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DEVELOPMENT OF THE DELAY LINES IN THE NUCLEUS LAMINARIS
OF THE CHICKEN EMBRYO REVEALED BY OPTICAL IMAGING

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Abstract—One strategy in localizing a sound source in the azimuthal plane is the comparison of arrival times of sound stimuli at the two ears. The processing of interaural time differences (ITDs) in the auditory brainstem was suggested by the Jeffress model in 1948. In chicks, binaural neurons in the nucleus laminaris (NL) receive input from both ipsilateral and contralateral nucleus magnocellularis (NM) neurons, with the axons of the latter acting as delay lines. A given neuron in the NL responds maximally to coinciding input from both NM neurons. To achieve maximum resolution of sound localization in the NL, the conduction velocity along these delay lines must be precisely tuned. Here, we examined the development of this velocity between embryonic days (E)12 and E18. Our optical imaging approach visualizes the contralateral delay lines along almost the complete NL of the chicken embryo. Optical imaging with the voltage-sensitive dye RH 795 showed no significant differences in the velocity between E12 and E15, but a significant increase from E15 to E18, at both 21 °C and 35 °C. Surprisingly, at 21 °C the conduction velocity in the dorso–lateral part of the NL was significantly higher compared to the situation in the ventro– medial part. The observed development in contralateral conduction velocity may be due to a developmental increase in myelination of the NM axons. Indeed, antibody staining against myelin-associated glycoprotein (α-MAG) showed no myelination of the NM axon branches within the NL at E12 and E15. On the other hand, a clear α-MAG immunoreactivity occurred at E18. Our results therefore describe the developmental physiological properties of the delay line in the chicken embryo. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: Jeffress model, nucleus magnocellularis, sound localization, RH 795, interaural time difference.

A chick hatchling strongly depends on its ability to localize and to characterize different maternal hen calls, not only for shelter and food, but also for memory formation (Field et al., 2007). The localization of a sound source in space is accomplished by processing the inputs from the two ears. Two parameters are involved in this process, interaural level differences and interaural time differences (ITDs, McAlpine and Grothe, 2003). For mammals and birds, ITDs are important cues for sound localization as neurons encode the phase difference of the stimulus sound wave and thereby gain information on the azimuthal position of the sound source (Moiseff and Konishi, 1981; Yin and Chan, 1990; McAlpine, 2005). In 1948, Jeffress proposed a hypothetical model on the basis of three features: (1) action potentials are phase-locked to a distinct phase of the stimulus waveform, (2) a set of coincidence detector neurons that fire action potentials only when they are excited nearly simultaneously by bilateral inputs and (3) delay lines that transcode the phase difference of the sound wave at both ears into a time difference (Jeffress, 1948). In birds, the first stage of ITD computing is the nucleus laminaris (NL) in the brainstem (Carr and Konishi, 1990). The NL receives bilateral excitatory input from the nucleus magnocellularis (NM), which in turn is innervated by the vestibulocochlear nerve (Takahashi and Konishi, 1988). Neurons of the NM are big and surrounded by a calyceal synapse which is optimized for high performance signal transduction (Boord, 1968; Whitehead and Moster, 1981; Feng and Moster, 2006). In fact, NL neurons carry out features of coincidence detectors, as shown by recordings of extracellular field potentials in the chick brainstem (Overy Holt et al., 1992; Hyson, 2005). Only the contralateral NM–NL projection acts as a delay line, whereas the ipsi-lateral projection conveys input to the entire NL simultaneously. Phase locking occurs in hair cells of the cochlea (Martin and Hudspeth, 2001), the auditory nerve, and neurons of the ventral cochlear nucleus (Woolf et al., 1981). The chick NL is arranged as an accurate monolayer of cells with symmetrical dendritic arbors forming dorsal and ventral fields (Smith and Rubel, 1979). Neurons located on an axis running from caudo–medial to rostro–lateral display the same morphology and fire preferentially to the same stimulus frequency, forming an isofrequency line of cells (Young and Rubel, 1983).

