Published in final edited form as:

Neuroscience. 2011 January 13; 172: 494–509. doi:10.1016/j.neuroscience.2010.10.038.

ELECTROPHYSIOLOGICAL PROPERTIES OF MORPHOLOGICALLY-IDENTIFIED MEDIAL VESTIBULAR NUCLEUS NEURONS PROJECTING TO THE ABDUCENS NUCLEUS IN THE CHICK EMBRYO

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Abstract

Neurons in the medial vestibular nucleus (MVN) show a wide range of axonal projection pathways, intrinsic firing properties, and responses to head movements. To determine whether MVN neurons participating in the vestibulocular reflexes (VOR) have distinctive electrophysiological properties related to their output pathways, a new preparation was devised using transverse brain slices containing the chicken MVN and abducens nucleus. Biocytin Alexa Fluor was injected extracellularly into the abducens nucleus so that MVN neurons whose axons projected to the ipsilateral (MVN/ABi) and contralateral (MVN/ABc) abducens nuclei were labeled selectively. Whole-cell, patch-clamp recordings were performed to study the active and passive membrane properties, sodium conductances, and spontaneous synaptic events in morphologically-identified MVN/AB neurons and compare them to MVN neurons whose axons could not be traced (MVN/n). Located primarily in the rostral half of the ventrolateral part of the MVN, MVN/AB neurons mainly have stellate cell bodies with diameters of 20–25 μm . Compared to MVNn neurons, MVN/ABi and MVN/ABc neurons had lower input resistances. Compared to all other MVN neuron groups studied, MVN/ABc neurons showed unique firing properties, including type A-like waveform, silence at resting membrane potential, and failure to fire repetitively on depolarization. It is interesting that the frequency of spontaneous excitatory and inhibitory synaptic events was similar for all the MVN neurons studied. However, the ratio for miniature to spontaneous inhibitory events was significantly lower for MVN/ABi neurons compared to MVN/n neurons, suggesting that MVN/ABi neurons retained a larger number and/or more active inhibitory presynaptic neurons within the brain slices. Also, MVN/ABi neurons had mEPSCs with slower decay time and half width compared to MVN/n neurons. Altogether, these findings underscore the diversity of electrophysiological properties of MVN neuron classes distinguished by axonal projection pathways. This represents the first study of MVN/AB neurons in brain slice preparations and supports the concept that the in vitro brain slice preparation provides an advantageous model to investigate the cellular and molecular events in vestibular signal processing.

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Keywords

VOR neurons; spike firing pattern; spontaneous synaptic events; brain slice preparation; biocytin dye injections; whole-cell recordings

Among the four main vestibular nuclei in vertebrates, the medial vestibular nucleus (MVN) is the one most extensively studied (for review, see Paterson et al., 2004). The MVN contains a wide diversity of neuron classes which project to the oculomotor nuclei, spinal cord, cerebellum, thalamus, contralateral vestibular nuclei, or function as interneurons (for review, see Straka et al., 2005; Highstein and Holstein, 2006). MVN neurons involved in the vestibuloocular reflexes (VOR) are further separated by their axonal pathways into those projecting to the abducens nucleus and controlling horizontal eye movements, and those projecting to the oculomotor and trochlear nuclei and controlling vertical eye movements. Collectively, MVN neurons show a high degree of plasticity in response to changing visual images, injury and disease, so that MVN neurons provide a popular cellular model to explore neuronal plasticity. However, the exact roles that specific MVN neuron classes play in normal signal processing and in the plasticity induced after lesions have hardly been explored.

Based on electrophysiological recordings, mammalian MVN neurons are distinguished routinely according to their spike waveform and afterhyperpolarizations (AHP) primarily as type A and type B neurons (Him and Dutia, 2001). However, the classification does not take into account diversity in their afferent inputs (e.g., Cox and Peusner, 1990) and efferent outputs (e.g., Cuccurazzu et al., 2007). Studies on other systems have shown that neurons which vary in inputs, outputs, and/or morphology process signals differentially (e.g., Feng et al., 1994; Ostapoff et al., 1994; Cossart et al., 2006). Unfortunately, the MVN has no laminar organization to assist in sorting out the neuron classes, and MVN neurons lack distinctive morphologies related to their inputs or outputs. Consequently, few electrophysiological studies have recorded from morphologically-identified vestibular nuclei neurons (Shao et al., 2009), and only exceptional studies target specific MVN neuron classes to study signal processing (Takazawa et al., 2004; Sekirnjac and du Lac, 2006; Bagnall et al., 2007; Malinvaud et al., 2010).

In the present study, MVN neurons projecting to the abducens nucleus (MVN/AB) were selected for recordings based on the presence of retrograde biocytin labeling after the dye was injected extracellularly into the abducens nucleus in transversely sectioned brain slices. A map of the chicken MVN was used to set the level of the brainstem containing both MVN neurons projecting to the abducens nucleus and the abducens nucleus itself (Gottesman-Davis and Peusner, 2010). A two-step, biocytin injection protocol was applied, with retrograde neuronal labeling followed by anterograde neuronal labeling during the recordings. Since this is the first study performing whole-cell patch-clamp recordings on chick MVN/AB neurons in brain slice preparations, a preliminary step was taken to record the basic input/output properties of MVN/ABi and MVN/ABc neurons and to compare them to other MVN neurons whose axons could not be traced (MVN/n). Passive membrane properties, spontaneous and evoked spike firing, and the underlying sodium conductances, as well as spontaneous synaptic events were recorded. After the recordings, all the brain slices were fixed and processed for biocytin visualization. All biocytin-injected and recorded MVN neuron cell bodies were visualized and their axons traced from their cell bodies of origin to either the ipsi- (MVN/ABi) or contralateral abducens nucleus (MVN/ABc), other ipsilateral (MVN/i) and contralateral (MVN/c) targets, or could not be traced completely (MVN/n). The results indicated that MVN/ABc neurons were distinguishable from other MVN neuron groups by the prevalence of type A-like firing pattern, lack of spontaneous

spike activity at resting membrane potential, and failure to fire repetitively on depolarization. MVN/ABi neurons were distinguished from other MVN neuron groups by their lower ratio of miniature to spontaneous inhibitory events, which suggests that more inhibitory presynaptic neurons and/or more active neurons were retained in the brain slices. Altogether, this study supports the concept that profiling vestibular nuclei neuron classes by their axonal outputs is a useful approach to establish a link between cellular electrophysiology and circuit function. Finally, this study presents fundamental electrophysiological properties of MVN/AB neurons in brain slice preparations of chick embryos, which provides an important first step for future studies on the development of MVN neuron and their plasticity after vestibular deafferentation.

Experimental procedures

Experimental animals

Experiments were performed on 16 day old white Leghorn chick embryos (E16) (*Gallus gallus*), purchased from CBT Farms (Chestertown, MD) as fertilized eggs and incubated in a laboratory egg incubator (GQF Manufacturing Company, model 1502, Savannah, GA) (n=102). Animal protocols were approved by the Institutional Animal Care and Use Committee of the George Washington University. Chick embryos were staged according to the Hamburger and Hamilton staging criteria (1951).

E16 was selected for experiments here, since it is known that chick tangential principal cells, a major group of second-order vestibular projection neurons in avians, acquire many basic morphological and electrophysiological properties at this age, and the neurons are exceptionally healthy in brain slice preparations, allowing long-term electrophysiological recordings (see Peusner and Giaume, 1997; Shao et al. 2003, 2006). In addition, the axonal pathways of embryonic vestibular nuclei neurons are considerably shorter than those in hatchlings, which facilitates tracing labeled axons to their target nuclei.

Brain slice preparation and solutions

Embryos were removed from the eggshell and decapitated. Under a dissecting microscope, the brainstem was dissected free from the cranium, periodic capsule, and cerebellum in a solution of chilled (4°C), oxygenated artificial cerebrospinal fluid (ACSF). Transverse sections (350 μ m) of the medulla oblongata were cut with a razor blade (Feather blue blades; Ted Pella Inc., Redding, CA) mounted on a microslicer (Leica VT1000S, Leica Microsystems CMS GmbH, Wetzlar, Germany) (Shao et al., 2003).

ACSF contained (in mM): 126 NaCl, 3.5 KCl, 1.2 NaH₂PO₄, 1.3 MgCl₂, 2 CaCl₂, 26 NaHCO₃, and 10 D-glucose. ACSF was bubbled with 95% O₂ and 5% CO₂ to maintain the pH at 7.2-7.4, and the osmolarity was adjusted to 310-320 mOsm.

