Electrophysiological and neurochemical studies of the vestibular nuclei of the rat in relation to the cerebellum

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Electrophysiological and Neurochemical Studies of the Vestibular Nuclei of the Rat in Relation to the Cerebellum

Submitted by

Yizhe Sun

In partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences

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December 29, 1999

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1999
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INTRODUCTION

It is well known that the vestibular system plays important roles in maintaining posture, balance, and coordination of movement between the head and eyes (Wilson and Jones, 1979). Within this system, second order vestibular neurons of the vestibular nuclear complex (VNC) occupy a significant place because of their connections. Although many studies using biochemical, pharmacological, electrophysiological, morphological and behavioral methods have been done (De Waele et al., 1995), especially in the last decade, a lot of questions about the basic neurochemistry and neuropharmacology of the VNC have remained unresolved. Balance-related disorders are common and very distressful to patients in clinical otolaryngology. From a clinical point of view, unless we have a better understanding of the vestibular system, the development of more effective drugs and treatments is unlikely. The purpose of this dissertation research is to explore the role of the neurotransmitter acetylcholine (ACh) in the VNC of normal rats, and of γ-aminobutyric acid (GABA) in the VNC normally and during cerebellar lesion-induced plasticity. The results will increase our understanding of physiological, pharmacological, and pathological conditions in the vestibular system and lay a foundation for future work.

In vitro electrophysiological recording from brainstem slices has led to insights into physiology and pharmacology (Dutia et al., 1992; Gallagher et al., 1985; 1992; Johnston et al., 1994; Kinney et al., 1994; Lin and Carpenter, 1993; Phelan and Gallagher, 1992; Serafin et al., 1991a, b; Smith et al., 1990; Ujihara et al., 1988; 1989)
and plasticity (Darlington et al., 1989, 1993; Kunkel and Dieringer, 1994; Smith and Curthoys, 1988) in the VNC. The presence of spontaneous neuronal activity in vitro is a distinguishing characteristic of some brainstem sensory regions (Lin and Carpenter, 1993; Waller and Godfrey, 1994). Changes in rates and patterns of spontaneous firing have been shown to be sensitive indicators of pharmacological effects in cochlear nucleus slices (Chen et al., 1994, 1995). Although a similar approach can be applied to the brainstem vestibular nuclei, all brain slice studies so far have been of the medial vestibular nucleus (MVN), and little is known of the characteristics of the lateral (LVN), superior (SuVN) or spinal (SpVN) nuclei. The first part of this dissertation research aimed to systematically investigate these four major vestibular nuclei in slices to determine the distribution and characteristics of their spontaneous activity in the VNC. The densities and patterns of spontaneously active neurons were compared among MVN, LVN, SuVN, and SpVN.

Several lines of evidence suggest that ACh plays neurotransmitter and/or neuromodulator roles in the vestibular nuclei (De Waele et al., 1995). In addition, the cholinergic system (that using ACh as a transmitter) in the VNC has been implicated in the vestibular-related symptoms of motion sickness (De Waele et al., 1995) and lesion-induced vestibular compensation (Torte-Hoba et al., 1996). Although the function of ACh in the MVN has been studied for decades, one major question concerns the types of ACh receptors involved in the VNC; there have been disagreements among previous reports (Ujihara et al., 1988, 1989; Phelan and Gallagher, 1992; Schwartz, 1986). Five muscarinic receptor subtype genes (m1-m5) have been cloned (Bonner, 1989), and four
pharmacologically-defined muscarinic receptor subtypes (M₁-M₄) have been identified in the central nervous system (CNS). A previous study (Dutia et al., 1990) reported that the M₁ muscarinic subtype may not exist in the MVN. A study of the ACh receptors and their predominant subtypes in the VNC should increase our understanding of its cholinergic mechanisms and form the basis for functional studies of cholinergic pathways in the vestibular system under normal and pathological conditions. In the second part of this research, we also employed in vitro experiments with a brain slice preparation and extracellular electrical recording with application of drugs. Muscarinic and nicotinic agonists and antagonists were used to identify the ACh receptors involved in the MVN. Comparisons were made of the responses to carbacol from neurons in the MVN, SuVN, and SpVN. Furthermore, we explored the effects of antagonists preferential for the different muscarinic receptor subtypes M₁-M₄ to estimate to what extent each is involved in cholinergic effects on MVN neurons.

Besides the inner ear, the VNC receives major inputs from the cerebellum (Rubertone et al., 1995). The VNC and the cerebellum are closely related to each other both anatomically and functionally. Biochemical, pharmacological, electrophysiological, and immunohistochemical studies have suggested GABA as a neurotransmitter involved in cerebello-vestibular projections (De Zeeuw and Berrebi, 1995; Fonnum et al., 1970; Houser et al., 1984; Obata et al., 1970). The cerebellum is considered to play an important role during the plasticity in the vestibular system following lesions (McCabe and Ryu, 1969; Smith and Darlington, 1991). Previous studies of cerebellar lesion effects focus only on GABA metabolism in the LVN, but no electrophysiological or
pharmacological study has been done of effects in the vestibular nuclei after removal of cerebellar input. The third and fourth parts of this dissertation were directed toward elucidating neurochemical and neuropharmacological changes at several different survival times after removal of cerebellar input by transection of the inferior cerebellar peduncle (ICP) on one or both sides. In the third part, quantitative mapping of amino acid distributions was done among different VNC regions in control rats and in rats with unilateral or bilateral ICP transection. Concentrations of 12 amino acids in samples from the VNC regions were quantitatively measured using microdissection of freeze-dried brain sections combined with high performance liquid chromatography (HPLC). In the fourth part, electrophysiological recording was made in the MVN to test GABA pharmacology in normal rats and its plasticity after bilateral transection of the ICP. Results of this study provide neurochemical and electrophysiological information about the degree of involvement of amino acid neurotransmitters in VNC functions under normal conditions as well as their modification during lesion-induced vestibular plasticity. They provide a foundation for further research about the mechanisms behind these changes.
LITERATURE

Background

The vestibular system maintains balance and equilibrium of the head and body in space by integrating both peripheral and central sensory and motor information from various sources. In mammals, the vestibular system is composed of peripheral and central components. The former include the vestibular end organs and vestibular nerve, and the latter consist of the vestibular nuclear complex, vestibulocerebellum and some other areas involved in vestibular functions.

Peripheral Vestibular System

Vestibular End Organs

The vestibular end organs are located in the inner ear, or labyrinth, which includes the bony labyrinth and the membranous labyrinth. The bony labyrinth contains the vestibular as well as the auditory sense organs. The membranous labyrinth is inside the bony labyrinth and is filled with endolymph, while the space between the membranous and the bony labyrinth contains perilymph. Endolymph has an ionic composition resembling intracellular fluid, while perilymph resembles extracellular fluid.
The vestibular labyrinth has two principal sets of structures: the otolith organs (utricle and saccule) and the semicircular canals (horizontal, anterior vertical and posterior vertical). Each of these structures responds to acceleration of the head. Different segments of the end organs respond to different aspects of acceleration. The three semicircular canals lie in three different planes that are mutually perpendicular. In three-dimensional space, they detect angular acceleration of the head in any direction. The otolith organs detect linear acceleration when the head moves, and they are also important for determining the position of the head with respect to gravity.

Each semicircular canal has an enlargement called the ampulla at its basal part. In part of the ampulla, the epithelium of each canal is thickened to form the ampullary crest, which is covered with a gelatinous mass called the cupula. The crest contains specialized receptor cells, the vestibular hair cells, and supporting cells. When the head is rotated, the endolymph pushes against the cupula, producing bending of the sensory hair bundles of the receptor cells. Bending of the hair bundles leads to changes of permeability of ion channels and receptor potentials in the hair cells of the crest. The receptor potentials elicit release of neurotransmitter from the basal parts of the hair cells, which alters the activity in the vestibular nerve fibers innervating them.

Portions of the otolith organs also are thickened and contain hair cells. These portions are called maculae. They are covered with a gelatinous substance in which crystals of calcium carbonate (otoliths) are embedded. When the head undergoes linear
acceleration, the otoliths deform the gelatinous mass, which in turn bends the hairs of receptor cells, resulting in changes of polarization of the hair cells.

The vestibular hair cells are the anatomical and physiological basis for normal function by transducing movement caused by hair displacement into receptor potentials. They are divided into two types: Type I and Type II. The hairs on hair cells, which are arranged in order from shorter to longer, consist of a single kinocilium and numerous stereocilia. Bending of the stereocilia toward and away from the kinocilium can depolarize and hyperpolarize the hair cell, respectively.

**Vestibular Nerve**

The 8th cranial nerve is composed of the vestibular and auditory nerves. The vestibular nerve consists of afferent axons of bipolar vestibular ganglion cells and efferent fibers to vestibular and auditory end organs. Both the afferent axons and the ganglion cell bodies are myelinated (Kandel and Schwartz, 1985). The peripheral axons are divided into two groups, the superior and inferior divisions, which have different innervation patterns. The superior division innervates the macula of the utricle, the anterior part of the macula of the saccule, and the cristae of the horizontal and anterior semicircular ducts. The inferior division innervates the posterior part of the macula of the saccule and the crista of the posterior semicircular duct. The vestibular nerve (primary afferent fibers) exhibits a high average level of spontaneous discharge and a wide range
of discharge regularities. Small fibers tend to discharge regularly, while large ones tend to discharge irregularly (Wilson and Jones, 1979).

The vestibular afferent fibers (with the auditory afferent fibers) run through the cerebellopontine angle to reach the lateral aspect of the caudal pons. They pass between the cochlear nucleus, then the restiform body, and the spinal trigeminal tract. These fibers project into the vestibular nuclear complex from its ventrolateral border and into the cerebellum (Barmack et al., 1993).

Central Vestibular System

Vestibular Nuclear Complex (VNC)

The VNC occupies a substantial portion of the medulla and pons beneath the floor of the fourth ventricle (Paxinos and Watson, 1998; Rubertone et al., 1995) and contains the second order neurons for the primary vestibular inputs. It has been postulated that the VNC was among the first cell groups to evolve out of the reticular formation (Mehler, 1972). The VNC has been described by several authors (Brodal and Pompeiano, 1957 a, b; Voogd et al., 1985) as consisting of superior (SuVN), lateral (LVN), medial (MVN), and spinal (SpVN) vestibular nuclei and smaller cell groups, which commonly include the interstitial nucleus of the vestibular nerve and groups F, L, X, Y, and Z.
Topography and Cytoarchitecture

SuVN  Rostrally, the SuVN is capped dorsally by the crescent-shaped superior cerebellar peduncle. The lateral border of SuVN is formed by the inferior cerebellar peduncle (icp). Medially, it is bounded by the locus coerulescens rostrally and by the medial vestibular nucleus caudally (Paxinos and Watson, 1986). The ventral border of SuVN is formed by fiber systems traversing the supratrigeminal region. The ventral part of the caudal pole of SuVN becomes replaced by the anterior portion of the lateral vestibular nucleus. Cytoarchitecturally, the SuVN is composed of darker staining medium-sized cells and paler small cells (Rubertone et al., 1995).

LVN  The rostral half of the LVN is capped dorsally by the SuVN and bounded medially by the MVN, while its caudal part is rostral to the oral pole of the SpVN (Rubertone et al., 1995). The lateral border of LVN is formed by the icp (Paxinos and Watson, 1998) and the medial border by the MVN. The LVN is characterized by a large number of giant cells, up to 60 µm in diameter in rats (Johnson et al., 1976). There are also many small and medium-sized cells in the LVN. Based on cytoarchitectural differences, primary afferent input, differential cerebellar projections, and commissural and intrinsic connections, the LVN is subdivided into dorsal (LVNd) and ventral (LVNv) divisions (Rubertone et al., 1983).

SpVN  The SpVN extends to the dorsal surface of the medulla for most of its rostrocaudal extent except at its oral pole, where it lies ventral to the caudal portion of
the LVN (Paxinos and Watson, 1998). Laterally, the SpVN is adjacent to the cuneate nucleus and the icp. The medial boundary of the SpVN is established throughout by the MVN. The SpVN contains cells of the same size as the MVN, i.e., small and medium-sized cells.

**MVN** The dorsomedial border of MVN lies beneath the ependyma of the 4th ventricle throughout its rostral-to-caudal extent, while its lateral border is made up of SuVN, LVN, and SpVN. At caudal levels, the MVN is bounded ventrally by the nucleus of the solitary tract and medially by the prepositus hypoglossal nucleus. At rostral levels, the MVN is bordered ventrally by the reticular formation and medially by the genu of the facial nerve and the suprageniculate nucleus (Paxinos and Watson, 1998). Although the MVN is the VNC nucleus that has been studied most, there are few reports describing the morphological classification of vestibular nuclear neurons (Hauglie-Hanssen, 1968). Nissl staining shows that the MVN is composed of small and medium-sized cells (Rubertone et al., 1985).

**Afferent Connections of the VNC**

Primary vestibular nerve fibers divide into two branches, one ascending to the cerebellum with collaterals to SuVN and MVN and one descending with collaterals to MVN, LVNv, and SpVN (Brodal, 1974). Some areas in which primary fibers do not terminate include the LVNd and small cell groups F, X, and Z. Group Y and the interstitial nucleus of the vestibular nerve, however, receive extensive input (Korte,
1979). Afferents from different parts of the end organs predominantly terminate in different parts of the ipsilateral VNC (Gacek, 1960). The SuVN receives primary afferent fibers principally from all semicircular canals. The rostral and the caudal MVN receive primary afferent projections from the semicircular canals and the utricle, respectively. The SpVN and the LVN are all recipients of primary afferent fibers mainly from the otolith organs. There are no primary afferent projections to the accessory VNC nuclei X, Z, and F, but Y may receive some fibers from the saccule. Synapses between primary afferent fibers and second-order neurons are generally thought to be excitatory (Wilson and Jones, 1979), although a recent report has suggested that thicker vestibular afferents may co-release glutamate/aspartate and glycine (Reichenberger et al., 1995).

Based on different responses to semicircular canal stimulation, second-order neurons can be divided into the following categories: type I, excited when stimulating the ipsilateral vestibular primary afferents; type II, excited when stimulating the contralateral vestibular primary afferents; type III, excited when stimulating the vestibular nerve on either side; and type IV, inhibited when stimulating the vestibular nerve on either side (Wilson and Jones, 1979).

Besides primary afferent projections, there are some non-primary afferent connections of the vestibular nuclei: (1) Spinovestibular connections: spinal projections to the VNC have been demonstrated in the rat (Mehler, 1969). There appear to be dual spinovestibular pathways: the lateral spinovestibular fibers course with the dorsal spinocerebellar tract to terminate in LVN, SpVN, and group X, whereas the medial
spinovestibular projection courses through the medial reticular formation to terminate in
the MVN (Rubertone et al., 1995). Injections of horseradish peroxidase (HRP) into the
VNC reveal the origins of spinal input. These include the central cervical nucleus,
bilateral intermediate gray cells, Clarke’s column, and dorsal horn. Injection of HRP into
the SuVN also revealed some labeled neurons in the cervical spinal cord (Vincent and
Rubertone, 1984). 2) Cerebellovestibular connections: cerebellar projections to the VNC
have dual origins: the Purkinje cells in the cortex and neurons in the deep cerebellar
nuclei. The anterior lobe vermis sends projections to LVNd (Eager, 1967), while the
fibers from the vestibulocerebellum (9-10) and the lobules of the posterior vermis (6-8)
especially ignore LVN and distribute widely but differentially throughout SuVN, SpVN,
MVN, and group X (Angaut and Brodal, 1967, Bigare and Voogd, 1977). Projections
from the cerebellar cortex to the VNC are bilateral. On the other hand, deep cerebellar
nuclei projections to the VNC in the rat originate chiefly from the medial cerebellar
nucleus and project most heavily to the contralateral LVNv and SuVN, only
contralaterally to the SpVN, and bilaterally to the MVN (Rubertone and Haroian, 1982).
(3) Other sources of vestibular nuclei afferent connections are the reticular formation
bilaterally with a contralateral dominance (Pompeiano et al., 1978), the inferior olivary
nucleus bilaterally (Balaban, 1988), the trigeminal nucleus ipsilaterally (Marfurt and
Rajchert, 1991), and the locus coeruleus bilaterally with an ipsilateral predominance
(Fung et al., 1987).

Efferent Connections of the VNC
Vestibulospinal projections: There are two vestibulospinal pathways, a lateral (LVST) and a medial (MVST) vestibulospinal tract (Brodal, 1974, Holstege and Kuypers, 1982). Recent studies of the rat and other species, however, suggest a reexamination of the classical point of view of vestibulospinal systems. The HRP injections into different levels of the spinal cord reveal that all four vestibular nuclei project to the entire rostrocaudal length of the spinal cord (Leong et al., 1984, Masson et al., 1991). Knowledge on the course and termination of the vestibulospinal systems also has been expanded. These axons are not restricted to ventromedial and ventrolateral funiculi to terminate only in the ventral horn, but, in rat, descend bilaterally in the lateral and dorsolateral funiculi and dorsal columns to terminate in the dorsal horns as well (Bankoul and Neuhuber, 1992).

Vestibulocerebellar projections: The cerebellum receives inputs from both the primary vestibular fibers and the VNC. All these inputs are considered to terminate as mossy fibers (Rubertone et al., 1995). The vestibulocerebellar projections influence the output of the cerebellum, which in turn has important effects on the VNC neurons (Wilson and Jones, 1979). The portion of the cerebellum which receives primary vestibular signals is called the vestibulocerebellum.

The projections from the primary vestibular afferents to the vestibulocerebellum are principally ipsilateral (Rubertone et al., 1985). This usually includes the lingula (lobule I), uvula (lobule IX), and nodulus (lobule X). The flocculus receives sparse input in rat. Whether the paraflocculus is part of the vestibulocerebellum remains uncertain.
(Brodal and Hoivik, 1964; Korte and Mugnaini, 1979; Mehler, 1969). In rat, terminal connections also appear in the caudal, small-cell part of the medial cerebellar nucleus.

Secondary vestibulocerebellar projections originate from the VNC except the LVN. After HRP injections into lobules II-X (Mehler, 1977; Rubertone and Mehler, 1981), labelled neurons were present bilaterally throughout SuVN, MVN, SpVN, the interstitial nucleus of the vestibular nerve, and groups X and Y. No labelled somata were found in the LVN of any of the animals (rat, cat, etc.) studied. Most of the secondary vestibulocerebellar projections in the rat are bilateral and reciprocate cerebellovestibular projections (Rubertone and Mehler, 1981). Some studies (Blanks et al., 1983; Paallysaho et al., 1991) suggested that there also are projections to the flocculus and lobules VII and VIII. Secondary vestibulocerebellar projections to the deep cerebellar nuclei appear to terminate mainly in the medial cerebellar nuclei in rat. The HRP injections of the medial cerebellar nucleus in rat show these projections originating bilaterally from MVN and SpVN but not LVN or SuVN (Haroian, 1984).

Vestibuloocular projections: the extraocular motor nuclei, which include the oculomotor nucleus, trochlear nucleus, and abducens nucleus, receive extensive projections from the VNC. Studies using HRP injection or noting retrograde degenerative changes (chromatolysis) following lesions of the brain stem above the vestibular nuclei (Brodal and Pompeiano, 1957b; Rubertone et al., 1995) suggested that the VNC neurons have connections with the extraocular motor nuclei via the medial longitudinal fasciculus and the superior cerebellar peduncle. In rat, not all vestibular nuclei project to the
extraocular motor nuclei. Fibers derive from SuVN (ipsilaterally), MVN (contralaterally), SpVN, and group Y (Rubertone et al., 1995). The relationship between the abducens nucleus and the VNC in rat is still uncertain. By means of these connections, which are either excitatory or inhibitory monosynaptic (Wilson and Jones, 1979), eye motion is correlated with vestibular input through vestibuloocular reflexes (VOR) (Highstein and Reisine, 1979).

