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Abstract:
The cerebellum is essential for performing accurate, smooth and coordinated movements in an ever-changing environment. While the cerebellum has been studied for well over one hundred years, many questions remain regarding how the underlying circuitry supports overall cerebellar function. To address questions related to anatomy and intrinsic excitability, a combination of neuronal tracing techniques and intrinsic electrophysiological recordings are used to study cerebellar circuit neurons. Experiments reveal that despite conveying different sensory, motor and cortical signals, precerebellar neurons in diverse precerebellar nuclei share most intrinsic excitability characteristics. In addition, neurons in the vestibular complex, a region well-connected with the cerebellum, have specializations in the conversion of current into firing rate related to their neurotransmitter content and specific anatomical projections. Finally, experiments reveal specific neurons participating in a cerebellar feedback loop connecting the nucleus prepositus hypoglossi to the inferior olive

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Anatomy and physiology of neurons supporting the cerebellar circuit

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Neurosciences by Kristine Elizabeth Champion

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2011
The Dissertation of Kristine Elizabeth Champion is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-chair

Chair

University of California, San Diego

2011
Dedicated to Nick. I love you forever. I like you for always.
Never give up, never surrender!

~ Jason Nesmith/Commander Peter Quincy Taggart in Galaxy Quest
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Chapter 2 is original work in preparation as Kolkman KE, McElvain LE, du Lac S. Shared electrophysiological signature of diverse precerebellar neurons and is included with permission from all the manuscript’s authors. The dissertation author was the primary author of this paper.

Chapter 3 is a reprint of the material as it appears in Kolkman KE, Moghadam S, du Lac S. Intrinsic physiology of identified neurons in the prepositus hypoglossi and medial vestibular nucleus. J Vestib Res Jan 1; 21(1):33-47. The dissertation author was the primary author of this paper.
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ABSTRACT OF THE DISSERTATION

Anatomy and physiology of neurons supporting the cerebellar circuit

by

Kristine Elizabeth Champion

Doctor of Philosophy in Neurosciences

University of California, San Diego, 2011

Professor Sascha du Lac, Chair

Professor William B. Kristan, Jr., Co-chair

The cerebellum is essential for performing accurate, smooth and coordinated movements in an ever-changing environment. While the cerebellum has been studied for well over one hundred years, many questions remain regarding how the underlying circuitry supports overall cerebellar function. To address questions related to anatomy and intrinsic excitability, a combination of neuronal tracing techniques and intrinsic electrophysiological recordings are used to study cerebellar circuit neurons. Experiments reveal that despite conveying different sensory, motor and cortical signals, precerebellar neurons in diverse precerebellar nuclei share most intrinsic excitability characteristics. In addition, neurons in the vestibular complex, a region well-connected with the cerebellum, have specializations in the conversion of current into firing rate related to their neurotransmitter content and specific anatomical projections. Finally, experiments reveal specific neurons participating in a cerebellar feedback loop connecting the nucleus prepositus hypoglossi to the inferior olive.
Chapter 1. Introduction

Among many functions, the cerebellum is necessary for performing smooth, accurate and coordinated movements in an ever-changing environment. Without this ability, animals become easy prey or unsuccessful predators, and therefore, this ability is essential for survival. The cerebellum has an important role in development, aiding coordination of muscle movements that give vertebrates the ability to run, swim, or eat. Likewise, the cerebellum is important in mature animals for adapting movement to changes in their environment, or the learning and fine-tuning of motor skills.

The necessity of proper cerebellar function is demonstrated most clearly when that function is diminished. In humans, lesions of portions of the cerebellum can cause a wide range of problems including impaired movement speed and accuracy (Kuper et al., 2011), vertigo, visual nystagmus (Ye et al., 2010), and even diminished semantic fluency (Schweizer et al., 2010). Medical science has witnessed and studied the consequences of cerebellar disease and dysfunction for over 200 years (Jackson, 1841), and yet many fundamental questions about how the cerebellum works remains.

Cerebellar circuitry

The circuitry within the cerebellum (Figure 1.1) is relatively stereotyped, almost crystalline, across this neural suborgan. Mossy fibers and neuromodulatory neurons project from precerebellar nuclei to the granule cell layer. There they contact granule cells as well as interneurons. Granule cells project parallel fibers to Purkinje cells, the sole output neurons of the cerebellar cortex. Purkinje cells also receive input from
climbing fibers originating in the inferior olive in the caudal brainstem. Purkinje cells then project either to the cerebellar nuclei or to the vestibular nuclei (Figure 1.2).

There are many neurons that are not located within the cerebellum per se but are equally a part of the cerebellar circuit. Precerebellar neurons located in multiple nuclei primarily in the brainstem and pons receive sensory, motor, and cortical signals that they convey to the cerebellum via mossy fibers (Figure 1.1). Likewise, climbing fibers originate with neurons in the inferior olive that receive sensory and/or motor information as well as feedback from the cerebellar and vestibular nuclei (Figure 1.1). In addition, some Purkinje cells project to neurons outside of the cerebellum, in the vestibular nuclei (Figure 1.2). These neurons are analogous participants in the cerebellar circuits to neurons in the cerebellar nuclei, and such neurons that send signals to or receive signals from the cerebellum are the focus of the work of this thesis.

*The medial vestibular and prepositus hypoglossi nuclei are pre- and post cerebellar nuclei*

The vestibular system has a unique relationship with the cerebellum (Figure 1.2). Purkinje cells in the vestibular cerebellum (flocculus, nodulus), project not to the cerebellar nuclei, but to the vestibular nuclei. In addition, neurons in the vestibular nuclei also project sensory and/or motor information to the cerebellum. The vestibular nuclei are both sensory and premotor nuclei that support several behaviors that undergo experience-dependent adjustments or learning via cerebellum-dependent processes. These learning behaviors include adaptation of the vestibulo-ocular and optokinetic reflexes, two reflexes that work in concert to stabilize images on the retina as an animal
These behaviors are among the simplest that “learn” in vertebrates, both in terms of circuitry and behavior. Both have very clear and measureable behavioral outputs that make them ideal for the study of learning. While these behaviors offer much promise for the connection of cellular changes in the brain to behavioral changes in the animal, there are still several anatomical and physiological questions to answer in order to connect activity at the cellular level and behavioral learning.

For the work in this thesis, I will focus on two of these vestibular nuclei that are involved in the processing of horizontal vestibular signals, the medial vestibular nucleus (MVN) and the nucleus prepositus hypoglossi (NPH). Though the NPH is not a vestibular nucleus in the strictest sense in that it does not receive direct sensory input from the vestibular nerve, I classify it as a vestibular nucleus with regard to the data for this thesis. The NPH is highly reciprocally connect to the MVN and projects to and receives projections from the same areas of the cerebellum. In addition, both nuclei support vestibular and oculomotor behaviors. In many ways, the nuclei work in concert to the degree that they could be considered specializations of the same nucleus. All three chapters of data in this thesis focus on these two nuclei, with chapter 2 focusing on an additional six precerebellar nuclei as well.

*Intrinsic excitability parameters in cerebellar circuit neurons*

The intrinsic excitability of a neuron governs how it converts its inputs into changes in membrane potential or firing rate. Neurons are electrical entities under the laws of physics, and the “rules” that govern the transformation of current into voltage change depend on three major factors: cellular anatomy, cellular history and ion channel
Neurons in the vestibular system have intrinsic properties endowing them with the ability to fire spontaneously in the absence of inputs. In addition, their transformations of current into firing rate follow a linear relationship over a very wide range of currents and firing rates. Many neurons in the vestibular system have fast sodium and potassium currents allowing them to fire at very high rates.

Although vestibular neurons share many intrinsic properties, they also have important differences. When comparing inhibitory neurons that project locally to non-GABAergic neurons that project outside of the vestibular system, many differences are apparent. The inhibitory neurons are overall smaller and have lower maximum firing rates, wider action potentials, and higher gains (slopes) of the current-to-firing rate relationship (Bagnall et al., 2007). While both express sodium currents allowing for fast firing rates (Gittis and du Lac, 2008), their potassium currents are balanced such that inhibitory neurons have proportionally more calcium-dependent and A-type currents, and non-GABAergic neurons have more voltage-gated (Kv) currents (Gittis and du Lac, 2007).

Studies examining specific subsets of neurons based on connectivity (inputs or projections) reveal even more specific intrinsic parameters. Vestibular neurons receiving an exceptionally large number of Purkinje cell terminals on their somas have higher spontaneous firing rates and more dramatic post-inhibitory rebound firing in response to release from hyperpolarization than many vestibular neurons (Sekirnjak et al., 2003). Premotor neurons projecting to the oculomotor nucleus had the ability to fire at exceptionally high firing rates and had little post-inhibitory rebound firing in response to release from hyperpolarization (Sekirnjak and du Lac, 2002).
The data presented in chapters 2 and 3 are also studies on neurons identified by connectivity or neurotransmitter content, and they examine the intrinsic excitability properties of these neurons with specific circuit roles. Here I will summarize the scientific context for the studies presented in chapters 2-4.

*Physiological characteristics of precerebellar neurons*

A major fundamental question about the cerebellum is how it makes sense of information from diverse sensory, motor and cortical areas. Previous studies have tackled this question using anatomy. They have clearly shown that certain precerebellar nuclei project to specific areas of the cerebellum, with nuclei such as the lateral reticular nucleus having very wide projections to many cerebellar lobules, and others such as the medial vestibular nucleus projecting only to a limited area of the cerebellum. An aspect to the question that is missing is whether precerebellar neurons that receive different sensory/motor/cortical signals have intrinsic physiological properties that are specialized to convert those signals into appropriate firing patterns for the cerebellum.

The question of whether intrinsic properties are tuned for the type of signal received requires examining precerebellar neurons from multiple nuclei that receive different sensory, motor and cortical signals under the same conditions. Until the study presented in chapter two, no study had compared more than two precerebellar nuclei at once. In fact, few studies had examined the intrinsic physiological properties in precerebellar neurons. The study presented in chapter two examines intrinsic excitability parameters for precerebellar neurons in both firing and subthreshold regimes. Pharmacological manipulation reveals some of the important ion channels that
support these physiological parameters. Finally, the intrinsic excitability of precerebellar neurons in eight different nuclei, with differing sensory, motor and cortical signals, are compared.

Intrinsic electrophysiological parameters of identified neurons in the NPH and MVN

The NPH and MVN both project to and receive projections from the cerebellar cortex as well as subserve related and unrelated functions in the vestibular and oculomotor systems. Because of their multifarious roles, the NPH and MVN have a wide variety of cell types as defined by either neuromodulator or connectivity. Previous studies have separated neurons in these nuclei into classes using action potential shape, transgenic mouse lines, transcripts and neuronal tracing.

In the study presented in chapter three, intrinsic excitability parameters in neurons in the NPH are examined using fluorescent transgenic mice expressing fluorescence in neurons differing in neurotransmitter content. The study is the first characterization of glycinergic neurons in the NPH, and demonstrates the diversity of GABAergic neurons, some of which inhibit locally, and others that project out of the vestibular complex.

Chapter three also includes data comparing and contrasting projection neurons in the MVN. Using neuronal tracing techniques combined with slice electrophysiology, the intrinsic parameters of two types of projection neurons, oculomotor-projecting and reticular formation/spinal cord-projecting are examined. Both projection neuron types, connect to motor areas, and their intrinsic properties were also quite similar. However,
this study highlights some key differences that may be important for their separate functions.

Anatomical examination of the MVN/NPH connection with the inferior olive

Climbing fibers are an important source of input to the cerebellum, and their firing rates are highly influential on the firing rates of Purkinje cells, one of their postsynaptic targets. Lesioning of the inferior olive has a variety of behavioral effects depending on the size and location of the lesion but can include motor deficits, problems with spatial memory, and inability to express and maintain classically conditioned memories (Gasabari et al., 2003; Voneida et al., 1990). The dominant model of cerebellum-dependent learning maintains that modifying the parallel fiber to Purkinje cell synapse underlies cerebellum-dependent motor learning. In this model, the climbing fiber synapses signal errors in motor movement that drive plasticity in the cerebellum, inducing a learning signal at the cellular level (Albus, 1971; Ito, 1982, Marr, 1969).

For most areas of the cerebellar cortex, Purkinje cells synapse onto neurons in the cerebellar nuclei, but for the horizontal-axis vestibular system, despite their location outside of the cerebellum, the MVN and NPH receive direct Purkinje cell input. Inhibitory neurons in the cerebellar nuclei send feedback signals to the inferior olive. An analogous connection has been identified for the MVN/NPH connection to the dorsal cap of Kooy, a subnucleus of the inferior olive that projects climbing fibers to the flocculus of the cerebellum (Figures 1.1 and 1.2). However, the GABAergic neurons in
the NPH represent a diverse population, and the subset of them that project to the dorsal cap have not been identified in previous studies.

In chapter four, I examine the connection between the NPH/MVN and the inferior olive using a combination of neuronal tracing, fluorescent transgenic mouse lines and immunocytochemistry. The techniques are employed to (1) identify neurons in the NPH and MVN that project to the inferior olive, (2) look for a connection from the dorsal cap of the inferior olive to the NPH, and (3) examine whether there are glycinergic terminals in the inferior olive.
Figure 1.1. Cerebellar circuitry. Sensory/motor/cortical information enters the cerebellum via mossy fibers in precerebellar nuclei. Signals denoting an “error” in motor function arrive to the cerebellum via climbing fibers from the inferior olive.
Figure 1.2. Circuitry of the flocculus of the cerebellum. Vestibular nuclei, including the medial vestibular nucleus and nucleus prepositus hypoglossi, both project to and receive projections from the cerebellar cortex. The green neuron represents a precerebellar neuron. The red neuron represents an olivary-projecting inhibitory neuron. The purple neuron represents a premotor neuron.
References


Chapter 2. Shared electrophysiological signature of diverse precerebellar neurons

Abstract

The cerebellum receives sensory, motor, and cortical signals via mossy fiber neurons. While the cerebellum is thought to perform similar computations on its inputs, the nature of the signals conveyed via precerebellar neurons varies widely. Little is known of whether they faithfully relay the signals they receive or perform more complicated computations. In this study, we examine the cellular properties of precerebellar neurons in eight precerebellar nuclei using patch-clamp recordings in mouse brain slices. Although the nuclei receive different combinations of sensory, motor and/or cortical signals, the cellular properties above spike threshold were remarkably similar. Precerebellar neurons had similar action potential profiles and linearly transformed current into firing rate over a wide range –properties supported by Kv3, SK and BK channels. While precerebellar neurons showed many similarities in their firing domains, subthreshold there were differences within and among precerebellar nuclei. Differences in spontaneous firing and the presence and magnitude of post-inhibitory rebound firing were highly variable within and between nuclei. We find that despite diversity in the nature of information they convey to the cerebellum, precerebellar neurons have a clear and predictable algorithm for converting currents into changes in firing rate, but differing responses to strong inhibition endow individual neurons with unique properties that may play a role in precerebellar processing.
Introduction

The cerebellum is important for motor control, motor learning, and cognitive tasks, but how cellular machinery transforms signals through cerebellar circuitry remains a source of study. The cerebellum is thought to perform a similar computation on the inputs it receives from numerous mossy fiber neurons in precerebellar nuclei located primarily in the pons and medulla. Each nucleus receives projections from CNS areas involved in sensory, motor, and/or cortical processing. Precerebellar neurons convey this information to the cerebellum where they contact granule, Golgi, unipolar brush and cerebellar nucleus neurons. A number of studies show the responses of precerebellar neurons to sensory stimulation and motor tasks (Bengtsson and Jorntell, 2009; Cheron et al., 1996; Eccles et al., 1971; Escudero et al., 1996; Garwicz et al., 1998; Noda, 1986; Rancz et al., 2007; van Kan et al., 1993). The sensory and motor evoked firing properties are highly variable for different sensory/motor modalities. For example, precerebellar neurons in the cuneate nucleus fire high frequency, short bursts of action potentials in response to somatosensory stimulation (Bengtsson and Jorntell, 2009). In contrast, medial vestibular nucleus mossy fiber neurons produce a graded change in firing rate that correlates with head velocity (Escudero et al., 1996). Because different sensory and motor modalities code signals in different ways, it may explain why precerebellar neurons have disparate firing patterns. However, it is also possible that the neurons have specialized cellular properties to create unique firing patterns. Decades of research on precerebellar neurons have failed to answer this question because few studies have compared mossy fiber neurons in precerebellar nuclei under identical conditions, and none have compared them across multiple nuclei. Also, the
vast majority of studies have been *in vivo* and thus have not allowed the properties of the neurons to be studied aside from their network influences. Since the cerebellum is thought to perform a common computation on inputs, and mossy fiber neurons produce a variety of firing patterns, a major question is are the intrinsic properties of mossy fiber neurons tuned to the types of signals they convey to the cerebellum? This study examines the intrinsic properties of precerebellar neurons by recording their responses to somatically injected currents. We find that precerebellar neurons in all nuclei recorded have a linear current-to-firing rate relationship over a very wide current and firing rate range, and that this relationship is dependent on SK and Kv3 channels. Precerebellar neurons differ, however, in post-inhibitory rebound firing properties. Therefore, precerebellar neurons transform current similarly in their firing domains with some differences subthreshold.

**Materials and Methods**

*Animals*

All protocols using animals were approved by the Animal Care and Use Committee at the Salk Institute in accordance with National Institute of Health guidelines. Neurons were imaged and recorded in wild-type and transgenic lines (GlyT2 (Zeilhofer et al., 2005), GIN (Oliva et al., 2000), YFP-16 (Feng et al., 2000), L7-GFP (Sekirnjak et al., 2003)) backcrossed to the C57BL/6 background.
**Tracer Injection**

Angle two stereotax and software (myneurolab.com; Richmond, IL) were used to target stereotaxic injections into the flocculus and vermis/hemisphere. For injections in adult mice (age P70+), the flocculus was targeted 3.32 mM lateral, 5.68 mM posterior, and 4.81 mM ventral of bregma using an angle of 30.69 degrees on the medial-lateral axis. For flocculus injections of youth mice (age P16-P23), the flocculus was targeted at 3.32 mM lateral, 5.08 mM posterior, and 4.81 mM ventral of Bregma using a 25 degree medial-lateral angle.

