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Transgenic mouse lines illuminate input and output processing streams in the medial vestibular and cerebellar nuclei

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Transgenic mouse lines illuminate input and output processing streams in the medial vestibular and cerebellar nuclei

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Neurosciences

by

Martha Whitney Bagnall

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Professor Massimo Scanziani

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Chair

University of California, San Diego

2008
This thesis is dedicated to my husband, Edward Han
whose splendid support, patience, and wisdom have been inadequately rewarded,
but everlastingly valued.
If pure scientists were motivated by curiosity alone, they should be delighted when someone else solves the problem they are working on— but that is not the usual reaction.

Alan Hodgkin
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ABSTRACT OF THE DISSERTATION

Transgenic mouse lines illuminate input and output processing streams in the medial vestibular and cerebellar nuclei

by

Martha Whitney Bagnall

Doctor of Philosophy in Neurosciences

University of California, San Diego, 2008

Professor Sascha du Lac, Chair

The cerebellum controls a vast array of coordinated reflexes and learned responses. All of its output to motor and premotor centers is funneled through the deep cerebellar and vestibular nuclei. Research on the information processing and internal circuitry of these nuclei has been hampered by the lack of available strategies for identifying and targeting functionally relevant subsets of neurons. I took advantage of several transgenic mouse lines in which distinct neuronal groups were labeled with fluorescent reporters to study the input and output characteristics of these regions. Experiments revealed that projection and local neurons in the medial vestibular nucleus exhibit different intrinsic physiological properties, and that their recruitment by sensory afferents is carefully tuned for the requirements of the vestibular system. Furthermore, both the medial vestibular and cerebellar nuclei make both excitatory and inhibitory projections to their motor nuclei targets. Finally, multiple types of inhibitory input are available to sculpt information processing in the vestibular nuclei.
I. Introduction

How does an animal time and coordinate its movements while navigating through its environment? The answers are of utmost importance to its survival. Vertebrates rely on the cerebellum to solve many of these problems, including both instantaneous challenges—how to compensate for an unexpected change in terrain?—and long-term ones—how to predict the onset of a stimulus from a learned cue?

Lesion and inactivation studies have shown that the cerebellum is critical for accomplishing these and many other tasks. Human patients with cerebellar damage exhibit deficits in posture (Lalonde and Strazielle, 2007), temporal precision of discontinuous movements (Spencer et al., 2003), and even nonmotor aspects of language production (Ivry and Justus, 2001). Damage to specific regions of cerebellar cortex prevents acquisition of the classically conditioned eyeblink reflex (Garcia et al., 1999) and modification of the vestibulo-ocular reflex (Lisberger et al., 1984), two well-studied responses that rely on the cerebellar circuit.

Despite the diversity of demands placed on this brain region, the cerebellar cortex is remarkably simple, even crystalline, in its structure (Fig. 1.1). Sensorimotor inputs from the pontine and vestibular nuclei excite tiny but numerous (~10\(^{11}\)) granule cells (and in some subregions, unipolar brush cells). A granule cell axon ascends to give rise to the parallel fibers making excitatory contacts onto 2000-3000 Purkinje cells, while each Purkinje cell receives ~200,000 parallel fiber contacts. Each Purkinje neuron is also contacted repeatedly by a single climbing fiber originating in the inferior olive, which is thought to provide an “error” signal for motor learning. In addition, four types
of inhibitory interneurons shape the patterns of neuronal activity in the cortex. Finally, the GABAergic Purkinje cells provide the only output from cerebellar cortex (Kandel et al., 1991; Zigmond et al., 1999).

All of the information carried by Purkinje cells is funneled through the deep cerebellar and vestibular nuclei, which are estimated to contain only 1/25th as many neurons as the Purkinje layer that feeds them, making these nuclei a site of massive convergence (Palkovits et al., 1977). In addition, the pontine projections to granule cells also send excitatory collaterals to the deep cerebellar nuclei, while the vestibular nuclei receive sensory information directly from the VIIIth nerve. (Ascending olivary axons also give off collaterals of unknown significance to the deep nuclei.) As a result, the cerebellar target nuclei must integrate both direct sensory information from excitatory pontine/vestibular fibers as well as a processed signal from inhibitory Purkinje cells. Understanding the properties of these two major inputs to the deep cerebellar and vestibular nuclei is therefore key to discovering how the cerebellum learns and controls such a diverse set of behaviors.

In contrast to the orderly cerebellar cortex, however, the deep cerebellar and vestibular nuclei are poorly organized. As in many other subcortical structures, such as the thalamus, the basal ganglia, and the brainstem nuclei, the absence of laminar structure complicates anatomical, and therefore functional, analysis. Not only is internal circuitry difficult to elucidate, even the identity of projection and nonprojection neurons is often ambiguous. In the cerebellar output nuclei, larger neurons are thought to be projection neurons, while smaller ones are presumed local interneurons (Chan-Palay,
1977; Epema et al., 1988); however, strategies for confident recognition of these groups and for division into finer subclasses based on their circuit roles have been scarce.

Recent advances in transgenic technology in mice offer an opportunity to surmount these problems. Within the last decade, many groups have begun inserting reporter genes under the control of cell-type specific promoters into the mouse genome. Initially, the most common reporter was the lacZ transgene, which had to be visualized with antibody staining to its gene product β-gal. The limitation of this approach is the requirement for tissue fixation for histology; a newer tactic is the use of the green fluorescent protein (GFP) gene, which has been modified by several groups to yield improved variants: a brighter construct known as enhanced GFP or eGFP (Heim et al., 1995; Reichel et al., 1996); color shifted variants including yellow and cyan fluorescent proteins (Heim and Tsien, 1996; Ormo et al., 1996); and functional appendages such as calcium sensitivity (Tsien, 1989).

Expression of these constructs under promoters delimiting neuronal subsets has informed neuroscience in a wide array of experiments (e.g., Feng et al., 2000). Within the cerebellar circuit, few groups have taken advantage of transgenic mice with fluorescently labeled subsets of neurons. Sekirnjak and colleagues addressed the problem of visualizing Purkinje cells in living tissue by creating a mouse line in which GFP is expressed under control of the L7 (Pcp2) promoter, which is specific for Purkinje cells. Following GFP-positive axons to their terminations in the medial vestibular nucleus, they found that only a small number (< 1%) of neurons there were heavily innervated by Purkinje cells, and that furthermore this subset exhibited striking physiological attributes, including rebound firing in excess of 100 spikes/s following
somatic hyperpolarization, that distinguished them from their uninnervated neighbors (Sekirnjak et al., 2003). This study both illuminated a clear relationship between anatomical role and physiological characteristics, and also provided a mouse tool for further investigation of this critically important inhibitory input to the vestibular nuclei.

For the work presented in this dissertation, I explored the use of this and other transgenic mouse lines to address several of the questions outlined above. Here I will summarize the scientific context that led to the studies presented in Chapters 2-5.

Intrinsic properties of vestibular nucleus neurons

As mentioned above, the loose organization of the vestibular nuclei precludes easy cell-type identification in slice preparation. Many investigators have attempted to use intrinsic neuronal physiological characteristics of neurons to differentiate the intermingled populations of the medial vestibular nucleus (MVN), which is the least myelinated and therefore the most amenable to physiological recordings of all the vestibular nuclei. Several groups reported that action potential shape and firing characteristics were widely variable across MVN neurons, and that neurons with wider action potentials, monophasic afterhyperpolarizations, and low maximum firing rates could be considered to be one group (“Type A”), while those with narrow action potentials, biphasic afterhyperpolarizations, and high maximum firing rates comprised a second group (“Type B”) (Serafin et al., 1991; Johnston et al., 1994). The specific partitions of these groups were labile and depended on the lab defining them, in large part because many neurons displayed characteristics that would be considered intermediate; and therefore the choice of parameters that divided the groups, as well as
the specific values of those parameters and the analysis techniques, were largely up to
the individual investigator (e.g., Beraneck et al., 2003). The presence of a “Type C”
group was debated among several labs (Straka et al., 2005), and it was questioned
whether neurons actually fell into discrete groups given that all of the parameters
analyzed were distributed continuously (du Lac and Lisberger, 1995; Sekirnjak and du
Lac, 2002). Furthermore, recording conditions (temperature, species, sharp vs. patch
pipettes, etc.) were different among the various labs engaged in these studies, further
complicating the question of group definition. Finally, several reports demonstrated that
most of these intrinsic parameters were even labile within a single neuron, following
induction of plasticity at either the neuronal level (Smith et al., 2002; Nelson et al.,
2003) or the organismal level, e.g. after vestibular nerve lesion (Beraneck et al., 2003;
Beraneck et al., 2004). These many challenges, coupled with the problem that no one
knew what functional categories these physiological types might correspond to, left the
field in contentious disarray.

Targeted recordings of fluorescent neurons in transgenic mouse lines with
complementary distributions of neurons, as described in Chapter 2, addresses these
problems as well as several more. Our data make clear that while physiological
parameters are continuously distributed, a well-defined boundary between distinct
groups can nonetheless be drawn. In addition, because one of the transgenic lines labels
GABAergic neurons while the other labels glutamatergic and glycinergic neurons, the
findings allow us to define the intrinsic characteristics in the context of the vestibular
circuit. As a result, we also demonstrate that the intrinsic properties of cortical and
hippocampal neurons, where local interneurons typically exhibit narrow action
potentials and high maximum firing rates (Cauli et al., 1997), do not provide a good blueprint for understanding the physiological characteristics of neurons in other regions of the brain. The rules for identification of projection and intrinsic neurons are reversed in the MVN, and well may be in other subcortical areas too.

Properties of vestibular afferents

Synaptic transmission is a highly complex process still poorly understood. At the presynaptic terminal, vesicle release relies on calcium concentration with approximately a 4-fold dependence, although the computed value ranges from 2-5 at different synapse types (Schneggenburger and Neher, 2005). The probability of single vesicle release following a presynaptic action potential is highly variable across synapses, perhaps in part due to the specific protein isoforms of the release machinery (Xu et al., 2007), while some synapses are able to release multiple vesicles simultaneously (Wadiche and Jahr, 2001). During repeated stimulation, the probability of release is influenced by: (1) build-up of calcium in the presynaptic terminal, which depends on the temporal proximity of the two stimuli, the amount of presynaptic calcium entry (Xu and Wu, 2005), and the presynaptic calcium buffering capacity (Blatow et al., 2003); (2) depletion of the readily releasable pool (Rosenmund and Stevens, 1996), which depends on the starting size of this pool and its speed of replenishment (Fernandez-Alfonso and Ryan, 2006); and (3) putative changes in the calcium sensitivity of the release machinery (Sullivan, 2007; Wolfel et al., 2007).

Postsynaptically, current flow depends on the number of transmitter receptors, their kinetics, and their availability, which can be limited by saturation or desensitization
(Trussell et al., 1993; Harrison and Jahr, 2003). The presence of enzymes to break down or take up free transmitter limits the duration over which transmitter is available, and also affects the success of transmitter spillover both to other postsynaptic sites and to presynaptic receptors, which can act to increase or decrease the probability of release of the next vesicle (Bergles et al., 1999; Zucker and Regehr, 2002). Given all of these factors, most of which have nonlinear effects on the process of transmission, it is unsurprising that synaptic properties vary so much from cell type to cell type, and from brain region to brain region.

In the face of this complexity, the vestibular afferent synapse onto vestibular nucleus neurons must solve an unusual problem. Excitatory vestibular fibers of the VIIIth nerve fire action potentials at rates ranging from ~20 to 100 spikes/s in mouse in vivo (Yang and Hullar, 2007), and the rate of spiking is linearly related to head velocity in this and in other species (Fernandez and Goldberg, 1976; Highstein et al., 2005). Postsynaptically, vestibular nucleus neurons are also constantly active at rates linearly related to head velocity (Shimazu and Precht, 1965). Therefore, the afferent synapse must drive postsynaptic firing at a rate linearly related to presynaptic activity. The system’s success can be seen through behavioral analysis of the vestibulo-ocular reflex (VOR), during which increasing head velocity yields linear increases in contraversive eye movement over at least a 30-fold range of velocities (Fig. 1.2).

The simplest solution to this problem is for each presynaptic release event to be large and robust enough to drive an action potential postsynaptically. This is the strategy adopted by the nearby auditory nuclei, where auditory nerve endings make large endbulb synapses onto projection neurons (bushy cells) in the anteroverentral
cochlear nucleus; each of the ~4 endbulb synapses per bushy cell contains 100-200 release sites and produces a large excitatory postsynaptic current (EPSC) which drives a single action potential postsynaptically (Trussell, 1999; Nicol and Walmsley, 2002).

The one-to-one presynaptic to postsynaptic transfer resulting from these calyceal afferents has the advantage of robustness, but the disadvantage of rigidity. Weaker inputs are unable to affect the postsynaptic response, driven as it is so completely by the endbulb synapses. In the vestibular system, unintentional head motion drives compensatory eye and trunk movements, but during deliberate head motion these movements become dysfunctional and must be suppressed. Therefore, activity in the vestibular nuclei cannot be as rigidly unmodifiable as it is in the auditory nuclei. While some vestibular nucleus neurons are driven by calyceal afferents from the vestibular nerve (e.g., Peusner, 1984), most must be able to integrate information regarding head movement intention from other brain regions, and thus cannot rely on this solution to the problem of synaptic linearity (Cullen, 2004). For the research described in Chapter 3, we recorded from vestibular nucleus neurons in slice while stimulating the vestibular nerve afferents in order to discover how the vestibular system produces robust linearity while maintaining its ability to respond to multiple types of inputs.

**Glycinergic output neurons in cerebellar nuclei**

As mentioned above, the absence of laminar organization in the deep cerebellar and vestibular nuclei has contributed to our ignorance about even so basic a question as transmitter phenotype of a given neuron. The medial vestibular nucleus, which mediates horizontal head movements, makes axonal projections to the nearby abducens nucleus,
whose motor neurons control the lateral rectus eye muscle (at the lateral edge of the eye). Injections of tritiated glycine into the abducens nucleus have suggested that MVN neurons projecting to the ipsilateral abducens are glycinergic, while those projecting to the contralateral abducens are glutamatergic (Spencer et al., 1989). Neurons sending axons to the abducens also typically send axon collaterals to the spinal cord (McCrea et al., 1980), suggesting that the vestibulospinal projection also uses glycine as a transmitter for ipsilateral connections. This schema represents a departure from the organization of the other deep cerebellar and vestibular nuclei, in which most projection neurons are thought to be glutamatergic or GABAergic (Kandel et al., 1991; Zigmond et al., 1999). To test whether the bauplan of the MVN was indeed distinct from that of other cerebellar target nuclei, it was necessary to have a tool for reliable identification of glycinergic neurons. However, histological analysis of transmitter phenotype is hindered by the fact that most proteins related to transmitter type (in this case, the neuronal glycine transporter GlyT2) cluster in the presynapse, rather than in the cell body; antibody stains for transmitter markers are often difficult to analyze as a result. Furthermore, most antibodies exhibit some degree of cross-reactivity with proteins other than the target protein, and can give misleading results. Therefore, I sought out another mouse transgenic line to aid in identification of glycinergic neurons in the cerebellar circuit.

The GlyT2-GFP mouse line, in which eGFP is expressed under control of the GlyT2 promoter sequence, was developed in the lab of J.M. Fritschy (Zeilhofer et al., 2005). Available evidence indicates that all glycinergic neurons in this mouse line express GFP, and that GFP is not spuriously expressed in any non-glycinergic neurons
(Zeilhofer et al., 2005). Therefore, it provides a useful tool for analysis of glycinergic projection neurons in the MVN, as well as in the other cerebellar target nuclei. Indeed, many medium-large neurons in the MVN were glycinergic (GFP+), but to our surprise the medial deep cerebellar nucleus, also known as the fastigial nucleus, contained several large GFP+ neurons as well. These observations cast doubt on the dogma that all projection neurons of the deep cerebellar nuclei are glutamatergic, and led us to carry out the research described in Chapter 4 in which we investigated the projection targets of glycinergic and presumed glutamatergic neurons in the deep cerebellar and vestibular nuclei. The results indicate that the cerebellum does not only influence output via glutamatergic neurons, but also through glycinergic projection neurons, suggesting that there are multiple processing streams in the encoding and delivery of information in the cerebellum.

**Distal dendritic inhibition from Purkinje cells**

The notion that the cerebellum achieves its effects through at least two types of projection neurons to motor regions, as shown in Chapter 4, raises the question of whether the model cerebellar circuit is in fact as “crystalline” as it is usually presented (Ito, 2006). Several computational analyses of cerebellar function have been presented in an effort to identify the loci of plasticity underlying cerebellar-dependent learning (Medina and Mauk, 2000; Steuber et al., 2007; Wetmore et al., 2007). These models usually focus on two forms of cerebellar learning: plasticity of behavioral gain (eye velocity divided by head velocity) of the VOR, which can be initiated through vestibular nerve lesion, magnifying or miniaturizing goggles, or visuo-vestibular
mismatch training (Miles and Eighmy, 1980); and acquisition and extinction of the
conditioned eyeblink reflex, which is achieved by pairing a tone or light stimulus with
an airpuff to the eye or periorbital shock, causing the animal to blink in anticipation of
the noxious stimulus (McCormick and Thompson, 1984; Kim and Thompson, 1997).
Both VOR learning and eyeblink conditioning are abolished by damage to the
appropriate regions of cerebellar cortex (De Zeeuw and Yeo, 2005), and both are
impaired, though not eliminated, in a transgenic mouse engineered to lack parallel fiber
long-term depression (De Zeeuw et al., 1998; van Alphen and de Zeeuw, 2002;
Koekkoek et al., 2003). In addition, cerebellar inactivation studies have indicated that
both VOR and eyeblink learning are initially dependent on Purkinje cell activity, but
that their long-term storage occurs downstream of Purkinje cells, in the deep cerebellar
and vestibular nuclei (Nagao and Kitazawa, 2003; Kassardjian et al., 2005; Ohyama et
al., 2006; Shutoh et al., 2006).

