Synaptic physiology in the cochlear nucleus angularis of the chick.

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Running Title: Synaptic physiology in nucleus angularis
Abstract

Nucleus angularis (NA), one of the two cochlear nuclei in birds, is important for processing sound intensity for localization and most likely has role in sound recognition and other auditory tasks. Because the synaptic properties of auditory nerve inputs to the cochlear nuclei are fundamental to the transformation of auditory information, we investigated the properties of these synapses onto NA neurons using whole-cell patch-clamp recordings from auditory brainstem slices from embryonic chickens (E16-E20). We measured spontaneous excitatory postsynaptic currents (EPSCs), and evoked EPSCs and excitatory postsynaptic potentials (EPSPs) by using extracellular stimulation of the auditory nerve. These excitatory EPSCs were mediated by AMPA and NMDA receptors. The spontaneous EPSCs mediated by AMPA receptors had submillisecond decay kinetics (556 µs at E19), comparable to those of other auditory brainstem areas. The spontaneous EPSCs increased in amplitude and became faster with developmental age. Evoked EPSC and EPSP amplitudes were graded with stimulus intensity. The average amplitude of the EPSC evoked by minimal stimulation was twice as large as the average spontaneous EPSC amplitude (~110 pA versus ~55 pA), suggesting that single fibers make multiple contacts onto each postsynaptic NA neuron. Because of their small size, minimal EPSPs were subthreshold, and we estimate at least 3-5 inputs were required to reach threshold. In contrast to the fast EPSCs, EPSPs in NA had a decay time constant of 12.5 ms which was heavily influenced by the membrane time constant. Thus, NA neurons spatially and temporally integrate auditory information arriving from multiple auditory nerve afferents.

Keywords: auditory, avian, magnocellularis, intensity, sound localization
Introduction

The synaptic properties of auditory nerve inputs onto their postsynaptic targets in the cochlear nuclei are fundamental to the transformation of auditory information. In birds, acoustic cues for sound localization are segregated into parallel streams: *in vivo* recordings showed that cochlear nucleus magnocellularis (NM) encodes timing cues, while cochlear nucleus angularis (NA) encodes intensity cues (Konishi et al. 1985; Sullivan and Konishi 1984; Takahashi et al. 1984). Recent work investigating the morphology, physiology and auditory responses in NA suggest this nucleus is also important for encoding sound for non-localization tasks, such as sound recognition and discrimination (Köppl and Carr 2003; Soares et al. 2002).

At the brainstem level, the cellular and synaptic specializations which allow temporal coding of sound phase for the computation of interaural time differences are well understood (Carr et al. 2001; Trussell 1999). Studies of nucleus magnocellularis neurons have revealed a suite of anatomical and physiological specializations that enable the precise encoding of the temporal properties of auditory nerve inputs, including large, calyceal synapses, very fast AMPA-receptor (AMPAR) mediated synaptic currents, short membrane time constants and fast synaptic potentials (Carr et al. 2001; Jhaveri and Morest 1982; Parks 2000; Trussell 1999; Zhang and Trussell 1994a,b). The time course of these AMPAR EPSCs is due to the presence of ‘flop’ splice variants of glutamate receptor subunits GluR3 and GluR4, and to the absence of subunit GluR2, which together confer faster desensitization kinetics, as well as permeability to calcium ions (Dingledine et al. 1999; Geiger et al. 1995; Parks 2000; Sugden et al. 2002).

In contrast, much less is known about the synaptic properties in nucleus angularis. Auditory nerve terminals in NA make anatomically smaller, bouton-like synapses (Carr and Boudreau 1991). Immunohistochemical evidence from the barn owl suggests GluR3 and GluR4 are the most prominent AMPAR subunits in NA (Kubke and Carr 1998; Levin et al. 1997). AMPA receptors expressed by chick NA neurons have fast desensitization kinetics (Raman et al. 1994) and are also calcium permeable (Zhou et al. 1995). These data suggest that the synaptically evoked responses should also display fast kinetics, like those in NM and other auditory brainstem areas. However, such fast kinetics would seem excessive and unnecessary in a nucleus whose neurons have comparatively slow membrane time constants (Fukui and Ohmori 2003; Soares et al. 2002) and is not thought to be involved in encoding timing cues.
We investigated the synaptic properties in NA using whole-cell intracellular recordings from auditory brainstem slices of chicken embryos during auditory nerve (AN) electrical stimulation. Both spontaneous and evoked AMPAR-mediated EPSCs in NA were very fast, with submillisecond decay time constants, similar to most other auditory brainstem nuclei. Excitatory EPSCs and EPSPs evoked by electrical stimulation of the AN were small and graded with stimulus intensity. Evoked excitatory postsynaptic potentials (EPSPs) in NA were much slower than the underlying synaptic currents due to filtering by the membrane time constant. We further provide physiological evidence that NA neurons receive inputs from at least several AN fibers, and that each fiber probably makes several synapses onto its postsynaptic target in NA. These data together show that NA neurons spatially and temporally integrate auditory information arriving from multiple AN afferents to a much greater degree than NM neurons.

