Development of Chloride-Mediated Inhibition in Neurons of the Anteroventral Cochlear Nucleus of Gerbil (*Meriones unguiculatus*)

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Milenković I, Witte M, Tureček R, Herinch M, Reinert T, Rübsamen R. Development of chloride-mediated inhibition in neurons of the anteroventral cochlear nucleus of gerbil (*Meriones unguiculatus*). *J Neurophysiol* 98: 1634–1644, 2007. First published June 27, 2007; doi:10.1152/jn.01150.2006. At the initial stages in neuronal development, GABAergic and glycineergic neurotransmission exert depolarizing responses, assumed to be of importance for maturation, which in turn shift to hyperpolarizing in early postnatal life due to development of the chloride homeostasis system. Spherical bushy cells (SBC) of the mammalian cochlear nucleus integrate excitatory glutamatergic inputs with inhibitory (GABAergic and glycineergic) inputs to compute signals that contribute to sound localization based on interaural time differences. To provide a fundamental understanding of the properties of GABAergic neurotransmission in mammalian cochlear nucleus, we investigated the reversal potential of the GABA-evoked currents (E_{GABA}) by means of gramicidin-perforated-patch recordings in developing SBC. The action of GABA switches from depolarizing to hyperpolarizing by the postnatal day 7 thereby providing the hyperpolarizing Cl\(^-\)-mediated inhibition in SBC.

INTRODUCTION

Spherical bushy cells (SBC) of the mammalian cochlear nucleus (CN) contain the first central synapses of the afferent auditory pathway that encode precise temporal information by providing excitatory input to the medial superior olive neurons (Smith et al. 1993). These large neurons integrate excitatory inputs from few auditory nerve terminals (endbulbs of Held) (Brawer and Morest 1975; Ryugo and Sento 1991) with acoustically driven, GABA- and glycine-mediated inhibition (Kopp-Scheinflug et al. 2002). The inhibitory inputs are provided by nonprimary innervation from sources including the dorsal cochlear nucleus, the superior olivary complex, and the contralateral cochlear nucleus (Wenthold 1991), and they appear to be glycineergic, glycine and GABAergic, and GABAergic only (Altsschuler et al. 1993; Juiz et al. 1996). Depolarizing GABAergic and/or glycineergic responses were described as a transient feature of immature mammalian auditory brain stem (Ehrlich et al. 1999; Kakazu et al. 1999; Kandler and Friau 1995; Löhrke et al. 2005), hippocampus (Ben-Ari et al. 1989; Cherubini et al. 1990), neocortex (Owens et al. 1996; Yuste and Katz 1991), cerebellum (Eilers et al. 2001), cultured midbrain neurons (Jarolimek et al. 1999; Titz et al. 2003), and retina (Huang and Redburn 1996). The excitatory responses are due to the elevated intracellular Cl\(^-\) concentration in the immature neurons; this is attributable to the prolonged postnatal development of the chloride homeostasis system (for review, see Ben-Ari 2002).

The K\(^+\)-Cl\(^-\)-extruding cotransporter KCC2 is expressed exclusively in neurons (Payne et al. 1996) and its upregulation is believed to underlie the functional switch from excitatory to inhibitory action of GABA (Owens and Kriegstein 2002; Rivera et al. 1999). Studies of the chick nucleus magnocellularis (NM; avian homologue to the CN) suggested that GABA\(_A\) receptor-mediated synaptic responses remain depolarizing throughout the animal’s life span due to the outward-directed electrochemical gradient for Cl\(^-\) at the resting membrane potential. However, depolarizing GABA-evoked responses prevent NM neurons from producing action potentials in developing as well as in mature auditory brain stem (Hyson et al. 1995; Lu and Trussell 2001; Monsivais and Rubel 2001). GABA\(_A\) receptors are primarily permeable to Cl\(^-\), yet also to HCO\(_3^-\) (relative permeability ca. 0.2) (Bormann et al. 1987; Kaila 1994), and a detailed characterization of the impact that GABA has on the membrane current flow must consider the actual intracellular anion concentration. In the present developmental study, we used the gramicidin-perforated-patch recordings because the membrane pores formed by gramicidin are exclusively permeable to monovalent cations and small, uncharged molecules, leaving the [Cl\(^-\)]\(_i\) undisturbed (Akaike 1996; Kyrozis and Reichling 1995). We determined the reversal potential of the SBC responses to GABA through the early postnatal development, up to the time of hearing onset (P12) (Woof and Ryan 1984) and studied the pattern of KCC2 expression from neonatal to subadult ages. Our results reveal a rather early (at P1) expression of the KCC2 protein in CN. However, the indications of its activity temporally correlate with hyperpolarizing GABA\(_A\)-mediated responses observed toward the end of the first postnatal week.
**METHODS**

**Animals and animal care**

This study was performed in the Neurobiology Laboratories of the Institute of Biology II at University of Leipzig. The Western blot analyses were done in the Neurophysiology Laboratories of the Paul-Flechsig Institute for Brain Research, University of Leipzig. All experimental procedures were approved by the Saxonian district Government, Leipzig. For experimental purposes, we used pigmented (agouti) Mongolian gerbils (Meriones unguiculatus) aging postnatal day one to 30 (P1-30), obtained from the institutes animal care facilities.