Commissural connections: an extensive commissural system reciprocally links most of the VNC of the two sides of the brainstem (Ketterer et al., 1990; Rubertone and Mehler, 1980). These commissural projections are not only point-to-point connections between homologous nuclei on each side of the brainstem but also multiple point-to-point connections. Based on the numbers of labeled neurons following HRP injections, the MVN establishes strong commissural connections, whereas those formed by SuVN, SpVN and group Y are moderate (Rubertone and Mehler, 1980). The LVNd projection is weak or nonexistent. It is still an unresolved question whether the rat LVNv participates in commissural connections or not. Basically, commissural fibers are inhibitory and possibly a property of canal-, but not macula-related central vestibular neurons (Wilson and Jones, 1979). There are two major mechanisms for commissural inhibition proposed between the MVNs on each side of the brain stem in cat. One is a projection from type I neurons on one side to inhibitory interneurons (type II neurons) on the contralateral side, which in turn form synapes on type I neurons (Nakao et al., 1982; Shimazu and Precht, 1966). The other involves direct monosynaptic projections from inhibitory type I neurons on one side to contralateral type I neurons (Kasahara et al., 1968; Mano et al., 1968).
However, both types of inhibition have not been shown in the same experiment. There is no evidence so far to support any projection of primary vestibular afferent fibers directly to the contralateral VNC (Walberg et al., 1958).

Centrifugal fibers in the vestibular nerve: Vestibular efferent neurons whose axons project in the eighth nerve to the end organs of the labyrinth are localized primarily in a region dorsolateral to the facial genu and ventromedial to MVN. A small group also appears medial to the genu, ventral to the suprageniculate nucleus (White and Warr, 1983). These centrifugal fibers may feed back information to the hair cells in the labyrinth to modulate their activities.

Other vestibular efferent projections: The VNC also sends projections to the thalamus, inferior olive, and reticular formation.

Intrinsic Vestibular Connections

The HRP injections into individual vestibular nuclei reveal highly organized, rather extensive internuclear connections in the rat (Rubertone et al., 1983). This intrinsic organization among different nuclei suggests a high degree of integrative communication within the VNC of each side of the brainstem.

Cerebellum
The cerebellum plays very important roles in control of movement (Wilson and Jones, 1979). It modulates the tone and contraction of muscles by a series of reflexes adjusting the output of the cerebral cortex, spinal cord, and sensory organs in light of the position and situation of the head and body in time and space.

The cerebellum is composed of an outer gray matter (cerebellar cortex), internal white matter, and three pairs of deep nuclei: the fastigial (medial), the interposed, and the dentate (lateral) nuclei (Voogd, et al., 1985). The input and output connections of the cerebellum run through three symmetrical pairs of tracts that connect to the brainstem: inferior cerebellar peduncle (or restiform body), middle cerebellar peduncle (or brachium pontis), and superior cerebellar peduncle (or brachium conjunctivum). Afferent systems enter the cerebellum mainly through the inferior and middle cerebellar peduncles (Rubertone et al., 1995).

The cerebellar cortex consists of three layers that contain only six principal types of neurons. The outermost or molecular layer is composed primarily of the axons of granule cells. It also contains scattered stellate and basket cells as well as the dendrites of the underlying Purkinje cells. The middle or Purkinje cell layer is composed of the large cell bodies of the Purkinje cells. The innermost or granular layer contains a vast number of densely packed small neurons, mostly small granule cells, and unipolar brush cells which are only present in vestibulo-cerebellum and are intermediate in size between granule and Golgi cells (Mugnaini and Floris, 1994). Golgi cells also are found at the outer border of this layer (Palay and Chan-Palay, 1974). Afferent pathways mainly from
two fiber systems, mossy fibers, and climbing fibers, synapse on neurons in both the deep cerebellar nuclei and cerebellar cortex. The cerebellar cortex also receives more diffuse projections, which are called aminergic systems, from the raphe and the locus ceruleus (Kandel and Schwartz, 1985). The deep cerebellar nuclei and the VNC transmit the entire output of the cerebellum. Activity of Purkinje cells is related inversely to activity of the deep cerebellar nuclei and is modulated by three types of inhibitory interneurons: stellate, basket, and Golgi cells. Unipolar brush cells are putatively excitatory and may function as relays in positive feedback from mossy fibers to the VNC.

Projections from the cerebellar cortex terminate bilaterally on VNC neurons (Rubertone et al., 1995), and these fibers have inhibitory effects on their targets. Projections from the fastigial, or medial deep cerebellar nucleus, which is also under inhibitory control from Purkinje cells, end mainly contralaterally on VNC neurons and exert excitatory effects (Wilson and Jones, 1979). The main connection between the cerebellum and the VNC travels in the inferior cerebellar peduncle (Rubertone, 1995).

Table I summarizes the afferent and efferent connections of each major vestibular nucleus.

Table I.1

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Afferent2</th>
<th>Efferent2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SuVN</td>
<td>Semicircular canals (ipsi)3</td>
<td>Spinal cord (bi)</td>
</tr>
<tr>
<td></td>
<td>Cervical spinal cord (bi)</td>
<td>Cerebellar cortex (bi)</td>
</tr>
<tr>
<td></td>
<td>Cerebellar lobules 6-10 (bi)</td>
<td>Oculomotor nucleus (bi)</td>
</tr>
<tr>
<td></td>
<td>Medial cerebellar nucleus (mostly contra)</td>
<td>Trochlear nucleus (ipsi)</td>
</tr>
<tr>
<td></td>
<td>Reticular formation (bi)</td>
<td>Thalamus (ipsi)</td>
</tr>
</tbody>
</table>

18
Inferior olivary nucleus (bi)  
Trigeminal primary afferent (ipsi)  
Locus coeruleus (bi)  

LVN  
Otolith organs (ipsi)  
Lateral spinovestibular projection (bi)  
Anterior lobe vermis (to LVNd, bi)  
Medial cerebellar nucleus (to LVNv, mostly contra)  
Trigeminal primary afferent (to LVNv, ipsi)  
Locus coeruleus (to LVNv, mostly ipsi)  
Reticular formation (bi)  
Inferior olivary nucleus (bi)  

SpVN  
Otolith organs (ipsi)  
Semicircular canals (ipsi)  
Lateral spinovestibular projection (bi)  
Cerebellar lobules 6-10 (bi)  
Medial cerebellar nucleus (bi)  
Reticular formation (bi)  
Inferior olivary nucleus (bi)  
Locus coeruleus (bi)  

Note: 1: Based on data from cat, monkey, rat, and other animals; References: Buttner-Ennever, 1992; Rubertone et al., 1995; Wilson and Jones, 1979.  
2: Intrinsic and commissural vestibular connections not included.  
3: bi=bilateral, contra=contralateral, ipsi=ipsilateral.

**Neurotransmitters in the Vestibular System**

**Acetylcholine**

Acetylcholine (ACh) has been known as a neurotransmitter for more than 60 yr (Kelly and Rogawski, 1985). In spite of the long history of work on this chemical, its neurotransmitter function in some brain areas is still not well understood. The ACh is synthetized in a reaction catalyzed by choline acetyltransferase (ChAT). Acetyl CoA and
choline are its substrates. The ACh is hydrolyzed by acetylcholinesterase (AChE). As is the case in peripheral synapses, cholinergic receptors in the CNS have been divided into two classes: muscarinic and nicotinic. Receptor binding studies have suggested that the concentration of muscarinic receptor in brain tissue is 100 times greater than that of nicotinic receptor (Kuhar and Yamamura, 1976; Wamsley et al., 1981). Electrophysiological data suggest that the muscarinic subtype of cholinergic receptor is predominant in the dorsal cochlear nucleus (Chen et al., 1995).

Five subtypes of muscarinic receptor (m₁-m₅) have been cloned (Bonner, 1989). All of them exhibit a slow response time (100-250 ms), are coupled to G proteins, and either act directly on ion channels or are linked to a variety of second messenger systems. When activated, the final effect can be to open or close K⁺ channels, Ca²⁺ channels or Cl⁻ channels (Goyal, 1989; Cooper et al., 1996). Cholinergic nicotinic receptors can be classified into two categories: muscle type and neural type (Papke, 1993). The nicotinic receptor acts by directly regulating the opening of a cation channel.

The pharmacological antagonists for defining different muscarinic receptor subtypes so far bind only to muscarinic M₁, M₂, M₃, or M₄ receptors. No drug selective for the M₅ receptor subtype has been found (see Table II).

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Currently Accepted Name</th>
<th>M₁</th>
<th>M₂</th>
<th>M₃</th>
<th>M₄</th>
<th>M₅</th>
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</tbody>
</table>
Although the idea that ACh might be the neurotransmitter for the primary vestibular afferents to the VNC has been discarded (Phelan and Gallagher, 1992; De Waele et al., 1995), the central cholinergic system is still thought to play an important role in the processing of sensory afferent information in the VNC. There is behavioral evidence for this point of view (Glaser, 1959; Wood and Graybiel, 1970; Schaefer and Meyer, 1973), and the cholinergic system in the VNC may be involved in vestibular compensation (Schaefer and Meyer, 1974; Torte-Hoba et al., 1996). Studies of ACh-related enzymes provide support for the neurotransmitter role of ACh in the VNC (Burke and Fahn, 1985; Carpenter et al., 1987; Tago et al., 1989; Barmack et al., 1992; Li et al., 1995), but there are some discrepancies between reports (Kimura et al., 1981; Armstrong et al., 1983; Sato et al., 1983). Muscarinic and nicotinic cholinergic binding sites have been detected in all vestibular nuclei, with the highest density within the MVN (Walmsley et al., 1981; Schwartz, 1986; Calza et al., 1992). Earlier in vivo studies of ACh in the VNC suggested that ACh has a depolarizing effect on neurons in the MVN and LVN (Yamamoto, 1967; Kirsten and Sharma, 1976b; Ito et al., 1981). The results of
in vitro experiments with slices are in agreement with the in vivo studies (Ujihara et al., 1988, 1989; Phelan and Gallagher, 1992), but the in vitro studies only focused on the MVN. Currently there is controversy about the receptors involved: is the spontaneous activity of the MVN regulated via muscarinic receptors alone, as suggested by extracellular brainstem slice studies (Ujihara et al., 1988, 1989), or are both muscarinic and nicotinic receptors involved, as proposed from intracellular slice recording data (Phelan and Gallagher, 1992). Concerning muscarinic receptor subtypes in the VNC, extracellular recordings in the MVN suggested that the \( M_1 \) subtype may not be involved in cholinergic mechanisms in this subnucleus (Dutia et al., 1990).

The sources of cholinergic innervation of the vestibular nuclei have not been accurately determined. Likely sources include cholinergic neurons in other regions, such as the reticular formation, upper cervical cord, local pontomedullary nuclei, tegmental dorsal nuclei (Woolf and Butcher, 1989; Brown, 1993), and contralateral inferior olive (Matsuoka et al., 1983). Some studies have suggested that cholinergic second order vestibular neurons send axons to the cerebellum (Barmack et al., 1992, 1993), and that some vestibulospinal projections also may be cholinergic (Jones et al., 1986).

There is evidence to support ACh as a neurotransmitter for the centrifugal projections from the VNC to the peripheral vestibular end organs (Godfrey et al., 1984; Schwarz et al., 1986).

**Amino Acid Neurotransmitters**
γ-aminobutyric Acid (GABA)

γ-aminobutyric acid, the predominant inhibitory transmitter in the CNS, is formed by the α-decarboxylation of L-glutamate, a reaction catalyzed by glutamic acid decarboxylase (GAD). The precursor of GABA, L-glutamate, can be formed from α-oxoglutarate, a Krebs cycle intermediate, by transamination or from glutamine converted by glutaminase. γ-aminobutyric acid is degraded by GABA-transaminase (GABA-T). Transmitter action of GABA is terminated mainly by uptake. In general, the localization of GAD in the CNS correlates quite well with the GABA content, but there does not appear to be a consistent relationship between GABA concentration and GABA-T activity (Cooper et al., 1996). Based upon pharmacological evidence, GABA receptors have been subdivided into two groups: the GABA_A receptor and the GABA_B receptor (Nicoll et al., 1990).

The GABA_A receptor is so far the most studied of the known GABA receptors (Nakayasu, et al., 1995). It contains an integral transmembrane ion channel that is gated by the binding of two agonist molecules and conducts Cl⁻ ions. Two important classes of drugs, the benzodiazepines and the barbiturates, can allosterically modulate the frequency and the duration of Cl⁻ channel opening (Cooper, et al., 1996). Compared with GABA_A, the GABA_B receptor seems to be coupled to Ca^{2+} or K⁺ channels via a GTP binding protein. The action of GABA_B receptor activation appears to be mediated through either an increase in K⁺ conductance or a decrease in Ca^{2+} conductance and elicits a slow inhibitory potential (Bowery, 1989).
There is increasing evidence for a novel GABA receptor (GABA\textsubscript{C}, or GABA\textsubscript{NANB}) that pharmacologically falls outside that defined by the GABA\textsubscript{A/B} classification (Johnston, 1994). It is similar to GABA\textsubscript{A}, since it regulates chloride channels, but is not influenced by either bicuculline or baclofen (Lukasiewicz, 1996).

There are different agonists and antagonists for subtypes of GABA receptors (See Table III).

<table>
<thead>
<tr>
<th>Receptor Class</th>
<th>Pharmacology</th>
<th>Second Messenger</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA\textsubscript{A}</td>
<td><em>Competitive</em> GABA, Musimol, Isoguvacine</td>
<td>Cl\textsuperscript{+}↑, None</td>
</tr>
<tr>
<td></td>
<td><em>Competitive</em> Bicuculline, GABA\textsubscript{Az}ine</td>
<td>Benzodiazepine, Barbiturates</td>
</tr>
<tr>
<td></td>
<td><em>Noncompetitive</em> Picrotoxin, TBPS</td>
<td>Steroids, DBI peptides</td>
</tr>
<tr>
<td>GABA\textsubscript{B}</td>
<td>Phaclofen, Saclofen, 2-OH-Saclofen, CGP-36742</td>
<td>K\textsuperscript{+}↑, Ca\textsuperscript{2+}↓, IP\textsubscript{3}/DAG</td>
</tr>
<tr>
<td>GABA\textsubscript{C}</td>
<td>GABA, CACA</td>
<td>cAMP</td>
</tr>
</tbody>
</table>

Modified from Cooper et al. (1996).

There is evidence to support a GABA afferent neurotransmitter role in the peripheral end organs (Felix and Ehrenberger, 1982; Lopez et al., 1992). Both GAD- and GABA-like immunoreactivities were observed in cytoplasm of type I and II hair cells, while immunoreactivity was absent from supporting cells (Meza et al., 1992). γ-aminobutyric acid has long been implicated in the control of vestibular reflex function.
(Curtis et al., 1970; Obata et al., 1967). Behavioural studies have demonstrated that
GABA_A and GABA_B receptors play important roles in central vestibular function (Cohen
et al., 1987; Dieterich et al., 1991; Straube et al., 1991).

The distribution of GABA shows significant variation in the VNC. There exists a
gradient of GABA concentration, decreasing from dorsal and rostral to ventral and caudal
(Li et al., 1994). GABA- or GAD-immunoreactive fibers and fine processes are found
throughout the VNC, especially in the LVN (Nomura et al., 1984; Walberg et al., 1990).
In the MVN, approximately 30% of the neurons have been reported to be GABA-
immunoreactive; many labeled axons from MVN neurons were observed to be directed
toward the midline, presumably constituting part of the commissural system (Holstein et
al., 1996). GABA- or GAD-immunoreactive neuron somata are generally small in size
and widely distributed, in agreement with general principles concerning the anatomical
organization of GABA-ergic neurons studied so far in the CNS. In an
immunocytochemical study of L-baclofen-sensitive GABA_B binding sites in the MVN,
evidence has been presented for the existence of both pre- and postsynaptic GABAergic
inhibition mediated by GABA_B receptors (Holstein et al., 1992).

In vitro electrophysiological data have suggested that GABA_A (Gallagher et al.,
1986, 1992; Smith et al., 1991; Dutia et al., 1992; Lin and Carpenter, 1993) and GABA_B
(Dutia et al., 1992, Vibert et al., 1995) receptors exist on many MVN neurons.
GABAergic neurons in the VNC are likely to contribute not only to inhibitory interactions within the VNC but also to efferent projections from the VNC to related structures. Firstly, extensive commissural inhibitory projections that link the two VNCs contain a GABA_A component (type II or inhibitory type I neurons) (Precht et al., 1973; Furuya et al., 1991). This was demonstrated in cats and guinea pigs. Secondly, the inhibitory projections from the VNC to the extraocular motoneurons (Spencer and Baker, 1992) and to the spinal cord (Curtis et al., 1984, Blessing et al., 1987) have been considered to be GABAergic. Furthermore, the GABA-containing fibers in the VNC probably include those from cerebellar Purkinje cells (Brodal, 1974; Obata et al., 1969, 1970). Ablation of the anterior cerebellar vermis resulted in a large reduction in the number of GAD-immunoreactive terminals (Houser et al., 1984) and a great loss in the activity of GAD assayed by a radiochemical method (Fonnum et al., 1970) in LVNd, whereas these were unaltered in LVNv. The LVN receives contralateral projections from the dorsal cap and β-nucleus of the inferior olive, and GABA might be one of the neurotransmitters associated with this pathway (Matsuoka et al., 1983). Finally, we might expect that inhibitory interneurons interconnecting nuclei on the same side in the VNC might also use GABA as a neurotransmitter.

Glycine

Glycine also is involved in central vestibular function (Spencer and Baker, 1989; De Waele et al., 1995). It was suggested that glycine concentrations in the VNC appear to be distributed oppositely to those of GABA (Li et al., 1994, 1996b). Glycine has been
suggested as the inhibitory neurotransmitter of some commissural fibers between the two VNCs (Precht et al., 1973; Furuya et al., 1991), of some premotor neurons which are related to horizontal eye movement (Spencer and Baker, 1992), and of vestibulospinal projections to motoneurons (Wilson and Jones, 1979; Triller et al., 1987). Recent study has shown that glycine may be colocalized with glutamate at the level of some primary vestibular afferent terminals (Reichenberger et al., 1995).

**Glutamate**

There is increasing evidence that glutamate is a neurotransmitter used by the primary vestibular afferent fibers (Raymond et al., 1984; Lewis et al., 1989; Doi et al., 1990; Kinney et al., 1994; Li et al., 1994, 1996a, b). The predominant role of the glutamatergic synaptic transmission between first- and second-order medial vestibular neurons was confirmed by in vitro whole brain intracellular electrophysiological recordings (Vidal et al., 1995). 6-Cyano-7-nitro-quinoxaline-2, 3-dione (CNQX, a selective antagonist of AMPA/kainate receptors) abolished the major part of the field potential or the EPSPs at monosynaptic latency in response to stimulation of the primary vestibular afferent fibers, demonstrating the important role of AMPA receptors in synaptic transmission between first- and second-order medial vestibular neurons, while APV (a selective antagonist of NMDA receptors) could block a variable part of the EPSPs which remained after CNQX perfusion, indicating a variable contribution of NMDA receptors. Immunocytochemical and binding studies showed the distribution of glutamate-containing terminals, receptors, or binding sites in all nuclei of the VNC, with
the highest densities observed in the MVN (Touati, et al., 1989; Walberg et al., 1990; Smith et al., 1990; Li et al., 1996a, 1997).

