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Hypothyroidism impairs chloride homeostasis and onset of inhibitory neurotransmission in developing auditory brainstem and hippocampal neurons

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ABSTRACT

Thyroid hormone (TH) deficiency during perinatal life causes a multitude of functional and morphological deficits in the brain. In rats and mice, TH dependency of neural maturation is particularly evident during the first 1-2 weeks of postnatal development. During the same period, synaptic transmission via the inhibitory transmitters glycine and GABA changes from excitatory depolarizing effects to inhibitory hyperpolarizing ones in most neurons (D/H shift). The D/H shift is caused by the activation of the K^+/Cl^- co-transporter KCC2 which extrudes Cl^- from the cytosol, thus generating an inward-directed electrochemical Cl^- gradient. Here we analyzed whether the D/H shift and, consequently, the onset of inhibitory neurotransmission, are influenced by TH. Gramicidin perforated-patch recordings from auditory brainstem neurons of experimentally hypothyroid rats revealed depolarizing glycine effects until postnatal day (P) 11, i.e., almost one week longer than in control rats, where the D/H shift occurs at about P5-6. Likewise, until P12-13, the equilibrium potential E_{Gly} in hypothyroids was more positive than the membrane resting potential. Normal E_{Gly} could be restored upon TH substitution in P11-12 hypothyroids. These data demonstrate a disturbed Cl^- homeostasis following TH deficiency and point to a delayed onset of synaptic inhibition. Interestingly, immunohistochemistry demonstrated an unchanged KCC2 distribution in hypothyroids, implying that TH deficiency does not affect KCC2 gene expression, but may impair the functional status of KCC2. Hippocampal neurons of hypothyroid P16-17 rats also demonstrated an impaired Cl^- homeostasis, indicating that TH may promote the D/H shift and maturation of synaptic inhibition throughout the brain.

INTRODUCTION

The thyroid hormone (TH) is an indispensable and very potent maturation signal, as demonstrated impressively by its triggering effect of amphibian metamorphosis. In mammals, TH plays important roles during brain development (Thompson & Potter, 2000; Forrest et al., 2002). Lack of TH during perinatal life results in functional deficits, including mental retardation and deafness (Uziel, 1986; Bernal et al., 2003). Conversely, hyperthyroidism induces an earlier onset of auditory function in neonatal rats, as evidenced by TH injections and measurement of auditory brainstem responses (Freeman et al., 1993). Aside from functional impairments, TH deficiency leads to several morphological changes in the neonatal rat brain, e.g. decreased axonal density, reduced number of dendrites and dendritic spines, and reduced synaptogenesis (reviews: (Thompson & Potter, 2000; Nunez et al., 2008). Here, we have assessed TH effects on the development of inhibitory neurotransmission. Our study was fostered by the fact that synaptic inhibition, which is mainly caused by glycine and GABA, undergoes a shift (D/H shift) from an initially depolarizing and excitatory character present in immature neurons to the hyperpolarizing, inhibitory nature classically seen in adults (reviews: Payne et al., 2003; Ben-Ari et al., 2007; Farrant & Kaila, 2007) . The D/H shift was reported in multiple neural systems and is very likely a ubiquitous phenomenon in the CNS. Interestingly, in the majority of systems, the D/H shift occurs during the first 1-2 postnatal weeks in rats, the same period when several neural maturation processes depend critically on TH (Oppenheimer & Schwartz, 1997; Thompson & Potter, 2000; Knipper et al., 2001).

The D/H shift is due to the activation of KCC2, an electro-neutral, neuron-specific K^+/Cl^- co-transporter which lowers the intracellular Cl^- concentration ($[Cl^-]_i$), thereby generating an inward-directed electrochemical gradient for Cl^-. This gradient forms the prerequisite that opening of glycine or $GABA_A$ receptors, both being ligand-gated Cl^- channels, results in hyperpolarization (Rivera et al., 1999; Balakrishnan et al., 2003). We focused our study on the lateral superior olive (LSO), a prominent auditory nucleus in the mammalian brainstem. LSO neurons receive a powerful inhibitory, glycinergic input from the contralateral ear which, together with an excitatory, glutamatergic input from the ipsilateral ear, enables them to detect interaural intensity differences, thus participating in sound localization. Rat and mouse LSO neurons are depolarized during the first postnatal days by glycine and GABA, yet become hyperpolarized at about postnatal day 5 (P5) (Kandler & Friauf, 1995; Ehrlich et al., 1999). In LSO neurons of KCC2 knockdown mice, the D/H shift fails to appear (Balakrishnan et al., 2003). By means of gramicidin perforated-patch recordings in hypothyroid rats, we here describe that perinatal TH deficiency delays the D/H shift by 7 days in the LSO, implying an impaired Cl^- homeostasis and pointing to a retarded development of synaptic inhibition. The effect can be counteracted by TH substitution. Immunohistochemistry demonstrated that KCC2 inoperability is not due to a lack of protein, yet likely caused by posttranslational mechanisms. Electrophysiological characterization of hippocampal neurons revealed that the participation of TH in generating a low $[Cl^-]_i$ may be a common phenomenon during neuronal maturation.

MATERIALS AND METHODS

Animals and Drug Administration

Starting 10 days after mating, the antithyroid, goitrogenic drug 1-methyl-2-mercapto-imidazole (MMI, 0.02%), which is routinely used to suppress plasma TH levels in animals and humans (Lind, 1997; Reid et al., 2007), was administered in the drinking water of the dams (Sprague Dawley or Wistar rats). MMI can cross the placenta (Calvo et al., 1992), and our administration ensures that TH levels are effectively suppressed to < 5 ng mL^{-1} L-thyroxine and < 0.15 ng mL^{-1} triiodothyronine (Knipper et al., 2000) at the onset of fetal thyroid gland function, which takes place at embryonic days 17-18 in rats (Bernal & Nunez, 1995). Treatment was continued postnatally until the pups were sacrificed between P5 and P17 (the day of birth was designated P0). In TH substitution experiments aimed to counteract experimentally induced hypothyroidism, pups received subcutaneous injections of triiodothyronine (0.3 µg g^{-1} body weight) every other day, starting at P1. Animal treatment was in accordance with the German law for conducting animal experiments and also followed the NIH guide for the care and use of laboratory animals. Protocols were approved by the responsible animal care and use committees (Regierungspräsidium Tübingen and Landesuntersuchungsamt Rheinland-Pfalz, Germany). All efforts were made to minimize the number of animals and their suffering.

