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MATURATION OF FIRING PATTERN IN CHICK VESTIBULAR NUCLEUS NEURONS

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Abstract

The principal cells of the chick tangential nucleus are vestibular nucleus neurons participating in the vestibuloocular and vestibulocollic reflexes. In birds and mammals, spontaneous and stimulus-evoked firing of action potentials is essential for vestibular nucleus neurons to generate mature vestibular reflex activity. The emergence of spike-firing pattern and the underlying ion channels were studied in morphologically-identified principal cells using whole-cell patch-clamp recordings from brain slices of late-term embryos (embryonic day 16) and hatchling chickens (hatching day 1 and hatching day 5). Spontaneous spike activity emerged around the perinatal period, since at embryonic day 16 none of the principal cells generated spontaneous action potentials. However, at hatching day 1, 50% of the cells fired spontaneously (range, 3 to 32 spikes/s), which depended on synaptic transmission in most cells. By hatching day 5, 80% of the principal cells could fire action potentials spontaneously (range, 5 to 80 spikes/s), and this activity was independent of synaptic transmission and showed faster kinetics than at hatching day 1. Repetitive firing in response to depolarizing pulses appeared in the principal cells starting around embryonic day 16, when < 20% of the neurons fired repetitively. However, almost 90% of the principal cells exhibited repetitive firing on depolarization at hatching day 1, and 100% by hatching day 5. From embryonic day 16 to hatching day 5, the gain for evoked spike firing increased almost 10-fold. At hatching day 5, a persistent sodium channel was essential for the generation of spontaneous spike activity, while a small conductance, calcium-dependent potassium current modulated both the spontaneous and evoked spike firing activity. Altogether, these *in vitro* studies showed that during the perinatal period, the principal cells switched from displaying no spontaneous spike activity at resting membrane potential and generating one spike on depolarization to the tonic firing of spontaneous and evoked action potentials.

Keywords

intrinsic membrane properties; spike activity; brain slice

The vestibuloocular (VOR) and optokinetic (OKR) reflexes are involved in stabilizing visual gaze during head movements, whereas the vestibulocollic (VCR) reflex stabilizes the head position in space. To perform these functions, the VOR generates compensatory eye movements in a direction opposite to the head movements, while the OKR produces eye movements in the same direction as the image motion, and the VCR stimulates the muscles of the neck to support the head during movements. Defined as the eye speed divided by head speed, the VOR gain is an important property, which is subject to change during different types of movements. For example, during natural head rotations, the gain of compensatory eye movements is larger than 1, but when there is image motion during head turning, the gain is smaller than 1. Thus, the VOR is an adaptive response, rather than an automatic reflex (for

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review, see Collewyn, 1989). Compensatory eye movements require the integration of vestibular, visual, proprioceptive and other behaviorally relevant signals at the level of the vestibular nucleus neuron (VNN). Due to the adaptive capabilities of VNNs, extensive *in vitro* electrophysiological studies have been performed to understand their firing pattern in adult animals of different species (e.g. rat: Johnston et al., 1994; chick: du Lac and Lisberger, 1995; guinea-pig: Serafin et al., 1991).

So far, studies on the maturation of VNN firing pattern have focused on neurons from the medial vestibular nucleus (MVN) in rat and mouse, and also on the principal cells of the chick tangential nucleus (Peusner and Giaume, 1997; Gamkrelidze et al., 1998) (for review, see Straka et al., 2005). In rat and mouse, VNNs showed significant changes in their spontaneous and evoked firing pattern around postnatal days 14–15 (P14–P15), when the eyes opened and visual stimuli were first detected (Johnston and Dutia, 1996; Murphy and du Lac, 2001). Firing pattern in VNNs is also subject to change in response to unilateral loss of peripheral vestibular input. In humans and animals, severe symptoms of the lesion subsided within about a week, a process known as “vestibular compensation” (Aldrich and Peusner 2002). During compensation, changes in the spontaneous and evoked-spike firing patterns were detected in both the ipsi- and contralateral VNN (Darlington et al., 2002). In the developmental and compensation studies performed on mammals, the recordings were combined from different neuron classes, so that information processing by identified classes of VNNs was not determined, as done in the auditory system (e.g. Rothman and Manis, 2003). Accordingly, the chick tangential nucleus was selected for study here, since its principal cells offer the opportunity to study a large number of morphologically-similar neurons. In the present study, the recorded neurons were injected with biocytin to insure that the recordings were obtained only from the principal cells.

Located in the lateral medulla oblongata, the chick tangential nucleus is the interstitial nucleus of the vestibular nerve in avians. The nucleus is dominated by its principal cells, which comprise 80% of the neuron population (Peusner and Morest, 1977). The principal cells receive synaptic terminals from the large and small diameter primary vestibular fibers, cerebellum, cervical spinal cord, and contralateral vestibular nuclei (Cox and Peusner, 1990a,b). The axons of the principal cells project to four major neuronal targets: oculomotor, trochlear and abducens nuclei, and motor neurons in the high cervical spinal cord, resulting in their classification as vestibulooculocollic neurons (Cox and Peusner, 1990a). In contrast to the mammalian interstitial nucleus of the vestibular nerve (Brodal and Brodal, 1985; Langer et al., 1985), the axons of the principal cells do not project to the cerebellum. Thus, according to their afferent and efferent connections, the chick tangential nucleus most closely resembles the lateral vestibular nucleus in mammals (see Discussion).

Unlike altricial mammals, the precocial chicken undergoes rapid vestibular development during the perinatal period (Peusner and Giaume, 1997). At embryonic day 16 (E16), the embryo is confined to the eggshell, and lacks vision (Wallman et al., 1982). During the first 24 h after hatching (H0), the chickens’ righting reflexes were absent (Heaton, 1975), but after 24 h (H1), the chickens could stand erect, walk, feed and drink (Decker, 1970). Perching was first observed in 4-to-5 day old hatchlings (H4–H5) (Heaton, 1975). Accordingly, the ages selected for study here were E16, H1 and H5.

Using the whole-cell patch-clamp technique, the spontaneous spike activity of the principal cells was recorded at resting membrane potential (RMP). Stimulus-evoked spike activity was induced by injecting 400 ms duration depolarizing currents at different intensities. Finally, since at H5 the spontaneous spike activity was mediated by intrinsic membrane conductances, a persistent sodium current (I_{NaP}), hyperpolarization-activated cationic current (I_h), and small conductance, calcium-dependent potassium current (SK) were investigated due to their

important roles in spike activity in other neurons (e.g. de Waele et al., 1993; Johnston et al., 1994; Bennett et al., 2000; Smith et al., 2002).