**Slice preparation**

Acute brain stem slices (200 μm) containing the rostral pole of the anterointernal cochlear nucleus (AVCN) were prepared from P3–P12 gerbils using vibratome (Microm HM 650, Walldorf, Germany) as previously described by Oertel (1983). The cold (3–4°C) preparation solution used for cutting contained (in mM) 125 NaCl, 2.5 KCl, 0.1 CaCl₂, 3 MgCl₂, 1.25 NaH₂PO₄, 25 NaHCO₃, 25 glucose, 2 sodium pyruvate, 3 myo-inositol, and 0.5 ascorbic acid, continuously equilibrated with 5% CO₂/95% O₂, pH 7.3 with KOH (280 mosM). The remainder of the pipette was back-filled with the same mixture. For recordings in which ASCF was exchanged with HEPES extracellular solution, sodium bicarbonate was substituted by 20 mM pyruvate, 3 myo-inositol, and 0.5 ascorbic acid, pH 7.4 when equilibrated with 5% CO₂/95% O₂ mixture. For recordings in which ASCF was exchanged with HEPES extracellular solution, sodium bicarbonate was substituted by 20 mM HEPES, NaCl increased to 135 mM, NaH₂PO₄ was omitted, and pH was adjusted to 7.4 with NaOH. Slices were transferred to a recording chamber (1 ml volume) mounted on the stage of an upright microscope (Axioskop 2, Zeiss, Germany) and continuously perfused at the rate of 2 ml/min with the extracellular solution. All experiments were performed at RT (21–23°C).

**Electrophysiology**

Patch pipettes were pulled from filamented borosilicate glass capillaries (Harvard Apparatus, Edenbridge, UK) to have resistances of 3–6 MΩ for whole cell- and 5–6 MΩ for gramicidin-perforated recordings. To measure $E_{\text{GABA}}$ in whole cell recordings, pipette solutions with two different Cl⁻ concentrations were used: high [Cl⁻]ᵢ solution for control experiments with Cl⁻ extruding capacity of KCC2 (Kakazu et al. 1999, 2000), K⁺-based internal solutions were used for perforated-patch recordings. Pipettes were tip-filled with (in mM) 140 KC1 and 10 HEPES, pH 7.3 with KOH to form Na⁺ and K⁺ solutions; or with solution in which 140 mM KCl was substituted by 145 mM K⁺-gluconate and 5 mM KCl (283 mosM). The remainder of the pipette was back-filled with the same K⁺-based solution including gramicidin (gramicidin A, Sigma, Deisenhofen, Germany). A 10 mg/ml stock solution of gramicidin was freshly prepared in dimethylsulfoxide, sonicated, and diluted to a final concentration of 50 μg/ml just prior to experiment.

Membrane currents and membrane potentials were recorded using a single-electrode amplifier (npi electronic, Tamm, Germany) in bridge, discontinuous current- or voltage-clamp mode. Switching frequency was 20 kHz, and signals were filtered at 1 kHz and digitized at 2–5 kHz using npi electronic hardware and software (CellWorks 5.0). Data analysis was performed with pClamp 9.0 software (Axon Instruments, Union City, CA). To gap-seal formation (>5 GΩ). Voltages were corrected off-line for junction potentials as follows: −8.8 mV (CsMeSO₄) and −12 mV (K-glucuronate-based pipette solution). During voltage-clamp recordings, extracellular solution was supplemented with 0.3 μM TTX and 25 μM CGP 4638 (both Tocris, Bristol, UK) to block Na⁺-dependent action potentials and to antagonize GABAₐ receptors, respectively. GABA (100 μM; Sigma) and muscimol (10 μM; Tocris) solutions were prepared in HCO₃⁻-containing extracellular solution and pressure applied (5 psi, 50 ms) over the soma of the recorded neuron through a wider-tip patch pipette mounted on a Picospritzer (General Valve, Fairfield, NJ). Although the application of agonists via a puff pipette slightly dilutes the antagonist when it is presented in the bath, the blocking effects were efficient with low antagonist concentration (Fig. 1). Recorded cells were characterized as SBC according to their firing of a single action potential at the start of depolarizing current step (Wu and Oertel 1984).

To determine $E_{\text{GABA}}$ the cells were voltage-clamped at 0 mV and stepped to various test potentials using an “up” protocol with ±2-mV interval between applications because it was previously shown not to cause the shifts in the [Cl⁻]ᵢ in similar experiments (Ehrlich et al. 1999). Step voltages $V_{\text{test}}$ ranged from −60 to 40 mV for CsCl-filled electrodes, from −89 to 11 mV for Cs-methanesulphonate, from −92 to −32 mV for K-glucose, and from −90 to −30 mV for KCl-filled pipettes. Fitting of the I-V relations was calculated with linear regressions and $E_{\text{GABA}}$ was determined as the voltage at the zero current. $V_{\text{rest}}$ was estimated as the mean potential of the cell at the zero current in gramicidin-perforated-patch configuration.

**Immunohistochemistry**

Gerbils were killed with sodium pentobarbitol (10 mg/kg body wt, ip), and the tissue was fixed through transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer. The brains (3–4 for each age investigated) were removed and kept in a fixative for 4 h. For cryoprotection, the brains were kept in 30% sucrose in 0.1 M phosphate buffer until they sank. The brains were shock-frozen to −70°C, and coronal brain stem sections (16 μm) were obtained by means of cryocut. Brain sections were washed thoroughly with PBS and PBS/0.3% Triton/1% DMSO solutions. After blocking of nonspecific binding sites with 5% normal goat serum (NGS) in PBS/0.3% Triton/1% DMSO (30 min at 37°C), the specimens containing AVCN were incubated overnight with polyclonal anti-KCC2 antibody (raised in rabbits), which is described to be highly specific for KCC2 (Ehrlich et al. 1998). Bound antibody was visualized with the avidin-biotin-peroxidase complex method (Alkaline phosphatase method) using a 1:500 dilution of DAB chromogen (Sigma, Deisenhofen, Germany). For visualization of KCC2, the sections were incubated overnight with polyclonal anti-KCC2 antibody (raised in rabbits); bound antibody was visualized with the avidin-biotin-peroxidase complex method using a 1:500 dilution of DAB chromogen (Sigma, Deisenhofen, Germany). For visualization of specific binding sites for GABA or GABA subtype receptors was performed using a polyclonal or monoclonal antibody, respectively.