Preparation of acute brainstem and hippocampus slices

Rat pups were deeply anesthetized, decapitated, and their brains were rapidly removed and dissected in a chilled solution (ca. 4°C) containing (mM): 25 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 260 D-glucose, 2 sodium pyruvate, 3 *myo*-inositol and1 kynurenic acid, pH 7.4, when gassed with 95% O_2 and 5% CO_2. 300-

µm-thick coronal slices at the level of the LSO or the hippocampus were cut with a vibratome (VT1000S; Leica, Nussloch, Germany), preincubated for 1 h at 37°C, and stored at 25°C until recording commenced. The storing solution was equivalent to the extracellular recording solution and contained (mM): 125 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 10 D-glucose, 2 sodium pyruvate, 3 *myo*-inositol and 0.4 ascorbic acid. Its pH was 7.4 when gassed with 95% O_2 and 5% CO_2.

Electrophysiology

Patch pipettes had resistances of 2-5 MΩ when filled with a solution containing (in mM): 140 KCl, 5 EGTA, 3 $MgCl_2$, 5 HEPES (pH 7.3 with KOH). They were front filled with gramicidin-free pipette solution for 2-3 min and then backfilled with this solution that additionally contained 2.5-10 µg mL^{-1} of gramicidin such that the pipette solution never contained more than 0.1% DMSO. Slices were transferred to a recording chamber which was continuously perfused at a rate of 1.5-2 mL min^{-1} with extracellular solution at room temperature. LSO and CA1 hippocampal neurons were visualized with DIC-infrared optics using a 40x (numerical aperture 0.80) water immersion objective on an upright microscope (Eclipse E600-FN, Nikon, Düsseldorf, Germany). Electrophysiological responses were recorded with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) and pClamp 8.0.2 software (Axon Instruments) or an EPC 10 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) and PatchMaster and FitMaster software (v2.20, HEKA). To obtain gramicidin perforated-patch recordings, a gigaohm seal (≥1 GΩ) was established and the progress of perforation was monitored until the access resistance had stabilized to ~200-300 MΩ (after 10-30 min). Neurons with a resting membrane potential (V_{rest}) >-50 mV were discarded from further analysis. The population of neurons with such a positive V_{rest} was very small and there was no age-related trend which could have compromised the developmental conclusions. The voltage-clamp protocol consisted of stepping the membrane potential from a holding potential of -70 mV to command potentials (V_{com}) ranging from -120 mV to 30 mV (1-3 s step duration). With a delay of 500 ms after the step onset, glycine (1 mM) or GABA (1 mM) was applied to LSO or hippocampal neurons with puffs (10 or 100 ms duration, ~25 kPa pressure). The puffs were applied through a wider tip patch pipette (~4 µm) mounted on a Picospritzer (General Valve, Fairfield, NJ) or a pneumatic drug ejection system (Model 2T, npi electronic, Tamm, Germany). Application intervals lasted 10 s, long enough to prevent lasting changes of $[Cl^-]_i$ and, consequently, artifacts in the reversal potential of glycine- or GABA-activated currents (E_{Gly} or E_{GABA}, respectively). Such artifacts may be caused by Cl^- loading or depletion at positive or negative command potentials, respectively (Ehrlich et al., 1999). The liquid junction potential was ~3 mV and therefore neglected. The peak amplitude of glycine- or GABA-activated currents was determined as the difference between the holding current and the maximally evoked current amplitude. Peak current responses for each voltage were plotted and the data were analyzed for best fitting regression functions by the statistic software Winstat for Excel (version 1999.3; Fitch Software, Zierenberg, Germany). E_{Gly} and E_{GABA} were determined from the x-intercept value of the regression line. The $[Cl^-]_i$ in LSO neurons was calculated with the Nernst equation ($E_{Cl} = RT/F \cdot \ln [Cl^-]_i/[Cl^-]_o$) by applying the measured E_{Gly}, the relevant value of RT/F, and $[Cl^-]_o$ = 133.5 mM. To analyze glycine- or GABA-induced membrane potential changes, current-clamp recordings were obtained at V_{rest} (0 pA current injection) and drugs were applied as described above. Data are given as mean ± standard error of the mean. Statistical analysis was performed by a Student's t test and significant differences between two data groups are depicted with asterisks in the diagrams (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

In small neurons, in which the input resistance (several GΩ) is not very much lower than the seal resistance, a leak conductance is introduced through the seal resistance that induces an artifact, namely a depolarization of the membrane potential (Barry & Lynch, 1991; Tyzio et al., 2003). As a consequence, this artifact imposes an error on the GABA and/or glycine driving force, the difference between E_{Cl} and the resting membrane potential. Nevertheless, it does not affect E_{Cl} and any conclusions concerning changes of intracellular chloride.

Immunohistochemistry and laser scanning confocal microscopy

Immunohistochemistry was performed as described (Blaesse et al., 2006). Briefly, rat pups were deeply anesthetized and perfused transcardially with phosphate-buffered saline (pH 7.4), followed by 2% paraformaldehyde/15% picric acid in 0.1 M phosphate buffer. Brains were removed and coronal sections of 30 µm thickness were cut on a freezing microtome and incubated with the polyclonal primary antibody nKCC2 (raised in rabbit against the N-terminus of KCC2; Blaesse et al., 2006) at a dilution of 1:500 and for 24 h at 6℃. The primary antibody was visualized with goat anti-rabbit Ig conjugated to Alexa Fluor 488 (1:1,000; Invitrogen, Karlsruhe, Germany). Sections were analyzed and photographed on a laser scanning confocal microscope (LSM 510, Zeiss, Oberkochen, Germany) equipped with an argon laser and appropriate excitation and emission filters (488 nm; 505–550 nm bandpass). Images of 2,048 x 2,048 pixels were obtained at 10x (Plan-Neofluar, 10x/numerical aperture 0.3; Zeiss) and 40x (Plan-Neofluar, 40x/numerical aperture 1.3 oil; Zeiss) and further processed with Zeiss LSM Image browser software 2.80. Figures were prepared with Adobe Photoshop 5.5.