**Other Neurotransmitters**

Some studies have suggested the existence of histamine (Kirsten and Sharma, 1976b; Phelan et al., 1990; Serafin et al., 1993), serotonin (Pazos and Palacios, 1985; Gallagher et al., 1992; Johnston et al., 1993; Licata et al., 1993), norepinephrine (Kirsten and Sharma, 1976a; Gallagher et al., 1992; Schuerger and Balaban, 1993), and neuropeptide (Darlington et al., 1993; Lin and Carpenter, 1994; De Waele et al., 1995) receptors in the VNC.

**Spontaneous Activity in the VNC**

Spontaneous activity represents the action potentials of neurons in the absence of any known stimulation. It is a common feature of many CNS neurons (Llinas, 1988). It may be related to synaptic transmission from other neurons, or it may be related to intrinsic membrane properties. Intrinsic mechanisms include noninactivating Na$^+$ channels (Schwindt and Crill, 1995) and slowly inactivating Ca$^{2+}$ current (Azouz et al., 1994). In vivo studies have reported spontaneous activity in MVN (Kawabata et al., 1990), LVN (Ito et al., 1981; Laurence et al., 1995), and SuVN neurons (Laurence et al., 1995). All mammalian brainstem-slice vestibular nuclear studies conducted so far have
focused on MVN neurons. Many MVN neurons have been shown to have resting spontaneous activity, classified into regular and irregular patterns, with firing rates usually between 15 and 40 spikes/sec (Gallagher et al., 1992). Development of spontaneous activity gradually reached adult values at postnatal day 30 (Dutia et al., 1995). The observations that the firing was present even in neurons completely isolated from exogenous fibers, and that spontaneous activity persists when synaptic transmission is blocked by superfusion with high Mg$^{2+}$, low Ca$^{2+}$ ACSF suggest that the resting activity is due to an intrinsic pacemaker property (Gallagher et al., 1985, 1992; Lin and Carpenter, 1993). Although intracellular studies have revealed a variety of voltage-dependent channels in MVN neurons, and although the spontaneous firing is exquisitely sensitive to small changes in membrane potential (Serafin et al., 1991a, b), the kinds of voltage-dependent ion channels responsible for spontaneous activity in MVN neurons in vitro remain undetermined. A component of the resting activity seems to be related to synaptic transmission within the slice (Lewis et al., 1989). Hence, MVN neuron resting activity may be influenced by a complex interaction between voltage-dependent and receptor-mediated channels.

Using intracellular investigations, Gallagher et al. (1985) reported, and Johnston et al. (1994) later confirmed, that there are two major types of neurons in the rat MVN in vitro. ‘Type A’ neurons have a single, deep afterhyperpolarization that appears to be mediated by TEA-sensitive K$^+$ conductance and apamin-insensitive Ca$^{2+}$-activated K$^+$ conductance. ‘Type B’ neurons have an early, fast afterhyperpolarization and a delayed, slow afterhyperpolarization. The former afterhyperpolarization was mediated by a TEA-
sensitive $K^+$ conductance, while the latter one appeared to be due to an apamin-sensitive $K^+$ conductance.

There are some limitations to the comparisons of in vivo preparations with in vitro brain slice preparations in the study of the VNC. Recently, several groups, using the in vitro whole brain (IWB) of frog (Kunkel and Dieringer, 1994) or guinea pig (Muhlethaler et al., 1993), have reported that IWB may be used to bridge the gap between results obtained in vivo and in vitro.

Spontaneous activity can be employed to examine the mechanisms of vestibular compensation. After primary vestibular deafferentation, the ipsilateral second order vestibular neuron spontaneous activity falls to nearly zero, while the contralateral vestibular neuron activity almost doubles. This results in asymmetry between the lesioned and unlesioned sides. At a certain time (differing among species) after deafferentation, the vestibular neurons regain approximately normal rates of resting activity. This may be the foundation for the compensation of static vestibular symptoms (Smith and Curthoys, 1989).

*Vestibular compensation*

Vestibular compensation is a process of behavioral recovery which occurs following removal of the vestibular receptor cells in the peripheral vestibular end organs or transection of the vestibular nerve. A complex syndrome of ocular motor and postural
symptoms can be observed during vestibular compensation since vestibulo-ocular and vestibulospinal reflexes are severely compromised following loss of primary vestibular input. In general, these symptoms have been classified into two categories: static and dynamic symptoms. The former represent those that exist in the absence of head movement, including tonic eye deviation, spontaneous ocular nystagmus, tonic head tilts, and circling or rolling toward the lesioned side (Smith and Curthoys, 1989). The dynamic symptoms are those that occur as a result of head movement, such as abnormal vestibulo-ocular and vestibulospinal reflexes (Darlington et al., 1991).

The development of vestibular compensation has been observed in many animals and in humans. The different species have different time courses of vestibular compensation with respect to different ocular motor and postural symptoms (Darlington et al., 1991). Although various hypotheses (Smith and Darlington, 1991) have been proposed for the mechanisms of vestibular compensation, they are still unconfirmed.

The cerebellum is thought to play an important role in vestibular compensation because it has complex afferent and efferent connections with the VNC and functions in motor coordination in normal animals. However, previous lesion studies of the cerebellum during vestibular compensation have given inconsistent results. Selective lesions of the flocculus (Jeannerod and Courjon, 1981; Kitahara et al., 1997), nodulus and uvula (Igarashi and Ishikawa, 1985) inhibit compensation of the static symptoms, but the effects of cerebellar input upon vestibular compensation remain controversial. Total cerebellectomy has been reported to prevent compensation (Smith and Curthoys, 1989).
However, the same procedure in another study demonstrated only a transient retardation (Schaefer and Meyer, 1974). Carpenter et al. (1959) have reported that ablation of the medial cerebellar nucleus severely retards compensation, whereas another study (Robles and Anderson, 1978) showed that the lesion of the deep cerebellar nuclei neither prevented compensation nor affected animals of the same species (cat) which had already compensated. Lesions of the anterior vermis were reported to retard compensation of the static ocular motor and postural symptoms less than lesions of the posterior vermis (Schaefer and Meyer, 1981).

Thus, the results of cerebellar lesion studies are contradictory regarding the role of the cerebellum in vestibular compensation. However, it seems likely that the compensation of dynamic vestibular symptoms is affected by cerebellar input since cerebellar lesions should have marked effects on the responses of VNC neurons to head movement during vestibular compensation (Schaefer and Meyer, 1981; Smith and Curthoys, 1989).
Spontaneous activity in rat vestibular nuclei in brain slices and effects of acetylcholine agonists and antagonists

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Abstract

Extracellular recording was used to investigate spontaneously active neurons in all four major nuclei of the rat vestibular nuclear complex (VNC) in brainstem slices. The density of spontaneously active neurons was highest in the medial vestibular nucleus (MVN), slightly lower in the superior (SuVN) and spinal (SpVN) nuclei, and lowest in the lateral vestibular nucleus (LVN). We compared the effects of acetylcholine agonists and antagonists on spontaneously discharging neurons in MVN, SuVN, and SpVN with those in the nearby dorsal cochlear nucleus (DCN). The proportion of neurons responding to carbachol was greatest in DCN and smallest in SpVN. Unlike in DCN, some neurons in MVN, SuVN, and SpVN showed decreased firing during carbachol or muscarine. Magnitudes of responses to carbachol and muscarine were closely correlated ($P<0.01$). MVN neurons possessed nicotinic as well as muscarinic receptors. Activation of either type was unaffected by blocking synaptic transmission. The IC$_{50}$ values for the muscarinic subtype-preferential antagonists were compared, and tropicamide, preferential for M$_4$, was the most potent. Our results suggest that: (1) the relative numbers of spontaneously active neurons in rat VNC differ among nuclei; (2) acetylcholine agonists elicit changes in mean firing rates of neurons in MVN, SuVN and SpVN, but fewer neurons respond, and responses are smaller than in DCN; (3) both muscarinic and nicotinic acetylcholine receptors are present on MVN neurons, but muscarinic receptors may be more prominent.

Theme: Motor system and sensorimotor integration
Topic: Vestibular system

Keywords: Extracellular recording; Muscarinic receptor; Nicotinic receptors; Cochlear nucleus; Tropicamide.
1. Introduction

Spontaneous firing is characteristic of many central nervous system neurons [38]. In vivo studies have reported spontaneous activity in the medial vestibular nucleus (MVN) [31,32,36,43], lateral vestibular nucleus (LVN) [29,31,32,35,43,63], superior vestibular nucleus (SuVN) [43] and spinal vestibular nucleus (SpVN) [46]. Changes in spontaneous activity have been implicated in vestibular compensation [13, 15, 42, 43, 48, 64]. So far, all electrophysiological studies of the vestibular nuclear complex (VNC) in mammalian brainstem slices have focused on MVN neurons [14,21,22,37,55,64]. Little has been known about in vitro spontaneous activity in the LVN, SuVN or SpVN.

Acetylcholine receptors have been pharmacologically categorized as two major types: muscarinic and nicotinic. Anatomical, biochemical and behavioral studies have suggested that cholinergic modulation plays an important role in the VNC and may be involved in vestibular compensation [54,58]. Muscarinic [6,62] and nicotinic [11] receptor binding sites have been reported in all vestibular nuclei, with the highest density in the MVN. Studies of acetylcholine-related enzymes support a neurotransmitter role for acetylcholine in the VNC [2,4,7,23,53], although not all studies agree [1,47].

Changes in spontaneous firing rates and patterns are sensitive indicators of pharmacological effects on neurons [8,60]. In vivo electrophysiology in the VNC demonstrated that LVN and MVN neurons increase firing during application of acetylcholine, physostigmine and muscarinic agonists [29,31,32], and that these effects can be blocked by the muscarinic antagonists atropine and scopolamine. The results of in

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vitro experiments with brain slices are generally consistent with the in vivo results [41,55,56] for the MVN, but no information is yet available for the LVN, SuVN or SpVN. Based on available studies [41,56], it is still not clear whether neuronal activity in the MVN is regulated by muscarinic receptors alone or also by nicotinic receptors.

Five subtypes of muscarinic receptor (m₁–m₅) have been cloned [3,30]. Some antagonists preferential for different muscarinic receptor subtypes have been developed, i.e. for M₁, M₂, M₃, or M₄, which correspond to molecular forms m₁–m₄ [25]. One report [18] has mentioned that the M₁ subtype might not exist in the MVN, but other subtypes have not yet been studied in the VNC.

In this study, we first mapped the distributions of spontaneously active neurons in all four major vestibular nuclei by extracellular recordings. Extracellular recordings enable access to a variety of cell sizes and do not affect spike generation, as may be the case with intracellular recordings. Next, we investigated the effects of agonists and antagonists of acetylcholine on regular neuron activity of MVN, SuVN and SpVN neurons, for comparison with those shown by regular neurons in the dorsal cochlear nucleus (DCN) of the same slices. Fewer neurons were tested in the DCN because data are already available from a previous study with the same methods [8]. Finally, we used subtype preferential muscarinic antagonists to suggest which subtypes predominate in the MVN.

Preliminary results from this study have been presented [50,51].
2. Materials and methods

2.1. Preparation of brain slices

Sprague–Dawley rats weighing 200–400 g, male or female, were anesthetized with sodium pentobarbital (52 mg/kg, i.p.) and decapitated. For each rat, the caudal part of the brain was exposed while rinsing the head in ice-cold artificial cerebrospinal fluid (ACSF) and removed into ice-cold ACSF, pregassed with 95% O\textsubscript{2} and 5% CO\textsubscript{2}. The overlying cerebellum was removed, and the part of the brainstem containing the VNC was serially sectioned with a vibratome at 450–550 μm thickness, in either the coronal or horizontal plane. Slices containing SuVN, SpVN, or MVN and LVN were transferred onto a nylon mesh in an interface chamber [24,27,60] perfused at about 1.2 ml/min with ACSF consisting of (mM): KH\textsubscript{2}PO\textsubscript{4} 1.25, KCl 5, MgSO\textsubscript{4} 2, CaCl\textsubscript{2} 2, NaCl 124, Glucose 10, and NaHCO\textsubscript{3} 26. Humidified gas (95% O\textsubscript{2}, 5% CO\textsubscript{2}) flowed continuously over the slices. The chamber temperature was maintained at 32–34 °C.

2.2. Electrophysiological recordings

After a recovery period of about 2 h, slices were explored with microelectrodes having 2–3 μm tip diameter, filled with 1 M NaCl. Extracellular discharges of spontaneously active vestibular neurons and DCN neurons (excluding bursting neurons) were displayed, recorded on magnetic tape, and led into a digital interface. Spike counts
and intervals were saved with 0.1 ms time resolution in binary files for later calculations of interval and autocorrelation histograms [60]. The locations of microelectrode penetrations were mapped onto a drawing of each slice, using the coordinates formed by the nylon mesh as a reference. From this drawing, the distance of each electrode penetration from the midline of coronally-cut slices or the midpoint of the rostral–caudal range of the MVN of horizontally-cut slices was measured. With the aid of an atlas of the rat brain [40], boundaries of the major vestibular nuclei were visualized with a dissecting microscope using both transmitted and reflected light. Extracellular spike amplitude ranged widely up to 1 mV. We excluded units with spike amplitudes below 50 μV from all studies because they were not reliably triggered and therefore unsuitable for pharmacological experiments. The identification of the firing pattern of a tested neuron was made by a combination of visual observation, interval histograms, autocorrelation histograms and measurement of coefficients of variation (CV) of interspike intervals. A neuron was classified as regular if its autocorrelation histogram displayed three or more clearly distinguishable peaks, and its CV was lower than 0.5. The CVs for irregular neurons were higher than 0.5.

2.3. Pharmacology of brain slices

In studying the pharmacology of brain slices, electrophysiological recordings were similar to those for the mapping study. Spontaneous firing of MVN, SuVN and SpVN neurons was recorded, and interval histograms and spike counts were obtained for
all neurons before, during, and after bath application of drugs. Changes in firing rates were analyzed with a computer program [60]. To test the effects of synaptic blockade on firing, neurons were recorded during exposure to low Ca\textsuperscript{2+}/high Mg\textsuperscript{2+} ACSF, which contains 0.2 mM Ca\textsuperscript{2+} and 7.8 mM Mg\textsuperscript{2+}. This low Ca\textsuperscript{2+}/high Mg\textsuperscript{2+} ACSF largely or completely blocks synaptic transmission [8,16,19,39,60,61]. For pharmacological studies, the response was defined as the increase in mean firing rate from control level to the largest value of the firing rate after drug application. The effects of agonists and antagonists were expressed as the percent increase or decrease from the control rate.

2.4. Drug applications

Drugs were usually dissolved in distilled water for stock solutions, diluted in the ACSF at different concentrations before the experiment, and applied to the slice by bath application. Acetylcholine-related drugs used, and their concentrations, based on previous studies [8,9], were carbamylcholine chloride (carbachol, 10 μM), muscarine chloride (10 μM), atropine sulfate (0.1-1 μM), pirenzepine dihydrochloride (1-30 μM), nicotine (free base, 10-50 μM), hexamethonium bromide (50-100 μM), mecamylamine hydrochloride (5-10 μM), D-tubocurarine chloride (1-2 μM), tropicamide (0.01-3 μM), all from Sigma; p-fluorohexahydro-siladifenidol (p-f-HHSiD, 1-30 μM) from RBI; and AF-DX 116 (1-30 μM) from Boehringer Ingelheim.
2.5. Data analysis

ANOVA and chi-square tests were used to analyze data for statistically significant differences among vestibular nuclei. For each ANOVA, post-hoc comparisons were made using Dunnette’s C tests. Correlations were examined using linear regression analysis. For all comparisons, $P<0.05$ was considered statistically significant.

The care and use of animals studied in this work were approved by the Medical College of Ohio Institutional Animal Care and Use Committee and the National Institutes of Health (NIDCD grant DC02550).
3. Results

3.1. Mapping study

The four major vestibular nuclei were mapped for spontaneous neural activity (Fig. 1). Spontaneously discharging neurons were found in each vestibular nucleus (Fig. 2), but the relative numbers of active neurons differed significantly among them. The density (neurons/penetration) of spontaneously active vestibular neurons was highest in the MVN and lowest in the LVN ($P<0.05$ for difference between MVN and any other vestibular region). All VNC regions showed substantially lower densities than the DCN ($P<0.001$ for difference between DCN and any vestibular region). Within the MVN, spontaneously active neurons were about twice as prominent in the rostral half as in the caudal half and in the medial half as in the lateral half. There were no obvious differences of densities between dorsal and ventral parts of the MVN.

Most of the active neurons in the VNC showed regular firing patterns: 97% in the MVN, 73% in the LVN, 88% in the SuVN, and 89% in the SpVN ($P<0.05$ for difference between MVN and LVN). The rest showed irregular patterns. Unlike the DCN, no neurons showed bursting patterns [60]. Therefore, to facilitate comparisons, bursting neurons were not included in the sample of DCN neurons either. There were no obvious differences among the vestibular nuclei with respect to spike waveforms. Mean firing rates (mean±S.E.M.) of SuVN (24.0±1.8) and MVN (21.3±0.71) neurons were significantly higher than those for LVN (17.4±2.7), SpVN (16.6±1.6) and DCN (17.2±1.5) (SuVN or MVN vs. SpVN, $P<0.005$; SuVN or MVN vs. DCN, $P<0.05$).
3.2. Effects of acetylcholine agonists on neurons in the vestibular nuclei

All neurons studied pharmacologically had regular firing patterns. Irregular neurons were not included because of their unstable firing rates. Drug effects were not tested in the LVN because of its low density of regular neurons.

In the DCN, ten out of 13 and seven out of eight regular neurons responded to 10 μM carbachol and 10 μM muscarine, respectively (Table 1). No neurons were found to decrease firing rate during carbachol. This is consistent with a previous study [8].

Of 252 neurons tested in the MVN, SuVN, and SpVN, the mixed acetylcholine agonist, carbachol, usually increased or did not affect firing rate (Table 1). Fewer neurons in SuVN and SpVN showed increased firing during carbachol than in MVN and DCN. Responses were smaller in magnitude in the SuVN and SpVN than in the MVN, but everywhere less than in the DCN. Differences were statistically significant between MVN or DCN and SuVN or SpVN (P<0.001 for all). The number of DCN neurons tested was insufficient for demonstrating a statistically significant difference between DCN and MVN. The percentage increases of firing rate for only those neurons showing increases during carbachol showed trends across regions similar to those in Table 1, but differences among regions were smaller. Fig. 3 shows typical responses to 10 μM carbachol and muscarine in MVN, SuVN, SpVN and DCN neurons. The mean responses in all the nuclei to the muscarinic acetylcholine agonist, muscarine, were similar to, although slightly smaller than, those to carbachol (Fig. 4). About a third of MVN neurons (Table
1) were also responsive to the nicotinic acetylcholine agonist, nicotine, although percentage increases in firing rate to the higher concentrations of nicotine were typically smaller than those to 10 μM carbachol or muscarine (Fig. 5). Mean increased firing rate (±S.D.), for the six responsive MVN neurons, to 10-50 μM nicotine was 57±24%. No MVN neurons showed decreased firing during nicotine.