Extracellular dye injections

The levels of the brain slices selected for recordings were determined by reference to a two-dimension map and a three-dimensional reconstruction of the chicken MVN (Gottesman-Davis and Peusner, 2010). Throughout most of its anteroposterior extent, the MVN is subdivided by the crossed cochlear bundle into dorsomedial (MVN_{DM}) and ventrolateral parts (MVN_{VL}), which are distinct in transverse sections. MVN_{VL} and MVN_{DM} merge at levels anterior and posterior to the crossed cochlear bundle. In the previous study, the location of VOR neuron cell bodies within the MVN was mapped by subdividing the MVN into five anteroposterior levels from posterior (1.0) to anterior (5.0). MVN/AB neurons are distributed from the level of the posterior tip of the crossed cochlear bundle (2.25) through the level of the anterior tip of nucleus laminaris (4.75). Brain slices, containing both the

MVN and abducens nucleus, were selected for recordings from the level of the central tangential nucleus (3.25) through the level of the anterior tip of the nucleus laminaris (4.75). MVN/AB neurons are located bilaterally, primarily within the MVN_{VL}, with few neurons situated in the MVN_{DM} or the MVN anterior to the disappearance of the crossed cochlear bundle (Gottesman-Davis and Peusner, 2010). From each E16 chick brainstem, 2-3 transverse brain slices of 350 μm thickness met the above criteria and were available for recordings. The level of the brain slice was identified by the structures present at the mid-point. At E16, the abducens nucleus measures about 300 μm mediolaterally and 400 μm dorsoventrally at the level of anterior tangential nucleus (3.75).

Ten minutes after sectioning, the brain slices were placed on filter paper (#2, Whatman LTD, Kent, England) dampened with oxygenated ACSF. Under a dissecting microscope (10x; Wild Heerbrugg M5A, Gais, Switzerland), the abducens nucleus on one side was punctured with the tip of a 33 gauge syringe needle. Crystals of biocytin Alexa Fluor 488, or 647 (Invitrogen, Carlsbad, CA) were placed on the tip of a second needle and inserted into the punctured abducens nucleus (see Bagnall et al., 2007). This procedure took 1-2 minutes, followed by transfer of the brain slice immediately to a Petri dish containing room temperature, oxygenated ACSF. Brain slices were incubated at least 30 minutes before transferring to a recording chamber.

After the recordings, the brain slices containing biocytin injected and recorded neurons were fixed and processed. The injection sites were characterized using conventional fluorescence and confocal imaging (see below). From a total of 122 extracellular biocytin injections targeting the abducens nucleus, most injections (96/122) were confined to the abducens nucleus, except for 9 which extended outside the nucleus and 17 which missed the nucleus completely. MVN neurons projecting to the abducens nucleus (MVN/AB) were included in the study only if the biocytin injection appeared to be restricted to the abducens nucleus.

Electrophysiological techniques

Brain slices were incubated in a small, glass bottom recording chamber (180 μl , Warner Instruments, Hamden, CT) mounted on a fixed-stage, upright microscope (Zeiss Axioskop FS2, Jena, Germany) equipped with differential interference contrast optics and a 40x water-immersion lens (NA, 1.0). Brain slices were held in place using a tight-fitting, U-shaped, flattened platinum wire with thin nylon threads glued in parallel. Preheated ACSF was perfused through the recording chamber at a rate of 2-3 ml/min, and ACSF was maintained in the recording chamber at 30-31°C using a temperature controller (TC324B, Warner Instruments). Visualization of the recorded neuron and pipet was achieved using an infrared light source (filter, 770 nm), detected by an infrared-sensitive CCD camera (Vidicon C2400, Hamamatsu, Hamamatsu City, Japan) and observed on a monitor (Sony, Tokyo, Japan). Image contrast and shading were adjusted using a camera controller (C2400, Hamamatsu). Fluorescent labeling of the injection sites and neurons in living brain slices were viewed using Lucifer yellow or Cy5 filters and excited by an AttoArc 2 mercury lamp (Zeiss Instruments).

MVN neurons retrogradely-labeled with biocytin after abducens nucleus injections and unlabeled MVN_{VL} neurons were recorded. Retrogradely-labeled MVN neurons were selected for recording based on: (1) presence of fluorescent label in the neuron cell body and (2) healthy neuron morphology, indicated by smooth surfaces of the living neurons under Nomarski optics. Unlabeled MVN neurons were recorded under two conditions: (1) when no healthy retrogradely-labeled MVN neurons were observed in the brain slices after biocytin was injected into the abducens nucleus, but a healthy unlabeled MVN_{VL} neuron was seen in the brain slice and had a 10-30 μm long, multipolar-to-elongate cell body (Gottesman-Davis

and Peusner, 2010) (n=36), and (2) when brain slices were used for recording which had not receive an extracellular biocytin injection in the abducens nucleus (n=35).

After the recordings, brain slices containing recorded MVN neurons were fixed and processed for biocytin visualization of all recorded neuron cell bodies, and their axons were traced to their target nuclei (for protocol, see below). Those recorded MVN neurons whose axons could not be traced were designated MVN/n neurons (n=70).

Micropipets (2-5 M Ω) were pulled from thin-walled, borosilicate glass tube (World Precision Instruments, Sarasota, Florida) using a Brown/Flaming horizontal puller (P-87, Sutter Instruments, Novato, CA). Due to our experimental design which required obtaining both spontaneous spike activity and excitatory and inhibitory synaptic events from the same neuron, we selected KCl pipet solution, a common patch pipet solution which allows both current- and voltage-clamp data to be recorded in the same cell (see Kay, 1992). Moreover, in previous experiments performed in this laboratory, KCl and Cs-gluconate pipet solutions provided similar frequencies for spontaneous synaptic events (Shao et al., 2003), and action potentials of tangential principal cells were recorded readily using KCl pipet solution (Gamkrelidze et al., 1998, 2000). KCl pipet solution contained (in mM): 130 KCl, 10 EGTA, 10 HEPES, 1.0 CaCl₂, 2.0 Mg-ATP salt. The pH of the pipet solution was adjusted to 7.2 using KOH, and the osmolality was adjusted to 270-290 mOsm.

Electrophysiological data acquisition and analysis

Signals were amplified using Axopatch-1D amplifier and the data acquired using Clampex 9.2 computer program (Molecular Devices, Sunnyvale, CA). The axopatch amplifier may distort the action potential waveform, most apparent in a modified shape of the first AHP following the action potential (Magistretti et al., 1996, 1998). In the present study, type A neurons were characterized by a single-phase AHP, while type B neurons had a biphasic AHP. Since both type A and type B neurons were distinguished by the number of AHP phases, any distortion of the waveform due to the amplifier should not affect the interpretation of the data.

Since type A and type B neuron classification originated in the mammalian system, here a modified nomenclature, type A-like and type B-like, was applied to chicken MVN neurons with similar waveforms to the mammalian neurons to avoid drawing conclusions on their functional roles in the chicken. Immediately after obtaining the whole-cell configuration, the resting membrane potential was read from the amplifier panel meter, and the input resistance was determined by injecting a +5 mV pulse using seal test function. Spontaneous and evoked spike activity was recorded in current-clamp mode, while I_{Na} , I_{NaP} , and spontaneous synaptic activity were recorded in voltage-clamp mode. Signals recorded in current-clamp mode were digitized at 20 kHz and filtered at 5 kHz, while signals recorded in voltage-clamp mode were digitized at 10 kHz and filtered at 2 kHz. Series resistance was not compensated. DC offset was corrected ($\pm 1-9$ mV) to determine the resting membrane potential, but the liquid junction potential (+3 mV) was not corrected. Biocytin (0.5%) (Sigma-Aldrich) was dissolved in the pipet solution weekly. Biocytin passively filled the recorded neurons during the recordings, which took 15-45 minutes.

Spontaneous spike activity was recorded at resting membrane potential for 3–4 min, and evoked spike activity was induced by applying 400 ms duration depolarizing current pulses in 0.1 nA steps for up to 1.5 nA injected current. Spontaneous and evoked spike activity was analyzed off-line using pClamp Clampfit 9.2 computer program (Molecular Devices). Frequency was expressed as the inverse of the mean interspike interval (ISI). The coefficient of variation (CV), defined as the standard deviation of the ISI divided by the mean ISI (Shao et al., 2006), was calculated for all the spontaneous spike firing neurons. The kinetics for

spontaneous action potentials were analyzed from averages of 15 spikes/neuron. The kinetics for evoked action potentials were calculated from the spikes generated on depolarization using the lowest intensity injected current steps required to obtain at least 3-5 spikes. Neurons that required higher depolarization resulted in bridge imbalance and were excluded from the study (Shao et al., 2006). Between 3-15 evoked action potentials were used for kinetics measurements. Thirteen neurons were excluded from the analysis of spike discharge on depolarization, including 9 neurons which failed to generate action potentials, and 4 neurons which required depolarization above -30 mV. Spike threshold was defined using Minianalysis computer program (Synaptosoft Inc., Decatur, GA), which determines spike threshold based on the value of dV/dt . Spike amplitude was defined as the potential difference between threshold and peak amplitude. Rise time was defined as the duration between threshold and peak amplitude during depolarization, while decay time was defined as the duration between peak amplitude and threshold during repolarization. Action potential width was measured as the duration between 50% spike amplitude during depolarization and repolarization. The gain for stimulus-evoked action potentials was defined as the ratio of firing rate relative to the injected current (Shao et al., 2009). AHP profile for type A-like and type B-like neurons was identified based on averaged spike waveforms.