**Materials and Methods**

**Brain slice preparation.**

Chicken embryos of ages E16-E20 were rapidly decapitated and an approximately 4 mm segment of the caudal skull containing the brainstem removed with a razor blade and quickly submerged in artificial cerebral spinal fluid (ACSF in mM: 130 NaCl, 26 NaH2CO3, 3 KCl, 2 CaCl2, 2 MgCl2, 1.25 NaH2PO4, and 10 dextrose). The brainstem segment was dissected out and transferred to a vibrating tissue slicer (Campden Instr., Leicester, U.K.) where it was mounted with cyanoacrylate glue, supported by a gel solution (4% agarose in H2O) and cut in ACSF. Transverse slices (250-300 µm) containing both NA and afferent fibers of the 8th nerve were collected and maintained in a holding chamber at room temperature (24-25°C) in oxygenated (95% O2-5% CO2) ACSF. For recordings, slices were placed in a submersion-type recording chamber continuously perfused with oxygenated ACSF (1-2 ml min⁻¹) heated to 28-31°C with a Warner TC 324B (Warner Instr., Hamden, CT), except for current-voltage relation experiments, which were conducted at room temperature. All experiments were performed in accordance with the guidelines approved by the University of Maryland Institutional Animal Care and Use Committee.

**Whole-cell recordings and physiological analyses.**
Whole-cell patch-clamp recordings were made from visually identified NA cells using IR/DIC (infrared/differential interference contrast) video microscopy (Stuart et al. 1993). Our standard potassium gluconate intracellular solution was (in mM): 120 potassium gluconate, 20 KCl, 0.1 EGTA, 2 MgCl₂, 2 Na₂ATP, 10 HEPES, and 0.1% biocytin. We also used a cesium intracellular solution for voltage clamp experiments to determine the IV curve of the synaptic currents (in mM): 70 cesium sulfate, 5 BAPTA, 10 HEPES, 1 MgCl₂, 1 Na₂ATP, 4 NaCl. All reagents were from Sigma unless otherwise indicated.

Electrophysiological recordings were made using an Axoclamp 2B or AxoPatch 200B (Axon Instruments, Foster City, CA) in voltage-clamp or fast current-clamp mode. Extracellular stimulation of the 8th nerve was produced with a tungsten metal monopolar or bipolar electrode. Stimulation and recordings were controlled by a computer running custom software written with IGOR Pro (Wavemetrics, Lake Oswego, OR) and delivered via a National Instruments A/D board (National Instruments, Austin, TX). Stimuli were precisely controlled biphasic waveforms run through an analog stimulus isolation unit (World Precision Instruments, Sarasota, FL).

We report data from a total of 105 individual NA neurons. Neurons were judged healthy if they had a resting potential below −50 mV, and rejected if the initial series resistance was >25 MΩ. For recordings with potassium gluconate electrodes, the resting potential determined immediately after establishing whole-cell mode was −60.3 ± 6.5 mV and whole-cell capacitance was 48.5 ± 15.0 pF. NA neurons were classified by their action potential responses to step current injections in current clamp. We limit our analysis to three broad groups: 1) single-spiking, which always fired one action potential at the step onset, 2) damped, which fired multiple action potentials that attenuated in amplitude with stronger stimulation, and 3) tonic, which fired multiple, full amplitude action potentials. Although tonically firing neurons may be further distinguished into three subgroups (Soares et al. 2002), we lump them here. A junction potential of −10 mV was subtracted posthoc.

Current-voltage experiments were carried out in 20 µM bicuculline and 3 µM strychnine; some were also done in 50-100 µM APV (2-amino-5-phosphovalerate). The synaptic reversal potentials were calculated from linear regression of fast AMPA (α-amino-3 hydroxy-5 methyl-4 isoxazole propionic acid) receptor component of the EPSC across all voltages, and the x-intercept of the linear fit of slow NMDA (N-methyl-D-aspartate) receptor component across voltages from 0 to +40 mV. The ratio of slow to fast EPSC
components was measured from average EPSCs during +40mV step; in cases in which a clear secondary current peak could not be distinguished, we measured the amplitude at 10ms. All other experiments were carried out on pharmacologically isolated AMPAR-mediated events (in the presence of APV, bicuculline, and strychnine) unless otherwise noted. Voltage-clamp investigation of evoked and spontaneous EPSCs was performed with series resistance correction set to 70-95%. Any recordings that could not be corrected to a residual series resistance of <4 MΩ were rejected. We used DNQX (6,7-dinitroquinoxaline-2,3-dione) and GYKI-52466 (Tocris Cookson, Ellisville, MO) for pharmacological identification of AMPA-receptor mediated currents.

Spontaneous EPSCs were recorded under voltage-clamp at –80 mV without tetrodotoxin (TTX); miniature EPSCs were recorded with the addition of 0.1-1 µM TTX. Spontaneous and miniature EPSC analysis was carried out with the program MiniAnalysis (Synaptosoft, Decatur, GA) using an amplitude threshold of 8-20 pA. All events were scanned by eye to remove errors and closely spaced EPSCs for kinetic analysis. A minimum of 30 events per cell were required to be included in the analysis, but typically 100-1000 events per cell were analyzed. Analysis of dendritic filtering was restricted to neurons with 80 or more events.

We used a minimal stimulation protocol similar to that used in hippocampal and other slice preparations (Allen and Stevens 1994). Briefly, the extracellular stimulus level was increased until a threshold level could be determined and a stable, small, evoked EPSC or EPSP could be elicited (e.g., see Fig. 8A). The response was probed with stimulus level steps of 5-15% of threshold level with 10-30 trials for each level, and an average failure rate and ‘potency’ (amplitude not including failures) was calculated. The minimal response was considered the lowest stable potency plateau with a range >10% of threshold level. In most cells plateau failure rates were non-zero (n = 9 of 11). In order to find an accurate threshold for a single fiber, we used ACSF containing 3 mM Ca^{2+} and 1 mM Mg^{2+} ACSF to maximize probability of release. However, this technique does not eliminate the possibility that the ‘minimal’ response actually represents two or more fibers with similar thresholds.

