Spontaneous Synaptic Activity Is Primarily GABAergic in Vestibular Nucleus Neurons of the Chick Embryo

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Spontaneous synaptic activity is primarily GABAergic in vestibular nucleus neurons of the chick embryo. J Neurophysiol 90: 1182–1192, 2003; 10.1152/jn.00076.2003. The principal cells of the chick vestibular nucleus are vestibular nucleus neurons participating in the vestibular reflexes. In 16-day embryos, the application of glutamate receptor antagonists abolished the postsynaptic responses generated on vestibular-nerve stimulation, but spontaneous synaptic activity was largely unaffected. Here, spontaneous synaptic activity was characterized in principal cells from brain slices at E16 using whole cell voltage-clamp recordings. With KCl electrodes, the frequency of spontaneous inward currents was 3.1 Hz at −60 mV, and the reversal potential was +4 mV. Cs-glucuronate pipette solution allowed the discrimination of glycine/GABA$_A$ receptor-mediated events according to their different reversal potentials. The ratio for spontaneous excitatory to inhibitory events was about 1:4. Seventy-four percent of the outward events were GABA$_A$, whereas 26% were glycine receptor-mediated events. Both pre- and postsynaptic GABA$_A$ receptor effects were shown, with presynaptic GABA$_A$ receptors inhibiting 40% of spontaneous excitatory postsynaptic currents (sEPSCs) and 53% of spontaneous inhibitory postsynaptic currents (sIPSCs). With TTX, the frequency decreased ~50% for EPSCs and 23% for IPSCs. These data indicate that the spontaneous synaptic activity recorded in the principal cells at E16 is primarily inhibitory, action potential-independent, and based on the activation of GABA$_A$ receptors that can be modulated by presynaptic GABA$_B$ receptors.

INTRODUCTION

The principal cells of the chick vestibular nucleus are second-order vestibular neurons in the medulla oblongata involved in the three-neuron vestibular-ocular and vestibulocollic reflexes, which stabilize gaze and head position during head and body movements (Fig. 1A). The principal cells represent the predominant cell population (80%) in this major avian vestibular nucleus (Peusner and Morest 1977). From intracellular current-clamp recordings at embryonic day 16 (E16), the postsynaptic responses generated in the principal cells on vestibular-nerve stimulation were blocked by the co-application of specific glutamate receptor antagonists, d-2-amino-5-phosphonoveric acid (d-APV) and 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) (Peusner and Giaume 1994). However, the spontaneous synaptic potentials were largely unaffected, suggesting that such basic activity could be important under physiological conditions. In neurons, spontaneous spiking activity can be produced either by synaptic events reaching threshold for firing action potentials or by nonsynaptic events generated from the intrinsic membrane properties of the neuron. For example, we have shown that in embryonic vestibular nucleus neurons, spontaneous synaptic activity does not necessarily reach the threshold for firing action potential (Peusner and Giaume 1994). Investigations on spontaneous activity in vestibular nucleus neurons have been performed mainly using extracellular recordings to characterize the spontaneous discharge rates from single units in the medial vestibular nucleus (MVN) that were not identified as to morphological neuron type (for review, see Darlington et al. 1995; Dutia et al. 1992; Johnston et al. 2001; Yamanaka et al. 2000). In these studies, GABA and/or GABA$_A$ and GABA$_B$ receptor agonists (e.g., muscimol, baclofen, respectively) inhibited the spontaneous discharge, whereas the GABA$_A$ receptor antagonists (e.g., bicuculline) typically diminished the inhibitory effects of GABA. The effects of GABA on spontaneous discharge rate in MVN neurons was age dependent with greater inhibition recorded in the older postnatal rats (Giardino et al. 2002; Him et al. 2001).

Using immunolabeling techniques, glycine has been identified in first-order vestibular neurons (cat, Godfrey et al. 1977; rat and frog, Reichenberger and Dieringer 1994). However, so far there have been no electrophysiological studies demonstrating monosynaptic inhibitory responses from vestibular nucleus neurons on vestibular-nerve stimulation (e.g., Straka et al. 1997). In diverse systems, electrophysiological studies have indicated that GABA and glycine may be coreleased at the same synapse (e.g., spinal cord interneurons, Jonas et al. 1998; hypoglossal motor neurons, O’Brien and Berger 1999; cerebellar Golgi cells, Dumoulin et al. 2001; abducent motor neurons, Russier et al. 2002). Further, a developmental shift from GABA to glycine transmission was reported in the developing auditory system (Kotak et al. 1998) and spinal cord (Gao et al. 2001). Accordingly, we suspect that GABA and glycine may play important and possibly changing roles in the developing and mature central vestibular system. Finally, there is mounting evidence for a crucial role of GABA receptor-mediated events in vestibular nucleus neurons during the recovery of function that follows unilateral peripheral vestibular lesions, known as vestibular compensation (e.g., Cameron and