EXPERIMENTAL PROCEDURES

Experimental animals

Experiments were performed on E16, and H1 and H5 chickens (*Gallus gallus*), obtained from CBT Farms (Chesterton, MD, USA). The animal protocols were approved by the Institution Animal Care and Use Committee of the George Washington University. All experiments conformed to international guidelines on the ethical treatment of animals. All efforts were made to minimize the number of animals used and their suffering. The age of the embryos was established by referring to the staging criteria of Hamburger and Hamilton (1951). The age of the hatchlings was determined by counting the day of birth as 0 days, and 24 h afterward as a 1 day old hatchling, H1.

Brain slice preparation and solutions

At E16, transverse sections (300 μm) of the medulla oblongata were cut in cold, artificial cerebrospinal fluid (ACSF) (4 $^{\circ}\text{C}$) using a microslicer (VT1000S; Leica Instruments, Bannockburn, IL, USA) and razor blades (Feather blue blades; Ted Pella Inc., Redding, CA, USA). The embryonic slices recovered for 1 h in ACSF at room temperature (21–22 $^{\circ}\text{C}$) in a Petri dish before they were transferred to the recording chamber. Transverse sections (240 μm) of the hatchling brainstem were cut in sucrose ACSF (1–4 $^{\circ}\text{C}$) using a sapphire knife (Delaware Diamond Knives, Wilmington, DE, USA). After sectioning, the hatchling slices were incubated in ACSF (37 $^{\circ}\text{C}$) for 40 min, and then maintained at 21–22 $^{\circ}\text{C}$ for at least 20 min before transferring to the recording chamber. Preheated ACSF was superfused through the recording chamber (volume, 180 μl ; Warner Instruments, Hamden, CT, USA) at a rate of 2–3 ml/min, while the ACSF in the recording chamber was maintained at 30–31 $^{\circ}\text{C}$ using a temperature controller (TC324B, Warner Instruments). Sucrose ACSF contained (in mM): 210 sucrose, 2.5 KCl, 7 MgSO_4 , 1.5 NaH_2PO_4 , 1 CaCl_2 , 26 NaHCO_3 , and 10 D-glucose. ACSF contained (in mM): 126 NaCl, 3.5 KCl, 1.2 NaH_2PO_4 , 1.3 MgCl_2 , 2 CaCl_2 , 26 NaHCO_3 , and 10 D-glucose. Both sucrose ACSF and ACSF were bubbled with 95% O_2 /5% CO_2 to maintain the pH at 7.2–7.4. The osmolarity was 310–320 mOsm.

All of the drugs, except CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), were dissolved in ACSF and then added to the bath ACSF to achieve the final concentration. The drugs included tetrodotoxin (TTX; 1 μM ; RBI, Natick, MA, USA) to block the voltage-dependent sodium currents, including the fast sodium current, I_{Na} , and the slow, I_{NaP} ; DL-2-amino-5-phosphovaleric acid (AP-5; 30 μM ; Sigma, St. Louis, MO, USA) to block the glutamatergic N-methyl-D-aspartate (NMDA) receptor-mediated currents; strychnine (1 μM ; Sigma) to block the glycine receptor-mediated currents; bicuculline methochloride (10 μM ; Tocris, St. Louis, MO, USA) to block the GABA_A receptor-mediated currents; and apamin (100 nM; Alomone) to block the SK; cesium chloride (CsCl ; 3 mM; Sigma) to block the Ih. CNQX (10 μM ; RBI) was used to block the glutamatergic, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated currents by dissolving the compound in dimethyl sulfoxide (DMSO; Fisher Scientific, Fair Lawn, NJ, USA) at a concentration of 20 mM, which was added to the bath ACSF to achieve the final concentration.

Electrophysiology

Slices were viewed on a fixed-stage, upright microscope (Zeiss Axioskop, FS-1, Jena, Germany) equipped with differential interference contrast optics and a 40 $\times$ water-immersion lens (NA, 0.75). Visualization of the recorded neuron and pipet was achieved using an infrared light source (filter, 770 nm), which was detected by an infrared-sensitive tube camera (Vidicon

C2400-01, Hamamatsu, Hamamatsu City, Japan) and observed on a monitor (Sony). A 4× lens was inserted between the microscope and camera, and the image contrast and shading were adjusted with a camera controller (C2400, Hamamatsu).

Microelectrodes (2–5 MΩ) were pulled from borosilicate glass tube (World Precision Instruments, Sarasota, FL, USA) using a Brown/Flaming horizontal puller (P-87, Sutter Instruments, Novartis, CA, USA). KCl ($n = 12$) and potassium methylsulfate (KMeSO₄) ($n = 12$) pipet solutions were used to record from the E16 principal cells, with no differences detected in the RMPs or kinetics for the evoked action potentials with either pipet solution. Therefore, all of the data acquired at E16 were pooled for analysis. All of the hatchling brain slices were recorded using KMeSO₄ pipet solution. KCl pipet solution contained (in mM): 130 KCl, 10 EGTA, 10 HEPES, 1.0 CaCl₂, 2.0 Mg-ATP. KMeSO₄ pipet solution contained (in mM): 120 KMeSO₄, 20 KCl, 10 HEPES, 0.5 EGTA, 2 MgCl₂, 4 Na₂ATP, 0.3 Tris GTP. For both pipet solutions, the osmolarity was 270–290 mOsm, and the pH was adjusted to 7.2 with KOH. Biocytin (0.5%) (Sigma) was dissolved in the pipet solution weekly and kept in the freezer between the experiments.

Signals were amplified using either Axopatch-1D or Axo-patch-200B in normal mode (Axon Instruments, Foster City, CA, USA). The Axopatch amplifiers may produce distortions in the action potential waveform (Magistretti et al., 1996, 1998), most apparent in the first action potential afterhyperpolarization (AHP). The distortion is diminished by using the fast mode. However, in fast mode, our recorded neurons became unstable and depolarized, and were lost within 15 min. Indeed, with 2–5 MΩ recording pipets, the normal mode permitted stable recordings in our preparations for 30 min or longer, which was necessary to complete our standard experimental protocols. Since all of the developmental studies performed here used the same approach at different ages, the same distortion, if present, applies to all of the experimental conditions equally, so it should not affect the conclusions. Current-clamp recordings were digitized at 20 kHz and filtered at 5 kHz, while voltage-clamp recordings were digitized at 10 kHz and filtered at 2 kHz. The spontaneous spike activity at RMP was recorded on a digital audiotape recorder (DTR-1201; Biologic Instruments, Claix, France) and acquired using Axoscope 8.1 (Axon Instruments). Evoked action potentials were induced by injecting depolarizing current pulses of 400 ms duration and varying intensities from RMP, and recorded using pClamp 6.0 computer program (Axon Instruments). The gain for the evoked action potentials (m) was defined as the ratio of the firing rate relative to the input current (Smith et al., 2002). I_{NaP} was induced using 800 ms voltage steps from −75 to −40 mV in 2.5 mV increments (before correcting for liquid junction potential). I_h was tested by applying an 800 ms voltage step from −60 to −75 mV. Finally, SK was induced using a 15 ms voltage step from −60 mV to −20 mV. In these voltage-clamp experiments, the voltage was controlled within the soma and proximal processes, but probably not sufficiently within the distal processes due to space-clamp problem. Space-clamp error could alter the magnitude of the recorded currents. However, in these experiments, space-clamp error did not change the results, since only the presence or absence of currents was tested.

