Physiol. Res. 60 (Suppl. 1): S15-S27, 2011

REVIEW

Development of the Chloride Homeostasis in the Auditory Brainstem

I. MILENKOVIĆ[1], R. RÜBSAMEN[1]

[1]Faculty of Biosciences, Pharmacy and Psychology, University of Leipzig, Leipzig, Germany

Received February 14, 2011
Accepted March 30, 2011
On-line July 19, 2011

Summary

Inhibitory neurotransmission plays a substantial role in encoding of auditory cues relevant for sound localization in vertebrates. While the anatomical organization of the respective afferent auditory brainstem circuits shows remarkable similarities between mammals and birds, the properties of inhibitory neurotransmission in these neural circuits are strikingly different. In mammals, inhibition is predominantly glycinergic and endowed with fast kinetics. In birds, inhibition is mediated by γ-Aminobutiric acid (GABA) and too slow to convey temporal information. A further prominent difference lies in the mechanism of inhibition in the respective systems. In auditory brainstem neurons of mammals, $[Cl^-]_i$ undergoes a developmental shift causing the actions of GABA and glycine to gradually change from depolarization to the 'classic' hyperpolarizing-inhibition before hearing onset. Contrary to this, in the mature avian auditory brainstem Cl^- homeostasis mechanisms accurately adjust the Cl^- gradient to enable depolarizing, but still very efficient, shunting inhibition. The present review considers the mechanisms underlying development of the Cl^- homeostasis in the auditory system of mammals and birds and discusses some open issues that require closer attention in future studies.

Key words

GABA • Glycine • Development • Inhibition • Auditory pathways

Corresponding author

Ivan Milenković, Faculty of Biosciences, Pharmacy and Psychology, Institute of Biology, University of Leipzig, Talstrasse 33, D-04103 Leipzig, Germany. Fax: +49 3419736848. E-mail: mili@uni-leipzig.de

Introduction

The central auditory system of mammals and birds encodes information about the location of a sound source by comparing – in a frequency-specific manner – the level of sound, the time of its arrival, and spectral disparities at the two ears. The interaural level and time differences (ILD and ITD) are first computed in brainstem nuclei where the inputs from both ears converge (Grothe *et al.* 2010, Konishi 2003). In the respective neuronal circuits, excitation is mediated by synaptically released glutamate while the inhibitory neurotransmission which strongly contributes to the processing of the sound source localization, is mediated by γ-Aminobutiric acid (GABA) and glycine (Grothe 2003). Despite the long separated evolution of the two vertebrate lines leading to the homeothermic birds and mammals, there are striking similarities in the organization of the auditory brainstem nuclei with respect to monaural and binaural signal processing in second- and third-order neurons of the ascending auditory system (Oertel 1999). This is mostly true if one considers excitatory aspects of the afferent processing system, but there are substantial differences in the properties of inhibitory neurotransmission in each system. The inhibitory neurotransmission in the avian auditory brainstem is mainly mediated by GABA and has slow kinetics (Howard *et al.* 2007, Howard and Rubel 2010, Lu and Trussell 2000, Monsivais *et al.* 2000); glycinergic synaptic inputs have been only recently described (Kuo *et al.* 2009) and their functional role is still unclear. In mammals, on the other hand, fast and predominantly glycinergic transmission is the characteristic feature of many brainstem nuclei (Awatramani *et al.* 2004, Grothe and Sanes 1994, Kandler

PHYSIOLOGICAL RESEARCH • ISSN 0862-8408 (print) • ISSN 1802-9973 (online)

Fax +420 241 062 164, e-mail: physres@biomed.cas.cz, www.biomed.cas.cz/physiolres

and Friauf 1995, Lim *et al.* 2003, Magnusson *et al.* 2005, Smith *et al.* 2000). Even more surprising is how the inhibitory effect is accomplished in the respective systems. In the mammalian brain, GABA and glycine are the main inhibitory transmitters evoking hyperpolarization in most mature neurons, due to Cl^- influx through $GABA_A$ and glycine receptors (Bormann *et al.* 1987, Kaila 1994, Sivilotti and Nistri 1991). The influx of Cl^- is maintained through an inward-directed electrochemical gradient at the resting membrane potential, effective due to a relatively low intracellular Cl^- concentration ($[Cl^-]_i$). The same applies for the adult auditory brainstem neurons, where the inhibitory action of GABA and glycine is achieved by both (i) shunting, i.e. reduction of the amplitude of excitatory postsynaptic potentials due to a local reduction of the input resistance in the vicinity of excitatory synapses and (ii) by membrane hyperpolarization, i.e. moving the membrane potential away from the action potential threshold. In prehearing mammals, on the other hand, the same auditory brainstem neurons show depolarizing inhibitory postsynaptic potentials (IPSPs) mostly evoked by GABA. Yet, in the avian auditory brainstem neurons, depolarizing GABAergic IPSPs are maintained also in the mature system (Hyson *et al.* 1995, Lu and Trussell 2001). The inhibitory effect is achieved, despite their depolarizing action, due to the shunting effect of the outward directed Cl^- conductance and subsequent activation of the low-voltage activated K^+ conductance (Howard *et al.* 2007, Monsivais and Rubel 2001). Thus, when comparing the avian and the mammalian brainstem circuits, the difference in the $[Cl^-]_i$ underlies the respective mechanism of inhibition (depolarizing vs. hyperpolarizing). In fact, the nature of the synaptic response evoked by GABA/glycine is determined by the sign of the difference between the reversal potential $E_{GABA/Gly}$ and the resting potential of the cell (V_{rest}). While substantial literature is available on the regulation of the Cl^- homeostasis in developing auditory brainstem of rats, mice, and gerbils, the mechanisms maintaining high $[Cl^-]_i$ in the homologue chick neurons receiving GABA/glycinergic synaptic inputs remain inconclusive.

Shift from depolarizing to hyperpolarizing inhibitory neurotransmission in the auditory brainstem of mammals

In forebrain areas of the mammalian CNS, depolarizing GABA/glycinergic responses were described as a transient feature of immature neurons of the hippocampus (Ben-Ari *et al.* 1989, Cherubini *et al.* 1990), hypothalamus (Chen *et al.* 1996), neocortex (Owens *et al.* 1996, Yuste and Katz 1991), cerebellum (Eilers *et al.* 2001), retina (Huang and Redburn 1996), spinal cord (Reichling *et al.* 1994, Wu *et al.* 1992) and of cultured midbrain neurons (Jarolimek *et al.* 1999, Titz *et al.* 2003). The respective excitatory responses are due to the elevated intracellular Cl^- concentration in the immature neurons which is attributable to the prolonged postnatal development of the chloride homeostasis system (Ben-Ari 2002, Ben-Ari *et al.* 2007, Owens and Kriegstein 2002). Depolarizations mediated by GABA/glycine can cause an increase in intracellular Ca^{2+} concentration, generally thought to promote synapse stabilization during the critical period of synaptic reorganization in developing neuronal circuits (Ben-Ari *et al.* 2007). A similar time course of the $[Cl^-]_i$ regulation was also described in auditory brainstem nuclei of rats, mice, and gerbils, where GABA/glycinergic responses shift from depolarizing to hyperpolarizing (D/H shift) during the early postnatal development (cochlear nucleus: Milenkovic *et al.* 2007, Vale *et al.* 2005; superior olivary complex: Balakrishnan *et al.* 2003, Ehrlich *et al.* 1999, Kakazu *et al.* 1999, Kandler and Friauf 1995, Löhrke *et al.* 2005). In the lateral superior olive (LSO), depolarizing GABA/glycinergic synaptic inputs can even trigger action potentials and activate voltage gated Ca^{2+} channels which might change the strength of GABA or glycinergic synapses in a synapse-specific manner (Kullmann *et al.* 2002, Kullmann and Kandler 2008). In this regard, noteworthy is also the higher potency of synaptic GABA to evoke intracellular Ca^{2+} signals with respect to glycine. This seems to be a general feature of mixed GABA/glycinergic synapses and probably due to longer GABAergic currents being more effective in triggering Ca^{2+} signals (Chery and De Koninck 1999, Jonas *et al.* 1998, Yoshimura and Nishi 1995). Referring to developmentally-regulated synaptic reorganization, it is interesting that synaptic depression evoked by depolarizing GABA, but not by glycine (Chang *et al.* 2003, Kotak and Sanes 2000), possibly precedes synapse elimination in the LSO and thereby contributes to the functional tonotopic refinement of developing auditory brainstem circuits (Kandler *et al.* 2009).

