MECHANISMS OF PLASTICITY IN THE VESTIBULAR SYSTEM

By

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BSc Hons

Submitted towards a PhD degree

at the Faculty of Medicine

the University of Edinburgh

2004

DECLARATION

The work described in this thesis was carried out by myself, apart from the experiments in Chapter 6 which were completed in collaboration with Dr J. M. Paterson and Dr D. Short. This work was conducted under the supervision of Dr M. B. Dutia in the Department of Biomedical and Clinical Laboratory Sciences, at the University of Edinburgh between October 2000 and September 2003.

Published work from this thesis

1. Guilding, C. Paterson, J.M., Dutia, M.B. (2002). Proteomic analysis of neuronal and synaptic plasticity in the vestibular system. *FENS Abstr.* Vol 1, A093.5
2. Guilding, C., Seckl, J.R., Dutia, M.B. (2003). 11β-Hydroxysteroid dehydrogenase type 1 activity and vestibular neuronal plasticity in rat. *British Neurosci. Assoc. Abstr.* 51, P04.08
3. Guilding, C. and Dutia, M.B. (2003). 11β-Hydroxysteroid dehydrogenase type 1 activity in medial vestibular nucleus and cerebellum after unilateral vestibular deafferentation in the rat. *Stress.* Accepted, May 2004.

ACKNOWLEDGMENTS

Firstly, I would like to thank my supervisor Dr Mayank Dutia for his time, support and for giving me the opportunity to work in his laboratory.

Thanks to all in the lab for their friendship, encouragement and help, and especially to Janet whose arrival brightened up my PhD.

I would like to thank my parents and sisters for their continuing support, enthusiasm and encouragement before, during and after my PhD, and my parents for installing in me the belief that I am capable of doing anything I put my mind to.

Finally, thanks to Andy for his love, friendship, support, and weekends of proofreading.

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ABSTRACT

Vestibular compensation (VC) is the process of behavioural recovery that follows damage to the vestibular receptors of one inner ear or vestibular nerve; it is an attractive model of adult central nervous system plasticity. The first, rapid phase of compensation has been associated with the recovery of a spontaneous resting activity in the ipsilateral vestibular nucleus complex. The mechanisms involved in this compensation are controversial, and a number of theories such as an up-regulation of ipsilateral excitatory postsynaptic receptors, substitution of non-vestibular sensory inputs, reactive synaptogenesis and modifications of the intrinsic properties of the deafferented neurones have been postulated. Vestibular compensation is a complex, heterogeneous process; rapidly developing compensatory mechanisms may be quite distinct from the longer term molecular and neuronal remodelling needed to sustain VC.

In the early stages of vestibular compensation in rat, neurons in the medial vestibular nucleus (MVN) on the lesioned side develop a sustained up-regulation of their excitability and a significant down-regulation of the functional efficacy of inhibitory GABA receptors. These adaptations are in the appropriate direction to promote the recovery of the resting activity of the deafferented MVN neurons *in vivo*, an essential first step in VC. The up-regulation of ipsilesional MVN neuronal excitability is dependent on the activation of glucocorticoid receptors (GR) which presumably occurs during the acute stress response that accompanies the vestibular deafferentation symptoms.

This thesis describes a series of experiments in rats, designed to investigate the underlying molecular causes of VC. The role of the stress system in the adaptive down-regulation of GABA receptors during compensation was investigated using biochemical and *in vitro* electrophysiological techniques. The results demonstrate that the down-regulation of GABA receptor efficacy in ipsilesional MVN neurones observed after 4h of compensation following a unilateral labyrinthectomy (UL) is dependent on activation of glucocorticoid receptors. The enzyme, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) regenerates active glucocorticoids

from inert forms, and is found to be present in the central vestibulo-oculomotor centres of the brainstem and cerebellum. This suggests that this modulatory enzyme is likely to be involved in regulating the exposure of these centres to circulating glucocorticoids. The results show that the levels of 11β-HSD1 activity in the vestibulo-cerebellum and MVN are stable over the 4h period after UL, disproving the hypothesis that changes in enzyme modulatory activity may occur in parallel with the deafferentation induced changes in the properties of the ipsilesional MVN neurones over this time.

The relative importance of intrinsic membrane properties versus synaptic inputs to the spontaneous firing rate of ipsilateral MVN neurones was investigated at varying times following UL. The endogenous activity of MVN neurones was assessed by their spontaneous activity recorded in brainstem slices perfused with a cocktail of neurotransmitter antagonists to block synaptic transmission. The results demonstrate a significant increase in excitability of lesioned rostral MVN neurones at 4h post-UL, which is maintained primarily by changes in the intrinsic pacemaker properties of these neurones. At 48h and 1wk post-UL the significant increase in excitability of lesioned rostral MVN neurones is sustained, however by this time it is maintained by an increased excitatory synaptic input onto the neurones. Thus different mechanisms are utilised in the initiation and maintenance of processes involved in VC.

Finally, the powerful techniques of quantitative two-dimensional gel analysis and mass spectroscopy were used to assess changes in protein expression in the MVN and vestibulo-cerebellum during VC. The results indicate changes between the ipsilesional and contralesional MVN, in the expression of several proteins at 1wk post-UL, and a number of known proteins which are expressed in the MVN are identified.

ABREVIATIONS

11β-HSD	11β-hydroxysteroid dehydrogenase
11-DHC	11-dehydrocorticosterone
2-DGE	2-dimensional gel electrophoresis
ACh	acetylcholine
aCSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
ADX	adrenalectomised
AVP	arginine vasopressin
CNS	central nervous system
CRF	corticotrophin releasing factor
DEX	dexamethasone
EPSP	excitatory postsynaptic potential
FTN	floccular target neuron
GAD	glutamic acid decarboxylase
GR	glucocorticoid receptor
h	hour
HPA	hypothalamo-pituitary-adrenocortical
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
i.c.v.	intracerebroventricular
IO	inferior olive
i.p.	intraperitoneal
IPG	immobilised protein gradient
IPSP	inhibitory postsynaptic potential
ir	immunoreactivity
ISH	*in situ* hybridisation
IVN	inferior vestibular nucleus
LTP	long term potentiation
LTD	long term depression
LVN	lateral vestibular nucleus

mGluR	metabotropic glutamate receptor
MLF	medial longitudinal fasciculus
MR	mineralocorticoid receptor
MS	mass spectrometry
MVN	medial vestibular nucleus
NO	nitric oxide
PKC	protein kinase C
Psol	parasolitary nucleus
RHT	roll head tilt
S.E.M	standard error of the mean
SN	spontaneous nystagmus
SVN	superior vestibular nucleus
UL	unilateral labyrinthectomy
UVD	unilateral vestibular deafferentation
VC	vestibular compensation
VOR	vestibulo-ocular reflex
VCR	vestibulo-colic reflexes
VN	vestibular nuclei
VNC	vestibular nucleus complex
VSR	vestibular-spinal reflexes
wk	week
YHT	yaw head tilt

INTRODUCTION

The maintenance of a stable gaze and controlled orientation of the body in space are essential aspects of survival. The vestibular system is the sensorimotor system concerned with the sensation of head movement and with generation of reflexes for stabilising gaze (vestibulo-ocular reflexes, VORs) and body posture (vestibulo-colic reflexes, VCRs; vestibulo-spinal reflexes, VSRs). The vestibular nuclei in the brainstem are the core of vestibular information processing; they receive inputs from the peripheral vestibular organs, visual system, somatosensory system, cerebellum and spinal cord and process appropriate motor commands. The vestibular nucleus complex (VNC) consists of four main nuclei: the medial, lateral, superior and inferior nuclei. Of these, the medial (MVN) and lateral vestibular nuclei (LVN) are most extensively studied.

The complex and often convergent processing of several types of sensory inputs requires the vestibular system to learn and adapt to often conflicting sensory cues following lesions or alteration of the subject's environment. A remarkable example of vestibular plasticity occurs after damage to one vestibular labyrinth (unilateral labyrinthectomy, UL) or nerve (unilateral vestibular deafferentation, UVD). This results in a severe syndrome of oculomotor and postural symptoms. These symptoms are divided into two categories: *static symptoms* persist in the absence of head movement (e.g. spontaneous ocular nystagmus, yaw and roll head tilts); and *dynamic symptoms* occur as a result of head movement (e.g. abnormal gain and phase of the VORs and VCRs) (for review, see Smith and Curthoys, 1989). Remarkably, many of the static symptoms of unilateral vestibular damage disappear over a few days in most species, as part of a process termed vestibular compensation. Vestibular compensation is independent of functional regeneration of the deafferented vestibular nerve and is therefore ascribed to central nervous system plasticity.

The study of vestibular compensation is important for a number of reasons. Firstly, understanding how the vestibular system functions following damage lends us information on how the system functions normally. Secondly, research into the components of vestibular compensation may contribute to the development of effective treatments for vestibular disorders in humans. Thirdly, vestibular

compensation represents an interesting model of central nervous system plasticity in adults: it has well defined and relatively simple inputs and outputs, which can be quantified both on neuronal and behavioural levels. It seems likely that mechanisms of plasticity defined in the vestibular system could extrapolate to plasticity in other brain regions. For these reasons vestibular compensation has been the focus of a large amount of research over the last 40 years. However, despite documentation of many behavioural, anatomical and physiological aspects of compensation, the mechanisms underlying functional recovery, and the position in CNS where they reside are not well understood.

The aim of this thesis is to investigate some of the neuronal, molecular and biochemical substrates underlying vestibular compensation, as outlined below. The thesis will be divided into 6 main chapters:

1) The general anatomy, pharmacology, electrophysiological properties and function of the vestibular system.

2) The neuronal, pharmacological and structural components of vestibular compensation, and a discussion of possible mechanisms of compensation.

3) An electrophysiological investigation into the role of the stress axis in the down-regulation of GABA receptors in the ipsilesional MVN during vestibular compensation.

4) An investigation into the presence of the glucocorticoid metabolising enzyme, 11β-hydroxysteroid dehydrogenase type 1, in the vestibular system.

5) An electrophysiological and pharmacological investigation into the mechanisms underlying changes in the neuronal discharge rates of MVN neurones *in vitro*, during vestibular compensation.

6) Development of a proteomics technique to examine changes in protein expression during compensation.

Chapter 1

VESTIBULAR FUNCTION

1.1 VESTIBULAR SYSTEM: ANATOMY AND FUNCTION

The vestibular system is the sensorimotor system which senses the position and movement of the head and body in space, and uses that information to control posture and stabilise vision. The peripheral vestibular organs are situated in the bony labyrinth of the temporal bone, and consist of the semicircular canals and otolith organs. There are three semicircular canals; horizontal, anterior and posterior, orientated approximately in each plane of three dimensional space, detecting angular (up-down, tilting and side to side) rotations of the head. The otolith organs consist of the utricle and saccule; they are orientated perpendicular to each other and signal linear acceleration and head orientation relative to earth's gravity. The arrangement of the semicircular canals and otolith organs allows the most efficient signalling of head position in three-dimensional space. Vestibular hair cells in the peripheral vestibular organs detect movement and position of the head. This information is relayed via the vestibular portion of the eighth cranial nerve to the vestibular nuclei in the brainstem. The primary vestibular afferents carried by the eighth nerve are bipolar cells, their cell bodies located in Scarpa's ganglion. There are four main vestibular nuclei on either side of the brainstem: the superior, inferior, medial and lateral vestibular nuclei. Each vestibular nucleus receives at least some primary afferent fibre input, which is partly endorgan specific. For example, the medial vestibular nucleus (MVN) receives afferents mainly from the horizontal canal and utricle, the superior vestibular nucleus (SVN) from the anterior and posterior canals, and the lateral vestibular nucleus (LVN) and inferior vestibular nucleus (IVN) from the otolith organs. This primary vestibular information converges in the vestibular nuclei with visual and somatosensory information to provide an internal 3-D representation of body position and movement in space.

The function of the vestibular system is the maintenance of a stable gaze and body posture, essential in many behaviours such as visuo-motor co-ordination, spatially orientated tasks and visual tracking of objects in the external world. In addition, the vestibular system appears to contribute to a cognitive perception of spatial orientation via a hippocampal projection which provides information on spatial environment and self-movement (Russell *et al.*, 2003). The vestibular nuclei

also provide efferent connections to target oculomotor and spinal motorneurones, which give rise to inter-related families of reflexes; the vestibulo-ocular reflexes (VORs), vestibulo-colic reflexes (VCRs) and the vestibulo-spinal reflexes (VSRs). VORs help stabilise the retinal image during head rotation, by rotation of the eyes in the orbits by a nearly equal and opposite amount to head movement. The most direct pathway from the peripheral vestibular organs to the eye muscles in the vertebrate, is the three-neurone VOR arc.

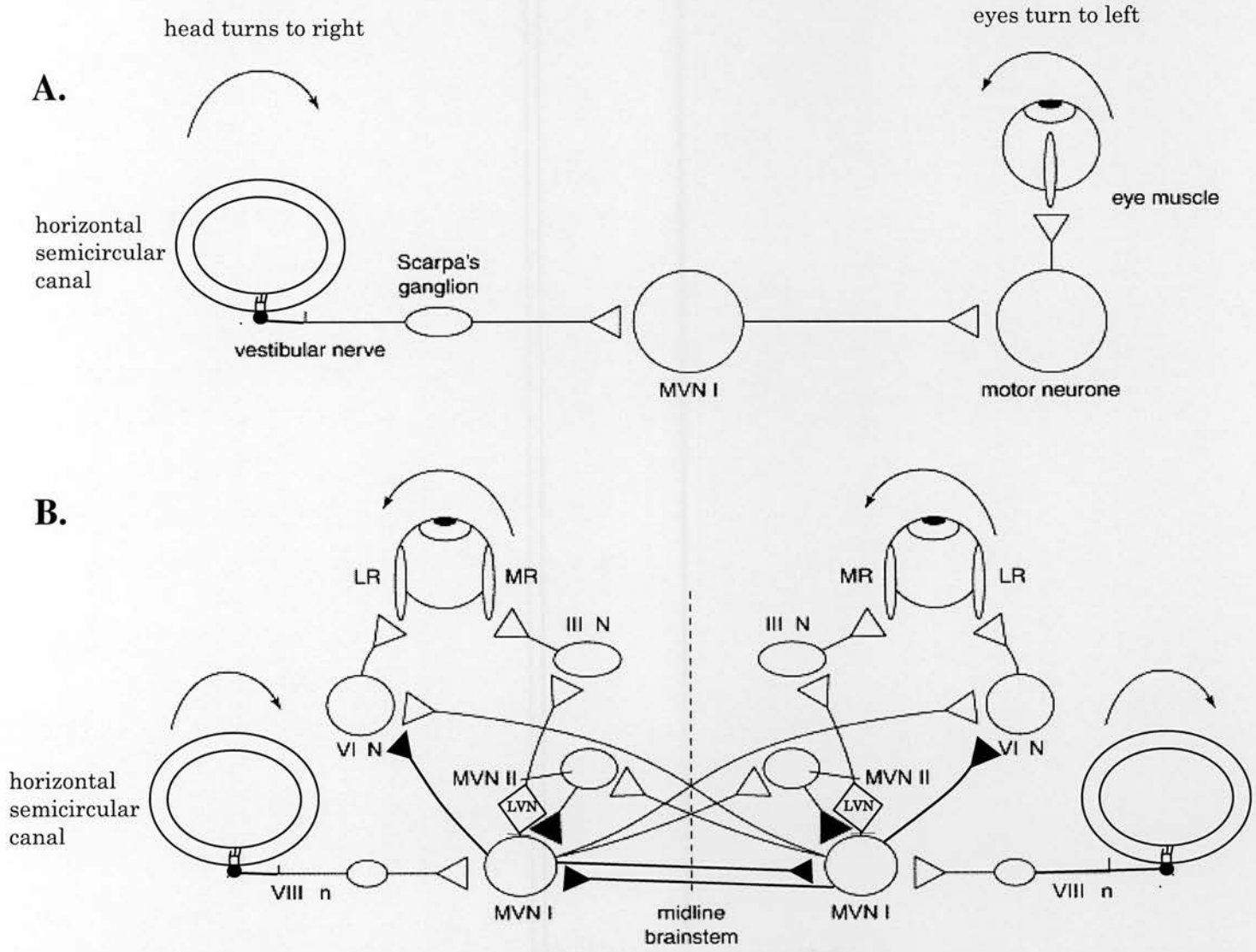
This three-neurone arc, schematically represented in figure 1.1 consists of:
1) First order canal afferents located in Scarpa's ganglion, connecting the sensory hair cells with neurones in the vestibular nuclei.
2) Second order vestibular afferents located in the vestibular nuclei, sending their axons via the medial longitudinal fasciculus to the oculomotor nuclei.
3) The motorneurones located in the oculomotor nuclei, innervating the various extra-ocular muscles.

Vestibular nuclei, with the exception of the SVN, project to the spinal cord via the lateral, caudal and medial vestibulospinal tracts. Vestibulo-spinal reflexes give rise to the reflex excitation of motorneurones of postural and extensor muscles to control the trunk and limbs against gravity during movement. Vestibulo-colic reflexes give rise to the reflex excitation of the appropriate groups of neck muscles to resist and counteract head displacement, thus acting to stabilise the head on the trunk. The integrated stabilisation of eyes, head and trunk through VORs, VCRs and VSRs provides and essential base on which to execute co-ordinated eye, head and body movements.

Figure 1.1 Schematic diagram of the neuronal network mediating the horizontal vestibulo-ocular reflex (hVOR)

A. Schematic diagram of the three synapses mediating the three neurone VOR arc.

B. Simplified schematic diagram of some of the pathways involved in the horizontal VOR. The medial vestibular nucleus type II neurone (MVN II) exhibits an increase in firing rate for horizontal angular acceleration to the contralateral side, and MVN I exhibits an increase in firing rate for horizontal angular acceleration to the ipsilateral side. LR, lateral rectus muscle; LVN, lateral vestibular nucleus; MR, medial rectus muscle; III N, oculomotor nucleus; VI N, abducens nucleus; VIII n, eighth nerve. Open triangles represent excitatory synapses, filled circles represent inhibitory synapses. Many other projections are activated, either directly or indirectly by vestibular afferent inputs and other pathways, which are not shown in this simplified diagram. (Adapted from Smith and Darlington, 1996)


A.
head turns to right
eyes turn to left
horizontal semicircular canal
Scarpa's ganglion
eye muscle
vestibular nerve
MVN I
motor neurone
B.
LR
MR
III N
MR
LR
III N
VI N
VI N
MVN II
MVN II
LVN
LVN
horizontal semicircular canal
VIII n
VIII n
MVN I
midline brainstem
MVN I

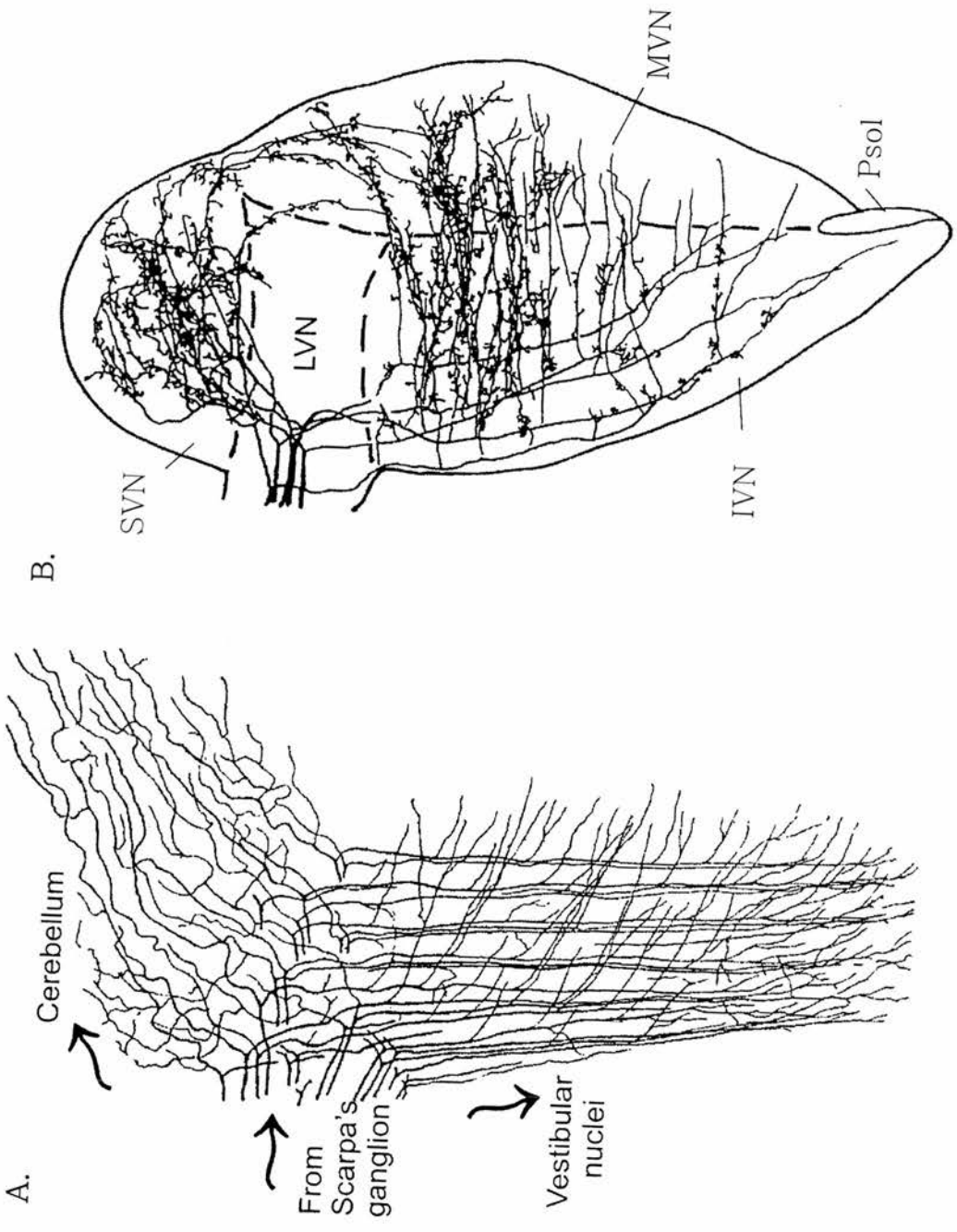
1.1.1 Afferent Connections of the Vestibular Nuclei

Primary vestibular afferents, originating from the vestibular organs and carried by the eight nerve, connect to the vestibular nuclei in the brainstem. There is a differential distribution of vestibular afferents within parts of each vestibular nucleus; portions not considered to receive primary vestibular afferents include the dorsal half of the LVN, peripheral parts of the SVN and caudal and medial parts of the MVN (see Figure 1.2B). Primary vestibular afferents bifurcate as they enter the brainstem into ascending and descending pathways. Ascending pathways project dorsomedially and rostrally through parts of the LVN to terminate in the SVN, rostral MVN and cerebellum; descending pathways project to the IVN issuing collaterals to the ventral LVN and the MVN (see Figure 1.2A) (Brodal, 1974; Korte, 1979; Mehler and Rubertone, 1985; Carpenter, 1988a; Sato *et al.*, 1989; Newlands and Perachio, 2003). Anatomical studies using axon degeneration or anterograde labelling have demonstrated that the VNC receives an endorgan specific distribution of primary vestibular afferents: ipsilateral semicircular canal afferents terminate predominantly in the rostral MVN and SVN, and otolith afferents terminate in the IVN, LVN and lateral MVN (Stein and Carpenter, 1967; Gacek, 1969; Brodal, 1972, 1974; Carleton and Carpenter, 1983; Sato *et al.*, 1989). Precht and Shimazu (1965) were the first to demonstrate that second order neurones in the MVN were monosynaptically and polysynaptically activated by electrical stimulation of the ipsilateral eighth nerve. Subsequently, Wilson *et al.* (1967) showed that cells responding monosynaptically to vestibular nerve stimulation were mainly concentrated in the rostral MVN, correlating with the anatomical data. Although the vestibular nerve provides a large portion of afferents to the vestibular nuclei, inputs to these nuclei are also derived from other sensory systems such as visual and somatosensory systems and other brain regions such as the cerebellum and brainstem nuclei (for reviews, see Brodal, 1974; Barmark, 2003). Indeed the cerebellum provides a major tract projecting to the vestibular nuclei (see Section 1.1.5).

Figure 1.2 Projection of primary vestibular afferents to the vestibular nucleus complex and cerebellum

A. Primary vestibular afferents bifurcate as they enter the brainstem into ascending and descending pathways.

B. An illustration of the terminal fields of five primary vestibular afferents in cat, intra-axonally labelled with HRP. The terminals are mapped onto a schematic that represents a horizontal section through the vestibular complex. IVN, inferior vestibular nucleus; LVN, lateral vestibular nucleus; MVN, medial vestibular nucleus; Psol, parasolitary nucleus; SVN, superior vestibular nucleus. (Adapted from Barmack, 2003; Sato and Sasaki, 1993)


A.
Cerebellum
From Scarpa's ganglion
Vestibular nuclei
B.
SVN
LVN
MVN
IVN
Psol

1.1.2 Efferent Connections from the Vestibular Nuclei

Vestibulo-ocular pathways

Physiological and anatomical studies have shown that efferent fibres from the vestibular nuclei, particularly the MVN and SVN, ascend in the medial longitudinal fasciculus (MLF) to the motorneurones in the oculomotor nuclei (Wilson *et al.*, 1968; Gacek, 1969, 1974; Tarlov, 1970; Isu and Yokota, 1983; Langer *et al.*, 1986). The six extraocular muscles of each eye are innervated by motorneurones in the oculomotor, trochlear and abducens nuclei via the IIIrd, IVth and VIth cranial nerves respectively (Wilson and Melvill Jones, 1979). The abducens and trochlear nuclei each supply only one muscle, the ipsilateral lateral rectus muscle and contralateral superior oblique respectively. The oculomotor nucleus innervates the ipsilateral inferior and medial recti, and inferior oblique, and the contralateral superior rectus. Fibres from the SVN enter the ipsilateral MLF and branch to the ipsilateral trochlear and oculomotor nuclei, and the contralateral oculomotor nucleus. Fibres from the MVN enter the ipsilateral and contralateral MLF and project bilaterally to the abducens and oculomotor nuclei and contralaterally to the trochlear nucleus (Baker *et al.*, 1969; Wilson and Melvill Jones, 1979). Electrical stimulation of the eighth nerve or horizontal canal afferents, causes a disynaptic excitatory postsynaptic potential (EPSP) in the contralateral abducens motorneurones and a disynaptic inhibitory postsynaptic potential (IPSP) in the ipsilateral abducens motorneurones (Precht *et al.*, 1969; Highstein, 1973; Schwindt *et al.*, 1973; Babalian *et al.*, 1997). Similar potentials, but monosynaptic in their latency could be evoked in the abducens by stimulation of the rostral MVN (Baker *et al.*, 1969). Thus the anatomical and physiological data indicate that one aspect of the horizontal VOR reflex, the three neurone VOR arc, originates in the horizontal canal, relays in the rostral MVN, which then projects to the oculomotor nuclei causing contractions of the extraocular muscles sufficient to rotate the eyes in an equal and opposite manner to head movement (see Figure 1.1).

Vestibulo-spinal pathways

Activation of the vestibular organs leads to various reflexes of the body and limbs sufficient to result in the stabilisation of head position and visual field. Afferents from the vestibular organs are processed in the vestibular nuclei and relayed to the spinal cord by vestibular efferents. The major links between the VNC and the spinal motor centres consists of the lateral vestibulospinal tract (LVST) and medial vestibulospinal tract (MVST), although activity is also relayed by the reticulospinal tract and the caudal vestibulospinal tract (Wilson and Melvill Jones, 1979). The LVST originates from the LNV and IVN and projects ipsilaterally to all segments of the spinal cord where it synapses on extensor motorneurone pools (Brodal *et al.*, 1962). The MVST originates primarily in the MVN, but the LVN and IVN also contribute. It projects bilaterally, predominantly to the ventral horn of the cervical spinal cord from C1 to C6 (Nyberg-Hansen, 1964; Wilson *et al.*, 1967). There are also fibres from the MVN innervating neck motorneurones (Wilson, 1972). These motorneurones receive disynaptic excitation and inhibition from all 6 semicircular canals and constitute the short latency pathway for the vestibulo-colic reflex.

1.1.3 Commissural connections

Anatomical studies using axonal degeneration techniques and anterograde and retrograde labelling techniques have demonstrated the presence of a commissural pathway that interconnects the bilateral MVN, SVN and IVN, but only the ventromedial portions of the LVN (Cat: Ladpli and Brodal, 1968; Gacek, 1978; Carleton and Carpenter, 1983; Ito *et al.*, 1985; Monkey: Carleton and Carpenter, 1983; Rat: Rubertone *et al.*, 1983; Rabbit: Epema *et al.*, 1988; Gerbil: Newlands *et al.*, 1989). Horseradish peroxidase (HRP) retrograde labelling techniques have identified commissural pathways linking contralateral vestibular nuclei, the MVN having the most extensive commissural network, projecting to the SVN, LVN and IVN (Rubertone *et al.*, 1983; Ito *et al.*, 1985; Epema *et al.*, 1988). Electrophysiological studies demonstrated that stimulation of the contralateral vestibular nerve evokes field potentials in the ipsilateral vestibular nuclei (Shimazu

and Precht, 1966; Nakao *et al.*, 1982; Furuya *et al.*, 1992). Midline incision which interrupted the commissural fibres, abolished this response (Precht *et al.*, 1966). In mammals the majority of commissural neurones are GABAergic (Furuya *et al.*, 1992; Holstein *et al.*, 1999b). Babalian *et al.* (1997) showed that in the guinea pig, contralateral vestibular nerve stimulation produced inhibitory synaptic potentials with either di- or trisynaptic latencies in 75% of second order MVN neurones. The remaining 25% of neurones responded with excitatory, or mixed excitatory and inhibitory synaptic potentials. In commissural pathways with disynaptic latencies, an inhibitory second order neurone in the contralateral MVN sends its axon in the commissural pathway to inhibit second order ipsilateral MVN neurones; in the trisynaptic pathway, excitatory commissural afferents (MVN I in Fig. 1.1) stimulate inhibitory interneurones in the ipsilateral MVN (MVN II in Fig. 1.1) which inhibit second order MVN neurones.

1.1.4 Intrinsic connections

Iontophoretic microinjections of HRP into individual vestibular nuclei have revealed highly organised and extensive internuclear connections between individual vestibular nuclei in rat (Rubertone *et al.*, 1983), cat, monkey (Carleton and Carpenter, 1983), and rabbit (Epema *et al.*, 1988). The SVN, IVN and MVN are reciprocally connected (Epema *et al.*, 1988). The IVN projects to the LVN and the LVN projects to the SVN but these connections are not reciprocated (Ito *et al.*, 1985); the MVN is reciprocally connected to the LVN (Rubertone *et al.*, 1983). In addition to the connections between individual subnuclei, there are reciprocal connections between the rostral and caudal MVN (Pompeiano *et al.*, 1978; Epema *et al.*, 1988).

1.1.5 Cerebellum

Cerebello-vestibular afferents

The vestibular nuclei receive extensive afferent projections from the area of the cerebellum known as the vestibulo-cerebellum, consisting of the uvula-nodulus,

flocculus, paraflocculus and fastigial nuclei. The uvula and nodulus differ in some respects in their vestibular projections. The uvula and nodulus both send fibres to the peripheral SVN, the ventrocaudal IVN and some sparse fibres to the LVN. The MVN receives no uvula fibres whereas the nodulus sends a dense projection of fibres to the caudal MVN (see Figure 1.3) (Brodal, 1974; Carleton and Carpenter, 1984; Shojaku *et al.*, 1987; Walberg and Dietrichs, 1988). Immunohistochemical tracing studies show that fibres from the flocculus terminate ipsilaterally, mainly in the central SVN and rostral region of the MVN, with some sparse labelling in the LVN and IVN (Fig. 1.3) (Anguat and Brodal, 1967; Brodal, 1974; Carleton and Carpenter, 1984; Langer *et al.*, 1985; Nagao *et al.*, 1997). The areas of the MVN and SVN that receive floccular fibres approximately coincide with the areas that receive primary vestibular afferents and that are involved in the VOR pathways (compare Figs. 1.2 and 1.3).

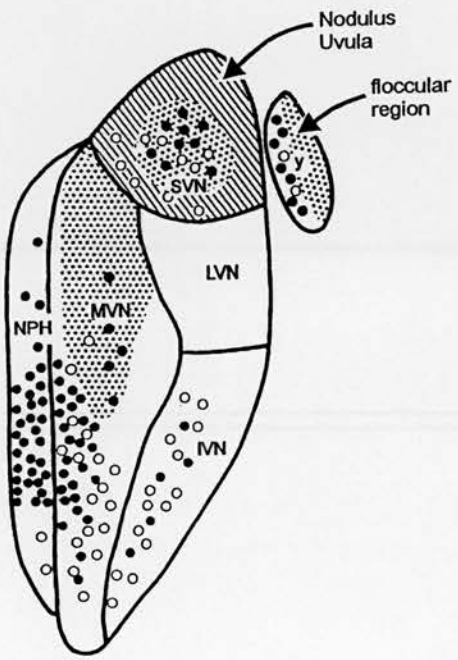


Figure 1.3 A schematic diagram summarising connections between the vestibulocerebellum and vestibular nuclei. Cells projecting to the floccular region are represented as filled circles, cells projecting to the nodulus and uvula as open circles. Areas where cerebellar efferents from the floccular region terminate are indicated by fine dots, and inputs from the nodulus and uvula are depicted by cross hatching. Abbreviations as in Fig. 1.2. NPH, nucleus prepositus hypoglossi; Y, Y-group.

Electrophysiological experiments have identified floccular Purkinje cells that inhibit both the excitatory and inhibitory branches of the VOR pathways to various oculomotor muscles. These actions are mediated through inhibitory monosynaptic projections to vestibular nucleus neurones called "flocculus target neurones" (FTNs). Babalian and Vidal (2000) used an *in vitro* whole brain study in guinea pig to assess floccular modulation of the horizontal VOR pathway. Stimulation of the ipsilateral flocculus significantly decreased the inhibitory field potential evoked in the abducens nucleus by ipsilateral vestibular nerve stimulation, but had no effect on responses evoked in abducens nuclei after contralateral nerve stimulation. Thus the flocculus exerts inhibitory actions on inhibitory second order vestibular neurones activated by the ipsilateral vestibular nerve and projecting to the ipsilateral abducens nucleus. Projections of the flocculus do not converge with vestibular afferent inputs on excitatory second order vestibular neurones projecting to the contralateral abducens nucleus. FTNs, identified electrophysiologically by the monosynaptic IPSPs recorded after ipsilateral floccular stimulation, constitute around 15 to 20% of all recorded second order rostral MVN neurones (Stahl and Simpson, 1995; Babalian and Vidal, 2000). Recently, a technique has emerged which enables the visualisation of FTNs *in vitro*, allowing an investigation of the electrophysiological properties of FTNs. Sekirnjak *et al.* (2003) used the Purkinje cell specific L7 protein promoter to express a green fluorescent protein (GFP) fusion protein, selectively in Purkinje cells. GFP positive axons and terminals were sparse in the MVN projecting onto just 1% of all MVN neurones, all of which were concentrated in the rostral region. Ipsilateral flocculectomy abolished the expression of GFP in rostral MVN neurones, indicating a floccular rather than nodular origin of these fibres. The intrinsic firing properties of the identified FTNs differed markedly from non-FTNs (the surrounding non-GFP labelled MVN neurones). FTNs had exceptionally high spontaneous firing rates, lower action potential thresholds and significantly smaller after-hyperpolarisations than non-FTNs. After a membrane hyperpolarisation, FTNs displayed dramatic postinhibitory rebound firing, whereas non-FTNs exhibit little or no rebound firing (Sekirnjak *et al.*, 2003).

Vestibulo-cerebellar afferents

Vestibular information is fed to the cerebellum through primary and secondary vestibulo-cerebellar projections. Primary vestibular projections consist of eighth nerve afferents (Fig. 1.2A). Several folia of the cerebellum receive scattered projections from primary vestibular afferents, however more than 90% of terminals are restricted to the ipsilateral uvula-nodulus (Korte and Mugnaini, 1979; Gerrits *et al.*, 1989; Barmack, 2003). Dow (1936) originally showed that the uvula-nodulus, but not the flocculus or paraflocculus receive primary vestibular afferent projections. Subsequent studies in monkey (Carleton and Carpenter, 1983; Nagao *et al.*, 1997) and cat (Brodal and Hoivik, 1964) using silver impregnation, retrograde axonal transport and autoradiography techniques, revealed a direct primary vestibular projection to the flocculus and paraflocculus. However the use of similar techniques in rabbit, failed to find any projections to the paraflocculus and either very sparse or no projections were observed to the flocculus (Gerrits *et al.*, 1989; Barmack *et al.*, 1993). In all studies, the projections to the uvula-nodulus terminated as mossy fibres exclusively on the ipsilateral side of the brain.

Secondary vestibulo-cerebellar afferents project to the uvula-nodulus, flocculus, paraflocculus and folia 1 of the anterior lobe (Carleton and Carpenter, 1984; Gerrits *et al.*, 1989; Barmack *et al.*, 1992c). The granule cell layer of the uvula-nodulus receives a bilateral vestibular projection from the MVN, IVN and SVN, a major fraction of which originates in the caudal MVN and is cholinergic (Barmack *et al.*, 1992a; Jaarsma *et al.*, 1997). Rubertone and Haines (1981) reported that in monkey, the ventromedial MVN projects to the ventral paraflocculus, and the dorsomedial MVN and dorsal IVN project to the dorsal paraflocculus. The flocculus receives secondary vestibular fibres from discrete regions of the vestibular nucleus. In cat, projections to the flocculus were traced from the ventromedial and dorsomedial MVN, the ventromedial rostral IVN and the central area of the SVN (Sato *et al.*, 1983). This and another study in cat could find no vestibular nucleus projection to the paraflocculus (Kotchabhakdi and Walberg, 1978; Sato *et al.*, 1983). The discrepancies of vestibulo-paraflocculus projections could be due to species differences between cat and monkey. Electrophysiological studies have demonstrated that rostral MVN neurones send fibres to either the ipsilateral or contralateral

flocculus, with a strong contralateral preference (Shinoda and Yoshida, 1975; Cheron *et al.*, 1996).

1.1.6 Inferior olive

The inferior olive (IO) is proposed to be involved in the learning and timing of motor behaviour. It is the sole source of climbing fibres innervating Purkinje cells in the cerebellum, while the Purkinje cells are themselves the sole source of the output signals from the cerebellum to the vestibular nuclei (for review, see De Zeeuw *et al.*, 1998b). The mammalian inferior olive is divided into very specific functional subgroups, composed of: the principle olive, the dorsal and medial accessory olives and several smaller subnuclei such as the ventrolateral outgrowth, dorsal cap of Kooy, β nucleus and the dorsomedial cell column (Whitworth and Haines, 1986). In general, each olivary subnuclei projects contralaterally to one or more cerebellar zones of Purkinje cells, which in turn project to a particular set of cerebellar or vestibular nuclei. Anatomical studies using HRP injections have mapped climbing fibre projections from the contralateral dorsal cap and ventrolateral outgrowth to the flocculus, and from the contralateral dorsal cap, dorsomedial cell column and β nucleus to the uvula-nodulus (Yamamoto, 1979; Sato and Barmack, 1985; Bernard, 1987; Maekawa *et al.*, 1989). Cerebellar nuclei project reciprocally to the olivary subnucleus from which they receive collaterals, but also have an additional variable ipsilateral component (Ruigrok, 1997). The olivary projections to the cerebellum are excitatory, probably glutamatergic, whereas the cerebellar projections to the IO are GABAergic (Barmack, 2000).

The vestibular nuclear complex and IO are connected, although the only direct connection from the four main vestibular nuclei is from the LVN to IO (Matesz *et al.*, 2002). The MVN and IVN provide secondary vestibular afferent projections into the ipsilateral parasolitary nucleus (Psol; a small GABAergic nucleus that receives a primary vestibular afferent projection), this projects ipsilaterally to the β-nucleus and dorsomedial cell column of the IO (Barmack, 1996). The β-nucleus and dorsomedial cell column project to the contralateral uvula-nodulus, which in turn provides an ipsilateral GABAergic projection to the Psol (Barmack and Yakhnitsa, 2000).

1.2 ELECTROPHYSIOLOGICAL PROPERTIES OF VESTIBULAR NEURONES

1.2.1 *In vivo* properties of vestibular nucleus neurones

Both primary vestibular afferents and second order vestibular neurones display spontaneous resting activity in the absence of head movement, enabling neurones to respond bi-directionally. MVN neurones *in vivo*, can be activated monosynaptically and polysynaptically by electrical stimulation of the vestibular nerve (Precht and Shimazu, 1965; Goldberg and Fernandez, 1971). In addition, many neurones in the rostral region of the MVN respond to angular acceleration of the head in both the horizontal and vertical plane (Shimazu and Precht, 1965; Markham, 1968; Curthoys and Markham, 1971). Neurones responding to canal stimulation have been divided into two main types according to whether it is the ipsilateral or contralateral canal that evokes them: a type I neurone increases its discharge frequency with ipsilateral canal stimulation and decreases it with contralateral stimulation whereas type II neurones have the opposite response. Shimazu and Precht (1965) recorded neuronal responses in the VNC of decerebrate cats after stimulation of the canals; 67% of neurones examined showed type I responses and 29% type II responses. Two smaller subsets of responses were recorded; type III neurones increased their discharge frequency with rotation in either direction, and constituted 3% of recorded neurones, and type IV neurones decreased firing with rotation in either direction and constituted 1% of recorded neurones. Melvill Jones and Milsum (1970) reported similar proportions of type I and type II responses *in vivo* in the cat, yet in the awake monkey, Fuchs and Kimm (1975) showed equal proportions of type I and II responses. Different nuclei show different proportions of type I and type II cells: the MVN is mostly type I whereas the LVN is mostly type II (Carpenter, 1988b).

Shimazu and Precht (1965; Precht and Shimazu, 1965) further classified type I neuronal responses into tonic and kinetic. Kinetic neurones comprised 20% of type I cells and were characterised by no resting discharge, monosynaptic excitation by ipsilateral vestibular nerve stimulation and an increase or decrease in their discharge frequencies along steep slopes during rotational stimulation. Tonic neurones comprised 80% of type I cells and were characterised by spontaneous activity at rest,

polysynaptic activation in response to a single electric shock to the ipsilateral vestibular nerve and less steep response sensitivities to activation or cessation of rotational stimulation.

1.2.2 *In vitro* properties of vestibular nucleus neurones

Gallagher *et al.* (1985), using extracellular and intracellular recording techniques in rat, were the first to demonstrate that MVN neurones fire spontaneous action potentials in brainstem slices *in vitro*, where all inputs to the nucleus are cut. Subsequent studies in the MVN have confirmed these findings, recording *in vitro* firing rates ranging from 0.5-60 spikes/s, with a mean firing frequency between 10-25 spikes/s (Lewis *et al.*, 1987; Ujihara *et al.*, 1988, 1989; Darlington *et al.*, 1989; Doi *et al.*, 1990; Smith *et al.*, 1990; Serafin *et al.*, 1991a,b; Dutia *et al.*, 1992; Johnston *et al.*, 1994). These firing rates are comparable with those recorded *in vivo* (Precht and Shimazu, 1965; Shimazu and Precht, 1965; Goldberg and Fernandez, 1971; Hamann and Lannou, 1987; Ris *et al.*, 1995), which was initially surprising as it was assumed that a large component of the resting activity of MVN neurones was provided by excitatory vestibular nerve afferents.

A major component of the spontaneous activity is generated by an intrinsic mechanism, evidenced by its persistence after blockage of synaptic transmission with calcium blocking agents or by perfusion of the slice with low Ca^{2+}/ high Mg^{2+} medium. From their intracellular recordings in rat, Gallagher *et al.* (1985) identified two spontaneously active subtypes of MVN neurones, distinguished by their differing after-hyperpolarisation potential (AHP) profiles: one group showing a single AHP the other a double (See Figure 1.4). Subsequently Serafin and colleagues (1991a,b), using intracellular recordings from guinea pig brainstem slices, classified three subtypes of MVN neurones on the basis of their AHP profiles. Type 'A' MVN neurones constituted about 30% of the spontaneously recorded cells and were characterised by a broad action potential and a single deep AHP. Type 'B' MVN neurones comprised about 50% of recorded cells and displayed a narrow action potential with an initial fast AHP followed by a delayed slow AHP. A third subtype of non-homogeneous cells, type 'C' was classified; their action potential shapes and

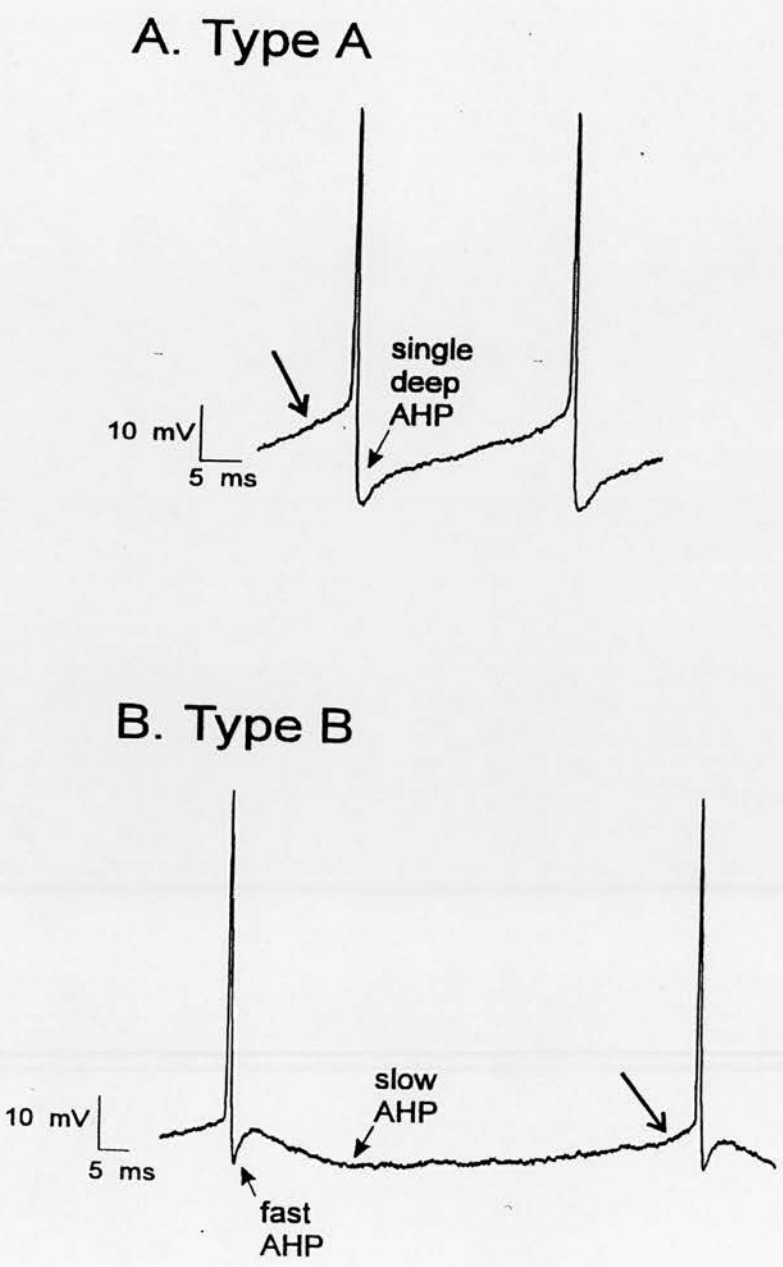


Figure 1.4 Intracellular recordings of spontaneous action potentials in type A and type B MVN neurones. Spontaneous action potentials arise from a gradual membrane depolarisation preceding the spike (arrows).

A. Type 'A' neurone, displaying a single deep AHP

B. Type 'B' neurone, displaying an initial fast, followed by delayed slower, AHP

(Adapted from the PhD thesis of A.R. Johnston (1995), University of Edinburgh)

AHP profiles indicated an intermediate subtype, unable to be classed as type A or type B (Serafin *et al*, 1991a, b).

In confirmation of Gallagher's original observations, subsequent detailed intracellular experiments using spike shape averaging, indicated the existence of only two subtypes of MVN neurones in rat (Johnston *et al.*, 1994; Him and Dutia, 2001). Type A neurones (33% neurones recorded by Johnston *et al.*, 1994) exhibit a single deep AHP mediated by an apamin-insensitive Ca^{2+} activated K^+ conductance and a tetra-ethyl ammonium (TEA)-sensitive K^+ conductance. Type B neurones (67% neurones recorded by Johnston *et al.*, 1994) exhibit an early fast AHP and a delayed slow AHP, mediated by a TEA-sensitive K^+ conductance and an apamin sensitive Ca^{2+} activated K^+ conductance respectively (see Fig. 1.4).

There is no difference between the firing rates of type A and type B neurones *in vitro*. As mentioned above the two MVN cell subtypes differ in their active membrane conductances, therefore different mechanisms regulate their tonic firing. Type A neurones have a TEA sensitive K^+ conductance (probably I_K), which is responsible for action potential repolarisation. An A-like outwardly rectifying K^+ conductance may also play a part in spike repolarisation as 4-aminopyridine slows the rate of repolarisation. The single deep AHP is mediated by a Ca^{2+} dependent outward conductance (perhaps I_C), evidenced by the fact that Ca^{2+} free Cd^{2+} medium has only a small effect on the initial rate of repolarisation but greatly reduces the AHP amplitude (Johnston *et al* 1994). In this medium, cells fire regularly indicating that a Ca^{2+} activated membrane conductance is not essential for the regularity of their tonic discharge. Depolarisation of the membrane between successive action potentials involves a non-inactivating persistent Na^+ conductance (I_{NaP}) (Serafin *et al.*, 1991a, b; Johnston *et al.*, 1994) although the rate of depolarisation and the tonic discharge may depend more on the rate of inactivation of K^+ channels.

In type B neurones TEA blocks the initial fast AHP and slows the rate of repolarisation. Rate of repolarisation was not much affected in a Ca^{2+} free medium, indicating that as in type A neurones normal repolarisation of type B neurones is largely mediated by a TEA sensitive K^+ conductance (Johnston *et al.*, 1994). The delayed slow AHP is TEA insensitive, abolished in Ca^{2+} free medium and blocked by apamin, and is probably due to a Ca^{2+} activated K^+ current, maybe involving K^+

influx through small conductance K^+ channels (SK channels). Blockage of SK channels leads to an irregular pattern of firing, thus the Ca^{2+} mediated interspike hyperpolarisation is important in regulating the timing of successive spikes. A non-inactivating sodium conductance (I_{NaP}) which is active near the resting membrane potential and continually depolarises the neurone towards firing threshold mediates the spontaneous activity of type B neurones. (Serafin *et al.*, 1991a, b; Johnston *et al.*, 1994). Around 30% these neurones exhibit a low threshold Ca^{2+} conductance that may contribute to membrane depolarisation between successive spikes (Johnston *et al.*, 1994). A small component of the resting activity of MVN neurones *in vitro* may come from synaptic inputs from within the same nucleus, since various neurotransmitters and neuromodulators can modulate the spontaneous firing rates of these neurones.