**RESULTS**

GABA_A receptor-mediated currents in spherical bushy cells. Whole cell patch-clamp recordings on P7 neurons with CsCl-filled electrodes. Currents evoked by a 50-ms pressure application of GABA or muscimol were blocked by 96 and 94%, respectively, after a bath application of the GABA_A receptor antagonist gabazine (SR95531).
against residues 932-1,043 of the rat KCC2; catalog No. 07–432; Upstate, Lake Placid, NY; 1:200 in blocking solution at 4°C). The antibody corresponds to the antibody published previously by Williams et al. (1999). After washing the specimens with PBS/0.3% Triton/1% DMSO, the secondary goat anti-rabbit Cy2-tagged antibody (20 μg/ml in blocking solution; Jackson ImmunoResearch Lab, Diaanova, Hamburg, Germany) was incubated for 2.5 h at RT. In animals of younger stages, additional counterlabeling of all cell nuclei was performed with Hoechst 33258 (1:1,000 in PBS, 30 min at RT; Molecular Probes, Leiden, Holland). After rinsing with PBS and dH2O, the sections were dehydrated in xylol, dried, and coverslipped with entellan. Additional staining of large presynaptic calyceal inputs (endbulbs of Held), which terminate at somata of SBC, was performed with goat anti-calretinin antibody (1:1,000 in blocking solution; Swant, Bellinzona, Switzerland) as previously published (Bawinský and Rübsamen 2000; Hättig et al. 2001). This step was conducted to visualize the postsynaptic localization of KCC2 in SBC, the signal of which underlies the calretinin labeling. For this purpose, Cy3-conjugated donkey anti-goat secondary antibody (20 μg/ml in blocking solution) was used. Each cytochemical procedure was controlled by the omission of primary antibodies and the subsequent identical processing of few sections causing the lack of marked structures. Additionally, the fluorophores related to the relevant markers were switched; for example, anti-KCC2 was also revealed by donkey anti-rabbit Cy3 and calretinin by donkey anti-goat Cy2 antibodies. The images were acquired using a confocal laserscanning microscope (LSM 510, Zeiss, Germany).

Western blots

Two experimental approaches were performed for Western blot analysis. First, we took advantage of a clear demarcation of the cochlear nucleus in the dorsolateral brain stem by the posterior cerebellar peduncle; this enabled us to exclusively dissect this nucleus in P1, P5, P9, P14, and P30 gerbils. Whole cell lysates were prepared from this tissue by using the Mammalian Cell Lysis-1 Kit containing 7.2 MILENKOVIC´ , WITTE, TURECˇEK, HEINRICH, REINERT, AND RU¨ BSAMEN

\[ \text{RESULTS} \]

\[ \text{GABA}_A \text{ receptor-mediated responses in SBC} \]

Whole cell patch-clamp recordings were performed to investigate the characteristics of the GABA-induced responses. Focally applied GABA or muscimol evoked inward currents at $V_{\text{hold}}$ of −60 mV and [Cl\textsuperscript{−}], biased to 140 mM by the patch pipette. The GABA- or muscimol-elicited currents were blocked by 96 ± 1% (n = 8) or by 94 ± 2% (n = 5), respectively, by a bath application of SR95531, a GABA\textsubscript{A} receptor antagonist (Fig. 1). Furthermore, GABA and muscimol-evoked large ionic currents reversed close to the equilibrium potential predicted for Cl\textsuperscript{−} (Fig. 2). Reversal potentials for $I_{\text{GABA}}$ (Fig. 2, A and B) and $I_{\text{muscimol}}$ (C and D) were consistent with activation of Cl\textsuperscript{−} channels ($E_{\text{GABA}}$ = 4.8 ± 1.7 mV, n = 9; $E_{\text{muscimol}}$ = 5.3 ± 1.6 mV, n = 7; Nernst potential for Cl\textsuperscript{−} = 1.2 mV) and were independent of age (data not shown). Finally, dependence of the GABA\textsubscript{A} receptor-mediated currents on [Cl\textsuperscript{−}] was further exemplified with a low Cl\textsuperscript{−} (17 mM) pipette solution, which yielded a shift in $E_{\text{GABA}}$ to −49.2 ± 0.8 mV (n = 7), which is in accordance with the Nernst prediction of −52.6 mV (Fig. 2, E and F).

