Morphology, Intrinsic Membrane Properties, and Rotation-Evoked Responses of Trochlear Motoneurons in the Turtle

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Jones MS, Ariel M. Morphology, intrinsic membrane properties, and rotation-evoked responses of trochlear motoneurons in the turtle. J Neurophysiol 99: 1187–1200, 2008. First published December 26, 2007; doi:10.1152/jn.01205.2007. Intrinsic properties and rotation-evoked responses of trochlear motoneurons were investigated in the turtle using an in vitro preparation consisting of the brain stem with attached temporal bones that retain functional semicircular canals. Motoneurons were divided into two classes based on intrinsic properties. The first class exhibited higher impedance (123.0 ± 11.0 MΩ), wider spikes (0.99 ± 0.05 ms), a single spike afterhyperpolarization (AHP), little or no spike frequency adaptation (SFA), and anomalous rectification, characterized by an initial “sag” in membrane potential in response to hyperpolarizing current injection. The second class exhibited lower impedance (21.8 ± 2.5 MΩ), narrower spikes (0.74 ± 0.03 ms), a double AHP, substantial SFA, and little or no rectification. Vestibular responses were evoked by horizontal sinusoidal rotation (1/12-1/3 Hz; peak velocity: 30–100°/s). Spiking in higher-impedance cells was recruited earlier in the response and exhibited a more limited dynamic range relative to that of lower impedance cells. Spiking evoked by injecting depolarizing current during rotation was blocked during contraversive motion and was consistent with a shunting inhibition. No morphological features were identified in neuropeptidin-filled cells that correlated with the two physiological classes. Recovered motoneurons were multipolar but exhibited a less-complex dendritic morphology than ocular motoneurons of similarly sized mammals. The two physiologically defined cell classes have homologues in other vertebrates, suggesting that intrinsic membrane properties play an important role in oculomotor processing.

INTRODUCTION

Motoneurons in the oculomotor, trochlear, and abducens nuclei comprise the common final output pathway for the control of eye movement. A variety of descending and brain stem projections converge on these motoneurons (Buttner-Ennever and Horn 1997), which transform a complex spatiotemporal pattern of synaptic input into a rate-coded spike train that drives the extraocular muscles in a coordinated and precise fashion. These neurons are not only responsible for the generation of voluntary eye movements but also participate in critical brain stem reflexes such as the vestibuloocular reflex (VOR), a compensatory movement of the eyes during motion of the head that acts to stabilize the visual scene on the retina (Wilson and Melvill-Jones 1976).

There has been intensive study of oculomotor physiology and the VOR over the last few decades, motivated not only by its clinical relevance but also the utility of the system as a model of neural control (Schwarz 1976; Young 1995). One issue that remains poorly understood is the role of intrinsic membrane properties in shaping motoneuron output. In the spinal cord, the role of intrinsic properties in influencing synaptic integration (Skydsgaard and Hounsgaard 1994), spiking patterns (Gustafsson 1974, Kernell 1965; Smith and Perrier 2006; Zengel et al. 1985), and recruitment (Gustafsson and Pinter 1985) is well established. However, the oculomotor, trochlear and abducens nuclei are located relatively deep within the brain and therefore present challenging targets for intracellular recording. Additionally, study of the VOR involves movement of the head; this compromises the mechanical stability required of intracellular techniques. Intrinsic properties of ocular motoneurons have been investigated in both slice preparations and the intact brain (Baker and Highstein 1975; Baker and Precht 1972; Baker et al. 1969a,b; Durand 1989a,b; Goldberg et al. 1974, 1976; Grantyn and Grantyn 1978; Gueritaud 1988; Gurahian and Goldberg 1987; Llinas and Baker 1972; Nelson et al. 1986; Nieto-Gonzalez et al. 2007; Precht and Baker 1972; Russier et al. 2003; Shall and Goldberg 1992; Uchino et al. 1978). In these studies, synaptic responses were evoked by electrical stimulation of different pathways when they were examined at all. As a rule, studies of the VOR employing natural stimulation have been restricted to extracellular recording (Blanks et al. 1978; Delgado-Garcia et al. 1986; Fuchs et al. 1988; Pastor and Gonzalez-Forero 2003; Pastor et al. 1991).

Our laboratory is studying vestibuloocular physiology using an in vitro preparation consisting of the intact brain stem of the pond turtle with attached temporal bones (Ariel et al. 2004; Fan et al. 1997; Jones and Ariel 2006). Because of the extraordinary resistance to hypoxia exhibited by the turtle, the brain can be isolated in this fashion, facilitating the use of intracellular recording techniques. However, because the semicircular canals remain intact, the preparation responds to natural vestibular stimulation.

In the present study, extracellular multiunit recording of the trochlear nerve was combined with whole cell patch recording of individual trochlear motoneurons. This allowed us to quantify intrinsic properties and morphology of representative ocular motoneurons in this species. Additionally, as recordings obtained using the whole cell patch technique exhibit a surprising degree of mechanical stability, we have been able to examine intracellular events during rotation of the preparation. This permitted us to investigate influences of intrinsic properties in shaping motoneuron output during natural vestibular stimulation and compare rotation-evoked activity of individual motoneurons with that of the simultaneously recorded popula-
tion response. The present results not only complement studies of ocular motoneurons in other species but also provide an opportunity for comparison of motoneurons in the ocular motor system with those of the spinal cord, the latter having been extensively studied in the turtle.

METHODS

All procedures were approved by the Saint Louis University Animal Care and Use Committee. Recordings were performed in adult pond turtles (Trachemys scripta elegans; carapace length: 15–20 cm). Animals were housed in a local aquarium with swimming and basking facilities, maintained at room temperature with a 16-h light/8-h dark cycle.

