Research Article

Heterogeneous Calretinin Expression in the Avian Cochlear Nucleus Angularis

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ABSTRACT

Multiple calcium-binding proteins (CaBPs) are expressed at high levels and in complementary patterns in the auditory pathways of birds, mammals, and other vertebrates, but whether specific members of the CaBP family can be used to identify neuronal subpopulations is unclear. We used double immunofluorescence labeling of calretinin (CR) in combination with neuronal markers to investigate the distribution of CR-expressing neurons in brainstem sections of the cochlear nucleus in the chicken (*Gallus gallus domesticus*). While CR was homogeneously expressed in cochlear nucleus magnocellularis, CR expression was highly heterogeneous in cochlear nucleus angularis (NA), a nucleus with diverse cell types analogous in function to neurons in the mammalian ventral cochlear nucleus. To quantify the distribution of CR in the total NA cell population, we used antibodies against neuronal nuclear protein (NeuN), a postmitotic neuron-specific nuclear marker. In NA neurons, NeuN label was variably localized to the cell nucleus and the cytoplasm, and the intensity of NeuN immunoreactivity was inversely correlated with the intensity of CR immunoreactivity. The percentage of CR+neurons in NA increased from 31 % in embryonic (E)17/18 chicks, to 44 % around hatching (E21), to 51 % in postnatal day (P) 8 chicks. By P8, the distribution of CR+neurons was uniform, both rostrocaudal and in the tonotopic (dorsoventral) axis.

Immunoreactivity for the voltage-gated potassium ion channel Kv1.1, used as a marker for physiological type, showed broad and heterogeneous postsynaptic expression in NA, but did not correlate with CR expression. These results suggest that CR may define a subpopulation of neurons within nucleus angularis.

Keywords: calretinin, NeuN, cochlear nucleus, avian, calcium binding protein, Kv1.1, potassium channel

Abbreviations: NA – Nucleus angularis; NM – Nucleus magnocellularis; NL – Nucleus laminaris; CR – Calretinin; PV – Parvalbumin; CaBP – Calcium-binding protein; NeuN – Neuronal nuclear protein; MAP2 – Microtubule-associated protein 2; IC – Inferior colliculus; ICc – Central nucleus of the inferior colliculus; MLd – Nucleus mesencephalicus lateralis pars dorsalis (IC); ITD – Interaural time difference; ILD – Interaural level difference; E – Embryonic (day); P – Postnatal (day)

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INTRODUCTION

In the avian auditory brain stem, acoustic timing and intensity cues are processed in separate ascending pathways, then reconverge in the midbrain to generate a map of auditory space (Carr and Code 2000; Knudsen and Konishi 1978a, 1978b; Konishi et al. 1985, 1988; Moiseff and Konishi 1983; Sullivan and Konishi 1984; Takahashi et al. 1984). The two

anatomically distinct cochlear nuclei, nucleus magnocellularis (NM) and nucleus angularis (NA), each receive information from the auditory nerve and specialize for encoding timing information (NM) and intensity, or sound level, information (NA) for the computation of sound location (Boord 1968; Carr and Boudreau 1991; Parks and Rubel 1978; Puelles et al. 2007; Reyes et al. 1994; Sullivan and Konishi 1984; Trussell 1999). Nucleus angularis is highly heterogeneous in terms of neuronal morphology, acoustic response types in vivo and intrinsic physiology in vitro, and has multiple ascending projections, suggesting that it performs multiple functions (Carr and Soares 2002; Fukui and Ohmori 2003; Häusler et al. 1999; Krützfeldt et al. 2010a; MacLeod and Carr 2007; Sachs and Sinnott 1978; Sato et al. 2010; Soares and Carr 2001; Soares et al. 2002; Wang and Karten 2010; Warchol and Dallos 1990). The present study was undertaken to better define the molecular characterization of neurons in NA.

The auditory brain stems of birds and mammals have been characterized by high expression levels of three calcium-binding proteins (CaBP): calretinin, calbindin, and parvalbumin (Braun 1990; Braun et al. 1985, 1991b; Celio and Heizmann 1981; Friauf 1994; Kubke et al. 1999; Lohmann and Friauf 1996; Parks et al. 1997). The calcium buffering capability of these CaBPs may play a neuroprotective role against high activity levels or degeneration during hearing loss (Camp and Wijesinghe 2009; D'Orlando et al. 2002; Idrizbegovic et al. 2006; Parks et al. 1997). In the avian brain stem, neurons in the timing pathways are characterized by calretinin (CR) immunoreactivity, which also demarcates the terminal fields of these pathways in the midbrain (Takahashi et al. 1987). In the intensity pathway, a subpopulation of neurons in NA was also shown to express CR immunoreactivity, but it was absent or weak in the terminal fields of the projection from NA to the midbrain, which were nonoverlapping in barn owl and chick (Takahashi and Konishi 1988; Wang and Karten 2010). In the mammalian brain, multiple CaBPs show complementary patterns of expression within and across auditory brainstem nuclei and have been associated with specific subsets of cell types (Förster and Illing 2000; Fredrich et al. 2009; Lohmann and Friauf 1996).

The chick cochlear nuclei exclusively express CR, but not parvalbumin or calbindin (Rogers 1989a), but whether CR was associated with specific subtypes of auditory neurons is unknown. Calretinin immunoreactivity was found in the neuropil, presumably in the axon terminals of the eighth nerve, but was also localized to the cytoplasm of cell bodies in NA and NM (Kubke et al. 1999; Parks et al. 1997). Over development, progressive CR expression coincides with the tonotopic axes in NA, NM, and NL. In NA,

the tonotopic axis has been shown to be oriented dorsoventrally, with high frequencies represented dorsally and low frequencies ventrally (Köppl 2001; Warchol and Dallos 1990). Neuropil and cellular immunolabeling appeared as early as E11 in the chick, but was restricted to the dorsal extreme. Over the course of development, the CR immunolabeling progressed from dorsal to ventral, reaching the ventral extreme by postnatal day (P) 1. Over development, the immunolabeling also became progressively less intense in the neuropil and more intense in the cell body. Previous studies used single antibody approaches and were performed prior to characterization of the different cell types in NA based on morphological analysis (Soares et al. 2002; Soares and Carr 2001). Therefore, the question of whether CR labeling was ubiquitous among the neuronal cell types in NA was not investigated, although the immunolabel intensity of the immunoreactivity was noted as heterogeneous. To address this question, we performed double label immunofluorescence and confocal microscopy to allow a quantitative assessment of the distribution of CR-expressing neurons in NA in conjunction with pan-neuronal markers neuronal nuclear protein (NeuN) and microtubule-associated protein 2 (MAP2). Calretinin clearly immunolabeled a subpopulation of neurons in NA, with ~30 % of the total neuronal population showing CR immunoreactivity at embryonic day 17/18. By postnatal day (P) 8, the proportion of CR+neurons had increased but was still limited to half of all neurons and these neurons were uniformly distributed. We conclude that CR may be a useful marker for a subpopulation of neurons in NA and further study is needed to establish a relationship to specific cell type and functional class.

MATERIALS AND METHODS

Subjects

Experiments were carried out on White Leghorn chickens (*Gallus gallus domesticus*) purchased from Charles River SPAFAS (Lebanon, CT). Tissue was obtained from chickens of either sex on embryonic day (E) 17–18 and E21 and postnatal day (P) 8. All animal procedures were approved by the University of Maryland Institutional Animal Care and Use committee and followed NIH guidelines.

Antibodies

The antibodies used in the present study were monoclonal mouse anti-NeuN (Millipore Cat#MAB377; clone A60), monoclonal mouse anti-MAP2 (Millipore Cat #MAB3418), polyclonal rabbit anti-Kv1.1 (Alomone Labs Cat#APC-009), monoclonal

mouse anti-Calretinin (Swant Cat#6B3), and polyclonal rabbit anti-Calretinin (Swant Cat#7699/4H) (Table 1).

Calretinin antibody specificity

The anti-calretinin antibodies were obtained from Swant (Bellinzona, Switzerland). Calretinin is an EF-hand calcium-binding protein whose distribution and localization have been well characterized. The rabbit-derived polyclonal antibody has been used extensively to characterize the auditory pathways in mammalian and several avian species including chicken (Rogers 1989; Kubke et al. 1999; Parks et al. 1997; Fredrich et al. 2009). The mouse monoclonal antibody was obtained by immunizing mice with recombinant human calretinin-22 k and its specificity verified by immunoblot in chickens according to the product information sheet provided by Swant. Both antibodies react with calretinin but not other calcium-binding proteins (i.e., parvalbumin or calbindin). Anti-CR label has been extensively characterized in chick, finch, and barn owl tissue, and our results qualitatively comport with published descriptions on labeling in the chick auditory brain stem.

NeuN antibody specificity

The NeuN antibody was a monoclonal antibody raised against the purified cell nuclei from mouse brain and originally characterized by Mullen et al. (1992), who showed the anti-NeuN was reactive in most neuronal cell types across a wide range of vertebrate tissue including chicken tissue. NeuN antibody was obtained from Millipore (MAB377; clone A60). Similar cellular expression profiles have been described in mouse and chicken, including a common lack of label in Purkinje cells of the cerebellum (Mezey et al. 2012; Weyer and Schilling 2003). Specificity has been verified with Western blots using chicken protein extracted from whole brains which showed specific bands at estimated molecular weights of 46 and 48 kDa (Mezey et al. 2012), similar to mouse (Lind et al. 2005). NeuN has been used extensively to label postmitotic neurons in developmental studies in chick (Mezey et al. 2012; Perez et al. 1999; Zeng et al. 2008) and in a study of cochlear nucleus neurons in mice (Mostafapour et al. 2002).