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To our knowledge, there have been no studies on spontaneous postsynaptic currents in embryonic vestibular nucleus neurons. Under physiological conditions, we do not know what rules govern spontaneous excitatory and inhibitory receptor-mediated events in vestibular nucleus neurons, although a link is expected between input and output. In the present study, we characterized spontaneous and miniature synaptic activity using whole cell patch-clamp recordings on identified principal cells of the tangential nucleus at E16. One objective was to define the characteristics of spontaneous synaptic activity at a critical age (E16) when developmental change occurs in the morphological and electrophysiological properties of the principal cells (Peusner and Giaume 1997). The second objective relates to lesion studies where some cells re-express developmentally regulated genes during recovery and then repeat certain aspects of their development (e.g., Han et al. 1996; Iwahashi et al. 1996; MacFarlane and Sontheimer 1997). Accordingly, our second objective was to add to our knowledge of the developmental schedule of synaptic properties in vestibular nucleus neurons, so that this data can be available for studying synaptic events during vestibular compensation.

M E T H O D S

B r a i n s l i c e p r e p a r a t i o n a n d p r e s e r v a t i o n

All of the observations were made on 16-day-old White Leghorn chick embryos (Gallus gallus) obtained from CBT Farms (Chesteron, MD) as eggs that were incubated in the laboratory until the desired age. The age of the embryos was established by reference to the staging criteria of Hamburger and Hamilton (1951). The animal protocols were approved by the Institutional Animal Care and Use Committee of George Washington University. Briefly, the embryos were removed from the egg and decapitated, and the part of the cranium containing the brain stem and cerebellum was placed in a dissecing dish containing oxygenated artificial cerebrospinal fluid (ACSF) chilled to 1–2°C. The cerebellum, choroid plexus, periotic capsule, and vestibular ganglia were removed from the brain stem. The brain stem was glued to a vibroslicer tray, immersed in ice-cold ACSF, and viewed under a dissection microscope while sections (300 μm thickness) containing the tangential nucleus were cut on a vibroslicer (VT1000S, Leica) using feather blades (Ted Pella). Because the anteroposterior extent of the tangential nucleus is ~450–500 μm at E16 (Peusner and Moster 1977), the tangential nucleus was contained in two transverse slices of the brain stem. The ACSF solution contained (in mM) 126 NaCl, 3.5 KCl, 1.2 NaH₂PO₄, 1.3 MgCl₂, 2 CaCl₂, 26 NaHCO₃, and 10 d-glucose. The pH of this solution was 7.2–7.4 after saturation with 95% O₂-5% CO₂ at room temperature. The osmolarity of the ACSF solution was 310 mOsm.

Brain slice superfusion and microscopy

After ~1 h recovery at room temperature, the slices were transferred to a small glass-bottom recording chamber (180 μl, Warner Instruments) and kept in place by nylon threads glued to a U-shaped, flattened platinum wire. The slices were superfused with heated ACSF (30–31°C) at a rate of 2–3 ml/min except for some experiments where the slices were perfused with room temperature ACSF (21–23°C). The slices were viewed on a fixed-stage microscope (Axioskop FS1, Zeiss Instruments), equipped with Nomarski differential interference contrast optics and a ×40 water-immersion lens (NA, 0.75). The recorded neuron and pipette movement were viewed using an infrared light source (filter, 770 nm) that was detected by an infrared-sensitive camera ( Videomax 2400-01, Hamamatsu) and observed on a monitor (Sony). The microscope image was further magnified by placing a ×4 lens between the microscope and the camera. Image contrast and shading were adjusted with a camera controller (C2400-01, Hamamatsu).

All of the drugs, except for CNQX and phaclofen, were prepared daily by dissolving in ACSF and then adding them to the superfusing ACSF to achieve the final concentrations. Tetrodotoxin (TTX, RBI) was applied in the voltage-clamp experiments to block voltage-dependent Na⁺ currents. Other drugs used included N₂-amino-5-phosphonovaleric acid (AP-5; Sigma), bicuculline methochloride (Tocris), strychnine (Sigma), and baclofen (Sigma). CNQX (RBI) and phaclofen (RBI) were dissolved daily in dimethyl sulfoxide (DMSO; Fisher Scientific) at a concentration of 20 and 500 mM, respectively, which were then added to the ACSF solution to achieve the final concentrations.

E lectrophysiology

Recording pipettes were pulled from borosilicate glass tubing (1.5 mm OD, 1.12 mm ID, thin-walled, World Precision Instruments) with a Brown/Flaming horizontal pipette puller (P-87, Sutter Instruments). The pipette resistance was 1–3 MΩ. KCl pipette solution contained (in mM) 130 KCl, 10 EGTA, 10 HEPES, 1.0 CaCl₂, and 2.0 Mg-ATP (Ganakreidize et al. 1998). The pH of the solution was adjusted to 7.2 with KOH (8.0 N). Cesium gluconate pipette solution contained (in mM) 135 Cs-glucurate, 1 EGTA, 10 HEPES, 0.1 CaCl₂, and 2 MgCl₂. The pH was adjusted to 7.2 with CsOH (Cossart et al. 2000). The osmolarity for both pipette solutions was adjusted to 290 ± 10 mOsm. Finally, 0.5% bicocytin was made fresh daily and added to the pipette solution.