1.3 PHARMACOLOGY OF THE VESTIBULAR SYSTEM

Neurotransmission and neuromodulation within the central vestibular system is mediated through a range of amino acids, monoamines and neuropeptides. Behavioural, biochemical and electrophysiological methods have been employed to study the pharmacology of the vestibular system.

1.3.1 Amino acids

The amino acids glutamate, GABA and glycine are mediators of fast excitatory and inhibitory transmission in the central nervous system, acting mainly through ionotropic receptors. These amino acids regulate the majority of neurotransmission and also have neuromodulatory roles in the central vestibular system.

Glutamate

The central nervous system contains two distinct groups of glutamatergic receptors: ionotropic and metabotropic receptors. The ionotropic receptors are subdivided into three groups: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainite, and N-methyl-D-aspartate (NMDA) glutamate receptor subtypes. Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors and regulate the production of intracellular messengers (Ozawa *et al.*, 1998). Both ionotropic and metabotropic glutamate receptors have been identified in the vestibular nucleus complex (VNC) (for review, see de Waele *et al.*, 1995).

A large body of evidence has accumulated to suggest that glutamate and/or aspartate is the major transmitter used between the primary vestibular nerve and the second order vestibular nuclei neurones. Raymond *et al.* (1984), showed that presynaptic primary vestibular nerve terminals in cat take up glutamate, and this uptake is significantly decreased after nerve section. Ligand binding studies have demonstrated high levels of glutamate binding sites in all vestibular nuclei, with the highest density observed in the MVN (Touati *et al.*, 1989; Walberg *et al.*, 1990). Glutamate receptor immunoreactivity is observed in vestibular afferents, and in cell bodies in all parts of the VNC in frog and rat (Reichenberger and Dieringer, 1994;

Reichenberger *et al.*, 1997). All four vestibular nuclei are densely stained with specific antibodies for AMPA-type glutamate receptors (Petralia and Wenthold, 1992), NMDA and kainite-type glutamate receptors (Petralia *et al.*, 1994a, b; for review of NMDA receptors, see Vidal *et al.*, 1996). *In situ* hybridisation studies have observed mRNA for a range of NMDA, non-NMDA and metabotropic glutamate receptor subunits in the VNC (Horii *et al.*, 2001). Microdialysis studies show that glutamate is released from the eighth nerve, and electrical stimulation of this nerve increases glutamate levels in the MVN (Yamanaka *et al.*, 1995b, 1997).

In vitro electrophysiological studies corroborate the biochemical evidence implicating glutamate as the major neurotransmitter between primary vestibular afferents and second order vestibular nuclei neurones, and have further been used to elucidate the glutamate receptor subtypes involved in this transmission. Extracellular and intracellular studies in brainstem slices have shown that synaptic responses evoked in neurones assumed to be second order MVN neurones, by electrical stimulation of the eighth nerve are blocked by AMPA or kainite receptor antagonists, but not by NMDA receptor antagonists (Cochran *et al.*, 1987; Lewis *et al.*, 1989; Doi *et al.*, 1990; Carpenter and Hori, 1992). However, subsequent patch clamp studies using slices from young rats (postnatal days 4-25), showed that excitatory postsynaptic potentials (EPSPs) induced in the MVN by electrical stimulation of the eighth nerve can be blocked by NMDA receptor antagonists at high levels of depolarisation (Kinney *et al.*, 1994; Takahashi *et al.*, 1994). These results may reflect the presence of NMDA receptor mediated transmission in response to high frequency vestibular nerve stimulation. However the discrepancy between the two groups of data may be due to the age of animals used, as developmental difference exist in the CNS in the expression of NMDA receptors (e.g. Tremblay *et al.*, 1988).

Using *in vitro* whole brain preparation of young adult guinea pigs, Babalian *et al.* (1997) reported that about a third of second order MVN neurones displayed NMDA receptor mediated transmission. Most of the field potentials in the MVN, and the EPSPs recorded from individual neurones were blocked by the AMPA/kainite receptor antagonist CNQX, but any remaining components were blocked with the NMDA receptor antagonist APV. Apart from their role in synaptic transmission, NMDA receptors probably play a role in information processing in the VNC as there

is evidence that they mediate commissural inputs from the contralateral VN, and inputs from the spinal cord (Cochran *et al.*, 1987; Knopfel, 1987).

Glutamate may also modulate the activity of vestibular nucleus neurones. Application of NMDA receptor antagonists to *in vitro* brainstem slices, decreased the firing rates of extracellularly recorded MVN neurones (Smith *et al.*, 1990; Smith and Darlington, 1992; Lin and Carpenter, 1993). This indicates that the tonic release of glutamate, acting on NMDA receptors, modulates the spontaneous firing of MVN neurones *in vitro*. MVN neurones recorded intracellularly from brainstem slices were depolarised by both NMDA and AMPA/kainite receptor agonists, with an increase in spontaneous discharge and an apparent decrease in membrane resistance (Lewis *et al.*, 1987, 1989; Serafin *et al.*, 1992). A behavioural study in alert guinea pig recorded that chronic infusion of the specific NMDA receptor antagonist D-APV into the vestibular nuclei on one side of the brain induced a postural and oculomotor syndrome similar to the one observed following acute vestibular deafferentation (de Waele *et al.*, 1990). The authors concluded that NMDA receptors contribute to the resting discharge of VN neurones and consequently may be involved in static vestibular reflexes.

GABA and glycine

Glycine and γ-aminobutyaric acid (GABA) are the main inhibitory neurotransmitters in the CNS. There are two types of GABA receptors in the vestibular nuclei. $GABA_A$ receptors are ligand-gated receptors which are directly associated with Cl^- ion channels in the membrane. $GABA_B$ receptors are linked to G-proteins, or intracellular effector systems, and inhibit adenylate cyclase activity. $GABA_B$ activation can also cause an increase in K^+ conductance, or a decrease in voltage-dependent Ca^{2+} currents resulting in neuronal inhibition (Bowery *et al.*, 2002). Glycine receptors are ligand-gated receptor channels that have similar permeability and conduction properties to the $GABA_A$ receptor channel (Barry *et al.*, 1999).

Immunohistochemical studies using antibodies raised against GABA, glycine or glutamic acid decarboxylase (GAD; the GABA synthesising enzyme), have identified GABA and glycine containing cells, fibres and granules throughout the

VNC in a range of species (Houser *et al.*, 1984; Nomura *et al.*, 1984; Kumoi *et al.*, 1987; Walberg *et al.*, 1990; Spencer and Baker, 1992; Zanni *et al.*, 1995; Reichenberger *et al.*, 1997; Holstein *et al.*, 1999a). In addition, GABA and glycine have been shown to co-localise in some MVN neurones (Walberg *et al.*, 1990; Lahjouji *et al.*, 1996; Russier *et al.*, 2002). GABA-immunoreactive (-ir) cells are mostly small and lie scattered throughout the VNC with highest levels observed in the MVN, where medium sized GABA-ir cells are also observed (Kumoi *et al.*, 1987; Walberg *et al.*, 1990). In agreement with these data, *in situ* hybridisation with probes for GAD and $GABA_A$ alpha 1 receptor subunit mRNA, localised these mRNAs throughout the VNC with highest levels of GAD mRNA in the MVN (Hironaka *et al.*, 1990; de Waele *et al.*, 1994; Zanni *et al.*, 1995). $GABA_A$, $GABA_B$ and glycine receptors are widely distributed post-synaptically on vestibular neurones, and $GABA_B$ receptors are also presynaptically located (Holstein *et al.*, 1992; Lapeyre and De Waele, 1995).

GABA and glycine mediate synaptic transmission at a number of inhibitory synapses in the vestibular nucleus complex. The tonic discharge of ipsilateral vestibular neurones in mammals is inhibited by stimulation of the contralateral eighth nerve via a commissural system linking the two vestibular nuclei (see Section 1.1.3). Precht *et al.* (1973) demonstrated that the ipsilateral IPSPs, generated by electrical stimulation of the contralateral eighth nerve in cat *in vivo*, were blocked by systemic injection of the $GABA_A$ agonist, bicuculline, or the glycine agonist, strychnine, but not by both. This suggested that commissural inhibition is mediated by either $GABA_A$, or glycine receptors. In contrast, more recent experiments in cat suggest that glycine only plays a minor role in commissural inhibition, the majority of this inhibition being GABAergic (Furuya and Koizumi, 1998). Furuya and Koizumi observed that bicuculline, applied iontophoretically to the ipsilateral vestibular nuclei neurones, abolished the field potential evoked by contralateral vestibular nerve stimulation, whereas strychnine did not. The discrepancy between the two studies may be due to the location of drug administration, as the systemic administration of Precht *et al.* (1973) could have affected other CNS regions. However, it is possible that both GABA and glycine together are involved in mediating the commissural

inhibition since both neurotransmitters co-localise in some MVN neurones as discussed below.

The vertical and horizontal canal vestibulo–ocular reflex (VOR) neurones are located in the SVN and MVN, respectively (see Section 1.1.2). The vertical VOR is mediated primarily via $GABA_A$ receptors, as vestibulo-evoked inhibition of trochlear motorneurones is blocked by intravenous bicuculline; in addition, lesioning of the medial longitudinal fasciculus (MLF) which carries all the inhibitory vertical VOR fibres causes a significant reduction in the concentration of GABA in the trochlear and oculomotor nuclei (Precht *et al.*, 1973). The short relay pathway of the horizontal VOR is a disynaptic pathway: first order vestibular neurones from the horizontal semicircular canal stimulate second order neurones in the MVN, which inhibit ipsilateral or stimulate contralateral, motorneurones in the abducens nuclei. Early electrophysiological data indicated that glycine was the inhibitory transmitter used in the horizontal VOR pathway: Glycinergic but not GABAergic antagonists blocked the disynaptic IPSP recorded in the ipsilateral abducens nucleus following stimulation of the ipsilateral vestibular nerve. In addition, immunohistochemical data revealed high levels of glycine-ir in the abducens nuclei with low levels in the oculomotor and trochlear nuclei, and high levels of GABA-ir in the oculomotor and trochlear nuclei with low levels in the abducens nuclei (Spencer *et al.*, 1989). However more recent studies have indicated that GABA and glycine may be co-localised in the vestibular and oculomotor nuclei (Walberg *et al.*, 1990). Immunocytochemical techniques revealed that 9% of individual synaptic terminals impinging on rat abducens nucleus motorneurones were immunoreactive for both GABA and glycine (Lahjouji *et al.*, 1996). In corroboration of this data, a whole cell patch clamp study in young rat abducens nucleus motorneurones, recorded a proportion miniature IPSP responses indicative of single evoked vesicles containing both neurotransmitters (Russier *et al.*, 2002). This GABA and glycine co-release was also identified during transmitter release evoked after stimulation of the ipsilateral MVN. Thus there may be a co-operation between GABA and glycine in the inhibitory transmission at the abducens nucleus, and perhaps in other efferent pathways from the MVN.

The majority of inhibitory vestibulo-spinal neurones are glycinergic but GABAergic projections exist in some species. Felpel (1972) demonstrated that intravenous injection of glycine, but not GABA antagonists, depressed the amplitude of the IPSP recorded in neck motorneurones in cat, after electrical stimulation of the vestibular nerve. Immunohistochemistry for GAD, combined with retrograde tracing from the spinal cord have identified GAD-ir neurones in the MVN, LVN and IVN which project to the spinal cord in rabbit and rat (Blessing *et al.*, 1987; Valla *et al.*, 2003). The above findings point to the involvement of both the GABAergic and glycinergic systems in the control of vestibulo-ocular and vestibulo-spinal pathways.

In addition to their involvement in efferent projections from the VN, GABA and glycine are likely to contribute to inhibitory interactions within specific vestibular nuclei. Using ultrastructural visualisation of neurones in the MVN, Holstein *et al.* (1999a) observed GABAergic cells which were involved in complex local synaptic interactions which they concluded may represent a class of vestibular interneurones. *In vitro*, electrophysiological experiments in brainstem slices containing the MVN have confirmed the presence of locally acting GABA and glycine within this region. Exogenous application of GABA or glycine to guinea pig MVN slices, inhibited the tonic firing rate in around 50% of neurones tested (Smith *et al.*, 1991). In contrast, Dutia *et al.* (1992) observed an inhibition in the firing rate of all MVN neurones in response to GABA or its agonists. This inhibition could be blocked by both $GABA_A$ and $GABA_B$ receptor antagonists, indicating that both receptor subtypes are present on MVN neurones (Dutia *et al.*, 1992). The discrepancy in GABA actions between the two experiments may be due to differences in the thickness of slices used. Dutia and colleagues found that low micromolar doses of GABA were less efficient at inhibiting neurones in thicker slices (350-500μM), but inhibited all neurones in slices of less that 350μM, probably due to a greater uptake and metabolism of GABA in thicker slices. The slices used by Smith and colleagues ranged from 400-600μM in thickness.

Intracellular studies by Vibert *et al.* (1995b,c), demonstrated that both type A and type B MVN neurones were hyperpolarized by the $GABA_A$ agonist, muscimol and the $GABA_B$ agonist, baclofen in the presence of tetrodotoxin (TTX); while in normal

medium some cells were hyperpolarized and some depolarised. This suggests that local inhibitory interneurones are spontaneously active *in vitro* and can modulate the activity of MVN neurones via $GABA_A$ and $GABA_B$ receptors. While the brainstem slice study of Smith *et al.* (1991) showed that 50% of MVN neurones were inhibited by exogenous glycine, Lapeyre and de Waele (1995) demonstrated that all MVN neurones *in vitro* were inhibited by glycine in a dose dependant manner. This inhibition persisted in low Ca^{2+}/high Mg^{2+} solution, indicating a postsynaptic site of action.

1.3.2 The cholinergic system

The cholinergic system is one of the most important modulatory transmitter systems in the brain. Acetylcholine (ACh) acts through two types of receptors: nicotinic and muscarinic. Nicotinic receptors are ligand gated ion channels, which act by directly regulating the opening of cation channels. Muscarinic receptors belong to the family of G-protein coupled receptors, and act through a range of second messenger systems (Pompeiano, 2000). Acetylcholine receptors are located both pre- and postsynaptically, including on presynaptic terminals of cholinergic neurones where they can inhibit ACh release via muscarinic receptors or enhance ACh release via nicotinic receptors.

Receptor binding and immunohistochemistry studies have revealed the presence of both muscarinic and nicotinic receptors throughout the VNC, particularly in the MVN. (Rotter *et al.*, 1979; Wamsley *et al.*, 1981; Schwartz, 1986; Swanson *et al.*, 1987; Calza *et al.*, 1992). *In situ* hybridisation studies have identified cells containing mRNA coding for the m_2 subunit type of muscarinic receptors (Vilaro *et al.*, 1992) and different types of nicotinic receptor subunits (Wada *et al.*, 1989) in the vestibular nuclei of rats. All vestibular nuclei have been shown to display choline-acetyltransferase (ChAT; the ACh synthesising enzyme) immunoreactivity, with highest levels found in the MVN (Carpenter *et al.*, 1987; Barmack *et al.*, 1992b). Indeed studies of ChAT localisation have been widely used to identify ACh as one of the main efferent neurotransmitter in the VNC (Barmack *et al.*, 1992c; Goldberg *et al.*, 2000).

Application of ACh to LVN and MVN neurones *in vivo*, modulates their activity, increasing the extracellularly recorded spontaneous firing rates of these cells (Kirsten and Schoener, 1973; Kirsten and Sharma, 1976a,b). These effects were blocked by muscarinic but not nicotinic receptor antagonists. The results of these *in vivo* experiments were confirmed using extracellular recordings *in vitro* in vestibular nucleus neurones (Ujihara *et al.*, 1988, 1989; Dutia *et al.*, 1990; Carpenter and Hori, 1992). Ujihara and colleagues showed that 66.7% of extracellularly recorded MVN neurones exhibit a dose dependant increase in spontaneous activity after application of the ACh agonist carbachol; 22.2% of neurones showed a decrease in spontaneous activity, with no change in the remaining neurones. The excitatory or inhibitory effects of carbachol were mimicked by muscarine, not nicotine, and were antagonised by atropine, indicating a role for muscarinic but not nicotinic receptors in modulation of vestibular neuronal activity. Other studies have recorded similar results (Dutia *et al.*, 1990; Carpenter and Hori, 1992).

In contrast, intracellular experiments recording from MVN neurones, have shown that ACh acts through both muscarinic and nicotinic receptors in the MVN (Phelan and Gallagher, 1992; Sun *et al.*, 2002). MVN neurones responded to ACh or carbachol with membrane depolarisation, and similar results were obtained with application of selective nicotinic or muscarinic agonists. The muscarinic induced depolarisations were linked to a decrease in conductance due to closure of K^+ channels, whereas the nicotinic induced depolarisations were linked to an increase in conductance due to the opening of cation channels. Both types of depolarisation persisted in low Ca^{2+}/high Mg^{2+} solution indicating a postsynaptic site of action (Phelan and Gallagher, 1992; Sun *et al.*, 2002). In addition to the excitatory effects, a minority of MVN neurones show a transient hyperpolarisation during muscarinic receptor agonist application (Dutia *et al.*, 1990; Phelan and Gallagher, 1992). This hyperpolarisation was blocked by antagonists of $GABA_A$ receptors, indicating that activation of muscarinic receptors either postsynaptically on GABA interneurones, or presynaptically on GABA terminals, causes an increase in this inhibitory neurotransmitter (Phelan and Gallagher, 1992).

`1.3.3 The monoaminergic system

Monoamines are modulatory transmitters in the central nervous system. The five monoamines (histamine, serotonin, dopamine, noradrenaline and adrenaline) have more dispersed and moderate effects on neurones than amino acids. Most only activate metabotropic receptors through second messenger systems and therefore have much slower effects upon neuronal activity.

Histamine

In mammals, all histaminergic neurones of the central nervous system are located in the tuberomammillary nucleus of the posterior hypothalamus, and their fibres are found in almost all regions of the brain. Three types of metabotropic histaminergic receptors have been identified. H_1 and H_2 receptors are located postsynaptically and generally induce neuronal excitability through positive coupling to phospholipase C and adenylate cyclase respectively. H_3 receptors are located on presynaptic terminals, predominantly of histaminergic neurones, where they exert a negative control on the synthesis and release of histamine (Vibert *et al.*, 2000b).

There are several lines of evidence suggesting that the histaminergic system modulates the function of central vestibular neurones. A direct projection from the tuberomammillary nucleus to the VNC is observed in rat (Takeda, 1987; Panula *et al.*, 1989; Steinbusch, 1991), guinea pig (Airaksinen and Panula, 1988), and cat (Tighilet and Lacour, 1996, 1997). Ligand binding and *in situ* hybridisation studies have located H_1, H_2, and H_3 binding sites in all vestibular nuclei, with highest levels observed in the SVN and MVN (Bouthenet *et al.*, 1988; Ruat *et al.*, 1991; Pillot *et al.*, 2002). Early *in vivo* electrophysiological studies by Kirsten and colleagues reported that iontophoretic application of histamine caused a decrease in firing rate in the majority of vestibular neurones in cat (Kirsten and Sharma, 1976a; Satayavivad and Kirsten, 1977). This inhibitory action of histamine was blocked by the H_2 receptor antagonist, metiamide but not by the H_1 receptor antagonist, diphenhydramine.

In contrast, *in vitro* electrophysiological studies in guinea pig using intracellular recording techniques, demonstrated that application of histamine predominantly produced membrane depolarisation and an increase in the tonic firing rate of MVN

neurones (Serafin *et al.*, 1993). These effects of histamine could be mimicked by the H_2 agonist, impromidine and were inhibited by the H_2 antagonist, cimetidine. The histamine induced depolarisations persisted in high Ca^{2+}/low Mg^{2+} solution, and were unaffected by application of a H_1 receptor antagonist or H_3 agonists or antagonists (Serafin *et al.*, 1993). The authors concluded that histaminergic depolarisation of MVN neurones is mediated through postsynaptic H_2 receptors. However, extracellular recordings in brainstem slices from rats demonstrated that histamine acts postsynaptically through both H_1 and H_2 receptors (Inverarity *et al.*, 1993; Wang and Dutia, 1995). The discrepancy between these studies may be due to species differences in either the anatomical distribution or the pharmacological properties of H_1 receptors.

The presence of H_3 receptors within the MVN, which may modulate vestibular neuronal activity was demonstrated by *in vivo* studies in the alert guinea pig (de Waele *et al.*, 1992; Yabe *et al.*, 1993). Unilateral perfusion of the H_3 agonist, alpha-methylhistamine, or the H_2 antagonist, cimetidine caused a postural and oculomotor syndrome directed towards the side of the infusion, similar to the syndrome observed after unilateral labyrinthine destruction. This suggests that histaminergic fibres reaching the vestibular nuclei are endowed with presynaptic H_3 receptors, the activation of which inhibits tonic histamine release inducing a decrease in firing rate of ipsilateral vestibular neurones.

Serotonin

Serotonergic neurones of the CNS are located within the brainstem reticular formation. Serotonin receptors are classified into 4 main groups: 5-HT_1, 5-HT_2 and 5-HT_4 are metabotropic receptors, and 5-HT_3 is an ionotropic receptor. 5-HT_1 receptors have a high affinity for serotonin and are divided into three subtypes (5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D}), which are located pre- and postsynaptically and are generally negatively coupled to adenylate cyclase. 5-HT_2 receptors are also divided into three subtypes (5-HT_{2A}, 5-HT_{2C} and 5-HT_{2C}), the receptors are located both pre- and postsynaptically and are positively coupled to phospholipase C. Immunohistochemical studies have demonstrated that the vestibular nuclei receive a dense serotonergic innervation, probably originating from the dorsal raphe nucleus

(Steinbusch, 1981; Giuffrida *et al.*, 1991; Zanni *et al.*, 1995; Halberstadt and Balaban, 2003). A recent *in vivo* microdialysis study has demonstrated the release of serotonin from nerve terminals within the MVN (Inoue *et al.*, 2002). Accordingly, several autoradiographic and *in situ* hybridisation studies have detected 5-HT_{1A}, 5-HT_{1B} and 5-HT_2 receptors (or corresponding mRNA) located in all vestibular nuclei (Pazos *et al.*, 1985; Pazos and Palacios, 1985; Zanni *et al.*, 1995).

Serotonin may modulate the function of central vestibular neurones. Intra-cerebroventricular injection of serotonin has been shown to increase the gain of the vestibulo-ocular reflex in rat (Ternaux and Gambarelli, 1987). *In vivo* electrophysiological studies have demonstrated excitatory, inhibitory or biphasic responses to iontophoretically applied serotonin in the MVN and SVN (Licata *et al.*, 1993a). *In vitro*, serotonin mainly depolarises MVN neurones in rat brainstem slices (Gallagher *et al.*, 1992; Johnston *et al.*, 1993). The observed depolarisation was mediated via 5-HT_2 receptors as application of a range of 5-HT_2 receptor antagonists blocked the depolarisation. A minority of cells *in vitro* were depolarised by serotonin acting through 5-HT_{1A} receptor subunits (Johnston *et al.*, 1993).

Dopamine

Dopamine can activate at least five distinct subtypes of metabotropic receptors. These five receptors are grouped into two classes according to their pharmacological properties (Civelli *et al.*, 1993). D_1-like receptors (D_1 and D_5) are located pre- and postsynaptically and are generally positively coupled to adenylate cyclase; the presynaptically located receptors mainly stimulate neurotransmitter release. D_2-like receptors (D_2, D_3 and D_4) are also pre- and postsynaptically located and are generally negatively coupled to adenylate cyclase; the presynaptically located receptors mainly inhibit neurotransmitter release (Starke *et al.*, 1989).

The vestibular nuclei have not been shown to receive any specific dopaminergic innervation (Moore and Bloom, 1978). However an autoradiography study in rat has identified D_2 receptors in the vestibular nuclei, with highest levels in the MVN (Yokoyama *et al.*, 1994), and an *in situ* hybridisation study has located D_2 receptor mRNA in the MVN (Bouthenet *et al.*, 1991). Electrophysiological experiments support the notion of dopaminergic modulation of the vestibular system. Application

of dopamine *in vitro*, depolarised MVN neurones in rat (Gallagher *et al.*, 1992) and guinea pig (Vibert *et al.*, 1995a). The dopamine induced depolarisations in the guinea pig MVN were mimicked by a selective D_2-like receptor agonist, but not by a D_1-like receptor agonist. When synaptic transmission was blocked in this preparation using low Ca^{2+}/high Mg^{2+} solution, the depolarising effect of the D_2-like receptor agonist was reversed to a hyperpolarisation. This hyperpolarizing effect was also observed in the presence of the $GABA_A$ receptor antagonist, bicuculline, indicating that dopamine may have a postsynaptic hyperpolarizing effect on MVN neurones, and can also act to depolarise MVN neurones by presynaptically inhibiting the tonic release of GABA.

Noradrenaline

Most noradrenergic neurones in the CNS originate from the locus coeruleus and the nucleus subcoeruleus. Adrenergic receptors can be classified into three main groups: α_1, α_2 and β receptors. $Alpha_1$ receptors (consisting of four distinct subtypes; α_{1A} to α_{1D}) are generally presynaptically located and are positively coupled to phospholipase C, increasing intracellular calcium. $Alpha_2$ receptors have at least three distinct receptor subtypes, are pre- and postsynaptically located and are generally negatively coupled to adenylate cyclase, decreasing transmitter release and hyperpolarising membranes. Beta receptors (consisting of three distinct subtypes; β_1, β_2 and β_3) are pre- and postsynaptically located, are generally positively coupled to adenylate cyclase, stimulating transmitter release and depolarising membranes.

It has been postulated that the noradrenergic system could play and important role in regulation of vestibulo-spinal and vestibulo-ocular reflexes (Pompeiano, 1989; Tan *et al.*, 1991), yet only recently has a noradrenergic projection to the VNC been found. Schuerger and Balaban (1993) demonstrated projections from the locus coeruleus and the A_4 region of the nucleus subcoeruleus to all regions of the VNC using antibodies raised against tyrosine hydroxylase and dopamine β-hydroxylase. The highest densities of noradrenergic fibres were found in the superior and lateral vestibular nuclei. Autoradiographic and immunocytochemical studies have revealed the presence of β and α_2 noradrenergic receptors in the VNC (Wanaka *et al.*, 1989;

Fernandez-Lopez *et al.*, 1990). While β receptors are abundant in the SVN and LVN, α_2 receptors are mostly concentrated in the MVN (Talley *et al.*, 1996). *In situ* hybridisation studies have located mRNA for the α_1, α_{2A} and α_{2C} receptor subtypes in all vestibular nucleus subnuclei (Nicholas *et al.*, 1993; Pieribone *et al.*, 1994).

Electrophysiological data support a role for noradrenergic modulation of vestibular neurones. *In vivo*, iontophoretic application of noradrenaline in cerebellectomised cats generally increased the resting activity of LVN neurones, whereas it mostly decreased it in MVN neurones (Yamamoto, 1967; Kirsten and Sharma, 1976b). In contrast, an *in vivo* study in rat found that iontophoretically applied noradrenaline inhibited most SVN and LVN neurones, acting through α_2 receptors (Licata *et al.*, 1993b). The discrepancy between these results may be due to the different *in vivo* preparations used. *In vitro* electrophysiological experiments have sought to elucidate the noradrenergic receptor subtypes present on vestibular neurones. Podda *et al.* (2001), recording extracellularly in MVN neurones from rats, found that the majority of neurones were responsive to application of noradrenaline, and in 87% of noradrenaline sensitive neurones excitatory responses were recorded. The excitatory effects were mimicked by α_1 and β receptor agonists and were antagonised by α_1 and β receptor antagonists. Inhibitory responses were antagonised by α_2 receptor antagonists. This data is in accordance with intracellular recordings from guinea pig MVN neurones (Vibert *et al.*, 2000b). The β receptor agonist, isoproterenol, depolarised 60% of MVN neurones while decreasing their membrane resistance. The α_1 receptor agonist, L-phenylephrine depolarised 60% of MVN neurones while increasing their membrane resistance and the α_2 receptor agonist, clonidine hyperpolarized most MVN neurones while decreasing their membrane resistance (Podda *et al.*, 2001). The hyperpolarizing effects of clonidine persisted in synaptic uncoupling conditions, indicating a postsynaptic site for α_2 mediated inhibitions, while most α_1 and β responses were indirectly modulated.

1.3.4 The neuropeptide system

Numerous neuropeptides have been identified in the central nervous system, most of which act through specific metabotropic receptors. Pharmacological and electrophysiological studies have provided evidence that several neuropeptides act as modulators of central vestibular neurones, including the opioid peptides, somatostatin, substance P and adrenocorticotropin (for review, see Saxon and Beitz, 2000).

The opioid peptides

Opioid peptides are classified into three different families; enkephalins, β-endorphins and dynorphins. These peptides act on three different types of opioid receptor; μ, δ and κ. Enkephalins have the highest affinity for δ receptors but can also bind to μ receptors, β-endorphins bind equally well to μ and δ receptors, whereas dynorphins have a high affinity for κ opioid receptors but can also bind to μ and δ receptors. All opioid receptors are coupled to G-proteins; μ and δ receptors mediate the inhibition of adenylate cyclase and the activation of inwardly rectifying potassium channels, and κ and δ receptors can also inhibit the opening of voltage dependant Ca^{2+} channels (Brownstein, 1993).

Enkephalin terminals and enkephalin immunoreactive cell bodies have been detected within the vestibular nucleus complex (Hokfelt *et al.*, 1977; Finley *et al.*, 1981a; Nomura *et al.*, 1984; Merchenthaler *et al.*, 1986). Cells positive for preproenkephalin (the precursor for Met- and Leu-enkephalin) mRNA were observed in the MVN and IVN (Saika *et al.*, 1993), and the proenkephalin peptide is found in the fibre plexuses of these same regions (Balaban *et al.*, 1989). Accordingly, the MVN contains neurones expressing both enkephalin mRNA and peptide, and is rich in opiate receptors (Zanni *et al.*, 1995). There is little evidence for the presence of endorphins within the vestibular nuclei, whereas low levels of dynorphins have been observed in the MVN and IVN using immunohistochemical techniques (Zamir *et al.*, 1983).

Electrophysiological studies have provided conflicting evidence relating to the physiological effects of opioids on vestibular neurones. Carpenter and colleagues

reported that 30% of tonically active rat MVN neurones *in vitro* were excited by iontophoretic application of both the specific μ receptor agonist, morphine, and by the δ receptor agonist, ala-leu-enkephalin (Carpenter and Hori, 1992; Lin and Carpenter, 1994). The excitation was blocked by the μ and δ receptor antagonist naloxone, and persisted in synaptic uncoupling conditions indicating the presence of postsynaptic μ and δ receptors on MVN neurones. In contrast, studies by Kawabata *et al.* (1990) and Sulaiman and Dutia (1998) observed mainly inhibitory effects of opiates on MVN neurones. Kawabata *et al.* (1990) demonstrated that enkephalin, applied iontophoretically to anaesthetised cats *in vivo*, inhibited the firing of type I MVN neurones responding to horizontal rotation. Using extracellular and whole cell patch clamp recordings *in vitro*, Sulaiman and Dutia (1998) observed that the large majority of MVN neurones were inhibited by the δ receptor agonists [D-Ala^2, D-Leu^5]-enkephalin (DADLE), and [D-Pen^2, D-Pen^5]-enkephalin (DPLPE). This inhibition persisted in synaptic uncoupling conditions and was blocked by the selective δ receptor antagonist naltrindole, indicating that it was mediated by postsynaptic δ receptors. Selective μ and κ opioid receptor agonists had no effect on the discharge of any MVN neurones tested. The discrepancy between these findings may be due to the method of opiate agonist application, as the iontophoretic application of Lin and Carpenter (1994) cannot calibrate the applied drug to the pH of the perfusing aCSF.

Somatostatin

There are four different types of somatostatin receptor, $SS_{1\text{-}4}$. They are negatively coupled via G-proteins to adenylate cyclase and can modulate Ca^{2+} and K^+ channels to cause a decrease in neuronal excitability. Somatostatin immunoreactive cell bodies and axons have been demonstrated throughout the vestibulo-cerebellum and VNC, with highest densities of fibres observed in the MVN (Finley *et al.*, 1981b; Johansson *et al.*, 1984; Vincent *et al.*, 1985; Zanni *et al.*, 1995). The vestibular nuclei display intermediate to high concentrations of high affinity binding sites for somatostatin, with greatest binding in the MVN (Uhl *et al.*, 1985).

Behaviourally, intraventricular injection of somatostatin in rat results in a postural syndrome similar to that seen after unilateral labyrinthine destruction, the effect of which is blocked by antimuscarinic drugs, suggesting an indirect action of somatostatin on vestibular function (Balaban *et al.*, 1989). Despite this, Chan-Palay *et al.* (1982) recorded that iontophoretic application of somatostatin in rabbits depressed the resting activity of LVN neurones. LVN neurones receive a large Purkinje cell input from the cerebellar vermis and these cells are somatostatin immunoreactive (Chan-Palay *et al.*, 1982), thus somatostatin may be released as a co-transmitter with GABA in vestibulo-cerebellar pathways.

Substance P

Substance P was the first neuropeptide discovered in the body and is now recognised as a neurotransmitter and neuromodulator in the central and peripheral nervous systems. Substance P is one of three tachykinins in the CNS, the others being neurokinin A and neurokinin B. These molecules act on receptors named NK-1, NK-2 and NK-3 respectively, all of which are G-protein coupled receptors. Many substance P immunoreactive (-ir) fibres and cell bodies have been demonstrated in the vestibular complex. Whilst substance P -ir fibres are found in all four main vestibular nuclei (Nomura *et al.*, 1984), substance P -ir cell bodies have been visualised exclusively in the caudal region of the MVN and throughout the IVN (Ljungdahl *et al.*, 1978; Nomura *et al.*, 1984). Since there are no substance P -ir cell bodies in the SVN and LVN, the immunoreactive fibres probably originate from the MVN, IVN or from primary vestibular neurones which are substance P -ir (Usami *et al.*, 1991). Receptor autoradiography and *in situ* hybridisation techniques have located substance P binding sites and NK-1 mRNA in the MVN (Maeno *et al.*, 1993; Zanni *et al.*, 1995).

Only one electrophysiological study has examined the effect of substance P on vestibular neurones *in vitro*. Vibert *et al.* (1996) recording intracellularly from guinea pig MVN neurones, demonstrated that bath application of substance P caused a depolarisation and increase in membrane resistance in around 50% of the neurones, while it inhibited and caused a decrease in membrane resistance in 10% of neurones. Both responses persisted under synaptic uncoupling conditions indicating a

postsynaptic site of action. The substance P induced depolarisations could not be reproduced with agonists of the three tachykinin receptor subtypes, nor was it blocked by specific NK-1 receptor antagonists, suggesting the involvement of a new, pharmacologically distinct 'NK-1-like' receptor. The observed hyperpolarisations were mimicked by specific NK-1 receptor agonists, suggesting that hyperpolarising effects are mediated via the typical NK-1 receptors observed in the MVN (Vibert *et al.*, 1996).

Adrenocorticotropic hormone

Adrenocorticotropic hormone (ACTH) belongs to the melanocortin family of peptides. Melanocortin receptors can be divided into 5 subtypes (MRC1-5), all are G-protein associated, and are positively coupled to adenylate cyclase (Tatro, 1996). Radioimmunoassay and immunocytochemical studies have failed to find ACTH binding sites or ACTH containing fibres in the VNC (Palkovits, 1984). Despite this, electrophysiological studies of MVN neurones *in vitro* have indicated that around 50% of these neurones respond to the 4-9 or 4-10 fragments of ACTH, mostly with a decrease in their resting discharge (Darlington *et al.*, 1992; Gilchrist *et al.*, 1994, 1996a). *In vivo*, $ACTH_{4\text{-}10}$ has been shown to accelerate behavioural recovery from unilateral labyrinthine destruction in a range of species (Flohr and Luneburg, 1982; Igarashi *et al.*, 1985; Gilchrist *et al.*, 1990; Gilchrist *et al.*, 1994). Thus ACTH fragments appear to have a significant effect on the vestibular system despite the lack of evidence for its presence in the vestibular system. It may be that $ACTH_{4\text{-}10}$ acts allosterically on non-melanocortin receptors, such as the glutamate NMDA receptor (Darlington *et al.*, 1996), or, as ACTH can be secreted directly into the CSF via the choroids plexus, it could be reaching the MVN via the CSF of the IVth ventricle (Saxon and Beitz, 2000).

Summary

The vestibular system is involved with both sensing and controlling movement and orientation in space. The peripheral vestibular organs detect angular and linear accelerations of the head in three dimensions, and this information is conveyed to the central vestibular nuclei. Here it converges with visual and somatosensory information from centres such as the cerebellum and inferior olive, and information is further modulated by the intrinsic properties of vestibular nucleus neurones themselves. Multiple neurotransmitter systems and neuromodulators converge on the vestibular nuclei, thus are involved in control of movement and posture. Excitatory and inhibitory amino acids and their receptors play a major role in vestibular function while acetylcholine, monoamines and neuropeptides play to some extent secondary neuromodulatory roles, the functions of which remain to be fully characterised.

Chapter 2

VESTIBULAR COMPENSATION

2.1 INTRODUCTION

Unilateral damage to the peripheral vestibular system, as a consequence of destruction of the vestibular receptor cells (unilateral labyrinthectomy, UL) or transection of the vestibular nerve itself (unilateral vestibular deafferentation, UVD), results in a characteristic syndrome of ocular motor and postural symptoms. These symptoms can be divided into two categories on the basis of their relationship to head movement. *Static symptoms* are those present in the absence of head movement, affecting posture and eye movements (e.g. spontaneous nystagmus, yaw and roll head tilts), whereas *dynamic symptoms* are elicited by head movements, and result in deficits in the amplitude and timing of vestibulo-ocular and vestibulo-colic reflexes. Over time many of these symptoms subside in a process of behavioural recovery known as vestibular compensation (VC). Vestibular compensation is a complex, heterogeneous process, with wide varieties in the time course and ultimate level of compensation for different symptoms, and strong inter-species variability regarding all aspects of compensation. Generally static symptoms undergo a remarkable degree of recovery, many symptoms disappearing within a few days following UL, while compensation of dynamic symptoms is much slower and often incomplete.

Vestibular compensation is independent of any regeneration of the peripheral vestibular receptors, or any functional recovery of the cells in Scarpa's ganglion, and therefore is attributed to central nervous system (CNS) plasticity. Vestibular compensation is a valuable model of CNS plasticity for two main reasons: First, the extent of the peripheral lesions can be precisely controlled and easily reproduced; second, the neuronal networks underlying the vestibular system are relatively simple, with input and output relations that can be quantified on both behavioural and neuronal levels.

Despite a tremendous amount of research over the last 40 years into the behavioural and neural correlates of compensation in different species, the exact mechanisms of vestibular compensation, and where they reside in the CNS, are poorly understood.

2.2 BEHAVIOURAL COMPONENTS OF VESTIBULAR COMPENSATION

2.2.1 Static Symptoms

The first description of the static oculomotor and postural symptoms resulting from unilateral labyrinthine destruction, and their gradual recovery, was recorded in 1823 by Flourens (cited in Schaeffer and Meyer, 1974). Sixty years later, Bechtrew (1883) provided evidence for the hypothesis that the symptoms of labyrinthine lesions are due to the loss of resting activity in one vestibular nucleus, and rebalancing of this neural activity is responsible for the disappearance of the static symptoms (cited in Schaeffer and Meyer, 1974). Bechtrew reported that if a second labyrinthectomy was performed on the remaining intact labyrinth of an animal a few days or weeks after the first, a reappearance of the original symptoms of UL are observed but are reversed in their direction. This phenomenon is known as "Bechtrew compensation" and indicates that neural rebalancing must have taken place in the interval between the two labyrinthectomies.

Spontaneous nystagmus

In mammals, the most easily quantified effect of UL is a high frequency sustained horizontal ocular nystagmus (SN) seen as a slow drift of the eyes towards the lesioned side (slow phase) followed by a fast beat towards the intact side (quick phase). It is the direction of the quick phase that is most easily observed, and frequency of SN is recorded as quick phase beats per minute. Soon after UL, the frequency of SN in rat and guinea pig is around 80-180 beats per minute (Schaefer and Meyer, 1974; Sirkin *et al.*, 1984; Magnusson *et al.*, 2002). In submammalian species such as frogs, SN does not occur (Dieringer, 1995). The frequency of SN begins to decrease immediately after UL, with the time required for the disappearance of SN remaining similar between mammalian species with complete disappearance observed from around 15 hours in rat to a few days in human (Maioli *et al.*, 1983; Maioli and Precht, 1984; Sirkin *et al.*, 1984; Curthoys *et al.*, 1988; Fetter and Zee, 1988; Cass *et al.*, 1992; Hamann *et al.*, 1998).

Ocular skew deviation

UL results in disturbances in the position of the eyes. In humans, this ocular skew deviation results in the eye on the affected, side moving down in its orbit relative to the eye on the intact side, which remains in a normal position (Curthoys and Halmagyi, 1999). In guinea pigs and rats, the eye on the side of the lesion deviates down, and the other eye deviates up (Schaefer and Meyer, 1974; Sirkin *et al.*, 1984). A comparable deviation is found in rabbits and frogs: the eye on the lesioned side bulges out while the eye on the intact side sinks into its orbit (Baarsma and Collewijn, 1975; Dieringer, 1995)

Head tilts

Most species exhibit disturbances of head and body posture after UL. Humans show relatively little head and body deviations, but common to many other species is a tilt of the head towards the lesioned side in the roll plane (roll head tilt; RHT), and a tilt of the head towards the lesioned side in the yaw plane (yaw head tilt; YHT) (Schaefer and Meyer, 1974; Curthoys *et al.*, 1988; Fetter and Zee, 1988; de Waele *et al.*, 1989; Hamann *et al.*, 1998). Other static symptoms of UL include a yaw curvature of the spine towards the lesioned side, circular walking and barrel rolling towards the lesioned side, head nystagmus and extension of the contralateral forelimbs (for a review, see Schaefer and Meyer, 1974).

2.2.2 Dynamic symptoms

Unlike the recovery of static symptoms, the recovery of dynamic symptoms is slow and incomplete. Dynamic symptoms result from abnormalities in the vestibulo-ocular and vestibulo-spinal reflexes in response to head movement. In all mammals, UL results in severe abnormalities in the gain, symmetry and phase of the horizontal vestibulo-ocular reflex (hVOR) (Baarsma and Collewijn, 1975; Maioli *et al.*, 1983; Fetter and Zee, 1988; Gilchrist *et al.*, 1998; Hamann *et al.*, 1998; Curthoys and Halmagyi, 1999). In normal animals, the gain of the hVOR (the ratio of eye velocity response to a given head movement) is around 1.0; thus the eye moves in an equal and opposite manner to head movement, stabilising the image of the world on the

fovea (see Figure 1.1). After UL, the eye velocity response to horizontal head rotation in either direction is decreased. There is also asymmetry in the gain of the horizontal VOR: there is a slower eye velocity gain in response to rotations towards the lesioned side than towards the intact side. In humans, during rotations towards the lesioned side, the VOR gain falls to <0.4 (Curthoys and Halmagyi, 1999) and in guinea pigs it falls to <0.3 (Gilchrist *et al.*, 1998). During rotations towards the intact side, the VOR gain falls to around 0.7 in humans and around 0.8 in guinea pig. Although there may be some recovery of VOR gain and symmetry in response to low velocity and low frequency rotational stimuli (Lasker *et al.*, 1999), VOR responses to higher frequency stimulation such as that which occurs during natural head movement appear to be impaired in the long term as they can be observed months and years following labyrinthectomy (Gilchrist *et al.*, 1998; Hamann *et al.*, 1998; Curthoys and Halmagyi, 1999).

2.3 NEURAL COMPONENTS OF VESTIBULAR COMPENSATION

In a groundbreaking study into the mechanisms of VC Precht *et al.* (1966), recording in the decerebrate cat showed that the appearance of the static symptoms following UL correlated with the loss of spontaneous resting activity in type I neurones ipsilateral to the lesion, and compensation of the static symptoms correlated with a restoration of activity in these neurones. This loss of spontaneous resting activity in VN neurones on the lesioned side and its gradual recovery over time as VC develops has subsequently been recorded *in vivo* in many different species (McCabe and Ryu, 1969; McCabe *et al.*, 1972; Dieringer and Precht, 1977, 1979a; Ried *et al.*, 1984; Hamann and Lannou, 1987; Smith and Curthoys, 1988a, b; Newlands and Perachio, 1990; Zennou-Azogui *et al.*, 1993; Ris *et al.*, 1995, 1997). These experiments suggest that the return of resting activity to ipsilesional VN neurones is important for the recovery from the static symptoms of UL.

Type I vestibular nucleus neurones are excited by ipsilateral horizontal canal afferents. Following UL an immediate reduction in the number of type I neuronal responses, and a reduction in the resting discharge of these neurones is recorded in the ipsilesional MVN. In the anaesthetised guinea pig, for up to 8 hours following UL, the ipsilesional MVN is almost devoid of type I neuronal activity (Smith and Curthoys, 1988a); however, by 52-60 hours post-UL type I neurones were encountered more frequently and their resting discharge had recovered to levels not significantly different from normal. In contrast, type I neurones in the contralesional MVN showed an increase in their resting discharge, probably due to disinhibition from the ipsilesional type I neurones (Smith and Curthoys, 1988b).

Type II vestibular neurones are excited by contralateral horizontal canal afferents via the commissural pathway, therefore ipsilesional type II neurones do not lose their main source of excitation following UL. Ipsilesional type II neurones, recorded up to 8 hours following UL in anaesthetised guinea pig, were encountered more frequently and their average resting discharge was significantly higher than normal (Smith and Curthoys, 1988a). This is presumably due to increased commissural activation of these neurones from the intact MVN. At 52-60 hours post-UL ipsilesional type II responses were encountered less frequently than in the 0-8 hour group and their

resting discharge rate was lower. In the contralateral nucleus at 0-8 hours post-UL, type II neurones were more difficult to find and their resting discharge rate was lower than in control animals (Smith and Curthoys, 1988b).

In a series of detailed studies in the alert guinea pig, Ris *et al.* (1995, 1997) demonstrated that immediately after UL, 69% of monosynaptic ipsilesional MVN, SVN and LVN neurones were silent and the remaining active units had a very low resting discharge. Recovery of this resting activity was first observed 12-16 hours post-UL (Ris *et al.*, 1997). One week after UL there were no silent units and the resting discharge had recovered to preoperative levels.

In addition to electrophysiological studies, experiments using [^{14}C]2-deoxyglucose (2-DG) uptake as an indicator for metabolic activity demonstrated large decreases of 2-DG uptake within the lesioned VNC from within a few hours after UL in rat, which persisted for up to 20 days post-UL (Patrickson *et al.*, 1985; Luyten *et al.*, 1986). Similar results have been recorded in frogs indicating that soon after UL neuronal activity is significantly decreased in the lesioned VNC (Flohr *et al.*, 1981; Flohr and Luneburg, 1989).

In parallel with the loss of resting activity in ipsilesional MVN neurones, their sensitivity to angular head accelerations is significantly decreased after UL (Precht *et al.*, 1966; Hamann and Lannou, 1987; Smith and Curthoys, 1988a; Ris *et al.*, 1995). However, in contrast with the recovery of resting activity, the sensitivity of ipsilateral MVN neurones to horizontal rotation remains decreased up to one year after UL, which correlates with the incomplete recovery of the horizontal VOR (Maioli *et al.*, 1983; Smith and Curthoys, 1988a).

Whilst the return of resting activity to once silent VNC neurones is assumed to be partly responsible for the compensation of static behavioural deficits following UL, it is unlikely to be the only mechanism because some discrepancies exist in the time course of neuronal and static behavioural recovery. For example in guinea pig, during the first 90 min following UL, animals are able to stand up and maintain their balance, while neuronal activity in the ipsilesional VNC in low or absent. During the first 10 hours post-UL before neuronal recovery has started *in vivo*, SN diminishes markedly (Ris *et al.*, 1995, 1997).

Summary

The development of VC for the static behavioural symptoms has been causally related to the recovery of spontaneous resting activity in the VNC ipsilateral to the lesion. This is evidenced by an approximate correlation between recovery of resting activity in ipsilesional VNC neurones and compensation of static symptoms post-UL. In addition, VNC neurones project directly to motorneurones controlling the oculomotor and skeletal muscles involved in the manifestation of static symptoms. While there may be an early phase of compensation which is not due to recovery of the resting activity in the ipsilesional VNC, it is generally assumed that recovery of this activity is essential for the maintenance of VC. The mechanisms that cause the return of resting activity to the lesioned VNC neurones are widely researched, and while many structural and neurochemical changes have been implicated there is no clear evidence favouring any one cause.

2.4 STRUCTURES INVOLVED IN VESTIBULAR COMPENSATION

Modulation of compensation may occur through areas of the CNS other than the VNC. Structures implicated in the compensation process include the vestibulo-cerebellum and inferior olive.

2.4.1 *Role of the cerebellum in vestibular compensation*

An important functional role for the cerebellum in VC is suggested by results from lesion studies. During the initial 90min following labyrinthectomy, compensation of SN in cat (Haddad *et al.*, 1977) and guinea pig (Schaefer *et al.*, 1979) was significantly reduced in animals with lesions of the flocculi, nodulus and uvula. In these studies, the long-term compensation of SN was not significantly different from cerebellar intact animals, implicating the cerebellum in only the rapid early recovery of static symptoms. This contrasts with subsequent studies in cat and squirrel monkey, which record significant long-term retardations in the compensation of SN following lesions of the flocculus (Jeannerod *et al.*, 1981; Courjon *et al.*, 1982), uvula and nodulus (Igarashi and Ishikawa, 1985). A more recent clinical study supports the importance of the vestibulo-cerebellum for compensation in humans (Furman *et al.*, 1997). Although the role of the cerebellum in recovery of static symptoms is unclear, it seems likely that it is involved in compensation of dynamic symptoms as cerebellar lesions affect the response of vestibular nucleus neurones to head movement during compensation, and retard recovery of certain postural symptoms (Haddad *et al.*, 1977; Schaefer *et al.*, 1979; Smith and Curthoys, 1989).

Biochemical studies have shown that the immediate early gene c-fos and its protein product Fos, are expressed in the uvula, nodulus, flocculus and ventral paraflocculus 3-24 hours following UL (Kaufman *et al.*, 1992; Cirelli *et al.*, 1993, 1996). Immediate early genes such as c-fos are rapidly induced in neurones in response to cell stimulation and are involved in the first wave of gene transcription (Hughes and Dragunow, 1995). Thus their presence in the vestibulo-cerebellum indicates that the imbalance in activity between the vestibular nuclei is rapidly detected in these regions.