Based on these findings, computational models have been developed to analyze
the expected physiological and behavioral results of the synaptic and intrinsic plasticity
identified in slice experiments. The anatomical and physiological parameters of most
connections in cerebellar cortex have been reasonably well described, providing a
strong foundation for this portion of any computational model. However, much less
information is available regarding the structural and physiological properties of the
outputs to the cerebellar target nuclei, as well as the processing within the internal
circuits of these nuclei. For these reasons, I studied the anatomical properties of the
Purkinje cell input to MVN neurons.
While the characteristics of MVN neurons receiving heavy innervation from the cerebellum have already been well described, these neurons represent less than 1% of the MVN population. For a series of reasons, we expect to see a much higher percentage of cerebellar target neurons in the MVN: first, that in vivo around 8% of MVN neurons in the cat are described as responsive to cerebellar floccular stimulation (Sato et al., 1988); second, that all heavily innervated MVN neurons are targets of floccular Purkinje cells (Sekirnjak et al., 2003), but another region of the cerebellum known as the nodulus also makes functional projections to the MVN, in particular to vestibulospinal neurons (Precht et al., 1976; Xiong and Matsushita, 2000); and third, that our data show that all heavily innervated neurons are glycinergic, not GABAergic (see Chapter 4), but prior anatomical work has indicated that some cerebellar target neurons in the MVN are GABAergic (De Zeeuw and Berrebi, 1995). These three observations provide strong evidence that the MVN must contain more cerebellar target neurons than those already described as heavily innervated floccular target neurons. Anatomical information regarding these “missing” neurons would provide a useful addition to our information about types of cerebellar processing, and might also suggest new avenues for modeling the downstream effects of Purkinje cells in the deep cerebellar and vestibular nuclei. While some anatomical analysis of this sort has already been carried out in the lateral (dentate) cerebellar nucleus, the dearth of tools for analyzing microcircuitry has hindered most such efforts in the vestibular nuclei. With this in mind, I used the L7-GFP transgenic mouse, in which Purkinje cells express GFP, to visualize Purkinje cell synapses onto MVN neurons with less dense innervation than those previously described. Furthermore, I used my prior correlation of cell type with
physiological characteristics (Chapter 2) to provide context for the intrinsic characteristics of these putative cerebellar target neurons. While analysis of synaptic transmission between Purkinje cells and MVN neurons remains out of reach for technical reasons, the anatomical results nonetheless provided some insight into the multiple types of cerebellar cortical output to target neurons, and suggested some new avenues for improved analysis of cerebellar function.
References


Fig. 1.1 The basic cerebellar circuit. Sensory information is processed directly by the deep cerebellar nuclei (DCN) and vestibular nuclei (VN), and also travels through a side loop defined by granule and Purkinje cells. So-called “error” signals arrive via climbing fibers arising in the inferior olive. Purkinje cells provide the only output of cerebellar cortex; their projections to the DCN and VN are exclusively GABAergic. Open triangles, predominantly glutamatergic synapses (see Chapter 4); closed circles, GABAergic synapses. (Adapted from Bagnall and du Lac, 2006)
Fig. 1.2  Linearity of the vestibulo-ocular reflex across a > 30-fold range of head velocities. Eye velocity in darkness was measured while applying sinusoidal head rotations at 1 Hz over a range of different angular velocities. Eye velocity is directed opposite to head motion and provides excellent compensation at all velocities tested. Straight line represents ideal compensation. The relationship between head velocity and eye velocity is highly linear. Figure adapted from Michael Faulstich.
II. Transgenic mouse lines subdivide medial vestibular nucleus neurons into discrete, neurochemically distinct populations

Abstract

Identification of neuron types within circuits is fundamental to understanding their relevance to behavior. In the vestibular nuclei, several classes of neurons have been defined in vivo on the basis of their activity during behavior, but it is unclear how those types correspond to neurons identified in slice preparation. By targeting recordings to neurons labeled in transgenic mouse lines, this study reveals that the continuous distribution of intrinsic parameters observed in medial vestibular nucleus (MVN) neurons can be neatly subdivided into two populations of neurons, one of which is GABAergic and the other of which is exclusively glycinergic or glutamatergic. In slice recordings, GABAergic neurons labeled in the GIN line displayed lower maximum firing rates (< 250 Hz) than glycinergic and glutamatergic neurons labeled in the YFP-16 line (up to 500 Hz). In contrast to cortical and hippocampal interneurons, GABAergic MVN neurons exhibited wider action potentials than glutamatergic (and glycinergic) neurons. Responses to current injection differed between the neurons labeled in the two lines, with GIN neurons modulating their firing rates over a smaller input range, adapting less during steady depolarization, and exhibiting less rebound firing than YFP-16 neurons. These results provide a scheme for robust classification of unidentified MVN neurons by their physiological properties. Finally, dye labeling in slices demonstrates that both GABAergic and glycinergic neurons project to the contralateral vestibular nuclei, indicating that commissural inhibition is accomplished through at least two processing streams with differential input and output properties.
Introduction

The diversity of neuron types in the brain supports the complex processing that underlies fine-tuned behaviors. Identification and classification of these neuron types represents a critical step in understanding the behavioral relevance of their interconnections. In some regions of the brain, such as cortex and hippocampus, anatomy and morphology provide sufficient clues for broad cell type classification. In other areas, including the brainstem and spinal cord, the lack of clear anatomical segregation of cell types has hampered progress in circuit analysis and, as a result, in pinpointing relationships between cellular properties and their consequences for behavior.

One well-characterized behavior which relies on a comparatively simple circuit is the vestibulo-ocular reflex (VOR). The VOR acts to stabilize the retinal image in spite of head motion by transforming vestibular input into oculomotor output, so that the eyes rotate to counteract the effects of head movement. Decades of research into the VOR have yielded a wealth of information on its underlying circuitry, which consists of the brainstem vestibular nuclei, the cerebellum, and several oculomotor nuclei (Highstein and Holstein, 2005; Straka et al., 2005). The vestibular nuclei have been implicated as a site of VOR learning, just as their homologs, the deep cerebellar nuclei, have been implicated in eyeblink conditioning (Lisberger et al., 1994; du Lac et al., 1995; Mauk et al., 1998; Medina et al., 2000; Attwell et al., 2002; Ohyama et al., 2006). As a result, the VOR circuit provides a useful system for elucidating direct relationships between changes in behavior and their physiological and molecular underpinnings.
Interpretation of \textit{in vitro} physiological experiments in the vestibular nuclei, however, has been limited by the absence of clear relationships to \textit{in vivo} circuitry. In most studies, cell types have been identified in slice recording primarily on the basis of their intrinsic physiology, without reference to circuit function. Because the many cell types in the vestibular nuclei span a continuum of physiological properties (du Lac and Lisberger, 1995; Straka et al., 2005), the inability to identify them with certainty in slice preparations has restricted our understanding of the available forms of neuronal plasticity in the brainstem.

We sought better tools for associating the physiology of neurons recorded in slice with their role in the VOR circuit. The mouse offers many advantages in genetic approaches to this problem, and its behavioral performance and adaptive capability in the VOR are excellent (De Zeeuw et al., 1998; Stahl et al., 2000; Faulstich et al., 2004). In this study, two lines of transgenic mice, GIN and YFP-16, are used to elucidate the intrinsic physiology of neurochemically delimited sets of neurons in the medial vestibular nucleus (MVN) without the need for extensive post-recording processing (Feng et al., 2000; Oliva et al., 2000). In addition, we demonstrate a simple method for identifying commissurally-projecting neurons in standard slices. Finally, we target identified commissural neurons in slice recording to show that glycinergic and GABAergic commissurally-projecting neurons in the MVN have distinctive intrinsic physiologies. The results provide a classification scheme of MVN neurons grounded in a molecular, rather than phenomenological, framework.
Methods

Mouse lines and morphological analysis

GABAergic neurons were identified in GIN mice, which express enhanced green fluorescent protein (eGFP) in a subset of GAD67-positive neurons (Oliva et al., 2000). To find a mouse line with a fluorescently labeled population distinct from that of GIN neurons, we examined mice generated by insertion of a modified thy1 promoter driving eYFP (enhanced yellow fluorescent protein; Feng et al., 2000). In the YFP-16 line, eYFP expression in large neurons in the deep cerebellar nuclei (Bagnall and du Lac, unpublished observations) suggested its potential utility in the homologous vestibular nuclei. We also used the GAD67-GFP line of mice, in which GFP is knocked in to the native Gad1 locus (Tamamaki et al., 2003), to target GABAergic neurons for recording.

One GIN and one YFP-16 animal were perfused transcardially with PBS followed by 4% paraformaldehyde. After removal of the brain and postfixation for ~30 min, the brain was sunk in 30% sucrose and sectioned at 30 µm on a freezing microtome. From each mouse line, the unilateral MVN from two sections, at approximately Bregma -5.88 and -6.24, was imaged with a 100x objective on a confocal microscope (Olympus FV300) in 0.4 µm steps. Neurons were included for morphological analysis if the entire soma was enclosed within the resulting z-stack.

Slice preparation and electrophysiology

Coronal slices of the brainstem (250-300 µm) were prepared using a DSK-1500E or Leica VT1000S vibratome. Slices were incubated in carbogenated ACSF at 34°C for 30 min and then at room temperature for > 30 min. ACSF contained (in mM)
124 NaCl, 26 NaHCO₃, 5 KCl, 1.3 MgCl₂, 2.5 CaCl₂, 1 NaH₂PO₄, 11 dextrose. For recording, slices from 17 to 28-day-old (P17-28, mean P21 ± 3 s.d.) YFP-16 and GIN mice were submerged in a recording chamber and perfused with 34° C carbogenated ACSF. An additional dataset from recordings made at room temperature from similar age animals (P16-37, mean P25 ± 5) is included for comparison in Table 2.2.

Fluorescence was imaged with a FITC filter and Optiquip illumination on an Olympus microscope using a 40x objective. MVN neurons labeled in the YFP-16 line display a wide range of intensities; dim neurons were not targeted for recording. Neurons were visualized under infrared illumination with differential interference contrast optics. Whole-cell current clamp recordings were made with an AxoClamp 2B or a MultiClamp 700B amplifier. Data were filtered at 10 kHz, digitized with an ITC-16 board at 40 kHz for action potential analysis and 20 kHz for all other measurements, and collected in Macintosh OS 9 using custom-written code in Igor Pro 4. Electrodes were filled with an internal solution of (in mM) 140 K gluconate, 10 HEPES, 8 NaCl, 0.1 EGTA, 2 MgATP, 0.3 Na₂GTP, and, in some cells, 0.1% biocytin (Sigma).

For commissural studies, 450-500 µm slices were made from P12-P17 animals. Afterwards, slices were temporarily removed from ACSF and placed on Whatman filter paper. A 27 G needle was used to poke several holes in the MVN on one side of the brainstem, particularly in the midventral region, which is the primary target of commissural fibers (Ladpli and Brodal, 1968; Epema et al., 1988). Crystallized fluorolabeled dextran was delivered by needle into the MVN of GIN (fluororuby) or YFP-16 (Texas Red, both 10,000 MW; Molecular Probes) slices. Slices were rinsed in ACSF, removed from filter paper, and returned to the incubation chamber. After 6-8 hrs
incubation at ~32°C, slices were either fixed in 4% paraformaldehyde for histology or used for electrophysiological recordings as above. Commissural electrophysiology was performed on slices from P14-17 animals.

**RT-PCR**

After recording neuronal physiology, negative pressure was applied as the pipette was removed from the slice. The electrode contents were expelled with pressurized air, and dNTPs (0.5 µL, 10 mM) and random hexamer primers (0.5 µL, 500 ng) were added. Following 5 min incubation at 65º C, samples were kept on ice until addition of RT buffer, Superscript III reverse transcriptase and dithiotreitol (all Invitrogen). Reactions were then incubated at 50º C for 1 hr, 75º C for 10 min, and stored at 4º C. Positive controls (whole-brain RNA preparations (RNeasy kit, Qiagen)), and negative controls (recording pipettes dipped in the slice and removed without attaching to a cell) were included in every round of RT-PCR.

Primers and sources are listed in Table 2.1. Primers were selected so that the primer or the product spanned an exon-intron boundary to eliminate the possibility of genomic contamination. For the first PCR reaction, primers to five gene products were multiplexed: VGlut1, VGlut2, GlyT2, GAD65, and GAD67. In some cases actin primers were included as well (data not shown). The entire volume of the RT reaction (~10 µL) was mixed with water, buffer, and 0.5 µL Taq (Eppendorf) and heated to 94º C for 2 min. Primers were added to the mixture for a final concentration of ~0.2 µM in a final reaction volume of 100 µL, and PCR continued for 20 cycles of 1 min 94º C, 1 min 56º C, 1 min 72º C. For the second PCR reaction, 2 µL of the first PCR reaction was mixed with 50 µM dNTPs, buffer, 0.5 µL Taq, and 0.2 µM primers for a single
gene product (usually nested or semi-nested; in some instances the same primer set was used for the first and second PCR reactions; Table 2.1). The PCR protocol was the same as above but for 35 cycles. ~10 µL of this reaction product was run on a 1.5 % agarose gel with TAE.

**Physiology analysis**

Recordings were targeted primarily to the rostral two thirds of the MVN in both magnocellular and parvocellular divisions, approximately Bregma -5.88 to -6.48. Neurons were included in this study only if they were capable of firing continuously during a 1 s depolarization, and if their spike height (threshold to peak) was greater than 50 mV (45 mV for commissural studies). Analysis was carried out as in Sekirnjak and du Lac (2006). For action potential analysis, cells were injected with hyperpolarizing or depolarizing current (if required) to force a rate of 8-12 spikes/s for 1-5 sec. These spikes were averaged together by alignment at their peaks. Spike threshold was defined as the $V_m$ at which $dV/dt$ reached 10 V/s. Spike width was measured at threshold, and spike half-width at the halfway point between threshold and peak. The maximum afterhyperpolarization (AHP) was defined as the difference between threshold and the most negative $V_m$ reached during the AHP; the time to maximum AHP was defined as the time difference between this point and the spike peak. The afterdepolarization (ADP) was quantified as the maximum value of the spike derivative during the 1 ms after the spike repolarized to threshold value.

To examine how neurons respond to various levels of inputs, steps of depolarizing current (1 s) were delivered in increasing amplitude until the neuron was unable to sustain action potentials across the whole step. Firing rate was taken as the
average of the reciprocal of inter-spike intervals across the entire step. In current steps that yielded a firing rate of 35-45 Hz at the end of the step, firing rate adaption was measured as the ratio of the firing rate over 100 ms at the end of the step versus the beginning of the step. The first 50 ms of firing was excluded from analysis (Sekirnjak and du Lac, 2006). Cellular gain was measured as the slope of the best-fit line to a graph of firing rate versus current step amplitude. Input resistance was measured at ~75 mV by injecting a small negative current step (-5 to -50 pA) to produce a 2-3 mV hyperpolarization. 6 repetitions were averaged for analysis.

A dataset from similar-aged wild-type mice (P21 - P25) which had been sham-operated 3 d prior for vestibular labyrinthectomy was analyzed for comparison (A.B. Nelson, M. Faulstich, S. Moghadam, unpublished data). All data were analyzed with custom-written code in Igor Pro 4 and statistical tests were carried out in KaleidaGraph 3.6. Significance was determined by Student’s unpaired t-test, and is reported as means ± standard deviation, except as noted.

Results

Two transgenic lines of mice label different subsets of neurons in the MVN: in the YFP-16 line (Feng et al., 2000), a modified thy1 promoter drives eYFP expression; and in the GIN line (Oliva et al., 2000), a portion of the Gad1 promoter (gene product, GAD67) drives eGFP expression. The gross distribution of eYFP+ (hereafter, YFP-16) and eGFP+ (hereafter, GIN) neurons in fixed tissue sections of the MVN was roughly complementary. YFP-16 neurons were located throughout the rostrocaudal extent of the MVN, with particularly heavy distribution in the more lateral, magnocellular regions
(Fig. 2.1A). In contrast, while GIN neurons were also found throughout the MVN, they were most concentrated in parvocellular areas close to the fourth ventricle, with a smaller scattering more laterally in the magnocellular region (Fig. 2.1B). Thus the territories of the two cell types are dissimilar but overlapping, in keeping with reports of intermingled cell type distribution in the MVN (Serafin et al., 1991; Sekirnjak and du Lac, 2002; Takazawa et al., 2004; Sekirnjak and du Lac, 2006).

Fluorescent neurons of both lines had heterogeneous morphology (Fig. 2.1C, 2.1D). Analysis with confocal microscopy of neurons in similar slices from each line (YFP-16, n = 108; GIN, n = 149) showed that YFP-16 neurons on average measured 17.5 ± 4.3 µm on the long axis and 9.2 ± 2.7 µm on the short axis, while GIN neurons were significantly smaller, with measurements of 14.9 ± 3.6 µm and 8.1 ± 2.1 µm (long axis, p < 0.0001; short axis, p < 0.001). The approximate somatic area, quantified as the product of long and short axes, was thus significantly larger in YFP-16 than GIN neurons (Fig. 2.1E; p < 0.0001). Most YFP-16 neurons (94%) were multipolar and typically extended 3-5 primary dendrites from the soma (average, 4.2 ± 1.2); in contrast, about 20% of GIN neurons were bipolar, and on average they extended only 3.3 ± 1.0 processes (Fig. 2.1F; p < 0.0001). Despite these significant differences in size and morphology, the two populations display a large degree of overlap (Fig. 2.1E, 2.1F), precluding simple anatomical identification in slice.

**YFP-16 and GIN neurons express different neurotransmitters**

GIN neurons comprise a subset of GABAergic cells (Oliva et al., 2000), but the transmitter phenotype of the majority of YFP-16 neurons is unknown (with the
exception of motor neurons, all of which are labeled (Walsh and Lichtman, 2003)). Antibody staining against protein markers of transmitter phenotype is complicated by the fact that most such proteins are only weakly expressed somatically. Therefore, we turned to reverse transcription PCR (RT-PCR). RT-PCR analysis of transmitter phenotype of single neurons targeted for recording revealed that 50% (9/18) of YFP-16 neurons were positive for the glycine transporter GlyT2, which is found in glycinergic neurons (Poyatos et al., 1997), while the remaining 50% were positive for the vesicular glutamate transporter VGlut2, which labels glutamatergic neurons (Fig. 2.2A) (Fremeau et al., 2001). Notably, no YFP-16 neurons were found to be positive for either GAD67 (0/16) or GAD65 (0/11), the two major isoforms of glutamate decarboxylase, nor primarily for VGlut1 (1/16), a vesicular glutamate transporter most commonly found in excitatory neurons with low probabilities of release (Fig. 2.2A) (Fremeau et al., 2004).

Having demonstrated that YFP-16 neurons are glutamatergic or glycinergic, but not GABAergic, we verified the transmitter phenotype of GIN neurons by the same method. RT-PCR analysis indicated that GIN neurons in the MVN expressed GAD67 (23/28) and GAD-65 (26/28) but did not express VGlut1 or VGlut2 (0/28), in contrast to YFP-16 neurons (Fig. 2.2B). In keeping with reports of colocalization of GABAergic and glycinergic markers in a subset of MVN neurons (Tanaka and Ezure, 2004), we found that 50% (14/28) of GIN neurons also expressed GlyT2 (Fig. 2.2B). Therefore, GIN and YFP-16 neurons represent neurochemically distinct populations within the MVN.
Intrinsic physiology

MVN neurons are physiologically diverse with respect to their responses to current injection and the shape of their action potentials (Serafin et al., 1991; Johnston et al., 1994). However, because neurons of similar physiology may be found in quite different regions of the vestibular nuclei, while neurons of dissimilar physiology may lie right next to each other, there has been a dearth of effective strategies for targeting particular subclasses of neurons in slice recordings. Because the GIN and YFP-16 lines label two distinct populations of neurons in the MVN, they provide excellent tools for identifying the physiology of GABAergic and non-GABAergic neurons. We targeted GIN and YFP-16 neurons for physiological recording in whole-cell patch clamp in the MVN, primarily in mid-rostral regions (Bregma -5.88 to -6.48). Recordings were made at 34°C in juvenile (P17-28; average, P21 ± 3) mice from 59 (GIN) and 49 (YFP-16) neurons. YFP-16 neurons vary widely in brightness; recordings were targeted to the most visible, and therefore brightest, neurons. We analyzed action potential shape, which has been used in several laboratories to classify MVN neurons into two or three groups (Serafin et al., 1991; Johnston et al., 1994). In addition, we characterized the response of the cells to depolarizing and hyperpolarizing current steps. A dataset of 61 cells recorded in separate experiments from wild-type animals (P21 - P25) was included in analyses to demonstrate how YFP-16 and GIN neuron physiological characteristics compare with those of unidentified neurons.
Action potential waveform is largely distinct between YFP-16 and GIN neurons

Fewer YFP-16 than GIN neurons fired spontaneously (22/49 vs 47/59; \( p < 0.001 \), Fisher’s exact test), but within spontaneously active neurons, YFP-16 neurons fired at higher spontaneous rates than GIN neurons (YFP-16, 24.5 ± 15.5 Hz [range, 3 - 71 Hz]; GIN, 16.3 ± 11.1 Hz [range, 1 - 60 Hz]; \( p < 0.05 \)). No difference was seen in the regularity of spontaneous action potentials, as measured by coefficient of variation (YFP-16, 0.14 ± 0.17; GIN, 0.11 ± 0.13; \( p > 0.4 \)). Examples of typical action potentials recorded in YFP-16 and GIN neurons are shown in Figs. 2.3A and B. As is characteristic of MVN neurons, these examples exhibit remarkably swift rising and falling phases of the action potential, with the total spike width usually < 1 ms. The afterhyperpolarization (AHP) in some instances is monophasic (e.g. Fig. 2.3B, top) and in others displays a fast and a slow component. Three differences are salient between the YFP-16 and GIN neuron spikes at the top of the panels: the width of the action potential, the magnitude of the AHP, and the presence or absence of an afterdepolarization (ADP) dividing the AHP into an early and late component. Although these examples are distinctive, some action potentials from each population bear strong resemblances to each other (examples in lower half of Fig. 2.3A, B).

To facilitate action potential comparisons across neurons, DC current was applied to force a firing rate of ~10 Hz, and the resulting spikes were averaged for analysis. Because MVN neurons have previously been subdivided based on the presence of one or two components to the AHP (Serafin et al., 1991; Johnston et al., 1994; Beraneck et al., 2003), the ADP, which separates these components, was analyzed (see Methods). When the ADP was plotted versus action potential half-width
(a slightly more robust measure than action potential width because it is less sensitive to threshold definition), YFP-16 and GIN populations were largely distinct on the resulting continuum (Fig. 2.3C). The ADP was significantly larger and the action potential half-width significantly narrower in the YFP-16 than in GIN neurons (Table 2.2). This correlation may reflect either that the currents necessary to produce both a rapid repolarization and an ADP are coregulated (e.g., MacLean et al., 2003) or simply that the ADP is evident only in cells whose action potential repolarizes swiftly. The ADP has been attributed, variously, to calcium channels (Autret et al., 2005; Metz et al., 2005), persistent sodium channels (Yue et al., 2005), or a contribution of current from multiple dendrites (Quadroni and Knopfel, 1994). Given that YFP-16 neurons extend significantly more dendrites than GIN neurons (Fig. 2.1F), our data are consistent with the last of those possibilities.