RESULTS

Glycine-evoked responses in developing LSO neurons of normal and hypothyroid rats

In order to assess TH effects on Cl$^-$ homeostasis and the maturation of inhibitory neurotransmission, we analyzed age-related changes in the polarity of glycine-evoked responses in LSO neurons of normal and experimentally induced hypothyroid rats. To do so, we applied the gramicidin perforated-patch technique, which preserves the native [Cl$^-$]$_i$ (Kyrozis & Reichling, 1995; Akaike, 1996). In current-clamp mode, glycine pulses (10 ms) applied to the somata of LSO neurons elicited depolarizing responses in control animals before P6, yet hyperpolarizing responses thereafter. Typical examples of such depolarizing and hyperpolarizing responses, obtained during the first and second postnatal week, respectively, are shown in Fig. 1A. These age-related changes in the polarity of the responses were consistent with previous findings, which had demonstrated the occurrence of the D/H shift at around P5 in the rat LSO (Ehrlich et al., 1999).

In contrast to the controls, the great majority of LSO neurons of hypothyroid rats became depolarized upon glycine application throughout the period analyzed. The depolarizing effect was present not only during the first postnatal week, but also at P8 and older ages (Fig. 1B). Between P5 and P12, the oldest age analyzed, depolarizing responses were seen in 72% of the neurons (18 out of 25). Amongst the remaining seven neurons (28%), four showed hardly any change of the membrane potential, whereas three displayed hyperpolarizing responses with amplitudes >5 mV. The latter responses were all recorded at P12. Together, the current-clamp data indicated that LSO neurons of hypothyroid rats aged P5-12 displayed abnormally high [Cl$^-$]$_i$ values and a disturbed Cl$^-$ homeostasis.

To further address the issue of abnormally high $[Cl^-]_i$ values, we performed gramicidin perforated-patch recordings in voltage-clamp mode and determined the equilibrium potential E_{Gly}, at which no net current is evoked. Because of the negligibly low permeability of glycine receptors to anions other than Cl^- (e.g. HCO_3^-), $E_{Gly} \approx E_{Cl}$ in the LSO (Ehrlich et al., 1999). Typical recordings, in which the membrane potential was stepped from –70 mV to V_{com} values ranging from -120 mV to 0 mV, are illustrated in Fig. 2. The two examples depict neurons from hypothyroid rats at P5 and P8 which displayed glycine-evoked inward currents at $V_{com} \leq -60$ mV, yet outward currents at $V_{com} \geq -30$ mV. In accordance with this, the current-voltage relation revealed E_{Gly} values of -34 mV and -44 mV, which were > 20 mV more positive than the V_{rest} values (-63 mV and -67 mV, respectively).

When we plotted E_{Gly} and V_{rest} values for all 25 LSO neurons of hypothyroid rats and determined the regression lines (a linear regression turned out to be the best fit), we detected age-related shifts for E_{Gly} towards more negative potentials, yet no changes for V_{rest} (Fig. 3B). We observed the occurrence of a D/H shift during the period analyzed but, importantly, it appeared not earlier than P12-13. Thus, the D/H shift was not prevented by hypothyroidism, but it was delayed by 7 days compared to the controls in which the D/H shift occurred at P5-6 (Fig. 3A).

We next categorized our data into two age groups (P4-7 and P9-12) to allow a statistical analysis (Fig. 3C). From these grouped data, several aspects became apparent: (1), at P4-7, E_{Gly} did not differ significantly from V_{rest} in the control animals (-55.1 ± 3.0 mV vs. -60.7 ± 1.9 mV, n = 13; p = 0.1), which is in accordance with the fact that the D/H shift takes place during this period. (2), in contrast to the control group, in the age-matched hypothyroid group, E_{Gly} was significantly more positive than V_{rest} (-43.3 ± 5.6 mV vs. -65.6 ± 1.0 mV, n = 9; p = 0.004), implying an abnormally high $[Cl^-]_i$ as a consequence of TH deficiency (calculation of $[Cl^-]_i$ yielded

17.1 ± 1.8 mM in the controls and 30.2 ± 6.6 mM in the hypothyroids). (3), by P9-12, E_{Gly} had become significantly more negative than V_{rest} in the controls (-80.0 ± 4.3 mV vs. -62.7 ± 1.4 mV, n = 15; p = 0.001), demonstrating a low $[Cl^-]_i$ (7.3 ± 1.2 mM) and an efficient Cl^- extrusion mechanism, i.e., powerful KCC2 activity at this age. (4), in contrast to the age-matched control group, hypothyroid rats at P9-12 displayed no significant difference between E_{Gly} and V_{rest} (-58.3 ± 7.9 mV vs. -66.8 ± 1.9 mV, n = 13; p = 0.3), implying again that Cl^- extrusion and KCC2 activity were impaired ($[Cl^-]_i$ was calculated to 22.3 ± 5.9 mM). Together, these results showed unanimously that hypothyroidism impairs Cl^- homeostasis and the onset of inhibitory neurotransmission in developing LSO neurons. We also analyzed our data regarding age-related effects of TH deficiency. Whereas the negative shift of E_{Gly} between P4-7 and P9-12 was significant in controls (p = 0.0001), this was not the case in hypothyroid rats (p = 0.2; Fig. 3C).