Whole cell recordings were obtained using an amplifier in voltage-clamp mode (Axopatch-1D, Axon Instruments). The recording pipette was advanced through the slice under visual guidance using a piezo-electric manipulator (PCS-5000, Burleigh Instruments). The R̅g com-

FIG. 1. A: diagram of transverse section of chick embryo medulla at the level of the inner ear. TN, tangential nucleus; VG, vestibular ganglion; NA, nucleus angularis; NL, nucleus laminaris; NM, nucleus magnocellularis; IV, fourth ventricle; D, dorsal; V, ventral. B and C, low- and high-power photomicrographs of a recorded principal cell (PC) stained with Streptavidin Alexa Fluor 647 (red). The primary vestibular fibers were counterstained with DiO (green). Dorsal to the top, and the lateral brain surface to the left. Scale bars: B, 200 μm; C, 40 μm.
pensation was set at 80% and lag 10 μs. Grounding was performed with an Ag-AgCl reference electrode. Using KCl pipette solution, the membrane potential of the principal cells was −66 ± 4 mV (range: −54 to −75 mV) and the input resistance was 63 ± 5 MΩ (range: 45–83 MΩ; n = 10). Using Cs-glucconate pipette solution, the input resistance of the principal cells averaged 78 ± 7 MΩ (range: 50–100 MΩ; n = 12).

Biocytin processing of slices

During the recordings, the living principal cells were visualized using the infrared camera, and their typical oval cell bodies were apparent between the primary vestibular fibers in the lateral medulla oblongata (Fig. 1, B and C). In this study, we intentionally selected to record from the principal cells and avoided recording from other neuron populations to accumulate a maximal number of recordings from a homogeneous class of neurons. Biocytin was loaded into the recording pipette so that all of the recorded neurons were filled passively with biocytin during the recording session. After recording, the slices were processed for conventional fluorescence staining to confirm the morphology and neuronal type of the recorded neuron. At E16, the principal cell exhibited three to seven primary dendrites that arborized extensively in the dorsoventral plane. The cell’s axon coursed in a medial direction, as already described (e.g., Peusner and Giaume 1997).

Due to proximity of the dye-injected neuron to the surface of the slice (50–100 μm), no further sectioning of the slice was necessary before processing for biocytin staining. After the recording, the intact slice was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (overnight, refrigerated), rinsed with 0.1 M phosphate buffer saline containing 0.1% Triton X-100 (PBS-T) (3 times for 20–60 min), incubated in 50% ethanol in PBS-T (20 min), and placed in Streptavidin Alexa Fluor 647 (1:200) in PBS-T (overnight, room temperature). After rinsing in PBS-T (20 min), the slice was counter-stained with DiO (2 mg/ml dissolved in N,N-dimethyl formamide; Molecular Probes; 40 s), to enhance the appearance of the primary vestibular fibers. The slices were mounted on gelatin-coated slides, coverslipped with Fluoromount G, and observed on a Nikon Optiphot (Molecular Probes; 40 s), to enhance the appearance of the primary vestibular fibers. The slices were mounted on gelatin-coated slides, coverslipped with Fluoromount G, and observed on a Nikon Optiphot microscope equipped with ×4 (NA, 0.2) and ×20 (NA, 0.65) objectives. Fluorescent images were captured with a slow-scan CCD camera (Spot RT, Diagnostic Instruments) connected to a personal computer. Alexa Fluor 647 was excited with a light of 600 to 650 nm wavelength, and the emission was filtered from 670 to 740 nm (filter set XF110–2NS32; Omega Optical). Immunostaining for Alexa Fluor 647 was visualized on the red channel, while DiO was visualized on the green channel.

Data acquisition and analysis

No correction was applied for the voltage offset observed after withdrawing the pipette from the cell (≤ ±3 mV) or for the liquid junction potential (−3 mV). The reversal potentials were determined by identifying the holding potential at which no synaptic current was observed. During the experiment, the input resistance and series resistance were determined by giving 5 mV hyperpolarizing pulses. If the membrane exhibited a peak amplitude change in the capacitance currents >25% in response to the 5 mV pulse, the neuron was excluded from study. All of the data were 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). Synaptic currents were analyzed off-line using MiniAnalysis program (version 5.6.3, Synaptosoft) with a minimal acceptable amplitude set at 15 pA. Analysis of the postsynaptic 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). The voltage-clamp data were digitized at 10 kHz and filtered at 2 kHz (pClamp program, Axon Instruments). All of the data are given as the means ± SE. Frequency differences were analyzed with the Student’s t-test for independent samples and considered significant with \( P < 0.05 \). Amplitude distributions were compared with the Kolmogorov-Smirnov (K-S) test with the significance level set at \( P < 0.05 \). Correlations were analyzed using Prism software program (Graphpad), with a two-tailed distribution and 95% confidence level.