Because of the well-known connectivity of the avian auditory brainstem nuclei and their easy accessibility, this system provides an excellent model for studies of synapse maturation and development (Boord and Rasmussen, 1963; Rubel and Fritzsche, 2002; Ryugo and Parks, 2003). The NM neurons receive functional synaptic input from embryonic day (E)7 on, while those in the vestibulo–cochlear nucleus receive latent input from E6 (Sato and
Momose-Sato, 2003; Momose-Sato et al., 2006). An immunohistochemical approach showed positive staining for synaptic vesicle protein 2 from E8 on (Hendricks et al., 2006). The NM–NL projection is functional from E10 on (Gao and Lu, 2008).

In the present study, we examined the NM–NL projection of the chick using an optical imaging approach with voltage-sensitive dyes. With the advantage of simultaneous recordings from most neurons along the NL, we could directly demonstrate the presence and absence of a delay line in the contralateral and ipsilateral NM–NL projection, respectively. Furthermore, the development of the contralateral conduction velocity was examined at three embryonic ages (E12, E15, E18) at 21 °C and 35 °C. Finally, immunohistochemistry with antibodies against myelin associated glycoprotein (a-MAG) was performed to assess the cytological basis for velocity increases.

EXPERIMENTAL PROCEDURES

Solutions and drug application

The preparation solution contained (in mM): NaCl, 125; NaHCO3, 25; NaH2PO4, 1.25; KCl, 2.5; MgCl2, 1; CaCl2, 2; glucose, 260; Na-pyruvate, 2; myo-inositol, 3; and kynurenic acid, 1. Storage and recording was performed in artificial cerebrospinal fluid (ACSF) composed of (mM): NaCl, 125; NaHCO3, 25; NaH2PO4, 1.25; KCl, 2.5; MgCl2, 1; CaCl2, 2; glucose, 10; Na-pyruvate, 2; myo-inositol, 3; ascorbic acid, 0.4. The loading solution equaled ACSF except that it contained 122.5 mM NaCl, 5 mM KCl, and 50 µM of the voltage-sensitive dye RH 795 (Molecular Probes, Leiden, Netherlands). All solutions were bubbled with carbogence (95% O2, 5% CO2) to ensure pH 7.4.

Egg incubation and tissue preparation

All protocols were approved by the animal care and use committee responsible for our institution (Landesuntersuchungsamt Rheinland-Pfalz, Germany). Treatment of the animals was in accordance with the German law for conducting animal experiments and also followed the NIH guide for the care and use of laboratory animals. Both the number of animals and their suffering were reduced to the possible minimum.

Fertilized eggs from White Leghorn chickens (Gallus gallus domesticus) were obtained from Lohmann Tierzucht (Cuxhaven, Germany) or from a local breeder and stored at 13–15 °C up to 2 weeks. Embryonic development was triggered by placing the eggs in an incubator (HEKA Euro-Lux E-I, HEKA, Rietberg, Germany). Embryos were sacrificed between E12 and E18, corresponding to the Hamburger–HAMILTON stages 38–44 (Hamburger and Hamilton, 1951). Thereof, eggs were placed on ice for 5 min, and then the embryos were removed and rapidly decapitated. The preparation procedure was completely carried out in 4 °C preparation solution. The brain was dissected, freed from remaining meninges, and the cerebellum was removed. A brainstem block was attached with the caudal side to the stage of a vibratome slicer (VT 1000S, Leica, Bensheim, Germany) using cyanoacrylate glue. Coronal 300-µm-thick slices containing NM and NL were cut in preparation solution and stored for 60 min in ACSF at 37 °C to remove pharmacological effects and restore physiological activity. Thereafter, the slices were held at room temperature until recordings began. Embryos aged E11–E12 are referred to E10, E14–E15 to E15, and E17–E18 to E18.

Voltage-sensitive dye loading and optical recording

Each slice was incubated for 50 min in the RH 795 loading solution (for details, see above). Thereafter, the loaded slices were kept for 30 min in ACSF to remove unbound dye molecules. Loading and washout were carried out at room temperature (~21 °C). The slices were then transferred to the recording chamber and constantly superfused with ACSF. Recordings at 35 °C were performed by heating the recording chamber and the solution via a temperature controller (LN Temperaturecontroller III, Luigs & Neumann, Ratingen).