Experimental preparation

Animals were cryoanesthetized at 4°C for ~1 h after which they were rapidly decapitated and the cranium dissected to isolate the brain stem and temporal bones, the latter retaining functional semicircular canals (Fig. 1A). Blood was flushed from the tissue by infusing the brain’s vasculature with 50 ml of chilled oxygenated physiological media (containing, in mM: 96.5 NaCl, 2.6 KCl, 2.0 MgCl₂, 31.5 NaHCO₃, 20.0 D-glucose, and 4.0 CaCl₂ bubbled with 95% O₂-5% CO₂) via the carotid arteries. The cerebral cortex was removed and the anterior portion of the optic tectum was opened to visualize the right trochlear nucleus (Ncl. IV) on the wall of the third ventricle (Fig. 3A). The left trochlear nerve (nIV) was cut 1–2 cm distal to its exit from the brain stem and prepared for recording by clearing it of connective tissue. The preparation was then secured dorsal side up in a superfusion chamber where it was bathed continuously in oxygenated physiological media throughout the remainder of the experiment. The superfusion chamber was placed at the center of a computer-controlled turntable (Contraves-Goerz, Pittsburgh PA) that rotated about the vertical axis with respect to gravity. The preparation was oriented so that the horizontal semicircular canals were approximately aligned in the plane of table rotation (Brichita et al. 1988).

Whole cell patch recording

Intracellular responses were recorded using the blind whole cell patch technique (Blanton et al. 1989). Electrodes were constructed from thick-walled borosilicate glass using a horizontal puller (P-87; Sutter Instruments, Novato, CA) and filled with a standard pipette solution (containing, in mM: 124 KMeSO₄, 2.3 CaCl₂, 1.2 MgCl₂, 10 HEPES, and 5 EGTA) with 0.2% Neurobiotin (Vector Laboratories, Burlingame, CA). Electrode impedance was 5–7 MΩ. Electrodes were advanced into the right Ncl. IV in ~2-µm steps using a remote-controlled microdrive mounted on the turntable. A positive pressure of ~300 mbar was established prior to placing a pipette in the bath to keep the tip free of debris. This pressure was maintained until the

FIG. 1. A: dorsolateral view of the in vitro turtle brain stem preparation. The skull is dissected and the cortex removed to expose the brain stem and temporal bones. The dorsal surface of the tectum and diencephalon are visible at center. B: transverse section of the turtle brain at the level of the trochlear nucleus. Right trochlear nucleus (Ncl. IV) motoneurons have been retrogradely labeled after application of horseradish peroxidase (HRP) to the contralateral trochlear nerve. The nucleus (filled arrow) consists of a dense cluster of neurons on the wall of the 3rd ventricle (3V) that caps the medial longitudinal fasciculus (mlf; indicated by asterisk). Stained axons (open arrow) can be seen exiting the nucleus. C: parasagittal section 100 µm to right of midline showing stained axons leaving the nucleus in separate fascicles that subsequently merge as they enter the trochlear decussation (Cb: cerebellum; 4V: 4th ventricle). D: higher-magnification coronal section showing dendrites from filled motoneurons extending ventrally into the mlf. E: an ipsilaterally projecting Ncl. IV motoneuron in the left Ncl. IV, which was labeled following application of HRP to the left trochlear nerve. Scale bar in C–E: 250 µm. F: thin section of a turtle trochlear nerve. This specimen contains 620 myelinated axons. G: histogram of axon sizes in the left trochlear nerve (nIV) specimen shown in F. Cross-sectional area of axons varied from 1.9 to 65 µm².
electrode had passed through the ependymal cells lining the ventricular surface. At a depth 50–100 μm, pressure was reduced to ~70 mbar, and the pipette was then advanced while pulses of negative current were injected to monitor electrode impedance (~1.0 nA at 1 Hz; pulse duration: 100 ms). When an increase in impedance indicated that the tip of the electrode was adjacent to a cell membrane, positive pressure was released, and an attempt made to form a gigaohm seal. Seals sometimes formed spontaneously and quickly (~<1 min) following release of positive pressure; however, it was usually necessary to apply negative pressure to the pipette and the seal formation occurred over the course of several minutes. If a seal did not form, the electrode was withdrawn, and the process repeated using a fresh pipette. When a gigaohm seal was obtained, negative pressure was applied to rupture the cell membrane and obtain a whole cell recording. The use of a moderately slow (~1 s) ramp of suction was found to be the most reliable technique for break-in. Recordings were made using an Axoclamp 2B amplifier (Molecular Devices, Union City, CA) configured in current-clamp mode. Intracellular signals were not corrected for junction potentials. At the termination of recording, the patch pipette was slowly withdrawn in 2-μm steps to allow the cell membrane to reseal for subsequent histological recovery.

Multunit nerve recording

Multunit activity (MUA) was recorded from the left nIV using a fire polished suction electrode referenced to the bath using a chlorided silver wire. Signals from the nerve were buffered through a unity-gain headstage, then amplified (~1,000) and band-pass analog filtered at 300–1,000 Hz using a differential amplifier (Model 1800, A-M Systems, Carlsborg, WA). The amplifier could also be configured to apply current pulses to the nerve to test Ncl. IV recordings for antidromic activation.

Data collection

All recordings were made at room temperature. Nerve MUA, intracellular potentials, and turntable tachometer signals were digitized at 10 kHz using commercial data-acquisition software (pClamp 10; Molecular Devices) and stored for subsequent analysis offline. Prior to obtaining rotation-evoked responses, 30 s of spontaneous activity, and responses to hyper- and depolarizing current, were recorded.