Previous studies have distinguished MVN cell types by examining the magnitude of the AHP (with respect to threshold) in relationship to the time at which the maximum is reached (Takazawa et al., 2004; Sekirnjak and du Lac, 2006). YFP-16 neurons fell primarily in two groups, based on whether the AHP reached a maximum before or after the ADP (< 1 ms or > 3 ms following the spike, Fig. 2.3D). Similarly, some GIN neurons repolarized swiftly (1-3 ms after spike), while others reached a maximum AHP > 4 ms after the action potential (Fig. 2.3D). Notably, however, no GIN neurons reached the deepest portion of the AHP as quickly as the earliest YFP-16 neurons, despite the fact that a few GIN neurons have comparable spike widths to YFP-16 neurons (Fig. 2.3C). Furthermore, GIN neurons on average exhibit significantly deeper maximum AHPs than do YFP-16 neurons (Fig. 2.3C, Table 2.2), an effect
mostly attributable to those neurons which repolarize in the 2-4 ms following the action potential (a time when very few YFP-16 neurons reach a maximum AHP). The larger magnitude of the AHP relative to spike threshold derives from two sources: first, that GIN neurons exhibit higher spike thresholds (-45.3 ± 4.4 mV) than YFP-16 neurons (-50.7 ± 4.0 mV, \( p < 0.0001 \)), accounting for 5.4 mV of the total 6.6 mV average AHP difference between the groups. The remaining 1.2 mV difference in average AHP magnitude is likely due to differences in the amplitude of repolarizing currents between YFP-16 and GIN neurons (A. Gittis and S. du Lac, unpublished observations). In all measures of action potential waveform, GIN and YFP-16 neurons span the same extent as unidentified neurons recorded in wild-type animals (Figs. 2.3C and D, dark gray dots), suggesting that the two populations taken together represent a high proportion of MVN neurons typically targeted for recording.

YFP-16 and GIN neurons display different signal transformation properties

Input-output characteristics are widely heterogeneous across MVN neurons (Serafin et al., 1991; Ris et al., 2001; Sekirnjak and du Lac, 2002). To explore the dynamic range of GABAergic and non-GABAergic neurons in the MVN, we applied depolarizing steps of current for 1 s, increasing the current on each step until the neuron was unable to sustain firing across the step. Fig. 2.4 shows the resulting firing patterns in a typical YFP-16 (Fig. 2.4A) and GIN (Fig. 2.4B) neuron. The YFP-16 neuron attained instantaneous firing rates of over 500 spikes/s, declining over the course of the current step to ~300 spikes/s, while the highest firing rate that the GIN neuron could sustain was only ~200 spikes/s. The maximum firing rate, an indication of the output
range of a neuron, was taken as the average of the firing rate on the highest step for which the neuron fired without fail. Maximum firing rates of YFP-16 neurons were significantly higher than those of GIN neurons, while their input resistances were lower (Fig. 2.4C; Table 2.2), in keeping with previous reports of an inverse relationship between these measures (Sekirnjak and du Lac, 2002). Comparison with data from wild-type animals revealed that the YFP-16 and GIN populations together span the same values of maximum firing rate and input resistance as unidentified neurons (Fig. 2.4C). The amplitude of the largest current step, a measure of the functional input range over which the neuron could sustain firing, was on average about three times larger in YFP-16 than GIN neurons (Fig. 2.4D). Together these data indicate that YFP-16 neurons have a wide dynamic range of inputs and outputs, while GIN neurons are constrained to a narrower operating range.

The transformation of current input to firing rate output not only occurs over a wider range in YFP-16 than GIN neurons, but also has different temporal properties. Firing rate declines appreciably over the course of the current step in the YFP-16 neuron shown in Fig. 2.4A, but less so in the GIN neuron (Fig. 2.4B). The adaptation ratio, defined as the firing rate at the end divided by the firing rate at the beginning of the 1 s step (see Methods), was significantly higher in GIN neurons (0.91 ± 0.09) than in YFP-16 neurons (0.81 ± 0.13, p < 0.0001), indicating less adaptation across the step in GABAergic than in nonGABAergic cells. These results are plotted in histogram form in Fig. 2.4E.

The current-firing rate relationship is linear in many MVN neurons (e.g. Fig. 2.4A and B, bottom) and can be described by a single gain value. In some cells, a
modest bilinearity can be discerned when comparing the gain below ~80 Hz or the gain above (the two line fits in each example neuron). Overall, YFP-16 and GIN neurons had comparable levels of excitability, whether gain was measured at firing rates below 80 Hz or above (Table 2.2). However, when comparing gain above 80 Hz to gain below, YFP-16 neurons were significantly less linear (ratio of high range gain to low range gain, 0.65 ± 0.19) than GIN neurons (0.88 ± 0.19, \( p < 0.0001 \)) (Fig. 2.4F). Furthermore, cellular excitability above spike threshold (gain) is not closely related to cellular excitability below spike threshold (input resistance): in YFP-16 neurons, the ratio of gain to input resistance was more than twice as high as in GIN neurons (Table 2.2).

Following a 1 s hyperpolarizing current step of ~30 mV, MVN neurons tend to fire at elevated rates compared to those prior to the step, a phenomenon termed rebound firing (Fig. 2.5A). High rates of rebound firing are associated with low input resistance and strong adaptation (Sekirnjak and du Lac, 2002). Furthermore, neurons with extensive innervation from the floccular lobe of the cerebellum (floccular target neurons, or FTNs), identified in slice in an L7-GFP transgenic mouse, have rebound firing ranging from 80-250 Hz, while non-FTNs usually display rebound of 0-100 Hz (Sekirnjak et al., 2003; Sekirnjak and du Lac, 2006). We found that YFP-16 neurons had significantly higher rebound than GIN neurons, in agreement with predictions from previous work (Sekirnjak and du Lac, 2002) (Fig. 2.5B). Comparison of rebound with the ADP magnitude revealed that the measures are highly correlated (Fig. 2.5C; \( R^2 = 0.61, \ p < 0.0001 \)). The correlation might arise because the same current underlies both characteristics (e.g. calcium, which has been shown to be related to rebound bursts in the deep cerebellar nuclei (Molineux et al., 2006)), or because of a coregulation of the
currents responsible for the characteristics. The many differences between the intrinsic physiology of the YFP-16 and GIN cell groups likely reflect a different distribution of ionic currents in the two cell types, particularly those activated above spike threshold (A. Gittis and S. du Lac, unpublished observations).

Subsets of both YFP-16 and GIN neurons expressed the glycinenergic marker GlyT2, but the presence or absence of GlyT2 did not distinguish cell types in either line (Table 2.3). Glycinergic YFP-16 neurons displayed physiological characteristics similar to glutamatergic YFP-16 neurons, and GIN neurons expressing GlyT2 were similar to GIN neurons that did not express GlyT2. In contrast, the intrinsic physiological properties of GlyT2-expressing GIN neurons differed substantially from those of GlyT2-expressing YFP-16 neurons (Table 2.3).

Both YFP-16 and GIN neurons project commissurally

GABAergic, glycinenergic, and glutamatergic neurons make commissural projections between the two halves of the MVN (Shimazu and Precht, 1966; Kasahara et al., 1968; Mano et al., 1968; Gacek, 1978; Dieringer and Precht, 1979b, 1979a; Spencer et al., 1989; Holstein et al., 1999). Commissural inhibition sets up the push-pull circuitry that underlies the bilaterality of the VOR and is thought to support recovery of the VOR following labyrinthectomy or canal plug (Bienhold and Flohr, 1978; Galiana et al., 1984; Furuya et al., 1992) (though see Smith et al., 1986). We hypothesized that the differences in physiology between YFP-16 and GIN neurons might reflect their circuit roles—perhaps that one sends projections contralaterally while the other does not. However, because of the topology of the vestibular nucleus it is rare to be able to
trace axons of cells recorded in slice preparations for substantial distances. As a result, neuronal fills are frequently uninformative in cell type identification. We turned instead to dye labeling of axon tracts (Sekirnjak and du Lac, 2006).

To determine whether any of the YFP-16 or GIN neurons project contralaterally, thick slices were made from young (P12-P17) animals and crystals of lipophilic dye (fluororuby [GIN mice] or Texas Red [YFP-16 mice]) were deposited into one side of the MVN, typically throughout the ventrolateral aspect. 6-8 hrs after injections, fluorescently labeled neurons were seen contralateral to the site of injection, suggesting that commissural axons had transported the dye retrogradely to cell bodies.

To verify that neurons were making commissural connections, we first examined the midline of the slice. Fig. 2.6B shows the fluorescent labelling in a slice in which the unilateral MVN had been injected ~6 hrs previously: on the left is the GFP signal of the fluorescent neurons; in the middle the fluororuby signal; and on the right the overlay. Double-labeled axons were seen coursing across the midline and entering or emerging from the medial longitudinal fasciculus (Fig. 2.6B, GIN; also in YFP-16, data not shown). Fig. 2.6C shows examples of retrograde commissural label in the side of the MVN contralateral to dye injection in YFP-16 slices, while Fig. 2.6D shows the same for GIN slices. Double-labeled neurons were found in each line of mice, indicating that some YFP-16 and GIN neurons made projections to the contralateral MVN. Neurons were located both close to the ventricle and in somewhat more lateral portions of the MVN.

The physiology of those neurons that did project commissurally was examined in slices incubated for > 6 hrs at ~32° C to allow for dye transport. Double labeled
Texas Red/YFP+ or fluororuby/GFP+ cells were easily identifiable under fluorescence and were targeted for recording. Fig. 2.7A shows example action potentials from a commissurally projecting YFP-16 neuron (left) and GIN neuron (right). Double-labeled neurons were analyzed and the results superimposed on data from previous figures. Cellular characteristics of double-labeled YFP-16 and GIN neurons were highly similar to those of their respective parent populations, both in spike shape and in firing characteristics (Fig. 2.7B and C). Slight differences between a commissural population and the overall population may reflect age-dependent changes in neuronal physiology (Murphy and du Lac, 2001), since the commissural experiments were carried out in somewhat younger animals (P14–P17). RT-PCR on a subset of commissurally labeled YFP-16 neurons indicated that all (6/6) were positive for GlyT2 but not VGlut2 ($p < 0.05$ in comparison to YFP-16 population as whole, Fisher’s exact test). Intriguingly, commissurally projecting GIN neurons also frequently expressed GlyT2 (7/10). Therefore, commissural inhibition between the two sides of the MVN is accomplished by at least two highly distinct groups of neurons: GIN neurons, with a near-linear transformation of input and output that operates over relatively smaller ranges, and which may inhibit its targets with GABA and/or glycine; and glycinergic YFP-16 neurons, with a less linear but broader range of outputs.

**Schema for cell type classification in the MVN**

It has been noted for several years that the intrinsic physiology of MVN neurons is heterogeneous, and that two canonical types can be defined on the continuum of characteristics by their action potential waveforms and other traits (Serafin et al., 1991;
Johnston et al., 1994). In this schema, action potential shapes have been grouped based on the profile of the AHP: Type A neurons, with monophasic AHPs, a “strong A-like rectification” (i.e. a slowed rate of rise in the AHP); Type B, with biphasic AHPs; and sometimes Type C, a mixed group with anomalous characteristics. These groupings are remarkably similar across species, including rat, chick, and guinea pig (Serafin et al., 1991; Johnston et al., 1994; du Lac and Lisberger, 1995).

Based on our recordings, GIN and YFP-16 neurons, respectively, appear to be excellent representatives of “Type A” and “Type B” groups, and using the data presented above we propose several criteria for sorting unidentified neurons into these two groups. We include data from GIN and YFP-16 neurons recorded at room temperature (23º C) both to facilitate comparisons with data from other studies and to evaluate the resilience of our criteria to recording conditions (Table 2.2). The single most useful factor for dividing the populations is maximum firing rate, measured as the average rate across the highest depolarizing 1 s step over which a neuron can sustain continuous firing (Fig. 2.4). A dividing line at 220 spikes/s divides the populations at 34º C with 89% accuracy, quantified as # neurons sorted correctly (74) divided by total # of neurons (83); and with 97% accuracy (33/34) in room temperature recordings. Although differences in the details of protocol such as species choice, electrode type, and internal/external solution composition may alter the precise cutoff somewhat, its robustness to changes in temperature make this attribute a good candidate for analysis by other laboratories.

When data on neuronal maximum firing rate is unavailable, the measure of ADP defined here (the maximum value of the derivative during the 1 ms following the action
potential repolarization to threshold) can be used both to divide the populations (Fig. 2.3C) and to provide an unbiased criterion for monophasic vs. biphasic AHP. At 34º C, ADP values above or below 0 V/s reliably classify 94% of neurons (102/108). When spontaneous spikes from the neurons in this study were examined (as opposed to the spikes at a forced 10 Hz rate), the ADP was still 91% (59/65) accurate at dividing the population, despite the fact that the spontaneous action potentials occurred at rates ranging from 3-70 Hz. At 23º C, however, only 83% of neurons (52/63) are classified correctly, suggesting that spike analysis of neurons at room temperature may be less reliable.

Although many other measures are different between the two populations of neurons (Table 2.2), the maximum firing rate and the ADP taken together are highly effective at dividing the population as follows:

Maximum FR + 25 x ADP < 240 \rightarrow \text{GIN (Type A)}

Maximum FR + 25 x ADP > 240 \rightarrow \text{YFP-16 (Type B)}

This equation predicted the identity of 95% (79/83) of the neurons in this study at 34º C and 100% (34/34) of neurons at 23º C. In addition, it correctly classified 96% (43/45) of neurons that underwent RT-PCR and were not included in the primary dataset used to derive this equation (Table 2.3). To verify the equation’s utility at identifying GABAergic neurons, we recorded from fluorescent neurons in the GAD67-GFP knock-in mouse, which carries eGFP in place of one of the native copies of the Gad1 gene. 92% (22/24) of eGFP+ neurons were classified correctly as GIN-like, or GABAergic, by the above equation (Fig. 2.8). Therefore, our classification scheme may be generally
useful to other laboratories attempting to distinguish classes of MVN neurons without the benefit of transgenic mouse lines.

Discussion

In this paper, we use transgenic mice with complementary subsets of fluorescently labelled neurons to supply a critical link between cell type and intrinsic physiology in the MVN. Glycinergic and glutamatergic neurons labeled in the YFP-16 mouse exhibit intrinsic membrane and firing properties distinct from those of GABAergic neurons in the GIN mouse, indicating that there exist at least two information processing streams that differentially transform incoming signals to firing rate output. Surprisingly, glycinergic YFP-16 neurons display characteristics similar to glutamatergic, rather than GABAergic, neurons, implying that intrinsic physiological properties are matched to circuit function, rather than to transmitter phenotype. Retrograde dye labeling indicates that both GABAergic and glycinerergic neurons play a role in commissural signalling to the contralateral vestibular nuclei. Our results supply a schema for using action potential or firing properties to classify unidentified MVN neurons as GABAergic or non-GABAergic.

Cell type classification in the MVN

Intracellular recordings of MVN neurons in brain slices revealed that action potential properties vary continuously throughout the neuronal population (Serafin et al., 1991; Johnston et al., 1994; du Lac and Lisberger, 1995). Initial attempts to classify neurons in vitro subdivided the population into two types, A and B, largely on the basis
of the presence of second component to the AHP (Serafin et al., 1991; Johnston et al., 1994). While action potentials characteristic of type A and B neurons have been observed in a variety of species (Straka et al., 2005), classification of neurons based on a continuous AHP profile requires semi-arbitrary subdivisions (Beraneck et al., 2003) and is not robust to changes in intrinsic physiological properties that occur with development (Dutia and Johnston, 1998; Murphy and du Lac, 2001), cellular plasticity (Nelson et al., 2003), or altered vestibular experience (Ris et al., 2002; Beraneck et al., 2003; Beraneck et al., 2004).

A significant advance in classifying MVN neurons in vitro used RT-PCR to identify the transmitter phenotype of physiologically characterized cells: Takazawa and colleagues (2004) demonstrated that neurons with a monophasic AHP were primarily GABAergic, and that glutamatergic neurons tended to exhibit an ADP. These results suggested that type A neurons were GABAergic but that neurons likely to be classified as type B based on their AHP profile could be either glutamatergic or GABAergic; glycinergic neurons were rarely encountered.

Data from two distinct sets of MVN neurons identified in transgenic lines indicates that GABAergic neurons can be differentiated from glycinergic or glutamatergic neurons using intrinsic physiological characteristics. Our results thus both extend and supplant prior classification schemes by providing objective criteria that subdivide MVN neurons into two types on the basis of maximum firing rate or ADP, while taking advantage of the tools of mouse genetics to avoid relying on these measurements in future studies. Consistent with previous findings, GABAergic neurons, which encompass type A, have relatively wide action potentials (Takazawa et al.,
YFP-16 neurons exhibit narrow action potentials described previously in type B neurons (Serafin et al., 1991; Johnston et al., 1994). Both glutamatergic and glycinergic YFP-16 neurons (Table 2.3) exhibit firing properties characteristic of glutamatergic neurons recorded by Takazawa et al. (2004) as well as of presumed glutamatergic oculomotor nucleus projecting neurons (Sekirnjak and du Lac, 2006). The spatial segregation of GIN and YFP-16 neurons into the parvocellular and magnocellular divisions of the MVN coupled with their transmitter phenotype suggest that YFP-16 neurons project to ocular motor nuclei and spinal cord, while GIN neurons provide intrinsic connections within the vestibular complex (Epema et al., 1988; Buttner-Ennever, 1992).

Our classification scheme also illuminates previous results that relied on the Type A/B division. Camp and colleagues (2006) reported that Type B MVN neurons receive both glycinergic and GABAergic inputs, while Type A receive only GABAergic inputs, suggesting that glutamatergic and glycinergic neurons are targets of glycinergic inputs, while GABAergic neurons are not. Unilateral labyrinthectomy differentially affects Type A and Type B neurons, triggering physiological changes which may cause one type to masquerade as the other (Him and Dutia, 2001; Beraneck et al., 2003; Beraneck et al., 2004); these results highlight the utility of the transgenic mice studied here for future studies.

**YFP-16 neurons in the MVN**

RT-PCR indicates that YFP-16 neurons targeted in this study express glycinergic or glutamatergic markers (Fig. 2.2). *In vivo* recordings and histology have
demonstrated that glycinergic neurons provide a major output of the MVN, namely that to the ipsilateral abducens nucleus, which controls the lateral rectus muscle (Spencer et al., 1989). We hypothesize that glycinergic YFP-16 neurons send axons to the abducens nucleus and/or the spinal cord, based on in vivo cell fills that show abducens-projecting MVN neurons also sending collaterals caudally (McCrea et al., 1980; Spencer et al., 1989). Glutamatergic neurons in the MVN are likely to make one of two major circuit projections: to motor neurons in the contralateral ocular motor nuclei; or as mossy fibers to the cerebellar flocculus (Graf et al., 1997; Graf et al., 2002). Based on the physiological similarity between YFP-16 neurons and oculomotor-projecting neurons (Sekirnjak and du Lac, 2006), we propose that the glutamatergic YFP-16 neurons identified here are sources of input to the contralateral oculomotor nuclei, and perhaps to the contralateral abducens. Glutamatergic floccular-projecting neurons in cats are located primarily in the dorsolateral, parvocellular MVN (Epema et al., 1990; Cheron et al., 1996), where YFP-16 neurons are sparse (Fig. 2.1A), arguing against the likelihood that YFP-16 neurons project to the flocculus, although further experiments will be needed to resolve this question.