Biocytin processing of slices and dendritic measurements

After the recordings, the slices were processed for biocytin visualization with Streptavidin Alexa Fluor 647 (Molecular Probes, Eugene, OR, USA) and counterstained with DiO (Molecular Probes), as previously described (Shao et al., 2003). The injected neurons were studied using a conventional fluorescent microscope (Nikon Optiphot, Nikon, Tokyo, Japan), equipped with a 20× objective (N.A. 0.5), and photographed using a Spot-RT camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). The identity of the principal cells was confirmed by studying the somatic shape, axonal morphology, and dendritic branching pattern.

Data acquisition and analysis

Immediately after obtaining the whole-cell configuration, the RMP was read from the amplifier panel meter, and the input resistance was determined by giving a +5 mV pulse using the seal test function. All of the data were corrected for liquid junction potential (KMeSO₄, +6 mV; KCl, +3 mV). The data were analyzed off-line using Clampfit 9.2 (Axon Instruments).

Spontaneous spike activity was analyzed from random trace samples of 3–4 min duration. The frequency was expressed as the inverse of the mean interspike interval (ISI). The coefficient of variation (CV) was calculated for all of the spontaneously-firing principal cells at H1 and H5 ($n = 24$). The CV was defined as the standard deviation (S.D.) of the ISI divided by the mean ISI. Since the frequencies were different in the cells sampled, the number of intervals used for the measurements varied from 560 to 14,000. The principal cells were distinguished as regular- or irregular-firing neurons, according to their CVs. Regular-firing principal cells were associated with a CV < 0.5, while the irregular-firing principal cells had a CV > 0.5 (Graf et al., 1993).

The kinetics for spontaneous action potentials were analyzed from averages of 15 spikes/cell. Spike threshold was defined either visually as the point in the voltage trace where a sharp inflection occurred just prior to generating the action potential, or using the Minianalysis computer program (Synaptosoft Inc., Decatur, GA, USA), which determines the spike threshold based on the value of dV/dt . There was no significant difference between the visual and calculated values for spike threshold as previously reported (Fricker et al., 1999). Spike amplitude was defined as the potential difference between threshold and peak amplitude. Rise time was defined as the duration between threshold and peak amplitude during depolarization, while decay time was defined as the duration between peak amplitude and threshold during repolarization. Action potential width was measured as the duration between 50% spike amplitude during depolarization and repolarization. AHP amplitude was defined as the difference in voltage between the spike threshold and peak negativity after a spike. The values were presented as mean $\pm$ standard error of the mean. Differences were considered significant when $P < 0.05$ (independent Student's t -test and paired t -test). The normal distribution of the samples was evaluated using Kolmogoroff-Smirnoff test with a significance level set at $P < 0.05$. Correlations were analyzed using Prism software program (Graphpad, San Diego, CA, USA), with a two-tailed distribution and 95% confidence level. The Pearson test was used when both samples showed a normal distribution, whereas a Spearman test was applied when either sample lacked a normal distribution.

RESULTS

Morphological identification of the principal cells

At all of the ages studied, the principal cells were characterized by striking oval cell bodies among the parallel bundles of primary vestibular fibers (not shown). From E16 to H5, the somata did not grow significantly (E16: $24 \pm 0.6 \times 17 \pm 0.8 \mu\text{m}$, $n = 12$; H1: $27 \pm 1 \times 20 \pm 1.1 \mu\text{m}$, $n = 16$; H5: $25 \pm 0.9 \times 19 \pm 0.7 \mu\text{m}$, $n = 21$) ($P > 0.05$). However, at E16 the somata exhibited many fine, radiating processes, which were absent at H1 and H2. Whatever the age, the principal cell axons followed the same course through the tangential nucleus. The smooth-surfaced axon originated medially, either from the cell body or from a dendrite, and ran medially through the tangential nucleus without forming any collateral branches. Two morphological subtypes of the principal cells were distinguished by their dendritic branching pattern. Most of the principal cells (E16, $n = 8/12$, 75%; H1, $n = 11/16$, 69%; H5, $n = 15/21$, 71%) had three-to-five primary dendrites radiating in the dorsal, ventral, medial and/or lateral direction, while the remaining cells had one lateral, primary dendrite which branched profusely. There was no difference detected for these two subtypes in location in the nucleus, input

resistance, or RMP, as found in an earlier study (Peusner and Giaume, 1997). In the conventional fluorescence microscope, the dendrites of the principal cell appeared to increase in length between E16 and hatching, as reported in a previous quantitative study (Peusner and Giaume, 1997).

Passive membrane properties

The whole-cell recordings presented here were obtained from a total of 61 morphologically-identified principal cells from the tangential nucleus at E16 ($n = 24$), H1 ($n = 16$), and H5 ($n = 21$). The RMP was -74 ± 1 mV at E16, -72 ± 2 mV at H1, and -66 ± 4 mV at H5. The input resistance was 86 ± 6 M Ω at E16, 94.8 ± 5.1 M Ω at H1, and 97.1 ± 7 M Ω at H5. These passive membrane properties did not differ significantly at the ages investigated ($P > 0.05$).

Spontaneous spike firing

At E16, none of the principal cells exhibited spontaneous spike activity at RMP ($n = 24$) (Fig. 1A), or after steady depolarization of the membrane potential up to -40 mV ($n = 9$) (data not shown).

However, in hatchlings (H1, H5), most of the principal cells ($n = 24/37$, 65%) fired action potentials spontaneously, with either a regular or irregular firing pattern (Fig. 1B). The mean firing rate for the irregularly-firing principal cells was 6.6 ± 2.3 spikes/s, ranging from 3 to 23 spikes/s ($n = 10$), while the regularly-firing principal cells showed a higher mean firing rate of 23.6 ± 5.1 spikes/s, and ranged from 7 to 80 spikes/s ($n = 14$). As expected, a negative correlation between firing frequency and CV was plotted (Spearman $r = -0.52$, $P < 0.05$) (Fig. 1C), indicating that the principal cells with higher frequencies of firing action potentials usually showed more regular firing pattern. Occasionally, neurons with high firing frequencies exhibited high CVs, as shown in other systems (see Goldberg and Fernandez, 1971). There was no correlation between firing frequency and RMP (Pearson $r^2 = 0.02$, $P > 0.05$) (Fig. 1D). The mean CV for the irregularly-firing principal cells was 1.22 ± 0.3 at H1, 2.49 ± 1 at H5, with no significant difference between the two ages ($P > 0.05$). However, the mean CV for the regularly-firing principal cells was 0.23 ± 0.08 at H1 and 0.18 ± 0.03 at H5, which was significantly different ($P < 0.05$). Thus, the principal cells, which fired regular action potentials at H5, had the highest firing rates.