Rotation-evoked responses

Vestibular responses were investigated using horizontal sinusoidal rotation of the preparation at 1/3, 1/6, and 1/12 Hz. This stimulation falls within the spectral content of head motions observed in the behaving turtle (Dieringer et al. 1983) and, as demonstrated in our previous study, evokes a robust response in the trochlear motoneuron population (Jones and Ariel 2006). This response likely reflects a combination of horizontal and vertical canal input to Ncl. IV, the latter being activated by horizontal rotation because of the nonorthogonality of the canal planes (Bricha et al. 1988). It is generally accepted that the trochlear nucleus receives excitatory drive primarily from the contralateral posterior canal, a plan that appears to be conserved in vertebrate physiology (Highstein 1988). Yet in the turtle, eliminating this input by cutting the posterior branch of the vestibular nerve has little effect on the Ncl. IV response to horizontal rotation (see Fig. 6C in Jones and Ariel 2006). Moreover, ongoing studies of eye movements elicited by electrical stimulation of the cranial nerves in the reduced turtle preparation indicate that contraction of the superior oblique induces substantial abduction in this species (J. R. Dearworth, personal communication). However, it is possible that the horizontal input is a weak or secondary component, say, as might be necessary to modulate contraction of the superior oblique during multi-dimensional head movements. This is relevant to the present study in that we may not be testing the full dynamic range of Ncl. IV motoneurons.

The finite mechanical stability of whole cell recordings placed constraints on peak velocity of the applied table motion. Consequently, spike responses were evoked by table rotation in only about half of the recordings obtained in the study. However, with one exception (shown in Fig. 7), rotation generated a clear subthreshold response that was quantifiable even if it did not generate spiking. Additionally, some whole cell recordings were obtained in preparations exhibiting MUA with abnormal phase or amplitude characteristics. Such recordings usually occurred at the end of long (~>12 h) experiment sessions. In these cases, the cell was considered acceptable for the measurement of intrinsic properties and morphological analysis, but its rotation-evoked responses were discarded. In some experiments, recordings were obtained from the left Ncl. IV and the right nIV. In these cases, rotation-evoked responses were shifted in phase by 180° to appear as if all recordings were obtained from the right Ncl. IV and the left nIV.

Numerical values are reported as means ± SE. Two-tailed t-tests were used for group comparisons except where noted.

Histology and morphological analysis

After each experiment, brains were fixed for 24–36 h in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), followed by 24 h in 30% sucrose-PB. The tissue was cut in 100-μm sections using a cryostat, rinsed in three changes of PB, incubated in avidin-biotin complex (ABC; Vector Laboratories) for 2 h, and then reacted using a heavy-metal-intensified dianaminobenzidine/H2O2 procedure in Tris Buffer (Adams 1981). Sections were mounted on gelatinized slides, air-dried, then cleared and coverslipped using Permount. Serial reconstruction of labeled trochlear motoneurons was performed using Neurolucida software (MicroBrightfield, Colchester, VT). Trochlear motoneurons were retrogradely labeled with horseradish peroxidase (HRP, Sigma Chemical, St. Louis, MO) so that the organization of Ncl. IV in this species could be examined prior to attempting intracellular recordings. Briefly, dissection was performed as described in the preceding text, and the left or right trochlear nerve was drawn into the tip of a snug-fitting glass capillary tube using gentle suction. A small quantity of concentrated HRP paste was introduced through the open end of the capillary and applied to the exposed end of the nerve, where it dissolved in a small volume of Ringer solution. The nerve was left secured in the capillary for 18–36 h after which the brain stem was sectioned and processed histochemically as described in the preceding text.

RESULTS

Anatomy of the turtle trochlear nucleus and nerve

Characteristic anatomical features of the trochlear nucleus are demonstrated in the histological material shown in Fig. 1. Figure 1B depicts a transverse section of the turtle brain stem at the level of the trochlear nucleus in which motoneurons in the right Ncl. IV have been labeled by retrograde transport of horseradish peroxidase that was applied to the nIV. The nucleus consists of a dense cluster of cells ~250 μm in extent (Fig. 1B; filled arrow), forming a protuberance on the wall of the third ventricle and bounded ventrally by the medial longitudinal fasciculus (mlf, asterisk). Some motoneurons extend dendrites laterally into the surrounding mesencephalic gray and/or ventrally into the mlf as discrete bundles (Fig. 1D). Axons from Ncl. IV motoneurons course dorsolaterally (Fig. 1B; open arrow) and then cross the midline and exit the brain stem as the contralateral trochlear nerve. In sagittal sections, it can be seen that axons ascend in three or four separate fasciciles.
then enter the trochlear decussation as a single bundle (Fig. 1C). The significance of this axon segregation is not known.

In many specimens, a small number of labeled cells were found in the ipsilateral nucleus. An example is shown in Fig. 1E in which a motoneuron in the left Ncl. IV was filled following application of HRP to the left trochlear nerve. Ipsilaterally projecting trochlear motoneurons have been observed in many species. In mammals, such cells comprise 2–4% of the Ncl. IV population (Murphy et al. 1986).

A representative thin section of the trochlear nerve is shown in Fig. 1F. The nerve is a flattened oval with a minor axis of ~175 μm. All axons appeared to be myelinated. A histogram of axon sizes measured in one nerve is shown in Fig. 1E. Cross-sectional areas (measured inside the myelin sheath) ranged from 1.9 to 65 μm² with a median value of ~20 μm². There was not an obvious clustering of axons into groups of different sizes. Axon counts in six specimens gave a mean value of 629 ± 12.