I_{NaP} was induced using 800 ms voltage steps from -80 to -47.5 mV in 2.5 mV increments, and was measured 100 ms after initiating the voltage step by subtracting the traces before and after TTX. I_{Na} was induced using 100 ms voltage steps from -100 to +10 mV in 10 mV increments, and was measured as the difference between baseline and the inward peak by subtracting the traces before and after TTX.

Spontaneous postsynaptic currents (sPSCs) were recorded at -60 mV holding potential and analyzed from samples of random traces of 3-4 min duration off-line using Minianalysis computer program (Synaptosoft Inc.). The threshold for detecting synaptic events was set at 3x the root mean square of baseline noise recorded at -60 mV (noise level range: 3.3–8.1 pA; mean: 4.9 ± 0.1 pA). Analysis of PSCs included peak amplitude, rise time (from 10 to 90% peak current), decay time (from 90 to 37% amplitude return to baseline) and half-width (duration of event at 50% of peak amplitude).

All drugs, except CNQX (6-cyano-7-nitroquinoxaline-2, 3-dione) and DNQX (6, 7-dinitroquinoxaline-2, 3-dione), were dissolved in ACSF and then added to the bath ACSF to achieve the final concentration. Tetrodotoxin (TTX; RBI, Natick, MA) was used to block voltage-dependent sodium currents, including the fast (I_{Na}) and slow persistent (I_{NaP}) sodium currents. Strychnine (Sigma-Aldrich, St. Louis, MO) was used to block glycine receptor-mediated currents. Bicuculline methochloride (Tocris, St. Louis, MO) was applied to block GABA_A receptor-mediated currents. CNQX or DNQX (Sigma-Aldrich) were used to block glutamatergic, alpha-amino-3-hydroxy-5-methyl- 4-isoxazolepropionic acid (AMPA) receptor-mediated currents by dissolving the compound in dimethyl sulfoxide (DMSO; Fisher Scientific, Fair Lawn, NJ) at a concentration of 20 mM, which was added to the bath ACSF to achieve the final concentration.

Processing biocytin-injected neurons in brain slices

After the recordings, the brain slices were fixed immediately in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) overnight in the refrigerator (Shao et al., 2003). Anterograde labeling of the recorded neurons was detected using streptavidin Alexa Fluor 488 or 647 (Vector Laboratories, Burlingame, CA). Brain slices which received extracellular injections of biocytin Alexa Fluor 488 crystals were processed with streptavidin Alexa Fluor 647 to visualize the anterograde labeling. Brain slices were rinsed in 0.1 M phosphate buffered saline (PBS) containing 0.1% Triton X-100 (PBS-T), treated with 50% alcohol in PBS (20 minutes), rinsed with PBS-T (three times, 5 minutes each) and incubated in

streptavidin/Alexa Fluor 647 or 488 overnight. Finally, the brain slices were then rinsed in PBS-T and mounted on glass slides, air dried, and cover-slipped with antifading, water-soluble medium (Fluoromount G; Electron Microscopy Sciences, Hatfield, PA). Since all the brain slices were taken from chick embryos of the same age (E16), shrinkage due to fixation and spreading after cover-slipping the brain slices should be equivalent, so these factors were disregarded.

Imaging

Conventional fluorescent microscopy—Low power images were taken of all biocytin-labeled neurons using an Olympus BX60 conventional fluorescent microscope, equipped with a 4x objective (NA 0.4) and Image Pro Plus computer program (Media Cybernetics, Bethesda, MD). In addition, low and high power images were taken on a Zeiss Axio Imager.2 conventional fluorescent microscope equipped with Axiovision computer software (Zeiss Instruments). The total axon length and direct distance from the injection site to the labeled neuron cell body were measured on the Zeiss Axio Imager.2 microscope using the 10x (NA .45) or 20x (NA 0.8) objective.

Confocal microscopy—Labeled neurons and fibers were observed on a Zeiss LSM 710 confocal laser scanning microscope (Zeiss Instruments) using a 10x Plan-Apo (NA 0.45) or 20x PlanApo (NA 0.8) objective. Excitation for Alexa Fluor 488 was achieved using an Argon laser emitting at 494-600 nm, and excitation for Alexa Fluor 647 was achieved using a HeNe633 laser emitting at 637-755 nm. The injection sites, retrogradely-labeled neuron cell bodies, and recorded and biocytin-injected neuron cell bodies were identified using the 10x objective, whereas cell body size and shape, and dendritic morphology were viewed using the 20x objective. Image J software (National Institutes of Health, Bethesda, Maryland) was used to measure the cell body sizes and axon diameters and count the number of primary dendrites. Axon diameters were measured 150 μm from the neuron cell body.

The nomenclature for vestibular nuclei and brainstem nuclei and tracts were adopted from Ramon y Cajal (1908), Peusner and Morest (1977a), Wold (1978), Cox and Peusner (1990), and Popratiloff and Peusner (2007). Also, several avian brain atlases were referred to (pigeon, Karten and Hodos, 1967; hatchling chicken, Kuenzel and Masson, 1988; Puellas et al., 2007).

Axons were traced using the Zeiss Axio Imager.2 microscope and confocal images (see above). Axon terminals could not be identified in these preparations due to destruction of the abducens nucleus during the biocytin injections. Based on projection target, the recorded MVN neurons were classified as (1) neurons projecting to the ipsilateral abducens nucleus (MVN/ABi, n=29), (2) neurons projection to the contralateral abducens nucleus (MVN/ABc, n=33), (3) neurons projecting to the ipsilateral brainstem, but not the abducens nucleus (MVN/i, n=13), or (4) neurons projecting to the contralateral brainstem, but not the abducens nucleus (MVN/c, n=21). Finally, MVN neurons whose axon could not be traced were designated as MVN/n (n=70).

When retrogradely-labeled MVN neurons were recorded from brain slices which had received discrete biocytin injections into the abducens nucleus, 78% (60/77) had axons which could be traced to the injection site, 21% (16/77) had axons which were significantly damaged or lost during fixation and processing of the brain slices, and one had an axon which was traced to the MLF adjacent to the injection site. When unlabeled MVN_{VL} neurons were selected for recordings based on cell body size and shape and location in the MVN_{VL}, 4% (3/71) had axons which could be traced to the abducens nucleus, 35% (25/71) had axons which were traced to other targets, and the remaining neurons had axons that

could not be traced. Thus, the retrograde neuronal labeling approach greatly improved the probability of recording from MVN/AB neurons. The extent of dye coupling between the injected neuron and other neurons and glial cells was not studied due to variability in the biocytin injection times used during the recordings.

Statistical analysis

Values are presented as mean $\pm$ standard deviation (SD), with differences significant with $P < 0.05$ (Student's *t*-test). Differences in percentages were considered significant with $P < 0.05$ (Fisher's exact test) (GraphPad InStat, GraphPad software, La Jolla, CA).

Results

The morphological and functional properties of MVN/AB neurons (MVN/ABi, $n=29$; MVN/ABc, $n=33$) were studied and compared to MVN neurons not projecting to the abducens nucleus (MVN/i, $n=13$; MVN/c, $n=21$), and MVN neurons whose axons could not be traced (MVN/n, $n=70$). In the living brain slices which received extracellular biocytin injections into the abducens nucleus, about 5-10 MVN neurons appeared strongly retrogradely-labeled on each side of the brainstem under the Zeiss FS2 microscope. MVN neurons which were anterogradely-labeled only ($n=60$) had the same resting membrane potential (-62.0 ± 7.7 mV) and input resistance (249 ± 170 M Ω) as MVN neurons which were labeled both anterogradely and retrogradely by the two-step process ($n=106$) (-63 ± 10.3 mV; 203 ± 133.9 M Ω), suggesting that the retrograde biocytin labeling protocol did not diminish neuron health. Depending on the anteroposterior level of the brain slice, other second-order vestibular neurons in the ventrolateral, descending, or tangential nucleus, were retrogradely-labeled bilaterally.

Morphology of MVN neurons

The majority of MVN/AB neurons had stellate cell bodies (41/53), while the remaining neurons had elongate cell bodies with the somatic long axis at least twice as long as the short axis (Table 1) (Gottesman-Davis and Peusner 2010). The percentage of stellate (77%) and elongate cells (23%) was similar in all MVN neuron groups studied ($P > 0.05$). The average long axis of MVN/ABi neuronal somata was 23.3 ± 3.9 μ m, which was not different from those of MVN/ABc neurons, or from other MVN neuron groups ($P > 0.05$). In general, MVN neurons with axons remaining ipsilateral (MVN/ABi, MVN/i) had more dendrites than those MVN neurons whose axons ran to the contralateral side of the brainstem (MVN/ABc, MVN/c) ($P < 0.05$). Finally, double-labeled MVN neurons exhibited longer dendrites with more branches (Fig. 1B₂, C₂) than those MVN neurons which were retrogradely-labeled only (Fig. 1A₂). However, no difference in dendritic morphology could be detected between MVN neurons which received double-labeling and those which were anterogradely-labeled only (not shown).