Following the depolarizing phase of the early postnatal development, $[Cl^-]_i$ is regulated to enable hyperpolarizing responses to GABA/glycine. As in forebrain structures, the activity of the K^+-Cl^--extruding cotransporter KCC2 mediates the D/H shift in the auditory brainstem neurons (Balakrishnan *et al.* 2003, Ehrlich *et al.* 1999, Milenkovic *et al.* 2007). KCC2 is a neuron-specific, secondary-active cotransporter which uses energy stored in the K^+-chemical gradient to move Cl^- against its chemical gradient out of the neuron *via* an electroneutral K-Cl transport (Payne *et al.* 2003). The increased KCC2 activity in developing neurons gradually renders E_{Cl} more negative than V_m and converts depolarizing and excitatory GABA responses to the well-established hyperpolarizing inhibition seen in the adult (Hübner *et al.* 2001, Rivera *et al.* 2005). In order to understand the functional significance of the dynamic Cl^- homeostasis regulation in auditory brainstem nuclei, it is important to refer it to the onset of acoustically evoked signal processing which in rats, mice, and gerbils occurs after postnatal day twelve (>P12) (Blatchley *et al.* 1987, Sonntag *et al.* 2009, Woolf and Ryan 1984).

Auditory nerve fibers convey excitatory input from the cochlear hair cells to different types of second-order neurons in all three partitions of the cochlear nucleus (CN). The spherical bushy cells (SBC) in the anteroventral CN are the starting point of the brainstem circuit that computes acoustic localization based on ITD in low-frequency sounds. These neurons integrate excitatory inputs from just a few auditory nerve terminals (endbulbs of Held) (Brawer and Morest 1975, Ryugo and Sento 1991) and likewise acoustically driven GABA- and glycine-mediated inhibitory inputs (Backoff *et al.* 1999, Caspary *et al.* 1994, Dehmel *et al.* 2010, Gai and Carney 2008, Kopp-Scheinpflug *et al.* 2002). In the SBC, the D/H shift is completed by the end of the first postnatal week (<P8) (Fig. 1A). The activity of the KCC2 gradually changes the reversal potential for $GABA_A$-mediated responses (E_{GABA}) from –42 mV (at P3-5) to –79 mV (at P10-12). Based on these data, the $[Cl^-]_i$ calculated according to the Goldman equation [considering the E_{HCO3} of –12 mV and permeability HCO_3^-/Cl^- of 0.2 (Bormann *et al.* 1987, Staley and Proctor 1999)] changes from ~ 23 mM to ~ 8 mM. The globular bushy cells (GBC) represent another type of second-order neurons in the ventral CN showing a higher degree of convergence of excitatory auditory nerve fiber inputs. These neurons are the starting point of the brainstem circuitry that processes sound source localization based on ILD of high-frequency sounds. In GBC, hyperpolarizing responses to glycine were recorded at P9-11 (E_{Gly} ~ –94 mV and estimated $[Cl^-]_i$ ~ 4 mM), which is consistent with the changes shown in SBC. However, the synaptic terminals of GBC that provide calyceal excitatory input to the contralateral medial nucleus of the trapezoid body (MNTB), maintain depolarized E_{Gly} with respect to V_m (–51 mV vs. –77 mV, respectively) (Price and Trussell 2006). These terminals apparently lack the KCC2 ($[Cl^-]_i$ ~ 21 mM) even after hearing onset. Hence, the activation of presynaptic GABA and glycine receptors depolarizes the terminal and facilitates glutamate release (Turecek and Trussell 2001). So, in cochlear nucleus bushy cells, the KCC2 within the somata changes the initially high $[Cl^-]_i$ to approximately 4-8 mM before hearing onset, while in the calyceal terminals of GBC the $[Cl^-]_i$ remains elevated due to the lack of this transporter. The axon terminals from SBC, on the other hand, provide binaural excitatory synaptic inputs to the neurons in the medial superior olive (MSO) which are capable of encoding ITD in the sub-millisecond range. In the MSO neurons, measurements using voltage-sensitive dye- and perforated-patch recordings revealed a D/H shift between P5-9 (Löhrke *et al.* 2005) (Fig. 2). Thus, the Cl^- regulation in the MSO neurons and CN bushy cells follows a similar time course.

The neurons considered so far convey excitatory information, but inhibitory projection neurons also contribute to the brainstem sound localization circuitry. Most notably these are the principal neurons (PN) from the MNTB which provide the major glycinergic input to a number of nuclei such as MSO, LSO and superior paraolivary nucleus (SPN). The PN receive a strong glutamatergic input from GBC, but they are also the target of inhibitory projections releasing glycine and/or GABA. While the sources of the latter inputs still remain inconclusive, it has been shown by means of perforated-patch recordings that the action of inhibitory inputs changes from depolarizing (E_{GABA} ~ –50 mV at P5-7) to hyperpolarizing (E_{Gly} ~ –80 mV at P13-15) (Awatramani *et al.* 2005). These data and data from Löhrke *et al.* (2005) suggest that the D/H shift in the MNTB occurs later in development than in the aforementioned excitatory neurons, i.e. just prior to hearing onset.

Principal neurons of the LSO are involved in the binaural processing of ILD, based on the integration of the excitatory input from the ipsilateral cochlear nucleus and inhibitory input from the MNTB, which is driven by sound from the contralateral ear. The LSO contains both excitatory and inhibitory principal neurons which establish ascending projections to the inferior colliculus in the midbrain. Concerning the inhibitory input to the LSO, the terminals from the MNTB not only undergo