For a detailed description of the recording setup and the functionality of RH 795, see Srivinasa et al. (2004). Briefly, the recording chamber was placed under a photodiode array (PDA, RedShirL Imaging, Fairfield, USA), composed of 464 single photodiodes. With the 40× objective used, one diode covered an area in the slice of approximately 19×19 µm. Optical control of the slice was ensured by a CCD camera (VC 45; PCO, Kelheim, Germany) and a PC frame grabber card (DT 3155, Data Translation, Bietigheim-Bissingen, Germany). During optical recordings, the slices were illuminated with light from a mercury arc light bulb (HB013; Otsram, Munich, Germany). Illumination duration was maintained by a shutter (VS25, Uniblitz; Vincent Associates, Rochester, NY, USA) which was controlled by the acquisition software (Neuroplex 3.01; RedShirL Imaging, Fairfield, USA). A set of fluorescent filters (excitation: 546:12 nm, beam splitter: 580 nm, emission: 590 nm; Zeiss, Jena, Germany) optimized the wavelength range of light according to the absorbance and emission maxima of RH 795. The fast voltage-sensitive dye RH 795 shows a fluorescence decrease upon membrane depolarization and a fluorescence increase upon hyperpolarization. In accordance to common electrophysiological conventions, optical signals were inverted so that upward and downward deflections correspond to membrane potential depolarizations and hyperpolarizations, respectively. For simplicity, optical signals are named “depolarizations” or “hyperpolarizations”. They are presented as the ratio Δf [% of the fluorescence change (Δf)] and the fluorescence baseline level (f0). The latter was measured for each diode immediately before the acquisition of the stimulus-induced optical signals. Optical signals were acquired with a sample frequency of up to 100 kHz. All data are given as averages of 10 consecutive measurements of the ratio Δf/. The time interval between measurements was 10 s. For the calculation of the conduction velocity, the stimulus-induced peaks were considered. Thus, the linear distance of the examined diodes (for example one diode from recording site 1 and one from recording site 4 in Fig. 1B) and the time difference of the occurrence of the peak amplitudes were measured. The average of the conduction velocity of three to five such pairs of diodes lying in the different areas gave the resulting conduction velocity for one data point in each group.

All datasets were statistically checked for outliers (P<0.05), and the bell-shaped distribution was observed (Kolmogorov–Smirnov test). Differences between two datasets were tested with Student’s t-test and were considered as significant when P<0.05 (*; higher levels are marked as P<0.01, **, and P<0.001, ***). All values illustrated represent mean±SEM.

Electrical stimulation

To excite NM–NL projections, electrical stimuli (square current pulses, 150–300 µA, 0.2 ms) were applied bilaterally via a modified stimulus generator (STG 1004, Multi Channel Systems, Reutlingen, Germany) and self-made glass pipettes (tip diameter ~5–10 µm, GB190-5P, Science Products, Hofheim, Germany). The pipettes were filled with ACSF and connected to the stimulus generator, with one wire inside and one wire coiled around the outside (distance to the tip ~2.5 mm). These stimulus electrodes provide a sharp-shaped electric field, suited for focal stimulation of only a small subset of axon fibers in the slice. The ipsilateral
Fig. 1. Virtually no delay in the ipsilateral NM–NL projection compared to the contralateral NM–NL projection. (A) Representative videomicrograph of an E15 brainstem slice showing the NM and NL as well as the position of the stimulation electrodes. Scale bar=200 µm. (B) Semischematic overview of the image shown in (A). d, dorsal; l, lateral; NL, nucleus laminaris; NM, nucleus magnocellularis; ise, ipsilateral stimulation electrodes; cse, contralateral stimulation electrodes; numbers represent different recording positions. (C) Examples of responses to ipsilateral (left) and contralateral (right) stimulations obtained at four different recording sites. Ipsilaterally, peak amplitudes jitter in a range of 600 µs (gray bar), whereas contralaterally, the time difference from recording site 1 to 4 amounted to 2050 µs (gray bar). (D) Correlation between the delay and distance from the first diode. Upon contralateral stimulation, a linear increase in the delay occurred, with farer distances from the first diode \( r=0.74 \). In contrast, no such correlation was found upon ipsilateral stimulation \( r=0.06 \). (E) The mean delay, normalized to 100 µm, was significantly higher at contralateral stimulation \( P=5 \times 10^{-4} \). The mean conduction velocity, calculated from distances and delay times for the contralateral NM–NL projection, amounted to 0.21±0.03 m/s. Measurements were performed at 21 °C.
NM–NL projection was activated by placing a stimulation electrode directly into the NM of the same side. The contralateral NM–NL projection was activated at the ventro–medial portion of the fiber tract by placing a stimulation electrode on axon bundles in the crossed dorsal cochlear tract.