Typically, axons of MVN/ABi neurons originated from the ventral aspect of the cell body (11/18), whereas most axons of MVN/ABc neurons originated from the medial side (12/20). Axon diameters of MVN neurons were about 2 μ m (Table 1). On average, axons of MVN/ABi neurons were 958 μ m long, whereas axons of MVN/ABc neurons were 1873 μ m long. When the ratio for axon length relative to the distance traveled between the cell body and abducens nucleus was calculated, no significant difference was detected between MVN/ABi and MVN/ABc neurons ($P > 0.05$). Occasionally, axons of MVN/ABi neurons ($n=2/21$) had long collateral branches which could be traced more than 200 μ m to terminate in the ipsilateral MLF or ventrolateral brainstem. Axons of MVN/ABc neurons did not produce collaterals.

Passive membrane properties

The average resting membrane potential for MVN/ABc neurons was -64.5 mV, which was significantly more hyperpolarized compared to MVN/c neurons ($P<0.05$) (Table 2). MVN/ABi (185.1 M Ω) and MVN/ABc (155 M Ω) neurons had significantly lower input resistance compared to MVN/n neurons (244.1 M Ω) ($p<0.05$). Also, MVN/ABc neurons had significantly lower input resistance compared to MVN/i and MVN/c neuron groups ($P<0.05$).

Spontaneous spike activity

According to their ability to generate action potentials at resting membrane potential, MVN neurons were subdivided into two subsets, spontaneous spike firing (SSF) and silent cells. While most neurons fired spontaneous spikes (66% MVN/ABi, 69% MVN/i, 62% MVN/c, and 54% MVN/n), it is notable that significantly fewer MVN/ABc neurons (21%) could fire spikes spontaneously (Fig. 2A). The mean spontaneous spike discharge rate for all spontaneous spike firing MVN neurons was 4.9 ± 5.6 spikes/s, with a CV of 0.74 ± 0.46 ($n=86$), and this rate did not change significantly among the different MVN neuron groups (Fig. 2B). Spontaneous spike firing and silent cells did not differ in their average cell body length, percentage of stellate and elongate cells, or number of primary dendrites. However, the spontaneous spike firing cells showed more depolarized resting membrane potentials and higher input resistances than silent cells (Table 2) ($P<0.05$).

Sodium conductances underlying spontaneous spike activity—To determine whether sodium conductances underlie the difference in spontaneous spike activity detected among the various MVN neuron groups, MVN neurons were exposed to 1 μ m TTX. All spike activity in the spontaneous spike firing neurons from all MVN neuron groups was abolished ($n=68$), demonstrating that sodium channels are responsible for spike generation (not shown). A previous study on the chick tangential principal cells showed that spontaneous spike firing neurons have larger amplitude I_{NaP} and a lower threshold for I_{Na} than the silent cells, contributing to their higher excitability (Shao et al. 2009). Therefore, both I_{NaP} and I_{Na} were investigated here.

A persistent inward current was induced in MVN neurons on exposure to 800 ms duration voltage steps from -80 to -47.5 mV. The slowly-activating inward current was revealed by subtracting outward current traces before and after exposure to TTX (Fig. 3A₁-A₃). From the I/V curve, the current was shown to activate around -60 mV and became larger with more depolarizing voltages (Fig. 3A₄). The inward current had all of the characteristics of I_{NaP} , including low activation threshold, slow inactivation, and TTX sensitivity (Llinas 1988). Although all MVN neurons had measurable I_{NaP} , spontaneous spike firing neurons ($n=21$) had significantly larger amplitude I_{NaP} at -47.5 mV compared to the silent cells ($n=25$) (Fig. 3A₄). Thus, I_{NaP} distinguished the spontaneous spike firing and silent MVN neurons.

On exposure to 100 ms duration voltage steps from -100 to $+10$ mV, MVN neurons exhibited a fast-activating inward current which was sensitive to 1 μ m TTX, revealing that it was an inward I_{Na} (Fig. 3B, C). I_{Na} activated at -40 mV for most spontaneous spike firing cells (21/23), but activated at or above -30 mV for 10/29 silent cells (Fig. 3B₄). Consistent with this finding, I_{Na} activated at -40 for almost all MVN/ABi neurons (15/16) and most MVN/n neurons (13/16), but activated at -30 mV for nearly half of MVN/ABc neurons (6/14) (not shown). Thus, spontaneous spike firing and silent MVN neurons showed different I/V curves, with the former showing significantly larger I_{Na} at -40 mV. Once I_{Na} was activated around -30 mV in the silent MVN neurons, no significant difference in I_{Na} amplitude was found between these two neuron classes. The lower I_{Na} activation threshold

for spontaneous spike firing MVN neurons made them more capable of generating action potentials than silent MVN neurons. Accordingly, spontaneous spike firing and silent cells MVN neurons were distinguishable by their sodium conductances.

Spike waveforms

In mammalian studies, MVN neurons are classified as type A or type B neurons, depending on whether the action potential is followed by a single deep AHP or an early fast AHP followed by a delayed slow AHP (e.g., Serafin et al. 1991; Johnston et al. 1994). Besides the classical type A-like (n=51) and type B-like neurons (n=89) (Fig. 4A, B) observed in the present study, another type B-like neuron subset, identified as type B₀-like, was found in the chicken MVN. Type B₀-like neurons lacked the early fast AHP (n=13), in keeping with immature mouse type B neurons (see Dutia and Johnston, 1998; Eugène et al. 2007). In addition, the action potentials generated in the chick type B₀-like neurons exhibited significantly smaller amplitude, and slower rise time, decay time and halfwidth compared to type B-like neurons (Table 3). Accordingly, these type B₀-like neurons were excluded from analysis. Finally, those neurons which did not fire action potentials at resting membrane potential or on depolarization (n=9), as well as other neurons whose action potentials were generated only by high current injections and lacked distinctive AHPs (n=4), were excluded from classification.

The action potentials generated from type A-like neurons had significantly lower amplitude, and slower rise time, decay time, and half-width compared to type B-like neurons, but spike thresholds were similar for both neuron groups (Table 3). Both type A-like and type B-like neurons were found throughout the MVN regions sampled, with type B-like neurons predominating among most MVN neuron groups (MVN/ABi, 19/28; MVN/i, 6/12; MVN/c, 13/19; MVN/n, 40/65), except for MVN/ABc where type A-like neurons prevailed (17/29). Type A-like and type B-like neurons showed the same percentage of stellate and elongate cells found in other MVN neuron groups. The majority of type B-like neurons (64/89) fired spontaneous spikes at resting membrane potential, while most type A-like neurons were silent (38/51). Also, most spontaneous spike firing neurons were type B-like neurons (62/85), while most silent neurons were type-A like neurons (38/68) (Fig. 4C). Consistent with this, type B-like neurons had more depolarized resting membrane potentials and higher input resistances than type A-like neurons ($P<0.05$, Table 2).

Spike discharge on depolarization

The majority of MVN neurons fired repetitively on depolarization (MVN/ABi, 22/29; MVN/i, 9/13; MVN/c, 16/21; MVN/n, 52/70), but only about one-third of MVN/ABc neurons could do so (12/32; 31%) (Fig. 5). In fact, spike firing on depolarization paralleled the neuron's ability to generate spontaneous spike activity (MVN/ABi, 66%; MVN/ABc, 21%; Table 5). Almost all the spontaneous spike firing MVN neurons fired repetitively on depolarization (81/86; 94%), whereas less than half of the silent MVN neurons fired repetitively on depolarization (28/79; 35%) (Fig. 5). Since most type B-like neurons exhibited spontaneous spike firing, it is not surprising that most type B-like neurons fired repetitively on depolarization (70/88; 80%). Likewise, most type A-like neurons were silent, and less than half of them fired repetitively on depolarization (24/50; 48%).

Among the MVN neurons which fired spikes repetitively on depolarization, there was a linear relationship between the injected current and evoked spike firing rate (gain). No significant difference was detected in the firing gains of different MVN neuron groups. The firing gains in spikes/s/nA were 22.4 ± 12.9 in MVN/ABi neurons (n=17), 14.9 ± 12.4 in MVN/ABc neurons (n=11), 23.2 ± 9.5 in MVN/I neurons (n=7), 19.6 ± 15.4 in MVN/c neurons (n=15), and 21.2 ± 12.4 in MVN/n neurons (n=48) ($P>0.05$). In addition, no

significant difference was found in the evoked spike firing gains between subsets of spontaneous spike firing (21.6 ± 13 , $n=73$) and silent MVN neurons (17.9 ± 11.9 , $n=25$), or between type A-like (19 ± 13.1 , $n=24$) and type B-like MVN neurons (21.3 ± 13.8 , $n=62$) ($P>0.05$).