All statistical analyses were performed in Microsoft Excel 2001 for Mac (Student’s t test) or with Statview (ANOVA’s). Values reported are mean ± s.d., unless otherwise specified.

Anatomy.
Slices containing biocytin fills were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer overnight, and then processed with Vectastain ABC (avidin-biotinylated HRP complex) Elite Kit (Vector Labs, Burlingame, CA). The boundary of NA, the location of filled neurons, and their morphological reconstructions were drawn using the computer-assisted tracing program Neurolucida (Microbrightfield Inc, Williston, VT), or photographed. Photographs were composited and adjusted for brightness and contrast in Adobe Photoshop.

Results

*Evoked excitatory postsynaptic currents: voltage dependence and pharmacology.*

Nucleus angularis was easily recognizable as a bean-shaped area of neuropil at the dorsolateral margin of the transverse slice through chick auditory brainstem (Fig. 1A). Individual neurons in NA were clearly identified with video microscopy using IR-DIC. Whole-cell recordings were established as previously described (Soares et al. 2002). A metal electrode was placed in the auditory nerve tract, which was clearly visible under light or DIC microscopy, at the dorsomedial or medial margin of NA where AN fibers enter the nucleus (Fig. 1B)(Boord and Rasmussen 1963; Fukui and Ohmori 2003; Köppl 2001). Because NA is known to receive GABAergic input locally and from the superior olive (Carr et al. 1989; Monsivais et al. 2000; Takahashi and Konishi 1988; Yang et al. 1999), and also stains weakly for glycine receptors (Code and Rubel 1989), all experiments were performed with pharmacological isolation of the excitatory current with 20 µM bicuculline methiodide and 3 µM strychnine. Two intracellular solutions were used: 1) a cesium solution for voltage clamp analysis of the current-voltage relationship, and 2) a potassium gluconate solution for all other current clamp and voltage clamp experiments. Most NA neurons were identified by physiological criteria and classified as single-spiking, tonically firing, or damped (see Methods; Fig. 1C). In some neurons, cell type was further verified by the inclusion of biocytin in the intracellular solution. We did not observe any differences by neuronal cell type or location within NA and therefore we report pooled data, except for one analysis (see spontaneous EPSC results and Table 1, below).

The voltage-dependence of the excitatory synaptic current in NA was examined in slices from E18 chick. Evoked EPSCs were recorded during a series of voltage steps in the presence of GABA-receptor
and glycine-receptor blockers (Fig. 2A). In the absence of APV, two components could be observed. First, there was a fast component, which peaked and decayed in less than 2 ms at negative voltages (Fig. 2A, filled square). This component had a linear IV relationship, which reversed at +5.3 mV (n = 6; Fig. 2B, filled squares). Second, there was a slowly-decaying, voltage-dependent component, sometimes observed as a secondary peak in the EPSCs during depolarizing voltage steps (Fig. 2A, open square). This slowly-decaying component had a nonlinear IV relationship with a negative peak at −30 mV and which reversed at +2.3 mV (n = 6; Fig. 2B, open squares), as would be expected for an NMDA receptor mediated current. The amplitude of the slowly-decaying current was large: the ratio of the fast EPSC component to slow EPSC component, measured during the voltage step to +40 mV, was 0.94 ± 0.34 (range, 0.44 to 1.42; n = 6; Fig. 2B). To determine the identity of these components, we applied pharmacological agents. When APV (75-100 µM) was bath applied, the slow component apparent at +40 mV was blocked (n = 4; upper traces, Fig. 2C), leaving a fast outward EPSC; APV had no effect on the inward EPSC at −80 mV (lower traces, Fig. 2C). The APV-resistant EPSC was reversibly blocked by the AMPA/kainate antagonist DNQX (5-20 µM, n = 8; Fig. 2D) and had an IV relationship similar to the fast component (n = 4). The fast, APV-resistant EPSC was also blocked by GYKI-52466, an AMPAR-specific antagonist (data not shown; n = 4). When DNQX was bath applied, the fast component of the control currents was blocked, but not the slow, voltage-dependent component (n = 4; Fig. 2E). Thus, the slow component is a direct NMDAR-mediated effect, and not due to polysynaptic activity. These results demonstrate that the excitatory AN synapses onto NA are glutamatergic, with a fast AMPAR-mediated component and a large, slow NMDAR-mediated component.

**Spontaneous excitatory postsynaptic currents.**

Previous work showed that the desensitization kinetics of AMPA receptors expressed by NA neurons are fast in response to glutamate exposure (Raman et al. 1994). To determine whether the kinetics of the synaptically evoked responses are also fast, we measured spontaneous excitatory postsynaptic excitory currents (sEPSCs) in 40 NA neurons from animals aged E16-20. These recordings were done in the absence of tetrodotoxin in order to determine the physiological firing properties: 22 tonically firing, 10 single-spike firing, 4 damped, and 4 undetermined). Neurons were voltage clamped at −80 mV and the
series resistance was corrected by 65-95%. Frequent spontaneous events could be observed at 29-31°C and in the presence of GABAR, glycine-R and NMDAR antagonists, which were completely blocked with DNQX, and thus were AMPA-receptor mediated (Fig. 3A). Event frequency ranged from 0.08 to 6.1 Hz among NA neurons (1.5 ± 1.5 Hz; see Fig. 3D for an interval histogram for one NA neuron). The results reported below are based on ~30-2000 individual events per cell (average: 490 events per cell).