Texas red dextran (10,000 MW) was used as the neuronal tracer. Injections were made using either dextran crystals or a near-saturating solution of dextran liquid diluted in DI water. Brain slices were made 2-4 days after surgery for electrophysiological recording in youth mice or 3-7 days later for histological analysis in adult mice.

**Fixed tissue preparation**

Animals were transcardially perfused with phosphate buffered saline (PBS) and subsequently 4% paraformaldehyde in PBS. The brains were removed and placed in 4% paraformaldehyde in PBS at room temperature for 30 minutes. The brains were then transferred to 4°C and placed in 30% sucrose in PBS until the brains sank (approximately 24 hours). Thin coronal sections (40 µm) were sliced with a freezing microtome (Microm) through the brainstem and pons. Slices were wet-mounted and coverslipped with 2.5% DABCO (1,4 diazabicyclo-[2.2.2]octane).
Slice Preparation for Electrophysiology

Before brain tissue was harvested, mice (P18-25) were deeply anesthetized with Nembutal and then decapitated. Their hindbrains were removed and dissected in ice-cold carbogenated (95% O₂ – 5% CO₂) artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 5KCl, 1.3 MgSO₄, 26 NaHCO₃, 2.5 CaCl₂, 1 NaH₂PO₄, and 11 glucose. When carbogenated, ACSF had a pH of 7.4 and an osmolarity to 300 mOsm. 250 uM thick coronal slices of the brainstem and pons were cut on either a Leica VT1000S or DSK-1500E vibratome. Slices were incubated in a holding chamber containing carbogenated ACSF at 34°C for 45 minutes and then carbogenated at room temperature before use.

Electrophysiological Recording

Recordings were made in a submersion chamber with constant perfusion of carbogenated ASCF at 34°C. Picrotoxin (100 µM) and strychnine (1 µM) were added to the ACSF for all recordings in order to block GABAergic and glycinergic transmission, respectively. Fluorescent cells were visualized using Olympus (U-LH100HG) illumination and a calcium crimson filter (for visualizing texas red). They were targeted for whole-cell patch clamp recording using a Dage-MTI or VE1000 camera mounted on a Olympus BX51WI infrared differential contrast microscope with 40x magnification.

Whole-cell patch clamp recordings were performed with an AxoClamp 2B or a MultiClamp 700B amplifier in current clamp mode. Micropipettes (4-7 MΩ) were from borosilicate glass (inner diameter 0.86 mm, outer diameter 1.50 mm) using a Sutter
Instruments P-97 puller. Solution filling the electrodes contained (in mM): 140 K gluconate, 10 HEPES, 8 NaCl, 0.1 EGTA, 2 MgATP and 0.3 Na$_2$GTP and was adjusted to pH 7.3-7.5 and 280-285 mOsm. Data were filtered at 10 kHz and digitalized at 40 kHz with an ITC-18 (Instrutech). Data acquisition and analysis was performed using code written in house in Igor 6. A liquid junction potential of 15 mV was subtracted from all membrane potential measurements.

*Analysis of electrophysiological parameters*

Action potential profiles were acquired at a rate of 8-12 spikes/s and then averaged over 1-5 s after aligning at their peaks. Action potential threshold was defined as the membrane potential where derivative of the voltage trace reached 10 V/s. The width of the action potentials was determined at threshold, and the halfwidth was defined as the width halfway between threshold and peak. Afterhyperpolarization (AHP) was determined as the different between threshold and the most hyperpolarized membrane potential between spikes. Neurons were excluded from the study if they could not fire throughout a 1 s depolarizing step or if their action potential height was less than 45 mV.

To measure excitability above threshold, the current-to-firing rate relationship was determined by applying 1 s depolarizing current steps of increasing magnitude until the neuron could not fire throughout the step, and recording the corresponding mean firing rate across the step. The gain of the relationship was defined as the best-fit line of the current versus mean firing rate curve and was calculated separately for mean firing rates above and below 80 spikes/s (Bagnall et al., 2007). Maximal firing rate was
defined as the mean firing rate across the 1 s step with the largest magnitude throughout which the neuron could fire.

Excitability below threshold was determined by measuring the input resistance. Input resistance was measured by hyperpolarizing the neurons to -75 mV and giving small hyperpolarizing steps. 6 steps were given and averaged for analysis.

Adaptation was measured in depolarizing steps that yielded a firing rate of approximately 150 spikes/s at the end of the 1 s step. Firing rate adaptation was defined as the ratio of the firing rate over the first and last 100 ms of the step after excluding the first 50 ms from the analysis (Sekirnjak and du Lac, 2002). Post-inhibitory rebound firing was measured by applying DC current to maintain neuronal firing at 10 spikes/s. 1 s steps that hyperpolarized the neurons by 30 mV were applied, and the rebound firing rate was determined as the difference in firing rate before and after the hyperpolarizing step.

To measure the firing rate response to sinusoidally modulating current, mossy fiber neurons were depolarized to fire at 30 spikes/s. Sinusoidally modulating current was delivered at 0.25, 0.5, 1, 2 and 4 Hz with an amplitude producing modulation of ± 10-15 spikes. The sinusoidal firing rate responses were fit using sinusoidal functions $f(t)=A+B\sin(2\pi ft+C)$. The frequency (f) was set to the input frequency, and A, B and C were chosen to minimize the mean standard error. Comparison of the fit sinusoid to the input sinusoid was used to determine gain and phase responses of the neurons.
Statistical Analysis

The eight precerebellar nuclei recorded were compared using the Kruskal-Wallis nonparametric multiple group comparison followed by the Dunn’s posthoc test (if P<0.05) performed in GraphPad Prism (version 4.0) software. Data from the eight nuclei were grouped together for pharamcological experiments. Data before and after pharamcological manipulation were compared using the paired Wilcoxon Rank Sum nonparametric test in Kaleidagraph (version 4.03). Values are reported as the mean ± standard deviation.

Results

Diversity and locations of precerebellar neurons

To identify precerebellar neurons in vitro, we made dextran tracer injections in the cerebellum. Injections targeting the flocculus (Figure 2.1A), vermis and hemispheres (Figure 2.1F) were confirmed by retrograde neuronal labeling in the contralateral inferior olive (Figure 2.1B and 1G). Flocculus injections also resulted in retrogradely-labeled neurons (Figure 2.1C-E) in the medial vestibular nucleus (MVN), nucleus prepositus hypoglossi (NPH), supragenual nucleus, the nucleus intercalatus/nucleus of Roller, nucleus reticularis tegmenti pontis (NRTP), and lateral reticular nucleus (LRN). Vermis and hemisphere injections resulted in retrogradely-labeled in the LRN, pontine nucleus, NRTP, nucleus of Roller/intercalatus, and external cuneate nucleus (Figure 2.1H-J).

Neurons in eight nuclei that project mossy fiber axons to the cerebellum were targeted for whole-cell patch recordings. These nuclei were selected to represent the
diversity of sensory, motor, and cortical signals entering the cerebellum. The external cuneate primarily processes sensory information with the somatosensory system supplying the dominant inputs. In contrast, the NRTP primarily processes motor information, receiving efference copies of eye movement commands. The LRN integrates proprioceptive and autonomic signals with descending motor commands. The MVN and NPH process a combination of sensory and motor information; head motion sensory signals from the inner ear and efference copies of eye movement commands. The supragenual and Roller nuclei process brainstem and cerebellar signals related to gaze. Finally, widespread regions of the cerebral cortex convey highly processed signals to the cerebellum via a major projection from precerebellar neurons in the pontine nuclei.

Several precerebellar nuclei project axons bilaterally to the cerebellum, including the MVN, NPH, supragenual nucleus, and nucleus of Roller/intercalatus. The intrinsic physiological properties of neurons retrogradely labeled ipsilaterally versus contralaterally to the injection site in cerebellar cortex were compared for MVN, NPH, nucleus of Roller/intercalatus and supragenual neurons projecting to the flocculus. Ipsilaterally- and contralaterally-projecting neurons in each nucleus were indistinguishable with respect to membrane and firing properties and thus were grouped.

*Action potential waveforms are similar across precerebellar neurons*

Some retrogradely-labeled neurons in most precerebellar nuclei fired spontaneously in whole-cell patch recordings, while other neurons recorded in the same
brain slice preparation and nuclei were silent at rest. The highest proportion of neurons firing spontaneously was found in the MVN (52%), while about 1/3 in the LRN, NPH, and Roller nuclei, and 1/5 of neurons in the external cuneate and supragenual nuclei fired spontaneously (Table 2.1). Only one (of 30) neuron in the NRTP fired spontaneously, and all pontine neurons had resting membrane potentials below spike threshold (Table 2.1).

Despite differences within and across nuclei in resting activity, the action potential profiles of precerebellar neurons were remarkably similar. Figure 2.2 shows representative action potentials for precerebellar neurons from each of the 8 nuclei targeted for recording. To standardize comparison across neurons, action potentials were measured at firing rates of 10 spikes/s, maintained as necessary by intracellular current injection. As evidenced by each of the example precerebellar neurons, the action potential comprises both a rapid rising and repolarizing phase and is followed by a biphasic afterhyperpolarization (AHP) consisting of early, rapid phase and a second, slower phase. The width of the action potential (measured at half-height; see Methods) averaged 0.35 ms, and the peak amplitude of the AHP averaged 17.6 mV. These parameters varied within nuclei, but their population values were indistinguishable statistically across nuclei. The similarity of action potential waveforms across eight precerebellar nuclei suggests similarities in the underlying ionic currents.

Firing responses to depolarizing current

Precerebellar neurons recorded in vivo exhibit a range of firing properties. To investigate whether these responses reflect differences in intrinsic excitability across
precerebellar nuclei, we assessed firing responses to intracellular depolarization. All precerebellar neurons fired continuously in response to 1 s of depolarization. Responses to a range input current amplitudes, plotted as instantaneous firing rate versus time, are shown for a typical precerebellar neuron (from the pontine nucleus) in Figure 2.3A. Following the stimulus onset, the neuron exhibited a rapid increase in firing rate that was proportional to the input amplitude and adapted modestly over the course of a second. In response to inputs > 3.5 nA, the neuron exhibited depolarization block and was unable to fire continuously throughout the 1 sec stimulus. Figure 2.3B plots the mean evoked firing rate as a function of the input current and demonstrates a typical feature of precerebellar neuronal input-output properties; firing rates are a bilinear function of input currents, exhibiting a change in gain (slope) at firing rates of 80 spikes/s. For the neuron shown in Figures 2.3A and B, the gain (evoked firing rate divided by input current) of the firing responses below 80 Hz was 79.9 spikes/s/nA, the gain of firing responses above 80 Hz was 50.5 spikes/s/nA, and the maximum firing rate (see Methods) was 200 spikes/s.

Firing responses to current steps ranged widely but were qualitatively similar across precerebellar neurons recorded in different nuclei. Maximum firing rates ranged widely within nuclei but did not differ significantly across nuclei; the average maximum firing rate was 170 spikes/s (Figure 2.3C). Almost all neurons exhibited two ranges of linear firing responses to inputs, such that the ratio of gains derived for values fit below versus above 80 spikes/s averaged between 1.5 and 1.9 (Figure 2.3F). Within both ranges, firing responses were almost perfectly linear functions of input current amplitude, as evidenced by correlation coefficients that averaged 0.99 (Figure 2.3D).
Gains in both ranges varied significantly between precerebellar nuclei (Figures 2.3E and F and Table 2.2). MVN and LRN neurons exhibited the highest and lowest gains, respectively, averaging 164 and 99 (spikes/s)/nA when measured below 80 spikes/s. The extent of firing rate adaptation also varied across precerebellar neurons within and across nuclei (Figure 2.3G, Table 2.2), but statistical posthoc tests failed to show individual differences. Differences in excitability within the firing domain across nuclei were not reflected in analyses of excitability below spike threshold; input resistances, which were typically between 100 and 200 MΩ, did not vary significantly across nuclei (Figure 2.3H).

**Firing responses to temporally-modulated inputs**

To examine firing responses to temporally-modulated inputs, we alternately depolarized and hyperpolarized neurons using sinusoidal current injection. To standardize the comparisons and to prevent firing rate from rectifying, temporally modulated current was delivered to neurons firing at baseline rates between 25 and 30 Hz (evoked by DC current injection), and sinusoidal current was constrained to produce peak-to-peak modulations that averaged 20 spikes/s. Firing responses of a typical precerebellar neuron, recorded in the nucleus of Roller/intercalatus, to sinusoidally modulated current are shown in Figure 2.4A. This and all other precerebellar neurons exhibited remarkably faithful modulation of firing rate in response to temporally modulated inputs, resulting in excellent fits of sine waves to instantaneous firing rate (middle panel, Figure 2.4A).
Peak-to-peak firing rate responses to sinusoidal inputs ranging from 0.25 to 4 Hz are plotted as a function of input frequency in figure 2.4B. Each data point represents the population average and standard error of the population responses for each of 8 different precerebellar nuclei. Firing responses to sine waves were qualitatively identical across neurons and nuclei. Response gains increased slightly and systematically as input frequency increased from 0.25 to 4 Hz (Figure 2.4B). Firing responses were nearly in phase with input currents, exhibiting a slight phase lead at the lowest input frequencies and a slight phase lag at the highest input frequencies (Figure 2.4C).

As with responses to depolarizing steps, precerebellar neurons from distinct nuclei exhibited quantitative differences in sinusoidal gain (Figure 2.4B, Table 2.2). MVN neurons had significantly higher gains (p<0.05) across all frequencies than did pontine, nucleus of Roller/intercalatus and NRTP neurons, which exhibited the lowest gains. In contrast, phase responses were quantitatively identical across frequencies for all precerebellar nuclei except at the lowest frequencies where the MVN had a slight but significant phase lead relative to the nucleus of Roller/intercalatus (0.25 and 0.5 Hz, p<0.05) and the LRN (0.25 Hz, p<0.05). Together, these results indicate that within the firing domain, precerebellar neurons exhibit qualitative similarities with some quantitative differences in neurons within and between nuclei.

SK channels decrease firing response gain and maximum firing rates

The data presented so far indicate that precerebellar neurons share similarities in their action potential waveforms, their ability to sustain high firing rates, and their
remarkably faithful and linear firing responses to input currents, suggesting that they share underlying conductances. What ion channels are required to generate linear firing responses over a wide range of inputs and firing rates?

Slow-conductance calcium-activated potassium (SK) currents influence excitability in many types of neurons (Sah, 1996), including unidentified neurons in the MVN (Smith et al., 2002). We examined the role of SK currents in precerebellar neurons by assessing the effects of pharmacological manipulation with the specific SK blocker apamin. Sub-saturating concentrations of apamin (50 nM) were used to preclude high frequency bursting. Partial blockade of SK currents had no effect on the rising and falling phases of the action potential but diminished both the fast and slow components of the AHP, as evidenced in a representative precerebellar neuron recorded in the LRN in figure 2.5A. The decrease in AHP amplitude was consistent across all precerebellar neurons, with an average decrease of 6 mV (Figure 2.5D, p<0.01). Firing responses to input current steps increased dramatically in the presence of apamin (Figure 2.5B), resulting in a large increase in gain across the entire firing range (Figure 2.5C). Partial blockade of SK channels increased gain 2 to 4 fold across all precerebellar neurons tested (Figure 2.5F, n=9; gain measured below 80 spikes/s, p<0.05). The increase in gain was accompanied by a significant increase in adaptation (Figure 2.5B and E, p<0.05). Interestingly, blockade of SK channels resulted in a greater than 50% increase in maximum firing rates and a decrease in the range of input currents over which neurons could sustain firing (Figure 2.5G, p<0.01 and p<0.05 respectively). Together, these results indicate that SK currents active during the interspike interval are crucial for the typical firing responses of precerebellar neurons.
and that they act to reduce excitability and increase the input range over which neurons fire proportionately with their inputs.

**BK channels decrease firing response gain**

Big-conductance calcium-activated potassium (BK) currents influence excitability in many cell types (Sah, 1996; Smith et al., 2002) and are targets for activity-dependent plasticity in MVN neurons (Nelson et al., 2003; Nelson et al., 2005). We examined the role of BK currents in intrinsic excitability of precerebellar neurons by assessing the effects of the BK channel blocker paxilline (1 µM). Similar to the results from blocking SK channels, blockade of BK channels with paxilline reduced the magnitude of the AHP, although the average magnitude of the decrease was half of that with SK blockade (Figure 2.6A, p<0.01). Firing response gains for firing rates less than 80 Hz were increased slightly in paxilline (on average by 19%; p<0.05, Figure 2.6D, n=10), but this effect was significantly less dramatic than the doubling to quadrupling of gain in apamin (compare with Figure 2.5F). Paxilline did not have a significant effect on input current range (Figure 2.6E) or maximum firing rate (Figure 2.6F). These results indicate that BK currents have a modest effect on precerebellar neuronal gain but are not essential for their canonical firing properties.

**Kv3 channels enable high firing rates**

Kv3 currents are critical for rapid neuronal firing (Gittis et al., 2010). We used TEA to examine the contributions of Kv3 currents to firing in precerebellar neurons. At low concentrations (1 mM), TEA blocks Kv3 channels, BK channels, and dendrotoxin-
sensitive channels, which do not contribute to excitability in MVN neurons (Gittis and du Lac, 2007). To influence Kv3 currents specifically, we first blocked BK currents with paxilline and then applied 1 mM TEA. Blockade of Kv3 currents had a dramatic effect on action potential repolarization and abolished the early phase of the AHP (p<0.01) but had no effect on the late phase of the AHP (Figure 2.6A). Firing response gain was reduced slightly in the presence of 1mM TEA at low firing rates (p<0.01) but reduced dramatically at higher firing rates (p<0.05, Figure 2.6C). As a consequence, the maximum firing rate decreased significantly (p<0.01, Figure 2.6C). Across precerebellar neurons, blockade of Kv3 currents produced an average decrease in maximum firing rate of 56 spikes/s, a decrease to 62% of the control value. In contrast, TEA had small, but significant, effect on the input current range (p<0.05, Figure 2.6E). These results indicate that Kv3 currents are essential for high firing rates attained by precerebellar neurons but have little influence on firing responses below 80 spikes/s.