Kv1.1 antibody specificity

The Kv1.1 antibody was a polyclonal rabbit anti-Kv1.1 obtained from Alomone Labs (Cat#APC-009). The antigen was a GST fusion protein corresponding to amino acid residues 416–495 of mouse Kv1.1 (accession P16388). Immunohistochemistry using antibody has been described previously in chicken brain stem tissue (Fukui and Ohmori 2004; Popratiloff et al. 2003). Our staining using enzymatic methods in NM and NL was consistent with published descriptions. When primary antibodies were preincubated with the supplied fusion protein, immunolabeling was not detected in the brain stem tissue (Fukui and Ohmori 2004; Popratiloff et al. 2003).

MAP2 antibody specificity

The MAP2 antibody was monoclonal anti-MAP2 from Millipore (clone AP20) that was raised in mouse using the bovine brain microtubule protein, cross reacts with chicken protein, and been shown to densely label cell bodies and primary dendrites of neurons in chicken brain stem (Shao et al. 2009; Wang and Rubel 2008).

Tissue preparation and immunohistochemistry

After cooling eggs several minutes at -20°C , E17–18 embryos were extracted and deeply anesthetized with pentobarbital IM. The E20–21 and P8 chickens were deeply anesthetized with isoflurane followed by IM injection pentobarbital. The embryos and hatchlings were then perfused transcardially with 0.9 % saline and heparin, followed by 4 % paraformaldehyde (PF) in 0.01 M phosphate buffered saline (PBS, pH 7.4). Brains were postfixed for 1–2 days, then cryoprotected in 30 % sucrose in PBS at 4°C overnight. Serial sections in the transverse plane were cut on a freezing microtome at 40- μm thickness and collected in PBS.

Free-floating sections containing the cochlear nucleus angularis, laminaris, and magnocellularis were washed in PBS then incubated in 10 % normal serum in 0.01 M PBS plus 0.5 % Triton X-100 for 1 h. Primary antibody solutions consisted of the appropriate antibody diluted in PBS containing 2.5 % normal

TABLE 1
Antibody characterization

<i>Antibody</i>	<i>Immunogen</i>	<i>Manufacturer and type</i>
Calretinin	Recombinant human calretinin	Swant 7699, rabbit polyclonal
Calretinin	Recombinant human calretinin	Swant Cat#6B3, mouse monoclonal
NeuN	Purified cell nuclei from mouse brain	Millipore Cat#MAB377; clone A60, mouse monoclonal
Kv1.1	Mouse Kv1.1 fusion protein (aa 416–495)	Alomone Labs Cat#APC-009, rabbit polyclonal
MAP2	Bovine brain MAP2	Millipore Cat #MAB3418, mouse monoclonal

serum plus 0.5 % Triton X-100. Sections were incubated in the primary solution for 1 h at room temperature then overnight at 4 °C. Dilution series were carried out to determine the effective concentrations used in this study (Table 2). Control sections were incubated in the solution containing normal serum where primary antibodies were omitted.

Visualization in single antibody experiments was achieved by enzymatic methods using biotinylated secondary antibodies (goat anti-rabbit or goat anti-mouse; Invitrogen) and the avidin–biotin–peroxidase technique. After incubation with secondary antibody solution (1:200) at room temperature for 1 h, sections were treated with a hydrogen peroxide solution (1 % H₂O₂ and 10 % methanol in PBS) for 10 min to eliminate endogenous peroxidase activity, then incubated in avidin/biotin/peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA) for 45 min. Sections were rinsed several times in PBS, and then the reaction product was visualized with 3,3-diaminobenzidine peroxidase substrate solution, intensified with nickel (DAB peroxidase Substrate Kit, Vector Laboratories). Sections were washed, mounted on gel-coated slides, dried overnight, and cover slipped with Cytoseal mounting medium (Thomas Scientific, Swedesboro, NJ).

For immunofluorescent labeling, sections were washed and incubated as described above in solutions simultaneously containing two primary antibodies raised in different host species in the combinations listed in Table 2. After rinsing in PBS, sections were incubated with the solution containing two secondary antibodies tagged with different fluorophores (with excitation wavelengths at 488 or 594 nm) diluted in PBS plus 0.5 % Triton X-100 for 2 h at room temperature. The secondary antibodies that were used were as follows: Alexa Fluor 488 donkey anti-rabbit IgG (Invitrogen CAT#A21206); Alexa Fluor 594 donkey anti-mouse IgG (Invitrogen Cat#A21203); DyLight 488 donkey anti-mouse (Jackson ImmunoResearch, Cat# 711-485-150); and DyLight 594 donkey anti-rabbit (Jackson ImmunoResearch, Cat#711-515-152). Sections were rinsed, mounted, and cover slipped immediately with Prolong Gold

containing DAPI mounting medium (Invitrogen Cat# P36935). Specificity of the secondary antibodies was verified by running controls in which one of the two primary antibodies were omitted. In these cases, no label was observed that related to the omitted primary species antibody. Immunolabeling patterns of the antibodies used in double labeling were compared with results obtained using single primary antibodies and visualized by enzymatic or fluorescent methods and found to be consistent, suggesting that the double immunolabeling did not result in nonspecific cross labeling.

Double immunofluorescence experiments were carried out on 13 embryonic and hatchling chickens: 8 animals for anti-CR/anti-NeuN (3 E17/18; 2 E21; 2 P8), 2 animals for anti-CR/anti-Kv1.1, and 3 animals for anti-Kv1.1/anti-MAP2.

Confocal microscopy and analysis

Images of immunofluorescently labeled sections were taken on a Zeiss LSM 710 confocal microscope using a×40 or×63 oil immersion lens. DAPI, FITC, and rhodamine lasers were optimized for digital offset and master gain to produce best images. Serial z-stack images (optical sections of 0.48–0.52 μm) were acquired encompassing the upper and lower limits of the tissue section. For each NA, four to five adjacent images were acquired to be later stitched together as a composite.

Quantitative analysis was done by importing multiple Zeiss LSM z-stack files to form a composite 3-D image in the Stereo Investigator software platform (MicroBrightField Inc.). A contour was drawn by eye around the perimeter of nucleus angularis. Dorsal, middle, and ventral subsections were defined by finding the long axis in the approximate dorsoventral orientation and dividing the nucleus into three subsections (bounded by NA perimeter) along equal points along this axis (see Fig. 4A). Cell density was defined as the number of counted neurons per subsection/nucleus area. Analysis comparing dorsal, middle, and ventral subsections was limited to the central portion of the rostrocaudal extent of NA that

TABLE 2
Double immunofluorescence experiments

Experiment	1 ° Antibodies	Dilution	Age(s)
1	Rabbit polyclonal α-CR	1:1000	E17/18
	Mouse monoclonal α-NeuN	1:500	E21 P8
2	Mouse monoclonal α-CR	1:1000	E21
3	Rabbit polyclonal α-Kv1.1	1:1000	E21
	Rabbit polyclonal α-Kv1.1	1:1000	
	Mouse monoclonal α-MAP2	1:200	

had a characteristic bean-like shape with full dorso-ventral extent (see Fig. 3). Focusing through the depth of the section, immunoreactivity and colocalization were assessed in the cell bodies of each neuron relative to background fluorescence. Total neuronal population consisted of the summed numbers of neurons that were judged to be CR+and/or NeuN+. While most of final images shown are 2-D maximum projections, cross sections through the centers of neuronal cell bodies and nuclei were assessed during counting to avoid erroneous colabeling judgments due to overlap of the fluorescence label in the *x-y* plane. Luminance analyses were performed using the Neurolucida software package from MicroBrightField, Inc. To quantify luminescence of individual neurons, single optical cross sections were used. For the CR versus NeuN luminance measures, the brightest calretinin and NeuN neurons were selected within a single section of NA. For the Kv1.1/MAP2 double labeled tissue, the MAP2 fluorescence delineated the extent of an individual cell body and was used to draw a perimeter contour; the Kv1.1+ immunolabel was then measured within this contour. Images were adjusted for brightness and contrast and assembled for figures in Adobe Photoshop (Adobe Systems). All values reported as mean $\pm$ s.d. unless otherwise indicated.

RESULTS

NeuN expression in the avian auditory brainstem

In order to identify and count the neuronal population in NA, the pan-neuronal molecular marker NeuN was used (Mezey et al. 2012; Mullen et al. 1992; Weyer and Schilling 2003). NeuN label was widely observed throughout the chick brain stem, with abundant labeling in the auditory areas NA and NM, as well as the target nucleus for NM involved in interaural timing difference coding, nucleus laminaris (NL; Fig. 1A). We observed strong subcellular labeling in the nuclei of all NM and NL neurons (Fig. 1C, D). In contrast, the NeuN expression in NA was heterogeneous, with some neurons showing only nuclear labeling while others showed both nuclear and cytoplasmic labeling (Fig. 1B). For the quantitative cell counts in this paper, anti-NeuN fluorescent label that was localized to the cell nuclei, cytoplasm, or both and had a brightness above background was considered positive.

Calretinin identifies a subpopulation of neurons in NA

To address the specificity and heterogeneity of CR expression, we sought to determine (1) the propor-

FIG. 1. NeuN immunohistochemistry labeled neurons in the avian auditory brainstem. **A** Low power image of dorsal quadrant of a transverse section containing NA, NM, and NL. Margins of NA are outlined; boxes in **A** indicate location of higher power images of NA (**B**), NM (**C**), and NL (**D**). The subcellular localization of the NeuN label

differed across the three nuclei, with NeuN immunolabel localized to the cell nucleus in NM and NL neurons, but variably immunolabeling the cytoplasm in addition to the cell nucleus in NA neurons. Scale bars: 200 μ m (**A**), 50 μ m (**B–D**). Enzymatic method (DAB) in an E21 chick.

tion of NA neurons that express CR and (2) the distribution of CR-positive neurons within the nucleus and across development.