**GABAergic neurons in the MVN**

Although GIN neurons comprise a subset of GAD67 expressing neurons (Oliva et al., 2000), their intrinsic physiological properties appear to be representative of GABAergic neurons more generally, as evidenced by recordings from GAD67-GFP knockin mice (Fig. 2.8). GIN neurons in spinal cord and cortex have highly variable intrinsic and morphological attributes, suggesting that they may comprise several
subgroups with different circuit roles (Heinke et al., 2004; Dougherty et al., 2005; Halabisky et al., 2006). GABAergic neurons in the MVN are thought to have five major targets: spinal motor areas; ipsilateral oculomotor and trochlear nuclei; inferior olive; ipsilateral MVN and prepositus neurons; and the contralateral MVN (Holstein, 2000). Because GIN neurons are found primarily in the parvocellular, intrinsically projecting region of the MVN (Fig. 2.1B) (Buttner-Ennever, 1992), and because their physiology is dissimilar to oculomotor-projecting neurons (Sekirnjak and du Lac, 2006), they are unlikely to fill the first two categories. Our data are consistent with the possibility that some GIN neurons project to the inferior olive (Gerrits et al., 1985; Barmack et al., 1993) and/or participate in feedforward inhibition from the vestibular nerve to other MVN neurons (Kinney et al., 1994; Straka and Dieringer, 1996). In addition, some GIN neurons project commissurally (Fig. 2.6); thus, they likely represent the source of contralateral GABAergic input that helps set up the push-pull dynamics of the VOR (Shimazu and Precht, 1966; Kasahara et al., 1968; Mano et al., 1968; Holstein et al., 1999).

Some GIN neurons, including those projecting commissurally, express GlyT2 (Fig. 2.2). GABA and glycine share the vesicular transporter VGAT (Wojcik et al., 2006), and a subset of MVN neurons colocalize GABAergic and glycine markers (Reichenberger et al., 1997; Takazawa et al., 2004; Tanaka and Ezure, 2004). Our data indicate that there are two types of glycineergic commissural neurons with distinct processing properties (Fig. 2.7C and D); it is possible that they target different populations of neurons in the contralateral MVN (Camp et al., 2006) and/or that the neurons colocalizing GABA and glycine appose postsynaptic neurons with selective
receptor expression (Precht et al., 1973), as occurs in the vestibulocerebellum (Dugue et al., 2005).

**Physiological diversity of neuronal types**

Throughout the cortex and hippocampus, GABAergic interneurons are readily distinguished from glutamatergic pyramidal neurons by their narrow action potentials and fast firing rates (Cauli et al., 1997; Jonas et al., 2004). Surprisingly, we find that the reverse is true in the MVN: GABAergic neurons exhibit broader action potentials and slower maximum firing rates than those of putative projection neurons in the MVN. Because GABAergic and glycinergic neurons can display markedly different physiological characteristics, while glutamatergic and glycinergic neurons exhibit similar ones, we propose that in the VOR circuit, cellular physiology is more closely related to the circuit role of a neuron rather than simply its neurotransmitters. The abducens and spinal cord, both of which are major components of vestibular circuits, receive glycinergic and glutamatergic input from the MVN; thus, the YFP-16 neurons, which are likely members of that circuit, share the responsibility of translating head motion into compensatory motor behavior, and as such require a wide dynamic operating range. In contrast, GABAergic neurons, putatively local interneurons whose influence does not extend beyond the bilateral vestibular nuclei, exhibit a significantly narrower dynamic range. While these intrinsic physiological attributes may reflect functional roles, they are labile and can change following experience (Straka et al., 2005; Gittis and du Lac, 2006). Thus the mouse lines described here should provide a natural springboard for studies of cell-type specific plasticity.
Acknowledgments

This chapter is a reprint of the material as it appears in Bagnall MW, Stevens RJ, du Lac S. Transgenic mouse lines subdivide medial vestibular nucleus neurons into discrete, neurochemically distinct populations. J Neurosci. 2007 Feb 28; 27(9):2318-30.

The dissertation author was the primary author of this paper.
References


Fig. 2.1  Distribution and morphology of YFP-16 and GIN neurons in the MVN. Coronal sections from the YFP-16 (A) and GIN (B) lines. MVNp, parvocellular MVN; MVNm, magnocellular MVN; NPH, nucleus prepositus hypoglossi; IV, fourth ventricle (darkened for contrast). Scale bar is 200 µm and applies to both panels. C, Magnified view of YFP-16 neurons and D, GIN neurons in MVN. Scale bar, 10 µm, applies to both. E, Histogram of soma size, measured as the product of length and width. YFP-16 neurons are significantly larger than GIN neurons ($p < 0.001$). F, Histogram of number of processes extending from cell body. On average, YFP-16 neurons extend significantly more primary dendrites than GIN neurons ($p < 0.0001$). N = 108 (YFP-16) and 160 (GIN) neurons for both graphs.
Fig. 2.2 Single cell RT-PCR indicates that YFP-16 neurons are glutamatergic or glycinergic, while GIN neurons are GABAergic. RT-PCR was carried out on YFP-16 and GIN neurons from slice recordings. Neurons were assayed for five indicators of transmitter content: VGlut1, VGlut2, GlyT2, GAD65, and GAD67. Two neurons are shown from each line. A, YFP-16 neuron A is VGlut2+ while neuron B is GlyT2+. B, GIN neuron A expressed the GABAergic markers GAD67 and GAD65, as well as the glycinergic marker GlyT2; GIN neuron B expressed only GAD67 and GAD65. RNA controls, right, were 0.5 ng RNA extracts from whole mouse brain processed alongside single cells.
Fig. 2.3  Action potentials differ between YFP-16 and GIN neurons. A, Example action potentials from two YFP-16 neurons (black). B, Examples from two GIN neurons (gray). C, Relationship between afterdepolarization following spike repolarization and action potential width at half-height. Each point represents one neuron (YFP-16, open squares, n = 49; GIN, gray triangles, n = 59; unidentified neurons from a separate study, dark gray dots, n = 61). D, Maximum depth of the AHP in YFP-16 and GIN neurons, plotted versus the time after the spike peak when that maximum is reached. Symbols and n as for C.
Fig. 2.4  Input-output properties in YFP-16 and GIN neurons. Examples of responses to depolarizing current steps in A, a YFP-16 neuron, and B, a GIN neuron. Steps of increasing amplitude were given until the neuron could no longer fire action potentials throughout the whole step. Bottom panels: mean firing rate evoked during depolarization is plotted versus the amplitude of the current step for each neuron shown above. The break in line fit indicates the measurement of gain below and above 80 Hz for each neuron. C, Scatterplot of maximum firing rate (average over whole step) versus input resistance measured below spike threshold. Each point represents one neuron (YFP-16, n = 40; GIN, n = 43; unidentified neurons, n = 61). D, Maximum input current for which a neuron could sustain action potentials during 1 s of depolarization. YFP-16 neurons can respond to roughly 3-fold more current than GIN neurons (p < 0.0001, Table 2.2). Means are indicated by black bars; three YFP-16 neurons whose maximum input current exceeds 4 nA are not shown here for graphical clarity. E, Histogram of adaptation ratio, defined as the firing rate at the end of the 1 s depolarization divided by firing rate at the beginning. YFP-16 neurons adapted significantly more than GIN neurons (p < 0.0001, Table 2.2). F, Ratio of cellular gain in high range (> 80 spikes/s) to gain in low range (< 80 spikes/s). GIN neurons were significantly more linear than YFP-16 neurons (p < 0.0001, Table 2.2).
Fig. 2.5  Post-inhibitory rebound firing in YFP-16 and GIN neurons. A, Examples of rebound firing following 1 s of hyperpolarizing current injection in YFP-16 (open squares) and GIN (closed triangles) neurons. Neurons were forced to fire 10-20 Hz and hyperpolarized ∼30 mV in order to facilitate comparisons across cells. B, Data from individual neurons is plotted against their input resistance. YFP-16 neurons exhibit significantly more rebound firing than GIN neurons, although there is large heterogeneity (Table 2.2). Each symbol represents one neuron (YFP-16, n = 38; GIN, n = 24). C, Rebound firing and ADP are positively correlated across all neurons (n as in B). One neuron with rebound of 300 spikes/s was excluded from analyses.
Fig. 2.6  Identification of MVN neurons that project commissurally. A, Diagram of dye labeling method. Crystals of fluorescently labeled dextran (fluororuby or Texas Red) were deposited unilaterally into the ventral aspect of the MVN in live slices. The dye was taken up by cut axons and transported retrogradely to cell bodies of origin. B, Section from a GIN animal, showing axons at the midline. Left, GFP; middle, fluororuby; right, merge. Scale bar 100 µm. C, Commissurally-projecting YFP-16 and D, GIN neurons in the MVN contralateral to the injection. Scale bar 50 µm for both.
**Fig. 2.7** Physiology of identified commissural YFP-16 and GIN neurons. 

**A**, Action potentials from a YFP-16 commissural neuron (left, blue) and a GIN commissural neuron (right, red). 

**B**, Data as in Fig. 3C; the ADP is plotted versus the action potential half-width for each neuron. Commissural neurons in both lines display similar action potential waveforms to their parent populations (YFP-16 commissural, blue squares, n = 16; GIN commissural, red triangles, n = 17). 

**C**, Reproduction of Fig. 4C with data from commissural neurons overlaid (YFP-16, n = 17; GIN, n = 16). YFP-16 and GIN commissural neurons resemble the unidentified overall population of YFP-16 neurons. Slight differences may arise from developmental changes, since commissural recordings were made in younger animals (P14-P17, compared to P17-P28).
Fig. 2.8 Measurements of intrinsic physiological characteristics successfully predict whether an MVN neuron is GABAergic or not. The action potential ADP value for each neuron is plotted versus its maximum firing rate; a line on the resulting graph accurately classifies 96% of YFP-16 and GIN neurons into their respective groups (see Results). The general utility of this dividing line was then evaluated by recording from GABAergic neurons labeled in the GAD67-GFP line, 92% of which are also classified correctly as GABAergic. YFP-16, n = 40; GIN, n = 43; Gad67-GFP, n = 24.
Table 2.1 Primers for single-cell RT-PCR. VGlut1 and VGlut2 used nested primers while the other products were semi-nested. F/R: forward/reverse primers.

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<td>Zhang et al., 2004</td>
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<tr>
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Table 2.2  Intrinsic characteristics of YFP-16 and GIN neurons in the MVN are distinct at both physiological and room temperature. Room temperature recordings: n for spike parameters is 26 (YFP-16) and 37 (GIN); for cell parameters, n = 14 - 37. With the exception of threshold and gain < 80 Hz, all parameters are significantly different between YFP-16 and GIN at 23º C (p < 0.05). Values indicate mean ± SD. *Some input resistance measurements were made in voltage clamp rather than current clamp.

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<th>P value</th>
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<td>GIN</td>
<td>YFP-16</td>
<td>GIN</td>
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<td>AP width (ms)</td>
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<td>Max deriv (V/s)</td>
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<td>444 ± 117</td>
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<td>Max. firing rate (Hz)</td>
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<td>242 ± 122</td>
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<td>PRF (spikes/s)</td>
<td>42 ± 40</td>
<td>12 ± 13</td>
<td>38</td>
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Table 2.3 Glutamatergic and glycinergic YFP-16 neurons exhibit similar physiology to each other, but not to glycinergic GIN neurons. Left columns, the physiology of YFP-16 neurons subjected to RT-PCR. Spike parameters, n = 9 (each group); cell parameters, n = 7 - 9. Right columns, the physiology of GIN neurons that underwent RT-PCR. Most neurons expressed both GAD67 and GAD65; the data here are divided into those neurons which did or did not co-express GlyT2 (spike parameters, n = 14, each group; cell parameters, n = 11-14). Data represent a separate pool of neurons than those analyzed in Table 2. Within YFP-16 neurons, only input resistance and PRF were significantly different between VGlut2+ and GlyT2+ neurons; within GIN neurons, only AHP magnitude was different between GlyT2+ and GlyT2- neurons (p < 0.05). Values indicate mean ± SD.

<table>
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<td>Max. firing rate (Hz)</td>
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<td>Adapt. ratio @ 40 Hz</td>
<td>0.72 ± 0.12</td>
<td>0.79 ± 0.08</td>
<td>0.91 ± 0.08</td>
<td>0.92 ± 0.09</td>
</tr>
<tr>
<td>Gain &lt; 80 Hz (Hz/nA)</td>
<td>178 ± 63</td>
<td>222 ± 74</td>
<td>240 ± 71</td>
<td>239 ± 120</td>
</tr>
<tr>
<td>Gain &gt; 80 Hz (Hz/nA)</td>
<td>98 ± 37</td>
<td>134 ± 37</td>
<td>202 ± 64</td>
<td>185 ± 99</td>
</tr>
<tr>
<td>Gain / Input resistance</td>
<td>2.27 ± 0.74</td>
<td>2.06 ± 0.44</td>
<td>0.97 ± 0.39</td>
<td>1.12 ± 0.44</td>
</tr>
<tr>
<td>PRF (spikes/s)</td>
<td>52 ± 32 *</td>
<td>21 ± 16 *</td>
<td>11 ± 8</td>
<td>8 ± 7</td>
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</table>
III. Activity at a primary afferent synapse drives postsynaptic firing with linear dependence on stimulation rate

Abstract

The vestibular system, which is responsible for transforming head motion into compensatory eye, head, and body movement, is capable of exceptional speed and linearity. During the vestibulo-ocular reflex, a ten-fold increase in head velocity must generate a ten-fold increase in contraversive eye velocity, or the visual scene will blur. How can central excitatory synapses, which are typically thought of as nonlinear elements, support a linear behavior over a wide dynamic range? In this study, we demonstrate that the glutamatergic vestibular afferent synapse can provide a linear transfer of sensory information to central neurons without relying on a one-to-one relationship between presynaptic and postsynaptic firing. In slice preparation, recording in voltage clamp from medial vestibular nucleus neurons while stimulating the vestibular nerve revealed steady-state depression of EPSCs to ~60% of their initial value over a wide range of frequencies (5 - 100 Hz) that correspond to in vivo dynamic range of the nerve. As a result, the total synaptic charge transfer per second was linearly related to presynaptic stimulation rates. This observation held true for recordings from both projection and intrinsic neurons in the vestibular nucleus, indicating that the two populations examined here are probably recruited in similar ways by head movements. Finally, we demonstrate that vestibular nucleus neurons are capable of transducing this synaptic input into linear increases in their firing rate output. The results indicate that
the vestibular system relies on a carefully tuned synapse in order to maintain linearity through the first stage of central processing in the vestibulo-ocular and vestibulospinal circuits.

**Introduction**

Vestibular reflexes are both fast and robust following head movements with velocities exceeding 150 °/s. During the vestibulo-ocular reflex (VOR), head motion elicits compensatory eye movements whose gain (eye velocity divided by head velocity) is close to 1 over a 30-fold range of head velocities (M. Faulstich, Fig. 1.2). The neural basis of the VOR is a three-synapse arc from the vestibular nerve to the vestibular nuclei, and from there to oculomotor nuclei and eye muscles. It is known that the individual fibers of the vestibular nerve code primarily for velocity, although some also include an acceleration component (reviewed in Highstein et al., 2005). Their postsynaptic targets, vestibular nucleus neurons, are reliable indicators of head velocity in mouse (Beraneck and Cullen, 2007) as in other species (du Lac et al., 1995). Therefore, the vestibular afferent synapse onto vestibular nucleus neurons must faithfully transmit a wide range of head velocity signals.

Although these excitatory inputs are responsible for driving several behaviors, including the VOR and the vestibulospinal reflexes, their synaptic properties have not been well described. *In vivo* recordings from the VIIIth nerve have revealed that each vestibular fiber exhibits sensitivity to head motion over the whole range of possible frequencies, in contrast with auditory fibers, which respond best to sounds of a characteristic frequency (Oertel, 1999; Highstein et al., 2005). In mouse, the sensitivity
of each fiber to rotation ranges from ~0.05 to 0.4 (spikes/s)/(deg/s), with only minimal increases in gain for increasing head rotation frequency, overlaid on a background firing rate of 40-60 spikes/s (Yang and Hullar, 2007). In order for vestibular nucleus neurons to be comparably modulated, the afferent synapse must successfully transmit a signal whose amplitude over a given time period scales linearly with head velocity, and thus with afferent firing rates.

In the neighboring auditory system, afferent fibers contact bushy projection cells in the anteroventral cochlear nucleus with large (~10 µm long) synapses known as the endbulbs of Held. These synapses contain ~100-200 release sites and drive postsynaptic action potentials after every release event (Trussell, 1999; Nicol and Walmsley, 2002). In contrast, vestibular afferent fibers make moderately sized synapses of < 3 µm onto both magnocellular (presumed projection) and parvocellular (presumed intrinsic) neurons in the medial vestibular nucleus (MVN) (Sato and Sasaki, 1993), and are not thought to be able to drive action potentials with every stimulus. Therefore, it is unclear whether the synaptic output of the vestibular nerve itself is a faithful readout of head velocity, or whether the synapse exhibits some nonlinearities which require compensatory postsynaptic processing in vestibular nucleus neurons. In this study we stimulate the vestibular afferents in mouse brainstem slices while recording in voltage and current clamp from MVN neurons, and examine the synaptic properties that produce this linear pre- to post-synaptic firing rate transformation.
Methods

Two transgenic lines of mice were used in this study: YFP-16 (Feng et al., 2000), in which glutamatergic and glycinergic neurons in the MVN are fluorescently labeled (Bagnall et al., 2007); and GIN, in which a subset of GABAergic MVN neurons are fluorescently labeled (Oliva et al., 2000; Bagnall et al., 2007). Mice aged 15-25 days postnatal (mean, P21 ± 0.3) were deeply anesthetized with Nembutal and decapitated.

After rapid dissection in ice cold Ringer’s solution (in mM: 124 NaCl, 5 KCl, 1.3 MgSO_4_, 26 NaHCO_3_, 2.5 CaCl_2_, 1 NaH_2PO_4_), 250 - 400 µm thick slices were cut on a DSK DTK-1500E or Leica VT1000S vibratome and allowed to recover at 34 ºC for 30 min. Slices rested at room temperature before being transferred to a recording chamber and perfused with carbogenated Ringer’s containing 1-10 µm strychnine and 100 µm picrotoxin at 34 ºC.

Patch pipettes were pulled from flame-polished glass (Warner) with resistances of ~2-4 MΩ. Pipette internal solution contained (in mM) 140 K gluconate, 10 HEPES, 8 NaCl, 0.1 EGTA, 2 Mg-ATP, 0.3 Na_2-GTP. Neurons were visualized with epifluorescence through a GFP filter as well as under infrared differential interference contrast illumination with Nomarski optics. A bipolar concentric stimulating electrode (FHC, Maine) was placed on the vestibular nerve lateral to the vestibular complex (Fig. 3.1A) and controlled via an Isoflex stimulus isolation unit (AMPI, Israel). Biphasic pulses, each half 0.1 ms in duration, were delivered to the electrode to avoid charge build-up during high frequency trains.

Data were acquired with a Multiclamp 700B filtering at 6 kHz for voltage clamp and 10 kHz for current clamp. Data were digitized at 40 kHz with an ITC-16
(InstruTECH). House-written code in Igor 5 was used for acquisition and analysis. Recordings were discarded if series resistance, tested with a small hyperpolarizing square pulse, exceeded ~15 MΩ. 5-10 sweeps (10-30 s interstimulus interval) of each stimulus were averaged together.

Because vestibular nerve afferents are heterogeneous with respect to diameter and myelination (Huwe and Peterson, 1995), conduction velocities vary across the afferent population. As a result, in many EPSCs an inflection point was visible in the rise, decay, or both, presumably representing the arrival of several different synaptic currents at slightly different latencies. All such EPSCs were monosynaptic, based on their latency (< 2 ms) and on the fact that they did not disappear during manipulation of external divalent ions intended to eliminate disynaptic activation (to 1 mM Ca$^{2+}$, 2.8 mM Mg$^{2+}$; or to 4 mM Ca$^{2+}$, 4 mM Mg$^{2+}$, data not shown). Larger diameter afferents should in theory have a lower threshold for recruitment than thinner diameter fibers. However, in our hands there was no clear relationship between stimulation intensity and the recruitment of these longer- or shorter- latency EPSC components. This may be due to heavier myelination of large afferents, which would increase the stimulation intensity required to activate them. Because of the impossibility of studying these components systematically, we instead treated each compound EPSC as a single unit, and analyzed its decay kinetics with a 90-10% fall time measure, rather than fitting an exponential tau of decay. During recordings, stimulation intensity was adjusted to produce a reliable EPSC, normally in the range of 1.5 - 3x the threshold intensity. During voltage clamp, the postsynaptic cell was clamped at -75 mV to isolate primarily an AMPA-mediated response; NMDA currents are thought to be small in MVN neurons at the ages recorded.
here (S.K. Lai et al., Soc. Neurosci. Abstr., 2007) Firing rates are reported as the average of the reciprocal of the inter-spike interval, and were averaged across the entire 1 s of synaptic stimulation.