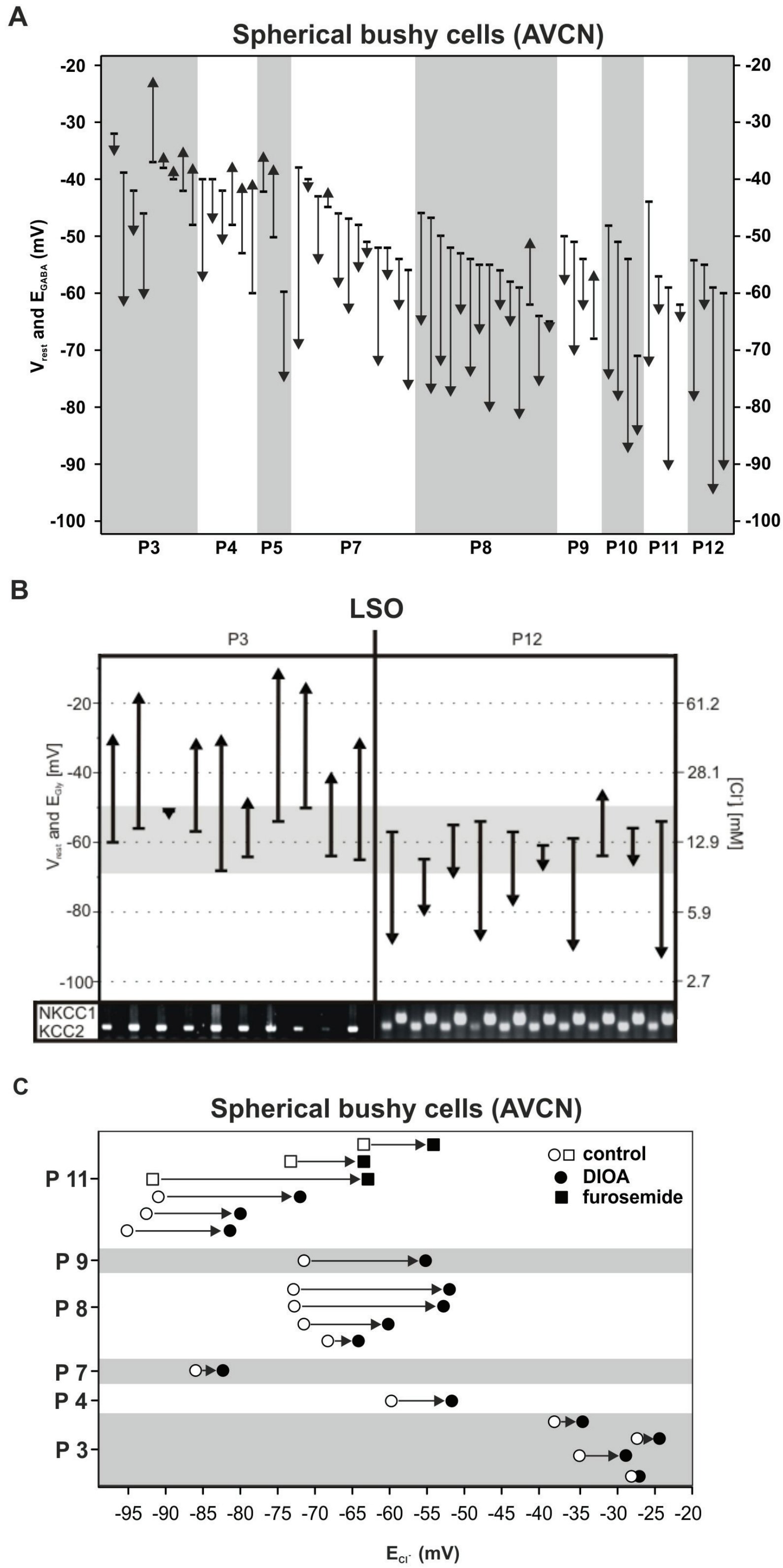

Fig. 1. Developmental shift of $E_{GABA/Gly}$ in SBC of the anteroventral cochlear nucleus (AVCN) and in LSO neurons. (**A, B**) Estimated V_{rest} and $E_{GABA/Gly}$ are plotted for each neuron recorded in gramicidin-perforated patch configuration and the cells were sorted by age of the animal. V_{rest} is indicated by the base of each arrow and $E_{GABA/Gly}$ by the corresponding arrow tip; arrows pointing upwards indicate the cells in which measured $E_{GABA/Gly}$ was depolarizied with respect to the V_{rest}, downward pointing arrows indicate hyperpolarized $E_{GABA/Gly}$. The presence of KCC2 and NKCC1 transcripts in individual LSO neurons was investigated by single-cell RT-PCR, depicted in the bottom part of the figure in B. Every neuron analyzed was positive for KCC2 mRNA, regardless of age. In contrast, no NKCC1 transcript was detected in the P3 group, whereas it was present in every neuron at P12. (**C**) The D/H shift in SBC is mediated by the KCC2 activity. The effects of KCC2 inhibitors, DIOA (50 μM) or furosemide (100 μM) on E_{Cl}. Arrows indicate the shift of E_{Cl} caused by DIOA (black circles) or by furosemide (black squares) for each cell recorded with its maturity given. Note that at P3 DIOA caused only minor changes of E_{Cl}. Gray and white backgrounds simply separate the cells from different postnatal days. (A and C from Milenkovic *et al.* 2007, B with kind permission by the authors from Balakrishnan *et al.* 2003).

a developmental change in neurotransmitter phenotype, from being primarily GABAergic to being glycinergic (Nabekura *et al.* 2004), but they also transiently release glutamate (Gillespie *et al.* 2005). Co-transmission with glutamate is most prevalent during the first postnatal week, the period when GABA and glycine are still depolarizing. This provides a favourable condition for the induction of NMDA-dependent developmental plasticity,

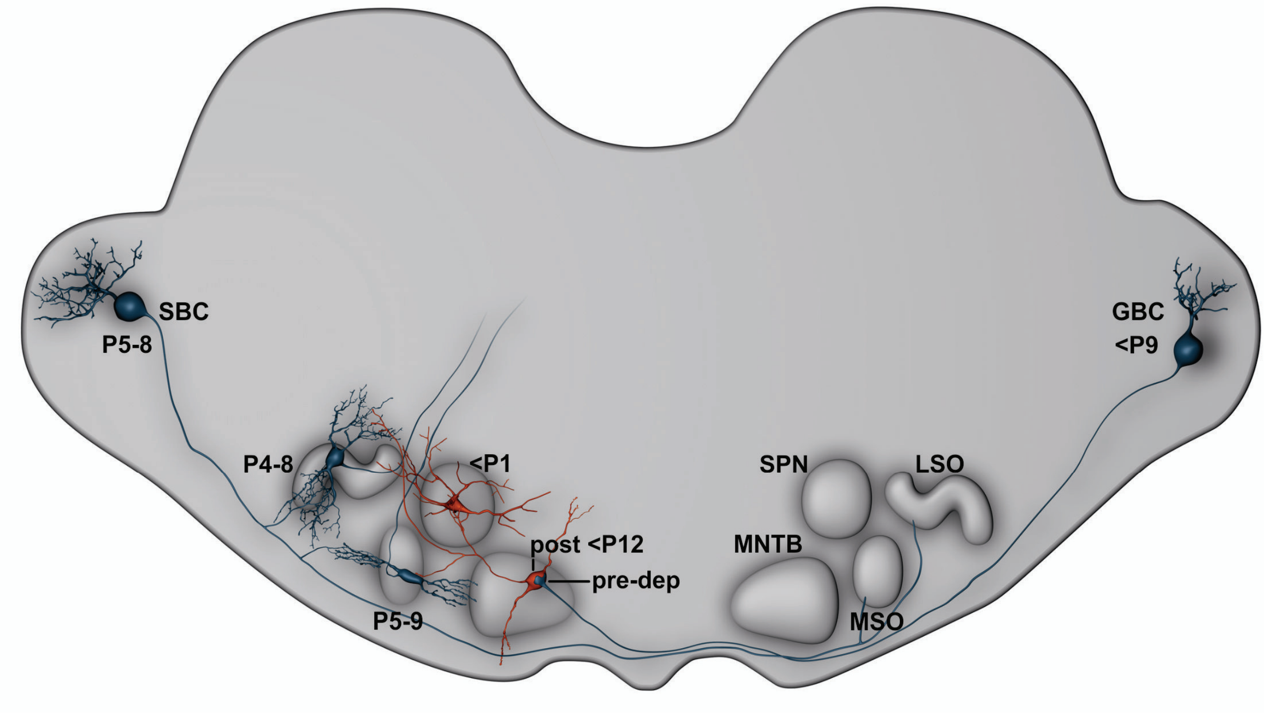

Fig. 2. Shift from depolarizing to hyperpolarizing actions of GABA and glycine in the auditory brainstem of mammals. The image summarizes currently available experimental evidence, assessed with gramicidin perforated-patch recordings or with voltage-sensitive dye measurements (see text for references). With an exception of the giant presynaptic terminal, the calyx of Held (pre-dep), where the depolarizing Cl^- gradient is maintained due to apparent absence of KCC2, the Cl^- gradient undergoes a developmental shift with different time courses in distinct nuclei, but it is completed before hearing onset (<P12). The inhibitory neurons release glycine (MNTB) or GABA (SPN), while all other neurons shown here use glutamate as neurotransmitter. SBC – spherical bushy cell and GBC – globular bushy cell in the cochlear nucleus; LSO – lateral superior olive; MSO – medial superior olive; SPN – superior paraolivary nucleus; MNTB – medial nucleus of the trapezoid body.