We used double label immunofluorescence using primary antibodies against NeuN and CR. Sections were counterstained with DAPI. Confocal z-stack images of the auditory nuclei were assembled into composite images from individual fields (Fig. 2A). We analyzed the brain stems of E17/E18 ($n=3$) and E21 ($n=3$) chick embryos and P8 juvenile chickens ($n=2$). For each animal, a complete rostrocaudal series of 40- μ m transverse sections were processed (Fig. 3). Neurons were determined to be either NeuN+only (CR-), double labeled for both (NeuN+/CR+) and, rarely, CR+only (NeuN-) (Fig. 2B).

CR+immunoreactivity was observed in many cell bodies in NA, extending into primary dendrites, as well as in processes throughout the neuropil. The presence of postsynaptic labeling was confirmed by optical sectioning through the cell bodies and observing consistent cytoplasmic staining throughout, showing that CR label in NA was not limited to CR+terminals but was also expressed in many of their neuronal targets. Consistent with previous reports, anti-CR strongly labeled NM cell bodies (Fig. 2C) and more weakly labeled NL cell bodies (not shown). In NA, CR expression was clearly heterogeneous, with many NeuN+neurons lacking any detectable CR (Fig. 2B, arrowheads, NeuN+/CR-). NA neurons that were CR+showed a broad range of label strength. The overwhelming majority of CR-positive cell bodies also were NeuN-positive (NeuN+/CR+). In addition, a small number of CR+cells lacked NeuN expression, despite other-

wise appearing like neurons in size, shape, and primary processes and resembling other nearby CR+/NeuN+cells (Fig. 2B, arrow, NeuN-/CR+). These CR+/NeuN-cells suggest that NeuN does not label all neurons in the brainstem. In NM, CR expression also tended to be strongest under the cell membrane, confirming the subcellular localization reported by Hack et al. (2000) (Fig. 2C). CR expression in NA tended to be more distributed throughout the cytoplasm and was not excluded from the cell nucleus.

To quantify the expression of CR in NA, we analyzed the overall incidence of CR expression (all CR+/total numbers across all sections), as well as the pattern of CR expression in the rostrocaudal and dorsoventral extent of NA (Fig. 3A, B). Outline contours containing NA were drawn and complete counts were made of the NA neurons that were positive for each label (for an example, see Fig. 4A). The percentage of NA neurons that were CR+varied with developmental age: 31 % at E17/18, 44 % at E21, and 51 % at P8 (Fig. 4B). For E21 animals, the percentage of CR+did not differ along the rostrocaudal dimension (Fig. 3C). These numbers demonstrated that CR was expressed in a subset of neurons in NA and that the heterogeneity of expression persisted in older animals.

Distribution of CR+neurons in NA along the tonotopic axis

To determine the distribution of CR as a percentage of the total neuronal number, we made cells counts by dividing NA into three subsections: (dorsal, middle, and ventral) and defined cell density as the number of

FIG. 2. Double fluorescence immunohistochemistry of CR (red, A i) and NeuN (green, A ii) and combined image (A iii). **B–E** Higher power image showing differences in cytoplasmic label of CR and NeuN using double immunofluorescence in NA (**B–D**) and NM (**E**). Only a subset of all NeuN+neurons was CR+(double label NeuN+/CR+, asterisks). Some NeuN+neurons had no discernable or very weak CR label (NeuN+/CR-, arrowheads) and tended to have stronger

cytoplasmic NeuN label. A small number of CR+cells had no discernable NeuN label but were identified as neurons based on size and shape (NeuN-/CR+, arrows). **E** NM neurons were homogeneously labeled by NeuN and calretinin. NeuN label was localized to the nucleus, while CR appeared localized to the cell membrane. Scale bars: 100 μ m (A iii), 20 μ m (**B–E**).

FIG. 3. **A)** Calretinin immunolabel in NA at E21 showed a dorsoventral gradient, but no rostrocaudal gradient. Caudorostral sequence of sections from same case from left to right, every third section (interval between sections shown: 120 μ m). NA borders are shown in outline. Scale bar: 200 μ m. **B)** Matched higher power images from dorsal (top row) and ventral (bottom row) areas from sections in A. Scale bar: 50 μ m. **C)** Distribution of fraction of CR+ positive neurons in rostrocaudal direction showed no gradient of

expression. Paired Student's *t* tests carried out on caudal, middle and rostral segments showed no significant differences: caudal vs middle, $p=0.26$, middle vs rostral, $p=0.43$, caudal vs rostral, $p=0.054$, $n=3$). Caudal: pooled sections 3 and 4; middle: pooled sections 7 and 8; rostral, pooled sections 10–11. Rostral and caudal extremes excluded due to variation in shape that limits comparisons and confounding effects of dorsoventral gradients (e.g., caudal section #1 in A).

neurons per subsection area. While there was little variation in %CR+ across comparable rostrocaudal sections (Fig. 3), there was a clear dorsoventral gradient of expression in younger animals (Fig. 4C, E). For E17/18 animals, CR+ cell density (Fig. 4C) and %CR+ (Fig. 4E) were highest in dorsal sections, and lowest in ventral sections. Total cell density, in contrast, showed a slightly higher density in the ventral sections relative to the dorsal (Fig. 4D). Similar patterns were seen with section from E21 chick. In P8 animals, in contrast, there was no gradient in CR+ cell density

dorsoventrally and a much weaker gradient in terms of %CR+. This is consistent with previous work that showed that with maturity, the CR+ expression gradient disappears in both chick and barn owl (Kubke et al. 1999; Parks et al. 1997). However, even in P8 animals and in the dorsal-most sections, the percentage of CR+ neurons was modest (56 %). These data suggest that in older animals, CR was expressed in a persistent subpopulation that was roughly evenly distributed dorsoventrally, rostrocaudally, and relative to the tonotopic axis in NA.

FIG. 4. Quantification of the distribution of calretinin expression along the dorsoventral axis and during development. **A)** Distribution of labeled neurons in one NA section shown in outline. Each section was divided into thirds along the long dorsoventral axis for cell counts. **B)** Overall proportions of CR+ and CR-neurons at three ages showed an increasing proportion of neurons as CR+ to a maximum ~55 % by P8. Total cell counts from a representative 4–5 sections per animal were: 2111, 1855, and 1559 from 3 animals aged E17/18; 1,119 and 1,584 (left, right sides in one animal; 2738 and 2309 (left, right sides in second animal) and 2303 from 3 animals aged E21; 2069, 1772 from two animals aged P8. For analysis in C–F, data from left and right sides were averaged within subjects. **C)** Density distribution of CR+neurons by age and dorsoventral subsection.

Dorsoventral gradient declined with age (E17/18 versus P8). **D)** Density distribution of all neurons shows a reverse gradient (more dense ventral) that declined with age. **E)** Distribution of CR+neurons expressed as a percentage of total number of neurons. Dorsoventral gradient declined with age. **F)** Neuropil areas were measured for the defined dorsal, middle, and ventral subsections (denominator for cell density measures). Subsection area showed that the nucleus size increased slightly, but not significantly, with age. All values in C–E are mean $\pm$ s.d. Statistical comparisons made using 2-way ANOVA ($\alpha=0.05$) and Tukey's posthoc multiple comparisons test; asterisks indicate differences ($p<0.05$).

Inverse relationship between CR and NeuN expression

In NA, the full range of NeuN expression was widely variable (e.g., Fig. 2C). Neurons with the strongest CR label were also observed to have the weakest NeuN label, and vice versa. To quantify the relationship of CR and NeuN expression, luminance measurements were made by selecting the CR+neurons with the visually brightest immunolabel (subjectively assessed) and measuring their respective NeuN immunolabel. Conversely, we selected the neurons with the strongest cytoplasmic NeuN immunolabel, and measured their respective CR immunolabel. We observed a strong negative correlation between luminance of CR and NeuN, suggesting complementary regulation and/or restricted localization of the NeuN signal to the nucleus in the presence of high levels of CR (Fig. 5A). Neurons with strong cytoplasmic NeuN immunolabeling and weak or absent CR immunolabeling ($n=22$) were smaller than neurons with strong CR immunolabel ($n=35$; $p<0.0001$, Student's *t* test; Fig. 5B).

Heterogeneous label for Kv1.1 throughout NA

We have shown a restricted expression of CR to a subset of NA neurons and that these neurons were distributed throughout NA in older animals. This pattern suggests that CR may define an identifiable subpopulation, as appears to be the case in the mammalian cochlear nucleus (see “DISCUSSION”) (Bazwinsky et al. 2008; Fredrich et al. 2009; Lohmann and Friauf 1996). To better define the CR subpopulation in NA, we investigated the expression of Kv1.1 in NA using double immunofluorescent labeling of anti-Kv1.1 along with anti-MAP2 (Fig. 6).