At H1, four principal cells exhibited irregular and four regular spontaneous spike firing (Fig. 2A), while the remainder were silent ($n = 8/16$, 50%). The average firing rate for irregular, spontaneously-firing principal cells at H1 was 6.8 ± 2.6 spikes/s, with a range from 3 to 12 spikes/s ($n = 4$), while the average for the regular, spontaneously-firing principal cells was 22.3 ± 5.6 spikes/s, and ranged from 13 to 32 spikes/s ($n = 4$). In all of the irregularly-firing principal cells, spontaneous spike firing was blocked completely in 3–4 min by a combination of excitatory (10 μ M CNQX and 30 μ M AP-5) and inhibitory (10 μ M bicuculline and 1 μ M strychnine) neurotransmitter receptor antagonists ($n = 4$) (Fig. 2B). In addition, this combination of antagonists blocked completely the spontaneous spike firing in 50% of the regularly-firing principal cells at H1 ($n = 2/4$, 50%; not shown). The average RMP of the principal cells was -70 ± 4 mV after the spontaneous spike firing was blocked completely with the combined neurotransmitter antagonists at H1, with no significant difference compared with the control RMP (-68 ± 5 mV; $n = 6$; paired t -test, $P > 0.05$). While all of the action potentials originated directly from baseline in the regularly-firing principal cells (not shown), two different waveforms were detected in the irregularly-firing principal cells at H1. One waveform was preceded by an excitatory postsynaptic potential (Fig. 2C, asterisk), while the other originated directly from the baseline (Fig. 2D). Altogether, these findings indicate that the majority of spontaneously-firing principal cells at H1 relied on synaptic transmission to trigger their spontaneous spike activity.

At H5, most of the principal cells ($n = 16/21$, 76%) showed spontaneous spike firing, including 6 cells with irregular and 10 with regular spike activity. Indeed, the principal cells which fired spontaneously when recorded in the whole-cell mode, also fired spontaneously under the non-invasive cell-attached configuration (3A₁). Since the cell-attached recordings were made for < 1 min, the analysis was more accurate for the regular-firing cells. In seven regularly-firing principal cells at H5, the mean frequency was 35.4 ± 10.8 spikes/s, with a CV of 0.21 ± 0.03 in the cell-attached mode, while the mean frequency of firing was 36 ± 10.9 spikes/s, with a CV of 0.12 ± 0.02 in the whole-cell configuration. Thus, there was a small change in the firing regularity between the cell-attached and whole-cell recordings, likely due to the effects caused by the diffusion of pipet solution into the cell's cytoplasm during the recordings. At H5, the mean firing frequency for the irregularly-firing principal cells was 12.8 ± 2.7 spikes/s, ranging from 5 to 23 ($n = 6$), while the mean firing frequency for the regularly-firing principal cells was 24.2 ± 7.1 spikes/s, ranging from 7 to 80 spikes/s ($n = 10$). During the recordings from the regularly-firing principal cells, the firing rate remained constant (up to 3–4 min) (Fig. 3B). On exposure to synaptic transmission antagonists, none of the principal cells tested at H5 showed decreased spontaneous spike firing ($n = 3$; one irregular and two regular) (Fig. 3A₂, A₃), suggesting that these cells have acquired intrinsic membrane properties underlying the spontaneous spike activity. Furthermore, from H1 to H5, there was a developmental change in the spike waveform (Fig. 3C) (see below).

Stimulus-evoked spike firing

At E16, two types of firing patterns were generated in the principal cells by injecting 400 ms duration depolarizing current pulses. Eighty-three percent of the cells (20/24) exhibited a single spike at the onset of the current injection, even when the membrane potential was depolarized to -30 mV with up to 1.6 nA of injected current (Fig. 4A). The few remaining principal cells fired repetitively on depolarization ($n = 4$) (Fig. 4B). At H1, most of the principal cells (14/16, 88%) fired repetitively (Fig. 4C). Finally, by H5, *all* of the principal cells (21/21) fired multiple spikes on depolarization (Fig. 4D). Accordingly, the proportion of principal cells firing multiple spikes on depolarization increased gradually during development. Table 1 contains the frequency of spike firing for the principal cells in response to current pulses of different intensities at the three ages studied. At all of the ages, the relationship between injected depolarizing current and firing frequency was linear (Fig. 5A). However, during development, the slope of the relationship for injected current and firing rate, or gain, increased dramatically from 38 spikes/s/nA at E16, to 204 spikes/s/nA at H1, and finally 313 spikes/s/nA at H5. In fact, at H1 and H5, all of the principal cells showed very regular spike firing on depolarization ($n = 36$, CV < 0.2 ; data not shown), regardless of the spontaneous spike firing pattern at RMP (regular: $n = 14$; irregular: $n = 10$; or no spontaneous spike firing at all: $n = 12$). Moreover, there was no accommodation in the frequency of evoked spike firing for depolarizing pulses up to 0.7 nA for most of the principal cells tested at H1 (12/14, 86%) and H5 (18/21, 86%).

Waveform of the evoked and spontaneous action potentials

At E16, on exposure to brief depolarizing current pulses, 50% of the single-spike firing principal cells showed only a fast AHP following the action potential (Fig. 5B₁), while the remaining cells exhibited both a fast and a slow AHP (6/12) (Fig. 5B₂, dark trace). When the principal cells with only the fast AHP at E16 were compared with those with two AHPs, those cells with two AHPs had lower spike thresholds (-39 ± 3.6 mV vs. -19 ± 3.2 mV), larger spike peak amplitudes (64 ± 5 mV vs. 40 ± 4 mV), and required less injected current to evoke the first spike (0.8 ± 0.1 nA vs. 1.4 ± 0.2 nA) ($P < 0.05$). However, there were no significant differences in their rise times, decay times, or action potential halfwidths ($P > 0.05$). When E16 principal cells with two AHPs were injected with high intensity depolarizing current pulses up to 1.6 nA, the second AHP disappeared in a few cells (2/6, 33%) (Fig. 5B₂, thick trace), but remained in most cells (4/6, 67%).

On comparing the kinetics for the spontaneous action potentials at H1 and H5, the rise time, decay time, and halfwidth of the action potentials were significantly faster at H5 than at H1 ($P < 0.05$) (Fig. 3C; Table 2). However, no significant differences were found in their spike threshold, peak amplitude, or AHP amplitudes ($P > 0.05$). In the vast majority of the principal cells recorded from at H1 ($n = 7/8$, 88%) and H5 ($n = 12/16$, 75%), the spontaneous action potentials were followed by a fast and a slow AHP. In the remaining principal cells, only a fast AHP was observed ($n = 5$). When hatchling principal cells firing spontaneous spikes followed by two AHPs at RMP were depolarized by current pulse injections from 0.1–0.7 nA, only the fast AHP remained ($n = 16/17$, 94%) (Fig. 5C). Accordingly, the spontaneous-spiking principal cells in these preparations exhibited a voltage-sensitive, slow AHP.

