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Intrinsic physiology of identified neurons in the prepositus hypoglossi and medial vestibular nuclei

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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.

Keywords

glycinergic; GABAergic; glutamatergic; vestibulospinal; oculomotor

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 [1]. 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: [10, 54]). 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 [6, 21, 27]. Neurons in the MVN and the ventral portion of the lateral vestibular nucleus additionally project to the cervical spinal cord and medulla [21]. 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 [13, 26, 29, 31, 40, 43]

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 [9, 23, 46, 54]. Although neurons were initially classified into two groups (A and B) on the basis of action potential waveform [23, 46], subsequent studies clarified that action potential properties were distributed continuously across neurons [9, 44]. Recordings targeted to MVN neurons in which the primary neurotransmitter was identified either by single cell PCR [55] or via fluorescence expression in transgenic mouse lines [3] 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 [45]. Collectively, published results indicate that intrinsic electrophysiological properties can differentially influence signal processing in MVN neurons [42, 44, 54], 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 [22]. 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 [50]. Although an extensive glycinergic projection from the NPH to the abducens nucleus has been documented [53], relatively few glycinergic neurons were identified in NPH recordings [50]. 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) promoter with GFP [35]. The YFP-16 transgenic mouse line, in which the thy-1 promoter drives YFP expression in a subset of neurons [15], was used to identify glutamatergic and glycinergic projection neurons [3, 30]. To identify glycinergic neurons, we used the GlyT2-GFP transgenic mouse line [58]. 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 [45], 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 μ m) 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, 5 KCl, 1.3 MgSO₄, 26 NaHCO₃, 2.5 CaCl₂, 1 NaH₂PO₄, and 11 dextrose. Carbogenated ACSF had an osmolality of 300 mOsm and a pH of 7.4. Coronal slices (250 μ m) 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 μ M) and strychnine (1 μ M) 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 M Ω) 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 osmolality 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 (V_m) 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 V_m difference between threshold and peak. The afterhyperpolarization was defined as the V_m 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 [3]. 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 [45]. 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 $\pm$ 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 [3]. In the YFP-16 line [15], 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. Fig. 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 [35] 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 [3]. The distribution of fluorescent neurons and processes in the GIN line differs strongly in the NPH and MVN (Fig. 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) promoter. This mouse line previously enabled the discovery of a unique subset of projection neurons in a cerebellar nucleus [4]. Fig. 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 [17]. 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. Fig. 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 (Fig. 2B). In contrast, none of the GIN neurons in the NPH expressed Kv3.3 (Fig 2C). Interestingly, a subset of the largest GlyT2 neurons within the NPH were immunopositive for Kv3.3, whereas no small GlyT2 neurons were (Fig. 2D). These results indicate that Kv3.3 subunits are differentially expressed in NPH neurons and predict that, as in the MVN [3], 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. Fig. 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 [7] or as AHPs+ [50]. Neurons with a similar action potential profile that play an important role in eye fixation have been observed in the NPH [32]. The lower panel of Fig. 3A action potentials illustrates the heterogeneity in action potential profiles within and across cell types. Most YFP-16 neurons exhibited a biphasic AHP, sometimes without an intervening depolarizing phase, as in the YFP-16 neuron illustrated in the lower panel of Fig. 3A. In contrast, many GIN and GlyT2 neurons exhibited a monophasic AHP, as illustrated in the lower panel of Fig. 3A, and would be classified as type A [7] or AHPs-[50].

Although action potential profiles exhibited qualitative similarities 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) (Fig. 3C and Table 1). In contrast, the magnitude of the AHP and the action potential threshold (Table 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 [5] but does not provide insights into how a neuron's complement of ion channels influences 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. Fig. 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 [3], 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 1).