**Antibody staining**

For antibody staining, the complete brain was kept for 1 to three days in 4% paraformaldehyde (Serva, Heidelberg, Germany) at 7 °C and then transferred into phosphate-buffered saline (PBS, containing 130 mM NaCl, 7 mM Na2HPO4, 3 mM NaH2PO4, pH 7.4). After cryoprotection with 30% sucrose, 60-μm-thick sections of the brainstem were prepared with a freezing microtome (HM 400R, Microm, Walldorf, Germany). Antibody staining was performed free floating. First, the tissue was blocked for 1 h in blocking solution containing 2% bovine serum albumin (BSA, Serva, Heidelberg, Germany), 3% normal goat serum (NGT, In Vitrogen, Karlsruhe, Germany) and NP-40 (Igepal CA-630, Sigma Aldrich, Steinheim, Germany). Tissue was then stained overnight at 4 °C with the α-MAG antibody (1:200 dilution; No. MAB1567, Chemicon International, Temecula, CA, USA) in a carrier solution containing 2% BSA and NP-40. After washing with PBS, slices were again blocked for 30–60 min in blocking solution and then incubated with the second antibody (goat-anti-mouse, Alexa Fluor546, A11030, 1:200, Molecular Probes, Eugene, USA). Tissue was then washed three times in PBS for 5–10 min, with the first washing step containing propidium iodine (1:1000). For analysis, videomicrographs were taken with a confocal laser scanning microscope (LSM-510, Zeiss, Jena, Germany).

**RESULTS**

Only the contralateral NM–NL projections form a delay line

To assess the suitability of the method for examination of the contralateral and ipsilateral NM–NL projection, we first performed proof of principle experiments with RH 795 and tissue from animals at E15 at 21 °C (Fig. 1). We positioned the stimulation electrode into the ipsilateral NM (Fig. 1A, B) and recorded optical signals from the NL of eight slices. This approach visualized the spread of activity along the ventro–medial to dorso–lateral extent of the NL (recording sites 1 to 4 in Fig. 1B). In all experiments, very short delays were observed between the peak amplitudes of optical signals along the NL. In three out of these eight experiments, the central aspects of the NL were the first areas to be excited and to reach their peak depolarization (cf. recording site 3 in Fig. 1C, left). In this example, the maximal jitter between the peak amplitude of optical signals amounted to 600 μs (gray bar in left panel of Fig. 1C). The remaining five experiments did not show this center-to-periphery excitation pattern. All in all, the mean delay between the diodes that were farthest apart along the NL amounted to 122±60 μs (n=8) when normalized to 100 μm. Furthermore, almost no correlation was found between the delay and distance from the first recording site (Fig. 1D; open symbols; r=0.06).

For the stimulation of contralateral NL inputs, the stimulation electrode was placed on the ventro–medial axon bundles near the NL (Fig. 1A, B). In response to stimulation, a temporal progression of the peak amplitudes of depolarizations was observed in every case (n=8), demonstrating a delay along the ventro–medial to dorso–lateral axis of the NL. Traces on the right hand side in Fig. 1C show a representative recording. The diodes located proximally to the stimulation electrode, that is, those sampling from ventro–medial positions in the NL (recording site 1), were the first to record a depolarization, while dorso–lateral diodes showed depolarization onset and peak amplitudes only after a delay. This delay was observed in all eight experiments with contralateral stimulation, and increased linearly with increasing sampling distance along the NL (Fig. 1D). The mean delay amounted to 536±70 μs (n=8) when normalized to 100 μm. In addition, and in contrast to ipsilateral stimulations, there was a strong correlation at contralateral stimulations between the delay and distance from the first recording site (Fig. 1D; filled symbols; r=0.74). Together, these results demonstrate that the contralateral, yet not the ipsilateral, NM–NL projection forms a delay line confirming previous results (Overholt et al., 1992).