Kitahara *et al.* (1997a,b; 1999) used a range of morphological and immunohistochemical techniques to demonstrate the importance of the flocculus in the initial stages of VC. Using immunoreactivity for Fos as a marker of neuronal activation, the ipsilateral flocculus was found to inhibit the contralateral MVN, and these inhibitory effects were up-regulated following ipsilateral labyrinthectomy. Correspondingly, the inhibition of ipsilateral MVN neurones by the contralateral flocculus decreased after an ipsilateral labyrinthectomy (Kitahara *et al.*, 1997b). Hence, the bilateral flocculus could restore the balance of vestibular nucleus activities after UL, by an increased inhibition of the overexcited contralesional MVN neurones and a disinhibition of the suppressed ipsilesional MVN neurones.

Nitric oxide is a free radical gas that is released from neurones by simple diffusion and directly activates intracellular second messenger systems in other neurones. Kitahara *et al.* (1997a) demonstrated that in rats, at 12 hours post-UL, NO synthase (NOS; the enzyme which synthesises NO from arginine) immunoreactivity was significantly increased in unipolar brush cells of the ipsilateral flocculus and ventral paraflocculus, with no change in NOS immunoreactivity seen in the uvula, nodulus or vestibular nuclei. NOS immunoreactivity reached its maximum intensity in the flocculus at 24 hours post-UL and gradually disappeared 7 days after surgery. In the second part of this study, rats were treated with a continuous perfusion of the NOS inhibitor L-NAME into the bilateral flocculi from 2 hours before a UL. These rats displayed a significantly higher frequency of SN and a delayed recovery of SN compared to UL animals that had received a saline perfusion. L-NAME perfusion also leads to an induction of Fos-like immunoreactivity in the contralateral MVN, indicating that the suppression of NO leads to the activation of the contralateral MVN, causing a more severe imbalance in neuronal activities after UL (Kitahara *et al.*, 1999). These results suggest that in the initial stages of VC, NO mediated signalling in floccular unipolar brush cells could be a driving force of the flocculus mediated inhibitory circuits, resulting in the restoration of balance between the bilateral vestibular nuclei.

In contrast to the above results in rat, subcutaneous L-NAME perfusion in guinea pig either had no effect on VC, or accelerated compensation of SN if administered prior to the labyrinthectomy (Smith *et al.*, 2001). In addition, NOS activity in the

ipsilesional MVN of guinea pigs was significantly decreased immediately following UL, with no recovery of levels recorded by 50h post-UL (Anderson *et al.*, 1998), whereas no change in MVN NOS levels were observed in rat at time-points from 12 hours to 2 weeks post-UL (Kitahara *et al.*, 1997a). Liu *et al.* (2003) observed a significant decrease in the endothelial form of NOS (eNOS), but not the inducible form of NOS (iNOS) in the ipsilesional MVN of rat 2 weeks post-UL but not before. Thus there may be large species differences between rat and guinea pig in NOS activity, the subtype of NOS expressed, and in the floccular/NO contribution to VC.

Activation of intracellular signalling pathways in the cerebellum following UL has been implicated in the process of VC. Goto *et al.* (1997) demonstrated a regionally selective asymmetric expression of protein kinase C (PKC) isoforms in Purkinje cells within 6 hours of a UL, which had returned to normal within 24 hour. PKC levels increased in floccular and nodular lobes of the vestibulo-cerebellum contralateral to the lesion, and decreased in nodular lobes ipsilateral to the lesion. Intracerebroventricular injection of PKC inhibitors in rat significantly retarded the loss of SN during the first 8 hours post-UL (Balaban *et al.*, 1999), and intrafloccular microinjection of PKC inhibitors prevented increase in the excitability of MVN neurones at 4 hours post-UL, which is though to contribute to the recovery of their resting activity early in compensation (Johnston *et al.*, 2002) (see Section 2.6.6). De Zeeuw *et al.* (1998a) reported that both cerebellar long term depression (LTD) and VOR adaptation were abolished in transgenic mice that express a pseudosubstrate PKC inhibitor, in Purkinje cells. Thus retardation of VC by PKC inhibitors is consistent with transient attenuation of LTD in the vestibulo-cerebellum.

2.4.2 *Role of the inferior olive in vestibular compensation*

The olivo-cerebellar projection has been suggested to be indispensable for VC as lesions of the inferior olive disrupt VC (Llinas *et al.*, 1975; Azzena *et al.*, 1979). Llinas *et al.* (1975) reported that chemical lesioning of the IO in rat, prevented compensation occurring, or caused decompensation in animals that had received a labyrinthectomy 6 months previously. Biochemical studies have reported increased immediate early gene and brain-derived neurotropic factor (BDNF; a member of the neurotropin family) expression after labyrinthectomy in compartments of the olivo-

cerebellar system that integrate and transmit vestibular related information. C-fos mRNA, Fos protein, and BDNF levels, are all increased in the contralateral β nucleus of the inferior olive within 1 hour of a UL (Kaufman *et al.*, 1992; Cirelli *et al.*, 1996; Sato *et al.*, 1997; Li *et al.*, 2001). The β nucleus of the IO projects to the contralateral uvula-nodulus, which in turn provides a bilateral GABAergic projection to the vestibular nuclei. BDNF has been shown to modulate synaptic transmission efficacy, and to induce structural changes in axons and dendrites (McAllister *et al.*, 1999). Thus after UL, the IO detects the imbalance between bilateral vestibular nuclei, via projections from the vestibular nuclei themselves and from abnormal visual stimuli from the dorsal cap. Functional reorganisation, or modifications in synaptic efficacy in the olivo-cerebellar system may recalibrate the imbalance between vestibular nuclei, facilitating VC.

Summary

Results from lesion studies are always difficult to interpret as failure to compensate, suggests the region is necessary for compensation but does not indicate that it is causal. Also CNS lesions can result in diaschisis, which could be mistaken for retarded compensation, or decompensation. Thus far the results from lesion studied do not identify a clear role for the cerebellum or IO in VC. Biochemical studies however, indicate that the cerebellum and IO may be involved in the rebalancing of resting discharge between the bilateral VNC neurones after UL.

2.5 NEUROCHEMICAL CHANGES DURING VESTIBULAR COMPENSATION

Three main types of method have been used to study the neurochemical components of VC: 1) behavioural studies, examining the effect of various drugs, administered systemically or via an implanted cannula, on the time course of behavioural compensation; 2) electrophysiological studies, recording the effect of drugs on the neuronal correlates of VC; 3) biochemical and pharmacological studies: receptor binding studies, phosphorylation studies or biochemical assays performed on tissue from animals at various stages of compensation.

At present, the majority of studies are behavioural, yet interpretation of such studies is difficult because of the inability to specify the exact site of drug action. Different transmitter systems may play a role in different phases of VC, and there may be many different neurochemical changes that contribute to any specific aspect of behavioural recovery.

2.5.1 *Glutamate*

The presence of glutamate as a major neurotransmitter and neuromodulator within the VNC has attracted the possibility that glutamate receptor subunits, especially the NMDA receptor, may be involved in vestibular compensation. Biochemical studies of glutamate in the VNC during vestibular compensation have utilized HPLC, binding, immunohistochemical and *in situ* hybridisation techniques. In a large-scale HPLC study, Li *et al.* (1996b) found a complex range of amino acid changes in the bilateral VNC between 2 and 30 days post-UVD in rat. Significant decreases in glutamate levels were observed in all ipsilesional subnuclei except the MVN by two days post-UVD, which were maintained through to 30 days as compared to control animals. Decreased glutamate levels were observed in the dorsal region of the ipsilesional MVN by 30 days post-UVD. Normal glutamate levels were found in the ipsilesional VNC at 10 months post-UVD in squirrel monkey (Henley and Igarashi, 1991). No change was found in the density of glutamate binding sites in the ipsilesional VNC between 2 weeks and one year post-UVD in rat (Raymond *et al.*, 1989). More recently, Li *et al.* (1997) reported large decreases in AMPA and

NMDA receptor binding in the ipsilesional VNC, relative to the intact side, between 2 and 30 days post-UVD. Immunohistochemical studies revealed small but significant decreases in the AMPA receptor subunits GluR2/3 immunoreactivity on the lesioned side of all VNC regions in rat except the dorsal region of the MVN, relative to the intact side at 7 days post-UVD (Li *et al.*, 1996a). Most of these asymmetries disappeared between 14 and 30 days post-UVD. Conversely, a more recent immunohistochemical study in rat, found a bilateral decrease in GluR3 receptor subunit immunoreactivity in the MVN three days after UL returning to normal by 8 days post-UL (Rabbath *et al.*, 2002). *In situ* hybridisation (ISH) studies have generally found no change in the NR1 subunit of NMDA receptors (which is a key subunit of NMDA receptors) in the ipsilesional VNC of rat between 5 hours and 2 weeks post-UVD (Horii *et al.*, 2002; King *et al.*, 2002; de Waele *et al.*, 1994; but see Sans *et al.*, 1997). A recent ISH study linked to real time quantitative PCR, found decreases in the NMDA receptor subunit NR2A, the AMPA receptor subunit GluR2 and the metabotropic glutamate receptor subunit mGluR7, in the ipsilesional VNC at 6 hours post-UL (Horii *et al.*, 2002). These decreases were reversed at 50 hours post-UL, a timepoint which in rat correlates with the disappearance of most of the static symptoms of UL. However the down-regulation of these glutamate receptor subunits may be the result of decreased glutamate levels after denervation, rather than being a causal factor of compensation.

Electrophysiological studies do not support any changes in glutamate receptors during VC. Knopfel and Dieringer (1988) reported that the increase in efficacy of the commissural system in frog, which is associated with vestibular compensation (Dieringer and Precht, 1979a), does not result from an increased NMDA receptor component. Similarly, Smith and Darlington (1992) found no difference in the responses of MVN neurones to NMDA antagonists in slices from normal guinea pigs, or from guinea pigs labyrinthectomised 3 days to 2 months previously. There have been no electrophysiological studies to date looking at the effect of non-NMDA glutamate receptors on compensation, or looking at compensated neurones from 0-3 days post-UL.

Most data implicating glutamate receptors in VC is behavioural. In guinea pig systemic injections of the NMDA receptor antagonists MK-801 or CCP either

disrupted the development of VC during the first 24 hours (Darlington and Smith, 1989), or caused decompensation when the injection was delivered at 2-3 days, but had no effect after 12 days post-UVD (Smith and Darlington, 1988). Similar results were obtained with systemic injections of MK-801 in frog (Flohr and Luneburg, 1993) and rat (Kitahara *et al.*, 1995b; Kim *et al.*, 1997). Again the decompensating effects were time dependant and could not be observed at 60 days post-UL in frog (Flohr and Luneburg, 1993), nor after 2 weeks in rat (Kitahara *et al.*, 1995b). Substances administered systemically may have effects on regions other than the VNC, affecting compensation through indirect physiological changes such as altered blood pressure or drowsiness. Cannula studies, with drugs administered directly into the VNC, have provided more direct evidence for NMDA receptor modulation of VC. Injection of the NMDA receptor antagonist, CCP, directly into the ipsilesional VNC caused decompensation in guinea pigs compensated for 2-3 days (Sansom *et al.*, 1990). De Waele *et al.* (1990) recorded similar results with the NMDA receptor antagonist DL-APV administered to posturally compensated guinea pigs. Surprisingly, systemic, i.c.v., or intra-VNC injections of MK-801 before UL has the opposite effect to post-UL injections, in that SN and postural symptoms of UL are reduced (Aoki *et al.*, 1996; Sansom *et al.*, 1992, 2000). Recently, Gliddon *et al.* (2000) investigated the involvement of group 1 metabotropic glutamate receptors (mGluRs) in VC in guinea pig. Perfusion of the group 1 mGluR antagonist AIDA into the ipsilesional VNC from 30 minutes before to 30 minutes after UVD, significantly reduced YHT and the frequency of SN, suggesting that this receptor subtype may be involved in the expression of postural and ocular motor symptoms post-UVD.

Taken together, the results from biochemical, electrophysiological and behavioural studies indicate that there are probably no long-term changes in the number, affinity or efficacy of glutamate receptor subunits that could be involved in the maintenance of VC. However results from behavioural experiments suggest that NMDA and mGluR receptors may play a key role in the induction or development of VC whereas after a limited time period, which varies between species, they become redundant. This is consistent with their role other forms of plasticity such as LTP,

which has recently been demonstrated in the MVN (Grassi *et al.*, 1998; Darlington and Smith, 2000; Grassi and Pettorossi, 2001; Puyal *et al.*, 2003).

2.5.2 *GABA*

Changes in the GABAergic system after unilateral vestibular deafferentation have been less extensively studied than those in the glutamatergic system. The GABAergic system is involved in vestibulo-ocular and vestibulo-spinal reflexes (see Section 1.3.1), and could play a role in vestibular compensation after deafferentation. In normal animals, unilateral microinjection of GABA receptor agonists and antagonists into the lateral and medial vestibular nuclei resulted in postural asymmetries (Luccarini *et al.*, 1992) and changes in the gain of the horizontal VOR (Straube *et al.*, 1991). Behavioural data from humans with labyrinthine dysfunctions, demonstrated that systemic injection of the GABA antagonist picrotoxin accelerated compensation of postural symptoms (Ehrenberger *et al.*, 1982) whereas systemic injections of GABA agonists to compensated frogs can cause decompensation (Flohr *et al.*, 1985).

Electrophysiological studies have investigated the responses of MVN neurones from control and labyrinthectomised rats to the $GABA_A$ and $GABA_B$ agonists, muscimol and baclofen. Yamanaka *et al.* (2000) observed a marked down-regulation of the functional efficacy of both $GABA_A$ and $GABA_B$ receptors in the rostral region of the ipsilesional MVN at 4 hours post-UL, marked by a rightward shift and a decrease in slope of the dose-response relationship for both agonists. The down-regulation of $GABA_B$ receptor efficacy persisted long term, while $GABA_A$ receptor responses had returned to normal by 7-10 days post-UL (Johnston *et al.*, 2001).

Biochemical studies have also noted changes in the GABAergic system following UVD. A study by Li *et al.* (1996b), using HPLC techniques, observed early decreases in GABA concentrations in the ipsilesional VNC post-UL, with levels recovering in most regions, and significantly increasing in the dorsal MVN and LVN by 30 days post-UL. Immunohistochemical studies found increased GABA levels in the ipsilesional MVN, IVN and LVN at 3-7 days post operation, with decreased levels in the contralesional LVN (Thompson *et al.*, 1986; Tighilet and Lacour, 2001). One year post-UL, a bilateral increase in GABA levels was found in

the MVN, with the higher increase observed on the ipsilesional side (Tighilet and Lacour, 2001). However, an *in situ* hybridisation study detected no imbalance or change in GAD mRNA levels between 5 hours and 3 weeks post-UL in rat (de Waele *et al.*, 1994). Using autoradiography, Calza *et al.* (1992) observed a decreased benzodiazepine binding in the ipsilesional MVN at 3-23 hours post-UL, which returned to normal within 3 days suggesting a transient down-regulation of $GABA_A$ receptors.

The data from electrophysiological and binding studies suggest that there is a rapid down-regulation in the functional efficacy of GABA receptors in the ipsilateral MVN following lesion. This down-regulation could help to overcome the excessive commissural inhibition from the contralesional MVN, contributing to the rebalancing of resting activity between the bilateral VN.

2.5.3 *Acetylcholine*

There are several lines of evidence suggesting that acetylcholine (ACh) is a possible neurotransmitter involved in vestibular compensation, the majority arising from behavioural studies. Systemic administration of ACh agonists to compensated frog and squirrel monkey, resulted in decompensation or increasing severity of the remaining static symptoms (Abeln *et al.*, 1981; Ishikawa and Igarashi, 1985). Correspondingly, systemic administration of ACh antagonists resulted in over-compensation, or a reduction in the severity of static symptoms. Acetylcholinesterase (AChE) inhibitors also elicit decompensation in fully compensated frog (Bienhold and Flohr, 1980; Flohr *et al.*, 1985). More recently Fukushima *et al.* (2001) investigated the role of cholinergic vestibulo-cerebellum fibres in the compensation process. They found that selective destruction of vestibulo-floccular and vestibulo-uvulonodular cholinergic mossy fibres resulted in much more severe vestibulo-ocular symptoms during the initial stages of compensation.

Biochemical studies have noted changes in levels of ACh and related chemicals throughout compensation. Torte-Hoba *et al.* (1996) investigated AChE immunoreactivity in the VNC of rat, at 6 hours, 3 weeks and 1 year post-UL. They observed significantly decreased AChE activity in the ipsilesional caudal MVN at 6 hours post-UL, with a weaker decrease in activity persisting at one year. Yamada *et*

al. (1988) found no change in levels of ACh, muscarinic ACh receptor binding sites, or activity of AChE in the LVN up to 7 days post-UL in rat. However, Calza *et al.* (1992) observed a slight increase in the density of muscarinic receptors in the ipsilesional LVN between 3 and 10 days post-UL. More significant was the decrease in density of muscarinic receptors in the ipsilesional MVN, observed from 3 hours to 90 days post-UL (Calza *et al.*, 1992).

The results from behavioural and biochemical data together do not necessarily suggest that there is any plasticity in the ACh system which is responsible for compensation. Systemic injections of drugs which potentiate ACh transmission (ACh agonists or AChE inhibitors) cause decompensation in already compensated animals, whereas drugs which decrease ACh transmission (ACh antagonists) improve compensation (for a review see Smith and Darlington, 1991). The asymmetries in normal ACh receptor levels could be the result of the UL and the ACh modulating drugs may be having their effects by acting on these asymmetries.

2.5.4 *Monoamines*

Vestibular related syndromes in humans are clinically treated with histaminergic medications, implying a role for histamine in functional recovery of the vestibular system (Tighilet *et al.*, 2002). Betahistine, a partial agonist at the H_1 receptor and an antagonist at the H_3 receptor (Arrang *et al.*, 1985), facilitates compensation of both postural and locomotor symptoms following UVD in cat (Tighilet *et al.*, 1995). A subsequent immunohistochemical study showed a significant bilateral decrease in histamine fibre staining in the vestibular and tuberomammillary nuclei at 1, 3 and 52 weeks post-UVD; treatment of control and compensating cats with betahistine resulted in a near-total lack of histamine staining in these regions (Tighilet and Lacour, 1997). Betahistine works by up-regulating histamine turnover and release from nerve terminals, probably by blocking presynaptic H_3 receptors (Tighilet *et al.*, 2002). These results indicate that the decreased histamine fibre staining after UVD results from an increased release of histamine into the VNC, which promotes functional vestibular recovery.

Modifications of the serotonergic, dopaminergic and noradrenergic systems during VC are less clear. Cransac *et al.* (1996) studied the concentrations of

serotonin, dopamine, noradrenaline and their metabolites in the MVNs of albino and pigmented rats 6 hours after UL. In albino rats there was a bilateral increase in noradrenaline metabolites and an ipsilateral increase of dopamine, whereas no such changes were observed in pigmented rats. No change in serotonin levels were found in either species of rat but an increased utilisation of serotonin was suggested in the pigmented rat (Cransac *et al.*, 1996). Behavioural data on the effects of these monoamines on vestibular compensation are also unclear. Systemic administration of dopamine antagonists to frog have been show to retard (Smith and Darlington, 1991) or have no effect on compensation (Bienhold *et al.*, 1981). Systemic injections of noradrenaline agonists in compensated frogs caused decompensation of roll head tilt, whereas noradrenaline antagonists caused overcompensation (Flohr and Luneburg, 1985).

2.5.5 *Neuropeptides*

The adrenocorticotropic hormone neuropeptide fragment, $ACTH_{4\text{-}10}$, enhances compensation of roll head tilt in frogs (Flohr and Luneburg, 1982). Compensation of spontaneous nystagmus and vestibulo-spinal reflexes was enhanced in squirrel monkey by treatment with $ACTH_{4\text{-}10}$ (Igarashi *et al.*, 1985). Accordingly, systemic administration of $ACTH_{4\text{-}10}$, or the $ACTH_{4\text{-}9}$ analogue Org 2766, significantly accelerated the compensation of spontaneous nystagmus in guinea pig (Gilchrist *et al.*, 1990, 1994, 1996b). *In vitro* electrophysiological studies have compared the effects of $ACTH_{4\text{-}10}$ on MVN neurones from labyrinthectomised and control guinea pigs (Darlington *et al.* 1992, 1993). No difference in the inhibitory effects of $ACTH_{4\text{-}10}$ on MVN neurones was found between control and labyrinthectomised animals, or between ipsi- or contralesional neurones from compensated animals. The opioid receptor antagonist naloxone, has been demonstrated to significantly reduce the frequency of SN in compensating guinea pig, but has little effect on compensation of postural symptoms (Dutia *et al.*, 1996).

Summary

Many neurotransmitter and neuromodulator systems undergo changes during VC. These neurochemical changes occur over a range of different time courses, which may be related to the different stages in the initiation, development and maintenance of VC. The glutamatergic system has been the most extensively studied during compensation; yet while behavioural studies indicate that there may be some short term species specific involvement of glutamate receptors, biochemical studies have failed to find any consistent changes in receptor affinity, efficacy or number post lesion. Thus far there is no convincing evidence that changes in any of the above systems are causal factors in VC.

2.6 POSSIBLE MECHANISMS OF VESTIBULAR COMPENSATION

The ipsilesional vestibular nucleus complex (VNC) is a vital centre for vestibular compensation, since lesions of this region are shown to prevent, or cause a loss of, compensation (Spiegel and Demetriades, 1925, cited in Schaeffer and Meyer, 1974). Concurrent with this is the generally accepted hypothesis that recovery from static symptoms during compensation is associated with the return of resting activity in ipsilesional VNC neurones (Precht and Dieringer, 1985). Since the vestibular nerve does not undergo any functional regeneration post UVD, the problem for researchers was how the VNC neurones replace the resting activity lost as a result of deafferentation. Results from animal experiments have led to several hypotheses for possible mechanisms which may restore the lost resting activity.

2.6.1 *Reactive synaptogenesis*

Reactive synaptogenesis is the sprouting of healthy synaptic boutons into the synaptic contacts left by the degenerating axons of neurones destroyed during labyrinthectomy. Evidence for axonal sprouting and the formation of new synapses on second order vestibular neurones has been found in frogs (Dieringer *et al.*, 1984; Goto *et al.*, 2000, 2001). The only evidence for new synapse formation in mammals was found by Korte and Friedrich in cats, where at 5 days post UVD a new group of small boutons with flattened vesicles appeared in the SVN following disappearance of the normal large boutons (Korte and Friedrich, 1979). Yet the authors themselves suggest reserve at this finding, proposing that the newly observed boutons may have been previously present but altered by physiological activity after UL. Other studies in cats have found inconclusive (Raymond *et al.*, 1991; Dieringer, 1995), or no evidence (Gacek *et al.*, 1988) of reactive synaptogenesis up to one year post operation. Even where structural changes are observed it is hard to make the distinction between it being a causal affect or the effect of the compensation process. Ultimately, reactive synaptogenesis, which is thought to begin 6 days post operation (Smith and Curthoys, 1989), is too slow to account for the rapid compensation of static symptoms which is complete within 2-3 days in most species.

2.6.2 *Denervation supersensitivity*

Vestibular nucleus neurones deprived of glutamate as a result of ablation of the eighth nerve, could develop denervation supersensitivity, up-regulating their glutamate receptors, thus resulting in a greater effect of any remaining inputs. Glutamatergic neurones from other areas, synapsing onto the up-regulated receptors of VN neurones may cause enough excitation to re-establish their resting activity. Some studies have indicated that an initial up-regulation of NMDA receptors may occur in the VNC (de Waele *et al.*, 1990; Pettorossi *et al.*, 1992). However, this up-regulation is not maintained in the long term as injection of NMDA receptor antagonists after 2 weeks in the guinea pig and 6 weeks in frog has no effect on loss of compensation (Smith and Darlington, 1992). Other electrophysiological (Knopfel and Dieringer, 1988; Smith and Darlington, 1992), biochemical (de Waele *et al.*, 1994; Rabbath *et al.*, 2002) and pharmacological studies (Raymond *et al.* 1989) in a range of species, have been unable to provide any evidence of an increase in NMDA or AMPA receptor efficacy, affinity, or numbers post UL.

2.6.3 *Cerebellar shutdown hypothesis*

The cerebellar shutdown hypothesis (McCabe and Ryu, 1969), suggests the cerebellum modulates its output to cause a reduction of activity in the vestibular nuclei following UL. This would reduce the high spontaneous activity in the contralesional nuclei, decreasing commissural inhibition and leading to the rebalancing of activity between the bilateral VNC. This conclusion was drawn from the observation that in cat, one day following UL, both MVN are electrically silent (McCabe and Ryu, 1969; McCabe *et al.*, 1972). This bilateral shutdown of activity was attributed to the cerebellum which had been partially removed in all previous *in vivo* recordings. However, it was subsequently demonstrated that between 12 and 20 hours following UL in guinea pig with intact cerebellum, vigorous type I neuronal activity is present in the contralesional MVN (Smith and Curthoys, 1989). The cat presents both ocular and postural symptoms immediately following a UL, suggesting that the contralesional MVN is active and it is the asymmetry in bilateral resting activity of the VN that is responsible for these symptoms. These results suggest that the cerebellar shutdown hypothesis may not be correct.

2.6.4 *Substitution of sensory inputs*

Neural recovery after UL could be due to the substitution of other sensory inputs for the missing labyrinthine inputs. Sensory inputs suggested for this include visual, neck proprioceptive and somatosensory (Smith and Curthoys, 1989). Visual deprivation in cat (Courjon *et al.*, 1977), guinea pig (Smith *et al.*, 1986) and rhesus monkey (Fetter *et al.*, 1988) significantly reduced the rate of compensation of a number of static and dynamic symptoms. Yet the role of vision may be species and symptom specific, for example complete visual deprivation retarded the recovery of SN in cat (Courjon *et al.*, 1977) but had no effect on the recovery of SN in guinea pig (Smith *et al.*, 1986). A level of compensation is eventually reached in all visually deprived animals, where SN is absent even in complete darkness (Haddad *et al.*, 1977).

Spinal afferents have been implicated in VC. Transection of the spinal cord before UL retards compensation of head deviation (Azzena, 1969) and transection post-UL causes decompensation (Azzena, 1969; Jensen, 1979). Using autoradiography, Dieringer *et al.* (1984), demonstrated an increased spinal afferent projection to the VNC in frogs, 4 months post-UL. On a shorter timescale (from 3 days post-UL), Vibert *et al.* (1999a) recorded an increased efficacy of spinal input into the ipsilesional MVN, and decreased input into the contralesional MVN, intimating the functional substitution of deficient vestibular inputs with spinal related reflexes. As with many of the above theories, most of the experimental data for sensory substitution is collected at a time at which recovery from static symptoms is almost complete.

2.6.5 *Intrinsic mechanism hypothesis*

Recently the search for mechanisms of neuronal recovery has moved away from external and substituting inputs, to focus on intrinsic properties of the neurones themselves. The intrinsic mechanism hypothesis (Darlington and Smith, 1996), states that the recovery of resting activity in the ipsilesional vestibular nucleus complex is largely the result of changes in the intrinsic membrane properties of vestibular nucleus neurones. *In vitro* brainstem slice studies have shown that vestibular

neurones retain the spontaneous firing activity observed *in vivo*, in the absence of vestibular nerve or other CNS inputs and even once synaptic transmission has been blocked (Gallagher *et al.*, 1985, 1992; Darlington *et al.*, 1989; Serafin *et al.*, 1991a, b; Johnston *et al.*, 1994; Vibert *et al.* 1999b; Ris *et al.*, 2001; Him and Dutia, 2001). MVN neurones demonstrate a persistent Na^+ current which may be partly responsible for the spontaneous firing of these cells *in vitro* (see Section 1.1.2). However, the contribution of these intrinsic membrane properties to the *in vivo* firing of MVN neurones is unknown.

Darlington and Smith (1996) originally proposed that VNC resting activity is lost following UL as a result of 'neural shock' or diaschisis (induced by elevated Ca^{2+} levels in these cells), and recovery is due to the return of function to the intrinsic membrane properties that normally provide this resting activity. There has been no experimental evidence to date to support the theory that 'neural shock' renders the neurones electrophysiologically unresponsive. Indeed, Cameron and Dutia (1997) revealed that even at 2 hours post-UL in rat, when the *in vivo* symptoms are near their maximum intensity, MVN neurones isolated *in vitro* had discharge rates no different from normal. This supported the view that silencing of MVN neurones *in vivo* was due to disfacilitation, after loss of excitatory eighth nerve input and subsequent increased commissural inhibition. More significantly, recording from the rostral region of the ipsilesional MVN in rat horizontal brainstem slices, they demonstrated that beginning between 2-4 hours post-UL there is a significant increase in neuronal excitability compared to slices from control animals. This was measured as an increase in mean spontaneous tonic discharge rate from 14.1 ±0.69 spikes/sec (mean ±S.E.M.) in MVN slices from control rats to 21.56 ±1.7 spikes/sec in MVN slices from rats labyrinthectomised 4 hours previously. The significant increase in excitability was maintained until 48 hours post-UL, whereupon it remained elevated but no longer significant versus control. Cameron and Dutia proposed that this significant increase in excitability of the ipsilesional MVN neurones is responsible for the restoration of the resting activity in the initial stages of VC, as it will act to overcome the disfacilitation and increased commissural inhibition the neurones are subject to *in vivo*.

In the guinea pig, Darlington and colleagues reported a trend towards higher tonic discharge rates of ipsilesional MVN neurones from brainstem slices of fully compensated animals, compared to MVN neurones in slices from normal animals (Darlington *et al.*, 1992; Smith and Darlington, 1992). Further *in vitro* experiments in guinea pig brainstem slices provide evidence for increased neuronal excitability in MVN neurones after UL. In coronal brainstem slices, Vibert *et al.* (1999b) noted an increase in firing rate of ipsilesional MVN neurones at 7 days post-UL, which had increased to a level significantly higher than control by 30 days post-UL. This rise in firing rate actually increased further in synaptic uncoupling conditions (low Ca^{2+}/ high Mg^{2+} solution), resulting in a significant increase in ipsilesional neuronal excitability by 7 days post-UL. The authors proposed that from one week after the lesion, VC increasingly relies on changes in the intrinsic membrane properties of ipsilesional vestibular neurones. Ris *et al.* (2001) also investigated the intrinsic pacemaker properties of MVN neurones in coronal brainstem slices from guinea pig. A cocktail of antagonists of the main neurotransmitters in the vestibular nuclei (glutamate, GABA and glycine) was used to block synaptic transmission. Under these conditions they recorded a significant increase in the number of spontaneously active MVN neurones encountered and an increased firing rate of these neurones, at 48 hours and one week after labyrinthectomy compared to neurones from unlesioned animals. This result provided further evidence for an increase in pacemaker activity of MVN neurones after UL, and provided additional support for the theory that increased pacemaker activity can lead to the restoration of spontaneous firing activity in the deafferented vestibular neurones after labyrinthectomy.

Mechanisms underlying increased excitability in ipsilesional vestibular neurones

Recent intracellular studies by Him and Dutia (2001), revealed that the increase in resting activity of rostral ipsilesional MVN neurones in rat following UL is due to changes in the intrinsic properties of type B but not type A neurones. At 24-72 hours post-UL type B neurones displayed an increase in input resistance, spike amplitude and spontaneous firing rate, and had more depolarised resting membrane potentials than control neurones. These changes may be the effect of modulation of the

expression or function of Na^+, K^+ or Ca^{2+} channels, and could increase the responsiveness of type B neurones to their own intrinsic membrane conductances and external excitatory inputs. A systematic voltage clamp experiment detailing all the currents in control MVN slices and investigating any changes in these currents post-UL is needed to elucidate the precise role of these membrane properties.

Another possible mechanism proposed to underlie the increased excitability in MVN neurones was an adaptive down-regulation of the functional efficacy of GABA receptors on these neurones (Cameron and Dutia, 1997). This theory is supported by the observation of an initial decrease in benzodiazepine binding in the ipsilesional MVN, 3-24 hours post-UL, which returns to normal within 3 days (Calza *et al.*, 1992). Electrophysiological studies demonstrated that within 4 hours of UL in rat, the neurones in the rostral region of the ipsilesional MVN showed a marked down-regulation in their responsiveness to the $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen *in vitro* (Yamanaka *et al.*, 2000). Changes in $GABA_B$ receptor efficacy persisted for at least one week post-UL, whereas $GABA_A$ receptor efficacy had returned to control levels by this time (Johnston *et al.*, 2001). A decreased responsiveness to glycine, at 3 days post-UL in guinea pig, has also been recorded (Vibert *et al.*, 2000a). Decreased responsiveness of ipsilesional MVN neurones to inhibitory amino acids may be functionally compensatory as neuronal responses to commissural and cerebellar inhibitory inputs will be blunted; this could counteract the disfacilitation and increased commissural inhibition that silences these cells immediately after UL.

There now appears not to be a causal link between the down-regulation of GABA receptor efficacy and the increased excitability in MVN neurones following UL, as there was no correlation between the *in vitro* discharge rates and the cells responses to GABA agonists (Yamanaka *et al.*, 2000). In agreement, an ipsilesional flocculectomy performed at the same time as UL was shown to prevent the increased excitability in MVN neurones, but did not prevent the down-regulation of the functional efficacy of GABA receptors (Johnston *et al.*, 2002), indicating the existence of two independent cellular mechanisms mediating the two post-lesional changes. The development of the increase in neuronal excitability in the rostral ipsilesional MVN post-UL appears to be dependant on an intact ipsilesional flocculus

(Johnston *et al.*, 2002). In addition, intra-floccular microinjection of the PKC inhibitor BIS-1 and the mGluR1 antagonist AIDA prevented the increase in excitability (Johnston *et al.*, 2002). The authors suggest that the increased excitability in MVN neurones post-UL may be the result of LTD in the ipsilesional flocculus.

Glucocorticoid receptors (GR) have been implicated in the development of the increased excitability in MVN neurones post-UL (Cameron and Dutia, 1999; Johnston *et al.*, 2002). In rats that were kept urethane anaesthetised for 4-6 hours after UL, thereby not experiencing the stress that normally accompanies UL, the increase in excitability of the ipsilesional MVN neurones did not occur (Cameron and Dutia, 1999). Significantly, administration of the synthetic GR agonist, dexamethasone, to urethane anaesthetised rats at the same time as the UL restores these changes. These results suggest that the acute stress response, that accompanies the severe behavioural syndrome following UL, plays an important role in facilitating the cellular changes that mediate the increased excitability in ipsilesional MVN neurones in rat. Johnston *et al.* (2002) indicated that the locus for this GR dependant plasticity was the ipsilesional flocculus, as intra-floccular microinjection of the GR antagonist RU38486 abolished the increased excitability.

Summary

The vestibular system displays remarkable plasticity in its ability to adapt to the permanent loss of one vestibular afferent input. The behavioural recovery that follows a peripheral vestibular lesion is the result of multiple different processes, throughout which, separate symptoms compensate at different rates and to various extents. There are inter-species variabilities in the importance of specific CNS structures in VC and the effect of various compounds on the rate of recovery. Data also indicates that different mechanisms of adaptation may be activated at different time points between species. A neural factor common to all species in the process of VC is the recovery of resting activity in the ipsilesional VNC which starts to develop very quickly following lesion. Static compensation is associated with, and may be partly caused by, the return of this resting activity. Mechanisms for the rebalancing of resting activity between the bilateral VN can be generally divided into pre- and postsynaptic categories. Presynaptic theories suggest changes in the synaptic input to the ipsilateral VNC, such as altered efficacy of non-vestibular sensory inputs and increased transmitter release, whereas postsynaptic theories favour changes in the ipsilateral VNC neurones themselves such as up-regulation of excitatory postsynaptic receptors. There is evidence for many complex biochemical changes occurring in the VNC and cerebellum following UL, and it is probable that both pre- and postsynaptic alterations occur to bring about compensation. Yet how these various factors are linked, and which of them have a causal relationship with the physiological changes underlying compensation, remains to be determined.

Chapter 3

STRESS AND NEURAL PLASTICITY IN THE VESTIBULAR SYSTEM

3.1 INTRODUCTION

An increasing number of studies suggest that stress hormones and their metabolites have an important role in the process of vestibular compensation (for review see, Seemungal *et al.*, 2001). During stress, glucocorticoid steroids are secreted from the adrenal cortex; they have potent effects on metabolism and gene expression in a wide variety of tissues. Anxiety and stress in patients with vertigo significantly delays the recovery from vestibular symptoms (Yardley *et al.*, 1994). Conversely, treatment of patients with the glucocorticoid steroid methylprednisolone, improves compensation of symptoms of vestibular neuritis (which rapidly damages the unilateral peripheral vestibular organs) (Ariyasu *et al.*, 1990; Kitahara *et al.*, 2003). Thus steroid hormones, and particularly glucocorticoid based therapies may be useful in the treatment of vestibular disorders.

Yamanaka *et al.* (1995a) showed that administration of the synthetic glucocorticoid agonist dexamethasone, prior to surgery facilitated the behavioural recovery following UL in rabbit; in contrast, administration of the glucocorticoid antagonist, RU38486, delayed compensation. Glucocorticoids may have a direct action on MVN neurones, as iontophoretic administration of dexamethasone to the VNC produced a dose dependent increase in the firing rate of type 1 MVN neurones, which could be blocked by RU38486 (Yamanaka *et al.*, 1995a). However in a recent study, systemic administration of dexamethasone was found to have no effect on the rate of compensation of spontaneous nystagmus in the guinea pig (Alice *et al.*, 1998).

More intriguingly the development of the increase in excitability of MVN neurones, observed from 4 hours post-UL in rat, is dependant on activation of glucocorticoid receptors. Thus, the development of increased excitability in rostral ipsilesional MVN neurons was prevented by systemic administration of the glucocorticoid receptor (GR) antagonist RU38486, and did not develop in animals that remain anaesthetised after UL. However, in anaesthetised animals that were treated with the synthetic GR agonist dexamethasone simultaneously with UL, the increase in excitability did occur (Cameron & Dutia, 1999). These data indicate that the acute stress that normally accompanies the severe behavioural symptoms immediately after UL may have an important role in the process of vestibular compensation in rat. This chapter investigates the role of the stress system in neuronal plasticity in lesioned rostral MVN neurones following UL.

3.2 LITERATURE REVIEW

3.2.1 The stress response

An essential requirement for the survival of any organism is its ability to maintain internal homeostasis in response to a variety of stressful conditions. Mammals have developed a complex neuroendocrine stress axis, the hypothalamo-pituitary-adrenocortical (HPA) axis (see Figure 3.1), which coordinates metabolic and behavioural responses to both physical and psychological stressors. The end product of this axis is the release of adrenal steroids from the adrenal cortex. The adrenal steroids are known collectively as corticosteroids and consist of mineralocorticoids and glucocorticoids. They exert effects on metabolism and gene expression in virtually every organ and tissue in the body. Corticosteroids exert their effects through two types of intracellular receptors: mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). Regulation of corticosteroid actions is achieved at the levels of steroid synthesis and metabolism. The HPA axis regulates steroid synthesis, while corticosteroids are metabolised in many tissues by enzymes, including 11β-hydroxysteroid dehydrogenase (11β-HSD), which specifically regulates the levels of active glucocorticoids within the body's tissues.

3.2.2 Corticosteroid synthesis

Corticosteroids are synthesised from cholesterol in the adrenal cortex. The adrenal cortex is subdivided into three morphologically distinct zones: the zona glomerulosa is responsible for the production of the major mineralocorticoid, aldosterone; the zona fasiculata and zona reticularis are responsible for the production of the glucocorticoids, cortisol (in humans) and corticosterone (in rodents). Glucocorticoid synthesis and release is controlled almost entirely by the binding of adrenocorticotropic hormone (ACTH) to specific receptors on the outer cell membrane of the adrenal cortex. ACTH is in turn released from corticotrophes in the anterior pituitary gland and its secretion is regulated by corticotrophin releasing factor (CRF), arginine vasopressin (AVP) and other agents released from the hypophyseal portal circulation in response to a variety of stimuli (Fig. 3.1. For a review, see Jones and Gillham, 1988). Glucocorticoids provide negative feedback

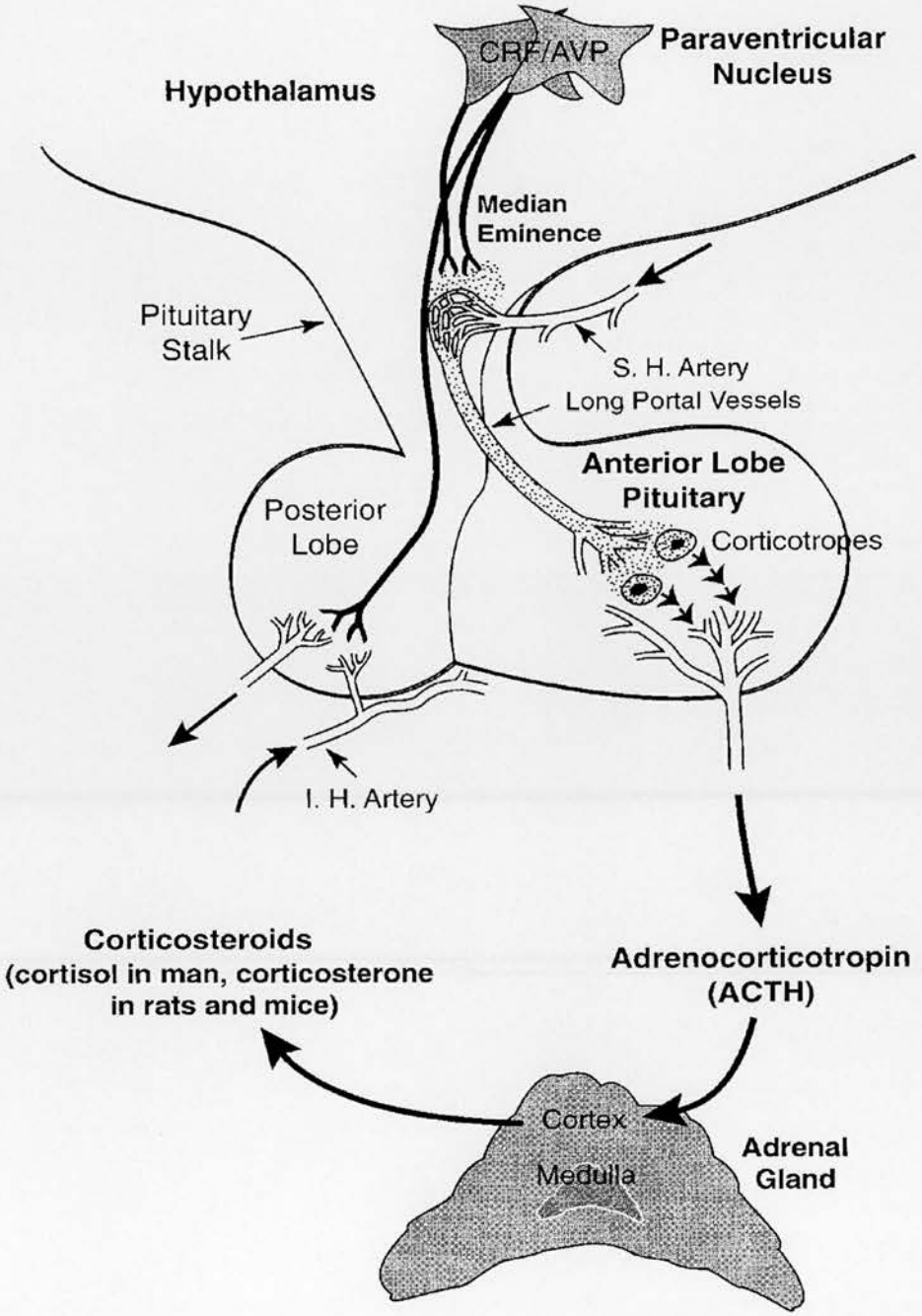


Figure 3.1 Schematic representation of the hypothalamic-pituitary-adrenal axis. CRF, corticotrophin releasing factor; AVP, arginine vasopressin; I.H., inferior hypophysial; S.H., superior hypophysial. (Adapted from Turnbull and Rivier, 1999)

onto the CRF and AVP neurones in the hypothalamic paraventricular nucleus (PVN), and also onto the corticotrophes in the anterior pituitary, thus completing a negative feedback route which regulates their plasma concentration (Orth, 1992).

In addition to the homeostatic negative feedback loop controlling glucocorticoid secretion, there are other factors known to affect glucocorticoid release. Glucocorticoid and ACTH secretion exhibit a circadian rhythm in mammals (Kant *et al.*, 1986). This is modified by sleep and light patterns such that pulse amplitude ACTH secretion reaches a peak a few hours before waking and declines throughout the day to reach a nadir in the evening. Plasma glucocorticoid levels rise and fall in response to ACTH secretion thus reaching maximal levels in the early morning. The mechanism of this circadian rhythm is probably a combination of an endogenous pacemaker in the suprachiasmatic nucleus of the hypothalamus and environmental stimuli (Jones and Gillham, 1988).

Both physical and psychological stress such as exercise, trauma, surgery, fear and depression activate the HPA axis by increasing hypothalamic secretion of CRF and AVP, stimulating ACTH and glucocorticoid secretion.

3.2.3 Glucocorticoid circulation and receptors

Glucocorticoid hormones are released immediately following synthesis, with only small amounts stored in the gland (Dickerman *et al.*, 1984). Circulating glucocorticoids are mostly bound to the plasma proteins, corticosteroid-binding globulin (CBG) or transcortin. Only a small amount (3-4%) travels as free hormone (Dunn *et al.*, 1981). Glucocorticoids have widespread and varied actions in the body and CNS. Within the body they modulate fuel homeostasis and salt balance, have profound immunosuppressive effects, effect bone metabolism and connective tissue function. In the central nervous system (CNS) they have multiple effects including modulation of sleep patterns, mood, cognition and reception of sensory inputs (De Kloet *et al.*, 1998).

Glucocorticoids act by binding to the intracellular MRs and GRs. These ligand-activated receptors function as nuclear transcription factors and modulate cell function by activating or repressing target genes or gene networks. These classical actions are usually slow in onset and long in duration. More recently, rapidly evoked

changes in electrical properties of neurones have been demonstrated by both steroids and neurosteroids, indicating the presence of a membrane receptor for the steroids (Joels, 1997).

Many of the known steroid receptors have been characterised by binding studies and cloning. Steroid receptor proteins have molecular masses of around 65 to 100 kDa (O'Malley, 1990). Each receptor protein binds a single steroid molecule, but the receptors usually dimerise when bound to DNA (Perlmann *et al.*, 1990; Baniahmad and Tsai, 1993). The most highly conserved domain is a cystine-rich DNA binding domain containing a zinc finger structure (Freedman *et al.*, 1988; Freedman and Luisi, 1993). Two zinc fingers confer specificity for the steroid response element and are required for, and stabilise the receptor-DNA interaction (Freedman and Luisi, 1993; Glass, 1994). Glucocorticoids are thought to enter cells by passive diffusion, and bind to the C-terminal ligand binding domain of their receptors. This leads to the dissociation of one of its associated heat shock proteins (hsp) hsp90, thus "activating" the receptor and enabling it to bind to DNA (Sanchez *et al.*, 1985; Denis *et al.*, 1988). The activated receptor-hormone complex translocates to the nucleus where it binds as homodimers to specific regulatory DNA sequences called glucocorticoid response elements (GREs) (Glass, 1994). GREs are frequently located near the promoter region of target genes, and receptor binding results in activation or suppression of transcription (Baniahmad and Tsai, 1993). Intriguingly, both GR and MR bind to the same DNA response elements (the DNA binding domain being highly conserved between MR and GR, with 94% homology), indicating that the specificity of genomic actions lies in DNA sequences outside the GRE, or in interactions with other transcription acting proteins (Pfahl, 1993).

The two receptors differ in their affinity for ligands. In rat, corticosterone binds with a high affinity (Kd~0.5nM) to the MR, and with an approximately 10-fold lower affinity to the GR (Joels, 1997). The daily variations in glucocorticoid levels, due to circadian fluctuations and acute stress, will alter the relative occupation of MR and GR. Under basal conditions there will be a predominant activation of MR, but upon increasing glucocorticoid levels, at the diurnal peak or in response to stress, GR will become additionally activated (Reul and de Kloet, 1985).

3.2.4 MR and GR distribution in the brain

Both MR and GR receptor types are expressed in the brain. GR are expressed fairly ubiquitously by most neurones and glia, with high levels found in the hippocampus, neocortex, thalamus, cerebellum and stress related nuclei of the hypothalamus and brainstem (Morimoto *et al.*, 1996; Kawata *et al.*, 1998). MR show a more restricted distribution, with high expression confined mainly to the limbic regions, and lower expression observed in the brainstem, sensory and motor nuclei and spinal cord (McEwen *et al.*, 1986; Reul and de Kloet, 1986; Ahima and Harlan, 1990).

3.2.5 Glucocorticoid actions in the brain

Glucocorticoids work at various levels to alter cellular excitability. They can alter ionic conductances, affect the efficacy of transmitter systems, and alter general cell properties such as metabolism and cell morphology. Glucocorticoids can alter the electrical properties of neurons by targeting voltage gated ion channels, signalling cascades activated by neurotransmitters (including ligand-gated ion channels and G-protein coupled receptors), and the pumps and transporters responsible for maintenance of ionic gradients across the cell membrane. Many studies of steroidal action have been carried out on the hippocampal brain slice, since hippocampal CA1 pyramidal neurons express a high density of both MR and GR. With use of selective ligands for MR and GR and artificial manipulations of corticosteroid levels in ADX rats, discrimination between the physiological effects mediated by either receptor subtype is possible.

Of the voltage-gated ion conductances investigated, Ca^{2+} currents are the most potently regulated by corticosteroids. Predominant MR activation as seen under rest at the circadian nadir, is associated with a small amplitude of voltage-gated Ca^{2+} conductances (Karst *et al.*, 1994). Additional activation of GR increases the amplitude of Ca^{2+} conductances (Kerr *et al.*, 1992; Karst *et al.*, 1994; Werkman *et al.*, 1997). Absence of any glucocorticoid receptor activation, as observed in ADX rats, also results in large Ca^{2+} conductances; thus a U-shaped dose-dependency relation exists for glucocorticoid effects on Ca^{2+} current amplitude (Werkman *et al.*, 1997). Glucocorticoid altered current amplitudes appeared in slices after a few hours

and effected mainly high voltage activated Ca^{2+} currents (Karst et al 1994). This time delay between manipulation of corticosterone levels and effect, and the fact that these changes rely on protein synthesis imply a genomic mechanism for action (Joels, 1997). Properties of Ca^{2+} currents, such as voltage dependence and their steady state inactivation, appeared to remain unaltered by corticosteroid treatment, leading to the suggestion that corticosterone acts to increase the number of Ca^{2+} channels rather than producing a change in Ca^{2+} channel structure (Werkman *et al.*, 1997). However Nair *et al.* (1998) recently characterised the mRNA expression of voltage and ligand gated Ca^{2+} channels in individual dissociated CA1 neurones in response to long term corticosterone, observing that this treatment altered the expression of mRNA to those subunits favouring enhanced Ca^{2+} influx (e.g. increased expression of NR2B over NR2A). Thus electrophysiological and molecular evidence supports the concept that voltage and ligand gated Ca^{2+} channel subunits are modulated to increase Ca^{2+} influx after GR activation.