Intrinsic membrane properties underlying spontaneous spike firing at H5

Sodium currents—As a first step in characterizing the intrinsic membrane properties underlying the spontaneous spike firing at H5, the principal cells were exposed to 1 μ M TTX in current-clamp mode. As reported at E16 and H1 (Gamkrelidze et al., 1998,2000), all of the spike activity was abolished (Fig. 6A₁, A₂), indicating that sodium channels contributed to action potential generation in the principal cells at H5. In the presence of TTX, the RMP was -48 ± 5.5 mV, which was not significantly different from the control (-63.4 ± 1.4 mV) ($n = 4$) (paired t -test, $P > 0.05$).

In voltage-clamp recordings in normal ACSF, a persistent inward current on the order of 10 pA was induced in the principal cells by applying an 800 ms depolarization voltage step from -66 to -51 mV (Fig. 6B₁). The inward current was abolished by adding TTX, and revealed an underlying, small outward current (Fig. 6B₂). A plot of the current-voltage relationship (I – V curve) for the inward current revealed a negative slope between -65 and -50 mV (Fig. 6C). When the TTX-insensitive current was subtracted from the control current, the inward current was found to activate between -65 and -50 mV (Fig. 6D). This inward current has all of the characteristics of I_{NaP} , including a low-activation threshold, slow inactivation, and TTX sensitivity (Llinas, 1988). Accordingly, I_{NaP} contributed to drive the principal cells at H5 toward their spike firing threshold (-41.4 ± 2.4 mV).

I_h—Since I_{NaP} activated between -65 and -50 mV, another current must be present in the principal cells which activated at potentials more negative than -65 mV for recovery from the AHP. Accordingly, we tested for the presence of a hyperpolarization-activated cationic channel, I_h , which typically activates at more hyperpolarized potentials than -65 mV (Bevan and Wilson, 1999). In current-clamp recordings from the principal cells at H5, a depolarizing “sag” was observed during a 0.5 nA hyperpolarizing current pulse ($n = 4$) (Fig. 7A₁). The sag was abolished in 3 mM CsCl, suggesting the presence of I_h (Fig. 7A₂). In voltage-clamp recordings, a slowly developing, inward current was evoked by an 800 ms voltage step from -66 to -81 mV (Fig. 7B₁). This inward current was blocked by CsCl (Fig. 7B₂). However, in current-clamp recordings, the principal cells exhibited no change in their firing frequency in the presence of CsCl (up to 6 min) (Fig. 7C₁, C₂), indicating that I_h was not involved in the generation of spontaneous spike firing at H5 ($n = 4$).

Calcium-dependent potassium currents: SK The role of SK in the spontaneous and evoked firing was investigated by applying apamin, a specific SK channel antagonist. Apamin (100 nM) produced several effects on the spontaneous spike activity (Fig. 8). On exposure to apamin, in current-clamp recordings, the membrane potential of both the irregularly ($n = 2$) and regularly-firing ($n = 3$) principal cells depolarized from -62 ± 2 mV to -54 ± 2 mV, a significant difference (paired t -test; $P < 0.05$) (Fig. 8A₁, and A₂). Also, the frequency of spontaneous spike firing increased from 11 ± 2.7 spikes/s to 20 ± 7.3 spikes/s, and the CV changed from 0.6 ± 0.3 to 0.4 ± 0.2 ($n = 5$). Finally, in current-clamp recordings, apamin

abolished the slow AHP in the principal cells (Fig. 8B), demonstrating that this slow AHP was mediated by the SK channel. In voltage-clamp recordings, a fast, 15 ms depolarizing voltage step from -60 mV to -20 mV induced an outward current, which could be abolished completely by 100 nM apamin ($n = 6$) (Fig. 8C).

In a second set of experiments, the effect of apamin on depolarizing-pulse evoked spike activity was investigated. On depolarization, apamin increased significantly the firing rate of the principal cells at H5 ($n = 6$) (Fig. 8D₁, D₂) (paired t -test; $P < 0.05$), so that the firing gain (m) increased from 223 spikes/s/nA to 333 spikes/s/nA ($n = 6$) (Fig. 8E). However, after exposure to apamin, the principal cells did not show a significant change in their CVs on depolarization (paired t -test, $n = 6$; Fig. 8F), which were already close to the lowest measurable CV value.

DISCUSSION

Like many sensory systems, the chick vestibular system is transformed at birth by the nature of its relevant stimuli. In our *in vitro* brain slice preparations, spontaneous spike activity was not detected in any of the principal cells at E16, but at H1 50% of these cells could fire spontaneous spikes, which depended on synaptic transmission. By H5, spontaneous spike activity, which was recorded in most principal cells (80%), was *independent* of synaptic transmission. In response to depolarizing pulses, $< 20\%$ of the principal cells could fire action potentials repetitively at E16, 90% at H1, and all of them by H5. Like the MVN neurons in mammals (Murphy and du Lac, 2001), the principal cells responded to depolarizing pulses in a linear way. Indeed, the gain for repetitive firing of evoked action potentials increased almost 10-fold, from 38 spikes/s/nA at E16 to 313 spikes/s/nA at H5.

The chick tangential nucleus in brain slice is a good model to study neuronal excitability

Studies on the intact animal permit a behavioral assessment of functional activity, and offer the closest approximation to physiological conditions for evaluating neuronal excitability, if the effects of anesthesia are discounted. However, *cellular* electrophysiology is difficult to perform on the intact animal due to the problems encountered in manipulating the ionic environment and determining the site of action and concentration of drugs on the target neurons. These issues are not relevant to the brain slice, which not only provides stable recording conditions, but also allows ready access to the recorded neurons for pharmacological manipulations. Since its introduction for neuronal recording (Li and McIlwain, 1957), the brain slice has provided considerable detail on neuronal electrophysiology and pharmacology in diverse brain regions. Disadvantages of the brain slice result from the removal of some fibers and structures during the slicing. Accordingly, a preliminary step in selecting neurons for study in the brain slice is to determine to what extent the remaining neuronal circuitry is functional.

Here, we propose that the chick tangential nucleus has several advantages for studying the emergence of spike firing pattern and the underlying membrane currents. During slicing, the vestibular ganglion, cervical spinal cord, and cerebellum are removed. However, removing these structures does not affect the intrinsic membrane conductances over the short-term of the recording session (2–4 h). Moreover, some synaptic inputs remain intact in these brain slices (Shao et al., 2004), since they could trigger spontaneous spike activity at H1 in the present study. Finally, the development of firing pattern in chick VNNs occurred rapidly, in about a week during the perinatal period, while the emergence of firing pattern in altricial mammals occurs over 3-to-4 weeks postnatally (e.g. Dutia and Johnston, 1998). Accordingly, since the chick vestibular system develops in a compressed schedule in young animals, it provides major advantages for brain slice studies.