For this contralateral delay line, we calculated the conduction velocity by relating the time difference of the occurrence of peak amplitude (Fig. 1C, right) to the distance between the two most distant recording sites (diodes at recording sites 1 and 4 in Fig. 1B). The mean conduction velocity amounted to 0.21±0.03 m/s (Fig. 1E, right column).

**Development of the velocity within the contralateral delay line**

Our determined conduction velocity along the contralateral delay line of 0.21 m/s at E15 and 21 °C (Fig. 1E) was much slower than the value obtained in a previous electrophysiological study on posthatch chicks (Overholt et al., 1992; 3.6 m/s). Therefore, we assessed the conduction velocity at various embryonic ages (E12, E15, E18) to evaluate the time course of this developmental increase and its possible physiological reasons. At 21 °C, no significant differences in conduction velocity were detected between E12 (0.25±0.02 m/s, n=12) and E15 (0.25±0.03 m/s, n=12; Fig. 2). However, by E18, the conduction velocity had increased with a high significance (0.57±0.09 m/s, n=12, P=0.004; Fig. 2B, cf. example in Fig. 2A).

At 35 °C, conduction velocities were significantly higher than at 21 °C for all ages (Fig. 2B; E12: 0.45±0.05 m/s, n=9; E15: 0.41±0.04 m/s, n=12; E18: 1.28±0.14 m/s, n=9). Conduction velocity did not change between E12 and E15, yet it increased significantly between E15 and E18 (P=2×10^-4). Thus, the developmental changes displayed the same pattern at both temperatures. One should, however, keep in mind that we measured the conduction velocity along a line, rather than a curvilinear, and might therefore have underestimated the conduction velocity. Regardless of this caveat, the data at both temperatures indicate that developmental changes influence conduction velocity not earlier than E15.

To further characterize the delay line, we compared the conduction velocity in the ventro–medial part (diodes at recording sites 1 and 2 in Fig. 1B) with those in the dorso–
lateral part of the NL (diodes at recording sites 3 and 4 in Fig. 1B). At E12 and E15, we found no difference in conduction velocity between these two parts of the NL (Fig. 2C). Surprisingly, the developmental increase in conduction velocity between E15 and E18 was significantly higher in the lateral part of the NL.

Development of the velocity correlates with myelination within the NL

Myelination is correlated with changes in action potential propagation speed and, therefore, may explain the developmental speed-up seen in the conduction velocity along the

Fig. 2. The contralateral delay line is temperature dependent and age dependent. (A) Exemplary traces of the three different age groups (E12, E15, and E18) obtained at 21 °C and 35 °C. The two traces in every test group represent the signals from recording site 1 (lower traces) and recording site 4 (upper traces). Gray bars mark the time difference between the two peak amplitudes (Δt). Given is always the distance between the two recording sites (Δs) and the calculated velocity (v). (B) All three age groups showed a significantly higher velocity at 35 °C (E12: 0.45±0.05 m/s, n=9; E15: 0.41±0.04 m/s, n=12; E18: 1.28±0.14 m/s, n=9) compared to 21 °C (E12: 0.25±0.02 m/s, n=12; E15: 0.25±0.03 m/s, n=12; E18: 0.57±0.9 m/s, n=12). There was no notable difference in the mean conduction velocity between E12 and E15, but a significant increase from E15 to E18 at both temperatures tested (21 °C: P=0.004; 35 °C: P=2×10⁻¹⁰). (C) At 21 °C, no significant differences was found between the medial and the lateral conduction velocity at E12 and E15, whereas at E18, a significantly higher velocity was measured in the lateral part of the NL (medial: 0.42±0.04 m/s, lateral: 0.68±0.11 m/s, P=0.04). In both the lateral and the medial part, significantly higher conduction velocities were found at E18 compared to E15 (medial: E15: 0.25±0.03 m/s, E18: 0.42±0.04 m/s, P=0.006, lateral: E15: 0.24±0.03 m/s, E18: 0.68±0.11 m/s, P=0.002).
contralateral delay line. Consequently, we looked for myelination of the NM axon bundles between E12 and E18. The antibody against myelin-associated proteins (α-MAG) in the chicken auditory brainstem showed only a weak staining at E12 (Fig. 3A, upper image). At both E15 and E18, a clear immunoreactive labeling was visible around the NL. Furthermore, at E18, also the fine axon branches within the NL were clearly labeled (Fig. 3C, left image in lower row). Within the NM, that is between the somata, no (E12; Fig. 3A) or even very rare (E18; Fig. 3C) immunoreactivity was found.

