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Localization of KCNC1 (Kv3.1) Potassium Channel Subunits in the Avian Auditory Nucleus Magnocellularis and Nucleus Laminaris during Development

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Abstract

The KCNC1 (previously Kv3.1) potassium channel, a delayed rectifier with a high threshold of activation, is highly expressed in the time coding nuclei of the adult chicken and barn owl auditory brainstem. The proposed role of KCNC1 currents in auditory neurons is to reduce the width of the action potential and enable neurons to transmit high frequency temporal information with little jitter. Because developmental changes in potassium currents are critical for the maturation of the shape of the action potential, we used immunohistochemical methods to examine the developmental expression of KCNC1 subunits in the avian auditory brainstem. The KCNC1 gene gives rise to two splice variants, a longer KCNC1b and a shorter KCNC1a that differ at the carboxy termini. Two antibodies were used: an antibody to the N-terminus that does not distinguish between KCNC1a and b isoforms, denoted as panKCNC1, and another antibody that specifically recognizes the C terminus of KCNC1b. A comparison of the staining patterns observed with the pan-KCNC1 and the KCNC1b specific antibodies suggests that KCNC1a and KCNC1b splice variants are differentially regulated during development. Although pan-KCNC1 immunoreactivity is observed from the earliest time examined in the chicken (E10), a subcellular redistribution of the immunoprotein was apparent over the course of development. KCNC1b specific staining has a late onset with immunostaining first appearing in the regions that map high frequencies in nucleus magnocellularis (NM) and nucleus laminaris (NL). The expression of KCNC1b protein begins around E14 in the chicken and after E21 in the barn owl, relatively late during ontogeny and at the time that synaptic connections mature morphologically and functionally.

Keywords

chicken; barn owl; ontogeny; time coding; outward current; high threshold

INTRODUCTION

Birds use interaural time differences (ITDs) to localize sounds in the azimuth. Chickens and owls represent two ends of a spectrum: chickens are auditory generalists and can hear up to about 4 kHz (Gray and Rubel 1985), while barn owls are auditory specialists with the ability

to hear an extended range of frequencies (up to 12 kHz; Konishi, 1973). Despite these behavioral differences, the time coding circuits in the brainstem of both birds are similar. The auditory branch of the eighth nerve enters the brainstem and bifurcates with one branch innervating nucleus magnocellularis (NM, Fig. 1) in a tonotopic manner. NM projects bilaterally to the second order nucleus laminaris (NL; Parks and Rubel 1975; Carr and Konishi, 1990; Overholt et al., 1992). The projection from NM to NL is also tonotopic and resembles the Jeffress model circuit for the detection of time differences in that the NM axons act as delay lines and NL neurons as coincidence detectors (Jeffress, 1948). Precisely timed action potentials phase-lock to the auditory stimulus and form the physiologic basis of ITD detection (Sullivan and Konishi, 1984).

The neurons that make up the time coding pathway in the brainstem of both birds have been extensively described in terms of their morphology, synaptic connectivity, and the sequence of events governing their assembly during ontogeny (Rubel and Parks, 1988; Kubke and Carr, 2000). Although much is known about morphologic changes during development, less is known about physiological development, especially changes in intrinsic electrical properties. Potassium conductances contribute significantly to the membrane properties of neurons in NM and NL (Hendriks et al., 1999a; see Oertel, 1999; Trussell, 1999, for reviews). The identification of potassium channel proteins that are expressed in embryonic avian brainstem neurons is important to our understanding of the development of electrical excitability.

Voltage-gated potassium (KCN) channels are a diverse group of channels and play a central role in neuronal excitability. They contribute to the determination of the resting membrane potential, shaping of action potentials, the regulation of frequency and pattern of action potential discharges, and the modulation of transmitter release (Hille, 1992). Channels formed by *Shaw*-like KCNC subunits activate rapidly at potentials positive to -10 mV (see reviews by Rudy et al., 1999, 2001). KCNC, a member of this subfamily, is highly expressed in auditory neurons (Perney et al., 1992, 1997; Grigg et al., 2000; Li et al., 2001; Parameshwaran et al., 2001). Computer simulations have suggested that the KCNC1 conductance improves postsynaptic temporal coding precision (Perney and Kaczmarek, 1997; Wang et al., 1998a) by reducing the width of the action potential without compromising its amplitude.

In the rat, alternative splicing of the KCNC1 gene gives rise to two proteins, KCNC1a and KCNC1b (Luneau et al., 1991). The splice variants differ in their carboxy termini but are physiologically indistinguishable in that they both produce high threshold delayed-rectifier currents (Yokoyama et al., 1989; Kanemasa et al., 1995). The *in vivo* expression of the two KCNC1 gene products in rat is temporally regulated during development such that KCNC1a is predominant in the embryonic and neonatal neurons, while levels of KCNC1b expression are up-regulated after postnatal day 10 (Perney et al., 1992). In the chicken, a high threshold potassium current with kinetics similar to that of KCNC1 has been reported in developing NM neurons *in vitro* (Rathouz and Trussell, 1998; Hendriks et al., 1999a, 1999b).

