effects are post-synaptic since they persist in high Mg^{2+}/low Ca^{2+} containing medium. Iontophoretic application of

acetylcholine *in vivo* increases the firing rate of vestibular nuclei neurones, and this effect is blocked by muscarinic receptor antagonists (Kirsten and Schoener, 1973; Kirsten and Sharma, 1976a,b).

Histamine

Three types of histaminergic receptors are identified in the mammalian central nervous system. Postsynaptic H_1 and H_2 receptors are positively coupled to phospholipase C and adenylate cyclase, respectively. H_3 receptors, which are located on the presynaptic nerve terminals, control the synthesis and the release of histamine from histaminergic terminals through G proteins (for review, see Arrang et al. 1995). There are several works suggesting that histaminergic system modulate the function of the vestibular neurones. Anatomical studies have shown histaminergic projections from the posterior hypothalamus to the vestibular nuclei, especially to the MVN (Steinbusch, 1991; Tighilet and Lacour, 1996). Histaminergic receptor agonists and antagonists have been used in humans for the symptomatic treatment of vertigo (Fisher, 1991; Rascol et al. 1995; Kingma et al. 1997). Autoradiographic and in situ hybridisation studies have disclosed the presence of H_1 and H_2 binding sites in all vestibular nuclei (Bouthenet et al. 1988; Vizuete et al. 1997). *In vivo* and *in vitro* electrophysiological experiments support the involvement of histaminergic receptors in vestibular function. In guinea pig slices, histamine depolarised the membrane potential of MVN neurones via postsynaptic H_2 receptors (Serafin et al. 1993) while in rats depolarisation was mediated postsynaptically by both H_1 and H_2 receptors (Phelan et al. 1990; Wang and Dutia, 1995). *In vivo* studies have further demonstrated that vestibular neuronal activity was modulated by histamine (Stayavivad and Kirsten, 1977; Takatani et al. 1983). Perfusion of the guinea-pig's vestibular nuclear complex with specific ligands of the H_2 and H_3 receptors induced postural changes supporting the view that MVN neurones are endowed with both H_2 and H_3 receptors (Serafin et al. 1993). In the same study it was shown that intraperitoneal injection of H_3 antagonists significantly decreased the horizontal vestibulo-ocular reflex gain demonstrating that histamine modulates the dynamics of the vestibular reflexes.

Serotonin (5-hydroxytryptamine)

Serotonin receptors are classified into four groups: 5-HT_1, 5-HT_2 and 5-HT_4 are metabotropic receptors, while 5-HT_3 is an ionotropic receptor (Bonate, 1991). The three subtypes of 5-HT_1 receptors (5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D}) have the highest affinity for serotonin and are negatively coupled with adenylate cyclase whereas 5-HT_2 receptors activate phospholipase C. A rich serotoninergic innervation of the vestibular nuclei has been demonstrated by immunocytochemical methods, probably originating from the dorsal raphe nuclei (Steinbusch, 1981). High-performance liquid chromatography (HPLC) experiments detected high levels of serotonin in the MVN (Cransac et al. 1996). Furthermore autoradiographic and in situ hybridisation studies have identified 5-HT_{1A}, 5-HT_{1B} and 5-HT_2 receptors in the vestibular nuclear complex (Pazos and Palacios, 1985; Pazos et al. 1985a,b; Zanni et al. 1995). *In vitro* electrophysiological recordings in rat brainstem slices showed that serotonin depolarised most of the MVN neurones. (Gallagher et al. 1992; Johnston et al. 1993). A small minority of MVN cells was inhibited by 5-HT_{1A} receptor agonists while the excitatory responses of the cells were produced by 5-HT_2 agonist. Microiontophoretic application of 5-hydroxytryptamine into the MVN of anaesthetised rats produced excitatory, inhibitory and biphasic responses in resting activity of vestibular neurones (Licata et al. 1993). Similar kinds of responses of the vestibular neurones were demonstrated to the electrical stimulation of dorsal raphe nuclei in rats (Licata et al. 1995).

Dopamine

Dopamine can bind to five distinct metabotropic receptors, D_1-D_5 receptors. They have been divided into two families according to their pharmacological molecular properties: D_1-like receptor family includes D_1 and D_5 receptors whereas D_2, D_3 and D_4 receptors form the D_2-like receptor family. D_1-like receptors are generally positively coupled to adenylate cyclase while D_2-like receptors are negatively coupled to adenylate cyclase. Both receptors types can be localised postsynaptically and presynaptically (Civelli et al. 1993).

The presence of D_2 receptors was demonstrated in vestibular nuclei of the rat by Yokoyama et al. (1994) using a radioligand binding method. However Bouthenet et al. (1987) failed to detect D_2 receptors within the vestibular nuclei using similar methods in the same species. In a later study they localised D_3 receptor mRNAs in the vestibular nuclei (Bouthenet et al. 1991). Cransac et al. (1996) demonstrated dopamine content in the MVN using HPLC electrochemical detection. Electrophysiological experiments are in support of the dopaminergic modulation of the vestibular system. *In vitro* studies in slices have demonstrated a depolarising action of dopamine in medial vestibular nucleus neurones mediated by D_2 receptors (Gallagher et al. 1992; Vibert et al. 1995c). Dopamine had an inhibitory effect on the MVN cells when synaptic transmission was blocked by high Mg^{2+}/low Ca^{2+} containing solution or in the presence of bicuculline, suggesting both a postsynaptic effect and a presynaptic modulation of GABA release (Vibert et al. 1995c).

Noradrenaline

Schuerger and Balaban (1993) have demonstrated noradrenergic projections from the locus coeruleus to the vestibular nuclei using antibodies raised against thyrosine hydroxylase and dopamine β-hydoxylase. The presence of α_2 and β_1 adrenergic receptor mRNAs was shown in the vestibular nuclei by in situ hybridisation experiments (Nicholas et al. 1993a,b). Radioligand binding studies have also demonstrated α_2 and β adrenergic receptors in the vestibular nuclei (Zanni et al. 1995). Electrophysiological experiments support the noradrenergic modulation of the vestibular neurones. Iontophoretic microinjection of noradrenaline in anaesthetised cats inhibited the resting discharge of the MVN cells while it increased the LVN cell firing (Kirsten and Sharma, 1976). In a more recent *in vivo* study in the rat, Licata et al. (1993b) showed that noradrenaline and α_2 agonists induce dose-dependant inhibition of the majority of the vestibular neurones. This inhibition was blocked by α_2 receptor antagonists. *In vitro* studies have reported that noradrenaline depolarised vestibular neurones (Gallagher et al. 1992). Using *in vitro* brainstem slices Podda et al. (2001) have recently demonstrated that noradrenaline excited the majority of

MVN neurones through both α_1 and β noradrenergic receptors. The remaining cells were inhibited by noradrenaline through α_2 noradrenergic receptors.

Opioid Peptides

Endogenous opioid peptides, enkephalins, β-endorphins and dynorphins, exert their pharmacological and physiological effects by specifically binding to G-protein-coupled opioid receptors which are classified into three distinct receptor types namely mu (μ), delta (δ) and kappa (κ). β-endorphins bind equally well to μ- and δ-receptors while enkephalins and dynorphins have high affinity for δ- and κ-opioid receptors, respectively (Grudt and Williams, 1995). Both enkephalin-immunoreactive cell bodies and enkephalin terminal endings have been detected within the vestibular nuclei (Nomura et al., 1984; Beitz et al., 1987; Saika et al., 1993; Zanni et al., 1995). The MVN contains the highest density of enkephalinergic neurones in the brain (Beitz et al., 1987). Dynorphin-immunoreactive sites have also been detected in the medial and lateral vestibular nuclei (Zamir et al., 1983). The vestibular nuclei neurones were shown to have both μ and δ receptors (Mansour et al., 1994; Zastawny et al., 1994). Electrophysiological experiments are rather contrasting as to the actions of the opiate peptides on the MVN neurones. *In vitro* studies on slices (Carpenter and Hori, 1992; Lin and Carpenter, 1994) have shown that morphine (a selective agonist of μ receptors) and [D-Ala^2]leucine enkephalin (a selective agonist of δ receptors) increased the resting firing of about 30% of the MVN cells. This excitation persisted in synaptic uncoupling conditions suggesting a post-synaptic action and was blocked by μ and δ receptor antagonist naloxone. However, in a recent study, Sulaiman and Dutia (1998) have demonstrated that the large majority of the MVN neurones were inhibited by δ receptor agonists [D-Ala^2, D-Leu^5]-enkephalin (DADLE) and [D-Pen^2, D-Pen^5]-enkephalin. This inhibition persisted after blockade of synaptic transmission and was blocked by selective δ receptor antagonist naltrindole suggesting that it is mediated by post-synaptic δ receptors. The selective μ and κ receptor agonists failed to elicit a response in MVN neurones. Iontophoretic application of enkephalin in anaesthetised cats *in vivo*

inhibited the firing of MVN cells responding to horizontal rotation (Kawabata et al., 1990).

Substance P

Numereous substance P immunoreactive fibres and cell bodies have been identified in the vesibular nuclei (Nomura et al., 1984). Substance P immunoreaction has been shown in the vestibular ganglion and substance P is suggested to be co-localised with glutamate in some primary vestibular afferents (Usami et al., 1991). In situ hybridisation and receptor autoradioraphy studies demonstrated the presence of substance P receptors in the vestibular nuclei (Maeno et al., 1993; Zanni et al., 1995). *In vitro* electrophysiological experiments reported both excitatory and inhibitory effects of substance P on the MVN cells. Vibert et al. (1996) demonstrated that bath application of substance P caused depolarisation in 50% of the MVN neurones, and hyperpolarisation in 10% of the cells. Both responses persisted under conditions of blockade of synaptic uncoupling indicating a postsynaptic action. The substance P-induced membrane depolarization could not be reproduced with the specific agonists of the NK-1, NK-1 and NK-3 tachykinin receptor subtypes, nor was it blocked by the specific NK-1 receptor antagonists. This depolarising effect could be due to the activation of a new, pharmacologically distinct, 'NK1-like' receptor.

Adrenocorticotrophic hormone

Systemic application of short fragments of adrenocorticotrophic hormone ($ACTH_{4-10}$) has been shown to enhance compensation of static vestibular symptoms after unilateral labyrinthine damage (Flohr and Luneburg, 1982; Gilchrist et al., 1990). Extracellular recordings in *in vitro* brainstem slices have shown that neuronal activity of the MVN cells was inhibited by $ACTH_{4-10}$ (Darlington et al., 1990, 1992, 1993). The mechanisms by which $ACTH_{4-10}$ inhibits MVN neuronal activity remain unclear at present.

1.3 Vestibular Compensation

Unilateral damage to one vestibular labyrinth, either as a result of surgical removal of the receptor cells (unilateral labyrinthectomy,UL) or transection of one VIIIth cranial nerve, (unilateral vestibular deafferentation, UVD), results in a behavioural syndrome which includes ocular motor and postural symptoms (for reviews see Smith and Curthoys, 1989; Curthoys and Halmagyi, 1995a). This syndrome includes static deficits that persist in the absence of head movements and affect posture and eye position (e.g. spontaneous ocular nystagmus, yaw head tilt, roll head tilt) and dynamic perturbations of the VOR and VCR elicited by head movements such as reduced velocity or amplitude (gain) and abnormal timing (phase) of these reflexes. Surprisingly many of these oculomotor and postural symptoms disappear over time in a process of behavioural recovery known as vestibular compensation. This recovery process is particularly striking in the case of static symptoms, which in many species disappear within a few days following the UL. On the other hand compensation of the dynamic deficits is generally much slower and some deficits appear to be permanent (Curthoys and Halmagyi, 1995b). Since the peripheral vestibular receptors do not regenerate once removed and the neurones of Scarpa's ganglion do not undergo any substantial functional recovery, vestibular compensation is assumed to be due to CNS plasticity and is widely used as a model of lesion-induced CNS plasticity. The UL does not result in deafferentation of the second order vestibular neurones since the central part of the vestibular nerve survives after the lesion and hence provides a non-deafferentation model. Using silver impregnation method Kunkel and Dieringer (1994) showed that UL does not result in degeneration of eighth nerve. As a consequence of this survival electric stimulation of the stump of the eiththh nerve evokes post-synaptic field potentials in the vestibular nulei (Kunkel and Dieringer, 1994; Sirkin et al., 1984; Jensen, 1983).

The advantage of vestibular compensation as a model of lesion-induced plasticity is twofold: First, the neuronal network underlying vestibular synergies has been well described and input-output relations can be easily quantified both at the cellular and

behavioural levels. Second, the extent of the peripheral lesions can be controlled and easily reproduced.

1.3.1 Behavioural component of vestibular compensation

Static Symptoms

In mammalian species among the static vestibular symptoms, the most prominent effect of the UL is a high frequency, mainly horizontal spontaneous nystagmus (SN). SN is seen as a slow drift of the eyes toward the lesioned side (slow phase) followed by a fast beat to the intact side (quick phase). It is the quick phase that is apparent to the observer and SN is usually expressed as the number of the quick phases per minute. The frequency of SN soon after the UL is in the range of 80-100 beats per minute in rats and guinea pigs (Sirkin et al., 1984; Curthoys et al., 1988; Hamann and Lannou, 1987; Kitahara et al., 1998). Immediately after the UL the frequency of SN starts to decrease. The recovery period of the spontaneous nystagmus after UL varies among species, from a few hours to few days for the rat (Hamann and Lannou, 1987) and guinea pig (Curthoys *et al.*, 1988), a few days for the cat (Maioli *et al.*, 1983; Maioli and Precht, 1985) and monkey (Fetter and Zee, 1988), and about a week for humans (Cass *et al.*, 1992).

In addition to the SN, there is a disturbance in the position of the eyes after the UL. In species such as guinea pigs and rats, the eye on the side of the lesion deviates down and the eye on the contralateral side deviates up (Dieringer and Precht, 1982; Sirkin *et al.*, 1984). In humans the UL results in maintained posture of the head and eyes called ocular tilt reaction. The head has a roll tilt towards the lesioned side, both eyes adopt a torted position by rolling around the optical axis toward the lesioned side, and the eye on the lesioned side moves down in the orbit relative to the position of the eye on the opposite side (Curthoys et al., 1991; Halmagyi et al., 1991).

Another common symptom of UL in most species is the disturbance of the head and body posture. Head is tilted down around the roll plane toward the operated side (roll

head tilt) and is horizontally deviated to the operated side (yaw head tilt). Changes in maintained head posture after UL are more prominent in species such as guinea pigs (Curthoys *et al.*, 1988; De Waele *et al.*, 1989), rats (Sirkin *et al.*, 1984; Hamann and Lannou, 1987), and monkeys (Fetter and Zee, 1988) than in human (Halmagyi *et al.*, 1991). In rats after UL, there is an ipsilateral flexion and contralateral extension of the forelimbs, and the animals show circular walking and barrel rolling toward the lesioned side, which subside within 6h (Hamann and Lannou, 1987; Cameron and Dutia, 1997). The time course and extent of the recovery from the postural symptoms is largely species specific and ranges from 6 h in the rat (Llinas et al., 1975; Hamann and Lannaou, 1987), a few days in the guinea pig (Curthoys et al. 1988; de Waele et al., 1989), and 3 weeks in the monkey (Fetter and Zee, 1988).

Dynamic Symptoms

As for dynamic deficits, UL results in severe abnormalities in the gain, symmetry and timing of the horizontal VOR. The gain of the VOR is smaller for head rotations toward the lesioned side than that of head rotations toward the intact side representing an asymmetric VOR gain. The gain of the VOR for head rotations to the intact side is also smaller than normal gain. Therefore eye movements are not sufficient in amplitude and phase to compensate for head movements (Maioli et al., 1983; Takahashi et al., 1984; Cremer et al., 1987; Fetter and Zee, 1988). In humans, during rotations to the lesioned side the VOR gain falls to <0.4. Rotations to the intact side show a higher gain around 0.7. Both are significantly smaller than normal VOR gain, which is around 1.0 (Halmagyi et al., 1990).

UL also causes vestibulo-spinal reflex deficits resulting in severe disturbance of locomotion (Lacour et al., 1979; Igarashi et al., 1981; Dutia, 1985). Unlike the recovery of static symptoms, the recovery of dynamic symptoms is much slower and usually incomplete. The majority of the studies of VOR during vestibular compensation suggest that the dynamic VOR gain does not recover to normal levels, although it may improve somewhat. A substantial recovery of VOR function has been reported using tests with low-frequency sinusoidal horizontal rotations (Takahashi et al., 1984; Jenkins 1985). However Curthoys et al. (1995b), using high

frequency natural head acceleration tests, have shown that there is little improvement in dynamic VOR function over time.

1.3.2 Neuronal component of vestibular compensation

There are remarkable changes in the resting activity and the response characteristics of the vestibular nucleus neurones during the course of vestibular compensation. The most significant change after UL is the loss of spontaneous activity of the vestibular nuclei neurones on the lesioned side and its gradual recovery over time as vestibular compensation develops. In anaesthetised guinea pigs, up to 8 h following UL, Smith and Curthoys (1988a,b), using horizontal head acceleration stimulations, reported that type I neurones on the ipsilesional side were rarely encountered and usually had no resting activity. However by 52-60 h, type I neurones were encountered more frequently, although not as frequently as in the normal animal, and their average resting activity had recovered to a level not significantly different from normal. In contrast type I neurones recorded in the contralateral MVN showed an increase in their resting discharge probably due to dis-inhibition from the ipsilateral type I neurones. However, up to 8 h following UL, on the lesioned side, type II neurones were encountered more frequently than normal and those encountered had an average resting activity that was significantly higher than normal, whereas on the intact side type II neurones were encountered much less frequent. In a detailed study in alert guinea pigs, Ris et al. (1995) demonstrated that immediately after UL, 69% of the monosynaptically activated second order vestibular nuclei (including MVN, LVN and SVN) neurones were silent and the remaining active units had a very low resting discharge (7.2 ± 13.9 (SD) spikes/s). One week after the UL, there were no silent units and the mean discharge of the recorded cells had recovered to the preoperative level of 41.0 ± 24.7 (SD) spikes/s.

In addition to electrophysiological studies the result of 2-deoxyglucose (2-DG) experiments also suggest that there is a large asymmetry in neuronal activity between the MVN of the two sides following UL; 2-DG uptake in the ipsilesional MVN was

below normal suggesting a decreased neuronal activity (Luyten et al., 1986). Apart from the MVN the activity in many areas of the brain including other vestibular nuclei, inferior olive (IO) and cerebellum is changed following UL as shown by 2-DG uptake studies (Patrickson et al., 1985; Luyten et al., 1986) and fos expression experiments (Kaufman et al., 1992; Kitahara et al., 1995; Cirelli et al., 1996).

In parallel with the loss of resting activity in the ipsilateral MVN cells, their sensitivity to the ipsilateral and contralateral angular head accelerations was also changed. Smith and Curthoys (1988a) have reported that the sensitivity of the ipsilesional type I neurones to ipsilateral head movements was significantly decreased after the UL. However in contrast to the recovery of resting activity, the sensitivity of the ipsilateral MVN neurones to horizontal rotation did not recover up to one year after UL, which correlates with the incomplete recovery of the horizontal VOR (Maioli et al., 1983; Takahashi et al., 1984). Ris et al (1995) also reported a decreased sensitivity of the ipsilateral type I and type II neurones to angular head accelerations which did not recover one week after the UL.

1.3.3 Mechanisms of vestibular compensation

Various findings in animal experiments have led to several hypotheses about the mechanisms of vestibular compensation; cerebellar shut-down (McCabe and Ryu, 1969), the substitution of other sensory inputs for the missing vestibular input (Putkenen et al, 1977), reactive synaptogenesis (Precht et al., 1966; Galiana et al., 1984; Igarashi, 1984), denervation supersensitivity (Precht et al., 1966; Xerri et al., 1983), calcium-induced diachisis hypothesis (Darlington and Smith, 1996). However there has been little or no experimental evidence to support any of these hypotheses as a mechanism of the fast neuronal recovery following the UL.

Temporal correlation between the recovery from the static oculomotor and postural symptoms of vestibular compensation and neuronal recovery in the vestibular nuclei, particularly in the MVN, in the lesioned side have led to the general consensus that

recovery of resting activity in the ipsilateral MVN has a major causative role in compensation of the static vestibular symptoms. There is a good reason to believe this since lesions of the ipsilateral vestibular nuclei have been shown to prevent compensation or cause loss of compensation (Spiegel and Demetriades, 1925). In addition to electrophysiological studies showing recovery of resting activity in the ipsilateral MVN (Precht et al., 1966; McCabe and Ryu, 1969; Ried et al., 1984; Smith and Curthoys, 1988a; de Waele et al., 1988) 2-DG uptake studies have demonstrated a recovery toward more symmetrical metabolic activity between the bilateral MVN (Luyten et al., 1986). In guinea pigs compensation of the static symptoms and recovery of resting activity in the deafferented MVN occur within approximately 2 days after UL (Smith and Curthoys, 1988a; de Waele et al., 1988).

Although the general assumption is that neuronal recovery is associated with a behavioural recovery, since first suggested by Precht et al. (1966), the cellular mechanisms responsible for recovery of resting activity is still undetermined. The experiments carried out during this period have led to several hypotheses but none of them received a general acceptance, although some have been cited more frequently.

Reactive synaptogenesis, the sprouting of new synapses in response to de-afferentation, is widely regarded as a plausible mechanism for vestibular compensation. There has been clear evidence for formation of new synapses in the de-afferented vestibular nuclei in frogs (Dieiringer et al., 1984; Goto et al., 2000) and in the cat (Korte and Friedrich, 1979; Raymond et al., 1991) which took at lest 5 days to appear after UL. However this is unlikely to be responsible for the early recovery from the static symptoms in mammalian species because compensation is complete within 2-3 days after UL in most mammalian species.

It has been suggested that following UL, neurones in the ipsilateral vestibular nuclei may gradually develop denervation supersensitivity to remaining excitatory inputs and that this might account for the return of resting activity to the de-afferented nuclei. However, to date, there is no direct evidence in support of this hypothesis. The results of binding studies do not support the idea of denervation supersensitivity

by means of an up-regulation of muscarinic ACh (Yamada et al., 1988) and glutamate receptors (Raymond et al., 1989). Moreover electrophysiological studies in the frog (Knopfel and Dieringer, 1988) and in the guinea pig (Smith and Darlington, 1992) did not show any increase in NMDA receptor efficacy.

According to the cerebellar shutdown hypothesis (McCabe and Ryu, 1969) after unilateral labyrinthine damage the cerebellum acts to reduce the activity in vestibular nuclei in both sides. Therefore a high spontaneous activity in the intact side will be reduced releasing its commissural inhibition on the deafferented nuclei, leading to gradual restoration of symmetric activity between the two nuclei. Recording in cats from the MVN after UL they demonstrated that MVN neurones in both sides were silent and after cerebellectomy activity in the intact MVN returned showing that it was under cerebellar inhibition. The reduction of the horizontal VOR gain for both directions (Fetter and Zee, 1988) is also in agreement with this hypothesis. However this hypothesis is questionable on the ground that SN is present soon after UL and further *in vivo* recordings by subsequent workers have showed that MVN neurones in the intact side were not silenced by UL both in cat (Shimazu and Precht, 1966; Markham et al., 1977) and in other species (Smith and Curthoys, 1988b; Ris et al., 1997, in the guinea pig; Hamann and Lannou, 1987, in the rat). However the molecular studies of Kitahara et al. (1997, 1998, 2000) suggest that the cerebellum, particularly floculus, is involved in vestibular compensation. They propose that during the acute stage after the UL, the flocculus reduces the imbalance in the activity of the MVN neurones of both sides by suppressing the neuronal activity on the contralesional MVN neurones, and during the chronic stage by reducing the inhibitory effect on the ipsilesional MVN neurones.

The substitution of other sensory inputs for the missing labyrinthine input was suggested as a likely mechanism for neuronal recovery after UL (Putkonen et al., 1977; Courjon et al., 1982). Using anatomical methods Dieringer et al., (1984), showed increased spinal afferent projections to the vestibular nuclei following UL. In monkeys the cervicoocular reflex (COR) was shown to potentiate after UL (Goldberg and Marsh, 1988). Deprivation of visual input (Courjon et al., 1977; Smith et al.,

1986; Fetter et al., 1988) and spinal afferent inputs (Azzena, 1969; Igarashi et al., 1969) were shown to delay vestibular compensation or cause decompensation. However, although in these studies disruption of non-vestibular sensory inputs affects the time course of behavioural recovery, vestibular compensation still occurs. A recent electrophysiological study in frogs presents evidence supporting the reactive synaptogenesis as a mechanism for vestibular compensation (Goto et al., 2000). Two months after the sectioning of the anterior branch of the eighth cranial nerve, there were more cells in vestibular nuclei responding to posterior canal stimulation and they responded with larger field potentials. These results suggest that the posterior canal- related afferent inputs expanded onto part of those vestibular neurones that were deprived of their afferent vestibular input.

Recently, it has been suggested that the intrinsic excitability of MVN cells may underlie the regeneration of resting activity after UL, the so-called 'intrinsic mechanism' hypothesis (Darlington and Smith, 1996). This hypothesis seems most reasonable since recent studies demonstrated that MVN neurones *in vitro* exhibit resting activity in the absence of synaptic input (Darlington et al., 1989, 1990; Lewis et al., 1989; Smith et al., 1991; Serafin et al., 1990, 1991a,b; Dutia et al., 1992) and have intrinsic membrane properties such as a persistent Na^+ conductance and low and high threshold Ca^{2+} conductance (Serafin et al., 1990, 1991a,b; Johnston et al., 1994). However to what extent this intrinsic pacemaker activity is responsible for *in vivo* resting activity has yet to be determined.

Recent advances in the analysis of cellular mechanisms of vestibular compensation

Since the finding that MVN neurones have pacemaker-like activity *in vitro* in the absence of any synaptic input, it has been suggested that intrinsic membrane properties contribute substantially to the *in vivo* resting activity of the vestibular neurones. Apart from having a role in normal resting neuronal activity these intrinsic membrane properties might also undergo compensatory changes during the course of vestibular compensation to restore resting activity in the deafferented vestibular

neurones. Recent *in vitro* experiments carried out in MVN slices presented direct evidence for changes in intrinsic excitability of MVN neurones after UL.

Cameron and Dutia (1997), recording from MVN slices prepared from animals at various time points after UL, demonstrated that MVN cells in the rostral region of the ipsilateral nucleus, where the lesioned primary afferents mainly terminate, develop a sustained increase in their excitability between 2-4 h after UL. This is seen as a significant increase in the mean intrinsically-generated tonic discharge rate of the rostral cells (21.56 ± 1.7 (s.e.) spikes/s) compared to the mean discharge of cells recorded in slices prepared from normal animals (14.1 ± 0.69 (s.e.) spikes/s). Cameron and Dutia have proposed that this increase in the intrinsic excitability of the ipsilateral MVN cells is responsible for the restoration of resting activity in these cells during the early stage of vestibular compensation, as it will serve to counteract and overcome their initial disfacilitation and increased commissural inhibition from the contralateral MVN.

The following experiments in guinea pig brainstem slices provided further support for changes in excitability of MVN neurones after UL; Vibert et al. (1999) reported an increase in spontaneous discharge rates of extracellularly recorded vestibular neurones in the lesioned side recorded one week after UL. This increase in firing rate was still evident when the slices were perfused with low Ca^{2+}-high Mg^{2+} solution, which is known to block synaptic transmission. The increase in firing rate was even more significant in slices prepared one month after UL. Recently, Ris et al. (2001) using a similar approach presented evidence for an increase in pacemaker activity of the MVN cells after UL. Recording spontaneous activity of the MVN neurones in the presence of a cocktail of neurotransmitter blockers, which freed them from synaptic influences, they showed that the mean number of spontaneously active neurones and the mean firing rate of the recorded neurones dramatically increased from 48h to 1 week after UL. It was suggested that an increase in pacemaker activity might be a factor responsible for the restoration of spontaneous activity in the vestibular nucleus after labyrinthectomy.

As for the mechanism underlying the increase in excitability of the MVN neurones in the lesioned side Cameron and Dutia (1997) suggested that the down-regulation of GABA receptors on these cells could occur, in response to the excessive commissural inhibition to which they are subjected *in vivo*. This is supported by the finding of Calza et al., (1992), who observed a decrease in benzodiazepine binding in the ipsilateral MVN within 3 h after UL. Subsequently Yamanaka et al. (2000) looked at the $GABA_A$ and $GABA_B$ receptor responses to muscimol and baclofen, $GABA_A$ and $GABA_B$ receptor agonists respectively, in MVN slices prepared from the rats 4 h after UL. They observed a marked down-regulation of the functional efficacy of both $GABA_A$ and $GABA_B$ receptors in the rostral region of the ipsilateral MVN, where an increase in the intrinsic excitability was demonstrated by Cameron and Dutia (1997). Therefore it is likely that the loss of excitatory input from the ipsilateral primary vestibular afferents is compensated for by a decrease in responsiveness of the MVN cells to inhibitory synaptic inputs. Recently Johnston et al. (2001) reported a differential regulation of the $GABA_A$ and $GABA_B$ receptor efficacy in rats compensated for labyrinthectomy for 7-10 days following UL; the efficacy of the $GABA_A$ receptors agonist returned to normal while $GABA_B$ receptors were still down-regulated in the rostral MVN cells in the lesioned side.

The increase in intrinsic excitability of the MVN neurones following UL was shown to be dependent on the activation of glucocorticoid receptors (Cameron and Dutia, 1999). In animals that were kept under urethane anaesthesia for 4-6h after UL, during which they did not experience the stress that normally accompanies UL, the compensatory changes did not occur in the intrinsic excitability of the MVN neurones. Significantly, administration of glucocorticoid receptor agonist, dexamethasone, to the anaesthetised animals restored the changes in excitability. Therefore these experiments suggest that activation of the hypothalamo-pituitary-adrenal stress axis following UL is an essential requirement for the expression of neuronal plasticity in MVN cells. Parallel to these findings Cameron and Dutia also demonstrated a high level of immunoreactivity for c-fos protein, an immediate early gene product, in the paraventricular nucleus of the hypothalamus over the first 6h after UL confirming the activation of the stress axis following UL.

1.4 Aims of the Thesis

The vestibular system undergoes remarkable adaptive changes during vestibular compensation. The most significant change at the cellular level after the UL is the loss of the spontaneous activity of the vestibular nuclei neurones on the lesioned side and its gradual recovery over time when vestibular compensation develops (Smith and Curthoys, 1988a,b; Ris et al., 1995). Although it is generally accepted that the resumption of the spontaneous activity of the ipsilesional MVN neurones leads to the recovery of the behavioural symptoms of the UL, the cellular mechanisms responsible for the recovery of neuronal activity in the MVN are yet to be fully understood. Recent *in vitro* experiments in brainstem slices described adaptive changes in intrinsic excitability and GABAergic inhibition in the MVN during vestibular compensation (Cameron and Dutia 1997; Yamanaka et al., 2000; Vibert et al. 1999). To date, all the electrophysiological studies of the vestibular compensation used extracellular single unit recording techniques by which the Tyep A and Type B cells could not be distinguished and limited information could be obtained about intrinsic membrane properties of the second order vestibular neurones, which may be important in the mechanisms of neuronal recovery after UL.

The aim of the first part of the experiments therefore was to analyse the intrinsic membrane properties and firing characteristics of the MVN neurones and to examine the changes in these properties during vestibular compensation using whole-cell patch clamp recording technique in *in vitro* brainstem slices.

In the second part, experiments were carried out to investigate the effects of ageing on the excitability and GABA responsiveness of MVN neurones in slices of the brainstem prepared from young adult and aged rats. The aim was to determine if the reported delay in the rate of vestibular compensation after UL in aged animals (Drago et al., 1996) could be due to age-related changes in the intrinsic activity or GABA receptor sensitivity of the MVN neurones in old animals.

Chapter 2

Intrinsic membrane properties of rat MVN neurones and their role in vestibular compensation

2.1 Introduction

Intrinsic membrane and discharge properties of the vestibular nuclei neurones largely determine how these neurones process the information they receive from their various sources such as vestibular, visual and proprioceptive input. The intrinsic properties of the individual neurones are also important in shaping the emerging characteristics of the vestibular neuronal networks underlying gaze and postural control. The studies on the intrinsic properties of the MVN cells started with Gallagher et al, (1985) who first demonstrated that the MVN cells had spontaneous activity in *in vitro* brainstem slices. The subsequent *in vitro* intracellular experiments classified MVN neurones according to their action potential shapes and described underlying ionic currents (Serafin et al. 1991a,b; Johnston et al 1994; du Lac and Lisberger 1995; Babalian et al. 1997). A detailed account of these experiments on the neuronal cell types and ionic currents described in the MVN neurones is given in the first chapter of this thesis.

In addition to their role in information processing within the neuronal network, intrinsic membrane properties of the MVN neurones may be involved in learning and adaptive plasticity of the vestibular system. The vestibular system undergoes remarkable adaptive changes during vestibular compensation, a behavioural recovery process that takes place after a unilateral peripheral vestibular lesion (UL). The most significant change at the cellular level after the UL is the loss of the spontaneous activity of the vestibular nuclei neurones on the lesioned side and its gradual recovery over time when vestibular compensation develops (Smith and Curthoys, 1988a,b; Ris et al., 1995) (for a detailed account of vestibular compensation see section 1.3). Although it is generally accepted that the resumption of the spontaneous activity of the ipsilesional MVN neurones leads to the recovery of the behavioural symptoms of the UL, the cellular mechanisms responsible for the recovery of neuronal activity in the MVN are yet to be fully understood.