Post-inhibitory rebound firing

The results presented thus far indicate that neurons in different precerebellar nuclei have similar firing responses to a wide range of inputs. At rest, however, some precerebellar neurons fire spontaneously while others are silent, indicating that ionic conductances near spike threshold may differ across precerebellar neurons and nuclei. To further explore such differences, we examined responses to stimuli that hyperpolarized neurons below spike threshold. To standardize the baseline conditions and to compare with previous literature in brainstem neurons (Sekirnjak and du Lac, 2003), hyperpolarizing currents steps that reduced average membrane potential by 30
mV were applied to neurons firing at 10 spikes/s (maintained as necessary with intracellular DC current). In most neurons, including the examples shown in Figures 2.7A1 and 2.7B1, hyperpolarization produced an initial decrease in membrane potential followed by a depolarizing sag in membrane hyperpolarization that is characteristic of the hyperpolarization-activated cationic conductance $I_H$. Following the offset of the hyperpolarizing step, most neurons exhibited an increase in firing rate above baseline levels. The magnitude and timecourse of this rebound firing varied considerably across neurons, as evidenced by the examples in Figure 2.7A1 and 2.7B1. A subset of neurons exhibited a transient decrease in firing rate following hyperpolarization offset; these neurons were concentrated in pontine nuclei and the NRTP.

Rebound firing responses were quantified across the population of precerebellar neurons both as the change in rate relative to baseline (Figure 2.7C; see Methods) and as the delay from the offset of hyperpolarization to the time of the next spike (Figure 2.7D). Rebound responses differed quantitatively both within and across nuclei. The most pronounced rebound responses were observed in the external cuneate and MVN, in which several neurons fired transiently at 30 to 100 spikes/s above the baseline rate of 10 spikes/s (Figure 2.7C). Neurons in both of these nuclei varied considerably, however, with many neurons little or no rebound firing; such diversity mirrors that observed in unidentified MVN neurons (Sekirnjak and du Lac, 2003). In contrast, pontine neurons tended to exhibit little or no rebound firing (Figure 2.7C). Furthermore, the time to the first spike after hyperpolarization offset was markedly longer in pontine neurons than in each of the other precerebellar nuclei (Figure 2.7D). These results are consistent with the activation of an outward current that opposes postinhibitory rebound
firing (Sekirnjak and du Lac, 2003), and they imply that the amplitudes or nature of the ionic currents near threshold vary considerably across precerebellar nuclei.

To examine mechanisms underlying rebound firing in precerebellar neurons, we pharmacologically blocked $I_{\text{H}}$, the dominant inward current activated by neuronal hyperpolarization. Application of the ZD7288, an $I_{\text{H}}$ blocker, reduced the depolarizing sag during steady hyperpolarization (Figures 2.7A2, B2). Blockade of $I_{\text{H}}$ also reduced the magnitude of rebound firing in the neurons that exhibited rebound firing in control conditions ($p<0.01$, Figure 2.7E), and it consistently increased the delay to the subsequent spike ($p<0.05$, Figure 2.7F). Interestingly, in the two neurons examined with exceptionally pronounced rebound firing (including the example in Figure 2.7B), blockade of $I_{\text{H}}$ had a relatively small influence on rebound firing. In summary, post-inhibitory rebound firing varies considerably within and across precerebellar nuclei, suggesting differential expression of ionic conductances activated at membrane potentials around or below spike threshold.

**Discussion**

In this study, we examine whether the cellular characteristics of precerebellar neurons are different depending on the types of sensory, motor, and/or cortical information that they convey. The cellular properties of precerebellar neurons from eight different precerebellar nuclei demonstrate similar properties above threshold including similar action potential profiles and linear current-to-firing rate relationships. SK, Kv3 and BK currents affect action potential characteristics that, in turn, influence the gain and maximum firing rate of precerebellar neurons. In contrast, precerebellar
neurons show pronounced heterogeneity in their post-inhibitory rebound firing properties. We conclude that precerebellar neurons process inputs using similar scaling computations, but that they vary in their transformation of current into voltage below action potential threshold.

**Similarities and differences among precerebellar neurons**

Precerebellar neurons in all nuclei recorded share the ability to fire at high rates. *In vivo*, recordings during sensory or motor tasks revealed heterogeneous precerebellar neuron responses ranging from modest graded changes in firing rate (Cheron et al., 1996) to short high frequency bursts of action potentials (Bengtsson and Jorntell, 2009; Garwicz et al., 1998; Rancz et al., 2007), to various other responses such as combinations of bursts and changes in tonic firing rate (Escudero et al., 1996; van Kan et al., 1993). The necessity of fast firing has been demonstrated for some precerebellar neurons recorded during sensory and/or motor stimulation (Bengtsson and Jorntell, 2009; Eccles et al., 1971; Garwicz et al., 1998; Rancz et al., 2007; van Kan et al., 1993). For other neurons, firing rates above 200 spikes/s have not been recorded *in vivo* (Escudero et al., 1996), but does this mean that these neurons have fundamental differences in their firing abilities? The data presented demonstrate that although not all precerebellar neurons may fire at rapid rates *in vivo*, all have the ability to fire quickly for up to a second. Precerebellar neurons in nuclei known to produce high frequency bursts did not have significantly higher maximum firing rates than those in other precerebellar nuclei. Precerebellar neurons can respond to a wide range of inputs that they possibly would never receive. Therefore differences across firing responses likely
reflect differences in the inputs driving precerebellar neurons rather than intrinsic cellular limitations.

Precerebellar neurons in all precerebellar nuclei also displayed a linear relationship between current inputs and firing rate over a very wide range of currents. Linear transformations of current into firing rate have been previously demonstrated in for precerebellar neurons in both the NRTP and Clarke’s nucleus, a precerebellar nucleus located in the spinal cord (Eide et al., 1969; Kitai et al., 1976). Additionally, some precerebellar neurons display linear firing rate responses to sensory input (Arenz et al., 2008; Cheron et al., 1996; Escudero et al., 1996; Gamlin and Clarke, 1995; Mackie et al., 1999; van Kan et al., 1993) suggesting they may linearly transform current into firing rate. Our study demonstrates that linearity in the current-to-firing rate relationship is not limited to mossy fiber neurons in select precerebellar nuclei. While the slope of this relationship differed across cells and nuclei, the wide current range over which neurons could convert current into firing rate did not. Therefore, regardless of their inputs, precerebeller neurons are intrinsically able to respond to both small and large current inputs with a conserved scaling computation.

Some aspects of precerebellar neuron physiology differed across nuclei, particularly those demonstrated subthreshold. The data presented demonstrate significant differences in post-inhibitory rebound firing within and between precerebellar nuclei. Some nuclei, such as the pontine nucleus and NRTP, show almost no post-inhibitory rebound, while others, like the external cuneate display a wide range of responses. In vivo, external cuneate neurons produce high frequency, well-timed bursts to certain types of sensory stimulation (Bengtsson and Jorntell, 2009; Mackie et
al., 1999), and perhaps these neurons employ post-inhibitory rebound firing combined with excitation to improve the timing of these bursts. Neurons without post-inhibitory rebound may differ in their balances of excitation and inhibition as well as the nature of the signals that they produce.

**Mechanisms of precerebellar neuron firing**

The data presented show that precerebellar neurons in eight different nuclei have strong similarities in their intrinsic properties. One remarkable feature of precerebellar neurons is their ability to fire at very high rates. This ability depends on fast sodium currents that can depolarize the neuron quickly and Kv3 channels, which are expressed by fast-firing neurons in many brain areas (Erisir et al., 1999; Lien and Jonas, 2003; Martina et al., 1998; McKay and Turner, 2004; Wang et al., 1998). There is evidence that persistent sodium currents contribute to the action potential in pontine neurons (Schwarz et al., 1997), and resurgent and persistent sodium currents contribute to fast-firing neurons in the MVN (which include mossy fiber neurons) (Gittis and du Lac, 2007; Gittis and du Lac, 2008). Kv3 acts to narrow the action potential width by mediating rapid repolarization that leads to rapid deinactivation of sodium channels (Gittis and du Lac, 2008). This ‘protection’ of sodium channels enables neurons to fire action potentials in quick succession and fire at high rates for an extended period of time. A recent study (Gittis et al., 2010) examined MVN neurons in the YFP-16 mouse line which includes MVN precerebellar neurons (McElvain et al., 2010); this study found that levels of Kv3.3 correlated with the width of the action potential and the ability to fire at high rates. Likewise, a study of YFP-16 neurons in the NPH (which
includes mossy fiber neurons), showed that these neurons also express Kv3.3 which was not expressed by slower inhibitory neurons (Kolkman et al., 2011).

Precerebellar neurons linearly transform current into firing rate over a very wide range of currents. This transformation depends on SK channels which limit the maximum firing rate of the neuron, but extend the current range over which it can fire. By decreasing the sensitivity to changes in current, SK allows the neuron to change firing rate over a wide range of input magnitudes. SK is the predominant current between action potentials (Smith et al., 2002), underlying a long AHP. This is consistent with previous findings that the AHP in the pontine and Clarke’s (a precerebellar nucleus in the spinal cord) nuclei is dependent upon calcium (Gustafsson et al., 1978; Schwarz et al., 1997) since SK is a calcium-dependent potassium channel. The data presented also demonstrates that BK significantly influences the gain of the current-to-firing rate relationship, without altering the current or firing rate range of the neuron. When blocked, neurons can still linearly transform current into firing rate over a wide range, so what functional role does BK current serve for precerebellar neurons? BK conductances can be modulated by activity in the MVN. Reduction of BK channels in these neurons increases the gain of the current-to-firing rate relationship and spontaneous firing rates (Nelson et al., 2003). BK current plasticity may similarly give precerebellar neurons flexibility in the sensitivity of the current-to-firing rate relationship, while still maintaining the wide dynamic range of linear processing.
Functional significance for mossy fiber synapses

Precerebellar neurons in all eight nuclei recorded have the ability to produce both low and high frequency signals, but are these signals faithfully transmitted to their postsynaptic targets? Mossy fibers arising from precerebellar neurons contact granule cells via glutamatergic synapses. Properties of the mossy fiber-granule cell (MF-GC) synapses allow a wide frequency range of presynaptic firing rates to modulate granule cell firing. Low frequency signals are enhanced via a large amount of glutamate spillover contributing about half of the synaptic conductance (DiGregorio et al., 2002). The MF-GC synapse also under tonic inhibition from GABAergic Golgi cells, and modulation of that inhibition can increase or decrease sensitivity to low frequency inputs (Chadderton et al., 2004). Transmission of high frequency signals is aided by fast vesicle reloading (Hallermann et al., 2010) and desensitization-resistant AMPA receptors (DiGregorio et al., 2007). These specializations make it possible for the synapse to signal both low and high frequency information.

Mossy fibers also synapse on Golgi and unipolar brush interneurons that provide feedforward inhibition and excitation respectively to cerebellar granule cells. The mossy fiber-Golgi cell synapse is strong, but stimulating mossy fibers beyond about 50 Hz does not lead to an increase in Golgi cell firing (Kanichay and Silver, 2008). Therefore, high frequency signals may be filtered at this synapse. Mossy fiber-unipolar brush synapses are specialized to entrap glutamate allowing for a prolonged glutamatergic signal lasting up to 5 seconds (Kinney et al., 1997). Little is known about the sensitivity of unipolar brush cells to presynaptic firing rate, but glutamate accumulation would smear the timing of a mossy fiber signal. The feedforward
excitation and inhibition are certainly important for processing in the cerebellar cortex, but the temporally precise wide range of current-to-firing rate produced in mossy fiber neurons is not preserved in these neurons.

*Mossy fiber neurons have a common developmental past that may lead to common ion channel expressions*

Mossy fiber neurons share not only a role in cerebellar circuits but also a common developmental origin. Born in the ventral portion of the rhombic lip, mossy fiber neurons express the same developmental markers until they eventually migrate along the rostral-caudal axis of the brain to their final locations in precerebellar nuclei (for review, (Ray and Dymecki, 2009)). The data presented provide strong evidence that these neurons share remarkable similarity in excitability and ion channel expression within their firing domain. Given their shared a common developmental history, a common transcriptional program may guide their maturation process and dictate their future ion channel repertoire. This may explain the pronounced similarities in these neurons that are physically located in distinct brain regions receiving different types of inputs. Differences in the amount and nature of post-inhibitory rebound firing reveal that precerebellar neurons have differences in their ionic currents subthreshold. Because of differences in rebound firing among different precerebellar nuclei, it is possible that the local environment of the neurons dictates the expression of certain ion channels. Variability in post-inhibitory rebound firing within individual nuclei may suggest that the inhibitory or excitatory connections specific to individual mossy fiber neurons may help to shape subthreshold current expression.
Our results demonstrate that mechanisms shown to be important for other fast-firing neurons such as those in the MVN (Gittis et al., 2010; Smith et al., 2002), DCN (Aizenman and Linden, 1999; Jahnsen, 1986a; Jahnsen, 1986b; Llinas, 1988; Raman et al., 2000), and cortical/hippocampal interneurons (McCormick et al., 1985; Rudy and McBain, 2001) are also important for precerebellar neurons. Therefore, a way to build a fast-firing neuron is conserved across brain regions with different inputs and functions. A neuron can linearly transform current into firing rate over a very wide dynamic range with fast sodium currents, Kv3 to repolarize neurons quickly, and SK to control the inter-spike interval and dictate the sensitivity of the current-to-firing rate relationship. Precerebellar neurons, along with other fast-firing neurons across the brain, may be considered a specific class of neurons defined by their ability to fire fast and the ionic mechanisms underlying that property.
Figures

Figure 2.1: Identification of precerebellar neurons in pons and medulla via retrograde labeling of red fluorescent dextrans. A, Tracer injection site (red) in the floccular lobe of the cerebellum in a coronal ~5.6 mm caudal to Bregma. Retrogradely labeled neurons from this injection are shown in coronal sections in B-E. B, Caudal medulla, ~7.75 mm caudal to Bregma, showing neurons in the inferior olive retrogradely labeled. C through E. Retrogradely labeled neurons in the MVN, NPH, nucleus of Roller/nucleus intercalatus, and supragenual nucleus in medullary sections 6.96, 6.5, and 5.8mm caudal to Bregma, respectively. F, Cerebellar injection, ~6.25 mM caudal to Bregma, which resulted in the pattern of retrograde labeling show in G-J. G, Retrogradely labeled neurons in the inferior olive and LRN, are shown in this medullary section ~7.56 mM caudal to Bregma. H. Retrogradely labeled neurons in the LRN, ~7.76 mm caudal to Bregma. I. Retrogradely labeled neurons in the external cuneate in this section ~ 7.35 mm caudal to Bregma. J. Retrogradely labeled neurons in the NRTP and pontine nuclei in a section ~ 4.25 mm caudal to Bregma.
Figure 2.2: Action potential profiles of mossy fiber neurons in 8 precerebellar nuclei are similar. A, Average action potential waveform from 8 retrogradely-labeled neurons recorded in the external cuneate, lateral reticular, medial vestibular, prepositus hypoglossi, reticular tegmentis pontis (NRTP), pontine, roller, and supragenual nuclei. B, Action potential width at half-height for all recorded neurons. C, AHP amplitude for all recorded neurons. E, external cuneate; L, lateral reticular; M, medial vestibular; N, nucleus prepositus hypoglossi; T, nucleus reticularis tegmenti pontis; P, pontine; R, nucleus of Roller/nucleus intercalatus; S, supragenual nucleus. Open symbols are individual data points. Black horizontal bars represent the mean value for each nucleus.
Figure 2.3: Precerebellar neurons transform input current into firing rate linearly. A, Instantaneous firing rate versus time for a retrogradely labeled neuron recorded in the NRTP in response to 1 s depolarizing inputs of increasing magnitude. B, The relationship between current and mean firing rate across 1 s depolarizing steps for the neuron shown in A. Lines represent best linear fits to firing rate responses below and above 80 spikes/s. C, Maximum firing rates for all recorded precerebellar neurons. D, Linear correlation coefficient (R2) for current-to-firing rate relationship below 80 spikes/s. E, Gain of the current-to-firing rate relationship below 80 spikes/s. Significant differences of p<0.05 were found for L vs. M or S and R vs. M, N, or S. F, Ratio of gain for the current-to-firing rate relationship under and over 80 spikes/s. F, Ratio of gain for the current-to-firing rate relationship below and above 80 spikes/s. G, Adaptation ratio indicates the ratio of the firing rate at the beginning versus the end of the 1 s current step measured at steady state firing rates of 150 spikes/s. H, Input resistance measured below spike threshold. E, external cuneate; L, LRN; M, MVN; N, NPH; T, NRTP; P, pontine; R, nucleus of Roller/intercalatus; S, supragenual nucleus. Open symbols are individual data points. Black horizontal bars represent the mean value for each nucleus.
Figure 2.4: Mossy fiber neuron firing rate modulation in response to sinusoidal inputs. 