Because we observed no GABA\textsubscript{C}-mediated effects on SBC (data not shown), different deactivation kinetics of the responses evoked by GABA and muscimol (Figs. 1 and 2) are most likely due to a different rate of ligand unbinding from respective binding sites at the GABA\textsubscript{A} receptor (Baumann et al. 2003; Jones et al. 1998).

\[ E_{\text{GABA}} \text{ in developing SBC} \]

To examine the influence of brief GABA applications on the excitability of SBC with intact [Cl\textsuperscript{−}], gramicidin-perforated-patch recordings were performed on brain stem slices containing the rostral pole of the AVCN and isolated from P3 to P12 gerbils. This period was previously shown to be critical for a shift from glycnergic depolarization to hyperpolarization in the superior olivary complex nuclei (SOMC) in mammalian brain stem (Kandler and Friau 1995; Löhrke et al. 2005). Taking advantage of the cation-specific pore-forming agent gramicidin (Akaike 1996; Kyrozis and Reichling 1995), we were able to observe a developmental change in responses of SBC to GABA. As shown in Fig. 3A, pressure ejection of GABA to the somatic region produced depolarizing responses from rest in P3 and P4 SBC. However, already at P4, GABA exerted hyperpolarization in three of six SBC recorded. GABA-induced hyperpolarization observed between P5 and P8 were followed by a slower depolarization tail. These biphasic responses disappeared by P9, and thereafter the repolarizing
correlation to the Nernst prediction
inward component dominated by HCO₃⁻ recorded with a low [Cl⁻] of 1.6 mV. Nernst prediction
neurons (Ehrlich et al. 1999). As shown in Fig. 3
neurons (Coredero-Erausquin et al. 2005), and in the LSO
observations were reported in neurons of the hippocampus
/ HCO₃⁻ ions permeating through GABAₐ channel during the response (Cordero-Erausquin et al. 2005; Staley and Proctor 1999).

To elaborate the development of chloride-mediated inhibition in SBC, we also scrutinized the relationship between reversal potential of GABA responses (E_{GABA}) and the resting membrane potential (V_{rest}) (Fig. 4). Fifty-one of sixty neurons depicted in Fig. 4 showed monophasic responses to GABA application and in nine cells biphasic currents when recorded at V_{hold} slightly depolarized from E_{Cl⁻}. In all cells, the peak amplitude of the current (in biphasic responses the peak outward current) was measured and plotted because this component represents the Cl⁻ flux through the GABAₐ receptor.

FIG. 2. Responses to GABA and muscimol are primarily determined by [Cl⁻]. A: voltage dependence of the GABA-evoked fast transient currents. Example of current records at the holding potentials from -60 to 20 mV (V_{hold}) from a P8 SBC; [Cl⁻] = 140 mM. B: mean ± SD peak current-voltage relationship of the GABA-evoked current that was determined in nine (P7-9) independent experiments. E_{GABA} = 4.8 ± 1.7 mV. Nernst prediction = 1.2 mV. C: GABAₐ receptor-mediated currents recorded on a P7 SBC with [Cl⁻] = 140 mM. V_{hold} varied in 20-mV increments from -60 to +40 mV. D: I-V relationships for peak muscimol-evoked currents (n = 7, P7-9) E_{GABA} = 5.3 ± 1.6 mV. Nernst prediction = 1.2 mV. E: GABA receptor-mediated currents recorded with a low [Cl⁻] (17 mM) solution. The voltage steps were in 10-mV increments. F: reversal potential of the GABA-evoked currents altered in dependence on the intracellular chloride. Reduction of the [Cl⁻] = 17 mM yielded a shift in E_{GABA} to -49.7 ± 0.8 mV (n = 7, P7-9) which is in close correlation to the Nernst prediction = -52.6 mV. B, D, and F show mean ± SD values.

phase of the GABA-evoked hyperpolarizations became faster with maturity [τ = 0.9 ± 0.5 s for P3-5 (n = 8); τ = 0.5 ± 0.1 s for P10-12 (n = 12)]. These data indicate that the developmental shift in the action of GABA on SBC temporally correlates to the depolarizing-hyperpolarizing change in the glycine action on the rat lateral superior olive (LSO) and medial superior olive (MSO) neurons (Ehrlich et al. 1999; Löhreke et al. 2005). Biphasic responses as seen in Fig. 3 (recordings were done in bicarbonate buffer) can be explained by a depolarizing gradient for HCO₃⁻, which shapes the late phase of the GABA response when the driving force for Cl⁻ collapses (Cordero-Erausquin et al. 2005). In those cells, in which biphasic responses to GABA were recorded under the voltage clamp, we addressed the possibility of the initial outward component representing the Cl⁻ flux followed by an inward component dominated by HCO₃⁻ efflux. Comparable observations were reported in neurons of the hippocampus (Kaila et al. 1997; Staley et al. 1995), in spinal dorsal horn neurons (Cordero-Erausquin et al. 2005), and in the LSO neurons (Ehrlich et al. 1999). As shown in Fig. 3a, the slow inward tail of the biphasic response, recorded at a V_{hold} slightly depolarized with respect to E_{Cl⁻}, was abolished in HEPES-buffered extracellular solution and the remaining outward current represents the Cl⁻ flux. Moreover, the biphasic response observed in bicarbonate buffer was reverted to a monophasic course by shortening the application time of GABA (Fig. 3b), most likely due to changes in concentrations of Cl⁻ and HCO₃⁻ ions permeating through GABAₐ channel during the response (Cordero-Erausquin et al. 2005; Staley and Proctor 1999).