Statistical tests were done with KaleidaGraph 3.6 (Synergy Software) and are reported as mean ± SEM except as noted.

Results

Two transgenic mouse lines label complementary neuronal distributions in the MVN: the YFP-16 line (Feng et al., 2000), in which a subset of glycinergic and glutamatergic neurons are fluorescent; and the GIN line (Oliva et al., 2000), in which a subset of GABAergic neurons are fluorescent (Bagnall et al., 2007). Physiologically, YFP-16 neurons, which predominate in the magnocellular MVN, resemble identified oculomotor-projecting neurons, with narrow action potentials and high maximum firing rates (Sekirnjak and du Lac, 2006). In contrast, GIN neurons, located primarily in the parvocellular MVN, have broad action potentials and lower maximum firing rates (Bagnall et al., 2007). Based on these observations, as well as tracer studies showing YFP-16 but not GIN projections to several major MVN target nuclei (B. Zingg, K. Kolkman, unpublished data; Epema et al., 1988), the YFP-16 population may be taken to represent projection neurons, the GIN population intrinsic or local neurons (including some commissural projections to the contralateral MVN) (Bagnall et al., 2007). Therefore, these two transgenic mouse lines permit analysis of the vestibular afferent synaptic characteristics with reference to circuit function (Fig. 3.1B).
Recordings were made from fluorescently-labeled neurons in brainstem slices from older juvenile mice (P21 ± 0.3). Stimulation of the vestibular nerve in brainstem slices at a point slightly lateral to the vestibular complex (Fig. 3.1A) elicited synaptic currents in the majority of vestibular nucleus neurons, consistent with in vivo results suggesting that the vestibular nerve contacts most MVN neurons (Goldberg and Fernandez, 1971; Straka and Dieringer, 1996; Babalian et al., 1997). In both YFP-16 and GIN neurons, EPSCs exhibited fast kinetics (Fig. 3.2A; YFP-16, black; GIN, gray). 10-90% rise times in most neurons were < 0.5 ms (Fig. 3.2B; YFP-16, 0.41 ± 0.05 ms; GIN, 0.39 ± 0.04 ms), and 90-10% decay times usually < 5 ms (Fig. 3.2C; YFP-16, 3.4 ± 0.34 ms; GIN, 3.3 ± 0.32 ms).

Increases in stimulation intensity yielded increases in EPSC amplitude due to the recruitment of more vestibular afferents in both YFP-16 and GIN neurons (sometimes visible as inflections in the rise or decay time; see Methods) (Fig. 3.3A). The largest synaptic currents elicited in YFP-16 neurons were frequently in excess of 500 pA, and in some cases ranged to several nA (median, 0.73 nA) (Fig. 3.3B). In contrast, the synaptic currents seen in GIN neurons were less than 1 nA, and in most cases never exceeded 500 pA (median, 0.18 nA; p < 0.0001, Wilcoxon unpaired test) (Fig. 3.3B). While the synaptic amplitudes recorded in slice preparation cannot be regarded as definitive due the likelihood that many axons are cut or damaged during slicing, these results are consistent with two other types of data: first, that the vestibular nerve axons taper and become more sparse, and the presynaptic boutons shrink, as the nerve progresses medially toward the 4th ventricle, where most GIN neurons are located (Huwe and Peterson, 1995); and second, that YFP-16 neurons have significantly lower
input resistances than GIN neurons [YFP-16, 107 ± 92 MΩ; GIN, 269 ± 137 MΩ (Bagnall et al., 2007)], indicating that they would need a larger synaptic input to drive firing. Therefore, it is highly probable that most vestibular contacts onto projection neurons are more powerful than those onto intrinsic neurons.

Throughout the nervous system there exists a wide variety of presynaptic release characteristics, with release probabilities ranging from low (e.g., the Schaffer collateral synapse to CA1 neurons) to maximal (e.g., the climbing fiber input to Purkinje cells). These presynaptic properties can shape the effective circuit role of different neuron classes by recruiting neurons immediately following the onset of activity at a high release probability synapse, or at longer latencies after prolonged activity of a low release probability synapse (Zucker and Regehr, 2002). We stimulated vestibular afferents at intervals ranging from 5 ms to 10 s and measured the ratio of the second EPSC to the first (Fig. 3.4A). In both YFP-16 and GIN neurons, the paired-pulse ratio was close to unity across all intervals tested (Fig. 3.4B). At the longest interval (10 s), no facilitation or depression was observed in either population; at intervals from 5 ms to 1 s, the paired-pulse ratio was ~0.85 in both types of neurons. This represents an unusually large range of frequency-independent response, in contrast to many synapses at which the presence of particular calcium buffers, synaptotagmin isoforms, and other as-yet unknown players endows the presynapse with a profile of frequency-dependent facilitation and depression (Blatow et al., 2003; Xu et al., 2007). The vestibular synapse instead appears largely resilient to changes in frequency.

In vivo, the mouse vestibular nerve fibers are constantly active at frequencies of 40-60 spikes/s and modulate above and below those frequencies during head motion
Therefore, we examined the postsynaptic response to trains of stimuli at frequencies from 0.1 to 200 Hz. Over the course of 20 pulses, EPSCs depressed during the first 5 - 10 stimuli and then reached a plateau of about 60% of their initial value for the remainder of the train (Fig. 3.5A, a synapse onto a YFP-16 neuron at 20 and 100 Hz; Fig. 3.5B, the same for a GIN neuron). Remarkably, this profile could be seen at stimulation frequencies ranging from 5 to 100 Hz, with slightly more depression occurring at 200 Hz (Fig. 3.5C), in both YFP-16 and GIN neurons. Within this frequency range, the number of stimuli, rather than the time course of their delivery, defined the downward slide into depression, as can be seen in plots of normalized EPSC amplitude relative to stimulus number (Fig. 3.5C). The range of inter-stimulus intervals over which the same plateau value occurs (Fig. 3.5D) suggests that in vivo, the vestibular inputs to populations of both projection and intrinsic neurons exist in steady-state depression.

Because the vestibular nerve fires action potentials at rates linearly related to head velocity (with a small acceleration signal) (Highstein et al., 2005), this means that the total charge transfer at each synapse over a given time period should be directly and linearly related to the head motion signal, i.e. the presynaptic rate of activity. We tested this idea by integrating the area under the average EPSC during the plateau phase (pulses 11-20) at several different frequencies. The resulting number, representing the charge transfer per steady-state EPSC, was normalized to the charge transfer at the first EPSC in the train in order to facilitate comparison across cells, and then multiplied by the rate of stimulation in order to obtain the total charge transfer during 1 s of steady-state activity at a given frequency. In both YFP-16 and GIN neurons, there was a linear
relationship between the rate of stimulation and the total synaptic charge transfer over the course of 1 s, for frequencies ranging from 5 to 100 Hz, with a moderate drop-off at 200 Hz (Fig. 3.5E).

Many synapses exhibit a “recovery” from high-frequency stimulation following a rapid transition to a lower rate of activity (Telgkamp and Raman, 2002). However, as the vestibular nerve modulates its activity rates relative to head motion, any synaptic recovery (or further depression) will compromise the ability of the postsynaptic neuron to provide time-invariant response to a given head velocity. To examine whether the afferent steady-state depression exhibited any sensitivity to transition between frequencies, we stimulated the vestibular nerve with two patterns of trains: 20 stimuli at 10 Hz followed by another 20 stimuli at 50 Hz (Fig. 3.6A), or vice versa (Fig. 3.6B). In both cases, the frequency shift did not cause any change in the EPSC amplitude, whether from low to high frequency or high to low. These results demonstrate that during normal modulation of head motion, the vestibular nerve faithfully transmits a synaptic signal whose single EPSCs are invariably the same size, and whose total charge transfer accurately reflects head velocity.

The data presented thus far demonstrate that the synaptic charge transfer at the vestibular nerve synapse onto different types of vestibular nucleus neurons is linearly related to presynaptic activity rates. The question remains, however, whether MVN neurons are able to preserve the linearity encoded at the synaptic input level in their firing rate output. To answer this question, we recorded in current clamp the response of MVN neurons to synaptic stimulation over a range of frequencies. DC depolarizing or
hyperpolarizing current was injected into MVN neurons to maintain a baseline firing rate of ~10 spikes/s, to provide a stable baseline and simulate the low end of in vivo firing rates (Beraneck and Cullen, 2007). Synaptic stimulation for 1 s at rates from 20 to 200 Hz caused increases in firing rates confined to the period of stimulation (Fig. 3.7A; left, YFP-16; right, GIN). The average firing rate over the course of 1 s of stimulation is plotted for two stimulation frequencies (20 and 100 Hz) for an example YFP-16 and GIN neuron in Fig. 3.7B. Fig. 3.7C shows the average firing rate over the stimulation period (averaged over two trials) at all stimulation rates in the two neurons in Figs. 3.7A and B. In both instances, the MVN neuron responds to increases in stimulation rate with a linear increase in firing rate, up through presynaptic stimulation rates of 150 Hz (YFP-16, R² = 0.992; GIN, R² = 0.994). Both populations exhibited highly linear responses across 20-150 Hz stimulation rates (YFP-16, median R² = 0.948; GIN, 0.978) (Fig. 3.7D). At 200 Hz, there was typically a slight to moderate drop in response linearity, as predicted from the greater synaptic depression visible at that stimulation frequency in voltage clamp (Fig. 3.5E). The slope of the line fit to the firing rate relative to stimulation rates of 20 - 150 Hz was variable across neurons, as expected due to variations in synapse size and postsynaptic capacitance and resistance, but on average insignificantly higher in YFP-16 than GIN neurons (YFP-16, median = 0.17 spikes/Hz; GIN, 0.09; p = 0.09, Wilcoxon unpaired test) (Fig. 3.7E). Thus it is clear that both GABAergic and nonGABAergic vestibular neurons are capable of faithfully reproducing a progressively faster synaptic input as a linearly and progressively higher firing rate output, not by relying on a powerful single input that always drives an action potential, as at the calyx of Held in the auditory brainstem, but by successfully summing
the excitatory input and maintaining a linear current-firing rate relationship over at least a 7-fold range of input frequencies.

**Discussion**

In this study we show that activity at the vestibular nerve synapse onto vestibular nucleus neurons depresses excitatory postsynaptic currents (EPSCs) to a steady state of ~60% of their initial value. This depression occurs independent of stimulation frequency, over a range from 5 - 100 Hz that matches the dynamic range of *in vivo* firing rates. As a consequence, the steady-state charge transfer at these synapses is linearly related to the rate of stimulation, within the range of physiological frequencies of the nerve. We furthermore show that vestibular synapses onto both nonGABAergic presumed projection neurons and local GABAergic neurons display these characteristics, although synaptic currents measured in nonGABAergic neurons were typically ~3-fold larger than those measured in GABAergic neurons. This study provides insight into the basic synaptic properties responsible for the linearity of the vestibulo-ocular reflex, and lays the groundwork for investigation into their mechanistic underpinnings.

**Synaptic kinetics and glutamate receptors**

The vestibular fibers of the VIIIth nerve, like their auditory homologs, fire at high rates *in vivo* (Yang and Hullar, 2007). In this study we show that their synapses onto vestibular nucleus neurons, like the homologous auditory synapses onto bushy cells in the anteroventral cochlear nucleus, exhibit rapid EPSC kinetics (Trussell, 1999).
As a result, there is very little summation of synaptic currents arriving at intervals of even 5 or 10 ms in the postsynaptic neuron (e.g. Fig. 3.5A, B). In these recording conditions, with the postsynaptic cell voltage clamped at -75 mV, very little activation of NMDA receptors would be expected, in accordance with the rapid fall times seen here. It will be of interest to see whether this synapse resembles the auditory synapse onto the chick nucleus magnocellularis in lacking the AMPA receptor subunit GluR2, which has slow kinetics (Otis et al., 1995). In its absence, the non-GluR2 forms of the AMPA receptor would provide a source of postsynaptic calcium entry, which could be relevant for synaptic plasticity. At the homologous synapse in frog, NMDA-receptor mediated currents are recruited at low stimulation intensities of the vestibular nerve, and become proportionally less significant with increasing stimulus intensities, suggesting that only certain low-threshold vestibular fibers are apposed to synapses containing NMDA receptors (Straka et al., 1996). While we did not examine the NMDA-receptor current, the linearity of the postsynaptic response in current clamp (Fig. 3.7) suggests that the NMDA current is trivial if present at all, because otherwise the significant postsynaptic depolarization with increasing stimulation rates would have driven a supra-linear response.

Linear relationship between presynaptic activity rate and steady-state charge transfer

Very few neuronal connections exhibit the type of frequency-independent steady-state depression detailed here. More commonly, there is an interaction between facilitation, thought to be caused by residual terminal Ca$^{2+}$, and depression, thought to be due to depletion of a readily releasable pool of vesicles and a change in Ca$^{2+}$
sensitivity of the release machinery (Zucker and Regehr, 2002; Wolfel et al., 2007). Synapses display a wide range of profiles of facilitation and depression with respect to frequency of presynaptic activity. The GABAergic projection from cerebellar Purkinje cells onto deep cerebellar nucleus neurons exhibits comparable frequency-independent depression to vestibular afferents, although with significant summation between IPSCs as well as a clear offset at shifts between frequencies (Telgkamp and Raman, 2002; Telgkamp et al., 2004) (cf. Fig. 3.5A, Fig. 3.6). Similarly, the cerebellar mossy fiber to granule cell synapse is robust over a range frequencies of stimulation, but again exhibits substantial summation and progressively more depression at increasing stimulation rates (Saviane and Silver, 2006).

The net effect of the frequency-independent steady state plateau is that at all physiologically relevant frequencies, the total charge transfer over 1 s will depend linearly on the afferent stimulation rate (Fig. 3.5E). These results stand in marked contrast to the relationship between afferent activity rate and charge transfer at the cortical layer 4 to 2/3 synapse, where depression progressively deepens with increasing stimulation rates in an exponential fashion. As a result, the steady-state charge transfer per unit time at that synapse is almost independent of the presynaptic rate of activity, and only fluctuations in presynaptic activity produce changes in charge transfer (Abbott et al., 1997). The data here show that the afferent synapse fits the unusual requirements of the vestibular system, in which increased head motion must be perfectly matched by increased compensatory eye and/or body motion to maintain image stability on the retina.
What are the possible mechanisms of the frequency-independent steady-state transmission documented here? The two primary options are that either the vestibular fiber releases the same amount of transmitter on each pulse in steady-state; or that frequency-dependent changes in the quantity of transmitter released are masked by postsynaptic changes in sensitivity, which could arise from AMPA receptor saturation or desensitization. At the Purkinje cell to deep cerebellar nucleus neuron synapse, presynaptic terminals ~2-4 µm long each contain about 10 distinct release sites, and as a result the IPSC maintains a relatively steady amplitude regardless of presynaptic stimulation rate (Telgkamp et al., 2004). The only anatomical study to date of vestibular nerve afferent terminals demonstrated that boutons were also on average ~2-3 µm long, and while the authors do not address the possibility of multiple release sites, there are several clearly visible in their high-quality electron micrographs (Sato and Sasaki, 1993, e.g. their Fig. 6). If vestibular afferents are analogous to Purkinje cell axons in this respect, then low probabilities of release at each release site would help provide a robust postsynaptic response, since at least one release site will typically be functional (Telgkamp et al., 2004). Alternatively, if vestibular afferents can recycle vesicles at the rates posited for the cerebellar mossy fiber to granule cell synapse (Saviane and Silver, 2006), then release may be robust simply because a loaded, docked vesicle is always available.

Post synaptic firing response

In vivo, most MVN neurons are both activated by ipsiversive head turns and inhibited during contraversive head turns, via inhibitory commissural projections from
the contralateral MVN (Shimazu and Precht, 1966). Therefore, the gains of these neurons with respect to synaptic input from the ipsilateral side will be amplified by a concomitant reduction in inhibitory drive from the contralateral side. It will be of interest to examine whether inhibitory commissural inputs obey the same synaptic properties as we have described here for excitatory nerve inputs. Furthermore, inhibition typically has two effects on postsynaptic activity: first, direct hyperpolarization due to the entry of chloride ions; and second, a current shunt accompanying the increased membrane conductance. Based on the linear relationship between membrane voltage and firing rate in MVN neurons, it is unclear whether shunting inhibition might create an additional, nonlinear dynamic. In addition, feedforward disynaptic inhibition from vestibular afferents has been described at this synapse in many MVN neurons (frog: Straka and Dieringer, 1996; guinea pig: Babalian et al., 1997); the function of this circuit element, and indeed its presence in mammals, is unknown.

Vestibular neurons are known to display a linear relationship between somatic current injection and firing rate over the entire dynamic range of the neuron, which can reach rates as high as 400-500 spikes/s (du Lac and Lisberger, 1995; Sekirnjak and du Lac, 2002). However, it is well established that excitatory synaptic inputs in other systems can result in sub- or supra-linear summation, depending on the spatial and temporal relationships of those synapses, as well as their amplitudes (e.g., Gasparini and Magee, 2006). The data shown here demonstrate that currents from several vestibular afferents can and do sum linearly in MVN neurons, regardless of their temporal proximity, to produce postsynaptic firing in as linear a manner as somatic current
injection (Fig. 3.7). It is not yet known how vestibular neurons regulate their composition of ionic currents to maintain this relationship between current input and firing rate output; however, individual neuronal gains can be modified by potassium channel regulation (Smith et al., 2002; Nelson et al., 2003), suggesting a cellular opportunity to adjust the synaptic input/firing rate relationship independent of synaptic plasticity.

**Acknowledgments**

Chapter 3 is original work in preparation as Bagnall MW, McElvain LE, du Lac S. Activity at a primary afferent synapse drives postsynaptic firing with linear dependence on stimulation rate and is included with permission from all the manuscript’s authors. The dissertation author was the primary author of this paper.
References


Fig. 3.1  Schematic of recording setup. A, Outline of coronal brainstem section with approximate locations of recording pipette (left) and stimulation electrode (right). VN, medial vestibular nucleus. B, YFP-16 neurons (Y) receive vestibular nerve input and project to a variety of target nuclei. GIN neurons (G) also receive vestibular nerve input but make projections within the vestibular complex, including in some cases to the contralateral MVN (B. Zingg, unpublished data). It is likely that GIN neurons provide inhibition onto local YFP-16 and other neurons (dotted line).
Fig. 3.2  Kinetics of EPSCs elicited by vestibular nerve stimulation in YFP-16 and GIN neurons. A, Example EPSCs recorded in YFP-16 (top) and GIN (bottom) neurons are characterized by rapid rise and decay times. B, Scatterplot of 10-90% rise times for EPSCs evoked at ~2x threshold intensity in YFP-16 (n = 21) and GIN neurons (n = 24). Rise times were typically less than 0.5 ms. C, Scatterplot of 90-10% fall times in the same population of neurons as in B. Fall times were usually under 5 ms.
Fig. 3.3  EPSC amplitudes are larger in YFP-16 than in GIN neurons. A, Increasing stimulation intensity produces increasingly larger EPSCs in YFP-16 (left) and GIN (right) neurons, but the maximum synaptic amplitude is larger in YFP-16 than in GIN neurons. B, Maximum synaptic current, in nA, elicited in neurons from the YFP-16 (n = 32) and GIN (n = 23) populations. One YFP-16 neuron with an EPSC size of 6 nA is not shown.
Fig. 3.4  The paired pulse ratio is close to unity in both YFP-16 and GIN neurons. A, EPSCs resulting from vestibular nerve stimulation at a 10 ms interval in a YFP-16 (left) and GIN (right) neuron. B, Paired pulse ratio (P2/P1) at interstimulus intervals ranging from 5 ms (200 Hz) to 10 s (0.1 Hz). Values hover around 0.85 at all ISIs shorter than 2 s, for EPSCs in both types of neurons.
Fig. 3.5  Trains of stimuli evoke frequency-independent short-term depression.  

A, EPSCs elicited at 5 Hz (top) and 100 Hz (bottom) in YFP-16 neurons.  

B, Same as A, in GIN recordings.  

C, Group data of normalized EPSC amplitude versus stimulus number at 5 Hz (top) for 6 YFP-16 and 5 GIN neurons, and at 100 Hz (bottom) for 7 YFP-16 and 8 GIN neurons.  

D, Average EPSC during plateau phase (EPSCs 11-20), normalized to first EPSC amplitude, at frequencies ranging from 0.1 to 200 Hz.  