A, Upper trace, voltage recording of a mossy fiber neuron firing during sinusoidal modulation. Middle trace, firing rate of the neuron. Lower trace, sinusoidal current input to the soma. B and C, Bode plots summary of gain (B) and phase (C) in mossy fiber neurons. Symbols represent the average response of neurons in the eight precerebellar nuclei. Error bars indicate the standard error of the mean. ExCun, external cuneate; LRN, lateral reticular; MVN; medial vestibular; NPH, prepositus hypoglossi; Roller, nucleus of Roller/nucleus intercalatus; Supragen, supragenual nucleus; NRTP, nucleus reticularis tegmenti pontis. E, external cuneate; L, lateral reticular; M, medial vestibular; N, nucleus prepositus hypoglossi; T, nucleus reticularis tegmenti pontis; P, pontine; R, nucleus of Roller/nucleus intercalatus; S, supragenual nucleus. Open dots are individual data points. Black horizontal bars represent the mean value for each nucleus.
Figure 2.5: SK channels constrain the gain and maximum firing rate of mossy fiber neurons. A, Example of an action potential before and after application of apamin (50 nM), a blocker of SK channels, in a precerebellar neuron recorded in the lateral reticular nucleus. B, Instantaneous firing rate versus time during a 1 s depolarizing current step before and after application of apamin. C, Relationship between current and mean firing rate over 1 s depolarizing steps before (open circles) and after (filled triangles) apamin application. Change in AHP amplitude (D) and adaptation ratio (E) after apamin perfusion. Ratio of current-to-firing rate gain (F), maximum firing rate and maximum current input (G) before and after apamin. D-G show results from precerebellar neurons recorded in the LRN (n=3), pons (n=1), NRTP (n=4) and Roller (n=2).
Figure 2.6: BK and Kv3 currents contribute to gain and maximum firing rates of mossy fiber neurons. A, Example of an action potential before and after application of paxilline (1 µM) and paxilline + TEA (1 µM + 1 mM respectively) in a precerebellar neuron recorded in the nucleus of Roller. B, Instantaneous firing rate vs time during a 1 s depolarizing current step before and after application of paxilline and paxilline+TEA. C, Relationship between current and mean firing rate over 1 s depolarizing steps before and after paxilline and paxilline/TEA application. Change in gain below 80 Hz (D), maximum input (E), and maximum firing rate (F) normalized to the control values. G, Change in AHP amplitude after paxilline and paxilline/TEA exposure. D-G show results from precerebellar neurons recorded in each of the 8 precerebellar analyzed in this manuscript (2 neurons each from NPH and Roller).
Figure 2.7: Post-inhibitory rebound firing and the effect of Ih blockade varies across and within precerebellar nuclei. A1, Example of firing responses to a 1 s hyperpolarization in a precerebellar neuron recorded in the lateral reticular nucleus. A2, Same neuron as in A1 after application of the IH blocker ZD7288 (50 µM). B1, Firing responses to hyperpolarizing inputs in a second exemplar, recorded in the external cuneate. B2, Same neuron as in A1 after application of the IH blocker ZD7288 (50 µM). Neurons were maintained at baseline firing rates of 10 spikes/s with DC current. C, Peak rebound firing rate (baseline subtracted) for all precerebellar neurons recorded. Significant differences p<0.05 are P vs. L, M, N, R, or S and T vs. R. D, Delay from hyperpolarization offset to the next spike in all precerebellar neurons recorded. E, Peak rebound firing rate after IH blockade with ZD7288 is plotted vs control rebound rate for precerebellar neurons recorded in the external cuneate (n=3), Roller (n=5), NRTP (n=2), and LRN and pontine nuclei (n=1 each). Diagonal line represents no change. F, Delay to first post-hyperpolarization spike after ZD7288 is plotted versus control delay for the same precerebellar neurons plotted in E. E, external cuneate; L, lateral reticular; M, medial vestibular; N, nucleus prepositus hypoglossi; T, nucleus reticularis tegmenti pontis; P, pontine; R, nucleus of Roller/nucleus intercalatus; S, supragenual nucleus.
### Tables

**Table 2.1.** Resting properties of precerebellar neurons recorded in 8 different nuclei.

<table>
<thead>
<tr>
<th>nucleus</th>
<th>n</th>
<th># firing spont (spikes/s)</th>
<th>spont FR ± SE (spikes/s)</th>
<th>Vrest ± SE (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>external cuneate</td>
<td>24</td>
<td>5</td>
<td>22 ± 8</td>
<td>-75.6 ± 1.2</td>
</tr>
<tr>
<td>LRN</td>
<td>13</td>
<td>4</td>
<td>8 ± 2</td>
<td>-66.8 ± 1.6</td>
</tr>
<tr>
<td>MVN</td>
<td>40</td>
<td>21</td>
<td>13 ± 2</td>
<td>-71.4 ± 1.1</td>
</tr>
<tr>
<td>NPH</td>
<td>21</td>
<td>6</td>
<td>11 ± 7</td>
<td>-74 ± 1.3</td>
</tr>
<tr>
<td>NRTP</td>
<td>30</td>
<td>1</td>
<td>9</td>
<td>-76 ± 1.0</td>
</tr>
<tr>
<td>pontine</td>
<td>20</td>
<td>0</td>
<td>na</td>
<td>-78 ± 1.6</td>
</tr>
<tr>
<td>Roller</td>
<td>27</td>
<td>8</td>
<td>16 ± 4</td>
<td>-72 ± 1.7</td>
</tr>
<tr>
<td>supragenual</td>
<td>23</td>
<td>4</td>
<td>12 ± 2</td>
<td>-73 ± 1.3</td>
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</table>

**Table 2.2.** Statistical analysis of comparisons across 8 precerebellar nuclei. P value obtained from Kruskal-Wallis nonparametric analyses.

<table>
<thead>
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<th>Intrinsic measure</th>
<th>p value</th>
</tr>
</thead>
<tbody>
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<tr>
<td>AHP</td>
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</tr>
<tr>
<td>gain (below 80 spikes/s)</td>
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</tr>
<tr>
<td>input resistance</td>
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<td>maximum firing rate</td>
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<td>firing rate adaptation</td>
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<td>sine gain 1 Hz</td>
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<td>sine phase 1 Hz</td>
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<td>postinhibitory rebound</td>
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<td>delay to rebound spike</td>
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</table>
Acknowledgements

Chapter 2 is original work in preparation as Kolkman KE, McElvain LE, du Lac S. Shared electrophysiological signature of diverse precerebellar neurons and is included with permission from all the manuscript’s authors. The dissertation author was the primary author of this paper.
References


Chapter 3. Intrinsic physiology of identified neurons in the prepositus hypoglossi and medial vestibular nucleus

Abstract

Signal processing in the vestibular system is influenced by the intrinsic physiological properties of neurons that differ in neurotransmitters and circuit connections. Do membrane and firing properties differ across functionally distinct cell types? This study examines the intrinsic physiology of neurons in the medial vestibular nucleus (MVN) and nucleus prepositus hypoglossi (NPH) which express different neurotransmitters and have distinct axonal projections. NPH neurons expressing fluorescent proteins in glutamatergic, glycinergic, or GABAergic neurons were targeted for whole cell patch recordings in brainstem slices obtained from transgenic mouse lines (YFP-16, GlyT2, and GIN). Recordings from MVN neurons projecting to the spinal cord, reticular formation, or oculomotor nucleus were obtained by targeting fluorescent neurons retrogradely labeled from tracer injections. Intrinsic physiological properties of identified neurons exhibited continuous variations but tended to differ between functionally defined cell types. Within the NPH, YFP-16 neurons had the narrowest action potentials and highest evoked firing rates and expressed high levels of Kv3.3 proteins, which speed repolarization. MVN neurons projecting to the spinal cord and oculomotor nucleus had similar action potential waveforms, but oculomotor-projecting neurons had higher intrinsic gains than those projecting to the spinal cord. These results indicate that intrinsic membrane properties are differentially tuned in MVN and NPH neurons subserving different functions.
Introduction

The vestibular and oculomotor systems provide excellent models for linking cellular mechanisms with circuit function, behavior, and clinical dysfunction. Decades of electrophysiological recordings in intact animals have provided a wealth of information about signal processing in central neurons during head and eye movements (Angelaki and Cullen, 2008). Insights about neuronal activity during normal behavioral performance have been complemented by analyses of neuronal firing responses during different stages of adaptive plasticity (for review, (du Lac et al., 1995; Straka et al., 2005)). As such, the vestibular and oculomotor systems are exceptionally well-suited for connecting cellular and molecular mechanisms with sensory-motor performance and learning.

Neurons in the circuitry subserving the vestibulo-ocular reflex (VOR) have been extensively studied both in vivo and in vitro. The VOR enables gaze stability during self-motion by producing compensatory eye movements. Neurons that process head and eye movement information during the VOR reside in the medial vestibular nucleus (MVN) and adjacent nucleus prepositus hypoglossi (NPH). Several complementary methods have been used to classify neurons in the MVN and NPH, including identification of axonal projections and recordings of signals carried by neurons in awake behaving animals. Neurons in both nuclei can project to ocular motor nuclei, the floccular lobe of the cerebellum, or locally within the bilateral vestibular/prepositus complex (Belknap and McCrea, 1988; Highstein and Holstein, 2006; McCrea and Baker, 1985). Neurons in the MVN and the ventral portion of the lateral vestibular nucleus additionally project to the cervical spinal cord and medulla (Highstein and
Holstein, 2006). MVN and NPH neurons have been distinguished electrophysiologically according to differences in firing responses during eye and head movements. Most recorded neurons tend to vary continuously in their firing responses during eye and head movements, although physiologically distinct cell types can be identified (Escudero et al., 1992; Lisberger and Pavelko, 1988; McCrea et al., 1999; McFarland and Fuchs, 1992; Ramachandran and Lisberger, 2008; Scudder and Fuchs, 1992).

Firing responses recorded in vivo reflect a combination of the signals carried by afferent neurons, synaptic mechanisms, and the intrinsic physiological properties of the postsynaptic neuron. To what extent do differences in the response properties of functionally distinct MVN and NPH neurons reflect differences in intrinsic electrophysiological properties? Can intrinsic physiology be used to identify functionally distinct cell types in vitro? Several published studies using intracellular or whole-cell patch recording techniques in brainstem slices have attempted to address these questions. Recordings from unidentified neurons in the MVN revealed differences in action potential waveforms and responses to intracellularly injected current across neurons (du Lac and Lisberger, 1995; Johnston et al., 1994; Serafin et al., 1991; Straka et al., 2005). Although neurons were initially classified into two groups (A and B) on the basis of action potential waveform (Johnston et al., 1994; Serafin et al., 1991), subsequent studies clarified that action potential properties were distributed continuously across neurons (du Lac and Lisberger, 1995; Sekirnjak and du Lac, 2002). Recordings targeted to MVN neurons in which the primary neurotransmitter was identified either by single cell PCR (Takazawa et al., 2004) or via fluorescence
expression in transgenic mouse lines (Bagnall et al., 2007) revealed that although GABAergic neurons tend to have wider action potentials than non-GABAergic neurons, both groups exhibit a wide range of action potential profiles and intrinsic firing properties. In contrast, neurons that project to a specific target (the oculomotor nucleus) exhibited relatively uniform membrane and firing properties (Sekirnjak and du Lac, 2006). Collectively, published results indicate that intrinsic electrophysiological properties can differentially influence signal processing in MVN neurons (Ris et al., 2001; Sekirnjak and du Lac, 2002; Straka et al., 2005), but intrinsic properties cannot be used to unambiguously classify neurons into functionally distinct groups.

NPH neurons recorded *in vitro* exhibit a range of action potential waveforms and intrinsic firing properties that are largely similar to those documented in the MVN, although a cell type with integrative properties appears to exist in the NPH but not the MVN (Idoux et al., 2006). NPH neurons expressing GABAergic markers tended to differ from those expressing glutamatergic markers, but as in the MVN, considerable overlap was found between these two distinct cell types (Shino et al., 2008). Although an extensive glycinergic projection from the NPH to the abducens nucleus has been documented (Spencer et al., 1989), relatively few glycinergic neurons were identified in NPH recordings (Shino et al., 2008). Do glycinergic neurons comprise a physiologically unique subset of NPH neurons? Do MVN neurons projecting to different target structures exhibit distinct intrinsic electrophysiological properties? To address these questions, this study uses transgenic mouse lines and stereotaxic dye injections to target whole-cell patch recordings to identified NPH and MVN neurons in brainstem slices.
Methods

Animals

All procedures using live animals were approved by the Salk Institute Animal Care and Use Committee in accordance with National Institute of Health guidelines. Neurons were imaged and recorded in three mouse lines backcrossed to the C57BL/6 background. GABAergic neurons were identified in the GIN transgenic mouse line which labels a subset of neurons expressing the glutamic acid decarboxylase (GAD-67) promotor with GFP (Oliva et al., 2000). The YFP-16 transgenic mouse line, in which the thy-1 promoter drives YFP expression in a subset of neurons (Feng et al., 2000), was used to identify glutamatergic and glycinergic projection neurons (Bagnall et al., 2007; McElvain et al., 2010). To identify glycinergic neurons, we used the GlyT2-GFP transgenic mouse line (Zeilhofer et al., 2005). For study of physiological properties, the following numbers of mice were used: 8 spinal or medullary reticular formation injected (P18-21 at recording), 9 YFP-16 (P16-24), 13 GIN (P17-29) and 3 GlyT2 (P18-19).

Tracer injection

The cervical spinal cord was targeted for injection of solid 10,000 MW texas red dextran crystals (Molecular Probes, Eugene, OR). Animals were anesthetized with isofluorane and placed in a stereotaxic apparatus. Pitch and roll of the head were leveled by eye, and the head was raised so that the neck was taut. An incision in the skin was made along the rostral-caudal axis from the interparietal plate to between the shoulders. Musculature covering the C1 vertebra was cut along the midline and pulled laterally to expose the base of the skull and C1 vertebra for the duration of the
procedure. A small incision was made in the dura mater allowing access to the space inbetween the skull and C1 vertebra. A custom-made microinjector equipped with a 33G hypodermic needle and an internal movable rod (0.2 mm outer diameter, 0.1 mm inner diameter) (Creative Instruments Development Company) was used to deliver dextran crystals. It was lowered 1.25 mm into the cervical spinal cord or very caudal brainstem at an angle perpendicular to the dorsal face of the cord. Thirty pulses (25 psi, 25 ms duration) were delivered to the internal piston with compressed nitrogen in order to deliver the crystals. The injector was then retracted and the wound sutured. The animal was given a subcutaneous injection of buprenorphine hydrochloride, a partial opiate agonist, post-operative to minimize discomfort. Survival time was 5 days for adults (>P70) injected for histological analysis and 2-3 days for juveniles (P16-19) injected for electrophysiological studies.

The electrophysiological results obtained from oculomotor nucleus-projecting (OMP) neurons were replotted from Sekirnjak and du Lac (Sekirnjak and du Lac, 2006), and the methods for (OMP) dextran injections are detailed there.

*Fixed tissue preparation*

Animals were transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. The brains and cervical spinal cords were removed and put in 4% paraformaldehyde in PBS for 30 minutes at room temperature. The brains were then moved to 30% sucrose in PBS for 24 hours at 4°C until they sank. Thin coronal sections (40 µm) were sliced on a freezing microtome (Microm) from the...
spinal cord through the MVN/NPH. After washing in PBS, sections were wet-mounted and coverslipped with 2.5% DABCO (1,4-diazabicyclo-[2.2.2]octane).

**Imaging**

Fluorescent images were acquired using a Hamamatsu CCD camera (C4742-95) attached to an Olympus BX61 microscope. Images were collected digitally using ImageJ software and then transferred to Adobe Photoshop. Colocalization of spinal or reticular formation-projecting (Sp/Ret) neurons with fluorescently labeled cells in transgenic lines was performed by eye at 20x magnification. For YFP-16 colabeling, 451 Sp/Ret neurons in 29 sections were examined. For GlyT2 colabeling, 122 Sp/Ret neurons in 12 sections were examined.

**Immunohistochemistry**

Mice (2 months old) were perfused transcardially with phosphate buffered saline (PBS), followed by 4% paraformaldehyde in PBS. Brains were removed and drop-fixed with the same fixative at 30 – 60 min. at room temperature then sank in 30% sucrose for 24 hr. at 4°C. Thin sections (30 μm) containing the NPH were made through the brainstem on a freezing microtome (Microm). 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 h, followed by primary antibody in working buffer (10-fold dilution of blocking buffer) overnight at 4°C. Sections were washed three times with working buffer and treated with fluoro-conjugated secondary antibody for 1 h at room temperature. After washes in PBS, sections were wet-mounted
and coverslipped with 2.5% DABCO (1,4-diazabicyclo-[2.2.2]octane) or Vectashield Hardset (Vector Laboratories). The primary antibody, obtained from Alomone, was rabbit anti-Kv3.3 (1:200); controls for antibody specificity were performed by the vendor using Western blot analyses of membrane fractions that were preincubated with purified antigen. Kv3.3 immunosignal was detected with Alexa Fluor 594-conjugated goat anti-rabbit (1:100-200, Molecular Probes).

**Electrophysiology**

Mice (age P16-P29) were deeply anaesthetized with Nembutal, and their hindbrains were removed and dissected in ice-cold artificial cerebrospinal fluid (ACSF) bubbled with carbogen (95% O₂-5% CO₂). ACSF contained (in mM): 124 NaCl, 5KCl, 1.3 MgSO₄, 26 NaHCO₃, 2.5 CaCl₂, 1 NaH₂PO₄, and 11 dextrose. Carbogenated ACSF had an osmolarity of 300 mOsm and a pH of 7.4. Coronal slices (250 µm) were cut through the brainstem on either a Leica VT1000S or DSK-1500E vibratome. The slices were transferred to a holding chamber in carbogenated ACSF and incubated at 34°C for 30-45 min and were then carbogenated at room temperature before use.

Recordings were made in a submersion chamber with carbogenated ACSF at 34°C constantly perfused over the recorded slice. Picrotoxin (100 µM) and strychnine (1 µM) were added to the ACSF for recordings to block GABAergic and glycinergic transmission, respectively. Fluorescence was visualized using a fluorescein isothiocyanate (for visualization of EGFP or EYFP) or calcium crimson (for visualization of texas red) filter and Olympus (U-LH100HG) illumination. Fluorescent cells were viewed using a fluorescent digital camera (DAGE-MTI, IR-1000) on an
Olympus microscope (BX51W1) with 40x magnification. Cells were patched under infrared illumination using differential interference contrast optics.

Whole-cell current clamp recordings were made with an AxoClamp 2B or a MultiClamp 700B amplifier. Patch-clamp micropipettes (4-7 MΩ) were pulled from borosilicate glass (outer diameter 1.50 mm, inner diameter 0.86 mm) with a Sutter Instruments P-97 puller. Solution filling the electrodes contained (in mM) 140 K gluconate, 10 HEPES, 8 NaCl, 0.1 EGTA, 2 MgATP and 0.3 Na₂GTP. The pH and osmolarity were adjusted to 7.3-7.5 and 280-285 mOsm respectively. Data were filtered at 10 kHz and digitalized at 40 kHz with an ITC-16 or 18 (Instrutech). Acquisition and analyses were performed with code written in house in Igor 6. The liquid junction potential of 15 mV was subtracted from membrane potentials.