The development of the avian auditory system has been approximately divided into four time periods in the context of specific developmental milestones (Rubel and Parks, 1988, embryonic date given for chicken embryos). (1) Early development including proliferation, neuronal migration (E1–E9), (2) synaptogenesis and onset of hearing (E10–E15), (3) pre-hatching synapse maturation (E16 to hatch), and (4) posthatch (P0 –adult). The expression of KCNC1 during early development of the inner ear and the acousticovestibular anlage of the rhombencephalon has been recently reported (Zhou et al., 2001). We describe the pattern of expression of KCNC1 in NM and NL of the chicken and the owl during the latter three periods of development. We show that in the chicken, the expression of KCNC1a and b has

distinct time courses. Moreover, expression of KCNC1b in both the chicken and the barn owl begins at the time that the synapses begin to mature (Jackson and Parks, 1982; Carr and Boudreau, 1996).

MATERIALS AND METHODS

Animal protocols were approved by the University of Maryland Animal Care and Use Committee and conform to NIH guidelines.

Antibodies and Immunoblot Analysis

The two antibodies used in this study, anti-KCNC1b and anti-panKCNC1, were characterized and purified as described elsewhere (Perney and Kaczmarek, 1997). The specificity of binding of the anti-KCNC1b antibody to the chicken KCNC1 protein has been demonstrated in a previous study (Parameshwaran et al., 2001). For immunoblot analysis of the panKCNC1 antibody, synaptosomes were prepared from adult rat and chicken (E17 and P30) brains as follows. Briefly, 1.5 g of brain tissue was homogenized with 10 strokes of a Dounce homogenizer in 15 mL of buffer containing 0.3 M sucrose, 10 mM sodium phosphate (pH 7.4), 0.5 M disodium ethylenediaminetetraacetic acid (EDTA), and a cocktail of protease inhibitors (20 µg/mL phenylmethyl sulphonyl fluoride, 1 µg/mL leupeptin, 1 µg/mL aprotinin, 1 µg/mL pepstatin A). Homogenates were centrifuged at $2000 \times g$ for 10 min to remove nuclei and debris. The supernatant was collected and centrifuged at $45,000 \times g$ for 45 min and the resultant pellet resuspended in 15 mL of low sucrose buffer (25 mM Tris, pH 7.4, 1 mM EDTA, and added protease inhibitors) by several passages through a 23-gauge needle. The resuspended pellet was centrifuged at $8000 \times g$ to remove mitochondria and then the collected supernatant was centrifuged at $45,000 \times g$ for 45 min. The pellet was resuspended in 3 mL of low sucrose buffer and the protein concentration determined using the BCA method (Pierce, Rockford, IL).

For immunoblots, 50 µg of membrane protein was added to reducing sample buffer, boiled for 20 min, and electrophoresed on 9% SDS PAGE gels. Protein was transferred to nitrocellulose membranes in Tris-glycine buffer with 0.1% SDS by rapid transfer (100 V) for 1 h at 4°C. Transfer of proteins was confirmed by Ponceau S (Sigma, St. Louis, MO) staining. The blots were blocked with 4% nonfat dry milk in TBST (0.1 M Tris-buffered saline and 0.05% Tween 20) for 2 h and then incubated with affinity purified antibody to panKCNC1 (1–2 µg/mL) for 2 h at room temperature. In some cases, the antisera were preabsorbed with 50 µM of synthetic peptide for 30 min. Blots were then washed in 4% milk/TBST and incubated with 1:5000 dilution of HRP conjugated goat anti-rabbit IgG (Jackson Laboratories, West Grove, PA) in TBST for 2 h. After washing, bound antibody was detected by enhanced chemiluminescence reaction (Pierce Super Signal kit, Pierce, Rockford, IL) following manufacturer's instructions.

Immunohistochemistry

Forty chicken embryos between embryonic days 10 and 20 were used. Chickens at postnatal day 2 ($n = 2$), postnatal day 17 ($n = 5$), and 15 weeks ($n = 3$) were also included in this study. The E10–E14 embryos were exsanguinated through the umbilical cord. Following decapitation, the heads were fixed in 4% electron microscopy (EM) grade paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) in 0.1 M phosphate-buffered saline (PBS), pH 7.2 for 48 h. Embryos older than E14 and the posthatch chickens were anesthetized with pentobarbital and perfused through the heart with PBS, followed by 30–75 mL 4% paraformaldehyde in PBS. All brains were dissected out, postfixed for 1 h in 4% paraformaldehyde, and then sunk overnight in 15% sucrose/PBS. Thirty-micron coronal sections were cut on a freezing microtome and collected into PBS. Endogenous peroxidase

activity was quenched by incubating sections for 10 min in 1% hydrogen peroxide/50% methanol. After washing with PBS, sections were blocked for 1 h in staining medium (DMEM; Gibco Life Tech., Gaithersburg, MD; 10% fetal bovine serum; 1% Triton X-100; 0.02% Na azide), and then incubated overnight at room temperature with either panKCNC1 or KCNC1b antibody (~2 µg/mL). After washing in PBS, the sections were incubated in a 1:400 dilution of HRP conjugated goat anti-rabbit IgG (Jackson Laboratories, Bar Harbor, ME) in PBS for 4 h and then washed again. Reaction product was visualized with diaminobenzidine (DAB, 0.03%) and hydrogen peroxide (0.003%), intensified with nickel sulfate (0.2%). Controls included preabsorption of antiserum with 50 µM of synthetic peptide for 30 min and omission of the panKCNC1 antibody.