**Morphology of Ncl. IV motoneurons**

Many neurobiotin-filled motoneurons were recovered following recording and histological processing, from which eight that appeared completely filled were selected for reconstruction and analysis. Two representative examples are shown in Fig. 2, A and B. Recovered cells were multipolar, giving off three to four primary dendrites that subsequently branched diffusely throughout the nucleus. Neurobiotin-labeled processes were typically found within one to three serial sections. Dendrites were preferentially oriented in the transverse plane with the dendritic tree of deeper neurons being more radially symmetric while that of cells located more superficially in the nucleus were necessarily constrained dorsomedially by the ventricular surface. The axon originated at the soma or proximally on a primary dendrite (arrowheads in Fig. 2, A and B) and coursed dorsolaterally without branching. No evidence of axon collaterals was found in any histological material.

Somata of filled cells were roughly elliptical, and exhibited aspect ratios (long axis/short axis) ranging from 1.2 to 2.8. Soma diameters [(long axis + short axis)/2] ranged from 16.5 to 25.1 μm. A comparison of the soma area of neurobiotin-filled cells to that measured in a sample of HRP-labeled material is shown in Fig. 2C. Although there is gross overlap of the two histograms, the distribution of neurobiotin-filled cells peaks at ~350 μm² in contrast to a broad distribution of soma areas from 300 to 600 μm² in the HRP material. While this is suggestive of a bias in the intracellular recordings toward smaller members of the Ncl. IV population, the means of the two distributions were not statistically different (P > 0.05).

A quantitative summary of soma and primary dendrite morphology is given in Table 1. The quantified parameters include the number of primary dendrites and the ratio of soma diameter to mean primary dendrite diameter. These have been tabulated in other studies, and permit a comparison of motoneuron morphology in the turtle with that of other species (see DISCUSSION).

**Fig. 2.** A and B: reconstructions of trochlear motoneurons that were intracellularly filled with neurobiotin. Cells positioned more deeply in the nucleus, such as the example shown in B, tended to exhibit more radially symmetric dendritic trees compared with superficially located cells such as that in A. A: axons. C: soma sizes of neurobiotin-filled cells (NB) compared with that of cells labeled by retrograde transport of HRP. Although it appears that the intracellular sample was biased toward smaller cells, the means of the 2 distributions did not differ statistically. D: depth profile of the 29 intracellular recordings obtained in the study. All but 2 recordings were obtained within 300 μm of the ventricular surface.
whole cell recordings were obtained deeper than 300 µm, but their physiological data were discarded. All recordings were included in the morphological analysis in the preceding text, but their physiological data were discarded. All recordings were obtained within 500 µm of the ventricular surface and only two recordings were obtained deeper than 300 µm (Fig. 2D).

A demonstration of simultaneous intracellular and suction electrode recording of a single trochlear motoneuron is shown in Fig. 3B. Action potentials evoked by injecting depolarizing current through the patch pipette (Fig. 3B; Ncl. IV) are clearly identifiable in the MUA recorded by the suction electrode (Fig. 3B; nIV). There is a brief (<1 ms) delay between corresponding spikes in the intracellular and extracellular recordings that is not apparent at this time scale. Because of the short conduction distance resulting from the intracranial placement of the suction electrode, no attempt was made to measure conduction velocity.

Unambiguous identification of individual spikes in the MUA was possible because of the low level of background activity exhibited by the turtle brain stem preparation. However, spikes from most motoneurons were not as prominent in the extracellular MUA as the example shown in Fig. 3B, and in approximately half of the intracellular recordings, spikes elicited by current injection were not apparent in the MUA at all. It seems unlikely this resulted from spike conduction failure given the short conduction distance and absence of branch points. Additionally, in recordings where spikes evoked by depolarizing current injection could be clearly identified in the extracellular record, conduction failures were never observed (n = 2,440 spikes). A simpler explanation is suggested by the ~30-fold variation in the sizes of axons making up the trochlear nerve (Fig. 1G): whereas prominent spikes in the MUA were recorded from large axons, spikes in smaller axons were likely smaller in amplitude and less likely to be detected in the ongoing, albeit low-level, background MUA.

**Intrinsic membrane properties**

CURRENT-VOLTAGE RELATIONS. Examination of the response to half-second hyperpolarizing pulses suggested that two classes of motoneurons occur in Ncl. IV. Such classification was based on the presence of anomalous rectification in a subset of recordings, revealed by a characteristic “sag” in membrane potential in response to hyperpolarizing current injection (Spain et al. 1987). An example of a cell exhibiting such rectification is shown in Fig. 4A. The amplitude of the membrane potential sag increased with current amplitude; however,
in most cells of this type, the rectification was apparent to some degree at all current levels. In some trials, rebound spikes were observed at the end of the current pulse (data not shown). The current-voltage dependencies of this rectification were not examined in detail in the present study, but it appeared qualitatively similar to the H-type current we previously examined in the basal optic nucleus of the turtle (Kogo and Ariel 1997), and which has been described in rat abducens motoneurons (Russier et al. 2003).

A representative example of a Ncl. IV cell in which anomalous rectification is not apparent is shown in Fig. 4B. No voltage sag occurs in response to any hyperpolarizing current injection even though the current steps shown here are larger than those applied to the cell in Fig. 4A.

Larger currents were required to evoke spiking in cells that did not exhibit rectification, as can be seen by comparing the current records in Fig. 4, A and B. This suggested that the two cell classes might also exhibit systematic differences in cell impedance (Rm). An analysis of this correspondence is shown in Fig. 4C in which Rm is plotted against the amplitude of the voltage sag for all 29 cells in the study. A group of low-impedance, low-sag-amplitude data points cluster at the lower left of the plot while a second group of higher-impedance data points cluster at upper right. All but three of the higher-impedance cells exhibited relatively large hyperpolarization sags.