E, Normalized total charge transfer over 1 s, calculated as the average normalized area under EPSCs 11-20 multiplied by the rate of stimulation.
Fig. 3.6 Steady-state EPSCs maintain their amplitude at transitions to higher or lower stimulation frequencies. **A**, 20 stimuli delivered to the vestibular nerve at 10 Hz, followed by 20 stimuli at 50 Hz. No further depression occurs at 50 Hz. All data from YFP-16 neurons (n = 7). **B**, 20 stimuli at 50 Hz followed by 20 stimuli at 10 Hz. No recovery from depression is seen following the switch. N = 8.
Fig. 3.7  Linear translation of synaptic input into postsynaptic firing rate output.  
A, Vestibular nerve stimulation for 1 s at 20 or 100 Hz. DC current was delivered to the postsynaptic neuron to force a baseline firing rate of ~10 Hz. On the left, an example YFP-16 neuron; right, GIN. B, Instantaneous firing rates during stimulation at 20 Hz (black) or 100 Hz (gray) for the corresponding neurons in A. Neither neuron fires action potentials in response to every synaptic stimulus. C, Average firing rate during 1 s of stimulation (average of two sweeps), versus the rate of synaptic stimulation. In both examples, there is a linear relationship between synaptic stimulation rate and postsynaptic firing rate over the range of 20 to 150 Hz, with moderate drop-off at 200 Hz. D, R² values of line fits as shown in C, for 7 YFP-16 and 8 GIN neurons. E, Slope of line fits to plots shown in C, across both populations.
IV. Projection neurons of medial cerebellar output nuclei are glycinergic

Abstract

The cerebellum funnels its entire output through a relatively small number of projection neurons in the deep cerebellar and vestibular nuclei. These neurons, which exert their influence over a variety of motor and premotor regions, have classically been thought to be glutamatergic. Using transgenic mice in which glycinergic neurons are labeled with a fluorescent marker, we demonstrate that many large projection neurons in the medial cerebellar (fastigial) and medial vestibular nuclei are in fact glycinergic. Transmitter identity of these neurons was verified with single-cell RT-PCR. Whole-cell patch recordings of large fastigial glycinergic neurons revealed that their intrinsic physiological characteristics, including maximum firing rate and post-inhibitory rebound firing, were similar to those of large glutamatergic projection neurons. To examine whether large glycinergic neurons project outside the cerebellar/vestibular complex, we made dye injections into the rostral cervical spinal cord, which yielded numerous retrogradely labeled glycinergic fastigial and medial vestibular neurons ipsilateral to the injection. In contrast, glutamatergic neurons in both nuclei projected to contralateral spinal tracts. This pattern of ipsilateral glycinergic and contralateral glutamatergic afferents is repeated in the brainstem abducens nucleus, and therefore may represent a widespread design. These data uncover a fundamental organizing principle of cerebellar output, which is that medial nuclei exert motor control by inhibiting ipsilateral and exciting contralateral premotor targets in the spinal cord and
brainstem in order to coordinate flexor-extensor muscle groups for eye, head, and trunk movements.

**Introduction**

The cerebellum sends its output to a wide variety of brain regions, including the thalamus, medulla, and spinal cord. Functions of the cerebellum are thought to include timing and coordination of complex movements, and perhaps even cognition (Kim et al., 1994; Spencer et al., 2003). Among both experimentalists and theorists, the cerebellar cortex is prized for its crystalline structure with limited numbers of cell types, of which only one, Purkinje cells, projects out of the cortex.

Despite the diverse functions of the cerebellum, all Purkinje cell output converges on a comparatively small number of neurons in the deep cerebellar and vestibular nuclei (Fig. 1.1). There are thought to be two primary types of projection neurons in the deep cerebellar nuclei: medium-sized GABAergic neurons providing feedback to the inferior olive; and large glutamatergic neurons, numbering ~10,000 in mouse (Roffler-Tarlov and Herrup, 1981; Heckroth, 1994), supplying the entire remaining output of the cerebellum. Thus it has long been assumed that Purkinje cells influence the rest of the brain via just these two processing streams.

In this study we show that the medial (fastigial) deep cerebellar nuclei contain a third population of projection neurons that are glycinergic and target ipsilateral spinal motor pools. In contrast, glutamatergic fastigial neurons descend contralaterally (Asanuma et al., 1983). The same pattern of ipsilateral inhibition and contralateral excitation is repeated in the projections of the medial vestibular nuclei to both spinal
and oculomotor nuclei, suggesting it may represent a widespread blueprint for motor control.

Methods

Materials

Chemicals were from Sigma (St. Louis MO) unless otherwise specified. Rabbit anti-calbindin and mouse anti-NeuN were diluted 1:200 (Chemicon, Temecula CA); Cy3 goat anti-rabbit (Chemicon) and Alexa 594 goat anti-rabbit (Molecular Probes, Invitrogen, Carlsbad CA) were diluted 1:100. GlyT2-eGFP transgenic mice were generated in the lab of Dr. J.M. Fritschy (Zeilhofer et al., 2005); L7-GFP mice were created in this lab (Sekirnjak et al., 2003).

Electrophysiology and reverse transcription PCR

Coronal cerebellar slices were cut from GlyT2-GFP mice aged P10-14 as previously described (Bagnall et al., 2007). Recordings were made at ~33°C using a combination of epifluorescence and infrared illumination with differential interference contrast to visualize large neurons in the fastigial nuclei. Data were collected and analyzed with custom-written code in Igor Pro 5. Ringer’s solution for slicing and recording consisted of (in mM): 124 NaCl, 26 NaHCO₃, 5 KCl, 1.3 MgCl, 1 NaH₂PO₄, 11 dextrose. Whole cell patch pipette internal solution: 140 K-gluconate, 10 HEPES, 8 NaCl, 0.1 EGTA, 2 MgATP, 0.3 Na₂GTP. Data are reported as means ± SEM. Single-cell RT-PCR was carried out as described previously (Bagnall et al., 2007), using primers to VGluT1, VGluT2, and GlyT2.
Surgery

Animals were deeply anesthetized with isoflurane until breathing slowed to ~1 breath/s and the foot-pinching reflex vanished. They were then placed on a stereotaxic apparatus with a bite bar (Benchmark Angle Two, MyNeuroLab.com) and given continuous inhalation anesthesia as needed to maintain status. A midline incision was made from the base of the skull along the length of the neck, and underlying muscles were blunt dissected to expose the spinal cord and C1. The pia was then opened at a point just rostral to the first vertebra, at the atlanto-occipital junction. A custom-made injector needle (0.2 mm OD, 0.1 mm ID, Creative Instruments Development Company; cidco@cox.net) was loaded with crystals of fluorolabeled dextran, either Texas Red, Cy3, or Cascade Blue, 10,000 MW (Molecular Probes) and the tip was sealed with melted bone wax (Sekirnjak and du Lac, 2006). The midline was visually identified and fiber tracts were targeted unilaterally within a range of 0-0.3 mm lateral to the midline. The needle was oriented roughly orthogonal to the spinal cord, then lowered to a depth of 1.25 mm, at which point the interior plunger was repeatedly depressed (~100 µm) with calibrated air pressure (25 psi, 25 ms) to deliver the crystals into the tissue. After waiting 1-2 min for the dye to settle, the needle was withdrawn and the skin sutured. Animals were treated post-surgery with Buprenex (1.5 µg in saline) to minimize discomfort. 4-5 days after injection, mice were sacrificed.

Tissue preparation

Adult animals (> P28) were anesthetized with Nembutal and perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde
in PBS (PFA) for 5 min. After removal of the brain from the skull, the tissue was post-fixed for 30-60 min in PFA, then sunk in 30% sucrose in PBS overnight at 4 °C. 20-40 µm coronal sections were cut on a freezing microtome (Microm) and washed in PBS or mounted directly onto slides. For immunocytochemistry of free floating sections, blocking buffer (2% normal goat serum, 1% bovine serum albumin, and 0.3% Triton X-100 in PBS) was applied for 1 hr, followed by primary antibody in working buffer (10-fold dilution of blocking buffer) overnight at 4 °C. Sections were washed 3 times with working buffer and treated with fluoro-conjugated secondary antibody for 1 hr at room temperature. Following washes in PBS, sections were wet-mounted and coverslipped with 2.5% DABCO or Vectashield Hardset (Vector Labs, Burlingame CA).

**Image acquisition and processing**

Epifluorescent images were recorded using a Hamamatsu CCD camera attached to a Olympus BX60 or BX61 light microscope with a 4x (NA 0.13) or 10x (NA 0.3) objective lens with SlideBook 4. Confocal images were acquired in 0.1 - 0.5 µm steps on a Leica TCS SP2 AOBS microscope using laser lines of 488 and 561 nm, with a 20x (NA 0.5) or 63x (NA 1.4) objective and in some cases 3x hardware zoom. In most cases images were collected by sequential scanning to avoid possible fluorophore crosstalk. Leica software was used to average sequential z-planes in most images (typically 2-6 planes representing < 3 µm total). Images were transferred to Adobe Photoshop for brightness/contrast adjustment and image overlay.
Results

A recently developed transgenic mouse line permits the visualization of glycinergic neurons in brain tissue by driving the fluorescent reporter eGFP under the promoter for the neuronal glycine transporter GlyT2 (Zeilhofer et al., 2005). In the cerebellum and vestibular brainstem, GFP expression is seen in three regions: a subset of Golgi interneurons in the molecular layer which co-express glycine and GABA (Simat et al., 2007); all three deep cerebellar nuclei; and the medial vestibular nuclei (MVN) (Fig. 4.1A). While GFP+ neurons in the interpositus and dentate nuclei are typically small (Fig. 4.1B), consistent with reports that the deep cerebellar nuclei contain a few glycinergic local interneurons (Rampon et al., 1996), the numerous large GlyT2-GFP+ neurons visible in the ventral portion of the fastigial nuclei were unexpected (Fig. 4.1C). These neurons had long diameters exceeding 15 µm and thus were comparable in size to the large projection neurons found in all three deep nuclei (Chan-Palay, 1977), suggesting that they might also project out of the cerebellum.

The literature and indeed textbooks refer exclusively to glutamatergic, not glycinergic, projection neurons of the deep cerebellar nuclei (Kandel et al., 1991; Zigmond et al., 1999), although some reports have noted large glycinergic neurons in the fastigius (Chen and Hillman, 1993; Tanaka and Ezure, 2004). Therefore, we first verified that GFP expression correctly reflected the transmitter contents of these neurons. Single neurons from the fastigial nuclei were subjected to reverse transcription PCR for three neurotransmitter markers: the vesicular glutamate transporters VGluT1 and VGluT2 (Fremeau et al., 2001), and the neuronal glycine transporter GlyT2 (Zeilhofer et al., 2005; Bagnall et al., 2007). GFP+ neurons from the GlyT2-GFP line
expressed GlyT2, but neither VGluT1 nor VGluT2, in all neurons assayed (Fig. 4.2, n = 7). In contrast, large GFP negative neurons from the fastigial and dentate nuclei expressed VGluT2, but not VGluT1 or GlyT2 (Fig. 4.2, n = 4). These data support the accuracy of GFP expression in the GlyT2-GFP mouse line and indicate that the large GFP+ neurons in the fastigial nuclei are indeed glycinergic.

Projection and local neurons in the deep nuclei display markedly different physiological characteristics (Jahnsen, 1986; Aizenman et al., 2003; Uusisaari et al., 2007). We assayed the intrinsic properties of large glycinergic fastigial neurons by recording from them and from neighboring large non-glycinergic neurons in cerebellar slices from young (P10-14) mice. Epifluorescence was used to target whole-cell patch recordings to both GFP-positive and -negative fastigial neurons whose diameters exceeded ~15 μm. The two groups exhibited no differences in their intrinsic physiological attributes (Fig. 4.3). Action potentials were narrow (width at half-height, Gly+: 0.27 ± 0.11 ms, n = 13; Gly–: 0.27 ± 0.07 ms, n = 9), consistent with reported values for large deep nuclear neurons (Fig. 4.3A) (Jahnsen, 1986; Aizenman et al., 2003; Uusisaari et al., 2007). Most neurons displayed significant post-inhibitory rebound firing (Fig. 4.3B), another distinctive characteristic of deep nuclear projection neurons (Jahnsen, 1986; Aizenman et al., 2003); glycinergic neurons were not significantly different from the non-glycinergic population (Gly+ 47 ± 45 spikes/s, n = 12; Gly–, 77 ± 85, n = 7) (Fig. 4.3B). Both groups of neurons also typically fired spontaneous action potentials in slice at rates of 10 - 100 spikes/s (9 out of 13 Gly+, average 37 ± 27 spikes/s; 8 out of 9 Gly–, average 21 ± 10) (Raman et al., 2000). Finally, depolarizing current steps of 1 s revealed that both Gly+ and Gly– large
neurons were capable of firing action potentials at maximum rates ranging from ~150 to 450 spikes/s (Gly+, 215 ± 92, n = 12; Gly−, 221 ± 119, n = 7). Taken together, these data indicate that large glycinergic neurons in the fastigius closely resemble their glutamatergic counterparts in their intrinsic properties (Aizenman et al., 2003). Notably, they are dissimilar from deep nuclear GABAergic neurons, whose action potentials are broader than those of their large nonGABAergic neighbors and whose firing usually saturates at ~50 spikes/s (Uusisaari et al., 2007), in contrast to the neurons recorded in this study. Therefore, the large glycinergic neurons exhibit intrinsic parameters most similar to those of other large presumed glutamatergic projection neurons, and not those of local or olivary-projecting inhibitory neurons.

We next assessed whether the large GlyT2-GFP+ neurons in the fastigius and MVN were targets of Purkinje cell innervation. Although virtually all large neurons in the deep cerebellar nuclei are highly innervated by Purkinje cells (Chan-Palay, 1977; De Zeeuw and Berrebi, 1995), only a sparse subset of MVN neurons are thus innervated (Sekirnjak et al., 2003). The targets of cerebellar cortical inhibition may be visualized using the transgenic L7-tau-GFP mouse line, in which Purkinje cells express GFP (Sekirnjak et al., 2003). However, because both mouse lines (L7-GFP and GlyT2-GFP) drive GFP expression, they cannot be usefully combined to determine whether Purkinje cell synaptic terminals are apposed to glycinergic neurons. A second strategy for visualizing these inputs is immunocytochemistry with the calcium binding protein calbindin d-28K (CaBP) found in Purkinje cells (Batini, 1990). Antibody staining revealed a near-perfect overlap between GFP expression in the L7-GFP mouse line and CaBP immunofluorescence in Purkinje cell terminals (Fig. 4.4A, fastigius; 4B, MVN).
Although there are a few CaBP-positive non-Purkinje afferents in the vestibular nuclei, these ghost-like neuronal outlines sketched by Purkinje afferents in the MVN are completely absent following flocculectomy (Sekirnjak et al., 2003) and may thus be assumed to be cerebellar projections.

CaBP immunocytochemistry in brain slices from the GlyT2-GFP mouse revealed that Purkinje cell axon terminals were present in high numbers surrounding all the large glycinergic neurons in the fastigial nucleus (Fig. 4.4C) and many medium-large glycinergic neurons in the MVN (Fig. 4.4D). In contrast, large innervated neurons in the dentate and interpositus nucleus were never GFP+, although small glycinergic neurons could be seen intermingled with larger glutamatergic ones (Fig. 4.4E, dentate). Taken together, these data provide proof that not all large Purkinje cell target neurons in the deep cerebellar nuclei are glutamatergic, as had been commonly assumed, but instead include a glycinergic population. In addition, they demonstrate that neurons strongly innervated by Purkinje cells in the MVN are also glycinergic, as previously inferred (Spencer et al., 1989). Thus it appears that the cerebellar nuclei have two distinct modes of output, one excitatory and one inhibitory, in addition to the known GABAergic feedback to the inferior olive.

Although many GlyT2-GFP positive neurons in the fastigial nuclei were comparable in size to the other large presumed projection neurons, it remained possible that they in fact were local projection neurons, similar to small glycinergic neurons in the deep nuclei (Chan-Palay, 1977). Because fastigial and vestibular neurons drive eye and trunk movements (Matsushita and Hosoya, 1978; McCrea et al., 1980; McCrea et al., 1987), we evaluated whether GlyT2-GFP+ neurons might send their axons to the
spinal cord. Fluorescently-conjugated dextrans were injected into the rostralmost spinal cord, using a plunger to deliver a bolus of crystals. Dextrans are taken up by damaged neuronal processes and transported retrogradely, such that somatic label in regions distant to the injection may be taken to indicate that those neurons project their axons to or through the injection location. Injections were sited just rostral to the first vertebra and extended about 0.3 mm medial-lateral, 1 mm dorsal-ventral, and 0.3 mm in the rostro-caudal axis (Fig. 4.5A). 4-5 days following surgery, mice were perfused. Brainstem and cerebellar sections were then examined with fluorescent microscopy for evidence of retrogradely labeled neurons.

The majority of spinal-projecting neurons were located, as expected, contralateral to the injection site (Matsushita and Hosoya, 1978; Asanuma et al., 1983). However, several retrogradely-labeled neurons were present ipsilateral to the site of injection in the fastigial nucleus (Xiong and Matsushita, 2000) (Fig. 4.5B) and MVN. When the injections were made in GlyT2-GFP mice, these neurons colocalized retrograde label and GFP. Fig. 4.5C shows a high-magnification confocal view of a fastigial neuron ipsilateral to the injection. Fluorolabeled dextran (magenta) is visible in the soma in the form of small dots, which likely represent lysosomal packages. A similar neuron in the MVN is shown in Fig. 4.5D. Both neurons express GFP, indicating that they are glycinergetic neurons that make ipsilateral projections to the cervical spinal cord or beyond. A schematic view of the typical ipsilateral label in these nuclei is shown in Fig. 4.5B. Double-labeled neurons were found primarily in the ventral portion of the fastigial nucleus and in the ventrolateral portion of the MVN. The topography of this projection was evaluated by examining the retrograde label patterns
from injections sited flush to the midline vs those slightly more lateral (> 0.3 mm from the midline). Fastigial neurons were labeled following more lateral injections, while MVN label was more common in the medial injections (schematic, Fig. 4.7).

While all neurons heavily innervated by Purkinje cells in the MVN are glycinergic (Fig. 4.4D), not all glycinergic neurons are innervated by Purkinje cells. To determine whether the ipsilaterally-projecting glycinergic neurons in the MVN were targets of Purkinje cell inhibition or not, we repeated the spinal injections in the L7-GFP mouse. Of the labeled MVN neurons ipsilateral to injection, approximately 2% (5/208, n = 2 mice) were highly innervated on their somata by Purkinje cell axons, while another 4% (8/208) were moderately somatically innervated; the remainder were not, although dendritic innervation was impossible to assess. Fig. 4.5E shows a low-magnification image of a spinal-projecting cerebellar target neuron in the midst of several spinal-projecting non-target neurons; another target neuron is shown magnified in Fig. 4.5F. These data suggest that there exist two populations of glycinergic ipsilaterally spinal-projecting neurons in the MVN—cerebellar targets and nontargets—in comparison to the large fastigial glycinergic neurons, all of which are heavily innervated by Purkinje cells (Fig. 4.4C).

In contrast, none of the contralaterally spinal projecting neurons seen in the fastigial or vestibular nuclei were GlyT2-GFP positive (Fig. 4.6A, fastigial; Fig. 4.6B, MVN). Many large multipolar neurons were visible in the dorsal portion of the fastigial nucleus, in a region distinct from the ventrally located ipsilaterally projecting fastigiofugal neurons (diagram, Fig. 4.7). MVN contra-projecting neurons were located in approximately the same region as the ipsi-projecting neurons, indicating that
glutamatergic and glycinergic vestibulospinal neurons cohabitate within the ventrolateral MVN, in concordance with previous work (McCrea et al., 1980).

Anatomical and physiological evidence indicates that many MVN neurons make projections to the contralateral abducens nucleus, where they drive the vestibulo-ocular reflex (McCrea et al., 1987), before their axons continue caudally to the spinal cord (McCrea et al., 1980) to guide the vestibulo-collic and vestibulo-spinal reflexes. Therefore, the spinal-projecting MVN neurons might send axon collaterals to the abducens nucleus. Following spinal dye labeling, labeled axons and synaptic boutons were visible in the abducens ipsilateral to the injection site. These fibers might arise from the glutamatergic MVN neurons contralateral to injection or from glycinergic neurons ipsilateral to the injection. Confocal microscopy at high magnification revealed that many of these fibers were not glycinergic (Fig. 4.6C, arrow); however, in some instances a glycinergic axon could be seen making putative synaptic contacts onto abducens neurons (Fig. 4.6D, arrow). While it was impossible to determine whether those axons arose specifically from cerebellar target neurons or from other glycinergic neurons ipsilateral to injection, it is nonetheless interesting that the pattern of inputs to the spinal cord—ipsilateral inhibition, contralateral excitation—also appears to hold true for the abducens nucleus, suggesting that this design of control over motor circuits may be widespread.

