Emergence of Action Potential Generation and Synaptic Transmission in Vestibular Nucleus Neurons

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INTRODUCTION

Vestibular nucleus neurons (VNNs) are second-order neurons in the three-neuron vestibulooculocordic reflexes, which regulate posture and balance. Vestibular nuclei are most often described as containing small, medium, and large neurons randomly dispersed in the nucleus (e.g., McCrea et al., 1987). However, among the vertebrate vestibular nuclei, the chicken tangential nucleus is architecturally striking because 80% of its neurons are principal cells, the large oval somata of which align in rows between the horizontal bundles of primary vestibular fibers near the lateral surface of the medulla oblongata (Peusner and Morest 1977). Besides their striking morphology and orderly arrangement, the principal cells are characterized by a rather unique one-to-one relationship between the primary vestibular fiber and the principal cells. Their presynaptic inputs, here we defined for the first time their spontaneous activity at these early developmental ages. Altogether, this information will lead to a better understanding of the synaptic development of VNNs and the emergence of second-order neuron excitability in the vestibular reflex pathways. In addition to this, understanding the developmental steps leading up to mature neuronal function may help in clarifying the process by which VNNs recover their electrical excitability after injuries to the peripheral vestibular end organ. Indeed, in many different adult systems, including the vestibular system, certain aspects of cellular development may be repeated during recovery of function after injury or lesions (for review, see Straka et al. 2005).

So far, the excitability of young embryonic principal cells and their presynaptic signals have not been investigated electrophysiologically. Accordingly, as a first step, the generation of action potentials on depolarization, the presence of postsynaptic currents on vestibular-nerve stimulation, and the spontaneous and miniature synaptic events in the principal cells were studied at E10 and E13, and compared with events recorded in older embryos (E16) and hatching chickens (H1) (Shao et al. 2003, 2004, 2006).

METHODS

Experimental animals

The experiments were performed on White Leghorn chick embryo eggs (Gallus gallus), obtained from CBT Farms (Chesterfield, MD), and incubated in a laboratory incubator with temperature and humidity control and an egg-rotation unit (Humidaire Incubator, No. 21), until the appropriate ages (E10, E13). The age of the embryos was morphogenesis to be traced in the chick embryo (Peusner and Morest 1977). Briefly, at embryonic day 7.5 (E7.5), the colosal fibers first contact the principal cells, forming immature chemical synapses (Petrailia and Peusner 1990), whereas the spoon endings first emerge from the colosal fibers at E10 and acquire their greatest expansion over the principal cell somata by E13 (Peusner 1981). Subsequently, the spoon terminals retract, covering about a third of the soma at E16, and ~10% at H1 (Peusner 1984). To our knowledge, synaptic transmission between the primary vestibular fibers and VNNs has not been investigated at early embryonic ages. Thus this system offers the opportunity to define for the first time the early steps in the emergence of synaptic transmission between first- and second-order neurons in the vestibular reflex pathways. In addition, because VNNs are known for processing multisensory inputs, here we defined for the first time their spontaneous synaptic activity at these early developmental ages. Altogether, this information will lead to a better understanding of the synaptic development of VNNs and the emergence of second-order neuron excitability in the vestibular reflex pathways. In addition to this, understanding the developmental steps leading up to mature neuronal function may help in clarifying the process by which VNNs recover their electrical excitability after injuries to the peripheral vestibular end organ. Indeed, in many different adult systems, including the vestibular system, certain aspects of cellular development may be repeated during recovery of function after injury or lesions (for review, see Straka et al. 2005).

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established by referring to the staging criteria of Hamburger and Hamilton (1951). The animal protocols were approved by the Institutional Animal Care and Use Committee of the George Washington University.

Preparation of brain slices

Briefly, the embryos were removed from the egg and decapitated, and the head was placed in a dish containing oxygenated artificial cerebrospinal fluid (ACSF) chilled to 1–4°C. Under a dissecting microscope, the brain stem was dissected out, mounted on the microslicer tray, and immersed in ACSF (for details, see Shao et al. 2003). Transverse sections of the brain stem containing the tangential nucleus were cut at 350 μm at E10 and 300 μm at E13, using a Leica microslicer (VT1000S) and razor blades (Feather blue blades; Ted Pella). When preparing the brain slices, the vestibular hair cells were removed, but the vestibular ganglia were kept attached in all slices prepared at E10 (n = 34) and some slices at E13 (n = 8/36). Indeed, the vestibular ganglion was retained easily in young embryos because the tissue around the ganglion is soft and readily dissected and sectioned without tearing. In the remaining E13 brain slices (n = 28), the vestibular ganglia were removed by cutting their central axons. Recordings from preparations with or without the vestibular ganglion allowed us to determine whether vestibular ganglion cells, in the absence of vestibular hair cell input, can fire spontaneously at E13. In addition, at E10, the vestibular ganglion provided a useful landmark to identify the tangential nucleus under the dissecting and fixed stage microscopes.

Solutions and drugs

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% O2–5% CO2 to maintain the pH at 7.2–7.4. The osmolarity was adjusted to 310–320 mOsm. All of the drugs, except 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, RBI), were dissolved in distilled water and added to the bath ACSF to achieve the final concentration. Tetrodotoxin (TTX; 1 μM; RBI) was used to block the voltage-dependent sodium current. D-Phenylalanine, DL-alanine, and choline chloride were added to the bath ACSF to achieve the final concentration. The threshold voltages were 10–40 V. Impulse duration was set at 0.2–3 ms at E10 and 0.2–2 ms at E13. Impulse frequency was set at 0.5–2 Hz at E10 and 0.5–2 Hz at E13.

Biocytin processing of brain slices

During recordings, biocytin diffused from the pipette into the recorded neuron. After the recordings, the slices were processed for biocytin detection with Streptavidin conjugated to Alexa Fluor 647 (Shao et al. 2003). For imaging with a conventional fluorescence microscope (Nikon Eclipse E600), the brain slices were counterstained with NeuroTrace (1:100 in PBS for 20 min; Molecular Probes), which provided Nissl-like images of the somata. The background staining assisted in establishing the boundaries of the tangential nucleus.