For discussion purposes, the two apparent classes of Ncl. IV motoneurons will be referred to as type-A and type-B, with type-A cells exhibiting higher impedance and more pronounced rectification. The two groups are indicated in Fig. 4C. Of 29 recordings, 13 were classified as type-A and 13 as type-B. Three higher-impedance, lower-sag-amplitude cells did not fit into either group and were left unclassified.

The mean impedances of type-A and type-B cells were 123.0 ± 11.0 and 21.8 ± 2.5 MΩ, respectively, and were statistically different (P < 0.001). These impedances are substantially higher than those reported for trochlear neurons in other species but are within the range of values reported for spinal motoneurons in the turtle (Russo and Hounsgaard 1996).

SPIKE AHP. Type-A and type-B cells exhibited systematic differences in other intrinsic membrane properties such as spike AHP as shown in Fig. 5. In these recordings, action potentials were evoked antidromically with the cell at its resting membrane potential. In all 29 recordings, nerve stimulation evoked antidromic spikes in an all-or-nothing fashion at a single latency. No evidence of recurrent axon collaterals was found, such as an emergence of additional spikes with increasing stimulus-intensity or the presence of excitatory postsynaptic potentials (EPSPs) when subthreshold stimulation was used.

Three types of AHPs were observed. The AHP in 13 cells consisted of a single slow component (Fig. 5A) usually separated from the falling phase of the spike by an inflection in the membrane potential (arrowhead in Fig. 5A). In some cells exhibiting this type of AHP, no inflection was present and the transition from spike to AHP was smooth. In none of the 13 cells was an afterdepolarization apparent. The single slow AHP was of relatively long duration, with hyperpolarization persisting for 70–100 ms before the membrane potential returned to its resting value.

**FIG. 4.** A and B: current-voltage relationships. Top traces in each panel show response to injection of hyper- or depolarizing current. Approximately half of the cell sample exhibited anomalous rectification, characterized by an initial sag in membrane potential in response to hyperpolarization, such as that shown in A. Cells with anomalous rectification also tended to exhibit higher impedance (note differences in current levels in A and B). C: log-log plot of impedance vs. anomalous rectification for all 29 cells in the study. The ordinate is the amplitude of the initial sag of the membrane potential in response to hyperpolarizing current injection divided by the amplitude of the current pulse. The clustering of the data suggests a classification of Ncl. IV motoneurons into 2 groups: type-A cells that have high-impedance and exhibit rectification and type-B cells that have low-impedance and exhibit little or no rectification. Three cells do not fit this scheme and were left unclassified.
The AHP in 14 cells exhibited both fast and slow components (Fig. 5B; arrowhead and double arrowhead, respectively). The amplitude of these components varied somewhat from cell to cell, but the fast component usually terminated while membrane potential was still positive relative to its resting value. The onset of the slower AHP was usually preceded by a distinct afterdepolarization (Fig. 5B; open arrow) after which the cell underwent a hyperpolarization that lasted 30–60 ms.

Two cells exhibited an AHP consisting of a single fast component (Fig. 5C). This was similar in latency and time course to the fast AHP shown in Fig. 5B but was of much larger in amplitude, exceeding 10 mV in both instances. A slow component was not observed in these cells. Instead membrane potential returned monotonically to resting value at the termination of the fast AHP.

There was a general correspondence between AHP and cell type although exceptions were noted. All except two type-A cells exhibited single AHPs such as that shown in Fig. 5A. All except one type-B cell exhibited double AHPs such as that shown in Fig. 5B. One type-A and one type-B cell exhibited single fast component AHPs of Fig. 5C. Additionally, one type-A cell exhibited an AHP with clear fast and slow components rather than a single deep AHP.

Three parameters were used to quantify the slower AHP in type-A and type-B cells for comparison (see inset in Fig. 5): the maximum hyperpolarization obtained (V_AHP), amplitude relative to resting potential (V_AHP - V_REST) and postspike latency at maximum hyperpolarization. V_AHP was not statistically different in type-A and type-B cells. However, AHP amplitude was larger in type-A cells (8.7 ± 1.3 vs. 2.7 ± 0.6 mV; P < 0.001) as was duration, with a mean value of 32.4 ± 3.2 ms measured for the type-A single AHP compared with 25.1 ± 1.2 ms for the slow component of type-B cells (P < 0.05).

SPIKE FREQUENCY ADAPTATION. Differences in frequency/current (f/I) relationships and spike frequency adaptation (SFA) were also observed between type-A and type-B cells. The initial slope of the f/I curve for a given cell was computed by injecting a 500-ms pulse of just-suprathreshold depolarizing current and measuring the resulting steady-state firing frequency. Repeating this 500-ms pulse using two higher current levels then gave three data points that were used to compute the primary f/I slope (data not shown). This value was significantly lower for type-B cells (17.0 ± 3.1 vs. 63.3 ± 8.5 Hz/nA; P < 0.001).

Spike frequency adaptation was quantified by comparing the instantaneous firing rate evoked at the onset and termination of the 500-ms depolarizing currents that were used to obtain the I-V relations shown in Fig. 4. Firing frequency in type-A cells tended to increase slightly during sustained depolarization (see Fig. 4A) although the effect was generally not pronounced. The relatively uniform evoked spiking shown in Fig. 4B was characteristic of some type-B cells. However, most type-B cells exhibited substantial spike frequency adaptation. This usually took the form of transient higher frequency firing occurring at the onset of depolarization that rapidly decreased to a lower rate persisting for the remainder of the response. An example of this is shown in Fig. 4B. The initial spike frequency in this type-B cell was ~125 Hz, which decreased to 44 Hz within 100 ms. Instantaneous spike frequency at the termination of the current pulse was 33 Hz. The trends in spike frequency adaptation shown in these representative examples were significant when examined across the two cell populations.