Excitatory and inhibitory synaptic transmission

Frequency of sEPSCs and sIPSCs—To determine whether the MVN neuron groups under study have different synaptic inputs, sPSCs were recorded at -60 mV where all the events appeared as inward currents. The frequency for the total inward currents was 2.8 ± 3.6 Hz ($n=143$). CNQX or DNQX, combined with bicuculine and strychnine, blocked all the sPSCs ($n=29$), suggesting that the events were mediated by AMPA/KA-, GABA_A-, and glycine receptors at this holding potential. In all experiments where the sPSCs were isolated pharmacologically, sEPSCs showed faster rise time, decay time, and half width compared to the sIPSCs ($P<0.05$) (Fig. 6A-D). Therefore, the frequency of sEPSCs and sIPSCs could be determined based on decay time. According to this analysis, the average sEPSC frequency was 0.7 ± 1.1 Hz ($n=125$), while the average sIPSC frequency was 2.5 ± 2.7 Hz ($n=82$). sEPSC and sIPSC frequencies did not vary significantly between MVN/AB and MVN/n groups (Fig. 7C). However, sEPSC and sIPSC frequencies were higher for spontaneous spike firing MVN neurons (sEPSC, 1 ± 1.04 Hz, $n=60$; sIPSC, 3.69 ± 4.22 Hz, $n=69$) compared to the silent cells (sEPSC, 0.55 ± 0.68 Hz, $n=59$; sIPSC, 1.15 ± 1.2 Hz, $n=65$) ($P<0.05$) (Fig. 7C). No difference was detected in sEPSC and sIPSC frequencies between type A-like and type B-like MVN neurons.

Frequency of mEPSCs and mIPSCs—Miniature synaptic events were observed after applying TTX ($1 \mu\text{M}$) to abolish the action potential-dependent activity (Fig. 7A, B). Generally, mEPSC frequencies were similar among the different MVN neuron groups tested. Likewise, no difference was found for mIPSC frequencies among the different MVN neuron groups (Fig. 7D). However, mEPSC frequency was higher for the spontaneous spike firing MVN neurons compared to the silent cells, and for type B-like MVN neurons compared to type A-like neurons. Finally, mIPSC frequency was not different between spontaneous spike firing and silent MVN neurons, or between type A-like and type B-like MVN neurons.

In all the MVN neurons tested, most sEPSCs were TTX-resistant mEPSCs, including those recorded in MVN/ABi (72%, $n=8$), MVN/ABc (76%, $n=6$), and MVN/n neuron groups (79%, $n=6$). Thus, few excitatory presynaptic neurons were spontaneously active in these brain slices. Concerning inhibitory events, less than half of sIPSCs were TTX-resistant in MVN/AB neurons (MVN/ABi, 37%, $n=11$; MVN/ABc, 49%, $n=13$). In contrast, more than half of sIPSCs were mIPSCs in MVN/n neurons (69%, $n=11$), which was significantly higher than MVN/ABi neurons ($P<0.05$). Thus, MVN/ABi neurons retained greater numbers and/or more active inhibitory presynaptic neurons in the brain slices compared to MVN/n neurons.

GABAergic and glycinergic IPSCs—Additional experiments were performed to distinguish the sIPSCs as GABAergic or glycinergic events ($n=14$). Most MVN neurons (9/14) have both GABAergic and glycinergic sIPSCs, with the remaining neurons exhibiting GABAergic events only. In our small sample of type A-like MVN neurons ($n=4$), some had both GABAergic and glycinergic events ($n=2$), while others had GABAergic only ($n=2$). Likewise, type B-like MVN neurons were characterized by combined GABAergic and glycinergic events ($n=6$), or GABAergic events only ($n=3$). Indeed, no MVN neurons were recorded with only glycinergic events.

On exposure to TTX and strychnine, most mIPSCs were not abolished (MVN/ABi, 66%, n=3; MVN/ABc, 71%, n=5; MVN/n 82%, n=7), indicating that they were mainly GABAergic events (Fig. 7B₄). The percentage of GABAergic mIPSCs was not significantly different among the MVN neuron groups investigated, or the subsets of spontaneous spike firing (82%, n=5) compared to silent MVN neurons (72% n=10), or type A-like MVN neurons (70%, n=5) compared to type B-like neurons (85%, n=7).

Kinetics of mEPSCs and mIPSCs—When mEPSCs kinetics were compared among the different MVN neuron groups, mEPSCs in MVN/ABi neurons were striking for their longer decay time and half width compared to the MVN/n neuron group, suggesting that the AMPA receptors of MVN/ABi neurons contained more GluR2 and/or less GluR4 subunits than MVN/n neurons (Popratiloff et al., 2004). In addition, mEPSC peak amplitude was larger in the subset of spontaneous spike firing neurons compared to the silent MVN neurons, which may contribute to their greater ability to generate spontaneous spikes (Table 4). The rise time of mIPSCs was significantly slower in MVN/ABi neurons compared to MVN/ABc and MVN/n neurons. Altogether, the mEPSCs of MVN/ABi neurons had slower decay time and halfwidth, and their mIPSCs had slower rise time compared to MVN/n neurons.

Discussion

This is the first study to characterize the electrophysiological properties of morphologically-identified MVN neurons projecting to the abducens nucleus in brain slice preparations. MVN/ABi and MVN/ABc neurons were distinguished by a catalog of electrophysiological properties in the late-term chick embryo. Specifically, MVN/ABi neurons showed differences in their synaptic activity compared to MVN/n neurons, including a significantly lower percentage of mIPSCs/sIPSCs and slower mEPSC and mIPSC kinetics. MVN/ABc neurons exhibited a different spike firing pattern compared to MVN/ABi and MVN/n neurons. In particular, most MVN/ABc neurons had type A-like waveform (59%), lacked spontaneous spike activity (79%), and failed to fire spikes repetitively on depolarization (69%) (Table 5). These differences in basic electrophysiological properties underscore the importance of performing functional analyses of signal processing on neuron classes defined by their axonal outputs. Moreover, this study provides fundamental information on the functional properties of MVN/AB neurons for future studies on their development and adaptation to peripheral vestibular lesions.

MVN neurons projecting to the abducens nucleus

Previous studies describe a regional segregation of MVN neurons projecting to diverse targets, but no sharp boundaries and some intermingling among neuron classes (for review, see Straka et al., 2005; Malinvaud et al., 2010). In avians and mammals, MVN/AB neurons are located in the rostral half of the MVN_{VL} bilaterally, interspersed among neurons projecting to other targets. In monkey and cat, neurons projecting to the abducens nucleus, which are retrogradely labeled by horseradish peroxidase (HRP) injections into the abducens nucleus, are found in adjacent regions of the MVN and ventrolateral vestibular nucleus (Langer et al., 1986). While MVN/ABi and MVN/ABc neurons tend to be located in complementary MVN regions in the cat, MVN/ABi neurons are more numerous. Thus, there is a bilateral distribution of MVN neurons projecting to the abducens nucleus in different species, with variable numbers of neurons on each side of the brain (Langer et al., 1986, McCrea et al., 1980, Spencer et al., 1989). Finally, the morphology of chicken MVN/AB neurons is similar to that described in mammals, with both displaying primarily stellate cell bodies with 3-5 primary dendrites (Gottesman-Davis and Peusner, 2010).

MVN/ABc neurons represent a glutamatergic, excitatory input to the abducens nucleus (e.g., Straka and Dieringer, 1993). In the cat, most MVN/ABi neurons are glycinergic (Spencer et al., 1989), but few glycinergic MVN neurons are found in the rat (Takazawa et al., 2004). The absence of glycinergic neurons in the latter study may be due to sampling neurons from MVN_{DM} (see Fig. 7, Takazawa et al., 2004), where neurons projecting to the trigeminal motor nucleus are concentrated (Cuccurazzu et al., 2007) while MVN/AB neurons are positioned more ventrally and laterally (Spencer et al., 1989). Immunocytochemical studies reveal that the oculomotor and trochlear nuclei contain many GABAergic terminals, but few glycinergic terminals, which are abundant in the abducens nucleus. Thus, GABA is thought to be the major neurotransmitter for inhibitory vestibular nucleus neurons involved in vertical eye movements, whereas glycine is considered the likely inhibitory neurotransmitter released by MVN/ABi neurons participating in horizontal eye movements (Spencer et al., 1989; for review see Straka et al., 2005). The specificity of this relationship underscores the importance of recording from MVN projection neurons whose targets are identified to understand their roles in signal processing.

In the present study, MVN/ABi and MVN/ABc neurons displayed major differences in basic electrophysiological properties. These differences could result from a delayed developmental schedule, since contralaterally projecting axons must traverse a longer distance, requiring more time to reach the target. However, this explanation seems unlikely because the axons of chick vestibular nuclei neurons emerge early in development, around E6 (Peusner and Morest, 1977b). Accordingly, it is likely that axons of MVN/AB neurons reached their targets long before the time of this study. Thus, we propose that other factors, besides developmental gradients, are likely responsible for the distinctive functional properties of MVN/AB neurons, such as neurotransmitter phenotype and/or role in signal processing in the vestibular neural circuitry. The finding of a small subset (<10%) of type B₀-like neurons which are clearly immature underscores that some, if not most, chick MVN neurons have yet to achieve the mature spike waveforms at E16. Indeed, compared to tangential principal cells at H5 (Shao et al., 2006), the spike waveform of type B-like MVN neurons displayed slower rise time, decay time and halfwidth, suggesting that they are immature compared to later stages. At postnatal day 15 (P15) in the mouse, 80% of type B MVN neurons have immature spike waveforms, but by P30 all type B neurons have mature spike waveforms (Dutia and Johnston, 1998). Accordingly, vestibular development in the E16 chick embryo is likely equivalent to a mouse stage somewhere between P15 and P30. Electrophysiological studies performed in hatchling chickens can address this issue specifically.