Of all K^+ conductances tested, only the inwardly rectifying K^+ conductance, which is active at very negative membrane potentials, was sensitive to corticosteroid receptor activation. Increasing occupation of GR in addition to MR was shown to up-regulate the amplitude of the inwardly rectifying K^+ current (I_Q) (Karst *et al.*, 1993), which generally serves to counteract temporary disturbances of the resting membrane potential.

Ligand-gated ion channels appear far less regulated by corticosterone than voltage-gated channels. In general, modulatory actions of corticosterone on synaptic transmission mediated by glutamate- or GABA receptors were seen with very high concentrations of the hormone (Joels, 2000). The mechanisms by which corticosteroids modulate amino acid transmission are unclear. When effective, corticosteroid effects are relatively fast in onset (< 20 min delay), but are reversible within 10-20 minutes. Activation of MR receptors appears to stabilise (Joels and de Kloet, 1993) or even enhance glutamate and GABA mediated synaptic transmission (Rey *et al.*, 1989), whereas GR activation decreases this. Untreated ADX rats also elicit a decrease in synaptic transmission, indicating again a U-shaped dose-dependency curve for glucocorticoid effects on amino acid transmission (Joels,

1997). Hence MR and moderate GR activation results in stabilised or enhanced synaptic transmission.

It has become clear in recent years that some metabolites of peripherally released steroids and brain derived neurosteroids can rapidly alter the excitability of neurones by binding to membrane receptors on ion channels. One of the most documented examples of a non-genomic action of steroids is the modulation of $GABA_A$ receptors by neurosteroids. The neurosteroid dehydroepiandrosterone (DHEA), and its sulphate derivatives, are secreted from the adrenal cortex during stress and can act as non-competitive modulators of the $GABA_A$ receptor (Demirgoren *et al.*, 1991). 5α derivatives of progesterone such as allopregnanolone and dihydroprogesterone bind to $GABA_A$ receptors at sites different from benzodiazepine and barbiturates and are allosteric agonists for $GABA_A$, inducing chloride currents (Lambert *et al.*, 1995). In different neuronal populations distinct neurosteroids may act as positive or negative modulators of $GABA_A$. For example, allopregnanolone has been shown to act *in vitro* to potentiate $GABA_A$ mediated responses, while pregnenolone sulphate negatively modulates the same receptors (Compagnone and Mellon, 2000). *In vivo*, allopregnanolone rapidly potentiated the extracellularly recorded inhibitory responses of cerebellar Purkinje neurones to GABA (Smith *et al.*, 1987). Iontophoretic application of the neurosteroid dehydroepiandrosterone sulphate (DHEAS), onto MVN neurones *in vivo*, had no effect on the spontaneous and rotation induced firings of type 1 neurones (Yamamoto *et al.*, 1998). However, DHEAS dose dependently blocked the decrease in firing which occurred when $GABA_A$ and $GABA_B$ agonists were applied to the neurones (Yamamoto *et al.*, 1998). These effects occurred within a few seconds of administration suggesting that the neurosteroid can block both $GABA_A$ and $GABA_B$ mediated signalling via a non-genomic, perhaps membrane action.

Following UL in rat, there are changes in the responsiveness of ipsilesional MVN neurones to GABA: A marked down-regulation of the functional efficacy of both $GABA_A$ and $GABA_B$ receptors is observed in the rostral region of the ipsilesional MVN, in brainstem slices taken from animals that have compensated for 4 hours following UL (Yamanaka *et al.*, 2000). This down-regulation may be

functionally compensatory as it will help the lesioned neurones overcome the excessive commissural inhibition they are subject to *in vivo*, following UL. Glucocorticoid receptors have been implicated in the up-regulation of excitability that occurs in ipsilesional MVN neurones from 4 hours following UL in rat (Cameron and Dutia, 1999; Johnston *et al.*, 2002). This chapter investigates the possibility that glucocorticoids play a role in the GABA receptor plasticity at this time.

3.3 AIMS AND EXPERIMENTAL RATIONALE

The following experiments were designed to investigate the role of the stress axis in the down-regulation of GABA receptor efficacy in lesioned rostral MVN neurones at 4h post-UL.

1. To investigate the effect of endogenous stress hormones on the down-regulation of GABA receptor efficacy *in vitro*, 14 animals received a left UL under urethane anaesthesia (UL) and were kept in a temperature regulated cage for 4 hours following surgery. These animals did not wake up at any time after UL and therefore experienced none of the stress normally associated with the vestibular deafferentation syndrome.

2. To determine whether the down-regulation of GABA receptor efficacy in MVN neurones was affected by the anaesthetic used, 14 animals received a sham operation under urethane anaesthesia (Sham), and were kept in a temperature regulated cage for 4 hours after surgery.

3. To mimic the endogenous activation of the stress axis that would normally occur in alert labyrinthectomised animals, the synthetic glucocorticoid receptor agonist, dexamethasone (DEX), was administered (5mg/kg i.p.), 15 min before the lesion to urethane anaesthetised labyrinthectomised animals (UL+DEX) (n=15).

4. To control for any direct effects of dexamethasone on the down-regulation of GABA receptor efficacy in MVN neurones, 14 sham operated urethane anaesthetised animals received the same protocol of dexamethasone administration as above (Sham+DEX).

3.4 METHODS

Animals

Male Sprague-Dawley rats 80-120g (Bantin & Kingman, UK) were used throughout these experiments. Animals were housed in litter groups under control conditions: ambient temperature 21-23 °C, humidity 50-60%, 12 hour light-dark cycle, food and water *ad libitium*. Animals were kept in the Medical Faculty Animal Area (MFAA) for at least 4 days before the start of experimental procedures, to accustom them to handling and minimise the effects of non-specific stress. Animal care and experimental procedures were conducted in accordance with UK Animals (Scientific Procedures) Act 1986.

Anaesthesia

For all experiments in this chapter, rats were given a single intraperitoneal (i.p.) injection of urethane (ethyl carbamate, 25% weight/volume solution; Sigma, UK) at a dose of 1.25g/kg, 15 min before the start of surgery. This produced the required level of anaesthesia for the duration of the experiments.

Surgery

Animals were anaesthetised with urethane anaesthesia. A sufficient level of surgical anaesthesia was determined by lack of reflex to a paw pinch. Surgery was carried out under aseptic conditions. For experiments using DEX, a single i.p. injection 5mg/kg was administered after anaesthesia, 15 min before the start of surgery. Animals were shaved at the back of the head between the two ears, and the area was cleaned with disinfectant iodine solution (Betadide, UK). Under a microscope (Wild ms-c, Heerburg, Switzerland) a 3 cm incision was made with a sterile scalpel above the left ear, and the surrounding connective tissue removed using sharp surgical scissors. The area was clamped using surgical clips. The middle ear was opened and the ossicles removed with fine forceps. Muscles attached to the caudal side of the lamboidal ridge were cleared and the temporal bone exposed. The bony duct of the horizontal semicircular canal was visualised, then exposed by drilling with a 0.7mm diameter dental drill (Precision P.C.B drill RS Components,

UK). The area was kept clear with a needle attached to a suction pump (Eschmann VP25, International Market Supply). The canal was opened above the point of exit of the seventh cranial nerve, and the drilling continued following the open canal duct anteriorally through the ampula into the vestibule. The contents of the vestibular cavity were rinsed with 100% ethanol (Sigma, UK). The wound was sutured and the animals returned to their home cages. Sham operated animals underwent the same procedure, but the canal was not opened and the inner ear was undamaged.

Preparation and maintenance of slice containing MVN *in vitro*

After 4 hours the animal was decapitated using a small animal guillotine. Scalp and neck muscles were removed, and fine curved scissors were used to make a midline incision in the scull extending from the occipital to the frontal bone, and an incision across the midline. The occipital, parietal and frontal bones were removed using fine bone rongeurs. The whole exposed brain was cleared of meninges and a fine scalpel used to cut the cranial nerves and tip the brain into a beaker of ice cold (4°C) artificial cerebrospinal fluid (aCSF; see solutions) bubbled with 95% O_2 and 5% CO_2. The brain was kept in the ice cold aCSF for one minute before being placed on a cold dissecting stage. The cortex extending rostrally from the inferior colliculus was removed from cerebellum and brainstem block. The brainstem containing the overlying cerebellum was placed on its rostral surface and the brainstem gently removed from the cerebellum by parting the two with a blunt scalpel blade and cutting through the cerebellar peduncles. This remaining brainstem block containing the MVNs sitting prominently either side of the IVth ventricle on the dorsal surface of the medulla, was cleared of adherent pia. The block was then cemented with the floor of the forth ventricle uppermost to the advancing stage of a Vibroslice (Campden Instruments Ltd, UK). The stage was secured into the chamber of the Vibroslice and submerged in ice cold aCSF pre-bubbled with 95% O_2 and 5% CO_2. The block of tissue containing the MVN was cut in the horizontal plane approximately horizontal to the floor of the fourth ventricle, thus containing the entire rostro-caudal length of the MVN. The first slice containing the top 100-200μM of the nuclei was discarded. The next slice was cut to a thickness of 350μM and was

the thickness used for all experiments. The slice contained both MVNs, sat either side of the fourth ventricle. This was trimmed down the midline, to give the ipsi- and contralateral nuclei. The ipsilateral (left) MVN was transferred to an interface-type chamber which was continuously perfused with aCSF bubbled with 95% O_2 and 5% CO_2 (pH 7.4, flow rate 1.8 ml/min) and maintained at a temperature of 33°C ± 0.2°C. A continuous stream of 95% O_2 and 5% CO_2 gas saturated with water vapour was passed over the slice to oxygenate the slice and keep it moist. Slices were incubated for at least an hour in the chamber before recording commenced.

Drugs and Solutions

1. Artificial cerebrospinal fluid (aCSF)

The aCSF used for the preparation and maintenance of the slices was made up fresh on the day of the experiment according to the following recipe (Llinas and Sugimori, 1980)(in mM): Sodium chloride (NaCl) 124; Potassium chloride (KCl) 5; Potassium dihydrogen ortophosphate (KH_2PO_4) 1.2; Calcium chloride 2-hydrate ($CaCl_2$) 2.4; Magnesium sulphate heptahydrate ($MgSO_4$) 1.3; Sodium bicarbonate ($NaHCO_3$) 26; D-(+)glucose 10. The aCSF was bubbled with a gas mixture of 95% O_2 and 5% CO_2 to oxygenate it and maintain its pH at 7.4.

2. Muscimol and Baclofen

The $GABA_A$ agonist muscimol (5-aminomethyl-3-hydroxyisoazole) and the $GABA_B$ agonist baclofen (4-amino-3[4-chlorophenyl]butanoic acid) were obtained from Sigma, UK. Small aliquots of stock solution were made in distilled water and frozen until required. Aliquots of the stock solutions were thawed and diluted to their final concentrations in oxygenated aCSF immediately before use. Drug solutions were applied to the slice by switching the perfusion inlet tube to the appropriate reservoir.

3. DEX

Dexamethasone 21-phosphate was obtained from Sigma, UK. Aliquots of 1mg/ml solution were made in distilled water and frozen until required. Dexamethasone was administered i.p. at a dose of 5mg/kg, 15 min prior to the start of surgery.

Data collection and analysis

A conventional electrophysiological set-up was used for data collection, storage and analysis: glass microelectrodes filled with 2M NaCl coupled to an Axoclamp 2B amplifier (Axon instruments, USA), were used to systemically explore the MVN for tonically firing cells. 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 fed to a CED 1402 *plus* analogue-to-digital interface (Cambridge Electronic Design, Cambridge, UK) connected to a microcomputer (DCS 386, UK), which was used to display the instantaneous discharge frequency, autocorrelation and interspike interval histogram in real time, in order to ensure stable recording conditions. All data analysis was completed off-line, except when measuring extracellular firing rates which was done on-line. Several tonically active cells were often recorded in the course of a single track and the location of each track was noted with reference to the boundaries of the MVN. For the purpose of the analysis the MVN was divided by eye into three regions: rostral third, middle third and caudal third.

In the experiments in this chapter, recordings of the mean tonic discharge rate of cells sampled in the rostral third of the MVN were made, and the location of each cell recorded. The response of these cells to a 60-second test pulse of agonist were measured, as the maximal decrease in discharge rate expressed as a percentage of the resting discharge rate of each cell. The mean inhibitory effects of each concentration of the two agonists were calculated for all the cells tested, and sigmoidal functions of the form y=Bottom + (Top-Bottom) / (1+10^((logEC50-X)*HillSlope)), were fitted to the dose-response relationship using GraphPad Prism 3.03 software (GraphPad Software Inc, San Diego, USA). Although values were assigned to 100% inhibition

for all responses where the tonic activity of the cells were completely inhibited by GABA agonists, the parameters of the curve fitting algorithm were not constrained to within this range. The dose response relationships were compared using two-way ANOVA with Student-Newman-Keuls post hoc testing using SigmaStat 3.0 (SPSS Inc, Chicago, USA). Significance was assumed when $p<0.05$. Results are given as mean ±S.E.M. throughout.

3.5 RESULTS

Extracellular recordings were made from tonically active neurones, in the rostral region of the MVN, in brainstem slices from rat. The rostral region of the MVN has been shown to receive a particularly dense innervation of primary afferents from the ipsilateral semicircular canal (Stein and Carpenter, 1967; Gacek, 1969; Brodal, 1974; Sato *et al.*, 1989). Previous studies (Yamanaka *et al.*, 2000; Johnston *et al.*, 2001) have shown that in slices prepared from 4h UL animals, MVN cells in the rostral region of the ipsilesional nucleus show a marked down-regulation of their response to both the $GABA_A$ agonist muscimol, and the $GABA_B$ agonist baclofen, seen as a rightward shift and a decrease in slope of the dose response relationships for the two agonists. The effect of the glucocorticoid agonist dexamethasone on the responsiveness of MVN neurones to GABA was investigated by bath application of muscimol and baclofen to MVN slices prepared from urethane anaesthetised animals that had received a sham labyrinthectomy or a left labyrinthectomy 4h previously either with or without administration of dexamethasone (DEX).

1. Effect of glucocorticoid agonist dexamethasone on the mean discharge rate of ipsilesional rostral MVN neurones *in vitro*

As shown in figure 3.2, the mean discharge rates of MVN cells from DEX treated, sham operated animals (Sham+DEX) was not different from Sham operated animals (Sham) (Sham+DEX: 14.9 ±0.96 spikes/sec, n=41 cells, versus Sham: 15.0 ±1.13 spikes/sec, n=38 cells); all animals remained anaesthetised for 4h following surgery. These data indicate that DEX treatment alone does not result in changes in the mean *in vitro* discharge rate of MVN neurones. The firing rate of neurones recorded from UL animals which remained anaesthetised for 4h following surgery (UL: 15.5 ±1.23 spikes/sec, n=48 cells) were not different from the Sham or Sham+DEX groups. This indicates that the lesion alone is not sufficient to induce the increase in excitability of MVN neurones. However, neurones recorded from DEX treated anaesthetised UL animals (UL+DEX) were found to be firing at a significantly higher mean discharge rate than all other groups (21.0 ±1.46 spikes/sec,

n=34 cells; $p<0.002$, one-way ANOVA). These data confirm the previous findings of Cameron and Dutia (1999) who recorded firing rates of neurones in the rostral ipsilesional MVN of: 15.22 ±1.44 spikes/sec in sham operated animals anaesthetised with avertin so they awoke after the surgery, increasing to 21.61±1.8 spikes/sec in avertin anaesthetised UL animals. Upon urethane anaesthesia, the firing rate in UL animals fell to 16.79 ±1.49 spikes/sec, but addition of DEX to urethane anaesthetised UL animals restored the firing rate to 22.31 ±1.8 spikes/sec. These data suggest that while the lesion alone is not sufficient to induce the increase in excitability of MVN neurones, administration of DEX to UL anaesthetised animals restores the large compensatory increase in excitability seen in alert animals.

2. Effect of glucocorticoid agonist dexamethasone on the responsiveness of MVN neurones to muscimol and baclofen following UL

As illustrated in figure 3.3, bath application of the $GABA_A$ agonist muscimol, and the $GABA_B$ agonist baclofen to spontaneously active MVN neurones, caused reversible, dose-related inhibition of all the MVN neurones tested. Table 3.1 shows the parameters of the sigmoidal curves fitted to the dose-response relationship (maximal inhibition, half-maximal agonist concentration (EC_{50}) and slope) for all the data in these experiments.

The effects of muscimol and baclofen on rostral MVN neurones in slices from anaesthetised animals having undergone a sham operation (Sham), sham operation with administration of DEX (Sham+DEX), or a left labyrinthectomy (UL), did not differ as illustrated by their respective dose response curves in figures 3.4 and 3.5. These data indicate that neither the lesion alone, or DEX treatment alone is sufficient to induce the down-regulation of GABA receptor efficacy seen in alert animals.

In slices prepared from anaesthetised animals that had been unilaterally labyrinthectomised 4h previously and administered the glucocorticoid receptor agonist DEX (5mg/kg i.p., 15 min before the lesion) (UL+DEX), cells in the rostral region of the ipsilesional nucleus showed a marked decrease in their responsiveness to both muscimol and baclofen. This was seen as a significant rightward shift in the dose response curve relationships for both agonists compared to all other groups (Fig. 3.4 and 3.5, $p<0.001$, two-way ANOVA). There was over a three-fold increase

in the EC_{50} value of the fitted dose response curves for muscimol, and a two and a half-fold increase in the EC_{50} value of the fitted dose response curves for baclofen when compared with any other experimental group, and these were accompanied by a decrease in slope in both cases (see Table 3.1). These data indicate that in urethane-anaesthetised labyrinthectomised animals, administration of DEX restores the down-regulation of GABA receptor efficacy as seen in alert animals.

The relationship between the *in vitro* resting discharge rates of the ipsilesional rostral MVN neurones and their inhibitory responses to the representative doses of 3μM baclofen and 10μM muscimol are illustrated in figure 3.6. In slices from UL+DEX animals, more cells were found with resting firing rates above 20 spikes/sec, reflecting the increase in the mean resting discharge rate of these cells after UL (Figures 3.6A and 3.6B, crosses). In parallel with this the inhibitory effects of the two agonists were uniformly reduced. There was no correlation of the inhibitory response and the resting discharge of the neurones within any individual experimental group. However, a significant negative correlation was found for responses to 3μM baclofen, when data from experimental groups which showed no significant difference in firing rates and % inhibition versus Sham control were pooled (Sham, Sham+DEX, UL; $p<0.005$, spearman rank correlation). When all data sets responding to 3μM baclofen were pooled (Sham, Sham+DEX, UL, UL+DEX), this significance increased to $p<0.0001$. No significant correlation was found with pooled data in response to 10μM muscimol.

Summary

These data show that the compensatory down-regulation of GABA receptor efficacy seen at 4h post-UL, in neurones in the rostral third of the ipsilesional MVN in alert UL animals, was abolished when animals were kept anaesthetised and did not experience the stress that normally follows UL. Significantly, in urethane-anaesthetised labyrinthectomised animals, administration of the synthetic GR agonist dexamethasone, restores the down-regulation of GABA receptor efficacy. Therefore, these experiments suggest that the increase in mean firing rate and down-regulation

of GABA receptors in rat rostral ipsilesional MVN neurones at 4h post-UL, adaptations which may promote the VC process, are dependant on GR activation.

	Muscimol			Baclofen		
Treatment	Max inhibition (%)	EC50 (μM)	Slope	Max inhibition (%)	EC50 (μM)	Slope
Sham	104.0 ±8.63	11.5 ±3.9	1.70 ±0.58	92.4 ± 4.1	2.7 ±1.2	1.15 ± 0.25
Sham+DEX	104.6 ±5.57	14.2 ±1.9	1.36 ±0.30	107.7 ± 6.5	2.45 ±1.3	1.03 ± 0.29
UL	105.3 ±3.35	10.1 ±1.4	1.35 ±0.15	100.5 ± 8.5	3.13 ±1.3	1.21 ±0.44
UL+DEX	111.7 ± 3.67	27.86 ±1.2	1.20 ±0.07	112.6 ± 27.4	9.2 ±1.6	0.74 ±0.47

Table 3.1 Parameters of the dose-response relationships fitted to the mean inhibitory responses of MVN cells to muscimol and baclofen, in each of the experimental conditions.

Summary of maximum inhibition, EC50 and slope ± S.E.M. from fitted dose response curves of MVN neurones from urethane anaesthetised: sham operated animals (Sham), sham operated animals administered DEX (Sham+DEX; 5mg/kg i.p. 15 min before surgery), left labyrinthectomised animals (UL), and left labyrinthectomised animals administered DEX (UL+DEX; 5mg/kg i.p. 15 min before surgery).

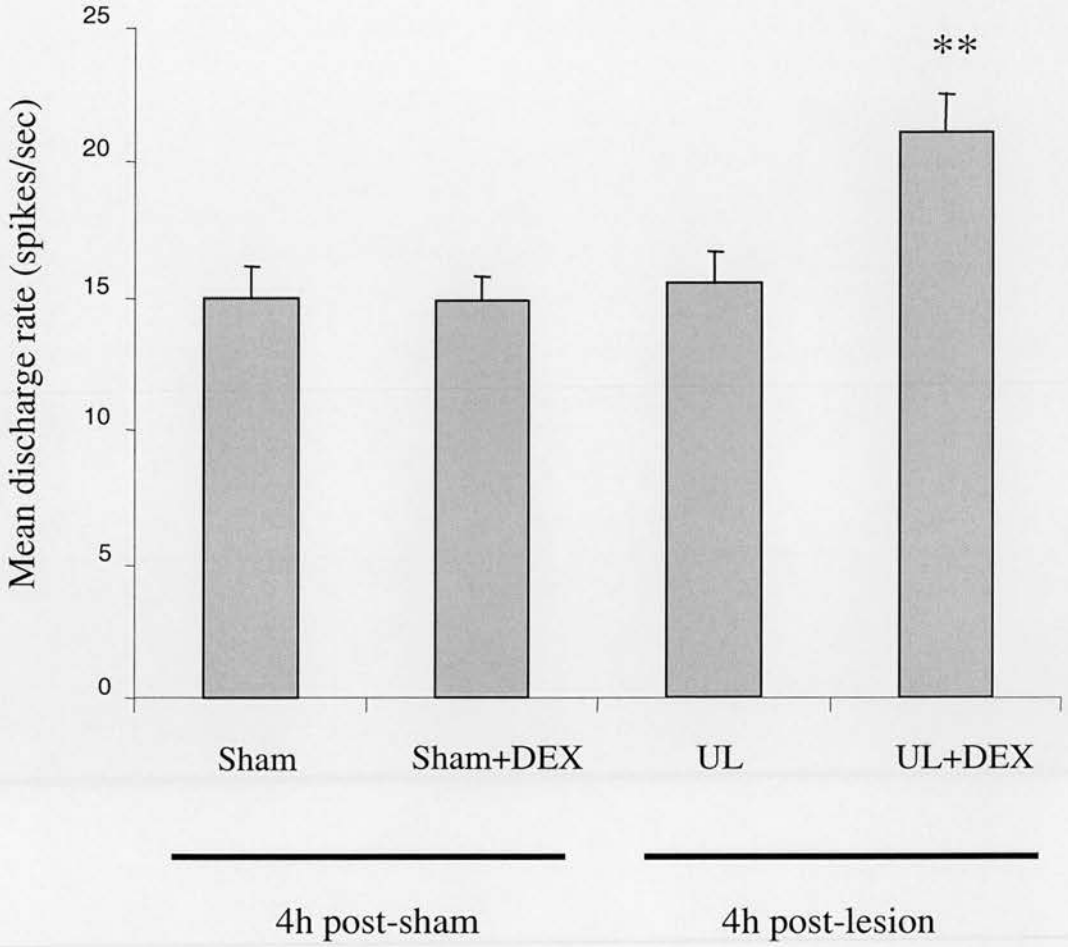


Figure 3.2 Effect of glucocorticoid agonist dexamethasone on the mean discharge rate of ipsilesional rostral MVN neurones *in vitro*

Histograms showing the mean ± S.E.M. *in vitro* tonic discharge rates of ipsilateral rostral MVN neurones in slices prepared from urethane anaesthetised animals that had received a sham operation or a left UL 4h previously, either with or without administration of dexamethasone (DEX: 5mg/kg, i.p.) 15 min prior to surgery.

Statistics: **P<0.002, versus MVN neurones from Sham, Sham+DEX and UL animals. One-way ANOVA.

Figure 3.3 Response of tonically active MVN neurones to $GABA_A$ and $GABA_B$ agonists.

A. Response of MVN neurone located in the rostral third of the MVN to 3, 10 and 30μM of the $GABA_A$ agonist, muscimol.

B. Response of MVN neurone located in the rostral third of the MVN to 1, 3 and 10μM of the $GABA_B$ agonist, baclofen.

In both A and B the 60s drug application is indicated by the bar above the data.
When a cell goes silent following drug application, the time taken to reach maximum inhibition is assumed to be the midpoint of the silencing. This is indicated by the arrow in the last response of figure A.


A.
Muscimol 3μM
Muscimol 10μM
Muscimol 30μM
Discharge rate (Spikes/sec)
30
20
10
0
60s
B.
Baclofen 1μM
Baclofen 3 μM
Baclofen 10 μM
Discharge rate (Spikes/sec)
40
30
20
10
0
60s

Figure 3.4 Fitted dose-response curves of the response of ipsilateral rostral MVN neurones to the $GABA_A$ agonist, muscimol

All animals were urethane anaesthetised and MVN slices made from animals that had received a sham operation or a left UL 4h previously, either with or without administration of dexamethasone (5mg/kg, i.p.) 15 min prior to surgery. Each data point represents the mean percentage inhibition for each concentration of muscimol, and dose response curves of y=Bottom+(Top-Bottom) / (1+10^((logEC50-X)*HillSlope)) were fitted to this data.

Filled diamonds represent data from sham operated animals (Sham)

Open squares represent data from sham operated animals administered DEX (Sham+DEX)

Open triangles represent data from UL animals (UL)

Crosses represent data from UL animals administered DEX (UL+DEX)

Statistics: **p<0.001, vs. dose response curves for Sham, Sham+DEX or UL animals; two-way ANOVA.

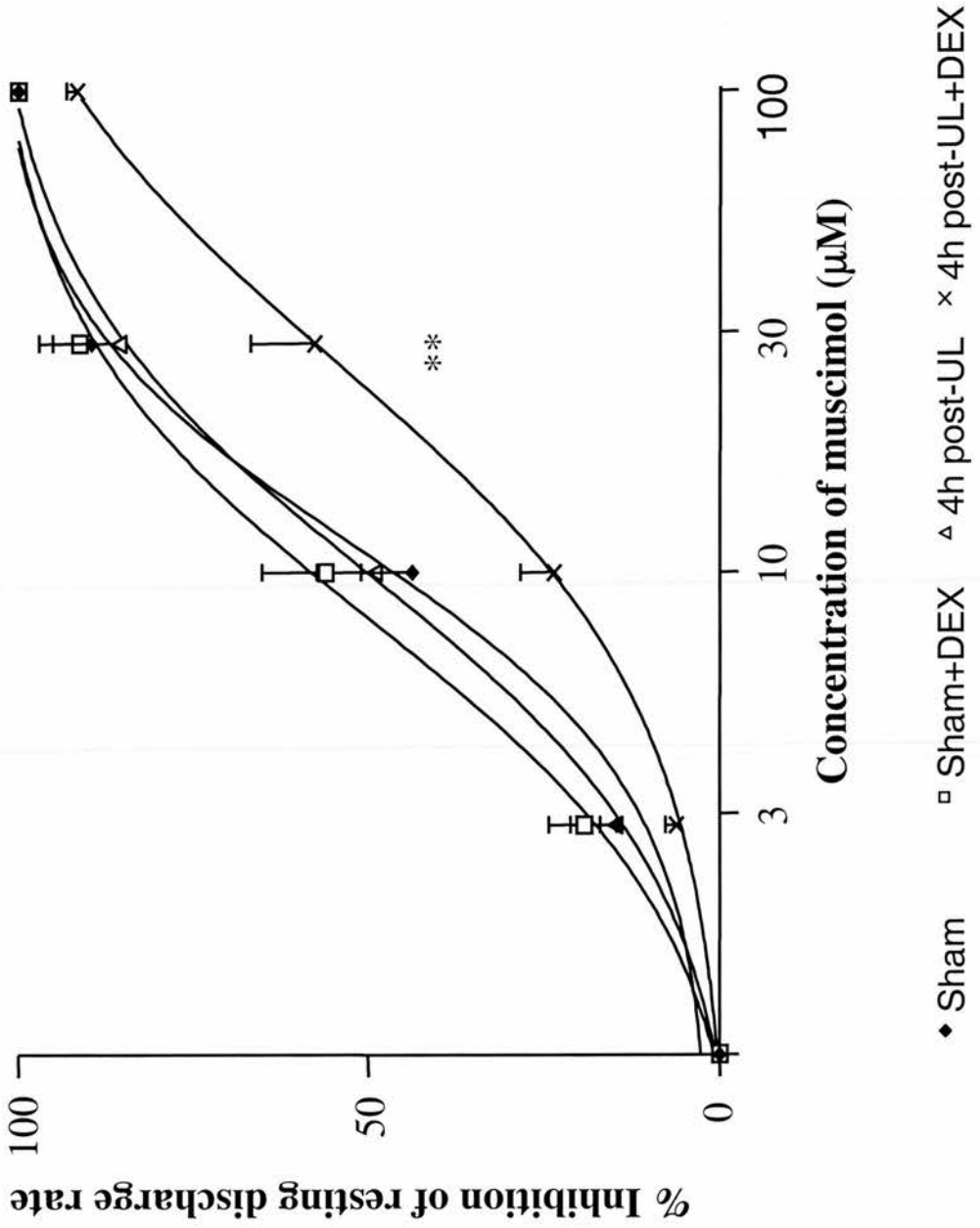

% Inhibition of resting discharge rate
100
50
0
3
10
30
100
Concentration of muscimol (μM)
**
◆ Sham
▫ Sham+DEX
▵ 4h post-UL
× 4h post-UL+DEX

Figure 3.5 Fitted dose-response curves of the response of ipsilateral rostral MVN neurones to the $GABA_B$ agonist, baclofen

All animals were urethane anaesthetised and MVN slices prepared from animals that had received a sham operation or a left UL 4h previously, either with or without administration of dexamethasone (5mg/kg, i.p.) 15 min prior to surgery. Each data point represents the mean percentage inhibition for each concentration of baclofen, and dose response curves of y=Bottom+(Top-Bottom) / (1+10^((logEC50-X)*HillSlope)) were fitted to this data.

Filled diamonds represent data from sham operated animals (Sham)

Open squares represent data from sham operated animals administered DEX (Sham+DEX)

Open triangles represent data from UL animals (UL)

Crosses represent data from UL animals administered DEX (UL+DEX)

Statistics: **p<0.001, vs. dose response curves for Sham, Sham+DEX or UL animals; two-way ANOVA.

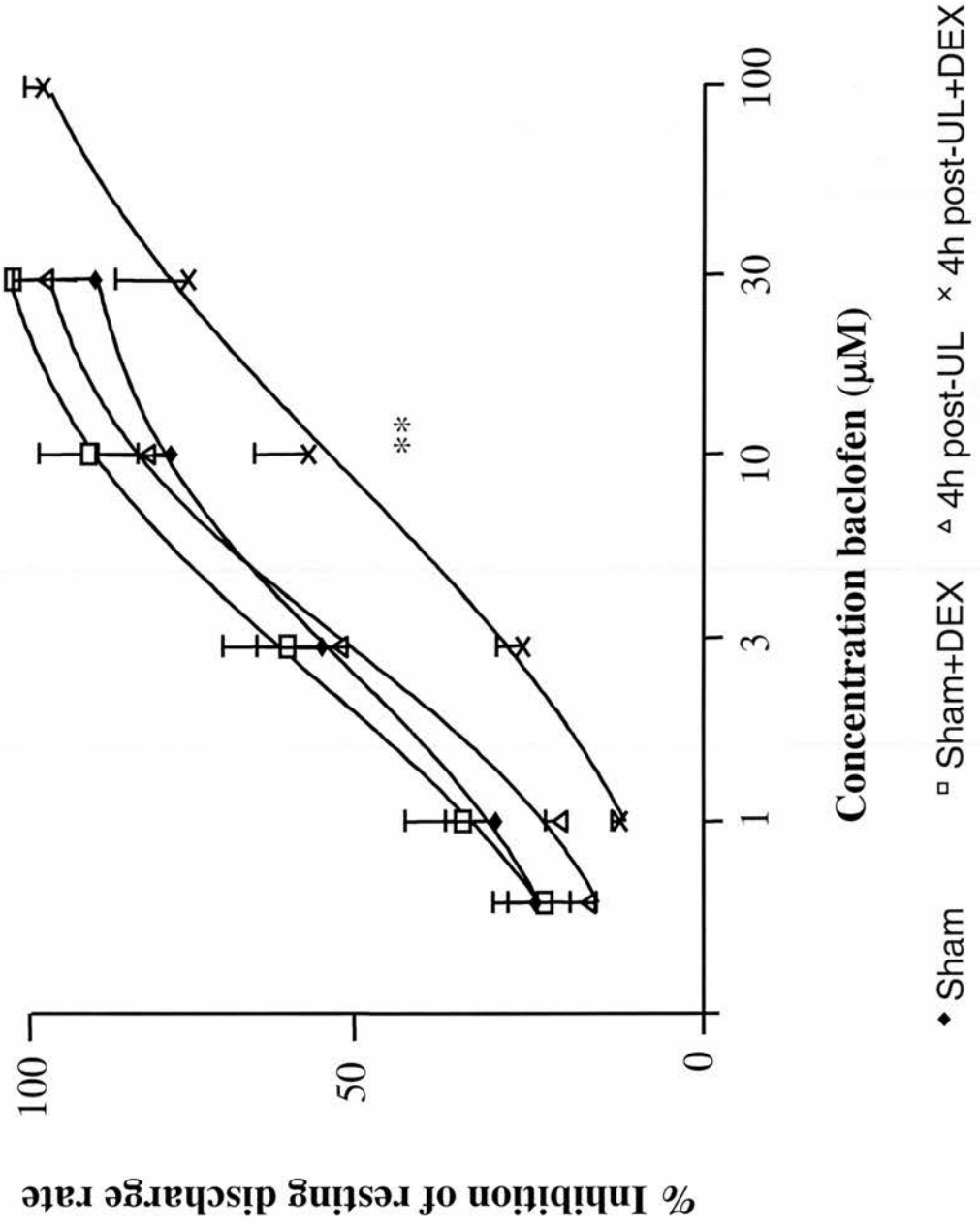

% Inhibition of resting discharge rate
100
50
0
1
3
10
30
100
Concentration baclofen (μM)
**
◆ Sham
▫ Sham+DEX
▵ 4h post-UL
× 4h post-UL+DEX

Figure 3.6 X-Y scatter graphs investigating correlation between the down-regulation of GABA receptor efficacy in ipsi-lesional MVN cells and their *in vitro* resting discharge rates

A. Distribution of the inhibitory responses of rostral MVN neurones to a 10μM dose of the $GABA_A$ agonist, muscimol

B. Distribution of the inhibitory responses of rostral MVN neurones to a 3μM dose of the $GABA_B$ agonist, baclofen

Filled diamonds represent data from sham operated animals (Sham)

Open squares represent data from sham operated animals administered DEX (Sham+DEX)

Open triangles represent data from UL animals (UL)

Crosses represent data from UL animals administered DEX (UL+DEX)

Statistics: No significant correlation was found within any individual experimental group.

† $p<0.005$, Spearman rank correlation; when data from experimental groups which showed no significant difference in firing rates and % inhibition versus Sham control were pooled (Sham, Sham+DEX, UL), a significant negative correlation was found for responses to 3μM baclofen.

No significant correlation was found with pooled data in response to 10μM muscimol.

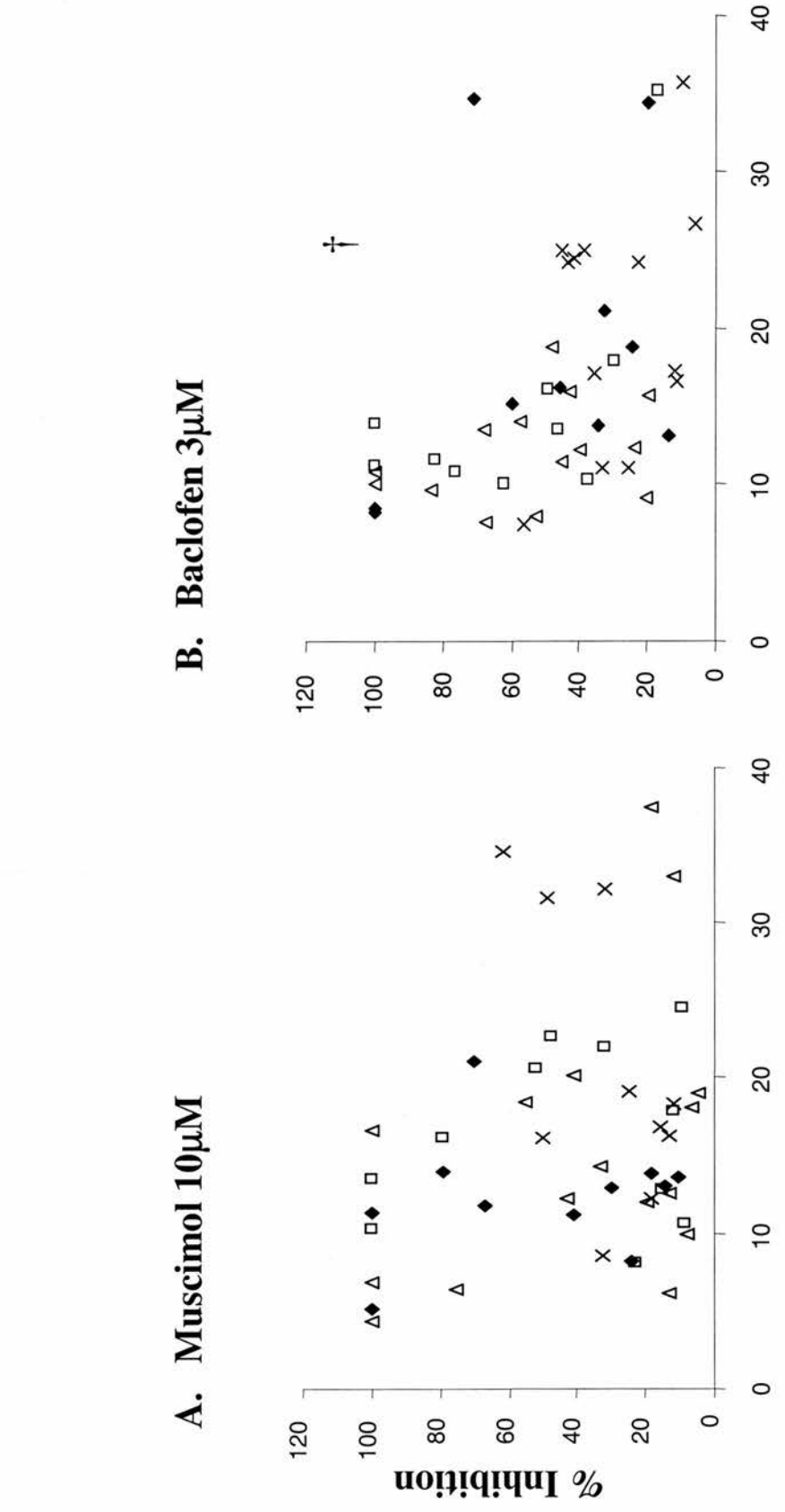

A. Muscimol 10μM
B. Baclofen 3μM
% Inhibition
Resting discharge (spikes/s)
0
20
40
60
80
100
120
0
10
20
30
40
◆ Sham
▫ Sham+DEX
▵ 4h post-UL
× 4h post-UL+DEX

Figure 3.7 Time to maximum inhibition for ipsilateral rostral MVN cells in response to muscimol

Histograms showing the time taken to reach maximum inhibition in response to the stated doses of the $GABA_A$ agonist muscimol.

Open bars represent data from MVN cells recorded from sham operated animals.
Single hatched bars represent data from MVN cells recorded from sham operated animals administered DEX (5mg/kg, i.p.) 15 min prior to surgery.
Horizontal striped bars represent data from MVN cells recorded from UL animals.
Single hatched bars represent data from MVN cells recorded from UL animals administered DEX (5mg/kg, i.p.) 15 min prior to surgery.

No statistical differences were found between any groups tested.

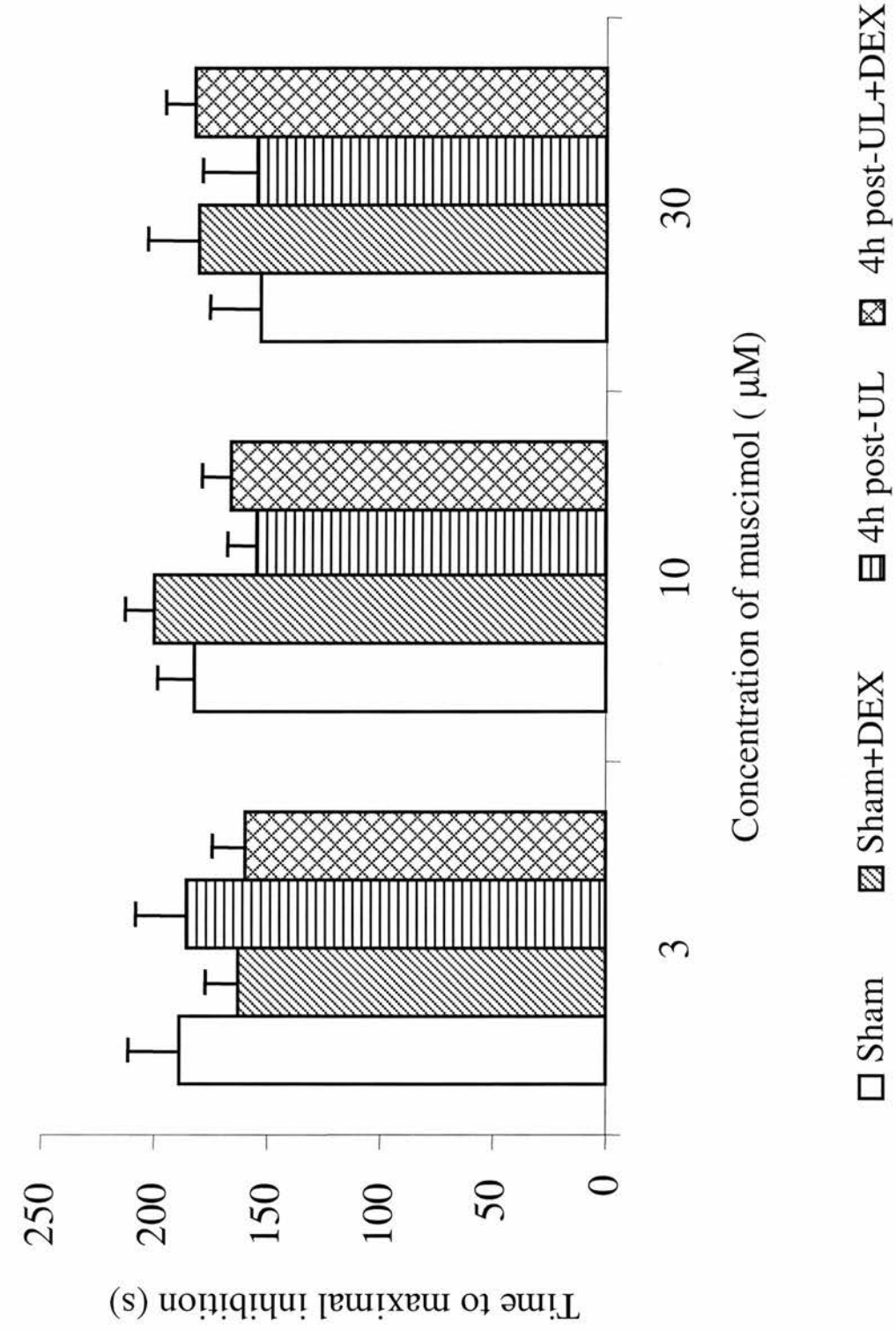

Time to maximal inhibition (s)
250
200
150
100
50
0
3
10
30
Concentration of muscimol (μM)
Sham
Sham+DEX
4h post-UL
4h post-UL+DEX

Figure 3.8 Time to maximum inhibition for ipsilateral caudal MVN cells in response to baclofen

Histograms showing the time taken to reach maximum inhibition in response to the stated doses of the $GABA_B$ agonist baclofen.

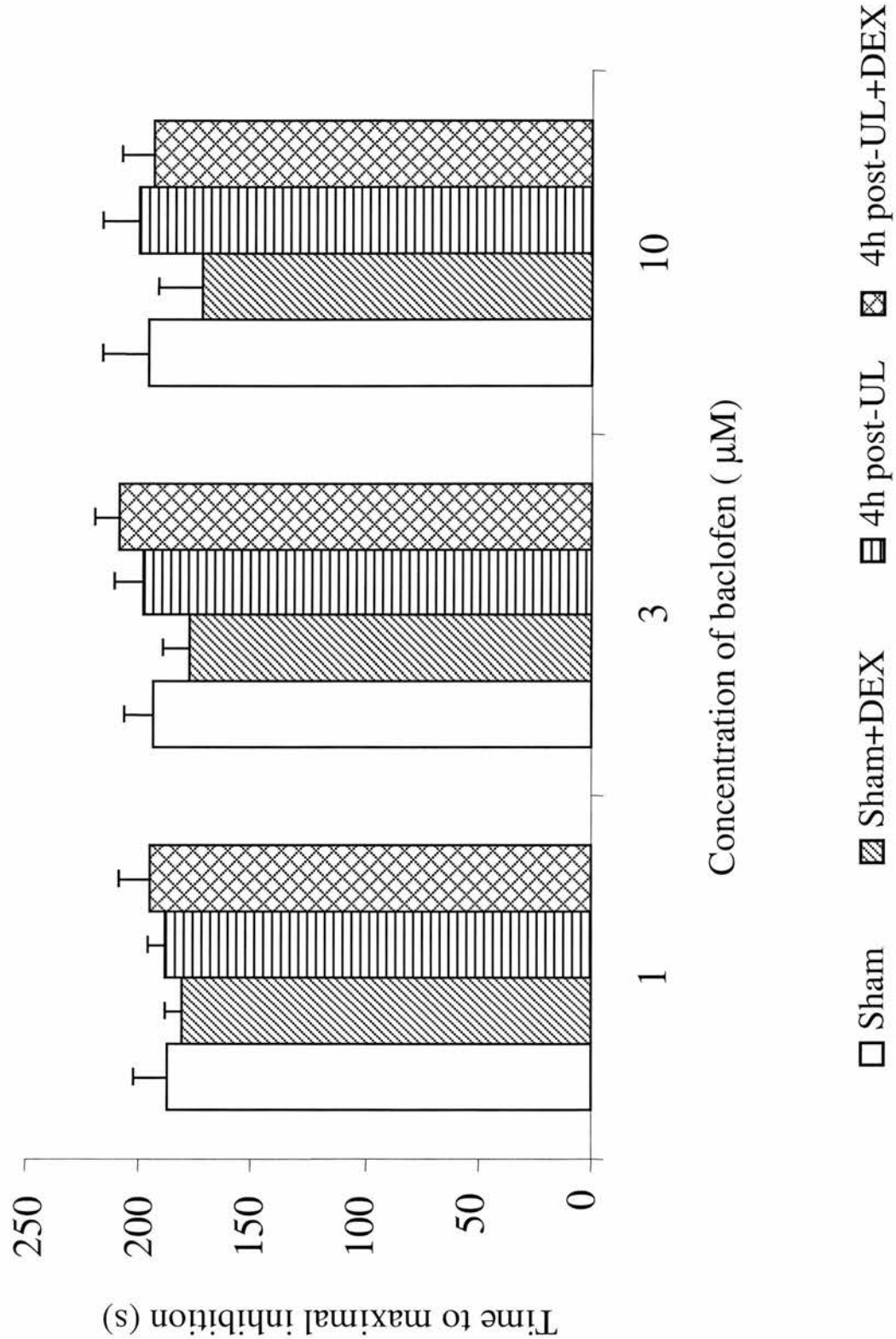
Open bars represent data from MVN cells recorded from sham operated animals.

Single hatched bars represent data from MVN cells recorded from sham operated animals administered DEX (5mg/kg, i.p.) 15 min prior to surgery.

Horizontal striped bars represent data from MVN cells recorded from UL animals.

Single hatched bars represent data from MVN cells recorded from UL animals administered DEX (5mg/kg, i.p.) 15 min prior to surgery.

No statistical differences were found between any groups tested.


Time to maximal inhibition (s)
250
200
150
100
50
0
1
3
10
Concentration of baclofen (μM)
Sham
Sham+DEX
4h post-UL
4h post-UL+DEX

3.6 DISCUSSION

The compensatory down-regulation of GABA receptor efficacy observed in rostral neurones of the lesioned MVN following UL was abolished in animals which were not allowed to recover from anaesthesia. Administration of the glucocorticoid agonist dexamethasone, to mimic activation of the stress axis in non-recovery UL animals restored the down-regulation of GABA receptor efficacy. Administration of dexamethasone to sham operated animals had no effect on GABA receptor efficacy. These experiments suggest that acute activation of the stress axis post-UL, the subsequent release of endogenous glucocorticoids and the activation of glucocorticoid receptors are necessary to induce the compensatory down-regulation of GABA receptor efficacy in rostral neurones of the lesioned MVN during the early stages of vestibular compensation (VC).

3.6.1 Mechanisms of changes in GABA receptor efficacy in the MVN following UL

In the rostral ipsilesional MVN, in slices taken from alert animals labyrinthectomised 4h previously, there is a marked down-regulation in the functional efficacy of both $GABA_A$ and $GABA_B$ receptors, seen as a significant rightward shift and decrease in slope for their dose-response relationships to muscimol and baclofen (Yamanaka *et al.*, 2000; Johnston *et al.*, 2001). These results demonstrated an adaptive down-regulation of the affinity or efficacy of postsynaptic GABA receptors on the MVN neurones. This down-regulation is functionally compensatory, in that it will reduce the sensitivity of the ipsilesional MVN neurones to the excessive commissural inhibitory synaptic drive that silences them *in vivo*. Consequently their intrinsic pacemaker-like conductances (Serafin *et al.*, 1991a; Johnston *et al.*, 1994) and remaining excitatory synaptic inputs *in vivo*, will be more effective in causing membrane depolarisation and the restoration of firing activity after UL. Thus the ipsilesional down-regulation of GABA receptor efficacy may help rebalance the excitability of the bilateral vestibular nuclei, promoting VC.