thereby contributing to synaptic refinement of inhibitory projections (Noh *et al.* 2010). Several studies in rats have shown that the D/H shift in LSO neurons occurs between P5 and P8 (Ehrlich *et al.* 1999, Kakazu *et al.* 1999, Löhrke *et al.* 2005). The experimentally determined value of the $[Cl^-]_i$ at P12 is 8±5 mM (Balakrishnan *et al.* 2003), consistent with estimated $[Cl^-]_i$ in SBC of the same age. Based on voltage-sensitive dye recordings, Löhrke *et al.* (2005) proposed that the D/H shift in the lateral, low-frequency limb of the LSO lags behind the changes in the medial, high-frequency limb by approximately two days. This observation raises the question of a frequency-specific gradient regarding the regulation of the Cl^- homeostasis. Previous studies using c-*fos* immunocytochemistry, tracer technique, and whole cell recordings in the auditory brainstem suggested that developmental processes reflecting the maturation of synaptic contacts, occur earlier in high-frequency regions than in corresponding low-frequency regions (Ford *et al.* 2009, Friauf 1992). However, the currently available data on development of the Cl^- homeostasis does not suggest a clear segregation in the maturation time course between the high-frequency ILD and the low-frequency ITD circuit. The earliest time point of the D/H shift is at E18-P1 in the SPN of the rat (Löhrke *et al.* 2005), but in the following two weeks the shift occurs in a staggered manner beginning in the cochlear nucleus bushy cells (P5-8) and the LSO (P5-8), followed by the MSO (P5-9), and shortly before the onset of hearing by the MNTB (P10-12) (Fig. 2). Therefore, the maturation pattern regarding the Cl^- homeostasis is not consistent with a notion of an earlier development in the high-frequency nuclei (ILD circuit), yet it does not rule out the possibility of gradual maturation along the frequency axes within one nucleus, as described for the LSO. Moreover, the Cl^- homeostasis first matures in the GABAergic SPN neurons, followed by the excitatory projection neurons (CN, LSO, MSO), and then by the glycinergic projection neurons from the MNTB. The signals that precisely control the Cl^- regulation system in each brainstem nucleus remain to be determined. Friauf and coworkers observed impaired efficiency of KCC2-mediated Cl^--

extrusion in hypothyroid rats and suggested that the functional status of KCC2 may be regulated by the thyroid hormone (Friauf *et al.* 2008). In all brainstem nuclei investigated so far, KCC2 seems to be the key molecule to render low $[Cl^-]_i$ to the "adult-like" level before hearing onset (Balakrishnan *et al.* 2003, Ehrlich *et al.* 1999, Kakazu *et al.* 1999, Milenkovic *et al.* 2007, Vale *et al.* 2005). The experimental evidence, based on immunohistochemistry, immunoblotting, electron miscroscopy, or mRNA analysis, strongly suggests the KCC2 expression in the brainstem neurons at, or shortly after birth. Yet, the mere early expression of KCC2 is not sufficient to trigger the D/H shift which emerges at different time points in some nuclei (Balakrishnan *et al.* 2003, Blaesse *et al.* 2006, Löhrke *et al.* 2005, Milenkovic *et al.* 2007, Vale *et al.* 2005) (Fig. 2). Besides KCC2, a weak expression of KCC1 and KCC4, though with a different time course, was observed in the superior olivary complex (SOC). While the amount of mRNA for KCC1 decreases, that of KCC4 increases from P3 to P12 (Becker *et al.* 2003). During the same developmental period, the amount of KCC2 mRNA and protein does not increase in auditory brainstem neurons (Fig. 1B), but what changes is the respective subcellular localization. During the period of the D/H shift, the KCC2 is gradually incorporated into the plasma membrane in perisomatic and peridendritic cellular regions. However, the expression and localization of KCC2 in the plasma membrane of auditory brainstem neurons does not reflect its functional state *per se*. This can be inferred from the major difference between the time course of the KCC2 protein expression in brainstem and in forebrain neurons. In the neocortex, hippocampus, and in retinal neurons, KCC2 expression increases up to the end of the second postnatal week, a period when E_{GABA} becomes hyperpolarizing with respect to V_m (Gulyas *et al.* 2001, Lu *et al.* 1999, Shimizu-Okabe *et al.* 2002, Vu *et al.* 2000). In the forebrain areas, the increase in the KCC2 gene expression is an immediate indicator of its functional activity (Khirug *et al.* 2005, Rivera *et al.* 2005, Stein *et al.* 2004). Nevertheless, there is also experimental evidence that developmental oligomerization of KCC2 is the rate-limiting step for the functionality of the transporter, both in the brainstem and also in forebrain structures (Blaesse *et al.* 2006). Also, posttranslational modifications in terms of (de-) or phosphorylation are conceivable as developmentally regulated steps towards achievement of the full transport activity (Kelsch *et al.* 2001, Stein *et al.* 2004, Strange *et al.* 2000, Vale *et al.* 2005). The onset of KCC2 activity in different auditory brainstem nuclei seems to be staggered in time, but in all nuclei the D/H shift is completed by the onset of hearing (P12), consistent with the prominent role of inhibition in processing of auditory information.

The time course of the D/H shift in the brainstem does not support the notion of chronologic maturation of the chloride homeostasis along the caudo-rostral axis i.e. early maturation in the spinal cord (Baccei and Fitzgerald 2004, Hübner *et al.* 2001, Wu *et al.* 1992), followed by the brainstem (Ritter and Zhang 2000, Singer *et al.* 1998), cerebellum (Brickley *et al.* 1996, Eilers *et al.* 2001), hippocampus (Fiszman *et al.* 1990, Khazipov *et al.* 2004, Rivera *et al.* 1999, Swann *et al.* 1989, Tyzio *et al.* 2006) and finally by the cerebral cortex (<P12) (Owens *et al.* 1996, Yamada *et al.* 2004). Thus, the chloride regulation in the auditory brainstem is perhaps tightly coupled to maturational processes of the auditory circuits, but it remains to be investigated what exactly determines the rather distinct time courses between some nuclei.