Immunohistochemical analysis of KCC2 in the developing LSO of normal and hypothyroid rats

The impaired Cl^- homeostasis in the LSO of hypothyroid rats and the delayed occurrence of the D/H shift suggested an ineffective KCC2 transport mechanism. To investigate whether TH effects on KCC2 activity were reflected by changes in KCC2 protein expression, we performed immunohistochemistry and analyzed the distribution of the protein at both the supracellular and the cellular level. Labeling was done at P12, when the population of LSO neurons in normal rats displays E_{Gly} values clearly more negative than V_{rest}, yet $E_{Gly} \approx V_{rest}$ in hypothyroid rats (Fig. 3). Immunofluorescent signals were present in the LSO and all other nuclei of the superior olivary complex (Fig. 4). At the supracellular level, the overall pattern and density of KCC2 labeling appeared qualitatively equal between control (Fig. 4A) and hypothyroid rats (Fig. 4B). Likewise, higher magnification of the LSO revealed no difference at the cellular level between both groups, as demonstrated by the fact that KCC2 immunoreactivity was consistently localized to the plasma membrane of somata (Fig. 4C,D, insets, arrow) and dendrites (Fig. 4C,D, insets, arrowheads). Thus, the distribution of KCC2 protein in the LSO did not appear to be affected by hypothyroidism. In other words, the delayed development of $[Cl^-]_i$ regulation was not paralleled by a delayed expression and/or membrane incorporation of KCC2, but rather suggest a posttranslational mechanism.

GABA-evoked responses in developing hippocampal neurons of normal and hypothyroid rats

To investigate whether the impairing effect of TH deficiency on Cl^- homeostasis is present in neuronal systems other than the LSO, we finally recorded from CA1 pyramidal neurons in acute slices of the hippocampus, a brain region in which seminal work on $[Cl^-]_i$ regulation has been published (Cherubini et al., 1990; Cherubini et al., 1991; Zhang et al., 1991; Ben-Ari et al., 1994; Rivera et al., 1999; Chudotvorova et al., 2005). Like in the LSO, gramicidin perforated-patch recordings were performed in both current-clamp and voltage-clamp mode. At P16-17, the great majority (9 of 12) of pyramidal neurons in control animals showed hyperpolarizing responses upon GABA application. The other three neurons displayed basically no voltage change (Fig. 5A). In contrast, neurons of hypothyroid rats were heterogeneous in that 6 became depolarized, 7 became hyperpolarized, and one displayed no voltage change (Fig. 5A). The statistical analysis revealed that E_{GABA} in the control animals was significantly more negative than V_{rest} (-77.5 ± 3.2 mV vs. -67.9 ± 2.6 mV, n = 12; p = 0.03; Fig. 5B), corroborating a low $[Cl^-]_i$ and, thus, effective KCC2 transport activity (very similar E_{GABA} values [-74 ± 3 mV] were recently obtained for rat CA3 pyramidal cells at P13-15; Tyzio et al., 2007). By contrast, in CA1 neurons of hypothyroid rats, E_{GABA} was virtually indistinguishable and not significantly different from V_{rest} (-72.4 ± 3.6 mV vs. -71.1 ± 1.5 mV, n = 14; p = 0.7; Fig. 5B). These results also point to an impaired Cl^- homeostasis as a consequence of TH deficiency and are in accordance with a previous *in vivo* study that demonstrated a suppression of GABA-mediated inhibition following TH insufficiency (Gilbert et al., 2007). Based on our results, it appears that the TH effects on Cl^- extrusion are not limited to the LSO, yet they are also present in the developing hippocampus.

TH substitution experiments in LSO neurons of hypothyroid rats

In a last series of analysis, we performed “rescue experiments” to investigate whether TH substitution can counteract the disturbing effects that experimentally induced hypothyroidism has on Cl^- homeostasis. To do so, MMI-treated rat pups received subcutaneous injections of triiodothyronine from P1 till P11-12, and gramicidin perforated-patch were subsequently performed in the LSO. Four out of five LSO neurons became hyperpolarized upon glycine application (Fig. 6A), whereas the fifth neuron displayed a depolarizing response. The statistical analysis showed a significantly more negative value for E_{Gly} than for V_{rest} (-87.5 ± 7.5 mV vs. -64.9 ± 3.1 mV, n = 5; p = 0.02; Fig. 6B), implying effective KCC2 transport activity and thus Cl^- extrusion. As E_{Gly} in these rescue experiments did not differ from E_{Gly} obtained in control rats (p = 0.4), these data also imply that normal Cl^- homeostasis was restored by TH substitution.

DISCUSSION

In this study, we investigated the maturation of glycinergic inhibition in the auditory brainstem nucleus LSO of hypothyroid rats. For comparison, we also examined the consequences of hypothyroidism on GABAergic inhibition in the developing rat hippocampus. We show that, in both brain regions, TH signaling is required for the normal maturation of synaptic inhibition mediated via glycine receptors or $GABA_A$ receptors. Our finding that TH deficiency retards the emergence of efficient Cl^- extrusion mechanisms, an effect that can be counteracted by TH substitution, identifies a novel signaling cascade associated with Cl^- homeostasis. In addition, the unaltered expression and plasma membrane location of the main neuronal Cl^- extruder, the K^+/Cl^- co-transporter KCC2, in hypothyroid LSO neurons is indicative of a posttranslational TH effect at the level of KCC2 protein function, probably by influencing a regulatory cascade. Collectively, our data add another facet to the general picture that TH deficiency typically produces immature features and retards the development, but does not lead to gross malformations (Forrest et al., 2002).

Effects of thyroid hormone on the developing auditory system

The retarded development of Cl^- homeostasis and the effects on the onset of inhibitory neurotransmission provide a new aspect to the manifold developmental aberrations of the auditory system in response to TH deficiency. So far, however, such aberrations were mainly identified in the periphery (cochlea) and manifested at the morphological, the physiological, and the molecular level. At the morphological level, TH affects the formation of the inner sulcus (Uziel et al., 1983a), the tectorial membrane and the tunnel of Corti (Uziel et al., 1985; Rüsch et al., 2001; Ng et al., 2004), and the synapses at outer hair cells (Uziel et al., 1983b) and inner hair cells

(Sendin et al., 2007). At the physiological level, TH affects a multitude of parameters, e.g. hearing thresholds (Knipper et al., 2000; Ng et al., 2004), a fast-activating K^+ conductance (Rüsch et al., 1998), as well as Ca^{2+} currents and the firing pattern of inner hair cells (Sendin et al., 2007; Brandt et al., 2007). Finally, at the molecular level, TH affects protein expression in the tectorial membrane (Knipper et al., 2001), in inner hair cells (Brandt et al., 2007) and outer hair cells (Weber et al., 2002; Winter et al., 2006; Winter et al., 2007), and in nonneuronal cells (Knipper et al., 1999). Elevated auditory brainstem response latencies in hypothyroid animals point to a developmental disturbance of synaptogenesis and/or myelination in the *central* auditory system (Knipper et al., 2000) as described for other brain areas (reviews: (Bernal & Nunez, 1995; Bernal, 2002; Leonard, 2007; Ahmed et al., 2008). Furthermore, throughout the central auditory system, thyroidectomized and hypothyroid rats exhibit low rates of glycose utilization (Dow-Edwards et al., 1986) and a higher amount of deiodinase type 2 mRNA, respectively (Dow-Edwards et al., 1986; Guadaño-Ferraz et al., 1999). Finally, mice lacking TH receptor beta are prone to audiogenic seizures (Ng et al., 2001). The results of the present study provide further evidence that TH influences the developing *central* auditory system and may help to yield some insight into the mechanisms underlying the susceptibility to audiogenic seizures.