**Analysis of electrophysiological parameters**

Action potential waveforms were acquired at a rate of 8-12 spikes/s over 1-5 s, maintained when necessary by current injection; action potentials were averaged after aligning at their peak. Action potential threshold was defined as the membrane potential (Vm) where the derivative of the voltage reached 10 V/s. The width of the spikes was determined at the threshold, and the half-width was defined as the width of the spike at half of the Vm difference between threshold and peak. The afterhyperpolarization was defined as the Vm difference between spike threshold and the most hyperpolarized membrane potential achieved between action potentials. Neurons were excluded from this study if they could not sustain firing throughout a 1 s depolarizing step or if their spike height was < 45 mV.
Neuron excitability was measured above threshold by determining the current-to-firing rate relationship and below threshold by determining the input resistance. Input resistance was measured by hyperpolarizing the neurons to -75 mV and injecting 6 small hyperpolarizing current steps. The 6 traces were averaged for analysis. The current-to-firing rate relationship was determined by injecting 1 s depolarizing current steps until the neuron could not fire through the step and determining the mean firing rate across each step. The gain was defined as the slope of the best-fit line of the input current versus mean firing rate. Gain was calculated separately for firing rates below and above 80 spikes/s (Bagnall et al., 2007). Maximal firing rate was defined as the average firing rate across the greatest magnitude 1 s current step through which the neuron could fire throughout the entire step.

Adaptation was assessed in steps that yielded a firing rate of approximately 150 spikes/s at the end of the 1 s step. Firing rate adaptation was measured as the ratio of firing rate for the first versus the last 100 ms of the trace. The first 50 ms of firing were excluded from analysis (Sekirnjak and du Lac, 2006). Post-inhibitory rebound firing was measured as the difference in firing rate before and immediately following a 1 s hyperpolarizing step of approximately 30 mV.

To measure firing rate and phase response to sinusoidally modulated current, neurons were first depolarized to fire at approximately 30 spikes/s. Sinusoidally oscillating current of a magnitude to modulate the neuron ±10-15 spikes/s was presented to the cells at 0.25, 0.5, 1, 2, and 4 Hz. Sinusoidal functions $f(t) = A + B\sin(2\pi ft + C)$ were fit to the sinusoidal firing rate responses of the modulated neurons. The frequency ($f$) was set to the input frequency, and $A$, $B$, and $C$ were chosen to minimize
the mean standard error. The fit sinusoid was compared to the input sinusoids to determine gain and phase responses of the neurons.

Statistical Analysis

YFP-16, GIN, and GlyT2 neurons were compared using Kruskal-Wallis nonparametric multiple group comparison followed by the Dunn’s posthoc test (if P<0.05) performed in GraphPad Prism (version 4.0) software. Sp/RetP and OMP neurons were compared using Wilcoxon Rank Sum nonparametric comparison test performed in Kaleidagraph (version 4.03). Values are reported as mean ± SD.

Results

Intrinsic physiology differs in neurochemically identified NPH neurons recorded in transgenic mouse lines

Transgenic mouse lines expressing fluorescent proteins in distinct subsets of neurons can distinguish two major classes of neurons in the MVN (Bagnall et al., 2007). In the YFP-16 line (Feng et al., 2000), yellow fluorescent protein (YFP) is expressed under the ubiquitous thy-1 promoter but is restricted to a major subset of glutamatergic and glycinergic neurons that are located primarily in the magnocellular portion of the MVN. Figure 3.1A shows that YFP-positive neurons in this line are also abundant within the NPH. YFP-16 neurons are distributed throughout the entire extent of the NPH. In the most caudal portions of the NPH (not shown), YFP-16 neurons tend to cluster near the ventrolateral portion of nucleus. The GIN mouse line (Oliva et al., 2000) labels a subset of GABAergic neurons located primarily within the parvocellular
portion of the MVN; their distribution in the MVN is complementary to that of YFP-16 neurons (Bagnall et al., 2007). The distribution of fluorescent neurons and processes in the GIN line differs strongly in the NPH and MVN (Figure 3.1B). A relatively small subset of NPH neurons express GFP in the GIN line, and these are distributed throughout the NPH rather than clustered along the 4th ventricle as in the MVN. In the GlyT2-GFP transgenic line, GFP expression is driven by the GlyT2 (glycine transporter 2) promotor. This mouse line previously enabled the discovery of a unique subset of projection neurons in the cerebellar nucleus (Bagnall et al., 2009). Figure 3.1C shows that both the NPH and the MVN express abundant GlyT2+ neurons. GFP-expressing neurons are labeled throughout the extent of the NPH in the GlyT2 line and are most densely distributed in the rostral 2/3 of the nucleus.

In the MVN, variations in action potential width within and between cell types arise predominantly from differences in the magnitude and kinetics of Kv3 currents (Gittis and du Lac, 2007). Immunohistochemical analyses indicate that Kv3.3 subunits are particularly differentially expressed; within the MVN many YFP-16 neurons, but no GIN neurons, express the Kv3.3 protein. To determine whether Kv3.3 is also differentially expressed across cell types within the NPH, immunohistochemistry was performed in transgenic mice. Figure 3.2A shows the pattern of Kv3.3 expression in the NPH; a subset of neurons in the ventral and lateral portions of the NPH were highly immunopositive for Kv3.3, exhibiting membrane labeling around the somata and proximal dendrites. In YFP-16 mice, Kv3.3 was coexpressed with NPH neurons that expressed YFP (Figure 3.2B). In contrast, none of the GIN neurons in the NPH expressed Kv3.3 (Figure 3.2C). Interestingly, a subset of the largest GlyT2 neurons
within the NPH were immunopositive for Kv3.3, whereas no small GlyT2 neurons were (Figure 3.2D). These results indicate that Kv3.3 subunits are differentially expressed in NPH neurons and predict that, as in the MVN (Bagnall et al., 2007), action potential repolarization rate will be faster in YFP-16 neurons than in GIN neurons.

To determine whether the intrinsic electrophysiological properties of NPH neurons differ as a function of neurotransmitter content, fluorescent neurons were targeted for whole cell patch recordings in acute brainstem slices obtained from each of the three mouse lines. Figure 3.3A shows typical action potentials from fluorescent neurons recorded in the YFP-16, GIN, and GlyT2 lines. To standardize the comparisons across neurons, action potentials were recorded when neurons were firing at an average rate of 10 spikes/s. To illustrate the variability in action potential profiles within cell classes, two examples are shown for each mouse line. In each case, the rapid rising phase of the action potential was followed by a rapid falling phase that undershot action potential threshold to produce a rapid afterhyperpolarization (AHP). The top row of action potentials illustrates examples of action potentials that exhibit a biphasic AHP, in which the rapid AHP was followed a slight depolarization and subsequently by second, slower AHP. These neurons would be classified as type B (Camp et al., 2006) or as AHPs+ (Shino et al., 2008). Neurons with a similar action potential profile that play an important role in eye fixation have been observed in the NPH (Navarro-Lopez Jde et al., 2004). The lower panel of Figure 3.3A action potentials illustrates the heterogeneity in action potential profiles within and across cell types. Most YFP-16 neurons exhibited a biphasic AHP, often without an intervening depolarizing phase, as in the YFP-16 neuron illustrated in the lower panel of Figure
3.3A. In contrast, several GIN and GlyT2 neurons exhibited a monophasic AHP, as illustrated in the lower panel of Figure 3.3A, and would be classified as type A (Camp et al., 2006) or AHPs- (Shino et al., 2008).

Although action potential profiles exhibited qualitative similarities similar across neurons, quantitative differences in action potential width were evident between cell types. Action potentials tended to be narrowest in YFP-16 neurons (half width: 0.33 ± 0.03 ms), intermediate in GIN neurons (0.49 ± 0.04 ms), and widest in GlyT2 neurons (0.69 ± 0.05 ms) (Figure 3.3C and Table 3.1). In contrast, the magnitude of the AHP and the action potential threshold (Table 3.1) did not differ across cell types. These results indicate that intrinsic properties of glycinergic, GABAergic, and glutamatergic neurons are similar but exhibit quantitative differences.

Action potential waveform can reflect the nature and distribution of ion channels (Bean, 2007) but does not provide insights into how a neuron's complement of ion channels influence the way it processes information. To determine whether differences in ionic conductances across cell types affect response properties, neurons were injected intracellularly with depolarizing current of different amplitudes for a duration of 1 s, and the resulting firing responses were assessed. At high current levels, neurons could not repolarize adequately to fire throughout the 1 s step; the maximum firing rate was defined for each neuron as the highest average firing rate prior to such depolarization block. Figure 3.3B shows typical current-to-firing rate results from a GlyT2 neuron recorded in the NPH; the mean firing rate evoked by depolarization is plotted as a function of current amplitude. As has been observed in MVN neurons (Bagnall et al., 2007), firing responses to depolarizing current steps increased linearly with current
amplitude at evoked firing rates of < 80 spikes/s. At higher firing rates, the relationship between input current and firing rate remained linear but exhibited a lower slope. Thus, for most neurons, two values of gain (slope) of the current-to-firing rate relationship were evaluated; above and below mean evoked firing rates of 80 spikes/s (Table 3.1).

NPH neurons targeted in different mouse lines ranged widely in gain (Figure 3.3C) and maximum firing rate (Figure 3.3D) but exhibited significant differences between cell types (Table 3.1). Figure 3.3C, which plots intrinsic gain (measured below 80 spikes/s) as a function of action potential width at half-height, shows that the gain of YFP-16 neurons recorded in the NPH tends to be lower than that of either GIN neurons or GlyT2 neurons. Figure 3.3D shows maximum firing rate plotted as a function of input resistance (measured with hyperpolarizing current pulses given below spike threshold). Although some overlap is evident among groups, the YFP-16 neurons tend to cluster; they have relatively low input resistances and high maximum firing rates. In contrast, GlyT2 neurons exhibit a wide range of input resistances but relatively low maximum firing rates, while GIN neurons are intermediary. Together, these data indicate that NPH neurons comprise a heterogeneous population that differs in action potential width, maximum firing rate, and intrinsic gain (Table 3.1). YFP-16 neurons tend to be the largest, fastest neurons with the narrowest action potentials and lowest gains, and GlyT2 neurons span a wide range of parameters but include the smallest, slowest neurons with the widest action potentials and the highest excitability.

The similarities and differences in intrinsic electrophysiological properties across cell types in the NPH are reflected in neuronal responses to sinusoidal current injection (Figures 3.3E, F). Neurons were maintained with DC current at firing rates
between 25 and 35 spikes/s and subjected to sinusoidal current modulated over a frequency range of 0.25 to 4 Hz. All NPH neurons examined responded with sinusoidal modulations in firing rate across this frequency range. Figure 3.3E and F plots the resulting gain (peak to peak firing rate divided by peak to peak current amplitude) and phase of average neuronal responses as a function of stimulus frequency for neurons recorded in each cell line. As with responses to step current, the gain of sinusoidal responses was highest in GlyT2 and GIN neurons and lowest in YFP-16 neurons (Figure 3.3E). YFP-16 neurons showed relatively little gain enhancement with increasing frequency, compared with GlyT2 and GIN neurons. In all 3 groups, responses were nearly in phase with the stimulus across the frequency range tested, with a slight phase lead at 0.25 Hz and phase lag at 4 Hz. Across all frequencies, GlyT2 neurons exhibited a modest phase lead (5-8 deg) relative to YFP-16 and GIN neurons (Figure 3.3F). These results indicate that NPH neurons have relatively similar intrinsic response dynamics but exhibit differences in response gain across cell types.

Comparison of intrinsic physiology in MVN neurons projecting to the spinal cord and oculomotor nucleus

The transgenic mouse lines described in Figure 3.1 of this study have been employed successfully to compare and contrast intrinsic neuronal physiology (Bagnall et al., 2007; Bagnall et al., 2009), underlying ionic currents (Gittis and du Lac, 2007, 2008; Gittis et al., 2010) and vestibular nerve synaptic properties and plasticity of different classes of neurons in both the cerebellar and medial vestibular nuclei (Bagnall et al., 2008; McElvain et al., 2010). In contrast with GIN neurons, which maintain
axonal projections within the bilateral MVN, YFP-16 neurons in the MVN have been demonstrated to project axons outside of the vestibular complex, to the cerebellar flocculus, thalamus, oculomotor nucleus, and medullary reticular formation (McElvain et al., 2010). This diversity of projections to functionally distinct targets raises the question of whether the intrinsic physiological properties of YFP-16 neurons differ across projection types. To address this question, MVN neurons projecting to distinct targets outside of the vestibular complex were retrogradely labeled from stereotaxic injections and targeted for whole-cell patch recordings in brainstem slices. In this study, properties of neurons projecting to the medullary reticular formation and/or spinal cord will be compared with those projecting to the oculomotor nucleus (data reanalyzed from (Sekirnjak and du Lac, 2006)). A separate study on MVN and NPH neurons projecting to the cerebellar flocculus (Kolkman et al., 2010) is in preparation.

Injections of fluorescent dextrans into the cervical spinal cord (Figure 3.4A) or medullary reticular formation caudal to the decussation of the pyramidal tract resulted in retrogradely labeled neurons that were distributed bilaterally within the lateral (magnocellular) portion of the MVN (Figure 3.4B), primarily in the middle third of the rostrocaudal extent of the MVN. They tended to be located more ventrally than neurons retrogradely labeled from the contralateral oculomotor nucleus (Sekirnjak and du Lac, 2006). The distribution within the MVN of neurons retrogradely labeled from cervical spinal or caudal medullary injections was similar, suggesting that medullary injections primarily labeled axons running through the medial vestibulo-spinal tract. Together, the retrogradely labeled neurons will be referred to as Spinal/Reticular-Projecting (Sp/RetP).
To determine whether Sp/RetP neurons are GABAergic, glycinergic, or glutamatergic, dextran injections were made in GIN, GlyT2, and YFP-16 mice. In YFP-16 mice, most Sp/RetP neurons were double labeled with YFP, both ipsilateral (250 colabeled of 283 total) and contralateral (163 colabeled of 188 total) to the injection site, indicating that Sp/RetP neurons are glutamatergic and/or glycinergic, but not GABAergic. Consistent with this finding, no Sp/RetP neurons coexpressed GFP in the GIN line. In contrast, dye injection into the cervical spinal cord in a GlyT2 mouse resulted in retrograde labeling that was largely coextensive with GFP on the side ipsilateral (51 colabeled of 71 total) to the injection but essentially devoid of GFP on the contralateral side (1 colabeled of 51 total). Together, these results indicate that Sp/RetP neurons projecting contralaterally are glutamatergic, while those projecting ipsilaterally are predominantly but not exclusively glycinergic.

MVN neurons that were retrogradely labeled from the spinal cord or caudal medulla had action potentials that were qualitatively similar to those of both unidentified YFP-16 neurons and OMP neurons. To compare physiological properties across projection neurons, Figure 3.5 shows intrinsic physiological properties of Sp/RetP and OMP neurons; the latter were replotted from Sekirnjak and du Lac (Sekirnjak and du Lac, 2006). Representative action potential profiles for the two projection neuron types are similar, as shown in Figure 3.5A, and include a rapidly repolarizing action potential followed by a biphasic AHP.

All neurons in both groups responded to steady intracellular depolarization current with maintained increases in firing rate. Although there was considerable overlap in the two populations, action potentials tended to be narrower in OMP versus
Sp/RetP neurons, and intracellular gains in response to 1 s depolarizing current steps tended to be higher in OMP vs Sp/RetP neurons, as indicated by the scatterplot in Figure 3.5B. Input resistance was similar in OMP and Sp/RetP neurons, but OMP neurons exhibited consistently higher maximum firing rates than did Sp/RetP neurons (Figure 3.5C, Table 3.2). Both projection neuron types exhibited modest gain increases (Figure 3.5D) and relatively constant phase (Figure 3.5E) with increasing frequency of sinusoidal current injection. Response gains and phases were nearly identical between the two groups at each stimulus frequency. AHP amplitude and action potential threshold were not significantly different (Table 3.2). These results indicate that the intrinsic physiological properties of MVN neurons projecting caudally and rostrally are largely similar but not completely identical, and that projection target cannot be inferred by action potential profile alone.

**Discussion**

Neurons in the prepositus hypoglossi and vestibular nuclei share several features of intrinsic excitability that differ from those of the more popularly studied neurons such as pyramidal cells in the hippocampus and cerebral cortex. In contrast with pyramidal cells, which are largely silent in the absence of synaptic inputs, NPH and MVN neurons fire spontaneously both in vivo and in vitro, and they respond to depolarizing inputs with sustained, linear increases in firing rate over an exceptionally wide range of input and output rates. Despite their similarities relative to other cell types in the brain, several aspects of intrinsic excitability differ between classes of NPH and MVN neurons defined by their neurotransmitter or axonal projection. Differences
across cell types, including firing range, gain, and dynamics, are interesting both because they influence behavioral signal transformations in distinct vestibular and oculomotor microcircuits, and because they indicate differences in the nature and distribution of underlying ion channels which ultimately reflect differences across cell types in both gene expression and the modification of ion channels by experience (Gittis and du Lac, 2006; Shao et al., 2009a; Straka et al., 2005).

Intrinsic physiology of NPH neurons defined by neurotransmitter

The NPH comprises morphologically diverse neurons (Belknap and McCrea, 1988; McCrea and Baker, 1985a; McCrea and Baker, 1985b) forming reciprocal connections with the perihypoglossal and vestibular nuclei, the medullary and pontine reticular formation, the extraocular motor nuclei, and the cerebellum as well as unidirectional connections with other areas (Belknap and McCrea, 1988; McCrea and Baker, 1985a). Intrinsic excitability in unidentified NPH neurons was investigated by Idoux et al. (Idoux et al., 2006) in guinea pigs, in which neurons were classified into 4 types on the basis of action potential waveform and firing patterns. A subsequent study by Shino et al. (Shino et al., 2008) in rats used a somewhat different physiological classification scheme to group NPH neurons into multiple types based on their spike afterhyperpolarization, firing patterns, and voltage response to hyperpolarization, with the addition of single cell PCR information identifying whether the recorded neuron was GABAergic or glutamatergic. The present study uses transgenic mice expressing fluorescent protein in GABAergic (GIN), glycinergic (GlyT2), and glutamatergic and glycinergic projection neurons (YFP-16) to target physiological recordings to distinct
types of neurons. The results indicate that although the intrinsic physiology of NPH neurons targeted in each of these mouse lines differs, neurons with a wide range of action potential and firing profiles can be glycinergic, GABAergic, or glutamatergic. As such, action potential profile can not be used in isolation to infer the neurotransmitter, circuit connections, or function of the recorded neuron.