RESULTS

Reversal potentials of the spontaneous currents

The whole cell recordings presented here were obtained from a total of 94 identified principal cells at E16. First, spontaneous currents were recorded in voltage-clamp mode at different holding potentials with pipettes containing KCl solution and in control ACSF (Fig. 2, A1 and A2). In these experiments, the mean reversal potential for all of the spontaneous synaptic activity was +4.2 ± 1.3 mV (n = 4) because the excitatory and inhibitory events had similar expected reversal potentials due to chloride loading of the cell when using KCl electrodes (Fig. 2B). The mean frequency for these spontaneous postsynaptic currents (sPSCs) was 3.1 ± 0.9 Hz at −60 mV (n = 13). It was apparent that the frequency of spontaneous synaptic activity was temperature dependent because at −60 mV, the mean frequency recorded at room temperature (21–23°C) was 1 ± 0.4 Hz (n = 5), which was significantly less than that recorded at 30–31°C (\( P < 0.05 \)).

To distinguish between excitatory and inhibitory events in the same cell without applying drug treatments that could interfere with the presynaptic circuitry, we carried out further experiments using electrodes filled with Cs-glucuronate solution. In these experiments, the spontaneous excitatory and inhibitory postsynaptic currents (sEPSCs and sIPSCs) recorded in the principal cells could be separated by their voltage dependencies (Figs. 3 and 4). The currents were measured at −60 and +10 mV, respectively, because these voltages approximated their reversal potentials under our experimental conditions. The small inward currents recorded in the principal cells at −60 mV had a reversal potential around +15 ± 2 mV (n = 5). Due to the difficulty in detecting the small inward currents from background noise starting around −40 mV, our estimated value for the reversal potential of the sEPSCs is likely higher than actual. Accordingly, it was not possible to plot reliably the I-V relations for the reversal potential of these sEPSCs. However, according to the Nernst equation, these currents were likely sEPSCs generated by glutamate (Hille 2001). In addition, the principal cells exhibited outward currents at a holding potential of +10 mV, which had a mean reversal potential at −72 ± 2.7 mV (n = 7), which is close to the chloride equilibrium potential according to the Nernst equation (Fig. 4B). Thus it is likely that these currents represented sIPSCs generated by a chloride conductance. In our subsequent experiments, the cells were recorded primarily at −60 or +10 mV to distinguish the excitatory and inhibitory spontaneous synaptic activity, respectively (Cossart et al. 2000).

Spontaneous synaptic activity is mainly inhibitory at E16

At a holding potential of −60 mV, the frequency of the inward currents was 0.6 ± 0.1 Hz (n = 27), whereas at
+10 mV holding potential, the frequency of the outward currents was $2.3 \pm 0.4$ Hz ($n = 30$; Fig. 3, $A_1$ and $A_2$, respectively). Thus the ratio for excitatory and inhibitory events was about 1:4, indicating that at E16 the spontaneous synaptic activity in the principal cells was primarily inhibitory (Fig. 3B).
**sEPSCs are mediated by the AMPA receptor**

At $-60$ mV holding potential, the inward currents were blocked completely by $10 \mu M$ CNQX, a specific AMPA/kainate (KA) receptor antagonist ($n = 5$; Fig. 3C). Typically, these sEPSCs exhibited the fast kinetics characteristic of AMPA events. However, in some cells ($5/27$) the CNQX-sensitive events included some currents with slower kinetics, which in other systems have been identified as KA receptor-mediated events (Fig. 3A, arrow) (Cossart et al. 1998, 2002; Frerking et al. 1998; Kidd and Isaac 1999). Accordingly, sEPSCs were identified as primarily AMPA receptor-mediated events with a few KA currents present.

**sIPSCs are mediated mainly by GABA>A-receptors**

At a holding potential of $+10$ mV, $74 \pm 3\%$ of the spontaneous outward currents were blocked completely by the GABA$_A$-receptor antagonist, bicuculline ($10 \mu M$). The addition of the glycine-receptor antagonist, strychnine ($4 \mu M$), blocked the remaining currents (Fig. 5A$_1$; 2,755 events, $n = 15$ cells). Consequently, the ratio for GABA to glycine receptor-mediated events was $3:1$ (Fig. 5B). Indeed, the ratio for GABA versus glycine receptor-mediated events was similar whether bicuculline or strychnine was applied first (Fig. 5A$_2$), suggesting that strychnine did not affect the postsynaptic GABA$_A$ receptors or presynaptic release in

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**FIG. 4.** A: averaged sEPSCs ($n > 50$) and spontaneous inhibitory postsynaptic currents (sIPSCs, $n > 100$) from the same principal cell recorded with Cs-glucosinate-pipette solution at different holding potentials, from $-60$ to $+10$ mV. B: current-voltage relationship in principal cells ($n = 7$) depicting averaged data that was normalized to the currents recorded at $+10$ mV. The extrapolated reversal potential for sIPSCs was $-72$ mV.