**Discussion**

In this study we show that projection neurons in two cerebellar output nuclei are glycinergic, using evidence from transgenic mouse lines, single-cell RT-PCR, and
immunostaining to verify the results. While glycinergic projection neurons have been shown to exist in the medial vestibular nucleus, they were unexpected in the fastigial (medial cerebellar) nucleus. We further establish that their axons travel ipsilaterally to at least two motor targets in the brainstem and spinal cord, in contrast with neighboring glutamatergic neurons whose projections are primarily contralateral (schematic, Fig. 4.7). These results suggest that two important motor control circuits rely on a combination of ipsilateral inhibition and contralateral excitation to accomplish coordinated movements of eye and trunk muscles on both sides of the midline.

The fastigial nucleus is known to make axonal projections to the rostral thalamus, the vestibular nuclei, the prepositus, the reticular formation, and the cervical spinal cord (Asanuma et al., 1983). The medial vestibular nuclei project to the thalamus, prepositus, reticular formation, and oculomotor nuclei in addition to the spinal cord and abducens (Highstein and Holstein, 2005). Glycinergic fibers are typically sparse in rostral brain regions, but it will be of interest to see whether any motor pools other than the abducens and spinal cord are targets of this matched ipsi/contra projection from either nucleus. Because axons of large deep nuclear neurons are thought to drop local collaterals before exiting the nucleus, it is also possible that the glycinergic neurons identified here inhibit their large fastigial neighbors, which have been shown to exhibit strychnine-sensitive synaptic currents (Kawa, 2003). It is clear from both anatomical and physiological studies that GABAergic deep nuclear neurons are small to medium sized and exhibit different intrinsic characteristics than large neurons, indicating that the
large glycinergic neurons examined here do not co-express GABA and therefore do not project to the inferior olive (Chen and Hillman, 1993; Uusisaari et al., 2007).

While transgenic mouse lines are susceptible to problems due to the presence of additional DNA, which can affect expression of native genes, and due to the repeated selection for genetic loci close to the transgene, we have three reasons for believing that the data presented here are not specific to the GlyT2-GFP mouse. First, we have seen ipsilateral retrogradely labeled neurons in both the fastigius and MVN following spinal injection in the L7-GFP mouse (Fig. 4.5E), as well as in four other separate lines of transgenic or knock-in mice (B. Zingg, unpublished data). Second, the functional separation of inhibitory and excitatory pathways into ipsi- and contra- projecting, respectively, is inconsistent with the possibility that neurons have been spuriously labeled from dye injection. Third, in situ hybridization for GlyT2 mRNA and glycine immunostaining in the rat have also demonstrated the presence of large fastigial glycinergic neurons (Chen and Hillman, 1993; Tanaka and Ezure, 2004).

Why have previous studies failed to reveal the existence of the ipsilateral fastigiospinal projection? Large glycinergic neurons appear to comprise ~15% of the estimated 1,250 large neurons in the unilateral fastigius ( Heckroth, 1994). Thus some sparse projections may have been overlooked, or left unpursued, by earlier research (Matsushita and Hosoya, 1978; Homma et al., 1995). It is also probable that because the interpositisus and dentate are devoid of large glycinergic neurons, and because the physiological characteristics of the large glycinergic neurons resemble those of large glutamatergic neurons (Fig. 4.3), researchers have not suspected the presence of a second distinct population. The physiological data shown here indicates that functional
circuit role, rather than transmitter type per se, is the key determinant of intrinsic characteristics in these nuclei (Bagnall et al., 2007). Finally, the emphasis on circuits of locomotion has perhaps led to some neglect of trunk, as opposed to limb, control.

What advantage might be conferred by controlling motor systems through two parallel pathways, and why might the fastigius and MVN, but not the other cerebellar and vestibular nuclei, rely on such an arrangement? The projections to the abducens nucleus suggest a possible answer. Abducens motor neurons control the lateral rectus eye muscle, whose activity drives temporally directed horizontal eye movements. During conjugate eye movements that do not involve vergence, the right and left lateral recti must act as a functional flexor-extensor pair, as horizontal eye movements necessitate contracting one lateral rectus while relaxing the other. However, because the motor nuclei are on opposite sides of the midline, in contrast to flexor-extensor pairs located unilaterally (e.g., the bicep and tricep), a bilateral system of motor control is required, here instantiated by the glycinergic ipsi/glutamatergic contra projections.

In this context, it is notable that the fastigial nucleus and the MVN both command motor pools that drive eye, neck, and trunk movements (Asanuma et al., 1983; Highstein and Holstein, 2005). In all of these central regions, flexion and extension must be coordinated across the midline because of the physical impossibility of moving one side of the neck, for example, without moving the other side; such a condition is not true for the limbs. The interpositus and dentate cerebellar nuclei, in comparison, control more lateral body parts, in particular the limbs, in which flexor-extensor pairs are unilaterally located (Ekerot et al., 1997; Dum et al., 2002). Therefore, in those pathways coordination of muscle flexion and extension can devolve
to the spinal cord, where local inhibition via Ia inhibitory interneurons accomplishes this job (Grillner, 2006). The data presented here demonstrate that the cerebellum uses two different strategies for directing midline and axial muscles; it will be of interest to explore whether the downstream spinal and medullary circuitry dedicated to central muscles is correspondingly distinctive.

Acknowledgment

Chapter 4 is original work in preparation as Bagnall MW, Zingg B, Moghadam S, du Lac S. Projection neurons of medial cerebellar output nuclei are glycinergic and is included with permission from all the manuscript’s authors. The dissertation author was the primary author of this paper.
References


**Fig. 4.1**  GlyT2-eGFP expression in brainstem and cerebellum. A, Low magnification image of coronal section of brainstem and cerebellum. Large glycinerergic neurons are visible in the fastigial (Fas) and medial vestibular nuclei (MVN), but not in the remaining cerebellar target nuclei: interpositus (Int), dentate (Den), lateral or superior vestibular nuclei (LVN, SVN). B, Confocal image of small glycinerergic neurons in the dentate nucleus. In this and subsequent figures, 2-3 µm of sequential images in the z-axis are averaged together. Scale bar 10 µm. C, Large glycinerergic neuron in the fastigial nucleus.
Fig. 4.2  Reverse transcription PCR of single cells verifies glycinergic identity of GFP-expressing neurons. Cellular contents of individual neurons were acquired during whole-cell patch recordings in the fastigial nucleus. Each neuron was assayed for two markers of glutamatergic function (VGLUT1 and VGLUT2) and a marker of glycinergic function (GlyT2). Left, a GFP-negative large fastigial neuron; middle, a GFP-positive large fastigial neuron; right, whole brain RNA positive control processed alongside. As expected, GFP-negative neurons expressed the glutamatergic marker VGLUT2 ($n = 4$) while GFP-positive neurons expressed the glycinergic marker GlyT2 ($n = 7$).
Large glycinergic and glutamatergic fastigial neurons resemble each other physiologically. A, Representative action potential from a glycinergic (top) and a glutamatergic (bottom) large fastigial neuron. Both had narrow action potentials with a slight afterdepolarization that preceded the slow afterhyperpolarization. B, Post-inhibitory rebound firing characteristics were similar in both neurons. DC current was delivered to force a baseline firing rate of ~10 spikes/s. Following a 1 s hyperpolarizing current step, neurons resumed firing at elevated rates compared to baseline. All intrinsic characteristics were found to be similar between large glycinergic and glutamatergic fastigial neurons.
**Fig. 4.4** Purkinje cells make synaptic contacts onto glycineric neurons in the fastigial and medial vestibular nuclei. **A**, Verification of calbindin (CaBP, magenta) as a marker for Purkinje cell terminals. Left, antibody stain to CaBP in the fastigial nucleus; middle, Purkinje cell processes visualized in the L7-GFP mouse line; right, merge. CaBP labels Purkinje cell axonal boutons surrounding an unlabeled large fastigial neuron. Scale, 10 μm. **B**, Same as **A**, in the MVN. Purkinje cell inputs are much sparser in the MVN than in the deep cerebellar nuclei, but are still recognizable with CaBP. **C**, Purkinje cell inputs labeled with an antibody to CaBP (magenta) on the soma of a glycineric large fastigial neuron labeled in the GlyT2-GFP line. Scale, 10 μm, applies to panels C-E. **D**, Same as C, in the MVN. Other glycineric neurons that are not contacted by Purkinje cells are also visible. **E**, Small glycineric neuron in the dentate (lateral cerebellar) nucleus. Nearby, the soma of a large presumed glutamatergic projection neuron (unlabeled) is outlined by Purkinje cell terminals (magenta).
**Fig. 4.5** Retrogradely labeled glycinergic neurons in the fastigial and MVN following *ipsilateral* spinal injections.  
A, Representative location of spinal fluorolabeled dextran injection.  
B, Low magnification view of retrogradely labeled neurons in the fastigial nucleus (magenta) in the GlyT2-eGFP mouse. Scale bar, 200 µm.  
C, Glycinergic fastigial neuron expressing GFP (left) is also retrogradely labeled following ipsilateral spinal cord injection (middle). Right, merge. Scale bar, 10 µm.  
D, Same as C in the MVN.  
E, Retrogradely labeled MVN neurons (magenta) in the L7-GFP mouse line show that Purkinje cell axons target a subset of vestibulospinal neurons. Scale bar, 100 µm.  
F, High resolution image of a vestibulospinal neuron with high levels of input from Purkinje cells. Scale bar, 20 µm.
Fig. 4.6  Retrogradely labeled glutamatergic neurons in the fastigial and MVN following contralateral spinal injections. A, Large fastigial neuron contralateral to spinal injection is not labeled in GlyT2-GFP mouse line (left) and is retrogradely labeled following spinal cord injection (middle). Right, merge. Scale bar, 10 µm. B, Same as in A, for an MVN neuron. C and D, Axon collaterals of neurons retrogradely filled following spinal injection are visible in the abducens nucleus ipsilateral to injection site. Some synaptic boutons arise from glutamatergic (non-GFP-expressing) neurons (C) while others are colabeled with GFP indicating that they arise from glycineergic neurons, presumably in the ipsilateral MVN or fastigial (D). Scale bar, 5 µm.
Fig. 4.7  Schematic of glycinergic and glutamatergic outputs from the fastigial and medial vestibular nuclei. Left: Glycinergic neurons (filled circles) in the medioventral aspect of the fastigial nucleus project ipsilaterally to axon tracts slightly off the midline of the spinal cord; glutamatergic neurons decussate (possibly within the cerebellum) before descending contralaterally. Right: a similar pattern of spinal projections is seen in MVN neurons, but their axons descend more medially. In addition, they send axon collaterals to the abducens nucleus (VI).
V. Distal dendritic inhibition from Purkinje cells onto neurons in the vestibular brainstem

Abstract

The sole output of cerebellar cortex, the Purkinje cell, makes GABAergic inhibitory synapses onto neurons in the deep cerebellar and vestibular nuclei. Recordings from large projection neurons in both the cerebellar and vestibular nuclei have shown that cerebellar stimulation causes a decrease in postsynaptic firing rates, sometimes followed by post-inhibition rebound. Anatomical studies of the deep cerebellar nuclei indicate that smaller, presumably GABAergic neurons do not receive much Purkinje cell input perisomatically, but instead sport Purkinje cell synapses on their dendrites. The goal of this study was to describe the anatomical distribution of Purkinje cell inputs to neurons in the medial vestibular nuclei which are homologs of the deep cerebellar nucleus neurons. Using a transgenic mouse in which Purkinje cells express a fluorescent reporter, we show that neurons in the vestibular nuclei exhibit a variety of patterns of Purkinje cell innervation. In several instances, distal dendrites are more heavily innervated than somata, suggesting that in these neurons, Purkinje cell activity will affect dendritic processing and integration of nearby excitatory inputs, rather than reducing somatic firing rates. These data compel an important addition to current models of cerebellar function and learning, namely that a subset of Purkinje target neurons will modulate synaptic integration, rather than firing rates, in response to changes in cerebellar activity.
Introduction

How does the nervous system control behavior? The cerebellum is critical for acquisition and maintenance of many forms of motor learning. The sole output neuron of cerebellar cortex is the Purkinje cell, which receives excitatory input from thousands of parallel fibers arising from granule cells, as well as from a single climbing fiber arising in the inferior olive. Conjunctive action of these two inputs produces long-term depression (LTD) at the parallel fiber synapse, both in vitro and in vivo, and selective block of the cellular mechanisms of LTD generally impairs acquisition of motor learning. Because Purkinje cells are GABAergic, parallel fiber LTD is thought to result in a disinhibition of downstream targets (Mauk et al., 1998; Medina et al., 2000; De Zeeuw and Yeo, 2005).

It is well established that Purkinje cells make both somatic and dendritic inhibitory contacts onto neurons in the deep cerebellar and vestibular nuclei (Chan-Palay, 1977). In theories of cerebellar function and learning, this inhibition has been assumed to cause a decrease in firing rates of all target neurons. Computer models of cerebellar activity during learning have focused exclusively on this aspect of inhibition, and as a result the firing rate reduction, as well as the post-inhibitory rebound firing, have been major elements of hypotheses regarding cerebellar processing (Medina et al., 2000; Wetmore et al., 2007). In turn, parallel fiber LTD is normally modeled as a global decrease in inhibition onto these target neurons, whose projections to motor and premotor regions are accordingly more active. Because all Purkinje cell output is funneled through the deep cerebellar and vestibular nuclei, at an approximate ratio of 26 Purkinje cells to each target neuron (Palkovits et al., 1977), one of the primary
challenges to these hypotheses has been the apparent absence of opportunity for input-specific changes. Decreases in Purkinje cell activity would be predicted to result in an increase in target neuron firing rate without regard for the activity of specific excitatory inputs from the mossy fibers or vestibular nerve.

It is unclear whether or not this model would be viable in producing the wide array of behaviors that the cerebellum is known to facilitate. In this study, we examine the patterns of Purkinje cell innervation of neurons in the medial vestibular nucleus (MVN) and show that Purkinje cells often selectively target distal dendrites of individual neurons, potentially providing a basis for highly specific forms of cerebellar learning. This second processing stream might arise from the cerebellar flocculus, nodulus, or both.

**Methods**

**Slice preparation and electrophysiology**

Coronal slices of the brainstem (250-300 µm) were prepared from L7-tau-GFP mice (Sekirnjak et al, 2003) and incubated in carbogenated ACSF at 34°C for 30 min and then at room temperature for > 30 min. ACSF contained (in mM) 124 NaCl, 26 NaHCO₃, 5 KCl, 1.3 MgCl₂, 2.5 CaCl₂, 1 NaH₂PO₄, 11 dextrose. Chemicals were from Sigma unless otherwise specified. For recording, slices from 17 to 24-day-old mice (average, P21) were submerged in a recording chamber and perfused with 33°C carbogenated ACSF with 2 mM kynurenic acid to block ionotropic glutamatergic transmission. In some experiments, picrotoxin (100 µM) was also included. The mid-
rostral, ventrolateral aspect of the MVN was preferentially targeted for recording because of the higher concentration of FTNs found there (Sekirnjak et al, 2003). Neurons were visualized under infrared illumination with differential interference contrast optics. Whole-cell current clamp recordings were made with an AxoClamp 2B or a MultiClamp 700B amplifier. Data were sampled at 40 kHz (ITC-16, InstruTech) and filtered at 10 kHz. Electrodes were filled with an internal solution of (in mM) 140 K gluconate, 10 HEPES, 8 NaCl, 0.1 EGTA, 2 MgATP, 0.3 Na₂GTP, and 0.1% biocytin.

Action potentials were recorded under DC depolarizing or hyperpolarizing current injection to force a 10 Hz firing rate; 1-5 s of action potentials were averaged together at their spike peaks and analyzed as described previously. Action potential properties were analyzed only for those cells whose spike height was greater than 45 mV, indicating access resistance of < 15 M\(\Omega\). To measure neuronal gain, 1 s long current steps were delivered with progressively larger amplitudes until the neuron was no longer able to fire action potentials continuously across the entire step. Firing rate was averaged across the step (Bagnall et al., 2007). Values reported are means ± SEM.

Postphysiology anatomy and analysis

Following recording, electrodes were slowly removed from the slice to leave the filled neuron intact. Slices were then placed in 4% paraformaldehyde for 1-3 days. In some cases, slices were agitated in 30% sucrose-PBS for >1 hr and resectioned at 80-100 \(\mu\)m on a freezing microtome to improve visibility. After washing with PBS, slices
were treated with Cy3-Streptavidin (Zymed) at 1:75 or 1:100. Tissue was mounted on slides with an anti-fade gel containing 2.5% DABCO and coverslipped.

Cells were rejected for analysis if the soma could not be found or appeared badly damaged or incomplete. Confocal images were acquired in 0.2 μm steps on a Leica TCS SP2 AOBS microscope using laser lines of 488 and 561 nm, with a 63x (NA 1.4) objective and in some cases 3x hardware zoom. During off-line analysis, Purkinje cell axon terminals making putative synaptic contacts onto MVN neurons were evaluated for size, shape, and profile. Because it was impossible to verify genuine synaptic connections, we instead assigned each putative contact a “percentage likely” score ranging from 10-100%. The score reflected the degree to which the GFP-positive process was sized and shaped similar to known Purkinje cell boutons (2-5 μm), and was closely apposed to the biocytin-filled postsynaptic neuron with no visible gap. M.W.B. and K.E.K. agreed on scoring principles and evaluated separate pools of neurons. A diagram of each neuron was made with putative appositions marked at representative locations. M.W.B. and K.E.K. assessed each diagram together. Percentage scores of all putative boutons were added to give a total innervation estimate. Somatic innervation may be underestimated because of damage caused to the tissue by the recording electrode. For display, several sequential frames were averaged together as indicated in figure legends, and resulting images were montaged.

Results

During whole-cell recordings from slices of L7-GFP mice, neurons in the MVN were filled with biocytin and afterwards examined with confocal microscopy for the
presence of putative synaptic boutons from Purkinje cells along their soma and dendrites. As previously described, a small percentage of neurons were heavily coated with Purkinje cell boutons that surrounded the soma and draped the dendrites (Sekirnjak et al., 2003). In other instances, neurons were strongly innervated perisomatically but innervation tapered off > 100 μm from the soma. An example of such a neuron is shown in Fig. 5.1. Purkinje cells boutons (green) are visible along all aspects of the soma and proximal dendrites (magenta). These neurons are distinct from the population described by Sekirnjak and colleagues (2003) based on their modest post-inhibitory rebound firing (see below). (For clarity of visualization, in this and all subsequent figures multiple planes of a confocal z-stack have been averaged together and montaged. Actual counting of probable synaptic contacts was performed by stepping through each z-image individually; the pictures here are the authors’ best two-dimensional representation of the results. Not all apparent appositions were judged as likely synaptic contacts; nor is every likely synaptic contact always visible.)

In addition to the neurons festooned with Purkinje cell inputs, we observed that the remainder of the MVN population exhibited varying degrees of putative Purkinje cell innervation. Strikingly, the patterns of innervation did not necessarily resemble scaled-back versions of the strong somatic and proximal dendritic innervation seen on the FTN shown in Fig. 5.1. In several instances, neurons with few perisomatic GFP+ inputs were garlanded with Purkinje axonal boutons on portions of distal dendrites. An example of this pattern is shown in Fig. 5.2A. In this neuron, the soma is contacted by an estimated 8 Purkinje boutons; the dendritic process that extends dorsally could be followed into two long branches (a more proximal offshoot was truncated by slicing).
One of those branches was largely uninnervated; in the inset Fig. 5.2A₁, a magnified version shows clearly that only 1 putative synaptic contact (asterisk) is visible. However, the other branch (inset, Fig. 5.2A₂), is draped with 9 likely synapses, in this case all apparently from a single Purkinje cell axon. This neuron received a total of 32 estimated Purkinje cell synapses over the surface left intact in slice, but as is clear from these images, those putative contacts were not evenly distributed over the cell membrane. A second short dendritic branch (at the upper right of the soma) was also heavily innervated, while the branch ventral to it was weakly, if at all, innervated by Purkinje cells. In the second neuron example (Fig. 5.2B), one dendritic segment > 100 µm from the soma is repeatedly contacted by Purkinje cells (inset, Fig 5.2B₁), and then as it continues more distally, sees few to no Purkinje cell synapses (inset, Fig 5.2B₂).