Immunolabel for Kv1.1 was broad and highly heterogeneous in NA (E21 chick embryo; similar label has been seen in P14 chicks, data not shown, Parameshwaran and Carr personal communication). Anti-Kv1.1 labeled both cell bodies and processes within the NA neuropil. The potassium channel marker label was weak in many cases but a few brightly labeled, large neurons in NA were observed (Fig. 6A, B). In the same sections, neurons of the NM

FIG. 5. Luminance measurements from double immunofluorescence showed an inverse correlation between CR+ and NeuN+ label strength ($n=57$; $R^2=0.39$). Individual neurons were selected based on having either strongest calretinin label (open squares) or strongest NeuN label (gray squares). Cell bodies were drawn as the region of interest, and then luminance values were taken for each corresponding fluorescent label and plotted against

each other (A). Cell selection was blinded for the opposite fluorophore. **B** Cell body size showed a significant effect when strong NeuN+ (NeuN luminance >10 ; $n=22$) were compared to strong CR+ (NeuN luminance <10 ; $n=35$; $p<0.0001$; Student's *t* test). All measurements were taken from fields that contained both strong NeuN+ and strong CR+.

were strongly labeled (Fig. 6C) and less variable. To quantify whether Kv1.1-like expression was more variable in NA than in NM, we measured single-cell luminance values of Kv1.1 label in NA neurons and NM neurons from matched sections. Average luminance values were significantly higher in NM than in NA (NM luminance median was 8.8, $n=71$; NA luminance median was 2.8, $n=70$) and only 14.3 % of NA neurons met or exceeded the 25th quartile luminance values for NM (mid-quartile range: 7.4–10.4; significantly different distributions, Kolmogorov-Smirnov test, $p<0.0001$). Luminance values in NA spanned a significantly broader range than those in NM (NM coefficient of variation (CV) was 0.25; NA CV was 0.88;) (Fig. 6D, inset). Cell body sizes for brighter Kv1.1-positive neurons were larger than those of weaker Kv1.1-positive neurons. Anti-MAP2 and anti-Kv1.1 co-labeled cell bodies, but their expression generally did not overlap in the processes, such as in the proximal dendrites invaded by MAP2 label. We commonly observed many thin processes in NA labeled with anti-Kv1.1 that were negative for MAP2 (see [Supplemental Video](#) clip), suggesting that Kv1.1 is present along many axonal processes in NA as well.

Most CR-positive neurons in NA co-label with Kv1.1

To determine whether CR expression was correlated with Kv1.1 expression, we performed double immunofluorescence experiments for the two markers. These experiments were performed in E21 chicks, so we restricted our analysis to the dorsal third of NA. Based on the MAP2/Kv1.1 double label results presented above, we found, not surprisingly, that most

CR+neurons (>90 %) were co-positive for Kv1.1 (Fig. 7A), as well as a population of Kv1.1+ neurons that were negative for CR (not shown). To determine whether the strength of CR and Kv1.1 label were correlated, we made single-cell luminance measurements of brightly labeled CR and Kv1.1 using the same procedure as above for CR and NeuN labeling (Fig. 7B). The Kv1.1 label varied continuously as did the CR label, with a weak negative correlation. Brightly labeled Kv1.1+ neurons were significantly larger than the brightest CR+neurons (Fig. 7C). In summary, we found that Kv1.1 labeling did not define a clear subpopulation of neurons in NA, and was not congruent, co-regulated, or excluded from CR-positive neurons in NA.

DISCUSSION

In an effort to molecularly characterize the subtypes of neurons in the avian cochlear nucleus angularis, we used double immunofluorescence and confocal microscopy to make a quantitative assessment of calretinin-expressing neurons in NA and their distribution. To quantify the proportion of CR-labeled neurons in the total neuronal population, we co-labeled with a pan-neuronal marker, NeuN. Slightly more than 50 % of NA neurons were immunopositive for CR by postnatal day 8. By this age, there was also no significant gradient in the proportion of CR-positive neurons in the dorsoventral, or tonotopic, orientation. These data suggest that calretinin may be a useful marker for a nearly uniformly distributed subpopulation of neurons within NA. The morpho-

◀ **FIG. 6.** Kv1.1 immunolabeling was weaker and more variable among NA neuron cell bodies than among NM neurons. Co-label of MAP2 (green) versus Kv1.1 (red) in NA (**A**, low power; **B**, high power of same section) and in NM (**C**). MAP2 luminance was similar in NA (**Ai**, **Bi**) and NM (**Ci**), but Kv1.1 luminance was overall lower and showed greater variation in NA (**Aii**, **Bii**) than in NM (**Cii**). All panels from the same section and imaged with identical parameters. Right panels (**Aiii–Ciii**) overlay MAP2 and Kv1.1 expression. Confocal images are maximum intensity projections. **D** Cumulative distribution of luminance values measured from individual cell bodies showed significant differences between NA ($n=70$) and NM ($n=71$; $p<0.0001$, two-sample Kolmogorov-Smirnov test). Cell bodies were identified with MAP2. Shaded boxes indicate the range of background neuropil label in NA (dark red: 1 s.d., light red, 2 s.d.). Inset: To compare distributions, luminance relative to the median value of each nucleus was plotted, showing a longer tail and greater variation in the NA luminance data.

logical and functional identification of this subpopulation remains to be determined.

Calretinin and parallel processing for sound localization

CR is one of a family of EF-hand CaBPs, along with parvalbumin and calbindin, that are highly expressed in neurons in the auditory brain pathways (Braun 1990; Braun et al. 1985, 1991a; Celio and Heizmann 1981; Celio et al. 1996; Friauf 1994; Kubke et al. 1999; Lohmann and Friauf 1996; Parks et al. 1997; Rogers 1989b). CaBPs have proved to be a useful marker for distinguishing the parallel pathways for sound source

localization in the avian and mammalian brain stem. Parallel pathways for auditory processing have been most clearly identified in the barn owl, but with many commonalities with the chicken and other avian species (Carr and Code 2000). In vivo and behavioral data from the barn owl have shown that cues for interaural time differences (ITD) and interaural intensity (or level) differences (ILD) underlie the binaural processing of sound location (Konishi 1973; Moiseff and Konishi 1981; Payne 1971). Timing and intensity cues are processed in separate ascending pathways beginning with the cochlear nuclei (Knudsen and Konishi 1978a, 1978b; Konishi et al. 1985, 1988; Moiseff and Konishi 1983; Sullivan and Konishi 1984; Takahashi et al. 1984). The avian cochlear nuclei, NM and NA, receive information from the auditory nerve (Boord and Rasmussen 1963; Carr and Boudreau 1991; Häusler et al. 1999; Krützfeldt et al. 2010b; Parks and Rubel 1978; Puelles et al. 2007; Soares and Carr 2001). NM specializes in encoding fine timing cues and projects to the binaural nucleus responsible for computing ITD, NL (Carr and Konishi 1990; Fukui et al. 2006; Hackett et al. 1982; Koyano et al. 1996; Nishino et al. 2008; Raman et al. 1994; Reyes et al. 1994; Smith 1981; Trussell 1999; Zhang and Trussell 1994). Sound intensity cues for interaural level differences are processed first in NA which projects to the binaural nucleus responsible for computing ILD (Adolphs 1993; Manley et al. 1988; Sato et al. 2010; Sullivan and Konishi 1984; Takahashi et al. 1984). Both NA and NL send a direct projections

FIG. 7. Kv1.1 expression overlapped with calretinin expression. **A** Double immunofluorescent labeling for calretinin (red) and Kv1.1 (green) showed a large overlap in expression in NA (combined). **B** Within KV1.1+/CR+ co-positive population, luminance measure-

ments revealed an inverse relationship ($n=24$, $R^2=0.29$). **C** The brightest Kv1.1+ neurons ($n=16$) tended to be larger than the brightest CR+ in the same field ($n=8$; $p=0.0001$, Student's *t* test).

to the midbrain, where they form nonoverlapping terminal fields in the central nucleus of the inferior colliculus (ICc) in chick and barn owl (Conlee and Parks 1986; Krützfeldt et al. 2010b; Takahashi et al. 1987; Wang and Karten 2010).

Some commonalities also arise in the patterns of CaBP expression in these auditory circuits across studies in barn owl, chick, zebra finch, and emu. In the brain stem nuclei, similar adult patterns of CR expression have been established in barn owl and chick, with strong labeling in the timing nuclei, NM and NL, and also in a subset of neurons in NA (Hack et al. 2000; Kubke et al. 1999; Parks et al. 1997; Takahashi et al. 1987). Both parvalbumin and CR were expressed in all three nuclei in emu (Kubke and Carr 2006; MacLeod et al. 2006), with heterogeneous expression in cell bodies in NA (MacLeod, Soares & Carr, unpublished observations). In the ascending parallel pathways in the barn owl, a CR-like immunoreactive fiber plexus was most strongly associated with the projections of NL in the lemniscus and the NL terminal field in the ICc, and was far weaker or absent in the projection areas of NA and the NA terminal field in the ICc (Kubke et al. 1999; Takahashi et al. 1987; Wagner et al. 2003). It seems likely that this pattern associating CR with the NL projection to midbrain is recapitulated in the chick (Puelles et al. 1994; Wang and Karten 2010), but studies combining tract tracing and immunolabeling are needed. Interestingly in the songbirds, while complementary patterns of CaBPs delineated subregions within IC (MLd, as termed in these studies), the terminal fields of NA and NL projections to MLd broadly overlapped, and thus did not appear to correspond to the immunohistochemical boundaries as in owl and, presumably, chick (Logerot et al. 2011; Zeng et al. 2008).