**FIG. 5.** A$_1$: sIPSCs were blocked almost completely by $10 \mu M$ bicuculline, whereas $4 \mu M$ strychnine combined with $10 \mu M$ bicuculline completely blocked the sIPSCs ($n = 9$). The traces were recorded from the same principal cell. A$_2$: sIPSCs were blocked partially by $4 \mu M$ strychnine, whereas $10 \mu M$ bicuculline combined with $4 \mu M$ strychnine blocked completely the sIPSCs ($n = 6$). The traces were recorded from the same principal cell. B: histogram of the normalized frequencies of sIPSCs in control conditions, in the presence of strychnine (GABAergic IPSCs), and in the presence of bicuculline (glycinergic IPSCs; $n = 15$).
this preparation. To further exclude the possibility that the high strychnine concentration (4 μM) could affect GABA₄ events, we performed experiments using 1 μM strychnine followed by 10 μM bicuculline (not shown). In the latter experiments, 32 ± 7% sIPSCs were sensitive to strychnine and 68 ± 7% events were sensitive to bicuculline (647 events; n = 4 cells). There was no statistically significant difference (P > 0.05) between the ratio for GABA to glycine receptor-mediated events obtained with 1 or 4 μM strychnine. Accordingly, the doses used for strychnine (1 or 4 μM) and bicuculline (10 μM) were considered to be specific for glycine and GABA receptor-mediated events, respectively, and there was no cross-reactivity detected in this preparation. Altogether, these data indicate that GABA was the major neurotransmitter mediating the inhibitory spontaneous synaptic activity in the principal cells at E16.

**Inhibition of sEPSCs and sIPSCs by GABA_B receptors**

To determine if postsynaptic GABA_B receptors are present on the principal cells using KCl pipette solution, we recorded the postsynaptic effects of the potent GABA_B receptor agonist, baclofen (10 μM), in the presence of 1 μM TTX and the receptor antagonists for GABA_A (10 μM bicuculline), glycine (1 μM strychnine), NMDA (30 μM AP-5), and non-NMDA (10 μM CNQX). In these experiments performed at a holding potential of −65 mV, baclofen was added to the ACSF solution and induced an outward current (n = 6; Fig. 6A). The amplitude of this current was small (range: 10–40 pA) and remained stable throughout the application of the drug. After washout with control ACSF, the current decreased slowly back to baseline. This current was not observed when Cs-glucuronate pipette solution was used (n = 3, not shown).

To test whether activation of presynaptic GABA_B receptors can modulate spontaneous synaptic activity, we recorded the presynaptic effects of baclofen using Cs-glucuronate pipette solution, which blocked the potassium channels and prevented the activation of postsynaptic GABA_A receptors. The effect of baclofen on the frequency and the amplitude of sEPSCs and sIPSCs was determined in the absence of TTX (Fig. 6B1 and B2). The frequency of sEPSCs decreased from 0.87 ± 0.3 to 0.52 ± 0.2 Hz (40 ± 7%; n = 5) and the frequency of sIPSCs decreased from 3.2 ± 1 to 1.5 ± 0.5 Hz (53 ± 7%; n = 8). Both decreases were significant (p < 0.05). In addition, 3/5 cells exhibited significant change in the amplitude distribution of sEPSCs and 4/8 cells showed significant change in the amplitude distribution of sIPSCs, according to K-S statistical analysis (p < 0.05). When the GABA_A receptor antagonist, phaclofen (500 μM) was added alone, no effect was observed on the frequency of sEPSCs (n = 3), but a slight increase in frequency of sIPSCs (6 ± 2%) was detected (n = 5; p < 0.05). Addition of baclofen (10 μM) 10 min after phaclofen application reduced the frequency of sEPSCs (26 ± 1%, n = 3) and sIPSCs (24 ± 4%, n = 5) but not so much as with baclofen alone (not shown). However, there was not statistically significant difference in the baclofen effect on sEPSCs frequency (26 vs. 40%) with or without the preapplication of phaclofen (p > 0.05), but there was a significant difference in the baclofen effect on sIPSCs frequency (24 vs. 53%; p < 0.05). These findings could be attributed to pharmacologically heterogeneous GABA_B receptors on the presynaptic terminals (see DISCUSSION). Altogether, both pre- and postsynaptic GABA_B receptors appear to modulate the spontaneous synaptic activity observed in principal cells at E16.