Recent *in vitro* experiments in brainstem slices described adaptive changes in intrinsic excitability and GABAergic inhibition in the MVN during vestibular

compensation. Cameron and Dutia (1997) showed that the MVN cells in the rostral region of the ipsilateral nucleus develop a sustained increase in their excitability within 4 h after the UL as evidenced by a significant increase in the mean intrinsically-generated tonic discharge rate of the rostral MVN neurones compared to the mean discharge of the neurones recorded in slices prepared from normal animals. This increase in intrinsic excitability of the MVN cells was later shown to last at least 10 days after the UL (Johnston et al., 2001). Yamanaka et al. (2000) demonstrated a marked down-regulation of the functional efficacy of $GABA_A$ and $GABA_B$ receptors in the rostral region of the ipsilateral MVN during the early stages of vestibular compensation in unilaterally labyrinthectomised animals. Similarly, an increase in spontaneous resting activity of the MVN cells and a down-regulation of glycinergic inhibition were reported in guinea pigs after the UL (Vibert et al. 1999). Both down-regulation of GABAergic and glycinergic inhibition and the increase in intrinsic excitability of the MVN neurones may be responsible for the restoration of resting activity in these cells during the early stage of vestibular compensation, as it will serve to counteract and overcome their initial disfacilitation and increased commissural inhibition from the contralateral MVN.

An increase in intrinsic excitability of MVN neurones has been proposed to be involved in the recovery of resting activity to MVN neurones after the UL (Darlington et al., 1989; Darlington and Smith, 1996, Cameron and Dutia, 1997; Vibert et al., 1999). These experiments were carried out using extracellular recording techniques which cannot explain the cellular mechanisms that underlie adaptive changes in the intrinsic properties of the MVN cells during vestibular compensation. It is therefore necessary to record intracellularly to investigate the role of the identified MVN cell types (Type A and Type B cell), which could not be distinguished in previous extracellular experiments, and to determine whether any of the wide repertoire of ionic conductances MVN neurones possess are affected by the UL. In this chapter of the thesis, using whole-cell patch clamp recordings, the intrinsic membrane properties and spike firing characteristics of rat MVN neurones in brainstem slices prepared from the normal animals were studied and compared

with the findings obtained from rats that were subjected to unilateral vestibular deafferentation.

2.2 Materials and Methods

2.2.1. Animals

Young adult male Sprague-Dawley rats (Bantin and Kingman, UK) weighing between 80 and 120g were used for the experiments. Animals were housed under controlled conditions of 21-23°C in a 12 hours light-dark cycle, relative humidity of 50-60%, and being fed *ad libitium*. Animals were kept in the Medical Faculty Animal Area (MFAA) for 3-4 days before any experimental procedure was carried out in order for them to get accustomed to handling and to minimise the effects of non-specific stress. Animals were collected from the MFAA on the day of the experiment. Animal care and experimental procedures were carried out in accordance with the UK legislation for the human care and use of laboratory animals.

2.2.2. Preparation and maintenance of MVN slices

The MVN slices were prepared from normal animals and animals that were subjected to unilateral labyrinthectomy operation beforehand. The rats were anaesthetised before culling. For anaesthesia, animals were placed in a small box containing halothane (Fluothane, May and Baker Ltd., UK) soaked cotton wool and covered with a Perspex lid. Once the animal became motionless the box was opened and the anaesthesia was confirmed by the absence of the limb withdrawal reflex to paw pinch. While under anaesthesia the animal was decapitated using a small animal guillotine. Using a pair of scissors the scalp was removed and neck muscles were cleared off. With a pair of pointed scissors a midline cut was made in the skull extending from the occipital bone to the frontal bone. Care was taken not to damage the brainstem with the tip of the scissors. The brain was exposed by removing occipital, parietal and frontal bones using fine bone rongeurs. The meninges were cut and cleared off the brain. After making a transverse cut anterior to the superior colliculus with a fine scalpel, the hind brain was lifted up and the cranial nerves were

cut. Free from all attachments the brain was dropped into a beaker of ice-cold (4°C) artificial cerebrospinal fluid (aCSF, for recipe see section 2.2.4. Solutions and drugs) bubbled with a gas mixture of 95% O_2 and 5% CO_2. This part of preparation was performed as quickly as possible, usually in less than a minute.

After keeping the brain in the ice-cold aCSF for a minute, it was placed on a cold dissecting stage with its dorsal surface uppermost. The dissecting stage consisted of a piece of filter paper moistened with ice cold aCSF and positioned on an upside down petri dish placed on ice. The cerebellum was then lifted gently by the help of a scalpel and the peduncles were cut. By trimming, a block of brainstem extending from the inferior colliculi to the obex was obtained and it was cleared of adherent pia using fine forceps. The MVN was easily recognised by its oval shape and grey colour sitting on the floor of the fourth ventricle. Using cynoacrylate glue the block of brainstem containing the MVN was then cemented on the advancing stage of a Vibroslice (Campden Instruments Ltd, UK) with the fourth ventricle facing up. The stage was placed into the reservoir of the Vibroslice and submerged in ice-cold aCSF pre-bubbled with a mixture of 95%O_2 and 5%CO_2. The block of brainstem containing MVN was cut in the horizontal plane approximately parallel to the floor of the fourth ventricle to obtain a slice that contained the entire rostro-caudal length of the MVN. The first slice containing the dorsal 100-200μm superficial layers of the MVN was discarded. The next slice was cut to a thickness of 400 μm and used for the experiment. This slice contained two MVNs sitting on each half of the slice. The two nuclei were separated by a section along the midline of the fourth ventricle. Using a fine paintbrush moistened with cold aCSF, the slices containing the left MVN (ipsilateral to the unilateral labyrinthectomy) was taken into a small petri dish filled with cold oxygenated aCSF and transferred to the recording chamber.

An interface-type recording chamber was used to maintain the slices and make intracellular recordings (Figure 2.2.1). The slice containing the MVN was placed on the fine nylon mesh stretched on the ramp of the chamber (see Figure 2.2.2 for a picture of a brainstem slice). The chamber was perfused with oxygenated aCSF at a temperature of 33 ± 0.2°C delivered and removed from the chamber at a rate of 1.6

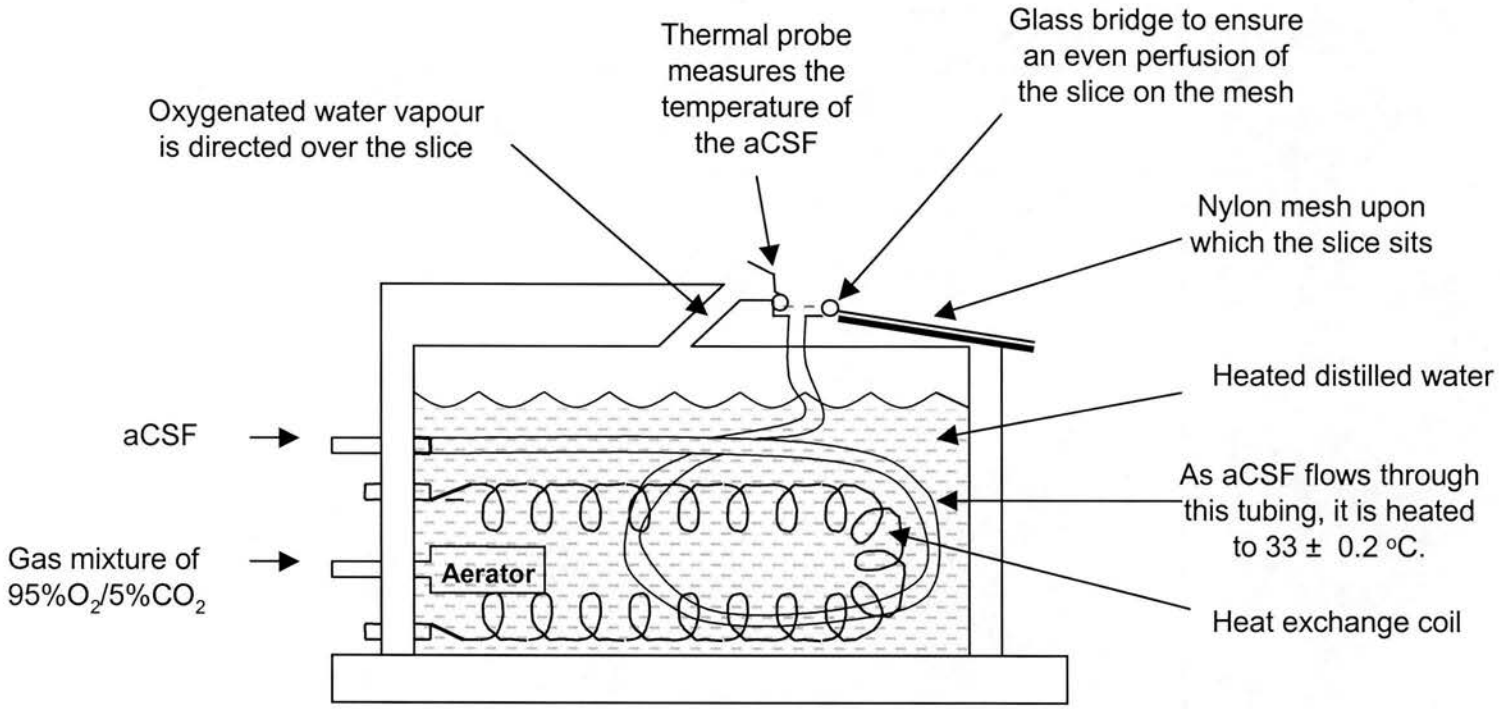

Figure 2.2.1. Schematic diagram of the interface type chamber used for maintaining the brainstem slices. (Adapted from Johnston, 1994).

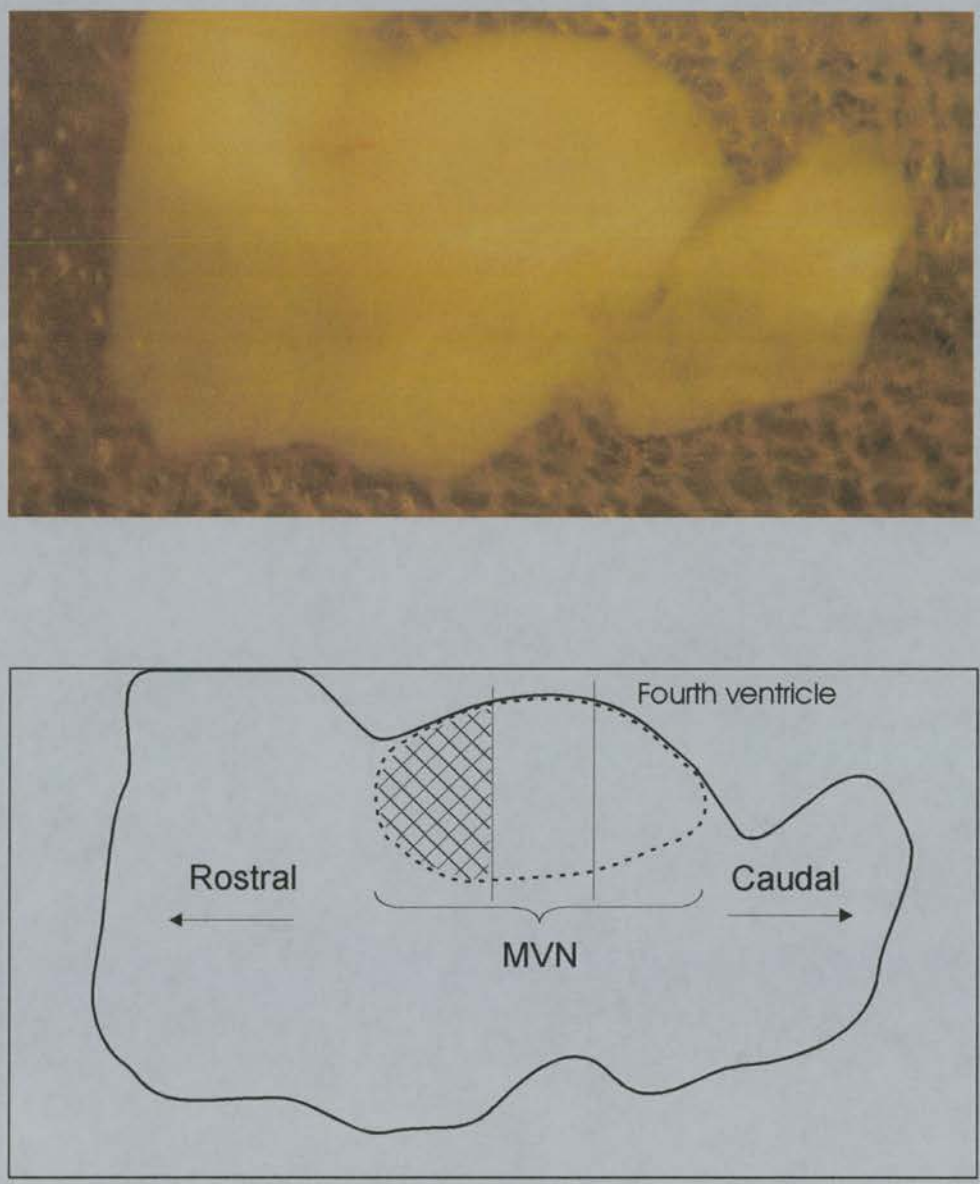

Figure 2.2.2. A photomicrograph (upper panel) and a drawing (lower panel) of a horizontal brainstem slice containing MVN. The photograph was taken during the incubation of the slice on a nylon mesh of the recording chamber. The drawing on the lower panel highlights the borders of the MVN with dotted lines. Hatched area indicates the rostral one third of the nucleus where the electrophysiological recordings were made. Medial side of MVN is facing upwards.

ml/min by means of a 2-channel peristaltic pump (Gilson, France) through PVC tubing (bore, 1.5 mm; wall, 1.0 mm, Altec, UK). The distilled water in the reservoir of the chamber was continuously bubbled with 95%O_2/ 5%CO_2 to produce a continuous stream of water saturated gas, which passed through an opening in the roof of the reservoir and flowed over the slice. In this interface type chamber, the slice was kept between the two layers: aCSF running beneath the slice on the nylon mesh and the gas layer over the slice. The slice was incubated for at least one hour in these conditions for equilibration before attempting electrophysiological recordings and drug applications.

2.2.3. Unilateral Labyrinthectomy

The surgery was carried out under avertin anaesthesia (Dyer et al., 1981) in the Medical Faculty Animal Area. The anaesthetic was made up according to the following recipe and stored in a fridge (at 4 °C) to use for up to a week. 2.5g tribromoethanol was added to 10 ml of ethanol and mixed until tribromoethanol dissolved completely. To this solution, 1.5 mls of 2-methlybutan-2-ol was added and subsequently made up to a final volume of 125 mls by the addition of normal saline (9g/l). The solution was injected intraperitoneally at a dose of 1 ml/100g body weight and provided surgical anaesthesia for 30-40 min.

After a sufficient level of surgical anaesthesia, as determined by a lack of the limb withdrawal reflex to a paw pinch, the area at the back of the head between the ears was shaved with clippers and cleaned with disinfectant iodine solution (Betadine, UK). An incision of about 4 cm long was made above the left ear with a sterile scalpel. The connective tissue was cut aside using sharp surgical scissors. The middle ear was then opened and the ossicles were removed with fine forceps. The temporal bone was exposed by cutting the muscles attached to the lamboidal ridge. By cleaning the remaining connective tissue with a dental blade the bony duct of the horizontal semicircular canal was visualised. The canal was then opened by drilling into the bony duct with a 1 mm diameter dental drill (Precision P.C.B. drill, RS

Component, UK). The blood and the fluid from the canal were aspirated by a blunted needle attached to a suction pump to keep the area clear. Drilling was continued anteriorly in the horizontal plane of the canal until the ampulla of the horizontal canal and the vestibule were opened. The contents of the vestibule were then aspirated and the cavity was rinsed with 100% ethanol (Sigma, UK). After the wound was sutured animals were returned to their home cages. The animals began to recover from anaesthesia about 30-40 min following induction, typically 20-30 min after the labyrinthectomy was completed. After recovery all the animals showed the characteristic severe symptoms of unilateral vestibular loss including tonic eye deviation, spontaneous nystagmus, barrel rolling, circular walking, loss of ipsilateral extensor tone and head deviation towards the side of the lesion. The animals were allowed to recover in their home cages for either 1-3 days (1-3d post-UL group) or 7-11 days (1w post-UL group), when they were re-anaesthetized with halothane and decapitated for the preparation of brainstem slices for electrophysiological recordings as described in section 2.2.2.

2.2.4. Solutions and drugs

The artificial cerebrospinal fluid (aCSF) used for the preparation and maintenance of the slices was made up fresh on the day of experiment according to the following recipe (Llinas and Sugimori, 1980) (in mM): Sodium chloride (NaCl) 124; Potassium chloride (KCl) 5; Potassium dihydrogen orthophosphate (KH_2PO_4) 1.2; Magnesium sulphate heptahydrate ($MgSO_4$) 1.3; Calcium chloride 2-hydrate ($CaCl_2$) 2.4; Sodium bicarbonate ($NaHCO_3$) 26; D-(+) glucose 10. The aCSF was oxygenated by continuously bubbling with a gas mixture of 95%O_2 and 5%CO_2.

Two kinds of modified aCSF solutions were used in some experiments:

(a) Ca^{2+}-free/Co^{2+} aCSF contained (in mM) NaCl 124; KCl 6.25; Cobalt chloride ($CoCl_2$) 2.4; Magnesium chloride ($MgCl_2$) 1.3; Tris 25; D-(+) glucose 10.

(b) The Ba^{2+}-containing aCSF had a composition of (in mM) NaCl 124; KCl 6.25, Barium chloride ($BaCl_2$) 2.4; $MgCl_2$ 1.3; $NaHCO_3$ 26, HEPES 1.25, D-(+) glucose 10.

Where indicated the following drugs were added to the bathing medium for pharmacological manipulation of ionic channels.

(a) Tetrodotoxin (TTX) was used at a concentration of 1 μM to block voltage-activated sodium channels (Johnston et al., 1994).
(b) Tetraethyl ammonium bromide (TEA), 10 mM, and 4-Aminopyridine (4-AP), 0.5 mM, are used to block voltage-activated potassium channels (Johnston et al., 1994).
(c) Apamin is used to block SK-type calcium-activated potassium channels at the concentration of 0.1 μM (Johnston et al., 1994).
(d) Nickel chloride ($NiCl_2$) is used to block T-type calcium channels at the concentration of 1 mM (Mouginot et al., 1997).
(e) Nifedipine is used to block L-type high voltage-activated calcium channels at a concentration of 10 μM (Morisset and Nagy, 1999).

All drugs were purchased from Sigma, UK. Stock solutions of the drugs were made in distilled water and kept frozen in eppendorf tubes. Aliquots of the stock solutions were thawed and diluted to their final concentrations in oxygenated aCSF prior to application.

Drug solutions were applied to the slice by switching the inlet tubing from the normal aCSF to the desired solution using three-way taps. Drugs were applied if the recordings are still stable after the measurents of the parameters described in section 2.2.6. The effects of the drugs were usually evident within 10 minutes of delivery. To see the reversibility of the drugs the recordings were continued in normal medium for about 30 minutes, by which time the cells tended to become unstable, and recordings were ceased.

2.2.5. Electrophysiological Recordings

A conventional electrophysiological recording set-up was used for data collection, storage and analysis. A schematic diagram of the recording set-up used in this study is illustrated in Figure 2.2.3. The signals from the recording electrode were fed into the Axoclamp 2A (Axon Instruments, USA) amplifier. The voltage output from the amplifier, which was amplified 10 times and filtered at 3 kHz, was passed to a two-channel storage oscilloscope (HM 205-3, Hameg, Germany) and monitored continuously. The voltage output was also fed to a CED 1401 Plus analogue-to-digital (A/D) interface (Cambridge Electronic Design, UK), which was then connected to a computer (P486, Dell, UK). Prior to entering A/D interface the voltage signals were amplified with a gain of x2 by conditioning amplifiers to optimise digitisation of the signals. Current commands were produced by the computer with pClamp 6.02 software (Axon Instruments, USA) and were delivered to the recording electrode through a DigiData 1200 interface (Axon Instruments, USA). All experimental data were stored on computer hard disk for off-line analysis.

Recording electrodes

Filamented standard-wall borosilicate glass pipettes (outer diameter1.5 mm, inner diameter 0.86 mm, Harvard Electronics, UK) were used for whole-cell patch-clamp recordings. The pipettes were prepared for recording just prior to the experiments using a horizontal three-stage programmable microelectrode puller (Model P87, Sutter Instruments, UK). After pulling the recording electrodes usually had an internal tip diameter of about 1 μM. The glass microelectrodes were then back-filled with an intracellular filling solution. The composition for the filling solution was (in mM) 145 potassium gluconate, 2 $MgCl_2$, 5 K_2ATP, 0.1 ethylene glycol-bis (β-amino-ethyl-ether)-N,N,N,'N'-tetraacetic acid (EGTA), 5 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) (Kinney et al., 1994). The osmolarity and the acidity of the solution were adjusted to 290 mosmol and pH 7.2, respectively. When filled with the recording solution the electrodes had a tip resistance of about 5-7 MΩ. The glass-

Figure 2.2.3. Schematic diagram of the experimental set-up. For whole-cell patch clamp experiments, the voltage signal output from the amplifier was fed into two A/D interfaces. The CED 1401 A/D interface was used with Spike 2 software to obtain average spike shape and related parameters of the action potentials. The DigiData 1200 A/D interface was used with pClamp software both to deliver current pulses and to record responses of the cell membrane potential. The Neurolog system, which consists of a filter, an amplifier and a trigger unit, was used for extracellular recordings of the firing responses of the cells to inhibitory neurotransmitters as explained in the third chapter of this thesis. In the extracellular experiments, the inhibitory neurotransmitter drugs were applied to the slice by switching the inlet tubing to the syringes containing the drugs using three-way taps.

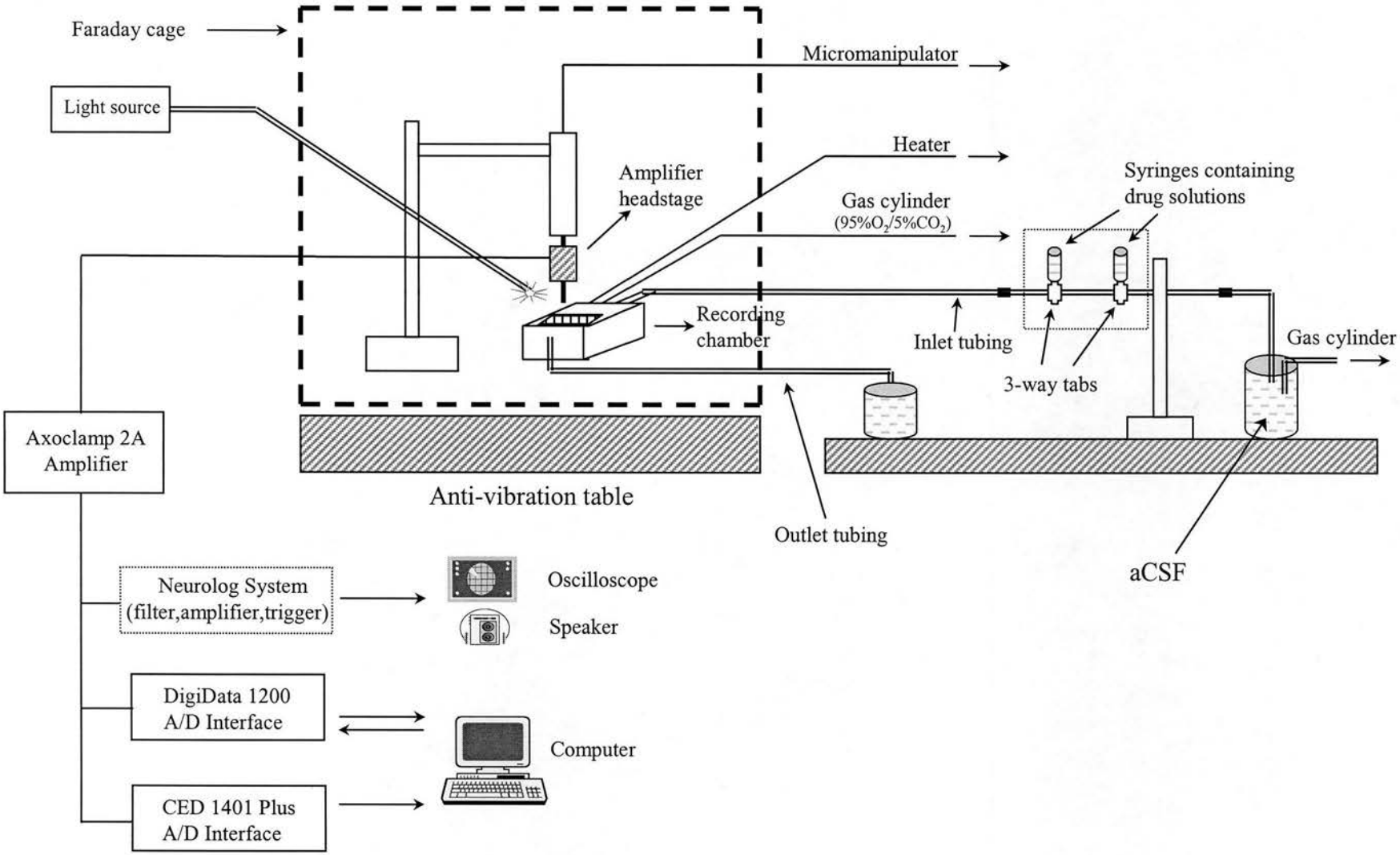

Figure 2.2.3. Schematic diagram of the experimental set-up.

recording electrode was inserted into a Perspex microelectrode holder that was linked to the unity gain headstage of the Axoclamp 2A amplifier. The electrical connection between the headstage and the pipette solution was provided by silver chloride-coated silver wire.

Whole-cell patch-clamp recording

Whole-cell patch-clamp recordings were made in the MVN slices with the 'blind' technique as described by Blanton et al. (1989). Under a binocular dissecting microscope (x16 magnification) and fibre optic illumination (Flexilux, Germany) the MVN was visualised and the recording electrode was positioned just over the MVN using a coarse manipulator. The microelectrode was then slowly advanced into the MVN with an inchworm controller (Burleigh 6000, Burleigh Instruments, USA). To prevent clogging of the electrode tip within the slice, a positive pressure was applied by gentle blowing to the PVC tubing attached to the side of the pipette holder. Once the recording electrode touched the slice, the offset potential between the reference electrode and the recording electrode was zeroed using the calibrated offset potentiometer control on the Axoclamp 2A amplifier. Using the DC current command controller of the amplifier with the bridge mode, -0.15 nA DC current pulses with 100 ms duration and 1 Hz frequency were delivered to the electrode. Because of the resistance of the electrode tip, current pulses caused a deflection on the voltage trace. The bridge balance control of the amplifier was advanced until the deflection on the voltage trace disappeared. The reading on the bridge balance control gave us the tip resistance of the recording electrode, which was typically around 5-7 MΩ. The electrode was lowered through the slice with 2 μM steps until a cell was encountered. The presence of a cell was indicated by extracellular action potentials and an increase in electrode resistance. If the electrode was in contact with a cell, letting go the positive pressure resulted in a further increase in resistance. With a gentle suction applied by mouth to the pipette, a gigaohm seal (1-3 GΩ) between the electrode tip and the cell membrane was usually formed within seconds. Once the gigaohm seal established, an additional suction was applied to the pipette to rupture the membrane patch under the electrode tip, which then gave a whole-cell

recording configuration. The technique for obtaining whole-cell patch-clamp recording is schematically illustrated in Figure 2.2.4.

Whole-cell recording provides a better signal-to-noise ratio and a better space clamp, and often gives a higher input resistance compared to intracellular sharp electrode recording (Staley et al., 1992). Therefore whole-cell patch clamp technique is a more sensitive method for measuring small synaptic events (Hestrin et al., 1990). One complication of the whole-cell recording technique is the alteration of intracellular homeostasis caused by diffusion of critical intracellular constituents into the electrode solution (Pusch and Neher, 1988). This could be important especially if intracellular calcium levels are changed and the energy source, ATP, is depleted. This drawback can be overcome by including calcium buffers and ATP in the recording electrode. Slow diffusion of the intracellular molecules to the recording electrode in whole-cell recording may also cause rundown of ionic currents especially in long recordings exceeding 30 min. The difusion between the cell interior and the recording electrode can also be an advantage providing a way to modify internal environment of the cell. Once a whole-cell recording is established it is also possible to proceed further to different recording configurations such as outside-out and inside-out patch recordings where single-channel data can be obtained. When combined with molecular biological techniques such as polymerase chain reaction it is also possible to genetically characterise the same cell by extracting the cytosol into the pipette and analysing it later. The techniques developed in this thesis provide the basis for future such applications to MVN neurones.

Figure 2.2.4. Schematic diagram of obtaining whole-cell patch clamp recording (left panels) and typical electrical signals observed during the patch clamping (right panels).

A. The pipette containing positive pressure is approaching the cell, but still remote from it. The amplitude of the deflection on the voltage trace (upper trace), in response to -0.15 nA current pulse (lower trace), indicates a pipette resistance of 7 MΩ (Ohm's law).

B. Advancing the pipette with positive pressure closer to the cell increased the pipette resistance as evidenced by a larger amplitude deflection on the voltage trace.

C. Releasing the positive pressure resulted in a further increase in the pipette resistance.

D. Within seconds after gentle suction is applied to the pipette, a gigaohm seal is formed between the pipette tip and the cell membrane. This is evident from the large amplitude deflection on the voltage trace, which is truncated here.

E. A further suction ruptured the membrane patch under the pipette tip resulting in whole-cell patch configuration where the contents of the pipette and the cell form a continuum. The voltage deflection is the result of the sum of the pipette resistance and cell membrane resistance.

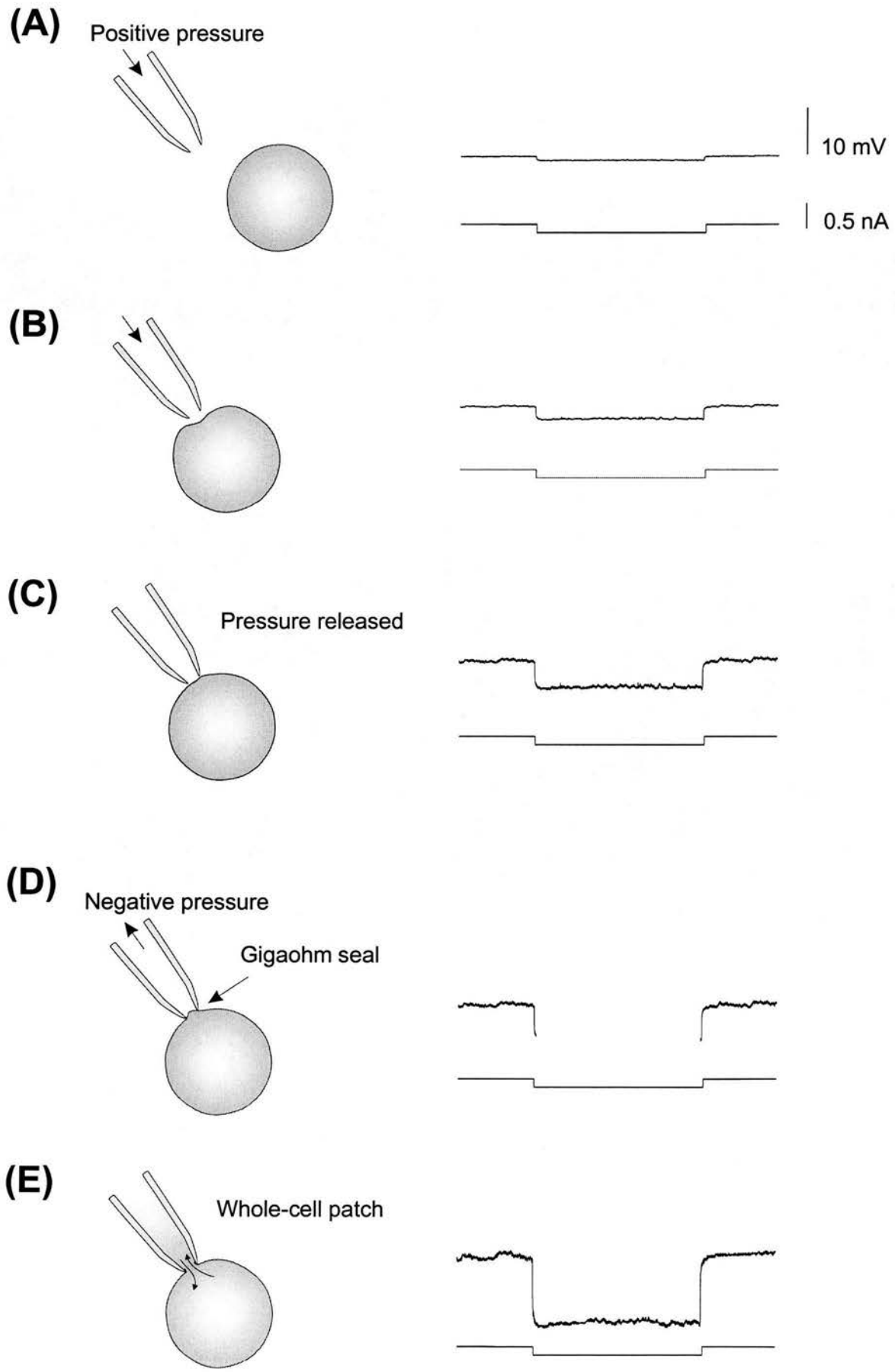

Figure 2.2.4. Schematic diagram of whole-cell patch clamp technique.

2.2.6. Data collection and analysis

All experimental data were collected using Spike2 software, version 3.00, (Cambridge Electronic Design, UK) and pClamp software, version 6.0.2 (Axon Instruments, USA). The data were recorded on the computer hard disk for off-line analysis. All figures of intracellular recordings were produced from selected data files using CorelDraw version 9.0 software (Corel Inc., Canada). The results were expressed as mean ± S.E.M. Statistical analysis of the results was performed using GraphPad Prism version 3.00 software (GraphPad Software Inc, San Diego, USA). Individual statistical methods used and the significance levels are indicated in the text.

The neurones included in this study had stable resting membrane potentials and spike amplitudes of greater than 50 mV. The recordings were made with the bridge mode of the Axoclamp 2A amplifier. The measurements obtained from individual MVN neurones are described below. All measurements were made within 15 minutes after the establishment of whole-cell patch clamp recording during which a stable recording was maintained.