FIG. 3. A: developmental changes of the GABA effects on SBC. Somatic gramicidin perforated-patch recordings of the GABA-evoked changes in V_{rest}. Application of GABA produced depolarizing or hyperpolarizing responses from resting membrane potential, depending on the postnatal age. Ba: in some neurons, at a V_{hold} slightly depolarized with respect to E_{Cl⁻}, the current response to 50-ms puff application of GABA was characterized by a fast outward and a slower inward component. The inward component was abolished in HEPES-buffered extracellular solution, consistent with the notion of the remaining outward current representing the Cl⁻ flux. Bb: same neuron also shown in Bb displayed monophasic current in response to 10 ms application, still the responses were biphasic when GABA was pressure ejected for ≥20 ms.
(Cordero-Erausquin et al. 2005; Kaila et al. 1997; Staley et al. 1995). Comparison of $V_{\text{rest}}$, estimated in current-clamp mode and of $E_{\text{GABA}}$ in the respective neurons (acquired as the interpolated zero current value in $I-V$ relations) revealed that in 56% SBC at age P3-5 ($n = 18$), $E_{\text{GABA}}$ was depolarized with respect to the $V_{\text{rest}}$ (Fig. 4B). On the other hand, only in 1 from 12 (8%) P7 SBC $E_{\text{GABA}}$ was found to be depolarized. Hyperpolarizing action of GABA was observed in all SBC from P10 ($n = 4$), P11 ($n = 4$), and P12 groups ($n = 4$; Fig. 4B). Figure 5 clearly demonstrates the significant shift in mean values for $E_{\text{GABA}}$ between developmental stages [P3-5: $E_{\text{GABA}} = -41.7 \pm 2.2 \text{ mV}$ ($n = 18$); P7-9: $E_{\text{GABA}} = -64.9 \pm 2.0 \text{ mV}$ ($n = 30$); P10-12: $E_{\text{GABA}} = -79.0 \pm 3.3 \text{ mV}$ ($n = 12$)]. Furthermore, from 14 SBC investigated at P8 in gramicidin-perforated-patch configuration, we recorded seven neurons with 140 mM Cl$^-$ and seven neurons with 5 mM Cl$^-$ in the pipette solution. The respective mean values for $E_{\text{GABA}}$ (low [Cl$^-$]$_{\text{pip}}$ = 67.9 ± 1.2 mV; high [Cl$^-$]$_{\text{pip}}$ = 71.2 ± 1.9 mV; $P > 0.05$) did not differ between the two experimental approaches, suggesting that our recordings were independent of the Cl$^-$ in the pipette and that gramicidin ionophores are impermeable to Cl$^-$.

The changes in estimated resting membrane potential between the P3-5 and P10-12 groups were less pronounced yet significant; $P < 0.05$: P3-5: $V_{\text{rest}} = -45.0 \pm 1.6 \text{ mV}$ ($n = 31$); P7-9: $V_{\text{rest}} = -53.4 \pm 0.9 \text{ mV}$ ($n = 47$); P10-12: $V_{\text{rest}} = -61.3 \pm 1.1 \text{ mV}$ ($n = 37$); Fig. 5). This observation is consistent with the notion of depolarized neuronal membrane potential being a general feature of immaturity (hippocampus: Cherubini et al. 1990; neocortex: Luhmann and Prince 1991; McCormick and Prince 1987; Owens et al. 1996; LSO: Kakazu et al. 1999).

Taken together, the data in Fig. 5 demonstrate the change from mainly depolarizing responses to GABA in SBC up to P5.
The preceding results suggest that by the end of the first postnatal week [Cl\(^-\)], in most SBC is lower than it would be otherwise outward \(I_B\), and \(E_{\text{Cl}}\) before and after the application of furosemide. Therefore only the cells showing monophasic \(I_{\text{GABA}}\) before and after the application of the blockers. Therefore only the cells showing monophasic responses to GABA (17 of 21 neurons recorded) were analyzed and represented in Fig. 6. The peak amplitudes of the currents, recorded at different holding potentials, were measured and plotted in Fig. 6C revealing a furosemide-induced shift in \(E_{\text{GABA}}\) by 13 mV in a P11 SBC. To avoid the loading of the cells with Cl\(^-\) by a repetitive activation of Cl\(^-\) conductance, which could mimic the effect of KCC2 inhibitors in our in vitro system, we reverted to the voltage protocol established by Ehrlich et al. (1999) and later used by Löhrecke et al. (2005), for similar experiments performed in SOC. In three recorded neurons at P11, furosemide caused a change of \(E_{\text{GABA}}\) toward more depolarized values (from \(-75.9 \pm 5.9\) to \(-60.7 \pm 3.2\) mV; \(P < 0.05\)). Besides interrupting the net outward Cl\(^-\) transport in older neurons, furosemide was also shown to perturb Cl\(^-\) accumulation mechanisms in immature neurons (Thompson and Gähwiler 1989; Owens et al. 1996; Jarolimek et al. 1999), but given the maturity of SBC, our finding is consistent with the activity of the furosemide-sensitive Cl\(^-\) extrusion mechanism. This notion is further supported by an effect of DIOA (Fig. 6D), a more specific KCC2 antagonist, which in P11 SBC caused a significant positive shift in \(E_{\text{GABA}}\) by \(15.1 \pm 2.1\) mV, \(P < 0.01\) (\(n = 3\)); in P7-9 SBC, the shift was \(12.5 \pm 3.0\) mV, \(P < 0.05\) (\(n = 6\)); but no significant change in \(E_{\text{GABA}}\) was observed at P3 [control: \(-33.2 \pm 6.0\) mV vs. DIOA: \(-29.0 \pm 4.9\) mV (\(n = 4\)]; paired Student’s \(t\)-test]. These data imply functional alterations regarding the KCC2 activity between P3 and P7-9 SBC.

**KCC2 expression in neonatal AVCN**

To assess the developmental pattern of the KCC2 protein expression in AVCN neurons, we performed immunohisto-
membrane-enriched fractions (mf) of P14 gerbils was determined. Note that all membranes in yield a weaker signal of the cochlear nuclei membrane preparation.