Heterogeneity of calretinin expression and functional diversity in NA

Previous immunohistochemical studies of CR in the NA of barn owl and chick using single antibody enzymatic labeling indicated heterogeneous expression patterns across individual neurons, with some strongly labeled and others more weakly labeled. These studies inspired us to examine the CR-expressing neuronal distribution relative to the overall neuronal distribution in NA. Our double label study using a pan-neuronal marker in addition to anti-CR expands on these previous findings by showing that a substantial fraction of NA neurons were immunonegative for CR. The CR-immunoreactive neurons thus comprised a distinct subpopulation that persisted into the late hatchling developmental stages. The functional or morphological identity of this subpopulation is unclear. NA is comprised of a

heterogeneous collection of neurons that have a broad range of neuronal morphology, physiological response types *in vivo* to sound stimuli, and intrinsic physiology as revealed by *in vitro* experiments. Using Golgi labeling methods and intracellular fills that allow dendritic ramifications to be observed, four different cell types have been identified in NA on morphological grounds in multiple avian species: planar, large radiate, vertical, and stubby (pigeon: Häusler et al. 1999; chick: Soares et al. 2002; barn owl: Soares and Carr 2001; emu: MacLeod, Soares, and Carr personal observations). *In vitro* physiological response types have been defined in the chick and include several varieties of repetitive-spiking cell types and a phasic, “single spiking” response types, the latter of which resembled neurons in the timing pathways (Carr and Soares 2002; Fukui and Ohmori 2003; Kuo et al. 2009; Soares et al. 2002); these intrinsic properties may have important implications for both level and spectrotemporal processing in NA (Kreeger et al. 2012). Physiological responses types *in vivo* corresponded closely to those found in the mammalian ventral cochlear nucleus, including “primary-like” (resembling phasic-tonic nerve discharges), “chopper” (characterized by intrinsic repetitive spiking and poor phase-locking to the carrier wave), and “onset” types (Hotta 1971; Köppl and Carr 2003; Sachs and Sinnott 1978; Warchol and Dallos 1990; see also Köppl and Carr (2003) for a discussion of these response types in the avian system). Together, these data indicate that NA likely supports multiple, parallel streams of acoustic processing besides level coding for binaural comparisons, including general spectrotemporal processing for sound recognition (Carr and Soares 2002; Köppl and Carr 2003). Since all of the morphological cell types appeared to project out of NA to higher order neural targets (Soares and Carr 2001), exactly which cell types contribute to the coding of intensity for sound localization versus spectrotemporal processing for sound recognition is still unknown. Further research on the functional role of different cell types would be aided by the availability of cell type-specific molecular markers. This study represents a step in identifying such markers by analyzing the expression pattern of one highly expressed protein in the chick auditory brainstem, calretinin.

Calretinin expression along the tonotopic gradient over development in the avian brain stem

Previous work showed a dorsoventral gradient of expression of CR label over development, suggesting a relationship to the tonotopic gradient that is oriented in the dorsoventral dimension in NA

(Köppl 2001; Kubke et al. 1999; Parks et al. 1997; Warchol and Dallos 1990) like a number of other maturational processes in the avian brainstem that also show systematic variations along the eventual tonotopic orientation (for review, see Kubke and Carr (2000)). However, many of these processes occurred independently of alterations in peripheral activity and prior to the establishment of frequency-tuned neuronal activity. For example, the expression levels of the CaBPs in the cochlear nucleus neurons was unaffected by removal of afferent inputs by removal of the otocyst in young embryos (Parks et al. 1997). In early embryonic tissue, the neuropil in NA showed diffuse labeling, while in later embryonic and post-hatch tissue, the neuropil label weakened and cell body label strengthened. These studies also showed a dorsoventral expression gradient that changes with development, wherein the dorsal-most areas showed early, stronger label that moved in a “front” toward the ventral pole. NM and NL are, comparatively speaking, morphologically and physiologically homogeneous nuclei with a single principal cell type (Carr and Boudreau 1993; Jhaveri and Morest 1982; Kubke and Carr 2000; MacLeod et al. 2006). Calretinin was also expressed in the timing nuclei in a developmentally regulated gradient, with high/early expression levels in high-frequency (rostromedial) regions and lower/later expression in lower frequency (caudolateral) regions. Aside from the larger scale gradient of expression, CR label in the timing nuclei was locally homogeneous, while expression in NA cell bodies appeared locally heterogeneous, with a subset of neurons showing strongest expression levels among the three auditory nuclei while others showed weak expression. Our data confirmed the dorsoventral gradient of expression that changes with development, qualitatively in overall expression strength and quantitatively in terms of the fraction of CR-positive neurons that expand ventrally. This ventral expansion was coincident in both the postsynaptic and neuropil labeling. By P8, however, we show that although there is no longer a gradient, CR-positive neurons form a subpopulation of ~50–60 % of the total number of neurons in NA that are uniformly distributed, both dorsoventrally and rostrocaudally.

Comparison with mammalian auditory brain stem expression

While calretinin was the only CaBP found in the chick auditory brain stem nuclei (Parks et al. 1997; Rogers 1989a), three members of the CaBPs family, calretinin, parvalbumin, and calbindin, have been found in the mammalian auditory system (Bazwinsky et al. 2005, 2008; Braun et al. 1991b; Braun and

Piepenstock 1993; Caicedo et al. 1996, 1997; Celio 1990; Förster and Illing 2000; Fredrich et al. 2009; Friauf 1993, 1994; Idrizbegovic et al. 2001, 2004, 2006; Kelley et al. 1992; Kulesza 2014; Logerot et al. 2011; Lohmann and Friauf 1996; Matsubara 1990; Vater and Braun 1994). These CaBPs formed complementary patterns of expression in the cochlear nuclei (Bazwinsky et al. 2008; Fredrich et al. 2009; Lohmann and Friauf 1996).

Interestingly, differential CaBP expression could indicate subpopulations even within previously morphologically and physiologically identified classes. Heterogeneity in CaBP expression in the auditory brain stem has been studied in detail in several mammals. Using double immunofluorescence labeling Bazwinsky et al. (2008) found that calbindin and calretinin were expressed in distinct subpopulations of globular bushy cells and octopus cells in the ventral cochlear nucleus of the gerbils and possum. Similarly, a study in rat brain stem showed that multipolar type I neurons in ventral cochlear nucleus expressed either PV or CR (Fredrich et al. 2009). Utilizing retrograde labeling combined with immunohistochemistry, Fredrich et al. (2009) also showed that particular CaBPs were associated with specific subpopulations of cell types based on their projection patterns; for example, PV+ multipolar Type I neurons projected to contralateral inferior colliculus, while CR+ multipolar Type I neurons projected to ipsilateral inferior colliculus. These studies together suggest there is no global heuristic regarding the association of CaBP expression and cell type, especially when other auditory brain stem nuclei are considered. Instead, subpopulations within the more classically defined cell types may be identified by highly specific combinations of neurotransmitter, CaBP expression, and projection patterns. Further study of the projection patterns of nucleus angularis in the avian brainstem combined with immunofluorescence will be needed to determine whether similar patterns exist in the bird cochlear nucleus.

Calretinin and low-threshold potassium channel expression in NA

In an effort to link CR expression with a functional cell type, we used an antibody against the low-threshold potassium channel Kv1.1. The Kv1.1 gene and protein product belongs to the Kv1 family of low-threshold potassium channels, whose activity defines the physiological responses of the single spiking neurons in the timing pathway (NM, NL) (Kuba 2007; Kuba et al. 2002; Rathouz and Trussell 1998; Reyes et al. 1994). The physiological differentiation of NA neuron subtypes *in vitro* have been partially defined by the expression of a dendrotoxin-sensitive,

low-threshold potassium current (Fukui and Ohmori 2003; Soares et al. 2002). While the Kv1 family channel protein expression had been quantified in the brain stem timing nuclei (Fukui and Ohmori 2004; Kuba et al. 2005; Lu et al. 2004), it had not been thoroughly investigated in NA. Combining Kv1.1 staining with MAP2 as a neuronal marker, we showed that Kv1.1-like immunoreactivity was broad and heterogeneous in NA, albeit at lower levels than in NM. Kv1.1 label was found localized in the cell bodies, but was also found in the neuropil. Since this made it difficult to distinguish process from cell body staining, we used single plane optical sections through the central portion of somata (as identified by MAP2 staining) to try to limit the contribution of neuropil staining to our estimates of postsynaptic, cytoplasmic label. Still, immunoreactivity levels in individual neuronal cell bodies in NA ranged from levels similar to the surrounding neuropil, up to levels as strong as that observed in NM neurons. The breadth and variability of the Kv1.1 label suggests there may be a large variation in the contribution by low-threshold potassium conductances to the physiological responses of NA neurons and that was not limited to the single spiking neurons. Were Kv1.1 staining limited to the single spiking neurons, which comprise only 20 % of the neuronal population (Soares et al. 2002), we would have expected far more restricted labeling. A role for the low-threshold potassium conductance in tonic firing neurons is supported by experiments demonstrating that α -dendrotoxin, a specific blocker of low-threshold K channels (Brew and Forsythe 1995; Rathouz and Trussell 1998; Rothman and Manis 2003), elevated firing in these neurons, in addition to converting single spiking neurons to multiple-spiking (Fukui and Ohmori 2003; Kreeger and MacLeod, unpublished observations). A dendrotoxin-sensitive current in putative Type I (repetitive firing) neurons has been described in the mammalian VCN as well; this current had a threshold slightly higher than the classic “low-threshold” current (Rothman and Manis 2003). How these currents play into the variation of firing properties observed in NA is open for debate.

Since both Kv1.1 and CR were broadly expressed, there was a high degree of overlap, with no clear correlation or exclusion between Kv1.1- and CR-positive profiles. We did observe a weak negative correlation in immunofluorescence luminosity between the two signals. The strongly Kv1.1-positive neurons also had larger cell bodies, which would be consistent, but not conclusive, with their identification as single spiking, “stubby” neurons, which had the largest soma when measured following intracellular fills (Soares et al. 2002). We attempted to define the morphological features that might be associated with

the CR expression, to no avail. Intracellular fills to label neurons during whole-cell patch recordings appeared to wash out the cytoplasmic CR from NA neurons (though not from NM neurons in control experiments): filled NA neurons were nearly all CR negative, although nearby unrecorded neurons could show strong CR-positive immunoreactivity. MAP2 label, while invading the proximal dendrites, was not sufficiently complete to distinguish morphological types.