Averaged Spike Shape

Using Spike2 software spontaneous action potentials were recorded at a high digitisation rate (40 KHz) for a period of two seconds (Johnston et al., 1994). To get an average shape of the spikes recorded during this period, the time of occurrence of each action potential peak was located in the digitised data, and the membrane potential across a 20 ms window around these times was averaged. Some of the measurements were calculated with reference to the firing threshold. However, it was not possible to make an objective measurement of the firing threshold since the membrane potential prior to the action potential showed a gradual depolarisation. The firing threshold, therefore, was determined by double differentiating the averaged action potential waveform and by evaluating the noise level on the second differential. The firing threshold was thus defined as the membrane potential at the point when the second derivative became twice as great as its own noise level (Figure 2.2.5) (Johnston et al., 1994).

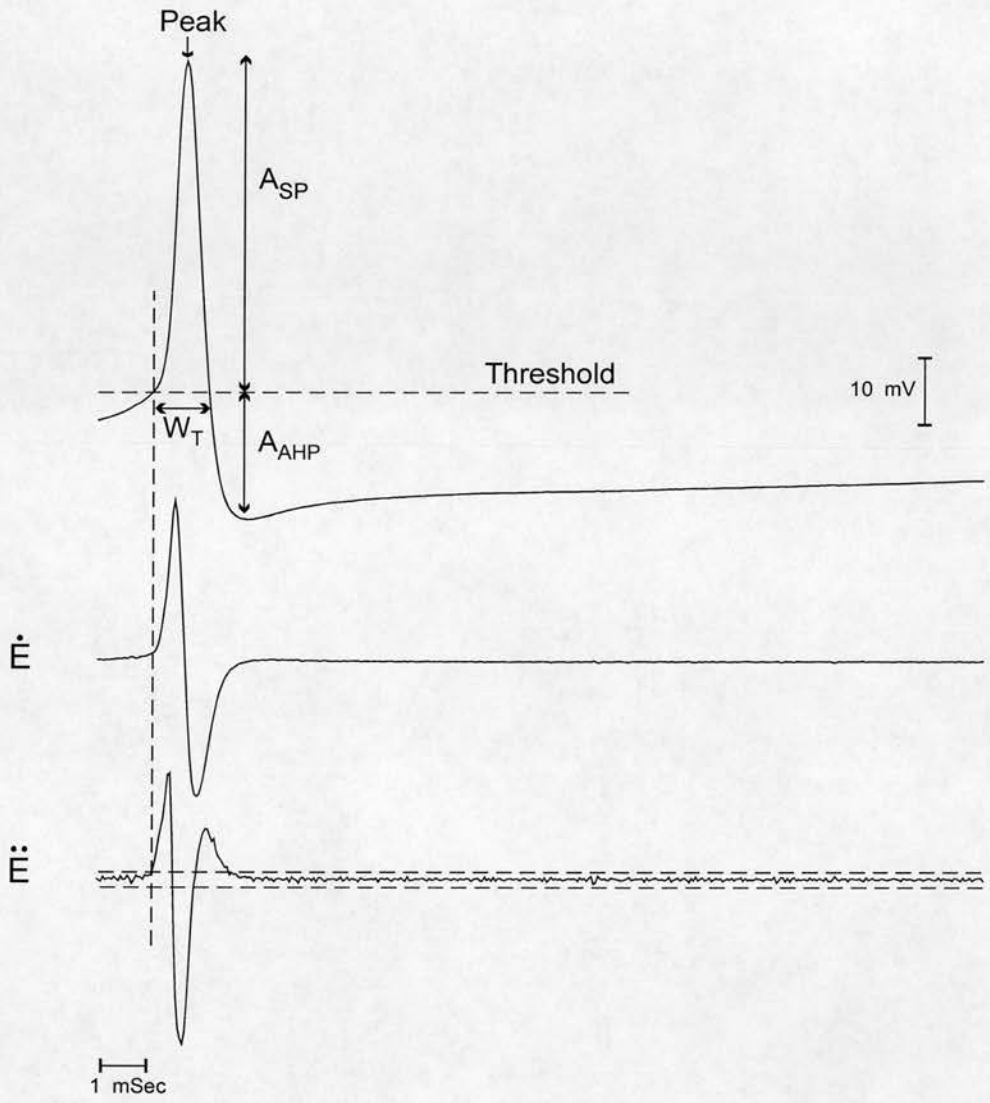

Figure 2.2.5. Analysis of the average action potential shape. Upper trace: Averaged action potential shape obtained by averaging membrane potentials of successive action potentials of spontaneously active neurones recorded over 2s period. Middle and lower traces: First (middle trace) and second (lower trace) differentials of the averaged spike shape calculated by taking simple differences between successive spikes. The horizontal dashed line in the lower trace indicates the level corresponding to twice the noise amplitude in the last 5 ms of this trace. The vertical dashed line indicates where the second differential becomes greater than this level. The horizontal dashed line on the averaged spike shape (upper trace) which crosses the vertical dashed line indicates the threshold level of this action potential. (Adapted from Johnston et al 1994). W_T, spike width at threshold; A_{SP}, spike amplitude; A_{AHP}, amplitude of the after hyperpolarisation.

The rise time of the action potential was measured as the time taken for membrane potential to reach the peak of the spike from the threshold. The fall time was calculated as the time taken to reach to the threshold from the peak of the spike. The duration of the action potential was measured as the width of the spike at the threshold. The amplitude of the action potentials and the afterpotentials were also measured with respect to the threshold (Figure 2.2.5).

The resting membrane potential

Since the MVN neurones included in this study were spontaneously active it was not possible to record a true resting membrane potential of the cells. Instead, the reading on the digital voltmeter on the front panel of the Axoclamp 2A amplifier was taken as the resting membrane potential. This reading was taken when there was no depolarising or hyperpolarizing current injection into the cell. This reading shows the time average of the membrane potential of the recorded cell. The value obtained from the voltmeter display was then corrected for an estimated liquid junction potential of –10 mV, which is due to different mobilities of the ions in the electrode and bath solutions, and was calculated according to the generalised Henderson equation (Barry and Lynch, 1991; Sakmann and Neher, 1995).

The membrane input resistance

To measure the membrane input resistance, the cells were first hyperpolarized until they stopped firing action potentials by injecting continuoes negative DC current through the recording electrode. At this holding potential, current pulses of 100 ms duration and 0.2 nA amplitude were applied using the pulse generator facility of the Axoclamp 2A amplifier. The bridge balance potentiometer on the amplifier was then advanced until the deflection on the voltage trace was at the same level as the holding membrane potential. The reading on the bridge balance potentiometer gave us the resistance of the electrode and the cell membrane, measured in MΩ. To obtain the input resistance of the cell membrane, the resistance of the electrode was subtracted from this value.

The spontaneous discharge rate

The number of spikes was recorded during the two-second sampling for spike shape averaging. Dividing the number of action potentials recorded during this period by two gave the resting discharge rate of the cell expressed as spikes/s.

Current injection protocols

Depolarising and hyperpolarising current commands were produced by the computer with pClamp 6.02 software (Axon Instruments, USA) and were delivered to the recording electrode through the DigiData 1200 interface (Axon Instruments, USA). Before current injections the cells were hyperpolarized below firing threshold by injecting negative DC current. The holding potential of the cells was typically –70 mV. To study the firing characteristics of the cells a series of constant depolarising current steps of increasing amplitude (50 pA increments from 10 pA) was injected for duration of 500 ms and/or 1000 ms. Depolarising and hyperpolarising current steps of 500 ms duration (25 pA decrements starting at +50 pA) were injected to study the current/voltage responses of the cells.

The accommodation/acceleration index (Δ Firing Rate)

The accommodation/acceleration indexes were obtained from the frequency responses of the cells to a 110 pA current injection delivered from a subthreshold holding potential (-70 mV). The Δ Firing Rate is calculated as the difference in the instantaneous firing rate obtained from the last interspike interval (ISI) and the first interspike interval. Therefore, positive Δ Firing Rate indicates a cell that shows spike frequency acceleration during the current pulse whereas a negative value means that the cell is producing more spikes at the beginning of the pulse and fewer spikes at the end, showing spike frequency accommodation.

Frequency/current (f/I) curves

Frequency/current (f/I) curves of the MVN cells were produced by plotting currents injected in increasing amplitude against the frequency responses of the cells. The frequency of the cells was calculated as the steady state frequency from the average of the last three interspike intervals ($ISI_1+ISI_2+ISI_3$) using the following formula:

Frequency (spikes/s) = $3/ISI_1+ISI_2+ISI_3$ X 1000

2.3 Results

The intrinsic membrane properties and spike firing characteristics of rostral MVN cells were investigated using whole-cell patch clamp recordings in brainstem slices prepared from 44 normal animals, and animals that had undergone vestibular compensation for either 1-3d (n= 34 animals) or 1w (n= 25 animals) after the UL.

2.3.1 Intrinsic membrane properties and spike firing characteristics of MVN neurones in normal rats

Whole cell patch clamp recordings were made from a total of 101 spontaneously active MVN neurones in slices prepared from normal young adult rats weighing between 80 and 120g. Based on their averaged spontaneous action potential shapes, as described previously by Johnston et al. (1994), and described in detail in section 2.2.6 of this thesis, each recorded cell was classified as either "Type A" having a single deep post-spike after-hyperpolarisation (Figure 2.3.1.1A), or as "Type B" with an early fast AHP followed by a delayed slow AHP (Figure 2.3.1.1B).

Type A MVN neurones

Of the 101 MVN cells recorded from the normal rats, 23 (23%) were classified as Type A cells (Figure 2.3.1.1A). The mean values of the passive membrane properties of the Type A cells obtained from the averaged action potential shapes are presented in Table 2.1. All Type A cells were spontaneously active firing with a mean rate of 15.8 ± 1.4 spikes/s. Type A cells had single deep AHPs ranging from 14.1 to 27.0 mV in amplitude, with a mean amplitude of 18.8 ± 1.1 mV. The mean resting membrane potential of Type A cells was -54.5 ± 0.5 mV. Their input resistance ranged from 79 to 181 MΩ with a mean value of 143.3 ± 7.3 MΩ. The mean amplitude of action potentials was 55.6 ± 1.6 mV. Type A cells had relatively longer action potentials compared to Type B cells. For Type A cells the duration of the

action potentials at the threshold was 1.08 ± 0.05 ms. whereas for Type B cells it was 0.81 ± 0.02 ms ($p<0.001$, Student's t-test). Similarly, the rise time (time taken for the membrane potential to reach the peak of the action potential from threshold) and fall time of the action potentials (time taken for the membrane potential to return to the threshold from the peak of the action potential) in Type A cells (0.53 ± 0.02 ms and 0.55 ± 0.03 ms respectively) were longer than that of Type B cells (0.45 ± 0.01 ms and 0.37 ± 0.01 ms, respectively)($p<0.01$, Student's t-test).

The dynamic membrane properties of the MVN cells were studied by measuring membrane potential responses to depolarising and hyperpolarising current pulses applied from a holding membrane potential just below firing threshold. All Type A cells fired repetitively in response to depolarising current pulses (Figure 2.3.1.2A). The frequency of discharge was proportional to the amount of current injected resulting in a linear frequency/current (f/I) relationship (Figure 2.3.1.2B, Figure 2.3.1.3). Type A cells did not show any distinctive spike frequency accommodation or spike frequency acceleration during the depolarising current pulses. The cells displayed a regular firing throughout the duration of the current injections. The accommodation/acceleration indexes for Type A cells (Δ Firing Rate)(described in methods section of this chapter) were evenly distributed around zero with a mean value of 0.2 ± 1 spikes/s (Figure 2.3.1.4).
When hyperpolarising current pulses were applied from a subthreshold holding potential most Type A neurones (88%) showed an inward rectification (arrow in Figure 2.3.1.5) seen as a sag in the voltage response of the membrane potential.

All Type A cells showed a passive return to the baseline at the end of the depolarising current pulses without any depolarising plateau potentials (Figure 2.3.1.2A). In some Type B cells, plateau potentials are seen at the end of depolarising current pulses without any pharmacological manipulations (Figure 2.3.1.7, Figure 2.3.1.8A, Figure 2.3.1.9A).

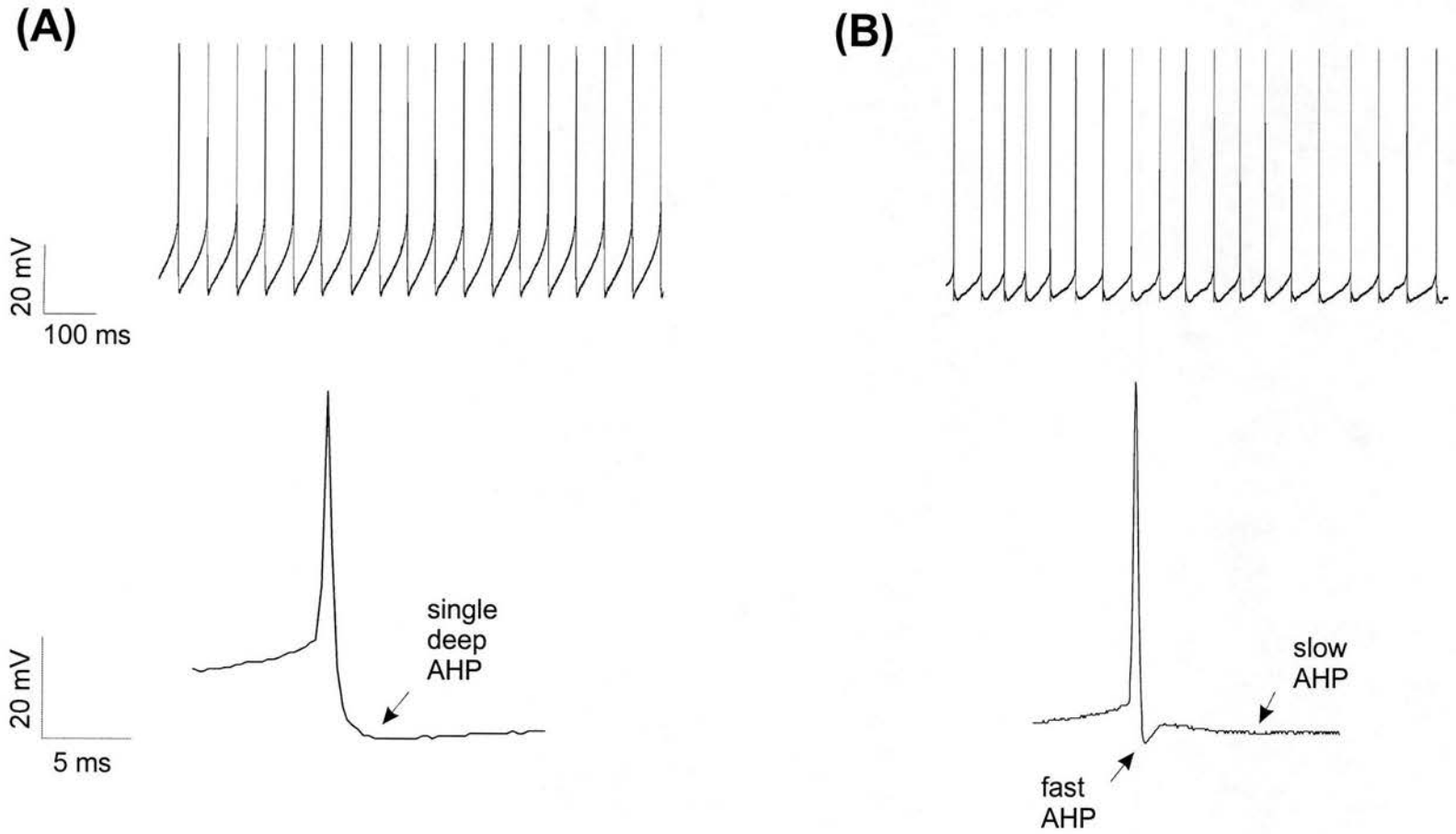

Figure 2.3.1.1. Examples of resting discharges and averaged spike shapes of a Type A (**A**) and a Type B (**B**) MVN neurone. Recordings were taken at the resting membrane potentials. Lower panels show the same cells as upper panels with expanded timescale.

Figure 2.3.1.2. The responses of a Type A MVN neurone to depolarising current pulses of increasing amplitude.

A. The cell displayed a regular firing throughout the duration of the current injections.

B. Linear f/I curves of the same Type A cell at different interspike intervals. The holding membrane potential of the cell is -65 mV.

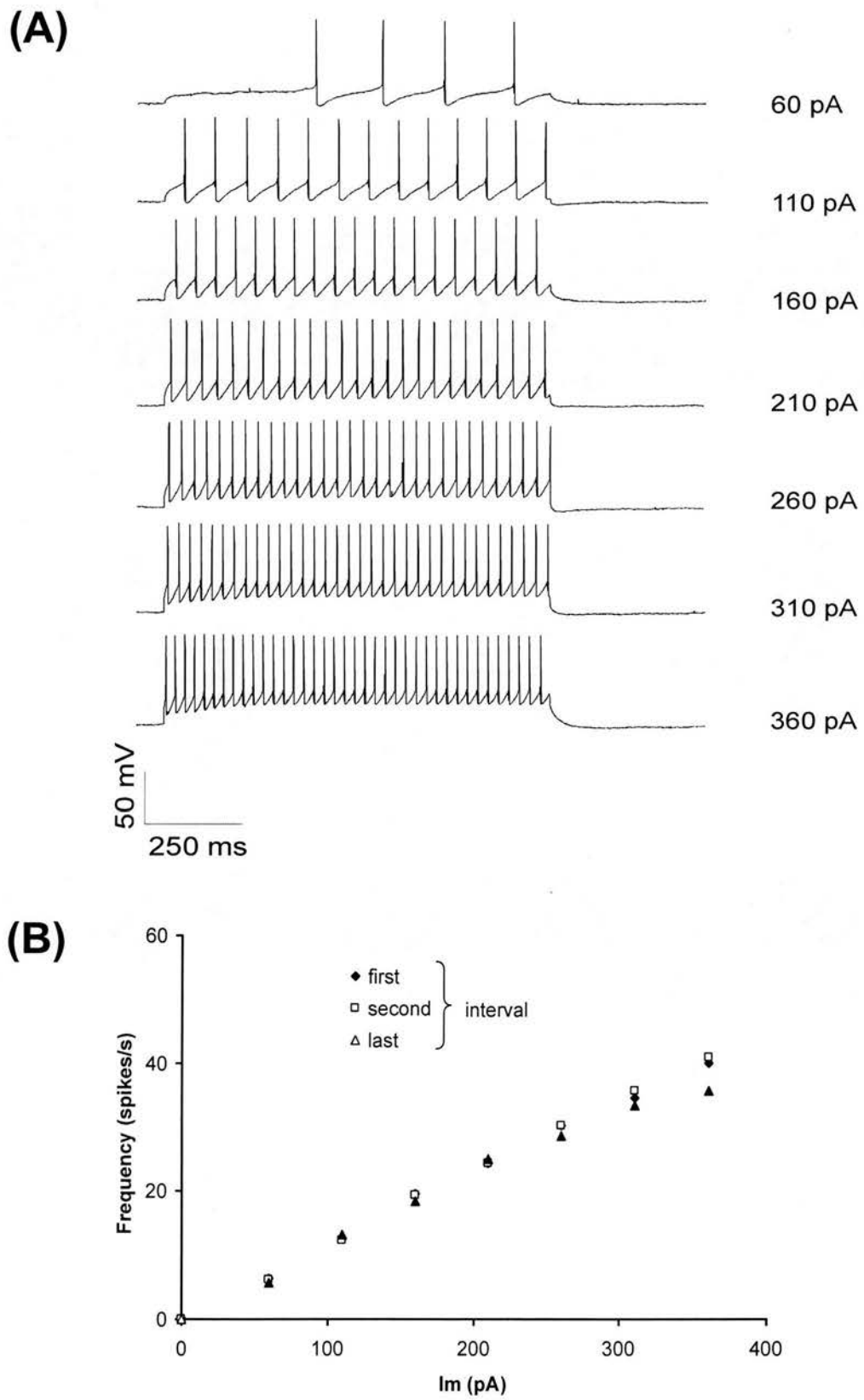

Figure 2.3.1.2. The responses of a Type A MVN neurone to depolarising current pulses of increasing amplitude.

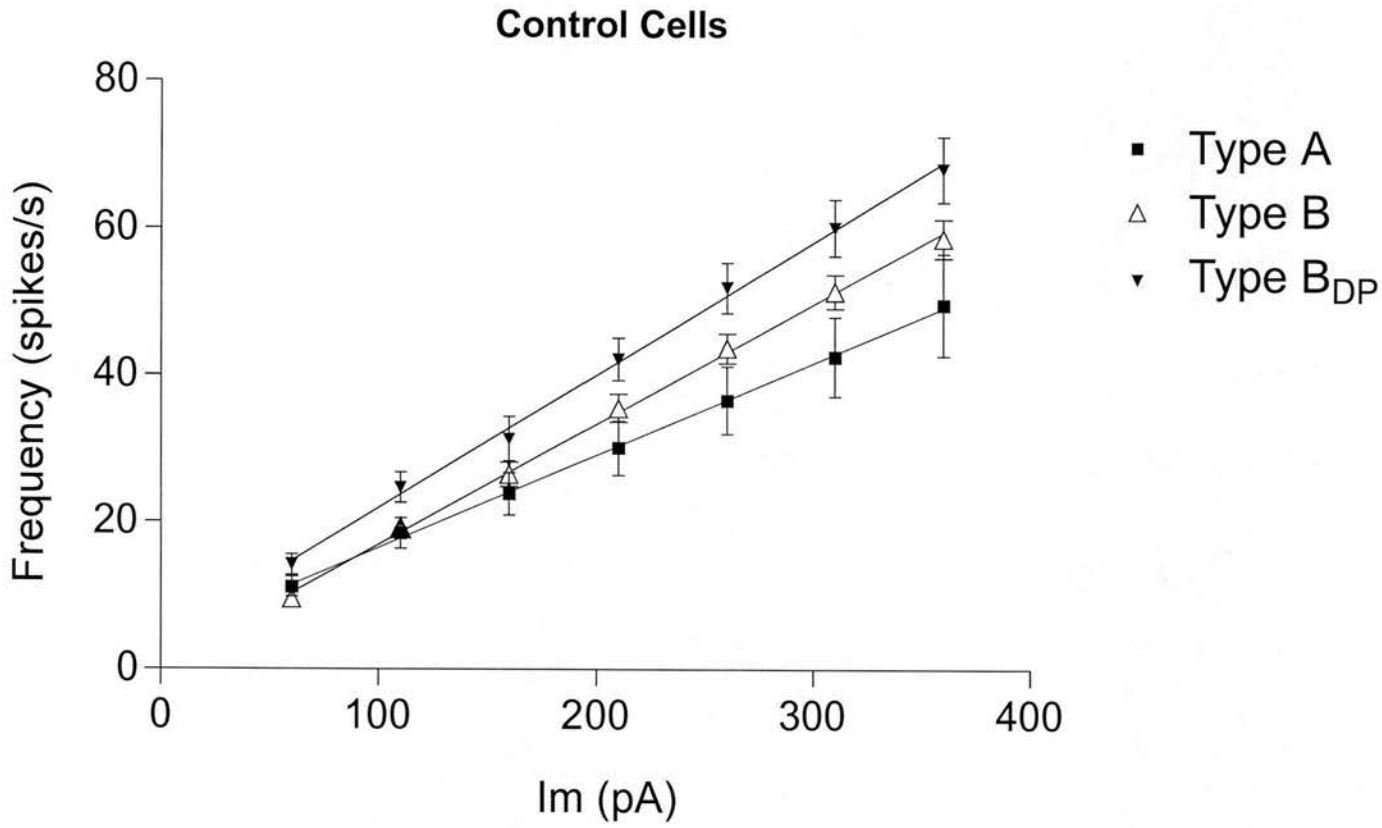

Figure 2.3.1.3. Frequency/current (f/I) relationships of the MVN cell types in control slices. The mean slopes (m_{slope}) of the linear regression line of Type B cells (m_{slope}= 0.17 ± 0.01 spikes/pA) and Type B_{DP} cells (m_{slope}= 0.20 ± 0.01 spikes/pA) are significantly greater than that of the Type A cells (m_{slope}= 0.14 ± 0.02 spikes/pA)($p<0.05$, Student's t-test)

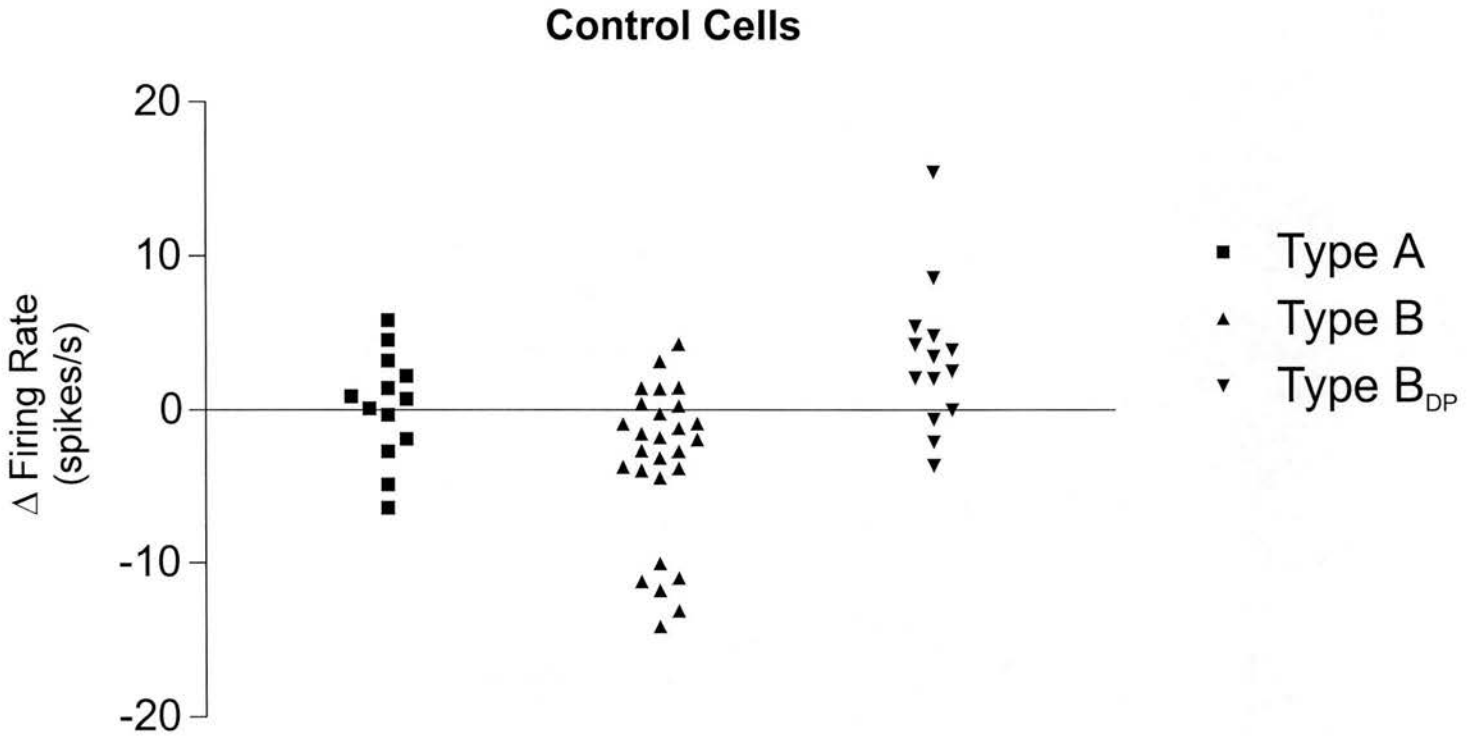

Figure 2.3.1.4. Scatter histogram of the spike frequency acceleration/accommodation indexes of the MVN cell types in control slices. Δ Firing Rate is the difference in firing rates obtained from the last interspike interval and the first interspike interval. Negative values indicate spike frequency accommodation and positive values indicate spike frequency acceleration. Type A cells do not show a distinctive spike frequency accommodation and acceleration. The majority of the Type B cells show spike frequency accommodation whereas most Type B_{DP} cells show spike frequency acceleration.

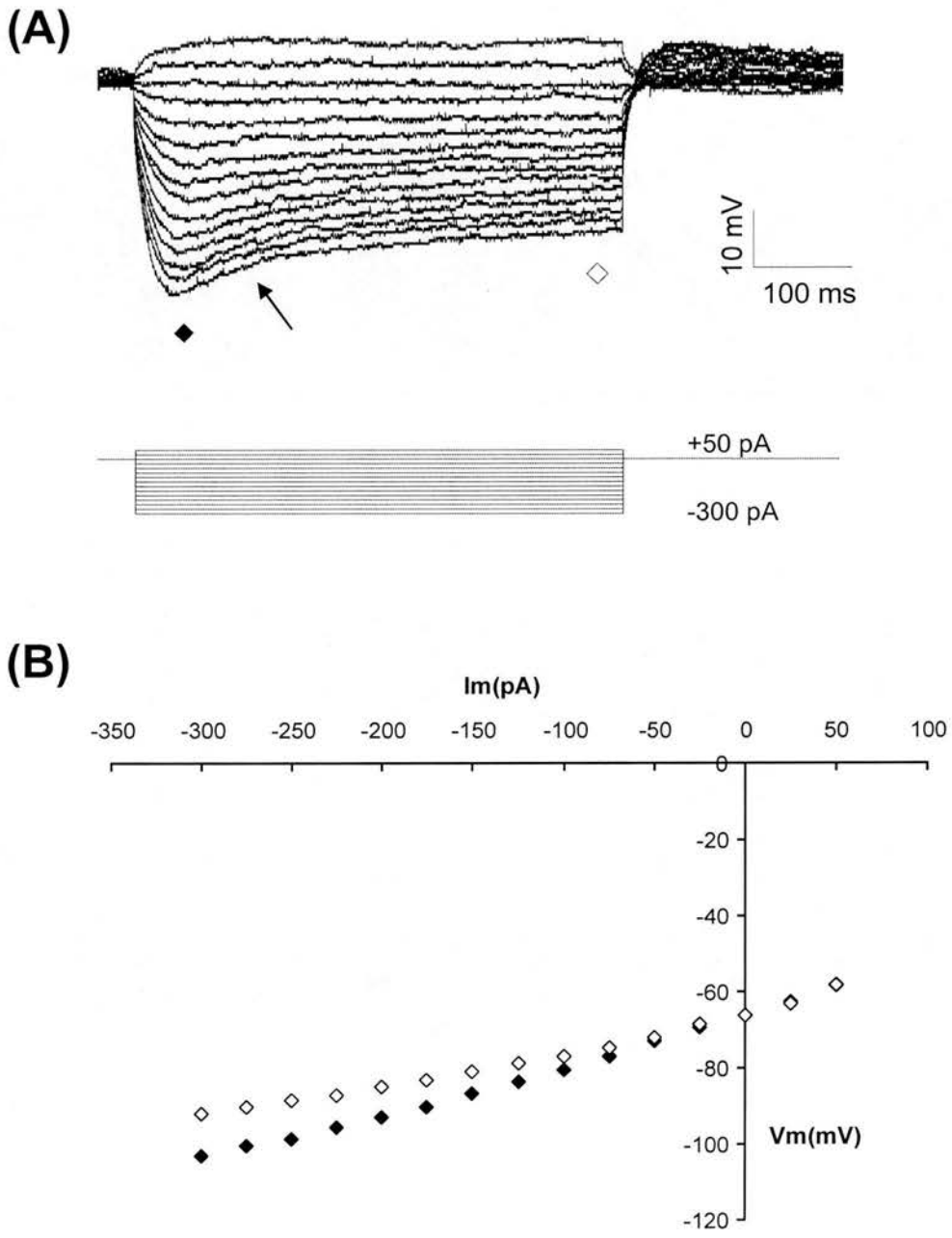

Figure 2.3.1.5. A.The response of a Type A cell to depolarising and hyperpolarising current pulses. Notice the large inward rectification seen as a sag in membrane potential (arrow). **B**. I/V relationship of the cell shown in A, before (filled symbols) and after (open symbols) inward rectification.

Table 2.1. Membrane properties of Type A and Type B MVN neurones in normal rats.

	Firing Rate (spikes/s)	Resting Membrane Potential (mV)	Input Resistance (MΩ)	Spike Amplitude (mV)	Spike Width (ms)	Spike Rise Time (ms)	Spike Fall Time (ms)	First AHP Amplitude (mV)
Type A (n=23)	15.8 ± 1.4	−54.5 ± 0.5	143.3 ± 7.3	55.6 ± 1.6	1.08 ± 0.05	0.53 ± 0.02	0.55 ± 0.03	18.8 ± 1.1
Type B (n=78)	17.0 ± 0.9	−54.0 ± 0.3	136.4 ± 4.9	56.6 ± 0.7	0.81 ± 0.02	0.45 ± 0.01	0.37 ± 0.01	14.0 ± 0.4
P value	NS	NS	NS	NS	< 0.001	< 0.01	< 0.001	< 0.001

Membrane properties of representative MVN cell types are obtained from averaged action potential shapes and are presented as mean ± SEM.
NS, not significant. P values are obtained using Student's t-test.

Type B MVN neurones

A total of 78 MVN cells out of 101 (77%) recorded in the normal rat brainstem slices were classified as Type B cells, according to their action potential shapes. Type B cells are easily distinguished from Type A cells with their AHP profiles. The early fast AHP is followed by a delayed slow AHP in Type B cells (Figure 2.3.1.1B). Another significant difference between Type B and Type A cells is the duration of the action potentials. For Type B cells, the average spike width measured at the threshold was 0.81 ± 0.02 ms, which is significantly shorter than spike width of Type A cells. All Type B cells were spontaneously active and had resting firing rates ranging from 4 to 34 spikes/s with a mean value of 17.0 ± 0.9 spikes/s. The input resistance (136.4 ± 4.9 MΩ) and resting membrane potentials (-54.0 ± 0.3 mV) of Type B cells were similar to Type A cells. The comparison of mean values of the passive membrane properties of Type B and Type A cells, obtained from the averaged action potential shapes, are presented in Table 2.1.