3.3. Effects of acetylcholine antagonists and synaptic blockade on MVN neurons

To test whether acetylcholine agonists were affecting neurons directly or through effects on other neurons, synaptic transmission was blocked in some cases with low Ca\(^{2+}\)/high Mg\(^{2+}\) ACSF. Of ten MVN neurons exposed to low Ca\(^{2+}\)/high Mg\(^{2+}\) ACSF without application of drugs before and after, firing rates of four were not affected, but discharge rates of the other six decreased or increased. No change of firing pattern was observed in any of the neurons. Carbachol and nicotine effects were compared in control ACSF and in ACSF with low Ca\(^{2+}\)/high Mg\(^{2+}\) (Fig. 6). The responses to carbachol (N=5) and nicotine (N=3) in the MVN always persisted in low Ca\(^{2+}\)/high Mg\(^{2+}\) ACSF, whereas a muscarinic antagonist, atropine, blocked carbachol effects (N=8), and tubocurarine (N=4) or mecamylamine (N=2), nicotinic antagonists, blocked nicotine effects. The effect of 10 μM carbachol on MVN neurons was not antagonized by the nicotinic blockers, mecamylamine, hexamethonium, and tubocurarine (N=3 for each antagonist) (Fig. 6 bottom). During application of many muscarinic and nicotinic blockers, most neurons showed small initial increases in firing rate. The recovery from atropine was usually
slow, and decreases in baseline firing rate were observed in all cases tested (Fig. 6, top and bottom).

3.4. Effects on MVN neurons of antagonists preferential for muscarinic receptor subtypes

Since our studies suggested that muscarinic agents were more effective than nicotinic agents upon the firing of VNC neurons, a more detailed investigation of muscarinic antagonists was done. Results were obtained from 38 regular MVN neurons tested with one or more concentrations of an antagonist. In all cases, one to three different concentrations of an antagonist were applied to the slice, each tested alone and in combination with 10 µM carbachol. Antagonists were applied in lower concentrations before higher to minimize residual effects in the slice, and only one antagonist was applied to a given slice. The control response to carbachol was routinely determined before and after application of different concentrations of the antagonist. Fig. 7 demonstrates the effects of four different antagonists on responses to carbachol. Pirenzepine decreased the response to 10 µM carbachol at 10 and 30 µM and AF-DX 116 at 30 µM. Tropicamide at 1 µM concentration completely blocked a neuron’s response to carbachol. Compared with that of tropicamide, the effect of p-f-HHSiD was of smaller magnitude but more prolonged. Concentration-response plots for the effects of the antagonists in several experiments are displayed in Fig. 8. The IC₅₀ value (concentration) for each antagonist was determined as the intersection of the least-squares line with 50% reduction of the test response. Although these available antagonists are not highly specific for particular subtypes, comparison of their IC₅₀ values with those measured
previously in the DCN (Table 2) suggests that the M₄ subtype predominates in MVN as in DCN, that the M₂ and M₃ subtypes are roughly equally represented in MVN and DCN, and that the M₁ subtype is relatively less prominent in MVN than in DCN. Comparison with $K_i$ values from the literature (Table 2) suggests that, as for the DCN, M₂ is the second most prominent subtype in MVN.
4. Discussion

Many reports have suggested that MVN neurons show regular spontaneous discharge in rat brain slices [14,21,42]. Our data support these previous results for the MVN and are the first to provide evidence that SuVN, SpVN, and LVN neurons possess generally similar patterns of spontaneous discharge in brain slices. Although the functional significance of spontaneous activity is not fully understood, there is evidence that it can be an important factor during vestibular compensation or other neuronal plasticity [15,42,48,64]. Spontaneous activities of neurons are useful to study in the brain slice preparation because the discharge is continuously measurable and is highly sensitive to agents that affect membrane channels, second messenger systems, and ion transport mechanisms [33,49,56].

Our finding of regular and irregular patterns of spontaneous activity in vestibular nuclei in slices resembles results for in vivo studies [43,45]. In cat, the majority of vestibular nuclear neurons fired irregularly, although their discharge rates (with the exception of higher rates for the LVN) were close to those measured here [45]. In guinea pig, vestibular nuclear neurons fired regularly and irregularly [43,57]. As to densities of spontaneous firing, no complete data are available from in vivo recordings to compare with our results. Since the spontaneous activities of most MVN neurons in our study were not greatly affected by blocking synaptic transmission with low Ca\(^{2+}\)/high Mg\(^{2+}\), their spontaneous activities in vitro may result predominantly from intrinsic membrane properties [38]. One would expect spontaneous firing to be altered to some degree in
brainstem slices compared to in vivo, because most fiber connections to and from the VNC were cut.

The factors underlying different densities of spontaneously active neurons among the various vestibular nuclei and in different parts of the MVN are not entirely clear. However, the very different densities of spontaneously active neurons in the LVN and MVN may be related to the different densities of neurons in these nuclei. The LVN has the lowest, whereas the MVN has the highest density of neurons among the main vestibular nuclei [52]. Concerning our finding of more spontaneously active neurons rostrally than caudally in the MVN, some studies have suggested that rostral and caudal MVN regions have different connections from or to labyrinthine, cerebellar, intrinsic, commissural, and spinal cord locations [5]. The evidence that large- and small-diameter primary vestibular afferents contribute differentially to rostral and caudal parts of the VNC may also provide an anatomical correlate for our results [28]. Further, it has been reported [52] that there is a decreasing gradient of MVN neuron soma size in the rostral-to-caudal direction. Different time courses for the post-natal development of rostral and caudal parts of MVN have been reported [17], with the most rostral cells exhibiting spontaneous activity before those located more caudally. Also, unilateral labyrinthectomy affects spontaneous activity more in rostral than in caudal parts of the ipsilateral MVN of brainstem slices [64]. The lack of difference in our study between dorsal and ventral parts of the MVN is somewhat surprising in view of previous evidence for differences between these regions with respect to morphology, physiology, and chemistry [23,26,34,44,59].
Our results support previous findings suggesting that MVN neurons possess muscarinic receptors [41,55,56] and that neuronal firing is often increased by the mixed acetylcholine agonist carbachol. In contrast to the MVN, fewer neurons in the SuVN and SpVN responded to acetylcholine agonists, and the responses were smaller. This is in agreement with higher activity of choline acetyltransferase, the enzyme of synthesis for acetylcholine [4,23], and higher muscarinic acetylcholine receptor binding density [62] in the MVN than in other vestibular nuclei. The smaller responses of VNC than of DCN neurons to carbachol [8] are consistent with higher average choline acetyltransferase activity in the DCN than in the vestibular nuclei [23]. The smallness of the nicotine effects, the correlation of the responses to muscarine with those to carbachol in all the nuclei tested, and the ability of muscarinic but not nicotinic antagonists to block carbachol effects suggest that receptors for acetylcholine in the vestibular nuclei may be mainly muscarinic, as in the DCN [8].

Carbachol may elicit either an increase or decrease of spontaneous firing among the vestibular nuclei, unlike in the DCN of brain slices. This effect in MVN neurons can be blocked by atropine but persists in low Ca\(^{2+}\)/high Mg\(^{2+}\) medium, suggesting effects directly on postsynaptic receptors. Muscarinic receptors produce their effects by activation of G proteins, which may affect ion channels directly or through second messenger systems [20,30]. Such activation results in either depolarization or hyperpolarization, depending upon which types of channels (K\(^{+}\) channels, Ca\(^{2+}\) channels, or Cl\(^{-}\) channels) are affected [3,12,20]. We conclude that the relative amounts of the various acetylcholine-gated ion channels may vary among MVN neurons.
Our finding that about a third of MVN neurons were responsive to nicotine is in agreement with previous studies. Nicotinic receptor binding sites have been detected in the VNC, with the highest density in the MVN [11]. Phelan and Gallagher [41], using intracellular recording, reported that selective nicotinic agonists could depolarize the membrane potential of MVN neurons, whereas, in a study with extracellular recording [56], concentrations of nicotine up to 100 μM were ineffective. This discrepancy might relate to fast desensitization of the nicotinic receptor, as proposed by Phelan and Gallagher [41]. Time courses for effective concentrations of nicotinic agonists to reach the recording site might differ for different experimental preparations. Furthermore, because of the small proportion of MVN neurons showing effects of nicotine, larger sample sizes are needed to be sure that the differences between the studies do not merely reflect differences in sampling bias.

Little or no difference in the effects of 10 μM carbachol or nicotine with synaptic transmission blocked in low Ca\(^{2+}\)/high Mg\(^{2+}\) ACSF, compared to control ACSF, suggests that these effects are through postsynaptic mechanisms, as for muscarinic receptors on DCN regular neurons [8].

The decrease in the baseline firing rate after application of atropine may be related to endogenous release of acetylcholine from cholinergic terminals, which could have a tonic excitatory effect on neuronal activity [10,31,32].
The determination of the prominence of muscarinic receptor subtypes is limited by the only preferential selectivity of available muscarinic subtype antagonists. However, comparisons of the IC₅₀ values for the muscarinic antagonists with their $K_i$ corresponding values enables the suggestion that, as for the DCN [9], the M₂ and M₄ subtypes are relatively more prominent than M₁ and M₃ in the MVN. Although mainly excitatory effects of activation of M₂ and M₄ receptors in the VNC and DCN would disagree with previous literature suggesting that these muscarinic receptors mediate inhibitory neuronal responses [12], this may be indicative of regional differences in the functions of muscarinic receptor subtypes. Our results for pirenzepine support the previous suggestion that the M₁ receptor subtype is not prominent in the MVN [18].
Acknowledgements

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References


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### Table 1
Effects of acetylcholine agonists on firing rates of MVN, SuVN, SpVN, and DCN neurons

<table>
<thead>
<tr>
<th>Location</th>
<th>Total (^a)</th>
<th>Increase (^b)</th>
<th>No change</th>
<th>Decrease (^c)</th>
<th>Mean change of firing rate ± SD (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVN</td>
<td>192</td>
<td>87 (45.3%)</td>
<td>89 (46.4%)</td>
<td>16 (8.3%)</td>
<td>43 ± 71%</td>
</tr>
<tr>
<td>SuVN</td>
<td>30</td>
<td>7 (23.0%)</td>
<td>20 (67.0%)</td>
<td>3 (10.0%)</td>
<td>11 ± 31%</td>
</tr>
<tr>
<td>SpVN</td>
<td>30</td>
<td>4 (13.3%)</td>
<td>25 (83.4%)</td>
<td>1 (3.3%)</td>
<td>13 ± 22%</td>
</tr>
<tr>
<td>DCN</td>
<td>13</td>
<td>10 (77.0%)</td>
<td>3 (23.0%)</td>
<td>0 (0.0%)</td>
<td>94 ± 80%</td>
</tr>
<tr>
<td><strong>Carbachol</strong> (10 µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MVN</td>
<td>18</td>
<td>8 (44.4%)</td>
<td>9 (50.0%)</td>
<td>1 (5.6%)</td>
<td>27 ± 38%</td>
</tr>
<tr>
<td>SuVN</td>
<td>13</td>
<td>2 (15.4%)</td>
<td>9 (69.2%)</td>
<td>2 (15.4%)</td>
<td>-1 ± 33%</td>
</tr>
<tr>
<td>SpVN</td>
<td>13</td>
<td>2 (15.4%)</td>
<td>10 (76.9%)</td>
<td>1 (7.7%)</td>
<td>9 ± 12%</td>
</tr>
<tr>
<td>DCN</td>
<td>8</td>
<td>7 (87.5%)</td>
<td>1 (12.5%)</td>
<td>0 (0.0%)</td>
<td>71 ± 54%</td>
</tr>
<tr>
<td><strong>Muscarine</strong> (10 µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MVN</td>
<td>19</td>
<td>6 (31.6%)</td>
<td>13 (68.4%)</td>
<td>0 (0.0%)</td>
<td>24 ± 27%</td>
</tr>
</tbody>
</table>

\(^a\) Only regular neurons are included.

\(^b\) Increase means change of firing rate to a value at least 30% higher than control.

\(^c\) Decrease means change of firing rate to a value at least 30% lower than control.
Table 2
Comparison of IC\textsubscript{50} and K\textsubscript{i} values for regular neurons in MVN and DCN

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>IC\textsubscript{50} (µM)</th>
<th>MVN</th>
<th>DCN*</th>
<th>K\textsubscript{i} (nM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>M\textsubscript{1}</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>9.20</td>
<td>12.20</td>
<td>443.00</td>
<td>165.00</td>
</tr>
<tr>
<td>AF-DX 116</td>
<td>28.68</td>
<td>920.00</td>
<td>139.00</td>
<td>979.00</td>
</tr>
<tr>
<td>p-f-HHSiD</td>
<td>12.00</td>
<td>55.70</td>
<td>132.00</td>
<td>19.80</td>
</tr>
<tr>
<td>Tropicamide</td>
<td>0.07</td>
<td>53.70</td>
<td>45.70</td>
<td>50.10</td>
</tr>
</tbody>
</table>

* Data and references from Chen et al. [9].
Figure Legends

Fig. 1: Tracings of coronal slices at three rostrocaudal levels of the vestibular nuclear complex (VNC) of a rat. Approximate boundaries of each nucleus were identified by correlating visual landmarks with an atlas of the rat brain [40]. Either one or two nuclei, depending on the rostrocaudal level, were mapped by a series of electrode penetrations alternately in dorsal-to-ventral and ventral-to-dorsal order. Numbers indicate how many neurons were encountered at each penetration site in the superior vestibular nucleus (SuVN), top tracing, medial vestibular nucleus (MVN) and lateral vestibular nucleus (LVN), middle tracing, and spinal vestibular nucleus (SpVN), bottom tracing. Top tracing is most rostral; dorsal is up and lateral right. DCN: dorsal cochlear nucleus; ECu: external cuneate nucleus; g7: genu of facial nerve; icp: inferior cerebellar peduncle; mlf: medial longitudinal fasciculus; PrH: prepositus hypoglossal nucleus; scp: superior cerebellar peduncle; sol: solitary nucleus; sp5: spinal trigeminal tract; VCN: ventral cochlear nucleus. Bar represents 1 mm.

Fig. 2: Densities of spontaneously active neurons in the vestibular nuclei and DCN, represented as mean±S.E.M. Number of neurons (penetrations) for each bar from left to right: 266 (1166), 42 (260), 37 (214), 17 (435), 128 (117). Bursting neurons were excluded from the sample of DCN neurons.
Fig. 3: Changes in mean firing rate during carbachol and muscarine for typical regular neurons in each region. Horizontal bars and vertical lines mark the times of drug application. A. The mean firing rate of a DCN neuron increased 266% in response to carbachol, 188% in response to muscarine; B. The mean firing rate of a medial MVN neuron increased 31% in response to carbachol, 27% in response to muscarine; C. The mean firing rate of a lateral SuVN neuron increased 46% in response to carbachol, 24% in response to muscarine; D. The mean firing rate of a medial SuVN neuron decreased 40% in response to carbachol, 39% in response to muscarine; E. The mean firing rate of a medial SpVN neuron increased 9% in response to carbachol, 11% in response to muscarine; F. The mean firing rate of a lateral SpVN neuron decreased 21% in response to carbachol, 14% (following an initial increase in firing) in response to muscarine. Bar width of plots: 30 s.

Fig. 4: Correlation between responses to carbachol and muscarine for neurons in each nucleus. The response of each neuron is represented as percent increase of mean firing rate from the control level preceding drug application. The dashed line is the least-squares line of best fit. The crossed horizontal and vertical lines mark the control levels (0% response). All regions showed similarly high coefficients of correlation ($r$) between responses to carbachol and muscarine. $n =$ Number of neurons.
Fig. 5: Effects of carbachol (carb), muscarine (musc) and nicotine (nic) on firing rate of an MVN neuron. Each agonist was applied for 3 min. The increase of firing rate to nicotine (34%) was smaller than to the same concentration of carbachol (140%) or muscarine (112%). Bar width of plot is 30 s. Times of drug applications are indicated by vertical and horizontal lines.

Fig. 6: Effects of synaptic block (0.2 mM Ca^{2+}, 7.8 mM Mg^{2+}) and muscarinic (atropine) or nicotinic [mecamylamine (meca), hexamethonium (hex), and tubocurarine (cur)] antagonists on responses of MVN neurons to 10 μM carbachol (carb) or 10 μM nicotine (nic). Times of application of drugs are indicated by vertical and horizontal lines. Bar width: 30 s.

Fig. 7: Effects of different muscarinic antagonists on responses of MVN neurons to 10 μM carbachol (carb) in four different experiments. A: pirenzepine (pir); B: AF-DX 116; C: p-f-HHSiD (HHS); D: tropicamide (trop). In A, B, and C, antagonists were applied for 7 min, starting 4 min before 3 min application of carbachol. In D, antagonist application continued for 4 min after application of carbachol. Bar width of plot is 30 s. Times of application of drugs are indicated by vertical and horizontal lines.
Fig. 8: Concentration-response curves for effects of four different muscarinic antagonists on responses of MVN neurons to carbachol. \( n \), number of tests (number of neurons in parentheses); \( r \), Pearson product–moment correlation coefficient; \( P \) is based on number of tests. Effects of different concentrations of an antagonist on the same neuron are connected by line segments, with different types of lines for different neurons. The solid lines without symbols are least-squares lines of best fit.
Fig. 2
Fig. 3 (A,B,C)
Fig. 3 (D,E,F)
Fig. 4

MEDIAL VESTIBULAR NUCLEUS

SPINAL VESTIBULAR NUCLEUS

n = 13
r = 0.88
p < 0.001

n = 13
r = 0.90
p < 0.001

SUPERIOR VESTIBULAR NUCLEUS

DORSAL COCHLEAR NUCLEUS

n = 13
r = 0.92
p < 0.001

n = 8
r = 0.86
p < 0.01
Fig. 6

- 0.2 mM Ca$^{2+}$, 7.8 mM Mg$^{2+}$
- 1 µM atropine

- 10 µM carb
- 10 µM nic
- 100 µM hex
- 2 µM cur
- 1 µM atropine
Fig. 7 (A, B)
Fig. 7 (C, D)
Fig. 8
Changes of Amino Acid Concentrations in the Rat Vestibular Nuclei after removal of Inferior Cerebellar Peduncle and midline commissural fibers

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Abstract

Using a combination of microdissection of freeze-dried brain sections and HPLC analysis, changes of amino acid concentration in the vestibular nuclear complex (VNC) were studied in rats after inferior cerebellar peduncle (ICP) transection. Distributions of 12 amino acids within the VNC were measured at 2, 4, 7, and 30 days after surgery and compared with the data from control and sham-lesioned rats. Concentrations of GABA decreased by 2 days after unilateral ICP transection in nearly all VNC regions on the lesioned side and showed partial recovery up to 30 days post-surgery. Most VNC regions on the unlesioned side also showed loss of GABA. Only MVNv showed no change on either side. Asymmetries between two sides of the VNC were maintained through 30 days. Changes of taurine concentrations roughly resembled those for GABA in LVN, but they were smaller. Glycine showed a small transient decrease in some regions but an obvious increase in the lesioned-side LVNd starting 4 days after lesion. Glutamate concentrations were reduced bilaterally in all regions of the VNC by 2 days and showed complete recovery in all of VNC regions except ipsilateral SuVN by 30 days. Aspartate changes were roughly similar to those of glutamate. Glutamine asymmetry was generally opposite to that of glutamate in that there was a large increase 2 days after surgery which recovered to control values by 30 days. Following bilateral ICP transection, there were larger reductions in concentration of the six major amino acids, especially GABA and glutamate. These results suggest that amino acid neurotransmitters are involved in plasticity of the VNC after ICP lesion, and that the contralateral ICP may enable compensation after unilateral ICP transection.
Key words: GABA, taurine, glycine, glutamate, aspartate, glutamine, cerebellum
Introduction

The vestibular nuclei and cerebellum are closely related in anatomy and function for the coordination of motor responses, eye movement, and posture. There are extensive bilateral projections from the cerebellum, both cortex and deep cerebellar nuclei, to the vestibular nuclear complex (VNC) (Brodal, 1974; Rubertone and Mehler, 1981). Direct projections that originate from the Purkinje cells in the anterior lobular vermis reach the dorsal part of the lateral vestibular nucleus (LVNd), whereas fibers from the cortex of the flocculonodular lobe and uvula project widely throughout the medial vestibular nucleus (MVN), superior vestibular nucleus (SuVN), and spinal vestibular nucleus (SpVN). Deep cerebellar nuclei, chiefly the medial cerebellar nucleus, send projections to most parts of the vestibular nuclei. The cerebellum also receives inputs from both primary and secondary vestibular fibers. Primary vestibulocerebellar projections are distributed to the lingula, uvula, nodulus, flocculus, and paraflocculus (Brodal and Hoivik, 1964; Carpenter et al., 1972), and degeneration studies (Carpenter, 1960) showed that most of the secondary vestibulocerebellar fibers reciprocate cortico-vestibular projections.