NeuN expression in the chick auditory brain stem

Originally identified as a neuronal specific nuclear protein in mouse (Mullen et al. 1992), NeuN has been used as a pan-neuronal marker in a number of studies of chick neuroanatomy and developmental studies in a variety of tissues, including forebrain, optic tectum, and spinal cord (Mezey et al. 2012; Perez et al. 1999; Yamagata et al. 2006; Zeng et al. 2008). While anti-NeuN labeled most neurons, including those in the murine cochlear nucleus (Mostafapour et al. 2002), it was notably absent in a few specific cell types: Purkinje cells in the cerebellum, mitral cells in the olfactory bulb, and photoreceptor neurons in the retina (Lind et al. 2005; Mullen et al. 1992; Weyer and Schilling 2003). We showed that neurons in the chick auditory brain stem were largely NeuN immunopositive, but that the subcellular expression profiles was heterogeneous in nucleus angularis. Nevertheless, NeuN was sufficiently universal to be a useful pan-neuronal marker for immunocytochemical studies in NA, labeling nearly all (>95 %) of neurons and providing clearer identification than possible with standard Nissl histological methods.

The anti-NeuN antigen has recently been identified as the gene product of *Rbfox3*, a member of the Fox-1 gene family of RNA splicing regulators (Kim et al. 2009). The *Rbfox3* protein is a neuron-specific RNA-binding protein which has been demonstrated to be required in the neuronal differentiation of postmitotic neurons in chick spinal cord (Kim et al. 2009, 2013). Alternative splice variations are common among neuronal proteins important in the auditory brain stem, especially glutamate receptors GluR1-4, where the flop variation of GluR3/4 enhances rapid signaling (Fabiana Kubke and Carr 1998; Levin et al. 1997; Parks 2000; Raman et al. 1994; Sugden et al. 2002). While this study utilized NeuN expression as a generic neuron-specific marker, the heterogeneity in anti-NeuN labeling we observed in NA might itself be related to neuronal diversity.

The inverse relationship between NeuN and CR expression suggests that NeuN may be actively regulated in a cell-specific manner. Differential subcellular

localization of NeuN label in NA may also be related to the finding that two isoforms of NeuN (molecular weights 46 and 48 kDa) are present in both mice and chick and both are labeled by the NeuN antibody (Lind et al. 2005; Mezey et al. 2012). In mice, these isoforms differ in their subcellular distributions, with the 48 kDa protein preferentially expressed in the cytoplasm, but whether differential isoform expression explains the heterogeneity in subcellular labeling in NA remains to be determined. Alternatively, anti-NeuN has been shown to be sensitive to phosphorylation states of the NeuN antigen (Lind et al. 2005), and therefore in our experiments, the differential label could possibly represent differential phosphorylation while the protein itself could be more uniformly distributed across the NA population.

In summary, calretinin immunoreactivity characterizes a subset of neurons in the avian cochlear nucleus. Further studies defining the projection pattern of these neurons or characterization of their morphology or physiological properties are needed to define this subset as a genuine subpopulation. Diversity of the immunolabeling with antibodies against the neuronal marker NeuN and the low-threshold ion channel Kv1.1 offer further evidence of the heterogeneous nature of the avian cochlear nucleus angularis.

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Conflict of Interest The authors declare that they have no conflict of interest.

REFERENCES

- ADOLPHS R (1993) Bilateral inhibition generates neuronal responses tuned to interaural level differences in the auditory brainstem of the barn owl. *J Neurosci* 13:3647–3668
- BAZWINSKY I, BIDMON H-J, ZILLES K, HILBIG H (2005) Characterization of the rhesus monkey superior olivary complex by calcium binding proteins and synaptophysin. *J Anat* 207:745–761. doi:10.1111/j.1469-7580.2005.00491.x
- BAZWINSKY I, HÄRTIG W, RÜBSAMEN R (2008) Characterization of cochlear nucleus principal cells of *Meriones unguiculatus* and *Monodelphis domestica* by use of calcium-binding protein immunolabeling. *J Chem Neuroanat* 35:158–174. doi:10.1016/j.jchemneu.2007.10.003
- BOORD RL (1968) Ascending projections of the primary cochlear nuclei and nucleus laminaris in the pigeon. *J Comp Neurol* 133:523–541. doi:10.1002/cne.901330410
- BOORD RL, RASMUSSEN GL (1963) Projection of the cochlear and lagenar nerves on the cochlear nuclei of the pigeon. *J Comp Neurol* 120:463–475
- BRAUN K (1990) Calcium-binding proteins in avian and mammalian central nervous system: localization, development and possible functions. *Prog Histochem Cytochem* 21:1–64
- BRAUN K, PIEPENSTOCK A (1993) Parvalbumin-immunoreactive neurons in the subcortical auditory pathway of the Mongolian gerbil (*Meriones unguiculatus*). *Acta Histochem Cytochem* 26:543–554
- BRAUN K, SCHEICH H, SCHACHNER M, HEIZMANN CW (1985) Distribution of parvalbumin, cytochrome oxidase activity and 14C-2-deoxyglucose uptake in the brain of the zebra finch. *Cell Tissue Res* 240:101–115. doi:10.1007/BF00217563
- BRAUN K, SCHEICH H, BRAUN S ET AL (1991A) Parvalbumin-, calretinin- and calbindin-D28k-immunoreactivity and GABA in a forebrain region involved in auditory filial imprinting. *Brain Res* 539:31–44
- BRAUN K, SCHEICH H, HEIZMANN CW, HUNZIKER W (1991B) Parvalbumin and calbindin-D28K immunoreactivity as developmental markers of auditory and vocal motor nuclei of the zebra finch. *Neuroscience* 40:853–869
- BREW H, FORSYTHE I (1995) Two voltage-dependent K⁺ conductances with complementary functions in postsynaptic integration at a central auditory synapse. *J Neurosci* 15:8011–8022
- CAICEDO A, D'ALDIN C, PUEL JL, EYBALIN M (1996) Distribution of calcium-binding protein immunoreactivities in the guinea pig auditory brainstem. *Anat Embryol* 194:465–487
- CAICEDO A, D'ALDIN C, EYBALIN M, PUEL JL (1997) Temporary sensory deprivation changes calcium-binding proteins levels in the auditory brainstem. *J Comp Neurol* 378:1–15
- CAMP AJ, WIJESINGHE R (2009) Calretinin: modulator of neuronal excitability. *Int J Biochem Cell Biol* 41:2118–2121. doi:10.1016/j.biocel.2009.05.007
- CARR CE, BOUDREAU RE (1991) Central projections of auditory nerve fibers in the barn owl. *J Comp Neurol* 314:306–318. doi:10.1002/cne.903140208
- CARR CE, BOUDREAU RE (1993) Organization of the nucleus magnocellularis and the nucleus laminaris in the barn owl: encoding and measuring interaural time differences. *J Comp Neurol* 334:337–355. doi:10.1002/cne.903340302
- CARR CE, CODE RA (2000) The central auditory system of reptiles and birds. In: Dooling RJ, Fay RR, Popper AN (eds) *Comparative hearing: birds and reptiles*. Springer-Verlag, New York, pp 197–248
- CARR CE, KONISHI M (1990) A circuit for detection of interaural time differences in the brain stem of the barn owl. *J Neurosci* 10:3227–3246
- CARR CE, SOARES D (2002) Evolutionary convergence and shared computational principles in the auditory system. *Brain Behav Evol* 59:294–311
- CELIO MR (1990) Calbindin D-28 k and parvalbumin in the rat nervous system. *Neuroscience* 35:375–475
- CELIO MR, HEIZMANN CW (1981) Calcium-binding protein parvalbumin as a neuronal marker. *Nature* 293:300–302
- CELIO MR, PAULS T, SCHWALLER B (1996) *Guidebook to the calcium-binding proteins*. Oxford University Press, Inc, Oxford
- CONLEE JW, PARKS TN (1986) Origin of ascending auditory projections to the nucleus mesencephalicus lateralis pars dorsalis in the chicken. *Brain Res* 367:96–113
- D'ORLANDO C, CELIO MR, SCHWALLER B (2002) Calretinin and calbindin D-28 k, but not parvalbumin protect against glutamate-induced delayed excitotoxicity in transfected N18-RE 105 neuroblastoma-retina hybrid cells. *Brain Res* 945:181–190
- FABIANA KUBKE M, CARR CE (1998) Development of AMPA-selective glutamate receptors in the auditory brainstem of the barn owl.