Because the owls are bred in small numbers in our animal facility, in most cases only one embryo was used to construct each time point. One each of the following ages was used in our study: E18, E21, E27, P0, and P6. Immunohistochemical procedures, identical to those described above for the chickens, were performed. Due to limited availability of embryos of equivalent ages, only KCNC1b antibody was used for the analyses.

Immunopositive neurons were characterized by purple-black reaction product. We have described the KCNC1 staining patterns on an arbitrary scale of intensity where intense > moderate > faint > barely detectable > undetectable. The tonotopic gradient is oblique to any transverse section (it runs from rostromedial to caudolateral), and thus each transverse section shows a gradient from higher best frequency (medial) to lower best frequency (lateral).

RESULTS

We have described our results with reference to existing physiological and anatomical studies. In both owl and chick, NM neurons receive endbulb terminals from the auditory nerve, and project bilaterally to NL (Rubel and Parks, 1988; Carr and Konishi, 1990). The cells in NM and NL are arranged tonotopically (Rubel and Parks, 1975; Takahashi and Konishi, 1988). High best frequencies (BFs) are represented rostromedially, while low BFs are represented caudolaterally. Intermediate frequencies are mapped across the mediolateral extent of the nucleus.

The KCNC1a Antibody Recognizes Identical Epitopes in Rat and Chicken

Alternative splicing of the KCNC1 gene gives rise to two isoforms that differ only at their carboxy termini (Luneau et al., 1991). The shorter form, KCNC1a (also called KCNC1β and Kv3.1a) arises from continuous transcription past the splice site and has a short tail of 10 amino acids after the splice site. The longer form, KCNC1b (also called KCNC1α and Kv3.1b) diverges from this splice site and has an 84 amino acid tail. One antibody was raised against a peptide corresponding to the terminal 19 amino acids of rat KCNC1b protein (Perney and Kaczmarek, 1997). Immunoblot analysis of adult rat and both E17 and P30 chicken brain membranes revealed that this antibody also recognized the chicken KCNC1 protein (Parameshwaran et al., 2001). A second antibody was raised against a peptide corresponding to the first 16 amino acids of the KCNC1 protein (Perney and Kaczmarek, 1997). Because the amino termini of both splice variants are the same, the antibody recognizes both of them. We have denoted the antibody that recognizes both KCNC1a and b as the panKCNC1 antibody and the antibody that recognizes only KCNC1b as the KCNC1b antibody. Western blot analysis with the panKCNC1 antibody revealed two prominent protein bands. One band migrated at an estimated molecular weight of 92 kDa, while the other band migrated further (84 kDa). Therefore, in both the rat and the chicken, panKCNC1 antibody recognized both splice variants, the 92 kDa KCNC1b and the 84 kDa KCNC1a protein [arrows, Fig. 2(A)]. Two additional higher molecular weight protein bands

in the rat and the chicken were also observed. Higher molecular weight bands have also been observed by other investigators (Hernandez-Pineda et al., 1999) in Western blots using antibodies to KCNC1 and KCNC2. No protein bands were detected when the antiserum was preincubated with 50 μ M antigenic peptide [Fig. 2(B)].

Immunostaining of chicken brainstem from P17 and 15 week old birds with either the panKCNC1 or KCNC1b antibody revealed similar patterns of staining [Figs. 3(A)–(E) and 4(G)], suggesting that the panKCNC1 immunostaining is indeed specific. This specificity was confirmed by demonstrating a lack of immunostaining when the panKCNC1 antibody was first preabsorbed with the antigenic peptide [Fig 3(F)]. The pattern of KCNC1 immunostaining in the 17 day and 15 week posthatch birds were comparable. We have, therefore, referred to the staining pattern in the P17 chicken as having reached the “mature” state.