Taken together, the immunohistochemical results clearly demonstrate the progress in myelination within the NM and the NL. These findings are in accordance with our measurements on the development of the conduction velocity within the contralateral delay line.

**Development of the synaptic transmission time of the NM–NL synapse**

To further investigate the properties of the NM–NL projection, we focused on the development of the synaptic transmission time. In most cases, our recordings showed two peaks, with the latter one being Ca$^{2+}$ sensitive and hence representing the postsynaptic excitation (Srinivasan et al., 2004; Fig. 2C). The time between the occurrence of the early presynaptic and the delayed postsynaptic depolarization was defined as the synaptic transmission time. We only included cases in which those two peaks were clearly discernable in our measurements (Fig. 4A). Upon contralateral stimulation, the synaptic transmission time significantly decreased with age (Fig. 4B; from $4.21\pm0.29$ ms at E12, $n=11$, to $2.71\pm0.19$ ms at E15, $n=13$, $P=2\times10^{-4}$, to $2.31\pm0.12$ ms at E18, $n=12$, $P=0.04$, all recordings at 21 °C).

**DISCUSSION**

The auditory brainstem nuclei of the chicken embryo form an easily accessible model system for studies of synapse maturation and development (Boord and Rasmussen, 1963; Rubel and Fritzsch, 2002; Ryugo and Parks, 2003). The existence of a delay line between the NM and the NL, as proposed by Jeffress (1948), has been verified in the medial and the lateral NL via simultaneous field potential recordings (Overholt et al., 1992). Here, we extend these investigations by employing voltage-sensitive dyes to obtain a more detailed description of this delay line and its developmental properties. Four main results were obtained from our measurements: (1) we confirmed the existence of the contralateral and the absence of the ipsilateral delay line in the chicken NM–NL projection; (2) the conduction velocity along the contralateral delay line remains unchanged between E12 and E15, whereas it increases significantly between E15 and E18; (3) the myelination of the NM afferents within the NL correlates with the developmental speed-up of the conduction velocity in the delay line; (4) the synaptic transmission time in the contralateral NM–NL projection decreases significantly between E12 and E18.

**Only the contralateral NM–NL projection forms a delay line**

A series of extracellular field potential recordings with one or two electrodes in 5–10-day-old chicks revealed a delay
line in the contralateral NM–NL projection, yet not in the ipsilateral projection (Overholt et al., 1992). There are two drawbacks of this approach: (1) the activation pattern in the complete NL could not be recorded simultaneously, but had to be inferred from a set of subsequent recordings; (2) only a small subset of NL neurons was used to calculate delays. In contrast, optical recordings in neuronal tissue allow the simultaneous registration of cellular membrane potential changes across a large population of neurons (Löhke et al., 2005; Srinivasan et al., 2004) as well as monitoring of subcellular polarization changes (Stuart and Palmer, 2006). Here, we show that this method is capable of analyzing the spread of excitation throughout the chick NL at sub-millisecond time resolution upon ipsilateral and contralateral stimulation. Our data confirm the earlier findings, obtained with simultaneous field potential recordings in the medial and the lateral NL, of a delay line in the contralateral projection yet not in the ipsilateral NM–NL projection (Overholt et al., 1992; Hyson, 2005).

However, there is a major difference to these earlier studies. The delay times that we found with optical imaging are around 40-fold longer for both the ipsilateral and the contralateral stimulation. After ipsilateral stimulation, we saw an average delay of 122±60 µs (n=8) per 100 µm at 21 °C, while Overholt and coworkers (1992) found a delay of 3.2±2.4 µs (n=3 slices) per 100 µm at 34 °C (values calculated from their data in Fig. 5A–C). Aside from being due to a temperature effect, this difference may result from age differences (Overholt et al.: 5–10-day-old posthatch chicks; this study: E15 embryos), and thus from differences in axon myelination and/or axon diameter.