The mechanisms that generate this down-regulation of GABA receptor efficacy are unknown; however most neurotransmitter receptors are subject to regulation

following activation by their agonists. The initial regulatory effect of agonist exposure is a rapid (seconds to minutes) desensitisation of the receptor, producing a loss of immediate responsiveness. Although there is an increased GABAergic input onto the ipsilesional MVN neurones post-UL, it is unlikely that the changes in GABA receptor efficacy are due to desensitisation, as all dose response measurements were made after a 1h incubation period in normal aCSF thus removing any excess GABA from the slices. If agonist exposure persists (minutes to hours), receptors are then removed from the cell surface into an internal membrane pool, in a process referred to as internalisation or sequestration. The internalised receptors can either be recycled to the surface or degraded. Chronic exposure to agonists (hours to days) can results in degradation of receptors and is often termed down-regulation. Direct evidence for a down-regulation of $GABA_A$ receptors post-UL was provided by Calza *et al.* (1992), who observed a decrease in benzodiazepine (^{3}H-flunitrazepam; ^{3}H-FNZ) binding in the ipsilesional MVN at 3-23h post-UL which returned to normal within 3 days. Simple internalization cannot account for the decreased ^{3}H-FNZ binding, as FNZ is membrane permeant, and labels both intracellular and extracellular $GABA_A$ receptors (Czajkowski and Farb, 1986). Whether the reduction in ligand binding in these neurones is correlated with a loss of receptor function remains to be determined.

The effects of chronic exposure of neurones to GABA have been widely investigated in neuronal cell cultures. Incubation of neuronal cultures derived from embryonic chick brain with 1mM GABA for 48h caused a reversible decrease of $GABA_A$ receptor numbers (Maloteaux *et al.*, 1987; Tehrani and Barnes, 1988; Roca *et al.*, 1990), and decreased the levels of mRNA encoding the α1, β2S, and γ1 $GABA_A$ receptor subunit isoforms with no change in mRNA turnover (Montpied *et al.*, 1991; Lyons *et al.*, 2000). The observed down-regulation of mRNA subunits preceded the receptor down-regulation; furthermore, GABA exposure inhibits $GABA_A$ receptor α1 and β1 subunit promoter activity (Lyons *et al.*, 2000). These data are consistent with a transcriptional mechanism of $GABA_A$ receptor down-regulation. Shorter term regulation of $GABA_A$ receptors by GABA has been recorded by Hirouchi *et al.* (1992), who observed a decrease in $GABA_A$ receptor α1 subunit

mRNA levels in mouse cortical neuron cultures following a 4h exposure to 10μM muscimol. A down-regulation of GABA receptor efficacy was recorded in rat MVN neurones after incubation of the brainstem slice in 10μM muscimol for 4h (Him, 1999; Masters thesis, Edinburgh University). These results suggest that exposure to increased GABA alone is sufficient to induce a down-regulation of at least $GABA_A$ receptors. However this is not the case for the down-regulation of GABA receptor efficacy in rostral neurones of the lesioned MVN post-UL, as the present results demonstrate that labyrinthectomy alone in anaesthetised animals did not result in this down-regulation. Immediately following labyrinthectomy, in anaesthetised and in alert animals *in vivo*, there is a silencing of the ipsilesional MVN neurones. This is due not only to the loss of tonic excitatory drive from the deafferented primary vestibular afferents, but also due to an enhanced commissural inhibition from the contralateral MVN neurones, which become hyperactive due to the decrease in commissural inhibitory drive from the lesioned side (Smith and Curthoys, 1988b). Hence, in anaesthetised animals there is an increased GABAergic input onto the ipsilesional MVN neurones *in vivo*, but this alone is not sufficient to stimulate the down-regulation of GABA receptor efficacy, as this was only observed in UL anaesthetised animals administered the glucocorticoid agonist DEX at the time of surgery (see Figures 3.4, 3.5). These data indicate that the down-regulation is dependant both on the vestibular lesion and on the activation of glucocorticoid receptors, during the acute stress response that follows UL.

3.6.2 Involvement of the stress system in vestibular compensation

The results presented here are in agreement with those of Yamanaka *et al.* (1995a) and Cameron and Dutia (1999), implicating a role for the stress system in vestibular compensation. Cameron and Dutia (1999) demonstrated that the increase in excitability of ipsilesional MVN neurones post-UL in rat, thought to facilitate the rebalancing of excitability between vestibular nuclei, and therefore VC, did not occur in animals which remained anaesthetised after UL so that they did not experience the stress normally associated with UL (see Fig. 3.2, compare with statistics of Cameron and Dutia (1999), outlined in Section 3.5.1). Administration of DEX to these animals

restored the increase in excitability; conversely, systemic administration of the GR antagonist RU38486 to alert animals prevented the increase. Yamanaka *el al.* (1995a) observed that systemic (i.v.) administration of DEX accelerated recovery of spontaneous nystagmus after UL in rabbit in a dose dependant manner, while RU38486 retarded it. However, Alice *et al.* (1998) observed that none of the three doses of DEX (5, 10, or 40mg/kg i.p.) administered to guinea pigs between 0 and 36 hours following UL (the injection schedule designed to mimic that of Yamanaka *et al.*, 1995a) had any effect on the rate of recovery of SN. The discrepancies between the two studies may be due to the fact that in guinea pig there are significant differences in various components of the pituitary-adrenal axis compared with other species (for reviews, see Funder, 1994; Keightley and Fuller, 1996). Guinea pigs have very high circulating levels of cortisol, and a low-affinity, low-capacity corticosteroid-binding globulin so that free cortisol levels are relatively high compared to other species. Guinea pigs also have very low affinity GRs, with approximately one-twentieth the affinity for DEX as those in the rodent (Kraft *et al.*, 1979). Thus, administration of DEX to guinea pigs may have little effect on the rate of VC as it is likely that the majority of GRs are already occupied in this species. Jerram *et al.* (1995) reported that systemic administration of the GR agonist methylprednisolone (30 mg/kg s.c.), before and 4h post-UL reduced the frequency of SN in guinea pigs. This result seems to contrast with that of Alice *et al.* (1998), yet although the frequency of SN was reduced by methylprednisolone, the rate at which it compensated was not, and no beneficial effects on postural compensation were observed. Methylprednisolone has a higher affinity for GRs than dexamethasone (Kawata, 1995) and the effects on SN observed by Jerram and colleagues could be due to the increased GR activation. Interestingly, the effects of methylprednisolone on SN followed an inverted U-shaped dose-response function; 30mg/kg decreased the rate of SN, but 15 or 60mg/kg had no effect on its rate (Jerram *et al.*, 1995). This inverted U-shaped dose-response relation, is a classic feature of glucocorticoid modulation of a range of cellular processes such as modulation of ion channel excitability (Joels, 1997; Section 3.2.5) and in the hippocampus, primed burst potentiation (Diamond *et al.*, 1992). Recently, Yamamoto *et al.* (2000) demonstrated that additional restraint stress applied to UL rats, delayed the recovery of SN as

compared to unstressed UL rats. Hence, glucocorticoids may be acting on the process of VC in an inverted U-shaped dose dependant manner, such that no, or low GR activation, or very high levels of GR activation are detrimental, but an optimum level of GR activation is required for the efficient development of VC. This theory could be tested on a behavioural and cellular level both *in vivo* and *in vitro*, by administration of varying concentrations of corticosterone pellets to adrenalectomised rats, thus artificially controlling circulating levels of corticosterone. After UL in these animals, *in vivo* recordings could be made measuring the time taken for the silenced ipsilesional MVN neurones to recover their spontaneous activity; *in vitro* recordings made, measuring the time course of the development of the increased excitability and down-regulation of GABA receptor efficacy in ipsilesional MVN neurones and these could be coupled with behavioural measurements.

3.6.3 Mechanisms of steroid modulation of GABA receptor efficacy following UL

Two types of action have been demonstrated to be mediated by steroid hormones. Classical genomic actions, via steroid binding to intracellular receptors are usually slow in onset and prolonged in duration, whereas rapidly evoked changes in electrical properties of cells have been observed and a membrane receptor for steroids has been implicated for these actions (see Section 3.2.5).

Genomic modulation of GABA function

The $GABA_A$ receptor is a pentamer, assembled from a diversity of subunits (α, β, γ, δ, ε, π and ρ), that gates a chloride conductance (Hevers and Luddens, 1998; Mehta and Ticku, 1999). Heterologous expression of $GABA_A$ receptor subunits has revealed that a combination of at least one α, one β and one γ subunit is required to form a channel with properties observed *in vivo* (DeLorey and Olsen, 1992). The receptor complex contains multiple, allosterically interacting binding sites, usually including sites for GABA, barbiturates, benzodiazepines (BZD), chloride channel antagonists, and endogenous steroids (Majewska *et al.*, 1986). Native and

recombinant $GABA_A$ receptors composed of different subunit combinations, differ in pharmacological properties such as channel kinetics and allosteric modulation (Vicini, 1999).

Much research into the effects of corticosterone application and stress paradigms on $GABA_A$ receptor function has been undertaken in the hippocampus, and has frequently yielded contradictory findings (for review, see Stone *et al.*, 2001). This may be due to a functional heterogeneity among hippocampal $GABA_A$ receptors resulting in different modulatory actions of glucocorticoids in different regions, as demonstrated by Orchinik *et al.* (2001). Even so, whilst restraint stress (Mosaddeghi *et al.*, 1993) and corticosterone applications (Stone *et al.*, 2001) have been shown to decrease BZD binding in the hippocampus, and swim stress selectively alters the $GABA_A$ complex preference for BZD antagonists over agonists (Park *et al.*, 1993), other studies have shown hippocampal BZD binding to increase (Wilson and Biscardi, 1994), remain unchanged (Motohashi *et al.*, 1993) or have bidirectional responses to stress (Medina *et al.*, 1983).

The $GABA_B$ receptor is a G-protein coupled receptor, which modulates synaptic transmission through intracellular effector systems (Kaupmann *et al.*, 1997; Bettler *et al.*, 1998; Bowery *et al.*, 2002). Both presynaptic and postsynaptic $GABA_B$ receptors exist. Presynaptic autoreceptors inhibit the release of GABA, whereas heteroceptors inhibit the release of glutamate, noradrenaline, dopamine, serotonin, substance P, cholecystokinin or somatostatin by suppressing neuronal Ca^{2+} conductances (for review, see Bowery, 2002). Postsynaptic $GABA_B$ receptors are coupled to K^+ channels by a G-protein, receptor activation leading to increased K^+ conductance and hyperpolarisation of cells. $GABA_B$ receptors can be modulated not only at the level of the membrane receptor itself, but also by modulation of G-protein coupled systems downstream of the receptor, such as protein kinase systems (Carman and Benovic, 1998). Hence, there are many regulatory mechanisms involved in the desensitisation and down-regulation of $GABA_B$ receptors which could be targeted by glucocorticoids.

The results presented in this thesis indicate that in the MVN following UL, DEX is acting in concert with the lesion to decrease the GABA receptor efficacy. It is unclear whether this is mediated by a genomic or membrane action of the steroid.

The $GABA_A$ receptor subunit γ2, is responsible for BZD binding (Gunther *et al.*, 1995), and decreases the sensitivity of $GABA_A$ receptors to GABA (Zhu *et al.*, 1996). Changes in BZD binding in the hippocampus in response to corticosterone applications were accompanied by changes in the level of γ2 subunit mRNA (Orchinik *et al.*, 1995, 2001; Stone *et al.*, 2001). These data indicated a transcriptional regulation of $GABA_A$ receptor function in response to corticosterone. It would be interesting to determine whether the decreased BZD binding in ipsilesional MVN neurones from 3h post-UL in rat (Calza *et al.*, 1992), and the down-regulation of $GABA_A$ receptor efficacy from 4h post-UL observed in this thesis, are accompanied by similar reductions in $GABA_A$ receptor subunit mRNAs.

Rapid modulation of GABA function

Many actions of glucocorticoids on GABA receptor function have been recorded, which are rapid in onset and relatively short in duration (Vidal *et al.*, 1986; Joels and de Kloet, 1993). While specific binding sites exist on the $GABA_A$ receptor complex for neurosteroids, the data in this chapter indicate that the down-regulation is dependant on glucocorticoid receptor activation, and a specific binding site for glucocorticoids on this complex has not been identified. Recent evidence in different species, suggests that rapid glucocorticoid actions are mediated by distinct membrane receptors and activation of non-genomic signalling mechanisms (for review, see Losel *et al.*, 2003). Glucocorticoids have been shown to bind specifically to cellular membrane sites (Suyemitsu and Terayama, 1975; Harrison *et al.*, 1979; Orchinik *et al.*, 1991) and to induce rapid influences on electrolyte movement across cellular membranes (Hua and Chen, 1989; ffrench-Mullen, 1995). Although the molecular identity of the membrane-associated receptors for corticosteroids is still unresolved, a high-affinity binding site for corticosterone, which appears to meet the criteria for a functional membrane-associated corticosteroid receptor, has been partially purified and characterised in neuronal membranes from amphibian brain (Evans *et al.*, 2000). This receptor has characteristics of a G-protein coupled receptor in that [^{3}H]corticosterone specific binding is enhanced by Mg^{2+} and inhibited by guanyl nucleotides (Orchinik *et al.*, 1992). Other studies indicate that a membrane

glucocorticoid receptor (mGR) similar to that found in amphibian brain may exist in mammalian brain (Harrison *et al.*, 1979; Sze and Towle, 1993).

The time point post-UL, at which the GR mediated down-regulation of GABA receptor efficacy first occurs, is not known. Experiments to determine if GABA receptor efficacy is down-regulated prior to the 4 hour timepoint in these experiments would give an initial indication of whether they were membrane or genomic mediated actions. At a 4 hour timepoint, it is more likely that the down-regulation in efficacy is due to phosphorylation of ion channels and receptors than to degradation of receptors or changes in GABA receptor subunits. This view is supported by experiments showing the early importance of protein kinases in vestibular compensation (Balaban *et al.*, 1999; Johnston *et al.*, 2002), and the observation that systemic injections of a protein synthesis inhibitor (cycloheximide) had no effect upon the early stages of resting activity recovery in the ipsilateral VNC of guinea pigs (Ris *et al.*, 1998). Compensation following UL in rats, is accompanied by transient, regionally selective changes in the distribution of protein kinase C (PKC) immunopositive Purkinje cells in the vestibulo-cerebellum, which parallel the time course of the disappearance of spontaneous nystagmus (Goto *et al.*, 1997; Balaban and Romero, 1998). Sansom *et al.* (1997), observed that MVN neurones following UL show changes in the phosphorylation of several likely PKC substrates. Inhibition of PKC significantly decreased the rate of recovery of SN in guinea pigs for up to 8 hours post-UL (Balaban *et al.*, 1999; Sansom *et al.*, 2000), and PKC inhibitors injected directly into the ipsilateral flocculus prevented the increase in excitability of rostral ipsilesional MVN neurones seen at 4 hours post-UL (Johnston *et al.*, 2002). These data indicate that PKC activation is an important requirement for vestibular compensation at an acute stage post-UL.

Administration of dexamethasone up-regulates PKC activity and the expression of γ and ε isozymes of PKC in the rat brain (Dwivedi and Pandey, 1999). It is possible that PKC activation is the result of glucocorticoid binding to membrane receptors. There is growing evidence that rapid actions of glucocorticoids can be mediated by coupling of a membrane receptor to intracellular signalling pathways (for review, see Chen and Qiu, 2001). Glucocorticoids have been shown to inhibit Ca^{2+} currents via a G-protein coupled activation of PKC in guinea pig hippocampal

CA1 neurones (ffrench-Mullen, 1995) and in PC12 cell lines (Chen and Qiu, 2001). In rat paraventricular nucleus neurones, a rapid suppression of glutamatergic inputs was generated by corticosterone and dexamethasone, an effect which could be blocked by G-protein and PKC inhibitors (Di *et al.*, 2003). The G-protein coupled effects of glucocorticoids, resulted from binding of a membrane receptor, as glucocorticoids conjoined to bovine serum albumin, rendering it membrane impermeable, were still effective in inhibiting Ca^{2+} currents (Chen and Qiu, 2001) and glutamatergic inputs (Di *et al.*, 2003). Other signalling pathways such as the G-protein-PKA pathway have been implicated in rapid membrane actions of glucocorticoids (Borski *et al.*, 1991; Chen and Qiu, 1999).

Thus, glucocorticoids may be affecting GABA receptor efficacy via modulation of intracellular kinase pathways, resulting in changed phosphorylation states of the receptors. Phosphorylation of $GABA_A$ receptor subunits via a PKC-dependant pathway results in a decrease in receptor activity (Brandon *et al.*, 2000; 2002), a reaction direction which ties in with the current observed down-regulation of GABA receptor efficacy. Desensitisation of G-protein coupled receptors such as $GABA_B$ receptors, are primarily mediated by kinases such as PKA and PKC (for review, see Carman and Benovic, 1998). PKC has been observed to suppress $GABA_B$ mediated responses in slices of cerebellar cortex (Taniyama *et al.*, 1992), and $GABA_B$ mediated inhibition of glutamate release (Perkinton and Sihra, 1998).

3.6.4 Time course of GABA receptor down-regulation

$GABA_A$ and $GABA_B$ receptors are differentially regulated following UL. While the functional efficacy of $GABA_B$ receptors remain significantly down-regulated at 7-10 days post-UL, $GABA_A$ receptor efficacy has returned to normal by this time (Johnston *et al.*, 2001). The transient down-regulation of $GABA_A$ receptors may be related to a transient activation of the stress axis in the few days post-UL. The time for which corticosterone levels remain elevated post-UL remains to be determined. The time course for the requirement of GR activation for the development of cellular plasticity could be determined in a converse set of experiments to those presented in this chapter: alert UL animals could be injected with the GR antagonist, RU 38486,

at regular intervals post-UL, and its effects on cellular and behavioural recovery recorded.

It is interesting to note the correlation observed between the resting discharge rate and response to 3μM baclofen in rostral MVN neurones when data from experimental groups is pooled (see Figure 3.6B). This indicates that the factors that regulate and maintain the down-regulation of $GABA_B$ receptor efficacy may be related to the factors that regulate the excitability in rostral MVN neurones. Thus the sustained down-regulation of $GABA_B$ receptor efficacy may be related to the sustained increase in excitability of rostral MVN neurones. However, ipsilateral flocculectomy was shown to prevent the increase in excitability of ipsilateral rostral MVN neurones post-UL, while the down-regulation of $GABA_B$ receptor efficacy remained, although the extent of this down-regulation was much less than for that of $GABA_A$ (Johnston *et al.*, 2002).

Summary

The present results demonstrate that the down-regulation of GABA receptor efficacy in rostral ipsilesional MVN neurones observed from 4h post-UL is dependent on the activation of glucocorticoid receptors. This down-regulation of GABA receptor efficacy is likely to be functionally compensatory as it will counteract the disfacilitation and excessive commissural inhibition to which these cells are subject *in vivo*, helping restore the balance in neuronal activity between the bilateral VN. Therefore, the acute stress that normally follows vestibular lesions appears to be important in the initiation of vestibular compensation.

The exact actions of glucocorticoids in inducing the down-regulation of GABA receptor efficacy are unknown. They may be acting at a genomic level to regulate GABA receptor subunit expression, or could be acting through membrane receptors and second messenger systems to alter the phosphorylation state of the receptors. Future experiments into the mechanisms of glucocorticoid action in vestibular compensation could provide parallels for glucocorticoid mediated plasticity in other brain regions.

Chapter 4

11β-HYDROXYSTEROID DEHYDROGENASE IN THE VESTIBULAR SYSTEM

4.1 INTRODUCTION

The previous chapter implicated glucocorticoid receptor activation in the development of plasticity in MVN neurones following UL. Until recently it was believed that glucocorticoid actions in the CNS were determined solely by the concentration of hormone in circulation (modulated by hormone binding to plasma proteins), and by the presence and relative density of the MR and GR receptor types in a particular tissue. However, it has become apparent that pre-receptor metabolism of glucocorticoids by the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes also potently regulate steroid access to receptors within certain tissues. Given the role of glucocorticoid receptor activation in the up-regulation of neuronal excitability and down-regulation of GABA receptor efficacy in lesioned rostral MVN neurones following UL, it is of interest whether glucocorticoids are modulated on a cellular level by 11β-HSD enzymes in the MVN and vestibulo-cerebellum.

11β-HSD catalyses the interconversion of active 11-hydroxycorticosteroids (cortisol in humans and most mammals, corticosterone in rats and mice) to their inert 11-keto forms (cortisone, 11-dehydrocorticosterone), which have little or no intrinsic affinity for MR or GR (Fig. 4.1). To date, two isozymes of 11β-HSD have been characterised which are the products of two distinct genes and differ considerably in tissue distribution and function (for reviews, see Seckl, 1997; Seckl and Walker 2001).

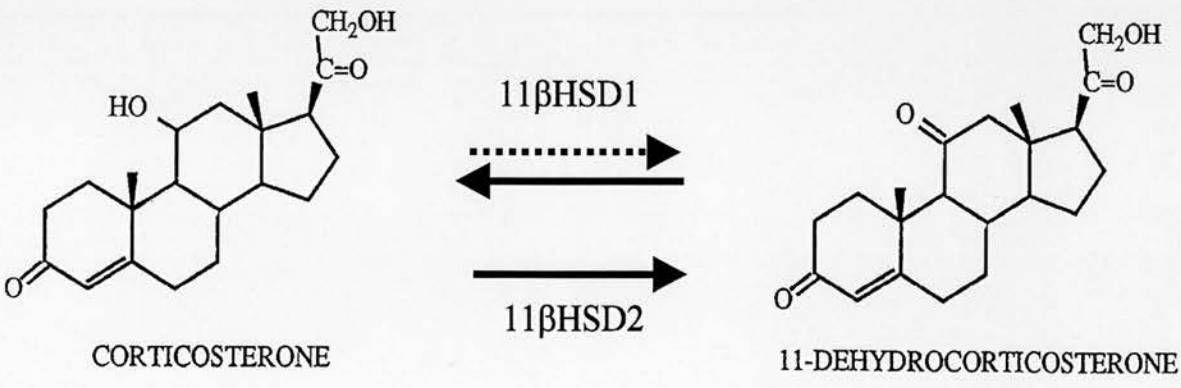


Figure 4.1 Biochemical reaction of the metabolism of glucocorticoids by the 11β-hydroxysteroid dehydrogenase enzymes, 11β-HSD1 and 11β-HSD2.

11β-HSD type 2

11β-HSD type 2 (11β-HSD2) is an NAD-dependant enzyme with a high affinity for glucocorticoids. The 11β-HSD2 gene is highly expressed in classical aldosterone selective target tissues (kidney, colon, pancreas, sweat glands) and the placenta. In the brain it is expressed only in discrete regions primarily associated with the central actions of aldosterone in salt appetite and blood pressure regulation (Robson *et al.*, 1998). 11β-HSD2 functions exclusively as an 11β-dehydrogenase, inactivating glucocorticoids (Fig. 4.1; Seckl, 1997). Mineralocorticoid receptors bind corticosteroids and aldosterone with equal affinity *in vitro* (Arriza *et al.*, 1988). The presence of 11β-HSD2 in these tissues allows selective access of aldosterone to these non-selective MRs *in vivo* (Sheppard and Funder, 1987). In the congenital absence of 11β-HSD2, glucocorticoids illicitly occupy mineralocorticoid receptors causing sodium retention, hypokalemia and hypertension (Ulick *et al.*, 1979; Stewart *et al.*, 1988).

11β-HSD type 1

11β-HSD type 1 (11β-HSD1) is a reversible NADP(H)-dependant enzyme with a low affinity for glucocorticoids. 11β-HSD1 is widely expressed, most actively in liver, testis and lung with lower levels of activity in brain, heart and vasculature. In the brain, high levels of mRNA expression, bioactivity and immunoreactivity are found in hippocampus, cerebellum and cortex, with lower levels demonstrated in the brainstem (Moisan *et al.*, 1990a, b; Lakshmi *et al.*, 1991; Sakai and Lakshmi, 1992). 11β-HSD1 is a 34kDa glycosylated protein first purified from rat liver in the 1980s (Lakshmi and Monder, 1985, 1988). In these original studies, 11β-HSD1 in the liver was shown to be bi-directional, although the reductase activity was unstable *in vitro* (Lakshmi and Monder, 1988). The function of 11β-HSD1 is considerably less clear-cut than that of 11β-HSD2. High levels of 11β-HSD1 activity are often found in sites where there is negligible MR, but where glucocorticoids play a key role in metabolism through activation of low affinity GR. Thus 11β-HSD1 may play a role in modulating glucocorticoid access to their receptors. The high 11β-HSD1 activity, mRNA expression and immunoreactivity in the cerebellum, which contains no

measurable MR, supports the role for 11β-HSD1 in the regulation of glucocorticoid access to GR (Moisan *et al.*, 1990b). 11β-HSD1 in the hippocampus is co-localised with MR and GR, thereby possibly modulating access to both types of receptor.

The key to understanding the functions of 11β-HSD1 in the brain came from studies of its reaction direction. Although 11β-HSD1 shows bi-directional activity in homogenates *in vitro*, it appears to act predominantly as a reductase in intact cells and *in vivo*. In intact mammalian COS-7 cells transfected with 11β-HSD1 cDNA, intact primary cultures of hepatocytes and in adipose tissue cell lines, 11β-HSD1 acts almost exclusively as a reductase, regenerating active glucocorticoids (Low *et al.*, 1994a; Stewart *et al.*, 1994; Napolitano *et al.*, 1998). In primary cultures of hippocampal cells (mainly neurones) 11β-HSD1 is the sole isozyme expressed and again acts predominantly as a reductase (Rajan *et al.*, 1996). Hippocampal neurones are sensitive to excess glucocorticoid, which produces atrophy of the dendrites, neuronal and cognitive dysfunction and neuronal loss (McEwen, 2000). The reductase activity of 11β-HSD1 has the potential to potentiate the neurotoxic effects of glucocorticoids. Ajilore and Sapolsky (1999) characterised the reaction direction of 11β-HSD *in vivo* in the hippocampus, using glucocorticoid neuroendangerment as an indirect assay of 11β-HSD1 function. 11-dehydrocorticosterone (11-DHC), exacerbated kainic acid toxicity in adrenalectomised (ADX) rats, indicating the activation of 11-DHC to corticosterone *in vivo*. This neuroendangering action of 11-DHC was prevented by inhibition of 11β-HSD1 with the non-selective 11β-HSD inhibitor, carbenoxolone (Ajilore and Sapolsky, 1999). To further study the function of 11β-HSD1 *in vivo*, mice homozygous for targeted disruption of the 11β-HSD1 gene were recently generated (Kotelevtsev *et al.*, 1997). Aged wild type (WT) mice developed elevated corticosterone levels that correlated with learning impairments in the watermaze (Yau *et al.*, 2001). Despite elevated corticosteroid levels throughout life, the glucocorticoid associated learning deficit was ameliorated in 11β-HSD1 knockout mice (Yau *et al.*, 2001). The 11β-HSD1 knockout mice also showed lower intra-hippocampal corticosterone levels than WT mice, presumably due to lack of 11-DHC activation.

11β-HSD1 can be regulated by a range of hormones in a tissue and species specific manner (Idrus *et al.*, 1996; Ricketts *et al.*, 1998). Factors influencing 11β-HSD1 activity include glucocorticoids, thyroid hormones, sex steroids, growth hormone, insulin and cytokines (for review, see Stewart and Krozowski, 1999). Many studies to date have pointed towards a positive regulation of 11β-HSD1 by glucocorticoids. The synthetic glucocorticoid, dexamethasone (DEX), induces 11β-HSD1 activity in rat cultured hippocampal neurones (Rajan *et al.*, 1996). *In vivo*, treatment of ADX rats with DEX for 10 days significantly increased hippocampal 11β-HSD1 activity and mRNA expression compared with sham or ADX controls (Low *et al.*, 1994b). Similar effects of DEX on 11β-HSD1 activity are observed in other brain regions including the cortex, cerebellum and hypothalamus of the rat (Seckl, 1997). Elevating corticosteroid levels artificially in rat, by long term (21 days) treatment with corticosterone in the drinking water, caused a significant increase in 11β-HSD1 activity in the pituitary, hippocampus and amygdala (Jellinck *et al.*, 1997). However, the effects of exogenous stressors, despite raising plasma corticosterone to the same level as those observed after administration, had mixed effects on 11β-HSD1 activity (Jellinck *et al.*, 1997). Chronic arthritic stress (14 days) significantly increased hippocampal 11β-HSD1 activity but not mRNA expression in rat (Low *et al.*, 1994b); chronic restraint stress (21 days) failed to change 11β-HSD1 activity in various regions of the rat brain including hippocampus (Jellinck *et al.*, 1997); and chronic psychosocial stress (28 days) attenuated hippocampal 11β-HSD1 activity in the tree shrew (Jamieson *et al.*, 1997). Induction of 11β-HSD1 in the brain during acute stress would help amplify intracellular glucocorticoid actions, whereas chronic attenuation of the enzyme activity may represent a homeostatic mechanism to ameliorate the otherwise excessive metabolic effects of glucocorticoid excess.

The acute stress that accompanies the severe behavioural symptoms of UL activates the HPA axis (Fig. 3.1) as demonstrated by the induction of Fos protein in the paraventricular nucleus of the hypothalamus (Cameron and Dutia, 1999) and raised salivary cortisol levels in guinea pig (Gliddon *et al.*, 2003). The resulting activation of glucocorticoid receptors appears to be essential for the expression a number of plastic changes in the MVN following UL in rat (see Chapter 3). If 11β-

HSD1 is expressed in the MVN and vestibulo-cerebellar regions, these glucocorticoid actions could be finely controlled at a cellular level by glucocorticoid metabolism. In addition, changes in 11β-HSD1 activity may contribute to this early stage of post-lesional plasticity, by potentiating or otherwise regulating the exposure of the MVN and cerebellum to glucocorticoids, during the time when the adaptations in MVN cell properties are taking place.

4.2 AIMS AND EXPERIMENTAL RATIONALE

The enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) regenerates active glucocorticoids from inert forms in certain tissues, modulating glucocorticoid effects in these tissue. The following experiments were designed to investigate 11β-HSD1 activity in central vestibular and vestibulo-cerebellar tissues from rat.

1. To investigate the presence of 11β-HSD1 in the MVN and vestibulo-cerebellum, its activity was assayed in the MVN, flocculus/paraflocculus (F/PF) complex and nodulus/uvula (NU) of normal animals (n=4).

2. To investigate whether changes in 11β-HSD1 activity contribute to the early post-lesional changes in vestibular plasticity, by regulating the exposure of the MVN and cerebellum to glucocorticoids, during the time when adaptations in MVN cell properties are taking place, activity was assayed in ipsilateral and contralateral MVN and F/PF, and in the NU from normal animals (n=8), sham operated animals (n=16) and animals that had undergone vestibular compensation for 4 hours after UL (n=16).

4.3 METHODS

Except for the following, all methods in this chapter are identical to those described in Chapter 3.

Anaesthesia

For all surgery in this section rats were given a single intraperitoneal (i.p.) injection of avertin (tribromoethanol; see drugs and solutions) 15 min before the start of surgery. This solution was injected at a dose of 1ml/100g body weight and provided surgical anaesthesia for 30-40 min. This produced the required level of anaesthesia for the duration of the surgery; a sufficient level of anaesthesia was determined by lack of the limb withdrawal reflex to a paw pinch.

Surgery

Animals began to show recovery from anaesthesia within 50-60 minutes of induction, typically 20-30 min after completion of the labyrinthectomy. All animals showed the severe characteristic symptoms of unilateral vestibular loss including spontaneous nystagmus, circular walking, barrel rolling and head deviation towards the side of the lesion. Sham operated animals underwent the same procedure, but the canal was not opened and the inner ear was undamaged. These animals did not exhibit any of the behavioural symptoms associated with unilateral vestibular loss. The animals were allowed to recover in their home cages for 4 hours, before they were re-anaesthetised with halothane (see drugs and solutions) by inhalation and decapitated for collection of brain tissue.

Tissue preparation

After decapitation, the skull was removed using fine bone rongeurs, with care taken on removal of the occipital bone to release the flocculus/parafloccculus (F/PF) complexes. After immersion of the released brain in ice cold aCSF for one minute, the F/PF complex was dissected from either side of the cerebellum, identifying the left (ipsilateral) and right (contralateral) nuclei. The cerebellar hemispheres were removed and the caudal most folia containing the nodulus and uvula (NU) were

dissected. 800μM thick horizontal slices of the dorsal brainstem containing the MVN were cut on a vibroslice, the left and right MVN were isolated by cutting along the midline and trimming. Immediately after dissection all tissue was frozen on dry ice and stored at -72°C until use.

11β-HSD1 assay

Tissue was homogenised on ice in C buffer (8% glycerol, 150mM NaCl, 1mM EDTA, 50mM Tris) in Dounce tissue homogenisers. Routinely four MVN and four F/PF were homogenised in 200μl C buffer, whilst four NU were homogenized in 800μl C buffer. Total protein concentration was estimated colorimetrically (BioRad protein assay kit, UK), and tissue samples were diluted to a final assay concentration of 0.2mg/ml with C buffer. Duplicate aliquots of homogenate containing 0.2mg/ml protein were incubated at 37°C with 400μM NADP and 10nM [^{3}H]-corticosterone ([^{3}H]-B) in a total volume of 250μl. To terminate the assay, 1ml of ethyl acetate was added to each sample and mixed to separate the steroids from the homogenate. The upper ethyl acetate layer was removed into HPLC tubes and dried down under air at 55°C. Steroids were resuspensed in 40μl ethanol containing 0.5mg/ml each of unlabelled corticosterone and 11-dehydrocorticosterone. Silica gel coated aluminium thin layer chromatography (TLC) plates (Merck Ltd, UK) were divided into lanes of 1.8cm width to allow application of ten samples onto each plate. Steroid samples were pipetted onto the plates 4μl at a time, allowing drying between applications until all 40μl of each sample had been plated. Corticosterone and 11-dehydrocorticosterone were separated by running the TLC plates in sealed tanks containing a mixture of chloroform:95% ethanol (92:8) for 45 minutes. Conversion of corticosterone (B) to 11-dehydrocorticosterone (A) was quantified by scanning densitometry using Aida image software (Raytest, Straubenhardt, Germany). Enzyme activity could be calculated as the percentage conversion of B to A using the equation A/(A+B)*100, because virtually no other products were formed under the conditions of incubation.

An initial assay used tissue pooled from 4 normal animals to assess the differing levels of 11β-HSD1 between brain regions (n=4). All tissue samples in this assay

were incubated in parallel for 90 minutes. From preliminary assays, the incubation time for each tissue type was adjusted such that the percentage conversion was within the range 10-50% for the given time. 11β-HSD1 was then assayed in ipsilateral and contralateral MVNs (incubation 330 min) and F/PF (incubation 45 min), and in the NU (incubation 60 min) of normal animals (n=8), sham operated animals (n=16) and animals that had undergone vestibular compensation for 4 hours after UL (n=16). For each assay, tissue was pooled from 4 animals and samples run in duplicate; four assays for each tissue type were processed in parallel, and the mean ±S.E.M. of these assays calculated. Differences in activity between control and surgery groups were tested for statistical significance using one-way ANOVA; difference between ipsi- and contralateral tissue were analysed by Students paired *t*-test. Results were considered significant at $p<0.05$.

Drugs and Solutions

1. Avertin

Avertin was made up fresh on the day of surgery, using the following recipe, and could be stored for up to 3 days at 4°C: 0.312 g tribromoethanol (Sigma, UK) was added to 1.25 ml ethanol (Sigma, UK) and mixed until the tribromoethanol dissolved completely. To this solution 0.187 ml tetra amyl alcohol was added followed by 14.25 ml sterile saline (0.9%). This solution was injected intraperitoneally (i.p.) at a dose of 1ml/100g body weight and provided surgical anaesthesia for 30-40 min.

2. Halothane

Prior to decapitation, animals were anaesthetised with halothane (Flurothane, May and Baker Ltd., UK) by inhalation. The anaesthetic was administered by placing the animals in a small Perspex box containing a piece of halothane soaked cotton wool. A sufficient level of anaesthesia was determined by lack of reflex response to a paw pinch.

4.4 RESULTS

The activity of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) was assayed in the MVN, flocculus/paraflocculus complex and nodulus/uvula of normal animals (n=4). Activity was then assayed in ipsilateral and contralateral brain regions from normal animals (n=8), sham operated animals (n=16) and animals that had undergone vestibular compensation for 4 hours after UL (n=16) to assess any possible changes in activity following labyrinthectomy.

1. 11β-HSD1 activity in vestibular related brain tissues

Figure 4.2 shows the results of an assay of 11β-HSD1 activity in MVN, flocculus/paraflocculus and nodulus/uvula tissue pooled from four normal rats. The samples were incubated in parallel for a standard time of 90 min, and each sample was assayed in duplicate. The values shown in Fig. 4.2 are thus the mean of two parallel, repeated assays for each tissue, carried out under identical conditions. High levels of 11β-HSD1 activity were observed in the flocculus/paraflocculus and the nodulus/uvula, while 11β-HSD1 activity in the MVN was five fold lower (**Flocculus/paraflocculus**: 65.6 ±0.25 % conversion to 11-dehydrocorticosterone; **Nodulus/uvula**: 46.7 ±2.2 % conversion to 11-dehydrocorticosterone; **MVN**: 10.3 ±0.3 % conversion to 11-dehydrocorticosterone). This finding is in line with previous reports showing high levels of 11β-HSD1 immunoreactivity, mRNA levels and bioactivity in cerebellum, and lower levels in the brainstem (Moisan *et al.*, 1990b; Lakshmi *et al.*, 1991). In addition, this result shows that high levels of 11β-HSD1 activity are found in the flocculus/paraflocculus and nodulus/uvula, which are cerebellar areas intimately involved with the control of vestibular reflexes and eye movements.

2. 11β-HSD1 activity in control and labyrinthectomised tissues

Figure 4.3 and table 4.1 show 11β-HSD1 activity in the MVN, flocculus/paraflocculus and nodulus/uvula in tissue samples taken from normal animals, sham-operated animals, and animals that had undergone vestibular

compensation for 4h after UL. In this assay the incubation time for each tissue type was adjusted taking into account the levels of activity demonstrated in the initial assay (Fig. 4.2), to maximise conversion of corticosterone without the reaction reaching saturation. Thus flocculus/paraflocculus samples were incubated for 40 min, nodulus/uvula samples for 60 min, and MVN samples for 330 min. There were no significant differences in the levels of 11β-HSD1 activity either between normal and post-UL tissues, or between the ipsilesional and contralesional MVN or flocculus/paraflocculus after UL (Table 4.1).

	MVN		Flocculus/paraflocculus		Nodulus/uvula
	Ipsi	Contra	Ipsi	Contra	
UL	20.5 ±3.3	16.9 ±2.2	27.6 ±1.8	27.6 ±1.8	29.8 ±1.8
Sham	20.2 ±1.6		35.2 ±4.6		33.4 ±2.9
Normal	15.6 ±0.9		32.2 ±1.8		36.6 ±1.9

Table 4.1. 11β-HSD1 activity assessed in the MVN, flocculus/paraflocculus and nodulus/uvula in normal animals, sham operated animals and animals which had undergone a left labyrinthectomy 4h previously (UL). Ipsi: ipsilesional side of the brain (left nuclei); contra: contralesional side of the brain (right nuclei). All values expressed as mean ± S.E.M., % conversion of corticosterone to 11-dehydrocorticosterone.

Figure 4.2 11β-hydroxysteriod dehydrogenase type 1 activity in the MVN and vestibulo-cerebellum of rat

A. Representative chromatogram showing conversion of corticosterone to 11-dehydrocorticosterone in duplicate samples from MVN, flocculus/paraflocculus complex (F/PF) and nodulus/uvula (NU), and in blank control which did not contain any brain homogenate.

B. 11β-HSD1 activity levels (expressed as % conversion of corticosterone to 11-dehydrocorticosterone), in MVN, F/PF and NU from normal rats (n=4). Columns indicate means ±S.E.M. of assays repeated in duplicate.

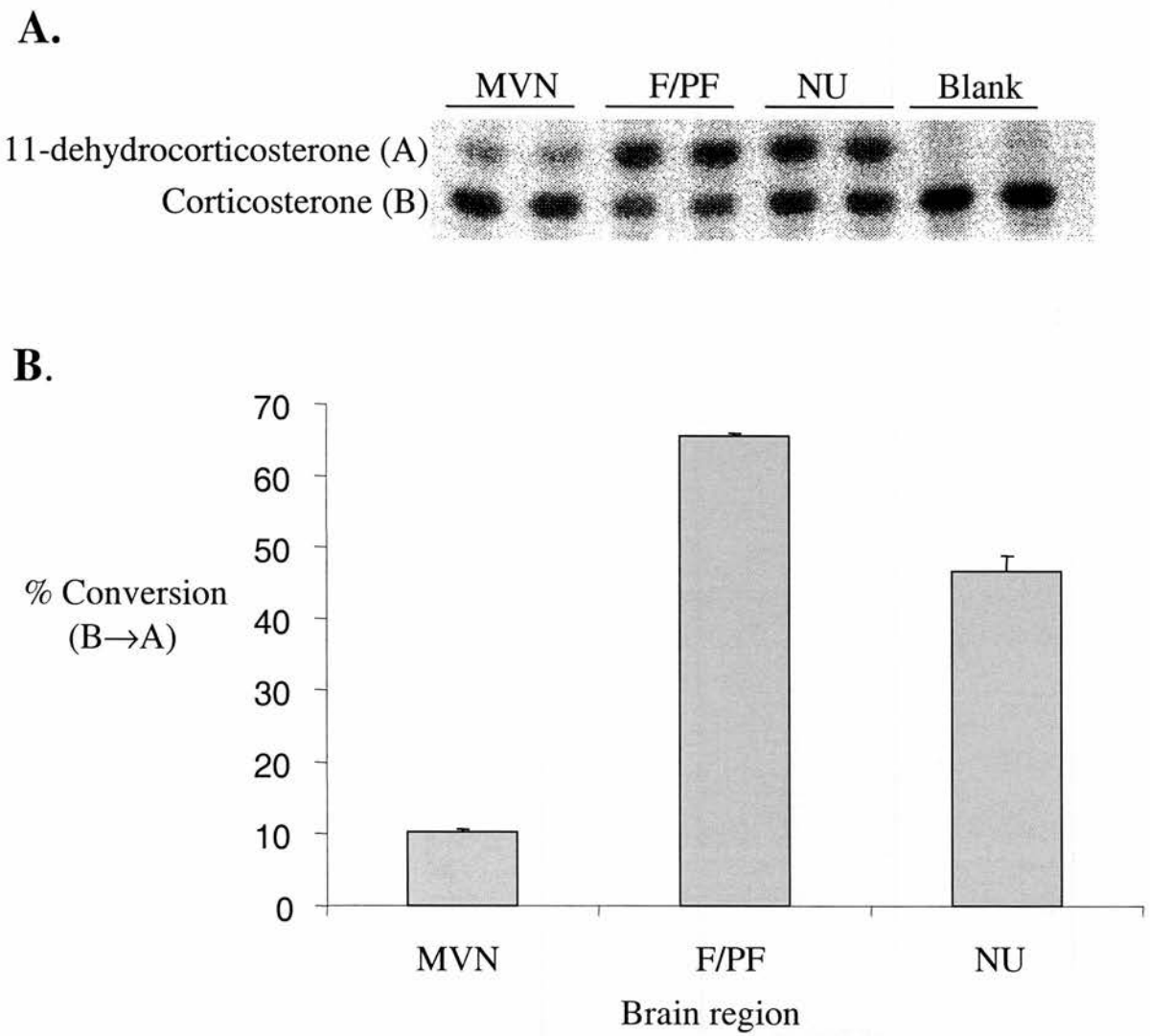
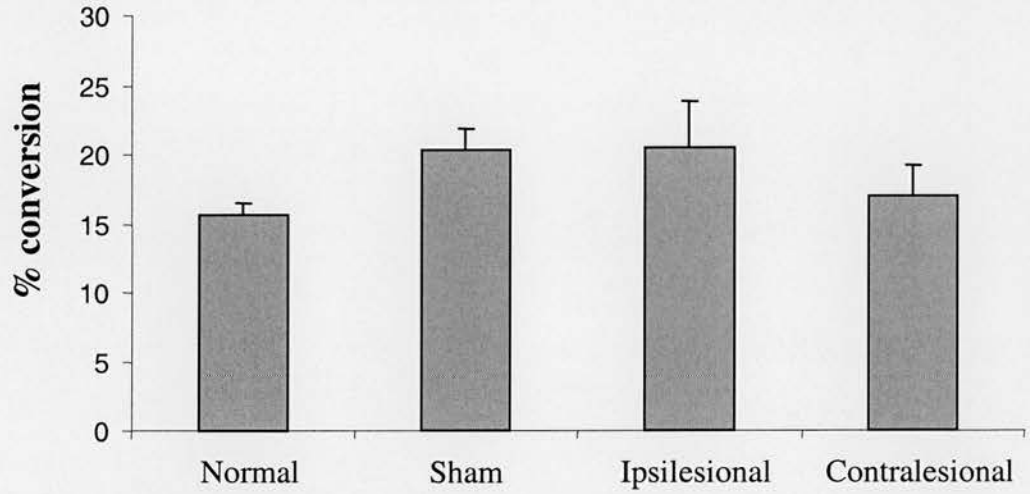

A.
MVN
F/PF
NU
Blank
11-dehydrocorticosterone (A)
Corticosterone (B)
B.
% Conversion
(B→A)
70
60
50
40
30
20
10
0
MVN
F/PF
NU
Brain region

Figure 4.3 11β-hydroxysteriod dehydrogenase type 1 activity following UL

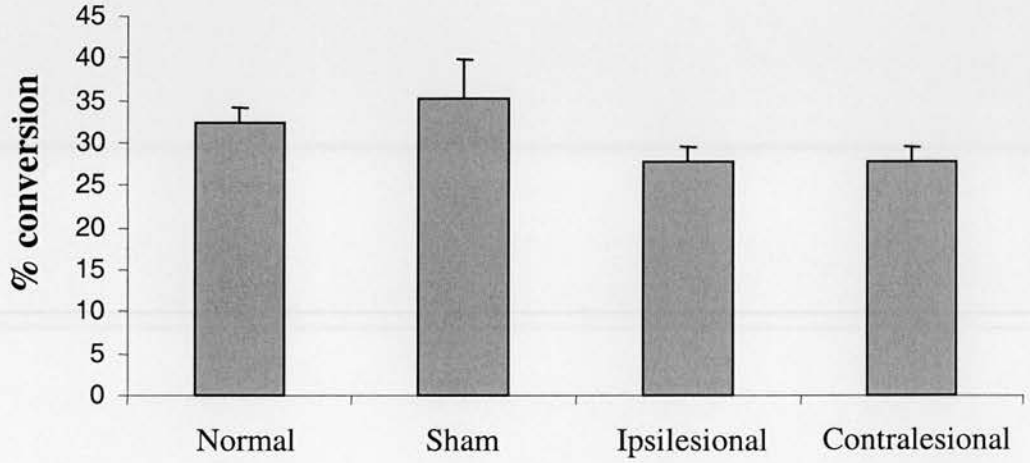
11β-HSD1 activity (expressed as % conversion of corticosterone to 11-dehydrocorticosterone) in ipsilesional and contralseional MVN (A), ipsilesional and contralseional flocculus/paraflocculus (B) and in nodulus/uvlua (C); from normal rats, sham operated rats and animals that underwent vestibular compensation for 4 hours post-UL

No statistical differences were found between any groups tested.

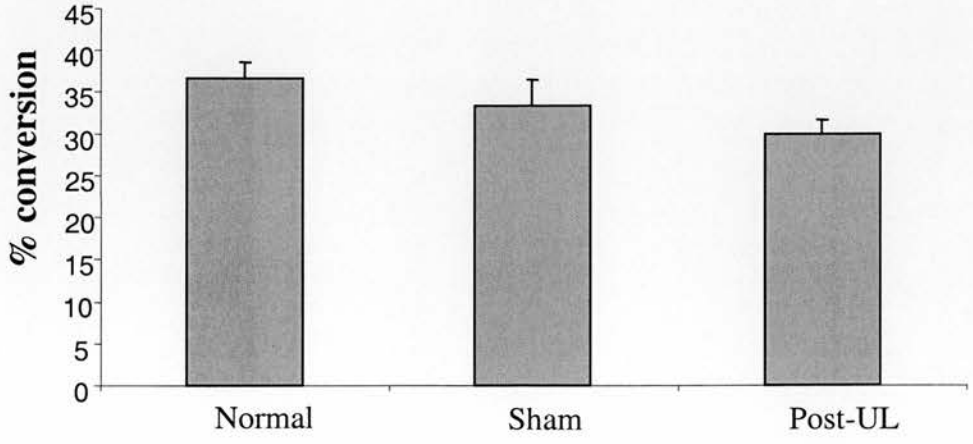
A. MVN



B. Flocculus/paraflocculus



C. Nodulus/uvula



4.5 DISCUSSION

The enzyme, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) regenerates active glucocorticoids from inert forms. The early deafferentation-induced changes in the properties of ipsilesional MVN cells observed at 4h post-UL in rat (the increase in mean firing rate and down-regulation of GABA receptor efficacy), are dependant on GR activation (Chapter 3; Cameron and Dutia, 1999). This study investigated the presence of 11β-HSD1 in the MVN and vestibulo-cerebellar tissues to determine if glucocorticoid actions could be finely controlled at a cellular level by glucocorticoid metabolism. It was hypothesised that changes in 11β-HSD1 activity may contribute to this early stage of post-lesional plasticity, by potentiating or otherwise regulating the exposure of the MVN and cerebellum to glucocorticoids, during the time when the adaptations in MVN cell properties are taking place.

This experiment is the first to specifically investigate 11β-HSD1 activity in central vestibulo-oculomotor centres of the brainstem and cerebellum. The results demonstrate the presence of 11β-HSD1 activity in the MVN, flocculus/paraflocculus and uvula/nodulus, and suggest that this modulatory enzyme is likely to be involved in regulating the exposure of these centres to circulating glucocorticoids. This could be of clinical significance since many treatments for vestibular disorders in man utilise steroid hormones (Seemungal *et al.*, 2001). The high levels of 11β-HSD1 activity in flocculus/paraflocculus and uvula/nodulus are of particular interest. These areas are likely to be rendered highly responsive to glucocorticoids, through the local amplification of active steroid concentrations by 11β-HSD1 activity. Indeed it was recently shown that the flocculus is an important site of glucocorticoid action in the initial stages of vestibular compensation after UL, where glucocorticoids may facilitate synaptic plasticity in the cerebellar cortex (Johnston *et al.*, 2002). The functional significance of the lower level of 11β-HSD1 activity in the MVN remains to be established, although the requirement of GR activation for GABA receptor down-regulation indicates a role for glucocorticoids specifically in the MVN (see Chapter 3).

