Presynaptic Na\(^+\) Channels: Locus, Development, and Recovery from Inactivation at a High-Fidelity Synapse

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Na\(^+\) channel recovery from inactivation limits the maximal rate of neuronal firing. However, the properties of presynaptic Na\(^+\) channels are not well established because of the small size of most CNS boutons. Here we study the Na\(^+\) currents of the rat calyx of Held terminal and compare them with those of postsynaptic cells. We find that presynaptic Na\(^+\) currents recover from inactivation with a fast, single-exponential time constant (24°C, \(\tau\) of 1.4–1.8 ms; 35°C, \(\tau\) of 0.5 ms), and their inactivation rate accelerates twofold during development, which may contribute to the shortening of the action potential as the terminal matures. In contrast, recordings from postsynaptic cells in brainstem slices, and acutely dissociated, reveal that their Na\(^+\) currents recover from inactivation with a double-exponential time course (\(\tau_{\text{fast}}\) of 1.2–1.6 ms; \(\tau_{\text{slow}}\) of 80–125 ms; 24°C). Surprisingly, confocal immunofluorescence revealed that Na\(^+\) channels are mostly absent from the calyx terminal but are instead highly concentrated in an unusually long (≈20–40 μm) unmyelinated axonal heminode. Outside-out patch recordings confirmed this segregation. Expression of Na\(_{\text{v}}\)1.6 α-subunit increased during development, whereas the Na\(_{\text{v}}\)1.2 α-subunit was not present. Serial EM reconstructions also revealed a long pre-calyx heminode, and biophysical modeling showed that exclusion of Na\(^+\) channels from the calyx terminal produces an action potential waveform with a shorter half-width. We propose that the high density and polarized locus of Na\(^+\) channels on a long heminode are critical design features that allow the mature calyx of Held terminal to fire reliably at frequencies near 1 kHz.

**Key words:** action potential; auditory; sodium; calyx of Held; high-frequency firing; heminode; nodes of Ranvier; presynaptic terminal

**Introduction**

Auditory brainstem neurons can fire action potentials (APs) at high frequencies and with remarkable precision, an ability thought to be crucial for the processing of sound signals (Oertel, 1999; Carr et al., 2001). The principal cells of the medial nucleus of the trapezoid body (MNTB) can follow afferent fiber stimulation rates of 200–300 Hz in postnatal day 9 (P9) rats (Borst et al., 1995), and some P14 principal cells can follow 800 Hz (Taschenberger and von Gersdorff, 2000). In mature mice, the calyx of Held nerve terminal can fire short AP trains at 1 kHz without failure (Wu and Kelly, 1993). Moreover, in cats, the afferent fibers of the calyces of Held can fire sound-triggered APs *in vivo* at rates reaching 0.5–0.8 kHz (Guinan and Li, 1990; Spirou et al., 1990; Smith et al., 1998b). To maintain discharge rates at such high frequencies, a fast recovery from Na\(^+\) channel inactivation is required (Kuo and Bean, 1994; Torkkeli and French, 2002). The biophysical properties, localization, and molecular identity of the Na\(^+\) channels on the calyx of Held synapse are, however, unknown.

Prolonged high-frequency firing tends to broaden APs and thus degrades their precise timing (Jackson et al., 1991; Geiger and Jonas, 2000). How does the calyx terminal avoid significant broadening of APs during high-frequency firing? What mechanisms are used to minimize Na\(^+\) current recovery time? Moreover, high-frequency firing may lead to a large and simultaneous increase in free intraterminal [Na\(^+\)]\(_i\), and [Ca\(^{2+}\)]\(_i\), which would then render the Na\(^+\)/Ca\(^{2+}\) exchanger ineffective in quickly extruding [Ca\(^{2+}\)]\(_i\) (Reuter and Porzig, 1995). How does the calyx terminal circumvent this problem, which could lead to desynchronized transmitter release and perhaps to toxic increases in [Ca\(^{2+}\)]\(_i\) via a reversal of the exchanger? In brief, what biophysical strategies and morphological specializations (Rowland et al., 2000) allow the calyceal axon and nerve terminal to fire reliably at the prodigious rates that have been observed *in vivo*?

Here we report that the rate of recovery from inactivation of calyx Na\(^+\) currents accelerates during early postnatal develop-
ment, in parallel with an increasing ability to cope with high-frequency firing. Immunocytochemical labeling revealed a steep gradient in $\text{Na}^+$ channel expression: strong staining on the pre-calyx axonal heminode and little to no staining on the calyx terminal. The expression of the node of Ranvier $\text{Na}_1.6$ subunit was found to increase during development, in accordance with an observed increase in axonal excitability with age. Biophysical modeling showed that exclusion of $\text{Na}^+$ channels from the calyx produces an AP with shorter half-width, making it more suitable to transmit precise timing information. We suggest that the high density of $\text{Na}^+$ channels on axonal heminodes allows for rapid extrusion of $[\text{Ca}^{2+}]_i$ (via $\text{Na}^+$/Ca$^{2+}$ exchangers) at the calyx during bouts of prolonged high-frequency firing.

**Materials and Methods**

*Brainstem slice preparation.* Transverse (200 $\mu$m thick) brainstem slices were obtained from P5–P18 Sprague Dawley rats. After rapid decapitation, the brainstem was immersed in ice-cold low-calcium artificial CSF (aCSF) containing the following (in mM): 125 $\text{NaCl}$, 2.5 $K\text{Cl}$, 3.0 $\text{MgCl}_2$, 0.1 $\text{CaCl}_2$, 25 glucose, 25 $\text{NaHCO}_3$, 1.25 $\text{NaH}_{2} \text{PO}_4$, 0.4 acetic acid, 3 myo-inositol, and 2 $\text{Na}-\text{pyruvate}$, pH 7.3 when bubbled with carbogen (95% $\text{O}_2$, 5% $\text{CO}_2$). After cutting in a Leica (Nussloch, Germany) VT vibratome, the slices were transferred to an incubation chamber containing normal aCSF bubbled with carbogen and maintained at 37°C for 45 min and thereafter at room temperature (RT). The normal aCSF was the same as the low-calcium aCSF, except that 1.0 mM $\text{MgCl}_2$ and 2.0 mM $\text{CaCl}_2$ were used.

Electrophysiology. Whole-cell patch-clamp recordings were performed in normal aCSF containing 200 $\mu$m $\text{CdCl}_2$ and 10 mM tetraethylammonium (TEA)-Cl. Currents were recorded at room temperature (22–24°C) except in some experiments shown in Figure 1 B, in which the bath temperature was increased to 34–36°C (average of 35°C) with a Warner Instruments (Hamden, CT) TC-324B automatic temperature controller. The internal solution consisted of the following (in mM): 90 $\text{Cs}$-methanesulfonate, 20 $\text{CsCl}$, 5 $\text{MgCl}_2$, 5 $\text{Na}_2$-phosphocreatine, 40 HEPES, 10 TEA-Cl, 0.2 EGTA, 2 $\text{ATP}$-Mg, and 0.2 GTP, pH 7.3 with CsOH (310 mOsm). Calyx terminals were identified visually by differential interference contrast (DIC) optics and sometimes by fluorescence of Lucifer yellow (0.25 mg/ml) added to the patch-pipette solution or by the presence contrast (DIC) optics and sometimes by fluorescence of Lucifer yellow (0.25 mg/ml) added to the patch-pipette solution or by the presence of prolonged high-frequency firing. Immunocytochemical labeling revealed a steep density of $\text{Na}^+$ channels in parallel with an increasing ability to cope with high-frequency firing. We suggest that the high density of $\text{Na}^+$ channels on axonal heminodes allows for rapid extrusion of $[\text{Ca}^{2+}]_i$ (via $\text{Na}^+$/Ca$^{2+}$ exchangers) at the calyx during bouts of prolonged high-frequency firing.