A broad range of sEPSC amplitudes was evident across individual traces (Fig. 3B), and in the amplitude histogram (Fig. 3C) for single NA neurons. In Figure 3, for example, the sEPSC amplitudes for this neuron ranged from 8.2 to 101.9 pA, with a mean of 43.8 ± 18.9 pA (n = 545 events). The amplitude histogram was highly skewed (skew, 0.84)(Fig. 3C), such that the median amplitude was less than the mean (38.9 pA). To determine the kinetics of synaptic transmission in NA we measured the 10-90% rise time and the exponential decay time constant of the sEPSCs. The decay of both individual and average sEPSCs in NA could be fit well by a single exponential (r > 0.99). The distributions of rise times (Fig. 3E) and decay time constants (Fig. 3F) were more symmetrical than those for amplitude; for the E18 NA neuron in Figure 3, the average rise time was 343 µs and the decay time constant was 820 µs. Over all NA neurons, spontaneous EPSCs had an average amplitude of 53.6 ± 20.4 pA, 10-90% rise time of 275 ± 85 µs, and exponential decay time constant of 809 ± 359 µs (n = 40). Thus the auditory nerve synaptic inputs to NA neurons had quantal current amplitudes and kinetics comparable to those reported in most other brainstem auditory areas (Barnes-Davies and Forsythe 1995; Bellingham et al. 1998; Gardner et al. 2001, 1999; Kuba et al. 2002).

To determine whether the spontaneous EPSCs measured in NA represented true miniature EPSCs (mEPSC), we measured spontaneously occurring EPSCs before and after the application of tetrodotoxin (TTX) to block spike-mediated release. Average EPSCs in each condition had similar amplitudes and time courses (n = 5; Fig. 4A). The amplitude distributions measured in the presence (mEPSC) and absence (sEPSC) of TTX also overlapped completely (Fig. 4B). The average amplitudes were 56.3 ± 23.6 pA for sEPSCs and 55.6 ± 23.1 pA for mEPSCs (p > 0.5, paired Student’s t). Furthermore, event frequencies were the same before and after TTX application (Fig. 4C). These data suggest that the amplitudes and kinetics measured from sEPSC are representative of quantal currents in NA.
Developmental effects on sEPSCs.

Developmental age contributed to the variability in the amplitude and kinetic measurements of sEPSCs from NA neurons. Average EPSC traces from E19 recordings were larger and faster than those from E17 (Fig. 5A). Analysis of variance of sEPSC amplitude showed that this effect was significant, with an age-dependent effect (p < 0.05, single factor ANOVA). Posthoc comparisons showed significant differences between E16 and E17 versus E18, and E16 versus E19 (p < 0.05, Fisher’s PLSD test, Fig. 5B). Across NA recordings, the amplitude of the average sEPSC increased with age, from 40.0 ± 9.3 pA at E16 to 57.6 ± 9.5 pA at E19 (p < 0.01; r = 0.43, simple regression; Fig. 5C). Analysis of variance also showed significant changes in 10-90% rise times and decay time constants (p < 0.05, single factor ANOVA). Posthoc comparisons for rise times and decay time constants showed significant differences between E16 and E17 versus E19 (p < 0.05, Fisher’s PLSD test, Fig. 5B). Rise times decreased from 320 ± 138 µs at E16 to 213 ± 28 µs at E19 (p < 0.01; r = -0.46; Fig. 5C). Decay taus decreased by 50%, from 1110 ± 464 µs at E16 to 556 ± 201 µs at E19 (p < 0.01; r = -0.48; Fig. 5C).

Spontaneous EPSCs by nucleus and cell type

Previous work has shown that mEPSCs in chick nucleus magnocellularis are among the fastest recorded synaptic currents (Raman et al. 1994; Trussell 1999). To make direct comparisons with the literature and our NA sEPSC data, we acquired sEPSCs from NM at E17 and E19. NM sEPSC amplitudes were significantly larger than those recorded from NA neurons at E19 (110.9 ± 23.3 pA for NM, n=3; p < 0.001, Student’s t; Fig. 6, A and B), although there was no significant difference at E17 (49.1 ± 13.2 pA for NA, n=15; 57.9 ± 31.9 pA for NM, n = 5; p > 0.05, Student’s t). The kinetics of the sEPSCs were significantly faster in NM than in NA at both E17 (not shown) and E19 (Fig. 6, A and B). At E17, the average decay time constant was 391 ± 92 µs for NM neurons, compared to 875 ± 279 µs for NA neurons (p < 0.01). At E19, the average decay time constant was 178 ± 19 µs for NM neurons, compared to 556 ± 201 µs for NA neurons (p < 0.01). These data suggest that while both sEPSCs become larger and faster with development in both nuclei, the size and speed of NA sEPSCs may lag behind those in NM. To determine whether there were differences in sEPSC properties within NA by cell type, we examined
amplitude, rise time, and decay time constants for sEPSCs recorded at E17. We found no significant
differences among this limited data set of tonically-firing (n = 6), single-spiking (n = 4), and damped (n =
3) neurons (Table 1).