11β-HSD1 activity is modulated by a range of hormones in peripheral tissues, and by glucocorticoids in peripheral and brain tissues (see Section 4.1 for detailed discussion). Adrenalectomy in rats significantly reduced 11β-HSD1 mRNA expression in the hippocampus and 11β-HSD1 activity in the hippocampus, cerebellum and cortex after ten days, but not after one. This decrease was reversed, to levels above those found in sham operated rats, by administration of the glucocorticoid agonist dexamethasone (Low *et al.*, 1994b). Chronic (15 days) arthritic stress has also been shown to increase hippocampal 11β-HSD1 activity. By contrast, chronic psychosocial stress (28 days) in the tree shrew, was found to attenuate hippocampal 11β-HSD1 activity (Jamieson *et al.*, 1997). Thus the effects of glucocorticoids on regulation of 11β-HSD1 follow a complex time course, could be species specific and may vary according to the stressor used (Jamieson *et al.*, 1999). Induction of 11β-HSD1 in the brain during acute stress would help amplify intracellular glucocorticoid actions, whereas chronic attenuation of the enzyme activity may represent a homeostatic mechanism to ameliorate the otherwise excessive metabolic effects of glucocorticoid excess. The present results show that the levels of 11β-HSD1 activity in the vestibulo-cerebellum and MVN are stable over the 4h period after UL. They therefore disprove the hypothesis that changes in enzyme modulatory activity may occur in parallel with the deafferentation-induced changes in the properties of the ipsilesional MVN neurons over this time. The present study does not however address the possibility that changes in 11β-HSD1 activity may occur at later times after UL, or in other vestibular nuclei such as the lateral vestibular nucleus or its related cerebellar areas, during vestibular compensation.

Chapter 5

MECHANISMS UNDERLYING ADAPTATIONS IN THE FIRING PROPERTIES OF MVN NEURONES *IN VITRO* DURING VESTIBULAR COMPENSATION

5.1 INTRODUCTION

The development of vestibular compensation for the static symptoms following UL has been correlated with a restoration of the spontaneous resting discharge in the ipsilesional VNC (for a discussion, see Chapter 2). Various theories have been proposed to explain how these neurones replace the resting activity lost as a result of deafferentation. One hypothesis suggests that rapid modifications in the intrinsic pacemaker properties of brainstem neurones may underlie this recovery (Darlington and Smith, 1996; see Section 2.6.5). In guinea pig, a significant increase in the excitability of ipsilesional MVN neurones in coronal brainstem slices *in vitro* is observed after 30 days of compensation (Vibert *et al.*, 1999b). However, blockage of synaptic transmission with low Ca^{2+}/ high Mg^{2+} solution within these slices resulted in a significant increase in excitability from 7 days post-UL, which increased further by 30 days post-UL (Vibert *et al.*, 1999b). Ris *et al.* (2001) observed an increase in excitability of ipsilesional MVN neurones in coronal brainstem slices *in vitro*, from 48 hours post-UL in guinea pig, when the slices were perfused with a cocktail containing antagonists of the major neurotransmitters in the MVN (CNQX, D-APV, strychnine and bicuculline). They used this method of blocking synaptic transmission because the low Ca^{2+}/ high Mg^{2+} solution used by Vibert *et al.* (1999b) is known to affect the intrinsic firing properties of neurones (Dutia *et al.*, 1992; Ris and Godaux, 2001). These data suggest that in the guinea pig, the later stages of VC rely increasingly on modifications of the intrinsic properties of the deafferented VN neurones.

An increase in excitability of ipsilesional MVN neurones *in vitro* is observed from within 4 hours post-UL in rat, specifically in the rostral region of the MVN. It is unknown whether these changes in neuronal excitability in rat are maintained by modification of the pacemaker properties of these neurones, or by alterations in the synaptic inputs onto these neurones. In this chapter the relative importance of intrinsic membrane properties versus synaptic inputs, to the spontaneous firing characteristics of ipsilateral MVN neurones are investigated at varying times following UL in rat.

5.2 AIMS AND EXPERIMENTAL RATIONALE

The following experiments were designed to investigate the mechanisms underlying changes in neuronal activity in horizontal brainstem slices containing the ipsilateral MVN during the process of vestibular compensation in rat.

1. To confirm the increase in firing rate observed previously in rostral but not caudal MVN neurones after UL, the firing rates *in vitro* of neurones from either the rostral or caudal third of ipsilateral MVN slices were recorded. Slices were taken from labyrinthine intact (control) animals, or animals labyrinthectomised 4 hours, 48 hours or 1 week previously.

2. To investigate the contribution of synaptic inputs to the firing rate of control MVN neurones *in vitro*, synaptic transmission was blocked by adding to the perfusing aCSF a cocktail of the specific antagonists of the main neurotransmitters in the vestibular nuclei (glutamate, GABA and glycine). The classic method of blocking synaptic transmission by perfusion of the brainstem slice with low Ca^{2+}/high Mg^{2+} solution was not used as this procedure can cause changes in the pacemaker properties of these neurones (Dutia *et al.*, 1992; Ris and Godaux, 2001).

3. To compare the contribution of intrinsic pacemaker properties to the firing rates of MVN neurones after labyrinthectomy, neurones were recorded in normal aCSF and in aCSF containing the cocktail of neurotransmitter antagonists. Recordings were taken from rostral and caudal regions of the MVN in slices from animals labyrinthectomised 4 hours, 48 hours and 1 week previously.

5.3 METHODS

Experimental data were obtained from 1234 MVN neurones from 42 male Sprague Dawley rats. Except for the following, the methods employed in this chapter of the thesis are identical to those described in the methods section of Chapter 3.

Anaesthesia

1. Avertin (tribromoethanol)

For all experiments the surgery was carried out under avertin anaesthesia (Dyer *et al.*, 1981). The anaesthetic was made up fresh at the beginning of each week according to the following recipe: 0.312 g tribromoethanol was added to 1.25 ml ethanol and mixed until tribromoethanol dissolved completely. To this solution 0.187 ml 2-methylbutan-2-ol and subsequently 14.25 ml sterile saline (0.9%) was added; the anaesthetic was stored at 4°C. The solution was injected i.p. at a dose of 1ml/100g body weight and provided surgical anaesthesia for 30-40 min. All Chemicals were purchased from Sigma, UK.

2. Halothane

Prior to decapitation, animals were anaesthetised with halothane (Flurothane, May and Baker, Ltd., UK) by inhalation. The anaesthetic was administered by placing the animal in a small perspex box containing a piece of halothane-soaked cotton wool. A sufficient level of anaesthesia was determined by lack of reflex response to a paw pinch.

Surgery and behavioural measurements

All surgery in this chapter was carried out under avertin anaesthesia. Each animal was observed following recovery from anaesthesia, which was complete within 50-60 min after induction. Unilaterally labyrinthectomised animals showed characteristic symptoms including tonic eye deviation, spontaneous ocular nystagmus, circular walking, head deviation towards the side of the lesion, extensor weakness in the ipsilesional limbs and barrel rolling.

Solutions

1. Cocktail aCSF

The following drugs were added to the normal perfusing aCSF to block the main neurotransmitters in the MVN:

(a) -(-) Bicuculline methobromide (20μM), to block $GABA_A$ receptor mediated transmission (Ris *et al.*, 2001)

(b) (2*S*)-(+)-5,5-Dimethyl-2-morpholineacetic acid (SCH 50911; 20μM), to block $GABA_B$ receptor mediated transmission (Xiong and Stringer, 2001)

(c) Strychnine hydrochloride (10μM), to block glycine receptor mediated transmission (Ris *et al.*, 2001)

(d) D-(-)-2-Amino-5-phosphonopentanoic acid (D-APV; 50μM) to block NMDA receptor mediated transmission (Ris *et al.*, 2001)

(e) 6-Cyano-7-nitroquinoxaline-2,3-dione disodium salt (CNQX; 20μM) to block AMPA/kainate receptor mediated transmission (Ris *et al.*, 2001)

All drugs were purchased from Sigma, UK, except SCH 50911 which was purchased from Tocris Cookson Ltd, UK. Stock solutions of the drugs were made in distilled water and kept frozen until use. Aliquots of the stock solutions were thawed and diluted to their final concentrations in oxygenated aCSF. The slice was perfused for at least 15 min with the cocktail aCSF before any recordings were taken.

Data collection and analysis

In these experiments both rostral and caudal thirds of the MVN were explored and the mean tonic discharge rate of sampled neurones recorded. Statistical comparisons of the mean discharge rates of either rostral or caudal MVN neurones in different groups were carried out using one- or two-way ANOVA with Student-Newman Keuls post hoc testing. Significance was assumed when $p<0.05$. Values are given as mean ±S.E.M. throughout.

5.4 RESULTS

Extracellular recordings from tonically active MVN neurones were made in brainstem slices prepared from normal animals or animals which had received a left UL 4h, 48h or 1wk previously. Each MVN was divided by eye into three approximately equal parts, namely rostral, middle and caudal parts. Recordings were made in the rostral and caudal parts of the MVN, and the location of each neurone was recorded. All MVN neurones included in this study (n=1234) showed a regular sustained discharge for 120s, then their firing rates were recorded for 60s. The firing rates of the cells were recoded in normal aCSF and in aCSF supplemented with a cocktail of antagonists of the major neurotransmitters in the vestibular nuclei (CNQX, D-APV, SCH 50911, strychnine and bicuculline).

Changes in spontaneous activity of MVN neurons at different stages of vestibular compensation

As shown in figure 5.1 and table 5.1 the mean discharge rate of cells in the rostral third of the MVN in slices perfused with normal aCSF and prepared from animals labyrinthectomised 4h (18.2 ± 0.95 spikes/sec, n=76 neurones), 48h (18.4 ±0.93 spikes/sec, n=82 neurones) or 1wk (20.7 ±0.93 spikes/sec, n=90 neurones) previously, were significantly higher than in slices prepared from control animals (15.2 ±0.9 spikes/sec, n=84 neurones, $p<0.0005$, one-way ANOVA, Fig. 5.1). This confirms the results of previous experiments, where a significant increase in the excitability of lesioned rostral MVN neurones is seen after UL. As shown in figure 5.2 and table 5.1, cells in the caudal third of the MVN showed no significant change in their mean discharge rate at any time after UL. The increase in excitability of lesioned rostral MVN neurones has been shown using intracellular recording techniques to be due to changes in type B rather than type A neurones (Him and Dutia; 2001). Thus it is likely that the increase in firing rate of rostral MVN neurones, recorded extracellularly here reflects an increase in type B neuronal activity, which will be masked to some extent by the recording of unchanged type A neuronal activity.

Effect of synaptic blockers on spontaneous activity of MVN neurons

All neurons, recorded in normal aCSF or in aCSF containing synaptic blockers displayed a regular discharge like that shown in figure 5.3C. These active neurons were found throughout the whole MVN. The discharge of neurons recorded in slices perfused with a cocktail of synaptic blockers (cocktail aCSF) was as regular as that of the neurons recorded in slices perfused with normal aCSF. Figure 5.3 presents histograms of the coefficient of variance (calculated as the standard deviation of the interspike interval divided by the mean of the interspike interval ±S.E.M) of neurones in rostral and caudal regions of the MVN from control animals recorded in normal aCSF (Fig. 5.3A) and in cocktail aCSF (Fig. 5.3B). The mean coefficient of variance (±S.E.M) was not different between populations: it was 0.095 ±0.014 (median =0.067) in control neurons in normal aCSF and 0.101 ±0.007 (median =0.080) in control neurons in cocktail aCSF (p=0.25, Mann-Whitney test).

The number of neurons recorded per 350μM track through the MVN slice provides an indicator of the relative number of spontaneously active neurons within a given slice. In both the rostral and caudal regions there is no significant difference in the number of spontaneously active neurons recorded in control slices perfused with either normal aCSF (rostral, 1.9 ±0.14; caudal 1.9 ± 0.2) or cocktail aCSF (rostral, 2.1 ±0.17; caudal, 2.0 ±0.13; Fig. 5.4, 5.5).

The mean firing rate of the neurones submitted to the cocktail of antagonists was not different from those recorded in normal aCSF in either the rostral or caudal region of the MVN (Table 5.1, Fig. 5.1, 5.2). The effect of the cocktail of antagonists was further documented by monitoring the firing rate of control neurones while the perfusion medium was shifted from normal aCSF to cocktail aCSF. The firing rate did not change in most neurones as illustrated in figure 5.7A, a few showed a transient increase in activity, followed by a decrease towards control levels, as illustrated in figure 5.7B.

Effect of synaptic blockers on spontaneous activity of MVN neurons at different stages of vestibular compensation

The number of neurones detected per track through the slice, remained static throughout vestibular compensation, when assessed under normal aCSF or under the antagonist cocktail (Fig. 5.4, 5.5). The discharge rates of neurones in all experimental conditions remained regular and there was no significant difference in the mean coefficient of variance between any experimental group (Fig. 5.6).

Neurones recorded in the rostral region of the MVN in slices prepared from animals labyrinthectomised 4h previously, and perfused with the antagonist cocktail, show an increase in their excitability equivalent to that observed in normal aCSF at this timepoint (neurones recorded in cocktail aCSF: control, 14.8 ±0.90 spikes/s; 4h UL, 18.5 ± 1.2 spikes/s, $p<0.05$, 2-way ANOVA. Table 5.1, Fig. 5.1). However, rostral MVN neurones recorded in slices prepared from animals labyrinthectomised 48h or 1 week previously, perfused with cocktail aCSF, showed firing rates equivalent to those recorded from control animals (Table 5.1, Fig. 5.1). This contrasts with increase in excitability observed in these neurones when the slices are perfused with normal aCSF (Fig 5.1). Thus the increase in excitability in rostral MVN neurones observed in slices from animals labyrinthectomised 48h or 1 week previously is lost upon addition of the cocktail of antagonists ($p<0.05$, 2-way ANOVA). This effect was documented by monitoring the firing rate of neurones from 48h or 1 week UL animals, while the perfusion medium was shifted from normal aCSF to cocktail aCSF. The firing rate did not change in some neurones, a few showed a transient increase in activity, followed by a decrease to a level below that of the initial rate, as illustrated in figure 5.8A, and many showed a decrease in activity upon addition of the cocktail (Fig 5.8B).

Neurones recorded in the caudal third of the MVN show no change in their mean discharge rate at any time during vestibular compensation, and no change upon addition of the cocktail of antagonists to the perfusing aCSF (Table.1, Fig. 5.2).

Treatment	aCSF	Rostral MVN Spikes/s ±SEM	*n*	Caudal MVN Spikes/s ±SEM	*n*
Control	Normal	15.2 ±0.90	84	16.4 ±0.81	81
	Cocktail	14.8 ±0.90	75	17.7 ±1.2	76
4h post-UL	Normal	**18.2 ±0.95**	76	17.4 ±1.1	70
	Cocktail	**18.5 ± 1.2**	70	18.6 ±1.2	70
48h post-UL	Normal	**18.4 ±0.93**	82	16.5 ±1.2	70
	Cocktail	15.7 ±0.90	80	19.1 ±1.3	70
1wk post-UL	Normal	**20.7 ±0.93**	90	16.5 ±0.90	72
	Cocktail	16.4 ±0.90	90	16.6 ±0.97	78

Table 5.1

The mean resting discharge rate of MVN neurons *in vitro* recorded from slices prepared from either labyrinthine intact animals (control) or animals which had previously received a left labyrinthectomy at the time stated. *n* is the number of neurones included in each sample. Values in bold indicate a significant difference when compared to the corresponding control region ($p<0.05$, two-way ANOVA).

Figure 5.1 Mean firing rates of rostral MVN neurones in normal and cocktail aCSF at different times following UL

Histograms showing the mean *in vitro* tonic discharge rates ±S.E.M. of MVN neurones in the rostral third of the ipsilateral nucleus in slices prepared at the time stated following unilateral labyrinthectomy

Open bars represent data recorded from slices perfused with normal aCSF

Filled bars represent data recorded from slices perfused with aCSF containing a cocktail of neurotransmitter antagonists

Statistics: * $p<0.05$ versus control slices, in normal aCSF
§ $p<0.05$ versus control slices, in cocktail aCSF
† $p<0.05$ slices in normal aCSF versus cocktail aCSF
} 2-way ANOVA

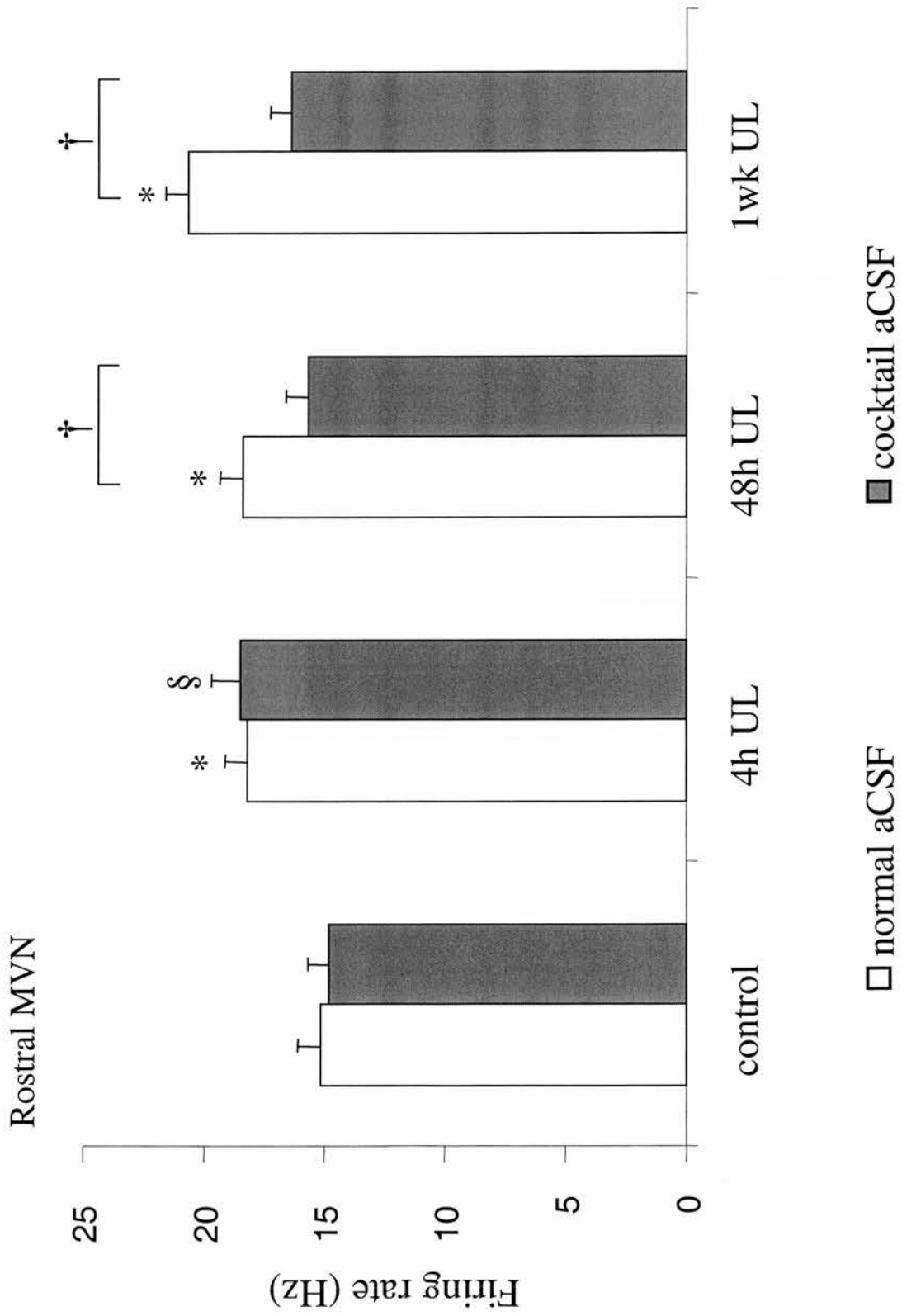

Rostral MVN
Firing rate (Hz)
25
20
15
10
5
0
control
4h UL
48h UL
1wk UL
*
§
†
normal aCSF
cocktail aCSF

Figure 5.2 Mean firing rates of caudal MVN neurones in normal and cocktail aCSF at different times following UL

Histograms showing the mean *in vitro* tonic discharge rates ±S.E.M. of MVN neurones in the caudal third of the ipsilateral nucleus in slices prepared at the time stated following unilateral labyrinthectomy

Open bars represent data recorded from slices perfused with normal aCSF

Filled bars represent data recorded from slices perfused with aCSF containing a cocktail of neurotransmitter antagonists

No significant differences were found in any group tested

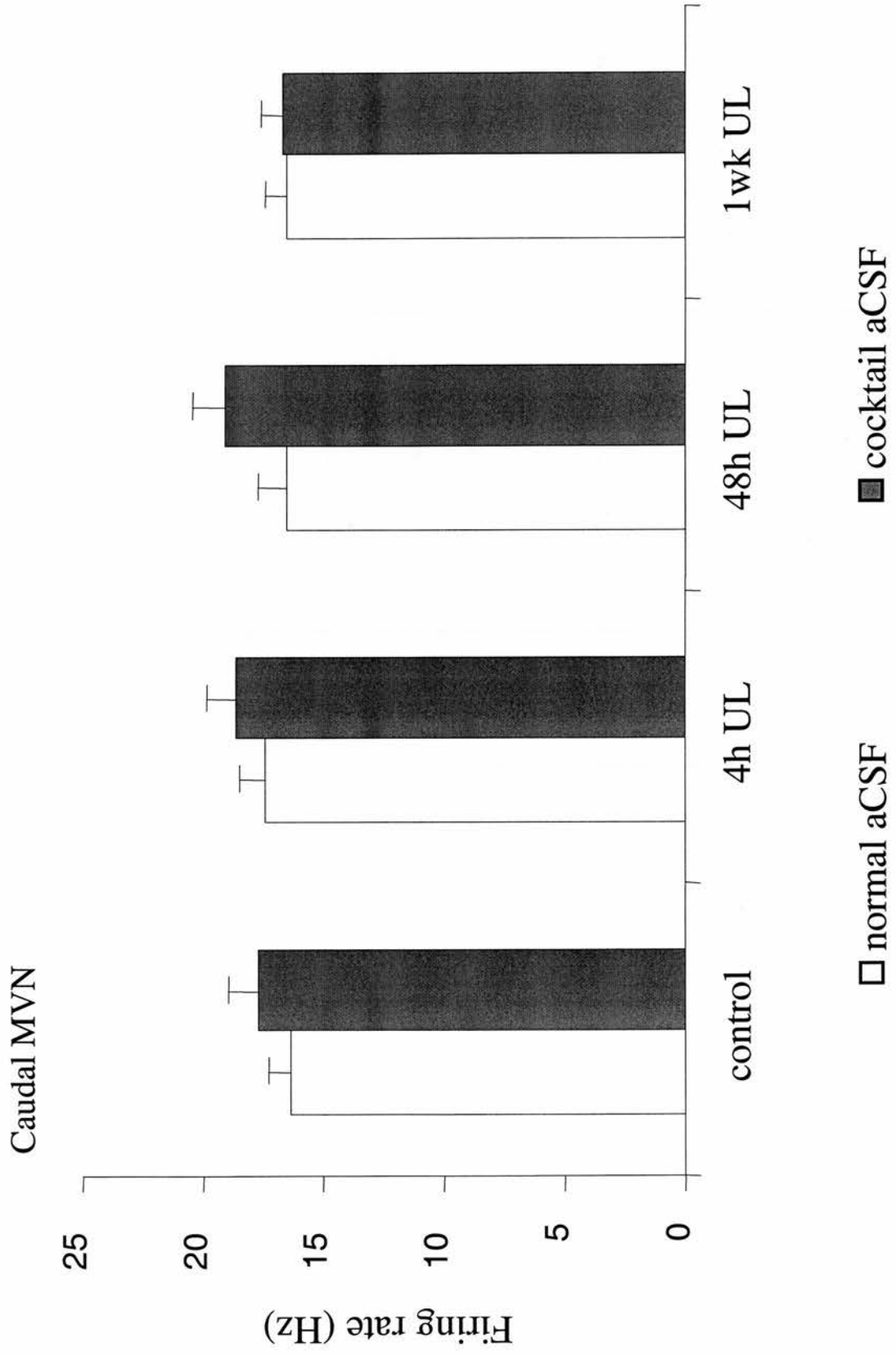

Caudal MVN
Firing rate (Hz)
25
20
15
10
5
0
control
4h UL
48h UL
1wk UL
normal aCSF
cocktail aCSF

Figure 5.3 Histograms of the coefficient of variance of interspike intervals of neurones from control animals in normal aCSF and in aCSF containing a cocktail of synaptic blockers

Comparison of the regularities of neuronal discharge of MVN neurones from unlesioned animals, recorded in normal aCSF and in aCSF containing a cocktail of antagonists of the main neurotransmitters in the MVN (cocktail aCSF).

Histograms of the coefficient of variance recorded in:

A. Normal aCSF

B. aCSF containing a cocktail of neurotransmitter antagonists

In each panel, the thick vertical bar corresponds to the median value

No significant difference was found between groups ($p=0.25$, Mann-Whitney test)

C. Oscilloscope trace of a regularly firing MVN neurone

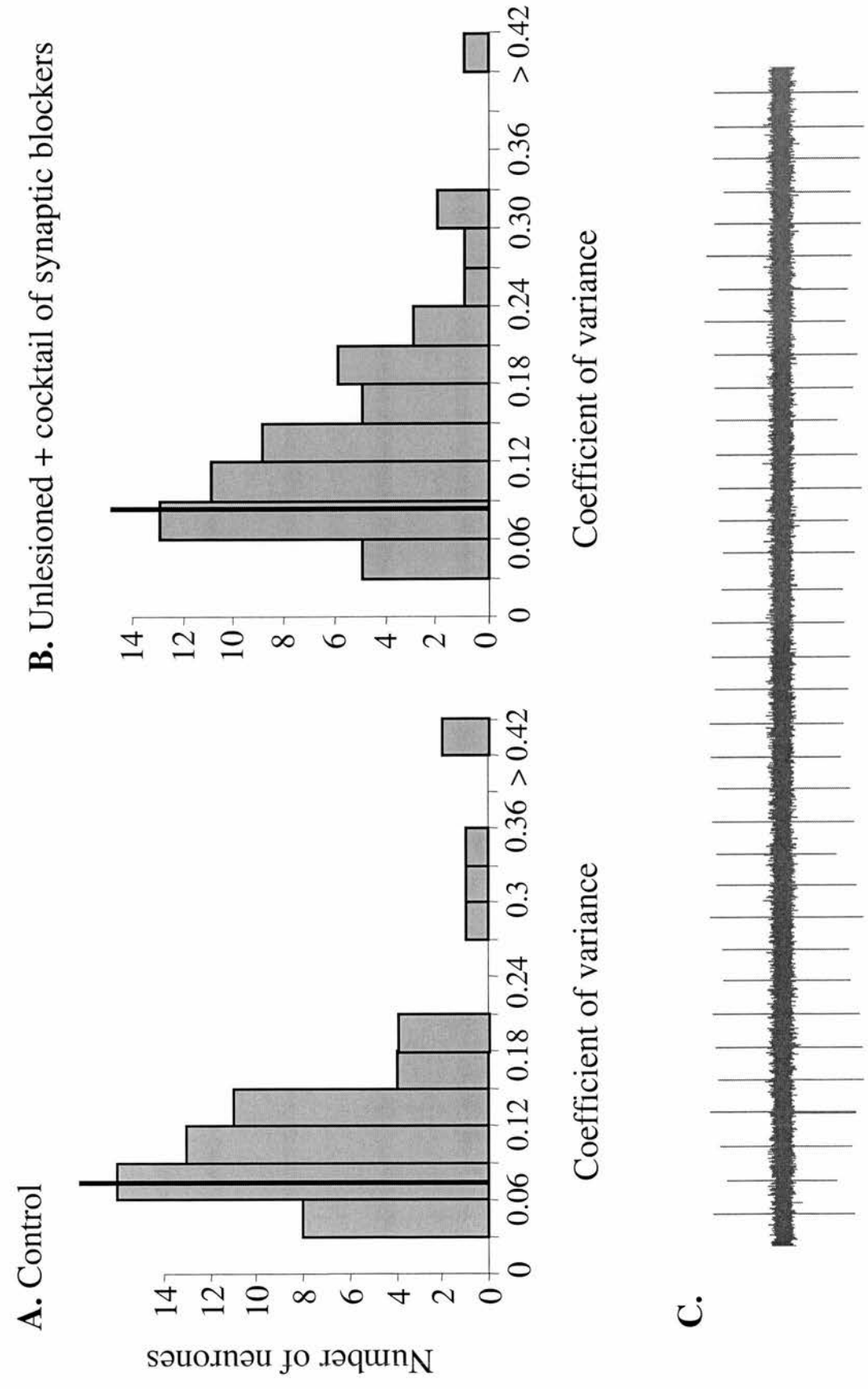

A. Control
B. Unlesioned + cocktail of synaptic blockers
Number of neurones
Coefficient of variance
Coefficient of variance
0
2
4
6
8
10
12
14
0
0.06
0.12
0.18
0.24
0.3
0.36
> 0.42
C.

Figure 5.4 Number of cells obtained per electrode track in the ipsilateral rostral MVN

The number of cells recorded from each 350μM track from the top to the bottom of each slice, averaged for each animal group.

Open bars represent data obtained from slices perfused with normal aCSF

Filled bars represent data obtained from slices perfused with a cocktail of neurotransmitter antagonists

No significant differences were recorded in any group tested

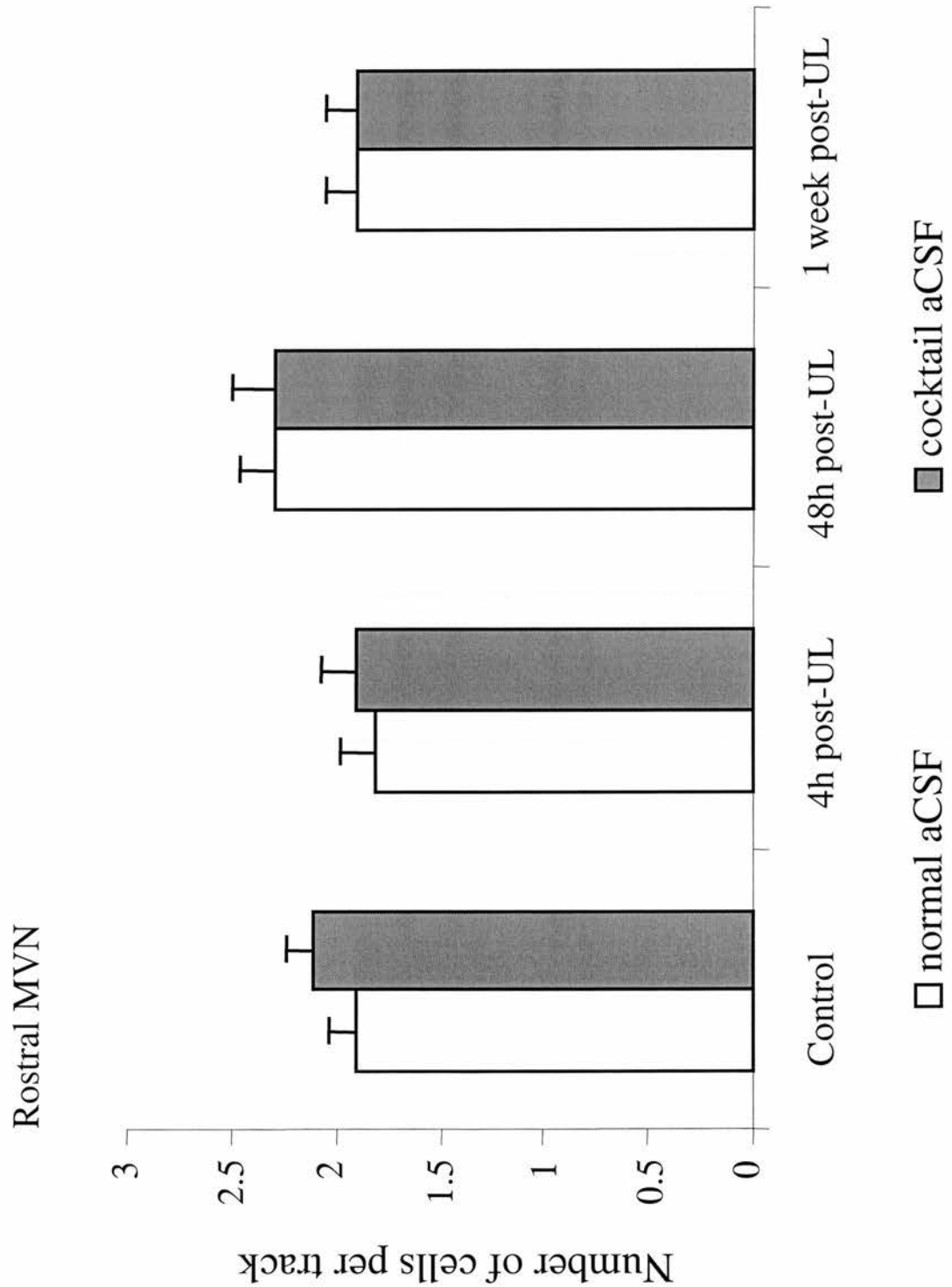

Rostral MVN
Number of cells per track
3
2.5
2
1.5
1
0.5
0
Control
4h post-UL
48h post-UL
1 week post-UL
normal aCSF
cocktail aCSF

Figure 5.5 Number of cells obtained per electrode track in the ipsilateral caudal MVN

The number of cells recorded from each 350μM track from the top to the bottom of each slice, averaged for each animal group.

Open bars represent data obtained from slices perfused with normal aCSF

Filled bars represent data obtained from slices perfused with a cocktail of neurotransmitter antagonists

No significant differences were recorded in any group tested

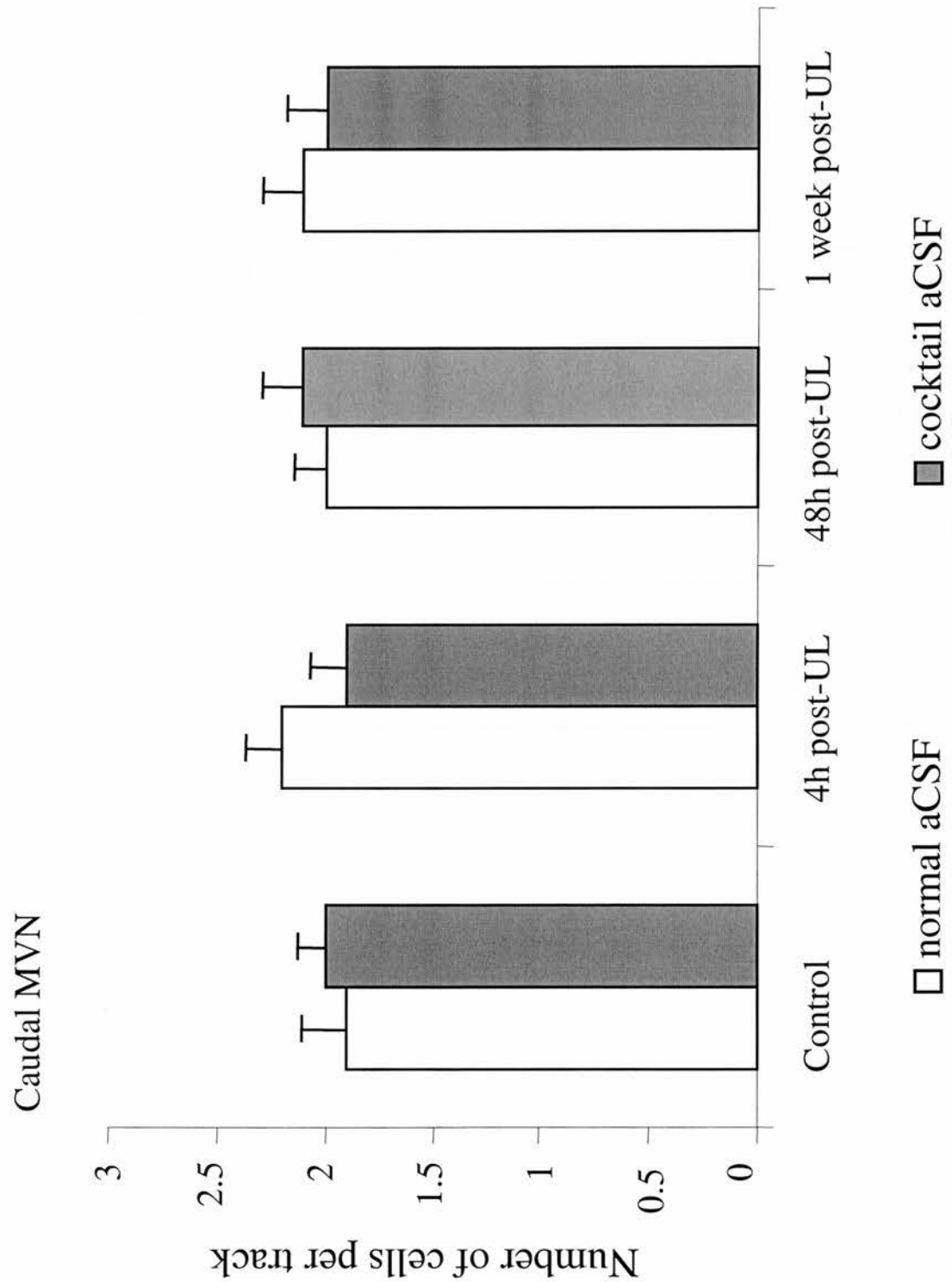

Caudal MVN
Number of cells per track
3
2.5
2
1.5
1
0.5
0
Control
4h post-UL
48h post-UL
1 week post-UL
normal aCSF
cocktail aCSF

Figure 5.6 Histograms of the mean coefficient of variance for MVN neurones recorded at varying time points following UL in normal perfusing medium and in medium containing a cocktail of neurotransmitter antagonists.

Mean ±S.E.M. coefficients of variance obtained from MVN neurones from animals at the time stated following UL.

A. Neurones recorded in the rostral region of the MVN
B. Neurones recorded in the caudal region of the MVN

Open bars represent data recorded in normal aCSF
Filled bars represent data recorded in aCSF containing a cocktail of neurotransmitter antagonists

No statistical differences were found between any groups tested

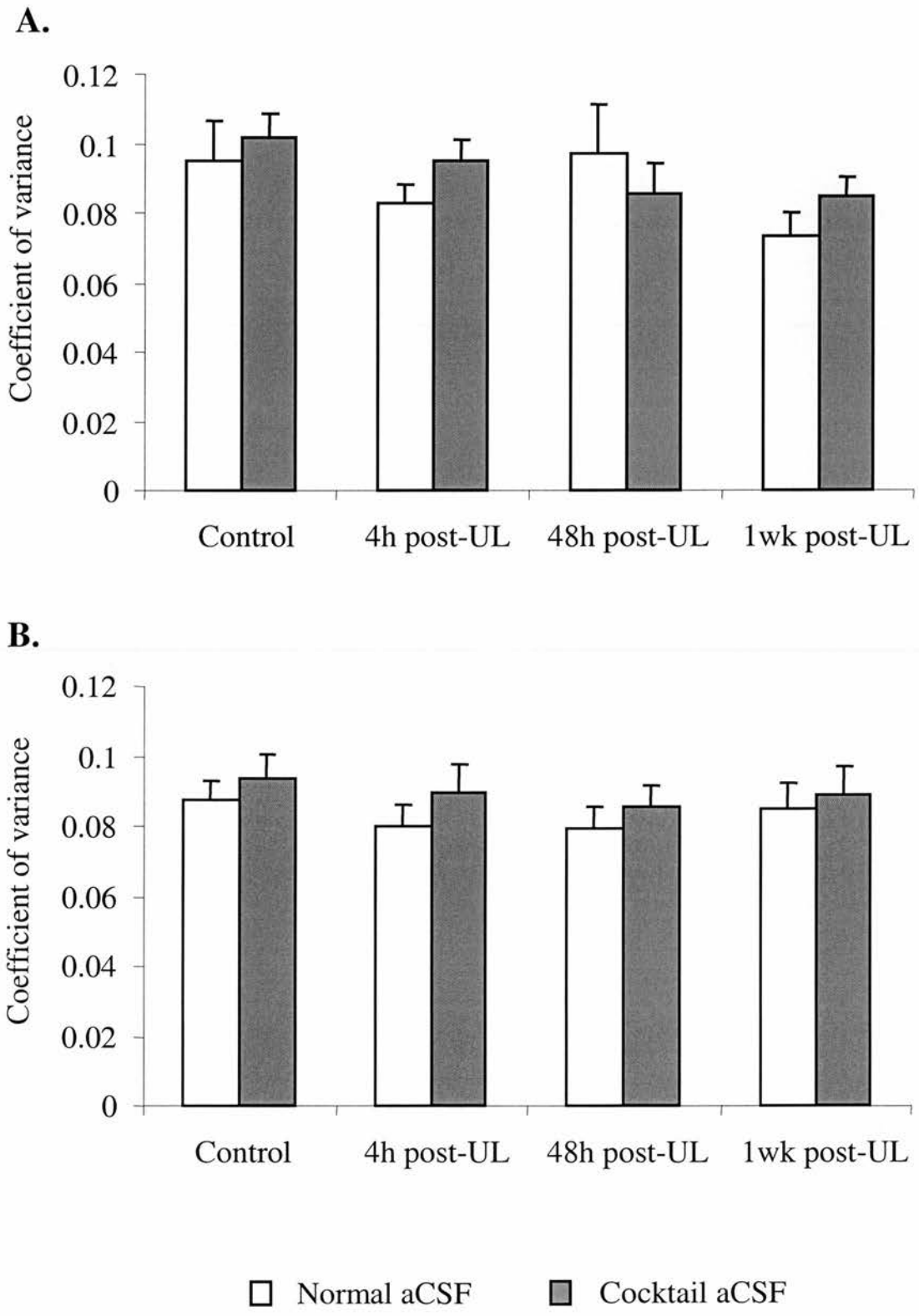

A.
Coefficient of variance
0.12
0.1
0.08
0.06
0.04
0.02
0
Control
4h post-UL
48h post-UL
1wk post-UL
B.
Coefficient of variance
0.12
0.1
0.08
0.06
0.04
0.02
0
Control
4h post-UL
48h post-UL
1wk post-UL
Normal aCSF
Cocktail aCSF

Figure 5.7 Effect of synaptic blockage on firing rate histograms of tonically active MVN neurones

Examples of the effect on the firing rates of tonically active MVN neurones in slices from control animals, upon addition of a cocktail of neurotransmitter antagonists to the perfusing aCSF.

A. Illustration of no change in firing rate upon addition of cocktail aCSF

B. Illustration of a transient increase in firing rate upon addition of cocktail aCSF

In both A. and B., addition of the cocktail of antagonists to the perfusing aCSF is indicated by the bar above each trace

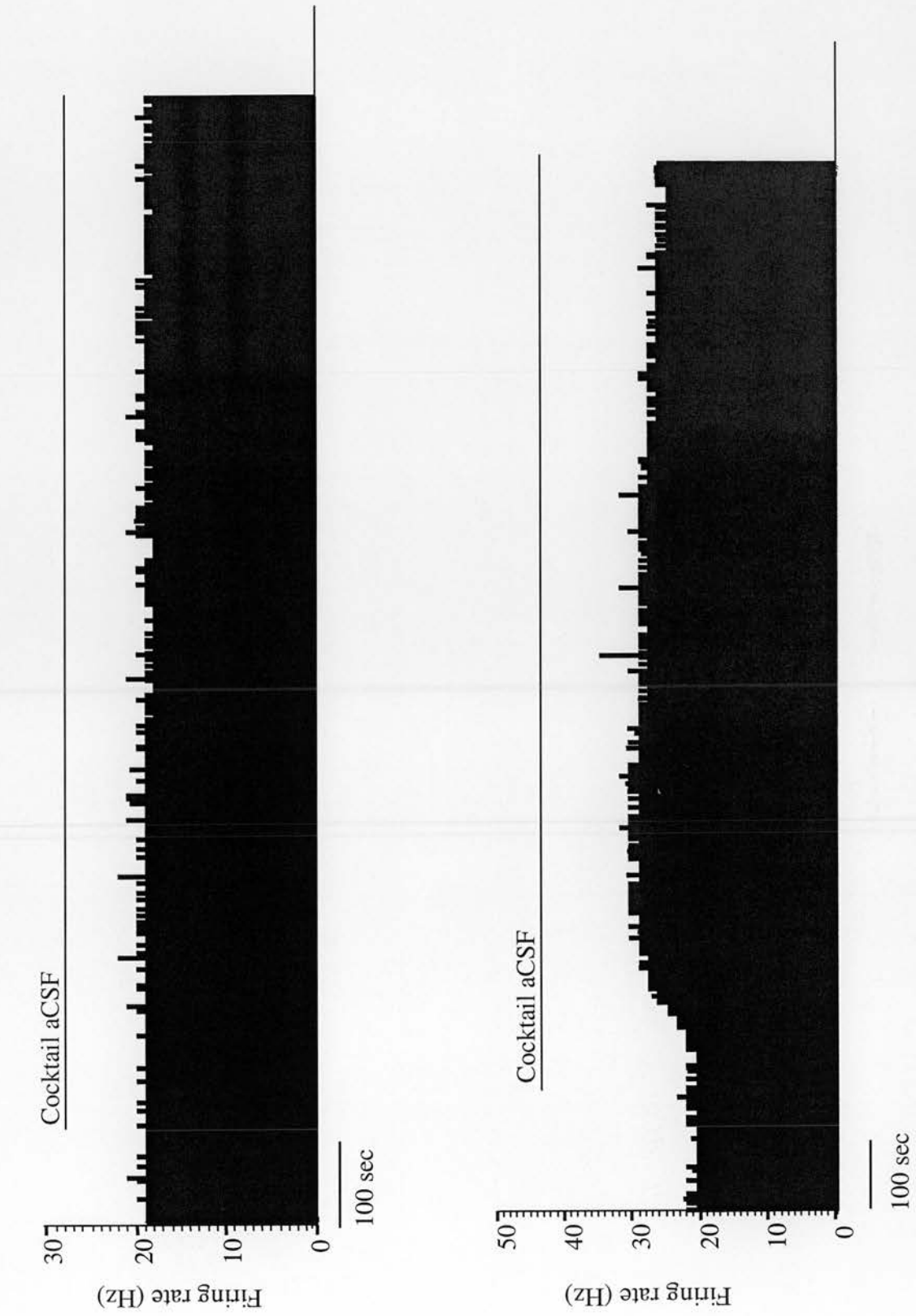

A.
Cocktail aCSF
Firing rate (Hz)
30
20
10
0
100 sec
B.
Cocktail aCSF
Firing rate (Hz)
50
40
30
20
10
0
100 sec

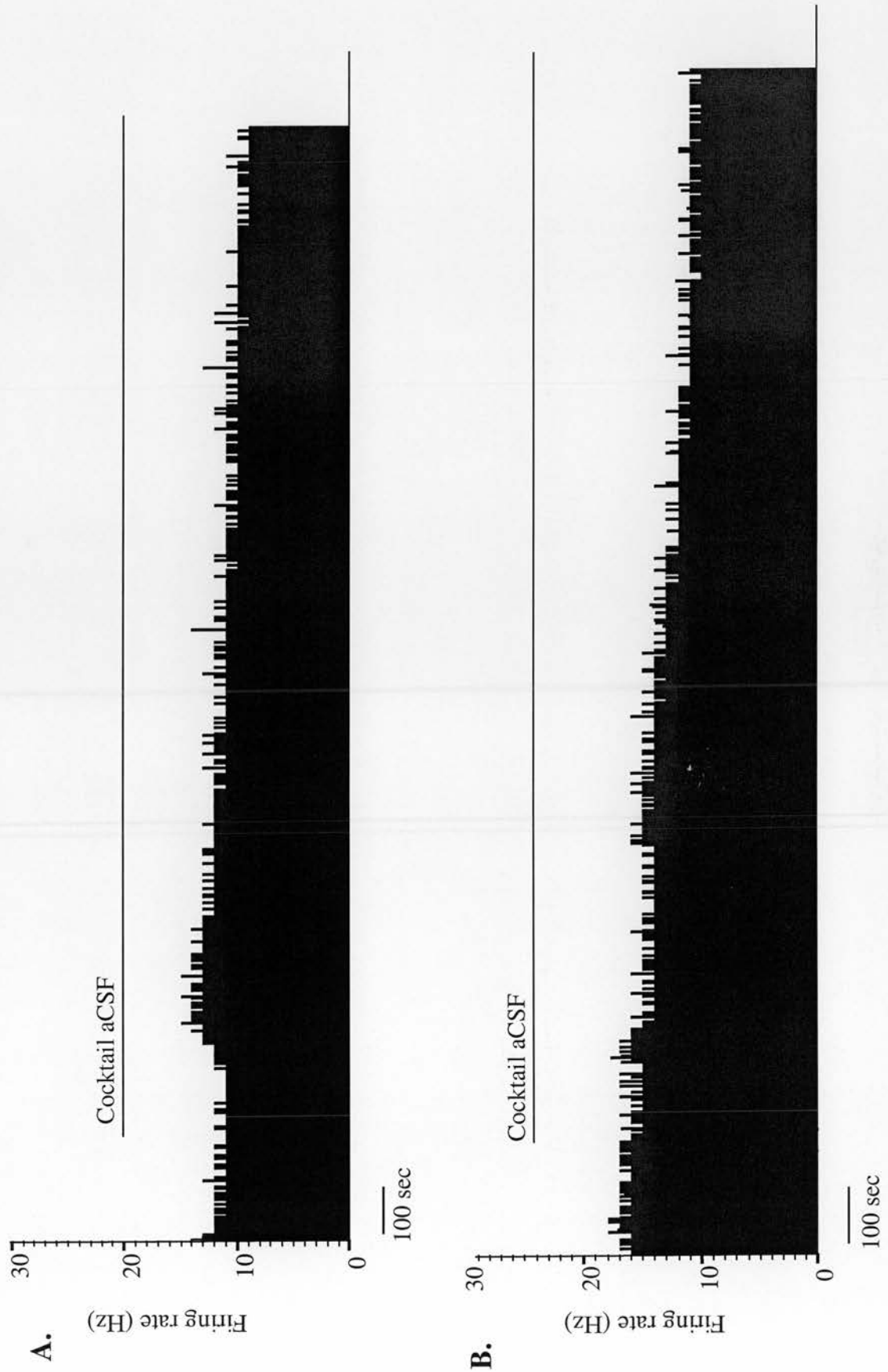
Figure 5.8 Effect of synaptic blockage on firing rate histograms of tonically active MVN neurones in slices from animals labyrinthectomised one week previously

Examples of the effect on the firing rates, of tonically active rostral MVN neurones in slices from animals labyrinthectomised one week previously, upon addition of a cocktail of neurotransmitter antagonists to the perfusing aCSF.

A. Illustration of a transient increase in firing rate followed by a decrease to a level below the initial rate upon addition of cocktail aCSF

B. Illustration of a decrease in firing rate upon addition of cocktail aCSF

In both A. and B., addition of the cocktail of antagonists to the perfusing aCSF is indicated by the bar above each trace


A.
Firing rate (Hz)
30
20
10
0
Cocktail aCSF
100 sec
B.
Firing rate (Hz)
30
20
10
0
Cocktail aCSF
100 sec

5.5 DISCUSSION

Perfusion of brainstem slices prepared from normal rats with a cocktail of neurotransmitters antagonists (CNQX, D-APV, SCH 50911, strychnine and bicuculline), had no effect on the number of spontaneously active neurones detected in either rostral or caudal regions of the MVN. The mean firing rate and the coefficient of variance of these neurones was also unchanged by addition of the cocktail of antagonists. These results suggest that in normal animals the firing activity of MVN neurones *in vitro*, in slices removed from their normal afferent inputs, is maintained primarily by the intrinsic pacemaker properties of these neurones.