**Most of the spontaneous synaptic activity consists of miniature events**

To test whether the spontaneous synaptic activity was evoked by presynaptic firing, TTX (1 μM) was applied to block the action potentials mediated by the activation of voltage-dependent sodium channels (Fig. 7). At a holding potential of −60 mV, we compared the spontaneous synaptic activity recorded in control conditions and after the addition of TTX to determine the percentage of spontaneous synaptic events that
are TTX-resistant. We found that 53 ± 7% of sEPSCs were TTX-resistant, the so-called miniature EPSCs, or mEPSCs (984 events; n = 7 cells; Fig. 7, A1 and A2). When the same protocol was applied while recording at a holding potential of +10 mV, 79 ± 4% of sIPSCs were TTX-resistant, the mIPSCs (3,969 events; n = 17 cells; Fig. 7, B1 and B2). These results indicated that the generation of sIPSCs in the principal cells was more independent of action potentials than sEPSCs.

In addition to decreasing the frequency of spontaneous synaptic activity in the principal cells, the application of TTX decreased the number of large-amplitude events recorded, which is indicated by a shift to the left of the cumulative probability curves for both EPSCs and IPSCs (Fig. 8, A and B). The mean amplitude was 35.2 ± 0.4 pA for sEPSCs and 23.3 ± 0.5 pA for mEPSCs (n = 7 cells), whereas the mean amplitude was 47.2 ± 0.7 pA for sIPSCs and 36.8 ± 0.4 pA mIPSCs (n = 6 cells), with no statistical difference (p > 0.05). While TTX did not produce a noticeable change in the kinetics of the mEPS Cs and mIPSCs as compared with their controls (Fig. 8, A and B, insets), the mEPSCs exhibited statistically faster rise time, faster decay time, shorter duration, and smaller amplitudes compared with the mIPSCs (p < 0.01; Table 1). Furthermore, the glycine-ergic mIPSCs exhibited significantly larger amplitudes and faster rise times compared with the GABAergic mIPSCs (p < 0.01). Also, there was a tendency for the glycine-ergic mIPSCs to exhibit shorter half-width and decay time than the GABAergic mIPSCs, but these differences were not statistically significant (p > 0.05).

To test whether the lack of space clamp affected the kinetics, correlation between the 10–90% rise time and 90–37% decay time and between 90–37% decay time and peak amplitude was plotted (Fig. 9). A lack of correlation between rise time and decay time, and the positive correlation between decay time and peak amplitude in all of the principal cells tested (for mIPSCs, n = 9; for mEPSCs, n = 5) supported the proposal that the kinetics differences were not due to space-clamp problems (Weiss et al. 1988).

**DISCUSSION**

This is the first work that defines the relative contribution of excitatory and inhibitory spontaneous synaptic activity in an identified class of embryonic vestibular nucleus neurons from acute brain slices. This analysis was performed by determining the occurrence of GABA/glycine and AMPA/kainate glutamate receptor-mediated spontaneous and miniature synaptic currents identified on the basis of their reversal potentials, kinetics, and sensitivities to their respective specific receptor antagonists. Knowledge of this contribution to the spontaneous synaptic activity helps to define the physiological properties of second-order vestibular neurons at E16, a critical age of embryonic development (Peusner and Giaume 1997). Because part of the phenotypic developmental schedule could be repeated in neurons during their recovery from injury or trauma (see INTRODUCTION), the present data could be relevant to studies of spontaneous synaptic activity in vestibular nucleus neurons during vestibular compensation.

**FIG. 8.** A and B: cumulative probability plots of the amplitudes of sEPSCs (984 events; n = 7) and mEPSCs (472 events; n = 7) and sIPSCs (1,478 events; n = 6) and mIPSCs (921 events; n = 6), respectively. On exposure to TTX, the proportion of large amplitude events decreased, as shown by a shift to the left of both the EPSC and IPSC curves. *Inset:* normalized EPSCs (A) and IPSCs (B) with and without TTX demonstrated that TTX did not change the kinetics.
The frequency of spontaneous synaptic activity is determined by pre- and postsynaptic factors. Presynaptic factors may include the frequency of action potentials entering the presynaptic terminals, the number of presynaptic terminals, and neurotransmitter metabolism. Postsynaptic factors include the activation of postsynaptic receptor subunits (Nicholls et al. 2001). Inhibitory receptors, including GABA and glycine receptors, and the excitatory receptors, AMPA/KA and NMDA receptors, have been demonstrated in vestibular nucleus neurons of different species (Dutia et al. 1992; Kinney et al. 1994; Smith et al. 1991). In the embryonic principal cells, the frequency of excitatory spontaneous synaptic activity was much less than that of inhibitory events (1:4), and the main neurotransmitter of the inhibitory receptor-mediated events was GABA. Indeed, at this age, the entire inhibitory response could be blocked by the co-application of bicuculline and strychnine, which suggests that GABA_A and glycine receptors are responsible for all of these events under our experimental conditions. Of course, these findings were obtained using pipettes containing Cs-glucuronate, which blocked the postsynaptic GABA_B receptors and putative synaptic events. A relatively low rate of action potential firing at vestibular presynaptic terminals at this age could underlie the low frequency of excitatory spontaneous synaptic activity recorded. Indeed, in the intact, ketamine-anesthetized newborn rat, vestibular nucleus neurons exhibited a spontaneous discharge rate of about 4.3 Hz (Lannou et al. 1979).