Some amino acids in the central nervous system are considered to be neurotransmitters besides their involvement in intermediary metabolism (Maycox et al., 1990). Many studies have supported the roles of glutamate, $\gamma$-aminobutyric acid (GABA), and glycine as neurotransmitters in the VNC. There is increasing evidence that glutamate is used as an excitatory transmitter by the primary vestibular afferents (Raymond et al, 1988; Lewis et al., 1989; Kinney et al., 1994; Li et al., 1996). Furthermore, glutamate is
considered to be a neurotransmitter for efferent pathways from the deep cerebellar nuclei (DCN). The majority of neurons in the DCN were glutamate-immunoreactive (Batini et al., 1992; Monaghan et al., 1986), and double-labeling studies have shown that projections to some precerebellar nuclei from the lateral and/or interpositus cerebellar nuclei, such as the red nucleus (Giuffrida et al., 1993) and the pontine nuclei (Schwarz and Schmitz, 1997), are glutamatergic. There is longstanding evidence for GABA as a neurotransmitter for the inhibitory input from the cerebellum (De Zeeuw and Berrebi, 1995; Fonnum et al., 1970; Houser et al., 1984; Obata et al., 1969, 1970) and for the inhibitory commissural connection from the contralateral VNC (Precht et al., 1973; Furuya and Koizumi, 1998; Holstein et al., 1999). GABAergic neurons in the VNC (Walberg et al., 1990) may also be involved in inhibitory projections from the VNC to the extraocular motoneurons (Spencer and Baker, 1992) and spinal cord (Blessing et al., 1987). Glycine has been suggested as the inhibitory neurotransmitter of some premotor neurons related to vestibuloocular reflexes (Spencer et al., 1989; Spencer and Baker, 1992), of vestibulospinal projections to motoneurons (Wilson and Jones, 1979; Triller et al., 1987), and of inhibitory commissural projections between the VNCs on each side (Furuya and Koizumi, 1998; Precht et al., 1973). It has been reported that glycine may be colocalized with glutamate in some primary vestibular afferent terminals (Reichenberger et al., 1995).

It is known that the vestibular system shows remarkable plasticity following lesions. This process is poorly understood but may be partly related to interactions between the VNC and cerebellum (McCabe et al., 1973; Smith and Darlington, 1991).
Previous studies of cerebellar lesion effects on the VNC focused on the change of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD) in the lateral vestibular nucleus (LVN) (Fonnum et al., 1970; Houser et al., 1984). One study had limited spatial resolution (Fonnum et al., 1970), whereas the other had limited quantitation (Houser et al., 1984). Therefore, a direct, high-resolution, comprehensive, quantitative study of effects of cerebellar lesions on amino acid concentrations in all the nuclei of the VNC is needed.

Direct measurement of amino acid concentrations in the VNC after unilateral labyrinthectomy (Henley and Igarashi, 1991) or ganglionectomy (Li et al., 1996) has demonstrated dynamic changes of amino acid concentrations during vestibular compensation. Microdissection of freeze-dried sections with analysis of amino acids by high performance liquid chromatography (HPLC) has the advantage that it allows the direct measurement of many amino acids simultaneously within the same samples from the VNC (Li et al., 1996; Ross et al., 1989). The present study found changes in amino acid concentrations in the four major nuclei of the VNC following removal of cerebello-vestibular connections on one or both sides, at several different survival times up to 30 days after transection of the inferior cerebellar peduncle (ICP).

A preliminary report of the results of this investigation has been presented previously (Sun et al., 1998).
Materials and Methods

All procedures and treatment of animals in this study were approved by the Medical College of Ohio Institutional Animal Care and Use Committee and the National Institutes of Health (NIDCD grant DC02550).

Surgery

Male Sprague-Dawley rats, weighing 330 – 350 g, were anesthetized with ketamine hydrochloride (Ketaset, 80 mg/kg) and xylazine (Rompun, 4 mg/kg), administered intramuscularly, and secured in a stereotaxic apparatus. The skin of the skull was incised, and parts of the occipital and parietal bones were removed. The dura and pia were cut open. For the ICP lesion, the main connections from the cerebellum to the vestibular complex were severed by transection of the ICP, on one or both sides, by lowering an L-shaped wire, with its horizontal portion 4 mm long, to the level at which the peduncle joins the brain stem, using stereotaxic coordinates, then turning the wire through 360° (Ross et al., 1983; Godfrey et al., 1985). After the surgery, the skin was sutured. Animals were kept in separate cages, usually woke up 1.5 – 2 hours after surgeries, and survived 2, 4, 7, or 30 days, with five rats per group. Survival times were chosen based on a previous study (Li et al., 1996) in which amino acid concentrations started to change in the VNC 2 days after vestibular ganglionectomy. Antibiotic (Liquamycin, 7 mg/kg, i.m.) was given daily up to 3 days after surgery. Because the animals could not stand up to reach the food and drink, daily injections of 15 ml of 5%
sucrose (subcutaneous) and 15 ml of 0.9% sodium chloride (intraperitoneal) were given. Also, orange slices and chocolate chunks were left in the cage for each animal during the first survival week. Body weights for lesioned animals after one week survival were about 10% less than those before surgeries. After one week, lesioned animals gained weights and gradually returned to pre-surgery levels. Three sham-lesioned rats received the same treatment except without ICP transection and four control rats received no surgery.

Preparation of sections for histology and microdissection

For the analysis of lesion-induced histological changes, each animal was deeply anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and decapitated. The brain was quickly removed and frozen within 3 minutes in Freeze-It (CMS) precooled to its freezing point (about -130 °C) with liquid nitrogen. The frozen brains were stored in a freezer at a – 80 °C until sectioning. Serial transverse sections 20 µm thick were cut in a Hacker cryostat at -20 °C. The sections containing the vestibular nuclei (Paxinos and Watson, 1998) were collected into 2 alternating sets. For histological verification of neuronal structures and evaluation of lesioned areas, one set was melt-mounted onto glass slides and stained with cresyl violet. For microdissection, the other set was placed into 5-hole aluminum racks and then into a vacuum tube for freeze drying at – 40 °C (20-24 hours) (Lowry and Passonneau, 1972). Freeze-dried sections were stored in the vacuum tube below -20 °C until analysed.
Microdissection

The methods used in this study have been described previously (Godfrey and Matschinsky, 1976). A Wild dissecting microscope with a camera lucida was used for microdissection. The outline of the freeze-dried section was traced and the boundaries of the vestibular nuclei identified by reference to adjacent Nissl-stained sections. Samples were dissected freehand from the section with reference to the identified nuclear boundaries under a binocular microscope with razor blade fragments (Lowry and Passonneau, 1972). The outlines of all the samples were traced onto the map of each section. Each sample was weighed on a quartz-fiber microbalance (Lowry and Passonneau, 1972) and loaded to the bottom of a 300 µl-capacity glass tube insert for assay.

HPLC quantification of amino acids

The amino acids were extracted from each sample into 15 µl of 50% (vol/vol) methanol. A pre-column ortho-phthalaldehyde (OPA) derivatization procedure was used (Ross et al., 1995). Aliquots (8µl) were withdrawn by a WISP autosampler and derivatized with 8 µl OPA, then injected onto a reversed-phase C8 column (MAC-MOD Analytical, Inc., Chadds Ford, PA) for separation of the fluorescent-derivatized amino acids by gradient elution. All gradient steps were controlled by a Maxima Chromatography Workstation.
The peaks of fluorescence were measured by a fluorescence detector and the data collected via a computer. The amino acids were identified based on their retention times and their concentrations calculated by comparison with calibrated amino acid standard solutions (Ross et al., 1989; Li et al., 1996).

Data Analysis

The concentration of each amino acid was expressed as millimoles per kilogram of dry tissue weight. The average concentration of each amino acid in each nucleus for each animal was based on 3 to 12 samples. The mean concentrations of amino acids and standard errors of the mean (SEMs) were calculated for the five rats at each survival time, compared with those from control (n = 4) and sham (n = 3) groups. For statistical comparisons, concentrations of amino acids were analyzed using two factor multiple-comparison analysis of variance (SPSS 8.0 software) followed by the posthoc test: Turkey. Differences having P < 0.05 were considered statistically significant.
Results

Histological observations in the VNC after ICP lesion

In all the lesioned animals, as observed in Nissl-stained sections, the ICP on the right side or on both sides (for unilateral or bilateral cuts, respectively) was completely transected just dorsal to the fourth ventricle (Fig. 1). No remarkable morphological changes were found in the Nissl-stained sections, and no obvious differences were noticed for any vestibular region between lesioned and unlesioned sides following the ICP cuts. Gliosis could be seen near the lesion (Fig. 1).

Amino acid concentrations in normal and sham-lesioned rats

The concentrations of six major (Table 1) and six other (Table 2) amino acids were measured in the VNC of control and sham-lesioned rats. There were no statistically significant differences between these two groups for any amino acid in any nuclear region (p > 0.05). The data for control rats were generally consistent among animals and generally in agreement with a previous report (Li et al., 1994). For GABA, relatively high concentrations were found in MVNd and LVNd (Table 1, Fig. 2). GABA levels in LVNd were twice as high as in LVNv, similar to the relative activities of GAD reported previously (Fonnum et al., 1970). Taurine concentrations paralleled GABA concentrations in LVN but were not high in MVNd. Concentrations of glutamate and aspartate didn’t vary as much as GABA among vestibular nuclei. Highest concentrations were in MVNd, and lowest in LVNd (Table 1, Fig. 3).
Changes in amino acid concentrations in the VNC following unilateral ICP transection

Changes in amino acid concentrations in the VNC were found on both the lesioned and unlesioned sides after ICP transection. These are represented in Figures 2-4.

GABA

In comparison with control rats, GABA concentrations were reduced on both sides in all VNC regions except in MVNv bilaterally and LVNv on the unlesioned side (Table 1, Fig. 2). Lesioned-side decreases in most of VNC regions, i.e., MVNd, LVNd, LVNv, SuVN, and SpVN, were statistically significant beginning as early as 2 days following surgery (Table 1), with the largest reduction in LVNd, and lasted through 30 days. All regions showed some recovery toward control values by 7 days after surgery (Fig. 2). On the other hand, unlesioned-side decreases began to recover 4 days after ICP transection, and by 30 days, only in LVNd and SpVN GABA levels were significantly different from control rat values. GABA concentrations in the unlesioned-side MVNv at 7 days after surgery were higher than those for control rats. Unilateral-lesion-induced GABA asymmetries were observed between lesioned and unlesioned sides in all VNC regions. They occurred in LVNd, LVNv, and SpVN at 2 days after surgery, and remained in LVNd, LVNv, and SuVN at 30 days (Fig. 2, 4).
Unlike control rats, with the highest GABA levels in LVNd, the lesioned sides of rats with unilateral ICP transections had highest GABA levels in MVNd, followed by MVNv and SuVN, with levels in LVNd among the lowest. After ICP lesion, the largest GABA losses were in LVNd, followed by LVNv and SpVN; this applied to both the lesioned and unlesioned sides (Fig. 2).

Taurine

Compared with control rats, taurine concentrations decreased bilaterally in LVNd and SpVN and ipsilaterally in LVNv by 2 days after surgery, then showed recovery toward control levels (Table 1). In MVN, taurine concentrations didn’t change significantly, whereas there was a small trend for an increase in the lesioned-side SuVN. In LVNd and SpVN, decreases in taurine concentrations paralleled those of GABA ($r = 0.75, p < 0.01$) but were smaller. Also, asymmetries between lesioned and unlesioned sides were not as consistent as for GABA.

Glycine

Bilateral decreases of glycine concentrations in MVNd, LVNv, and a decrease in SuVN on the lesioned side were found 2 days after unilateral ICP transection (Table 1). Glycine levels in these regions started to recover at 4 days and showed no statistically significant differences through 30 days. Glycine concentration in LVNd on the lesioned side increased 4 days after ICP lesion and remained at elevated levels through 30 days.
Significant asymmetries of glycine concentration between lesioned and unlesioned sides occurred at 4 and 7 days in LVNd (Fig. 4).

Glutamate

Two days after unilateral ICP transection, glutamate concentrations decreased bilaterally in all VNC regions except in the unlesioned side LVNd (Table 1, Fig. 3). In general, reductions of glutamate concentration were more evenly distributed among VNC regions than were those of GABA. They ranged from 24 to 37%, and from 13 to 26% of control for lesioned and unlesioned sides, respectively. Larger losses on the lesioned side occurred in LVNd, LVNv, and SpVN, smaller in MVN. The largest glutamate decreases on the unlesioned side were in SpVN, the smallest in LVNd. Recovery of glutamate concentrations toward control values began 4 days after ICP transection. By 30 days, the only statistically significant difference from control was a 12% reduction in the lesioned-side SuVN. Statistically significant bilateral asymmetries for glutamate concentrations (Fig. 4) were largest in LVNd at 2 and 4 days after surgery and remained uncompensated in SuVN through 30 days.

Aspartate

Like glutamate, aspartate concentrations showed significant bilateral decreases at 2 days after surgery (Table 1) and began to recover 4 days after surgery. There were no statistically significant differences from control by 30 days except bilaterally in SpVN.
As for glutamate, lesion-induced asymmetries (Fig. 4) were largest in LVNd and most persistent in SuVN.

Glutamine

Unlike all other major amino acids, glutamine showed bilateral increases (Table 1) in all regions 2 days after surgery. Levels began to return toward control values starting 4 days after ICP transection and were not significantly different by 30 days. Relatively slow returns toward control were on the lesioned sides of the more dorsally located nuclei, LVNd and SuVN. In most cases, glutamine concentrations on the lesioned side were higher than those on the unlesioned side (Fig. 4).

Other amino acids

Six other amino acids, including serine, threonine, arginine, alanine, asparagine, and tyrosine, have lower concentrations in the VNC regions of normal rats (Table 2). There were some post-surgery increases, especially at 7 days.

Changes in amino acid concentrations in the VNC following bilateral ICP transection

Bilateral ICP transections (n = 2) and sham-lesions (n = 1) were done with a survival time of 7 days. There were no statistically significant differences between the bilaterally sham-lesioned rat and control rats (all $p > 0.05$). After surgery, in general,
concentrations of the six major amino acids were reduced to lower levels than after unilateral transections. Fig. 5 shows comparisons for the two most important neurotransmitters associated with the cerebello-vestibular pathways, GABA and glutamate, with the data from control, sham and unilaterally-lesioned rats. For all VNC regions except SpVN and MVNv, GABA concentration was lower than those for the unilateral transection. The mean glutamate concentration was lower with bilateral transections in all regions except in SpVN. The differences in concentrations for both GABA and glutamate were statistically significant ($p < 0.05$ to $p < 0.001$) for each region except those mentioned above.
Discussion

Amino acids have multiple roles in the central nervous system (CNS); they are involved in energy metabolism, protein synthesis, and neurotransmission (Maycox et al., 1990). Direct assays of free amino acid concentrations in regions of CNS represent their total concentrations, present in all pools, in neuronal somata, dendrites, axons, and terminals, in glia, and in any blood vessels present. It has been reported that only 20-30% of glutamate is associated with glutamatergic nerve terminals (Fonnum, 1993), while the transmitter compartment contains about 30% of GABA (Martin, 1993). Measurements of the distributions of neurotransmitter-related amino acids within a brain region in specific conditions such as after transection of pathways (Minchin and Fonnum, 1979; Fonnum and Henke, 1982) or during induced pathological states (Fonnum and Paulsen, 1990), combined with knowledge about their roles in neurotransmission and in intermediate metabolism, can provide insight into their synaptic regulation.

Amino acids are probably the most prominent neurotransmitters in the CNS from a quantitative point of view since their concentrations are on the order of mmol/kg rather than μmol/kg. (Fykse and Fonnum, 1996). Amino acids have been divided into two general classes with respect to their roles in neuronal transmission, excitatory and inhibitory (Cooper et al, 1996). Of 12 amino acids measured in this study, GABA, taurine, glycine, glutamate, glutamine, and aspartate are much more concentrated than the rest in the VNC of normal rats and have been suggested to be neurotransmitters or closely related to neurotransmitters in the CNS (Cooper et al., 1996; Fonnum and Paulsen, 1990;
Ottersen et al., 1988). In lesioned rats, these amino acids underwent changes during the period of observation and are discussed individually below.

**GABA**

The importance of GABA as a neurotransmitter in the VNC is demonstrated by biochemical (Fonnum et al., 1970), immunohistochemical (Kumoi et al., 1987; Nomura et al., 1984; Walberg et al., 1990), and pharmacological data (Furuya et al., 1998; Obata and Takeda, 1969; Precht et al., 1973). Previous reports have suggested changes of GABA function plays a role in the VNC during vestibular compensation (Calza et al., 1992; Henley and Igarashi, 1991; Li et al., 1996) and in mutant mice (Bäurle et al., 1992, 1997). The most noticeable result of the present study was the marked reduction of GABA concentration in most VNC regions following ICP transection. GABA concentrations gradually recovered on both sides, with a faster rate on the unlesioned side, and asymmetries between the two sides decreased by 30 days.

Our observations on GABA concentrations in the LVN were compatible with previous studies. Immunocytochemical (Houser et al., 1984) and biochemical (Fonnum et al., 1970) studies have shown more GAD dorsally than ventrally in the LVN. In these two studies, anterior lobe ablations reduced GAD-positive terminals by 73% or GAD activity by 50-70% in the LVNd. Concentrations of GABA in the CNS correlate well with GAD activity (Austin et al., 1979; Cooper et al., 1996; Dick and Lowry, 1984; Godfrey et al., 1994; Minchin and Fonnum, 1979). Limited lesions in the cerebellum, such as ablation of
anterior lobe vermis (Fonnum et al., 1970; Houser et al., 1984), result in only partial
deafferentation to the vestibular nuclei because, for examples, LVNd not only receives
major projections ipsilaterally from the anterior lobe vermis but also a less prominent
input from the posterior lobe (Carleton and Carpenter, 1983).