At 4h post-UL, an increase in the mean firing rate of MVN neurones is observed solely in the rostral region of the ipsilateral MVN. Addition of the cocktail of antagonists to the slice had no effect on the firing rate firing of these neurones, i.e. the firing rate remained increased compared to control values. The number of neurones in the rostral MVN and the regularity of their firing were not different from control levels at 4h post-UL, either in normal aCSF or in aCSF containing the antagonists. These results suggest that the increase in excitability of rostral MVN neurones at 4h post-UL is maintained primarily by modifications in the intrinsic pacemaker properties of these neurones. Neurones in the caudal region of the MVN displayed no significant difference in any of the tested parameters, at any time post-UL.

At 48h and 1 week post-UL, an increase in the mean firing rate of spontaneously active rostral ipsilesional MVN neurones is observed in slices perfused with normal aCSF. When assessed under the action of the cocktail of antagonists, the mean firing rate of the spontaneously active neurones decreased to a level not significantly different from control. The number of neurones in the rostral MVN and the regularity of their firing were not different from control levels at either time post-UL, either in normal aCSF or in aCSF containing the antagonists. These experiments suggest that by 48h post-UL, the increase in excitability of rostral MVN neurones is maintained by alterations in the neurotransmitter inputs onto these neurones.

5.5.1 Basis of spontaneous activity in the MVN

Vestibular nucleus (VN) neurones display spontaneous activity both *in vivo* and *in vitro*. *In vivo* much of this activity was presumed to be a result of the excitatory input from the vestibular afferents. Yet experiments in bilaterally labyrinthectomised guinea pigs revealed only a 50% decrease in firing rate of VN neurones compared to control (Ris and Godaux, 1998). Thus, *in vivo*, a substantial amount of the activity in the vestibular nuclei is maintained either by excitatory inputs from sources other than the primary vestibular afferents, or by an intrinsic capacity of the neurones to generate action potentials (pacemaker properties). *In vitro*, MVN neurones maintain a considerable amount of resting activity in brainstem slices without their normal afferent inputs. In order to determine whether this spontaneous activity is due to intrinsic as opposed to synaptic influences, various 'synaptic uncoupling' media have been used within brainstem slices. The established method of blocking synaptic activity in slices is by perfusion with low Ca^{2+}/ high Mg^{2+} medium. This suppresses all synaptic inputs, because calcium entry into the presynaptic terminal is a prerequisite of transmitter release. Spontaneous discharge persists in some MVN neurons submitted to this treatment (for review, see Ris and Godaux, 2001), suggesting that a pacemaker-like mechanism is involved in the generation of tonic activity in these neurones. The measure used to assess the level of spontaneous activity in MVN neurones is the mean firing rate, and blockage of synaptic transmission with low Ca^{2+}/ high Mg^{2+} medium has been used to assess the extent of involvement of pacemaker properties in generating this activity (Vibert *et al.*, 1999b). The weakness of this technique is that some of the pacemaker properties of MVN neurones are maintained by Ca^{2+} currents (see Section 1.2.2), hence blockage of Ca^{2+} may alter the neuronal firing properties. Prolonged exposure (more than 20 min) to a low Ca^{2+}/ high Mg^{2+} solution alters the discharge patterns of many vestibular neurones causing them to fire in regular bursts (Dutia *et al.*, 1992; Vibert *et al.*, 1999b; Ris and Godaux, 2001); thus the use of mean firing rate to assess the level of spontaneous activity in MVN neurones is questionable in these conditions.

The present method uses a cocktail of neurotransmitter antagonists (modified from the methods of Ris *et al.*, 2001) to block synaptic transmission. The advantage

of this technique is that it blocks synaptic transmission without affecting the intrinsic membrane properties of the neurones. The continued regularity of firing in the cocktail aCSF justifies the use of mean discharge rate to quantify the activity of these neurones. The weakness of this technique is that there are many neurotransmitters and neuromodulators within the vestibular nuclei (see Section 1.3 for a detailed review) which may affect the spontaneous firing rate of the neurones, which are not blocked by the cocktail.

In the present study, in normal animals the firing activity of MVN neurones in slices removed from their normal afferent inputs, remains unchanged by the addition of the cocktail of antagonists to the perfusing medium (Table 5.1), indicating that the major neurotransmitters within the MVN do not maintain the spontaneous discharge of these neurones *in vitro*.

5.5.2 Mechanisms regulating spontaneous activity in the MVN during vestibular compensation

Recent intracellular experiments have revealed that the increase in excitability of rat MVN neurones post-UL is due to an increase in excitability of type B but not type A neurones (Him and Dutia, 2001). Type B neurones can be distinguished from type A by their narrower action potentials, and a two-component AHP: a first, fast and small component followed by a delayed and slower one (see Figure 1.4; Section 1.2.2 for a detailed discussion). The fast and small AHP is mediated by a tetra-ethyl ammonium (TEA)-sensitive potassium current (I_K) and a transient A-type current (presumably I_A blocked by 4-aminopyridine). In contrast, the delayed slow AHP is insensitive to TEA but abolished by Ca^{2+} free medium and bath-application of apamin; it is therefore mainly mediated by an apamin-sensitive Ca^{2+}-activated potassium current ($I_{K(Ca)}$, via SK channels) (Johnston *et al.*, 1994). These SK mediated K^+ currents control the regularity of the firing rate of these cells (De Waele *et al.*, 1993). Type B neurones have narrow action potentials, generated in part by a tetrodotoxin (TTX)-sensitive sodium current (the voltage-gated I_{Na}). Long-lasting subthreshold plateau potentials, which are TTX-sensitive and therefore ascribed to a persistent sodium current (I_{NaP}), have also been detected in more than half of type B neurones in guinea pig (Serafin *et al.*, 1991a, b). This persistent inward current will

drive the membrane potential towards depolarisation between successive spikes. In addition, 30% of rat type B neurones possess low threshold Ca^{2+} channels which will further contribute to membrane depolarisation (Johnston *et al.*, 1994). The combination of these currents in MVN neurones allows for, and controls, the spontaneous pacemaker activity: Na^+ and Ca^{2+} currents driving the neurones towards depolarisation, K^+ currents opposing this and stabilising the pacemaker activity. The increase in intrinsic pacemaker activity detected here at 4h post-UL could result from changes in a variety of channels: there could be a down-regulation in the number or efficacy of K^+ channels, or and up-regulation of channels carrying inward current (e.g. I_{Na}, I_{NaP} and low voltage gated Ca^{2+} channels).

Recently Patk *et al.* (2003), investigated changes in the expression of voltage gated Na^+ channels and the Ca^{2+}-activated potassium channels (SK channels) after UL in rat. They found no change in mRNA levels for the investigated subunits in the vestibular nuclei, between 1 and 30 days post-UL. Unfortunately they did not examine an earlier timepoint than 1 day, as the present data suggest that in rat, the increase in excitability is only maintained by changes in the intrinsic pacemaker properties of MVN neurones for a transient period following UL. The data from Patk *et al.* (2003) initially appears to corroborate the present data, indicating no changes in ion channels involved in controlling pacemaker properties of the neurones from 48h post-UL. However, the *in situ* hybridization technique is only sensitive to about a 30% change in subunit expression which would not detect subtle transcriptional changes. In addition, changes in pacemaker conductances may be maintained by translational rather than transcriptional regulation. Membrane ionic conductances other than those mediated by voltage gated Na^+ channels and the Ca^{2+}-activated potassium channels may be modulated post-UL. Indeed within 24h post-UL in rat, an increase in the proportion of type B neurones which display low threshold rebound potentials (LTS-like potentials), mediated by low threshold Ca^{2+} channels (perhaps by T-type Ca^{2+} channels), was observed in the ipsilesional MVN (Him and Dutia, 2001). Thus there may be an up-regulation of low threshold Ca^{2+} channels in a population of MVN neurones post-UL. At 24-72h post-UL the mean input resistance and spike amplitudes of type B neurones were significantly greater than control (Him and Dutia, 2001), perhaps mediated by changes in the channels controlling

pacemaker conductances, or perhaps due to a decreased chloride conductance through GABA receptors which are down-regulated at this time (see Chapter 3).

The increase in firing rate of MVN neurones in rat, detected here from 48h post-UL, cannot be due to, as was previously suggested (Darlington and Smith, 1996), an increase in the pacemaker properties of these neurones, because the increase is blocked by the cocktail of neurotransmitter antagonists (Table 5.1, Fig. 5.1). These results suggest that there is either an increase in the amount of excitatory synaptic input onto these neurones, or that the neurones have become more receptive to the remaining excitatory inputs. It is less possible that the increased excitability is due to a decrease in the inhibitory inputs onto these neurones, because the cocktail of antagonists did not affect the firing rate of neurones in control slices, indicating that the synaptic inputs remaining in the slice are not sufficient in control conditions to alter the firing rate of the neurones, therefore a decrease in the inhibitory inputs would have even less chance of influencing cellular excitability. However, this theory could be tested by repeating the experiment using either excitatory or inhibitory antagonists, and continued further by breaking down the cocktail into individual receptor antagonist components. This would allow identification of the specific receptors mediating the increase in excitability.

The resting membrane potentials of all type B MVN neurones from 24h to 10 days post-UL were significantly more depolarised than those of control type B neurones (Him and Dutia, 2001). This increased depolarisation may be a factor involved in the increase in the firing rate of MVN neurones, observed in the present experiments at 48h and 1 week post-UL. Increased membrane depolarisation would render these neurones more responsive to excitatory synaptic inputs from remaining nerve terminals within the slice, thus synaptic input that previously was not enough to generate spikes, could now be sufficient to generate additional action potentials in these neurones. The same effect could be achieved if there was an up-regulation of excitatory postsynaptic receptors on the neurones, with remaining synaptic inputs initiating a larger depolarising effect through the up-regulated channels. Previous studies revealed asymmetric changes in a number of glutamatergic receptor subunits from within 6 hours post-UL (Li *et al.*, 1997; Horii *et al.*, 2001; Rabbath *et al.*, 2002; see Section 2.5.1 for detailed discussion). However, these demonstrate either no

change, or a decrease in glutamate subunit expression in the ipsilesional VNC, which is in the opposite direction to that which would restore the excitability in MVN neurones. Ipsilesional decreases in mRNA for the glutamatergic receptor subunits NR2A, GluR2 and mGluR7 at 6h post-UL, had returned to normal levels by 50h post-UL (Horii *et al.*, 2001), thus may be a consequence of a transient ipsilesional reduction in glutamate levels after the lesion (see below). Other electrophysiological (Knopfel and Dieringer, 1988; Smith and Darlington, 1992), biochemical (de Waele *et al.*, 1994; Rabbath *et al.*, 2002) and pharmacological studies (Raymond *et al.* 1989) in a range of species, have been unable to provide any evidence of an increase in NMDA or AMPA receptor efficacy, affinity, or numbers following UL.

An alternative explanation for the increase in excitability detected here from 48h post-UL, could be an increase in excitatory synaptic input onto MVN neurones. A recent *in vivo* microdialysis study in rat provided evidence for an increase in excitatory synaptic input onto MVN neurones within 12h post-UL. Inoue *et al.* (2003) revealed that the concentration of glutamate in the ipsilesional MVN decreased significantly to a nadir at 4h post-UL, presumably due to the deafferentation of the primary glutamatergic inputs, and recovered to a level higher than basal at 12, 24 and 48h post-UL, though this difference was not significant. The error signal promoting the increase in intrinsic excitability at 4h post-UL may involve the decreased glutamate levels in the ipsilesional MVN; increasing glutamate levels may supersede the increase in intrinsic excitability as the mechanism enhancing the *in vitro* excitability of these neurones. The restoration of glutamate levels to control values (indeed slightly higher than control) by 12h post-UL provides evidence that there is an enhancement of glutamatergic input into the MVN, presumably from remaining sensory inputs. Microdissection of the VNC linked to HPLC revealed decreased glutamate levels in all ipsilesional VNC nuclei except the MVN between 2 and 30 days post-UVD (Li *et al.*, 1996b). This decrease in glutamate levels was ascribed to degeneration of the vestibular nerve terminals (Li *et al.*, 1996b). If this is the cause of the decreased glutamate levels, a similar decrease should be observed in the MVN which is rich in primary vestibular terminals (see Section 1.1.1). The lack of decreased glutamate levels in the MVN may be indicative of an increased glutamatergic input from other sensory systems. An increased

efficacy of spinal input onto the ipsilesional MVN neurones, and decreased input onto the contralesional MVN was recorded from 3 days post-UL in an isolated whole brain preparation of the guinea pig (Vibert *et al.*, 1999a), indicating a functional substitution of deficient vestibular inputs with spinal related reflexes. The above experiments and the data recorded in this chapter, provide evidence for the theory that the increase in excitability observed in ipsilesional MVN neurones from 48 hours post-UL in rat is maintained by an increased excitatory synaptic input onto these neurones.

5.5.3 Comparison with other studies

The question of whether spontaneous activity in the MVN *in vitro*, on the side of a previous UL, is due to intrinsic as opposed to synaptic influences has been investigated in two previous studies, both in guinea pig (Vibert *et al.*, 1999b; Ris *et al.*, 2001). An important factor to consider when comparing this study with previous studies blocking synaptic input, is that in normal perfusing medium there are species differences regarding changes in mean firing rate of MVN neurones *in vitro* after UL. Initial experiments in guinea pig reported that the mean resting discharge rate of ipsilesional MVN neurones in brainstem slices from 6 to 8 week compensated animals increased, although this increase was not significantly higher than control (Darlington *et al.*, 1989). However, the number of neurones recorded in this experiment (15 neurones from 7 slices) was too low for definite conclusions to be drawn. Later studies by the same group did find statistically significant increases in the firing rates of ipsilesional MVN neurones from animals compensated for between 3 days and 8 weeks compared to control (Darlington *et al.*, 1992; Smith and Darlington, 1992). However, the data from all stages of compensation was pooled; therefore it was not possible to determine when the increase in excitability first occurred, or to examine changing excitability levels over time. The earliest time at which the excitability of MVN neurones has been assessed *in vitro* post-UL in guinea pig is at 1 day (24 hours). Vibert and colleagues, using brainstem slices from guinea pigs, found no significant increase in the firing rate of ipsilesional MVN neurones between 1 and 7 days post-UL compared to control, but an imbalance in firing rate between the ipsi- and contralesional sides of UL animals was observed from 7 days

and a significant increase in firing rate was recorded at 30 days post-UL (Vibert *et al.*, 1999b; 2000a). An increase in the firing rate of ipsilesional MVN neurones *in vitro* is observed at 4 hours post-UL in rat, which persists for at least 7-10 days (Cameron and Dutia, 1997; 1999; Yamanaka *et al.*, 2000; Him and Dutia, 2001; Johnston *et al.*, 2001; present data). However, this increase was only observed in the rostral third of the MVN in horizontal brainstem slices. Recordings of MVN neuronal activity in guinea pigs *in vitro*, have been made from coronal brainstem slices, which do not necessarily contain the rostral region of the MVN; indeed Vibert *et al.* (1999b) took several coronal slices per brainstem therefore probably recorded from the entire rostro-caudal length of the MVN. Thus it is unknown whether the increase in excitability of ipsilesional MVN neurones at 30 days post-UL in guinea pig is occurring across the entire MVN, or in specific regions as occurs in rat. No recordings of the excitability of MVN neurones in brainstem slices from rat, have been made after 10 days of compensation, hence no comparisons can be made with data from the guinea pig at 30 days post-UL.

To investigate the contribution of intrinsic pacemaker properties in deafferented vestibular neurones during VC, Vibert and colleagues perfused guinea pig brainstem slices with low Ca^{2+}/ high Mg^{2+} solution to block all synaptic inputs. They observed large increases in the resting activity of ipsilesional MVN neurones from animals labyrinthectomised 7 days previously compared to MVN neurones from labyrinthine-intact animals (Vibert *et al.*, 1999b). Under the synaptic uncoupling conditions, the increase in ipsilesional MVN excitability had increased further by 30 days post-UL. The authors concluded that from 7 days after the lesion, VC relies increasingly on changes in the intrinsic properties of the deafferented vestibular neurones. Despite the side effects of low Ca^{2+}/ high Mg^{2+} solution (see Section 5.5.1), the firing rates of neurones recorded on the contralateral side of the slice, therefore submitted to the same solution, were significantly lower than the firing rates of ipsilesional neurones, validating the result. In a more recent study, Ris *et al.* (2001) used a cocktail of the main neurotransmitters in the MVN (CNQX, D-APV, strychnine and bicuculline) to reduce synaptic transmission without affecting the intrinsic properties of the neurones. In contrast to the synaptic uncoupling studies, they recorded a significant increase in the firing rate of ipsilesional MVN neurones from 48 hours post-UL

compared to MVN neurones from labyrinthine-intact animals in slices perfused with the cocktail of antagonists. Unfortunately they did not record the firing rate of deafferented MVN neurones in normal perfusing medium, so it is unclear if this increase in excitability under neurotransmitter blockage is translated to an increased excitability in normal medium. The most striking effect of perfusion of MVN neurones from unlesioned animals with the cocktail of antagonists was a dramatic reduction in the number of neurones recorded per slice compared to neurones recorded in normal aCSF (mean number of neurones ±SD of 9.5 ±3.5 in cocktail aCSF compared to 30.7 ±11.1 in normal aCSF) (Ris *et al.*, 2001). This is in marked contrast to the present data in rat where no change in the number of spontaneously active neurones per track is found either in normal or cocktail aCSF in the rostral or caudal region of the MVN at any time after UL (Fig. 5.4, 5.5). The decrease in the number of neurones detected per slice in guinea pig, was paralleled by a significant reduction in the mean discharge rate of the remaining MVN neurones (Ris *et al.*, 2001). Neither the present data (Fig. 5.1, 5.2, Table 5.1), nor that of Vibert *et al.* (1999b), detected any difference in the mean firing rate of MVN neurones when recording in normal medium compared to medium blocking synaptic transmission. The discrepancies between these experiments may lie in the specific neurotransmitters blocked. Vibert *et al.* (1999b) blocked all excitatory and inhibitory synaptic inputs, Ris *et al.* (2001) blocked $GABA_A$, glycine, NMDA and AMPA/kainite receptor mediated transmission, whereas the current methods also included an antagonist for $GABA_B$ receptors. It appears that in normal conditions *in vitro*, the excitatory and inhibitory synaptic influences are not of sufficient amplitude to alter the excitability of vestibular neurones. However, if all excitatory inputs are removed, the remaining inhibitory inputs may hyperpolarise neurones to an extent that can affect the number of action potentials generated. Thus the decrease in mean firing rate of control ipsilesional MVN neurones, elicited by the cocktail of Ris *et al.* may be the result of GABAergic inhibition through $GABA_B$ receptors, hyperpolarising neurones in the absence of excitatory depolarisations to an extent that decreases the number of action potentials generated by the intrinsic pacemaker properties. The increase in firing rate observed at 48h post-UL, in the cocktail

conditions (Ris *et al.*, 2001), but not in conditions of synaptic uncoupling (Vibert *et al.*, 1999b), may be related to a down-regulation of $GABA_B$ receptor efficacy as observed in rat post-UL (see Chapter 3).

The increase in excitability of ipsilesional rat MVN neurones from 4h post-UL may not have been observed in guinea pigs because the rostral region where these changes occur has never been specifically investigated. While the data from these experiments indicate that there is an increase in the pacemaker properties of these neurons at 4h post-UL, it is important to note that the cocktail does not block all excitatory neurotransmission in the MVN, and the increased excitability may be maintained by an increase in excitatory synaptic inputs other than those mediated via NMDA, AMPA and kainate receptors (see Sections 1.3, 2.5 for discussion of neurotransmitters present in the VNC).

The data from studies in rat and guinea pig appear to present opposing cellular changes post-UL. In guinea pig an increase in firing rate of ipsilesional MVN neurones is recorded between 48h and 30 days post-UL in conditions of synaptic blockade (Vibert *et al.*, 1999b; Ris *et al.*, 2001). The current data show that synaptic blockade in rat at these times actually causes a decrease in the firing rate of rostral MVN neurones. This decrease lowers the firing rate to the level obtained in control neurones perfused with either normal or cocktail aCSF. There are no increases in firing rate of the caudal MVN neurones at any time after UL in rat which would correlate with those observed in guinea pig. However, it may be that the initial increase in intrinsic excitability in the rat MVN is a transient change related to the compensation of horizontal spontaneous nystagmus, and an overall increase in intrinsic excitability equivalent to that seen in guinea pig would be recorded at later timepoints that those utilized in these experiments.

VC is a complex, heterogeneous process and it would be unsurprising if different species utilised diverging mechanisms of recovery at different times. For example, activation of glucocorticoid receptors enables the initial stages of VC in rat, whilst they play no role in compensation in guinea pig, presumably due to the large difference in the stress systems between species (see Chapter 3). In rat, recovery of the resting discharge of deafferented MVN neurones initially appears to be promoted by modifications of the pacemaker properties of the neurones; this is superseded by

adaptations in the intact sensory and vestibular-related inputs onto these neurones. The opposite mode of compensation has been proposed for guinea pig: initial substituting sensory inputs being increasingly replaced by changes in the pacemaker properties of the deafferented neurones (Vibert *et al.*, 1999b).

Summary

The present results demonstrate that the significant increase in excitability of lesioned rostral MVN neurones at 4 hours post-UL is maintained primarily by changes in the intrinsic pacemaker properties of these neurones. This increase may be maintained by changes in the subunit expression or phosphorylation state of ion channels involved in generating pacemaker activity. By 48 hours post-UL the significant increase in excitability of lesioned rostral MVN neurones appears to be maintained by an increased excitatory synaptic input onto these neurones. Thus different mechanisms are utilised in the initiation and maintenance of processes involved in VC.

Chapter 6

PROTEOMIC INVESTIGATION OF THE VESTIBULAR SYSTEM DURING VESTIBULAR COMPENSATION

6.1 INTRODUCTION

Proteomics is generally defined as the systematic identification of every protein expressed from the genome in a cell or tissue, as well as the prominent properties of each protein e.g. post-translational modifications, comparative abundance etc. The entire protein complement in a given cell or tissue is defined as the 'proteome'. Proteomics is a powerful and evolving experimental process that largely investigates changes in protein expression, resulting from various disorders or the effect of external factors such as brain trauma or surgical lesions. In neuroscience, proteomic techniques have been applied to a host of applications, including determination of the proteome of individual brain areas in health and disease (Rohlff, 2000; Butterfield *et al.*, 2003; Rohlff and Hollis, 2003). Its goals include the detection of novel drug targets, diagnostic markers and the investigation of biological events (for review see Banks *et al.*, 2000). Determination of protein rather than RNA levels has major advantages as it is the proteins that carry out functions in a cell, and there is often a low correspondence between changes at the transcription level and changes at the proteomic level (Ideker *et al.*, 2001).

Proteomics consists mainly of two steps: 1) separation of proteins usually by two-dimensional gel electrophoresis and 2) protein analysis and identification, mainly by mass spectrometry. A critical initial process is the extraction and solubilisation of proteins from cells or tissues. Once extracted the proteins are denatured into their polypeptide subunits and separated in two dimensions. In the first dimension, proteins are separated on the basis of differences in their net charge, by isoelectric focusing (IEF): on the application of a current, the charged polypeptide subunits migrate in a polyacrylamide gel strip that contains an immobilised pH gradient until they reach the pH at which their overall charge is neutral (isoelectric point or pI). This produces a gel strip containing discrete protein bands along its length. In the second dimension, the polypeptides in the gel strip are solubilised and denatured in the presence of a negatively charged detergent, sodium dodecyl sulphate (SDS); the proteins lose their three-dimensional structure and assume a net negative charge. The gel strip is applied to a standard polyacrylamide gel and the polypeptides migrate in an electric current towards the anode, thus are separated according to their

size (molecular weight). After separation and staining, the spots (each of which corresponds to a protein of unknown identity) are excised from the gel, digested using trypsin and the peptide fragments are characterised my mass spectroscopy. Proteins are then identified by comparing the masses of the peptide fragments with predictions from either DNA or protein databases (see Figure 6.1).

The application of proteomic techniques to investigate changes in protein expression and post-translational modifications during the process of vestibular compensation (VC), is potentially a very powerful method of examining the molecular mechanisms underlying plasticity. At present the molecular mechanisms of vestibular compensation are largely unknown, but probably involve changes in protein expression and/or phosphorylation. The adaptations in receptor efficacy in MVN neurons (see Section 2.5; Chapter 3), may occur through changes in receptor expression or subunit composition, and/or in the phosphorylation state of the receptor subunits. If the increase in excitability of MVN neurons from 48h post-UL relies on an increase in synaptic inputs (see Chapter 5) we may expect adaptations in a variety of proteins such as cytoskeletal and intermediary metabolic proteins, which are easily identified in proteomic maps of the brain (Lubec *et al.*, 2003). Several recent studies indicate that activation of kinase pathways may play an important role in VC (see Section 3.6) and several currently unidentified guinea-pig brainstem proteins undergo phosphorylation early in VC (Sansom *et al.*, 1997). In addition, although the protein synthesis inhibitor cyclohexamide does not prevent the early recovery of resting activity in the guinea-pig (Ris *et al.*, 1998), 2-D gel electrophoresis has revealed increased expression of several currently unidentified proteins 1 week post-UL in this species (Ris *et al.*, 1999). Numerous studies have documented changing expression of proteins involved in gene expression regulation, such as the cAMP/calcium response element binding protein (Kim *et al.*, 2000) and immediate early gene proteins (Kaufman *et al.*, 1992; Kitahara *et al.*, 1995a; Cirelli *et al.*, 1996) in MVN neurones over hours and days after UL in the rat. In the present study, to investigate changes in protein expression and phosphorylation post-UL, proteomic techniques were applied to brain tissue from regions of the central vestibular system and vestibulo-cerebellum in rat.

The methodologies of proteomics are continually developing, and the techniques of protein solubilisation, separation and visualisation require optimisation for any specific tissue. Over the last three years in the present study, a wide variety of solubilisation, separation and visualisation techniques were applied to MVN, floccular and cerebellar tissue. Initial experiments were performed using Coomassie blue staining to visualise proteins; this ensured that the extraction and solubilisation techniques used were effective. Coomassie blue stain has a protein detection limit of >100 ng (Herbert *et al.*, 2001) and therefore is not sensitive enough to visualise low abundance proteins. Subsequent experiments were performed using silver staining techniques, which can detect protein spots of between 1-10ng on gels. This increased the detection sensitivity to 10 times that of Coomassie and allowed the visualisation of around 800 proteins on 2-D gels of MVN tissue (see Figure 6.2). However, silver staining is not a quantitative staining technique; larger spots do not absorb an equivalent amount of dye to match their size compared to smaller spots, hence it is not suitable for quantitative comparisons of densities between spots, even between two gels stained at the same time. Its other disadvantage is that it is not an end point stain: termination of the silver reaction requires several, usually precisely timed manual interventions which affects reproducibility between different runs of gels. SYPRO Ruby (Bio-Rad Life Sciences, Hertfordshire, UK), is a fluorescence based stain with a minimum protein detection limit of around 10ng. It is a linear, quantitative, end point staining technique that enables the comparison of protein expression between different experiments; therefore this stain was adopted for the following experiments. Continuing from the preparatory experiments, this chapter describes the optimal proteomic method developed to date. Changes in protein expression between the ipsilesional and contralesional MVN one week after labyrinthectomy were investigated, and 69 proteins expressed in the MVN were identified. The experiments in this chapter were performed in collaboration with Dr Janet Paterson (Biomedical Sciences, Edinburgh University) and Dr Duncan Short (Fujisawa Institute for Neuroscience, Edinburgh), who performed the mass spectrometry.

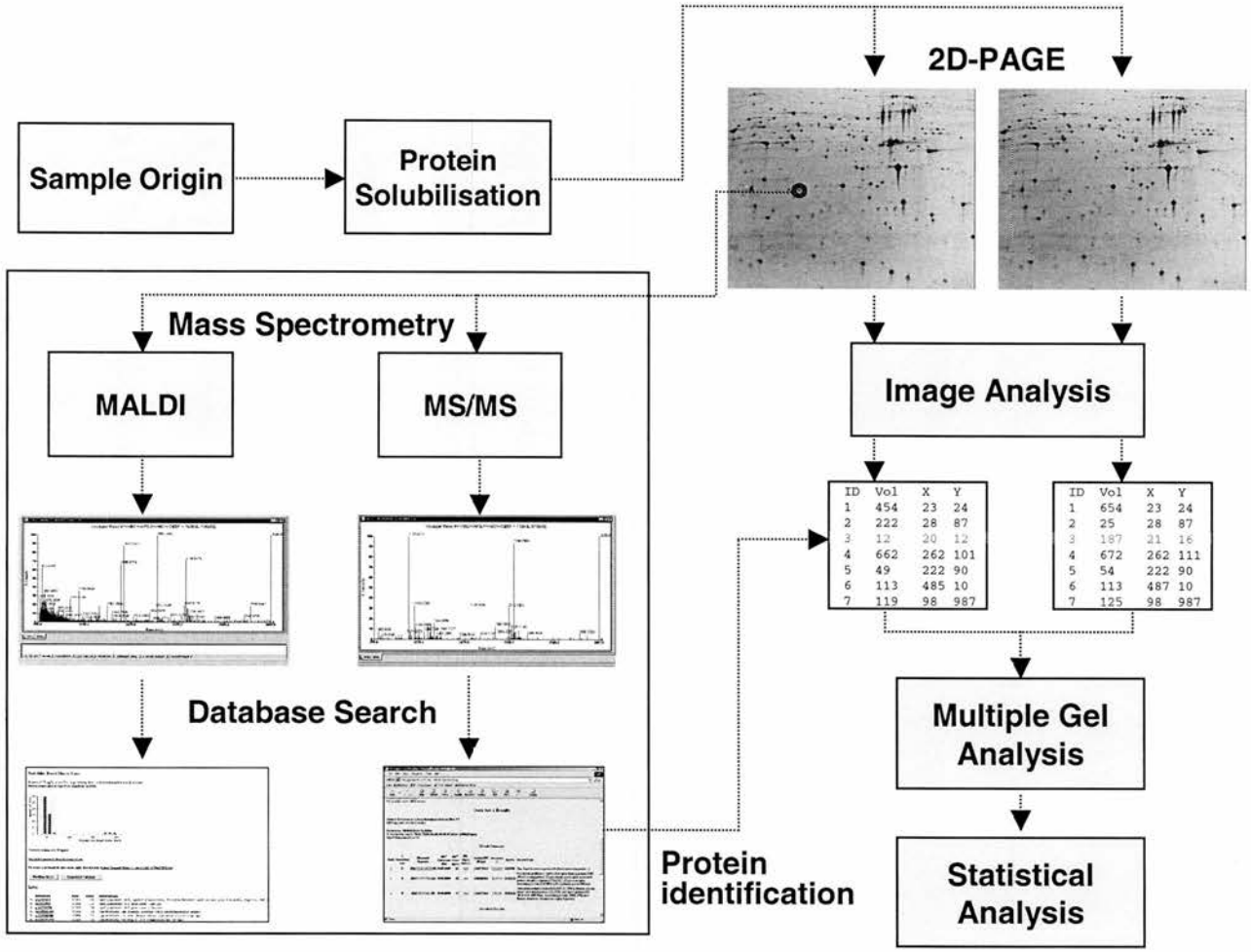


Figure 6.1 A workflow for a typical proteomics experiment. Proteins are separated in two dimensions by gel electrophoresis and subsequently identified by mass spectrometry, accompanied by searches of DNA and protein sequence databases. The gels are scanned and the images are analysed by software that detects properties of the gel spots, such as an estimate of the volume of protein on the gel.

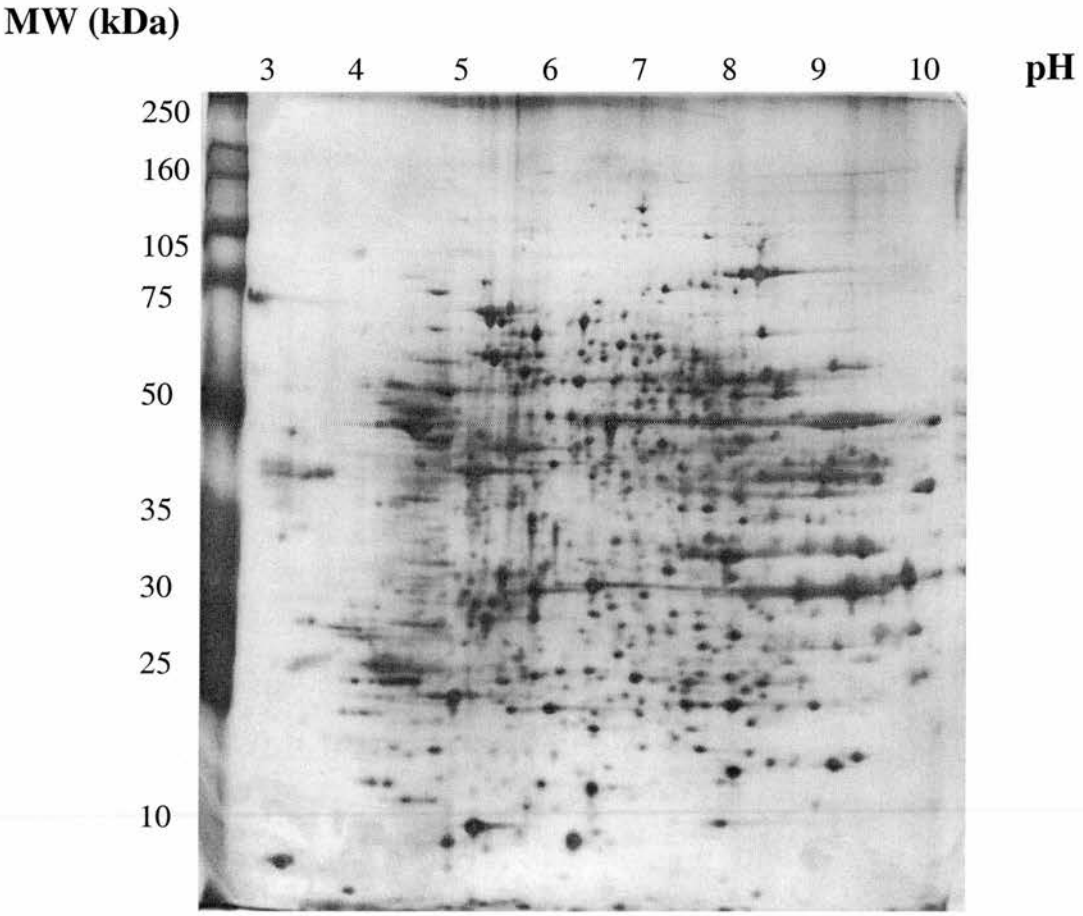


Figure 6.2 Example of a silver stained 2D gel of MVN tissue

Tissue was solubilised in a one step separation process: 6 MVNs from 3 normal rats were homogenised in 200µl of lysis buffer (50mM Tris-HCl pH 7.2, 2mM EDTA, 1% v/v Triton X-100, 100µg/ml trypsin inhibitor, 60µg/ml aprotinin) and centrifuged at 13,000g for 15 min. Supernatants were subject to 2D gel electrophoresis, using pH 3-10 strips in the first dimension, performed as documented in methods section (Section 6.3). Separated proteins were detected using sensitive silver staining (Amersham Biosciences, Buckinghamshire, U.K.) according to manufacturers instructions.

6.2 AIMS AND EXPERIMENTAL RATIONALE

Determination of the proteome of the MVN would provide a major opportunity to identify differences in protein expression between tissues from normal and previously labyrinthectomised animals. This could elucidate mechanisms involved in the compensation process and has the potential to identify new therapeutic targets for the treatment of vestibular disorders. The following experiments were designed to systematically analyse the proteome of the MVN and assess changes in protein expression in the MVN during vestibular compensation (VC).

1. To assess post-lesional changes in protein expression in the MVN during the process of VC, differential two-dimensional gel analysis techniques were applied to ipsilesional and contralesional MVN tissue collected from animals that had been allowed to compensate for one week after UL (n=18).

2. To determine the identity of proteins whose expression may change during the process of VC, these were subjected to mass spectrometry and searches of protein sequence databases.

3. To construct a reference database that catalogues proteins expressed in the MVN, mass spectroscopy and protein database searches were applied to MVN proteins which did not show significant changes after UL in the initial analysis.

6.3 METHODS

Except for the following, the methods employed in this chapter of the thesis are identical to those described in the methods section of Chapter 3. Three groups of six male Sprague Dawley rats, weighing 100-120g, were subjected to unilateral labyrinthectomy under avertin anaesthesia as described in detail earlier. The animals were allowed to recover in their home cages for one week post-UL, when they were anaesthetised with halothane and decapitated.

Tissue preparation

The brain was removed into ice-cold artificial cerebrospinal fluid (aCSF) and the brainstem isolated. Horizontal slices of the dorsal brainstem (650μM thick, taken approximately parallel to the floor of the IVth ventricle) were cut in ice-cold aCSF, and the left and right MVN were dissected. Care was taken to unambiguously identify the ipsilesional (left) and contralesional (right) sides, and to trim the MVN so as to take consistently similar samples of tissue from each animal. The MVNs were frozen on dry ice and stored at –72°C until required.

Homogenisation and protein solubilisation

For each of the three tissue batches, six ipsilesional and six contralesional MVNs were separately lysed in Dounce glass tissue homogenisers, with 300μl ice-cold sucrose buffer (see solutions). The lysate was centrifuged at 356,160*g* for 10min at 2°C, and the supernatant (cytoplasmic fraction) retained. The pellet was then homogenised in 300μl ice-cold detergent solution, which contained n-octyl glucoside, a strong non-ionic detergent which solubilises hydrophobic membrane proteins and minimises protein aggregation through hydrophobic interactions (see solutions). The lysate was centrifuged as before, and the second supernatant (membrane fraction) retained. Soluble protein content of the supernatants was determined using the Pierce BCA protein assay kit (Perbio Science, Cheshire, U.K.). Protein extracts were frozen on dry ice and stored at –72°C until required.

2-dimensional gel electrophoresis, visualisation and digitisation

For each experiment, three 2-dimensional gels were run for each batch of cytoplasmic and membrane fractions of the ipsilesional and contralesional MVN to assess and control for intra-assay variability. Isoelectric focussing (IEF) was performed using an Amersham Biosciences system (Amersham Biosciences, Buckinghamshire, U.K.), according to the manufacturer's instructions: protein extract was mixed with IPG strip rehydration buffer (see solutions) to obtain a concentration of 100µg protein per 250µl. In each case, 250µl of protein/buffer mixture was transferred to a 13cm IPG strip holder, with a pH 3-10 IPG immobiline drystrip to offer a wide overview of protein expression. Strips were rehydrated at 20°C for 14 hours on an Amersham IPGphor flat-bed unit, followed by IEF at 20°C starting at 500V for 1 hour, 1000V for 1 hour then increasing voltage to 8000V for a total of 17500 volt-hours. Following IEF, strips were incubated for 15min in dithiothreitol (DTT) equilibration solution, then for 15min in iodoacetamide equilibration solution (see solutions).

Second dimension SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out at 19.5°C using self-poured 16.5×19cm linear 10% acrylamide gels (see solutions), run at 20 mA per gel. Electrophoresis was terminated when the dye front reached approximately 1mm from the gel base. Molecular masses were estimated by running standard protein markers (Protein MW standards, Amersham Biosciences Buckinghamshire, U.K.), covering the range 6.5–205 kDa. Gels were incubated with agitation in fixing solution (10% v/v ethanol, 7% v/v acetic acid) for 30min, then incubated with agitation in Bio-Rad SYPRO Ruby protein gel stain (Bio-Rad Life Sciences, Hertfordshire, UK) for at least 3h. Following staining, gels were transferred to fixing solution for 1h, then washed in dH_2O. The gels were placed on a black, non-fluorescent glass plate, kept moist with dH_2O and imaged at 302nm using an Alpha Innotech Fluorchem 8900 Imaging System (Alpha Innotech Ltd., Cannock, UK). The acquisition time varied from 6-20sec and was chosen to have the overall strongest signal, while the intensity of the most abundant spot remained within the linear range of the camera and the background level was minimal. The images were

captured using the Fluorchem 8000 software package (Alpha Innotech Ltd., Cannock, UK).

Image analysis

Image analysis was performed using the Imagemaster 2-D Elite 3.10 image analysis software (Amersham Biosciences, Buckinghamshire, UK). In each experiment, 3 ipsilesional and 3 contralesional gels were produced, for both the cytoplasmic and membrane fractions of the MVN tissue. Within a given experiment, 10-15 large protein spots, expressed in all gels were tagged, and the images were morphed so the corresponding proteins overlaid each other in all gels. This corrected for differences in protein migration between gels. Gel images were systematically compared, in $10mm^2$ sections displayed in a montage window, and changes in the presence or density of expressed proteins that were consistent between the 3 ipsilesional and 3 contralesional gels were recorded. This experiment was repeated twice more, to determine any consistent changes between the 9 ipsilesional and 9 contralesional gels in the either cytoplasmic or membrane fraction. Systematically reproducible changes were sought for further analysis and quantification.

Sample preparation for mass spectrometry

Spots of interest were excised from the gel, and placed in Eppendorf centrifuge tubes. Gel pieces were incubated in 300µl of 200mM NH_4HCO_3 (ABC)/50% acetonitrile (ACN) at 30°C for 30min. This was repeated twice to remove SDS. Gel pieces were then incubated in 300µl 20mM DTT, 200mM ABC/50% ACN at 30°C for 1h to reduce the protein, then washed three times in 300µl 200mM ABC/50% ACN. Cysteines were alkylated in fresh 100µl 50mM iodoacetamide (IAA) and 200mM ABC/50% ACN at room temperature in the dark for 20min. Gel pieces were then washed three times in 500µl 20mM ABC/50% ACN. Gel pieces were spun at 13,000g for 2min then covered with ACN until they turned white (indicates dehydration). ACN was decanted and gel pieces allowed to dry. For tryptic digestion, gel pieces were swollen in 10µl of 12.5µg/ml trypsin in 50mM ABC at 4°C. Gel

pieces were placed in 96 well microplates, the tops sealed with Nescofilm and incubated at 32°C for 16-24h.

Protein identification by mass spectrometry

One microlitre of supernatant from the microplate wells was mixed with 1µl of 10mg/ml α-cyano-4-hydroxycinnamic acid in 50% ACN/0.1% trifluoroacetic acid and the mixed solution was spotted onto a 96-spot MALDI (Matrix assisted laser desorption/ionisation) plate. Samples were analysed on a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosciences, California, USA). The resulting peptide mass fingerprints were used to search the SWISSPROT protein database (http://us.expasy.org/sprot/), with MASCOT (http://www.matrixscience.com/): a search tool which compares the experimentally determined tryptic peptide masses with theoretical peptide masses calculated for proteins contained in the SWISSPROT database. A minimum level of 4 peptide matches and approximately 30% sequence coverage was set for unambiguous protein identification (see Figures 6.1, 6.8).

Solutions

1. Sucrose buffer

Sucrose 0.25M

HEPES 10mM, pH 7

EDTA 1mM

Sigma P8340 protease inhibitor cocktail 1% v/v

2. Detergent buffer

n-octyl glucoside 2% v/v

Aminohexanoic acid 0.5M

3. IPG strip rehydration solution

Urea 7M

Thiourea 2M

Triton X-100 0.5% v/v

Bromophenol blue 0.1%w/v

DTT 65mM

IPG buffer 0.5% v/v

4. DTT equilibration solution

Tris-HCl 50mM

Urea 6M

Glycerol 30% v/v

SDS 2% w/v

DDT 1% w/v

Bromophenol blue 0.1% w/v

5. Iodoacetamide equilibration solution

Tris-HCl 50mM

Urea 6M

Glycerol 30% v/v

SDS 2% w/v

Iodoacetamide 2.5% w/v

Bromophenol blue 0.1% w/v

6. Resolving gel solution (10% acrylamide)

Tris-HCl 375 mM

40% Acrylamide/Bis solution 25% v/v

SDS 0.1% w/v

Ammonium persulphate 0.05% w/v

TEMED 0.03% v/v

6.4 RESULTS

Two-dimensional gel electrophoresis (2-DGE) maps were constructed for rat MVN proteins. Differential 2-DGE was used to analyse changes in protein expression between the ipsilesional and contralesional MVNs of rats that had compensated for 1 week post-UL. A total of 36 gels were analysed in this experiment. Ipsilesional and contralesional MVN tissue (separately pooled from the same 6 rats) was subject to a two-step extraction procedure separating homogenates into a cytoplasmic (isotonically-extracted) and a membrane (detergent treated) fraction. The first dimension of the 2-DGE, involved isoelectric focusing, separating proteins according to their charge using pH 3-10 range IPG strips to obtain a wide overview of the proteins present in the MVN. In the second dimension the proteins were separated according to their molecular weight by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were visualised by SYPRO Ruby staining which can detect from around 10ng of protein in a spot. Approximately 800 protein spots were resolved in such a gel by computer-aided image analysis.

Representative 2-D gel images of the cytoplasmic and membrane associated fractions from the rat MVN tissue are illustrated in figures 6.4 and 6.5 respectively. Each batch of MVN tissue was separated 3 times by 2-DGE, either the cytoplasmic or membrane fraction, prepared from either the ipsilesional or contralesional MVN. Upon observation of a significant difference between ipsilesional and contralesional gels, these images can be morphed and the density of the spots between gels can be averaged for purposes of quantification. Figure 6.3 illustrates non-morphed gels produced from three separate batches of the cytoplasmic fraction of ipsilesional MVNs, each pooled from 6 rats.

Within the first two repeats of the experiment, consistent changes in the expression of four ipsilesional MVN proteins after VC were observed (Fig. 6.4, 6.5, A-D, runs 1-2). However, the third repeat of the experiments did not reveal the same consistent change in protein expression (Fig. 6.4, 6.5, A-D, run 3). More repeats of the experiment are needed to determine the validity of the initial observations, but time constraints mean these cannot be completed within the scope of this thesis.

Spots A, B and D (Fig. 6.4 and 6.5) were identified using MALDI-TOF analysis of their peptide mass fingerprints after trypsin digestion. Spot A was identified as glutamate dehydrogenase (accession number: p10860, MW: 61.4 kDa, pI 8.1. Spot number 3 in Figure 6.6 and Table 6.1). The protein indicated by the arrow in Figure 6.4B (spot B) as well as the larger spot to its immediate right were identified as fructose biphosphate aldolase C (accession number: p09117, MW 39.3 kDa, pI: 6.7. Spot number 2 in Figure 6.6 and Table 6.1). Spot D was identified as phosphoglycerate kinase (accession number: p16617; MW 44.5 kDa; pI 7.5. Spot number 27 in Figure 6.7 and Table 6.2). No changes in protein expression between the ipsilesional and contralesional MVN at 1 week post-UL in rat were revealed during analysis of the region of the MVN proteome corresponding to region encompassing the 3 protein changes detected in guinea pig (pI: 5.8-6.10, MW: 58-63 kDa. Ris *et al.*, 1999)

A further 66 MVN proteins which in the initial analysis do not show significant changes after UL were also identified on the basis of their peptide mass fingerprints. Identified proteins spots from the cytoplasmic MVN fraction are numbered in Figure 6.6 and the corresponding protein identification data are summarised in Table 6.1. Identified proteins spots from the membrane MVN fraction are numbered in Figure 6.7 and the corresponding protein identification data are summarised in Table 6.2. The confidence of the identification is determined by the number of matching peptides and the coverage of the proteins sequence by the matching peptides: a minimum of 4 peptide matches and approximately 30% sequence coverage is necessary for unambiguous protein identification. Where more than one significant protein hit per digested gel spot was returned from the database search, both proteins were recorded providing their pI and MW matched that of the region of the gel from which the protein spot was excised (see spot numbers 6 and 18: Fig. 6.7, table 6.2). Functions of the identified MVN proteins are outlined in table 6.3. The data for protein spot number 4 (Pyruvate kinase, M1 isozyme), from the cytoplasmic MVN fraction, is selected as an example: Figure 6.8 contains the MALDI-TOF peptide mass fingerprint spectrum of the trypsin digested protein, the predicted tryptic peptides identified from a SWISSPROT database search and an illustration of the sequence coverage of the peptides over the identified protein.

Figure 6.3 Examples of two-dimensional gels produced from 3 separate batches of MVN tissue.

2-D gel images produced from the cytoplasmic fraction of ipsilesional MVN tissue, collected from rats at 1 week post-UL. Each gel image is produced from a separate homogenate of MVN tissue pooled from 6 rats, and run in different experiments. Protein (100μg), was separated by 2-D gel electrophoresis using pH 3-10 strips in the first dimension, and the proteins were visualised using SYPRO Ruby staining.