- Microsc Res Tech 41:176–186. doi:[10.1002/\(SICI\)1097-0029\(19980501\)41:3<176::AID-JEMT2>3.0.CO;2-S](https://doi.org/10.1002/(SICI)1097-0029(19980501)41:3<176::AID-JEMT2>3.0.CO;2-S)
- FÖRSTER CR, ILLING RB (2000) Plasticity of the auditory brainstem: cochleotomy-induced changes of calbindin-D28k expression in the rat. *J Comp Neurol* 416:173–187
- FREDRICH M, REISCH A, ILLING R-B (2009) Neuronal subtype identity in the rat auditory brainstem as defined by molecular profile and axonal projection. *Exp Brain Res* 195:241–260. doi:[10.1007/s00221-009-1776-7](https://doi.org/10.1007/s00221-009-1776-7)
- FRIAUF E (1993) Transient appearance of calbindin-D28k-positive neurons in the superior olivary complex of developing rats. *J Comp Neurol* 334:59–74
- FRIAUF E (1994) Distribution of calcium-binding protein calbindin-D28k in the auditory system of adult and developing rats. *J Comp Neurol* 349:193–211. doi:[10.1002/cne.903490204](https://doi.org/10.1002/cne.903490204)
- FUKUI I, OHMORI H (2003) Developmental changes in membrane excitability and morphology of neurons in the nucleus angularis of the chicken. *J Physiol Lond* 548:219–232
- FUKUI I, OHMORI H (2004) Tonotopic gradients of membrane and synaptic properties for neurons of the chicken nucleus magnocellularis. *J Neurosci* 24:7514–7523. doi:[10.1523/JNEUROSCI.0566-04.2004](https://doi.org/10.1523/JNEUROSCI.0566-04.2004)
- FUKUI I, SATO T, OHMORI H (2006) Improvement of phase information at low sound frequency in nucleus magnocellularis of the chicken. *J Neurophysiol* 96:633–641. doi:[10.1152/jn.00916.2005](https://doi.org/10.1152/jn.00916.2005)
- HACK NJ, WRIDE MC, CHARTERS KM ET AL (2000) Developmental changes in the subcellular localization of calretinin. *J Neurosci* 20:RC67(1–5)
- HACKETT JT, JACKSON H, RUBEL EW (1982) Synaptic excitation of the second and third order auditory neurons in the avian brain stem. *Neuroscience* 7:1455–1469
- HÄUSLER UH, SULLIVAN WE, SOARES D, CARR CE (1999) A morphological study of the cochlear nuclei of the pigeon (*Columba livia*). *Brain Behav Evol* 54:290–302
- HOTTA T (1971) Unit responses from the nucleus angularis in the pigeon's medulla. *Comp Biochem Physiol A Comp Physiol* 40:415–424
- IDRIZBEGOVIC E, CANLON B, BROSS LS ET AL (2001) The total number of neurons and calcium binding protein positive neurons during aging in the cochlear nucleus of CBA/CaJ mice: a quantitative study. *Hear Res* 158:102–115
- IDRIZBEGOVIC E, BOGDANOVIC N, WILLOTT JF, CANLON B (2004) Age-related increases in calcium-binding protein immunoreactivity in the cochlear nucleus of hearing impaired C57BL/6J mice. *Neurobiol Aging* 25:1085–1093. doi:[10.1016/j.neurobiolaging.2003.11.004](https://doi.org/10.1016/j.neurobiolaging.2003.11.004)
- IDRIZBEGOVIC E, SALMAN H, NIU X, CANLON B (2006) Presbycusis and calcium-binding protein immunoreactivity in the cochlear nucleus of BALB/c mice. *Hear Res* 216–217:198–206. doi:[10.1016/j.heares.2006.01.009](https://doi.org/10.1016/j.heares.2006.01.009)
- JHAVERI S, MOREST D (1982) Neuronal architecture in nucleus magnocellularis of the chicken auditory system with observations on nucleus laminaris: a light and electron microscope study. *Neuroscience* 7:809–836
- KELLEY PE, FRISINA RD, ZETTEL ML, WALTON JP (1992) Differential calbindin-like immunoreactivity in the brain stem auditory system of the chinchilla. *J Comp Neurol* 320:196–212. doi:[10.1002/cne.903200205](https://doi.org/10.1002/cne.903200205)
- KIM KK, ADELSTEIN RS, KAWAMOTO S (2009) Identification of neuronal nuclei (NeuN) as Fox-3, a new member of the Fox-1 gene family of splicing factors. *J Biol Chem* 284:31052–31061. doi:[10.1074/jbc.M109.052969](https://doi.org/10.1074/jbc.M109.052969)
- KIM KK, NAM J, MUKOUYAMA Y-S, KAWAMOTO S (2013) Rbfox3-regulated alternative splicing of Numb promotes neuronal differentiation during development. *J Cell Biol* 200:443–458. doi:[10.1083/jcb.201206146](https://doi.org/10.1083/jcb.201206146)
- KNUDSEN EI, KONISHI M (1978A) A neural map of auditory space in the owl. *Science* 200:795–797
- KNUDSEN EI, KONISHI M (1978B) Center-surround organization of auditory receptive fields in the owl. *Science* 202:778–780
- KONISHI M (1973) How the owl tracks its prey. *Am Sci* 61:414–424
- KONISHI M, SULLIVAN WE, TAKAHASHI T (1985) The owl's cochlear nuclei process different sound localization cues. *J Acoust Soc Am* 78:360–364
- KONISHI M, TAKAHASHI T, WAGNER H ET AL (1988) Neurophysiological and anatomical substrates of sound localization in the owl. In: Edelman GM, Gan WE, Cowan WM (eds) *Neurophysiological and anatomical substrates of sound localization in the owl*. Wiley, New York, pp 721–745
- KÖPPL C (2001) Tonotopic projections of the auditory nerve to the cochlear nucleus angularis in the barn owl. *J Assoc Res Otolaryngol* 2:41–53
- KÖPPL C, CARR CE (2003) Computational diversity in the cochlear nucleus angularis of the barn owl. *J Neurophysiol* 89:2313–2329. doi:[10.1152/jn.00635.2002](https://doi.org/10.1152/jn.00635.2002)
- KOYANO K, FUNABIKI K, OHMORI H (1996) Voltage-gated ionic currents and their roles in timing coding in auditory neurons of the nucleus magnocellularis of the chick. *Neurosci Res* 26:29–45
- KREEGER LJ, ARSHED A, MACLEOD KM (2012) Intrinsic firing properties in the avian auditory brain stem allow both integration and encoding of temporally modulated noisy inputs in vitro. *J Neurophysiol* 108:2794–2809. doi:[10.1152/jn.00092.2012](https://doi.org/10.1152/jn.00092.2012)
- KRÜTZFELDT NO, LOGEROT P, KUBKE MF, WILD JM (2010A) Connections of the auditory brainstem in a songbird, *Taeniopygia guttata*. II. Projections of nucleus angularis and nucleus laminaris to the superior olive and lateral lemniscal nuclei. *J Comp Neurol* 518:2135–2148. doi:[10.1002/cne.22324](https://doi.org/10.1002/cne.22324)
- KRÜTZFELDT NOE, LOGEROT P, KUBKE MF, WILD JM (2010B) Connections of the auditory brainstem in a songbird, *Taeniopygia guttata*. I. Projections of nucleus angularis and nucleus laminaris to the auditory torus. *J Comp Neurol* 518:2109–2134. doi:[10.1002/cne.22334](https://doi.org/10.1002/cne.22334)
- KUBA H (2007) Cellular and molecular mechanisms of avian auditory coincidence detection. *Neurosci Res* 59:370–376. doi:[10.1016/j.neures.2007.08.003](https://doi.org/10.1016/j.neures.2007.08.003)
- KUBA H, KOYANO K, OHMORI H (2002) Development of membrane conductance improves coincidence detection in the nucleus laminaris of the chicken. *J Physiol Lond* 540:529–542. doi:[10.1113/jphysiol.2001.013365](https://doi.org/10.1113/jphysiol.2001.013365)
- KUBA H, YAMADA R, FUKUI I, OHMORI H (2005) Tonotopic specialization of auditory coincidence detection in nucleus laminaris of the chick. *J Neurosci* 25:1924–1934. doi:[10.1523/JNEUROSCI.4428-04.2005](https://doi.org/10.1523/JNEUROSCI.4428-04.2005)
- KUBKE MF, CARR CE (2000) Development of the auditory brainstem of birds: comparison between barn owls and chickens. *Hear Res* 147:1–20
- KUBKE MF, CARR CE (2006) Morphological variation in the nucleus laminaris of birds. *Int J Comp Psychol* 19:83–97
- KUBKE MF, GAUGER B, BASU L ET AL (1999) Development of calretinin immunoreactivity in the brainstem auditory nuclei of the barn owl (*Tyto alba*). *J Comp Neurol* 415:189–203
- KULEZA RJ (2014) Characterization of human auditory brainstem circuits by calcium-binding protein immunohistochemistry. *Neuroscience* 258:318–331. doi:[10.1016/j.neuroscience.2013.11.035](https://doi.org/10.1016/j.neuroscience.2013.11.035)
- KUO SP, BRADLEY LA, TRUSSELL LO (2009) Heterogeneous kinetics and pharmacology of synaptic inhibition in the chick auditory brainstem. *J Neurosci* 29:9625–9634. doi:[10.1523/JNEUROSCI.0103-09.2009](https://doi.org/10.1523/JNEUROSCI.0103-09.2009)
- LEVIN MD, KUBKE MF, SCHNEIDER M ET AL (1997) Localization of AMPA-selective glutamate receptors in the auditory brainstem of the barn owl. *J Comp Neurol* 378:239–253
- LIND D, FRANKEN S, KAPPLER J ET AL (2005) Characterization of the neuronal marker NeuN as a multiply phosphorylated antigen with discrete subcellular localization. *J Neurosci Res* 79:295–302. doi:[10.1002/jnr.20354](https://doi.org/10.1002/jnr.20354)