The most direct explanation for decreases in GABA concentrations in VNC
regions on the lesioned-side after ICP transection is that they are directly related to loss
of GABAergic innervation. After lesions of the striato-nigral pathway, the GABA
concentration in rat substantia nigra decreased by 70-80% compared with normal rats
(Minchin and Fonnum, 1979). Besides the primary vestibular nerve afferent fibers, the
quantitatively most important afferents to the VNC come from the vestibulocerebellum
and contain both axons of Purkinje cells of the cerebellar cortex and axons of the deep
cerebellar nuclei neurons, mostly, the fastigial nucleus (Carleton and Carpenter, 1983;
Rubertone and Mehler, 1981; Rubertone et al., 1995). Several studies support GABA as
the inhibitory neurotransmitter of the Purkinje axon terminals. Stimulation of the
cerebellar cortex increased release of GABA, but not of total amino acids, into the fourth
ventricle (Obata and Takeda, 1969). GABA, when applied iontophoretically, could
produce inhibitory postsynaptic potential changes in Deiters' neurons, mimicking the
natural neurotransmitter (Obata et al, 1970). Morphological and biochemical observations
(Fonnum et al., 1970; Houser et al., 1984; De Zeeuw and Berrebi, 1995) also support this
point of view. There have been no clearly quantitative description of distributions of
Purkinje cell axon terminals in the VNC, but two studies (Carleton and Carpenter, 1983;
Hauglie-Hanssenn, 1968) showed patterns of distribution to ipsilateral LVNd, MVN,
SpVN and SuVN which resemble distributions of GABA reductions in our data. Although it is not generally agreed that LVNv receives direct cerebellar cortical projections (Walberg and Jansen, 1961; Rubertone et al., 1995), our finding of loss of GABA in LVNv on the lesioned side supports a projection of cerebellar corticovestibular fibers not only to the LVNd but also to the LVNv (Andersson and Oscarsson, 1978a, b). There is additional evidence from an electrophysiological study (Akaike, 1983): stimulation of the anterior lobe vermis produced inhibition in all regions of the LVN, while stimulation of fastigial neurons had excitatory influences upon neurons in the LVNv. Dense GABA-immunoreactive fibers have been reported in MVNd as well as LVNd (Nomura et al., 1984), but our data showed only a moderate decrease of GABA concentrations in MVNd after ICP surgery. This may reflect that MVNd receives many GABAergic fibers from other extrinsic sources, such as commissural fibers from the contralateral VNC (Holstein et al., 1999; Furuya et al., 1998) and projections from the inferior olivary complex (Balaban, 1988; De Zeeuw and Berrebi, 1995), in addition to GABAergic intrinsic connections.

The rapid GABA reductions in the VNC on the unlesioned side suggest that the Purkinje cell projections through each inferior cerebellar peduncle innervate to some extent contralateral as well as ipsilateral VNC regions. Unilateral ICP transection may also affect the contralateral VNC indirectly at later post-surgery times through effects on commissural connections.
The GABA changes are not likely to have resulted from nonspecific effects of the lesions because no other amino acid showed similar changes. Also, there were no obvious lesion-induced morphological changes in the VNC in our Nissl-stained sections, and no major differences in size of the vestibular nuclei between lesioned and unlesioned sides. Transneuronal degeneration seems unlikely to contribute to the early decreases of GABA concentrations detected at 2 days after surgery (Fonnum and Henke, 1982), but it could contribute to later effects.

Mechanisms underlying the recovery of GABA concentrations in the VNC are not clear. One possibility is an influence from the contralateral VNC. Each VNC receives commissural fibers from the contralateral VNC (Rubertone et al., 1995), which are partly GABAergic (Furuya and Koizumi, 1998; Holstein et al., 1999; Precht et al., 1973). The major difference in effect between unilateral and bilateral lesions would be the influence of fibers traversing the contralateral ICP. These could promote recovery following unilateral ICP transections by an effect on commissural pathways or through a direct effect of fibers sprouting to replace synapses that had derived from the transected peduncle. Our results for bilateral lesions support the proposal that lost synapses are replaced by new GABAergic synapses from contralateral ICP fibers and/or from commissural fibers influenced by contralateral ICP fibers. After bilateral ICP cut, GABA levels in most of the VNC regions decreased to much lower levels at the same survival time (7 days after surgery) than following unilateral lesion. There is evidence that the commissural fiber system and/or cerebellum on the unlesioned side may be affected by
unilateral ICP lesion (McCabe et al, 1973; see review by Ito, 1984). Further study of this possibility is needed.

Taurine

Taurine has been suggested to be co-localized with GABA in the Purkinje cell terminals (Ottersen et al., 1988). The largest change for taurine after unilateral ICP lesion occurred in the LVNd, where there was the largest change for GABA. An immunohistochemical study showed that taurine-immunopositive fibers were more concentrated in LVNd than in LVNv (Walberg et al., 1990). This is consistent with its concentration distribution in our study. Our results support the concept that taurine is a possible modulator of GABA function in Purkinje cell terminals. However, taurine concentration changes in other regions, notably SuVN, did not parallel GABA changes after ICP transection. This suggests that the relation between taurine and GABA is more complicated than just a joint function in Purkinje cell terminals.

Glycine

Glycine has been suggested to be an inhibitory neurotransmitter of many VNC neurons (Spencer and Baker, 1992; Triller et al., 1987; Wilson and Jones, 1979). Besides vestibular nerve fibers (Reichenberger et al., 1995) and commissural fibers (Furuya and Koimuzi, 1998; Precht et al., 1973), there is no evidence for other sources of glycinergetic projections to the VNC. After ICP transection, the small changes of glycine
concentrations in MVNd, LVNv, and SuVN suggest only a minor role in plasticity there. However, the increased glycine concentration in LVNd on the lesioned side 7 days after surgery, continuing through 30 days, may partially compensate (as an inhibitory transmitters) large decrease in GABA concentration. An immunocytochemical study showed a massive increase in the number of glycine-immunoreactive neurons in VNC of Purkinje cell-deficient mutant mice accompanied by an increase in terminal size and density (Bäurle et al., 1997). The source of such new glycine terminals remains to be discovered.

Glutamate

Glutamate has been suggested to be the neurotransmitter for several pathways related to the VNC, such as primary vestibular nerve afferents (Raymond et al., 1988) and some commissural fibers (Cochran et al, 1987). Immunohistochemical studies showed glutamate labeling of both neuronal terminals and somata in the VNC (Walberg et al., 1990). Pharmacological results suggest that glutamate receptors contribute to synaptic function in the VNC normally (Doi et al., 1990; Gallagher et al., 1992; Kinney et al., 1994; Lewis et al., 1989; Smith, et al., 1990) and during vestibular compensation (Smith and Darlington, 1991). Following unilateral ICP transection, the decreases by 24-37% in VNC regions were comparable to 15-30% reduction in the VNC after unilateral vestibular ganglionectomy (Li et al., 1996), about 33% decrease in lateral septum after fornix/fimbria lesion (Fonnum and Walaas, 1978), and 21-34% loss in the dorsal horn of spinal cord after dorsal rhizotomy (Potashner and Dymczyk, 1986). The absence of
significant concentration changes of other amino acids such as serine, threonine, arginine, and asparagine in the VNC at 2 days after surgery argues against the glutamate decreases resulting from some nonspecific effect such as an overall metabolic decline. This rapid change for glutamate is likely to be related to the cerebellar nucleofugal fibers to the VNC. Electrophysiological studies have reported that these projections exert excitatory effects on LVN neurons (Ito, 1984; Wilson and Jones, 1979). The transmitter involved in this pathway could be glutamate (Batini et al., 1992; Giuffrida et al., 1993; Monaghan et al., 1986; Schwarz and Schmitz, 1997). Unilateral ICP caused to some extent damage to hook bundle sending off from the lesioned side to the unlesioned side. Therefore, reduction of glutamate levels on the unlesioned side following unilateral ICP lesion may result from damage to the hook bundle, and possibly through indirect effects through commissural fiber connections and/or through the involvement of the cerebellum (Ito, 1984).

There were remarkable differences between unilateral ICP transections and unilateral vestibular ganglion ablations (Li et al., 1996) in their effects on VNC glutamate concentrations. Whereas unilateral ICP transections led to bilateral reductions in glutamate concentrations at 2 days after surgery and bilateral recovery starting 4 days after surgery, unilateral vestibular ganglionectomies led to only ipsilateral reductions in lost regions at 2 days that showed no later recovery; rather, there were later reductions on the contralateral side in some VNC regions. The rapid recoveries after unilateral ICP transection appear to depend at least partly on fibers entering the VNC from the contralateral ICP because bilateral ICP transection resulted in larger ICP reductions in
glutamate concentrations at 7 days in almost all vestibular nuclei as compared to unilateral transections. Whether glutamatergic fibers from the deep nuclei presumed in the contralateral ICP directly replace synapses that had been made by transected ICP fibers or whether they act by affecting commissural fibers or interneurons, the results suggest a remarkably rapid compensation.

An alternative explanation for the glutamate change would be a remarkably rapid and transient bilateral transneuronal effect of unilateral ICP transection on the glutamate concentrations in VNC neurons. More experiments will be needed to decide among the possibilities.

Aspartate

It was reported (Kumoi et al., 1987) that the VNC contained aspartate-immunoreactive neurons in the guinea pig. Aspartate may be one of neurotransmitters for the primary vestibular nerve fibers (Raymond et al., 1988), but there is no evidence so far about its concentrations in Purkinje cell terminals, although a previous study (Fonnum, 1990) suggested that aspartate is sometimes more prominent in GABAergic rather than in glutamatergic terminals. Our results from lesioned rats showed aspartate concentrations to behave similarly to glutamate concentrations, as also found after removal of vestibular nerve input (Li et al., 1996), although the aspartate concentration changes were relatively larger in our study. Since aspartate and glutamate are metabolically closely related to each other through the action of aspartate aminotransferase (Godfrey, et al., 1994). The
reduction of aspartate concentrations in the VNC after ICP transection could result from the decreased glutamate concentrations.

Glutamine

The changes for glutamine concentrations after ICP transection opposite to those of glutamate in VNC regions are consistent with some previous reports. Following lesions of the striato-nigral pathway (Minchin and Fonnum, 1979) and destruction of dorsal hippocampus (Fonnum and Walaas, 1978), glutamine concentrations increased in substantia nigra and lateral septum. In the VNC after unilateral vestibular ganglion ablation, there was not an increase of glutamine concentrations, but concentrations did become higher ipsilaterally than contralaterally opposite to glutamate (Li et al., 1996). Metabolically, glutamine and glutamate are closely related to each other (Cooper et al., 1996). Glutamine is converted into glutamate by glutaminase in nerve terminals (Godfrey et al., 1994). Glutamine is also an important precursor for the synthesis of GABA through glutamate (Paulsen et al., 1988). Increased glutamine levels in the VNC on both sides after lesion, combined with decreased glutamate and GABA concentrations, could result from decreased glutaminase activity.
Acknowledgements

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References


### TABLE 1. Concentrations of six major amino acids (mmol/kg dry weight) in rat VNC at different survival times after transection of the right ICP

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Control</th>
<th>Sham</th>
<th>2 days</th>
<th>4 days</th>
<th>7 days</th>
<th>30 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L (mmol/kg dry weight)</td>
<td>R (mmol/kg dry weight)</td>
<td>L (mmol/kg dry weight)</td>
<td>R (mmol/kg dry weight)</td>
<td>L (mmol/kg dry weight)</td>
<td>R (mmol/kg dry weight)</td>
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<tr>
<td>GABA</td>
<td></td>
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<tr>
<td>LVNd</td>
<td>18.41 ± 0.49</td>
<td>18.76 ± 0.66</td>
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<tr>
<td>LVNv</td>
<td>8.78 ± 0.49</td>
<td>9.72 ± 0.88</td>
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<tr>
<td>MVNd</td>
<td>16.95 ± 0.44</td>
<td>16.94 ± 0.46</td>
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<tr>
<td>MVNv</td>
<td>8.81 ± 0.29</td>
<td>9.21 ± 0.32</td>
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<tr>
<td>SuVN</td>
<td>13.03 ± 0.31</td>
<td>12.84 ± 0.31</td>
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<tr>
<td>SpVN</td>
<td>11.27 ± 0.51</td>
<td>11.20 ± 0.58</td>
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<tr>
<td>Taurine</td>
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<tr>
<td>LVNd</td>
<td>20.90 ± 0.63</td>
<td>20.64 ± 0.80</td>
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<tr>
<td>LVNv</td>
<td>10.10 ± 0.52</td>
<td>10.44 ± 0.76</td>
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<tr>
<td>MVNd</td>
<td>10.14 ± 0.22</td>
<td>9.97 ± 0.27</td>
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<tr>
<td>MVNv</td>
<td>7.29 ± 0.16</td>
<td>7.43 ± 0.14</td>
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<tr>
<td>SuVN</td>
<td>8.67 ± 0.17</td>
<td>8.33 ± 0.16</td>
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<tr>
<td>SpVN</td>
<td>9.46 ± 0.36</td>
<td>8.92 ± 0.44</td>
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<tr>
<td>Glutamine</td>
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<tr>
<td>LVNd</td>
<td>22.52 ± 0.39</td>
<td>20.64 ± 0.56</td>
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<tr>
<td>LVNv</td>
<td>26.82 ± 0.78</td>
<td>26.23 ± 0.92</td>
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<tr>
<td>MVNd</td>
<td>34.76 ± 0.54</td>
<td>33.02 ± 0.50</td>
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<td>MVNv</td>
<td>29.11 ± 0.70</td>
<td>28.90 ± 0.61</td>
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<tr>
<td>SuVN</td>
<td>32.01 ± 0.65</td>
<td>29.22 ± 0.49</td>
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<tr>
<td>SpVN</td>
<td>29.06 ± 0.58</td>
<td>27.11 ± 0.31</td>
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<tr>
<td>Aspartate</td>
<td></td>
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<tr>
<td>LVNd</td>
<td>13.75 ± 0.35</td>
<td>12.38 ± 0.45</td>
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<td></td>
</tr>
<tr>
<td>LVNv</td>
<td>11.38 ± 0.23</td>
<td>10.52 ± 0.24</td>
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Data are presented as mean ± SE. Averages are based on number of rats, which was 4 for control, 3 for sham, and 5 for all other groups except 4 for glutamine at 2 days.

\( ^a p < 0.05, ^b p < 0.01, ^c p < 0.001 \) for comparison with control data.
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Data are presented as mean ± SE. Averages are based on number of rats, which was 4 for control, 3 for sham, and 5 for all other groups except 3 for arginine at 7 days.

* p < 0.05, ** p < 0.01, and *** p < 0.001 for comparison with control data.
Figure legends

Fig. 1 Photograph of the lesion resulting from an inferior cerebellar peduncle transection in a transverse section. L. Left. R. Right. CB. Cerebellum; Arrow points to the lesion. MVN and LVN can be seen in this section. Bar represents 1 mm.

Fig. 2. Maps of GABA concentrations in transverse sections at three rostral (top) to caudal (bottom) levels (dorsal is up and right is right) of the vestibular nuclei from a control rat, a sham-lesioned rat, and rats with survival times of 2, 4, 7, and 30 days after transection of the inferior cerebellar peduncle on the right side. Dotted lines show boundaries of the different vestibular nuclei. Solid lines represent cuts in microdissection of samples. Concentrations are pattern-coded. The ? symbols represent samples for which no data were obtained.

Fig. 3 Maps of glutamate concentrations in transverse sections at three rostral (top) to caudal (bottom) levels of the vestibular nuclei from a control rat, a sham-lesioned rat, and rats with survival times of 2, 4, 7, and 30 days after transection of the inferior cerebellar peduncle on the right side. Details as in Fig. 2.
Fig. 4 Comparisons of six amino acid concentrations in individual vestibular nuclei between the lesioned and unlesioned sides after unilateral cerebellar peduncle transection. Amino acid concentrations on lesioned sides were presented as percent changes of differences compared with the unlesioned sides. Means ± SE were averaged from five rats in each group. For paired t test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 5 Means plus standard errors of GABA and glutamate concentrations in the vestibular nuclei of two rats with 7 days survival after bilateral inferior cerebellar peduncle transection, compared to controls, one-side sham-lesioned rats, and rats with unilateral transections at 7 days survival times.
Figure 2  Control
Figure 2  Sham
Figure 2  2 d
Figure 2 4 d
Figure 3 Sham
Figure 3  2 d
Figure 3 4 d
Figure 3  7 d
Figure 3  30 d
Figure 4
Figure 5
Plasticity of γ-aminobutyrate receptors in the medial vestibular nucleus of rat after inferior cerebellar peduncle transection

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Abstract

Extracellular single unit recordings were made from regularly discharging medial vestibular nucleus neurons in brain slices from control rats and from rats surviving 7 days after bilateral transection of the inferior cerebellar peduncle. Decreases in firing rate during perfusion with the $\gamma$-aminobutyric acid (GABA) agonists, muscimol ($\text{GABA}_A$) and baclofen ($\text{GABA}_B$), were greater in lesioned rats than in control rats. For the grouped data, the half-maximally-effective concentrations of muscimol and baclofen were 3.2 $\mu$M, as compared with 19.6 $\mu$M for control, and 0.8 $\mu$M, as compared with 2.7 $\mu$M for control, respectively. The antagonists bicuculline ($\text{GABA}_A$) and 2-OH-saclofen ($\text{GABA}_B$) only minimally affected the spontaneous firing rates of neurons in lesioned rats, significantly less than in control rats. The data suggest that the decreases of endogenous GABA levels in the medial vestibular nucleus after inferior cerebellar peduncle transection are accompanied by up-regulation of $\text{GABA}_A$ and, to a lesser extent, $\text{GABA}_B$ receptors.

Keywords: GABA$_A$ receptor, GABA$_B$ receptor, Muscimol, Baclofen, Bicuculline, 2-OH-saclofen
1. Introduction

In the central nervous system (CNS), the neurotransmitter γ-aminobutyrate, or γ-aminobutyric acid (GABA), mediates much of the inhibitory synaptic transmission, and its physiological functions are elicited mostly through ionotropic receptors designated GABA<sub>A</sub> and metabotropic receptors designated GABA<sub>B</sub>. GABA<sub>A</sub> receptors are ligand-gated chloride channels, whereas GABA<sub>B</sub> receptors are G protein-coupled and modulate Ca<sup>2+</sup> and /or K<sup>+</sup> conductances by affecting adenylate cyclase activity [6,11].

GABA has been shown to be involved in the control of vestibular function [35,36,43,44,48,57,60]. The vestibular nuclear complex (VNC) is a major site of action. Anatomical studies have revealed a dense innervation of all vestibular nuclei by GABA-immunoreactive fibers [61], and high GABA concentrations have been measured in vestibular nuclei [35]. In situ hybridization and immunocytochemical techniques have suggested that vestibular neurons possess both GABA<sub>A</sub> and GABA<sub>B</sub> receptors, and that 30% of medial vestibular nucleus (MVN) neurons express glutamate decarboxylase (GAD), the enzyme for GABA synthesis [24,25,60]. Pharmacological studies in brain slices found that MVN neurons were inhibited by GABA through both GABA<sub>A</sub> and GABA<sub>B</sub> receptors [16,37,53,58,59], and changes of efficacy for both GABA<sub>A</sub> and GABA<sub>B</sub> receptors have been suggested in MVN neurons during vestibular compensation [29,65].
The VNC has connections with numerous other parts of the CNS, including an important connection with the cerebellum through the inferior cerebellar peduncle (ICP) [7,8,49]. The VNC is a major target of GABAergic cerebellar projections. In addition, there are many commissural connections, especially between the two MVNs, and intrinsic connections, some of which appear to utilize GABA as a neurotransmitter [18,25,28,43,44,60].