Electrophysiology

After 1 h recovery at room temperature, the brain slices were placed in a small, glass-bottom recording chamber (180 μl, Warner Instruments) and held in place with nylon threads glued to a U-shaped, flattened platinum wire. The recording chamber was mounted on a fixed-stage upright microscope (Zeiss Axioskop FS-1) and the slices were superfused continuously with heated ACSF (30–31°C) at a rate of 2–3 ml/min. The region of the tangential nucleus was identified under a ×2.5 objective, while the neurons were selected for recordings using a ×40 water-immersion lens (NA, 0.75), DIC optics, and a ×4 lens mounted between the microscope and an infrared tube camera (Vidicon C2400-01, Hamamatsu). Infrared image contrast and shading were adjusted with a camera controller (C2400-01, Hamamatsu).

Microelectrodes were pulled from borosilicate glass tube (1.5 mm OD, 1.12 mm ID, thin-walled, World Precision Instruments) using a Brown/Flaming horizontal pipette puller (P-87, Sutter Instruments). Cesium gluconate (Cs-gluconate) pipette solution, used to record the spontaneous synaptic activity and the postsynaptic currents on vestibular-nerve stimulation in voltage-clamp mode, contained (in mM) Cs-gluconate 115, 1 EGTA, 10 HEPES, 0.1 CaCl2, and 2 MgCl2. The pH was adjusted to 7.2 with CsOH. Potassium methylsulfate (KMeSO4) pipette solution, used for the current-clamp recordings and the detection of sodium currents in the voltage-clamp recordings, contained (in mM) 120 KMeSO4, 20 KCl, 10 HEPES, 0.5 EGTA, 2 MgCl2, 4 Na2ATP, and 0.3 Tris GTP. The pH was adjusted to 7.2 with KOH. For both pipette solutions, the osmolarity was 270–290 mOsm. Biocytin (0.5%) was made weekly in the pipette solution and kept frozen. The pipette resistance was 1–3 MΩ with Cs-gluconate or KMeSO4 pipette solutions containing biocytin.

Stimulation of the vestibular ganglion was achieved using a bipolar electrode made of silver wire coated with Teflon except at the tips (125 μM diam, WPI). The tips of the stimulating electrode were relatively large compared with the vestibular ganglion and the vestibular nerve at the ages investigated to provide adequate stimulation and minimize the possibility of failure due to the position of the electrode. The stimulation intensity was 1.2–2 times threshold, with the threshold voltages between 10 and 40 V. Impulse duration was set at 20 μs with a stimulus isolation unit (DS2, Digitimer LTD). The stimulating electrode was placed on the vestibular ganglion or the vestibular nerve ~200–350 μm (E10) or 400–500 μm (E13) away from the recording electrode in the tangential nucleus.

Whole cell patch-clamp recordings were performed using an Axopatch 200B amplifier in normal mode (Axon Instruments). The axo-patch amplifier may produce distortions in the action potential waveform, most apparent in the first action potential afterhyperpolarization (AHP) (Magistretti et al. 1996, 1998). However, because all the waveforms were recorded with the same amplifier, the same distortion, if present, applies to all of the waveforms obtained at different ages, so this should not affect the conclusions (Shao et al. 2006). The recording pipette was advanced through the slice under visual guidance using a piezoelectric manipulator (PCS-5000, Burleigh Instruments). The Rs compensation was set at 80%, lag 10 μs. The voltage-clamp recordings were digitized at 10 kHz and filtered at 2 kHz, whereas the current-clamp recordings were digitized at 20 kHz and filtered at 5 kHz.

Data acquisition and analysis

Immediately after obtaining the whole cell configuration, the resting membrane potential (RMP) was read from the amplifier panel.
mV), or for the liquid junction potential of Cs-gluconate pipette solution (+3 mV) (Neher 1992).

The current-clamp recordings of responses to depolarizing pulses and the voltage-clamp recordings of the postsynaptic currents generated on vestibular-nerve stimulation and sodium currents were recorded directly on the computer (Dell Pentium 4) using the pClamp program (version 9.2). Spontaneous and miniature synaptic activity was obtained using pClamp program (version 6.0.3; Axon Instruments), recorded with a DAT tape recorder (DTR-1201, Biologic Instruments), and acquired with Axoscope 8.1 software (Axon Instruments).

The kinetics for action potentials, generated by depolarizing current pulses of 400 ms and various intensities, were analyzed from averages of three to five spikes per cell. Spike threshold was defined as the point in the voltage trace where a sharp inflection appeared just before generating the action potential (see Shao et al. 2006). Spike amplitude was defined as the potential difference between threshold and peak amplitude. Action potential rise time was defined as the duration between threshold and peak amplitude during depolarization. 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 amplitude of the AHP was defined as the difference in voltage between spike threshold and the peak negativity after the spike. Silent principal cells were defined as neurons which generated no action potentials on depolarization up to ~30 mV using depolarizing pulses. Inward sodium currents were induced using 100 ms voltage steps from −100 to −10 mV, in 10 mV increments, starting from −60 mV. The amplitude of the inward sodium currents was measured as the difference between baseline and the inward peak at a holding potential of ~30 mV.

The postsynaptic currents generated on vestibular-nerve stimulation and the spontaneous synaptic currents were analyzed using MiniAnalysis program (version 6.0.3, Synaptosoft). The percentage of miniature events was defined as the proportion of spontaneous synaptic events that was not abolished on exposure to TTX. Silent principal cells were defined as neurons which generated no action potentials on depolarization. The latency for the evoked postsynaptic response was measured from the start of the stimulus artifact to the start of the response. The analyses of vestibular-evoked and spontaneous synaptic currents 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). For the spontaneous synaptic events, the minimal acceptable amplitude for detecting an event (threshold) was set at two to three times the root mean square of the baseline noise, which was 1–3 pA at −60 mV, and 2–5 pA at +10 mV. To define the proportion of GABA and glycine sIPSCs, recordings with relatively high-frequency of postsynaptic events were selected for the analysis. The data are presented as the means ± SE. The differences were analyzed using the independent Student’s t-test, with the significance level set at P < 0.05.