Recovery from inactivation. A standard double-pulse protocol was used to measure recovery of Na currents from inactivation (Hille, 2001). The calyx and principal cell of the MNTB were pre-stepped to $-100$ mV for 50–100 ms to remove Na channel inactivation, stepped to the test potential ($-30$ mV except when otherwise stated) for 5 or 10 ms to measure the maximum Na current, stepped to the interpulse potential ($-100$ mV except when otherwise stated) for variable periods to allow recovery from inactivation to proceed, and finally stepped to the test potential to measure the fractional recovery from inactivation that occurred during the interpulse interval. Currents shown are normalized to the current available after the pre-step to $-100$ mV, even when the interpulse potential was different from $-100$ mV. When measuring recovery from inactivation at $-80$ mV and especially $-60$ mV, there was significant steady-state inactivation at the interpulse potential, and thus the currents did not recover to 100%. A single-exponential function was fit to the recovery from inactivation of the presynaptic Na current. The time constants obtained from these fits give a first estimate of the time course of recovery of the underlying Na conductance.

Series resistance voltage errors could also distort the measured rates of recovery from inactivation if the open probability of the Na channels was not saturated during the test depolarizations. We tested this possibility by measuring recovery from inactivation in the same cell using test depolarizations to $-30$ mV (the apparent peak of the $V-I$ curve) or $-10$ mV. Under these conditions, the measured rates of recovery of the currents were not different (1.76 ± 0.07 vs 1.77 ± 0.13 ms; P6; P8; n = 4).

Preparation of dissociated cells. Isolated neurons from rat MNTB (P8–P10) were freshly dissociated using enzyme treatment and trituration in a procedure modified from Raman and Bean (1997) and Kanold and Manis (1999). Briefly, 300-$\mu$m-thick transverse brainstem slices were cut in ice-cold low-calcium aCSF and incubated in MEM (buffered with 20 mM HEPES) containing 40 U/ml papain ( Worthington, Freehold, NJ), 1 mM cysteine, and 0.5 mM EGTA (31°C, 20 min). After the digestion was blocked by adding trypsin inhibitor (1 mg/ml), the MNTB was micro-dissected out under a dissecting scope, transferred to aCSF buffered with 10 mM HEPES, and triturated with a fire-polished Pasteur pipette. Then the suspension of cells was transferred to the recording chamber, and the cells were allowed to settle for 30 min. The chamber was perfused with

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normal aCSF with 10 mM TEA-Cl and 200 μM CdCl₂ included to block K⁺ and Ca²⁺ current. In voltage-clamp recordings the holding membrane potential was −70 mV. The series resistance in whole-cell recordings was 6.8 ± 1.5 MΩ (n = 11) and was compensated by 90% (100 μs lag). The resting capacitance was 16.4 ± 3.4 pF (n = 11). The pipette solution was the same as for slice recordings of postsynaptic Na⁺ currents.

**Afferent fiber stimulation thresholds.** A bipolar tungsten stimulation electrode (parallel 80 μm wires, 200 μm apart; Frederick Haer Company, Bowdoinham, ME) was placed across the midline fiber tract, and voltage stimulation was delivered (100 μs duration, 0.1 Hz) using an IsoFlex stimulator driven by a Master 8 pulse generator (A.M.P.L., Jerusalem, Israel). Patch pipettes with resistances of 2–3 MΩ filled with recording saline were pressed against MNTB principal cells to form loose seals of 8–12 MΩ. The fiber tract was then stimulated at 20–25 V at 0.1 Hz, and cells with intact calyx inputs were identified by the presence of extracellula APs with appropriate kinetics and timing after the stimulation artifact. The same bipolar stimulation electrode was used for all of these recordings. The AP threshold was determined by decreasing stimulation voltage and monitoring the extracellula APs until failure occurred. The closest value of voltage above threshold within the range of 0.2–0.9 V was reported as the threshold. Thus, the threshold was determined with a resolution of <0.9 V. The extracellular AP size was invariant above threshold to increases in stimulation voltage, indicating that a unitary AP was being evoked (Bergsman et al., 2004). Sample sweeps were filtered at 3 kHz.

**Computational modeling.** The calyx reconstruction used in modeling studies was taken from an adult cat. Globular bushy cells in the calyx express the putative calcium binding protein PEP-19 (peptide protein 19) in all cellular compartments, and MNTB cells do not express this protein. The same bipolar stimulation electrode was used for all of these recordings. The AP threshold was determined by decreasing stimulation voltage and monitoring the extracellula APs until failure occurred. The closest value of voltage above threshold within the range of 0.2–0.9 V was reported as the threshold. Thus, the threshold was determined with a resolution of <0.9 V. The extracellular AP size was invariant above threshold to increases in stimulation voltage, indicating that a unitary AP was being evoked (Bergsman et al., 2004). Sample sweeps were filtered at 3 kHz.

**Immunocytochemistry.** Animals were injected with an overdose of Nembutal (50 mg/kg, i.p.). Rats were perfused transcardially with 40 ml of 0.1 M PB, pH 7.4, followed by 60 ml of 4% paraformaldehyde in 0.1 M PB. Brains were removed, cryoprotected in a 30% sucrose solution, and immersed in a series of alcohols, defatted, and coverslipped with Permount (Fisher Scientific, Houston, TX) in photobleaching-protective medium (Vectashield; Vector Laboratories, Burlingame, CA). Digital photomicrographs were acquired at a series of focal planes with a Nikon (Tokyo, Japan) PM–2000 laser scanning confocal microscope. Plates were assembled using Adobe Photoshop software (Adobe Systems, San Jose, CA).
approximately P8–P10 calyx terminal has a resting membrane potential of approximately −80 mV (Borst et al., 1995), and this very negative resting membrane potential likely aids high-frequency firing by maintaining the recovery period. In recordings from 22 calyces from P6–P14 rats, Na currents recovered almost completely (98.2 ± 0.6%) within 7.5 ms, after a single-exponential time course with \( \tau \) of 1.44 ± 0.08 ms at RT (\( \tau \) of 0.98 ± 0.06 ms in the uncorrected raw data (see Materials and Methods); \( V_{m} \) of −100 mV) (Fig. 1B). In three cells recorded at a more physiological temperature (35°C), the recovery was complete (99 ± 0.11%) at 1.5 ms and had a single-exponential time course (\( \tau \) of 0.50 ± 0.06 ms) (Fig. 1B). Na currents recorded from the calyx of Held were blocked by tetrodotoxin (3 μM; data not shown).