Intrinsic properties of the 29 cells included in the study are summarized in Table 2. In addition to the differences of type-A and type-B cells described in the preceding text, type-A cells also exhibited wider spikes than type-B (width at half-amplitude: 0.99 ± 0.05 vs. 0.74 ± 0.03 ms; P < 0.001). Spike amplitude and resting membrane potential were not statistically different in the two groups.

Histological material was examined for morphological properties of filled motoneurons that differed in the two physiologically defined cell classes. No such features could be identified. Type-A and type-B cells were of similar size and exhibited a similar number of primary dendrites and S:D ratios. Additionally, the depth distribution of recordings from the two cell types overlapped, suggesting that the populations are not segregated in the nucleus.
**Rotation-evoked responses**

Horizontal rotation evoked robust unit and population responses, the latter being indistinguishable from those recorded in our previous study. These exhibited flat gains, and increased phase lag with increasing rotation frequency (data not shown; see Fig. 7 in Jones and Ariel 2006). To a first approximation, population responses were aligned with the peak of contraversive table velocity (e.g., responses in the left trochlear nerve were aligned with rightward velocity). As the phase shift imposed by the eye plant during the low rotation frequencies used here is probably negligible (Skavenski and Robinson 1973), such results predict evoked eye movements that align with head velocity rather than head position—an apparently incorrect compensatory response. However, responses exhibiting similar phase characteristics have been observed in other nonmammalian species when deprived of visual feedback (Pantle and Dieringer 1998; Rohrregger and Dieringer 2002), perhaps reflecting the subsidiary role vestibular input plays in guiding ocular behaviors in these species (Dieringer and Precht 1986). As such, observing responses aligned with table velocity is not unexpected in the present study given that the eyes were not present in the in vitro preparations.

A representative example of the response of a trochlear motoneuron to horizontal rotation is shown in Fig. 6. Table rotation evoked a suprathreshold response in all four cycles of motion. Details of the response in one cycle are shown at bottom left of Fig. 6. The three pairs of numbers below the traces indicate instantaneous spike frequency and table velocity at the onset, peak, and termination of the response. In this cell, a variation in table velocity of ~20% produced a ~110% variation in spike frequency, with a threshold firing rate of 12.5 Hz evoked at 45°/s increasing to 26.3 Hz at the peak table velocity of 55°/s. Spiking frequency then decreased to 8.0 Hz before the response terminated. Spike threshold exhibited asymmetry with respect to table velocity with unequal values noted at onset (45°/s) and termination (48°/s) of the response.

Most cells exhibited a less-pronounced spike frequency modulation than the twofold variation demonstrated by the example shown in Fig. 6A. A summary of this effect is shown in Fig. 6B for 14 cells. Each entry depicts the mean and range of firing frequencies for one motoneuron evoked using 1/6-Hz stimulation with peak velocity adjusted to evoke spiking in four consecutive cycles. The results are ordered left-to-right by increasing cell impedance. The left-most entry is the cell shown in Fig. 6A, which exhibited a minimum, mean and maximum firing rate of 2.3, 12.1, and 26.9 Hz, respectively. This was representative of the greater modulation observed in lower-impedance cells. In higher-impedance cells, spiking was evoked using lower table velocities; however, the firing frequency was more constant once spike threshold was reached. This type of response was characteristic of type-A cells, which appear to the right of the 50 MΩ cutoff indicated in Fig. 6B. In some of these cells, evoked spiking varied only by 1 or 2 Hz from its mean firing rate. These impedance dependencies were found to be statistically significant when tested using linear regression (mean spike frequency vs. $R_m$; $r = −0.61, P < 0.05$; variance of spike frequency vs. $R_m$; $r = −0.51, P < 0.05$).

Table 2: Intrinsic cell properties

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<th>All (29)</th>
<th>Type-A (13)</th>
<th>Type-B (13)</th>
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<tr>
<td>$R_m$ (MΩ)</td>
<td>74.7 ± 10.5</td>
<td>123.0 ± 11.0</td>
<td>21.8 ± 2.5</td>
<td>**</td>
</tr>
<tr>
<td>$V_{REST}$ (mV)</td>
<td>−59.4 ± 1.3</td>
<td>−59.5 ± 1.5</td>
<td>−61.4 ± 1.9</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Spike amplitude (mV)</td>
<td>83.6 ± 2.6</td>
<td>83.6 ± 4.1</td>
<td>84.2 ± 4.1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Spike width (ms)</td>
<td>0.86 ± 0.04</td>
<td>0.99 ± 0.05</td>
<td>0.74 ± 0.03</td>
<td>**</td>
</tr>
<tr>
<td>Primary $f_t$ (Hz/mA)</td>
<td>48.2 ± 7.0</td>
<td>63.3 ± 8.5</td>
<td>17.0 ± 3.1</td>
<td>**</td>
</tr>
<tr>
<td>Spike adaptation (%)</td>
<td>−4.8 ± 9.5</td>
<td>+17.9 ± 8.9</td>
<td>−52.4 ± 13.0</td>
<td>**</td>
</tr>
<tr>
<td>$V_{AHP}$ (mV)</td>
<td>−66.2 ± 0.8</td>
<td>−68.4 ± 1.0</td>
<td>−65.3 ± 1.3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Amplitude $A_{AHP}$ (mV)</td>
<td>5.6 ± 0.9</td>
<td>8.7 ± 1.3</td>
<td>2.7 ± 0.6</td>
<td>**</td>
</tr>
<tr>
<td>Duration $A_{AHP}$ (ms)</td>
<td>28.6 ± 2.8</td>
<td>32.4 ± 3.2</td>
<td>25.1 ± 1.2</td>
<td>*</td>
</tr>
</tbody>
</table>