The possibility cannot be ruled out that some recorded neurons, identified as MVN/AB neurons in the present study, could be MVN neurons projecting to the MLF or to the contralateral vestibular nuclei, since some of these latter neuron groups have axons traversing the abducens nucleus (Wold, 1978, 1979), although many of them travel directly beneath the fourth ventricle (Cox and Peusner, 1990).

What role could the distinctive electrophysiological properties displayed by MVN/ABi and MVN/ABc neurons play at E16? The developing vestibular system is involved in inhibiting excessive motor activity until just before hatching, when the vestibular system regulates rotary head movements essential for hatching (Gottlieb, 1968). From this, it could be expected that inhibitory circuitries develop in advance of the excitatory connections. Thus, MVN/ABi neurons, which may utilize glycinergic neurotransmission, have spike firing patterns more responsive to synaptic inputs at E16 than the putative excitatory, glutamatergic MVN/ABc neurons, which generate little spike activity at this time.

MVN neurons projecting to the oculomotor nucleus

In the young mouse, MVN neurons projecting to the contralateral oculomotor nucleus are multipolar with 3-4 dendrites, on average, and have larger cell bodies than nearby unlabeled MVN neurons (Sekirnjak and du Lac, 2006). Indeed, chicken MVN neurons which project to the oculomotor nucleus have larger cell bodies than those neurons projecting to the abducens nucleus, and both neuron populations reside within overlapping MVN regions (Gottesman-Davis and Peusner, 2010). In the monkey, the superior vestibular nucleus plays a key role in vertical eye movements, with MVN neurons less involved (Highstein and Holstein, 2006; Straka et al., 2005). After biocytin was injected within the chicken oculomotor nucleus in brainstem preparations, the majority of retrogradely-labeled neurons were observed in the contralateral MVN, lateral vestibular, descending vestibular, and tangential nucleus, with fewer neurons residing in the superior vestibular nucleus ipsilaterally (Gottesman-Davis and Peusner, 2010). Thus, vertical eye movements in the chicken may be regulated by neurons within diverse vestibular nuclei rather than consolidated within the superior vestibular nucleus as in mammals.

In the juvenile mouse, MVN neurons projecting to the contralateral oculomotor nucleus show type B waveform exclusively, and are more likely to generate spontaneous spike firing and show higher spontaneous spike firing rates than MVN neurons sampled at random (Sekirnjak and du Lac, 2006). Since MVN neurons projecting to the contralateral oculomotor nucleus likely utilize excitatory, glutamatergic neurotransmission, like MVN neurons projecting to the contralateral abducens nucleus (for review, see Highstein and Holstein, 2006), the differences in electrophysiological properties between these two classes of excitatory VOR neurons in juvenile mouse and chick embryo could be due to differences in developmental ages, afferent fiber inputs, and/or efferent target neurons. In both juvenile mouse (Faulstich et al., 2004) and newly hatched chicken (Wallman et al., 1982), the VOR gain is considerably lower than that found in adults. VOR gain in the chick embryo has not been studied.

Comparison of MVN/AB neurons and tangential principal cells

Multiple lines of evidence indicate that the principal cells of the chick tangential nucleus project mainly to the contralateral oculomotor, trochlear or abducens nucleus (e.g. Wold, 1978; Gottesman-Davis and Peusner, 2010), with some neurons sending collaterals to cervical spinal cord (Cox and Peusner, 1990). Accordingly, principal cells are mainly VOR neurons, and/or vestibulo-ocular-colic neurons. In addition to this, some principal cells send axons ipsilaterally to the cervical spinal cord (Cox and Peusner, 1990). Thus, like MVN/ABc neurons, a subset of the tangential principal cells project to the contralateral abducens nucleus (Cox and Peusner, 1990; Gottesman-Davis and Peusner, 2010).

After biocytin is injected extracellularly into the chicken oculomotor nucleus, the majority of labeled neurons appear within a mediolaterally-extended band of vestibular nuclei neurons on the contralateral side. First described in E11 chick embryos (Pettursdottir, 1990), this band of retrogradely-labeled vestibular nuclei neurons cuts across nuclear boundaries to include neurons within the MVN, ventrolateral vestibular, descending vestibular, and tangential vestibular nuclei (Gottesman-Davis and Peusner, 2010). The number of MVN neurons projecting to the chick oculomotor nuclei was less than expected, perhaps due to the fact that VOR neurons are dispersed among several chick vestibular nuclei rather than concentrated within the MVN as in mammals. Thus, the tangential nucleus, which has no counterpart in mammals, may represent a lateral extension of the chicken MVN, since the principal cells are primarily VOR and/or vestibulo-ocular-colic neurons.

At E16, MVN/ABc neurons mirror the tangential principal cells in many basic features. Both neuron classes have axons that project to the contralateral abducens nucleus. In addition, most principal cells and MVN/ABc neurons are incapable of generating spontaneous spike firing and repetitive spike firing on depolarization (Shao et al., 2006). In the case of the principal cells, high levels of dendrotoxin-sensitive potassium current (I_{DS}) are present at E16, which block repetitive firing of action potentials on depolarization (Gamkrelidze et al., 1998). However, after hatching, I_{DS} amplitude decreases so that most principal cells can fire repetitively on depolarization (Gamkrelidze et al., 2000). Whether MVN/ABc neurons have high levels of I_{DS} , like principal cells, and unlike MVN/ABi neurons, remains to be determined. It is interesting that both principal cells and MVN/ABc neurons receive primarily GABAergic spontaneous synaptic activity in a ratio of 4:1 compared to excitatory events (Shao et al., 2003). Moreover, both neuron classes share similar EPSC and IPSC kinetics (Shao et al., 2003). Finally, the principal cells transform into highly excitable neurons after hatching when spontaneous spike firing and repetitive spike firing on depolarization are recorded in most principal cells (Shao et al., 2006). Future experiments performed on the hatchling chicken can address whether a similar transformation in spike firing pattern occurs in MVN/ABc neurons, and whether the differences between MVN/ABi and MVN/ABc persist. Already, it is known from intracellular recordings made on unidentified chick MVN neurons that almost all of them generate spontaneous spike activity from H1-H12 (du Lac and Lisberger, 1995).

Excitatory and inhibitory inputs to MVN/AB neurons

In the present study, both sEPSCs and sIPSCs were recorded in the same MVN neuron. This is the first analysis of sEPSCs in MVN neurons, which revealed that all the excitatory events were AMPA/kainate receptor-mediated at -60 mV in the presence of normal extracellular magnesium. The lower ratio for mIPSC/sIPSCs in MVN/ABi neurons compared to MVN/n neurons suggests that the inhibitory inputs to MVN/ABi neurons may be more intact in brain slice preparations. Accordingly, MVN/ABi neurons may retain greater numbers and/or more spontaneously active inhibitory presynaptic neurons in brain slice preparations compared to MVN/n neurons.

About two-thirds of the pharmacologically-tested MVN neurons received both GABAergic and glycinergic inputs, whereas one-third received GABAergic inputs only, and none of the recorded neurons received glycinergic inputs only. In the chick embryo, no clear association was found between type A-like and type B-like neurons and their inhibitory synaptic inputs, as found in the young mouse (Camp et al., 2006). In the mouse, type A neurons receive almost exclusively GABAergic mIPSCs, whereas type B neurons receive either GABAergic or glycinergic activity, or both. These differences may be due to the location of the recorded neurons, since the mouse MVN neurons were recorded primarily from the dorsal MVN at all anteroposterior levels, in contrast to the present study where recordings were made mainly from the rostral MVN_{VL}. The kinetics of mIPSCs in chick MVN neurons were similar to those recorded for mouse MVN neurons, which suggests that their postsynaptic receptor subunits may share similar composition. In the hippocampus, the decay times recorded for mEPSCs and mIPSCs in interneurons are related to their axonal connections (Cossart et al., 2006). In the present study, mEPSCs of MVN/ABi neurons showed significantly slower decay time compared to MVN/n neurons, but no difference was detected in the decay time between MVN/ABi and MVN/ABc. Finally, more than 50% of sIPSCs were TTX-dependent, indicating that many inhibitory presynaptic neurons reside within the brain slices and can fire action potentials. These inhibitory presynaptic neurons could be local MVN interneurons, or vestibular nuclei neurons whose axons form commissural inputs. GABAergic neurons are the principal source of inhibition, since most mIPSCs were GABAergic.

In summary, distinctive electrophysiological properties for ipsilateral and contralateral projecting MVN/AB neurons were revealed here by sorting out the neuron classes according to their axonal projections. Future electrophysiological investigations of these and other MVN neuron classes according to their axonal output pathways will assist in clarifying their special roles in signal processing under normal conditions and after vestibular deafferentation.