The age-dependent speed-up in the conduction velocity within the delay line starts at around E15

The outgrowing projections of the NM reach the NL at E8 (Hendricks et al., 2006) and are functional at least from E10 on (Gao and Lu, 2008). Here we investigated the functionality of the contralateral delay line and the development of the corresponding conduction velocity. Interestingly, we found no difference in the conduction velocity between E12 and E15. On the other hand, we detected a significant increase in the conduction velocity between E15 and E18. This developmental pattern between E12 and E18 was observed at 21 °C as well as at 35 °C, with faster velocities at the higher temperature, implying a possible start of the conduction velocity development at around E15. Whereas in post-hatch chicks (P5–P15), conduction velocity amounted to 3.6±0.6 m/s (n=3 slices, values calculated from the data in Fig. 7B–D of Overholt et al., 1992), we observed a strikingly slower value in our E18 animals (v=1.3±0.1 m/s at 35 °C, n=9, Fig. 2B). While the synaptic properties of NL neurons are thought to be mature by E18 (Gao and Lu, 2008), the propagation of action potentials along the NM axons forming the delay line still speeds up between E18 (present study) and P5 (Overholt et al., 1992).

The maximal detectable delay for 5–15-day-old chickens is approximately 180 µs, as calculated from the data of Overholt et al. (1992; at 40 °C, NL length about 1 mm). Our measurements, with an approximate NL length of about 750 µm and a conduction velocity of 1.3 m/s (at 35 °C), predict a maximal detectable interaural delay of 580 µs. Interestingly, for a chicken with a head diameter of about 20 mm, the maximal interaural time difference is around 75 µs (Hyson, 2005). The clue for this discrepancy seems to be the acoustic coupling of the two middle ear cavities through an interaural canal (Hyson et al., 1994). Depending on the frequency of the sound stimuli, the maximum interaural delay is increased up to 180 µs (Hyson et al., 1994). This is in good accordance with the values obtained by Overholt et al. (1992) but still just a third of the maximally possible delay in the NL of the developing E18 chick.

The development of myelination within the delay line of the chicken NL

Our antibody staining with α-MAG indicated only a weak myelination around the NL at E12, yet a strong myelination at E18 (Fig. 3A–C). Myelination within the delay line, that is the fine NM axon branches leading to the terminals inner-
vating NL neurons, was not visible at E12 and E15, but
was observed at E18. Regarding our voltage-sensitive dye
(VSD) findings on conduction velocity, we conclude that
the significant increase between E15 and E18 (Fig. 2B) is
due to the myelination of the fine NM axon branches
leading to the terminals. Recently, the development of the
myelin in the chick auditory brainstem (E10 until E19) was
investigated with antibodies against FluoroMyelin and my-
elin basic protein (MBP, Korn and Cramer, 2008). The
authors observed a continuous increase in the MBP im-
munoreactivity around the NM and NL from E10 until E19,
which is considerably earlier than suggested by our re-

sults. However, the MBP targeted by Korn and Cramer
(2008) consists of a family of four molecules that are
important structural components of myelin (de Ferra et al.,
1985). Compared to MAG, which is located at the interface
between myelin and the axon (Bartsch, 1996), MBP is
more widely expressed in the CNS (Nakahara et al.,
2001). In addition, MBP expression is necessary for the regulation
of MAG (Nakahara et al., 2001). The first MAG expression
might therefore be visible slightly after or with the expres-
sion of MBP. Thus, our data corroborate the findings of
Korn and Cramer (2008). We found that myelination first
occurs along the axons outside of the NL, while the fine
NM axon branches within the NL are initially spared. The
latter become myelinated in a second step after E15. These findings of a general myelination of NM axons,
followed by a myelination of the fine NM axon branches in
the NL (so called delay line myelination) are in accordance
with the two-step development found in the barn owl.
There, first the myelination of NM axons occurs at E18–
E32, followed by the myelination of the delay lines at
P2–P30 (Cheng and Carr, 2007).