Maintenance of high intracellular chloride in auditory brainstem neurons

While in the auditory brainstem of mammals, KCC2 seems to be the key transporter to mediate the D/H shift and hence render GABA and glycine hyperpolarizing during development, the mechanisms generating the initially depolarizing transmembrane gradient for Cl^- remain elusive. In mammals, elevated intracellular Cl^- is a transient feature of developing nervous system, but in the auditory brainstem nuclei of birds high $[Cl^-]_i$ is maintained up to adulthood. In both cases, depolarizing actions of GABA and glycine are mainly caused by Cl^- efflux, consistent with active Cl^- uptake mechanisms rendering $[Cl^-]_i$ above the predicted electrochemical equilibrium. Estimated values of neuronal $[Cl^-]_i$ in different forebrain areas of postnatal rodents are in the range of 20-40 mM, depending on the respective structure and the developmental stage (Ikeda *et al.* 2003, Kilb *et al.* 2002, Owens *et al.* 1996, Rohrbough and Spitzer 1996, Shimizu-Okabe *et al.* 2002, Sipila *et al.* 2006, Yamada *et al.* 2004). In auditory brainstem neurons, the approximate $[Cl^-]_i$ lies in the same range prior to the D/H shift (Balakrishnan *et al.* 2003, Kakazu *et al.* 1999, Milenkovic *et al.* 2007). The accumulation of intracellular Cl^- in various neuron types is thought to be mediated by an inward-directed Cl^- pump, the Na^+-driven K^+-Cl^--cotransporter NKCC1 (Achilles *et al.* 2007, Delpire *et al.* 1999, Plotkin *et al.* 1997, Sung *et al.* 2000). The continuous down-regulation of NKCC1 during development, alongside with an up-regulation of KCC2, is believed to mediate the observed D/H shift (Plotkin *et al.* 1997, Yamada *et al.* 2004). In the auditory system, early Cl^- accumulation in the cochlear nucleus SBC is most likely dominated by NKCC1, the expression of which appears to be down-regulated later in development (personal unpublished observations). Nonetheless, NKCC1 is probably not the only contributing factor to Cl^- accumulation (Delpy *et al.* 2008, Rocha-Gonzalez *et al.* 2008, Zhang *et al.* 2007). In the LSO, the NKCC1 expression pattern does not match the developmental profile of depolarizing effects of inhibitory neurotransmitters. Although the mRNA for NKCC1 is barely expressed in neonatal LSO, its expression is augmented when glycine is already hyperpolarizing (Fig. 1B). So, NKCC1 seems not to be the prime contributor to Cl^- accumulation in early LSO neurons (but see also Kakazu *et al.* 1999), which suggests that, besides or instead of NKCC1, other Cl^--accumulating transporters might act in a nuclear-specific pattern in the superior olivary complex (SOC). Becker *et al.* (2003) have pointed to the HCO_3^-/Cl^- exchanger AE3 as a possible candidate to render high $[Cl^-]_i$ in the SOC. According to their study performed in rats, AE3 was the only secondary active Cl^--accumulating transporter abundantly present during the first two postnatal weeks. However, we still lack experimental evidence of the physiological activity of AE3 in the LSO. The respective role of AE3 has been documented in the pyramidal neurons of the mouse hippocampus and in the embryonic spinal motoneurons of the chick, where the AE3 appears to support the Cl^- intrusion predominantly driven by NKCC1 (Gonzalez-Islas *et al.* 2009, Pfeffer *et al.* 2009). Similar to the development in mammals, neurons in the chick spinal cord also undergo the D/H shift which is thought to be mediated by reduction in NKCC1 function and concurrent up-regulation of KCC2 (Chub *et al.* 2006, Delpy *et al.* 2008, Jean-Xavier *et al.* 2006, Obata *et al.* 1978, Xu *et al.* 2005). This is in contrast to the development in the auditory brainstem of the chick, where neurons in the nucleus magnocellularis (NM, homologue to mammalian cochlear nucleus), nucleus laminaris (NL, homologue to mammalian MSO) and nucleus angularis (NA, processing of sound level information) maintain the outward-directed electrochemical gradient for Cl^- at resting membrane potential throughout the animal's life span (Howard *et al.* 2007, Hyson *et al.* 1995, Kuo *et al.* 2009, Lu and Trussell 2001, Monsivais and Rubel 2001, Tang *et al.* 2009). Here, GABA is effective through depolarizing inhibition which is achieved by an activation of the Cl^-- and subsequently K^+-conductance, and the inactivation of Na^+ channels, jointly contributing to shunting and action potential threshold accommodation (Howard and Rubel 2010). With the exception of NA neurons, which are subject to co-transmission of GABA and glycine (Kuo *et al.* 2009), the inhibitory neurotransmission in the chick auditory brainstem is solely GABAergic (Funabiki *et al.* 1998, Lu and Trussell 2001, Lu *et al.* 2005, Monsivais *et al.* 2000, Yang *et al.* 1999). The primary source of GABAergic inhibitory inputs to NM, NL, and NA neurons resides in the superior olivary nucleus (SON) (Burger *et al.* 2005, Nishino *et al.* 2008). Performing gramicidin perforated-patch recordings in the same slice preparation, Monsivais and Rubel (2001) revealed depolarizing E_{GABA} in the NM neurons, E_{GABA} around V_{rest} in the SON neurons and E_{GABA} hyperpolarized with respect to V_{rest} in the reticular formation. Hence in the chick brainstem, the Cl^- homeostasis in neurons involved in sound localization, seems to be under specific control that renders inhibition depolarizing, but very potent in eliminating spikes to asynchronous inputs, and thus in sharpening the temporal response properties (Howard and Rubel 2010). To our best knowledge, the mechanisms underlying Cl^- regulation in the avian auditory brainstem neurons are still inconclusive. Given the different $[Cl^-]_i$ in NM, NL, and NA neurons on one side, and SON and reticular formation neurons on the other, the chicken brainstem slice preparation may be a very attractive model to study the underlying mechanisms of the Cl^- homeostasis. Still, it should be adduced that cell-type-specific regulation of the Cl^- homeostasis is not unique to avian brainstem nuclei; excitatory and inhibitory effects of GABA were also described in distinct mature neuronal networks of mammals (Marty and Llano 2005). In the cortex and amygdala, interneurons have more depolarized E_{GABA} than pyramidal cells due to the prominent activity of NKCC1 or KCC2, respectively (Martina *et al.* 2001). Also in the thalamus, retina, and the suprachiasmatic nucleus (which largely drives day-night cycle in mammals), depolarizing Cl^- gradient is restricted to specific cell types or even to restricted cell compartments and it appears to be finely tuned by the cation-chloride cotransporters (Albus *et al.* 2005, Belenky *et al.* 2008, Ulrich and Huguenard 1997, Vardi *et al.* 2000).

Conclusions

It has been understood for many years that in mammalian auditory brainstem the action of inhibitory neurotransmitters GABA and glycine undergoes the D/H shift as a result of the cellular mechanisms regulating Cl^- homeostasis during development. However, the nature of the respective control mechanisms is still not completely resolved. While the Cl^--extruding cotransporter KCC2 seems to play a pivotal role in rendering the hyperpolarizing gradient for Cl^-, it remains intriguing why the respective D/H shift occurs at different time points in distinct but closely adjacent nuclei. The significance of NKCC1 in intracellular chloride accumulation is still unclear, but at least in the LSO, its expression pattern does not correlate with depolarizing Cl^- gradient. Moreover, despite the strong evidence for abundant expression of the Cl^- accumulating anion-exchanger AE3 in the SOC, the confirmation of its physiological activity is lacking. Thus, the Cl^- accumulation remains an issue in early postnatal neurons of mammals, but also in the mature auditory brainstem of the chick. In the latter, yet unknown homeostasis mechanisms accurately adjust the Cl^- gradient to enable a depolarizing, but still very efficient shunting inhibition. As we learn more about these mechanisms, it becomes clear that the precise regulation of the Cl^- content may endow neurons or even discrete compartments of such neurons with distinct physiological responses to a limited number of transmitters.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

Our work was supported by the Deutsche Forschungsgemeinschaft grants (MI 954-1/2 and RU 390/19-1) to I.M and R.R., respectively. Graphic contribution by EFFIGOS AG is gratefully acknowledged.

References

ACHILLES K, OKABE A, IKEDA M, SHIMIZU-OKABE C, YAMADA J, FUKUDA A, LUHMANN HJ, KILB W: Kinetic properties of Cl uptake mediated by Na^+-dependent K^+-2Cl cotransport in immature rat neocortical neurons. *J Neurosci* **27**: 8616-8627, 2007.

ALBUS H, VANSTEENSEL MJ, MICHEL S, BLOCK GD, MEIJER JH: A GABAergic mechanism is necessary for coupling dissociable ventral and dorsal regional oscillators within the circadian clock. *Curr Biol* **15**: 886-893, 2005.

AWATRAMANI GB, TURECEK R, TRUSSELL LO: Inhibitory control at a synaptic relay. *J Neurosci* **24**: 2643-2647, 2004.

AWATRAMANI GB, TURECEK R, TRUSSELL LO: Staggered development of GABAergic and glycinergic transmission in the MNTB. *J Neurophysiol* **93**: 819-828, 2005.

BACCEI ML, FITZGERALD M: Development of GABAergic and glycinergic transmission in the neonatal rat dorsal horn. *J Neurosci* **24**: 4749-4757, 2004.

BACKOFF PM, SHADDUCK PALOMBI P, CASPARY DM: Gamma-aminobutyric acidergic and glycinergic inputs shape coding of amplitude modulation in the chinchilla cochlear nucleus. *Hear Res* **134**: 77-88, 1999.

BALAKRISHNAN V, BECKER M, LÖHRKE S, NOTHWANG HG, GURESIR E, FRIAUF E: Expression and function of chloride transporters during development of inhibitory neurotransmission in the auditory brainstem. *J Neurosci* **23**: 4134-4145, 2003.

BECKER M, NOTHWANG HG, FRIAUF E: Differential expression pattern of chloride transporters NCC, NKCC2, KCC1, KCC3, KCC4, and AE3 in the developing rat auditory brainstem. *Cell Tissue Res* **312**: 155-165, 2003.

BELENKY MA, YAROM Y, PICKARD GE: Heterogeneous expression of gamma-aminobutyric acid and gamma-aminobutyric acid-associated receptors and transporters in the rat suprachiasmatic nucleus. *J Comp Neurol* **506**: 708-732, 2008.

BEN-ARI Y, CHERUBINI E, CORRADETTI R, GAIARSA JL: Giant synaptic potentials in immature rat CA3 hippocampal neurones. *J Physiol* **416**: 303-325, 1989.

BEN-ARI Y: Excitatory actions of gaba during development: the nature of the nurture. *Nat Rev Neurosci* **3**: 728-739, 2002.

BEN-ARI Y, GAIARSA JL, TYZIO R, KHAZIPOV R: GABA: A pioneer transmitter that excites immature neurons and generates primitive oscillations. *Physiol Rev* **87**: 1215-1284, 2007.