Thyroid hormone and neuronal excitability

In recent years, it has become increasingly clear that an imbalance between the strength of excitatory and inhibitory neurotransmission, particularly a reduction of synaptic inhibition, is the reason for several neurological disorders encompassing increased excitability, such as epilepsy and chronic pain (review: (De Koninck, 2007). For instance, audiogenic seizures are associated with a reduced efficacy of GABA-mediated inhibitory neurotransmission (Faingold, 2002). Changes in receptor expression, decreases in transmitter levels, and loss of inhibitory synapses may be the underlying cause. Based on our results, it is tempting to speculate that the basis of audiogenic seizures may as well be an impaired Cl^- homeostasis, eventually caused by an insufficient maturation of KCC2 transport activity. This would be analogous to human patients in which mesial temporal lobe epilepsy is associated with perturbed Cl^- homeostasis and reduced expression of KCC2 (Huberfeld et al., 2007).

Severe hypothyroidism during the neonatal period leads to structural alterations in the brain, including hypomyelination and defects of cell migration and differentiation, with long-lasting, irreversible effects on behavior and performance (Bernal, 2002). In contrast to these profound TH effects, surprisingly few genes have so far been found in the CNS to be under the direct transcriptional control of TH (Flamant and Samarut, 2003). It may be challenging though to consider that some of the deteriorative effects in the CNS that occur in the absence of TH are a consequence of the delayed emergence of inhibitory control described in the present study.

Concerning TH effects in the hippocampus, an up-regulation of Na^+ currents as well as increased amplitudes and frequencies of action potentials have been described *in vitro* (Potthoff & Dietzel, 1997; Hoffmann & Dietzel, 2004). Moreover, hypothyroidism impairs long term potentiation and decreases the level of c-fos, a marker protein for neuronal activity (Dong et al., 2005). These results all imply that TH *enhances* the neuronal excitability. By contrast, the present study demonstrates the requirement of TH to establish effective inhibition, i.e., to *reduce* neuronal excitability. Our results are in line with a recent report that TH is required in the early postnatal period to establish proper inhibitory function *in vivo* in the rat CA1 region (Gilbert et al., 2007). The opposite impacts lisrted above may seem paradoxical, yet they are in accordance with the manifold physiological TH effects and point to an orchestrating action of TH, ultimately resulting in fine-tuned excitability levels.

Molecular mechanisms of impaired Cl^- homeostasis with hypothyroidism

The age-dependent regulation of $[Cl^-]_i$ is achieved by an interplay between transporters mediating Cl^- uptake and Cl^- extrusion. Lowering of $[Cl^-]_i$ requires KCC2 activity in all neurons studied so far, including the two brain regions studied here (LSO: Balakrishnan et al., 2003; hippocampus: Rivera et al., 1999). Based on our results, we propose that TH affects proteins which inhibit the functional status of KCC2, i.e. its transport capacity, either in a direct or an indirect manner. This proposed posttranslational mechanism is unusual because most often, a reduced KCC2 expression is observed with disturbed Cl^- extrusion, for example after axotomy of motoneurons (Nabekura et al., 2002; Toyoda et al., 2003), in a model of neuropathic pain (Coull et al., 2003), and in epileptic patients (Palma et al., 2006; Huberfeld et al., 2007). However, an unaltered KCC2 expression was observed in surgically induced deafness when transporter function became disrupted (Vale et al., 2003) and a recent study indicates that phosphorylation of KCC2 through protein kinase C increases the rate of ion transport by the protein (Lee et al., 2007). These results, together with ours, demonstrate that KCC2 transport function can be effectively regulated by posttranslational mechanisms, rather than merely by gene expression. In register with this, during early development, when GABA and glycine still exert depolarizing effects, KCC2 protein is present in auditory brainstem areas at amounts which are indistinguishable from the mature situation (Balakrishnan et al., 2003; Vale et al., 2005; Löhrke et al., 2005; Blaesse et al., 2006).

Apart from a reduced, ineffective KCC2 transport activity caused by hypothyroidism, an alternative scenario may explain the delayed D/H shift. This is the possibility of an increased inward-directed Cl$^-$ transport activity in response to TH deficiency. However, we consider this possibility rather unlikely, because the lack of TH delays the maturation of synaptic inhibition in both LSO and hippocampus, whose neurons differ in their Cl$^-$ uptake mechanisms: in hippocampal neurons, the Na$^+$/K$^+$/Cl$^-$ cotransporter NKCC1 appears to be the main Cl$^-$ inward transporter (Sipilä et al., 2006), whereas it is absent in immature LSO neurons (Balakrishnan et al., 2003). Thus, if hypothyroidism would increase Cl$^-$ inward transporter activity, TH would have to affect various types of Cl$^-$ inward transporters and thus would need to act via separate cascades.