Development of spontaneous spike activity in VNNs *in vivo* and *in vitro*

First described from studies of catfish peripheral nerves (Hoagland, 1932), spontaneous spike activity occurs in many first- and second-order sensory neurons, including vestibular neurons (e.g. Goldberg and Fernandez, 1971; du Lac and Lisberger, 1992; Jones and Jones, 2000). Spontaneous spike activity is essential for VNNs to transmit reliably and accurately incoming vestibular stimuli (du Lac and Lisberger, 1995). From extracellular, single unit recordings performed on second-order vestibular neurons in the intact rat, spontaneous spike activity was irregular and low in frequency during the first few days after birth, but increased in regularity and frequency so that the adult values were recorded by 30 days (Lannou et al., 1979, 1980).

Spontaneous spike activity could result from the spatio-temporal summation of many subthreshold postsynaptic excitatory events, which bring the neuron to threshold, and/or from the neuron's intrinsic membrane properties. So far, studies on the development of spontaneous spike activity have been performed on heterogeneous neuron classes in the MVN using extracellular and intracellular recordings from mouse brain slices (Dutia et al., 1995; Johnston and Dutia, 1996; Dutia and Johnston, 1998). In agreement with the studies performed on the intact animal, the *in vitro* studies demonstrated that the frequency of spontaneous spike firing increased to adult values by the first postnatal month.

In the present study, we showed that during the developmental period from H1 to H5, the proportion of principal cells with a *regular* firing pattern increased from 25% to 50%, and their CVs decreased significantly. Altogether, our results suggest a developmental increase in the number of principal cells with a regular firing pattern, and an increase in their discharge regularity and firing rate. To our knowledge, the relationship between the regularity of spontaneous spike activity in VNNs and activity within the third-order neurons of this pathway has not been explored. However, studies have shown no correlation between the proportion of regular- and irregular-firing primary vestibular fibers innervating a mature VNN and their output firing pattern (Highstein et al., 1987).

Development of spike waveform

At H5, the *spontaneous action potentials* exhibited faster kinetics than at H1. This modification would allow the principal cells to fire spontaneous spikes more frequently, resulting in faster and more precise vestibular reflexes in older animals. At E16, 50% of the principal cells exhibited one fast AHP, while the remaining cells showed a fast AHP followed by a slower one. In hatchlings (H1, H5), 80% of the principal cells showed the fast and slow AHPs. The waveform transformation may result from the emergence of a calcium-dependent potassium channel, SK, which could be regulated developmentally (see Klocker et al., 2001). However, it is unclear whether the principal cells represent a single cell population undergoing developmental change from one to two AHPs, or two separate cell populations changing in proportions. Although the slow AHP is not characterized as voltage-dependent (Hille, 2001), our results suggested a depolarization-dependence of the AHP, perhaps due to the activation of voltage-dependent calcium and/or sodium currents during depolarization (Llinas, 1988).

Synaptic transmission-dependent spontaneous spike activity at H1

At H1, 50% of the principal cells could fire spontaneous spikes, which in most cells (6 of 8) were synaptically-driven. The synaptic influence was apparent by the presence of an EPSP preceding the action potential (Fig. 2C). Accordingly, the synaptically-driven, spontaneous spike activity at H1 appeared to be an intermediate stage in the development of the mature form of spontaneous spike activity. The intermediate stage was preceded by E16, when spontaneous spike activity was lacking, and postdated by H5, when spontaneous spike activity was intrinsically-driven.

Coincident with the emergence of spontaneous spike activity at H1, spontaneous *postsynaptic* currents exhibited significant changes in the principal cells studied in brain slices (Shao et al., 2003,2004). Compared with E16, glutamatergic, excitatory postsynaptic currents (EPSCs) exhibited higher frequencies and faster kinetics at H1. Although GABAergic and glycinergic events were found at both ages, the *proportion* of IPSCs switched from primarily GABAergic at E16 to primarily glycinergic at H1. This developmental shift in types of inhibitory events may produce decreased shunting, since glycinergic IPSCs exhibit faster kinetics than GABAergic events (Shao et al., 2004). Altogether, the combined increase in glutamatergic EPSCs and glycinergic IPSCs at H1 could contribute to the appearance of synaptic transmission-*dependent*, spontaneous spike firing at this age.

Intrinsic conductances underlying spontaneous spike activity at H5

By H5, spontaneous spike activity was transformed into a synaptic transmission-*independent* event, since the activity was insensitive to a cocktail of excitatory and inhibitory neurotransmitter blocking agents. In other preparations, intrinsically-mediated, spontaneous spike firing was generated by specific currents, including I_{NaP} (e.g. Serafin et al., 1991; Bevan and Wilson, 1999), calcium-dependent potassium current (I_{KCa}) (e.g. Raman et al., 2000), and I_h (e.g. Bennett et al., 2000). In the present study, TTX abolished spontaneous spike firing by blocking at least two different sodium channels, I_{NaP} and the fast sodium channel. In the principal cells, I_{NaP} activated between -65 and -50 mV, so this conductance could be responsible for driving the membrane potential closer to the firing threshold, where the fast sodium channel, activating around -40 mV, could contribute to the depolarizing phase of the action potential in the principal cells (Gamkrelidze et al., 1998).

Studies performed on neurons in the rodent and avian MVN have shown that spontaneous spike activity may be modulated by several potassium channels, including the calcium-dependent BK and SK channels, TEA-sensitive delayed-rectifier potassium current (I_K), and 4-AP-sensitive transient potassium current (I_A) channels (Johnston et al., 1994; du Lac, 1996; Nelson et al., 2003). In our preparation, the SK channel was found to underlie the second, slow AHP in both regularly- and irregularly-firing principal cells at H5. Characteristically, the slow AHP may prevent the cell from reaching firing threshold (Hille, 2001). After blocking the SK channel, the principal cells exhibited depolarized membrane potentials, and higher firing frequencies of spontaneous and evoked action potentials. Unlike other systems (Dutia and Johnston, 1998; Bevan and Wilson, 1999), the principal cells also showed increased regularity.

CsCl blocked I_h without changing the frequency of firing action potentials in the principal cells. I_h could help the principal cell to maintain the RMP, despite strong hyperpolarizing drive from inhibitory inputs. Thus, based on our experiments, both I_{NaP} and SK, but not I_h , are crucial to regulate the spontaneous spike firing in the principal cells at H5.