With the exception of the GlyT2 mouse line, which labels nearly all glycinergic neurons in the MVN and NPH (T. Kodama, unpublished results), the mouse lines used in this study express fluorescent protein in only a subset of neurons expressing a specific neurotransmitter. Although fluorescent neurons were thoroughly sampled, unlabeled GABAergic or glutamatergic neuronal types were not examined. However, while it is possible that some neurons were excluded, recording in the GlyT2-EGFP transgenic mouse line provided new information about glycinergic neurons which, although abundant in the NPH (Rampon et al., 1996; Spencer et al., 1989), were undersampled in a previous study examining NPH physiology with post-recording analysis of transmitter type (Shino et al., 2008).

The action potential profiles of NPH neurons reported in guinea pig (Idoux et al., 2006) and rat (Navarro-Lopez Jde et al., 2004; Shino et al., 2008) were observed in the present study in mouse. Neurons with monophasic AHPs and relatively wide action potentials, as observed in the lower panels of GIN and GlyT2 neurons in Figure 3.3A., would be classified as "type A" according to Idoux et al. and as AHPs- by Shino et al. (Shino et al., 2008), who observed this profile in GABAergic but not glutamatergic neurons. Consistent with the findings of Shino et al. (Shino et al., 2008), neurons with monophasic AHPs were not evident in the YFP-16 line. Given that such monophasic
action potential profiles were abundant in the GlyT2 line but were not universal in the GIN line, where many neurons exhibited biphasic AHPs, these results collectively indicate that NPH neurons with monophasic AHPs are either glycinergic or GABAergic.

Navarro-Lopez et al. (Navarro-Lopez Jde et al., 2004), found that a subset of NPH neurons with biphasic AHPs receive glutamatergic synapses from the paramedian pontine reticular formation and play a role in the generation of eye position signals via an acetylcholine-dependent mechanism (for review, (Delgado-Garcia et al., 2006)). Such neurons would be classified by Idoux et al. (Idoux et al., 2006) as "type B" and as AHPs+ by Shino et al. (Shino et al., 2008), who found that neurons of this profile could be either glutamatergic or GABAergic. Consistent with and extending on the latter findings, the present study found that neurons recorded in all three mouse lines could have biphasic AHPs. Neurons with the fastest action potentials tended to predominate in the YFP-16 line, which includes glutamatergic neurons that project to the cerebellar flocculus (McElvain et al., 2010). Neurons that fired erratically during sustained depolarization which would be classified as type D by Idoux et al. (Idoux et al., 2006) were not observed in the mouse NPH and were rare in the rat NPH (Shino et al., 2008), perhaps reflecting a role for such neurons in oculomotor integration (Idoux et al., 2006), which is robust in the guinea pig (Escudero et al., 1993) compared with the mouse (van Alphen et al., 2001).

Combining information about the physiological properties of NPH neurons expressing different transmitters together with the response properties and projections of NPH neurons reported in the literature provides a basis for distinguishing several cell
types in the NPH. The predominant glutamatergic projections of NPH neurons are to the cerebellar flocculus, the ipsilateral abducens motor nuclei, and the superior colliculus (Belknap and McCrea, 1988). High levels of Kv3.3 expression in YFP-16 neurons in the NPH (Figure 3.2), which are likely to target axons to these oculomotor-related structures, suggests a basis for the nystagmus that is prominent in spinocerebellar ataxia type 13, in which the gene encoding Kv3.3 is mutated (Waters et al., 2006).

The predominant glycinergic projection of NPH neurons is to the contralateral abducens nucleus (Spencer et al., 1989), yet most glycinergic neurons in the NPH have wide action potentials, low maximum firing rates, and high input resistances more characteristic of local inhibitory neurons than of projection neurons (Bagnall et al., 2007; McElvain et al., 2010). We propose that the NPH contains at least two classes of glycinergic neurons: a premotor projection to the abducens nucleus and local interneurons that maintain their axons within the NPH and/or MVN. GABAergic neurons in the NPH project to the inferior olive and locus coruleus, and make local circuit connections with the MVN. The intrinsic physiology of the subset of GABAergic neurons labeled in the GIN line was relatively heterogenous (Figure 3.2), consistent with the diverse circuit of GABAergic neurons in the NPH.

*Intrinsic physiology of projection neurons in the MVN*

MVN neurons that project to the oculomotor nucleus, thalamus, and cerebellar flocculus express fluorescent protein in the YFP-16 mouse line (McElvain et al., 2010). This study demonstrates that Sp/RetP neurons are also labeled in the YFP-16 mouse.
line, confirming this line as a marker for projection neurons. To determine whether neurons projecting to different target structures share intrinsic physiological properties, spinal-projecting neuronal physiology was compared to that previously published for MVN neurons projecting to the oculomotor nucleus (Sekirnjak and du Lac, 2006). The two populations were largely similar when compared with glycinergic neurons in the NPH, GABAergic neurons in the MVN (Bagnall et al., 2007), or unlabeled neurons near retrogradely-labeled oculomotor-projecting neurons (Sekirnjak and du Lac, 2006). Nonetheless, and despite striking similarities in intrinsic response dynamics (Figure 3.5), significant differences between OMP and SpRetP neurons were evident, with OMP neurons tending to have higher gains and maximum firing rates compared with SpRetP neurons. These intrinsic similarities and differences could be tuned for the functional needs of eye movements versus head movements. Some overlap between the two populations would be expected from the observation that some MVN neurons have axons that collateralize and project to both the oculomotor nucleus and the cervical spinal cord. Premotor vestibular nucleus neurons projecting to both ocular motor nuclei and the cervical spinal cord have been observed in a variety of species, including primates, cats, and chicks (Gottesman-Davis and Peusner, 2010).

Sp/RetP neurons were labeled bilaterally in the YFP-16 line but ipsilaterally in the GlyT2 line. This finding supports the idea that MVN neurons projecting ipsilaterally to caudal structures are predominantly glycinergic, while those projecting contraterally are glutamatergic. This glycinergic-ipsi, glutamatergic-contra pattern was previously established for MVN projections to the abducens (Spencer and Baker, 1992) and for fastigial (medial cerebellar) neurons receiving Purkinje cell synapses and
projecting to the caudal medulla (Bagnall et al., 2009). Given that MVN neurons make bilateral glutamatergic projections to the cerebellum; however, the pattern of ipsilateral glycinergic neurons appears to be confined to premotor projections.

**Ion channel basis of intrinsic physiological differences across cell types**

Differences between neurons in action potential waveform were observed in this and previous studies of MVN and NPH neurons. Predominant variations are in the speed of action potential repolarization (correlated with spike width) and the interspike waveform (i.e. monophasic versus biphasic AHP). Voltage clamp studies have revealed the mechanisms underlying action potential generation as well as the nature of differences in ion channels across cell types. While sodium currents are similar in fast- and slow-firing neurons (Gittis and du Lac, 2008), the density and kinetics of potassium channels differ (Gittis and du Lac, 2008).

The action potential clamp technique, in which a neuron's own action potential serves as a voltage clamp stimulus (Khaliq and Bean, 2010), has revealed the ionic currents flowing during the action potential when MVN neurons fire at different rates (Gittis et al., 2010). Two currents contribute to repolarization: Kv3 currents and big conductance type calcium-activated potassium (BK) currents. Variations in action potential speed (width and repolarization rate) across MVN neurons can be accounted for by variations in Kv3 current amplitudes and kinetics (Gittis and du Lac, 2007; Gittis et al., 2010) because repolarizing Kv3 currents contribute to sodium channel availability by transitioning sodium channels directly from open to closed states, bypassing inactivation (Gittis et al., 2010). The fastest neurons, which include most YFP-16
neurons, have higher density and faster repolarizing Kv3 potassium currents than the slower neurons, which include most GIN neurons (Gittis and du Lac, 2007; Gittis et al., 2010). Slower neurons have relatively more interspike potassium currents, including calcium activated potassium and A-type currents (Gittis and du Lac, 2007), that reduce action potential speed by maintaining the membrane potential at hyperpolarized levels. In slower neurons, BK currents dominate repolarization at the highest rates, compensating for the voltage-dependent reduction in Kv3 currents that occurs when neurons become increasingly depolarized at higher firing rates. Interestingly, although BK currents flowing during the action potential are similar across cell types, the kinetics of Kv3 currents differ, reflecting differential expression of the Kv3.3 subunit in YFP-16 but not GIN neurons (Gittis et al., 2010). Within the NPH, Kv3.3 subunits are also differentially expressed across cell types and are particularly strong in the fastest firing population of neurons (YFP-16). Thus, the predominant difference across neurons that have different action potential speeds is in the density and kinetics of Kv3 currents.

A major variation in action potential waveform across neurons is the waveform of the interspike interval directly after the action potential, with much attention devoted in the literature to whether neurons have a single or a biphasic AHP. Because Kv3 currents predominate during action potential repolarization, the waveform during the first component of the AHP will depend largely on the amplitude and kinetics of Kv3 currents and partially on BK currents (Gittis et al., 2010; Smith et al., 2002). The predominant interspike current in MVN neurons is the slow conductance type calcium-activated potassium (SK) current, which is required for the second phase of the biphasic AHP (Dutia and Johnston, 1998; Smith et al., 2002). Thus, the higher the expression of
SK current, the more likely a neuron will exhibit a slow component of the AHP.

In some MVN and NPH neurons with biphasic AHPs, an afterdepolarization (ADP) that separates the fast and slow AHPs is evident. In a modeling study, the ADP was proposed to reflect current propagating along dendrites (Quadroni and Knopfel, 1994); alternatively or in addition, the ADP could reflect an inward current mediated either by low threshold calcium channels (Serafin et al., 1991) or rapid relief of sodium channel inactivation. An ADP is evident only in neurons with rapid action potential repolarization and is absent in the slowest firing MVN and NPH neurons.

In summary, the action potential waveform of MVN and NPH neurons reflects the functional interaction of ionic currents that exhibit graded expression across neurons. Neurons with the most rapid action potentials which exhibit a biphasic AHP with an intervening ADP (type B or AHPs+) strongly express fast repolarizing currents via channels that include Kv3.3 subunits. Neurons with the slowest action potentials with monophasic AHPs only weakly express Kv3 currents via channels that are devoid of Kv3.3 subunits, but they strongly express A currents (Gittis and du Lac, 2007), which slow the timing of the peak of the AHP and precludes the rapid interspike depolarization that is required for the SK component of the AHP to become evident. Graded expression of the underlying ion channels across neurons accounts for the long standing (du Lac and Lisberger, 1995) and present observations that the intrinsic physiology of MVN and NPH neurons form a continuum rather than discrete types.

What can we learn about a neuron's identity from its action potential profile? As clarified by Takazawa et al. (Takazawa et al., 2004), Bagnall et al. (Bagnall et al., 2007), and Shino et al. (Shino et al., 2008), neurons that have wide action potentials and
monophasic AHPs (type A/AHPs-) are predominantly non-glutamatergic. The present study indicates that in the NPH, such neurons can be either GABAergic or glycinergic. Given lack of overlap of these and YFP-16 neurons, we propose that the neurons with the widest action potentials are local inhibitory neurons. On the other hand, neurons with rapid action potentials and biphasic AHPs (type B/AHPs+) can be glycinergic, GABAergic, or glutamatergic, and can project to the oculomotor nucleus, cerebellar flocculus, thalamus, spinal cord, or contralateral MVN. Thus, information about action potential waveform can be loosely informative (local inhibitory versus projection neuron) but does not otherwise provide insights into the functional role of the recorded neuron.

Ion channels are highly subject to modifications by neuronal activity and neuromodulators, providing a substrate for adaptive changes in neuronal excitability. Recordings of vestibular nucleus neurons after loss of peripheral vestibular function have demonstrated changes in the intrinsic excitability of MVN neurons (reviewed by (Dutia, 2010; Straka et al., 2005)). Dendrotoxin-sensitive potassium currents (Kv1.1 and 1.2), which are developmentally regulated (Popratiloff et al., 2003), are altered by vestibular deafferentation in vestibulo-oculo-spinal neurons (Shao et al., 2009a; Shao et al., 2009b). BK type calcium-activated potassium currents in MVN neurons are decreased by repeated hyperpolarization (Nelson et al., 2005; Nelson et al., 2003), resulting in a persistent increase in intrinsic excitability. Kv3 currents in auditory brainstem neurons can be regulated by auditory experience and are likely to be modified by phosphorylation in MVN neurons as well (Gittis et al., 2010). Each of the other currents that are expressed in MVN neurons, including Na (Gittis and du Lac, 2008;
Serafín et al., 1991), Ca (Johnston et al., 1994), CNG (Podda et al., 2008), and EAG (Pessia et al., 2008), are also subject to regulation by neuromodulators or activity. As such, the intrinsic excitability of MVN and closely related NPH neurons provides a flexible substrate for experience-dependent changes in vestibular and oculomotor behaviors.
Figure 3.1. Distribution of YFP-16, GIN, and GlyT2 neurons in the NPH. Coronal sections are from the YFP-16 (A), GIN (B), and GlyT2 (C) lines. NPH, nucleus prepositus hypoglossi; MVN, medial vestibular nucleus; IV Ventricle, fourth ventricle. Scale bar, 500 µm. Sections are approximately 6.12 mm caudal to bregma.
Figure 3.2. Kv3.3 expression in the NPH. A, Immunostaining to Kv3.3 reveals large neurons in the ventral and lateral portion of the NPH with Kv3.3 immunopositive membranes. B, Kv3.3 immunostain (purple) in a coronal section from a YFP-16 mouse; YFP label is green, overlap in the two labels is white. All YFP-16 neurons are labeled with the Kv3.3 antibody. C, Kv3.3 immunostain (purple) in a coronal section from a GlyT2 mouse; GFP label is green, overlap in label is white. Large, but not small GlyT2 neurons are labeled with anti Kv3.3 antibodies. D, Kv3.3 immunostain (purple) in a coronal section from a GIN mouse line. No GIN neurons are immunopositive for Kv3.3. Scale bar, 50 μm.
Figure 3.3. Electrophysiological properties of YFP-16, GIN, and GlyT2 neurons in the NPH. A (top and bottom), Examples of two different action potential waveforms each from YFP-16 (red), GIN (green), and GlyT2 (blue) neurons. B, Example from a GlyT2 neuron of the relationship between current and mean firing rate across 1 s depolarizing steps. C, Relationship between action potential width at half height and gain under 80 spikes/s. Each point represents one neuron [YFP-16 (red triangles), n=27; GIN (green circles), n=20; GlyT2 (blue squares), n=19]. D, Relationship between maximum firing rate and input resistance (Rin). Symbols and n are the same as those in C. E and F, Bode plots summary of YFP-16 (n=10), GIN (n=7), and GlyT2 (n=6) neurons. Symbols represent the average response of YFP-16 (red triangles), GIN (green circles), and GlyT2 (blue squares) neurons. Error bars indicate the standard error of the mean.
Figure 3.4. Spinal cord injection and retrograde labeling in the MVN.  

A, Texas red dextran crystal (purple) injection site in the cervical spinal cord of a YFP-16 (green) mouse.  Scale bar, 500 µm.  

B, Coronal section from the injection in A showing retrograde labeling of neurons (purple) in the MVN.  NPH, nucleus prepositus hypoglossi; MVN, medial vestibular nucleus.  Scale bar, 200 µm.
Figure 3.5. Electrophysiological properties of Sp/RetP and OMP neurons in the MVN. 