The dynamic membrane properties of Type B cells were studied by measuring membrane potential responses to depolarising and hyperpolarising current pulses applied from a holding membrane potential below firing threshold. All Type B cells fired repetitively in response to depolarising current pulses. Spike-frequency accommodation over the duration of the current pulse was seen in most Type B cells (Figure 2.3.1.6). The mean Δ Firing Rate of these cells was therefore -3.4 ± 1.0 spikes/s, which is indicative of spike frequency accommodation (Figure 2.3.1.4). The cells fired faster action potentials at the beginning of the pulses and therefore f/I relationships for the first and second interspike intervals were different from the f/I curve of the last interval (Figure 2.3.1.6B). In the majority of Type B cells (62/78 Type B cells, 80%), there was a passive return of the membrane potential to baseline at the termination of the depolarising current injections. Fifteen out of 78 Type B cells, however, showed long lasting depolarising plateau potentials with superimposed spikes at the end of depolarising current pulses, and these cells were named as Type B_{DP} cells. One cell had low threshold spikes (LTS), which were evoked at the termination of hyperpolarizing currents pulses. This cell was named as

Type B_{LTS}. The passive membrane properties and currents underlying LTS-like spikes and plateau potentials in Type B_{LTS} and B_{DP} cells were further investigated and were presented below.

Type B_{DP} cells

Nineteen percent of the Type B neurones recorded in MVN slices prepared from normal rats were identified as Type B_{DP} cells according to the their responses to depolarising current pulses. In these cells, positive current injections delivered from a holding membrane potential below firing threshold elicited long lasting depolarising plateau potentials, which were evident as a delay to the baseline at the end of the current pulses (Figure 2.3.1.7, arrows). The cells often continued firing during plateau potentials. The duration and the amplitude of the depolarising plateaus were related to the amplitude of the depolarising current pulse, and often lasted 200-1000 ms beyond the end of current injection (Figure 2.3.1.7). In the majority of these cells (12/15 cells, 80%) there was an acceleration of spike firing over the duration of the depolarising current pulse, in contrast to the spike-frequency accommodation seen in the majority of Type B cells (Figure 2.3.1.4). The mean Δ Firing Rate of these cells was therefore 3.3 $\pm$ 1.3 spikes/s. Spike frequency acceleration was more apparent during small depolarising current injections (Figure 2.3.1.7, upper trace). Spike frequency acceleration can also be seen in f/I relationships of the cells as exemplified in Figure 2.3.1.8B. Type B_{DP} cells were also different from the other type B cells in respect to their firing rates and resting membrane potentials (Table 2.3). The mean spontaneous *in vitro* discharge rate of Type B_{DP} cells in normal slices (21.6 $\pm$ 1.9 spike/s) was significantly higher than that of the remaining normal Type B cells (15.7 $\pm$ 1.0 spikes/s). Type B_{DP} cells had a mean resting membrane potential of -52.9 $\pm$ 0.7 mV, which is more depolarised than normal Type B cells' membrane potentials (-54.3 $\pm$ 0.3 mV).

Type B_{DP} cells were further studied to understand the currents underlying depolarising plateau potentials. For this purpose, in some experiments calcium-substituted aCSF was used to determine if Ca^{2+} was involved as a charge carrier for

plateau potentials. In this modified medium calcium is replaced with cobalt (Ca^{2+}-free/Co^{2+} medium). Some other cells were tested in a medium containing barium instead of calcium. Ba^{2+} is a better charge carrier for Ca^{2+}-channels and goes through the cannels more readily. Type B_{DP} cells were further tested with depolarising current pulses in the presence of nifedipine, a Ca^{2+}-channel blocker specific to L-type channels. Finally a modified medium containing TTX, TEA, 4-AP and apamin was used to block major Na and K currents, leaving calcium currents undisturbed, to determine if the calcium currents are responsible for the plateau potentials.

The effects of Ca^{2+}-free/Co^{2+} aCSF on the plateau potentials were tested in two Type B_{DP} cells in normal slices. In both cells, depolarising plateau potentials and after-discharges evoked by positive current injections were abolished after 5 min in Ca^{2+}-free/Co^{2+} medium (Figure 2.3.1.9).

The plateau responses of two Type B_{DP} cells were recorded in a medium containing barium as a replacement for calcium. In these neurones, plateau potentials were potentiated in Ba^{2+}-containing medium (Figure 2.3.1.10). Depolarising current injections produced plateau potentials with longer duration and bigger amplitude. The after-discharges riding on the plateaus also became more prominent when recorded in Ba^{2+}-containing medium (Figure 2.3.1.10B).

In 6 Type B_{DP} cells the effects of 10 μM nifedipine on the plateau potentials was tested. In all cells tested, application of nifedipine into the bath abolished the depolarising plateaus and accompanying after-discharges (Figure 2.3.1.11). The effect was evident within 5 minutes of drug application.

In one cell, when major sodium and potassium currents were blocked with a modified medium containing TTX, TEA, 4-AP and apamin, the depolarising plateau potentials were still observed while fast sodium action potentials were abolished (Figure 2.3.1.12). The plateaus were larger in amplitude presumably because of the blockade of potassium currents.

Type B_{LTS} Cells

One cell in control slices expressed low-threshold spikes (LTS) when tested with hyperpolarizing current pulses delivered from a hyperpolarised holding potential. Low-threshold spikes with superimposed action potentials were triggered at the termination of the hyperpolarizing current injections (arrows in Figure 2.3.1.13A). The hyperpolarizing current pulses with small amplitude were not enough to trigger LTS. The low threshold spikes could be revealed better when the fast action potentials were blocked with 1 μM TTX applied to the bathing medium (arrows in Figure 2.3.1.13B). Since there was only one cell with low threshold spikes among control, more cells from post-UL slices were used to study its underlying ionic currents (See section 2.3.2).

In four Type B_{LTS} cells recorded in post-UL brainstem slices, the effect of T-type Ca^{2+} channel blocker blocker Ni^{2+} was tested. In three cells, application of 1 mM Ni^{2+} to the bathing medium abolished the low-threshold spikes evoked after hyperpolarizing current pulses (Figure 2.3.1.14B) while there was not any effect in one cell. In another Type B_{LTS} cell, Ca^{2+}-free/Co^{2+} medium was used to further investigate the involvement of Ca^{2+} as a charge carrier in low threshold spikes. After 10 min in Ca^{2+}-free/Co^{2+} medium, low threshold spikes were abolished (Figure 2.3.1.15B).

Figure 2.3.1.6. The responses of a Type B MVN neurone to depolarising current pulses of increasing amplitude.

A. The cell displayed faster firing at the beginning of the current injections and showed spike frequency accommodation.

B. Linear f/I curves of the same Type B cell at different interspike intervals. Note the presence of a secondary range for the first and second intervals.

The holding membrane potential of the cell was -70 mV.

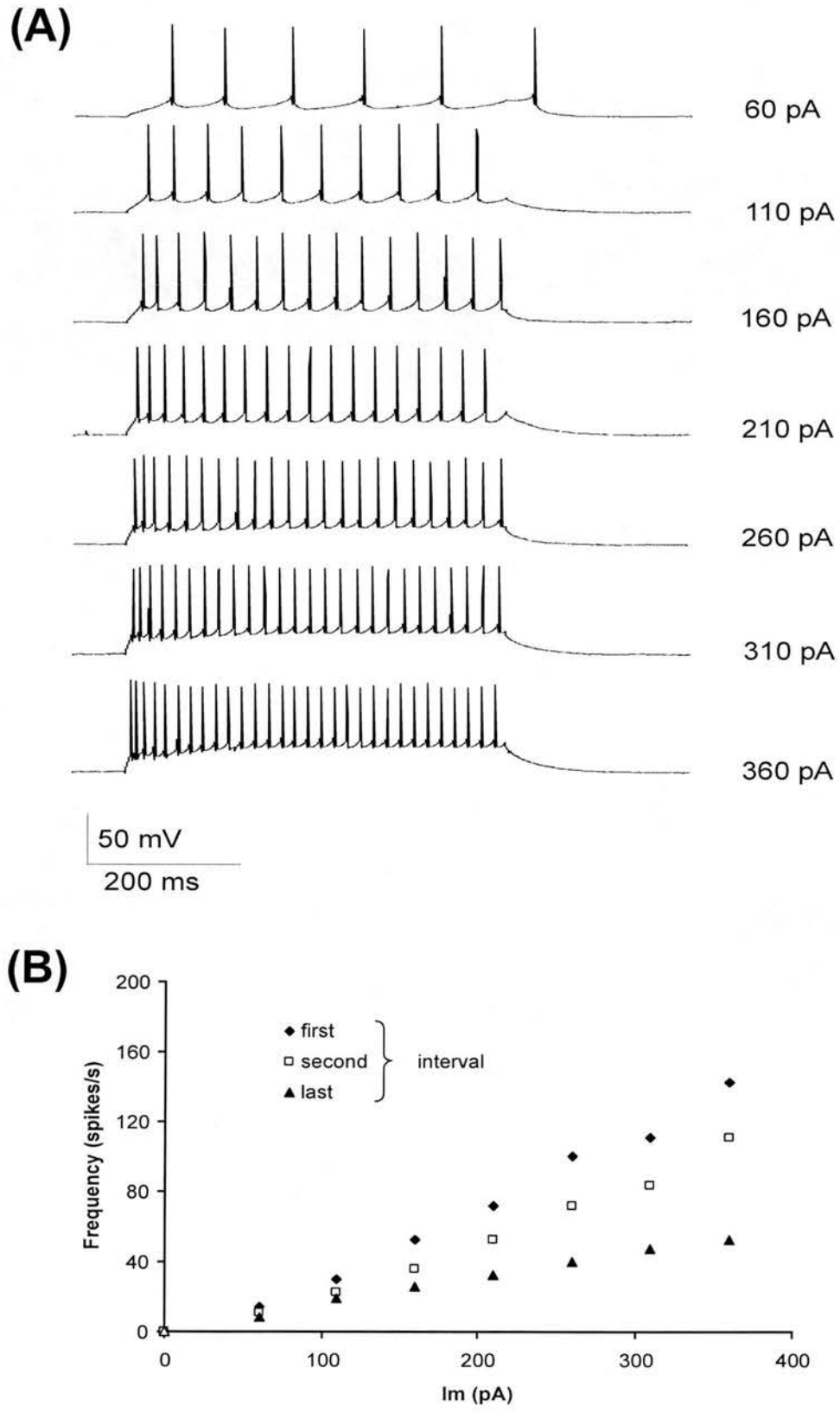

Figure 2.3.1.6. The responses of a Type B MVN neurone to depolarising current pulses of increasing amplitude.

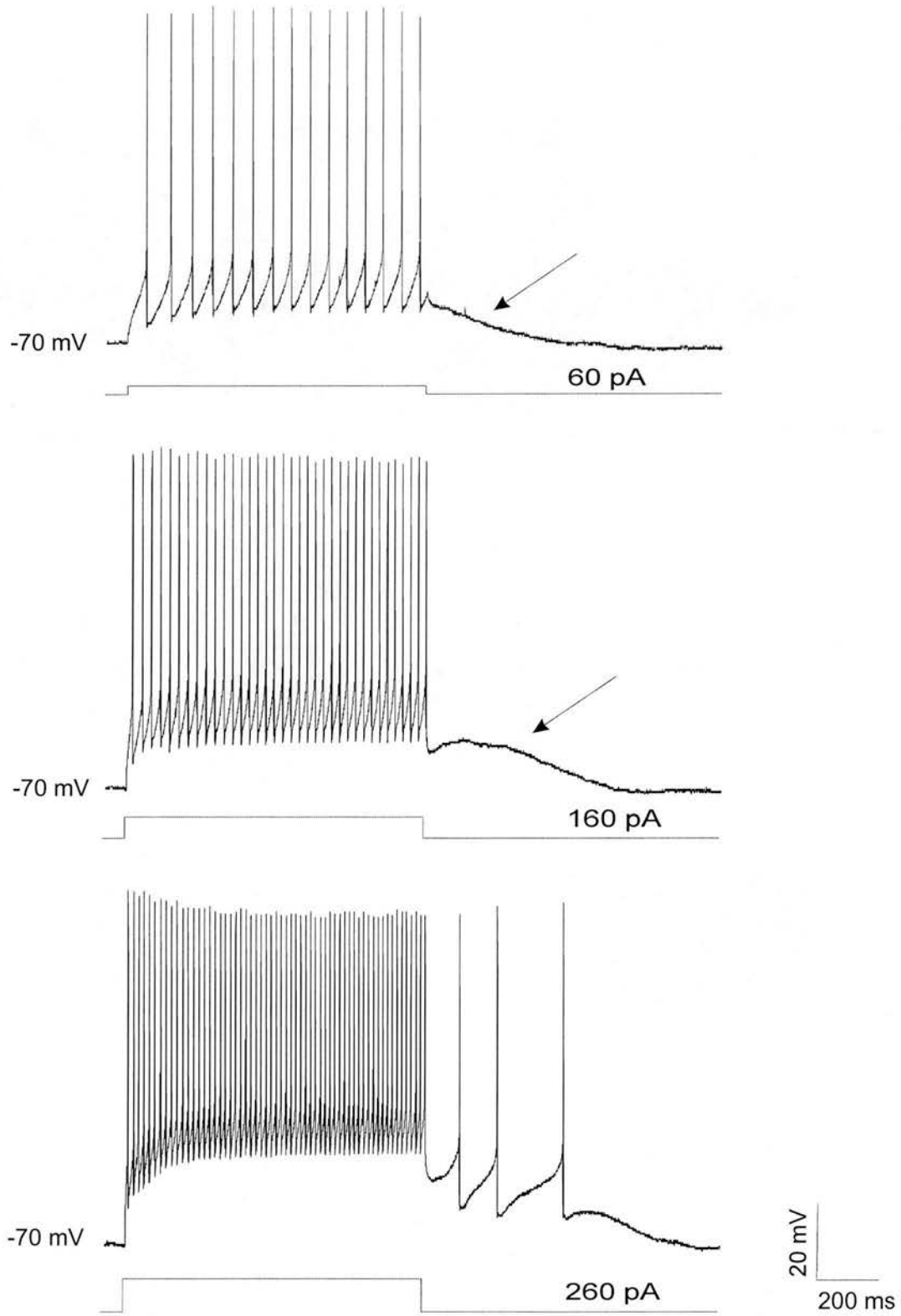

Figure 2.3.1.7. An example of a Type B_{DP} MVN neurone. The arrows indicate depolarising plateau potentials. Bigger positive current pulses evoke bigger and longer plateaus. On the lower trace, action potentials are riding on a depolarising plateau. The spike frequency acceleration is evident in the upper trace.

Figure 2.3.1.8. The responses of a Type B_{DP} cell to depolarising current pulses of increasing amplitude.

A. The cell displayed spike frequency acceleration over the duration of the pulses and showed long lasting depolarising plateaus at the end of current injections (arrows). Plateau potentials were more apparent with bigger current pulses.

B. Linear f/I curves of the same type B cell at different interspike intervals. Note that there is always higher frequency for last interspike intervals compared to first and second intervals.

The holding membrane potential of the cell was -70 mV.

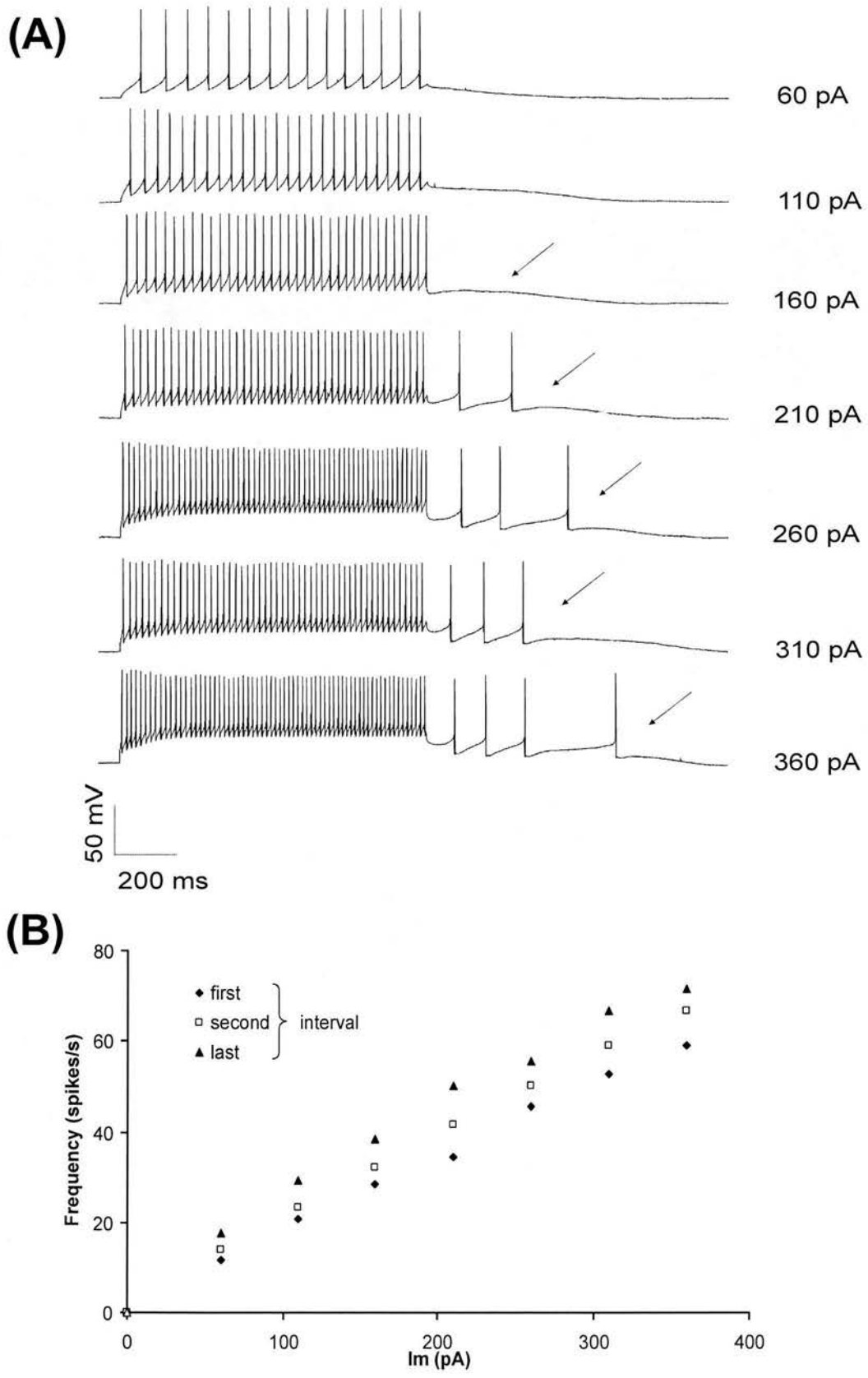

Figure 2.3.1.8. The responses of a Type B_{DP}cell to depolarising current pulses of increasing amplitude.

Figure 2.3.1.9. Effect of Ca^{2+}-free/Co^{2+} medium on the plateau potentials in a Type B_{DP} cell.

A. Injection of positive current pulses of increasing amplitude produced depolarising plateau potentials with after-discharges. Note the spike frequency acceleration on the upper trace.

B. After 5 min in Ca^{2+}-free/Co^{2+} medium, plateau responses of the membrane potential and after-discharges were abolished.

Current injections, (pA), 110, 210, 310.

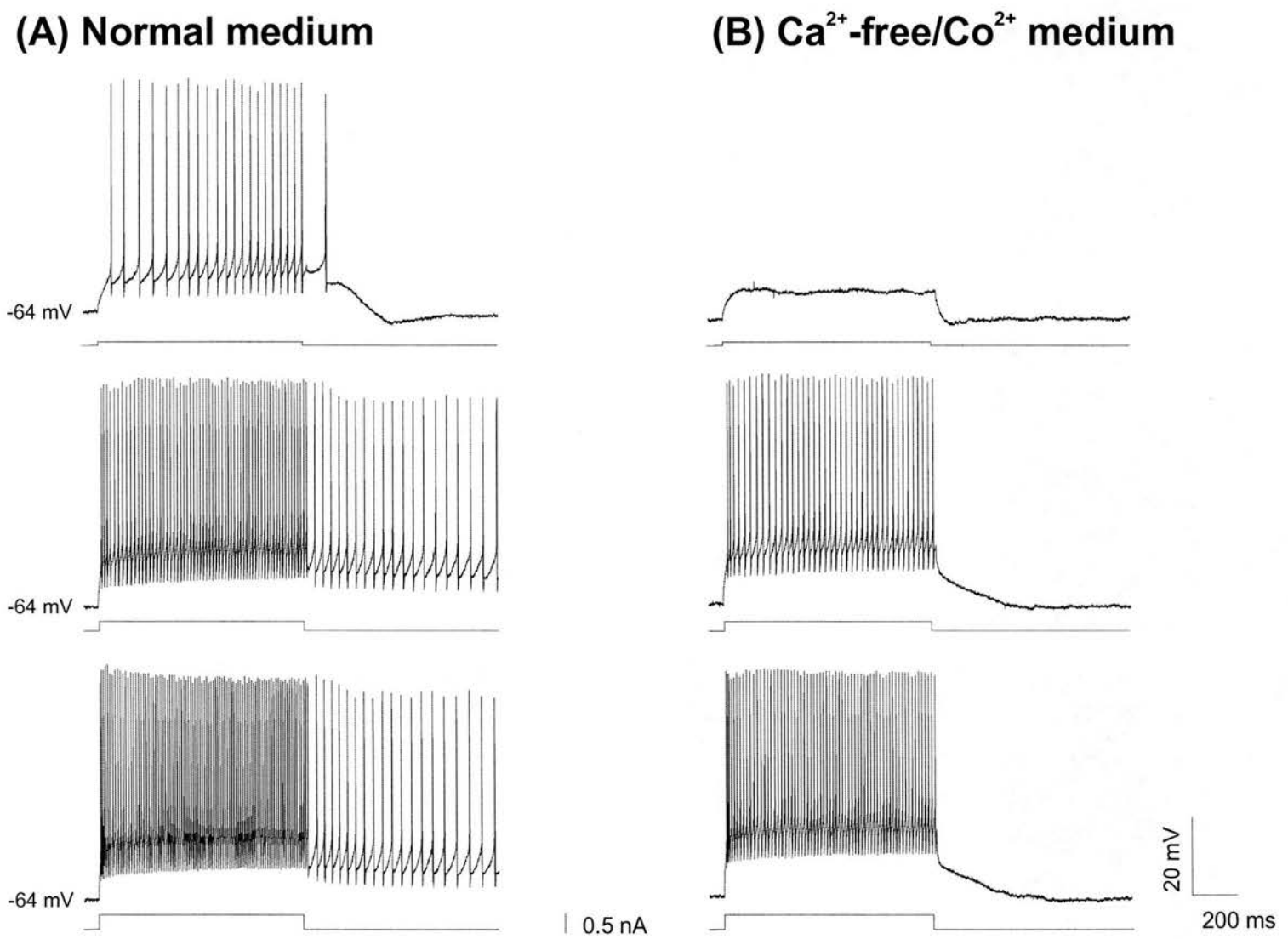

Figure 2.3.1.9. Effect of Ca^{2+}-free/Co^{2+} medium on the plateau potentials in a type B_{DP} cell.

Figure 2.3.1.10. Effect of Ba^{2+}-containing medium on the plateau potentials in a Type B_{DP} cell.

A. Injection of positive current pulses of increasing amplitude produced depolarising plateau potentials with after-discharges.

Current injections, (pA), 260, 310, 360.

B. After 5 min in Ba^{2+}-containing medium, even smaller positive current pulses produced plateau responses of longer duration and larger amplitude with more after-discharges.

Current injections, (pA), 110, 160, 210.

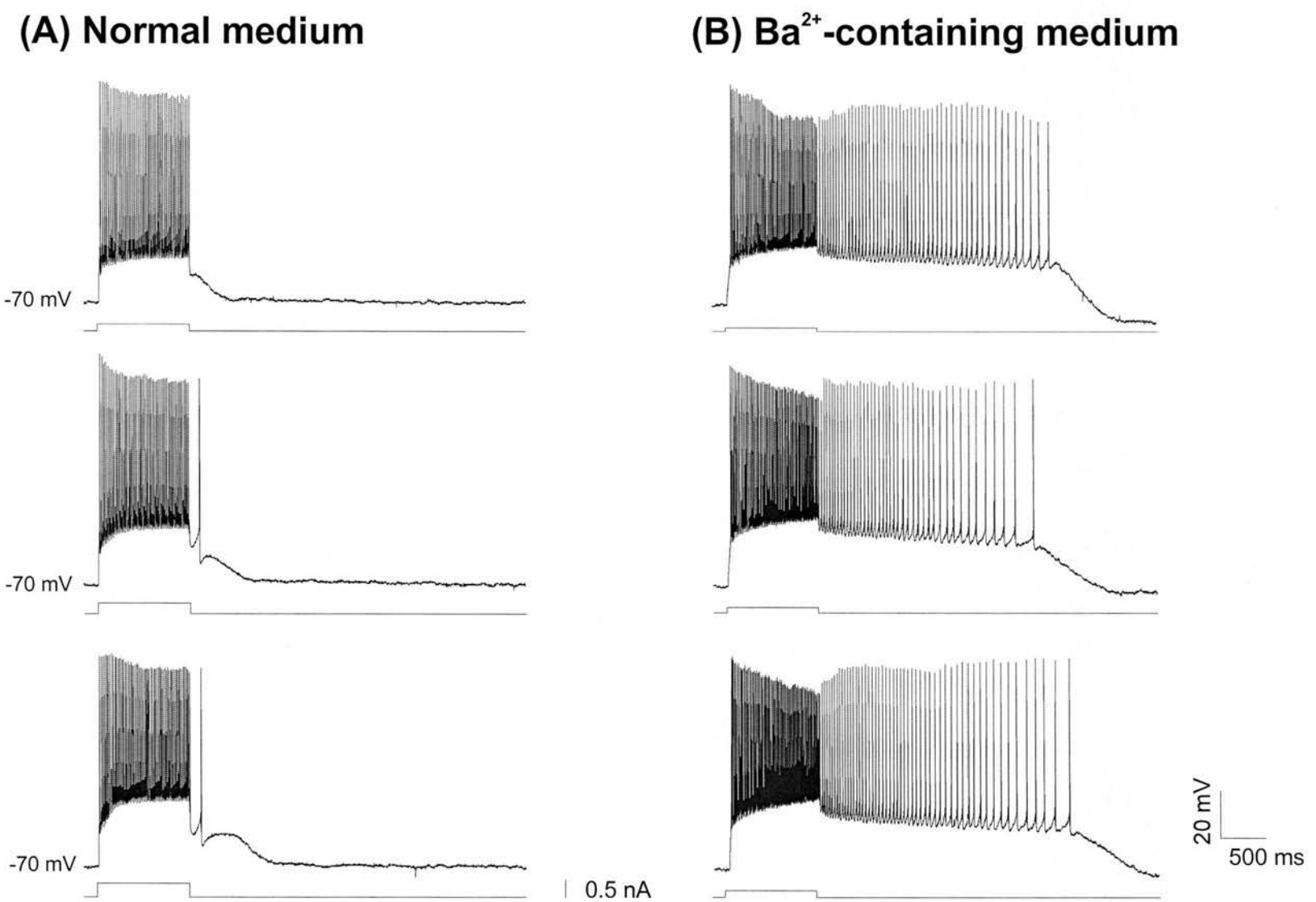

Figure 2.3.1.10. Effect of Ba^{2+}-containing medium on the plateau potentials in a Type B_{DP}.

Figure 2.3.1.11. Effect of L-type Ca-channel blocker nifedipine on plateau potentials in a Type B_{DP} cell.

A. Injection of positive current pulses of increasing amplitude produced depolarising plateau potentials with after-discharges.

B. After 5 min in 10 mM nifedipine applied into the bathing medium, plateau responses of the membrane potential and after-discharges were abolished.

Current injections, (pA), 110, 210, 310.

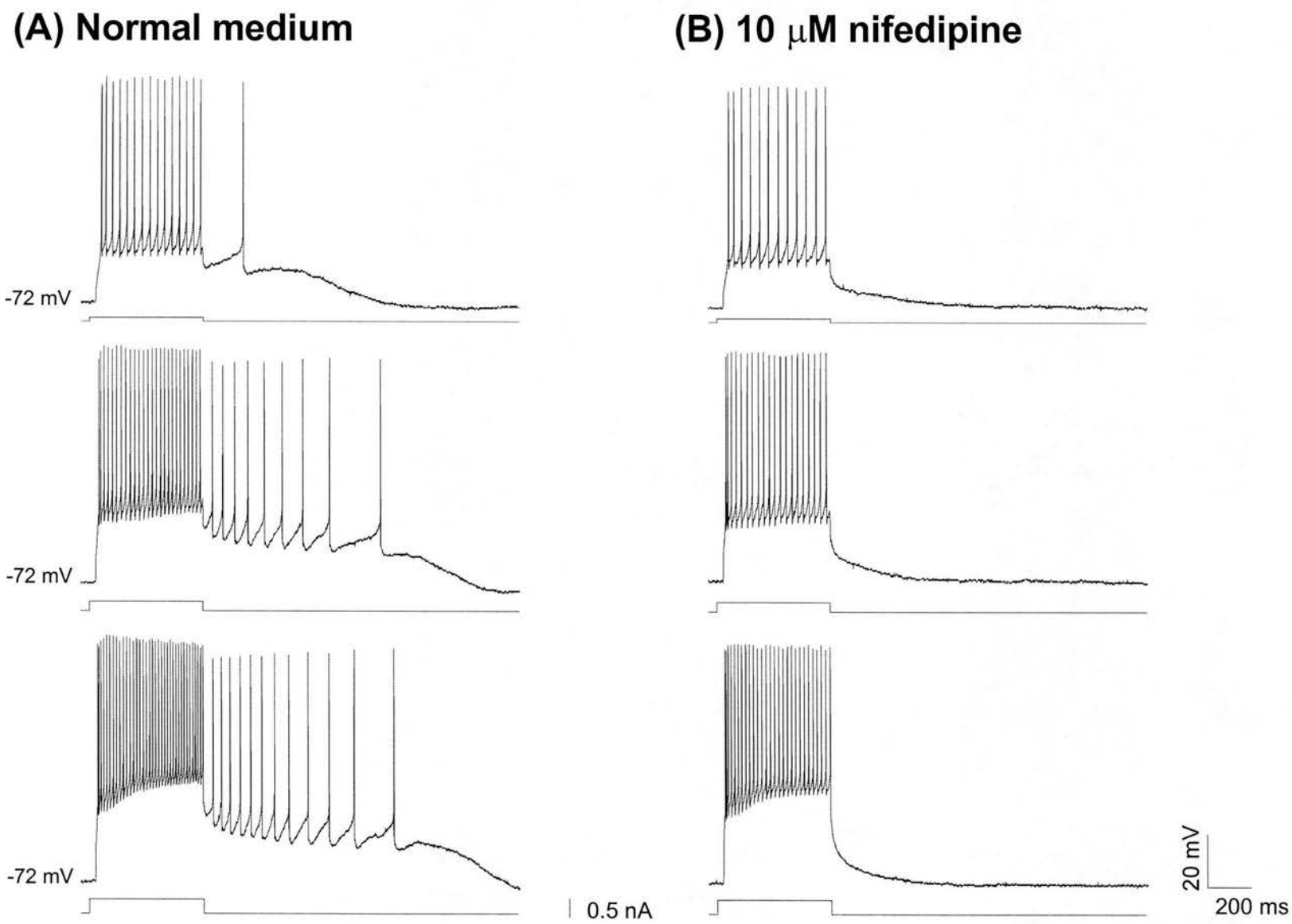

Figure 2.3.1.11. Effect of L-type Ca-channel blocker nifedipine on the plateau potentials in a type B-DP

Figure 2.3.1.12. Effect of the medium containing TTX, TEA, 4-AP and apamin on plateau potentials in a Type B_{DP} cell.

A. Injection of positive current pulses of increasing amplitude produced depolarising plateau potentials with after-discharges (arrows).

B. After 10 min in the medium containing TTX (1μM), TEA (10 mM), 4-AP (0.5 mM) and apamin (0.1 μM), action potentials were abolished but plateau responses of the membrane potential became even stronger (arrows).

Current injections, (pA), 110, 210, 310.