Pan-KCNC1 Immunolabel Is Detected Early in the Chicken Auditory Brainstem

Immunohistochemical studies with the panKCNC1 antibody indicated KCNC1 expression in chick NM at the earliest time point examined. At E10, moderate immunostaining was observed along the entire mediolateral and rostrocaudal extent of NM. An immunoreactive cluster of cell bodies appeared above a background of immunoreactive neuropil [Fig. 4(A)]. Immunostaining of NM axons in the vicinity of the contralateral NL was also observed [Fig. 5(A), arrows]. Two days later, at E12, levels of immunoreactivity were higher and NM was readily discerned as an intensely stained crescent with slightly higher levels of expression in the medial high BF region compared to lateral low BF regions [Fig. 4(B)]. The staining was diffuse and observed in both the somata and the neuropil of the NM [Fig. 4(C)]. The neuropil consists of the auditory nerve axons as well as the axons and dendrites of NM neurons. KCNC1 positive axons were clearly labeled as they exited the nucleus [arrows above and below NM in Fig. 4(B)]. At the level of the light microscope, however, it was not possible to determine whether the NM dendrites and auditory nerve fibers also contribute to the immunoreactivity of the neuropil [Fig. 4(C)]. At E14, a gradient of pan-KCNC1 staining could be observed across the mediolateral extent of NM, with the medial high BF regions more darkly stained. At high magnification, the immunoprotein outlined many cell bodies in the high BF regions [Fig. 4(E)]. Stained NM axons were also immunolabeled [arrow, Fig. 4(D)]. At E16, the majority of the NM staining was confined to the cell bodies and was now observed along the entire rostrocaudal extent, although the intensity of KCNC1 immunostaining was especially high in the medial high BF region [arrows in Fig. 4(F)]. The immunoprotein was distributed throughout the somatic cytoplasm, and it was difficult to discern specific membrane bound immunoreactivity at the level of the light microscope. The neuropil was faintly immunoreactive. This staining pattern persisted through hatching. By P17, when development of NM is complete (Rubel and Parks, 1988; Kubke and Carr, 2000), all NM neurons were intensely immunoreactive for KCNC1. The neuropil was faintly stained [Fig. 4(G)].

panKCNC1 staining in NL was also observed at the earliest time point examined. At E10, the multilayered collection of cells expressed moderate levels of KCNC1 immunoprotein [Fig. 5(A)]. At E12, the KCNC1 positive cells in NL were arranged in a monolayer, except at the future low BF region [arrow, Fig. 5(B)]. A faint level of immunoreactivity was also observed in the dendritic regions of the neuropil [Fig. 5(B), arrowheads]. At E14, the levels of KCNC1 immunoreactivity in the cell body layer remained unchanged while levels in the dendritic neuropil increased [Fig. 5(C), arrowheads]. By E17, except for the very low BF regions, the cell body layer in NL appeared devoid of KCNC1 immunoreactivity while the intense staining in the dendritic neuropil persisted [Fig. 5(D)]. At all embryonic ages examined, intense levels of panKCNC1 immunostaining were observed in the axon fibers from NM to NL (double arrows in Fig. 5). The pattern of panKCNC1 immunostaining in NL

at P17 was indistinguishable from the adult chicken [Figs. 5(E) and 3(D)]. The NL dendrites are intensely stained and the NM axons are faintly labeled. Only cell bodies in the high BF regions are KCNC1 immunoreactive.

The Onset of KCNC1b Immunolabeling in the Chicken Is Delayed during Development

Immunostaining with the KCNC1b antibody did not reveal any expression of the KCNC1b subunit in the NM and NL neurons of the chicken from E10 through E13/14 [Fig. 6(A) and (B)]. At E13/14, staining above background could be barely detected in NM [Fig. 6(B)]. At E15, neurons in the medial high BF region of NM were intensely KCNC1b immunoreactive [Fig. 7(A)], while in the lateral low BF region, immunoreactivity was diffuse and the somata and neuropil indistinguishable [arrows, Fig. 7(A)]. At higher magnification, the immunostaining in the somata appeared patchy with most of the dark label associated with the circumference of the neuron [arrows, Fig. 7(B)]. Immunolabeled processes were also observed in the neuropil [Fig. 7(B), arrowheads]. The label observed at E14 appears to be beginning of a rostromedial to caudolateral wave of KCNC1b expression that characterizes many aspects of auditory brainstem development (Rubel and Parks, 1988). By E17, KCNC1b immunoreactivity became detectable in the more lateral cells, such that all but the most caudolateral neurons in NM were now KCNC1 immunoreactive [Fig. 7(D)]. A few stained processes were observed emanating from the NM cell bodies which might be axons or dendrites [arrows, Fig. 7(E)]. KCNC1b associated immunoreactivity in the neuropil was faint [Fig. 7(D) and (E)]. By E19, intense KCNC1b staining throughout NM was observed [Fig. 7(G) and (H)], similar to that seen in 17-day posthatch birds [Fig. 3(A)]. Thus, a wave of KCNC1b expression appeared to begin in high best frequency regions at about E15, and to be complete by E19.

KCNC1b immunoreactivity in NL only became discernable at E15 and only in more rostral sections [compare Fig. 6(A) and (B) with Fig. 7(C)] with the more caudolateral regions of NL only faintly immunoreactive (data not shown). The cell body layer of NL at E15 was largely devoid of KCNC1b immunoprodukt. The KCNC1 immunostaining in the dorsal and the ventral neuropil sandwiching the cell body layer, however, was intense. It was unclear with light microscopy whether the staining in the NL neuropil could be attributed to the dendrites of NL neurons alone (as in mature chickens; Parameshwaran et al., 2001) or whether the terminals of the stained NM axons [Fig. 7(C), double arrow] were also KCNC1b positive at this age. In the sections containing high BF NM, staining in the lateral neuropil region appeared more extensive (but not more intense) than at the more medial extent [Fig. 7(C)]. Dendrites in caudolateral NL are longer and more arborized than in more rostromedial regions. At E17, staining of the NM axons diminished and majority of the staining was concentrated on the dorsal and ventral neuropil regions and along the entire extent of NL [Fig. 7(F)]. Although the somatic monolayer was largely un-stained, faintly stained somata were observed in the very low BF regions similar to what has been observed in the P17 chicken [Fig. 5(E); Parameshwaran et al., 2001]. At E19, KCNC1 immunostaining in NL was intense in the neuropil, moderate in the low BF encoding somata and comparable to NL at P17 [Figs. 7(I) and 3(A) and (C)].