BLAESSE P, GUILLEMIN I, SCHINDLER J, SCHWEIZER M, DELPIRE E, KHIROUG L, FRIAUF E, NOTHWANG HG: Oligomerization of KCC2 correlates with development of inhibitory neurotransmission. *J Neurosci* **26**: 10407-10419, 2006.

BLATCHLEY BJ, COOPER WA, COLEMAN JR: Development of auditory brainstem response to tone pip stimuli in the rat. *Brain Res* **429**: 75-84, 1987.

BORMANN J, HAMILL OP, SAKMANN B: Mechanism of anion permeation through channels gated by glycine and gamma-aminobutyric acid in mouse cultured spinal neurones. *J Physiol* **385**: 243-286, 1987.

BRAWER JR, MOREST DK: Relations between auditory nerve endings and cell types in the cat's anteroventral cochlear nucleus seen with the Golgi method and Nomarski optics. *J Comp Neurol* **160**: 491-506, 1975.

BRICKLEY SG, CULL-CANDY SG, FARRANT M: Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of GABAA receptors. *J Physiol* **497** (Pt 3): 753-759, 1996.

BURGER RM, CRAMER KS, PFEIFFER JD, RUBEL EW: Avian superior olivary nucleus provides divergent inhibitory input to parallel auditory pathways. *J Comp Neurol* **481**: 6-18, 2005.

CASPARY DM, BACKOFF PM, FINLAYSON PG, PALOMBI PS: Inhibitory inputs modulate discharge rate within frequency receptive fields of anteroventral cochlear nucleus neurons. *J Neurophysiol* **72**: 2124-2133, 1994.

CHANG EH, KOTAK VC, SANES DH: Long-term depression of synaptic inhibition is expressed postsynaptically in the developing auditory system. *J Neurophysiol* **90**: 1479-1488, 2003.

CHEN G, TROMBLEY PQ, VAN DEN POL AN: Excitatory actions of GABA in developing rat hypothalamic neurones. *J Physiol* **494** (Pt 2): 451-464, 1996.

CHERUBINI E, ROVIRA C, GAIARSA JL, CORRADETTI R, BEN ARI Y: GABA mediated excitation in immature rat CA3 hippocampal neurons. *Int J Dev Neurosci* **8**: 481-490, 1990.

CHERY N, DE KONINCK Y: Junctional versus extrajunctional glycine and GABA(A) receptor-mediated IPSCs in identified lamina I neurons of the adult rat spinal cord. *J Neurosci* **19**: 7342-7355, 1999.

CHUB N, MENTIS GZ, O'DONOVAN MJ: Chloride-sensitive MEQ fluorescence in chick embryo motoneurons following manipulations of chloride and during spontaneous network activity. *J Neurophysiol* **95**: 323-330, 2006.

DEHMEL S, KOPP-SCHEINPFLUG C, WEICK M, DORRSCHEIDT GJ, RUBSAMEN R: Transmission of phase-coupling accuracy from the auditory nerve to spherical bushy cells in the Mongolian gerbil. *Hear Res* **268**: 234-249, 2010.

DELPIRE E, LU J, ENGLAND R, DULL C, THORNE T: Deafness and imbalance associated with inactivation of the secretory Na-K-2Cl co-transporter. *Nat Genet* **22**: 192-195, 1999.

DELPY A, ALLAIN AE, MEYRAND P, BRANCHEREAU P: NKCC1 cotransporter inactivation underlies embryonic development of chloride-mediated inhibition in mouse spinal motoneuron. *J Physiol* **586**: 1059-1075, 2008.

EHRLICH I, LÖHRKE S, FRIAUF E: Shift from depolarizing to hyperpolarizing glycine action in rat auditory neurones is due to age-dependent Cl⁻ regulation. *J Physiol* **520** (Pt 1): 121-137, 1999.

EILERS J, PLANT TD, MARANDI N, KONNERTH A: GABA-mediated Ca^{2+} signalling in developing rat cerebellar Purkinje neurones. *J Physiol* **536**: 429-437, 2001.

FISZMAN ML, NOVOTNY EA, LANGE GD, BARKER JL: Embryonic and early postnatal hippocampal cells respond to nanomolar concentrations of muscimol. *Brain Res Dev Brain Res* **53**: 186-193, 1990.

FORD MC, GROTHE B, KLUG A: Fenestration of the calyx of Held occurs sequentially along the tonotopic axis, is influenced by afferent activity, and facilitates glutamate clearance. *J Comp Neurol* **514**: 92-106, 2009.

FRIAUF E: Tonotopic order in the adult and developing auditory system of the rat as shown by c-fos immunochemistry. *Eur J Neurosci* **4**: 798-812, 1992.

FRIAUF E, WENZ M, OBERHOFER M, NOTHWANG HG, BALAKRISHNAN V, KNIPPER M, LÖHRKE S: Hypothyroidism impairs chloride homeostasis and onset of inhibitory neurotransmission in developing auditory brainstem and hippocampal neurons. *Eur J Neurosci* **28**: 2371-2380, 2008.

FUNABIKI K, KOYANO K, OHMORI H: The role of GABAergic inputs for coincidence detection in the neurones of nucleus laminaris of the chick. *J Physiol (Lond)* **508**: 851-869, 1998.

GAI Y, CARNEY LH: Influence of inhibitory inputs on rate and timing of responses in the anteroventral cochlear nucleus. *J Neurophysiol* **99**: 1077-1095, 2008.

GILLESPIE DC, KIM G, KANDLER K: Inhibitory synapses in the developing auditory system are glutamatergic. *Nat Neurosci* **8**: 332-338, 2005.

GONZALEZ-ISLAS C, CHUB N, WENNER P: NKCC1 and AE3 appear to accumulate chloride in embryonic motoneurons. *J Neurophysiol* **101**: 507-518, 2009.

GROTHE B, PECKA M, MCALPINE D: Mechanisms of sound localization in mammals. *Physiol Rev* **90**: 983-1012, 2010.

GROTHE B, SANES DH: Synaptic inhibition influences the temporal coding properties of medial superior olivary neurons: an in vitro study. *J Neurosci* **14**: 1701-1709, 1994.

GROTHE B: New roles for synaptic inhibition in sound localization. *Nat Rev Neurosci* **4**: 540-550, 2003.

GULYAS AI, SIK A, PAYNE JA, KAILA K, FREUND TF: The KCl cotransporter, KCC2, is highly expressed in the vicinity of excitatory synapses in the rat hippocampus. *Eur J Neurosci* **13**: 2205-2217, 2001.

HOWARD MA, BURGER RM, RUBEL EW: A developmental switch to GABAergic inhibition dependent on increases in Kv1-type K^+ currents. *J Neurosci* **27**: 2112-2123, 2007.

HOWARD MA, RUBEL EW: Dynamic spike thresholds during synaptic integration preserve and enhance temporal response properties in the avian cochlear nucleus. *J Neurosci* **30**: 12063-12074, 2010.

HUANG BO, REDBURN DA: GABA-induced increases in $[Ca^{2+}]i$ in retinal neurons of postnatal rabbits. *Vis Neurosci* **13**: 441-447, 1996.

HÜBNER CA, STEIN V, HERMANS-BORGMEYER I, MEYER T, BALLANYI K, JENTSCH TJ: Disruption of KCC2 reveals an essential role of K-Cl cotransport already in early synaptic inhibition. *Neuron* **30**: 515-524, 2001.

HYSON RL, REYES AD, RUBEL EW: A depolarizing inhibitory response to GABA in brainstem auditory neurons of the chick. *Brain Res* **677**: 117-126, 1995.

IKEDA M, TOYODA H, YAMADA J, OKABE A, SATO K, HOTTA Y, FUKUDA A: Differential development of cation-chloride cotransporters and Cl^- homeostasis contributes to differential GABAergic actions between developing rat visual cortex and dorsal lateral geniculate nucleus. *Brain Res* **984**: 149-159, 2003.

JAROLIMEK W, LEWEN A, MISGELD U: A furosemide-sensitive K^+-Cl^- cotransporter counteracts intracellular Cl^- accumulation and depletion in cultured rat midbrain neurons. *J Neurosci* **19**: 4695-4704, 1999.