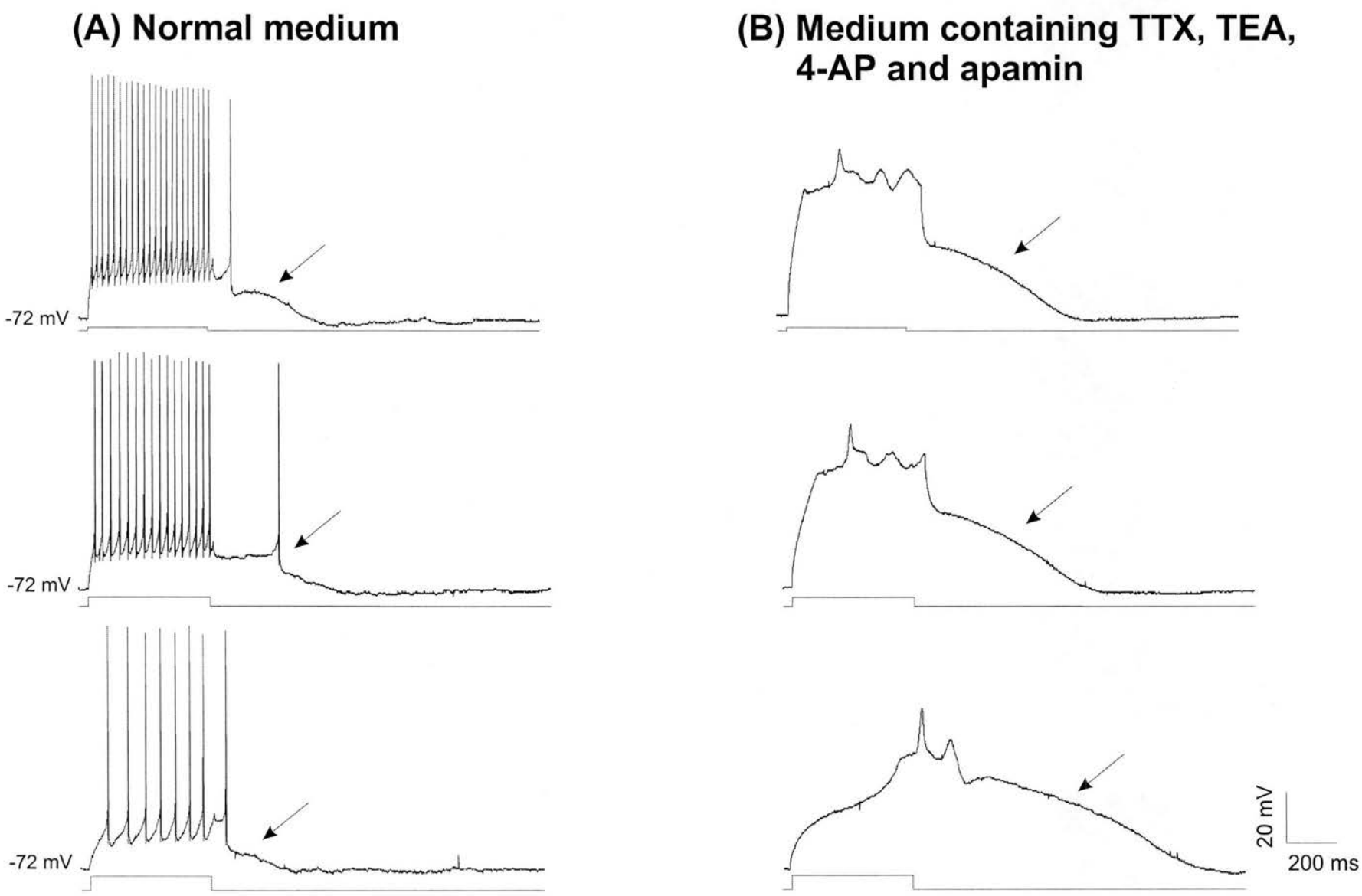

Figure 2.3.1.12. Effect of the medium containing TTX, TEA, 4-AP and apamin on the plateau potentials in a type B-DP cell.

Figure 2.3.1.13. Low threshold spikes in a Type B_{LTS} cell.

A. Low-threshold spikes with superimposed action potentials (arrows) were elicited after Injections of negative current pulses.
B. After 5 min in 1 mM TTX-containing medium, fast action potentials were blocked, leaving TTX-resistant low-threshold spikes (arrows).
The holding membrane potential of the cell is -66 mV.
Current injections, (pA), 50, 0, -50, -150, -225.

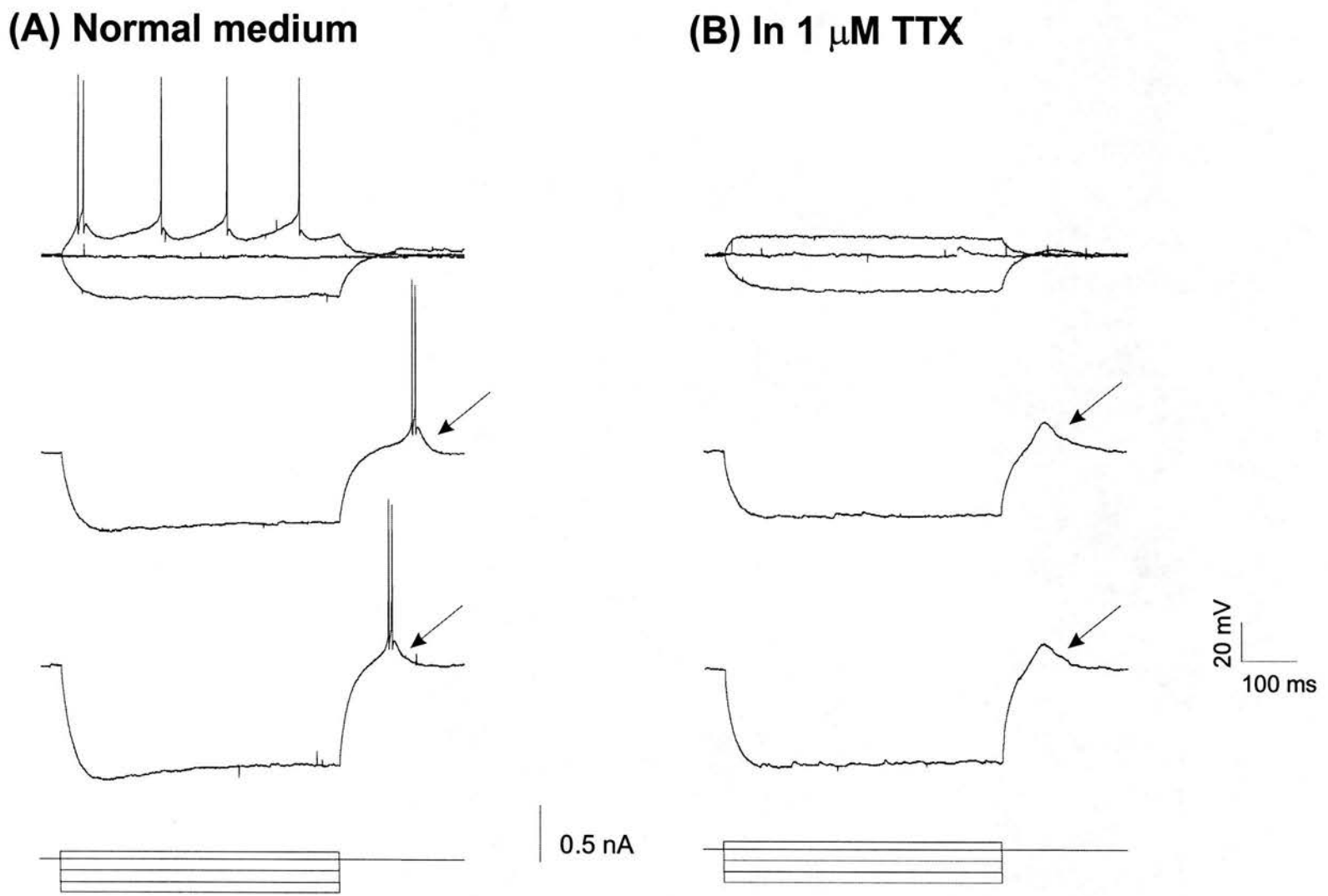

Figure 2.3.1.13. Low threshold spikes in a Type B_{LTS} cell.

Figure 2.3.1.14. Blockade of low threshold spikes with Ni^{2+} in a Type B_{LTS} cell

A. Low-threshold spikes with superimposed action potentials (arrows) were elicited after injections of negative current pulses.

B. After 5 min in 1 mM Ni^{2+}-containing medium, low-threshold spikes were abolished.

The holding membrane potential of the cell is -66 mV.

Current injections, (pA), 25, 0, -25, -75, -125.

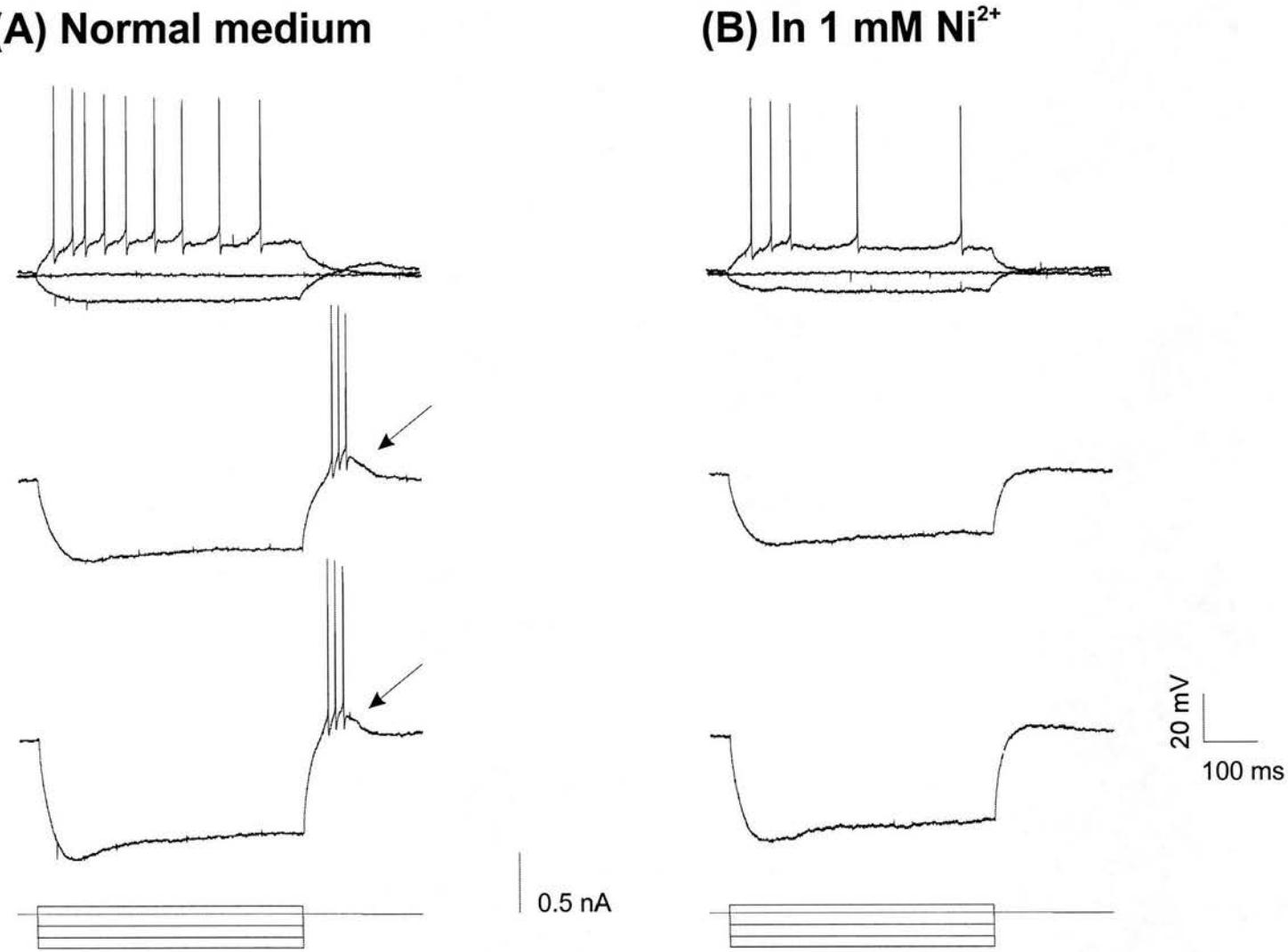

Figure 2.3.1.14. Blockade of low threshold spikes with Ni^{2+} in a Type B_{LTS} cell.

Figure 2.3.1.15. Blockade of low threshold spikes in Ca^{2+} -free medium in a Type B_{LTS} cell.

A. Low-threshold spikes with superimposed action potentials (arrows) were elicited after injections of negative current pulses.

B. After 10 min in Ca^{2+}-free/Co^{2+} medium, low-threshold spikes were abolished.

The holding membrane potential of the cell is -60 mV.

Current injections, (pA), 50, -50, -150, -250.

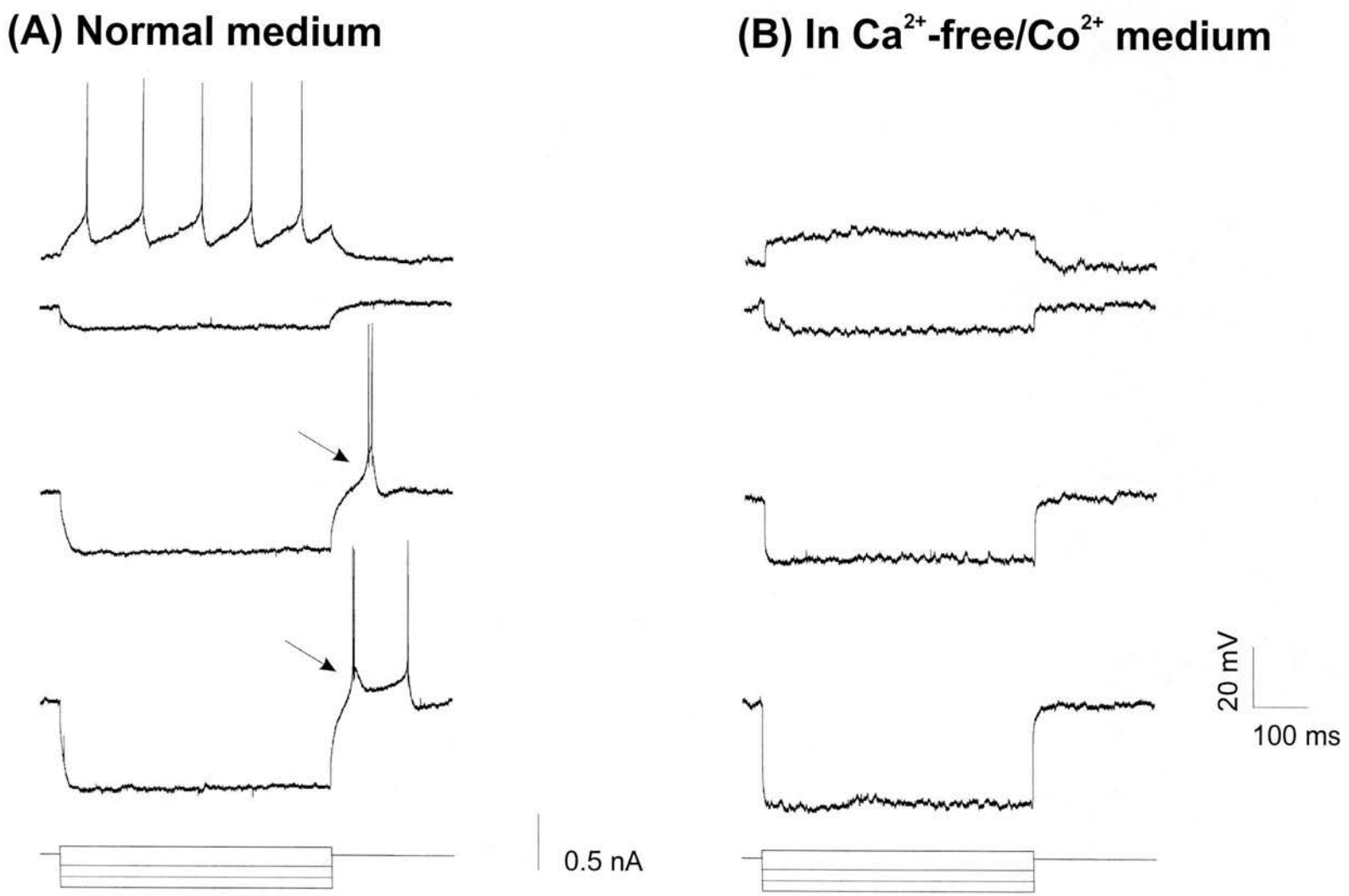

Figure 2.3.1.15. Blockade of low threshold spikes in Ca^{2+} -free medium in a Type B_{LTS} cell.

2.3.2 Intrinsic membrane properties and spike firing characteristics of MVN neurones after UL

Whole cell patch clamp recordings were made from a total of 174 spontaneously active MVN neurones in slices prepared from rats that received unilateral labyrinthectomy. Ninety cells were recorded in slices prepared 1-3d after the UL, and 84 cells were recorded 1w after the UL. Based on their averaged spontaneous action potential shapes, as described previously by Johnston et al. (1994), each recorded cell was classified as either Type A or Type B MVN neurones.

Type A MVN neurones in post-UL slices

Of the 90 MVN cells recorded from 1-3d post-UL group, 20 (22%) were classified as Type A cells. In the 1w post-UL group, 16 cells were identified as type A cells (19%). The mean values of the passive membrane properties of these Type A cells obtained from the averaged action potential shapes and comparisons with the control type A cells are presented in Table 2.2. The relative proportion of type A cells in control and post-UL slices were similar (23% in control vs 22% and 19% in 1-3d and 1w post-UL group). All Type A cells were spontaneously active and the mean discharge rate of Type A cells was not different from that of Type A cells in slices prepared from control rats at either time point post-UL (14.3 ± 1.8 spikes/s in 1-3d post-UL, and 16.1 ± 1.6 spikes/s in 1w post-UL vs 15.8 ± 1.4 spikes/s in control). Type A cells in 1-3d post-UL group had a mean input resistance of 162.3 ± 11.8 MΩ. Although this was higher than control (143.3 ± 7.3 MΩ) it was not significantly different. In 1w post-UL group the mean input resistance of Type A cells (148.4 ± 8.7 MΩ) was similar to control.

When dynamic membrane properties of the Type A cells were analysed, significant changes were found in 1-3d post-UL group. While Type A cells in control slices did not show any distinctive spike frequency accommodation or spike frequency acceleration during the depolarising current pulses, the majority of Type A cells in 1-

3d post-UL slices showed spike frequency accommodation (Figure 2.3.2.1). The mean Δ Firing Rate of these cells was -4.4 ± 1.5 spikes/s, which is significantly different from the control (0.2 ± 1 spikes/s) ($p<0.05$, Student's t-test), and is an indicative of spike frequency accommodation. The type A cells in 1w post-UL slices had a mean Δ Firing Rate of -1.4 ± 1 spikes/s, which is not significantly different from the control.

The averaged value of the slopes (m_{slope}) for the f/I curve of the type A cells in 1-3d post-UL group was significantly higher than the control ($m_{slope} = 0.22 \pm 0.03$ in 1-3d post-UL vs $m_{slope} = 0.14 \pm 0.02$ in control) ($p<0.05$, Student's t-test), while it was not different in 1w post-UL slices ($m_{slope} = 0.13 \pm 0.01$) (Figure 2.3.2.2).

Type B MVN neurones in post-UL slices

In 1-3d post-UL group, 70 cells out of 90 cells were classified as Type B MVN cell, (including Type B_{DP} and Type B_{LTS} cells), according to their action potential shapes. The relative proportions of Type B cells were similar to their proportion in control slices (78% in 1-3d post-UL vs 77% in control). The mean values of the passive membrane properties of these Type B cells obtained from the averaged action potential shapes and comparisons with the control Type B cells are presented in Table 2.3. The mean spontaneous firing rate of all Type B cells was 20.3 ± 1.2 spikes/s, which is significantly higher than that of the control (17.0 ± 0.9 spikes/s) ($p<0.05$, Student's t-test). The higher resting discharge of Type B cells of this group was accompanied by significantly more depolarised resting membrane potentials compared to control (-52.5 ± 0.4 mV vs –54.0 ± 0.3 mV in control, $p<0.001$, Mann-Whitney ranked sum test). The mean input resistance of all Type B cells in 1-3d post-UL group (153.8 ± 5.3 MΩ) was also significantly higher than that of the control (136.4 ± 4.9 MΩ) ($p<0.05$, Mann-Whitney ranked sum test). The mean spike width, spike rise time and spike fall time of these cells were not different from the control, whereas the mean action potential amplitude was significantly higher in 1-3d post-UL group (61.8 ± 0.7 mV vs 56.6 ± 0.7 mV, $p<0.01$, Student's t-test).

In 1w post-UL group, a total of 68 cells out of 84 MVN cells were identified as Type B MVN cells (including Type B_{DP} and Type B_{LTS} cells). The relative proportion of all Type B cells in this group was also similar to their proportion in slices prepared from control rats (81% in 1w post-UL vs 77% in control). The mean spontaneous firing rate of all Type B cells was significantly higher in 1w post-UL slices (22.9 ± 1.1 spikes/s vs 17.0 ± 0.9 spikes/s in control, $p<0.01$, Student's t-test). The Type B cells in this group had more depolarised resting membrane potentials compared to control (–51.1 ± 0.2 mV vs –54.0 ± 0.3 mV in control, $p<0.001$, Mann-Whitney ranked sum test). The rest of the parameters of the Type B cells obtained from averaged action potentials shapes were not different in 1w post-UL slices.

Type B cells were further classified as Type B_{DP} and Type B_{LTS} according to their firing properties. The intrinsic membrane properties and dynamic membrane characteristics of these subpopulations of the MVN cells and the remaining Type B cells in post-UL groups were analysed and compared to control.

Type B_{DP} MVN neurones

Twenty-three MVN cells in 1-3 d post-UL group and 17 cells in 1w post-UL group were identified as Type B_{DP} neurones. The relative proportions of Type B_{DP} cells among all Type B cells were not statistically different from their proportion in control (Table 2.4) (33% in 1-3d post-UL, 25% in 1w post-UL, vs 19% in control). The mean spontaneous discharge rate of Type B_{DP} cells in post-UL slices were not different from the mean discharge rates of Type B_{DP} cells in slices prepared from normal rats (Table 2.3). However they had significantly more depolarised resting membrane potentials both in 1-3d and 1w post-UL groups (–50.2 ± 0.5 mV in 1-3d post-UL, –50.8 ± 0.4 mV in 1w post-UL, vs –52.9 ± 0.7 mV in control, $p<0.05$, Student's t-test). The mean input resistance, the amplitude and duration of action potentials of the Type B_{DP} cells recorded in post-UL slices were not different from control values. Similarly there was not any change in the amplitude of the after-hyperpolarisations in post-UL groups.

The firing characteristics of the Type B_{DP} cells in post-UL groups and in control were also compared. The mean slope of f/I curve was 0.21 ± 0.02 for 1-3 d post-UL group and 0.19 ± 0.02 for 1w post-UL group. When compared to the mean slope of control Type B_{DP} cells (0.19 ± 0.01), these were not significantly different, although it was higher than control in 1-3d post-UL slices (Figure 2.3.2.3).

Similar to Type B_{DP} cells in control slices, most Type B_{DP} cells in 1-3 d post-UL group also showed spike frequency acceleration during the depolarising current pulses (Figure 2.3.2.4). The mean Δ Firing Rate of these cells in 1-3 d post-UL group was 3.4 ± 1.7 spikes/s, which is not significantly different from the control (3.3 ± 1.3 spikes/s) ($p<0.05$, Student's t-test). In 1w post-UL group, however, there were fewer cells that showed spike frequency acceleration (Figure 2.3.2.4). Although more cells in this group showed spike frequency accommodation rather than acceleration, the mean Δ Firing Rate of these cells was not significantly different from the control (−0.8 ± 2.2 spikes/s in 1w post-UL vs 3.3 ± 1.3 spikes/s in control).

Type B_{LTS} MVN neurones

Ten cells in 1-3d post-UL slices and 10 cells in 1w post-UL slices were identified as Type B_{LTS} neurones showing prominent LTS-like rebound potentials at the end of hyperpolarizing current injections (Table 2.4). The relative number of Type BLTS cells found in 1-3d and 1w post-UL slices was significantly higher than in control slices. (14% in 1-3d post-UL, 15% in 1w post-UL vs 1% in control)($P<0.05$, χ^2 test). These figures do not include the cells that showed both depolarising plateaus and LTS-like responses. One cell in 1-3 d post-UL and 5 cells in 1w post-UL group were identified as having both Type B_{DP} and Type B_{LTS} characteristics (Table 2.4). With these cells included, the relative proportions of the Type B cells with LTS-like responses in post-UL groups become even higher compared to control (16% in 1-3d post-UL, 22% in 1w-post-UL group).

Since there were only one Type B_{LTS} neurone in the control group, it was not possible to compare intrinsic membrane properties and firing characteristics of these cells in control and post-UL slices. It was also not possible to compare a single Type B_{LTS} cell with the rest of the Type B cells in the control group. Therefore intrinsic membrane properties and firing characteristics of Type B_{LTS} cells recorded in post-UL slices were compared with the Type B cells that did not show either depolarising potentials or LTS-like responses in control slices.

The mean spontaneous discharge rate of Type B_{LTS} cells in post-UL groups was not significantly different from the mean discharge rate of control Type B cells (12.3 ± 2.6 spikes/s in 1-3d post-UL, 13.5 ± 2.1 spikes/s in 1w post-UL, vs 15.7 ± 1.0 spikes/s in control) (Table 2.3). However the Type B_{LTS} cells in 1-3 d post-UL slices had significantly greater mean input resistance and spike amplitude compared to control Type B cells ($p<0.05$, Student's t-test). In 1w post-UL group, while these parameters were not different from the control, the mean resting membrane potentials of these cells were significantly more depolarised (–51.6 ± 0.7 mV vs –54.3 ± 0.3 mV in control, $p<0.05$, Student's t-test)(Table 2.3).

The gain of the Type B_{LTS} cells recorded in post-UL slices were higher than the gain of Type B cells in control slices as revealed by a significantly higher mean slope of the f/I curve for Type B_{LTS} cells (m_{slope} = 0.29 ± 0.05 in post-UL group vs m_{slope} = 0.17 ± 0.01 in control, $p<0.05$, Student's t-test) (Figure 2.3.2.5).

Type B_{LTS} cells showed initial bursts at the onset of depolarising current injections, and spike frequency accommodation over the duration of the pulses. The mean Δ Firing Rate of these cells was therefore significantly higher compared to control Type B cells (–108.8 ± 34.3 spikes/s in post-UL group vs –3.4 ± 1.0 spikes/s in control Type B cells)(Figure 2.3.2.6).

Remaining Type B MVN neurones

When Type B cells that did not show either depolarising plateaus or low-threshold spikes were analysed separately, significant changes were found in mean spontaneous firing rate and resting membrane potentials of these cells in post-UL slices compared to Type B cells in control slices (Table 2.3). The mean spontaneous firing rate of these remaining Type B cells was 19.0 ± 1.4 spikes/s in 1-3d post-UL slices and 23.4 ± 1.4 spikes/s in 1w post-UL slices. While the mean resting membrane potential in 1-3 d post-UL group (−53.2 ± 0.4 mV) was similar to control (−54.3 ± 0.3 mV, it was significantly more depolarised in 1w post-UL group (−51.3 ± 0.3 mV) (Table 2.3). The mean spike amplitude of the remaining Type B cells in 1-3d post-UL slices (63.8 ± 5.4 mV) was significantly greater compared to control (56.6 ± 0.8 mV) ($p<0.001$, Student's t-test), but it was not different from control in 1w post-UL group (56.9 ± 0.7 mV).

The gain and the frequency accommodation/acceleration properties of the remaining Type B cells in post-UL groups were not different from the control Type B cells. There were not significant differences in the slopes of f/I relationships in control and post-UL slices (Figure 2.3.2.7). Similar to the control, most of the remaining Type B cells in post-UL groups showed spike frequency accommodation upon depolarising current injections and the mean Δ Firing Rate of these cells were not different from the control (-7.5 ± 2.1 spikes/s in 1-3d post-UL, -6.1 ± 1.7 spikes/s in 1w post-UL, and -3.4 ± 1.0 spikes/s in control)(Figure 2.3.2.8).

Table 2.2. Membrane properties of Type A MVN neurones in normal and post-UL slices.

	Number of cells recorded	Firing Rate (spikes/s)	Resting Membrane Potential (mV)	Input Resistance (MΩ)	Spike Amplitude (mV)	Spike Width (ms)	Spike Rise Time (ms)	Spike Fall Time (ms)	AHP Amplitude (mV)
Control	23	15.8 ± 1.4	−54.5 ± 0.5	143.3 ± 7.3	55.6 ± 1.6	1.08 ± 0.05	0.53 ± 0.02	0.55 ± 0.03	18.8 ± 1.1
1-3d post-UL	20	14.3 ± 1.8	−53.3 ± 0.4	162.3 ± 11.8	60.9 ± 2.3	1.07 ± 0.05	0.54 ± 0.03	0.53 ± 0.03	18.1 ± 0.6
1w post-UL	16	16.1 ± 1.6	−53.1 ± 0.3	148.4 ± 8.7	60.9 ± 2.3	1.05 ± 0.05	0.54 ± 0.03	0.52 ± 0.02	17.5 ± 0.4

Membrane properties of the MVN cells are obtained from averaged action potential shapes and are presented as mean ± SEM.

Table 2.3. Membrane properties of Type B MVN neurones in normal and post-UL slices.

	n	Firing Rate (spikes/s)	Resting Membrane Potential (mV)	Input Resistance (MΩ)	Spike Amplitude (mV)	Spike Width (ms)	Spike Rise Time (ms)	Spike Fall Time (ms)	AHP1 Amplitude (mV)	AHP2 Amplitude (mV
Control										
Type B (all)	78	17.0 ± 0.9	−54.0 ± 0.3	136.4 ± 4.90	56.6 ± 0.7	0.81 ± 0.02	0.45 ± 0.01	0.37 ± 0.01	14.0 ± 0.4	13.9 ± 0.5
Type B_{DP}	15	21.6 ± 1.9	−52.9 ± 0.7	150.7 ± 11.6	56.4 ± 1.4	0.90 ± 0.06	0.49 ± 0.03	0.41 ± 0.03	15.9 ± 1.0	13.0 ± 1.0
Type B_{LTS}	1	24	−53	120	55.3	0.5	0.3	0.23	14.8	
Type B	62	15.7 ± 1.0	−54.3 ± 0.3	133.2 ± 5.5	56.6 ± 0.8	0.80 ± 0.03	0.44 ± 0.02	0.36 ± 0.01	13.4 ± 0.5	14.0 ± 0.6
1-3d post-UL										
Type B (all)	70	**20.3 ± 1.2***	**−52.5 ± 0.4**$^{\#\#}$	**153.8 ± 5.3**$^{\#}$	**61.8 ± 0.7****	0.76 ± 0.03	0.42 ± 0.02	0.34 ± 0.01	14.9 ± 0.5	13.5 ± 0.6
Type B_{DP}	23	24.4 ± 2.1	**−50.2 ± 0.5****	157.6 ± 9.3	58.5 ± 5.4	0.79 ± 0.16	0.43 ± 0.12	0.36 ± 0.02	16.9 ± 4.3	14.3 ± 1.6
Type B_{LTS}	10	12.3 ± 2.6	−55.4 ± 1.3	175.0 ± 16.2	62.0 ± 5.7	0.66 ± 0.17	0.38 ± 0.12	0.30 ± 0.08	14.4 ± 4.3	12.7 ± 1.2
Type B	36	**19.0 ± 1.4***	−53.2 ± 0.4	144.4 ± 6.9	**63.8 ± 5.4****	0.74 ± 0.19	0.41 ± 0.12	0.33 ± 0.02	13.8 ± 4.0	13.5 ± 0.6
1w post-UL										
Type B (all)	68	**22.9 ± 1.1****	**−51.1 ± 0.2**$^{\#\#}$	140.6 ± 7.3	56.5 ± 0.6	0.86 ± 0.03	0.47 ± 0.02	0.38 ± 0.01	14.1 ± 0.4	12.9 ± 0.4
Type B_{DP}	17	25.5 ± 2.2	**−50.8 ± 0.4***	150.3 ± 11.7	56.1 ± 1.1	0.86 ± 0.04	0.48 ± 0.02	0.39 ± 0.02	15.9 ± 0.9	13.1 ± 1.0
Type B_{LTS}	10	13.5 ± 2.1	−51.6 ± 0.7	161.1 ± 31.6	53.0 ± 2.1	0.78 ± 0.08	0.44 ± 0.05	0.34 ± 0.05	11.0 ± 1.2	9.9 ± 1.1
Type B	36	**23.4 ± 1.4****	**−51.3 ± 0.3**$^{\#\#}$	120.6 ± 5.8	56.9 ± 0.7	0.82 ± 0.04	0.46 ± 0.02	0.36 ± 0.02	13.6 ± 0.5	13.4 ± 0.5

Membrane properties of the MVN cells are obtained from averaged action potential shapes and are presented as mean ± SEM. Values in bold are significantly different compared to control, *P<0.05, **P<0.01, Student's t-test; $^{\#}$P<0.05, $^{\#\#}$P<0.01, Mann-Whitney ranked sum test.

Table 2.4. Numbers and relative proportions of the MVN cell types in control and post-UL slices.

	Control		1-3d post-UL		1w post-UL		χ^2 test
	n	%	n	%	n	%	
Type A	23	23	20	22	16	19	NS
Type B (all)	78	77	70	88	68	81	NS
Type B_{DP}	15	19	23	33	17	25	NS
Type B_{LTS}	1	1	10	14	10	15	P<0.05
Type B_{DP+LTS}	0	0	1	1	5	7	-
Type B	62	80	36	52	36	53	P<0.05

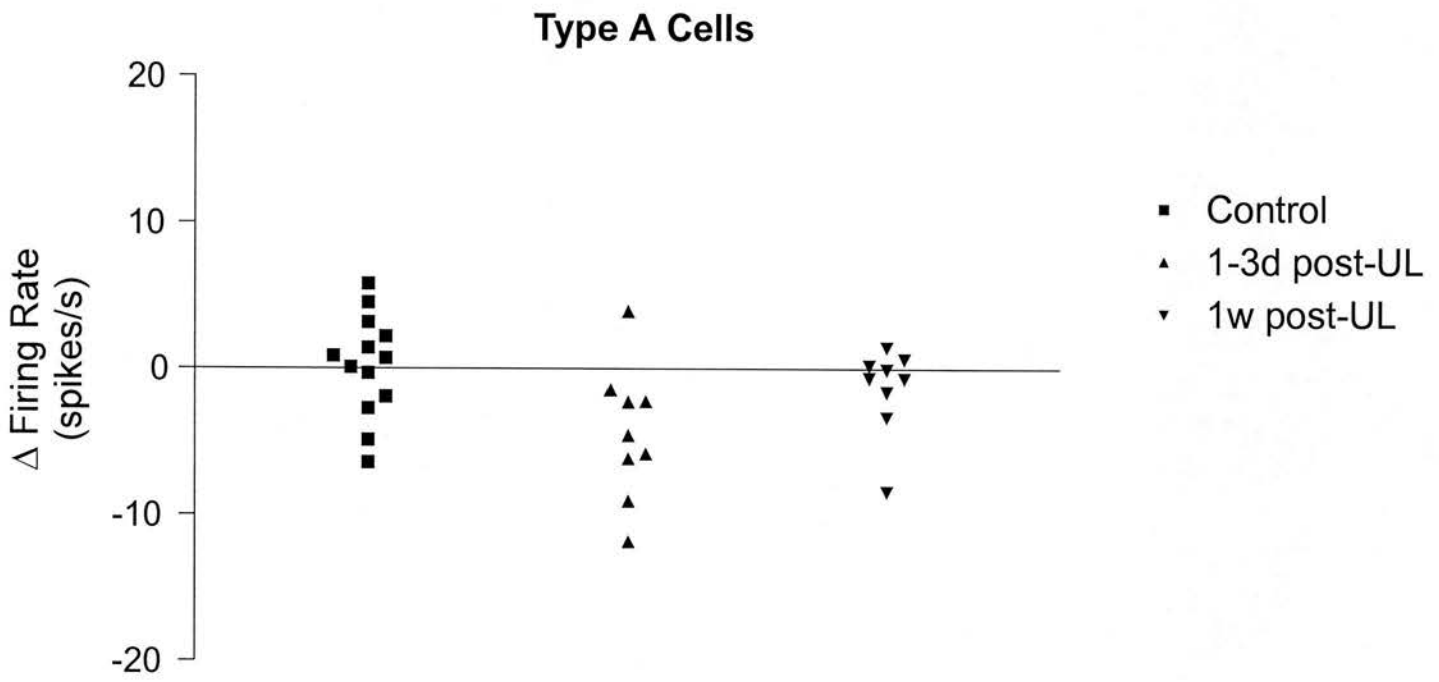

Figure 2.3.2.1. Scatter histogram of the spike frequency acceleration/accommodation indexes of the Type A cells in control and post-UL slices. Δ Firing Rate is the difference in firing rates obtained from the last interspike interval and the first interspike interval. Negative values indicate spike frequency accommodation and positive values indicate spike frequency acceleration. The majority of Type B cells in 1-3d post-UL group show spike frequency accommodation. The mean Δ Firing Rate of the cells in 1-3d post-UL group (-4.4 ± 1.5) is significantly different from the control (0.2 ± 1)($p<0.01$, Student's t-test) while it was similar to control in 1w post-UL group (-1.4 ± 1).