Research highlights

- Electrophysiological properties of MVN neurons differ according to outputs.
- Chick MVN neurons are characterized as type A-like and type B-like neurons.
- MVN neurons are labeled with extracellular biocytin injected in brain slices.

Acknowledgments

This work was supported by NIH grant R01 DC000970, NIH predoctoral fellowship (1 F31 DC008715), and NIH shared instrumentation grant (S10RR025565). The Authors gratefully acknowledge Dr. Anastas Popratiloff for advice on the confocal imaging and Mr. Xudong Cai for excellent technical assistance.

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Abbreviations

ACSF	artificial cerebrospinal fluid
AHP	afterhyperpolarization
I_{Na}	fast sodium current
I_{NaP}	slow persistent sodium current
ISI	interspike interval
mEPSCs	miniature excitatory postsynaptic currents
mIPSCs	miniature inhibitory postsynaptic currents
MVN	medial vestibular nucleus
MVN/AB	MVN neurons projecting to the abducens nucleus
MVN/ABc	MVN neurons projecting to contralateral abducens nucleus
MVN/ABi	MVN neurons projecting to ipsilateral abducens nucleus

MVN/c	MVN neurons with axons contralateral that do not project to the abducens nucleus
MVN/i	MVN neurons with axons ipsilateral that do not project to the abducens nucleus
MVN/n	MVN neurons with axons that could not be traced
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline containing 0.1% Triton X-100
sEPSCs	spontaneous excitatory postsynaptic currents
sIPSCs	spontaneous inhibitory postsynaptic currents
sPSCs	spontaneous postsynaptic currents
VOR	vestibuloocular reflex

Fig. 1.

(A₁-C₁) Fixed brain slices processed for biocytin visualization. (A₁) Extracellular biocytin was injected into the abducens nucleus (AB) (level 3.6); (B₁, C₁) Low power, double-labeled MVN/ABi and MVN/ABc neurons, respectively, which were anterogradely and retrogradely labeled with biocytin; arrows, labeled axons (level 3.75). (A₂) High power of MVN/ABi neurons taken from boxed-in region in A₁. Note stellate cell bodies (arrowheads). (B₂, C₂) High power of MVN/ABi and MVN/ABc neurons from B₁ and C₁, respectively, showing stellate cell bodies. Arrows point to axons of the injected neurons. IV, fourth ventricle. MVN_{VL}, ventrolateral part of MVN; MVN_{DM}, dorsomedial part of MVN; MLF, medial longitudinal fasciculus; NM, nucleus magnocellularis; NL, nucleus laminaris. Dashed lines indicate the midline and crossed cochlear bundle. Borders of the nuclei are dotted in in A. D, dorsal; L, lateral; V, ventral; M, medial. Scale bar for A₁-C₁, 400 μm. Scale bar A₂-C₂, 50 μm.

Fig. 2.

Spontaneous spike firing in different MVN neuron groups. (A) Percentage of MVN/ABc neurons firing spontaneous spikes at resting membrane potential was significantly lower (*) than all other MVN neuron groups ($P < 0.05$). In this and all subsequent figures, * denotes a significant difference. (B) Spike firing rate for spontaneous spike firing neurons in the various MVN neuron groups was not significantly different. Spike discharge rates in spikes/s and CVs are: MVN/ABi, 4.3 ± 6.5 , $CV = 0.9 \pm 0.5$; MVN/ABc, 7.4 ± 7.9 , $CV = 0.7 \pm 0.3$; MVN/i, 3.1 ± 3.3 , $CV = 1 \pm 0.4$; MVN/c 3.9 ± 3.2 , $CV = 0.74 \pm 0.42$; MVN/n 5.4 ± 5.6 , $CV = 0.64 \pm 0.44$.

Fig. 3.

I_{NaP} and I_{Na}. (A₁-A₃) Voltage-clamp recordings from a silent MVN/ABc neuron showing a slowly-inactivating current when the holding potential was switched from -60 mV to -47.5 mV (A₁, control) and after TTX (A₂). (A₃) subtracting A₂ from A₁ revealed I_{NaP}. Scale bar applies to A₁-A₃. (A₄) The amplitude of I_{NaP} at -47.5 mV was significantly greater in the spontaneous spike firing MVN neurons compared to the silent MVN neurons. (B₁, C₁) The fast-activating inward current, I_{Na}, recorded in a silent (B₁) and spontaneous spike firing (SSF) MVN/ABi neurons on exposure to 100 ms duration depolarizing voltage steps from -60 to +10 mV. (B₂, C₂) After TTX. (B₃, C₃) subtracting B₂ from B₁, and C₂ from C₁, revealed I_{Na}. Scale bar applies to traces in B₁-B₃ and C₁-C₃. Neurons in A, B and C were type B-like neurons. (B₄) I_{Na} activated at -40 mV in 91% of spontaneous spike firing MVN neurons, but activated at or above -30 mV in 35% of silent MVN neurons.

Fig. 4.

Spike waveforms of MVN neurons. (A) Type A-like and (B) Type B-like neurons. Scale bar applies to traces in A, B. (C) Most MVN neurons were type B-like, except for neurons in the MVN/ABc group, which contained mainly type A-like neurons. Not all percentages equal 100% because a small number of neurons failed to fire spikes.

Fig. 5.

Evoked spike firing on depolarization in MVN neurons. (A) Single spike response of a MVN/ABc neuron with type A-like waveform, and (B) repetitive spike firing response of a MVN/ABi neuron with type B-like waveform. Depolarizing current pulses of 400 ms duration were injected. (C) The percentage of neurons with repetitive spike firing on depolarization is significantly lower for MVN/ABc compared to all other MVN neuron groups. Most spontaneous spike firing neurons fired repetitively on depolarization, whereas only 35% of silent neurons could generate repetitive spike firing ($P < 0.05$). The percentage of repetitive spike firing neurons was lower among type A-like compared to type B-like neurons ($P < 0.05$). Not all percentages equal 100% because a small number of neurons failed to fire spikes.

Fig. 6.

Kinetics of sEPSCs and sIPSCs. (A₁-A₃) Voltage-clamp recordings at -60 mV from a MVN/n neuron. (A₁) Control showing fast events (*), which were blocked by CNQX (20 μ M) (A₂) indicating that they were sEPSCs. The remaining events were sIPSCs, since they were blocked by bicuculline (40 μ M) and strychnine (1 μ M). Scale bar in A₃ applies to A₁ and A₂. (B) Average sEPSCs (dotted) and sIPSC traces (solid). Traces in A and B were taken from same neuron. Inset: Scaled, superimposed sEPSC (dotted) and sIPSC (solid) traces to show the faster kinetics for sEPSCs. (C) Cumulative distribution curves of decay time for sEPSCs (dotted line, 1510 events; n=15 neurons) and sIPSCs (solid line, 2731 events; n=6 neurons) show that sEPSCs have significantly faster decay time. sIPSCs with decay time >50 ms (3%) were excluded from the plot. (D) Histogram of average decay time for pharmacologically isolated sEPSCs (n=34 neurons) and sIPSCs (n=19 neurons). The average decay time for sEPSCs (2.35 ± 0.9 ms) was significantly faster than for sIPSCs (16.4 ± 9.2 ms).

Fig. 7. sPSCs and mPSCs in MVN neurons. Voltage-clamp recordings at -60 mV from MVN/n (A₁-A₄) and MVN/ABi neurons (B₁-B₅). (A₁) Control. (A₂) Bicuculline (40 μ M) and strychnine (1 μ M) blocked sIPSCs. (A₃) Addition of TTX (1 μ M) revealed mEPSCs. (A₄) Addition of DNQX blocked all remaining activity. (B₁) Control. (B₂) DNQX (20 μ M) blocked sEPSCs. (B₃) Addition of TTX (1 μ M) revealed mIPSCs. (B₄) Addition of strychnine (1 μ M) revealed GABAergic mIPSCs. (B₅) Addition of bicuculline (40 μ M) blocked the remaining activity. (C) sIPSC frequencies were higher than sEPSC frequencies in all MVN neuron groups. Spontaneous spike firing neurons had significantly higher frequency of sIPSCs and sEPSCs than silent cells. (D) mEPSC and mIPSC frequencies did not differ significantly for any MVN neuron group. mEPSC frequency was higher in the spontaneous spike firing compared to the silent MVN neurons, and in type B-like neurons compared to type A-like MVN neurons. Numbers in the bars indicate the n values.