A, Examples of action potentials from Sp/RetP (green) and OMP (blue) neurons. B, Relationship between action potential width at half height and gain over 80 spikes/s. Each point represents one neuron [Sp/RetP (green circles), \(n=30\); OMP (blue squares), \(n=37\)]. C, Relationship between maximum firing rate and input resistance (Rin). Sp/RetP, \(n=32\); OMP, \(n=36\). Symbols are the same as those in C. D and E, Bode plots summary of Sp/RetP (\(n=25\)) and OMP (\(n=21\)) neurons. Symbols represent the average response of Sp/RetP (green circles) and OMP (blue squares) neurons. Error bars indicate the standard error of the mean. OMP data was previously published (Sekirnjak and du Lac, 2006).
### Tables

**Table 3.1.** Electrophysiological properties of GlyT2, YFP-16 and GIN neurons in the NPH

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GLYT2 (n=19)</td>
<td>YFP-16 (n=27)</td>
</tr>
<tr>
<td>AP width (ms)</td>
<td>1.54±0.39</td>
<td>0.80±0.30</td>
</tr>
<tr>
<td>AP half-width (ms)</td>
<td>0.688±0.19</td>
<td>0.333±0.15</td>
</tr>
<tr>
<td>AHP (mV)</td>
<td>24.3±4.00</td>
<td>21.7±3.37</td>
</tr>
<tr>
<td>Threshold (mV)</td>
<td>-46.1±5.50</td>
<td>-47.8±9.51</td>
</tr>
<tr>
<td>Input res. (MΩ)</td>
<td>927±703</td>
<td>160±128</td>
</tr>
<tr>
<td>Max. firing rate (spikes/s)</td>
<td>107±54.3</td>
<td>204±94.0</td>
</tr>
<tr>
<td>Gain &lt; 80 Hz (spikes/µA)</td>
<td>343±155</td>
<td>190±52.1</td>
</tr>
<tr>
<td>Gain &gt; 80 Hz (spikes/µA)</td>
<td>254±82.0</td>
<td>128±50.6</td>
</tr>
<tr>
<td>Adaptation ratio</td>
<td>0.84±0.07</td>
<td>0.83±0.07</td>
</tr>
<tr>
<td>PRF (spikes/s)</td>
<td>12.0±20.1</td>
<td>22.2±29.9</td>
</tr>
</tbody>
</table>

Abbreviations: AP: action potential; AHP: afterhyperpolarization; Input res: input resistance; PRF: post inhibitory rebound firing.
Table 3.2. Electrophysiological properties of two types of MVN projection neurons

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>N</th>
<th></th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OMP</td>
<td>Sp/RetP</td>
<td>OMP</td>
<td>Sp/Ret</td>
</tr>
<tr>
<td>AP width (ms)</td>
<td>0.72 ± 0.13</td>
<td>0.72 ± 0.29</td>
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<tr>
<td>AP half-width (ms)</td>
<td>0.31 ± 0.07</td>
<td>0.30 ± 0.14</td>
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<tr>
<td>AHP (mV)</td>
<td>19.2 ± 3.2</td>
<td>18.8 ± 2.8</td>
<td>37</td>
<td>33</td>
</tr>
<tr>
<td>Threshold (mV)</td>
<td>-52.3 ± 3.9</td>
<td>-50.3 ± 5.0</td>
<td>37</td>
<td>33</td>
</tr>
<tr>
<td>Input res. (MΩ)</td>
<td>160 ± 100</td>
<td>136 ± 104</td>
<td>36</td>
<td>32</td>
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<tr>
<td>Max. firing rate (spikes/s)</td>
<td>327 ± 94</td>
<td>236 ± 110</td>
<td>36</td>
<td>33</td>
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<tr>
<td>Gain &gt; 80 Hz (spikes/s/nA)</td>
<td>193 ± 57</td>
<td>133 ± 56</td>
<td>37</td>
<td>30</td>
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<tr>
<td>Adaptation Ratio</td>
<td>0.89±0.05</td>
<td>0.90±0.06</td>
<td>36</td>
<td>27</td>
</tr>
<tr>
<td>PRF (spikes/s)</td>
<td>12.4±9.4</td>
<td>14.1±14.4</td>
<td>28</td>
<td>29</td>
</tr>
</tbody>
</table>

Abbreviations: OMP: oculomotor-projecting; Sp/RetP: spinal or caudal reticular formation projecting; AP: action potential; AHP: afterhyperpolarization; Input res: input resistance; PRF: post inhibitory rebound firing.
Acknowledgements

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References


Chapter 4. Anatomical study of the connection between the MVN/NPH and dorsal cap of the inferior olive

Abstract

The inferior olive (IO) provides a major input to the cerebellum—the climbing fibers. These fibers directly contact Purkinje cells and also send collaterals to neurons in the cerebellar nuclei. It has been established that neurons in the cerebellar nuclei and the nucleus prepositus hypoglossi (NPH), a brainstem nucleus that also receives direct Purkinje cell input, project an inhibitory feedback signal to neurons in the IO. While the macroanatomy of this connection is well established, the subnuclear location of the neurons mediating this inhibitory feedback is not known. In addition, the existence of a climbing fiber collateral connection to the medial vestibular nucleus (MVN) and NPH has been debated in the literature. In this study, I use retrograde and anterograde neuronal tracing techniques to locate the neurons in the NPH and MVN that project to the IO. I also establish the reciprocal connection from the IO to the NPH. Finally, I show the presence of glycinergic terminals in the dorsal cap of the IO, implying that glycine release in the IO may be important for the olivary circuit.
Introduction

Climbing fibers, originating in the inferior olive (IO) of the brainstem, provide a major input to the cerebellum. They directly synapse onto Purkinje cells, the output cell of the cerebellar cortex and strongly influence their activity. Most olivary neurons project both to Purkinje cells as well as to neurons in the deep cerebellar nuclei. Climbing fiber signals are thought to play a very important role in cerebellum-dependent motor learning as well. The dominant model maintains that modification of the parallel fiber to Purkinje cell synapse underlies cerebellum-dependent learning. In this model, the climbing fibers signal motor errors, and the timing of that signal drives plasticity at the parallel fiber synapse. Therefore, they may act as an initiator for a cellular correlate of learning (Albus, 1971; Ito, 1982; Marr, 1969).

The vestibular and oculomotor systems undergo cerebellum-dependent learning changes, and both project signals to the flocculus of the cerebellum. The dorsal cap of Kooy is a subnucleus of the IO that projects to this cerebellar region. Olivary neurons in the dorsal cap receive visual signals via a glutamatergic projection from the nucleus of the optic tract, a forebrain nucleus receiving a direct retinal projection. Increases in dorsal cap olivary neuron firing rate can be correlated with errors in eye movements as perceived as a full-field “retinal slip” of vision, and the subnucleus is certainly involved in compensatory eye movements (for review, (Barmack, 2006)).

While the vast majority of the IO receives inhibition from cerebellar nucleus neurons, the dorsal cap receives inhibition from the nucleus prepositus hypoglossi (NPH) (De Zeeuw et al., 1993) and medial vestibular nucleus (MVN). As in the cerebellar nuclei, both the NPH and MVN receive direct Purkinje cell inhibition, and
olivary-projecting neurons in these nuclei may be among the direct recipients of Purkinje cell synapses. As such, olivary-projecting neurons in the NPH and MVN would serve an analogous circuit role as cerebellar nucleus olivary-projecting neurons in a cerebellar feedback loop in which receipt of inhibition from Purkinje cells would disinhibit the IO and affect climbing fiber firing rates (for review, (Bengtsson and Hesslow, 2006).

Many questions regarding the connections of the NPH/MVN with the IO remain. One line of questions relates to the neurotransmitter released. There is already evidence that not only GABA but also acetylcholine is released in the IO (Barmack et al., 1993; Caffe et al., 1996). However, it is unknown whether glycine may also be released as has been shown in other caudally projecting inhibitory projections in the MVN (Bagnall et al., 2009; Kolkman et al., 2011; Spencer and Baker, 1992). Also, because of the challenges related to labeling connections to the dorsal cap without labeling those to the surrounding medial longitudinal fasciculus, the subnuclear localization of neurons in the NPH and MVN projecting to the dorsal cap is unknown. Finally, while most olivary neurons project collaterals to the cerebellar nuclei, the existence of analogous collateral projections from neurons in the dorsal cap to the MVN and NPH have been debated (Balaban, 1984; Balaban, 1988; Ruigrok and Voogd, 2000; Sugihara et al., 1999). Do such olivary collaterals exist?

The overall goal of this study is to identify whether the principles of cerebellar anatomy, in particular the anatomical feedback loop, apply to the vestibular/oculomotor systems. To this end, we employ transgenic mice, immunocytochemistry, and neuronal tracing techniques to confirm the presence of GABAergic and cholinergic terminals as
well as identify glycinergic terminals in the dorsal cap of Kooy. We also determine the location of olivary-projecting neurons in the NPH and identify for the first time, a reciprocal connection from the IO to the NPH.

Materials and Methods

Animals

All protocols using animals were approved by the Animal Care and Use Committee at the Salk Institute in accordance with National Institute of Health guidelines. Neurons were imaged and recorded in wild-type and transgenic mouse lines (GlyT2 (Zeilhofer et al., 2005), GIN (Oliva et al, 2000), YFP-16 (Feng et al., 2000), L7-GFP (Sekirnjak et al., 2003)) backcrossed to the C57BL/6 background.

Tracer Injections and Fixed Tissue Preparation in Adult Mice

To target the dorsal cap, MVN or NPH for stereotaxic injection in adult (P70+) mice, an Angle two stereotax with corresponding software (myneurolab.com; Richmond, IL) were used. The dorsal cap of the IO was targeted at 7.56 mm caudal, 5.60 mm ventral, and 0 mm lateral to bregma using an 11 degree angle on the rostral/caudal axis, targeting the olive dorsally and rostrally. The MVN and NPH were targeted dorsally perpendicular to the bregma-lamda axis with small injections within combinations of the following coordinates with regard to bregma: 5.88-6.84 mm caudal, 0-1.25 mm lateral, and 3.73-4.50 mm ventral.

Texas red dextran (3,000 or 10,000 MW) crystals (Molecular Probes, Eugene, OR) were used as the neuronal tracer. Animals were anesthetized with isoflurane and
placed in a stereotaxic apparatus. Pitch and roll of the head were leveled by eye. The skull was exposed and a small hole drilled to allow entry of the injector. A custom-made microinjector equipped with a 33G hypodermic needle and an internal movable rod (0.2 mm outer diameter, 0.1 mm inner diameter) (Creative Instruments Development Company) was used to deliver dextran crystals. It was lower to the target, and thirty pulses (25 psi, 25 ms duration) were delivered to the internal piston with compressed nitrogen in order to deliver the crystals. The injector was then retracted, the skull hole filled with bone wax, and the wound sutured.

7 days after IO injection or 7-21 days after MVN or NPH injection, animals were transcardially perfused with phosphate buffered saline (PBS) and subsequently 4% paraformaldehyde diluted in PBS. The brains were removed and placed in 4% paraformaldehyde in PBS at room temperature for 30 minutes. They were then placed in 30% sucrose in PBS and transferred to 4 degrees C until the brains sank approximately 24 hours later. Thin coronal sections (40 µm) were sliced using a freezing microtome (Microm) through the brainstem. Slices were wet-mounted and coverslipped with 2.5% DABCO (1,4 diazabicyclo-[2.2.2]octane).

Tracer Injections in Youth Mice

Mice (age P14-16) were anaethetized with nembutal, and their hindbrains were removed and dissected in ice-cold artificial cerebrospinal fluid containing (in mM): 124 NaCl, 5KCl, 1.3 MgSO4, 26 NaHCO3, 2.5 CaCl2, 1 NaH2PO4, and 11 dextrose. ACSF was aerated with carbogen (95% O2-5% CO2), and aerated ACSF had an osmolarity of 300 mOsm and a pH of 7.4. Thin coronal slices were cut from the caudal face of the
hindbrain until the most caudal portion of the dorsal cap of the IO was visible. Reconstituted texas red dextran (MW 3000) crystals were prepared as described in (Glover et al., 1986). A small cut was made rostrally into the tissue with microdissection scissors along the dorsal and medial-lateral border of the dorsal cap unilaterally (see figure 4.4A). Crystals were placed by hand into the incision, and excess crystals were rinsed off of the tissue. A coronal cut was then made approximately 3.5 mm rostral to the IO.

The very thick slice was transferred to a holding chamber in carbogenated ACSF at room temperature for 8-12 hours. Halfway through the incubation time, fresh ACSF was exchanged. The slice was then transferred to 4% paraformaldehyde in PBS for 24 hours and then put in 30% sucrose in PBS until the slice sank. The thick coronal slice was resectioned into 100 µm coronal sections on a freezing microtome (Microm), and washed in PBS. The slices were then wet-mounted and coverslipped with 2.5% DABCO.

**Immunocytochemistry**

Mice (2 months old) were deeply anaesthetized with Nembutal, and their hindbrains were removed and dissected in artificial cerebrospinal fluid containing (in mM): 124 NaCl, 5KCl, 1.3 MgSO₄, 26 NaHCO₃, 2.5 CaCl₂, 1 NaH₂PO₄, and 11 dextrose. The hindbrains transferred to 4% paraformaldehyde in PBS for 2 days at 4°C and then transferred to 30% sucrose diluted in PBS for 1-2 days until they sank. Thin coronal sections (30 µm) were sliced using a freezing microtome (Microm) through the IO. For immunocytochemistry, sections were washed in PBS with 0.25% Triton.
Blocking buffer (0.1M PBS, 5% donkey serum, and 0.1% Triton) was applied for one hour, followed by primary goat antibody against choline acetyl transferase (ChAT) (1:100; Chemicon, Temecula, CA) antibody in blocking buffer for two days. Sections were rinsed, then treated with biotinylated donkey anti-goat IgG (1:250; Jackson ImmunoResearch, West Grove, PA) in blocking buffer for 3 hours. Sections were then rinsed and treated with Cy3-conjugated streptavidin antibody (1:100, Molecular Probes) for 1 hour at room temperature. After washes in PBS, the sections were wet-mounted and coverslipped with 2.5% DABCO.

**Imaging**

Fluorescent images were acquired using a CCD camera (Hamamatsu, model C4742-95) attached to an Olympus BX61 microscope. Images were collected digitally using ImageJ software and then transferred to Adobe Photoshop. Identification of neurons labeled from injections was performed by eye at 20x magnification. Terminals in the IO were examined at 60x magnification.

**Results**

*GABAergic, cholinergic and glycinergic terminals are found in the dorsal cap of the IO*

To identify the non-glutamatergic neurons projecting to the dorsal cap of the IO, I examined the neurotransmitter content of synaptic terminals in the olive using fluorescently labeled transgenic mice as well as immunocytochemistry. To find GABAergic terminals, I imaged coronal brainstem slices of the dorsal cap in transgenic mice that label GABAergic neurons with the fluorescent molecule EGFP (Oliva et al.,
As shown in figure 4.1A, fluorescently labeled GABAergic terminals were numerous in the olive. Terminals generally were concentrated away from cell somas, which appear as dark ovals in figure 4.1. To identify cholinergic terminals in the dorsal cap, immunocytochemical labeling of choline acetyltransferase, a marker of acetylcholinergic neurons. Figure 4.1B is a coronal image of the IO, showing cholinergic terminals and fibers. Cholinergic terminals were less numerous than GABAergic ones (see figure 4.1A) indicating they comprise either a distinct population or a subpopulation of GABAergic terminals which corelease transmitter (Caffé et al., 1996). Brainstem olivary slices were examined for colabeling with cholinergic and GABAergic terminals (n=1); however, no colabeled terminals were identified, suggesting that acetylcholine and GABA are not co-released.

In addition to GABAergic and cholinergic terminals in the olive, glycinergic terminals were also identified using a transgenic mouse in which EGFP labeling is driven by the GlyT2 promotor (Zeilhofer et al., 2005), a marker of glycinergic neurons. Figure 4.1C shows a coronal image of the dorsal cap of the olive in this mouse line. Numerous glycinergic terminals were found in the dorsal cap. Some neurons in the MVN contain both GABA and glycine (Bagnall et al., 2007), so it is possible that these may be co-released in the IO as has been seen in the auditory brainstem (Kuo et al., 2009) and synapses onto Golgi cells in the cerebellum (Dumoulin et al., 2001).

Retrogradely labeled neurons from IO injections

The neuronal tracer texas red dextran was placed in the IO in order to locate upstream neurons. Several days (3-7) after the injection, retrogradely labeled neurons
were identified in the MVN and NPH. Figures 4.2A and 4.2B show representative examples of IO injections in coronal sections of the caudal brainstem. The injection in figure 4.2A was performed in a GlyT2-EGFP transgenic mouse, and the injection in figure 4.2B was performed in a GIN transgenic mouse in which a specific subset of GABAergic neurons are labeled with GFP (Oliva et al., 2000). Both injections were targeted toward the middle of the rostral-caudal axis of the IO with similar spread of the crystals. The texas red tracer crystals were largely confined to the IO in both of these injections. The injection in Figure 4.2A resulted in some crystals being placed in the raphe pallidus, but tracer injections that missed the olive and were rather targeted in this more ventral nucleus did not result in retrograde labeling in either the NPH or the MVN.

Despite the similarities in these two injections, the patterns of retrogradely labeled neurons in the MVN and NPH was different as shown in the examples in Figures 4.2C and 4.2D which are from the same injections as Figures 4.2A and 4.B respectively. The coronal sections in figures 4.2C and 4.D are from approximately the same rostral-caudal location. In figure 4.2C, there are large neurons labeled in the magnocellular, ventrolateral portion of the MVN, with some smaller neurons labeled in the more dorsally located parvocellular portion of the MVN. There are two faintly labeled neurons in the NPH. In contrast, in figure 4.2D, retrogradely labeled neurons are primarily located in the parvocellular, dorsal portion of the MVN where large, as well as, small neurons are labeled. There are few neurons in the ventral magnocellular MVN region. Also in contrast to figure 4.2C, there are several well-labeled neurons in the NPH.
The lack of labeling of a common pool of neurons as shown by the two examples in figure 4.2 was also evident when comparing the other seven Texas red tracer injections that hit the dorsal cap of the IO, but were relatively confined to the IO. Many of the labeled neurons were likely labeled because of contamination of the medial longitudinal fasciculus, a fiber tract surrounding the dorsal-medial portion of the IO. Neurons in the MVN are known to project caudally into this fiber tract (Ishii and Yokota, 1983; Kolkman et al., 2011; Minor et al., 1990), and axons in this tract could be broken and take up dye during the injection process.

*Anterograde injections in the NPH and MVN reveal IO projection areas*

In order to distinguish between areas of the NPH and MVN that project to the IO and those that project to the surrounding medial longitudinal fasciculus, small injections were performed targeting subregions of the NPH and MVN, and caudal brainstem sections were examined for anterogradely labeled fibers in the dorsal cap and surrounding tissue.

Examples of 2 subnuclear injections are shown in figure 4.3A and B. Figure 4.3A is a coronal section through the MVN and NPH. Dextran neuronal tracer (purple) was injected into the ventromedial MVN at approximately 5.85 mm caudal to bregma. Figure 4.3C shows a coronal section through the IO from this injected animal. Labeled fibers from the injection are clearly seen in the MLF but are absent from the IO. The injection in Figure 4.3B, on the other hand, is located at the border between the NPH and MVN with the bulk of the crystals located in the NPH. This injection is approximately 6.30 mm caudal to bregma. Examining the olive from this injection
(figure 4.3D) shows clear labeling of fibers in the dorsal cap of the IO with little-to-no labeling in the surrounding regions.

Pooling all the subnuclear NPH and MVN injections together reveals that fibers in the medial half of the NPH and a dorsal-medial portion of the MVN project to the dorsal cap of the IO (see figure 4.3E, olive-projecting region labeled in blue). More lateral injections in the MVN and NPH either did not produce any labeling in the IO or projected to surrounding areas (Figure 4.3E, non olive-projecting region labeled in orange). These results indicate that the somas and/or fibers of IO-projecting neurons are located near the ventricle in the medial NPH and MVN. Retrograde labeling from olivary projections that labeled neurons in the lateral MVN were likely due to contamination from the MLF or other areas surrounding the IO. These NPH and MVN injections also confirm previous findings that the connection from the NPH/MVN to the IO is bilateral to the caudal IO (De Zeeuw et al., 1993).