The mechanisms underlying motor deficits associated with cerebellar disorders are not well understood. Because of their interconnections, pathological changes in the cerebellum should lead to changes in the VNC. Ablation of the anterior vermis of the cerebellum resulted in a large reduction of GAD in the lateral vestibular nucleus (LVN) of cat [17] and rat [26]. Experiments have also suggested remarkable changes of GABAergic terminals in the LVN in both mutant mice and hemicerebellectomized wild types [4,5]. The cerebellum also imposes an inhibition on MVN neurons contralateral to a unilateral labyrinthectomy at a critical stage of vestibular compensation, thereby reducing the asymmetry of activity [39].

We have previously found decreased GABA concentrations in the four major vestibular nuclei after unilateral or bilateral transection of the ICP [54,55]. We hypothesized that these decreases, which presumably at least mostly result from transection of GABAergic Purkinje cell axons, may lead to changes of GABA receptors in VNC nuclei. Although the decreases of GABA concentrations were largest in the LVN, that nucleus was not favorable for study in our slice preparation because we have
found spontaneously active neurons to be extremely sparse there [56]. Spontaneously active neurons are much more prevalent in the MVN [56], wherein GABA concentrations were reduced by about a third one week after bilateral ICP transaction ([54] and unpublished results). The purpose of this study was therefore to investigate GABA receptors in the MVN of brain slice preparations after bilateral transection of the ICP, at a survival time when there should be rather complete degeneration of the transected fibers, to increase understanding of cerebello-vestibular interactions and the underlying mechanisms for vestibular deficiencies which occur in patients suffering from cerebellar disorders [13,62].

Preliminary results of this study have been reported in abstract form [54].
2. Experimental procedures

All experimental procedures were in accordance with the NIH guide for care and use of laboratory animals and approved by the Medical College of Ohio Institutional Animal Care and Use Committee.

2.1. Inferior cerebellar peduncle (ICP) transection

Adult Sprague-Dawley rats, usually males of 330–350 g body weight, were anesthetized with ketamine (Ketaset, 80 mg/kg) and xylazine (Rompun, 4 mg/kg), administered intramuscularly, and secured in a stereotaxic apparatus. The skin over the skull was incised, and parts of the occipital and parietal bones were re-moved. After cutting the dura and pia, transection of the ICP on both sides was completed by lowering an L-shaped 0.375 mm-diameter wire, with the 2.0 mm-long horizontal part of the wire pointed laterally, to the level at which the peduncle joins the brain stem, using stereotaxic coordinates, then turning the wire through 360 (Fig. 1) [19]. After the surgery, the skin was sutured. Animals were kept in separate cages for 7 days until decapitation. During this time, because the rats had difficulty moving to obtain food and water, daily injections of 5% sucrose (10 ml subcutaneous) and 0.9% saline (10 ml intraperitoneal) were given for the entire 7 days. Also, antibiotic (Liquamycin, LA-200, Pfizer, New York, 0.1 ml/kg intramuscularly) was given for the first 3 days.
Sham lesions were not included since our previous study indicated that they did not affect amino acid chemistry in the VNC [55]. Reconstruction of lesion locations could not be done for the animals in this study because of the procedures for preparation of slices, including separation of the cerebellum from the brain stem. However, our previous experience with these transections [54,55] indicated that our procedure consistently produced complete interruption of the ICP.

2.2. Electrophysiology and pharmacology

For preparation of brain slices, each rat was anesthetized with sodium pentobarbital (52 mg/kg, i.p.) and decapitated. The brain stem was exposed while rinsing the head in chilled oxygenated artificial cerebrospinal fluid (ACSF), then rapidly removed and immersed in chilled ACSF prebubbled with 95% O₂ and 5% CO₂. The overlying cerebellum was removed, and a block of tissue containing the VNC was glued to the cutting stage of a vibratome and serially sectioned into 350–500 µm-thick transverse or horizontal slices. Slices containing the MVN were immediately transferred onto a nylon mesh in an interface chamber that was perfused at about 1.2 ml/min with ACSF consisting of (mM): KH₂PO₄ 1.25, KCl 5.0, MgSO₄ 2.0, CaCl₂ 2.0, NaCl 124, glucose 10 and NaHCO₃ 26. The interval of time between decapitation and placement of slices into the chamber was about 13–15 min. Humidified gas flowed continuously over the slices. Temperatures of ACSF and gas were maintained at 32–34 °C by a temperature-controlled water bath.
After a recovery period of 1.5–2 h, slices were explored with 2–3 µm tip diameter glass micropipettes filled with 1 M NaCl. Extracellular discharges of spontaneously active neurons in the VNC were displayed, recorded on magnetic tape, and also led into a digital computer interface for storage of continuous counts of discharges in consecutive 1 sec periods. Spike counts and measurements of intervals were done before, during, and after bath application of drugs, with a time resolution of 0.1 msec, and saved as binary files for further calculations. Firing rates were plotted as the average for each 30 sec period vs. time. Boundaries of the MVN were determined visually with a dissecting microscope using both transmitted and reflected light [56,63], with reference to an atlas of the rat brain [47].

One to four different concentrations of drugs were applied into the ACSF and tested on each recorded neuron. They were selected through a series of stopcocks, with an approximately one-minute time delay between the change of a solution and 50% rise of concentration in the chamber [20]. Agonists and antagonists used (all from RBI) were the GABA_A agonist muscimol hydrobromide (0.3–100 µM), the GABA_A antagonist (-)-bicuculline methochloride (10–20 µM), the GABA_B agonist (±)-baclofen (0.1–10 µM), and the GABA_B antagonist 2-OH-saclofen (100–200 µM). GABA (0.1–5 mM, from Sigma), used in a few cases, was freshly prepared before each experiment. When the blocking of agonist effect by an antagonist was tested, the antagonist was applied for 3–6 min before and throughout the time of application of the agonist.

2.3. Data analysis
All data are from recordings of regularly firing neurons in the MVN, which comprise the vast majority of those present in slices [56]. Mean firing rates of MVN neurons were compared before and after application of GABA-related chemicals. The magnitude of each drug effect was calculated as the firing rate during the 30 sec period showing maximal change from control within 6 min of the start of drug application, divided by the average firing rate for the 3 min period immediately preceding drug application. The concentration of each agonist which reduced the average firing of the tested group of neurons to half was designated as $I_{50}$. Statistical comparisons were made by t-tests, with significance taken as $p<0.05$, but taking into account the Bonferroni correction for multiple comparisons [46]. Statistical comparisons of linear regressions were made by t-tests as described by Zar [66].
3. Results

3.1. Comparisons of mean baseline firing rate between control and lesioned groups

The results of this study are based on recordings from 87 neurons in slices from 44 control rats and 69 neurons in slices from 30 lesioned rats. The spontaneous, or baseline, firing rates of these neurons ranged from 4.8 to 40 spikes/s, with most having firing rates between 10 and 30 spikes/s. There were no statistically significant differences in mean spontaneous firing rates among the different neuron groups. Mean ± S.E.M. baseline firing rates for neurons tested with GABA_A agonists or antagonists were 20.4 ± 0.8 for control and 19.1 ± 1.8 for lesioned rats. For GABA_B agonists or antagonists, baseline firing rates were 19.8 ± 1.0 for control and 20.3 ± 1.6 for lesioned rats.

3.2. Effect of GABA in control rats

Results with the endogenous agonist, GABA, were less consistent than those with its analogs, even with slices prepared from unanesthetized rats. GABA usually caused a decrease in firing rate of MVN neurons, but sensitivity varied considerably among neurons. The average percentage decrease in firing for 5 out of 7 MVN neurons during 0.1–5 mM GABA was, as mean ± S.E.M., 63 ± 16. Two neurons showed no change during bath application of GABA. Because more consistent results were obtained with GABA agonists than with GABA itself, experiments on lesioned rats did not include GABA.
3.3. Effects of GABA receptor agonists and antagonists in control rats

The GABA\textsubscript{A} receptor agonist muscimol was applied to 14 different neurons in control rats, at 2–4 different concentrations ranging from 3 to 100 µM. All the neurons responded to muscimol, but with different sensitivities. During muscimol, there was a reduction in firing rate, which gradually recovered after washout of the drug from the recording chamber (Fig. 2(A)). The concentration for reduction of average firing rate to half in the control group (I\textsubscript{50}) was estimated as 19.6 µM.

For the GABA\textsubscript{B} receptor agonist baclofen, a concentration-response relationship was also established. All 33 neurons tested for their response to superfusion of baclofen, at concentrations of 0.3–10 µM, were inhibited. During baclofen, firing rate decreased more rapidly than during muscimol, and this effect was quickly reversible after washout of the drug (Fig. 2(B)). Repeated applications of baclofen at the same concentration produced similar responses. The I\textsubscript{50} for the control rats was estimated as 2.7 µM.

To test whether inhibitory effects of muscimol and baclofen on spontaneously active neurons in the MVN were through a postsynaptic mechanism, muscimol (n = 4) or baclofen (n = 4) was applied after 8 min of perfusion with 0.2 mM Ca\textsuperscript{2+} /7.8 mM Mg\textsuperscript{2+} ACSF to block synaptic vesicle release [63]. An additional 9 neurons were tested with 0.2 mM Ca\textsuperscript{2+} /7.8 mM Mg\textsuperscript{2+} ACSF alone. Perfusion with 0.2 mM Ca\textsuperscript{2+} /7.8 mM Mg\textsuperscript{2+} ACSF had no obvious effect on firing pattern and, in about half the neurons, had a relatively small effect on firing rate (< 25% change, Fig. 3(A)). For about a quarter of the neurons,
there was an increase in firing rate during 0.2 mM Ca$^{2+}$/7.8 mM Mg$^{2+}$ ACSF, and for the remaining quarter a decrease (Fig. 3(A)). For 6 of the 8 neurons tested with muscimol or baclofen in 0.2 mM Ca$^{2+}$/7.8 mM Mg$^{2+}$ ACSF and in normal ACSF, the reduction of firing during the GABA agonist was similar in both media (effect in 0.2 mM Ca$^{2+}$/7.8 mM Mg$^{2+}$ 82–99% of control effect, Fig. 3(B)). Two neurons showed a larger effect of the agonist in 0.2 mM Ca$^{2+}$/7.8 mM Mg$^{2+}$, one each for muscimol and baclofen (Fig. 3(B)). Overall, these results suggest that the actions of both muscimol and baclofen on MVN neurons are mostly postsynaptic.

The selective GABA$_A$ receptor antagonist bicuculline blocked the responses of MVN neurons to muscimol ($n = 4$) (Fig. 2(A)). In all these cases, once the spontaneous activity had returned to baseline after muscimol, the effects of muscimol were retested in the presence of bicuculline. The neuron shown in Fig. 2(A) decreased its firing rate to 60% of baseline in response to 20 µM muscimol. During 20 µM bicuculline, the mean firing rate increased to 130% of control. This firing rate continued when 20 µM muscimol was applied, then it decreased to the control level after return to control ACSF. Bicuculline applied alone led routinely to a rapid increase in firing rate of MVN neurons that was reversible on washout: for 11 neurons studied with 20 µM bicuculline, firing rates increased to 127 ± 4% (mean ± S.E.M.) of basal values (range 109–159%).

The selective GABA$_B$ receptor antagonist 2-OH-saclofen antagonized the responses of MVN neurons to baclofen ($n = 3$). The neuron shown in Fig. 2(B) decreased its firing rate to 48% of control in response to 3 µM baclofen. During 200 µM 2-OH-
saclofen, the mean rate increased to 134% of control. When 3 µM baclofen was applied in the presence of saclofen, the response was reduced and delayed. Application of 2-OH-saclofen alone led routinely to a rapid increase in firing rate of MVN neurons: for 12 neurons tested with 200 µM 2-OH-saclofen, firing rates increased to 123 ± 2% (mean ± S.E.M) of basal values (range 107–133%).

3.4. Effects of GABA receptor agonists and antagonists in lesioned rats

We compared the responses of MVN neurons to GABA receptor agonists and antagonists in slices from rats with bilateral ICP transection to those from control rats. Concentration-response relationships of MVN neurons for GABA agonists were often shifted so that comparable responses were obtained at lower concentrations for lesioned-rat neurons as compared with control-rat neurons (Fig. 4). Responses to GABA receptor antagonists showed the opposite trend: responses of MVN neurons in slices from lesioned rats were routinely less than in those from control rats (Fig. 5).

For 30 lesioned-rat MVN neurons to which muscimol was applied (concentration range 1–10 µM) and 24 to which baclofen was applied (concentration range 0.3–3 µM) to study concentration-response relations, I_{50}s for control and lesioned groups were determined from least-squares lines of best fit (Fig. 6). I_{50}s for muscimol and baclofen in the lesioned group were lower than those in the control group: 3.2 µM for muscimol, as compared with 19.6 µM in control rats, and 0.8 µM for baclofen, as compared with 2.7 µM in control rats. Differences between lesioned and control rat data at the same
concentrations were highly significant for muscimol, as was the difference between the elevations of the linear regression lines. The differences between lesioned and control rats at individual concentrations did not reach more than a marginal level of statistical significance for baclofen, but the difference between the elevations of the linear regression lines was highly significant. These results suggest increased potency of the agonists in the lesioned rats.

When GABA receptor antagonists were tested on MVN neurons, bicuculline at 20 µM (n = 8) and 2-OH-saclofen at 200 µM (n = 7), the mean responses in lesioned rats were 110% of baseline for bicuculline, as compared with 127% in control rats, and 110% of baseline for 2-OH-saclofen, as compared with 123% in control rats (Fig. 7). Both differences were statistically significant.
4. Discussion

The present study confirms the presence of numerous spontaneously active neurons in the rat MVN *in vitro* [14,37]. As with previous MVN slice studies of others, we can not be sure of the morphological characteristics of the neurons in our study, including particularly their innervation densities from Purkinje cell axons. Heterogeneity with regard to this innervation density may partially underlie the diversity of sensitivities to GABA agonists and antagonists among the neurons, although the existence of other GABAergic inputs besides those from Purkinje cells, such as from interneurons and commissural connections, complicate any conclusions. Our evidence that most of the effects of the GABA agonists do not depend on synaptic activity suggests that they are predominantly related to GABA receptors on the neurons being recorded from and not receptors on other neurons that then influence the recorded neurons. The comparable rates and patterns of spontaneous activity in the neuron samples from control and lesioned rats support an assumption of similar relative amounts of different neuron types in the sampled populations from the lesioned and control rats.

Activation of both GABA\(_A\) and GABA\(_B\) receptors by their selective agonists, muscimol and baclofen, respectively, reduced the firing of the MVN spontaneously active neurons more consistently than GABA itself, in agreement with findings from other groups [16,27,59]. Our results concerning the persistence of inhibitory effects of muscimol and baclofen on MVN neurons in 0.2 mM Ca\(^{2+}\) /7.8 mM Mg\(^{2+}\) ACSF are in agreement with a previous report that both GABA\(_A\) and GABA\(_B\) receptors occur on
postsynaptic membranes of MVN neurons [16], although there also are reports of GABA<sub>B</sub> receptors on presynaptic terminals of GABAergic fibers in the MVN [16,24]. As in our study, low Ca<sup>2+</sup>/high Mg<sup>2+</sup> has previously been found to result in either increased, decreased, or no change in firing rate of MVN neurons [37] as well as regularly firing neurons of the dorsal cochlear nucleus [63]. Since the low Ca<sup>2+</sup>/high Mg<sup>2+</sup> should block all synaptic input to the neurons, these results suggest that their spontaneous activity is maintained by an endogenous mechanism that is modulated by synaptic input [37].

Neurons whose predominant synaptic input is excitatory should decrease firing in low Ca<sup>2+</sup>/high Mg<sup>2+</sup>, whereas neurons whose predominant synaptic input is inhibitory should increase firing in low Ca<sup>2+</sup>/high Mg<sup>2+</sup> ACSF. The two neurons in our study which showed an increased effect of muscimol or baclofen in low Ca<sup>2+</sup>/high Mg<sup>2+</sup> ACSF were both ones that had increased spontaneous firing in low Ca<sup>2+</sup>/high Mg<sup>2+</sup>. The increased effect of muscimol or baclofen in these neurons in low Ca<sup>2+</sup>/high Mg<sup>2+</sup> might therefore represent greater sensitivity to the agonists when the release of endogenous GABA, which would otherwise occupy some of the receptors, is decreased. To the extent that the effects of the agonists were presynaptic, they should have been reduced in the low Ca<sup>2+</sup>/high Mg<sup>2+</sup> ACSF, but this occurred to only a small degree in most of the neurons tested.

The potencies for muscimol (I<sub>50</sub> 19.6 µM) and baclofen (I<sub>50</sub> 2.7 µM) in our study are not very different from those reported previously (as EC<sub>50</sub>s) for MVN neurons [65]: 9–13 µM for muscimol and 3–5 µM for baclofen. They are also comparable to EC<sub>50</sub>s reported elsewhere in the rat CNS: 17 µM for muscimol in hippocampus [12] and 3.6 and 4 µM for baclofen in striatum and substantia nigra and cerebellum [50,64]. Both the
GABA$_{	ext{A}}$ antagonist bicuculline and the GABA$_{	ext{B}}$ antagonist 2-OH-saclofen, applied at the concentrations at which they blocked the inhibitory effects of their corresponding GABA receptor agonists, increased the basal firing rates of MVN neurons. This suggests that endogenous GABA release in the slices tonically inhibits MVN neurons through both GABA$_{	ext{A}}$ and GABA$_{	ext{B}}$ receptors, in agreement with a previous study [16]. Although our data for bicuculline (GABA$_{	ext{A}}$) resemble those of another report [37], their results for phaclofen as a GABA$_{	ext{B}}$ antagonist differ from ours for 2-OH-saclofen. This discrepancy may result from the different antagonists and concentrations used. It has been reported that 2-OH-saclofen has a higher selectivity for GABA$_{	ext{B}}$ receptors than does phaclofen [32].

One of the very important GABAergic inputs to the VNC comes from the cerebellum [17,43,44,60]. The MVN receives direct projections from lobules VIII, IX, and X [7,49], and we have found GABA concentrations decreased by about a third in the MVN after bilateral ICP transection ([54] and unpublished results). Our results for GABA antagonists in the present study suggest that a tonic influence of endogenously released GABA on spontaneous activity in MVN neurons is reduced after ICP transection, so that there is less ongoing GABA inhibition for the antagonists to block. This could also contribute to the increased potency of the GABA agonists after the lesions, since less of the neuronal GABA receptors would be occupied by endogenous GABA. Endogenous release of GABA from nerve terminals has been reported in brain slices of hippocampus [45] and substantia nigra [30]. The release of endogenous GABA in substantia nigra brain slices was reduced by 80% following a striatal lesion [31].
results add to the evidence for GABAergic inputs to the MVN from the cerebellum [15] and suggest that ongoing activity of these inputs modulates the spontaneous activity of MVN neurons. The decreased GABA input after ICP transection appears to be balanced by the increased sensitivity of the GABA receptors so that there is little overall change in rates of spontaneous activity.