Significant differences between type-A and type-B cells are indicated in the right-most column (*$P < 0.05$; **$P < 0.001$). Parentheses enclose number of cells. The last three rows refer to features of the slow afterhyperpolarization (AHP) defined in Fig. 5. These were measured in 11 type-A cells, 11 type-B cells, and 3 unclassified cells. Values are means ± SD.
RECRUITMENT. Additional characteristics of the rotation-evoked response are shown in Fig. 8. In Fig. 8A, the phase of the onset of spiking is plotted as a function of impedance for 13 cells. In each case, the response was obtained using a 1/6-Hz stimulus with the same peak velocity. Each data point represents the mean phase of the onset of spiking averaged across four cycles of motion (see inset in Fig. 8A). There is a positive correlation of phase with cell impedance ($r = 0.80$, $P < 0.005$), indicting that higher-impedance cells are recruited earlier in the response. This was not due to systematic phase...
differences in synaptic drive, as the phase of the underlying population EPSP and input impedance were uncorrelated (Fig. 8B; \( r = -0.49, P > 0.05 \)). These data suggest there may be an orderly recruitment of trochlear motoneurons according to increasing cell size as has been previously demonstrated in other motor systems.

INHIBITION. The trochlear nucleus receives inhibitory projections (Melendez-Ferro et al. 2000; Spencer et al. 1989) that may act in concert with excitatory drive to shape the motoneuron response. A demonstration of putative inhibition of a Ncl. IV motoneuron is shown in Fig. 9A. The top trace depicts the rotation-evoked response in a cell that was brought to threshold by injecting constant depolarizing current (note spiking at end of record in the absence of table motion). This spiking persists during rightward table motion. However, during leftward motion, there is suppression of the depolarization-evoked spiking, suggesting there is inhibition of the motoneuron during motion in this direction.

Conductance changes that occurred during table motion were examined in five cells by injecting brief hyperpolarizing current pulses during rotation (Fig. 9B). The cell shown in this example had a resting impedance of 21 MΩ. Injecting current pulses of 0.5 nA resulted in membrane deflections of ~7–10 mV that varied systematically during rotation (Fig. 9B; top trace). When these voltage deflections are converted to impedance (Fig. 9B; bottom trace), it can be seen that table rotation resulted in a biphasic modulation of cell impedance. One relative minimum was roughly aligned with peak leftward velocity, as would be consistent with a shunting inhibition that would suppress spiking during this half of the motion cycle. A second impedance minimum was aligned with peak rightward motion, presumably reflecting glutamatergic activation of cation channels during the excitatory phase of the response (Babalian et al. 1997; Straka and Dieringer 1993). The impedance changes during left- and rightward motion were comparable in amplitude and were equal to ~25% of the baseline cell impedance. A similar pattern was observed in all five cells tested using this protocol with a mean impedance decrease of 33.0 ± 5.9% measured during leftward motion.
This provides an opportunity for a comparison of the turtle oculomotor system with that of other species, especially those species exhibiting more complex visual behaviors.

**Morphology of the turtle Ncl. IV**

The turtle trochlear nucleus contains ~650 motoneurons, inferred from axon counts of the trochlear nerve. The trochlear nucleus consists of ~100 motoneurons in the frog (Knöpfel et al. 1984), 400 in rat (Eberhorn et al. 2006), and 900 in rabbit (Miyazaki 1985; Murphy et al. 1986). Thus the turtle Ncl. IV population is intermediate between rat and rabbit; this is concomitant with the relative eye size in these animals. Additionally, ocular motoneurons in these species exhibit a systematic increase in soma size and dendritic complexity. In the rat, a bimodal distribution of soma size has been reported with mean diameters of ~18 and 13 μm measured for trochlear motoneurons supplying singularly innervated and multiply innervated extraocular muscles, respectively (Eberhorn et al. 2006). Trochlear motoneurons in the guinea pig have a mean soma diameter of ~27 μm and five to nine primary dendrites, and those in rabbit have a mean diameter of ~40 μm and six to nine primary dendrites (Evinger et al. 1987). The 20- to 25-μm soma diameters observed in the turtle Ncl. IV are consistent with these observations, again being intermediate between rat and rabbit.

Relative to mammals, turtle Ncl. IV motoneurons have fewer primary dendrites for their size. Motoneurons in the rat abducens and oculomotor nucleus have five to eight primary dendrites (Durand 1989a,b) compared with the three to four found here in the turtle Ncl. IV. This difference may reflect simpler processing demands of the turtle oculomotor system relative to mammalian species as the eyes of the turtle are not strongly yoked (Ariel 1990). Alternatively, this may reflect an evolutionary divergence of warm- and cold-blooded animals. Whereas in heterotherms there is a trend toward augmenting the number of dendrites rather than their diameters, the opposite is observed in poikilotherms (see discussion in Evinger et al. 1987). Such a hypothesis is supported by the S:D ratios reported in Table 1, with the mean value of ~3.8 in the turtle approaching the lower bound of values reported in a number of mammalian species (Durand 1989a,b; Evinger et al. 1987) yet somewhat larger than that reported in goldfish (Graf and McGurk 1985) and flounder (Graf and Baker 1985). It is interesting to note that the NIV motoneurons in the turtle are smaller and exhibit less dendritic branching than those of the spinal cord (30–60 μm diameter, 3–6 primary dendrites; Hougsaard et al. 1988). A similar pattern has been noted in the cat and has been attributed to the greater demands of synaptic integration in the spinal cord compared with that of oculomotor processing (Grantyn and Grantyn 1978).