Emergence of evoked spike firing in VNNs and development of VOR

Our results showed that most principal cells generated a single spike on depolarization at E16, while most of the hatchling principal cells fired repetitively without accommodation in response to long-lasting depolarizing pulses. In addition, the gain in the principal cells firing multiple spikes on depolarization increased considerably at H5 compared with H1. This suggests that older principal cells are capable of more efficient signal transmission, especially in response to sustained stimuli, resulting in more complete compensation of eye movements.

The establishment and maintenance of synaptic connections and/or functional activity in central sensory pathways have been shown to depend critically on the influence of natural sensory stimuli (e.g. visual system: Hubel and Wiesel, 1970; somatosensory system: Killackey et al., 1995). By way of the first-order, vestibular ganglion cells, the VNNs receive natural

sensory stimuli from the hair cells of the three semicircular canals, the utricle and saccule. From studies performed on the developing rodent hair cells, different types of voltage-dependent ionic currents emerged during the first postnatal week (Rusch et al., 1998), at the time when the sensitivity of the vestibular ganglion cells increased to angular acceleration (Curthoys, 1982). Finally, although the types of ionic currents present in hair cells of the hatchling chick semicircular canals were similar to those recorded in the mature chicken, their kinetics, voltage-dependence and pharmacology continued to mature in older animals (Masetto et al., 2000,2003).

While the effects of angular acceleration on the development of the vestibular system can be studied using a turntable (Lannou, 1979;Curthoys, 1982), there is no simple way to investigate the effects of gravity on the otolithic components of the vestibular system. Since gravitational stimulation cannot be readily removed on Earth, space-flight offers a unique opportunity to study the role of gravity on the emergence of spontaneous and evoked spike firing in VNNs.

Role of dendrotoxin-sensitive potassium current (I_{DS}) in evoked spike firing

At E16, several potassium currents have been recorded in the principal cells, including a I_K , I_A , I_{KCa} , and I_{DS} (Gamkrelidze et al., 1998; Peusner et al., 1998). However, at E16, only I_{DS} was involved in changing the depolarizing pulse-evoked firing pattern in the principal cells. On exposure to the snake venom, dendrotoxin (DTX), I_{DS} was abolished, and the single-spike firing principal cells were converted into those capable of firing multiple spikes on depolarization. By H1, I_{DS} was developmentally down-regulated, since the current represented 45% of the total outward potassium currents in the principal cells at E16, but only 20% at H1 (Gamkrelidze et al., 2000). These results support our present finding that the vast majority of the principal cells convert to firing multiple spikes on depolarization at H1.

What are the mammalian neuron counterparts of the chick tangential principal cells?

According to synaptic terminal contacts, the principal cells of the chick tangential nucleus most closely resemble neurons of Deiter's nucleus of the mammalian lateral vestibular nucleus. Indeed, the primary vestibular fibers form large, axosomatic spoon terminals on the principal cells, which resemble the large axosomatic terminals formed on Deiter's neurons in the rat (Sotelo and Palay, 1971).

Until now, most studies on the central vestibular system have been performed on the MVN (Darlington et al., 1995). In the chicken, the laterally-situated tangential nucleus is regionally separate and distinct from the MVN, which borders the fourth ventricle (Wold, 1975,1976;Cox and Peusner, 1990b). Unlike the tangential principal cells, the chick MVN neurons receive input only from the small- and medium-diameter primary vestibular fibers (Cox and Peusner, 1990a). In the few studies in which spontaneous spike activity was compared in the MVN and the lateral VNNs in the same species (rat: Sun et al., 2002; guinea-pig: Uno et al., 2003), it was found that: (1) more MVN neurons showed spontaneous spike activity; (2) MVN neurons exhibited more regular firing patterns; (3) MVN neurons exhibited higher firing frequencies. In brain slice preparations from the hatchling chick MVN of H1-12, almost all of the neurons fired spontaneously in a very regular pattern ($CV < 0.05$), with a mean frequency of 20 Hz (du Lac and Lisberger, 1995). In our experiments performed on the chick tangential nucleus, about 80% of principal cell could fire spontaneous spikes at H5, with a mean frequency of 20 Hz, including 50% with a regular pattern and mean CV of 0.18, and 30% with an irregular firing pattern and mean CV of 2.5. Thus, the hatchling tangential principal cells are less likely to show regular, spontaneous spike activity, characteristic of hatchling MVN neurons.

Does the establishment of stimulus-evoked and spontaneous firing patterns coincide with vestibular synaptogenesis, the appearance of vestibular postsynaptic responses, or the appearance of vestibular reflex activity?

The first sign of vestibular spoon ending formation occurs at E8-9, when immature chemical synapses appeared at the large vestibular fibers apposed to the principal cell bodies (Peusner, 1981). However, the spoon endings first emerged at E10, and by E16, the spoon endings formed both chemical synapses and gap junctions (mixed synapses) on the principal cell bodies (Peusner, 1984). On vestibular-nerve stimulation, about half of the principal cells generated postsynaptic currents at E10, while most of them fired action potentials at E13 and E16 (Peusner and Giaume, 1994; Peusner et al., 2005). Repetitive firing on depolarization was lacking in the principal cells at E10 and E13, and first appeared in some cells at E16 (Peusner et al., 2005). Accordingly, the gradual transition from single to repetitive spike firing on depolarization began around E16, coincident with the appearance of mixed synapses at the spoon terminals (Peusner and Giaume, 1994).

The chick can stand and initiate vestibular-controlled head movements necessary to its survival within hours of birth (Decker, 1970). In newborn hatchlings, the VOR gain was < 0.1 , but increased to 0.55 in 4-to-6 week old chickens (Wallman et al., 1982). Indeed, after only 2 h of testing, inexperienced hatchling chicks adjusted their VOR gain. In this study, spontaneous spike firing first appeared at birth, coincident with the onset of standing, feeding and drinking movements.

Altogether, there are three major steps for the tangential principal cells to establish their mature firing pattern necessary for vestibular reflex activity. First, repetitive spike firing on depolarization begins to emerge in the late-term embryo (E16), when spoon terminals develop into sites containing mixed synapses. Second, spontaneous spike activity, which depends on synaptic transmission, appeared at birth (H1), when terrestrial movements controlled by vestibular reflexes begin to function. Third, spontaneous spike firing is driven by intrinsic membrane properties at H5, when robust vestibular-mediated movements, such as perching, are acquired. (Shimazu, 1965).

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Abbreviations

ACSF	artificial cerebrospinal fluid
AHP	afterhyperpolarization
AP-5	DL-2-amino-5-phosphovaleric acid
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CV	the coefficient of variation
E	embryonic day
EPSC	excitatory postsynaptic current

H	hatching day
I_A	transient potassium current
I_{DS}	dendrotoxin-sensitive potassium current
I_h	hyperpolarization-activated cationic current
I_k	delayed-rectifier potassium current
I_{KCa}	calcium-dependent potassium current
I_{NaP}	persistent sodium current
ISI	interspike interval
KMeSO₄	potassium methylsulfate
MVN	medial vestibular nucleus
OKR	optokinetic reflex
P	postnatal day
RMP	resting membrane potential
SK	small conductance, calcium-dependent potassium current
TTX	tetrodotoxin
VCR	vestibulocollic reflex
VNN	vestibular nucleus neuron
VOR	vestibuloocular reflex

Fig 1.