The kinetics of recovery from inactivation depended on the membrane potential during the recovery period (Fig. 1C). Time constants for recovery from inactivation were faster at hyperpolarized potentials (\( \tau \) of 0.68 ms at −120 mV; \( \tau \) of 2.4 ms at −80 mV; and \( \tau \) of 9.4 ms at −60 mV). The steady-state inactivation curve of the presynaptic current was well fitted by a Boltzmann function (\( V_{1/2} \) of −62 ± 1 mV; slope factor of 7.8 ± 1 mV). The P8–P10 calyx terminal has a resting membrane potential of approximately −80 mV (Borst et al., 1995), and this very negative resting membrane potential likely aids high-frequency firing by maintaining the recovery period. In recordings with a particularly low series resistance, we could measure calyx Na currents with acceptable voltage control as assessed by the gradual activation of the current–voltage relationship (Fig. 1B, inset, P10 rat). In this case, recovery from inactivation occurred with a \( \tau \) of 1.13 ms, similar to that obtained in the other calyx recordings. This observation supports the notion that recovery from inactivation is less sensitive to the voltage-clamp control.

In an attempt to study kinetic properties of voltage-gated Na channels under improved voltage-clamp conditions, we pulled outside-out patches from calyx terminals. Surprisingly, we observed that most (70%; \( n = 24 \) patches) of the calyx outside-out patches did not have Na currents, although they all had voltage-dependent K currents (Fig. 2A). Patches without Na currents were recorded at RT (22–24°C; ○) or at 35°C (●). The lines represent a single-exponential fit with time constant \( \tau \). Left inset, An example of pairs of Na currents elicited as in A from a P10 calyx. The \( I–V \) relationship from the same calyx is shown in the top right inset and, in the bottom right inset, a plot of the fraction of Na current that recovered from inactivation at −100 mV. The data were fit to a single-exponential function with \( \tau \) of 1.13 ms. C, Voltage dependence of the recovery from the inactivation. The fraction of Na current recovered is measured, using the same protocol as in A, but, after the first test pulse, various time intervals (from 0.1 to 7.5 ms) at different voltages (−80, −100, and −120 mV; \( n = 6 \) terminals from P10–P12) are given before the second test pulse. The lines are fits to single-exponential functions with time constants \( \tau \) shown in the labels. The fraction of Na current recovered increased with the amount of hyperpolarization.

Results

Fast recovery from inactivation of presynaptic Na\(^{+}\) currents

Na currents recorded in the calyx of Held activated and inactivated almost completely within −3 ms (Fig. 1). Although an analysis of the activation properties of this current may be impaired by the limited space- and voltage-clamp control, measurement of the inactivation and recovery of Na currents should be less compromised. A standard double-pulse protocol was used to study recovery from inactivation whereby two test pulses to −30 mV were separated by a variable recovery period, as can be seen in Figure 1A. In recordings from 22 calyces from P6–P14 rats, Na currents recovered almost completely (98.2 ± 0.6%) within 7.5 ms, after a single-exponential time course with \( \tau \) of 1.44 ± 0.08 ms at RT (\( \tau \) of 0.98 ± 0.06 ms in the uncorrected raw data (see Materials and Methods); \( V_{m} \) of −100 mV) (Fig. 1B). In three cells recorded at a more physiological temperature (35°C), the recovery was complete (99 ± 0.11%) at 1.5 ms and had a single-exponential time course (\( \tau \) of 0.50 ± 0.06 ms) (Fig. 1B). Na currents recorded from the calyx of Held were blocked by tetrodotoxin (3 μM; data not shown).

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contained exclusively high-voltage threshold K⁺ currents that activated at membrane voltages more than −30 mV. In contrast, the K⁺ current present in patches with Na current activated at more negative potentials, indicating the presence of low-voltage-activated K currents. Previous studies have shown that high-voltage threshold Kv3-type channels are exclusively located to the calyx terminal, whereas low-voltage threshold K⁺ channels of the Kv1.2-type are located exclusively in the axonal heminode (Doddson et al., 2003; Ishikawa et al., 2003). Thus, the observed correlation of Na⁺ current with low-threshold K⁺ current and not high-threshold current suggests that presynaptic Na⁺ channels are mostly excluded from the calyx terminal but are present on the axonal heminode. The presence of K⁺ currents in patches without Na⁺ currents also indicates that they were not attributable to vesicles formed in pipette tips by rescaling of the inner membrane. Interestingly, 37% of the outside-out patches excised from pituitary “beads-on-a-string” nerve terminals also have no Na currents, suggesting that Na channels may be highly clustered in these terminals (Jackson and Zhang, 1995).

Some outside-out patches (30%) displayed sizeable Na⁺ currents (260 ± 107 pA; n = 7) (Fig. 2B). In these patches, the Na⁺ current I–V relationship indicated good voltage control (data not shown). Recovery from inactivation in outside-out patches occurred with an average time constant of τ of 1.8 ± 0.2 ms (Vh of −100 mV; 24°C; n = 7) (Fig. 2C). This value was slightly larger than what we observed in whole-cell calyx recordings (τ of 1.34 ms) after series resistance correction of τ. However, we also note that this larger time constant might be attributable to a disruption of the Na channel phosphorylation after patch excision (Johnston et al., 1999; Cantrell and Catterall, 2001). In addition, we note that patch excision has also been shown to change Na current activation and inactivation properties compared with whole-cell recordings (Jackson and Zhang, 1995; Shcherbatko et al., 1999). By comparison, recovery from inactivation in outside-out patches from mossy fiber boutons was much slower (τ of 3.6 ms at Vh of −120 mV (Engel and Jonas, 2004)) than that recorded here for the calyx of Held.

Presynaptic Na⁺ currents: developmental changes

Presynaptic action potentials acquire a faster upstroke and shorter half-width as the calyx of Held matures (Taschenberger and von Gersdorff, 2000; Joshi and Wang, 2002). This may be attributable, in part, to developmental changes in the Na currents that contribute to the AP waveform. We observed that discharge failures often occurred in immature calyx synapses when theafferent fibers were challenged with high stimulation frequencies (e.g., 300 Hz; average of two to three failures for P5–P7 calyces after 15 stimuli; n = 6) (Fig. 3A), suggesting that the presynaptic Na currents were not optimized for high rates of firing. Note also that the P6 AP train rides on a small, depolarized plateau that is absent in the P13 train. This may be attributable to a lower density of K⁺ channels and/or a slower inactivation rate for Na⁺ channels in immature terminals. To further investigate this hypothesis, we compared the inactivation properties of Na channels recorded from presynaptic terminals at P6–P8 and P10–P14.