JEAN-XAVIER C, PFLIEGER JF, LIABEUF S, VINAY L: Inhibitory postsynaptic potentials in lumbar motoneurons remain depolarizing after neonatal spinal cord transection in the rat. *J Neurophysiol* **96**: 2274-2281, 2006.

JONAS P, BISCHOFBERGER J, SANDKÜHLER J: Corelease of two fast neurotransmitters at a central synapse. *Science* **281**: 419-424, 1998.

KAILA K: Ionic basis of GABAA receptor channel function in the nervous system. *Prog Neurobiol* **42**: 489-537, 1994.

KAKAZU Y, AKAIKE N, KOMIYAMA S, NABEKURA J: Regulation of intracellular chloride by cotransporters in developing lateral superior olive neurons. *J Neurosci* **19**: 2843-2851, 1999.

KANDLER K, FRIAUF E: Development of glycinergic and glutamatergic synaptic transmission in the auditory brainstem of perinatal rats. *J Neurosci* **15**: 6890-6904, 1995.

KANDLER K, CLAUSE A, NOH J: Tonotopic reorganization of developing auditory brainstem circuits. *Nat Neurosci* **12**: 711-717, 2009.

KELSCH W, HORMUZDI S, STRAUBE E, LEWEN A, MONYER H, MISGELD U: Insulin-like growth factor 1 and a cytosolic tyrosine kinase activate chloride outward transport during maturation of hippocampal neurons. *J Neurosci* **21**: 8339-8347, 2001.

KHAZIPOV R, KHALILOV I, TYZIO R, MOROZOVA E, BEN-ARI Y, HOLMES GL: Developmental changes in GABAergic actions and seizure susceptibility in the rat hippocampus. *Eur J Neurosci* **19**: 590-600, 2004.

KHIRUG S, HUTTU K, LUDWIG A, SMIRNOV S, VOIPIO J, RIVERA C, KAILA K, KHIROUG L: Distinct properties of functional KCC2 expression in immature mouse hippocampal neurons in culture and in acute slices. *Eur J Neurosci* **21**: 899-904, 2005.

KILB W, IKEDA M, UCHIDA K, OKABE A, FUKUDA A, LUHMANN HJ: Depolarizing glycine responses in Cajal-Retzius cells of neonatal rat cerebral cortex. *Neuroscience* **112**: 299-307, 2002.

KONISHI M: Coding of auditory space. *Annu Rev Neurosci* **26**: 31-55, 2003.

KOPP-SCHEINPFLUG C, DEHMEL S, DORRSCHEIDT GJ, RUBSAMEN R: Interaction of excitation and inhibition in anteroventral cochlear nucleus neurons that receive large endbulb synaptic endings. *J Neurosci* **22**: 11004-11018, 2002.

KOTAK VC, SANES DH: Long-lasting inhibitory synaptic depression is age- and calcium-dependent. *J Neurosci* **20**: 5820-5826, 2000.

KULLMANN PH, ENE FA, KANDLER K: Glycinergic and GABAergic calcium responses in the developing lateral superior olive. *Eur J Neurosci* **15**: 1093-1104, 2002.

KULLMANN PH, KANDLER K: Dendritic Ca^{2+} responses in neonatal lateral superior olive neurons elicited by glycinergic/GABAergic synapses and action potentials. *Neuroscience* **154**: 338-345, 2008.

KUO SP, BRADLEY LA, TRUSSELL LO: Heterogeneous kinetics and pharmacology of synaptic inhibition in the chick auditory brainstem. *J Neurosci* **29**: 9625-9634, 2009.

LIM R, OLESKEVICH S, FEW AP, LEAO RN, WALMSLEY B: Glycinergic mIPSCs in mouse and rat brainstem auditory nuclei: modulation by ruthenium red and the role of calcium stores. *J Physiol* **546**: 691-699, 2003.

LÖHRKE S, SRINIVASAN G, OBERHOFER M, DONCHEVA E, FRIAUF E: Shift from depolarizing to hyperpolarizing glycine action occurs at different perinatal ages in superior olivary complex nuclei. *Eur J Neurosci* **22**: 2708-2722, 2005.

LU J, KARADSHEH M, DELPIRE E: Developmental regulation of the neuronal-specific isoform of K-Cl cotransporter KCC2 in postnatal rat brains. *J Neurobiol* **39**: 558-568, 1999.

LU T, TRUSSELL LO: Inhibitory transmission mediated by asynchronous transmitter release. *Neuron* **26**: 683-694, 2000.

LU T, TRUSSELL LO: Mixed excitatory and inhibitory GABA-mediated transmission in chick cochlear nucleus. *J Physiol* **535**: 125-131, 2001.

LU Y, BURGER RM, RUBEL EW: GABA(B) receptor activation modulates GABA(A) receptor-mediated inhibition in chicken nucleus magnocellularis neurons. *J Neurophysiol* **93**: 1429-1438, 2005.

MAGNUSSON AK, KAPFER C, GROTHE B, KOCH U: Maturation of glycinergic inhibition in the gerbil medial superior olive after hearing onset. *J Physiol* **568**: 497-512, 2005.

MARTINA M, ROYER S, PARE D: Propagation of neocortical inputs in the perirhinal cortex. *J Neurosci* **21**: 2878-2888, 2001.

MARTY A, LLANO I: Excitatory effects of GABA in established brain networks. *Trends Neurosci* **28**: 284-289, 2005.

MILENKOVIC I, WITTE M, TURECEK R, HEINRICH M, REINERT T, RUBSAMEN R: Development of chloride-mediated inhibition in neurons of the anteroventral cochlear nucleus of gerbil (Meriones unguiculatus). *J Neurophysiol* **98**: 1634-1644, 2007.

MONSIVAIS P, YANG L, RUBEL EW: GABAergic inhibition in nucleus magnocellularis: implications for phase locking in the avian auditory brainstem. *J Neurosci* **20**: 2954-2963, 2000.

MONSIVAIS P, RUBEL EW: Accommodation enhances depolarizing inhibition in central neurons. *J Neurosci* **21**: 7823-7830, 2001.

NABEKURA J, KATSURABAYASHI S, KAKAZU Y, SHIBATA S, MATSUBARA A, JINNO S, MIZOGUCHI Y, SASAKI A, ISHIBASHI H: Developmental switch from GABA to glycine release in single central synaptic terminals. *Nat Neurosci* **7**: 17-23, 2004.

NISHINO E, YAMADA R, KUBA H, HIOKI H, FURUTA T, KANEKO T, OHMORI H: Sound-intensity-dependent compensation for the small interaural time difference cue for sound source localization. *J Neurosci* **28**: 7153-7164, 2008.

NOH J, SEAL RP, GARVER JA, EDWARDS RH, KANDLER K: Glutamate co-release at GABA/glycinergic synapses is crucial for the refinement of an inhibitory map. *Nat Neurosci* **13**: 232-238, 2010.

OBATA K, OIDE M, TANAKA H: Excitatory and inhibitory actions of GABA and glycine on embryonic chick spinal neurons in culture. *Brain Res* **144**: 179-184, 1978.

OERTEL D: The role of timing in the brain stem auditory nuclei of vertebrates. *Annu Rev Physiol* **61**: 497-519, 1999.

OWENS DF, BOYCE LH, DAVIS MB, KRIEGSTEIN AR: Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. *J Neurosci* **16**: 6414-6423, 1996.

OWENS DF, KRIEGSTEIN AR: Is there more to GABA than synaptic inhibition? *Nat Rev Neurosci* **3**: 715-727, 2002.

PAYNE JA, RIVERA C, VOIPIO J, KAILA K: Cation-chloride co-transporters in neuronal communication, development and trauma. *Trends Neurosci* **26**: 199-206, 2003.

PFEFFER CK, STEIN V, KEATING DJ, MAIER H, RINKE I, RUDHARD Y, HENTSCHKE M, RUNE GM, JENTSCH TJ, HÜBNER CA: NKCC1-dependent GABAergic excitation drives synaptic network maturation during early hippocampal development. *J Neurosci* **29**: 3419-3430, 2009.

PLOTKIN MD, SNYDER EY, HEBERT SC, DELPIRE E: Expression of the Na-K-2Cl cotransporter is developmentally regulated in postnatal rat brains: a possible mechanism underlying GABA's excitatory role in immature brain. *J Neurobiol* **33**: 781-795, 1997.