NPH neurons targeted in different mouse lines ranged widely in gain (Fig. 3C) and maximum firing rate (Fig. 3D) but exhibited significant differences between cell types (Table 1). Fig. 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. Fig. 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 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 (Figs 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. Figs. 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 (Fig. 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 (Fig 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 Fig. 1 of this study have been employed successfully to compare and contrast intrinsic neuronal physiology [3, 4], underlying ionic currents [17–19] and vestibular nerve synaptic properties and plasticity of different classes of neurons in the medial vestibular nucleus [2, 30]. 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 [30]. 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 [45]). A separate study on MVN and NPH neurons projecting to the cerebellar flocculus [25] is in preparation.

Injections of fluorescent dextrans into the cervical spinal cord (Fig 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 (Fig 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 [45]. 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 [3]. 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, Fig. 5 shows intrinsic physiological properties of Sp/RetP and OMP neurons; the latter were replotted from Sekirnjak and du Lac [45]. Representative action potential profiles for the two projection neuron types are similar, as shown in Fig. 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 Fig. 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 (Fig. 5C, Table 2). Both projection neuron types exhibited modest gain increases (Fig. 5D) and relatively constant phase (Fig. 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 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 [16, 48, 54].

Intrinsic physiology of NPH neurons defined by neurotransmitter

The NPH comprises morphologically diverse neurons [6, 27, 28] 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 [6, 27]. Intrinsic excitability in unidentified NPH neurons was investigated by Idoux et al. [22] 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. [50] 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 [41, 53], were undersampled in a previous study examining NPH physiology with post-recording analysis of transmitter type [50].

The action potential profiles of NPH neurons reported in guinea pig [22] and rat [32, 50] 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 Fig 3A., would be classified as “type A” according to Idoux et al. and as AHPs- by Shino et al. [50], who observed this profile in GABAergic but not glutamatergic neurons. Consistent with the findings of Shino et al. [50], 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. [32], 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, [8]). Such neurons would be classified by Idoux et al. [22] as “type B” and as AHPs + by Shino et al. [50], 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 [30]. Neurons that fired erratically during sustained depolarization which would be classified as type D by Idoux et al. [22] were not observed in the mouse NPH and were rare in the rat NPH [50], perhaps reflecting a role for such neurons in oculomotor integration [22], which is robust in the guinea pig [14] compared with the mouse [56].

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 [6]. High levels of Kv3.3 expression in YFP-16 neurons in the NPH (Fig. 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 [57].

The predominant glycinergic projection of NPH neurons is to the contralateral abducens nucleus [53], 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 [3, 30]. 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 coeruleus, and make local circuit connections with the MVN. The intrinsic physiology of the subset of GABAergic neurons labeled in the

GIN line was relatively heterogeneous (Fig. 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 [30]. 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 [45]. The two populations were largely similar when compared with glycinergic neurons in the NPH, GABAergic neurons in the MVN [3], or unlabeled neurons near retrogradely-labeled oculomotor-projecting neurons [45]. Nonetheless, and despite striking similarities in intrinsic response dynamics (Fig. 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 [20].

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-contralateral pattern was previously established for MVN projections to the abducens [52] and for fastigial (medial cerebellar) neurons receiving Purkinje cell synapses and projecting to the caudal medulla [4]. 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 [18], the density and kinetics of potassium channels differ [18].

The action potential clamp technique, in which a neuron's own action potential serves as a voltage clamp stimulus [24], has revealed the ionic currents flowing during the action potential when MVN neurons fire at different rates [19]. 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 [17, 19] because repolarizing Kv3 currents contribute to sodium channel availability by transitioning sodium channels directly from open to closed states, bypassing inactivation [19]. 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 [17, 19]. Slower neurons have relatively more interspike potassium currents, including calcium

activated potassium and A-type currents [17], 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 [19]. 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 [19, 51]. 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 [12, 51]. 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 [39]; alternatively or in addition, the ADP could reflect an inward current mediated either by low threshold calcium channels [47] 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 [17], 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 [9] 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. [55], Bagnall et al. [3], and Shino et al. [50], 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 [11, 54]). Dendrotoxin-sensitive potassium currents (Kv1.1 and 1.2), which are developmentally regulated [38], are altered by vestibular deafferentation in vestibulo-oculo-spinal neurons [48, 49]. BK type calcium-activated potassium currents in MVN neurons are decreased by repeated hyperpolarization [33, 34], resulting in a persistent increases 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 [19]. Each of the other currents that are expressed in MVN neurons, including Na [18, 47], Ca [23], CNG [37], and EAG [36], 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.