Figure 7, more pronounced. Figure 7, perisomatic and peridendritic immunoreactivity becoming

B–F, neurons at P0 (Gulyas et al. 2001). As illustrated in Fig. 7, appeared to be restricted to neuropil at P1 (Fig. 7

Hoechst dye, which marks the cell nuclei. The KCC2 signal P1 and P8 was present in areas which are not labeled by the AVCN. Counterstaining of cell nuclei at younger stages (P1–P8) was performed with Hoechst 33258 (blue, A–E) for better identification of the neurons. A: strong KCC2 immunoreactivity was present in the CN at P1. B–E: between P5 and P8, the labeling pattern changes from putative neuropol to perisomatic (arrows) and peridendritic (arrowheads) staining. At P9 (F), P14 (G), and P30 (H), pronounced perisomatic immunoreactivity of the putative SBC cell membrane (arrows) and dendrites was found (arrowheads). I: anti-calretinin staining of the endbulbs of Held (arrowheads) outlines the KCC2 immunoreactivity (arrows), ruling out a possible presynaptic KCC2 localization. Scale bar: 10 μm.

chemical and Western blot studies. Immunohistochemical staining was conducted on three to four brains per age group and revealed abundant KCC2 protein expression in the AVCN already at P1 (Fig. 7A). The KCC2 immunoreactivity between P1 and P8 was present in areas which are not labeled by the Hoechst dye, which marks the cell nuclei. The KCC2 signal appeared to be restricted to neuropil at P1 (Fig. 7A). Similar observations were reported for the rat SOC (Balakrishnan et al. 2003; Blaesse et al. 2006; Löhcke et al. 2005) and hippocampal neurons at P0 (Gulyas et al. 2001). As illustrated in Fig. 7, B–F, the staining pattern changed from P5 to P9 with the perisomatic and peridendritic immunoreactivity becoming more pronounced. Figure 7, F–H, indicates that the distribution of KCC2 reaches an “adult-like” pattern at P9, characterized by weak cytoplasm labeling, strong perisomatic, proximal and, possibly distal dendrite labeling. Additionally, the postsynaptic KCC2 immunoreactivity was confirmed by labeling of presynaptic endings (endbulbs of Held) with calretinin (Bazwinsky and Rübsamen 2000), revealing thick labeling pattern around the somata of SBC (Fig. 7I).

The investigation of the KCC2 by means of Western blot also showed continuous protein expression throughout the postnatal period investigated (P1–P30; Fig. 8). Consistent with the earlier work of Williams et al. (1999) and Stein et al. (2004), a strong immunoreactivity was restricted to a ~140-kDa band. These authors used the membrane-enriched fraction from tissue suspension, whereas our preparations were made from the whole cell lysates (due to the small size of the cochlear nucleus). Therefore we additionally compared our data to Western blots of membrane fractions isolated from cerebellum, cortex, brain stem, and dissected cochlear nuclei. A closer inspection of the data in Fig. 8B showed no qualitative difference between the bands obtained from the two different preparations. Together, immunohistochemical and Western blot data strongly corroborate the notion that the KCC2 is expressed in SBC before GABA attains its hyperpolarizing action in these neurons.

**DISCUSSION**

The purpose of the present study was to examine the time course of the [Cl⁻]i homeostasis in neurons of the cochlear nucleus. Our findings provide compelling evidence for the developmentally regulated negative shift of E_gaba indicating the maturation of inhibitory transmission in the cochlear nucleus, the first stage of signal processing in the mammalian central auditory system. The immunoreactivity of the Cl⁻-extruding cotransporter KCC2 can be visualized in SBC at P1, yet the switch from depolarizing to hyperpolarizing action of GABA occurs around P7 at the same time when physiological indications of KCC2 activity were found. Furthermore, the present results reveal that at the first central synapse of the mammalian auditory pathway GABA utilizes different signaling mechanisms than previously shown for the homologue synapse in the avian auditory system.

**Inhibitory neurotransmission in cochlear nucleus of mammals and birds**

Despite the independent evolution of the neuronal brain stem system for processing of interaural time differences (ITDs) in birds and mammals (reviewed in Grothe 2003), there are striking similarities in the organization of the auditory brain stem nuclei between the two homeothermic vertebrates with respect to monaural and binaural signal processing in second- and third-order neurons (reviewed in Oertel 1999). Still there are differences in how inhibition is linked into this system.

**FIG. 8.** Western blot analysis of the samples prepared from cochlear nuclei whole cell lysates (A), and membrane fractions (B), from cerebellum (CB), cortex (CX), brain stem (BS), and cochlear nuclei (CN). A: whole cell (wc) protein lysates were obtained from cochlear nuclei at P1, P5, P9, P14, P30, and constant amounts of protein (20 μg) were subjected to the Western blot analysis with anti-KCC2 antibody. B: for comparison, the KCC2 expression from the membrane-enriched fractions (mf) of P14 gerbils was determined. Note that all membranes in B except the cochlear nuclei (5 μg) were loaded at 20 μg; this yielded a weaker signal of the cochlear nuclei membrane preparation.