We examined sEPSCs from NA neurons for evidence of dendritic filtering by plotting the rise
time for each event against its decay time constant and amplitude (Fig. 7, A and B). While in a few cells a
correlation between rise and decay tau was observed (e.g., in Fig. 7A), it was relatively weak overall (r
values ranged from -0.22 to 0.58; average, 0.22 ± 0.18; n = 33). Data from most NA neurons showed no
correlation between rise time and amplitude (r values ranged from -0.19 to 0.51; average, 0.15 ± 0.14).
Thus, only weak dendritic filtering, at best, was observed in our recordings of spontaneously occurring
synaptic events. However, substantial variance in the kinetics and amplitude of quantal EPSCs at
individual sites may obscure filtering effects observable with this method (Bekkers and Stevens 1996).

**Evoked synaptic currents by minimal stimulation of auditory nerve.**

In NM neurons, stimulation of single auditory nerve fibers evokes large, all-or-none synaptic
currents, due to their giant calyceal synaptic terminals (Hackett et al. 1982; Trussell et al. 1993). In NA,
however, contacts are made by small, bouton-like synapses (Carr and Boudreau 1991). To determine the
impact of a typical auditory nerve input to NA, we recorded EPSCs from 11 NA neurons while
extracellularly stimulating the nerve tract at the dorsolateral or lateral margin of NA. These neurons were
composed of the following cell types: 1 single-spiking, 3 damped, 5 tonic, and 2 undetermined.
Minimal stimulation protocols were used to measure responses from single, or a small number, of input
fibers (Fig. 8A; see Methods). Evoked excitatory postsynaptic currents from putative single fibers were
recorded at a stimulation level 10-20% above threshold stimulation (~75-200 trials; average 123 ± 52
trials). Minimally evoked EPSCs from one NA neuron are shown in Figure 8, B and C. In this neuron, the
minimal stimulation strength always evoked an EPSC with no failures. Minimal stimulation more typically
evoked an EPSC with a probability of less than 1 (range: 0.15 to 0.71) despite the relatively high calcium
levels (see Methods)(n = 9 of 11).

For all NA neurons, the peak amplitude of the evoked AMPAR-mediated synaptic current,
excluding failures, was 109.3 ± 48.6 pA at a voltage of −80 mV, which corresponds to a peak conductance
of 1.36 ± 0.6 nS (n = 11; Fig. 8D). The average amplitude, including failures, was 87.3 ± 50.0 pA. The average coefficient of variation of the amplitude, excluding failures, was 0.35 ± 0.07 (range: 0.25 to 0.50). The decay of the average evoked EPSC was fit well with a single exponential (Fig. 8C). The evoked synaptic responses had a 10-90% rise time of 461 ± 151 µs and a single-exponential decay time constant of 1182 ± 341 µs (Fig. 8, C and D). The onset latency (10% rise time) was 1.7 ± 0.3 ms, while the peak latency was 2.2 ± 0.4 ms (Fig. 8C).

To compare the minimal evoked EPSC with the quantal EPSC, we also recorded sEPSCs from the same neurons in between evoked responses (Fig. 8, E and F). Evoked EPSCs were larger than these spontaneous EPSCs (54.2 ± 11.5 pA; p < 0.01; n = 11). We calculated that the average ratio of evoked EPSC amplitude to spontaneous EPSC amplitudes was 2.3 (range: 0.9 to 5.5), suggesting that each auditory nerve fiber makes at least two synaptic contacts, on average, onto its postsynaptic target.

The kinetics of evoked EPSCs and spontaneous EPSCs recorded in the same neurons were identical (sEPSCs rise times: 454 ± 81 µs; p > 0.05; sEPSCs decay time constants: 1179 ± 338 µs, p > 0.05; n = 11). Both rise times and decay time constants were well correlated between evoked EPSCs and spontaneous EPSCs within the same neurons (rise time, r = +0.65; decay time constant, r = +0.88; n = 11).

**Excitatory postsynaptic potentials in NA are graded and subthreshold.**

The large amplitude of auditory nerve inputs onto NM neurons results in reliable, superthreshold postsynaptic responses (Hackett et al. 1982; Zhang and Trussell 1994a). We now show that NA neurons receive graded subthreshold excitatory inputs, consistent with the observation of smaller terminals from the auditory nerve onto NA neurons (Carr and Boudreau 1991).

In current clamp recordings from NA neurons, subthreshold excitatory postsynaptic potentials (EPSPs) were always evident with low to moderate levels of stimulation, prior to reaching action potential threshold at higher stimulation levels (n = 19). In 11 NA neurons, we analyzed EPSPs from a large number of trials (100-200) across the full range of postsynaptic responses, from failure to spiking. Figure 9 shows that for one cell, at least two EPSP amplitude levels can be observed before reaching action potential threshold (Fig. 9, A and B), with a large variation in amplitude within each level. In 8 of 11 NA neurons, EPSPs responses could be grouped into 2-4 discrete, but broad, clusters according to peak amplitude (with
high variance in amplitude within a cluster). These results suggest that NA neurons receive and must sum at least 3-5 active auditory nerve fiber inputs to reach spiking threshold. In the remaining NA neurons, the range of subthreshold amplitudes did not show discrete clusters, and instead blended into a continuous range. Multiple EPSC amplitude levels were also routinely observed in voltage clamp recordings of evoked EPSCs with increasing stimulus intensity (Fig. 9, C and D).