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Figure 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 μ m. Sections are approximately 6.12 mm caudal to bregma.

Figure 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 μ m.

Figure 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 (R_{in}). 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 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 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 (R_{in}). 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).

Table 1

Electrophysiological properties of GlyT2, YFP-16, and GIN neurons in the NPH

Parameter	Mean $\pm$ SD				P value	
	GLYT2 (n=19)	YFP-16 (n=27)	GIN (n=20)	GlyT2 vs. YFP-16	GlyT2 vs. Gin	GIN vs. YFP-16
AP width (ms)	1.54 $\pm$ 0.39	0.802 $\pm$ 0.30	1.08 $\pm$ 0.34	<0.001	<0.05	
AP half-width (ms)	0.688 $\pm$ 0.19	0.333 $\pm$ 0.15	0.492 $\pm$ 0.18	<0.001		<0.05
AHP (mV)	24.3 $\pm$ 4.00	21.7 $\pm$ 3.37	22.1 $\pm$ 3.42			
Threshold (mV)	-46.1 $\pm$ 5.50	-47.8 $\pm$ 9.51	-46.3 $\pm$ 3.59			
Input res. (M Ω)	927 $\pm$ 703	160 $\pm$ 128	469 $\pm$ 318	<0.001		<0.01
Max. firing rate (spikes/s)	107 $\pm$ 54.3	204 $\pm$ 94.0	109 $\pm$ 55.1	<0.001		<0.01
Gain < 80 Hz (spikes/s/nA)	343 $\pm$ 155	190 $\pm$ 52.1	261 $\pm$ 108	<0.001		<0.05
Gain > 80 Hz (spikes/s/nA)	254 $\pm$ 82.0	128 $\pm$ 50.6	176 $\pm$ 93.4	<0.001	<0.001	
Adaptation ratio	0.84 $\pm$ 0.07	0.83 $\pm$ 0.07	0.85 $\pm$ 0.14			
PRF (spikes/s)	12.0 $\pm$ 20.1	22.2 $\pm$ 29.9	13.4 $\pm$ 26.9			

Abbreviations: AP: action potential; AHP: afterhyperpolarization; Input res: input resistance; PRF: post inhibitory rebound firing.

Table 2

Electrophysiological properties of two types of MVN projection neurons

	Mean $\pm$ SD		N		P value
	OMP	Sp/RetP	OMP	Sp/Ret	
AP width (ms)	0.72 $\pm$ 0.13	0.72 $\pm$ 0.29	37	33	0.091
AP half-width (ms)	0.31 $\pm$ 0.07	0.30 $\pm$ 0.14	37	33	0.019
AHP (mV)	19.2 $\pm$ 3.2	18.8 $\pm$ 2.8	37	33	0.809
Threshold (mV)	-52.3 $\pm$ 3.9	-50.3 $\pm$ 5.0	37	33	0.062
Input res. (M Ω)	160 $\pm$ 100	136 $\pm$ 104	36	32	0.206
Max. firing rate (spikes/s)	327 $\pm$ 94	236 $\pm$ 110	36	33	0.001
Gain > 80 Hz (spikes/s/nA)	193 $\pm$ 57	133 $\pm$ 56	37	30	<0.0001
Adaptation Ratio	0.89 $\pm$ 0.05	0.90 $\pm$ 0.06	36	27	0.776
PRF (spikes/s)	12.4 $\pm$ 9.4	14.1 $\pm$ 14.4	28	29	0.981

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.