Late Onset of KCNC1b Expression in the Barn Owl Auditory Brainstem

Because the barn owl is altricial and the chicken precocial, developmental studies of auditory processes warrant comparisons with respect to biological time rather than physical time (Kubke and Carr, 2000). The owl embryo hatches at E32, while the chick embryo hatches at E21. Furthermore, the time periods during which specific developmental changes occur in the owl are protracted and extend beyond hatching (Starck, 1993; Kubke and Carr, 2000). Due to the limited availability of embryonic owl tissue, and because the time coding circuits mature late, we determined the onset and time course of expression of the KCNC1b

splice variant that predominated in the adult (Perney et al., 1992; Kubke and Carr, 2000; Parameshwaran et al., 2001).

At E21, despite extended exposure to the DAB-chromagen, none of the neurons in NM displayed the KCNC1b immunoprodukt [Fig. 8(A)]. At E27, many neurons in the more rostral high BF regions of NM were moderately immunoreactive [Fig. 8(B)]. NM axons that cross the midline to the contralateral NL were also faintly labeled [arrows in Fig. 8(B)]. Five days later at P0, all but the most caudal neurons in NM were intensely KCNC1b immunoreactive [Fig. 8(C), (E), and (F)]. The wave of KCNC1b expression appeared to follow the tonotopic axis, beginning in high BF regions and progressing to lower BF regions [Fig. 8(E)]. At P0, neurons in the very lowest BF region of NM did not express KCNC1b [Fig. 8(E), arrows]. The staining of individual NM neurons appeared patchy and concentrated at the membrane [Fig. 8(F)]. Moderately labeled processes were also observed in the neuropil [arrows, Fig. 8(F)]. The levels of staining seen in the NM axons appeared darker at P0 than at E27 [Fig. 8(C)]. By P6, the adult pattern of NM staining emerged, and KCNC1b immunoreactivity extended further along the tonotopic axis into lower BF regions [Fig. 8(G)]. However, labeling of NM axons not seen in the adult was still evident at P6 (Parameshwaran et al., 2001).

The onset of KCNC1b immunoreactivity in the owl NL paralleled that observed in the NM. At E21, immunostaining in the cell bodies in the most rostral regions of NL was barely detectable (not shown). In the central and caudal regions immunostaining was not apparent [Fig. 8(A)]. At E27, KCNC1b immunoreactivity in NL followed the frequency axis such that the staining in the medial high BF region was moderately high and diminished in the more lateral low BF regions [Fig. 8(H)]. It was difficult to distinguish whether all the staining was in the neuropil or whether the membranes of NL neurons were also stained. At P0, the overall levels of KCNC1b immunoreactivity had increased. The gradient of KCNC1b expression persisted even as the more lateral regions of NL became KCNC1b positive [Fig. 8(C)]. At higher magnification [Fig. 8(I)], KCNC1b staining in NL neuropil was intense. Interspersed within the immunostained neuropil were dark immunoreactive rings indicative of the NL neurons. At P6, all but the low frequency “hook” region of NL was KCNC1b immunoreactive [arrowhead, Fig. 8(D)]. The low-frequency region only contained moderately stained neuropil while the rest of the nucleus consisted of NL neurons outlined in a background of intensely immunoreactive neuropil [Fig. 8(J)]. Thus, a wave of KCNC1b expression proceeded from rostromedial (E21) to caudolateral (P6) in NL.

DISCUSSION

Development of the nervous system is controlled by the orderly expression of genes directing neurogenesis, neurite outgrowth, differentiation, and synapse formation and function. Activity arising spontaneously within developing neural circuits or from environmental stimulation can regulate gene expression and thereby tailor development according to functional requirements (Ribera and Spitzer, 1992). The intrinsic membrane properties of neurons undergo dynamic changes during development. The maturation of the action potential is determined in part by the differentiation of various potassium currents that contribute towards the outward current (Spitzer and Ribera, 1998).

Differential Expression of KCNC1 in Auditory Neurons of the Chicken during Ontogeny

The pattern of KCNC1 expression during development of the time coding pathway in the chicken brainstem has been summarized in Figures 9 and 10. Several points about the KCNC1 immunostaining pattern during auditory development in the chicken are noteworthy. Like the rat, expression of KCNC1a and KCNC1b in the chicken follows different time courses (Perney et al., 1992). Immunostaining with the panKCNC1 antibody

(that does not distinguish between KCNC1a and b) was observed *in vivo* at the earliest times examined, E10. KCNC1b immunoreactivity, on the other hand, appeared around E14/15. Based on the time course of expression of panKCNC1 and KCNC1b, we infer that expression of KCNC1a during development begins early. KCNC1a is expressed in NM and NL at E10 and is likely to be the predominant splice variant until E14, when KCNC1b expression first appears. After E14, KCNC1a is co-expressed with KCNC1b with KCNC1a levels possibly remaining the same and KCNC1b expression levels gradually increasing until adult-like levels are reached by P17. The results reported here on the time course of KCNC1 expression in the chicken are largely consistent with and extend those of Zhou et al. (2001). These authors provide evidence for pan-KCNC1 staining early during embryogenesis when the precursor cells proliferate, migrate, and form axons. In accordance with the immunohistochemical observations, a KCNC1-like high threshold current has been observed in neurons of the acoustico-vestibular anlage as early as embryonic day 2 in culture (Hendriks et al., 1999a).