The short time course of the EPSPs in NM, due to the fast underlying kinetics coupled with the short membrane time constant, is thought to contribute to efficient phase-locking of NM neurons to the auditory nerve input by maintaining a narrow window for temporal integration (Zhang and Trussell 1994a). We have investigated the time course of the evoked EPSPs in NA with minimal stimulation. A comparison of the kinetics of the EPSPs with those of the EPSCs reported above suggests a strong filtering effect due to the membrane time constant of NA neurons. We made recordings from 16 NA neurons in current-clamp, that had the following cell types: 3 single-spiking, 3 damped, 9 tonically-firing, 1 undetermined. We measured the kinetics of small EPSPs likely to result from a single or small number of AN fiber inputs, using the same minimal stimulation methods described above, and in the presence of GABA-, NMDA- and glycine-receptor blockers (Fig. 10). These EPSPs had an onset (10% rise) latency of 2.1 ± 0.7 ms, a peak latency of 3.6 ± 0.8 ms, and average amplitude of 4.5 ± 3.1 mV, excluding failures (n = 16; range, 0.6 to 11.8 mV)(Fig. 10B). Resting voltage was –64.5 ± 4.5 mV. Compared to the evoked EPSCs, these evoked EPSPs had a slower time course with a 10-90% rise time of 1.2 ± 0.5 ms, and single-exponential decay time constant of 12.5 ± 7.0 ms (range, 4.6 to 28.5 ms)(Fig. 10, A and B). The decay time constant was similar to the membrane time constant of 10.1 ± 3.0 ms (no significant difference, p > 0.05, paired Student’s t). There was a clear relationship between EPSP decay time constant versus membrane time constant (r = 0.82; Fig. 10C). These data suggest substantial filtering of the EPSP due to the membrane time constant of the NA neurons.

**Discussion**

To better understand how sound information is encoded in the avian cochlear nuclei, we have identified and characterized the excitatory synaptic inputs from the auditory nerve onto chick nucleus angularis neurons. Stimulation of the auditory nerve evoked glutamatergic, excitatory postsynaptic
potentials that were small, graded, and subthreshold. Spontaneous and evoked AMPAR-mediated EPSCs had fast kinetics, similar to those that characterize other auditory brainstem regions in both avian and mammalian systems. Each NA neuron appeared to receive multiple small EPSPs from a number of AN fibers, which were filtered by the time constant of the NA membrane and summed to reach firing threshold. Evoked EPSCs also had a substantial NMDA-receptor mediated component.

The small size of the evoked EPSCs and EPSPs is consistent with the anatomical observation of small, bouton-like and en passant synapses in NA, unlike the large, calyceal synapses in NM (Carr and Boudreau 1991). We observed individual evoked currents as small as 14 pA, and evoked EPSPs as small as 0.2 mV. We used minimal stimulation procedures to estimate the evoked EPSC and EPSP amplitudes from the input of a single AN fiber. Since the minimal evoked EPSC amplitude in NA neurons was larger than that of the spontaneous EPSC amplitude, we conclude that the each auditory nerve fiber may make multiple synaptic contacts onto its postsynaptic target. While the data shows that number of contacts to be at least 2, this is a lower bounds estimate; the actual number should be higher because the probability of release is most likely <1 under normal physiological conditions. Because this procedure cannot guarantee stimulation of single fibers, however, we cannot exclude the possibility that the larger evoked EPSCs are composed of inputs from multiple fibers with similar thresholds, even though we took care to reject clearly compound EPSCs. The graded nature of the EPSPs and EPSCs with increasing stimulus intensity shows that each NA neuron also likely receives input from multiple auditory nerve fibers. Our results illustrating the small, graded excitatory inputs are similar to those found for T-stellate and octopus neurons in the mammalian ventral cochlear nucleus (VCN)(Ferragamo et al. 1998; Golding et al. 1995).

NA neurons express fast AMPA-receptor kinetics, both in spontaneous and evoked EPSCs. In the older, E19 embryos, spontaneous EPSCs had a mean 10-90% rise time of $213 \pm 28 \mu s$, with some cells on average as fast as $170 \mu s$. The EPSC decays could be well fit by a single exponential with a mean time constant of $556 \pm 201 \mu s$, with some cells as fast as $300 \mu s$. These results place NA AMPAR currents well within the category of fast excitatory currents that characterize the auditory brainstems of birds and mammals, which, for comparison, have decay time constants of: $\sim 200 \mu s$ in NM (Raman et al. 1994; Zhang and Trussell 1994b); $\sim 600 \mu s$ in chick nucleus laminaris (Kuba et al. 2002); $\sim 340-400 \mu s$ in the VCN (Bellingham et al. 1998; Gardner et al. 2001, 1999); $\sim 700 \mu s$ in the mammalian medial nucleus of the
trapezoid body (Barnes-Davies and Forsythe 1995). It should be noted that some variation among these
results is due to temperature and age differences. Our data are consistent with previous studies on GluR
subunit distributions (Levin et al. 1997) and receptor desensitization kinetics (Raman and Trussell 1992) in
NA. These ‘auditory’ currents (Parks 2000) are faster than most AMPAR-mediated currents reported from
many other areas in the brain, which have decay time constants of several milliseconds or more. These
include synapses in hippocampus (Hestrin et al. 1990; Jonas et al. 1993; Walker et al. 2002), cerebellum
(Llano et al. 1991; Silver et al. 1992), neocortex (Hestrin 1992, 1993; Stern et al. 1992), and non-auditory
brainstem (Raman et al. 1994; Titz and Keller 1997), with few exceptions (but see Silver et al. (1996)).
Even using techniques designed to circumvent voltage-clamp error (Häusser and Roth 1997; Walker et al.
2002), most decay time constants of EPSCs recorded outside the auditory brainstem are not in the sub-
millisecond range.