Finally, data analysis included comparing the data obtained at E10 and E13 to our previously published data on the principal cells obtained at E16 and H1 (Shao et al. 2003, 2004, 2006). Student’s t-test was used to detect significant differences from E10 to H1.

RESULTS

Morphology and passive membrane properties of the principal cells

Whole cell patch-clamp recordings were obtained from a total of 70 principal cells at E10 (n = 34) and E13 (n = 36). The identity of all of the recorded neurons was confirmed by studying the morphology of the somata, dendrites, and axons. These included the silent principal cells recorded from. At E10, the oval somata of the principal cells measured 23 ± 0.6 × 12.7 ± 0.7 μm (n = 34), and by E13, they had grown significantly to 30 ± 0.7 × 19 ± 0.6 μm (n = 36) (P < 0.05). At E10, the principal cells often exhibited a thickened lateral process (↓↓↓), and many fine processes (*) radiating in all directions from the soma and lateral process (Fig. 1A, 1B, 1C and 1). By E13, the principal cells had increased numbers of fine processes, which were often longer than those seen at E10 (Fig. 1B, 1C and 1D, *). At E13, two morphological subtypes of principal cells were distinguished based on their dendritic branching pattern, as described already at E16 and H1 (Peusner and Giaume 1997). The vast majority of the principal cells (n = 27/36; 75%) had three to four primary dendrites radiating in the dorsal, ventral, medial and lateral directions (Fig. 1B1), while the remaining principal cells had one lateral, primary dendrite that branched profusely (Fig. 1B2). There was no significant difference in the location, RMP, or input resistance between these two neuron subtypes. In addition to the heavily labeled, biocytin-injected principal cells, the slices routinely contained lightly labeled neuron and glial cell bodies (Fig. 1, A), which were stained likely by the passage of biocytin dye through the gap junction channels joining the injected principal cell with the lightly labeled cells (Popratiloff et al. 2005).

Using KMeSO₄, the RMP of the principal cells was −67.8 ± 1.8 mV at E10 (n = 10), and −73.4 ± 1.9 mV at E13 (n = 10). The input resistance was 199 ± 16 MΩ at E10 (range: 76–450 MΩ; n = 34), and 90 ± 7 MΩ at E13 (range: 43–204 MΩ; n = 36). Compared with E16 and H1 (Shao et al. 2003, 2004, 2006), no significant differences were found in the RMPs among the four ages, but there was a significant decrease in the input resistance of the principal cells between E10 and all of the other ages (Table 1).

Responses to depolarizing current pulses

At E10, in current-clamp mode, 6/10 of the principal cells fired a single spike on depolarization (Fig. 2A1), whereas the remaining four cells were silent (Fig. 2B1). In voltage-clamp, the spike-firing principal cells exhibited a fast-rising, inward current on depolarization (Fig. 2A2; 686 ± 217 pA, range: 122–1300 pA, n = 6). The inward current was sensitive to 1 μM TTX (n = 2, data not shown), indicating that it was a sodium current. However, the silent principal cells exhibited no net inward currents (Fig. 2B2; n = 4), although they exhibited a slight inward deflection on depolarization (Fig. 2B2 *).

At E13, 5/10 of the principal cells fired single spikes on depolarization (Fig. 2C1), whereas the remaining cells were silent (Fig. 2D1). Compared with the principal cells generating single spikes at E10, the single-spike firing principal cells at
E13 had larger amplitude sodium currents (2132 ± 690 pA, range: 535–3725 pA, n = 5; P < 0.05; Fig. 2C2), which were sensitive to TTX (n = 2, data not shown). As found at E10, the silent principal cells at E13 expressed no net inward sodium currents (Fig. 2D2) but showed an increased inward deflection on depolarization (Fig. 2D2,*). The I-V curve plotted at E10 showed a steeper slope than at E13, corresponding to the higher input resistance measured in E10 principal cells (Fig. 2E).

At E10 and E13, no significant differences were found in the morphology, cell location, RMP, or input resistance between silent and single-spike firing principal cells. In addition, on

TABLE 1. Membrane properties at E10, E13, E16, and H1

<table>
<thead>
<tr>
<th>Age</th>
<th>RMP, mV</th>
<th>Input resistance, MΩ</th>
</tr>
</thead>
<tbody>
<tr>
<td>E10</td>
<td>–67.8 ± 1.8 (10)</td>
<td>199 ± 16 (34)*</td>
</tr>
<tr>
<td>E13</td>
<td>–73.4 ± 1.9 (10)</td>
<td>90 ± 7 (36)</td>
</tr>
<tr>
<td>E16</td>
<td>–74 ± 1 (24)</td>
<td>78 ± 7 (12)</td>
</tr>
<tr>
<td>H1</td>
<td>–72 ± 2 (16)</td>
<td>67 ± 12 (30)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Number of cells is in parentheses. RMP was measured using KMeSO4, input resistance was measured using Cs-gluconate electrode solution. *Student’s t-test, significant difference between E10 and all the other ages studied. The data for E16 and H1 were taken from Shao et al. (2003, 2004, 2006). RMP, resting membrane potential.
exposure to 200 nM DTX, the firing pattern of silent (E13, \( n = 2 \)) and single-spike firing principal cells did not change (E10, \( n = 2; \ E13, n = 3 \); data not shown). These experiments suggested that DTX-sensitive potassium channel, if present, was insufficient to change the firing pattern in the principal cells as found at E16 (Gamkrelidze et al. 1998).