**Responses to head rotation**

**PUSH-PULL SYNAPTIC INPUTS.** The suppression of Ncl. IV motoneuron output during ipsiversive motion shown in Fig. 9A (ipsiversive relative to the cranial nerve, e.g., of the left trochlear nerve during leftward rotation) is consistent with a “push-pull” configuration of the VOR as has been consistently demonstrated in other species (Blanks et al. 1978; Graf et al. 1997; Highstein 1971; Precht and Baker 1972; Uchino et al. 1978). This is functionally relevant to ocular behavior as...
activity in the motoneuron pool during this phase of rotation would act against the contraction of antagonistic muscles in the orbit. The present results suggest the participation of an active, presumably GABAergic (Melendez-Ferro et al. 2000; Spencer et al. 1989) inhibition in this process and not merely the cessation of excitatory drive. This is supported by the results of Fig. 9, which shows both a silencing of spiking evoked by depolarizing current injection during leftward rotation (Fig. 9A) and an accompanying conductance modulation (Fig. 9B).

Alternate interpretations of this data such as a depolarization block seem unlikely, as such a mechanism would be expected to also silence spiking during excitatory (i.e., rightward) rotation. A similar conductance modulation has been demonstrated in the turtle spinal cord in studies of the fictive scratch reflex (Alaburda et al. 2005) and has been attributed to phasic inhibitory input. However, inhibition in turtle ocular motoneurons appears to be weak by comparison. The mean 33% conductance increase observed here in Ncl. IV is

![Figure 9](https://example.com/image.png)
substantially smaller than that reported in spinal motoneurons, where some cells more than double their baseline conductance.

**IMPEDEANCE-RELATED FEATURES.** Although differences in rotation-evoked activity of type-A and type-B cells were not noted per se, features of the response did correlate with cell impedance. The first of these was recruitment order, with spiking in higher-impedance cells occurring earlier in the response than those of lower-impedance cells. Mechanisms that underlie the orderly recruitment of motoneurons have been extensively investigated in the skeletal system (Binder and Mendell 1990), wherein motoneuron size has been proposed as being the primary determinant. However, whether the size principle operates in the ocular motor system remains unclear. Many studies have found the correlation between measures of ocular motoneuron size and recruitment order to be weak or nonexistent (Delgado-Garcia et al. 1986; Fuchs et al. 1988; Pastor and Gonzalez-Foreto 2003; Pastor et al. 1991). Yet a relatively strong correlation is observed in the data shown in Fig. 8. It has been suggested that intrinsic membrane properties and not literal cell size may play the definitive role in determining recruitment order (Gustafsson and Pinter 1985). Such properties would be included in a direct measurement of cell impedance as performed in the current study but not when indirect measurements of cell size such as antidromic latency or EMG amplitude are employed as has been performed in studies in which a weaker correlation was observed.

A second impedance-related feature of the rotation-evoked response was that higher-impedance cells tended to exhibit a more restricted spike frequency modulation (Fig. 6B). This effect may be related to the physical or electrotonic compactness of these cells. Increased excitation of a neuron can be accomplished by increasing the number of active synaptic inputs and the firing frequency of each. A physically smaller neuron may have fewer synaptic contacts, thus one of the two mechanisms that contribute fine-resolution modulation of spiking frequency is compromised. Alternatively, the limited range of spiking frequency in these cells may only be coincidentally related to impedance. Cells with higher impedance (type-A) exhibited a more pronounced AHP, which has been demonstrated to regulate firing frequency in spinal motoneurons (Gustafsson 1974; Kernell 1965; Zengel et al. 1985). A possible interpretation, then, is that the high-impedance of type-A motoneurons resulted in these cells being brought to spike threshold consistently and early in the response, with intrinsic properties of the cell then acting to limit the modulation of the spike train output.

**Significance of two motoneuron classes**

A consistent finding of intracellular studies of motoneurons in the oculomotor, trochlear or abducens nucleus is that these cells can be segregated according to their intrinsic properties (Baker and Precht 1972; Durand 1989a,b; Grantyn and Grantyn 1978; Gueritaud 1988). In a recent report, Nieto-Gonzalez and co-workers were able to categorize ~90% of motoneurons in the rat oculomotor nucleus into two groups that bear a strikingly similarity to our type-A and type-B in terms of AHP, spike width, spike frequency adaptation, and dynamic range (Nieto-Gonzalez et al. 2007). A nearly identical classification scheme has been applied to neurons in the vestibular nucleus (Serafin et al. 1991)—cells that make up the immediate premotor component of the VOR. This raises the possibility that motoneurons may be matched to properties of their synaptic precursors, perhaps to maintain “channels” in the vestibulo-motor transformation (Beraneck et al. 2007; Lisberger et al. 1983) or to the separate processing of velocity- and acceleration-like signals that are present in canal afferents (Brichta and Goldberg 1996).

Alternatively, motoneuron intrinsic properties may be related to contractile properties of the innervated muscle unit. Two separate subpopulations of trochlear motoneurons target singly innervated (i.e., twitch) or multiply innervated (non-twitch) muscle fibers in both in both monkey and rat (Buttner-Ennever et al. 2001; Eberhorn et al. 2006). This suggests a possible anatomical substrate for the two cell classes found presently, although a segregation of type-A and type-B cells in the nucleus, as was reported in mammals, was not observed. Although trochlear motoneurons innervating twitch and non-twitch motor units have been distinguished in intracellular recordings made in cats, their intrinsic properties were not reported (Nelson et al. 1986). Indeed much of our current knowledge of motoneuron intrinsic properties has come from in vitro preparations, which necessarily rely on indirect arguments to relate cell properties to those of the extraocular muscles. An advantage of the turtle brain stem preparation is that it may be possible to include muscles of the orbit in future experiments so that such correlations can be tested directly.

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**REFERENCES**