We observed that Na⁺ currents recorded in more mature terminals inactivated considerably faster than those in immature terminals (fast time constant of inactivation for P6–P7, τin of 0.63 ± 0.06 ms, n = 6; for P10–P12, τin of 0.34 ± 0.02 ms, n = 11; p < 0.01; 22–24°C) (Fig. 3B), which may contribute to the shorter presynaptic APs in mature calyces. Only Na⁺ currents with relatively small and similar amplitudes (e.g., those during the first steps of recovery from inactivation or in the presence of 200 nM TTX) were compared to exclude a distortion of the current waveform caused by series resistance errors (Zhang and Trussell, 1994). Peak Na⁺ current amplitudes recorded from more mature terminals tended to be larger than those from immature terminals [5.7 ± 2.0 nA for P6–P9 (n = 10) vs 12.2 ± 2.6 nA for

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**Figure 2.** Presynaptic Na⁺ currents in outside-out patches. A, Two examples of calyx outside-out patches: one contained only potassium currents (top; representative of −70% of the patches) and the other with Na⁺ and K⁺ currents (bottom; representative of −30% of the patches). B, Example of recovery from inactivation for an outside-out patch with unusually large Na⁺ current amplitude. The recovery followed a single-exponential function with τ of 2.4 ms (RT; P11 rat). C, Recovery from inactivation from outside-out patches that contained Na⁺ currents. Patches were held for −100 mV for 50 ms before a 5 ms test pulse to −30 mV and kept at −100 mV for varying amounts of time (from 0.5 to 14 ms) before testing the recovery from inactivation with a second −30 mV pulse. Data collected from seven patches that contained isolated Na⁺ currents. Rat slices ranged in age from P8 to P13 (RT is 22–24°C).
Figure 3.  Maturation of presynaptic Na\(^+\) currents. A, Action potential trains recorded in the calyx terminal triggered by afferent fiber stimulation. Examples from a P6 and a P13 rat. Asterisks represent failures in firing of the action potential during a 300 Hz train of 15 stimuli to the fibers in the axon length (cut during slicing) and partly attributable to differences in voltage-clamp quality. Such large currents may be necessary to quickly charge the large calyx membrane clamp during the AP.

Apart from their faster inactivation, Na currents in mature calyces also recovered significantly faster from inactivation (\(\tau\) of 1.34 ± 0.1 ms, \(n = 10\), P10–P14 vs \(\tau\) of 1.93 ± 0.06 ms, \(n = 6\), P6–P8; \(p < 0.05\); measured at a recovery potential of −100 mV) (Fig. 3C). These series resistance corrected values of \(\tau\) (for a given age group) did not correlate with the peak amplitude of the Na currents (which ranged from 1.5 to 15 nA for P6–P8 and from 2 to 25 nA for P10–P14; see above). Recovery from inactivation started after an initial delay (latency) of 0.1–0.9 ms (Fig. 3D). This delay may reflect the time needed for the channel to move back to the closed state (deactivate) to be able to open again (Kuo and Bean, 1994). The length of this latency period depended on the age of the animal. It was significantly shorter in more mature synapses compared with immature ones (0.28 ± 0.07 ms in P10–P14 vs 0.6 ± 0.12 ms in P6–P8) (Fig. 3E).

Developmental changes in axonal excitability

Developmental changes in axonal Na channel properties and density may affect the excitability of the afferent fibers that give rise to the calyx of Held. We thus stimulated the afferent fibers to the MNTB at 0.1–10 Hz to measure extracellular AP currents generated at the calyx of Held synapse during postnatal development with a loose-seal patch pipette (Fig. 4). Extracellular recordings of APs (or action currents) provide a relatively noninvasive examination of AP and synaptic timing with very high temporal resolution (Guinan and Li, 1990; Barnes-Davies and Forsythe, 1995; Borst et al., 1995; Smith et al., 1998b). Both presynaptic and postsynaptic APs can be detected at the MNTB, as evidenced by the fact that decreasing extracellular divalent ions to 0.1 mM Ca\(^{2+}\) and 0 Mg\(^{2+}\) selectively eliminated the postsynaptic waveform and also significantly reduced the presynaptic AP stimulation threshold (from 10.5 V to 9.3 V; \(p = 0.013\); paired \(t\) test; \(n = 4\) cells; see supplemental material, available at www.jneurosci.org), perhaps by relieving divalent screening of
Na\(^+\) channels (Hille et al., 1975). The threshold for AP generation was very sharp, with very small (e.g., 0.2 V) changes in voltage resulting in a shift from no responses (subthreshold) (Fig. 4 A, top gray traces) to fully reliable AP generation (suprathreshold) (Fig. 4 A, black traces). An increase in stimulation voltage did not result in any change to the shape or timing of the AP current, suggesting that we were activating only one afferent input (Bergsman et al., 2004). Shown are sample traces from recordings in young (P5) and mature (P18) rats. Note that the presynaptic and postsynaptic “AP waveforms” are shorter in P18 than in P5. Latencies between peaks of the presynaptic and postsynaptic spikes decreased significantly from P5 to P18 (3.2 ± 0.1 vs 0.92 ± 0.1 ms; \(p = 0.0001\); paired \(t\) test). Postsynaptic timing jitter, measured as the difference in fastest and slowest postsynaptic waveform peaks during a 10 stimuli train at 10 Hz, was also greatly reduced during development (e.g., 1.9 ms in P5 and 225 \(\mu s\) in P18 for Fig. 4 A). These developmental changes are all consistent with previous whole-cell AP recordings (Taschenberger and von Gersdorff, 2000).

AP threshold of MNTB afferent fibers decreased with age from an average of 10–15 V (P5–P7) to <5 V (P16–P18) (Fig. 4 B). A coordinated series of maturational events occurs in the second postnatal week. The onset of myelination occurs at approximately P8–P9 in rat MNTB (E. Friauf, personal communication), Na\(^+\) channels show an increase in inactivation speed by P10 (Fig. 3 B), hearing begins by P12 (Blatchley et al., 1987), and the morphology of the rat calyx becomes similar to that of adults by P14 (Kandler and Friauf, 1993). When stimulation thresholds are grouped according to these developmental milestones (P5–P9, P10–P12, and P14–P18), the mean thresholds of the three groups are significantly different from each other (\(p < 0.0001\); Kruskal-Wallis ANOVA). We suggest that this decrease in AP threshold is attributable to a combination of functional changes that occur during development: improved myelination of the axon, which reduces axonal capacitance, possible changes in axonal diameter, and insertion and clustering of Na\(^+\) channels at the nodes of Ranvier (Salzer, 2003).

**Comparison with postsynaptic Na\(^+\) currents**

Using the same protocols used to study calyx currents, we found that Na\(^+\) currents in the MNTB principal cells show a double-exponential recovery from inactivation with \(\tau_{\text{fast}}\) of 1.23 ± 0.04 ms and \(\tau_{\text{slow}}\) of 125 ± 28 ms (Fig. 5 A, B). The slow time constant did not change during development. However, the fraction of slow recovery from inactivation did decrease during development from 12 ± 1.7% in P5–P6 to 4 ± 1.4% in P8–P12 (Fig. 5 C). A similar developmental change in principal cell Na\(^+\) currents is also seen in the mouse MNTB (Ming and Wang, 2003).

A striking difference between presynaptic and postsynaptic Na\(^+\) currents is the presence of a TTX-sensitive persistent (i.e., non-inactivating) component in principal cells (Fig. 5 D). This current was on average 9.2 ± 1.3% (0.5 ± 0.06 nA) of the peak Na\(^+\) current at \(-30\) mV and was also present during a partial repolarization to +30 mV after more depolarized pulses (>0 mV; \(n = 5\); data not shown). The fraction of the persistent current increased during development from 6.5 ± 0.8% of the peak current (P7–P8) to 12.8 ± 1.9% (P9–P12; \(p < 0.01\); \(n = 5\)). A similar developmental increase in a slowly inactivating Na\(^+\) current component has also been seen in neocortical neurons (Huguenard et al., 1988).