Precisely targeted tracer injections reveal IO-projecting neurons

The data presented thus far reveal that olivary-projecting neurons, or at least their processes, are located near the ventricle in the NPH and very medial MVN. In order to definitively locate neurons projecting to the dorsal cap while avoiding labeling of the surrounding structures, tracer injections were performed in a very thick coronal brain slice containing the caudal IO on the caudal face and the rostral brainstem/caudal pons on the rostral face (figure 4.4A). In order to minimize the tracing distance and maximize slice health, brains from youth (P14-16) mice were used. Olivary-projecting neuron axons were cut and dextran crystals were placed by hand into the dorsal cap of
Kooy (see methods and figure 4.4A, (Glover et al., 1986)). Slices were incubated for 8-12 hours and then fixed and later resectioned to view NPH/MVN coronal slices.

In 2 injections (of 14), retrogradely labeled neurons were present in the NPH and MVN near the fourth ventricle. In both injections, the number of labeled neurons was very small (17 and 9 cells total in the NPH and 6 and 1 cells total in the MVN). The neurons were among the smallest in size as well, with soma diameters averaging 15 \( \mu \text{m} \).

_Tracer injections in the NPH reveal olivary projections to the NPH_

IO neurons projecting climbing fibers from the principle IO send collaterals to neurons in the cerebellar nuclei (Sugihara et al., 2001; Van der Want et al., 1989). However, such a climbing fiber collateral connection to the NPH/MVN has been greatly debated in the literature because of conflicting experimental results (Balaban, 1984; Balaban 1988; Ruigrok and Voogd, 2000; Sugihara et al., 1999). Balaban found evidence of terminals from the olive in the MVN in rabbit (Balaban, 1984; Balaban, 1988). In contrast, subsequent studies in rat (Ruigrok and Voogd, 2000; Sugihara et al., 2000) have failed to identify this connection. In this study, some small injections into the NPH not only revealed anterogradely labeled fibers in the dorsal cap, but also retrogradely labeled neurons in the dorsal cap of the IO. Figure 4.5A shows an example of a neuronal tracer injection localized in the NPH. Figure 4.5B shows labeling in the dorsal cap of the IO for the same injections, and tracer labeled the somas of several contralateral neurons. The data presented show the existence of a reciprocal projection from the IO to the NPH. No neurons were retrogradely labeled in the IO from
injections in the MVN (n=21). Therefore, this study also failed to find a connection from the IO to the MVN, but rather identified a projection from the IO to the NPH that may play a homologous role in the cerebellar circuit as the olivary collaterals to the cerebellar nuclei.

**Discussion**

The data presented in this study provide an anatomical description of the connection between the NPH/MVN and the dorsal cap of the IO. This study not only confirms the presence of GABAergic and cholinergic terminals in the olive but also shows that glycinergic neurons project to the region as well. In addition, the neurons that project to the dorsal cap of the olive are exceptionally small and located near the ventricle in the NPH and the very medial MVN. Finally, the data presented show that the dorsal cap of the IO does indeed project back to the NPH, showing homogeneity with the cerebellar nucleus circuit.

*Role of GABA in the IO*

GABAergic terminals contact both the somas of olivary neurons and their dendrites (De Zeeuw et al., 1993), and GABA release affects the firing of olivary neurons via at least two different mechanisms. GABA release in the olive decreases spontaneous firing rate of olivary neurons (Arts et al., 2000), and GABA release in the IO can decouple electrotonically connected olivary neurons (Lang et al., 1996; Llinas and Sasaki, 1989), in turn, decreasing the firing synchrony of Purkinje cell neurons in the cerebellum.
How do changes in climbing fiber firing rate affect cerebellar processing?

Increases or decreases in firing rate have direct consequences for complex spikes in Purkinje cells. The timing and frequency of complex spikes is related to multiple types of plasticity onto Purkinje cells that affect signal flow out of the cerebellar cortex (Hansel and Linden, 2000; Ito, 2001; Mathy et al., 2009; Vogt and Canepari, 2010). In addition, climbing fibers contact other neurons in the cerebellar cortex and nuclei and may affect processing in those neurons as well (Jorntell and Ekerot, 2003; Ruigrok, 1997; Sugihara et al., 1999). Another way that climbing fiber rates can affect cerebellar processing is by induction of plasticity mechanisms. Concomitant firing of mossy fibers and climbing fibers result in large calcium transients in Purkinje cells (Cousmans et al., 2004) and can result in parallel fiber to Purkinje cell LTD (Cousmans et al., 2004; Ito and Kano, 1982; Konnerth et al., 1992), a form of plasticity thought to be important for cerebellum-dependent learning. By affecting the firing rate of climbing fibers, olivary-projecting neurons indirectly control cerebellar plasticity.

How does synchrony of olivary neurons influence cerebellar processing? A complete lack of electrotonic coupling in the IO leads to abnormal firing patterns and lack of precision in timing that can lead to deficits in the timing of conditioned motor responses (Van Der Giessen et al., 2008). It has also been suggested that coupling between olivary neurons may help to coordinate movements involving different parts of the body (Llinas and Welsh, 1993). Further studies are likely to shed more light on these two mechanisms and on the role of the NPH/MVN-olivary connection.
Climbing fiber collaterals to the NPH

Cerebellar nucleus neurons receive collaterals from single olivocerebellar axons (Sugihara et al., 1999), but an anatomically analogous collateral connection to the MVN and NPH has been debated. Cerebellar cortex to cerebellar nucleus and nucleooolivary and olivonuclear projections are largely consistent topographically (Buisseret-Delmas et al., 1993; Groenewegen and Voogd, 1977; Ruigrok and Voogd, 1990; Ruigrok and Voogd, 2000; Voogd, 1967), but it is not clear whether all portions of the cerebellar circuit are organized in a similar manner. The MVN and NPH receive projections from the flocculus and project to the dorsal cap of the IO. If the circuitry is analogous to the cerebellar nuclei, then they should receive collaterals of climbing fiber neurons from the dorsal cap. Evidence for olivary collaterals to the MVN have been mixed. Using retrograde labeling from vestibular nucleus injections (Balaban, 1984) and anterograde labeling from olive injections (Balaban, 1988), Balaban found evidence supporting the presence of the olive to MVN connection in rabbit. However, later studies in rat (Ruigrok and Voogd, 2000; Sugihara et al., 1999) did not confirm these results but rather concluded that the olivary-MVN connection did not exist. However, dorsal cap olivary neurons sent collaterals to the ventral dentate (cerebellar) nucleus in the cerebellum.

The data presented in this study show evidence for a projection from the dorsal cap of the IO not to the MVN, but to the NPH. This is the first study to date to show this projection. Why might other studies have missed it? A likely reason that this projection had not been identified is because other studies either looked for terminals or traced climbing fibers from the olive. Looking just for labeled terminals from an
olivary projection could be deceiving. Most anterograde tracers also label retrogradely, so injections in the inferior olive would label not only anterograde fibers in the NPH but also olivary-projecting neurons. In such a case, it would take very careful tracing to confirm that terminals seen in the NPH after dorsal cap injection were not due to olivary-projecting neurons. Secondly, because the olivary-to-NPH projection was observed only in NPH projections, it is likely that it does not follow the same projection pattern as the climbing fibers that travel dorsally along the lateral edge of the brainstem to the cerebellar peduncles. It is more likely that the projection follows the same path as the NPH-to-dorsal cap projection, traversing the midline of the brainstem and entering the NPH from its ventromedial border. If the projection rather traversed laterally in the brainstem, labeling from MVN injections might have been expected because of the projecting fibers being broken.
Figures

**Figure 4.1.** GABAergic, cholinergic and glycinergic terminals in the dorsal cap of the IO. A, GABAergic terminals in the dorsal cap labeled by the GAD67-GFP transgenic mouse line. B, Cholinergic terminals in the dorsal cap labeled via CHAT immunohistochemistry. C, Glycinergic terminals in the dorsal cap labeled by the GlyT2-GFP transgenic mouse line. GAD67 is GABA amino decarboxylase 67. ChAT is choline acetyltransferase. GlyT2 is glycine transporter 2. Scale bar represents 10 μm.
Figure 4.2. Tracer injections in the IO produced diverse retrograde labeling in the MVN and NPH. A, Dextran tracer crystal injection (red) in a coronal section of caudal brainstem in a GlyT2 transgenic mouse (green). B, Dextran tracer crystal injection (red) in a coronal caudal brainstem in a GIN transgenic mouse (green). C, Retrogradely-labeled neurons in the MVN and NPH from the injection in A. D, Retrograde labeling in the MVN and NPH from the injection in C. Scale bar for A and B represents 250 µm. Scale bar for B and C represents 100 µm.
Figure 4.3. Neuronal tracer injections in the MVN and NPH reveal nucleus subregions that project to the dorsal cap of the IO. A, Dextran crystal injection (purple) in the MVN in a L7-GFP transgenic mouse (green). B, Dextran crystal injection (purple) at the medial border between in the NPH and MVN in a GIN-GFP transgenic mouse (green). C, Anterograde fiber labeling (purple) from the injection in A in the medial longitudinal fasiculus surrounding the dorsal cap. No fiber labeling was seen in the IO. D, Anterograde fiber labeling (purple) from the injection in C. Fibers were concentrated in the dorsal cap of the IO. Scale bar for A and C represents 250 µm. Scale bar for B and D represents 50 µm. E, Diagram of a coronal section of the NPH and MVN with the rostro-caudal axis collapsed. Blue labeling represents the areas where neuronal tracer injection sites produced anterograde fibers in the dorsal cap of the IO. Red labeling represents the areas where neuronal tracer injection sites did not produce anterograde fibers in the dorsal cap of the IO. Unlabeled areas represent areas of the NPH and MVN that were not well sampled with small neuronal tracer injections.
Figure 4.4. Dextran injections targeted to the dorsal cap in young mice reveal retrogradely labeled olivary-projecting neurons in the medial NPH and MVN. A, Diagram of the thick slice in youth mice used for injections. Slices contained the IO on the caudal end and included the NPH and MVN. Dextran crystals (red) were inserted directly into the dorsal cap without contaminating the surrounding medial longitudinal fasciculus. B, Coronal section showing retrogradely labeled neurons in the medial NPH and MVN from a youth brain slice injection. Scale bar represents 25 µm.
Figure 4.5. Neuronal tracer injections in the NPH reveal olivary neurons that project to the NPH. A, Dextran tracer injection in the nucleus prepositus hypoglossi (NPH). Scale bar represents 50 µm. B, Tracer labeling in the dorsal cap of the IO reveals retrogradely labeled neurons from the injection in A. Scale bar represents 25 µm.
References


Chapter 5. Conclusion and Future Directions

The anatomy of the cerebellar circuit has been studied in detail for well over a hundred years. However, there are still many fundamental questions challenging scientists today. One line of investigations explores differences in circuitry in cerebellar subregions and the reasons for this diversity. The study on neurons in diverse precerebellar nuclei that project mossy fibers to the cerebellum presented in chapter two provides important information regarding this question of similarity versus diversity in the cerebellar circuit. The data gives evidence that despite the great diversity, both in firing patterns and in sensory/motor/cortical modalities, precerebellar neurons have remarkably similar intrinsic properties that allow them to linearly transform currents into firing rate.

Another important area of cerebellum circuit research involves the study of vestibular nuclei in the brainstem – nuclei that are unique in both projecting to and receiving projections from the cerebellar cortex. These nuclei have tremendous workloads as they must both serve their cerebellar circuitry roles as well as support the vestibular and oculomotor systems. The study presented in chapter three sheds light on similarities and differences in cellular processing in specific cell types defined either by projection or neurotransmitter content within two of these nuclei, the MVN and NPH.

Finally, another major line of cerebellar research revolves around a major input to the cerebellum – the climbing fibers from the inferior olive. The data presented in chapter four represents progress toward understanding a form of feedback to the
cerebellum in the form of the connection between the NPH and the dorsal cap of the inferior olive. The data indicate a glycinergic input, previously unidentified to the dorsal cap and provides evidence for a novel feedback signal from the dorsal cap of the olive to the NPH. Finally, the sub-nuclear location of the neurons projecting from the NPH/MVN to the olive is identified.

Below, I present summaries of the studies presented in this dissertation with my commentary on the importance of the data and directions for future research.

Similarities and differences among precerebellar neurons in eight precerebellar nuclei

Precerebellar neurons project sensory, motor and cortical information to the cerebellum, and the precerebellar nuclei, or sometimes subgroups within those nuclei, convey signals derived from specific sensory/motor/cortical modalities. For example, there are two types of precerebellar neurons in the MVN – those that convey vestibular sensory information, and those that convey vestibular sensory information combined with eye movement signals (Cheron et al., 1996). Many types of precerebellar neurons have been recorded in vivo, and their firing patterns vary widely (Bengtsson and Jorntell, 2009; Cheron et al., 1996; Eccles et al., 1971; Escudero et al., 1996; Garwicz et al., 1998; Noda, 1986; Rancz et al., 2007; van Kan et al., 1993). This is perhaps expected since the experimental conditions vary as well.

These differences in firing patterns lead to some interesting questions about the circuit though. Are the precerebellar neurons specialized to process a particular type of signal or produce a particular firing pattern? Surprisingly, the study presented in chapter two really supports the opposite scenario. While some differences existed in
nuclei when subthreshold, the precerebellar neurons fire spontaneously \textit{in vivo}, and the precerebellar neurons all share the ability to linearly transform current into firing rate over an extremely wide range of currents.

This finding provides deep insight into the cerebellar circuit as a whole. The cerebellum has more neurons by far than the rest of the brain combined, and most of those neurons are granule cells, each one receiving specialized signals from particular mossy fiber neurons. One way nature could have dealt with diversity of signals that the cerebellum needs would be to make each neuron very unique. It would have only the ion channels it needed to convey its signal. It would also be very important for it to connect with just the right granule cells in order to properly convey the signal. On the other hand, nature could develop one neuron that could handle any signal and perform a simple current to firing rate computation. The neurons could send their signals to multiple granule cells that are then more specialized for the types of information they receive. This second scenario has a lot of advantages over the first and indeed seems to be the way the system evolved. From an energy perspective, it may be conservative. If you have 7 jobs you could hire seven people to do them, or you could hire one incredible person to do all 7 and then pay that person 4 times more than the rest. Making a neuron that can perform the same calculation on a wide range of inputs requires maintaining a diverse ion channel repertoire. However, it also means that one neuron can handle many tasks.

One interesting difference in precerebellar neurons was the gains (slopes) of the current to firing rate relationship. There was considerable variability both within and among nuclei recorded. The pharmacology presented shows that the calcium activated
potassium channels, SK and BK, contribute to setting the gain. Interestingly, a form of intrinsic plasticity discovered in the MVN, firing rate potentiation, depends on the activity of BK channels and causes the gain of the current to firing rate relationship to change (Nelson et al., 2003). Because precerebellar neurons play a very important role in the cerebellar circuit, it would be interesting if experiences that drive cerebellum-dependent learning employ this form of intrinsic plasticity on precerebellar neurons. Changing the gain of the signal entering the cerebellum could influence an entire circuit and may be a way to stabilize memory. Future studies could examine whether firing rate potentiation can occur in precerebellar neurons in slice, and finally, whether it does occur in a learning situation.

*Similarities and differences in physiological parameters of identified MVN and NPH neurons*

The data presented in chapter three compares neurons in the NPH identified by their neurotransmitter and neurons in the MVN compared by their projections. As shown previously for populations of MVN neurons, these comparisons reveal far more similarities than differences. Regardless of neurotransmitter content or projection, neurons in the MVN and NPH fire spontaneously, have the ability to fire at exceptionally high rates, and have a linear relationship between current and firing rate over a very wide range of each. When examined more closely, however, differences in cell types with disparate circuit roles became apparent. YFP-16 neurons, a subset of glutamatergic and glycinergic neurons, tended to the largest and fastest neurons and had the narrowest action potentials and lowest current-to-firing rate gains. Glycinergic
neurons as a population tended to be the smallest, slowest neurons with wide action potentials and high current-to-firing rate gains. GIN neurons, a subset of GABAergic neurons had more of an intermediate physiological phenotype.

In the MVN, reticular/spinal-projecting neurons were compared with oculomotor-projecting neurons, an interesting comparison because both cell types are projections out of the MVN to motor areas. Although more intrinsic parameters were very similar with these two neuronal types, despite the homologous circuit functions, significant differences were apparent between the two populations. Oculomotor-projecting neurons tended to have narrower action potentials and could fire at faster rates.

This study makes it clear that no one or two physiological parameters should be used to define a cell type with regards to circuit function. There is tremendous variability from cell to cell, and neurons playing different roles in behavioral circuits often have many specializations that may be related to their specific tasks.

The role of olivary inhibition in the cerebellar circuit

The NPH and MVN receive Purkinje cell input from the cerebellar cortex, project mossy fibers to the cerebellum and also project to the dorsal cap of the inferior olive (and thereby influence the climbing fibers that project to the cerebellum). The connection with the inferior olive is the focus of the data presented in chapter 4. Using three anatomical tracing techniques, olivary-projecting neurons were identified in the medial NPH and ventromedial corner of the MVN. GABAergic and cholinergic terminals in the olive were confirmed, and a novel glycnergic projection was
discovered. Finally, the data presented give strong evidence for a feedback connection from the dorsal cap of the inferior olive to the NPH.

These anatomical results are important in themselves as they enrich knowledge of this aspect of the cerebellar circuitry. However, their greatest usefulness will likely be to guide future experiments on this system. The homologous GABAergic connection from the cerebellar nuclei to the inferior olive has been studied during delay eyeblink conditioning, a cerebellum-dependent form of learning (Medina et al., 2002) where the connection plays a specific role in signaling the extinguishing of memory. Homologous experiments have not been published with regard to forms of cerebellar learning and memory dependent on the cerebellar flocculus and MVN/NPH. The role of the inferior olive itself in such behaviors is debated (Ke et al., 2009). Manipulating the MVN/NPH-to-dorsal cap connection in vivo would certainly contribute an interesting piece to this line of research.

All behavioral and most cellular work on this connection has purely focused on the GABAergic component of the input, which is perhaps fair since lesioning the cerebellar nuclei mimics GABA blockade with regard to complex spike activity (Lang et al., 1996), but the data presented clearly show a cholinergic and glycinergic component as well. It would be interesting to pharmacologically explore the behavioral relevance of these connections.
References