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In the avian NM, GABAergic terminals were found on neuron somata, and GABAergic inhibition originates from intrinsic neurons and from neurons in the superior olivary nucleus (SON) (Carr et al. 1989; Lachica et al. 1994; Yang et al. 1999). The SON seems to serve comparable functions as the mammalian periolivary nuclei. The fact is that glycine is hardly present in the superior olivary complex and from the medial nucleus of the trapezoid body (Benson and Potashner 1990; Ostadoff et al. 1990, 1997), whereas inhibitory projections originating from the ipsilateral dorsal cochlear nucleus and from contralateral CN are exclusively glycineric (Wenthold 1987; Wicksberg and Oertel 1990). Glycineric synaptic activity was demonstrated in several in vitro studies in the rat AVCN (P12-16) (Lim et al. 1999, 2000, 2003). In vivo electrophysiology using acoustic stimulation reported significant effects of both GABAergic and glycineric signal transmission on the precisely timed electrical signaling of bushy cells (Backoff et al. 1997, 1999; Caspary et al. 1994; Kopp-Scheinplug et al. 2002). Recordings of spontaneous and stimulus-evoked currents before and after the onset of hearing, suggest functional synaptic inputs on SBC mediated through both GABA_A and glycine receptors (unpublished data).

In the avian nucleus laminaris neurons, GABAergic inhibition is achieved by membrane depolarization throughout the life span (Funabiki et al. 1998; Yang et al. 1999), whereas in mammals, both MNTB and SOC neurons show a shift from depolarizing to hyperpolarizing gradients for Cl^- before the onset of hearing (Löhrke et al. 2005). To get a better understanding of the development of GABAergic inhibition in the cochlear nucleus, we tracked the postnatal changes in responses of SBC. In the chick, the GABA_A-receptor-mediated responses inhibit NM neurons via a depolarizing shunting inhibition (Hyson et al. 1995; Monsivais and Rubel 2001; Lu and Trussell 2001), yet in the gerbil GABA hyperpolarizes SBC by the end of the first postnatal week. This is consistent with the recent data by Price and Trussell (2006), who recorded hyperpolarizing responses to glycine from rat globular bushy cells at P9-11. The present study shows the time course of maturation of inhibitory neurotransmission in mammalian anteroventral cochlear nucleus and provides evidence on the underlying cellular mechanisms causing a shift from depolarization to hyperpolarization before the onset of hearing.

**Hyperpolarizing GABA responses correlate with the activity of KCC2**

The neuron-specific K^+^-Cl^-cotransporter KCC2 was proposed as the key molecule to render low [Cl^-]_i during development in various CNS regions and, as a result, to gradually convert depolarizing and excitatory GABA responses to the well-established hyperpolarizing inhibition seen in the adult (Rivera et al. 2005). In the neocortex, hippocampus, and retinal neurons of rats, KCC2 expression increases postnatally up to the end of the second postnatal week, a period during which E_{GABA} becomes hyperpolarizing with respect to V_m (Gulyas et al. 2001; Lu et al. 1999; Rivera et al. 1999; Shimizu-Okabe et al. 2002; Vu et al. 2000). In the auditory brain stem SOC, the shift from depolarizing to hyperpolarizing action of glycine is staggered in time and occurs over a period of almost 2 weeks (Ehrlich et al. 1999; Kandler and Friauf 1995; Löhrke et al. 2005; Sanes and Friauf 2000). The effect is due to an age-dependent decrease in [Cl^-], in SOC neurons, and KCC2 was proposed to be the key mediator of this change (Ehrlich et al. 1999; Kakazu et al. 1999). Our immunohistochemical and Western blotting data from the CN in gerbils show KCC2 expression already at P1. A similar study focusing on the CN in rats also found an early KCC2 expression at P3 (earliest time point examined) and constant expression levels throughout subsequent auditory development (Vale et al. 2005). According to our data, at P1 the KCC2 immunoreactivity appears to be mainly distributed in the neuropil and then gradually becomes perisomatic and possibly peridendritic between P5 and P9 (Fig. 7, B–F), indicating subcellular changes of the KCC2 expression.

Interestingly, neither our nor previous studies of the auditory brain stem (LSO, Balakrishnan et al. 2003; CN, Vale et al. 2005; SOC, Löhrke et al. 2005) showed any increase in the KCC2 protein level throughout the postnatal development, which has been described as the rate-limiting step for KCC2 functionality in forebrain areas (Khirug et al. 2005; Rivera et al. 1999; Stein et al. 2004). In accordance with our endeavor, a broader developmental study in mice by Stein et al. (2004) reported adult-like protein levels of KCC2 at birth in the spinal cord as well as in the brain stem.

Considering our data and those of Balakrishnan et al. (2003) and Löhrke et al. (2005), the mechanisms underlying Cl^- extrusion in CN and SOC neurons could be very similar: although the expression of KCC2 occurs early in postnatal development, its efficiency in Cl^- extrusion still seems to be very low (Fig. 6D). At this early developmental stage, inhibitory neurotransmitters GABA and glycine cause membrane depolarization in CN neurons (M. Witte, unpublished observations). The KCC2 activity in SBC, possibly accomplished through phosphorylation (Kelsch et al. 2001; Vale et al. 2005), is most likely achieved by the end of the first postnatal week. In a recent study of the rat LSO, Blaesse et al. (2006) reported that oligomerization of KCC2 protein correlates with its activation. The present study affirms the notion that, unlike in forebrain areas, KCC2 expression in auditory brain stem neurons does not reflect its functional state per se.

This hypothesis is corroborated by our gramicidin-perforated-patch-clamp experiments. We could demonstrate that in SBC the KCC2 inhibitor DIOA (Coull et al. 2003) or furosemide (Ehrlich et al. 1999; Jarolimek et al. 1999; Kakazu et al. 1999; Martina et al. 2001) reverse E_{GABA} toward depolarized values. The experiments with DIOA also suggest that KCC2 most likely contributes to Cl^- extrusion at P7 and P11, whereas it does not seem to be functional at P3 when GABA is still depolarizing.