Studies in cultured hippocampal neurons also revealed two components of recovery from inactivation (\(\tau_{\text{fast}}\) of 3.9 ms and \(\tau_{\text{slow}}\) of 938 ms) in whole-cell soma recordings of Na\(^+\) currents and nucleated patches (\(\tau_{\text{fast}}\) of 7.7 ms and \(\tau_{\text{slow}}\) of 923 ms (He et al., 2002)). The Na\(^+\) current recovery from inactivation was thus somewhat slower in nucleated patches, and this suggests that the fast recovery from inactivation may be subject to some corrections for voltage- and space-clamp escape. We attempted nucleated patches but had no success, probably because of the large size of the MNTB principal cell nucleus. However, after whole-cell recording of Na\(^+\) currents, we did obtain outside-out patches from the principal cells. These patches contained small Ca\(^{2+}\) and K\(^+\) currents, but, after blocking these with 200 \(\mu\)M Cd\(^{2+}\) and 10 mM TEA, no Na\(^+\) currents were observed (\(n = 5\) patches), indicating an absence, or very low density, of Na\(^+\) channels in the principal cell soma. This agrees with the lack of strong immuno-
mV induced a transient Na (Banks and Smith, 1992; Forsythe, 1994). Depolarization to example of Na was measured as 15.6 mV and corrected after the recording. An good for these dissociated cells. Here the liquid junction potential displayed in Figure 6 quite similar to the slice recordings (Leao et al., 2000), and separated by different time intervals from 0.5 to 7.5 ms. Note the non-inactivating current. B, Complete recovery from inactivation of the postynaptic Na current after 500 ms. Plot of the fraction of the Na current recovered after various time intervals (1–600 ms). The line is a double-exponential fit to the points (n = 5 for P7–P10 rats). C, Developmental difference in the fraction of slow-recovering Na current between cells from P5–P6 and P8–P12 rats. The fraction recovered at 15 ms was 88 ± 1.7% in P5–P6 (n = 5) and 96 ± 1.4% in P8–P12 (n = 6; p < 0.05). D, An example of a TTX-sensitive Na current recorded from a principal cell that shows a fast-inactivating component and a persistent current.

Labeling for PAN-NaCh (PAN-Na channels) in the postsynaptic principal cell (see Figs. 7B, 9). We suggest that postsynaptic Na channels may be highly clustered near the axon hillock (or farther down the axon initial segment), which was probably missed by our outside-out patches (Colbert and Pan, 2002).

To determine the recovery from inactivation with better voltage-clamp control, we acutely dissociated the principal cells. Figure 6A shows a typical DIC image of a P9 principal cell from the MNTB with a diameter of 20–25 μm and one short dendrite (Sätzler et al., 2002). In current-clamp mode, these neurons fired one AP for small- and large-current amplitude injections (n = 5) (Fig. 6B). This is characteristic of MNTB principal cells (Banks and Smith, 1992; Forsythe, 1994). Depolarization to −30 mV induced a transient Na current with average amplitude of 5.67 ± 1.80 nA (n = 11), which was completely blocked by 5 μM TTX (n = 2) (Fig. 6C). A typical I–V curve of Na current is displayed in Figure 6D, showing that the voltage control was good for these dissociated cells. Here the liquid junction potential was measured as 15.6 mV and corrected after the recording. An example of Na currents elicited by paired depolarizations to −30 mV with different time intervals (0.5–7.5 ms) is shown in Figure 6E. Using this paired-pulse protocol with dissociated neurons (step depolarizing pulses to −30 or 0 mV), we obtained the recovery from inactivation (Fig. 6F). Again, as in slice recordings, a double exponential was necessary to fit the data with τfast of 1.5–1.7 ms and τslow of 80–86 ms. Thus, pulses to −30 or 0 mV give similar values for τfast and τslow, and these values are also quite similar to the slice recordings (τfast of 1.23 and τslow of 125), suggesting that the time constant of recovery from inactivation may not be so sensitive a parameter to less than ideal voltage- and space-clamp control.

Na+ channel locus
As described above, most synaptically induced outside-out patches did not express Na+ currents. This suggested that Na+ channels may be highly clustered on the calyx or there may be a steep gradient in Na+ channel expression with a higher density on the axonal heminode compared with the calyx membrane. We determined the locus of Na+ channels by double labeling the MNTB with antibodies against the Na+ channel α-subunit and the synaptic vesicle protein SV2 (Fig. 7). We first used an antibody that recognizes all known voltage-gated Na channel α-subunits (PAN-NaCh) (Dugandzija-Novakovic et al., 1995). Immunocytochemistry with the PAN-NaCh antibody revealed a dense labeling in MNTB neuronal processes (Fig. 7B) that was abolished by preabsorption of the antibody with the antigenic peptide. Conversely, immunolabeling for SV2 revealed continuous structures surrounding the principal cells (Fig. 7C) (Wu et al., 1999). No overlap was observed when the PAN-NaCh and SV2 staining were merged (Fig. 7D, example arrowhead shows an axon segment, and the arrow shows a putative calyx). Calyceal axons of the MNTB often have a characteristic bend before they form the calyx, as shown in Figure 7A (Lucifer yellow-stained calyx and axon) (Kubawara et al., 1991). This characteristic bend or twist of calyceal axon was sometimes observed with the Na+ channel labeling (Fig. 7F, asterisk), and, in some confoocal sections, the membrane of the thick calyceal axon could be clearly delineated in a longitudinal cross section (Fig. 7F, arrow). PAN-NaCh staining was concentrated in axonal processes that could often be traced back through confoocal sections to a calyx or principal cell (Fig. 7E,F).

We observed intense SV2 staining around principal cells (Fig. 8A) and long bar-like structures with strong Na channel labeling in the MNTB (Fig. 8B), as can be seen also in Figure 7B. These bar-like structures were not found in the fibers passing through the trapezoid body. These axon-like structures were 20–40 μm long when traced through successive confoocal sections, and they suggested an unusually long nonmyelinated heminodal region before the formation of the calyx nerve terminal. We suggest that our ~30% outside-out patches with Na currents probably come from patches containing membrane from this heminode region rich in Na channel protein.

Na+1.6 (but not Na+1.2) is present in the MNTB
We observed a developmental change in the inactivation kinetics of the Na currents (Fig. 3B). It is possible that Na channel α-subunit composition changes during development in the MNTB, and this could account for these functional changes. Among the different α-subunits present in the CNS, Na+1.2 and Na+1.6 are abundantly expressed in axons and nodes of Ranvier (Westenbroek et al., 1989; Caldwell et al., 2000; Krzemien et al., 2000), and a developmental switch from Na+1.2 to Na+1.6 has been described by Boiko et al. (2001) and Kaplan et al. (2001). These authors suggested that nerve myelination induces the clustering of Na channels in the nodes of Ranvier. Myelination of the

Figure 5. Recovery from inactivation of postsynaptic Na+ currents. A, Pairs of Na+ currents elicited by step depolarizations and separated by different time intervals from 0.5 to 7.5 ms. Note the non-inactivating current. B, Complete recovery from inactivation of the postsynaptic Na+ current after 500 ms. Plot of the fraction of the Na+ current recovered after various time intervals (1–600 ms). The line is a double-exponential fit to the points (n = 5 for P7–P10 rats). C, Developmental difference in the fraction of slow-recovering Na+ current between cells from P5–P6 and P8–P12 rats. The fraction recovered at 15 ms was 88 ± 1.7% in P5–P6 (n = 5) and 96 ± 1.4% in P8–P12 (n = 6; p < 0.05). D, An example of a TTX-sensitive Na+ current recorded from a principal cell that shows a fast-inactivating component and a persistent current.
rat brainstem starts after approximately P7, and a developmental switch from one α-subunit to another could be responsible for some of our developmental changes in the Na current properties (Fig. 3). To address this question, we investigated the distribution of Na\textsubscript{1.2} and Na\textsubscript{1.6} subunits in the MNTB of rats in different age groups.