Surprisingly, we found a significantly higher conduction
velocity at E18 in the lateral part than in the medial part of
the NL (Fig. 2C), although there is no obvious difference in
MAG staining along the delay line within the NL at this age
(Fig. 3C). Orthogonally to the tonotopic axis, with high
frequencies being represented rostro–medially and low
frequencies caudo–laterally, the NL is arranged in a series
of isofrequency bands (Rubel and Parks, 1975). Depend-
ing on the rostro–caudal position, coronal slices may
therefore comprise NL neurons of different electrophysio-
logical properties and characteristic frequencies, increas-
ing from medial to lateral (Kuba et al., 2005). These differ-
ences may influence the conduction velocity and may
therefore explain the differences in velocity seen between
medial and lateral positions at E18. However, it does not
explain why such a difference in conduction velocity be-
tween medial and lateral cannot be observed at E15 and
E12. Further, Overholt et al. (1992) used coronal slices as
well, without observable differences in conduction velocity
between the medial and lateral NL in 5 to 15 day old
chicken. Currently, we can only hypothesize a temporal
effect of the different isofrequency lines between medial
and lateral that diminishes during development.

Differences in conduction velocity can also be due to
differences in the distance of the nodes of Ranvier as
described for sternachid fishes (Waxman et al., 1972). In
the chicken, NM fibers display internodal distances of
about 160 μm in the crossed dorsal cochlear tract which
shorten to about 55 μm within the NL (Seidl et al., 2010).
This difference, plus an increased axon diameter, speeds
up the conduction velocity of the contralateral NM–NL
projection which compensates for the temporal offset in-
ferred from the different axon lengths of the ipsilateral and
the contralateral NM–NL pathway (Seidl et al., 2010). Such
fine tuning of axon properties could also occur within the
NL and may therefore explain differences in the conduction
velocity between the medial and the lateral part of the NL.

Currently, we have no explanation of why the medial
part of the NL does not show a higher conduction velocity,
as would be expected from the progression of myelination
from ventro–medial to dorso–lateral (Korn and Cramer,
2008, this study). One speculation is that the conduction
velocity is primarily influenced by the myelination of the
fine branches of the NM axon terminals orthogonal to the
NL, and not by the myelination along the NL. It is also
possible that the optical signals from the fine branches
mask the optical signals of the axons along the NL. To
further investigate this problem, VSD measurements at a
higher spatial magnification are needed.

The development of the synaptic transmission time
Srinivasan et al. (2004) found that VSD signals obtained
from auditory brainstem neurons in the rat consist of a pre-
and a postsynaptic component. Here, we show a develop-
mental decrease in the synaptic transmission time in the
Nm–NL projection of the chicken embryo between E12 and
E18. Age-related changes in synaptic transmission time
could arise from changes in transmitter release, receptor
activation and/or changes in the width of the synaptic cleft.
Contradictory to our findings of 2.31 ms (Fig. 4B), Hackett
et al. (1982) reported a time of about 0.45 ms between the
occurrence of the presynaptic volley and the beginning of the
excitatory postsynaptic potential (EPSP). (E18-20,
room temperature, field potential recordings). We reexam-
ined their figures and determined the synaptic transmis-
sion time, that is the delay between the presynaptic volley
and the peak of the postsynaptic response. This resulted in
a value of about 0.9 ms, which is still less than half of the
value we measured with VSDs. Overholt et al. (1992), also
via field potential recordings, found synaptic transmission
times in the same range, even though they used older
animals and worked at higher temperature (34 °C), which
both should considerably shorten the transmission time.
The differences in transmission time between Overholt et
al. (1992) and our study might be explained by the age and
temperature differences, but we cannot explain the discrep-
ancy with the findings of Hackett et al. (1982). However, we
also cannot rule out methodological issues as we have not
compared imaging and field potential data directly.

CONCLUSION

In conclusion, we have shown for the first time that the
delay lines in the auditory brainstem of the chicken embryo
can be visualized with optical imaging methods. Further-
more, our VSD measurements are sensitive enough to assess the maturation of the cellular circuits that govern ITD computation. With the simultaneous imaging of almost the complete NL, we could demonstrate the development of the conduction velocity within the contralateral delay line in the ventro–medial and the dorso–lateral part of the NL. We assessed that the conduction velocity begins to increase at around E15. Together with the immunohistochemical corroboration of myelination, our findings offer new insights into the developmental properties of the NM–NL circuit.

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