It is likely that AMPA/KA receptors mediate input from the vestibular nerve and may at least partially transmit input from the contralateral vestibular nuclei via brain stem commissural fibers (Doi et al. 1990; Kinney et al. 1994) or from the spinal cord (frog: Knopfel 1987; rat: Doi et al. 1990). Where are the GABAergic inputs coming from? So far, there is no immunohistochemical evidence directly demonstrating inhibitory connections to the tangential nucleus. However, from extracellular horseradish peroxidase injections in the tangential nucleus, we know that there are inputs originating from the cerebellar flocculus (Cox and Peusner 1990), which release GABA in the cat (Fonnum et al. 1970), chick (du Lac and Lisberger 1992) and guinea pig (Babalian 2000). Other major inputs to the tangential nucleus originate from cervical levels of the spinal cord and the vestibular nuclei on the ipsi- and contralateral sides (Cox and Peusner 1990). GABA and glycine immunoreactive neurons have been stained in the vestibular nuclei of many species, including frog (Reichenberger et al. 1997), rat (Houser et al. 1984; Nomura et al. 1984), mouse (Ottersen and Storm-Mathisen 1984), cat (Tighilet et al. 2001; Walberg et al. 1990), guinea pig (Kumoi 1987) and monkey (Holstein et al. 1996). In addition, GABA immunolabeled fibers and putative synaptic terminals have been found throughout the vestibular nuclei (Houser et al. 1984; Kumoi 1987; Nomura et al. 1984; Walberg et al. 1990). Accordingly, it is likely that some GABA and glycine neurons are located in or around the chick tangential nucleus or in other vestibular nuclei and could contact the principal cells. Indeed, neurons that corelease GABA and glycine in the vestibular nuclei are another possibility (Dumoulin et al. 2001; Jonas et al. 1998; O’Brien and Berger 1999; Russier et al. 2002).

**Inhibitory effect of GABA_B receptor**

It was reported that both GABA_A and GABA_B receptor agonists decreased the frequency of spontaneous spiking activity in vestibular nucleus neurons in young and old rats (Him et al. 2001) and after unilateral labyrinthectomy (Johnston et al. 2001). From autoradiographic studies performed on the vestibular nuclei of young rats, the presence of GABA_B receptors was confirmed (Turgeon and Albin 1994). In the present study using KCI pipette solution, we showed that postsynaptic GABA_B receptors are present on the principal cells by applying the GABA_B agonist baclofen which induced an outward current. The current could be blocked by intracellular Cs; this allowed us to observe the presynaptic effects of baclofen. In these experiments, the frequency of sEPSCs and sIPSCs was decreased by baclofen ~40 and 53%, respectively. When phaclofen, a specific GABA_B receptor antagonist, was added alone, there was no effect on sEPSCs frequency, but there was a slight increase in sIPSCs frequency (6%). This finding suggests that in our system sEPSCs and sIPSCs are generated by separate presynaptic terminals. It seems that the presynaptic GABA_B receptors on the excitatory terminals are not activated tonically by ambient GABA, whereas the presynaptic GABA_B receptors on the inhibitory terminals can be activated. Finally, inhibitory postsynaptic currents, respectively. * Student’s t-test, statistically significant values are indicated in bold (p < 0.01).
when phaclofen was preapplied, baclofen still reduced the frequency of sEPSCs (26%) and sIPSCs (24%) but to lesser extents. Altogether, these experiments indicate that there may be different subtypes of GABA<sub>B</sub> receptors present at various presynaptic terminals in the tangential nucleus (for review, see Ong and Kerr 2000). Accordingly, GABA<sub>B</sub> receptors may play crucial roles in modulating the input to the principal cells and could be involved in the compensation process occurring after peripheral vestibular lesions.

GABA<sub>B</sub> receptors show different functions according to their pre- and postsynaptic locations. In the case of presynaptic action, baclofen could regulate the release of GABA, glutamate, and glycine (Kabashima et al. 1997; Lim et al. 2000) by either inhibiting the calcium current necessary for transmitter release (Deisz and Lux 1985; Doze et al. 1995), activating a potassium channel that indirectly inhibits the calcium current (Doze et al. 1995) or directly inhibiting quantal release by affecting protein kinase C (Jarolimek and Misgeld 1997). For postsynaptic inhibition, GABA<sub>B</sub> receptors are coupled to G proteins and mediate postsynaptic effects by increasing the potassium conductance (Dutar and Nicoll 1988).