Figure 8C–F depicts the immunoreactivity profiles generated with specific antibodies directed against both α-subunits at two distinct developmental stages (P6 and P13). We found no substantial Na\textsubscript{1.2} immunolabeling of the MNTB in either age group (Fig. 8D,F) despite the fact that other brainstem structures, such as the pyramids, displayed marked immunoreactivity levels (data not shown). These labeled structures thus served as internal positive controls of our immunocytochemical procedure. Similar results were also observed with immunofluorescence with anti-Na\textsubscript{1.6} (data not shown). Thus, unlike other PNS and CNS areas, the near-universal Na\textsubscript{1.2} α-subunits do not seem to be expressed in the MNTB.

Immunocytochemistry directed at Na\textsubscript{1.6} revealed a marked increase in expression levels with age. We found that, although some Na\textsubscript{1.6} immunoreactivity could be detected at P6, a substantial increase in immunolabeling occurred between this age and P13 (Fig. 8C,E). When immunofluorescence was used, structures similar to those observed with the PAN antibody were seen with the anti-Na\textsubscript{1.6} in MNTB from P13 and P10 rats (Fig. 8G). However, no significant immunofluorescence labeling was observed in P7 rats. Na\textsubscript{1.6} labeling was also less intense than labeling observed with the nonselective PAN-NaCh antibody. Based on these results, we suggest that an increase in Na\textsubscript{1.6} subunit expression occurs during development in the MNTB, perhaps replacing other slower gating isoforms. This increase in expression could account, at least in part, for the developmental changes observed with our electrophysiological experiments [e.g., the increase in upstroke speed of the calyx AP with increasing age (Taschenberger and von Gersdorff, 2000)]. Interestingly, recent electrophysiological data comparing the properties of Na\textsubscript{1.2} and Na\textsubscript{1.6} shows that Na\textsubscript{1.6} has a faster activation kinetics and is more resistant to cumulative inactivation during stimulus trains, indicating that Na\textsubscript{1.6} is better suited for high-frequency firing than Na\textsubscript{1.2} (Zhou and Goldin, 2004).

Although we cannot completely exclude the possibility that some of the 20- to 40-μm-long, bar-like structures seen with PAN-NaCh and Na\textsubscript{1.6} immunolabeling may represent axons leaving the postsynaptic principal cell, several lines of anatomical evidence support the conclusion that they are pre-calyx axonal heminodes. First, the calyceal axons are the largest fibers in the trap-ezoid body, having 4–15 μm diameters depending on species (Spirou et al., 1990; Kubawara et al., 1991), whereas the principal cell axons have a thinner diameter of 2–8 μm near the principal cell body (Kubawara and Zook, 1991; Smith et al., 1998b). The calyceal axons are thus much thicker than the principal cell axons. Our electron microscopy also indicates that the principal cell axon is relatively thin for P14 rats (1.0–1.5 μm diameter; see below). A curved pathway in the MNTB with an abrupt bend before forming the calyx is a characteristic of the calyceal axon (Kubawara et al., 1991) (Fig.
Figure 7. Na⁺ channels are excluded from the calyx. A, Lucifer yellow staining of the calyx of Held and axon from a P8 rat. The calyx was filled with dye via a patch-pipette recording. B–F, Confocal sections of MNTB slices labeled with anti-Na PAN antibody (red) and anti-SV2 antibody (green). B, C, Single excitation of the anti-Na channel–PAN–Alexa 488 conjugate (B; PAN-NaCh) and anti-SV2–Alexa 568 conjugate (C). D, Merging of the images in B and C showing the lack of overlap of Na channels and SV2 proteins. The arrow shows a calyceal terminal, revealed by the SV2 labeling, that appears connected to an axon-like structure formed by the Na channel labeling (arrowhead). Slice from a P7 rat. E, Example of another confocal plane from a different slice, in which many calyces can be seen (green) not overlapping with Na⁺ channels (red). An axon-like structure can be seen in the image inside the square. Slice from a P10 rat. F, Magnification of the area in the square in E, in which the labeling of the axonal membrane by the anti Na-PAN antibody (red) can be clearly seen. The thick axon (arrow) can be traced through successive confocal sections. To the right, it ends adjacent to a calyx-like terminal (green). Following the axon staining away from the terminal reveals that it abruptly ends inside the middle of the slice, perhaps attributable to myelination. The asterisk makes a sharp bend in the axon, a characteristic of the calyceal axons as can be seen in the Lucifer yellow-filled axon in A. Scale bars: A–E, 20 µm; F, 10 µm.

Figure 8. Confocal reconstruction of the calyceal (A) and axonal (B) structures in the MNTB. The dashed line represents the boundary of the MNTB. Image from a P7 rat. Scale bar, (in B) 40 µm. C–F, Immunolabeling of the MNTB for Na₁, 6 subunit (C, E) but not for the Na₁, 2 subunit (D, F). Expression of Na₁, 6 is especially clear in the P13 slice. G, Immunofluorescence for Na₁, 6 and SV2. A confocal plane through the MNTB from a P10 rat labeled for SV2 (green) and Na₁, 6 (red). The white arrowhead indicates a putative heminode that ends to the right on a putative calyx. Scale bars: C–F, 40 µm; G, 20 µm.

7A). In contrast, the principal cell axons tend to curve one time inside the MNTB and follow a relatively straight pathway toward the lateral superior olivary nucleus (Kubawara and Zook, 1991). The structures shown in Figure 7F, labeled by the PAN-NaCh and Na₁, 6 antibodies, match these descriptions of the calyceal axon. They are not seen outside the MNTB (Fig. 8B). In addition, these structures were often closely associated with a putative calyx terminal (Figs. 7D, F, 8G). Furthermore, a series of immunofluorescent confocal images with PAN-NaCh staining of a rat brainstem slice (P13) shows that the bar-like structures (Fig. 9A) are present completely inside the slice at an age when myelination is already significant (Fig. 9B–D). This indicates that the bar-like structures are not attributable to the cutting of axons by the slicing procedure, which often leaves long stretches of axon intact (Fig. 7A). We thus conclude that they probably reflect the axonal heminode region of the calyx.