When we compared spontaneous EPSC kinetics in NA with those we recorded in NM under
similar conditions, reproducing the results of (Zhang and Trussell 1994b), we found that the kinetics in NA
were significantly slower than in NM. There could be several reasons for this difference. Receptors in the
two nuclei may be composed of a different complement of GluR subunits, such that NM receptors are
simply faster. This may also be a developmental difference (Lawrence and Trussell 2000; Sugden et al.
2002), where the NA AMPAR currents simply lag NM AMPAR current development. Another possible
explanation for slower EPSC kinetics in NA is that the dendrites of NA neurons may lead to voltage-clamp
error, or dendritic filtering. However, we found only weak evidence for dendritic filtering, and no
correlation between decay time constant measurements and any voltage clamp settings (e.g., series
resistance, whole-cell capacitance, or speed of voltage clamp). Further experiments with older animals and
improved techniques will be needed to resolve this discrepancy.

We observed no differences in spontaneous EPSC receptor kinetics or amplitude by NA neuronal
cell type. Similarly, bushy, T-stellate, tuberculoventral and octopus cells in the mammalian VCN were
reported to have nearly identical kinetics to one another (Gardner et al. 1999). Neither did we observe any
bimodal kinetic distributions, as have been found in some fusiform cells of the dorsal cochlear nucleus
(DCN), where the population of slower EPSCs are thought to have a non-auditory origin (Gardner et al.
1999).
We also used minimal stimulation to estimate the time course of the AMPA-receptor mediated EPSP. The 10-90% rise times and exponential decay time constants of the EPSP were much slower than those of the underlying synaptic currents, and we showed that the decay time constant was directly correlated to the membrane time constants in NA neurons (Fukui and Ohmori 2003; Soares et al. 2002). Due to their shorter membrane time constants, the EPSP decay times reported for NM neuron (with a half-decay of < 1ms) (Zhang and Trussell 1994a) are faster than even the fastest we report here for NA (~4 ms). Thus, while all these neuron all receive fast underlying synaptic currents, the membrane time constant is the governing factor for how these EPSPs decay. The broad range of EPSP decay time constants we show across different NA neurons, combined with differential expression of voltage-dependent currents (Fukui and Ohmori 2003; Soares et al. 2002), could furthermore lead to differential integration of auditory inputs within NA. NA neurons with the slower EPSPs are precluded from encoding the precise temporal information present in the auditory nerve inputs, thus contributing to the segregation of acoustic information in the two nuclei.

It is unclear why a nucleus that appears to be better suited for integration would be equipped with receptors that display such fast kinetics. We propose that fast kinetics may be required in order to manage the high rates of input without saturation: in hatchling chick, the driven rates for AN fibers exceed 100-300 spikes/sec (Saunders et al. 2002). EPSP kinetics at synaptic sites distant from the soma may be considerably faster than measured and contribute to local dendritic computation. Faster EPSCs in NA might contribute to the in vivo firing responses of some NA neurons having onset and primary-like properties, which are characterized by temporal precision (Köppl and Carr 2003; Sachs and Sinnott 1978; Sullivan 1985; Warchol and Dallos 1990). Alternatively, with fast currents, the degree of temporal integration is largely determined by the time constant of the membrane, which is known to decrease over development (Fukui and Ohmori 2003), or could be easily modulated or voltage-dependent, providing a greater degree of computational flexibility.

In mammalian cochlear nucleus, Tstellate cells are thought have recurrent excitatory connections (Ferragamo et al. 1998; Oertel et al. 1990) and provide feed forward excitation to neurons in the DCN (Zhang and Oertel 1993a, b). In contrast, we did not observe any evidence of intrinsic circuitry, such as large numbers of delayed or polysynaptic EPSPs, as seen in T-stellate recordings (Ferragamo et al. 1998).
In many NA neurons for which the morphology was recovered, axons were observed to leave the nucleus, but no axon collaterals within or near the NA neuropil were found (personal observation). Furthermore, there is no avian cytoarchitectural division like the mammalian dorsal cochlear nucleus (Carr and Soares 2002). However, *in vivo* recordings from NA demonstrate a similar range of response types to sound stimuli as in mammalian cochlear nucleus (Köppl and Carr 2003). These data suggest that NA may contain a simplified circuit which still retains essential coding features to sound.

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**References**


**Figure 1.** A) Transverse view of chick auditory brainstem, stained with an antibody to potassium channel Kv1.2 (Alamone, Israel). NA, nucleus angularis; NM, nucleus magnocellularis; NL, nucleus laminaris. B) Schematic of experimental setup. C) Biocytin-filled radiate neuron in NA. This neuron was firing type tonic III (see Soares et al., 2002).

**Figure 2.** Voltage-dependence and pharmacology of the evoked excitatory postsynaptic current. All currents in A and B, and control currents in C-E are in the presence of bicuculline and strychnine. A) Current traces at different holding voltages. Note the fast component (filled square) and a secondary peak indicating the slow component with depolarization (open square). Averages of 20 traces each. B) Voltage-dependence of fast (filled squares) and slow (open squares) components. Both currents reversed between +2 to +5 mV. C) Top two traces: APV blocked the large slow component evident at a depolarized voltage (+40 mV), while the fast component remained (control, thick line; 100 µM APV, thin line). Bottom traces: APV had little effect on the fast component at a hyperpolarized voltage (-80 mV). D) The fast component was reversibly blocked by DNQX. Top: traces show complete blockade of synaptic response with DNQX. Bottom: The time course of the EPSC amplitude during blockade with DNQX and washout. Data taken at 10 second intervals (total length ~50 minutes). Traces taken at arrows. E) Top traces: DNQX blocks the fast, but not slow, current component during a depolarizing step (+40 mV)(control, thick line; 5 µM DNQX, thin line). Bottom traces: In the same cell, DNQX completely blocked the EPSC at hyperpolarized voltage (-80 mV).