- LOGEROT P, KRÜTZFELDT NOE, WILD JM, KUBKE MF (2011) Subdivisions of the auditory midbrain (N. mesencephalicus lateralis, pars dorsalis) in zebra finches using calcium-binding protein immunocytochemistry. *PLoS ONE* 6:e20686
- LOHMANN C, FRIAU E (1996) Distribution of the calcium-binding proteins parvalbumin and calretinin in the auditory brainstem of adult and developing rats. *J Comp Neurol* 367:90–109. doi:10.1002/(SICI)1096-9861(19960325)367:1<90::AID-CNE7>3.0.CO;2-E
- LU Y, MONSIVAIS P, TEMPEL BL, RUBEL EW (2004) Activity-dependent regulation of the potassium channel subunits Kv1.1 and Kv3.1. *J Comp Neurol* 470:93–106. doi:10.1002/cne.11037
- MACLEOD KM, CARR CE (2007) Beyond timing in the auditory brainstem: intensity coding in the avian cochlear nucleus angularis. *Prog Brain Res* 165:123–133. doi:10.1016/S0079-6123(06)65008-5
- MACLEOD KM, SOARES D, CARR CE (2006) Interaural timing difference circuits in the auditory brainstem of the emu (*Dromaius novaehollandiae*). *J Comp Neurol* 495:185–201. doi:10.1002/cne.20862
- MANLEY GA, KÖPPL C, KONISHI M (1988) A neural map of interaural intensity differences in the brain stem of the barn owl. *J Neurosci* 8:2665–2676
- MATSUBARA JA (1990) Calbindin D-28 K immunoreactivity in the cat's superior olivary complex. *Brain Res* 508:353–357
- MEZEY S, KRIVOKUCA D, BÁLINT E ET AL (2012) Postnatal changes in the distribution and density of neuronal nuclei and doublecortin antigens in domestic chicks (*Gallus domesticus*). *J Comp Neurol* 520:100–116. doi:10.1002/cne.22696
- MOISEFF A, KONISHI M (1981) Neuronal and behavioral sensitivity to binaural time differences in the owl. *J Neurosci* 1:40–48
- MOISEFF A, KONISHI M (1983) Binaural characteristics of units in the owl's brainstem auditory pathway: precursors of restricted spatial receptive fields. *J Neurosci* 3:2553–2562
- MOSTAFAPOUR SP, DEL PUERTO NM, RUBEL EW (2002) bcl-2 Overexpression eliminates deprivation-induced cell death of brainstem auditory neurons. *J Neurosci* 22:4670–4674
- MULLEN RJ, BUCK CR, SMITH AM (1992) NeuN, a neuronal specific nuclear protein in vertebrates. *Development* 116:201–211
- NISHINO E, YAMADA R, KUBA H ET AL (2008) Sound-intensity-dependent compensation for the small interaural time difference cue for sound source localization. *J Neurosci* 28:7153–7164. doi:10.1523/JNEUROSCI.4398-07.2008
- PARKS TN (2000) The AMPA receptors of auditory neurons. *Hear Res* 147:77–91
- PARKS TN, RUBEL EW (1978) Organization and development of the brain stem auditory nuclei of the chicken: primary afferent projections. *J Comp Neurol* 180:439–448
- PARKS TN, CODE RA, TAYLOR DA ET AL (1997) Calretinin expression in the chick brainstem auditory nuclei develops and is maintained independently of cochlear nerve input. *J Comp Neurol* 383:112–121
- PAYNE RS (1971) Acoustic location of prey by barn owls (*Tyto alba*). *J Exp Biol* 54:535–573
- PEREZ SE, REBELO S, ANDERSON DJ (1999) Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo. *Development* 126:1715–1728
- POPATILOFF A, GIAUME C, PEUSNER KD (2003) Developmental change in expression and subcellular localization of two Shaker-related potassium channel proteins (Kv1.1 and Kv1.2) in the chick tangential vestibular nucleus. *J Comp Neurol* 461:466–482. doi:10.1002/cne.10702
- PUELLES L, ROBLES C, MART NEZ-DE-LA-TORRE M, MART NEZ S (1994) New subdivision schema for the avian torus semicircularis: neurochemical maps in the chick. *J Comp Neurol* 340:98–125. doi:10.1002/cne.903400108
- PUELLES L, MARTINEZ-DE-LA-TORRE M, PAXINOS G, ET AL. (2007) The chick brain in stereotaxic coordinates: an atlas featuring neuromeric subdivisions and mammalian homologies. *Acad Press*
- RAMAN I, ZHANG S, TRUSSELL L (1994) Pathway-specific variants of AMPA receptors and their contribution to neuronal signaling. *J Neurosci* 14:4998–5010
- RATHOUZ M, TRUSSELL L (1998) Characterization of outward currents in neurons of the avian nucleus magnocellularis. *J Neurophysiol* 80:2824–2835
- REYES AD, RUBEL EW, SPAIN WJ (1994) Membrane properties underlying the firing of neurons in the avian cochlear nucleus. *J Neurosci* 14:5352–5364
- ROGERS JH (1989A) Immunoreactivity for calretinin and other calcium-binding proteins in cerebellum. *Neuroscience* 31:711–721
- ROGERS JH (1989B) Two calcium-binding proteins mark many chick sensory neurons. *Neuroscience* 31:697–709
- ROTHMAN JS, MANIS PB (2003) Differential expression of three distinct potassium currents in the ventral cochlear nucleus. *J Neurophysiol* 89:3070–3082. doi:10.1152/jn.00125.2002
- SACHS MB, SINNOTT JM (1978) Responses to tones of single cells in nucleus magnocellularis and nucleus angularis of the redwing blackbird (*Agelaius phoeniceus*). *J Comp Physiol A* 126:347–361
- SATO T, FUKUI I, OHMORI H (2010) Interaural phase difference modulates the neural activity in the nucleus angularis and improves the processing of level difference cue in the lateral lemniscal nucleus in the chicken. *Neurosci Res* 66:198–212. doi:10.1016/j.neures.2009.11.001
- SHAO M, POPATILOFF A, YI J ET AL (2009) Adaptation of chicken vestibular nucleus neurons to unilateral vestibular ganglionectomy. *Neuroscience* 161:988–1007. doi:10.1016/j.neuroscience.2009.04.027
- SMITH ZD (1981) Organization and development of brain stem auditory nuclei of the chicken: dendritic development in N. laminaris. *J Comp Neurol* 203:309–333. doi:10.1002/cne.902030302
- SOARES D, CARR CE (2001) The cytoarchitecture of the nucleus angularis of the barn owl (*Tyto alba*). *J Comp Neurol* 429:192–205
- SOARES D, CHITWOOD RA, HYSOON RL, CARR CE (2002) Intrinsic neuronal properties of the chick nucleus angularis. *J Neurophysiol* 88:152–162
- SUGDEN SG, ZIRPEL L, DIETRICH CJ, PARKS TN (2002) Development of the specialized AMPA receptors of auditory neurons. *J Neurobiol* 52:189–202. doi:10.1002/neu.10078
- SULLIVAN WE, KONISHI M (1984) Segregation of stimulus phase and intensity coding in the cochlear nucleus of the barn owl. *J Neurosci* 4:1787–1799
- TAKAHASHI TT, KONISHI M (1988) Projections of nucleus angularis and nucleus laminaris to the lateral lemniscal nuclear complex of the barn owl. *J Comp Neurol* 274:212–238
- TAKAHASHI T, MOISEFF A, KONISHI M (1984) Time and intensity cues are processed independently in the auditory system of the owl. *J Neurosci* 4:1781–1786
- TAKAHASHI TT, CARR CE, BRECHA N, KONISHI M (1987) Calcium binding protein-like immunoreactivity labels the terminal field of nucleus laminaris of the barn owl. *J Neurosci* 7:1843–1856
- TRUSSELL L (1999) Synaptic mechanisms for coding timing in auditory neurons. *Annu Rev Physiol* 61:477–496
- VATER M, BRAUN K (1994) Parvalbumin, calbindin D-28 k, and calretinin immunoreactivity in the ascending auditory pathway of horseshoe bats. *J Comp Neurol* 341:534–558. doi:10.1002/cne.903410409
- WAGNER H, GÜNTÜRKÜN O, NIEDER B (2003) Anatomical markers for the subdivisions of the barn owl's inferior-collicular complex and adjacent peri- and subventricular structures. *J Comp Neurol* 465:145–159. doi:10.1002/cne.10826
- WANG Y, KARTEN HJ (2010) Three subdivisions of the auditory midbrain in chicks (*Gallus gallus*) identified by their afferent

- and commissural projections. *J Comp Neurol* 518:1199–1219. doi:[10.1002/cne.22269](https://doi.org/10.1002/cne.22269)
- WANG Y, RUBEL EW (2008) Rapid regulation of microtubule-associated protein 2 in dendrites of nucleus laminaris of the chick following deprivation of afferent activity. *Neuroscience* 154:381–389. doi:[10.1016/j.neuroscience.2008.02.032](https://doi.org/10.1016/j.neuroscience.2008.02.032)
- WARCHOL ME, DALLOS P (1990) Neural coding in the chick cochlear nucleus. *J Comp Physiol A* 166:721–734
- WEYER A, SCHILLING K (2003) Developmental and cell type-specific expression of the neuronal marker NeuN in the murine cerebellum. *J Neurosci Res* 73:400–409. doi:[10.1002/jnr.10655](https://doi.org/10.1002/jnr.10655)
- YAMAGATA M, WEINER JA, DULAC C ET AL (2006) Labeled lines in the retinotectal system: markers for retinorecipient sublaminae and the retinal ganglion cell subsets that innervate them. *Mol Cell Neurosci* 33:296–310. doi:[10.1016/j.mcn.2006.08.001](https://doi.org/10.1016/j.mcn.2006.08.001)
- ZENG S, LIN Y, YANG L ET AL (2008) Comparative analysis of neurogenesis between the core and shell regions of auditory areas in the chick (*Gallus gallus domesticus*). *Brain Res* 1216:24–37. doi:[10.1016/j.brainres.2008.04.012](https://doi.org/10.1016/j.brainres.2008.04.012)
- ZHANG S, TRUSSELL L (1994) A characterization of excitatory postsynaptic potentials in the avian nucleus magnocellularis. *J Neurophysiol* 72:705–718