Our results do differ from those of Zhou et al. (2001) in some respects. We did not observe staining of auditory brainstem structures with KCNC1b before E14, while Zhou et al. report moderate staining between E5–E14. We also did not observe the biphasic pattern of expression of KCNC1b: first an increase in staining levels, followed by a dip, and then resurgence in expression levels. The major technical difference between our two studies was that Zhou et al. carried out their immunocytochemical studies on sections thaw-mounted onto slides. Although this method is needed for early embryonic material, it does increase background staining.

The onset of physiological activity in the chicken brainstem begins around E10/11, coinciding with the early stages of synaptogenesis (Saunders et al., 1973; Rubel and Parks, 1988). At this time, NM and NL neurons have extended dendritic processes and appear to receive synapses from far more auditory nerve fibers than they finally retain (Jackson and Parks, 1982; Jhaveri and Morest, 1982). PanKCNC1 immunoreactivity at this stage appeared diffuse and included the neuropil as well as somata. Around E14, the synapses begin to mature (Fig. 9) and the first “behavioral” responses to sound are obtained (behavior refers to motility monitored with platinum electrodes inserted beneath the shell membrane; Saunders et al., 1973; Jackson and Rubel, 1978; Jackson and Parks, 1982). Because sophisticated behavioral tests have not been developed for chicken embryos, the above experiment provides the only reliable evidence of responses to different frequencies of sound as a function of age. At this time point (E14), KCNC1 immunoreactivity condensed around the somata. This redistribution was more apparent in the medial high BF regions and correlates with the retraction of NM dendrites observed by Jhaveri and Morest (1982) and with the appearance of KCNC1b immunoreactivity. At E16, the gradient observed in levels of panKCNC1 immunoreactivity in NM [Fig. 4(F)] may, in part, reflect the restricted onset of the KCNC1b protein in the high BF region, overlying expression of KCNC1a across the nucleus [compare Fig. 4(F) with 7(A)].

At E15, dendrites in the rostromedial region of NL are maturing (Smith and Rubel, 1979; Smith 1981; Rubel and Parks 1988). This process of maturation continues into the posthatching period. Between E15–E17, the panKCNC1 staining in the NL cell body layer diminishes and appears to become restricted to the dendritic region that received synaptic connections from NM axons. Thus, the change in the pattern of KCNC1 immunoreactivity from the somatic to the dendritic region of NL is correlated with the onset of dendritic maturation and synaptic refinement in NL. Although both panKCNC1 and KCNC1b immunostaining was observed in axons before E17, KCNC1b immunolabel was not observed in NM axons of older birds. Prominent axonal staining reported in the developing rat auditory system, was also absent in the adult (Li, 2000).

There are several possible explanations for the dense staining observed in the NM axons from E10–E17. First, the observation that the panKCNC1 antibody labeled the neuropil more extensively than the KCNC1b antibody indicates that KCNC1a may be preferentially sorted to the axonal domain of neurons (Weiser et al., 1995; Ozaita et al., 2000). Consistent with this idea, KCNC1a has been found to sort to the apical region of Madin-Darby canine kidney cells in culture (Li, 2000). Apical sorting in epithelial cell lines has been equated to axonal compartmentalization (Dotti and Simons, 1990). Second, the reduction in axonal staining may reflect a redistribution of KCNC1 channel over the course of myelination (Cheng and Carr, 1992). Third, a combination of the above two explanations may apply. Although KCNC1a and b are physiologically identical, the divergent amino acid sequences at the C-termini of the two isoforms may be potential targets for differential modulation. It remains to be determined if preferential targeting of KCNC1a and b isoforms to somatodendritic and axonal compartments, respectively, would affect the physiology of the local synapses.

During the period from E14 to E18, when levels of KCNC1 immunoreactivity increased in the auditory nuclei (Zhou et al., 2001; this study), the magnitude of a KCNC1-like high threshold current in NM neurons in culture also increased dramatically (Hendriks et al., 1999a). The maturing of tone-elicited and electrically elicited responses from the brainstem auditory nuclei occurs during this time. Response latencies are reduced, the responses become less fatigable, thresholds decrease, and progressively higher frequencies become effective (Saunders et al., 1973; Jackson and Parks, 1982).

KCNC1b Expression in the Barn Owl

Because the barn owl is altricial, comparisons of developmental processes with the precocial chicken were made with respect to biological time rather than physical time (Kubke and Carr, 2000). In the owl, the time over which specific developmental changes occur is protracted and can extend beyond hatching (Starck, 1993). The onset of KCNC1b expression in chicken and barn owl NM and NL coincided with synapse maturation and progressed from the more medial high BF region to the lateral low BF regions.