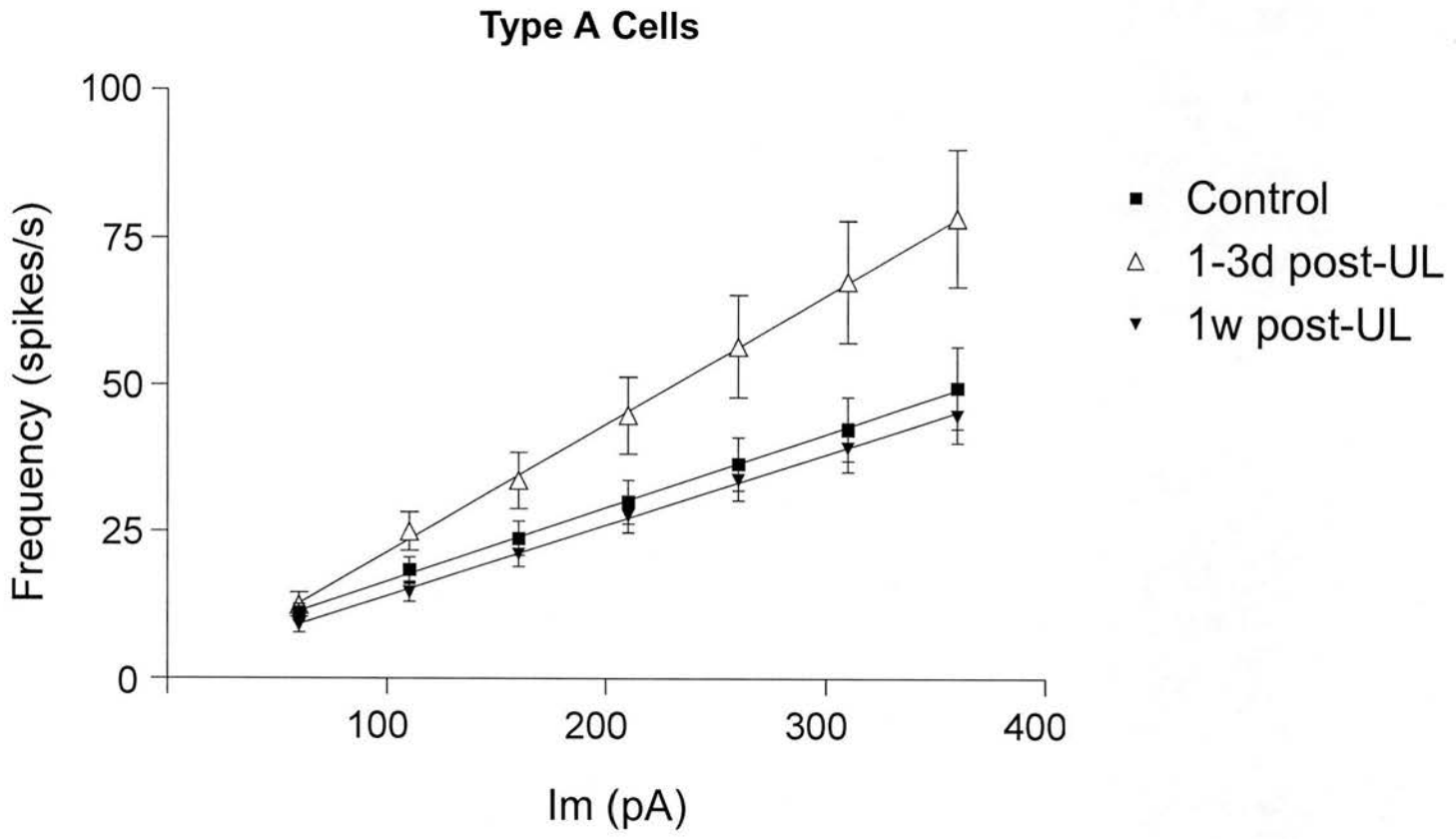

Figure 2.3.2.2. Frequency/current (f/I) relationships of the Type A cells in control and post-UL slices. The mean slope (m_{slope}) of the linear regression lines of the Type A cells in 1-3d post-UL group (m_{slope}= 0.22 ± 0.02 spikes/pA) is significantly different from the control (m_{slope}= 0.14 ± 0.02 spikes/pA)($p<0.01$, Student's t-test). Type A cells in 1w post-UL group has a mean slope of 0.13 ± 0.01 spikes/pA, which is not different from the control.

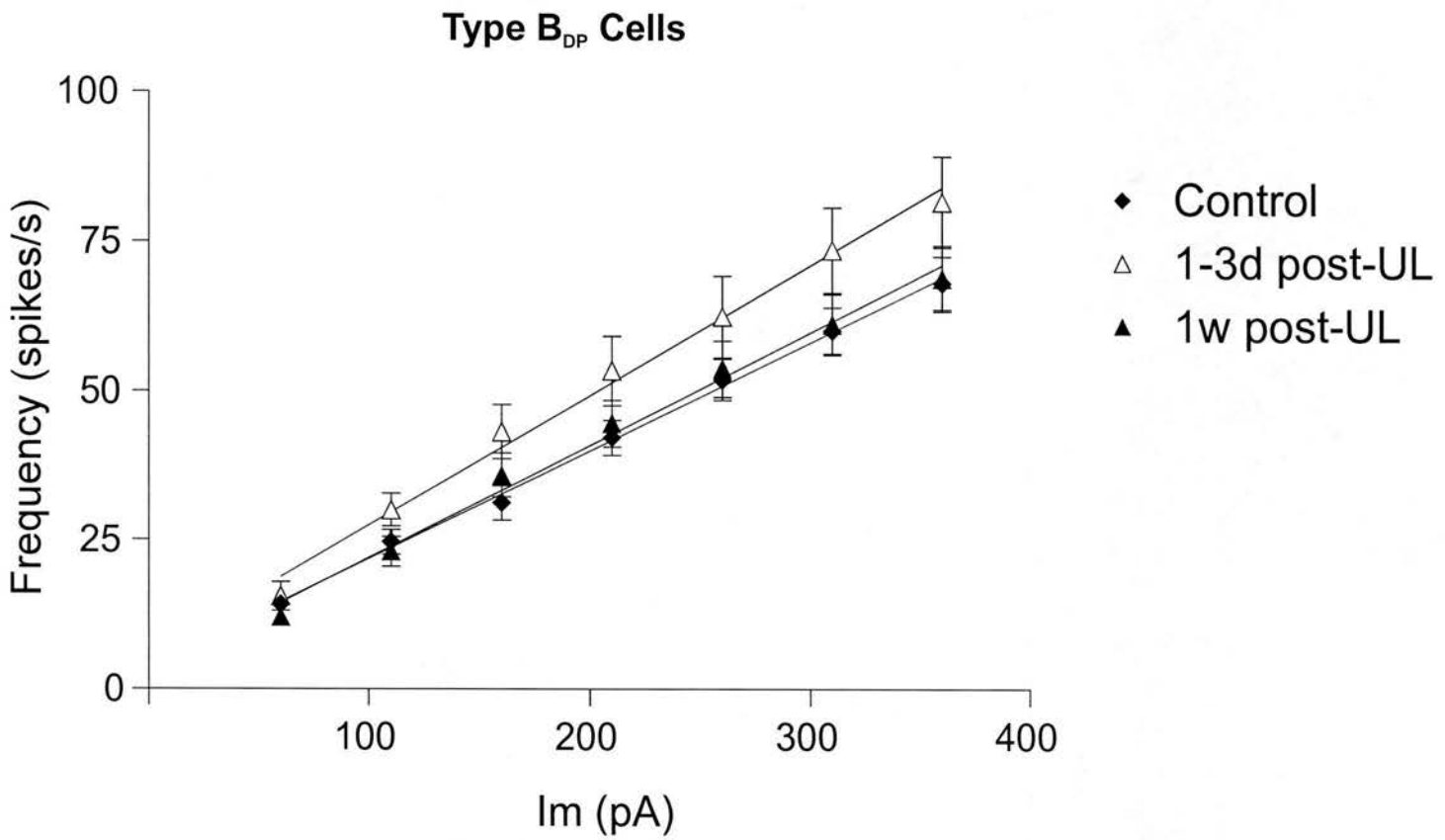

Figure 2.3.2.3. Frequency/current (f/I) relationships of the Type B_{DP} cells in control and post-UL slices. The mean slopes (m_{slope}) of the linear regression lines of the Type B_{DP} cells in control (m_{slope}= 0.20 ± 0.01 spikes/pAd), in 1-3d post-UL group (m_{slope}= 0.21 ± 0.02 spikes/pA), and in 1w post-UL group (m_{slope}= 0.19 ± 0.02 spikes/pA) are not significantly different.

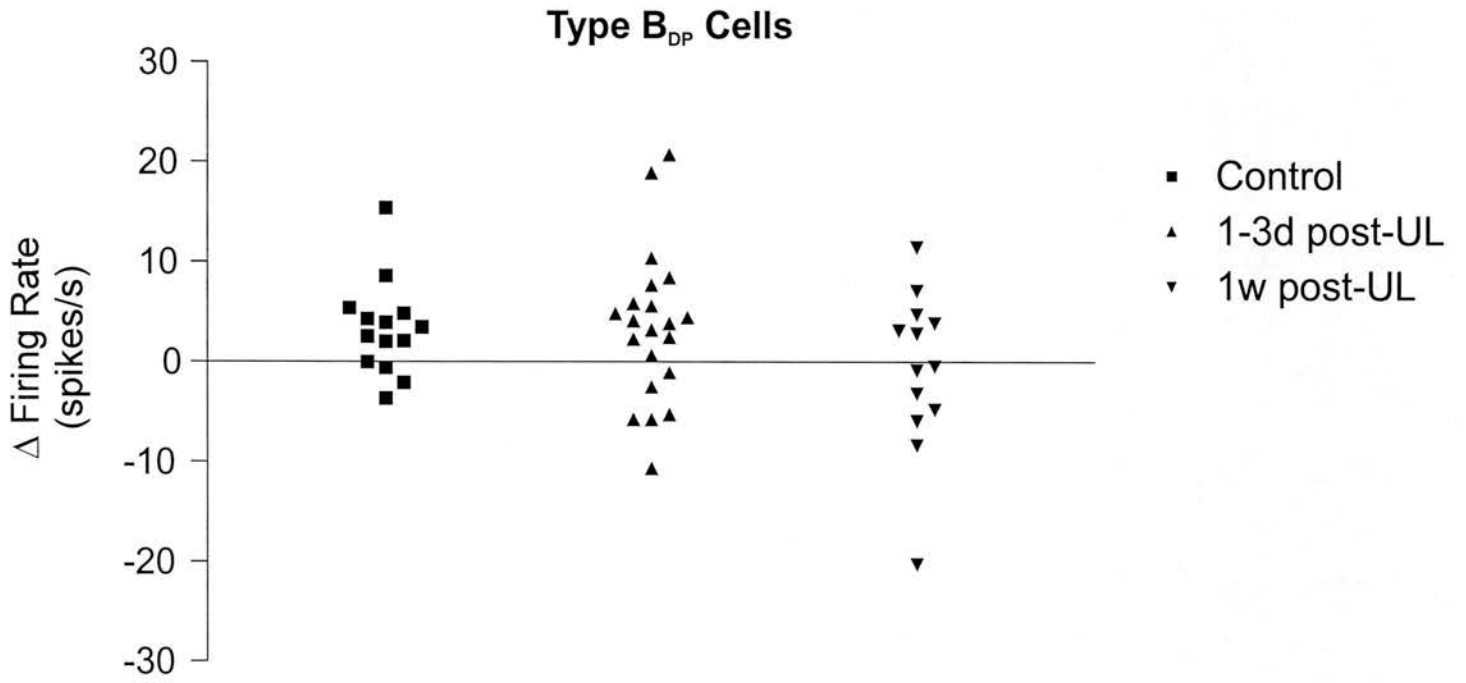

Figure 2.3.2.4. Scatter histogram of the spike frequency acceleration/accommodation indexes of the Type B_{DP} cells in control and post-UL slices. Δ Firing Rate is the difference in firing rates obtained from the last interspike interval and the first interspike interval. Negative values indicate spike frequency accommodation and positive values indicate spike frequency acceleration.

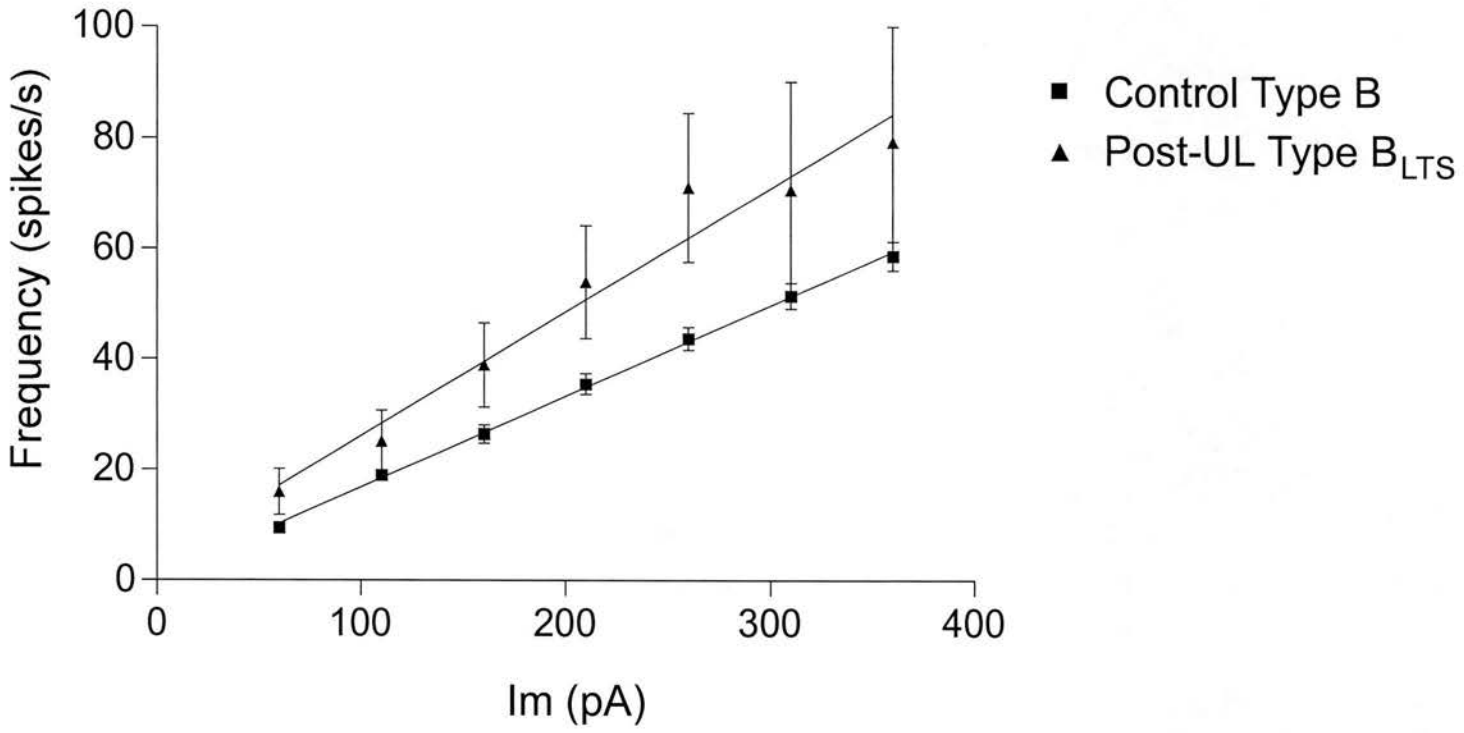

Figure 2.3.2.5. Frequency/current (f/I) relationships of the Type B cells in control slices and Type B_{LTS} cells in post-UL slices. The mean slopes (m_{slope}) of the linear regression lines of the two groups are significantly different (m_{slope}= 0.17 ± 0.01 spikes/pA in control vs m_{slope}= 0.29 ± 0.05 spikes/pA in post-UL group, $p<0.05$, Student's t-test).

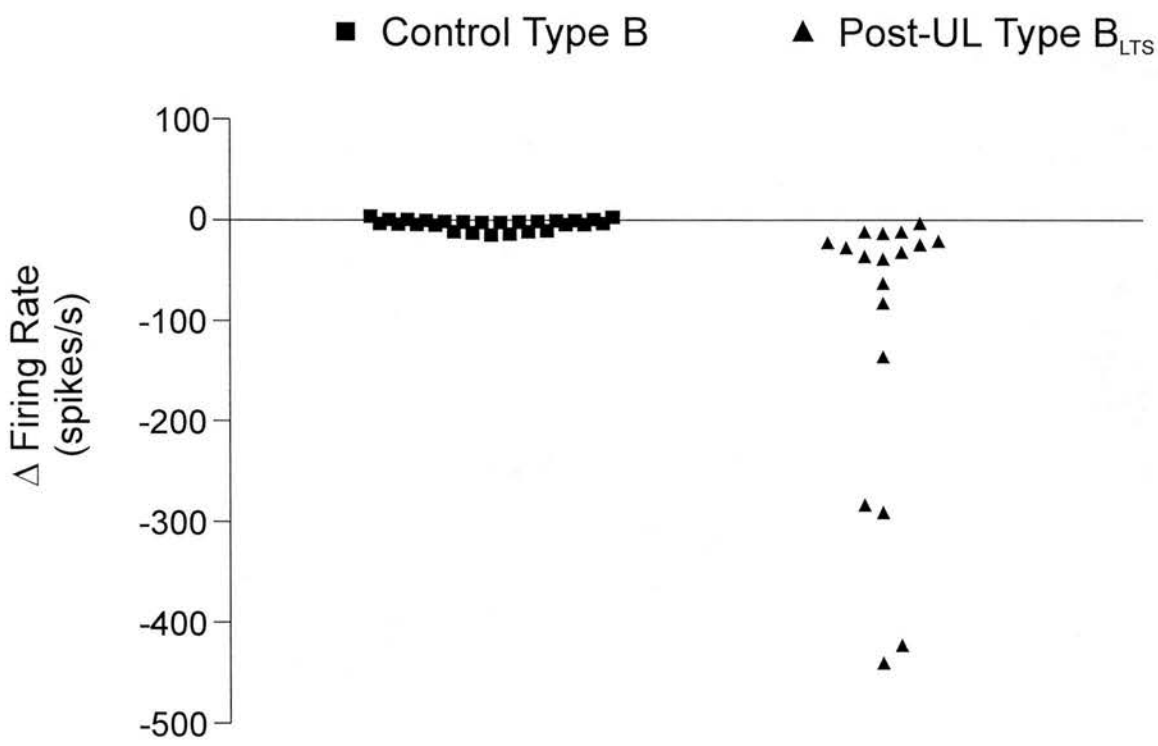

Figure 2.3.2.6. Scatter histogram of the spike frequency acceleration/accommodation indexes of the Type B cells in control slices and Type B_{LTS} cells in post-UL slices. Δ Firing Rate is the difference in firing rates obtained from the last interspike interval and the first interspike interval. Negative values indicate spike frequency accommodation and positive values indicate spike frequency acceleration.

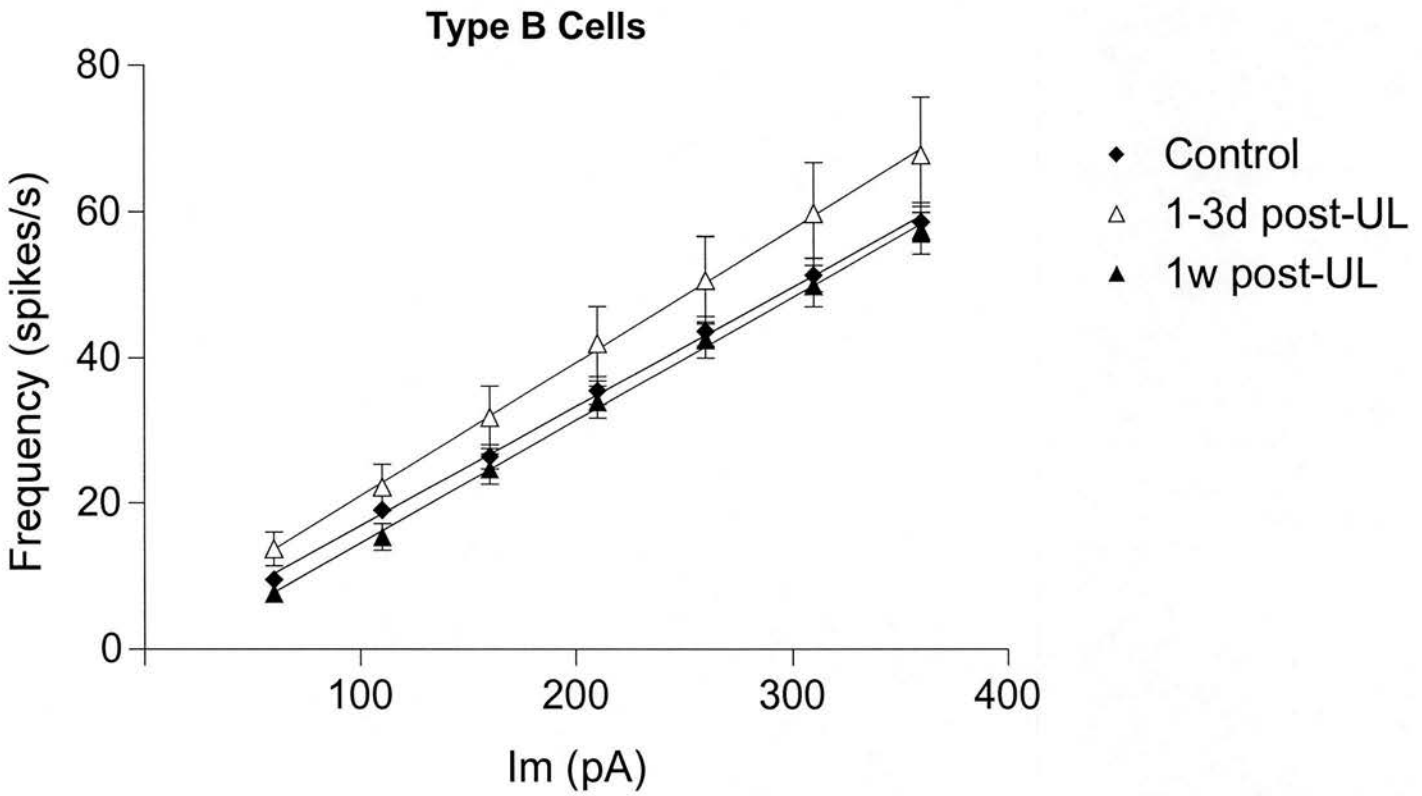

Figure 2.3.2.7. Frequency/current (f/I) relationships of the Type B cells in control and post-UL slices. The mean slopes (m_{slope}) of the linear regression lines of the 1-3d and 1w post-UL groups are not significantly different from the control (m_{slope}= 0.17 ± 0.01 spikes/pA in control, m_{slope}= 0.19 ± 0.02 spikes/pA in 1-3 d and 0.18 ± 0.01 spikes/pA in 1w post-UL group).

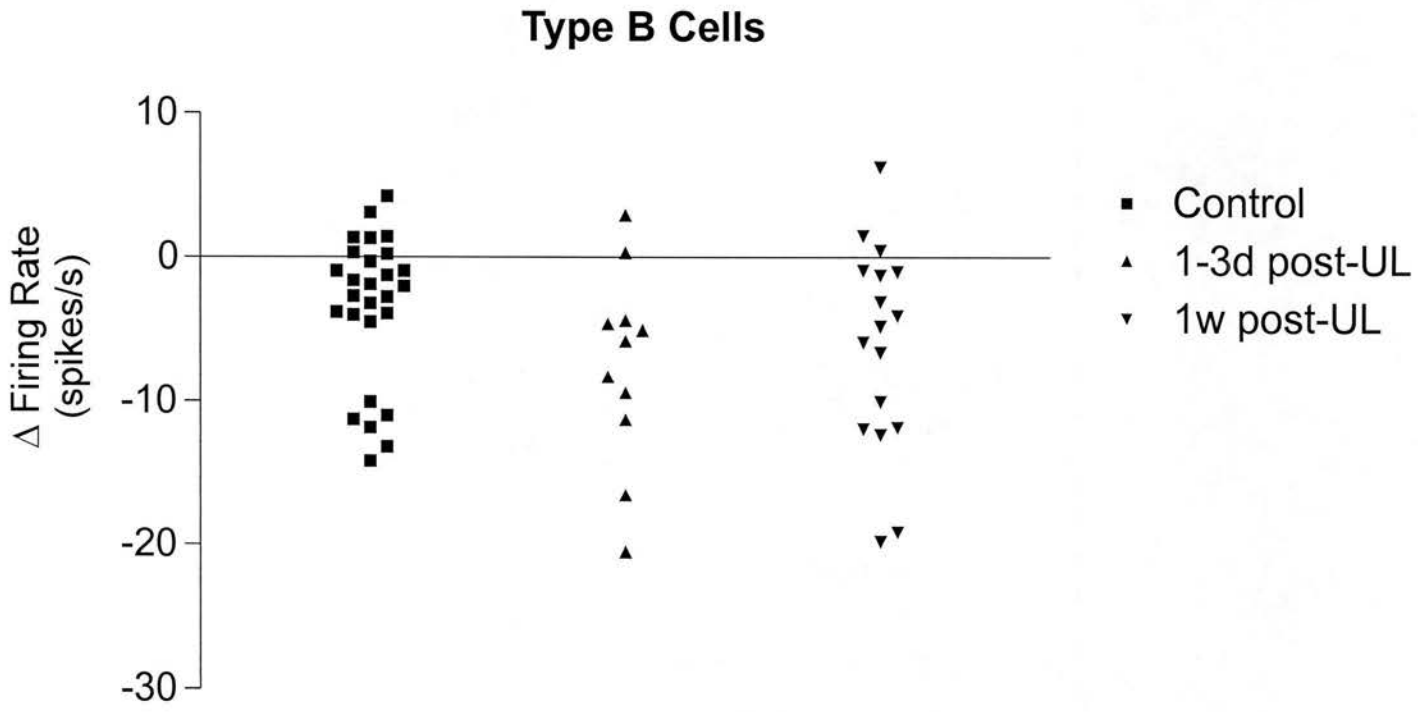

Figure 2.3.2.8. Scatter histogram of the spike frequency acceleration/accommodation indexes of the Type B cells in control and post-UL slices. Type B cells in these groups do not include Type B_{DP} and Type B_{LTS} cells. Δ Firing Rate is the difference in firing rates obtained from the last interspike interval and the first interspike interval. Negative values indicate spike frequency accommodation and positive values indicate spike frequency acceleration.

2.4 Discussion

2.4.1 Intrinsic membrane properties and spike firing characteristics of MVN neurones in normal rats

Based on action potential shapes, 23% of MVN neurones were identified as Type A while 77% were identified as Type B. The relative proportions and electrophysiological properties of the MVN cell types in the present study were similar to the earlier findings with sharp electrodes in rats (Johnston et al., 1994). There were no significant differences in the spontaneous resting discharge rates, resting membrane potentials, input resistances and action potentials amplitudes of Type A and Type B MVN neurones. However, the duration of the action potential of Type A cells was significantly longer than that of Type B cells. Similarly the mean spike rise time and fall time were longer in Type A cells. The amplitude of the first AHP in Type B neurones was significantly smaller than the AHP amplitude of Type A cells. Similar electrophysiological properties of Type A and Type B cells were reported in guinea pigs and chicks (Serafin et al., 1991a; du Lac and Lisberger, 1995). However, in chick MVN slices, du Lac and Lisberger (1995) reported that the membrane properties of MVN neurones were distributed continuously across the population of recorded neurones, and Type A and Type B cells are two extremes of this population. In guinea pigs, Serafin et al. (1991a) described a third cell type, Type C MVN neurone, which corresponds to about 20% of the population. Type C cells were a heterogeneous group that was not readily classified as Type A or Type B, and had properties of both cell types. In the present study in rats, however, all cells were readily classified as either Type A or Type B by using spike shape averaging. These results provide quantitative support for the view that Type A and Type B cells are two distinct subsets of MVN neurones rather than extremes of a single broad population.

The similarity of the cell types recorded in these experiments and the previous intracellular sharp electrode recordings in the rat (Johnston et al., 1994) and guinea

pigs (Serafin et al., 1991a,b; Babalian et al., 1997) suggest that neuronal sampling is not biased by using whole-cell patch clamp technique. Johnston et al. (1994) also reported similar proportion of Type A and Type B cells recorded with sharp electrodes (33% and 67%, respectively).. Small to medium size neurones were described in the MVN of the monkey (Holstein et al., 1999), the guinea pig (Serafin et al., 1993) and the chinchilla (Newman et al., 1992). In the guine pig, Serafin et al. (1993) studied the morphology of Type A and Type B cells in the MVN by injecting Lucifer yellow and reported that Type A and Type cells did not differ in size. Type A cells had two to three dentrites while type B cells had three to five dentrites with similar shapes. Unpublished observations with biocytin staining by Johnston (personal communications) also suggest that MVN of the rat also have small to medium size neurones, which have diameters of 10-30 microns. Type A and type B cell may not be distinguished simply based on their morphology since they show a great degree of overlapping in terms of cell size and dentritic branching.

In rat MVN slices using whole-cell patch clamp recording it was possible to make one or two successful recording per slice. Not every cell was viable for patch-clamp recording. In about one cell out of ten a successful whole-cell patch clamp recording could be obtained, which gave a better success rate compared to sharp electrodes in the same preparation. The use of in vitro slice technique may not cause a bias in therms of the population of the cells recorded since in the whole-brain preparations where the integrity of the MVN is preserved similar proportion of Type A and Type B cells were reported (Babalian et al., 1997).

Most Type A neurones showed an inward rectification (arrow in Figure 2.3.1.5) seen as a sag in the voltage response of the membrane potential in response to hyperpolarising current pulses. This is possibly due to hyperpolarisation activated cation currents as it is blocked by caesium (Serafin et al., 1991b). Type A cells fired repetitively in response to depolarising current pulses. The frequency of discharge was proportional to the amount of current injected resulting in a linear frequency/current (f/I) relationship (Figure 2.3.1.2B, Figure 2.3.1.3). These cells

displayed a regular firing throughout the duration of the current injections without any spike frequency acceleration or spike frequency accommodation. All Type A cells showed a passive return to the baseline at the end of the depolarising current pulses without any plateau potentials. These non-linear membrane properties are reminiscent of tonic vestibular neurones described *in vivo* as discussed below.

Most Type B cells showed spike frequency accommodation in response to depolarising current pulses whereas Type A cells responded tonically with a regular discharge all through the duration of current pulses (Figures 2.3.1.2, 2.3.1.4 and 2.3.1.6). Spike frequency accommodation in Type B cells ranged from weak to strong accommodation, which may be dependent on the degree of expression of voltage gated calcium channels and calcium activated potassium channels. The presence of both types of ionic currents was shown in Type B MVN neurones in guinea pigs (Serafin et al., 1991b) and rats (Johnston et al., 1994). The SK-type calcium activated potassium current underlies the delayed slow AHP in Type B cells and was suggested to be important in timing of successive action potentials (Johnston et al., 1994). However, accommodation of spike firing has not been described in MVN cells. In other areas of the brain, such as the rat hippocampus (Lancaster and Adams, 1986; Chitwood and Jaffe, 1998) and autonomic neurones (Sacchi et al., 1995), spike frequency accommodation was shown to be mediated by calcium activated potassium channels. Application of apamin, an SK-type calcium activated potassium channel blocker, in these neurones abolished spike frequency accommodation and increased the firing response of the cells to positive current pulses. This means that Type B cells that show spike frequency accommodation will have a brief response at the onset of depolarisations. This may provide second order Type B cells in MVN with the ability to respond reliably to high frequency vestibular stimulations. Although SK-type calcium activated potassium currents were shown to be present in all Type B cells, a minority of Type B cells did not show spike frequency accommodation. A depolarisation and/or calcium activated positive conductance that counteract the hyperpolarizing potassium current could be preventing the expression of spike frequency accommodation in these cells. This

kind of current may in fact be one of the currents underlying plateau potentials seen in some Type B cells, as discussed in more detail below.