Plasticity in the CNS may involve various cellular mechanisms, including the modulation of synaptic efficacy. The changes of GABA receptor function in the VNC in our study may result from the loss of GABAergic synapses from the cerebello-vestibular pathway. After bilateral ICP transection, MVN neurons displayed increased sensitivity to GABA agonists, particularly the GABA_A agonist muscimol. Since the concentration of GABA decreased in the MVN after ICP transection [54,55], it is reasonable to suggest that denervation supersensitivity [11] might be one of the mechanisms responsible. It has been reported that up- or down-regulation of GABA receptor subunits occurs following lesions in rat cerebral cortex [40] and in monkey visual cortex [23]. In hippocampus, an upregulation of GABA_A postsynaptic receptor density led to enhanced synaptic efficacy [42]. Increased densities of GABA_A receptor binding sites have been reported in the spinal cord dorsal horn after peripheral neurectomy [10] and in other regions during lesion-induced plasticities [34,41,51]. A similar effect may occur for GABA_B receptors. Up- or down-regulations of GABA_B receptors were suggested in different regions of the CNS after pretreatment with a GABA_B agonist or antagonist [38]. Since the GABA_B receptor is G-protein-coupled [6], alterations of G proteins could be involved in its plasticity [2,3].
GABA has been suggested to be involved in plasticity in the VNC during vestibular compensation [9,29,36,57,65], and in certain types of mutations in mice [4,5,21,22]. Changes in sensitivity of MVN neurons to GABA could facilitate recovery of the imbalance in excitability of the vestibular nuclei bilaterally after unilateral labyrinthectomy [52,58]. Previous studies have found decreased MVN neuron responses to muscimol and baclofen after unilateral labyrinthectomy [29,65], which persisted through one week for baclofen [29]. This evidence for downregulation of GABA receptors after removal of excitatory vestibular nerve input contrasts with our evidence for upregulation, of similar magnitude, after removal of inhibitory cerebellar input. In line with previous evidence for major cerebellar influences on vestibular compensation [1,33,39], our results provide further support for important roles of cerebello-vestibular projections in mechanisms of plasticity in the MVN.
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Figure Legends

Fig. 1. Schematic drawing of transverse section of the cerebellum and brain stem showing the lesion placements (thick horizontal lines) for bilateral transection of the ICP, or restiform body (RB). Other abbreviations: 7n, facial nerve root; CB, cerebellum; CN, cochlear nucleus; g7, genu of facial nerve; MVN, medial vestibular nucleus (approximate lateral boundary indicated on the left by dotted line); sp5, spinal trigeminal tract. Scale bar: 2 mm.

Fig. 2. Effects of the GABA$_A$ and GABA$_B$ receptor antagonists bicuculline (Bic) and 2-OH-saclofen (2-OH-Sac) on responses to muscimol (Mus) and baclofen (Bac) of 2 MVN neurons in slices from control rats. Times of application of drugs in the perfusion medium are marked by horizontal and vertical lines. Bar width of each histogram plot is 30 sec.

Fig. 3. Histograms showing effects of perfusion with 0.2 mM Ca$^{2+}$/7.8 mM Mg$^{2+}$ ACSF on spontaneous firing rates of MVN neurons (A) and on effects of muscimol (10 or 30 µM) and baclofen (1 or 3 µM) on firing rates (B). Each bar represents the number of neurons showing a percentage firing rate or change in firing rate within the range represented by the numbers on the horizontal axis. In A, gray bars represent neurons also
tested later with muscimol or baclofen (B), whereas black bars represent neurons tested
only with 0.2 mM Ca\(^{2+}\)/7.8 mM Mg\(^{2+}\) ACSF for comparison to control ACSF. In B, gray
bars represent neurons tested with muscimol, whereas black bars represent neurons tested
with baclofen. The neuron whose muscimol effect was increased by 31% (B) had a 22%
increase in baseline firing rate in 0.2 mM Ca\(^{2+}\)/7.8 mM Mg\(^{2+}\) ACSF (A). The neuron
whose baclofen effect was increased by 48% (B) had a 27% increase in baseline firing
rate in 0.2 mM Ca\(^{2+}\)/7.8 mM Mg\(^{2+}\) ACSF (A).

Fig. 4. Firing rates of MVN neurons of control and lesioned rats during muscimol (Mus)
and baclofen (Bac). Bar width of each histogram plot is 30 sec. Times of application of
drugs in the perfusion medium are marked by horizontal and vertical lines. A: Responses
of MVN neuron in a control rat to 1–2 mM GABA and 10–100 µM muscimol. B:
Responses of MVN neuron in a lesioned rat to 1–10 µM muscimol. C: Responses of
MVN neuron in a control rat to 1–3 µM baclofen. D: Responses of MVN neuron in a
lesioned rat to 0.1–1 µM baclofen.

Fig. 5. Comparisons of the effects on MVN neurons of control and lesioned rats of the
GABA\(_A\) agonist muscimol (Mus) and antagonist bicuculline (Bic) and of the GABA\(_B\)
agonist baclofen (Bac) and antagonist 2-OH-saclofen (2-OH-Sac). Times of application
of drugs in the perfusion medium are marked by horizontal and vertical lines. Bar width
of each histogram plot is 30 sec. A: Responses to 10 µM muscimol and 20 µM
bicuculline in a control rat. B: Responses to 10 µM muscimol and 20 µM bicuculline in a lesioned rat. C: Responses to 3 µM baclofen and 200 µM 2-OH-saclofen in a control rat. D: Responses to 1 µM baclofen and 200 µM 2-OH-saclofen in a lesioned rat.

Fig. 6. Average concentration-response linear regressions for the GABA\textsubscript{A} agonist muscimol (A) and the GABA\textsubscript{B} agonist baclofen (B). Data, as mean ± S.E.M., were pooled from all experiments for control rats (•) and lesioned rats (○). The numbers of measurements averaged for each data point are: muscimol, control (14 neurons), 6, 11, 11, and 8 for 3, 10, 30, and 100 µM; muscimol, lesioned (30 neurons), 11, 19, and 20 for 1, 3, and 10 µM; baclofen, control (33 neurons), 5, 7, 31, and 5 for 0.3, 1, 3, and 10 µM; baclofen, lesioned (24 neurons), 11, 17, and 10 for 0.3, 1, and 3 µM. Solid or dotted lines extending to the abscissa or ordinate are least-squares lines of best fit for control and lesioned groups, respectively. Scaling for the abscissa is logarithmic. The horizontal line represents 50% of baseline firing rate, and \textit{I}_{50}s were determined by its intersections with the regression lines for the control and lesioned groups. The slopes of the lines for control and lesioned groups were not significantly different, but the elevation differences were highly significant for both muscimol (\textit{p} < 0.001) and baclofen (\textit{p} = 0.005). The response differences between control and lesioned rats for muscimol at 3 and 10 µM were statistically significant at \textit{p} = 0.005 and \textit{p} < 0.001, respectively. The response difference between control and lesioned rats for baclofen at 3 µM was marginally statistically significant (\textit{p} = 0.04).
Fig. 7. Comparison between control and lesioned rats of responses (mean ± S.E.M.) to the GABA_A receptor antagonist bicuculline (Bic, 20 µM, n = 11 for control and 8 for lesion) and the GABA_B receptor antagonist 2-OH-saclofen (2-OH-Sac, 200 µM, n = 12 for control and 7 for lesion). Responses are shown as percentage increase relative to baseline firing rate. Statistical significances of differences between control and lesioned groups: *p < 0.01, **p < 0.001.
Figure 1
Fig. 2
A

Spontaneous activity

Percent of firing rate in control ACSF

Number of neurons

0 25 50 75 100 125 150 175 200

B

Agonist Effect

Percent of relative firing rate change in control ACSF

Number of neurons

70 80 90 100 110 120 130 140 150 160

Fig. 3
Figure 4 (A,B)
Figure 4 (C,D)
Fig. 5 (A,B)
Fig. 5 (C,D)
Figure 6

A

Muscimol

Response (Percentage of Baseline Firing Rate)

0 20 40 60 80 100

Concentration (µM)

0.1 1 10 100 1000

Control Group

Control Regression

Lesioned Group

Lesioned Regression

B

Baclofen

Response (Percentage of Baseline Firing Rate)

0 20 40 60 80 100

Concentration (µM)

0.1 1 10 100 1000

Control Group

Control Regression

Lesioned Group

Lesioned Regression

Figure 6
DISCUSSION/SUMMARY

Spontaneous Activity in the VNC

Spontaneous activity is a striking feature of neurons in some regions of the CNS (Llinás, 1988) and has proved useful for studying synaptic neurotransmission (Darlington et al., 1995) and compensation (Smith and Curthoys, 1989; Smith and Darlington, 1991) in the vestibular nuclei. Although the underlying mechanisms for spontaneous activity are not totally clear, it may be partly related to intrinsic membrane properties involving noninactivating Na$^+$ channels (Schwindt and Crill, 1995) and slowly inactivating Ca$^{2+}$ current (Azouz et al., 1994). It was reported recently that study of the membrane properties of MVN neurons in vitro may be suggestive of their dynamic properties in that the regular type A MVN neurons would correspond to regular, tonic MVN neurons in vivo, whereas the irregular type B MVN neurons would correspond to the irregular, phasic MVN neurons in vivo (Vidal et al., 1995).

In vitro spontaneous activity of MVN neurons has been studied in several species (Darlington et al., 1995), but that of the LVN, SpVN, and SuVN has not. Our results showed that spontaneous activity is present in the LVN, SpVN, and SuVN as well as the MVN, with moderate densities of spontaneously active neurons in SpVN and SuVN, but low density in LVN. Our results for ACh effects on MVN, SpVN, and SuVN neurons support the concept that spontaneous activity in vestibular nuclei can be used as a
sensitive indicator for pharmacological studies (Chen et al., 1994, 1995; Waller and Godfrey, 1994) and lay a foundation for future work in this area.

There have been few in vivo studies about spontaneous activity in the VNC. Spontaneously active neurons in four major vestibular nuclei of cat were divided into four categories (Ryu and McCabe, 1973): regular, random (irregular), pulse-train (typical complex spikes), and cyclic (atypical complex spikes). Firing patterns of major vestibular nuclear neurons in our data fell into only two groups: regular and irregular. The preponderance of firing patterns was regular, as in previous slice studies of MVN. Such differences between in vivo and in vitro preparations are common but remain to be explained. One possible explanation involves methodology. Several important nerve fibers to and from the VNC, such as connections with the cerebellum and peripheral vestibular system, are cut when preparing brain slices, thereby changing the inputs to the VNC neurons. Firing rates of many MVN neurons decreased or increased when they were exposed to low Ca$^{2+}$/high Mg$^{2+}$ ACSF, and decreased during blockers of GABA receptors. This suggests that spontaneous activity in MVN neurons is affected by synaptic transmission as well as intrinsic membrane properties (Gallagher et al., 1985). Neurons in the VNC receive both excitatory and inhibitory inputs, and the balance of between these determines their output (de Zeeuw and Berrebi, 1995). Chemical signals may modify, by depolarizing or hyperpolarizing the membrane, the pattern and rate of discharges of spontaneously discharging neurons in a manner dependent on vestibular stimulation. Therefore, in vitro spontaneous activity may only represent a portion of the
in vivo spontaneously active neurons, as suggested by a recent study (Laurence et al., 1995).

**Cholinergic System in the VNC**

The origins of cholinergic fibers innervating the VNC remain to be clarified. They could be either extrinsic or intrinsic. Extrinsic sources may come from the cholinergic neurons in several regions, such as the reticular formation, tegmental dorsal nuclei, and contralateral inferior olive neurons (Brown, 1993; Matsuoka et al., 1983; Woolf and Butcher, 1989). Intrinsic sources of cholinergic fibers send their projections to other regions in the CNS, such as the cerebellum (Barmack et al., 1992) and the spinal cord (Jones et al., 1986).

Resemblance of responses of neurons in the MVN, SpVN, and SuVN to carbachol and muscarine suggested that the muscarinic receptor is the predominant type in these nuclei. The responses of MVN neurons to carbachol or muscarine were larger than those of SpVN or SuVN neurons, but smaller than those of the DCN (Chen et al., 1994). Receptor binding studies showed more binding sites for the muscarinic receptors (Wamsley et al., 1981) and for ACh receptors (Schwartz, 1986) in the MVN than in other nuclei of the VNC. As far as the MVN is concerned, the nature of the ACh receptors involved is controversial. While Ujihara et al. (1988, 1989) have proposed that the spontaneous activity of MVN neurons in slices is regulated through muscrinic receptors, another study showed that both muscarinic and nicotinic receptors were involved (Phelan
and Gallagher, 1992). Our findings are in agreement with the latter point of view: MVN neurons possess nicotinic as well as muscarinic receptors, and cholinergic effects on the spontaneous activity of MVN neurons are principally through muscarinic receptors because of the following observations: (1) a larger proportion of the neurons responded to muscarine than to nicotine (although this is much lower compared with the proportion of DCN neurons responsive to muscarine); (2) amplitudes of responses to carbachol resembled those to muscarine; (3) responses to carbachol could be greatly or totally blocked by the general muscarinic antagonist, atropine.

Interpretation of the data from the studies of muscarinic receptor subtypes is difficult because of poor selectivity of the muscarinic antagonists for muscarinic receptor subtypes. No drug shows a potency to one subtype that is 10-fold higher than that to the rest of the subtypes (Chen et al., 1995). Among the muscarinic antagonists tested on MVN neurons, tropicamide, preferential for the M_4 subtype, blocked the responses of MVN neurons to carbachol at relatively low concentrations. However, results from the effects of other antagonists preferential for the rest of muscarinic subtypes on activation of neurons by carbachol showed higher concentrations needed to reach the same reduction of firing rate as tropicamide does. The order of relative potencies of the muscarinic receptor subtypes is: M_4 > M_2 > M_3 > M_1. This is similar to the findings in the DCN (Chen et al., 1995), which may reflect the close relationships between the peripheral vestibular and auditory systems. The functional significance of the muscarinic receptor subtypes is unclear. There have been behavioral studies suggesting that the cholinergic system in the VNC could be involved in vestibular compensation (Calza et al., 1992).
Changes of ACh-related enzymes were reported in the VNC after hemilabyrinthectomy (Torte-Hoba et al., 1996; Yamada et al., 1988). Systemic injection of cholinergic antagonists or cholinomimetics, following unilateral labyrinthectomy, produced a posture deficit or facilitated recovery of vestibular function, respectively (De Waele et al., 1995). The existence of cholinergic projections from the VNC to the cerebellum and spinal cord (Barmack et al., 1992; Jones et al., 1986) may explain the behavioral observations from the effects of ACh-related drugs. Therefore, more behavioral studies are needed to test the roles of the antagonists preferential for muscarinic receptor subtypes during vestibular compensation.

**Concerning ICP Transection and Aspiration of the Cerebellum**

ICP transection was firstly introduced in a lesion study of the cochlear nucleus (Godfrey et al., 1985), and aspiration of the cerebellum is a commonly used method to cut connections between the cerebellum and the VNC. We compared the two ways to deafferent the VNC from the cerebellum, and preferred ICP transection as a better way to make disconnection between the cerebellum and the VNC since it produce less damage and less stress to the rats. Lesioned rats recovered more quickly from ICP transection than from aspiration of the cerebellum and had a higher survival rate.

**Plasticity in the MVN after ICP Lesion**
Shortly after unilateral ICP lesion, both GABA and glutamate concentrations decreased in nearly all VNC regions on both lesioned and unlesioned sides. The time course and the degree of reduction for GABA and glutamate in the VNC after this surgery resembled lesion studies in other regions of the CNS (Minchin and Fonnum, 1979; Fonnum and Walaas, 1978; Potashner and Dymczyk, 1986), and in the VNC after unilateral vestibular ganglionectomy (Li et al., 1996). Rapid recovery of GABA and glutamate levels on the unlesioned side, and much larger reduction of GABA and glutamate levels after bilateral ICP transections at 7 d, point to contributions of the contralateral ICP and/or commissural fibers to plasticities in the VNC. A problem that we couldn’t solve in this study is whether the commissural fibers play a role alone or together with the contralateral cerebellum and ICP. Bilateral transections of ICPs at 2 d, and ICP transection combined with transection of the commissural fibers would help to resolve this problem. For a better understanding of the distribution and alteration of amino acid neurotransmitters after ICP lesion, silver impregnation of degenerated fibers from the cerebellum should be examined. The most direct explanation for the remarkable changes of GABAergic pharmacology in lesioned animals is the up-regulation of receptor resulting from decreased endogenous GABA. However, other possible mechanisms concerning the GABA$_B$ receptor subtype need to be explored.

In summary, the results of this study support the brain slice preparation as useful for studying neuropharmacological actions on neurons in the MVN, SpVN, and SuVN. The results of our study of ACh pharmacology in the VNC increase our understanding of cholinergic function involved in normal and pathological conditions. Results from the
ICP transection study provide further evidence that GABA is a neurotransmitter for the Purkinje axon terminals and that glutamate may be a transmitter for cerebello-nucleofugal pathways. Lesion-induced plasticity in the VNC after ICP transection involves changes of both excitatory and inhibitory amino acids on both the lesioned and unlesioned sides. Exploration of the roles of cerebellar and/or commissural fibers in plasticity after ICP transection needs further study. Up-regulation of GABA\textsubscript{A} and GABA\textsubscript{B} receptor subtypes in the MVN after ICP transection can contribute to our understanding of cellular and molecular mechanisms underlying pathological conditions such as cerebellar ataxia.


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ABSTRACT

We employed extracellular recording from rat brain slices to compare the spontaneous activity in the four major nuclei of the vestibular nuclear complex (VNC), and to investigate the roles of acetylcholine (Ach) and γ-aminobutyric acid (GABA) in the VNC of normal rats. We also employed microdissection of freeze-dried brain sections combined with HPLC analysis to measure the distributions of amino acid neurotransmitters in the VNC of normal rats. Next, we used these same techniques, combined with surgical transections of the inferior cerebellar peduncle (ICP), to study changes of amino acid distributions and GABA pharmacology after loss of the major cerebellar input to the VNC.

The mapping study of spontaneously active neurons in the four major nuclei showed that spontaneous firing was present in all subnuclei of the VNC, with the highest density in the medial vestibular nucleus (MVN) and the lowest in the lateral vestibular nucleus (LVN). Amounts are sufficient, except in the LVN, for pharmacological studies. There were regional differences of spontaneous firing in the MVN.

Results with the effects of ACh agonists and antagonists showed that cholinergic effects on spontaneous activity of MVN, spinal vestibular and superior vestibular neurons are mostly through muscarinic receptors. Nevertheless, MVN neurons were also somewhat responsive to nicotine. Effects of subtype-preferential muscarinic antagonists on MVN neurons suggested that the most prominent subtype is M₄.
Quantitative distributions of 12 amino acids in the VNC were measured in both control rats and in rats with transection of the ICP. After the unilateral cut, concentrations of GABA and glutamate decreased 2 days after surgery in nearly all VNC regions. Glutamate levels completely recovered by 30 days, whereas GABA showed only partial recovery on the lesioned side. The degrees of reduction of GABA and glutamate in different VNC regions varied. Larger losses of GABA and glutamate occurred 7 days after bilateral transections than 7 days after unilateral transections of the ICP.

Study of the GABA pharmacology in the MVN of bilaterally lesioned rats showed a higher sensitivity to both GABA_A and GABA_B agonists than for MVN neurons of control rats, suggesting these receptor subtypes are up-regulated. Responses of MVN neurons to GABA antagonists alone were smaller in lesioned than in control rats.