Through the whole population of imaged neurons, there were no clear patterns regarding amount or distribution of Purkinje cell terminals. Some neurons with no or little apparent innervation somatically were more heavily innervated on the dendrites, and vice versa. We reasoned that there might be either an anatomical subregion or a physiological profile associated with stronger Purkinje cell innervation. Therefore, we divided the population of filled neurons into three classes: those with low Purkinje cell innervation (< 15 total boutons over whole neuronal surface); moderate Purkinje cell innervation (15 - 50 boutons; e.g. Fig. 5.2); and high Purkinje innervation (> 50 boutons; e.g., Fig. 5.1). Locations of these neurons within the MVN are plotted in Fig. 5.3 on a representative slice outline. Low innervation neurons are shown as gray circles, moderate innervation neurons are marked with dark gray triangles, and high innervation
neurons are shown as open rectangles. There is no discernable relationship between the amount of innervation and the anatomical location of the neuron.

Extremely heavily innervated neurons exhibit a distinct physiological phenotype, consisting of high (> 40 spikes/s) spontaneous firing rates in slice, and unusually large rebound firing (> 120 spikes/s) following a 1 s hyperpolarizing step (Sekirnjak et al., 2003). Furthermore, previous evidence suggests that functional subclasses of MVN neurons tend to have distinct physiological characteristics (Takazawa et al., 2004; Bagnall et al., 2007). Therefore, we examined the intrinsic properties of the neurons studied here. Action potential waveforms, which tend to be narrower in nonGABAergic presumed projection neurons than in GABAergic neurons (Takazawa et al., 2004; Bagnall et al., 2007), are quantified in Fig. 5.4A. There is no obvious distinction between the spike profiles of the three groups. Comparison with Fig. 2.3C shows that neither the low nor the moderate innervation group falls within the boundaries sketched by the GABAergic and nonGABAergic groups from previous data. We furthermore analyzed the response of cerebellar target neurons to depolarizing and hyperpolarizing steps. NonGABAergic neurons typically display low input resistances and high maximum firing rates compared to GABAergic neurons (Bagnall et al., 2007). Again, there was no particular distinction between groups of cerebellar target neurons with moderate or low innervation (Fig. 5.4B). In addition, post-inhibitory rebound firing was no greater in strongly innervated than weakly innervated neurons (Fig. 5.4C). These data make clear that the special physiological characteristics attributed to extremely heavily innervated neurons are not shared by other members of the cerebellar target neuron group. Furthermore, it is apparent that models of cerebellar function
which do not distinguish among Purkinje cell target neuron classes will need revision, especially in light of recent models emphasizing the potential role of rebound firing in driving memory formation (Wetmore et al., 2007).

**Discussion**

The data presented here strongly suggest that many neurons in the MVN receive monosynaptic contacts from Purkinje cells concentrated on their dendrites, in contrast to other MVN neurons whose innervation is heavily perisomatic. Without further histological, physiological or ultrastructural evidence, it is impossible to be certain that these contacts visible at the light microscopic level are functional synapses. However, several lines of evidence suggest that they are unlikely to be artifactual. First, a single Purkinje cell was seen making repeated contacts with a single dendrite, an event unlikely to occur by chance given the absence of laminar structure within the vestibular nuclei (Fig. 5.2). Second, Chan-Palay (1977) noted that in the rat dentate nucleus, smaller presumed GABAergic neurons were significantly less likely than large presumed glutamatergic neurons to receive Purkinje cell input on their somata, with a full 50% of neurons devoid of axosomatic Purkinje contacts. To our knowledge, no investigator to date has described the properties of the Purkinje cell to GABAergic deep nuclear neuron synapse, leaving an open question whether some local GABAergic interneurons, as well as those that project to the inferior olive, might exhibit a different response to Purkinje activity than their large glutamatergic and glycinergic neighbors. Thirdly, it is known that Purkinje cells contact GABAergic olivary-projecting neurons in the MVN (De Zeeuw and Berrebi, 1995), but all of the heavily innervated neurons
there are glycinergic, not GABAergic (S. Moghadam; Chapter 4); therefore, some remaining population of MVN neurons must be targets of Purkinje cell innervation.

If the synaptic contacts described here are indeed real, then their functional implications are striking. As schematized in Fig. 5.5, neurons receiving dendritic vs. perisomatic innervation will respond quite differently to changes in Purkinje cell output. First, distal dendritic inhibition will be unlikely to cause much, if any, reduction in overall somatic firing rates; in contrast, somatic inhibition has been shown to decrease or silence the firing of cerebellar target neurons in several preparations (Lisberger et al., 1994; Aizenman and Linden, 1999). Thus, neurons whose distal dendrites, but not somata, are innervated by Purkinje cells may not appear in vivo to be cerebellar targets, since the criterion is always limited to changes in firing rates visible to extracellular electrodes.

Second, neurons with heavy somatic inhibitory contacts will experience a nonselective reduction of all excitatory drive to the soma during periods of high Purkinje cell activity; in contrast, distal dendritic inhibition will selectively “gate off” excitatory inputs arising distal to the inhibited segment, without influencing the somatic effect of excitatory inputs from other dendritic branches. As a result, the cerebellum will be able to determine which excitatory inputs are “heard” postsynaptically and which are not. This presents a significant opportunity for plasticity in the cerebellar system that has previously not been incorporated into models of cerebellar learning.

If the distribution of Purkinje terminals on MVN (and neighboring nucleus prepositus hypoglossi) neurons is in fact similar to that seen in the deep cerebellar nuclei, then the neurons shown here with more dendritic than somatic innervation are
analogous to the GABAergic and glycinergic deep nuclear neurons, which project locally and/or to the inferior olive. According to the model described above, then, these inhibitory neurons will not necessarily show firing rate changes during Purkinje cell activity modulation, but instead will see excitatory inputs selectively gated off. Small deep nuclear neurons in the rat dentate receive ~50% of their dendritic inputs from excitatory neurons, including mossy fiber collaterals (Chan-Palay, 1977), and are therefore well situated to provide the circuit with an input-selective signal, in contrast to the large projection neurons.

Two other possibilities bear mentioning: first, it is possible that this distal dendritic inhibition in fact provides a sufficiently broad conductance shunt that postsynaptic firing rates do indeed decrease significantly. If this is the case, in vivo recordings would likely show these neurons as weak cerebellar target neurons whose firing is damped but not abolished by Purkinje cell activity (Lisberger et al., 1994). A second possibility is that GABAergic synapses are depolarizing in vivo, due either to entry of bicarbonate, rather than chloride (Gulledge and Stuart, 2003), or to subcellular distribution of the dominant chloride transporter in adult neurons, KCC2, whose expression levels influence the local chloride concentrations and as a result the direction of chloride flux (Woodin et al., 2003). Although no in vivo experiments have documented action potentials in MVN neurons driven by cerebellar stimulation, the close proximity of the vestibular nerve to the flocculus would lead most investigators to assume that any such effect was due to spurious current spread to the brainstem.

Finally, in light of data indicating that different intrinsic physiological characteristics are associated with different functional neuron classes in the MVN
(Takazawa et al., 2004; Bagnall et al., 2007), it is interesting to see that there are no obvious physiological distinctions between neurons with low and high grade Purkinje cell innervation (Fig. 5.4). Though very heavily innervated neurons display distinctive traits, including high spontaneous firing rates and unusually large post-inhibitory rebound firing (Sekirnjak et al., 2003), the neurons recorded here were a heterogeneous group and did not sort cleanly into previously described classes (Bagnall et al., 2007). This is likely due in part to the inadequacies of anatomical description at the light microscopic level, and in part to the fact that several classes of MVN neurons, presumably with different associated physiological parameters, receive Purkinje cell inputs (De Zeeuw and Berrebi, 1995); some of the neurons identified here may be targets of the nodulus, rather than the flocculus, and as such might participate in the vestibulospinal circuit (Xiong and Matsushita, 2000). Superior identification of neuron types in the MVN, including such information as transmitter contents and postsynaptic targets, will undoubtedly improve our understanding of the relationship between Purkinje cell input and vestibular function.

Acknowledgment

Chapter 5 is original work in preparation as Bagnall MW, Kolkman KE, du Lac S. Distal dendritic inhibition from Purkinje cells onto neurons in the vestibular brainstem and is included with permission from all the manuscript’s authors. The dissertation author was the primary author of this paper.
References


**Fig. 5.1** Strong somatic and proximal dendritic Purkinje cell innervation of an MVN neuron. **A**, Filled MVN neuron (magenta) with extensive Purkinje cell input (green) perisomatically. Montage of several confocal images, each consisting of an average of 2 to 5 μm of z-axis images. Scale bar, 20 μm. **B**, Sequential confocal images of the soma, each an average of 2 μm in the z-direction (10 frames), showing intensive Purkinje cell innervation. Scale bar, 10 μm, applies to all.
**Fig. 5.2** Distal dendritic Purkinje cell innervation. **A**, Filled MVN neuron (magenta) with moderate Purkinje cell input (green) perisomatically. Scale, 20 µm. *Inset A1*, a distal dendrite with one putative Purkinje cell contact (asterisk); **A2**, a single Purkinje cell axon makes 9 probable synapses on this stretch of dendrite. Scale, 10 µm for both. **B**, Another MVN neuronal fill. *Inset B1*, an estimated 7 Purkinje synapses on a segment of dendrite ~100 µm from the soma; **B2**, the dendrite continues distally to the segment shown in *B1*, without much apparent Purkinje cell innervation.
Fig. 5.3  Location of reconstructed neurons. A, Schematic coronal section of the brainstem and cerebellum at the approximate rostrocaudal level of most recordings (Bregma -6.00). Boxed region is enlarged in B. Cells with a total of < 15 Purkinje cell putative synapses over the entire neuronal surface are marked with gray circles, left. 15-50 total synapses, dark gray triangles, right. Two neurons with strong innervation (> 50) as in Fig. 5.1, open squares. No pattern of anatomical distribution is evident.
Intrinsic physiological parameters do not distinguish between neurons with low or moderate levels of Purkinje cell innervation. A, Maximum spike afterdepolarization immediately following fast afterhyperpolarization, plotted versus action potential width at half-height. Analysis as for Fig. 2.3C. B, Scatterplot of maximum sustained firing rate in spikes/s over a 1 s step depolarization, plotted against input resistance measured by small hyperpolarizing steps at -75 mV. Same symbols as in A. Analysis carried out as in Fig. 2.4. C, Post inhibitory rebound firing does not distinguish any of the cell classes as grouped here. Analysis as in Fig. 2.5.
Fig. 5.5  Schematic of predicted effects of somatic compared to distal dendritic inhibition. Left, a neuron with strong perisomatic inhibitory input (green circles); right, a neuron with strong inhibitory input on only one dendrite. Both neurons receive excitatory dendritic inputs (a and b). During periods of low Purkinje cell inhibition, both neurons will fire at high rates (black) while integrating information from both dendrites a and b equally. When Purkinje cells increase their activity, the neuron with perisomatic inhibition will decrease its firing rate, and both excitatory inputs will appear smaller somatically. In the neuron with dendritic inhibitory input, however, firing rate will be slightly reduced, but the somatic effect of excitatory input b will be greatly attenuated or even abolished. In contrast, input a will be unaffected.
VI. Conclusions

The work presented here explores several different aspects of cerebellar and vestibular function. I used five lines of transgenic mice to address questions regarding the characteristics of projection and intrinsic neurons in the vestibular nuclei, and their modulation by vestibular nerve activity; the circuit function of unexpected glycinergic projection neurons in the medial deep cerebellar nucleus, and their homologs in the medial vestibular nucleus; and the anatomical properties of cerebellar inhibitory synapses. Together, these studies illuminate the multiple processing streams found within cerebellar target nuclei: the distinction between projection and intrinsic neuronal temporal processing, the presence of multiple subclasses of projection neurons, and the diverse roles of Purkinje cell inhibition.

The paucity of strategies for identifying and examining these information channels has led to oversimplification of theories regarding the cerebellar circuit. For example, no computational models as yet incorporate local inhibitory circuits within the cerebellar target nuclei, despite the fact that intrinsic GABAergic and glycinergic neurons are known to exist there. Two critical pieces of information—how those neurons modulate their activity during behavior, and how they influence neighboring projection neurons—are completely unknown. The cerebellum is an excellent model system for understanding the links between neuronal plasticity and behavior, because the inputs are fairly well characterized, the circuit is relatively simple, and the cerebellar cortical anatomy is well described. However, until we can define the properties of information processing in the cerebellar output nuclei, our understanding of the forms
of cerebellar plasticity will remain impoverished. My research has both clarified several aspects of cell-type specific processing and initiated the use of several tools for further research on these questions.

What are the broader inferences to be drawn from the work presented here? Although the preceding chapters address some of the possibilities, here I take the opportunity to speculate more generally on implications and future directions.

Intrinsic characteristics of projection and local neurons

The most surprising finding presented in Chapter 2 was that GABAergic interneurons exhibited lower maximum firing rates and broader action potentials than either glutamatergic or glycinergic projection neurons. In the cortex and hippocampus, where the reverse is generally true, interneurons are thought to confer precision timing on pyramidal activity by sculpting excitatory postsynaptic potentials (EPSPs). Because these interneurons have faster kinetics and higher maximum firing rates than pyramidal neurons, they can achieve a finer temporal resolution and therefore are well suited to this role (Jonas et al., 2004; Glickfeld and Scanziani, 2006). In contrast, MVN local circuit neurons provide a coarser-grained picture of activity than projection neurons do. Similar results have been seen for GABAergic neurons in the deep cerebellar nuclei (Uusisaari et al., 2007). These data suggest that local neurons in cerebellar target nuclei are not responsible for fine control of projection neuron timing, but that instead the projection neurons themselves are capable of temporal precision. No study has yet explored whether vestibular or deep cerebellar projection neurons exhibit low temporal
jitter in response to synaptic input, but it seems likely that they do, given their capacity for maintaining firing rates upwards of 300 spikes/s.

What then is the job of the GABAergic local neurons, if it is not timing-related? We are hampered by a dearth of information about local patterns of connectivity. A few studies in isolated whole-brain preparation have demonstrated that many MVN neurons receive apparent feedforward or feedback inhibition from both GABAergic and glycinergeric neurons (Straka and Dieringer, 1996; Babalian et al., 1997), although these experiments were carried out in current clamp and are therefore somewhat difficult to interpret. Behavioral data suggest that activity in a population of vestibular nucleus neurons is gated off during the VOR (Minor and Goldberg, 1991) but direct evidence, which would require some knowledge of internal circuitry, is absent. No study to date has reported paired recordings between any two neurons in either the vestibular or the deep cerebellar nuclei. Clearly, now that certain cell types can be targeted for patch recordings in the transgenic lines described here, these data should be noticeably easier to achieve and interpret. I hypothesize that one important role of local inhibitory neurons will be to adjust the activation and gain of the VOR during deliberate (planned) head motion (Cullen, 2004). If interneurons participate in this circuit suppression, then they are also probable sites of plasticity during learned increases or decreases of the VOR. In this scenario, interneurons provide not a phasic temporal control but instead a tonic gain control (Mitchell and Silver, 2003).
Excitatory vestibular afferent input

Vestibular afferents, which provide excitatory input to nearly every neuron in the ipsilateral vestibular nuclei, are active at high tonic rates of ~20-100 Hz (Highstein et al., 2005; Yang and Hullar, 2007). The research presented in Chapter 3 details their properties of synaptic transmission and demonstrates a frequency-independent depression of EPSCs over the range of physiologically relevant firing rates. How is this accomplished? Although it is possible that nonlinear aspects of release are compensated for by nonlinear postsynaptic effects, the simplest and therefore likeliest explanation is that vestibular nerve terminals release the same amount of neurotransmitter regardless of recent activity, within a physiologically relevant dynamic range.

Based on what is known about synaptic transmission, I suggest that this synapse has two critical properties: first, exceptional presynaptic calcium buffering and extrusion, such that higher rates of activity do not lead to a build-up of calcium and an accompanying increase in release probability; second, vesicular recycling speeds no slower than 10 ms (Saviane and Silver, 2006). The experimental challenge of assessing presynaptic release is that the cell bodies of the vestibular nerve reside in the bone-encased vestibular ganglion, and are as such quite difficult to load with any type of indicator (such as synaptopHluorin or FM 1-43). I suggest that one fruitful approach will be reverse transcription profiling of RNA expression in the vestibular ganglion, with particular emphasis on calcium binding proteins, to search for candidate genes important to presynaptic buffering capacity. This screening process may also reveal quantitative differences in the amount or type of various proteins involved in vesicular recycling. Finally, following either dye injection or histological stains to distinguish
vestibular afferent synapses, electron microscopy of the terminal ultrastructure will likely reveal specializations related to its unusual functional properties.

**Glycinergic and glutamatergic projections**

While prior data suggested that projection neurons in the MVN to abducens and spinal targets were likely glycinergic (Spencer et al., 1989), our discovery that some fastigial (medial cerebellar nucleus) projection neurons are also glycinergic is surprising. Our working hypothesis, as detailed in Chapter 4, is that control over central eye, neck, and trunk muscles may require bilateral cerebellar cooperation to ensure coordinated activation of muscles on both sides of the midline. A second hypothesis, which we are currently trying to evaluate, is that any cerebellar output nuclei with direct projections to motor neurons contains both an excitatory and an inhibitory projection pathway (Straka et al., 2005), while output nuclei projections to premotor structures like the thalamus or red nucleus are purely glutamatergic. This hypothesis is consistent with known evidence, but as of now we have no data regarding the specific targets of glycinergic fastigiospinal and vestibulospinal neurons.

I suggest that a second interesting line of experiments would be to examine the local connectivity of these neurons. Large neurons in the deep nuclei are thought to drop local axon collaterals before projecting out of the cerebellum (Chan-Palay, 1977). If these glycinergic neurons do the same, then their local synapses will presumably be inhibitory. Several alternative effects of glycinergic neurons include: a) that they do not in fact drop local collaterals; b) that they project commissurally to the contralateral, rather than ipsilateral, fastigial/medial vestibular nucleus (consistent with commissural
data in Chapter 2); or c) that they depolarize local targets, either because of a shifted local chloride balance or postsynaptic expression of the purely glycine-sensitive NR3 NMDA receptor subunit. Cell fills of glycinergic neurons or paired recordings would help to support or eliminate these and other possibilities.

**Purkinje cell dendritic inhibition of downstream targets**

Despite recent recordings targeted to inhibitory deep cerebellar nucleus neurons (Uusisaari et al., 2007), no one has yet reported on any aspects of Purkinje cell inhibition of non-projection neurons. Given the anatomical data in both the cerebellar (Chan-Palay, 1977) and vestibular nucleus (Chapter 5), it is clear that there are multiple forms of Purkinje inhibition onto different kinds of cerebellar target neurons. With the advent of tools for identifying different types of GABAergic and glycinergic neurons, it will be both straightforward and illuminating to stimulate Purkinje inputs to local inhibitory neurons in the deep nuclei. Do these synapses exhibit the same type of frequency-independent depression seen in large projection neurons (Telgkamp et al., 2004)? Do dendrite-targeting Purkinje cells succeed in hyperpolarizing or reducing the firing rate of the postsynaptic neuron, or is their influence primarily on input integration?

The anatomical limitations of the MVN, where it is difficult to selectively stimulate Purkinje cell axons, suggest that the easiest approach to these questions will be in the deep cerebellar nuclei. However, new tools for activating subsets of neurons will be of great use in the MVN. For example, expression of channelrhodopsin, a light-responsive channel, in Purkinje cells would permit selective activation of these axons
while recording from MVN neurons. In combination with electrical stimulation of vestibular nerve afferents, this would allow a direct test of whether Purkinje inhibition affects dendritic integration of vestibular information. As gene expression profiling aids us in labeling and stimulating other types of inputs to the vestibular nuclei, including those from the “error signal” of the accessory optic system, it will be of great interest to examine the rules of dendritic integration in MVN neurons. To date, efforts to understand dendritic computation have focused almost exclusively on neurons with very low spontaneous activity (e.g. hippocampal and cortical pyramidal neurons); MVN neurons, with their high spontaneous firing rates, might well follow different rules.
References