PRICE GD, TRUSSELL LO: Estimate of the chloride concentration in a central glutamatergic terminal: a gramicidin perforated-patch study on the calyx of Held. *J Neurosci* **26**: 11432-11436, 2006.

REICHLING DB, KYROZIS A, WANG J, MACDERMOTT AB: Mechanisms of GABA and glycine depolarization-induced calcium transients in rat dorsal horn neurons. *J Physiol* **476**: 411-421, 1994.

RITTER B, ZHANG W: Early postnatal maturation of GABAA-mediated inhibition in the brainstem respiratory rhythm-generating network of the mouse. *Eur J Neurosci* **12**: 2975-2984, 2000.

RIVERA C, VOIPIO J, PAYNE JA, RUUSUVUORI E, LAHTINEN H, LAMSA K, PIRVOLA U, SAARMA M, KAILA K: The K^+/Cl^- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature* **397**: 251-255, 1999.

RIVERA C, VOIPIO J, KAILA K: Two developmental switches in GABAergic signalling: the K^+-Cl^- cotransporter KCC2 and carbonic anhydrase CAVII. *J Physiol* **562**: 27-36, 2005.

ROCHA-GONZALEZ HI, MAO S, ALVAREZ-LEEFMANS FJ: Na^+,K^+,$2Cl^-$ cotransport and intracellular chloride regulation in rat primary sensory neurons: thermodynamic and kinetic aspects. *J Neurophysiol* **100**: 169-184, 2008.

ROHRBOUGH J, SPITZER NC: Regulation of intracellular Cl^- levels by Na(+)-dependent Cl^- cotransport distinguishes depolarizing from hyperpolarizing GABAA receptor-mediated responses in spinal neurons. *J Neurosci* **16**: 82-91, 1996.

RYUGO DK, SENTO S: Synaptic connections of the auditory nerve in cats: relationship between endbulbs of held and spherical bushy cells. *J Comp Neurol* **305**: 35-48, 1991.

SHIMIZU-OKABE C, YOKOKURA M, OKABE A, IKEDA M, SATO K, KILB W, LUHMANN HJ, FUKUDA A: Layer-specific expression of Cl^- transporters and differential $[Cl^-]_i$ in newborn rat cortex. *Neuroreport* **13**: 2433-2437, 2002.

SINGER JH, TALLEY EM, BAYLISS DA, BERGER AJ: Development of glycinergic synaptic transmission to rat brain stem motoneurons. *J Neurophysiol* **80**: 2608-2620, 1998.

SIPILA ST, SCHUCHMANN S, VOIPIO J, YAMADA J, KAILA K: The cation-chloride cotransporter NKCC1 promotes sharp waves in the neonatal rat hippocampus. *J Physiol* **573**: 765-773, 2006.

SIVILOTTI L, NISTRI A: GABA receptor mechanisms in the central nervous system. *Prog Neurobiol* **36**: 35-92, 1991.

SMITH AJ, OWENS S, FORSYTHE ID: Characterisation of inhibitory and excitatory postsynaptic currents of the rat medial superior olive. *J Physiol* **529** (Pt 3): 681-698, 2000.

SONNTAG M, ENGLITZ B, KOPP-SCHEINPFLUG C, RUBSAMEN R: Early postnatal development of spontaneous and acoustically evoked discharge activity of principal cells of the medial nucleus of the trapezoid body: an in vivo study in mice. *J Neurosci* **29**: 9510-9520, 2009.

STALEY KJ, PROCTOR WR: Modulation of mammalian dendritic GABA(A) receptor function by the kinetics of Cl^- and HCO_3^- transport. *J Physiol* **519** (Pt 3): 693-712, 1999.

STEIN V, HERMANS-BORGMEYER I, JENTSCH TJ, HÜBNER CA: Expression of the KCl cotransporter KCC2 parallels neuronal maturation and the emergence of low intracellular chloride. *J Comp Neurol* **468**: 57-64, 2004.

STRANGE K, SINGER TD, MORRISON R, DELPIRE E: Dependence of KCC2 K-Cl cotransporter activity on a conserved carboxy terminus tyrosine residue. *Am J Physiol Cell Physiol* **279**: C860-C867, 2000.

SUNG KW, KIRBY M, MCDONALD MP, LOVINGER DM, DELPIRE E: Abnormal GABAA receptor-mediated currents in dorsal root ganglion neurons isolated from Na-K-2Cl cotransporter null mice. *J Neurosci* **20**: 7531-7538, 2000.

SWANN JW, BRADY RJ, MARTIN DL: Postnatal development of GABA-mediated synaptic inhibition in rat hippocampus. *Neuroscience* **28**: 551-561, 1989.

TANG ZQ, GAO H, LU Y: Control of a depolarizing GABAergic input in an auditory coincidence detection circuit. *J Neurophysiol* **102**: 1672-1683, 2009.

TITZ S, HANS M, KELSCH W, LEWEN A, SWANDULLA D, MISGELD U: Hyperpolarizing inhibition develops without trophic support by GABA in cultured rat midbrain neurons. *J Physiol* **550**: 719-730, 2003.

TURECEK R, TRUSSELL LO: Presynaptic glycine receptors enhance transmitter release at a mammalian central synapse. *Nature* **411**: 587-590, 2001.

TYZIO R, COSSART R, KHALILOV I, MINLEBAEV M, HÜBNER CA, REPRESA A, BEN-ARI Y, KHAZIPOV R: Maternal oxytocin triggers a transient inhibitory switch in GABA signaling in the fetal brain during delivery. *Science* **314**: 1788-1792, 2006.

ULRICH D, HUGUENARD JR: Nucleus-specific chloride homeostasis in rat thalamus. *J Neurosci* **17**: 2348-2354, 1997.

VALE C, CAMINOS E, MARTINEZ-GALAN JR, JUIZ JM: Expression and developmental regulation of the K^+-Cl^- cotransporter KCC2 in the cochlear nucleus. *Hear Res* **206**: 107-115, 2005.

VARDI N, ZHANG LL, PAYNE JA, STERLING P: Evidence that different cation chloride cotransporters in retinal neurons allow opposite responses to GABA. *J Neurosci* **20**: 7657-7663, 2000.

VU TQ, PAYNE JA, COPENHAGEN DR: Localization and developmental expression patterns of the neuronal K-Cl cotransporter (KCC2) in the rat retina. *J Neurosci* **20**: 1414-1423, 2000.

WOOLF NK, RYAN AF: The development of auditory function in the cochlea of the mongolian gerbil. *Hear Res* **13**: 277-283, 1984.

WU WL, ZISKIND-CONHAIM L, SWEET MA: Early development of glycine- and GABA-mediated synapses in rat spinal cord. *J Neurosci* **12**: 3935-3945, 1992.

XU H, WHELAN PJ, WENNER P: Development of an inhibitory interneuronal circuit in the embryonic spinal cord. *J Neurophysiol* **93**: 2922-2933, 2005.

YAMADA J, OKABE A, TOYODA H, KILB W, LUHMANN HJ, FUKUDA A: Cl^- uptake promoting depolarizing GABA actions in immature rat neocortical neurones is mediated by NKCC1. *J Physiol* **557**: 829-841, 2004.

YANG L, MONSIVAIS P, RUBEL EW: The superior olivary nucleus and its influence on nucleus laminaris: a source of inhibitory feedback for coincidence detection in the avian auditory brainstem. *J Neurosci* **19**: 2313-2325, 1999.

YOSHIMURA M, NISHI S: Primary afferent-evoked glycine- and GABA-mediated IPSPs in substantia gelatinosa neurones in the rat spinal cord in vitro. *J Physiol* **482** (Pt 1): 29-38, 1995.

YUSTE R, KATZ LC: Control of postsynaptic Ca^{2+} influx in developing neocortex by excitatory and inhibitory neurotransmitters. *Neuron* **6**: 333-344, 1991.

ZHANG LL, DELPIRE E, VARDI N: NKCC1 does not accumulate chloride in developing retinal neurons. *J Neurophysiol* **98**: 266-277, 2007.