The threshold for spike generation did not change significantly (\( P > 0.05 \)) between E10 (\(-33.3 \pm 2.7 \) mV, \( n = 6 \)) and E13 (\(-35 \pm 1.4 \) mV, \( n = 5 \)). However, the action potentials generated on depolarization in the principal cells at E13 had significantly larger amplitudes (52.2 \( \pm 5.4 \) vs. 34.2 \( \pm 2.8 \) mV), faster rise times (0.57 \( \pm 0.06 \) vs. 1.08 \( \pm 0.1 \) ms), faster decay times (0.64 \( \pm 0.03 \) vs. 1.48 \( \pm 0.2 \) ms), and shorter half-widths (0.65 \( \pm 0.03 \) vs. 1.45 \( \pm 0.2 \) ms; \( P < 0.05 \); Fig. 2F, I and 2). All of the spikes generated on depolarization showed a single fast AHP (Fig. 2F, I and 2, ↑), with an amplitude of 5.6 \( \pm 0.8 \) mV at E10 and 21.6 \( \pm 1.9 \) mV at E13, which were significantly different (\( P < 0.05 \)). Indeed, a second, slow AHP first appears in the principal cells at E16, which is mediated by a calcium-dependent potassium current (SK) (Shao et al. 2006). Accordingly, here we tested whether SK currents were present before the appearance of the second AHP in the principal cells. Using a 15-ms voltage step from \(-60 \) to \(+20 \) mV in voltage-clamp mode, no outward currents were induced, indicating that there was no SK current present in the principal cells at E10 and E13 (Shao et al. 2006; \( n = 4 \); data not shown).

During later development, the firing threshold became more negative, with significant differences between E10 and H1 and E13 and H1 (\( P < 0.05 \); Table 2). However, the action potential amplitude and kinetics recorded at E13 did not change up to hatching. In addition, at E16, 50% of the principal cells acquired a second, slow AHP, which followed the fast one, and

### Table 2. Action potential waveforms at E10, E13, E16, and H1

<table>
<thead>
<tr>
<th>E10</th>
<th>E13</th>
<th>E16</th>
<th>H1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threshold, mV</td>
<td>33.3 ± 2.7</td>
<td>35 ± 1.4</td>
<td>39 ± 3.6</td>
</tr>
<tr>
<td>Amplitude, mV</td>
<td>34.2 ± 2.8</td>
<td>52.2 ± 5.4*</td>
<td>64 ± 5</td>
</tr>
<tr>
<td>Rise time, ms</td>
<td>1.1 ± 0.1</td>
<td>0.6 ± 0.1*</td>
<td>0.5 ± 0.04</td>
</tr>
<tr>
<td>Decay time, ms</td>
<td>1.5 ± 0.2</td>
<td>0.6 ± 0.03*</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Halfwidth, ms</td>
<td>1.5 ± 0.2</td>
<td>0.7 ± 0.03*</td>
<td>0.6 ± 0.04</td>
</tr>
<tr>
<td>First AHP, mV</td>
<td>5.6 ± 0.8</td>
<td>21.6 ± 1.9*</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>Second AHP, mV</td>
<td>none</td>
<td>none</td>
<td>8.8 ± 1.3</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Student's t-test, significant difference compared to all younger ages. **Student's t-test, significant difference compared to E10 and E13. The data for E16 and H1 were adapted (E16) or taken (H1) from Shao et al. (2006). The action potential waveforms were averaged from the first spike evoked on depolarization at E10, E13, and E16, whereas it was measured from spontaneous firing at H1. AHP, afterhyperpolarization.
90% of the principal cells had two AHPs at H1 (Fig. 2F, 3 and 4, 111; Table 2).

**Postsynaptic responses on vestibular-nerve stimulation**

At −60 mV holding potential, using Cs-gluconate pipette solution in voltage-clamp mode, half of the principal cells at E10 (n = 4/8), and most of the recorded cells at E13 (n = 5/6) displayed postsynaptic currents on vestibular-nerve stimulation (Fig. 3, A1 and B1). At both ages, these events were abolished completely by 10 μM CNQX, indicating that they were AMPA receptor-mediated events (Fig. 3, A2 and B2). The latency for the postsynaptic response generated in the principal cells was 1.9 ± 0.1 ms at E10 and 1.8 ± 0.1 ms at E13 without significant difference (P > 0.05). In addition, at E10, the amplitude of the evoked EPSCs was 70 ± 32 pA, the rise time was 0.9 ± 0.1 ms, the decay time was 4.5 ± 1.1 ms, and the half-width was 4.4 ± 1.2 ms. At E13, the amplitude of the evoked EPSCs was 400 ± 120 pA, whereas the rise time was 0.5 ± 0.2 ms, the decay time was 2.3 ± 1.3 ms, and the half-width was 2.4 ± 1.2 ms, which were significantly different from E10 (P < 0.05). Accordingly, as early as E10, the primary vestibular fibers fired action potentials on galvanic stimulation, which generated postsynaptic responses in the principal cells.

**Properties of spontaneous synaptic activity**

Using Cs-gluconate pipette solution and different holding potentials, the spontaneous excitatory (sEPSCs) and inhibitory postsynaptic currents (sIPSCs) could be separated within the same cell and identified by applying pharmacological agents. At E10, at a holding potential of −60 mV, all of the events recorded in the principal cells were inward currents, with a mean frequency of 0.2 ± 0.06 Hz (n = 21; Fig. 4A1). These events were blocked completely by 10 μM CNQX (n = 3; Fig. 4A2), indicating that they were sEPSCs mediated by glutamatergic AMPA receptors. When the holding potential was changed to +10 mV, all of the events were recorded as outward currents, with a mean frequency of 0.6 ± 0.12 Hz (n = 22; Fig. 4B1). These currents were blocked totally by 10 μM bicuculline (n = 8), indicating that they were sIPSCs mediated by GABA_A receptors (Fig. 4B2). Thus as early as E10, the principal cells exhibited sEPSCs mediated entirely by AMPA receptors, and sIPSCs mediated completely by GABAergic receptors.