**Possible significance of inhibitory inputs**

In the young postnatal brain, GABA functions primarily as an inhibitory neurotransmitter, although it may produce depolarizing effects in embryonic auditory nuclei (Hyson et al. 1995; Lu and Trussell 2001). During embryonic development, GABAergic inhibition may provide a continuous background level of inhibition to the principal cells, driving the membrane potential away from firing threshold and maintaining a relative steady state. Indeed, there was no rhythmic bursting observed in the embryonic tangential principal cells at E13 and E16 using intracellular and patch-clamp recordings (Gamkrelidze et al. 1998; Peusner and Giaume 1994, 1997). This contrasts to the spontaneous action potential firing recorded from E14 to 18 chick auditory neurons in the nucleus magnocellularis and nucleus laminaris (Lippe et al. 1994). Inhibitory spontaneous synaptic activity could contribute to the typical response of most embryonic principal cells of a single spike on depolarization at E16 (Gamkrelidze et al. 1998; Peusner and Giaume 1997). In some systems, GABA acts as a trophic factor during nervous system development, influencing multiple diverse events including proliferation, migration, differentiation, synapse maturation, cell death and GABA<sub>A</sub> receptor expression (Öwens and Kriegstein 2002). At E16, the tangential nucleus undergoes a major morphological transition because the spiny dendrites retract and acquire gap junction channels, and dendritic outgrowth in the principal cells becomes exuberant as compared with just a few days earlier at E13.

**Miniature activity**

In this study, most spontaneous synaptic activity was TTX-resistant, the so-called miniature activity, which is thought to be due to quantal neurotransmitter release (Nicholls et al. 2001). The quantal release theory attributes each mPSC to the release of the neurotransmitter content from a single vesicle. The experiments demonstrating a higher percentage of TTX-resistant inhibitory events than excitatory events suggest that excitatory events were more dependent on action potentials than inhibitory events. Because the kinetics of mIPSCs were longer than those of mEPSCs, it is conceivable that at this age excitatory events are shunted by inhibitory events and do not reach the threshold for firing action potentials.

There is accumulating evidence that cotransmission of GABA and glycine occurs from the same synaptic terminal with distinctive kinetics (e.g., Dumoulin et al. 2001; Jonas et al. 1998; O’Brien and Berger 1999; Russier et al. 2002). In our study, small differences in the kinetics of GABA and glycine (decay time and duration) were not statistically significant. The differences in the kinetics between mEPSCs and mIPSCs and between GABAergic mIPSCs and glycineergic mIPSCs may be attributed to the intrinsic receptor properties, variability in synaptic structures, including uptake system, barriers for diffusion, and/or transmitter concentration in the synaptic cleft (Clements 1996; Weiss et al. 1988). In our experiments, we excluded the possibility of inadequate space clamp because there was no correlation between rise time and decay time and a positive correlation between decay time and peak amplitude. The reasons for kinetics differences in our study as compared with other works may be due to variations in the slice preparations, receptor properties of the neurons, recording temperatures, pipette solutions, holding potentials and the use of flunitrazepam or pentobarbital.

**What is the significance of spontaneous synaptic activity in second-order vestibular neurons?**

While evoked synaptic transmission is accepted as crucial for nervous system function, the exact physiological significance of spontaneous synaptic activity is uncertain at present. First described in 1932 by Hoagland, spontaneous activity has been reported to occur in just about all first- and second-order sensory neurons, including vestibular sensory neurons (e.g., Goldberg and Fernández 1971). Excitability is a crucial property of mature vestibular nucleus neurons because most of them fire action potentials tonically (see Peusner et al. 1998 for review). This feature is essential for them to transmit reliably vestibular stimuli and to perform normal reflex functions (du Lac and Lisberger 1995). Even when there is no angular or linear acceleration, vestibular sensory neurons fire spikes actively so that there is a continual flow of action potentials along the primary vestibular fibers and vestibular nucleus neurons. In mature or nearly mature vestibular sensory neurons, spontaneous activity results in spike firing. However, in the embryonic principal cells, the spontaneous synaptic activity does not reach firing threshold. Despite small amplitudes, we suspect that the spontaneous synaptic activity may provide sufficient inhibitory and possibly excitatory drive to modulate the vestibular glutamatergic transmission in principal cells as shown for other glutamate-releasing systems (e.g., Cossart et al. 2002). Spontaneous synaptic activity may influence many aspects of developing excitatory cells, including formation of their basic neural circuitry, neurogenesis, generation of space maps, shaping of the tuning curve in the auditory system, and the expression of myogenic factors (e.g., Dallman et al. 1998; Frija and Lohmann 1999).

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