Type B cells are also more sensitive to positive current injections compared to Type A cells as demonstrated by their steeper f/I relationships (Figure 2.3.1.3). This is possibly due to their non-linear dynamic membrane properties since the mean input resistance did not differ significantly between the two cell types. The difference in sensitivity of Type A and Type B cells support the view that they could correspond to tonic and kinetic second order MVN cells described *in vivo* (Precht and Shimazu, 1965; Shimazu and Precht, 1965; Goldberg et al., 1987; Highstein et al., 1987; Sato and Sasaki, 1993). Tonic cells have regular spontaneous activity and less sensitivity to vestibular stimulations while kinetic cells have irregular resting activity and more sensitivity to vestibular stimulations. It is suggested that tonic vestibular neurones mainly encode low-frequency head movement, and would be involved in static postural and oculomotor control. The kinetic cells would in contrast encode high-frequency stimuli, and would play a role in the stabilization of gaze and posture during fast, transient postural perturbations. Although kinetic vestibular neurones have irregular firing *in vivo*, all Type B cells recorded in slices showed regular spontaneous activity. This could be due to the loss of a major part of the synaptic input to the vestibular neurones in slices. Recent experiments in *in vitro* isolated whole brain preparations, where much of the connectivity of the MVN is preserved, demonstrated irregular spontaneous activity of Type B cells (Babalian et al., 1997).

Plateau potentials in MVN neurones

Plateau potentials, or more generally regenerative depolarisations outlasting the duration of a stimulus, have been described in many neuronal types in both invertebrates (Golowash and Marder, 1992; Zhang and Harris-Warrick, 1995; Mills and Pitman, 1997) and vertebrates (Hounsgaard and Mintz, 1988; Kiehn, 1991; Russo and Hounsgaard, 1996; Rekling and Feldman, 1997), and the mechanisms are diverse. In the rat MVN slices of the present experiments, around 20% of Type B

cells showed plateau potentials as evidenced by a delayed return to baseline following depolarising current pulses delivered from subthreshold membrane potentials. These cells were classified as Type B_{DP} MVN cells. Pharmacological experiments on Type B_{DP} cells suggest that plateau potentials are mediated by calcium currents, as these potentials were abolished in Ca^{2+}-free/Co^{2+} medium and potentiated in a medium containing Ba^{2+}, a more potent charge carrier for voltage gated calcium channels. Moreover, depolarising plateaus were potentiated when major sodium and potassium currents were blocked with a modified medium containing TTX, TEA, 4-AP and apamin, which leaves calcium channels undisturbed. Blockade of plateau potentials with nifedipine suggests that the L-type of high voltage activated calcium channels mediate the plateau potentials. The size and the duration of plateau potentials varied between cells and only a minority of Type B cells developed plateau potentials. However, in guinea pig MVN slices, it was reported that all Type B cells show high threshold plateau potentials if potassium currents were blocked by TEA and 4-AP (Serafin et al., 1991b). Potentiation of plateau potentials in barium medium indicates that calcium-dependant potassium conductances are activated during the current pulses. Unlike calcium, barium does not activate calcium-dependant potassium conductances and blocks most potassium channels. This suggests that the expression of plateau potentials could be dependent on the equilibrium between calcium conductances and various counteracting voltage-dependant and/or calcium-dependant potassium conductances that each MVN neurone possesses.

Depolarising plateau potentials were not observed in Type A MVN neurones in this study. In guinea pig MVN neurones, however, depolarising plateau potentials were revealed in some Type A cells when sodium and potassium channels were blocked and calcium was replaced with barium (Serafin et al., 1991b). Without any pharmacological intervention Type A cells in guinea pigs did not show plateau potentials. In the present study since the dynamic membrane properties of the MVN cells were the main interest the detailed study of the ionic currents present in these cells were not pursued. Nevertheless the absence of plateau potentials in Type A cells does not suggest that these cells do not possess voltage activated calcium

currents, which underlie plateau potentials in other MVN cell types. It is likely that Type A cells in rats also have voltage gated calcium channels as they do in guinea pigs. However, it may not be large enough or was counteracted by hyperpolarizing potassium currents more effectively so that positive current pulses failed to elicit plateau potentials.

The majority of Type B_{DP} cells showed spike frequency acceleration over the duration of depolarising current pulses. This kind of response has not been reported in MVN neurones before. Ionic conductances responsible for spike frequency acceleration in these cells were not investigated in the present study. However, Ca^{2+} entry through voltage-gated calcium channels could activate calcium-dependent depolarising conductances, such as a calcium-activated non-specific cationic conductance (I_{CAN}) (Partridge et al., 1994). In dorsal horn neurones of rat spinal cord, depolarising plateau potentials were shown to be mediated by both voltage-gated calcium currents and I_{CAN}, the latter being responsible for spike frequency acceleration (Morriset et al., 1999). Firing acceleration and the duration of afterdischarges in Type B_{DP} cells were variable depending on the neurone. This suggests that the balance of the intrinsic conductances, relative weight of L-type calcium current and I_{CAN}, and calcium-dependent potassium current, is probably set differently in different neurones.

Plateau potentials in second order MVN neurones could play an important role in processing of vestibular related synaptic inputs. The fact that plateau potentials are dependent on the amplitude of the depolarising current pulses suggests that these cells could have an activity-dependent mechanism that tends to prolong spike firing, which may potentially lengthen the time-constant of their response to excitatory synaptic inputs. This may afford these cells the capability to "store" information transmitted from sensory afferents. Therefore it is possible that temporal summation of long lasting plateau potentials could be one of the neuronal mechanisms underlying velocity storage in vestibulo ocular networks. Velocity storage is known as a central neural integrator that underlies the longer time constant of oculomotor responses to head rotations than that of the vestibular canal signals driving it (Raphan

et al., 1979; Galiana and Outerbridge, 1984; Katz et al., 1991). Although the neurones mediating vestibular reflexes have been described neurophysiologically, the mechanism of velocity storage is still not completely understood. Some network models suggested recurrent inhibitory feedback loops between vestibular neurones as velocity storage mechanism (Anastasio, 1991; Arnold and Robinson, 1991, 1997). Recent studies showed that midline lesions (Katz et al., 1991; Holstein et al., 1999) and application of GABA receptor agonists and antagonists (Niklasson et al., 1994; Mettens et al., 1994; Yokota et al., 1996; Arnold et al., 1999) disrupt the velocity storage suggesting the involvement of inhibitory commissural connections. The contribution of specific ionic currents, such as calcium currents underlying plateau potentials, to velocity storage has yet to be investigated.

Low threshold spikes in MVN neurones

In 1 out of the 78 Type B cells (1%), low-threshold spikes (LTS) were elicited at the end of hyperpolarising current pulses delivered from a subthreshold holding membrane potential. This cell was called 'Type B_{LTS} cell' after Serafin et al. (1991b). Blockade with Ca^{2+}-free medium and Ni^{2+} ione, and the voltage sensitivity of the LTS suggest that T-type calcium currents are involved. The presence of LTS in MVN cells was previously described in guinea pigs (Serafin et al., 1991b). In guinea pigs, however, about 10-15% of normal MVN cells expressed LTS, which is much greater than the proportion of the Type B_{LTS} cells in the present study in rats. The functional relevance of Type B_{LTS} in vestibular physiology is still poorly understood. It was proposed that LTS could participate *in vivo* to the burst of discharge recorded in second order MVN cells during the fast phase of nystagmus (Serafin et al, 1990). However, *in vivo* studies showed that at least 50% of MVN cells bursted during fast phases of nystagmus (Ris et al., 1995), whereas Type B_{LTS} cells represented only 10-15% of all MVN cells. Moreover, bursting behaviour can be induced by pharmacological manipulations in Type B_{LTS} cells as well as other Type B cells. This suggests that LTS is not a prerequisite for phasic activity in MVN cells *in vivo*. The functional importance of LTS in MVN neurones remains therefore to be determined.

2.4.2 Intrinsic membrane properties and spike firing characteristics of MVN neurones after UL

All recordings of vestibular neurones after the UL were made in the MVN ipsilateral to the lesion. However, during the preparation of brainstem slices the vestibular input from the intact labyrinth is also cut. This is often mistakenly thought to be similar to 'the Bechterew phenomenon'. If the intact labyrinth is removed a few days after a UL, the animal shows ocular and postural deficits that are mirror image of those after the first lesion. This behavioural pattern is called the Bechterew phenomenon and is stong evidence that neural balancing in the vestibular network must have taken place between the two labyrinthectomies. The difference between brainstem slice preparation here and the Bechterew phenomenon is that MVN neurones in slices are not subject to the input from the MVN of the other side since the commissural connections are cut during slice preparation. However, in vivo, the rebalanced system is unbalanced again through the commissural system by the second labyrinthectomy. The MVN slices in this study were therefore not subject to the Bechterew phenomenon that is observed only *in vivo* and the changes in the properties of MVN neurones are not due to the removal of the second labyrinth during slice preparation.

Type A MVN neurones

Intrinsic membrane properties of Type A neurones in post-UL slices were similar to control. There were no changes in the mean firing rate, resting membrane potential and input resistance of Type A cells at either time point after UL (Table 2.2, section 2.3.2). The relative proportion of Type A cells in post-UL groups was also not different from control.

Significant changes were observed in dynamic membrane characteristics of Type A cells after UL. While Type A cells in control slices did not show any distinctive spike frequency accommodation or spike frequency acceleration during depolarising current pulses, the majority of Type A cells in 1-3d post-UL slices showed spike frequency accommodation (Figure 2.3.2.1). The slope of the f/I curve of Type A cells

was also significantly greater than control at 1-3d post-UL indicating an increase in their gain (Figure 2.3.2.2). Although these changes did not have any affect on their spontaneous activity in slices it could have functional implications in their behaviour *in vivo*. Superimposed on their pacemaker conductances, the increase in gain may lead to a higher level of neuronal activity of Type A cells *in vivo* by increasing their sensitivity to remaining synaptic excitations. This may contribute to the resumption of resting activity of these cells which decreased dramatically immediately after UL.

The greater number of Type A cells that showed spike frequency accommodation suggests that there is an up-regulation in the expression of calcium activated potassium channels after UL. This may not necessarily be a compensatory change in terms of the recovery of neuronal activity during vestibular compensation since up-regulation of calcium activated potassium currents will tend to reduce neuronal excitability. In hippocampal and autonomic neurones, pharmacologically blocking calcium activated potassium channels was shown to increase their excitability as evidenced by a larger spike firing response of the cells to positive current pulses (Lancaster and Adams, 1986; Chitwood and Jaffe, 1998; Sacchi et al., 1995). Nevertheless, the expression of calcium activated potassium conductance would change dynamic properties of Type A cells, which may assign them new roles in information processing during the early stages of vestibular compensation.

The finding that firing characteristics of Type A cells in 1w post-UL group were not different from control indicates that the changes seen at 1-3d are transient, and the properties of Type A cells recover to normal in the longer term.

Type B MVN neurones

These results show that in the early stage (1-3d post-UL) and in the later stage (1w post-UL) of vestibular compensation, there are significant changes in intrinsic membrane properties of Type B neurones in the MVN. Thus, the mean *in vitro* spontaneous discharge rate of Type B cells as a group was significantly higher than that of control Type B cells at 1-3 d post-UL, and remained higher than control at 1w

post-UL. In line with this the resting membrane potentials of Type B cells at both time-points after UL were significantly more depolarised than those of control Type B cells. In the short term after UL (1-3d post-UL) the mean input resistance of Type B cells was also significantly higher than that of control Type B cells, and their mean spike amplitude was greater, but by 1w post-UL these parameters had returned to normal.

The changes in membrane potential, input resistance and spike amplitude after UL in Type B cells may be mediated by modulation of ionic conductances involved in spike generation and repolarisation. An up-regulation of the expression or function of persistent sodium conductance (INaP) (Crill, 1996), and/or up-regulation of hyperpolarisation activated cation conductance (Ih) (Pape, 1996) would increase the pacemaker activity of the cells. A persistent sodium conductance was shown to amplify synaptic currents in apical dendrites of neocortical neurones (Schwindt and Crill, 1995), providing a mechanism for increased efficacy of tonic, excitatory synaptic input. Modulation of potassium channels can also account for changes in spontaneous discharge of neurones: Potassium currents oppose depolarisation, and hence have a stabilising influence counteracting pacemaker activity (Llinas, 1988; Hille, 1992). A decrease in the number and/or in the efficacy of voltage and/or calcium activated potassium channels, therefore, would favour an increase in spontaneous firing of these cells as observed after UL. All the above-mentioned ionic conductances are present in vestibular neurones (Serafin et al., 1991b; Johnston et al., 1994), making them probable candidates for modulation during vestibular compensation.

Down-regulation of GABA receptor efficacy and glycine receptors was reported in ipsi-lesional MVN neurones after UL (Yamanaka et al., 2000; Vibert et al., 2000). A decrease in chloride conductance through GABA and glycine receptors could also increase membrane input resistance and spontaneous firing rate of MVN cells. Up-regulation of pacemaker conductance in MVN cells after UL has recently been suggested in guinea pigs following extracellular experiments where an increase in resting firing of MVN cells was shown in synaptic uncoupling conditions (Vibert et

al., 1997; Ris et al., 2001). However, in these extracellular experiments in guinea pigs and those in rats (Cameron and Dutia, 1997), increase in excitability was reported in MVN neurones regardless of their type. The present study is the first to report an increase in *in vitro* excitability in a specific subset of MVN neurones, namely Type B cells, and modification of their intrinsic membrane properties during vestibular compensation after UL. Previous extracellular experiments lead to an under-estimate of the increase in resting discharge and assumed that all MVN cells increase their excitability after UL.

The relative proportions of Type A and Type B cells in post-UL groups were not different from control (Table 2.4). However, when Type B cells were further analysed with respect to their firing characteristics, significant changes were found in the proportions of Type B cells expressing low threshold spikes. The relative number of Type B_{LTS} neurones dramatically increased both in 1-3d post-UL and 1w post-UL groups. The increase in the proportion of Type B_{LTS} cells after UL could be due to an increased expression of low-threshold voltage activated calcium channels in some Type B cells. The decrease in the number of Type B cells that did not show either plateau or LTS in post-UL groups suggests that some of these cells may have become Type B_{LTS} cells by newly expressing low-threshold voltage activated calcium channels. The appearance of a greater proportion of Type B_{LTS} cells after UL could be related to the re-organisation of floccular inbibitory input to the vestibular nuclei during vestibular compensation. A decrease in the floccular inhibition on the ipsi-lesional MVN neurones after the UL has been shown by Kitahara et al. (1998, 2000), which may induce the changes in the expression of low-threshold voltage activated calcium channels. Floccular target neurones in the MVN of the guinea pig were shown to be all Type B cells suggesting a possible role for the flocculus in the expression of ion channels in their target neurones (Babalian and Vidal, 2000). However, the mean spontaneous discharge rate of Type B_{LTS} cells in post-UL groups was not significantly different from the mean discharge rate of control Type B cells, suggesting that Type B_{LTS} cells do not contribute to the increased excitability of MVN cells found in *in vitro* slices. Nevertheless, Type B_{LTS} cells may have important roles in re-organisation of vestibular networks during vestibular

compensation. Evident from their steeper f/I curve (Figure 2.3.2.5), these cells have higher gains compared to the rest of the Type B cells resulting in their responses being greater to synaptic inputs. Therefore, synaptic input that previously was not enough to generate spikes, would now, after UL, be sufficient to generate low threshold spikes in these cells, leading to the firing of bursts of action potentials. This may conceivably restore irregular, synaptically driven activity in these cells after UL *in vivo*. Such irregular activity has been observed in the isolated guinea pig brain during vestibular compensation by Vibert et al. (1999), who found that up to 33% of MVN neurones in the ipsi-lesional nucleus showed bursting behavior after 3 days of compensation.

The proportions of Type B_{DP} cells in post-UL groups were not different from control. The mean resting discharge rate of Type B_{DP} cells did not change after UL. There were also no significant changes in their gain and accommodation/acceleration indexes. However, like the rest of the Type B cells they had more depolarised resting membrane potentials after UL. However, depolarized membrane potentials were not reflected in their spontaneous discharge rates. This was in contrast to the overall mean discharge rate of Type B cells as a group, which was increased significantly above normal at both time points after UL.

The 'remaining' Type B cells, which did not show either plateau potentials or low threshold spikes, and made up around 52% of Type B cells, had significantly higher resting firing rates both in 1-3d and 1w post-UL groups compared to control. The resting membrane potentials of these cells also became significantly more depolarized 1w after UL. Thus, the overall increase in mean discharge rate of Type B cells observed after UL in the present and earlier studies, is in fact due to the increased intrinsic excitability of these remaining Type B cells. From the current study the mechanisms underlying the increase in *in vitro* firing rate of these remaining Type B cells are not clear. It could be due to up-regulation of pacemaker conductances as suggested by earlier studies (Darlington and Smith, 1996; Vibert et al., 1997; Ris et al., 2001). Functionally, the increase in *in vitro* excitability of these Type B cells is likely to be important in the early stages of vestibular compensation,

when the ipsi-lesional MVN cells are inhibited into silence through a combination of disfacilitation and excessive commissural inhibition after UL *in vivo*. In particular, increased pacemaker conductance and membrane depolarisation would render these cells more responsive to excitatory synaptic inputs from their remaining extra-vestibular afferents, and therefore contribute to resumption of neuronal activity in the MVN after UL by increasing their resting discharge.

From the current experiments it is difficult to answer why there is an increase in *in vitro* excitability of only Type B neurones after UL. It is necessary to know the synaptic connectivity of Type A and Type B neurones, and their roles in processing of vestibular related signals before understanding the significance of selective effects of UL on intrinsic properties and firing characteristics of MVN neurones. Although different cell types were described in MVN over a decade ago in *in vitro* studies, at present, there is little information related to physiological function of Type A and Type B cells. Future experiments that focus on the projections of individual cell types in MVN to various vestibular related structures will be valuable in understanding the mechanisms of plastic changes during vestibular compensation. Related to the connectivity of MVN cells, it is possible that Type B cells receive excitatory synaptic input from the ipsi-lateral VIIIth nerve ('Type I cells'), while Type A cells are inhibitory interneurones that are excited predominantly by the contralateral labyrinth ('Type II' cells). In this case it would be expected from experiments *in vivo* that Type B cells would be silenced after UL, while the resting activity of Type A cells would either remain unchanged or be increased (Smith and Curthoys, 1989; Newlands and Perachio, 1990a,b; Ris et al., 1995). The adaptive changes in neuronal excitability consequent to their silencing would then occur specifically in Type B cells, as seen here. The relative proportions of Type A and Type B cells found in slices (~30% and 70% respectively) (Johnston et al., 1994; Serafin et al., 1991a) are comparable to the proportions of Type I and Type II cells found *in vivo,* supporting this possible interpretation. However Babalian et al. (1997) have recently shown in the isolated guinea pig brain that both Type A and Type B cells receive monosynaptic excitation from the ipsilateral VIIIth nerve. This may argue against the above interpretation, but does not exclude it since electrical

stimulation of the whole VIIIth nerve activates convergent pathways from different peripheral end-organs. Thus a selective innervation pattern of the two subtypes of MVN cell by horizontal semicircular canal afferents, as suggested above, may be concealed by the projections of, for example, other canal and otolith afferents in the VIIIth nerve. In any case, other explanations for the selective effects of UL on the intrinsic excitability of Type B cells are possible, and direct experiments to demonstrate the character and connectivity of Type A cells and Type B cells are necessary.

Chapter 3

Tonic activity and GABAergic inhibition in aged rat MVN neurones

3.1 Introduction

The vestibular nuclear complex is a major visual-vestibular interaction centre mediating head-eye coordination and balance of the body. Age-related changes in vestibular nucleus neurones could have particularly important implications because of their direct involvement in vestibular related synergies.

Disequilibrium, impaired visual acuity, dizziness, vertigo and falls are extremely common problems with serious clinical consequences in older people and are often accompanied by deficits in vestibular function (Tinetti et al., 1986, 1988; Rubenstein et al., 1988; Tinetti and Speechley, 1989; Sloane et al., 1989; Fife and Baloh, 1993; Baloh et al., 1994). Deficits in multiple sensory systems and their integration are probably responsible for balance problems in the elderly. However it is most likely that age-related changes in the vestibulo-ocular reflexes (VORs) and deficits in visual-vestibular interactions may contribute to the above-mentioned impairments in the elderly, which makes the study of the effects of ageing on the vestibular system important.

Vestibular deterioration with ageing has been known to occur both anatomically and physiologically. Anatomical studies of vestibular ageing have mainly focused on the changes in the number of neurones in vestibular related areas of the brain and the receptors in the inner ear. The effects of ageing on vestibular function have been studied most extensively in VOR since the reflex is relatively easy to stimulate and evoked eye movements can be recorded easily even in old patients.

Anatomical studies of ageing in vestibular system

Degenerative changes in the peripheral and central parts of the vestibular system have been documented in aged animals and humans. In the periphery, Rosenhall (1973) quantified the number of hair cells in surface membrane preparations and

showed that the total number of hair cells decreases with increasing age. In his aged group, the reduction in the number of sensory hair cells amounted to 40% on the cristae and about 20% on the maculae. Using a different technique, serial temporal bone sections, Richter (1980) demonstrated that the density of hair cells in humans older than 30 years of age was lower than younger subjects. Similar changes in the number of peripheral vestibular neurones have been reported in old humans: Bergstrom (1973) showed that the number of nerve fibres peripheral to Scarpa's ganglion was reduced in aged individuals. Richter (1980) counted the neuronal cell bodies in Scarpa's ganglion in young and old people. Nerve counts from aged subjects were typically lower. He reported a decline of approximately 9% of the cell population per decade following 58 years of age. More recently, Park et al. (2001) quantified human vestibular ganglion cells in archival nonpathological temporal bone specimens using the optical fractionator and found that there was a statistically significant age-related decrease in the number of vestibular ganglion neurones.

Age-related changes were also observed in the central part of the vestibular system. Sturrock (1989) identified an age-related decline in neuronal number in the lateral vestibular nucleus of the mouse. There was about 40% decrease in the number of large vestibular neurones between the ages of 25 and 31 months. Lopez *et al.* (1997) reported an age-related loss of neurones in the vestibular nucleus complex of humans, with an approximately 3% neuronal loss per decade from age 40 to 90 years. Loss of neurones was preceded by an accumulation of lipofuscin in their soma and a loss of Nissl substances. Alvarez *et al.* (1998) studied the effects of ageing on the number of MVN neurones. They showed that the number of neurones in the MVN decreased about 38% from age 35 to 89. There was also a decrease in the nuclear diameter of the MVN neurones with increasing age. In addition the cerebellum, which plays an important role in the adaptive control of postural and oculomotor reflexes, shows age-related changes in the number and density of Purkinje cells. Hall et al. (1975) estimated the total number of Purkinje cells in the cerebellum of 90 normal human subjects. Although wide individual variations of the number of Purkinje cells were found at all ages, they found a mean reduction of 2.5% per decade over the age range of 0-100 years with greater change beyond the sixth

decade. A decrease in Purkinje cell density with increasing age was also demonstrated in cerebellar vermis of human subjects (Torvik et al., 1986). In accordance with studies that showed neuronal loss in the cerebellum, computerised tomography studies have showed that cerebellar atrophy occurs in aged individuals (Koller et al., 1981a,b).

Unlike the vestibular nuclei, quantitative studies of human brainstem nuclei have found relatively little age-related neuronal loss in other vestibular related brainstem nuclei. Vijayashankar and Brody (1977a,b) studied the trochlear and abducens nuclei in young and older humans, and showed that there were no significant differences in the number of neurones with advancing age in these nuclei. Similar observations were made in the inferior olivary nuclei (Moatamed, 1966; Monagle and Brody, 1974) and the cochlear nuclei (Konigsmark and Murphy, 1972) of human subjects.

Functional studies of ageing in the vestibular system

The studies of the effects of ageing on the physiological function of the vestibular system have focused mainly on the VOR. The VOR normally stabilizes gaze to reduce retinal slip during natural activities that induce head perturbations (see section 1.1 for more on the VOR). A deficient VOR would not maintain images steadily on the retina during head motion, leading to impairment of visual acuity (Demer et al., 1994; Herdman et al., 1998; Hillman et al., 1999) and perhaps impairing orientation, balance and postural control during head movements associated with daily activities. These vestibular related problems are often reported in the elderly as complaints of impaired visual acuity, dizziness, vertigo and falls (Tinetti et al., 1986, 1988; Rubenstein et al., 1988; Tinetti and Speechley, 1989; Sloane et al., 1989; Fife and Baloh, 1993; Baloh et al., 1994).

Despite the anatomical deterioration in the peripheral and central vestibular system laboratory measures of VOR function correlate poorly with the above-mentioned symptoms in older people. Most prior studies of the VOR in the elderly found only a

small decrease in VOR gain with age, with substantial overlap in performance between younger and older people (Van der Laan and Oosterveld, 1974; Mulch and Petermann, 1979; Wall et al., 1984; Stefansson and Imoto, 1986). An increase in VOR gain with age has even been reported (Yagi et al., 1983). However, more recent studies reported a significant decrease in VOR gain along with a decrease in the time constant of the VOR (Peterka et al., 1990a,b; Paige, 1992; Baloh et al., 1993, Hirvonen et al., 1997; Tian et al., 2001). The difference in VOR gain between younger and older people was greater when tested with higher head velocities (Paige, 1992; Baloh et al., 1993).

Two important factors may be responsible for the discrepancy between earlier and later studies. First, earlier studies of VOR in the elderly have employed stimuli with a narrow range of frequency and modest velocities or used non-physiological stimuli, such as caloric stimulus. These may not have challenged the VOR sufficiently to reveal an age dependant decline in performance. Second, the VOR is known to have an adaptive plasticity whenever visual-vestibular mismatch arises during head movement (Gonshor and Melvill Jones, 1976; Miles and Eighmy, 1980; Lisberger, 1984, Lisberger and Pavelko, 1988). The mismatch generates retinal image instability, which is then used to modify VOR performance in order to eliminate the mismatch and restore a stable image. Adaptive VOR recalibrations have been shown to occur when visual feed back is manipulated with magnifying or miniaturising goggles (Miles and Eighmy, 1980; Collewijn et al., 1983; Lisberger et al., 1981) or with vestibular lesions (Allum et al., 1988; Black et al., 1989; Fetter and Zee, 1988; Jenkins, 1985; Paige, 1989). The vestibular deterioration due to ageing may generate a gradual stimulus to the VOR recalibration mechanism that corrects overall VOR performance over time, thereby masking the underlying anatomical deterioration. However, potential deterioration in adaptive capabilities of the vestibular machinery might accompany ageing. Thus the progressive age-related decline in anatomical vestibular structures may become increasingly uncorrectable with ageing, resulting in progressive functional deficits in vestibular driven reflexes, which may be manifested only at high amplitude and/or frequencies of head rotations, as shown in

recent studies (Peterka et al., 1990a,b; Paige, 1992; Baloh et al., 1993, Hirvonen et al., 1997; Tian et al., 2001).

It seems plausible that age-related changes observed in the VOR may also be present in other vestibular reflexes, such as the vestibulospinal reflexes. Although deterioration in balance and gait have been well documented with ageing (Baloh et al., 1994, 1995, 1998; Norre et al., 1987; Kerber et al., 1998), it is difficult to distinguish changes in vestibulospinal reflexes from the well-documented changes that occur in the visual systems.

Vestibular compensation in the ageing vestibular system

In young adults the vestibulo-ocular system shows a high degree of synaptic and neuronal plasticity, which enables a substantial behavioural adaptation after drastic changes in peripheral afferent input. Two well-described examples of such vestibular plasticity are learning in VOR and vestibular compensation. Adaptation in VOR has been mentioned above and a detailed account of vestibular compensation was given in Section 1.3 of Chapter 1.

The plasticity in the central vestibular system is presumably also involved throughout life in compensating for deficits that arise from degenerative changes that occur with ageing. However, it has been shown that both VOR adaptation and vestibular compensation are impaired in the elderly. Paige (1992) studied the adaptation of the VOR gain by 2x-magnifying lenses in younger and older people. The older subjects showed consistently poorer gain adaptation compared to younger subjects. The deficit in VOR adaptation was more remarkable when tested with higher frequencies of head rotations. At 4 Hz, the older subjects displayed no adaptive gain enhancement. Barion and Andretta (1988) studied patients with unilateral labyrinthine deficits of sudden onset and observed that, in patients younger than 40 years of age, spontaneous nystagmus disappeared by the eighth day, while in those

over 40 years it persisted for at least 15 days. One year after an episode of vertigo, only 8% of patients younger than 40 complained of dizziness, whereas 87% of patients older than 40 had persistent symptom of dizziness. Poor compensation of vestibular related symptoms after unilateral labyrinthine lesions has been shown in aged rats by Drago et al. (1996). The decline of spontaneous nystagmus in aged animals after UL was slower than that of young rats. Moreover, motor performance and coordination in aged rats improved less rapidly than those of young controls during vestibular compensation.

Recent experiments on the cellular mechanisms of vestibular compensation have identified two synergistic, adaptive responses of medial vestibular nucleus neurones to de-afferentation. Within 4 hours after UL, MVN neurones up-regulate their intrinsic membrane excitability, and down-regulate the functional efficacy of their $GABA_A$ and $GABA_B$ receptors (Cameron and Dutia, 1997; Yamanaka et al., 2000; Johnston et al., 2001) (More detailed account of these mechanisms are given in Section 1.3.3 of Chapter 1). These changes are likely to be important in vestibular compensation, as they are appropriate to counteract the disfacilitation and increased commissural inhibition to which the MVN neurones are subjected immediately after UL (more detailed discussion of these mechanisms is given in Section 1.3.3 of Chapter 1). In this part of the thesis, the intrinsic spontaneous activity of MVN neurones *in vitro*, and their responsiveness to the $GABA_A$ and $GABA_B$ agonists muscimol and baclofen were investigated, in slices of the brainstem prepared from young adult and aged rats. Experiments were carried out, in particular, to determine if the reported delay in the rate of vestibular compensation after UL in aged animals (Drago et al., 1996) could be due to age-related changes in the intrinsic activity or GABA receptor sensitivity of the MVN neurones in old animals.

3.2 Materials and Methods

Animals

Young adult (8-12 weeks old, weighing 200-250 g) and aged (24 months old, weighing 500-600 g) male Lister Hooded rats (Charles River, UK) were housed under conditions of controlled lighting (lights on 07.00 - 19.00h) and temperature (22° C), with free access to food and tap water in the Medical Faculty Animal Area (MFAA) for 3-4 days before experiments. Aged rats had been housed in similar conditions before transferring to MFAA, and had not been subjected to other experimentation. Animals were collected from the MFAA on the day of the experiment. A total of 5 young adult and 5 aged rats were used for experiments. Animal care and experimental procedures were carried out in accordance with the UK legislation for the human care and use of laboratory animals.

Preparation and maintenance of MVN slices

The preparation and maintenance of MVN slices from young and aged rats were similar to the method described in section 2.2.2 of Chapter 2. The difference here is that, during the preparation of brainstem slices, brains were removed into a modified ice-cold artificial cerebrospinal fluid (aCSF) that composed of (mM) NaCl (25), KCl (1.9), KH_2PO_4 (1.2), $MgSO_4$ (10), $NaHCO_3$ (26), sucrose (188) and D-glucose (25). In this medium NaCl was replaced by sucrose and calcium was omitted to reduce cell damage during tissue preparation (Aghajanian and Rasmussen, 1989). Another difference here is that both left and right MVN in the brainstem were used for extracellular recordings. Electrophysiological recordings were undertaken in normal aCSF as described in methods section in chapter 2.

Extracellular recordings

Extracellular recordings were made from MVN neurones using conventional electrophysiological equipment as shown schematically in figure 2.2.3. Glass microelectrodes were pulled on a three-stage horizontal microelectrode puller (Sutter Intruments, UK) using filamented standard-wall boroslicate glass tubes (1.5 mm outer, 0.86 mm inner diameter (Clark Electromedical Instruments, UK). Microelectrodes were filled with 2M-sodium gluconate using a microelectrode filler (WPI, UK). The resistance of the electrodes ranged from 10-20 MΩ. The filled microelectrode was inserted into the headstage of the Axoclamp 2B amplifier (Axon Instruments, USA). Under a binocular dissecting microscope (x16 magnification) and fibre optic illumination (Flexilux, Germany) the MVN was visualised and the electrode was positioned just over the MVN using a coarse manipulator. The microelectrode was then slowly advanced into the MVN with a hydraulic micromanipulator (Narishige, Japan). The signals from the electrode were fed into the Axoclamp 2B amplifier. The amplified voltage signal (x10) was then delivered to a Neurolog System (Digimeter, UK) containing a filter module (low frequency cut-off 500Hz; high frequency cut-off 5000Hz), a high gain AC amplifier (set at a gain of 200), and an audio amplifier. The extracellular signal, filtered and amplified, was monitored on a 2-channel storage oscilloscope (Gould, UK) and fed to a spike discriminator/triggering module of the Neurolog System. To obtain a proper triggering of the extracellular action potentials, the discriminator was set to a level of amplitude greater than that of the background noise but below that of the peak of the extracellular action potential. Triggered action potential signals were fed to a CED1401 Plus laboratory interface (Digimeter, UK) linked to a microcomputer (P486, Dell, UK) running a spike collection and analysis programme, SCAP90, written by Dr. MB Dutia. Using SCAP90, the rate of discharge of the spontaneously active MVN neurones was displayed on the computer in the form of rate/time histogram (binwidth 1000msecs) and analysed on line.

Data collection and analysis

SCAP90 was used to produce spike frequency histograms of recorded cells and measure mean firing rates and responses of the neurones to GABA receptor agonists. Once a stable recording of spontaneous activity of a neurone had been obtained for 3-minute duration, its mean firing rate was calculated on line during a 60 second period and expressed as spikes/s. The $GABA_A$ agonist muscimol (5-aminomethyl-3-hydroxyisoxazole) and $GABA_B$ agonist baclofen (4-amino-3-[4-chlorophenyl]-butanoic acid) were purchased from Sigma (Dorset, UK). Aliquots of stock solutions of the drug in distilled water were frozen until used. Test solutions of each agonist were made up by diluting the stock solution in oxygenated aCSF immediately before use, and applied to the slices by switching the perfusion inlet to a reservoir containing the agonist for 60 s.