Electron microscopy of calyceal heminodes

To confirm that there are extended lengths of unmyelinated axon preceding the calyx of Held terminal, we performed serial EM reconstructions of ultrathin and semithin sections from P14 animals. Fortuitous sections revealed long unmyelinated axonal segments leading into the calyx (Fig. 10A). Because of bending of the calyceal axon (Fig. 7A) (see Discussion), we were able to trace axons to the set of paranodal loops in only three cases, yielding heminode lengths of 12, 32, and 36 µm (latter axon shown in Fig. 10B–D). An additional nine axons were traced for >10 µm from the calyx terminal without reaching the onset of myelination (12, 13, 17, 19, 21, 22, 29, and 46 µm) before extending outside of our range of sections. A three-dimensional reconstruction of a completely traced axon (Fig. 10B–D) reveals its bending trajectory en route to its target calyx synapse (Fig. 10E). The average diameter of these axons was 2.16 ± 0.46 µm (mean ± SD; n = 12). In one case, we were able to trace an MNTB principal cell axon from the cell body to the initial set of paranodal loops, covering a distance of 20 µm; the axonal diameter varied from 1.0 to 1.5 µm. These axonal diameters are in good agreement with previous DiI and Lucifer yellow confocal fluorescence images, which also show that the calyceal axon is clearly thicker than the principal cell axon (Forsythe, 1994). Finally, note the large density of mitochondria throughout the heminode region of the axon, which probably provides the Na⁺/K⁺ pumps on the heminode with a ready supply of ATP during high-frequency firing.
The immunocytochemical experiments demonstrate an absence of Na⁺ channels at the calyx of Held terminal. To examine the functional consequences of such asymmetric distribution of Na⁺ channels in the calyx terminal, we performed a modeling study using the program NEURON (Hines and Caravale, 2001). A previously recorded calyx of Held action potential waveform was used to voltage clamp the axon close to the calyx, and the resulting induced potential was recorded in the calyceal stalks and swellings (for details, see Materials and Methods). Figure 8F shows that the calculated AP with shortest half-width occurred when the Na⁺ conductance was set to zero in the calyx. Increasing the Na⁺ conductance led to successive broadening of the calyx AP waveform (Fig. 10F) [similar AP simulations were performed for lizard neuromuscular junction (NMJ) motor nerve terminals, which also have a very low density of Na⁺ channels, and they give the same result (Lindgren and Moore, 1989)]. The AP half-width in the calyx calculated from the swelling indicated in Figure 10F significantly increased from 0.278 to 0.333 ms when the Na⁺ conductance changed from 0 to 0.25 S/cm² on the calyx, whereas the AP overshoot changed from 38 to 49 mV (the original AP in the axon had a half-width of 0.254 ms and overshoot of 45.7 mV). The AP overshoot changed from 38 to 49 mV (the original AP in the calyx calculated from the swelling indicated in Figure 10F) [similar AP simulations were performed for lizard neuromuscular junction (NMJ) motor nerve terminals, which also have a very low density of Na⁺ channels, and they give the same result (Lindgren and Moore, 1989)]. The AP half-width in the calyx calculated from the swelling indicated in Figure 10F significantly increased from 0.278 to 0.333 ms when the Na⁺ conductance changed from 0 to 0.25 S/cm² on the calyx, whereas the AP overshoot changed from 38 to 49 mV (the original AP in the axon had a half-width of 0.254 ms and overshoot of 45.7 mV and was recorded from a P12 rat). Consequently, it seems that restricting Na⁺ channels to the heminode still ensures a fast and potent depolarization that can spread uniformly to the calyx without the necessity of distal Na⁺ channels on the calyceal fingers.

Note that the passive attenuation in height of the presynaptic AP as it propagates through the calyx structure to reach a distal tip is relatively small (change in overshoot from 45.7 to 38 mV) (Fig. 10F). Placing Na⁺ channels in the terminal boosts the height of the original AP from 45.7 to 49 mV. Such a small increase in AP height may, however, have a significant effect on release probability (Wu et al., 2004). Interestingly, outside-out patches from mossy fiber nerve terminals express a relatively high density of Na⁺ channels, and biophysical simulations show that presynaptic Na⁺ channels boost the height of the AP (and thus release probability) at these en passant beads-on-string boutons (Engel and Jonas, 2004).

It is also noteworthy that internode distances along a myelinated nerve often become progressively shorter as one approaches the preterminal region, in which they can be as short as 30 μm (Waxman, 1975; Quick et al., 1979). This may avoid impedance mismatch by allowing more nodes to contribute depolarizing current to a large branched nerve terminal. This design feature of myelinated axons is thought to thus facilitate failsafe AP invasion from the myelinated truck axon to a large nonmyelinated terminal. By the same logic, the extensive calyceal membrane presents the invading AP with a large capacitor that must be quickly charged. One solution is to reduce the pre-calyx axonal internode distance to zero or, in other words, remove myelin from an extended heminode region in which Na⁺ channels can be concentrated to provide the fast and large Na⁺ current needed to quickly charge the calyceal capacitor, thus generating a fast AP.

Discussion

Na⁺ currents have been recorded from neuronal somas, dendrites, and axons, but the properties of presynaptic Na⁺ channels are less well known (Trimmer and Rhodes, 2004). At the neuromuscular junction, controversy exists as to whether Na⁺ channels are expressed on the nerve terminal (Brigant and Mallart, 1982). For example, lizard NMJ nerve terminals contain very little, if any, Na⁺ current (Lindgren and Moore, 1989). However, a small TTX-sensitive extracellular current was observed in mouse motor terminals (Konishi, 1985). In the CNS cell-attached recordings from pinceau-type nerve terminals of cerebellum revealed a high density of Na⁺ current (38 pA/μm²) (Soutain and Robertson, 1998) and Na⁺ current is also present in mossy fiber boutons (Engel and Jonas, 2004), whereas cell-attached recordings from single neocortical synaptosomes revealed no Na⁺ current (Smith et al., 2004). Here we have shown that presynaptic Na⁺ current inactivation and recovery from inactivation becomes faster as the calyx of Held matures. Na⁺ channels were not detected in the calyx but were highly concentrated in the axonal heminode. What are the functional implications of these findings?

Presynaptic Na⁺ currents: fast recovery from inactivation

Fast inactivation of Na⁺ channels ensures that APs are brief, but this also creates a refractory period during which neurons cannot fire APs. The duration of this refractory period depends on the time necessary for the inactivated Na⁺ channels to move back to the resting state after the repolarization of the membrane (Kuo and Bean, 1994). Thus, neurons with Na⁺ channels that recover rapidly from inactivation are able to fire at higher frequencies (Torkkeli and French, 2002). We found time constants for the recovery from inactivation of ~0.5 ms at ~100 mV (35°C). Although similar time constants for recovery from inactivation have been reported for the Na current of the squid giant axon (0.6 ms at ~130 mV) (Bezanilla and Armstrong, 1977) and also for a TTX-resistant Na⁺ current in dorsal root ganglion C-type neurons [1 ms at ~100 mV (Cummins and Waxman, 1997)], the
values obtained here are faster than those reported from several neuronal somata and axons. Examples include rat motor neurons [16 ms at −80 mV (Ogata and Tatebayashi, 1993)], rat hippocampal basket cells [2 ms at −120 mV (24°C) (Martina and Jonas, 1997)], and rat myelinated peripheral fibers [0.9 ms at −118 mV (37°C) (Schwarz and Eikhof, 1987)].