**Figure 3.** Spontaneous EPSCs from a single E18 NA neuron. A) Raw current traces show many spontaneous events in control (top; in bicuculline, strychnine, and APV) which are eliminated with the addition of DNQX (bottom). B) Overlay of individual sEPSC events (thin lines) and their average (thick line). C-F) Frequency histograms of individual sEPSC amplitudes, inter-event intervals, 10-90% rise times, and single exponential decay time constants for this neuron.
**Figure 4.** Comparison of spontaneous EPSCs in control conditions with miniature EPSCs (mEPSCs) recorded in the presence of tetrodotoxin (TTX). A) Average EPSCs from 5 NA neurons before (sEPSC) and after bath application of TTX (mEPSC). B) Cumulative histograms of sEPSC and mEPSC amplitudes were nearly identical (mean ± s.e.m.; n = 5) C) sEPSCs and mEPSCs showed no differences in event frequency (p > 0.5, paired Student’s t; n = 5).

**Figure 5.** The effect of developmental age on sEPSC amplitude and kinetics. A) Grand average traces of NA sEPSC at E17 (n = 15, thin lines) versus E19 (n = 10, thick line). B) Bar plots of average amplitude, 10-90% rise time, and decay time constant with age. Significance, p < 0.05, posthoc Fisher’s PLSD test. C) Scatter plots of amplitude, rise time, and exponential decay time constant versus age. Each open circle represents one NA neuron: E16, n = 6; E17, n = 15; E18, n = 7; E19, n = 10; E20, n = 1.

**Figure 6.** A) Grand average traces of NM sEPSCs at E19 (n = 3; thick line) versus NA sEPSCs at E19 (n = 10; thin line). Dashed line, NA average sEPSC scaled to match NM sEPSC amplitude. B) Bar plots of NA and NM sEPSC average amplitude, rise time, and decay time constant (at E19; p < 0.01, Student’s t)

**Figure 7.** Plots of 10-90% rise time versus decay time constant or amplitude provide little evidence for dendritic filtering. Data for all events from one E18 NA neuron. A) Rise time and decay tau were weakly correlated in this neuron. B) Rise time and amplitude were uncorrelated for the same neuron.

**Figure 8.** Evoked EPSC properties under minimal stimulation. A) Failure rate (top) and evoked EPSC amplitude (bottom) versus extracellular stimulation level. Open symbols, individual EPSC amplitudes; closed symbols, average EPSC amplitude (mean ± s.d., n = 20 trials per stimulus level). B) Individual EPSCs from one NA neuron. C) Average evoked EPSC from the same neuron in B with single exponential fit of the decay. D) Summary data of average evoked EPSC amplitude, peak latency, 10-90% rise time, and exponential decay time constant from 11 NA neurons (mean ± s.d.). E) Average spontaneous EPSC from the same neuron in C was smaller, but had a similar single exponential decay time constant as the evoked EPSC. F) Scaled overlay of evoked EPSC and sEPSC from C and E.
**Figure 9.** NA neurons received EPSPs and EPSCs of graded amplitude with extracellular stimulation strength. A) Overlay of individual subthreshold EPSPs evoked with increasing extracellular stimulation strength. Note clustering of traces in distinct groups. Asterisk: action potential, clipped for clarity. B) Subthreshold EPSP amplitudes versus stimulation strength for cell in A. Threshold stimulation occurred at ~330 µA; at least two discrete EPSP amplitude clusters are apparent, with large variability within these. C) Overlay of individual EPSCs evoked with increasing stimulation strength. Different neuron than in A,B. D) EPSC amplitudes versus stimulation strength for cell in C.

**Figure 10.** Kinetics of evoked EPSPs in NA. A) Average minimal EPSP from one NA neuron and the single exponential fit to its decay. B) Summary data of average amplitude, peak latency, 10-90% rise time, and exponential decay time constant for NA neurons (n = 16). C) Scatter plot shows the correlation between minimal EPSP decay time constant and neuronal membrane time constant. Open symbols, minimally stimulated EPSPs; line, linear fit of minimal EPSPs (y = 1.48x − 2.50, r = 0.82; n = 16); closed symbols, supraminimal EPSPs shown for comparison (n = 7).
Table 1. Spontaneous EPSC properties by NA cell type

<table>
<thead>
<tr>
<th>Cell type</th>
<th>n</th>
<th>Amplitude (pA)</th>
<th>Rise time (µs)</th>
<th>Decay Τ (µs)</th>
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<tbody>
<tr>
<td>tonic</td>
<td>6</td>
<td>46.3 ± 10.3</td>
<td>400 ± 68</td>
<td>933 ± 206</td>
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<tr>
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<td>53.6 ± 17.9</td>
<td>489 ± 67</td>
<td>1281 ± 258</td>
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<tr>
<td>single</td>
<td>4</td>
<td>46.9 ± 16.6</td>
<td>371 ± 95</td>
<td>941 ± 260</td>
</tr>
</tbody>
</table>

Data from 13 E17 NA neurons.
Rise time: 10-90% rise time; Decay τ, n: initial fit.
Figure 1
Figure 2
Figure 3
Figure 4

Figure 5
Figure 8