Table 1

Morphology of MVN neurons

	MVN/ABi	MVN/ABc	MVN/i	MVN/c	MVN/n
Cell body long axis (μm)	23.3 $\pm$ 3.9 n=24	23.6 $\pm$ 4.6 n=29	21.9 $\pm$ 3.6 n=11	23.4 $\pm$ 5.4 n=17	23.1 $\pm$ 4.4 n=48
Number of stellate/elongate cells	19/5	22/7	8/3	12/5	41/7
Number of primary dendrites ^a	4.5 $\pm$ 0.9 n=25	3.7 $\pm$ 1.1 n=28	4.7 $\pm$ 0.7 n=11	3.8 $\pm$ 0.4 n=19	4.5 $\pm$ 1.1 n=53
Axon length (μm) ^b	958 $\pm$ 160 n=16	1873 $\pm$ 525 n=20	n/a	n/a	n/a
Direct distance to target (μm)	778 $\pm$ 185 n=15	1359 $\pm$ 327 n=20	n/a	n/a	n/a
Axon length: direct distance to target	1.2 n=15	1.5 n=20	n/a	n/a	n/a
Axon diameter (μm)	2 $\pm$ 0.4 n=15	2 $\pm$ 0.4 n=20	2 $\pm$ 0.3 n=8	2 $\pm$ 0.4 n=15	n/a

^aThe number of primary dendrites was significantly higher for ipsilateral (MVN/ABi, MVN/i) compared to contralateral-projecting neurons (MVN/ABc, MVN/c) ($P<0.05$).

^b Axons were significantly longer for MVN/ABc neurons compared to MVN/ABi neurons due to the longer distance from the target ($P<0.05$), but the ratio of axon length to direct distance was similar, indicating that ipsilateral- and contralateral-projecting axons followed equally direct paths.

In this and all subsequent tables, n refers to the number of cells

Table 2

Passive membrane properties

	Axon pathway				Spike firing		AP waveform		
	MVN/ABi n=29	MVN/ABc n=33	MVN/i n=13	MVN/c n=21	MVN/n n=70	SSF cells n=86	Silent cells n=80	Type A-like n=51	Type B-like n=70
RMP (mV)	-61.8±5.2	-64.5±7.1 ^a	-59.7±8.2	-60±6.5	-63.1±8.4	-58.0±3.7 ^a	-67.3±7.2	-64.9±7.9 ^a	-60.4±5.0
IR (MΩ)	185.1±102.9 ^b	155±83.8 ^b	285.9±168.1	242.7±157.4	244.1±169.4	279.8±157.6 ^b	156.1±98.3	188.8±114.3 ^b	251.4±133.9

^aResting membrane potential (RMP) was significantly more hyperpolarized for MVN/ABc neurons compared to MVN/c neurons, as well as for silent cells compared to spontaneous spike firing (SSF) cells, and type A-like cells compared to type B-like cells ($P<0.001$).

^bIR was significantly lower for MVN/ABc and MVN/ABi neurons compared to MVN/n neurons. Also, IR was significantly lower in MVN/ABc compared to MVN/i and MVN/c neurons, as well as for silent cells compared to SSF cells, and type A-like cells compared to type B-like cells ($P<0.001$).

Table 3

Spike waveforms

	Axon pathway				Spike firing			AHP waveform	
	MVN/ABi n=18	MVN/ABc n=19	MVN/i n=11	MVN/c n=16	MVN/n n=48	SSF cells n=69	Silent cells n=44	Type A-like n=38	Type B-like n=57
Threshold (mV)	-39.9±3.9	-40.8±4.3	-38.4±4.3	-40.4±5.3	-40.0±4.7	-40.2±3.7	-39.3±5.6	-40.2±5	-37.9±4.4
Peak ampl. (mV)	66.9±11.2 <i>a</i>	57.9±9.9	59.0±13.7	63.9±12.1	61.3±12.3	64.7±11.3 <i>b</i>	57.1±11.8	56.8±12.9	66.5±10.6 <i>c</i>
Rise time (ms)	0.7±0.2 <i>a</i>	1.0±0.3	0.9±0.3	0.8±0.3	1.0±0.3	0.9±0.3	0.9±0.3	1.0±0.4	0.8±0.2 <i>c</i>
Decay time (ms)	0.8±0.3 <i>a</i>	1.0±0.3	0.9±0.3	0.8±0.3	1.0±0.4	0.9±0.4	1.0±0.3	1.0±0.3	0.8±0.3 <i>c</i>
Half width (ms)	0.8± 0.2 <i>a</i>	1.0±0.3	1.0±0.4	0.9±0.3	1.0±0.3	0.9±0.3	1.0±0.3	1.0±0.3	0.8±0.2 <i>c</i>
Amplitude first phase AHP (mV)	-22.6±5.4	-21±5.8	-22.8±5.8	-23.0±7.7	-21.8±7.8	-23.3±7.2 <i>b</i>	-20.3±5.9	-21.1±6.1	N/A
Amplitude second phase AHP (mV)	-24.3±3.7	-23.9±4.4	-23.9±3.9	-23.5±6.4	-22.8±5.6	-23.8±5.1	-21.2±5.2	N/A	-23.0±5.4

SSF, spontaneous spike firing; AHP, afterhyperpolarization.

a MVN/ABi had significantly larger peak amplitude, faster rise time, and half width compared to MVN/ABc neurons. MVN/ABi had significantly faster rise time, decay time and half width compared to MVN/n neurons (*P*<0.05).

b Spikes of SSF cells had significantly larger peak amplitude and larger first phase AHP than silent cells (*P*<0.05).

c Spikes of type B-like neurons had significantly larger peak amplitude, faster rise time, decay time, and half width compared to type A-like and TypeB0-like neurons (*p*<0.05).

d Spikes of type B0-like neurons had significantly slower decay time compared type A-like neurons (*p*<0.05).

Table 4

Kinetics of mPSCs

	mEPSCs					mIPSCs					GABAergic mIPSCs				
	A (pA)	RT (ms)	DT (ms)	HW (ms)	n	A (pA)	RT (ms)	DT (ms)	HW (ms)	n	A (pA)	RT (ms)	DT (ms)	HW (ms)	n
MVN/ABi	18.6±3.7	0.7±0.3	2.0±0.9 ^a	2.2±0.5 ^a	8	47.6±13.3	1.8±0.5 ^b	15.1±22.9	11.2±11.1	10	37.9±13.8	1.4±0.6	7.9±3.6	7.8±4.0	3
MVN/ABc	17.6±1.1	0.7±0.3	1.7±0.7	2.0±0.56	5	57.4±30.2	1.2±0.3 ^b	9.2±3.4	7.3±1.9	9	47.5±22.5	1.4±0.1	7.1±3.3	5.3±2.9	5
MVN/n	18.2±4.4	0.5±0.1	1.2±0.3 ^a	1.6±0.3 ^a	7	50.1±14.5	1.4±0.5 ^b	10.5±5.3	9.4±5.2	16	49.4±11.4	1.4±0.5	8.2±2.2	7.7±2.2	7
SSF cells	20.9±4.5 ^c	0.5±0.2	1.6±0.7	1.9±0.5	18	52.1±14.6	1.5±0.5	8.9±3.3	8.6±3.2	12	47.1±13.8	1.4±0.5	8.4±2.6	8.3±2.7	5
Silent cells	17.4±2.6 ^c	0.7±0.3	1.4±0.6	1.8±0.5	11	50.5±21.6	1.5±0.6	12.7±14.7	9.7±8.0	25	46.2±18.9	1.4±0.4	7.5±3.0	6.3±3.0	10
Type A-like	17.7±3.0	0.6±0.2	1.2±0.29	1.7±0.4	6	49.3±25	1.6±0.4	11.7±5.8	10.2±6.2	9	46.6±22.1	1.6±0.3	7.1±2.9	6.4±2.8	5
Type B-like	20.5±4.9	0.5±0.1	1.6±0.7	1.9±0.5	17	51.7±18.7	1.5±0.6	11.9±15.1	9.5±7.5	23	49±13.4	1.4±0.5	8.5±1.5	7.6±2.8	7

A, peak amplitude; RT, rise time; DT, decay time; HW, half width;

^aDecay time and half width of mEPSCs was significantly slower in MVN/ABi neurons compared to MVN/n neurons.^bRise time of mIPSCs was significantly slower in MVN/ABi neurons compared to MVN/ABc and MVN/n neurons.^cPeak amplitude of mEPSCs was significantly higher in spontaneous spike firing MVN neurons compared to silent neurons.

Table 5**Summary**

	MVN/ABi	MVN/ABc	MVN/n
Number of primary dendrites	4.5±0.9 n=25	3.7±1.1 ^a n=28	4.5±1.1 n=53
% SSF neurons	66% n=29	21% ^a n=39	54% n=70
% type B-like neurons	68% n=28	38% ^a n=29	62% n=65
% repetitive spike firing on depolarization	76% n=29	30% ^a n=32	74% n=70
% mIPSCs	37% n=11 ^b	49% n=13	66% n=11
mEPSC decay time and halfwidth	2.0 ± 0.9 2.2± 0.5 n=8 ^c	1.7±0.7 2.0±0.6 n=5	1.2±0.3 1.6±0.3 n=7
mIPSC rise time	1.8 ± 0.5 n=10 ^c	1.2±0.3 n=9	1.4±0.5 n=16

^a indicates significant different between MVN/ABc neurons and MVN/ABi and MVN/n neurons.

^b Percentage of mIPSCs was significantly lower for MVN/ABi neurons compared to MVN/n neurons ($P<0.05$).

^c mEPSCs of MVN/ABi neurons had slower decay time and halfwidth compared to MVN/N neurons, and their mIPSCs had slower rise time compared to MVN/ABc and MVN/n neurons.