Signaling pathways of thyroid hormone

An open question concerns the signaling pathway through which TH may affect Cl$^-$ homeostasis. The only known protein which modulates Cl$^-$ transporter activity and whose gene expression is regulated by TH is the brain-derived neurotrophic factor BDNF (Koibuchi et al., 1999). Interestingly, BDNF effects on KCC2 expression in the hippocampus encompass down-regulation in mature neurons (Rivera et al., 2002; Rivera et al., 2004; Wake et al., 2007), yet up-regulation in fetal neurons (Aguado et al., 2003). These opposite effects are explained by the activation of distinct signaling cascades downstream to TrkB binding of BDNF (Rivera et al., 2004). They are further in harmony with the finding that regulation of BDNF gene expression by TH is heterogeneous, occurring in a promoter-, age-, and brain region-specific manner (Koibuchi et al., 1999). Whether BDNF-TrkB cascades play a role in the TH-mediated promotion of the D/H shift and maturation of synaptic inhibition needs to be elucidated by future work.

Recently, it was shown that oxytocin, an essential maternal hormone for labor, triggers a transient switch in the action of GABA from excitatory to inhibitory shortly before birth, due to a shift of E_{Cl} towards more negative values (Tyzio et al., 2006). The effect is thought to protect the neurons from stress caused by anoxic-aglycaemic episodes during delivery. Moreover, the effect appears to involve inhibition of the Cl^- inward transporter NKCC1, because it could be mimicked and occluded by bath application of bumetanide, a selective blocker of NKCC1 at low doses (Khazipov et al., 2008). Whether thyroid hormone can also modulate E_{Cl} directly, or rather involves general developmental retardation, is an open question to be addressed in the future.

Concluding remarks

Several studies describe the deleterious effects of TH deficiency on the development of the *central* auditory system, including abnormal neuronal proliferation, migration, decreased dendritic densities, and dendritic arborizations (Bernal & Nunez, 1995; Bernal, 2002; Rivas & Naranjo, 2007; Leonard, 2007; Ahmed et al., 2008). Taking into account that only few TH target genes have been identified so far in the mammalian brain (Oppenheimer & Schwartz, 1997; Thompson & Potter, 2000; Quignodon et al., 2007; Takahashi et al., 2008), it is tempting to speculate that there may be a causal relation between a retarded D/H shift and some of the other neural deficits that are caused by TH deficiency.

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ABBREVIATIONS

$[Cl^-]_i$	intracellular Cl^- concentration
D/H shift	depolarizing/hyperpolarizing shift
E_{GABA}	reversal potential of GABA-induced currents
E_{Gly}	reversal potential of glycine-induced currents
KCC	K^+/Cl^- co-transporter
LSO	lateral superior olive
MNTB	medial nucleus of the trapezoid body
MSO	medial superior olive
SPN	superior paraolivary nucleus
P	postnatal day
TH	thyroid hormone
V_{com}	command potential
V_{rest}	resting membrane potential

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FIGURE LEGENDS

FIG. 1. Glycine-evoked responses in LSO neurons of hypothyroid rats remain depolarizing until P11. Gramicidin perforated-patch recordings obtained from six neurons between P5-11 in the current-clamp mode. (A) Depolarizing responses were present at P5 in control rats, turning into hyperpolarizing responses by P8 and P11 in virtually every neuron. (B) By contrast, depolarizing responses persisted until P11 in hypothyroid rats. Arrowheads indicate the time of puff application (10 ms duration, 1 mM glycine). Values at the beginning of each trace depict the resting membrane potential ($V_{rest,}$ in mV) of each neuron.

FIG. 2. E_{Gly} values in LSO neurons of hypothyroid rats remain more positive than V_{rest} during the 2nd postnatal week. Upper panels illustrate typical voltage-clamp recordings obtained at P5 (A) and P8 (B) from two LSO neurons of hypothyroid rats in response to glycine application (triangles, 10 ms duration, 1 mM glycine). Neurons were held at five different command potentials (V_{com}), varying in steps of 30 mV and ranging from -120 mV to 0 mV. Lower panels depict corresponding current-voltage relations. E_{Gly} values of -34 mV (A) and -44 mV (B) were determined for the P5 (V_{rest} = -63 mV) and the P8 neuron (V_{rest} = -67 mV), respectively.

FIG. 3. The age-dependent D/H shift of E_{Gly} is delayed in LSO neurons of hypothyroid rats. (A,B) E_{Gly} and V_{rest} values from LSO neurons of control rats (n = 45, modified from Ehrlich et al., 1999) and hypothyroid rats (n = 25) were plotted against age; closed and open circles mark E_{Gly} and $V_{rest,}$ respectively. The regression lines of E_{Gly} and V_{rest} were calculated and are depicted by solid and dotted lines, respectively. In both groups, V_{rest} remained nearly constant throughout the period analyzed. In

hypothyroid rats, E_{Gly} became more negative with age, and the D/H shift occurred at P12-13. This is in clear contrast to the situation present in control animals, where the D/H shift occurred at P5-6. Values for $[Cl^-]_i$ were calculated from the Nernst equation and are depicted at the right Y axes. (C) Quantitative analysis of E_{Gly} and V_{rest} in LSO neurons of control and hypothyroid rats categorized into P4-7 and P9-12 groups. Several age-related and pharmacologically induced effects point to impaired Cl^- regulation under hypothyroidism (see text for details).

FIG. 4. The amount and distribution of KCC2 protein in the LSO is not affected by hypothyroidism. (A,B) Overview of the superior olivary complex of a control (A) and a hypothyroid (B) rat at P12, illustrating KCC2 immunofluorescent signals in the LSO, MNTB (medial nucleus of the trapezoid body), MSO (medial superior olive), and SPN (superior paraolivary nucleus). None of the nuclei in the hypothyroid rats displayed a change of the KCC2 immunosignal. LSO, MSO, and SPN showed strong signals which were evenly distributed, and MNTB showed a relatively weak signal. (C,D) Part of the LSO at higher magnification. Again, we observed no difference between control (C) and hypothyroid rats (D), i.e., KCC2 immunoreactivity was localized to the plasma membrane of somata (arrows in insets) and dendrites (arrowheads in insets). Dorsal is up and lateral to the right; bar in B equals 200 µm (A,B), 50 µm (C,D), and 25 µm (insets).