Many morphologic and biochemical features of the hindbrain auditory nuclei in the chicken and the barn owl develop along a rostromedial to caudal lateral gradient that corresponds to moving down the future tonotopic axis (Smith, 1981; Rubel and Parks, 1988; Carr and Boudreau, 1996; Massoglia, 1997; Carr et al., 1998; Kubke et al., 1999; Kubke and Carr, 2000). The emergence of the KCNC1b gradient in immunostaining in both chick and owl during development may reflect in part this general rostromedial to caudolateral maturation. The persistence of the gradient in the adult birds, however, indicates that this differential distribution along the tonotopic axis is not merely a developmental phenomenon. The KCNC1 gradient in the adult is more intense in the barn owl than the chicken, but is present in both species (Parameshwaran et al., 2001).

The pattern of KCNC1 expression during development of the time coding pathway in the barn owl brainstem is summarized in Figure 10. In the barn owl NL, KCNC1b expression reached detectable levels between E21 and E26 when synaptic connections between NM and NL become morphologically mature (Carr and Boudreau, 1996; Massoglia, 1997). Intense KCNC1 staining filled the neuropil until P0, and it was often difficult to distinguish the outline of NL neurons. The subsequent decrease in neuropil staining was coincident with the growth of NL, the associated decrease in cell density, and the decrease in NL dendritic length (Carr and Boudreau, 1996; Carr et al., 1998). The myelination of NM and NL axons (Cheng and Carr, 1992; Kubke and Carr, 2000) within the nucleus may also have resulted in reduced neuropil staining at later ages. The reduction in dendritic arborization and consequent redistribution of synapses from distal dendrites to more proximal locations in

barn owl NL may contribute to the discrete localization of KCNC1 immunoreactivity around NL somata (Parameshwaran, et al., 2001).

In the barn owl, the expression of KCNC1b in the time coding neurons followed a time line similar to that of the calcium binding protein, calretinin, and AMPA-type glutamate receptor expression (Kubke and Carr, 1998; Kubke et al., 1999). The expression of calretinin in the auditory brainstem nuclei begins as early as E17 and reaches adult levels by P14, while AMPA receptor expression reached adult levels by P14. KCNC1b expression appeared to begin 1–2 days later than calretinin, because faint levels could first be detected in rostral NL at E21, and reached approximate adult levels after P6.

Functional Role of KCNC1 during Development

Insights into the functional role of KCNC1 during development can be gained from a number of studies. Early in auditory development, before the onset of synaptic function, suppression of a high threshold KCNC1-like current with TEA and 4-AP resulted in a reversible block of migration in cultured neuroblasts (Hendricks et al., 1999b). In embryonic *Xenopus* spinal neurons, a developmental increase in delayed rectifier currents, specifically KCNC1, is necessary for the maturation of the action potential (Gurantz et al., 2000). Antisense suppression of KCNC1 channel expression reduced the amplitude and rate of activation of the outward potassium current. Significantly, it also increased the width of the action potential by 184% (Vincent et al., 2000). It has been argued that the absence of the KCNC1 current could extend the developmental period during which Ca^{2+} spikes are spontaneously generated by the neurons and ultimately affect neuronal differentiation (Gu and Spitzer, 1995). In immature mammalian auditory neurons, the amplitude of action potentials near 0 mV is similar to that of the adult. The duration of the action potentials is, however, two to three times longer than that of the adult (Sanes, 1993; Kandler and Friauf, 1995a; Taschenberger and von Gersdorff, 2000). The prolonged duration of the spikes could result in increased levels of calcium influx. Neural activity in the developing brainstem auditory pathway of the chicken embryo is dominated by a rhythmic pattern of spontaneous discharge. Rhythmic bursting is present as early as E14, shortly after the onset of functional synaptogenesis, and gives way to more mature, steady level of firing on E19, 2 days prior to hatching (Lippe, 1994; Jones and Jones, 2000). The increase in the periodicity of bursts and the presence of large calcium permeable, glutamate activated, AMPA receptor mediated synaptic currents in chick NM neurons are likely to necessitate extensive calcium buffering ability in auditory neurons (Lippe, 1995; Otis et al., 1995; Zhou et al., 1995). Calretinin is expressed early during development and has been implicated in restricting calcium transients (Parks et al., 1997; Kubke et al., 1999; Hack et al., 2000). The rapid repolarization of the action potential mediated by the KCNC1 conductance may be another mechanism to limit the amount of Ca^{++} entering the cells (Rudy and McBain, 2001).

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Figure 1. Schematic coronal section through the brainstem of (A), chicken, and (B), owl. The medial branch of the VIIIth nerve innervates NM. NL receives bilateral projections from NM. [Part (A) modified from Rubel and Parks, 1988].

Figure 2.

(A) Western blot using the rat panKCNC1 antibody. The arrows point to two bands that correspond to the 92 kDa KCNC1b protein and a shorter 84 kDa KCNC1a protein. The horizontal bars indicate the apparent molecular weights of prestained protein standard. (B) Control: left lane displays protein bands recognized by the panKCNC1 antibody and the right lane shows that preadsorption of the antibody with the antigenic peptide abolishes all protein bands.

Figure 4.