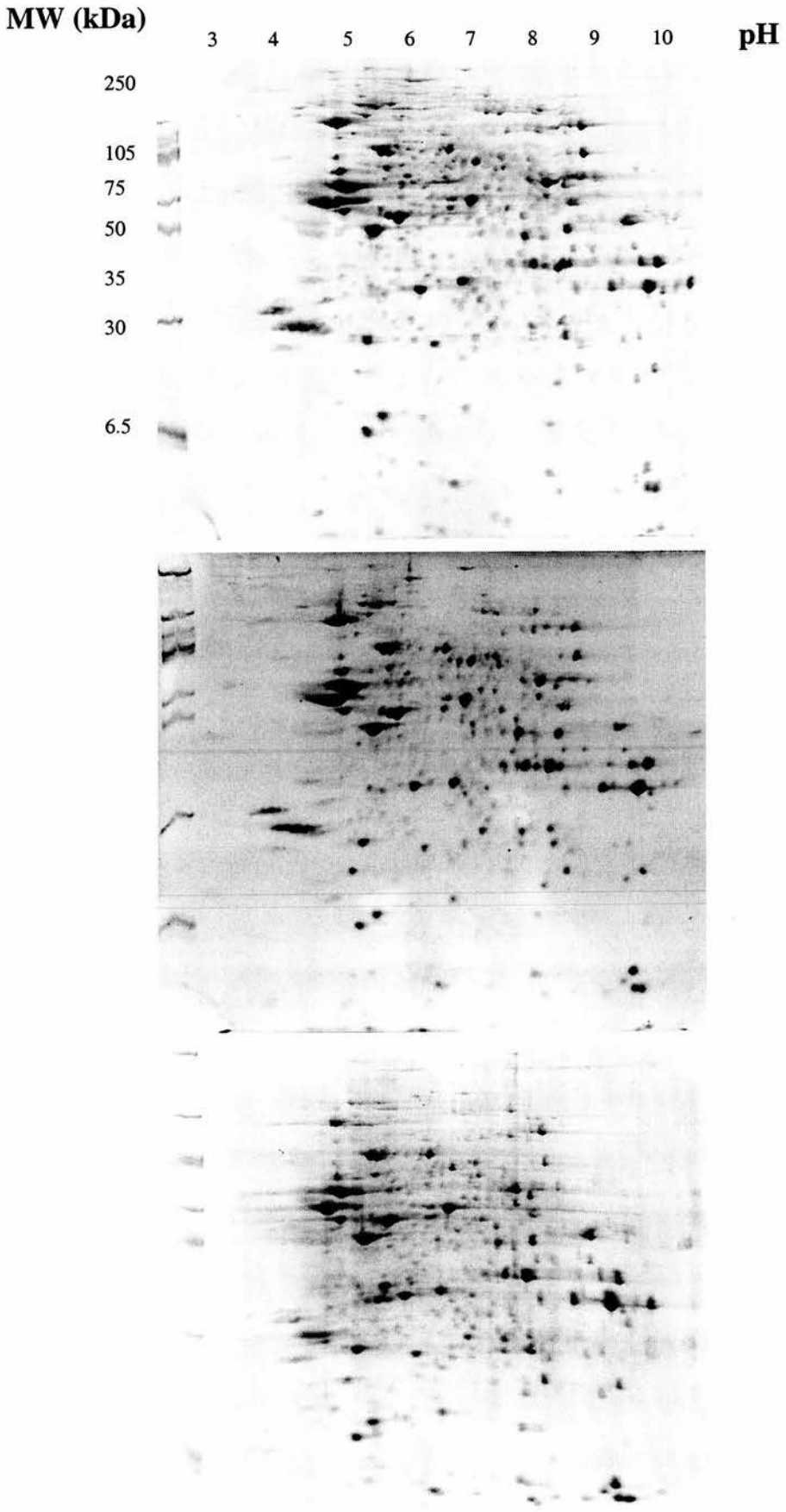
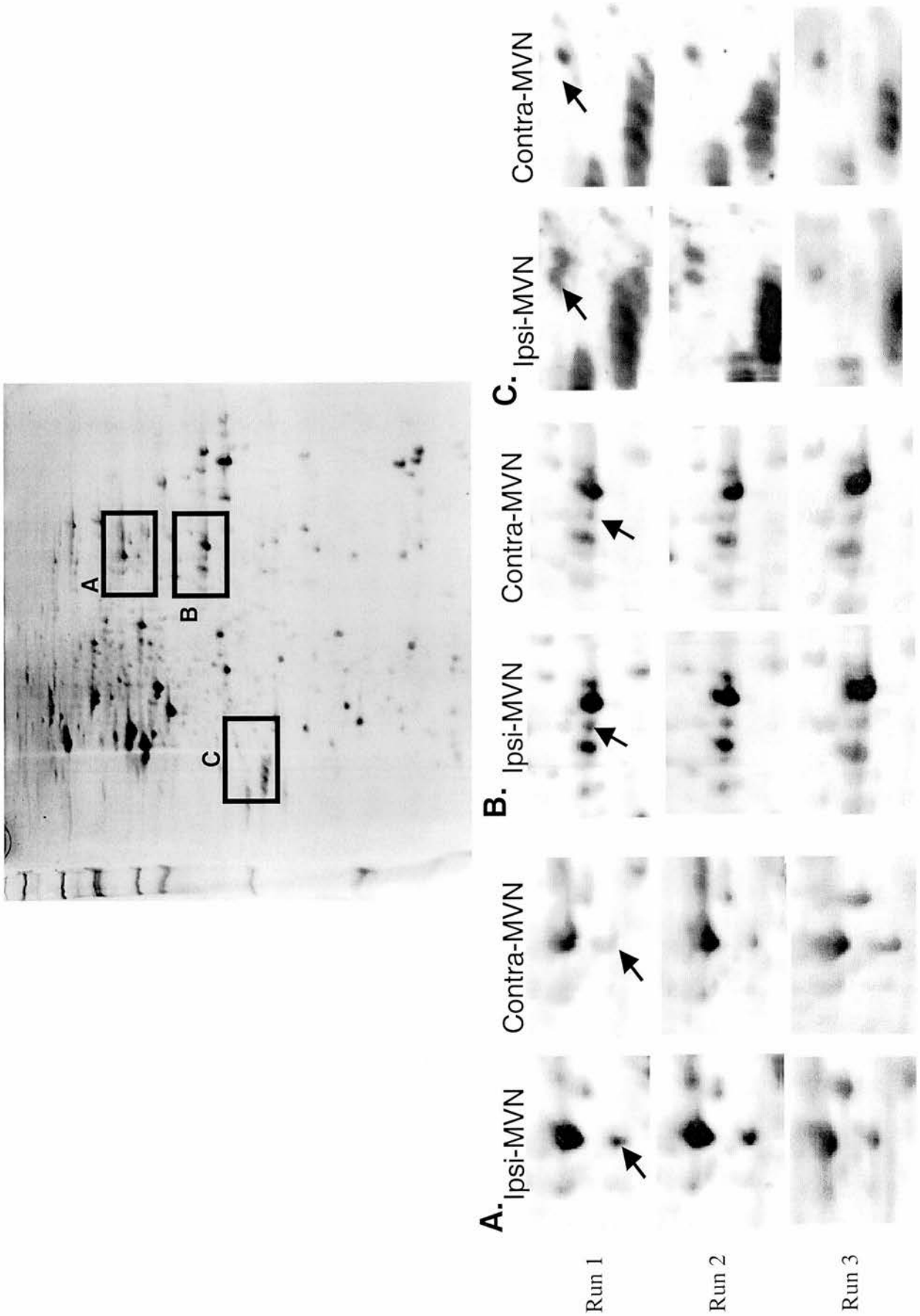

MW (kDa)
3
4
5
6
7
8
9
10
pH
250
105
75
50
35
30
6.5

Figure 6.4 Representative 2-D gel of the cytoplasmic associated rat MVN proteome

The cytoplasmic protein fraction from rat MVN tissue (pooled from 6 animals) was subject to 2-D gel electrophoresis and proteins visualised with SYPRO ruby, as documented in the methods section. Rectangles A-C indicate regions containing proteins whose expression appeared to significantly change in the first two repeats of the experiment (Runs 1-2). The left column shows ipsilesional MVN protein, the right shows contralesional MVN protein from the same animals. The gels were morphed using 2-D Imagemaster software and changes in protein expression were detected using the 'montage' display facility. Spot A (indicated by arrow in A, left panel) was identified as glutamate dehydrogenase. The protein indicated by the arrow in B, as well as the larger spot to its immediate right are identified as fructose biphosphate aldolase C. Spot C has yet to be identified.


A
B
C
A.
Ipsi-MVN
Contra-MVN
B.
Ipsi-MVN
Contra-MVN
C.
Ipsi-MVN
Contra-MVN
Run 1
Run 2
Run 3

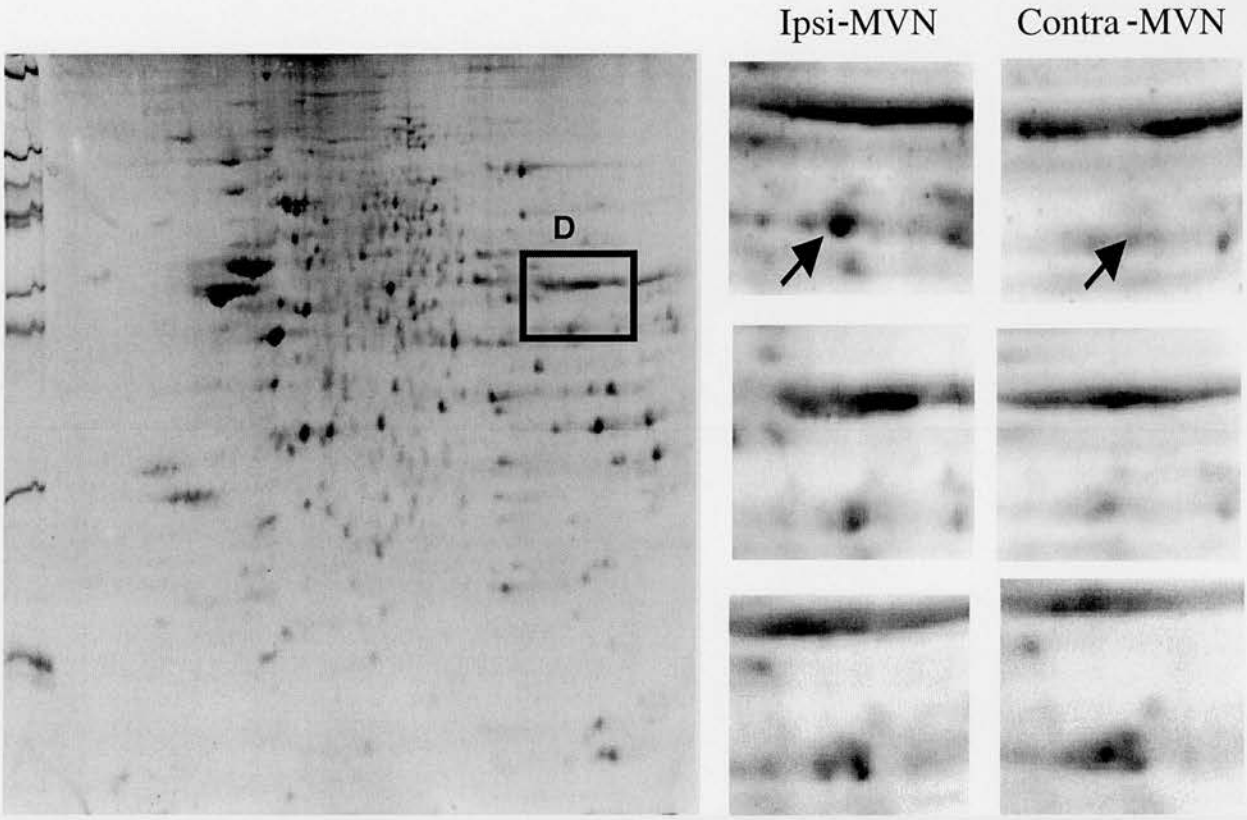


Figure 6.5 Representative 2-D gel of the membrane associated rat MVN proteome

The membrane protein fraction from rat MVN tissue (pooled from 6 animals) was subject to 2-D gel electrophoresis and proteins visualised with SYPRO Ruby, as documented in the methods section. The left column shows ipsilesional MVN protein, the right shows contralesional MVN protein from the same animals. The gels were morphed using 2-D Imagemaster software and changes in protein expression were detected using the 'montage' display facility.

Rectangle D indicates a region containing a protein, identified as phosphoglycerate kinase, whose expression appeared to significantly change in the first two repeats of the experiment (top two panels).

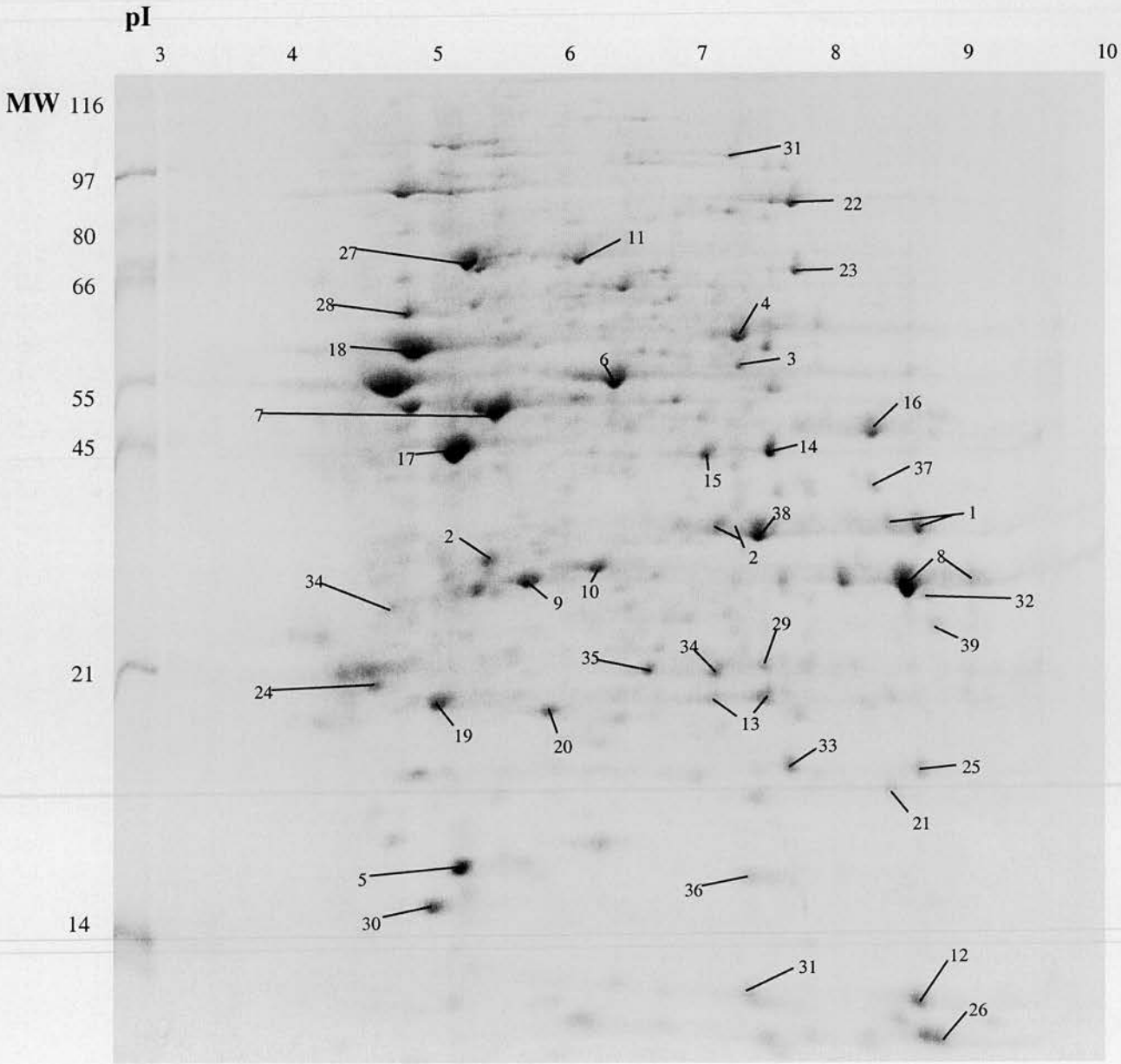


Figure 6.6 2-D gel image of the cytoplasmic fraction of the rat MVN proteome

Protein (100μg) was separated by 2-D gel electrophoresis and proteins were visualised by SYPRO Ruby stain. The protein spots identified in this study by MALDI-TOF MS are numbered; numbers refer to Table 6.1.

Spot number	Protein name	Species	SWISPROT accession code	MW	pI
1	Fructose bisphosphate aldolase A	rat	p05065	39.3	8.3
2	Fructose bisphosphate aldolase C	rat	p09117	39.3	6.7
3	Glutamate dehydrogenase (MP)	rat	p10860	61.4	8.1
4	Pyruvate kinase, M1 isozyme	rat	p11980	57.8	6.6
5	Phosphatidylethanolamine binding protein	rat	p31044	20.8	5.5
6	Alpha enolase	rat	p04764	47.2	5.8
7	Creatine kinase B-chain	rat	p07335	42.7	5.3
8	Glyceraldehyde 3-phosphate dehydrogenase	rat	p04797	35.8	8.4
9	L-lactate dehydrogenase B chain	rat	p42123	36.6	5.7
10	Malate dehydrogenase	mouse	p14152	36.4	6.2
11	Serum albumin precurser	rat	p02770	68.7	6.1
12	Cofilin (non-muscle isoforms)	rat	p45592	18.5	8.2
13	Triosephosphate isomerase (TIM)	rat	p48500	26.9	6.4
14	Aspartate aminotransferase	rat	p13221	46.3	6.3
15	Glutamine synthetase	rat	p09606	42.3	6.6
16	Phosphoglycerate kinase	rat	p16617	45.6	7.5
17	Actin	all		41.6	5.4
18	Tubulin α-6	human	q9bqe3	49.9	5
19	Ubiquitin carboxyl-terminal hydrolase isozyme L1	rat	q00981	24.7	5.1
20	Peroxiredoxin 6 (antioxidant)	rat	o35244	24.8	5.6
21	Peroxiredoxin 1	rat	q63716	22.1	8.3
22	Aconitate hydratase (MP)	bovine	p20004	85.4	8.1
23	Transketolase (TK)	rat	p50137	67.6	7.2
24	14-3-3 protein gamma	human	p35214	28.3	4.8
25	Carbonic anhydrase	rat	p27139	29.1	6.9
26	D3 Dopamine receptor	rat	p19020	49.5	9.4
27	Heat shock cognate 71kDa protein	human	p11142	70.9	5.4
28	Rab GDP dissociation inhibitor alpha	rat	p50398	50.5	5
29	Carbonic anhydrase II	rat	p27139	29.1	6.9
30	Nucleoside diphosphate kinase B	rat	p19804	17.3	6.9
31	Elongation factor 2 (EF2)	rat	p05197	95.3	6.4
32	L-lactate dehydrogenase A chain	rat	p04642	36.5	8.4
33	ATP synthase B chain (MP)	rat	p19511	28.9	9.4
34	Annexin V	rat	p14668	35.7	4.9
35	Enoyl-CoA hydratase (MP)	rat	p14604	31.5	8.4
36	Synaptosomal-associated protein 29 (SNAP 29)	rat	q9z2p6	29.1	5.3
37	Aconitate hydratase (MP)	human	q99798	85.4	7.4
38	Superoxide dismutse	rat	p07632	15.9	5.9
39	Na/K transporting ATPase alpha-2 chain precursor	rat	p06686	112	5.4

Table 6.1 Identified proteins from the cytoplasmic fraction of rat MVN proteome

Proteins from the rat MVN cytoplasmic proteome, identified by tryptic mapping and a SWISSPROT database search. MP; mitochondrial precursor. Ref. Figure 6.6.

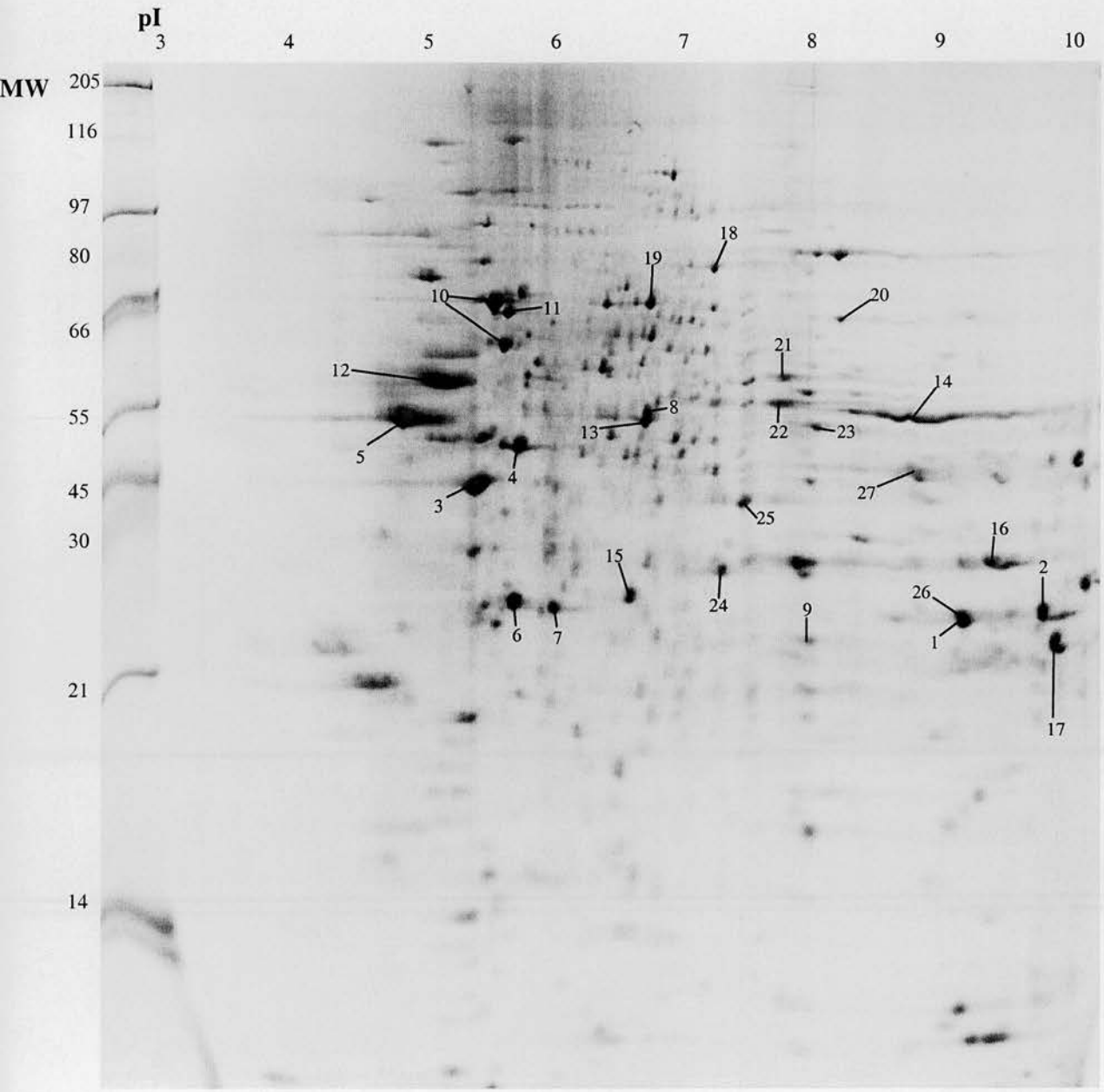


Figure 6.7 2-D gel image of the membrane fraction of the rat MVN proteome

Protein (100μg) was separated by 2-D gel electrophoresis and proteins were visualised by SYPRO Ruby stain. The protein spots identified in this study by MALDI-TOF MS are numbered; numbers refer to Table 6.2.

Spot number	Protein name	Species	SWISPROT accession code	MW	pI
1	Glyceraldehyde 3-phosphate dehydrogenase	rat	p04797	35.8	8.4
2	Malate dehydrogenase (MP)	rat	p04636	35.7	8.9
3	Actin	all		41.6	5.4
4	Creatine kinase, b-chain	rat	p07335	42.7	5.3
5	ATP synthase beta-chain (MP)	rat	p10719	56.3	5.2
6	L-lactate dehydrogenase	rat	p42123	36.6	5.7
	Guanine nuceotide-binding protein	bovine	p11017	35.6	6.1
7	L-lactate dehydrogenase	rat	p42123	36.6	5.7
8	Mitogen-activated protein kinase 8	rat	q63562	52.8	5.7
9	Beta-adrenergic receptor kinase 1	rat	p26817	79.9	6.6
10	Heat shock related protein	rat	p14659	69.5	5.4
11	Vacuolar ATP synthase cat sub A	p,b,h,m	q29048, p	68.3	5.4
12	Tubulin alpha 1chain	all	p05216, m	50	5
13	Alpha-enolase	rat	p04764	47.2	5.8
14	ATP synthase beta-chain (MP)	rat	p15999	58.8	9.2
15	Heat shock related protein	rat	p14659	69.5	5.4
16	Fructose bisphosphate aldolase A	rat	p05065	39.4	8.3
17	Voltage-dependent anion-selective channel protein 1	rat	q9z2l0	32.6	8.3
18	Vesicle-fusing ATPase	h,m	p46459, h	82.7	6.4
	Dynamin 2	rat	p39052	98	7
19	Succinate dehydrogenase (MP)	h,b,p	p31040, h	72.7	7.1
20	Transketolase	rat	p50137	67.6	7.2
21	Pyruvate kinase M1/M2 isozyme	rat	p11980/11981	57.8	6.6/7.2
22	Glutamate dehydrogenase	rat	p10860	61.4	8.1
23	4-aminobutyrate aminotransferase (MP)	rat	p50554	56.5	9
24	Solute carrier family 12 member	rat	q63632	120	6.2
25	Glutamine synthetase	rat	p09606	42.3	6.6
26	Protein kinase C	rat	p09217	67.7	5.5
27	Phosphoglycerate kinase	rat	p16617	45.6	7.5

Table 6.2 Identified proteins from the membrane fraction of rat MVN proteome

Proteins from the rat MVN membrane proteome, identified by tryptic mapping and a SWISSPROT database search. Gel spots can contain overlapping proteins, which when picked, digested into peptide fragments and used to search the protein databases can return multiple protein hits. When this is found to occur, all proteins whose theoretical pI and MW match the pI and MW range of the gel region from which it was picked are recorded (c.f. numbers 6 and 18). MP: mitochondrial precursor; h: human; m: mouse; b: bovine; p: pig. Ref. Figure 6.7.

Table 6.3 Functions of identified MVN proteins

Intermediary metabolism enzymes
Fructose bisphosphate aldolase A
Fructose bisphosphate aldolase C
Glutamate dehydrogenase (MP)
Pyruvate kinase, M1/M2 isozyme
Phosphatidylethanolamine binding protein
Alpha enolase
Creatine kinase B-chain
Glyceraldehyde 3-phosphate dehydrogenase
L-lactate dehydrogenase A chain
L-lactate dehydrogenase B chain
Malate dehydrogenase
Triosephosphate isomerase (TIM)
Aspartate aminotransferase
Glutamine synthetase
Phosphoglycerate kinase
Acetonitrate hydratase
ATP synthase B chain (MP)
Transketolase (TK)
Carbonic anhydrase II
Rab GDP dissociation inhibitor alpha
Vacuolar ATP synthase cat sub A
Succinate dehydrogenase (MP)
Transketolase
4-aminobutyrate aminotransferase (MP)
Cytoskeletal proteins
Cofilin (non-muscle isoform)
Actin
Tubulin α-6
Neuron-specific proteins
Synaptosomal-associated protein 29
Voltage-dependent anion-selective channel protein 1
Dynamin 2
Signalling proteins
14-3-3 protein gamma
Nucleoside diphosphate kinase
Guanine nuceotide-binding protein
Beta-adrenergic receptor kinase 1
Protein kinase C

Antioxidant proteins
Peroxiredoxin 6
Peroxiredoxin 1

Calcium binding proteins
Annexin V

Cell defence
Superoxide dismutse [Cu-Zn]

Transcription, splicing and elongation proteins
Elongation factor 2

Heat shock proteins and chaperones
Heat shock cognate 71kDa protein
Heat shock related protein

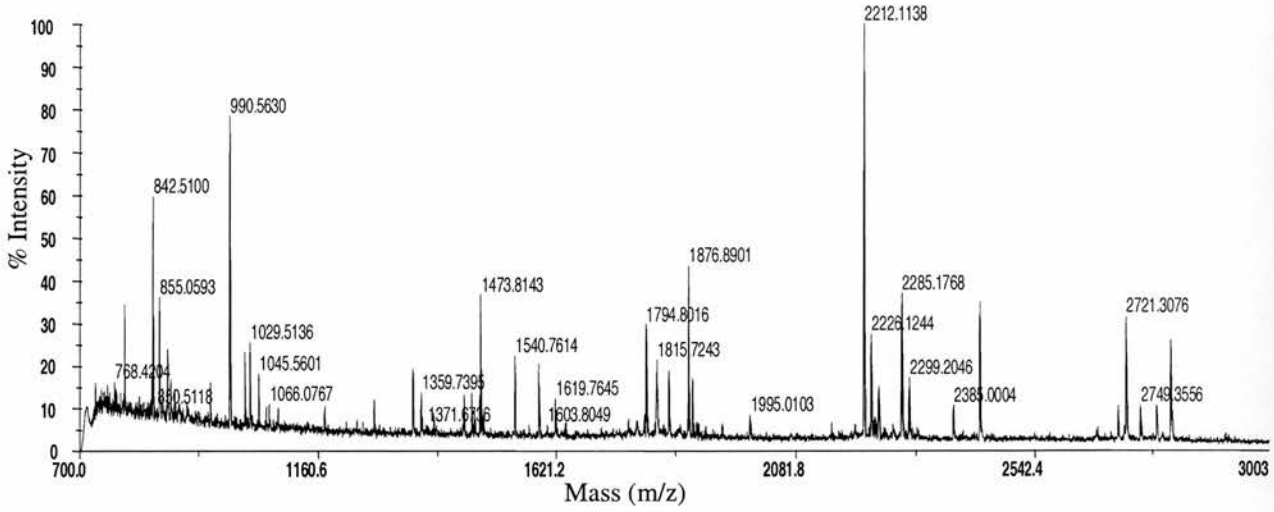
Ubiquitination and proteasome related proteins
Ubiquitin carboxyl-terminal hydrolase isozyme L1

Neuronal signal transmission
D3 Dopamine receptor

Other
Serum albumin precursor
Enoyl-CoA hydratase
Na/K transporting ATPase alpha-2 chain precursor
Mitogen-activated protein kinase 8
Vesicle-fusing ATPase
Solute carrier family 12 member

Table 6.3 continued. Functions of identified MVN proteins

A.



B.

Name: Pyruvate kinase, M1 isozyme (Pyruvate kinase muscle isozyme)

Accession number: P11980; **Species:** rat

MW: 57818 Da; **pI:** 6.6

m/z submitted	MH^+ matched	Database sequence
754.4967	754.4688	(K) KPRPTR (A)
787.4360	787.4215	(R) QAHLYR (G)
840.5240	840.5307	(R) APIIAVTR (N)
884.4904	884.4776	(R) MQHLIAR (E)
953.5058	953.4805	(K) IENHEGVR (R)
990.5634	990.5624	(R) LLFEELAR (A)
1019.5242	1019.5162	(K) GDYPLEAVR (M)
1029.5179	1029.5118	(R) EAEAAVFHR (L)
1235.5720	1235.6240	(K) EMIKSGMNVAR (L)
1359.7438	1359.7055	(R) NTGIICTIGPASR (S)
1462.8084	1462.8157	(K) IYVDDGLISLQVK (E)
1473.8158	1473.8099	(K) CLAAALIVLTESGR (S)
1587.7701	1587.7655	(K) DAVLDAWAEDVDLR (V)
1837.9088	1837.9118	(R) RFDEILEASDGIMVAR (G)
1875.8974	1875.8951	(K) FGVEQDVDMVFASFIR (K)
1883.9027	1883.9040	(R) LNFSHGTHEYHAETIK (N)
2435.2986	2435.2822	(R) AATESFASDPILYRPVAVALDTK (G)

C.

PKPDSEAGTA FIQTQQLHAA MADTFLEHMC RLDIDSAPIT ARNTGIICTI GPASRSVEML KEMIKSGMNV
ARLNFSHGTH EYHAETIKNV RAATESFASD PILYRPVAVA LDTKGPEIRT GLIKGSGTAE VELKKGATLK
ITLDNAYMEK CDENILWLDY KNICKVVEVG SKIYVDDGLI SLQVKEKGAD YLVTEVENGG SLGSKKGVNL
PGAAVDLPAV SEKDIQDLKF GVEQDVDMVF ASFIRKAADV HEVRKVLGEK GKNIKIISKI ENHEGVRRFD
EILEASDGIM VARGDLGIEI PAEKVFLAQK MMIGRCNRAG KPVICATQML ESMIKKPRPT RAEGSDVANA
VLDGADCIML SGETAKGDYP LEAVRMQHLI AREAEAAVFH RLLFEELARA SSQSTDPLEA MAMGSVEASY
KCLAAALIVL TESGRSAHQV ARYRPRAPII AVTRNPQTAR QAHLYRGIFP VLCKDAVLDA WAEDVDLRVN
LAMNVGKARG FFKKGDVVIV LTGWRPGSGF TNTMRVVPVP

6.5 DISCUSSION

The results in this thesis represent the first systematic analysis of the MVN at the proteomic level during vestibular compensation in rat. A previous study of changes in protein expression during VC in the guinea pig detected an up-regulation in the ipsilesional vestibular nuclei in the expression of 3 proteins at 1 week post-UL, which were almost absent in control animals (Ris *et al.*, 1999). The identities of these proteins are unknown because N-terminal Edman degradation, rather than mass spectrometry identification techniques were used. Edman degradation determines the amino acid sequence of the N-terminal of the protein, which is then used to search protein sequence databases. However, only two out of the three proteins which exhibited changes were able to be N-terminal sequenced, and the length of the amino acid sequences determined for these two proteins (9 and 11 amino acids) were not long enough to generate a significant hit in the databases searched (Ris *et al.*, 1999). In addition, Ris and colleagues were assessing changes in guinea pig proteins; the majority of mammalian protein and gene sequence data identified to date and available to search online corresponds to rat, mouse and human proteins. The low level of guinea pig protein sequence data available in these databases is a limiting factor for proteomic studies using guinea pig.

No changes in protein expression between the ipsilesional and contralesional MVN at 1 week post-UL in rat were revealed during analysis of the region of the rat MVN proteome corresponding to region encompassing the 3 protein changes detected in guinea pig (pI: 5.8-6.1, MW: 58-63 kDa. Ris *et al.*, 1999). This is unsurprising because it is probably different regions of the vestibular nuclei being analysed between studies, since it is unclear in the Ris paper from where in the vestibular nuclei the tissue samples were taken. In addition, the species analysed are different, and the current study uses a two-step separation technique as opposed to the single step used by Ris and colleagues.

The results from the present study indicate that there may be changes in the expression of a number of proteins between the ipsilesional and contralesional MVN at 1 week post-UL (Fig. 6.4 and 6.5, runs 1-2). However the observed changes in expression are only consistent between two out of the three repeats of this

experiment to date. There are a number of possible reasons for the observed discrepancies between the three experiments including variability in: sample (biological differences between rats), sample preparation, environmental conditions, batch of reagents and manual inputs. Because of these limitations in reproducibility when comparing small numbers of gels, the current experimental protocol utilised an 'all or nothing' approach, investigating qualitative rather than quantitative differences in protein expression. In protocols using SYPRO Ruby stains, quantitative changes, assessed by measuring the optical density of spots, are generally considered significant when the change is observed in three out of four gels and the change is over threefold (Choe and Lee, 2003). Thus, *at least* one more repeat of the current experiment is desirable to establish consistent and quantifiable changes in protein expression. These experiments are ongoing within the laboratory, however due to time limitations they cannot be included within the scope of this thesis.

What the present data demonstrate, is the potential power and utility of a proteomics approach to the investigation of vestibular compensation and indeed other forms of brain plasticity. Three of the proteins that showed changes in protein expression during the first two repeats of the experiment may be of particular interest to the process of VC. Mitochondrial glutamate dehydrogenase (Fig. 6.4 spot A; Fig 6.6 spot 3) and phosphoglycerate kinase (Fig. 6.5 spot D; Fig 6.7 spot 27) appear to be up-regulated in the ipsilesional MVN compared the contralesional MVN at 1 week post-UL. These proteins belong to a group defined as intermediary metabolism enzymes (see Table 6.3) and their presence can be used to determine the metabolic status of tissues (Lubec *et al.*, 2003); thus an up-regulation of these enzymes on the ipsilesional side would be indicative of significantly increased metabolic activity, specifically on the lesioned side of the brain. In the CNS, glutamate dehydrogenase appears to function in both the synthesis and the catabolism of glutamate (Mavrothalassitis *et al.*, 1988). An increase in glutamate dehydrogenase expression at 1 week post-UL could be related to a re-modeling or increased efficacy of the remaining glutamatergic inputs into the lesioned MVN; this in turn may be related to the increase in firing rate of rostral ipsilesional MVN neurons observed at this time *in vitro* (see Chapter 5).

Spot B as well as the larger spot to its immediate right (Fig. 6.4; Fig 6.6 spots numbered 2), have been identified as aldolase-C, also known as zebrin, a molecular marker that reveals a striped zonal organisation of the cerebellar cortex, and whose distribution appears to correspond to the spatial distribution of mossy fibre afferents within the cerebellar cortex (Hawkes and Herrup, 1995; Marzban *et al.*, 2003). Phosphorylation of a protein changes its charge; therefore the same protein in different phosphorylation states can often be found spotted horizontally across a 2-D gel. The change in aldolase-C expression between the first two repeats of gels suggests a change in the phosphorylation state of this protein during compensation. Altered phosphorylation may be due to changes in the MVN itself or may be due to changes in the Purkinje cell inputs from the flocculus, since aldolase-C is expressed in Purkinje cell axons and terminals. Alteration in the phosphorylation state of proteins after UL, in MVN projecting Purkinje neurones, is of interest as it may indicate a link between cerebellar cortical plasticity and VC. Indeed, a recent study showed the importance of protein kinase activity in the flocculus, for the increase in excitability of neurones that occurs in the MVN after UL (Johnston *et al.*, 2002; see Sections 2.1.4, 3.6 for discussion).

Figures 6.6-6.7 and tables 6.1-6.2, illustrate proteins present in the MVN which have been identified by mass spectroscopy. The functions of these identified proteins are outlined in table 6.3. The generation of this database of proteins expressed in the MVN will allow for increasingly rapid screening of protein changes during different stages of compensation, and can be extended to the investigation of protein changes in other forms of vestibular plasticity such as adaptive changes in the vestibulo-ocular reflex or recovery from bilateral labyrinthectomy. A number of recent studies have utilised proteomic techniques to examine mammalian brain proteomes (Fountoulakis *et al.*, 1999; Gauss *et al.*, 1999), and in 2002 an initial analysis of the mouse cerebellum proteome was published, with the view to constructing a web-based database to be used as a resource for comparative proteomics studies (Beranova-Giorgianni *et al.*, 2002). However, the majority of proteins identified in the mouse cerebellum and in the rat MVN in the present study, can be categorised as high abundance proteins, which generally do not include membrane receptor and signalling proteins, both of which have been implicated in

the process of VC. This is one of the limitations of a 2-D gel electrophoresis (2-DGE) approach to proteomics, which will be discussed below.

Limitations of 2-DGE techniques

1) Low abundance proteins

This is an important group of proteins because it encompasses receptors, signal transduction and regulatory proteins, modifications of which may carry significant information on the mechanisms of plasticity in the vestibular system. A major disadvantage of 2-DGE is its inability to visualise low copy number proteins in the presence of highly abundant proteins. The proteome of a cell can contain at least 10,000-30,000 different proteins (Denslow *et al.*, 2003), however depending on the 2-DGE technique used, only 1000-10,000 proteins can be visualised on a silver stained gel (Gauss *et al.*, 1999). Only some stained proteins are present at levels sufficient for mass spectrometric identification and it is likely that most of these are high abundance proteins. Increasing the amount of protein loaded onto the gel can increase detection sensitivity. However, with high protein loads, high abundance proteins can obscure low abundance proteins with similar pI values and MW. This problem can be combated with the use of multiple overlapping narrow IPG strips in the first dimension, and with the use of larger gels in the second, but this requires a significant increase in the quantity of tissue for analysis, which is not practical for the analysis of small quantities of brain tissue. Thus, abundant protein removal is the primary requirement of any technique used to concentrate low abundance proteins, and this is achieved by fractionation. Due to limited quantity of tissue, the current method uses a two step fractionation procedure, the sucrose buffer removes the most abundant soluble proteins, and the pellet which is re-solubilised is enriched with membrane and low abundance proteins. Where greater amounts of protein are available for analysis, multiple fractionation or affinity purification methods can be applied to enrich the proportion of the protein of interest (Krapfenbauer *et al.*, 2003). Kits to affinity purify specific groups of proteins such as phosphoproteins are now available commercially.

2) Hydrophobicity of proteins

Hydrophobic proteins are an important target in the investigation of mechanisms underlying VC as they are mainly represented by membrane proteins. The initial problem is the extraction and solubilisation of proteins from the membrane. The current method addresses this issue with a two-step separation process and the use of powerful detergents to solubilise integral membrane proteins. However, problems with the separation process exist once solubilised: hydrophobic proteins are generally alkaline (pI > 8) (Wilkins *et al.*, 1998), and are therefore not detected on standard 2-D gels which cover the pH 4-7 range. In the present experiment a wide ranging pH 3-10 IPG strip is used to visualise a broader range of proteins. A third problem with hydrophobic proteins is that they are poorly soluble in the aqueous solution used for isoelectric focusing. Even with the inclusion of Triton X-100 and thiourea to the IPG rehydration solution to solubilise the membrane proteins, these proteins are designed to be soluble in lipid bi-layers and not water, therefore migrate poorly into the IPG gel in the first dimension and the SDS gel in the second. Partial or limited extraction of these proteins may result, which creates problems for the quantitative analysis of differences between gels (for review, see Santoni *et al.*, 2000).

3) High and low molecular weight proteins

High and low molecular weight proteins can be poorly represented by proteomic techniques. These proteins can be excluded from 2-D gels due to difficulties migrating through the gel matrix, both in the first and second dimension. Further problems exist with the identification of low molecular weight peptides by MS, because the peptide mass fingerprint often contains too few peptide fragments for a significant hit to be generated from gene or protein databases. An alternative to 2-DGE combines capillary electrophoresis with tandem mass spectroscopy to detect low abundance and low molecular weight proteins (Javerfalk-Hoyes *et al.*, 1999).

These limitations aside, 2-DGE and mass spectroscopy are the most powerful techniques to date to study the global expression of proteins in a given tissue. They are also the only separation process with sufficient resolution, and reliable identification process, which can identify changes in post-translational modifications.

For example, phosphorylation changes protein charge and is often indicated by a horizontal streaking across a 2-D gel (see Fig. 6.6, spots numbed 2: fructose bisphosphate aldolase C). If there is sufficient protein the amino acid sequence of the modification can be determined by tandem MS. The ability to determine post-translational modifications in vestibular tissues is of importance in the study of mechanisms underlying VC since adaptations of phosphoproteins in particular are likely to be directly involved in post-lesional plasticity (see Chapter 3 for discussion).

This study represents the development of a novel technique within our laboratory to analyse the mechanisms involved in the process of VC. The protocol used in this study represents a global investigation of changes in protein expression, using basic tissue extracts and a large pH range to separate proteins. The results indicated that there may be changes in protein expression and/or phosphorylation in the MVN at 1 week post-UL, however no definite conclusions can be drawn since the third repeat of the experiment presented different results from the first two. The study indicates that changes in the MVN are likely to be too subtle to be detected with a global approach, and an approach targeted at analysing specific classes of proteins implicated in VC is planned. Here, the proteomic method complements other techniques used in this thesis: proteins such as membrane receptors implicated in VC by electrophysiological experiments can be selectively targeted for proteomic analysis. For example, a pull-down immunoprecipitation column could be created using subunits of the $GABA_B$ receptor to analyse changes in $GABA_B$ proteins, and proteins interacting with the $GABA_B$ receptor (the "interactome") during VC. Affinity columns are commercially available to purify phosphoproteins, which can then be separated by 2-DGE. Less specifically, the separation of tissue into distinct fractions (e.g. different molecular weight ranges using molecular weight filters and centrifugation) and using narrower pH ranges to target and expand the areas of gel which in experimental runs 1 and 2 suggest changes, will allow clearer visualisation of proteins of interest. New candidate mechanisms of plasticity indicated by changes in protein expression can be further investigated by behavioural, pharmacological or electrophysiological methods.

Summary

This study involved the development and implementation of a new experimental technique to study the mechanisms of VC in rat. A proteomics protocol was established which allowed for wide ranging visualisation of proteins present in the MVN. A database of proteins present in the MVN, identified by MS was constructed, which will allow for rapid screening of protein changes during different stages of compensation, and can be extended to the investigation of protein changes in other forms of vestibular plasticity such as adaptive changes in the vestibulo-ocular reflex or recovery from bilateral labyrinthectomy. The study demonstrates that the proteomic technique can be extended to investigate other forms of plasticity in the CNS.

Changes in protein expression and/or phosphorylation between the ipsilesional and contralesional MVNs in rats that underwent VC for 1 week post-UL were analysed. The results suggest that changes in protein expression and phosphorylation may be occurring in the MVN at this time; however the changes observed were not consistent throughout all runs of the experiment therefore more repeats are necessary before definite conclusions can be drawn. This preliminary study leads the way into experiments targeted at changes in specific classes of proteins, such as membrane proteins and phosphoproteins, during VC.

RESUME AND FUTURE EXPERIMENTS

Chapters 1 and 2 introduced the areas of general vestibular system anatomy and function, and reviewed the current understanding of the underlying causes and mechanisms of vestibular compensation.

In Chapter 3 it was demonstrated that the significant down-regulation of both $GABA_A$ and $GABA_B$ receptor efficacy in rostral ipsilesional MVN neurones was abolished if animals remained anaesthetised for the 4 hours following UL. In anaesthetised animals that had been treated with the glucocorticoid agonist, dexamethasone, the significant down-regulation of $GABA_A$ and $GABA_B$ receptor efficacy was restored. These experiments suggest that acute activation of the hypothalamo-pituitary-adrenal stress axis, the subsequent release of endogenous glucocorticoids and the activation of glucocorticoid receptors are necessary to induce the down-regulation of GABA receptor efficacy in rostral ipsilesional MVN neurones during the early stages of vestibular compensation.

The mechanisms by which glucocorticoids modulate GABA receptor efficacy following UL are unclear. Corticosteroids can act via both genomic and membrane receptors to affect GABA mediated transmission. Acting via genomic receptors, corticosteroids can modulate the transcription of specific $GABA_A$ receptor subunits (e.g. the $\gamma 2$ receptor subunit: Orchinik *et al.*, 1995; Stone *et al.*, 2001). Experiments to determine whether the down-regulation of $GABA_A$ receptor efficacy from 4h post-UL, is accompanied by changes in $GABA_A$ receptor subunit mRNAs that have been shown to be regulated by corticosteroids, may elucidate the mechanisms underlying these changes. Corticosteroids have been shown to bind to membrane receptors, and activate non-genomic signalling mechanisms such as the PKC pathways. Thus, glucocorticoids may be affecting GABA receptor efficacy via modulation of intracellular kinase pathways, resulting in changed phosphorylation states of the receptors. It is not know when the down-regulation of GABA receptor efficacy first occurs after UL; further experiments are now necessary to establish the exact time course over which this occurs, which will enable experiments focused at investigating specific second messenger pathways and molecular events involved in this process.

Chapter 4 demonstrated the presence of the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1, which regenerates active glucocorticoids from their inert 11-keto forms) in the MVN, flocculus/paraflocculus and nodulus/uvula. It is the first experiment to specifically investigate 11β-HSD1 activity in vestibular related brain tissue. The results suggest that this modulatory enzyme is likely to be involved in regulating the exposure of these centres to circulating glucocorticoids, by glucocorticoid metabolism at a cellular level. The results also show that the levels of 11β-HSD1 activity in the MVN and vestibulo-cerebellum remain stable over the 4h period after UL, disproving the hypothesis that changes in enzyme modulatory activity may occur in parallel with the deafferentation induced changes in the properties of the ipsilesional MVN neurones at this time.

In Chapter 5, the mechanisms underlying changes in neuronal activity of ipsilesional MVN neurones *in vitro*, during the process of VC, was investigated by perfusion of brainstem slices from rat with aCSF containing antagonists of the major neurotransmitters in the VNC. Perfusion of brainstem slices from normal animals with the cocktail of antagonists, had no effect on the number of neurones detected per slice, the regularity of firing of these neurones or the mean discharge rate of these neurones. These results suggest that in normal animals the firing of MVN neurones *in vitro* is maintained primarily by the intrinsic pacemaker properties of these neurones.

At 4h post-UL an increase in excitability of rostral ipsilesional MVN neurones is observed in normal aCSF, which is maintained in the presence of the neurotransmitter antagonists. This indicates that the increase in excitability of rostral ipsilesional MVN neurones at 4h post-UL is maintained primarily by modifications in the intrinsic pacemaker properties of these neurones. There are a variety of membrane channels and conductances which could be modified following UL to maintain this change in excitability: there could be a down-regulation in the number or efficacy of K^+ channels carrying outward current, or an up-regulation of Na^+ and Ca^{2+} channels carrying inward current. A systematic voltage clamp experiment,

investigating individual ionic currents before and after UL, is necessary to elucidate the mechanisms of this increase in excitability.

At 48h and 1 week post-UL, the increase in mean firing rate of rostral ipsilesional MVN neurones observed in normal aCSF, is lost upon addition of the neurotransmitter antagonists to the perfusing medium. This indicates that one or more of the neurotransmitter receptors blocked by the antagonist cocktail, is maintaining the increase in excitability. The specific neurotransmitter receptors mediating the increase in excitability should be investigated by repeating the experiment, firstly using either the excitatory or inhibitory receptor antagonists, and secondly by breaking the cocktail down into individual receptor components. The results provide evidence for the theory that the increase in excitability observed in ipsilesional MVN neurones from 48h post-UL is maintained by an increase in excitatory input onto these neurones, perhaps from remaining intact sensory systems. The increase in excitability of ipsilesional MVN neurones *in vitro* is likely to be functionally compensatory, as it will help overcome the loss of primary excitatory inputs from the deafferented vestibular nerve, and the excessive commissural inhibition from the contralesional MVN *in vivo*. Thus, different mechanisms are utilised in the initiation and maintenance of processes involved in VC.

The results also provide evidence for species differences in factors regulating neuronal excitability of MVN neurones following UL. In rat, recovery of the resting discharge of deafferented MVN neurones initially appears to be promoted by modifications of the pacemaker properties of the neurones. This is superseded by adaptations in the intact sensory and vestibular-related inputs onto these neurones. The opposite mode of compensation has been proposed for guinea pig: initial substituting sensory inputs being increasingly replaced by changes in the pacemaker properties of the deafferented neurones (Vibert *et al.*, 1999b).

Chapter 6 presents the first systematic investigation of the rat MVN proteome during VC. A novel proteomics protocol was developed and implemented which allowed for wide ranging visualisation of proteins present in the rat MVN. A database of proteins expressed in the MVN, visualised by 2-DGE and identified by MS, was constructed. Further MS experiments are now required to establish a

comprehensive and complete database of the rat MVN proteome. This will allow rapid screening of protein changes, identified in future differential 2-DGE experiments of MVN tissue. The proteomic techniques developed in this study, are currently being extended to the investigation of proteins present in the flocculus/paraflocculus complex and the uvula/nodulus, and protein changes in these areas during VC.

Results from the differential 2-DGE experiments of MVN tissue, indicate that there may be changes in the expression and/or phosphorylation of a number of proteins between the ipsilesional and contralesional MVN at 1 week post-UL. However, no definite conclusions can be drawn yet, because the third repeat of the experiment did not corroborate the first two; therefore more repeats of the experiment are now needed. Proteomic techniques provide a powerful complement to the established methods of investigating VC. For example, one of the proteins identified which displayed an up-regulation of activity in the ipsilesional MVN during the first two repeats of the proteomic experiment, was glutamate dehydrogenase. The results from Chapter 5 indicate that at 1 week post-UL, the increase in excitability observed in ipsilesional MVN neurones is maintained by an increased excitatory synaptic input, probably glutamatergic input, onto these neurones. An increase in glutamate dehydrogenase expression at 1 week post-UL could be related to a re-modeling or increased efficacy, of the remaining glutamatergic inputs into the lesioned MVN, leading to the increase in firing rate of rostral ipsilesional MVN neurons observed at this time in the electrophysiology experiments.

Continued systematic proteomic analysis of MVN tissue should uncover molecular mechanisms mediating the post-lesional plastic changes investigated in this thesis. In addition, analysis of the MVN and vestibulo-cerebellum should reveal new candidate mechanisms of plasticity, which can inform the design of future behavioural, pharmacological and electrophysiological experiments. Further proteomic approaches, targeted at specific classes of proteins implicated by electrophysiological and systems level studies of VC, should provide significant new information on the mechanisms of plasticity in the vestibular system.

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