The response of a cell to the drug was measured as the maximal decrease in discharge rate expressed as a percentage of the resting discharge rate. Once the cell responded with maximal inhibition (%100) to the drug the higher concentrations of the drug were not applied and the responses to the higher concentrations were assumed to be maximal. Data was analysed after being extrapolated with this assumption. Two Way Repeated Measures ANOVA was used to analyse the effect of ageing on the responses of MVN cells to muscimol and baclofen, significance being assumed when $P< 0.05$.

All data analysis was completed on line and the data for GABA response was stored in the computer hard disk for future reference. Statistical analyses of the results were performed using GraphPad Prism for Windows (GraphPad Software, USA) and graphics were produced in Microsoft Excel. Values are presented as mean ± SEM where appropriate.

3.3 Results

Extracellular recordings of tonically active MVN cells were made in brainstem slices of young and aged rats. Each MVN was divided under visual control into three approximately equal parts, namely rostral, middle and caudal parts (Figure 2.2.2). The recordings were made in the rostral and caudal parts of MVN. All MVN cells included in this study showed a regular sustained discharge for 180 s. The effects of muscimol and baclofen were tested on cells that had stable resting discharges. Drugs were applied at increasing concentrations starting from the lowest dose.

Spontaneous Firing Rate of MVN neurones

MVN neurones in slices from both young and aged rats showed a regular spontaneous *in vitro* discharge. The mean resting discharge rates of rostral and caudal MVN cells in slices from aged animals were not statistically different from those recorded in slices from young animals. In slices from young rats the mean firing rate of rostral MVN cells was 13.8 ± 0.8 spikes/s (n = 73 cells), while that of caudal MVN cells was 14.7 ± 0.9 spikes/s (n = 60 cells). In aged rats, mean firing rate of cells in the rostral region was 15.3 ± 1.4 spikes/s (n = 46 cells), while that of caudal cells was 16.5 ± 1.8 spikes/s (n = 31 cells) (Figure 3.1).

GABA responses of MVN neurones

In both young and aged rats, bath application of the $GABA_A$ receptor agonist muscimol (3-100 μM) and the $GABA_B$ receptor agonist baclofen (0.3-30 μM) caused a reversible, dose-dependent inhibition of the spontaneous discharge of the MVN neurones. Examples of inhibitory responses of MVN cells to GABA agonists can be seen in Figure 3.2 and Figure 3.3. There were significant differences in the efficacy of $GABA_A$ receptors in slices prepared from young and aged rats. The inhibitory effects of 10 μM muscimol on MVN cells in the aged rats were significantly greater

than those in young rats ($p < 0.01$, Student's t-test) and there was a leftward shift in the dose-response curve (Figure 3.4B). The mean values of GABA responses in young adult and aged animals are given in Table 3.1. The inhibitory effects of baclofen on the MVN neurones from aged animals were also consistently greater than those in young adults, but this difference was not statistically significant ($p > 0.05$, Two Way Repeated Measures ANOVA) (Table 3.1, Figure 3.5).

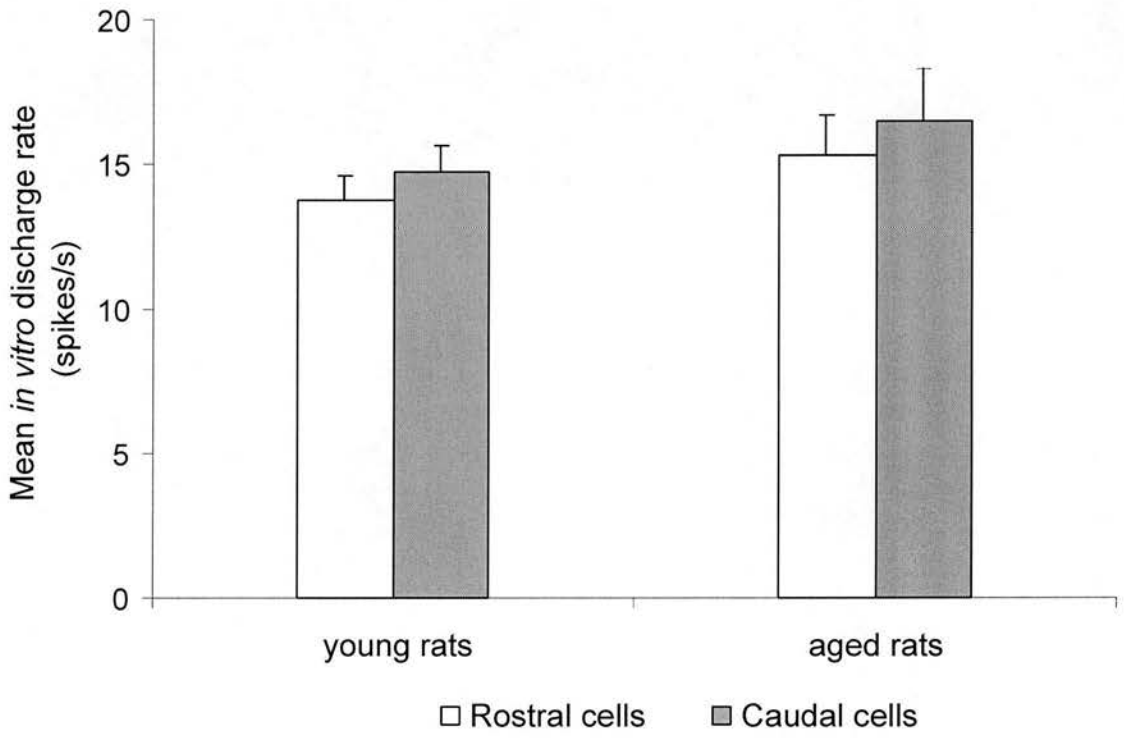

Figure 3.1. Histogram showing *in vitro* spontaneous discharge rate of MVN neurones recorded in brainstem slices from young and aged rats. There was no significant difference between the mean firing rates of rostral and caudal MVN neurones in young and aged animals. There was also no difference in mean firing rates of MVN neurones between young and aged animals..

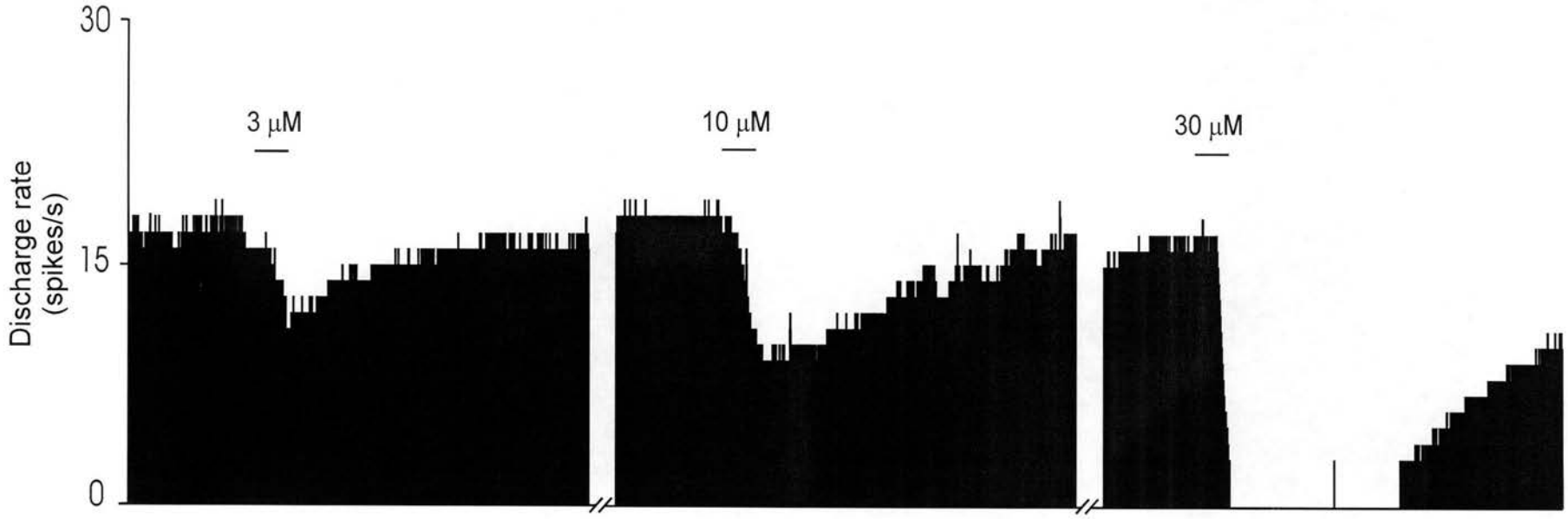

Figure 3.2. An example firing rate histogram showing the inhibitory effect of $GABA_A$ receptor agonist muscimol on the tonic activity of an MVN neurone recorded in a brainstem slice of an aged rat. The 60s periods of bath application of the drug are indicated by the bars above the data.

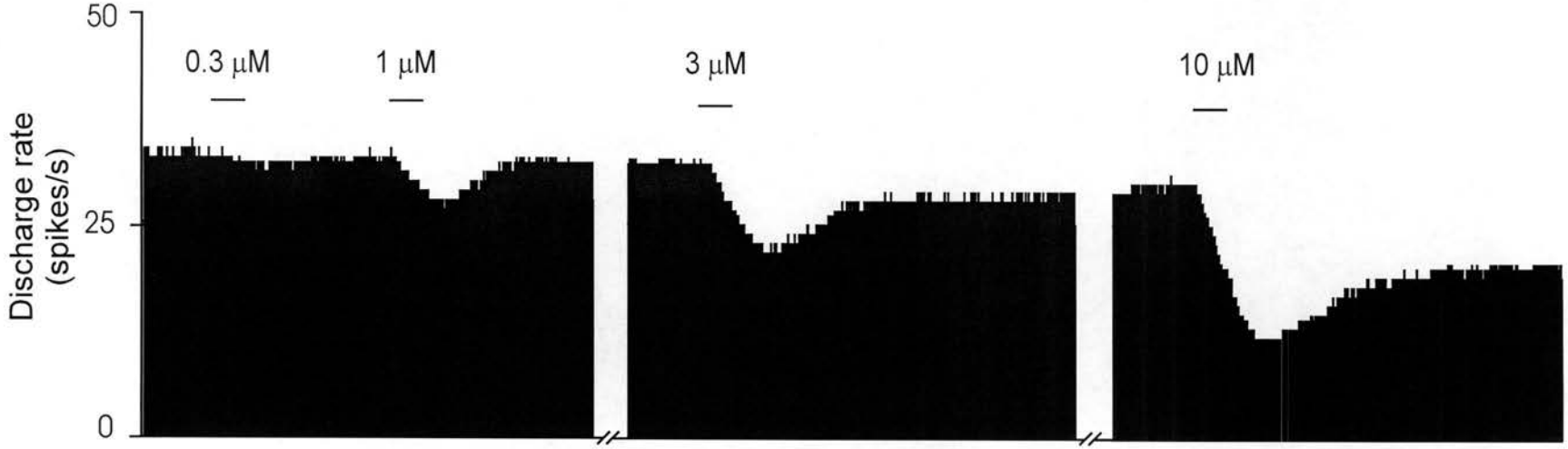

Figure 3.3. An example firing rate histogram showing the inhibitory effect of $GABA_B$ receptor agonist baclofen on the tonic activity of an MVN neurone recorded in a brainstem slice of an aged rat. The 60s periods of bath application of the drug are indicated by the bars above the data.

Figure 3.4. Dose-response relationships of the inhibitory responses to the $GABA_A$ receptor agonist muscimol of MVN neurones in slices of young and aged rats.

A. Scatter plot of the inhibitory effects of increasing concentrations of muscimol on the resting activity of MVN neurones. Horizontal bars on the data indicate the median response for each concentration in young and aged rats. The medians of the inhibitory responses in young rats are consistently lower than those in aged rats.

B. Dose-response curves fitted to the mean inhibitory responses for each concentration of agonist using a least-squares method.

The inhibitory effects of 10 μM muscimol was significantly greater in the aged rats compared to the effects in young adult rats ($p < 0.01$, Student's t-test). Statistical analysis was performed only for the effect of 10 μM muscimol since the measurements for all concentrations were not repeated on the majority of the cells.

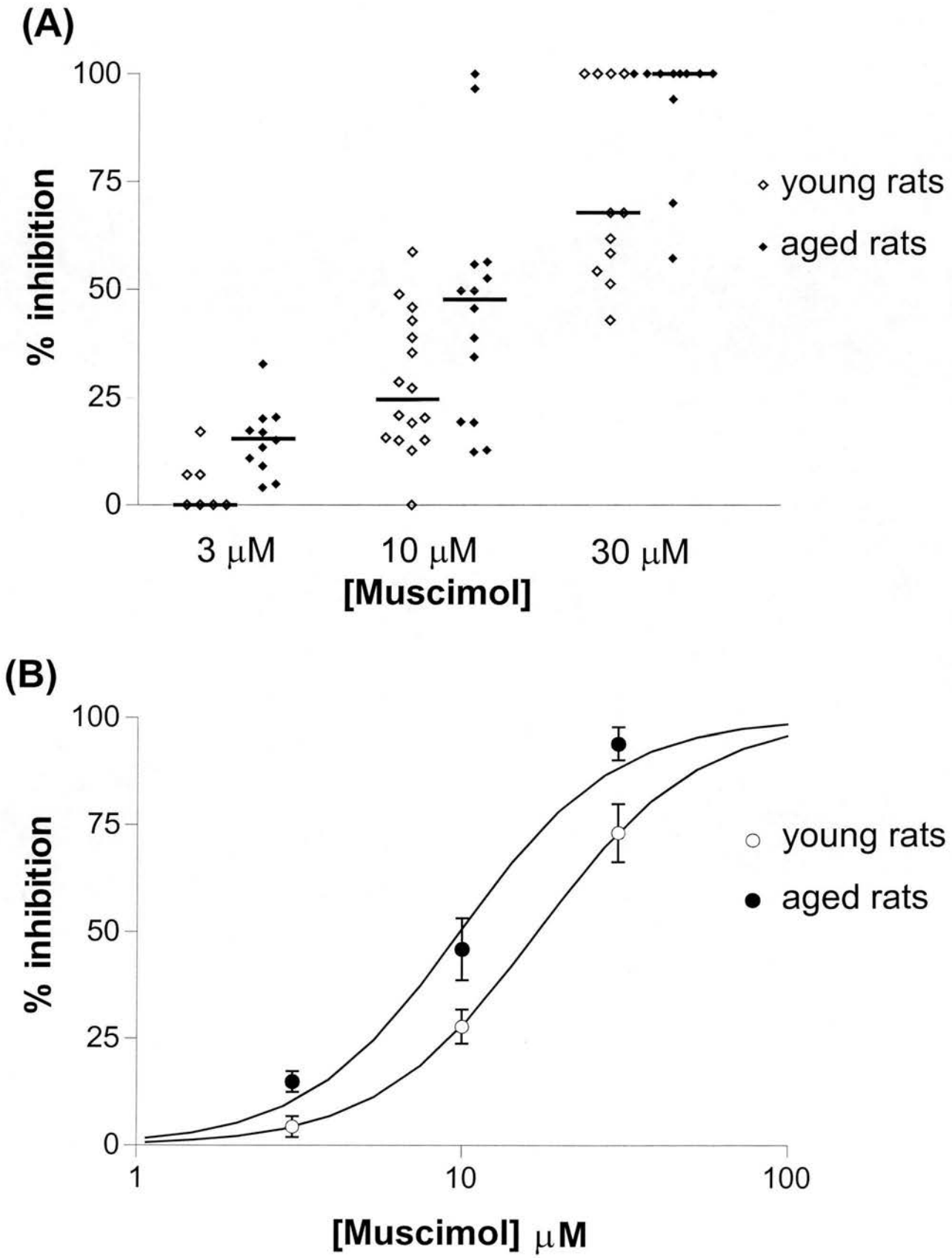

Figure 3.4. Dose-response relationships of the inhibitory responses to $GABA_A$ receptor agonist muscimol on MVN neurones in slices of young and aged rats.

Figure 3.5. Dose-response relationships of the inhibitory responses to the $GABA_B$ receptor agonist baclofen of MVN neurones in slices of young and aged rats.

A. Scatter plot of the inhibitory effects of increasing concentrations of baclofen on the resting activity of MVN neurones. Horizontal bars on the data indicate the median response for each concentration in young and aged rats.

B. Dose-response curves fitted to the mean inhibitory responses for each concentration of agonist using a least-squares method.

Although inhibitory effect of baclofen in the aged rats was consistently greater than those in young adults, this difference was not statistically significant ($p < 0.05$, Two Way Repeated Measures ANOVA). Extrapolated data was used for the analysis (see section 3.2, Data collection and analysis).

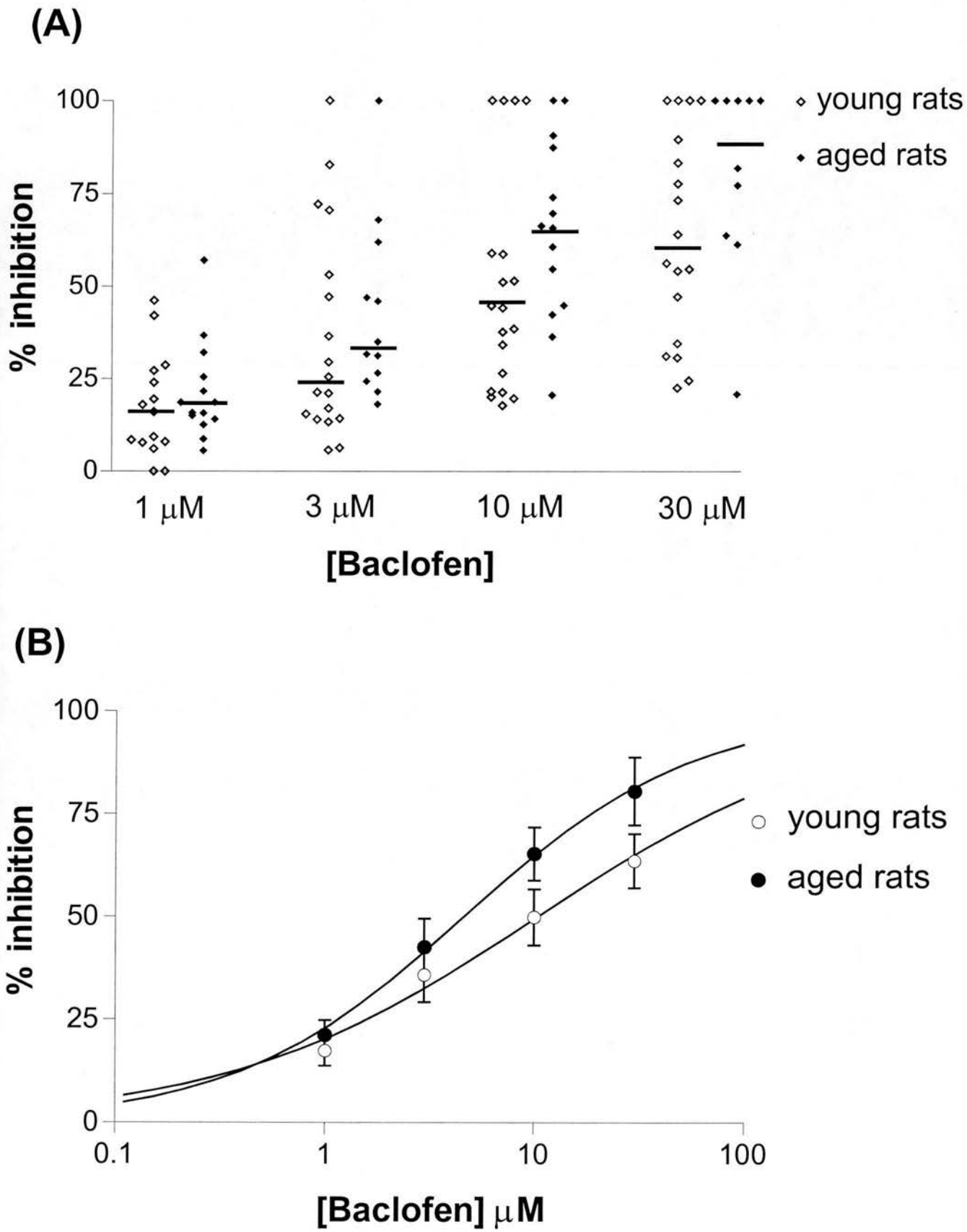

Figure 3.5. Dose-response relationships of the inhibitory responses to $GABA_B$ receptor agonist baclofen on MVN neurones in slices of young and aged rats.

Table 3.1. Inhibitory responses of MVN neurones to $GABA_A$ receptor agonist muscimol and $GABA_B$ receptor agonist baclofen in young adult and aged rats. The inhibitory responses to 10 μM muscimol are significantly greater in aged rats compared to the responses in young adult rats ($p < 0.01$, Student's t-test). Statistical analysis was performed only for the effect of 10 μM muscimol since the measurements for all concentrations were not repeated on the majority of the cells.

The responses to baclofen are not significantly different between the two groups ($p<0.05$ Two Way Repeated Measures ANOVA). Extrapolated data were used for the analysis (see section 3.2, Data collection and analysis).

	% inhibition ± S.E.M.			
Muscimol	Young adult rats	Number of Cells	Aged rats	Number of Cells
3 μM	4.4 ± 2.4	7	15.0 ± 2.4	11
10 μM	27.8 ± 4.0	16	46.0 ± 7.3	14
30 μM	73.1 ± 6.8	11	93.9 ± 3.8	13
Baclofen				
1 μM	17.4 ± 3.6	15	21.3 ± 3.6	14
3 μM	36.0 ± 6.8	18	42.6 ± 6.9	12
10 μM	49.8 ± 6.8	19	65.3 ± 6.4	14
30 μM	63.5 ± 6.6	18	80.5 ± 8.2	10

3.4 Discussion

Tonic activity of aged rat MVN neurones

These results show that the intrinsic spontaneous activity of MVN neurones in the aged Lister Hooded rat is similar to that in young animals of the same strain, and also similar to that seen in young Sprague Dawley animals in previous studies (Dutia et al., 1992; Cameron and Dutia, 1997, 1999). In earlier studies the intrinsic membrane properties of MVN neurones that underlie their tonic activity have been partially characterised, and include a persistent sodium current that causes membrane depolarisation and spontaneous spiking, as well as a range of voltage- and calcium-activated potassium conductances (Serafin et al., 1991b; Johnston et al., 1994). The intrinsic properties of MVN neurones are likely to be important in generating their resting discharge *in vivo*, and in shaping their response characteristics to afferent inputs (Graham and Dutia 2001). The similarity in spontaneous resting activity of MVN neurones in old and young rats indicates that the *in vitro* excitability of MVN neurones is maintained in the aged animals. The maintenance of cellular excitability is important in ensuring that an individual MVN neurone is neither induced to fire action potential excessively nor driven to silence.

The mechanisms by which MVN neuronal excitability is maintained despite the anatomical deterioration of peripheral and central vestibular system with ageing are not clear. It is likely that broadly similar membrane ion conductances are responsible for generating the resting activity of MVN neurones in old and young animals. However, this does not exclude the possibility that age-related changes occur in particular ion conductances in older animals. A more detailed investigation using intracellular recording techniques is however necessary to determine if individual ion channels are modified with ageing. The present studies have established that *in vitro* slice preparation and recording techniques are viable with MVN tissue from aged rats, and intracellular recording experiments may now be pursued.

The maintenance of MVN neuronal excitability could be important for vestibular compensation in the aged animals. It is likely that *in vitro* excitability of MVN cells in the aged animals might also undergo compensatory changes during vestibular compensation as seen in the young adult animals (Cameron and Dutia, 1997, 1999; Vibert et al., 1999; Ris et al., 2001).

GABA responses of aged rat MVN neurones

These results showed that age-dependent changes occur in the efficacy of inhibitory GABA receptors in the aged rat MVN neurones. The responsiveness of the MVN neurones of the aged rats to the $GABA_A$ receptor agonist muscimol was significantly greater than that of the young adult rats, while their responsiveness to the $GABA_B$ receptor agonist baclofen was not significantly different from those in young animals. Inhibitory modulatory inputs play an important role in regulating vestibular reflex function: in particular the functionally inhibitory vestibular commissures that link the MVN of the two sides (Furuya et al., 1992; Babalian et al., 1997; Holstein et al., 1999), and the inhibition of MVN neurones by Purkinje cells in the cerebellar flocculus (Sato et al., 1988; Lisberger et al., 1994; Stahl and Simpson, 1995; Babalian and Vidal, 2000), are important both in the generation of the normal VOR and in adaptive plasticity of the VOR (Lisberger, 1984, Lisberger and Pavelko, 1988). Age-dependant degenerative changes in the peripheral and central parts of the vestibular system have been documented in aged animals and humans. In aged animals there is a progressive loss of MVN neurones (Sturrock, 1989), as well as cerebellar Purkinje neurones (Hall et al., 1975; Torvik et al., 1986), which may have implications for the functional efficacy of these inhibitory modulatory inputs. These degenerative changes in vestibular system may cause a decline in the performance of the commissural and cerebellar inhibitory systems. The increased responsiveness of MVN neurones to $GABA_A$ receptor mediated inhibition, as observed here, may serve to counteract such possible decrease in efficacy of the commissural and cerebellar inhibitory systems with ageing.

The increase in functional efficacy of $GABA_A$ receptors in aged MVN neurones may reflect either an increase in receptor number or alterations in receptor subunit composition. Age-related changes in $GABA_A$ receptors have been observed in a number of studies with rather conflicting results. Several investigations of GABA receptors in the aged animals reported age-related decreases in the number of receptors in several parts of the brain (Govoni et al., 1980; Concas et al., 1988; Nabeshima et al., 1994; Araki et al., 1996; Ruano et al., 1996). In hippocampus of aged rats, Gutierrez et al. (1996) reported an increase in the expression of mRNA for $\alpha 1$ subunit of $GABA_A$ receptors, accompanied by an increase in receptor binding. Milbrandt et al. (1996) found no significant age-related change in $GABA_A$ receptor binding in the inferior colliculus of 3 and 26 months-old rats. However, Caspary et al. (1999) have found an increase in the expression of $\gamma 1$ subunit of $GABA_A$ receptors in the aged rat inferior colliculus, which resulted in an increase in the efficacy of $GABA_A$ receptors to flux chloride ions. It seems that the subunit composition and hence the functional efficacy of $GABA_A$ receptors are changed in the aged animals and the direction of modulation is different depending on the site of the brain studied. Further studies using in situ hybridisation methods are necessary to determine which $GABA_A$ receptor subunits are expressed in MVN neurones, and whether changes in receptor subunit composition occur during vestibular compensation.

Although the up-regulation of $GABA_A$ mediated inhibition in the aged animals may be a compensatory response to age-related deterioration in the vestibular system, it may also be implicated in the impairment of vestibular compensation in older animals. Recent experiments in young adult rats showed that GABA receptors in MVN neurones undergo significant plasticity during vestibular compensation after unilateral labyrinthectomy (Yamanaka et al., 2000; Johnston et al., 2001). There is a rapid down-regulation in functional efficacy of $GABA_A$ and $GABA_B$ receptors in the de-afferented MVN neurones, together with an up-regulation of their intrinsic excitability (Cameron and Dutia, 1997, 1999; Yamanaka et al., 2001; Johnston et al., 2001). In aged rats (Drago et al., 1996) and humans (Barion and Andretta, 1988), vestibular compensation is known to be significantly slower than in young adults.

While behavioural compensation is certain to involve many component adaptive processes, it is possible that the alteration in $GABA_A$ receptor function in aged rats is implicated in the impairment of compensation in older animals. Thus, if $GABA_A$ receptor subunit composition is significantly different in aged MVN neurones, it is possible that the rapid changes in $GABA_A$ receptor efficacy that occur in the early stage of vestibular compensation in young animals may take place less readily in the older brain. Altered $GABA_A$ receptor subunit composition in aged MVN neurones may also result in changes in their sensitivity to modulation by drugs such as benzodiazepines. Further experiments are necessary to investigate the molecular basis and functional implications of the change in $GABA_A$ receptor function in aged MVN neurones.

Conclusions and Future Experiments

The results presented in this thesis have shown that the horizontal slices of the dorsal brainstem containing the medial vestibular nucleus are viable preparations to study the electrophysiological characteristics of MVN neurones in aged rats and to study cellular mechanisms of vestibular compensation in unilaterally labyrinthectomised animals.

In the second chapter, whole-cell patch clamp recording experiments showed that, based on their action potential profiles, Type A and Type B cells are two distinct subsets of MVN neurones rather than extremes of a single broad population. Type A cells fired repetitively in response to depolarising current pulses without any spike frequency acceleration or spike frequency accommodation. All Type A cells showed a passive return to the baseline at the end of the depolarising current pulses without any plateau potentials. In response to depolarising current pulses, most Type B cells showed spike frequency accommodation that ranged from weak to strong. According to their firing behaviours in response to depolarising and hyperpolarising current pulses, Type B cells can be further classified into Type B_{DP} cells and Type B_{LTS} cells. Type B_{DP} cells showed long lasting plateau potentials at the end of depolarising current pulses delivered from subthreshold membrane potentials. Pharmacological experiments on Type B_{DP} cells suggest that plateau potentials are due to calcium currents mediated by L-type of high voltage activated calcium channels. The majority of Type B_{DP} cells showed spike frequency acceleration over the duration of depolarising current pulses. Ionic conductances responsible for spike frequency acceleration in these cells were not investigated in the present study. However, Ca^{2+} entry through voltage-gated calcium channels could activate calcium-dependent depolarising conductances, such as a calcium-activated non-specific cationic conductance (I_{CAN}). Further intracellular experiments will be useful to identify ionic currents underlying spike frequency acceleration in these cells. In the discussion part of Chapter 2, it was suggested that Type B_{DP} cells could be involved in the velocity storage in vestibulo ocular networks. The exact mechanisms of velocity storage are poorly understood. Further *in vivo* experiments investigating the contribution of Type

B_{DP} cells, particulary high-threshold calcium currents, to the velocity storage will clarify the physiological significance of plateau potentials in these cells. Type B_{LTS} cells showed low-threshold spikes (LTS) at the end of hyperpolarising current pulses delivered from a subthreshold holding membrane potential. Pharmacological experiments on Type B_{LTS} cells suggest that LTS are mediated by calcium currents through T-type low-threshold calcium channels.

The results of the present study demonstrate that pronounced adaptive changes take place in intrinsic membrane properties and firing characteristics of specific subpopulations of vestibular neurones in the rostral region of the ipsilateral MVN during vestibular compensation following UL. Type B cells had significantly higher resting discharges in post-UL slices while Type A cells did not show any change in their *in vitro* firing rates. The increase in *in vitro* excitability shown in previous extracellular experiments is therefore due to changes in only Type B vestibular neurones. The increase in excitability of Type B cells could be due to up-regulation of persistent sodium conductance and/or down-regulation of various potassium conductances. Further voltage-clamp experiments that can quantify individual ionic currents before and after the UL are necessary to understand the mechanisms of the increase in intrinsic excitability during vestibular compensation. Significantly greater number of Type B cells showed low threshold spikes in MVN slices of labyrinthectomised rats suggesting that T-type calcium currents were up-regulated in some MVN cells during vestibular compensation. During the early stage after UL, a greater number of Type A cells expressed spike frequency accommodation, and there was a significant increase in the gain of Type A cells. Although the *in vitro* firing rate of Type A cells did not increase after UL, the changes in their dynamic membrane properties could be important in recovery of neuronal activity within MVN. The results show that the up-regulation of intrinsic excitability in Type B cells is a sustained adaptation in their electrophysiological properties that appears soon after they are subjected to the disfacilitation and excessive commissural inhibition that silences them after UL *in vivo,* and persists through and beyond the period of vestibular compensation when the initial symptoms abate.

The neural network models explaining the mechanisms of vestibular compensation (Cartwright et al., 1999; Cartwright and Curthoys, 1996; Anastasio, 1992; Galiana, 1986; Galiana et al., 1984) have mainly focused on changes of synaptic strength in neuronal networks after UL. These models relied on the assumption that the properties of MVN neurones after the UL are the same as before. The present results question this assumption and clearly show that intrinsic membrane properties of post-synaptic neurones are subject to modulation during vestibular compensation.

The physiological significance of selective increase of excitability in only Type B cells is not clear. The answer to this question may lie in the synaptic connections of Type A and Type B cells. It was suggested that Type A cells may represent Type II cells whereas Type B cells may represent Type I cells. This could be readily tested by electrophysiological experiments in *in vitro* preparations where vestibular nerve and commissural connections are preserved. Future experiments that focus on the projections of individual cell types in MVN to various vestibular related structures will also be valuable in understanding the mechanisms of plastic changes during vestibular compensation as well as physiological function of Type A and Type B cells.

In the third chapter, the tonic activity and GABA receptor function of MVN neurones were studied using extracellular recording technique in slices prepared from young adult and aged rats. It was shown that the spontaneous resting activity of MVN neurones in old and young rats was similar suggesting that the *in vitro* excitability of MVN neurones is maintained in the aged animals. However, there was a selective change in the efficacy of GABA receptors in the aged rats. The responsiveness of the MVN neurones of the aged rats to the $GABA_A$ receptor agonist muscimol was significantly greater than that of the young adult rats, while their responsiveness to the $GABA_B$ receptor agonist baclofen was not significantly different from those in young animals. The increase in $GABA_A$ receptor efficacy may serve to counteract the possible decrease in efficacy of the commissural and cerebellar inhibitory systems with ageing. An increase in receptor number and/or alterations in receptor subunit composition may be responsible for functional

changes in $GABA_A$ receptors. Further experiments using in situ hybridisation methods are necessary to determine which $GABA_A$ receptor subunits are expressed in MVN neurones in young and aged rats and whether changes in receptor subunit composition occur during vestibular compensation. It is not known whether GABA receptors undergo plasticity during vestibular compensation in the aged animals as they do in young rats. A functional study of GABA receptors after the UL in the aged animals will be helpful in understanding the cellular mechanisms of vestibular compensation in the older subjects and may give useful data for future strategies for improving vestibular rehabilitation in old people.

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