In another set of earlier studies, it was shown that KCC2 and the Cl^-accumulating cotransporter NKCC1 both display sensitivity to furosemide and also that they are reciprocally regulated during development (Kanaka et al. 2001; Shimizu-Okabe et al. 2002; Yamada et al. 2004). We acquired our furosemide data from P11 SBC, when in all cells the application of GABA
had a hyperpolarizing effect, consistent with the active \( \text{Cl}^- \) extrusion by KCC2. Thus we assumed that the observed DIOA- and furosemide-induced depolarizing shifts of \( E_{\text{GABA}} \) in SBC are due to the inhibition of KCC2 activity.

**Technical consideration**

We observed a sustained shift in \( E_{\text{GABA}} \) toward negative values (from approximately \(-42 \) to \(-79 \) mV) from P3-5 up to the time of hearing onset at P12 (Woolf and Ryan 1984). During this period, the resting membrane potential gradually became less depolarized, and the difference in \( V_{\text{rest}} \) was significant between the P3-5 and P10-12 groups (\(-45 \) mV compared with \(-61 \) mV, respectively). We estimated \(-61 \) mV for \( V_{\text{rest}} \) at P10-12, which is comparable to the respective values for bushy cells in rats (\(-62 \) mV, P9-11) (Price and Trussell 2006) and mice (\(-64 \) mV, P16-19) (McGinley and Oertel 2006). More depolarized values of the membrane potential seem to be a general feature of neuronal immaturity (Ben-Ari et al. 1989; Lo Turco et al. 1991; Luhmann et al. 2000; Zhou and Hablitz 1996). Still it should not be concealed that estimation of \( V_{\text{rest}} \) by gramicidin-perforated-patch measurements could potentially show a small systematic bias as demonstrated by Tyzio et al. (2003). These authors showed a trend toward more depolarized values of the membrane potential during perforated-patch and whole cell recordings in small neurons with high-input resistances like hippocampal pyramidal cells. The SBC recorded here, however, are significantly larger and have considerably lower input resistances throughout early postnatal development (McGinley and Oertel 2006; Wu and Oertel 1987). Still we cannot rule out the possibility that the recorded values for \( V_{\text{rest}} \) at P3-5 are slightly shifted to more depolarized values, but such a bias would not interfere with the description of the developmental change of \( E_{\text{GABA}} \) shown here. More important, slightly augmented depolarized \( V_{\text{rest}} \) values in younger animals would not interfere with our conclusion on the action of GABA: at P2-5 the driving force for \( \text{Cl}^- \) is depolarizing while already at P7-9, when \( E_{\text{GABA}} \) reaches \(-65 \) mV, the driving force for \( \text{Cl}^- \) is hyperpolarized even to the \( V_{\text{rest}} \) of P10-12 neurons (\(-61 \) mV).

**Physiological relevance**

Throughout the early developmental period GABA could have a trophic effect and promote stabilization of synapses in the CN as previously shown in various neuronal structures (reviewed in Ben Ari 2002; Lauder 1993; Owens and Kriegstein 2002). In the present study, we show functional GABA\(_A\) receptors at P3 (the earliest time point investigated), which mediate depolarizing responses. Such depolarizing responses to GABA and also to glycine were shown to induce transient elevation of cytoplasmic Ca\(^{2+}\) by activating voltage-dependent Ca\(^{2+}\) channels (Reichling et al. 1994; Takahashi 1984; Wu et al. 1992). However, GABAergic currents last longer than glycineric ones and thus are thought to be more effective in triggering intracellular Ca\(^{2+}\) signals (Chery and de Koninck 1999; Yoshimura and Nishi 1995; unpublished observations in CN neurons). The increased [Ca\(^{2+}\)], might be causally related to mechanisms underlying development and stabilization of functional neuronal networks (Kocsis et al. 1993; Spitzer 1994). In rat cultured hippocampal neurons, for example, the shift from depolarizing to hyperpolarizing effects of GABA is mediated by the GABA\(_A\) receptors through the regulation of the mRNA level for KCC2 (Ganguly et al. 2001; but see also Ludwig et al. 2003 and Titz et al. 2003). Furthermore, a recent report on rat hippocampal cultures suggests that early KCC2 expression enhances the formation of functional GABAergic synapses (Chudotvorova et al. 2005). Thus it is tempting to assume the utilization of similar mechanisms for strengthening the inhibitory neurotransmission in the CN.

In adult CN, the precise temporal information given by bushy cells is determined by the interaction between excitatory (auditory nerve fibers) and noninhibitory (both GABAergic and glycineric) inputs, as concluded from in vivo electrophysiological studies (chinchilla, Backoff et al. 1997, 1999; Caspary et al. 1994; gerbil, Kopp-Scheinpflug et al. 2002). Through this inhibition SBC improves the temporal precision of postsynaptic neuronal discharges as seen from the neurons’ phase locking accuracy to pure-tone stimuli. Such temporal precision in the tenth of microsecond range is beneficial for the ITD processing in the MSO (reviewed in Grothe 2003).

In conclusion, the present study suggests a change from depolarizing to hyperpolarizing GABA responses in SBC by the end of the first postnatal week and points to the possible involvement of KCC2 in rendering GABA hyperpolarizing. Although KCC2 is already expressed at birth, the onset of its activity coincides with changes of \( E_{\text{GABA}} \) and, thus it is likely to play a role in the reduction of the \([\text{Cl}^-]\) to the “adult-like” level before hearing onset.

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**REFERENCES**