At E13, at a holding potential of −60 mV, all of the events recorded from the principal cells were inward currents, with a mean frequency of 0.7 ± 0.1 Hz (n = 18; Fig. 5A1). These currents were blocked completely by 10 μM CNQX (n = 5; Fig. 5A2), which indicated that they were sEPSCs mediated by AMPA receptors. At a holding potential of +10 mV, all of the events recorded from the principal cells were outward currents with a mean frequency of 0.8 ± 0.2 Hz (n = 23; Fig. 5B1). However, the outward currents were blocked completely by 10 μM bicuculline in only 3/10 of the principal cells (data not shown). In the remaining seven principal cells, 10 μM bicuculline blocked 75 ± 8% of the outward current, and the remaining events were blocked by 1 μM strychnine (Fig. 5B,

**FIG. 4.** Spontaneous EPSC (sEPSCs) and inhibitory postsynaptic currents (sIPSCs) recorded in principal cells at E10. A: at −60 mV, all of the events appeared as inward currents (A1), which could be blocked completely by 10 μM CNQX (A2). B: at +10 mV, all of the events appeared as outward currents (B1), which could be blocked completely by 10 μM bicuculline (B2). These results indicated that the principal cells exhibited AMPA receptor-mediated sEPSCs, and GABAergic sIPSCs at E10. AMPA receptors, and sIPSCs mediated completely by GABAergic receptors. At E13, at a holding potential of −60 mV, all of the events recorded from the principal cells were inward currents, with a mean frequency of 0.7 ± 0.1 Hz (n = 18; Fig. 5A1). These currents were blocked completely by 10 μM CNQX (n = 5; Fig. 5A2), which indicated that they were sEPSCs mediated by AMPA receptors. At a holding potential of +10 mV, all of the events recorded from the principal cells were outward currents with a mean frequency of 0.8 ± 0.2 Hz (n = 23; Fig. 5B1). However, the outward currents were blocked completely by 10 μM bicuculline in only 3/10 of the principal cells (data not shown). In the remaining seven principal cells, 10 μM bicuculline blocked 75 ± 8% of the outward current, and the remaining events were blocked by 1 μM strychnine (Fig. 5B,

**FIG. 5.** sEPSCs and sIPSCs recorded in principal cells at E13. A: at −60 mV, all of the events were recorded as inward currents (A1), which could be blocked completely by 10 μM CNQX (A2). B: at +10 mV, all of the events appeared as outward currents (B1), which could be blocked partially by 10 μM bicuculline (B2), whereas the remaining currents were abolished completely by 1 μM strychnine (B3). Thus at E13, the principal cells exhibited AMPA receptor-mediated sEPSCs and both glycinergic and GABAergic sIPSCs.
Dependence of spontaneous synaptic events on presynaptic action potentials

TTX (1 μM) was applied to test the dependency of spontaneous synaptic activity on presynaptic action potentials. At E10, after TTX exposure, the frequency was 0.23 ± 0.1 Hz for mEPSCs (Fig. 6A, I and 2; n = 8), and 0.56 ± 0.2 Hz for mIPSCs (Fig. 6B, I and 2; n = 8). Compared with the control, TTX did not change significantly the frequency of EPSCs (98 ± 9%) or IPSCs (106 ± 7%; P > 0.05). Accordingly, there were few, if any, postsynaptic action-potential-dependent events generated in the principal cells at E10.

However, at E13, after TTX exposure, the frequency was 0.77 ± 0.2 Hz for mEPSCs (Fig. 7A, I and 2; n = 8), and 0.57 ± 0.1 Hz for mIPSCs (Fig. 7B, I and 2; n = 12). Compared with the control, TTX decreased the frequency of the sampled sEPSCs (0.88 Hz) by 12 ± 3% and sampled sIPSCs (0.69 Hz) by 17 ± 4% (P < 0.05). Thus at E13, spontaneous synaptic activity was a mixture of action-potential-dependent and independent events. These results indicated that at E13, but not at E10, both excitatory and inhibitory presynaptic neurons were present within the brain slice preparation, and these neurons generated action potentials that were propagated to the presynaptic terminals contacting the principal cells. At E13, in brain slices with the vestibular ganglion attached, the frequency of sEPSCs was 0.64 ± 0.3 Hz (n = 6), whereas the frequency was 0.78 ± 0.1 Hz (n = 12) in slices lacking the vestibular ganglion, which was not significantly different (P > 0.05). These results suggest that the vestibular ganglion cells were not firing spontaneously at E13 in these brain slice preparations and did not contribute to the action potential-dependent sEPSCs recorded in the principal cells.

Amplitude distribution of the EPSCs and IPSCs

When the cumulative probability of the amplitude distributions were plotted at E10 and E13, the plots for sEPSCs (Fig. 8A) and mEPSCs (B) showed shifts to the right from E10 to E13, indicating that there were more of the large-amplitude EPSCs at E13 than at E10. The mean amplitude of the sEPSCs was 17.4 pA at E10 and 23 pA at E13. The mean amplitude of the mEPSCs was 15.5 pA at E10 and 18.2 pA at E13 (Table 3). Similarly, the plots for sIPSCs (Fig. 8C), mIPSCs (D), and GABAergic mIPSCs (E) showed shifts to the right from E10 to E13, supporting that there were more of the large-amplitude IPSCs at E13 than at E10. The mean amplitude of the sIPSCs was 24.8 pA at E10 and 48.9 pA at E13. The mean amplitude of the mIPSCs was 25.8 pA at E10 and 41.8 pA at E13. The mean amplitude of the GABAergic mIPSCs was 25.8 at E10 and 33.6 at E13 (Table 3). All of the data exhibited significant differences between E10 and E13 (P < 0.05). In addition, when glycinergic events first appeared at E13, the mean amplitude of their mIPSCs was 52.1 pA, which is significantly larger than the GABAergic mIPSCs at this age (P < 0.05).