FIG. 5. Hypothyroidism affects Cl^- homeostasis in hippocampal neurons. (A) Exemplary gramicidin perforated-patch recordings obtained from four neurons at P16 in the current-clamp mode. In control animals, GABA applications (triangles; 1 mM for 100 ms) typically induced hyperpolarizations (upper trace; n = 9/12); in three cases, no voltage changes were observed (lower trace). By contrast, in the

hypothyroid group, depolarizations (upper trace; n = 6/14) as well as hyperpolarizations (lower trace, n = 7/14) were present. Values at the beginning of each trace depict V_{rest} in mV. (B) Quantitative analysis of E_{GABA} and V_{rest} in hippocampal neurons of control and hypothyroid rats at P16-17. Whereas E_{GABA} was significantly more negative than V_{rest} in the controls ($p = 0.03$), there was no difference in the hypothyroid animals ($p = 0.7$), demonstrating that TH deficiency leads to impaired Cl^- regulation.

FIG. 6. Normal E_{Gly} is restored upon TH substitution in LSO neurons of P11-12 hypothyroid rats. (A) Exemplary gramicidin perforated-patch recordings in the current-clamp mode obtained from two neurons of hypothyroid rats which received TH to substitute for the MMI-induced hormone deficiency. Both neurons displayed hyperpolarizations in response to glycine application (triangles; 1 mM for 100 ms). Values at the beginning of each trace depict V_{rest} in mV. (B) Quantitative analysis of E_{Gly} and V_{rest} in five LSO neurons of hypothyroid plus TH-substituted rats at P11-12. E_{GABA} was significantly more negative than V_{rest} ($p = 0.02$), demonstrating that effective Cl^- extrusion was reconstituted by TH substitution.

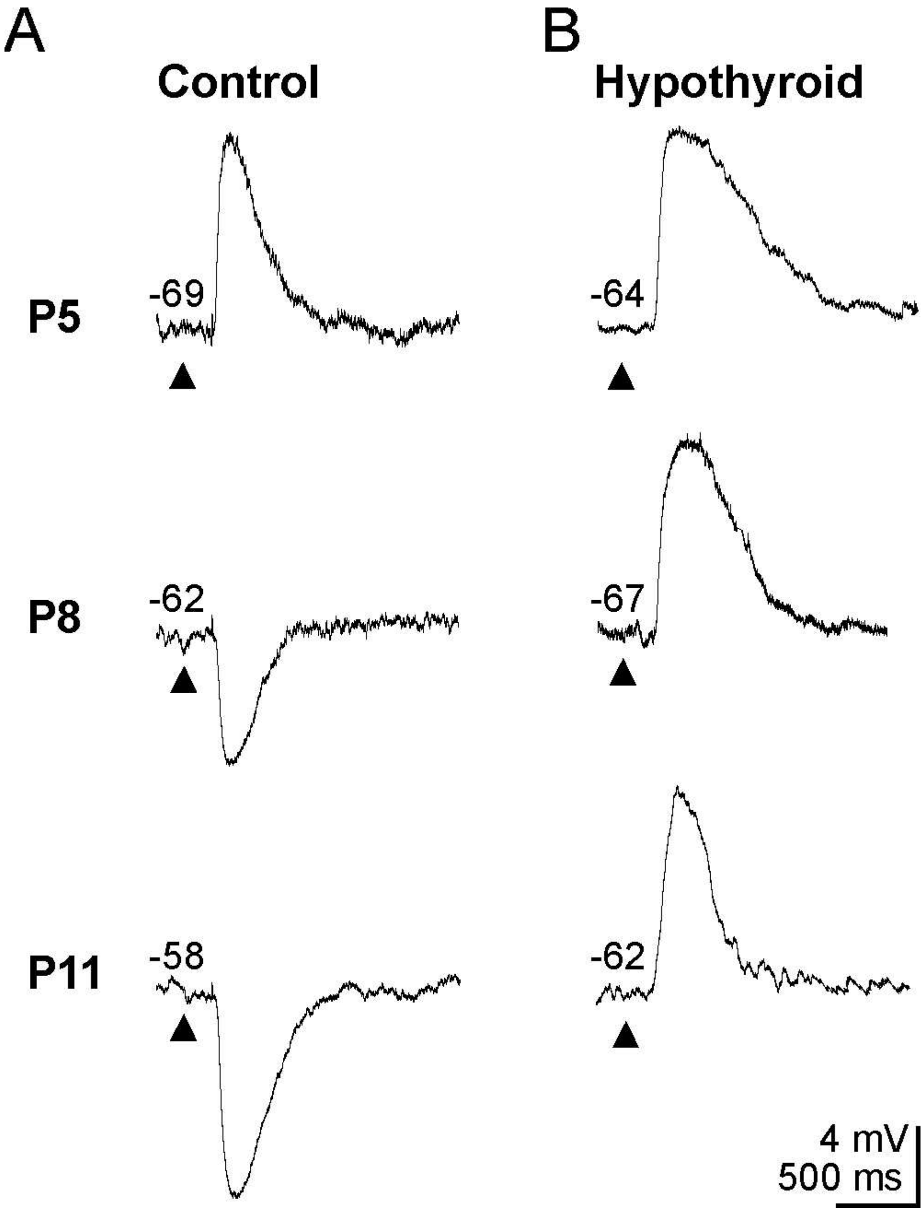

FIG. 1. Glycine-evoked responses in LSO neurons of hypothyroid rats remain depolarizing until P11. Gramicidin perforated-patch recordings obtained from six neurons between P5-11 in the current-clamp mode. (A) Depolarizing responses were present at P5 in control rats, turning into hyperpolarizing responses by P8 and P11 in virtually every neuron. (B) By contrast, depolarizing responses persisted until P11 in hypothyroid rats. Arrowheads indicate the time of puff application (10 ms duration, 1 mM glycine). Values at the beginning of each trace depict the resting membrane potential (Vrest, in mV) of each neuron.