(A) At E16, there was no spontaneous spike activity found in any of the recorded principal cells. (B) At H1 and H5, principal cells with spontaneous spike activity exhibited two patterns of firing action potentials, regular and irregular, which differed in their CVs. (C) For most of the principal cells, there was a negative correlation between spontaneous spike firing frequency and CV. (D) There was no correlation between RMP and spontaneous spike firing frequency.

Fig 2.

Spontaneous spike firing in principal cells at H1. (A) A principal cell with irregular, spontaneous-spike firing. Note that the principal cell exhibits two kinds of action potentials (see C, D). * Indicates action potentials arising from EPSPs (amplified in C). (B) Coapplication of synaptic transmission antagonists (bicuculline, strychnine, CNQX, and DL-APV) blocked completely the spontaneous spike firing. (C, D) Four, superimposed, truncated action potentials with (C) and without EPSPs (D).

Fig 3. Spontaneous spike firing in principal cells at H5. (A) The frequency of spontaneous spike firing was comparable for individual principal cells in the cell-attached (A₁) and whole-cell mode (A₂). On exposure to synaptic transmission antagonists, the spontaneous spike firing did not decrease (A₃). The recordings in A₁–A₃ were taken from the same cell. (B) Normalized frequency from regular, spontaneous-spike firing principal cells at RMP. The normalized firing rate was calculated as the ratio of the frequency for each 10 s interval compared with the total time of 120 s. (C) The spontaneous spike waveforms generated at H1 (thin trace) and H5 (thick trace) differ in rise time, decay time, and width of the action potential.

Fig 4.

The responses of principal cells to depolarizing current pulses of 400 ms duration and 0.1, 0.3, 0.5 and 0.7 nA intensities at different developmental ages. (A, B) At E16, most principal cells fired a single spike (A), while some fired multiple spikes on depolarization (B). (C) At H1, most principal cells fired repetitively. (D) At H5, all of the principal cells fired repetitively. Note that at RMP the principal cell in C exhibited no spontaneous spike activity, but the cell in D showed regular, spontaneous spike activity.

Fig 5. (A) Change in the mean evoked firing rate of principal cells plotted against depolarizing current pulses at different developmental ages. The gain (m) was 38 spikes/s/nA at E16, 204 spikes/s/nA at H1 and 313 spikes/s/nA at H5. (B) In response to depolarizing pulses, principal cells at E16 exhibited either one (B₁) or two AHPs (thin trace) (arrow) (B₂). On further depolarization, the principal cell in B₂ showed only a fast AHP (thick trace). (C) The spontaneous spikes generated from an H5 principal cell exhibited both fast and slow AHPs (arrow). However, on depolarization, only the fast AHP was seen.

Fig 6.

I_{NaP} in spontaneous-spike firing principal cells at H5. (A) In a spontaneously-firing principal cell (A_1), 1 μ M TTX blocked the activity (A_2). (B) In voltage-clamp, I_{NaP} , a persistent inward current, was induced by using an 800 ms voltage step from a holding potential of -66 mV to -51 mV (B_1) and was abolished by 1 μ M TTX (B_2). A and B were recorded from the same principal cell. (C) I/V curve obtained by measuring I_{NaP} 100 ms from the beginning of the voltage step. In control solution, there is a negative slope conductance from -65 to -50 mV, which is TTX-sensitive. (D) Average I_{NaP} obtained by subtracting the TTX-sensitive currents from the control currents in spontaneous-spike firing principal cells ($n = 4$). V_{cmd} , the command voltage, or voltage step, applied to the cell membrane in voltage-clamp recordings.

Fig 7.

Ih in spontaneous-spike firing principal cells at H5. (A) A sag was induced by a hyperpolarizing current pulse of 400 ms duration and 0.5 nA intensity (A₁), which was sensitive to CsCl, indicating Ih was present (A₂). (B) Ih was induced by applying an 800 ms voltage step from -60 to -80 mV (B₁) which was abolished by 3 mM CsCl (B₂). (C) As compared with the control (C₁), no change was observed in the spontaneous-spike firing frequency of the cell in the presence of 3 mM CsCl (C₂). These findings indicate that Ih was not involved in setting the rate of firing in the spontaneous-spike firing principal cells at H5. A, B and C are recorded from the same principal cell.

Fig 8. SK in principal cells at H5. (A) On exposure to apamin, a spontaneous-spike firing principal cell was transformed from irregular (A₁) (CV = 0.61) to regular (A₂) (CV = 0.32) firing at higher frequency. (B) In the same principal cell, apamin abolished the second, slower AHP. (C) In voltage-clamp, apamin abolished the outward current in the principal cell. Each trace is the average of six responses. (D) As compared with the control (D₁), the principal cells increased their firing rates in response to depolarizing current pulses after apamin exposure (D₂). Note the spontaneous spike activity in this principal cell. (E) On exposure to apamin, the principal cells showed increased firing gains. (F) Apamin did not change significantly the CVs for firing on depolarization ($n = 6$).

Table 1

Evoked spike frequency using different intensity depolarizing current pulses at E16, H1 and H5

Depolarization current (nA)400 ms duration pulse	E16	H1	H5
	Values, spikes/s (mean $\pm$ SEM)		
0.1	8 $\pm$ 1.4	32 $\pm$ 9	57 $\pm$ 6
0.3	13 $\pm$ 2	62 $\pm$ 10	122 $\pm$ 17
0.5	23 $\pm$ 6	103 $\pm$ 9	180 $\pm$ 21
0.7	30 $\pm$ 7	154 $\pm$ 17.4	245 $\pm$ 24
n	4	13	19

Table 2

Properties of spontaneous action potentials

	H1	H5
Firing threshold (mV)	-42.9 ± 2.7	-41.4 ± 2.4
Spike amplitude (mV)	56.4 ± 2.8	57.9 ± 4.2
Rise time (ms)	0.77 ± 0.1	$0.39 \pm 0.02^*$
Decay time (ms)	0.53 ± 0.1	$0.36 \pm 0.02^*$
Width (ms)	0.63 ± 0.1	$0.4 \pm 0.03^*$
AHP #1 amplitude (mV)	33.4 ± 4	36.1 ± 1.6
AHP #2 amplitude (mV)	18 ± 0.1 (n = 7)	19.9 ± 1.8 (n = 12)
n	8	16

All kinetics are measured at resting membrane potential. Values are mean $\pm$ SEM.

* Statistically different, Student's *t*-test ($p < 0.05$). AHP, after hyperpolarization.