During development, the incidence of presynaptic AP failures during high-frequency stimulation fell dramatically (Fig. 3A). The increased rate of Na current recovery from inactivation may provide a mechanism for this observation. We also observed that trains of calyx APs at P6 result in a small depolarizing plateau, which is absent in P13 calyx AP trains (Fig. 3A). This standing depolarization will likely slow down the recovery from inactivation of subsequent APs (Fig. 1C). A putative increase in presynaptic voltage-dependent K current density, and the faster inactivation rate of Na currents, may both play a role in eliminating this plateau depolarization and in the shortening of AP half-width during development (Taschenberger and von Gersdorff, 2000).

Finally, we note that a large surplus of Na channels, beyond what is needed to trigger an AP and ensure that its waveform is not altered, helps neurons to fire reliably at higher frequencies (Madeja, 2000). Accordingly, we observed an increase in the expression levels of the Na\textsubscript{1.6} subunit with age (Fig. 8C,E) and an improved ability of calyx APs to follow higher stimulation frequencies with increasing age (Fig. 3A). An axonal heminode of 40 μm in length may contain half a million Na\textsuperscript{+} channels [assuming a uniform rat node of Ranvier density of 2000 channels/μm\textsuperscript{2} (Chiu, 1980)]. This large number may be necessary to ensure the fidelity of high-frequency firing.

**Postsynaptic Na\textsuperscript{+} currents: transient and persistent components**

Na\textsuperscript{+} currents recorded from the postsynaptic principal cell also recovered rapidly from inactivation, but a fraction (5–10%) of the current recovered significantly slower. Although \(\tau_{\text{rev}}\) did not change during development, the contribution of the slowly recovering fraction decreased with age. The postsynaptic Na\textsuperscript{+} current also exhibits a persistent TTX-sensitive component that increased with development. A similar increase of the persistent Na\textsuperscript{+} current was also observed for cortical pyramidal neurons (Alzheimer et al., 1993). This persistent current might augment small EPSPs, which might arise during synaptic depression (von Gersdorff et al., 1997), by increasing the depolarization during the interspike interval and amplifying the current flowing through glutamate receptors (Grill, 1996).

**Na\textsuperscript{+} channel subunits in the MNTB**

The Na\textsubscript{1.6} subunit is present in the MNTB, and its expression increased with development. In immature MNTB, detection of Na\textsubscript{1.6} required sensitive enzymatically amplified DAB immunocytochemistry (Fig. 8C), whereas in older animals it could be detected using immunofluorescence. Na\textsubscript{1.2} is characteristic of
unmyelinated axons and immature nodes of Ranvier (Boiko et al., 2001), but it was not detected in the immature MNTB with either technique. Recombinant Na,1.6 expressed in Xenopus oocytes with auxiliary β-subunits, or in mouse DRG neurons, has a relatively slow recovery from inactivation [\( f = 3.8 \, \text{ms at} \, -100 \, \text{mV} \) (Smith et al., 1998a; Herzog et al., 2003)] compared with Na currents measured in the calyx of Held. If Na,1.6 is responsible for the Na currents in the calyx, some factor must contribute to speeding up the recovery from inactivation (Zhou and Goldin, 2004). The increased expression of Na,1.6 with age in the MNTB could also account for the increased expression of the postsynaptic persistent Na current (Raman et al., 1997; Smith et al., 1998a), although Na,1.6 expression in the calyx does not produce this current. We conclude that the Na,1.6 α-subunit (together with a particular β-subunit) could be responsible for the major portion of the Na current seen in the calyx and perhaps in the postsynaptic cell as well.

**Na**⁺ channel locus: the heminode

Labeling with the PAN-NaCh antibody did not detect Na⁺ channels in the calyx. Instead, they were concentrated on the heminode. Using antibodies against the Na,1.6 subunit, Kremzien et al. (2000) also reported an absence of Na⁺ channels in rat NMJ nerve terminals. Caldwell et al. (2000) found that, although Na,1.6 immunofluorescence was not detected in cerebral cortex terminals (identified by double labeling with anti-SV2), it could be detected by immuno-EM. These authors suggested that the intense labeling of the anti-SV2 could be masking a small immuno-reactivity signal attributable to a sparse population of Na⁺ channels in the terminal. In our results, however, even when we captured separately the signals from the different fluorophores used to label the Na⁺ channels and the SV2 protein (Fig. 7 B, C), we did not see any overlap of the labels, suggesting no significant colocalization of Na⁺ channels and synaptic vesicles. In addition, outside-out patches from the terminal often did not contain any Na⁺ current, although K⁺ currents could always be detected when potassium was present in the pipette solution. We conclude that Na⁺ channels in the calyx of Held are strongly concentrated in the heminode, and they steeply drop in density at the calycal terminal.

**Functional advantages to presynaptic Na⁺ channel segregation**

What is the physiological importance of excluding Na⁺ channels from the calyx of Held terminal? Besides making the AP waveform briefer by avoiding a longer depolarization of the terminal attributable to the opening of distal Na⁺ channels after the arrival of the passive depolarization from the heminode (Fig. 10 F), an absence of Na⁺ channels in the terminal avoids a potentially massive presynaptic Na⁺ ion influx during prolonged high-frequency firing. Indeed, an estimate of sodium influx into the terminal indicates that it can be significant. A P9 calyx of Held AP (Borst and Sakmann, 1999) has a half-width of 0.5 ms and an overshoot of +40 mV, parameters that are very similar to the AP of the squid giant axon, in which Na⁺ influx is 4 pmoI/cm² per impulse (Keynes and Lewis, 1951). A P9 calyx has a surface area of 2500 μm² and volume of 0.48 pl (Sätzler et al., 2002). Thus, a calyx with a modest Na channel density equivalent to the squid giant axon [0.06 S/cm² or 300 channels/μm² (Rosenthal and Bezanilla, 2002)] produces a single AP that elevates [Na⁺], by 0.2 mV. Fifty APs at 500 Hz would raise [Na⁺], by 10 mV in 100 ms. This transient accumulation of intraterminal Na⁺ could disturb neurotransmission because a reduced local Na⁺ gradient would hamper the extrusion of Ca²⁺ by slowing down the rate of activity of the Na⁺/Ca²⁺ exchanger. The Na⁺/Ca²⁺ exchanger, with its low affinity for Ca²⁺ and fast turnover rate, is particularly well suited to rapidly extrude high levels of Ca²⁺ at active zones (Reuter and Porzig, 1995; Regher, 1997). Indeed, the Na⁺/Ca²⁺ exchanger plays a major role in extruding Ca²⁺ at the calyx of Held (Chuhma and Ohmori, 2002) [70% of [Ca²⁺]], is extruded via Na⁺/Ca²⁺ exchangers (Kim et al., 2004), and it is located on the calyx of the chick ciliary ganglion (Juhaszova et al., 2000). A reduced Na⁺ gradient could lead to poor Ca²⁺ extrusion from the terminal and, consequently, to asynchronous release and a loss in temporal fidelity of synaptic transmission. Higher and more prolonged intraterminal [Ca²⁺], transients may also lead to an undesired facilitation of release, and thus to premature vesicle pool depletion, and thereby an inability to follow higher stimulation frequencies. Thus, Na⁺ channel polarization may constitute an important specialization to support sustained high-frequency firing.

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