Kinetics of the miniature events

For mEPSCs and GABAergic mIPSCs, the rise time, decay time, and half-width were significantly faster at E13 compared with E10 (P < 0.05; Table 4). When glycinergic mIPSCs first appeared at E13, the rise time was 1.1 ms, decay time was 17.4 ms, and half-width was 15 ms (n = 6), which were similar to the kinetics of GABAergic mIPSCs at this age (P > 0.05; Table 4).

To gain a better appreciation of the developmental change in synaptic transmission in the principal cells, we compared the data obtained at E10 and E13 with those previously published during the perinatal period (E16 and H1) (Shao et al. 2003, 2004).

Developmental change in sEPSCs and sIPSCs frequency

From E10 to H1, sEPSCs frequency increased gradually, with significant changes occurring between E10 and E13, and
E16 and H1. At the same time, sIPSCs frequency showed the largest increase between E13 and E16 (Fig. 9A). The frequency of GABAergic sIPSCs increased significantly from E10 to E16 and then decreased at H1. In addition, the frequency of glycinergic sIPSCs followed a slowly increasing trend, starting with no measurable current at E10, to the first appearance at E13, and increasing significantly by E16 (Fig. 9B). Accordingly, both GABAergic and glycinergic events contributed to the major increase in sIPSCs frequency from E13 (0.8 Hz) to E16 (2.3 Hz).

During development, GABA and glycine events contributed different proportions to the total composition of the sIPSCs. The percentage of GABAergic sIPSCs progressively decreased, whereas the percentage of glycinergic sIPSCs increased, with significant changes between E10 and E13 and E16 and H1 (Fig. 9C).

Developmental change in mEPSCs and mIPSCs frequency

The frequency of mEPSCs increased significantly from E10 to E13, whereas from E13 to E16, the frequency of mIPSCs increased dramatically (Fig. 9D). In addition, both GABAergic and glycinergic mIPSCs increased significantly from E13 to E16. However, subsequently GABAergic mIPSCs decreased significantly by H1 without any change in the glycinergic mIPSCs frequency (Fig. 9E). Accordingly, the significant increases in mIPSCs from E13 to E16 involved both GABAergic and glycinergic miniature events.

When the ratio of miniature/spontaneous events was compared during the developmental period from E10 to H1, the proportion of mEPSCs decreased continuously, which was significant between all ages tested (Fig. 9F). Also, the percentage of mIPSCs decreased, with significant differences at E10 and E13, and E13 and H1 (Fig. 9F). These results showed that synaptic transmission became more dependent on presynaptic action potentials in older animals.

Developmental change in EPSCs and IPSCs amplitude

From E10 to H1, the mean amplitude for sEPSCs and mEPSCs increased continuously, with significant differences between E10 and E13 and E13 and E16 (Fig. 10, A and B). For sIPSCs and mIPSCs, the mean amplitude also increased gradually, with significant differences between E10 and E13 and E13 and E16 and H1 (Fig. 10, C and D). The amplitude of GABAergic mIPSCs increased significantly from E10 to E13 (Fig. 10E), whereas the amplitude of glycinergic mIPSCs exhibited greater increase between E16 and H1 (Fig. 10F).

Developmental change in mEPSCs and mIPSCs kinetics

The rise time, decay time, and half-width of mEPSCs decreased significantly between E10 and E13 and E16 and H1 (Fig. 11A). In addition, both GABAergic and glycinergic mIPSCs significantly decreased between E13 and H1 (Fig. 11B). However, GABAergic mIPSCs decreased significantly from E10 to E13 (Fig. 11A). For GABAergic mIPSCs, there was a significant decrease in rise time, decay time, and half-width between E10 and E13, and a significant decrease in decay time and half-width between E13 and E16 (Fig. 11B). For glycinergic mIPSCs, there was a significant decrease in rise time, decay time, and half-width from E13 to E16, and a significant decrease in rise time from E16 to H1 (Fig. 11C).

![Figure 8](http://jn.physiology.org/)

**Fig. 8.** Amplitude distribution plots for sEPSCs (A), mEPSCs (B), sIPSCs (C), mIPSCs (D), and GABAergic mIPSCs (E) showed shifts to the right from E10 to E13, indicating increased number of large-amplitude events from E10 to E13.

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<tr>
<th>TABLE 3. Amplitude of EPSCs and IPSCs at E10 and E13</th>
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Values are means ± SE. Student’s t-test showed significant differences in all of the amplitude measurements made between the two ages. sEPSC and mEPSC, spontaneous and miniature excitatory postsynaptic current; sIPSC and mIPSC, spontaneous and miniature inhibitory postsynaptic current.
slice studies showed were compared systematically to the activity recorded at E16 and H1 (Shao et al. 2003, 2004, 2006) to evaluate the long-term developmental pattern. Altogether, these in vitro brain slice studies showed 1) at E10 and E13, ~50–60% of the principal cells generated action potentials on depolarization. The “silent” principal cells, i.e., those that cannot generate spikes on depolarization, were a major functional class (40–50%) at these ages. However, the number of silent cells declined significantly at E16 (13%) (Gamkrelidze et al. 1998), and H1 (0%) (Shao et al. 2006), coinciding with major changes in sodium ($I_{Na}$) and potassium currents ($I_{ks}$, $SK$). 2) On vestibular-nerve stimulation, 50% of principal cells generated EPSCs at E10, and 82% at E13. Apparently, the first-order primary vestibular axons generate spikes in advance of signaling at the synapses between hair cells and afferent vestibular axons in the inner ear (see following text). 3) Miniature events composed all of the sEPSCs and sIPSCs recorded at E10, and most of them at E13, but their relative contribution to the spontaneous synaptic activity decreased during subsequent development. Accordingly, the miniature events, which predate the appearance of activity-dependent spontaneous synaptic activity, could play a crucial role in the subsequent emergence of synaptic transmission.