Immunolabel with the panKCNC1 in the developing chicken NM. Left in all figures is medial and right is lateral. (A) E10; (B) E12, arrows point to stained NM axons exiting the nucleus; (C) E12 NM magnified to show neuropil; (D) E14, arrow points to stained NM axons; (E) E14 NM magnified, arrows point to stained patches on the membrane; (F) E16. KCNC1 staining is more intense in the medial high BF region, arrows; (G) P17 NM. Normalized optical density (OD) values were measured in central NM for E10. There was an increase in normalized OD from lateral to medial (i.e., with increasing best frequency). Normalized OD measures could not be interpreted after E14, because the pan antibody

recognizes both KCNC1a and b, and KCNC1b is expressed at detectable levels after E14. All scale bars are 100 μ m.

Figure 5. Immunostaining in the developing chicken NL detected with the panKCNC1 antibody. Left in all figures is medial and right is lateral. (A) E10; (B) E12, arrows point to the stained neurons in the low BF region of NL; (C) E14; NM axons projecting into NL are stained (arrows); (D) E17; (E) P17, arrowheads point to the dendritic region. All scale bars are 100 μ m.

Figure 6. Expression of KCNC1b during early development in the chick auditory brainstem. (A) At E10, KCNC1b immunolabel is absent; (B) at E14, very faint KCNC1b immunostaining is detected. Scale bars are 100 μ m.

Figure 7.

Expression of KCNC1b during development of NM and NL in the chicken. (A) E15 NM. Arrows point to low BF region of NL that has diffuse KCNC1 immunolabel. (B) E15 NM neurons at higher magnification showing stained processes of NM neurons (arrowhead) and arrowheads point to concentrated patches of immunolabel. (C) E15 NL. Arrows point to the stained NM axons projecting to NL. (D) E17 NM. (E) E17 NM neurons at high magnification. Arrows point to stained NM processes extending out from the somata. (F) E17 NL. (G) E19 NM. (H) E19 NM neurons at high magnification. (I) E19 NL. There was an increase in normalized OD from lateral to medial (i.e., with increasing best frequency). Scale bars: (B), (E), and (H) are 50 μm ; (A), (D), (G), (C), (F), and (I) are 100 μm .

Figure 8.

Expression of KCNC1b in the developing time coding circuit of the barn owl brainstem. (A) E21 NM and NL. (B) E27 NM and NL. Arrows point to stained NM axons. (C) P0 NM and NL. Arrows point to stained NM axons. (D) P6 NM and NL. Arrows point to stained NM axons, arrowhead points to lateral low BF NL. (E) Montage of P0 NM, arrows point to the caudolateral region where somata are not KCNC1b immunoreactive. (F) KCNC1 staining in individual NM neurons, arrows point to stained processes in the neuropil region. (G) Extent of immunolabel along the tonotopic axis in P6 NM. Arrows point to low BF tail of NM. (H) Gradient of staining in E27 NL. (I) P0 NL. Arrows point to KCNC1 label outlining individual somata. (J) P6 NL. Arrows point to KCNC1 label outlining individual somata.

There was an increase in normalized OD from lateral to medial (i.e., with increasing best frequency). Scale bars: (A), (B), (C), (D), and (G) are 500 μm ; (E) is 1 mm; (F) is 50 μm ; (H), (I), and (J) are 100 μm .

	CHICKEN		OWL
	E14/15		E27/28
	KCNC1a	KCNC1b	KCNC1b
	&b		
8th NERVE	---	---	---
NM SOMA	+++	++	+
NM NEUROFIL	+++	+/-	+
NM AXONS	+++	+++	+

	E16		P0/P1
	KCNC1a	KCNC1b	KCNC1b
	&b		
8th NERVE	---	---	---
NM SOMA	+++	+++	++
NM NEUROFIL	+	+/-	+
NM AXONS	+	+/-	++

	E17/18		P6
	KCNC1a	KCNC1b	KCNC1b
	&b		
8th NERVE	---	---	---
NM SOMA	+++	+++	+++
NM NEUROFIL	+/-	+/-	+
NM AXONS	+/-	+/-	++

	ADULT		ADULT
	KCNC1a	KCNC1b	KCNC1b
	&b		
8th NERVE	---	---	---
NM SOMA	+++	+++	+++
NM NEUROFIL	+/-	+/-	+
NM AXONS	+/-	+/-	++

Figure 9.

Summary of Kv3.1 expression in NM. Symbols denote level of staining: + + +, intense; + + moderate; +, faint; +/- barely detectable; ---, undetectable. The drawings depicting the morphology of the NM neuron and its afferent synapse are taken from Jhaveri and Morest (1982). The corresponding structures in the chick and barn owl have similar morphologies during development. The afferent auditory nerve is depicted in black and the NM neuron is shaded a light gray.

Figure 10.

Expression of Kv3.1 in NL. Symbols denote level of staining: + + +, intense; + + moderate; +, faint; +/- barely detectable; - - -, undetectable. The drawings depicting the morphology of the NL neurons and its dendritic arbors during ontogeny are taken from Smith and Rubel (1981) for the chicken, and from Carr and Boudreau (1996) for the barn owl.