All of the present studies were performed on brain slice preparations. The most important structures cut off in these brain slices were the vestibular hair cells and bipolar vestibular ganglion cells. However, no action potential-dependent sEPSCs were recorded at E10 with the vestibular ganglia attached, and sEPSC frequency was not affected by the presence or absence of the vestibular ganglion cell bodies at E13, suggesting that the vestibular ganglion cells could not fire action potentials in the absence of hair cell signaling at these ages. Indeed, vestibular hair cells are most likely too immature at these embryonic ages to transmit signal to the brain (see following text). Accordingly, cutting off the hair cells in our embryonic brain slice preparations did not change the sEPSC frequency in the VNNs, although hair cell removal can modify the spontaneous spike activity of primary vestibular afferents in older animals (Li and Correia 1998). Besides input from the vestibular nerve, there are numerous other inputs to the principal cells which originate from central neurons (see Cox and Peusner 1990a,b; Doi et al. 1990; du Lac and Lisberger 1992; Gross 1985; Sato et al. 1997). Therefore the few sEPSCs recorded in the principal cells at E13 most likely originated from excitatory inputs other than primary vestibular fibers.

Based on our findings, the following topics will be discussed: the development of action potential generation and firing pattern, the development of signaling between the vestibular inner ear and VNNs, and the development of synaptic transmission in VNNs and its relation to vestibular system function.

**FIG. 9.** Developmental change in frequency of spontaneous and miniature synaptic events. A: frequency of sEPSCs increased during development with significant differences between E10 and E13 and E16 and H1. Also, sIPSC frequency increased during development with a significant increase between E13 and E16. B: frequency of GABAergic sIPSCs increased significantly between E10 and E16 but decreased significantly by H1. The frequency of glycinegic sIPSCs increased significantly between E13 and E16. C: during development, the percentage of GABAergic sIPSCs decreased continuously, whereas at the same time the percentage of glycinegic sIPSCs increased continuously with significant differences between E10 and E13 and E16 and H1. D: there was a significant increase in mEPSCs frequency from E10 to E13 and a significant increase in mIPSCs frequency from E13 to E16. E: frequency increased significantly for both GABAergic and glycinegic mIPSCs from E13 to E16. F: percentage of mEPSCs decreased during development with significant differences at all ages. The percentage of mIPSCs also decreased with significant differences between E10 and E13 and E16 and H1. These results support that synaptic transmission is more dependent on the presynaptic action potentials in the older chickens. The data for E16 and H1 were taken (A, C, D, and F) or adapted (B and E) from previously published papers (Shao et al. 2003, 2004). * significant difference between 2 columns at $P < 0.05$.
Development of action potential generation and firing pattern

It is interesting that the number of silent principal cells decreased from about half at E10 and E13 to 13% at E16 (Gamkrelidze et al., 1998) and disappeared after hatching (Shao et al., 2006). The silent cells were not glia because biocytin-injections revealed neurons with the morphological features of principal cells. In addition, glial cells are characterized by low input resistances (Gamkrelidze et al., 1998) and disappeared after hatching (Shao et al., 2005). Silent principal cells could be due to cell damage, perhaps due to dendrotoxin-sensitive, low-threshold potassium channels. During our recordings, the input resistance and RMP were monitored (see Methods) so that all of the neurons included in the study had stable input resistances and RMP less than ~60 mV, ruling out that the recordings came from damaged neurons. At E10, the silent principal cells exhibited inward deflections on depolarization, which became larger at E13. Similar inward deflections on depolarization recorded from silent principal cells at E16 were shown to be TTX sensitive (Gamkrelidze et al., 1998). This finding suggests that silent principal cells have some functional sodium channels, which are insufficient to generate action potentials on depolarization. Finally, in contrast to E16 and H1 (Gamkrelidze et al., 1998, 2000), exposure to DTX did not change the firing pattern of the principal cells at E10 and E13. Accordingly, $I_{DS}$, a dendrotoxin-sensitive, low-threshold sustained potassium current was not functional at E10 or E13, perhaps due to 1) its absence, 2) the levels of $I_{DS}$ present were insufficient to repolarize the membrane potential to remove sodium channel inactivation, or 3) the channel subunit composition differed at these young ages and was insensitive to the α-dendrotoxin applied.

About half of the principal cells generated single action potentials on depolarization at E10 and E13 and 70% at E16 (Gamkrelidze et al., 1998). In voltage-clamp recordings, a fast rising, TTX-sensitive inward current was induced from all of this subclass of principal cells. Accordingly, a fast sodium current is responsible for generating action potentials at E10 and E13. $I_{DS}$ is present in the principal cells at E16 and plays a crucial role in blocking the repetitive firing of action potentials (Gamkrelidze et al., 1998). However, 17% of principal cells at E16 can generate repetitive spike firing on depolarization and 90% at H1 (Gamkrelidze et al., 1998; Shao et al., 2006), coincident with a substantial decrease in the proportion of $I_{DS}$ found in the principal cells. At the same time, half of the principal cells exhibit spontaneous spike firing at H1 and 76% by H5.

When comparing the action potential waveforms at different ages, it was striking that the second, slow AHP and its underlying SK current recorded in principal cells at E16 and H1 (Shao et al., 2006) were never observed at E10 and E13. The slow AHP was voltage sensitive because it disappeared on further depolarization (Shao et al., 2006). In other systems, SK current limits the firing rate or produces spike frequency...
adaptation (Sah 1996). Because at E10 and E13, the principal cells never showed repetitive spike firing on depolarization, it could be expected that SK current would be lacking. The emergence of SK current during development may depend on adding more voltage-dependent calcium channels to increase calcium entry into the neurons during spike generation, which in turn could activate SK channels.

The action potential waveforms generated at E10 were distinct from those recorded at E13 and older ages because they had exceptionally small amplitudes and slow kinetics, typical for immature neurons (e.g., spinal cord motor neurons) (Gao and Ziskind-Conhaim 1998). Moreover, at H1, action potential waveforms had more negative thresholds; this may contribute to the repetitive firing of the principal cells and generation of spontaneous spike activity, two features essential for mature vestibular reflex function. The emergence of action potentials and their underlying currents have been investigated in many developing systems. Like in our system, action potentials were sodium-dependent at the onset of excitability in rat and chick spinal motor neurons (Gao and Ziskind-Conhaim 1998; McCobb et al. 1990) and chick ciliary ganglion cells (Bader et al. 1983). In these preparations, the amplitude of the sodium currents increased gradually with simultaneous changes in action potential amplitude and kinetics.

Accordingly, there are at least six steps for developing principal cells to acquire their mature firing pattern. 1) Silent principal cells were recorded in decreasing numbers from E10 to E13 and E16. Silent cells lacked sufficient sodium currents to generate spikes on depolarization. 2) About half of the principal cells recorded from at E10 and E13 were single-spike cells. These cells had sufficient inward sodium currents to generate spikes but lacked functional I\(_{DS}\) and SK currents. 3) At E16, single-spike principal cells predominated. They had sufficient inward sodium currents to generate spikes with large amounts of I\(_{DS}\), which prevented repetitive firing, and SK current underlying the slow AHP, which modulated repetitive firing when present. 4) From E16 to H1 and H5, an increasing number of principal cells had repetitive firing on depolarization, resulting from loss of I\(_{DS}\) and persistence of SK current (Shao et al. 2006). 5) At H1, principal cells acquired spontaneous spike firing driven by synaptic transmission (Shao et al. 2006). 6) At H5, spontaneous spike firing in principal cells was driven by intrinsic membrane properties (Shao et al. 2006).

Development of signaling between the vestibular inner ear and VNN

Synaptic transmission between the vestibular receptors in the inner ear and the brain stem VNNs involves two synaptic connections, the first one between the hair cells and peripheral axons of vestibular ganglion cells and the second one between the ganglion’s central axons and the VNNs. Although the colossal vestibular fibers form morphologically mature calycine endings in the crista ampullaris by E13 (Fink and Morest 1977), it is not known when the vestibular hair cells begin to transmit signals to the brain. However, as early as E10, the chick vestibular hair cells exhibit inward calcium and outward potassium currents with the signature potassium currents first expressed at E17 (Massetto et al. 2000) at about the same time (E19) when the potassium ion concentration in endolymph becomes sufficiently high to permit transmitter release from the hair cells (Massetto et al. 2005) and when compound action potentials are recorded from chick primary vestibular fibers (E18–E19) (Jones and Jones 1996). Accordingly, chick vestibular hair cells likely transmit signals to the brain just before hatching but not at E10 and E13.

The primary vestibular fibers enter the chick embryo medulla oblongata at E3 (Knowlton 1967), but the first signs of their synaptogenesis occurs at E7.5 when the colossal vestibular fibers form chemical synapses on principal cells (Petralia and Peusner 1990). Indeed, the principal cells receive their first synaptic contacts at E5 from longitudinally coursing fibers within the brain stem (Petralia and Peusner 1990), which are axons belonging to central neurons (Petralia et al. 1991). So far, no patch-clamp recordings have been performed at these early ages. However, using voltage-sensitive dyes and optical recordings, chick VNNs in the dorsomedial medulla oblongata were found to generate postsynaptic responses on vestibulonerve stimulation as early as E7 (Sato and Momose-Sato 2003). In the present study, half of the principal cells at E10 generated postsynaptic responses on vestibular-nerve stimulation. Accordingly, on galvanic stimulation the primary vestibular fibers are electrically excitable before transmitting signals from the peripheral vestibular hair cells to the VNNs. Finally, there may be a peripheral-to-central sequence of synapse formation in the motor portion of the chick VOR, since the neuromuscular junction develops at E3-E4 while the synaptic connections between second- and third-order VOR neurons form after E5 (Glover 2003).

Development of synaptic transmission in VNN and its relation to vestibular system function

At E10, spontaneous synaptic activity was composed entirely of miniature events, indicating that the first type of synaptic activity generated between developing first- and second-order vestibular neurons consisted of miniature synaptic events. Indeed, miniature synaptic events are preeminent for the assembly of many different emerging sensory circuits (see Zucker 2005 for a review). Despite the widespread nature of miniature events, presently little is understood about their influence on the networks generating the activity. However, compensatory changes in frequency and amplitude of the miniature events have been induced by blocking spontaneous spike activity or applying synaptic transmission antagonists (e.g., Gonzalez-Islas and Wenner 2006; Turrigiano et al. 1998). Indeed, the maintenance of appropriate levels of miniature activity at a single synapse may be crucial to the sensory pathway beyond the synapse recorded from, in a similar way to how activity in the retinal pathway influences the specification of synaptic connections in the cerebral cortex (e.g., Hubel and Wiesel 1970; Shatz 1990; for review, see Space Studies Board 1998).

At E10 and E13, the frequencies of sEPSCs and sIPSCs were very low, as expected because the principal cells receive a limited number of small synaptic terminals and the large spoon terminals contacting them form only a few immature chemical synapses (Peusner 1981, 1984). At E16, the frequency of IPSCs increased dramatically, mainly due to GABAergic but also glycineric events. Thus the vestibular system in the chick embryo may be designed to inhibit excessive motor activity and control rotary head movements until they become necessary for hatching (Rogers 1995). At H1, the
most dramatic changes in synaptic activity recorded in the principal cells were an increased frequency of EPSCs and the predominance of glycinergic IPSCs over GABAergic IPSCs (Shao et al. 2003, 2004). These changes will expedite the emergence of spontaneous spike activity and continued development of repetitive firing on depolarization postnatally (Shao et al. 2004) and must contribute to the chick’s ability to stand and walk shortly after the birth, and the robust nature of vestibular-mediated movements at HS (Shao et al. 2006).

In summary, action potential generation and synaptic transmission in VNNs undergo a series of changes spanning the entire developmental period studied. Gaining a better appreciation of the specific nature of these electrophysiological events is crucial for understanding how the vestibular system is assembled during development and essential for devising new treatments for abnormal vestibular development. In addition, this information may provide important clues for evaluating synaptic plasticity occurring after injury or diseases affecting the vestibular system.

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