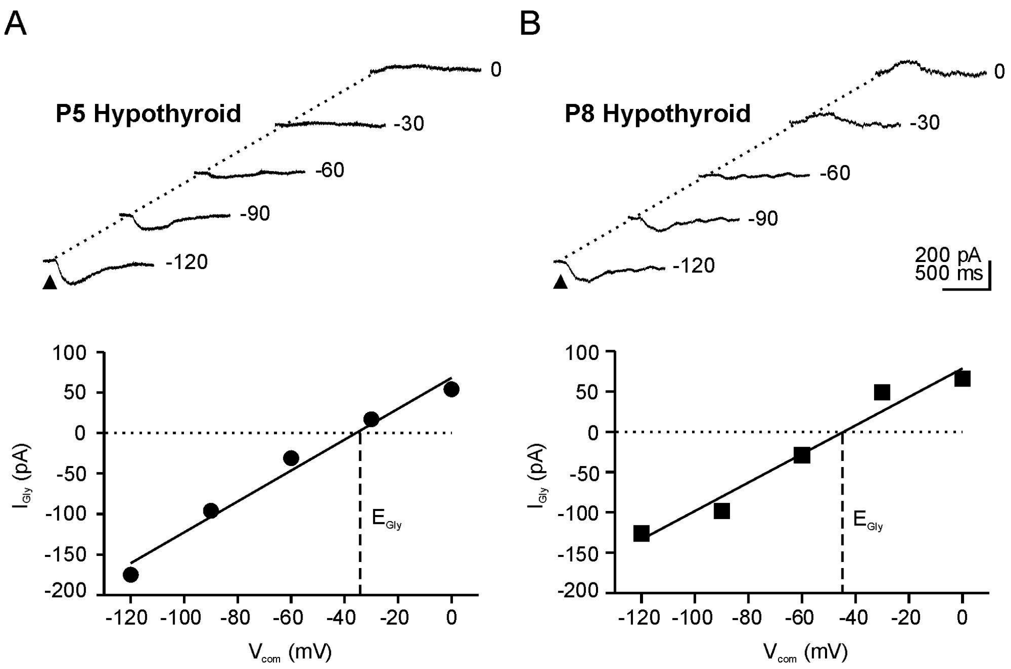

FIG. 2. EGly values in LSO neurons of hypothyroid rats remain more positive than Vrest during the 2nd postnatal week. Upper panels illustrate typical voltage-clamp recordings obtained at P5 (A) and P8 (B) from two LSO neurons of hypothyroid rats in response to glycine application (triangles, 10 ms duration, 1 mM glycine). Neurons were held at five different command potentials (Vcom), varying in steps of 30 mV and ranging from -120 mV to 0 mV. Lower panels depict corresponding current-voltage relations. EGly values of -34 mV (A) and -44 mV (B) were determined for the P5 (Vrest = -63 mV) and the P8 neuron (Vrest = -67 mV), respectively.

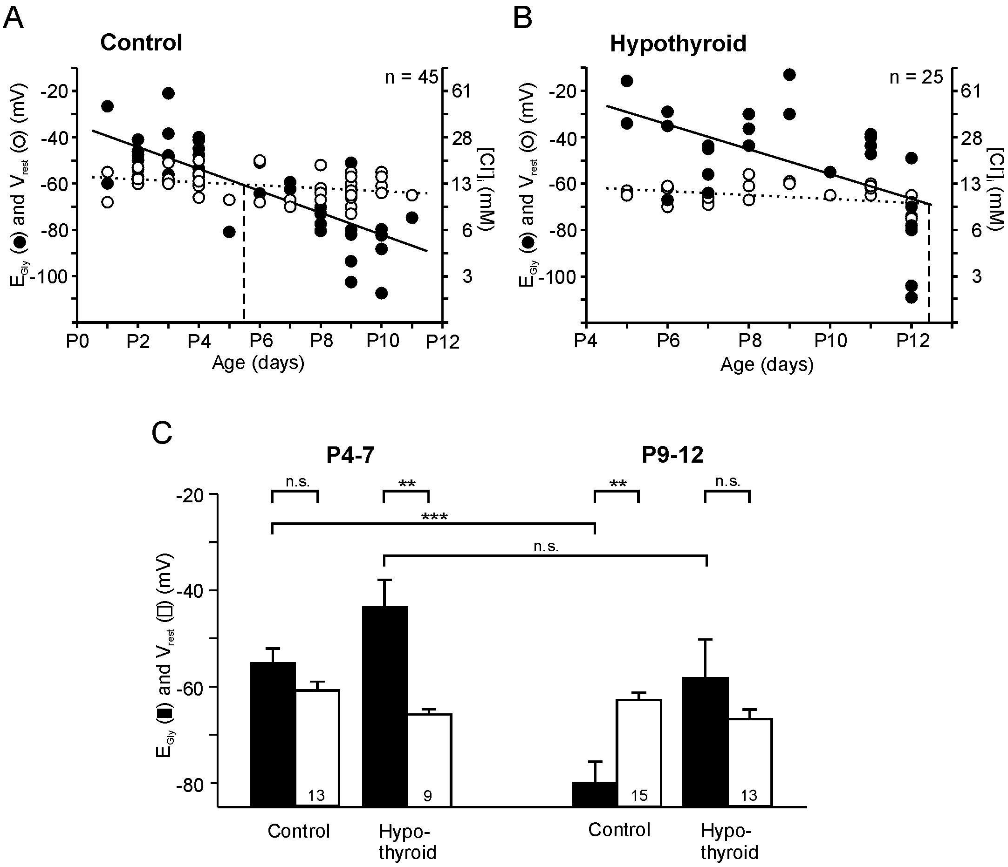

FIG. 3. The age-dependent D/H shift of EGly is delayed in LSO neurons of hypothyroid rats. (A,B) EGly and Vrest values from LSO neurons of control rats (n = 45, modified from Ehrlich et al., 1999) and hypothyroid rats (n = 25) were plotted against age; closed and open circles mark EGly and Vrest, respectively. The regression lines of EGly and Vrest were calculated and are depicted by solid and dotted lines, respectively. In both groups, Vrest remained nearly constant throughout the period analyzed. In hypothyroid rats, EGly became more negative with age, and the D/H shift occurred at P12-13. This is in clear contrast to the situation present in control animals, where the D/H shift occurred at P5-6. Values for [Cl-]i were calculated from the Nernst equation and are depicted at the right Y axes. (C) Quantitative analysis of EGly and Vrest in LSO neurons of control and hypothyroid rats categorized into P4-7 and P9-12 groups. Several age-related and pharmacologically induced effects point to impaired Cl- regulation under hypothyroidism (see text for details).

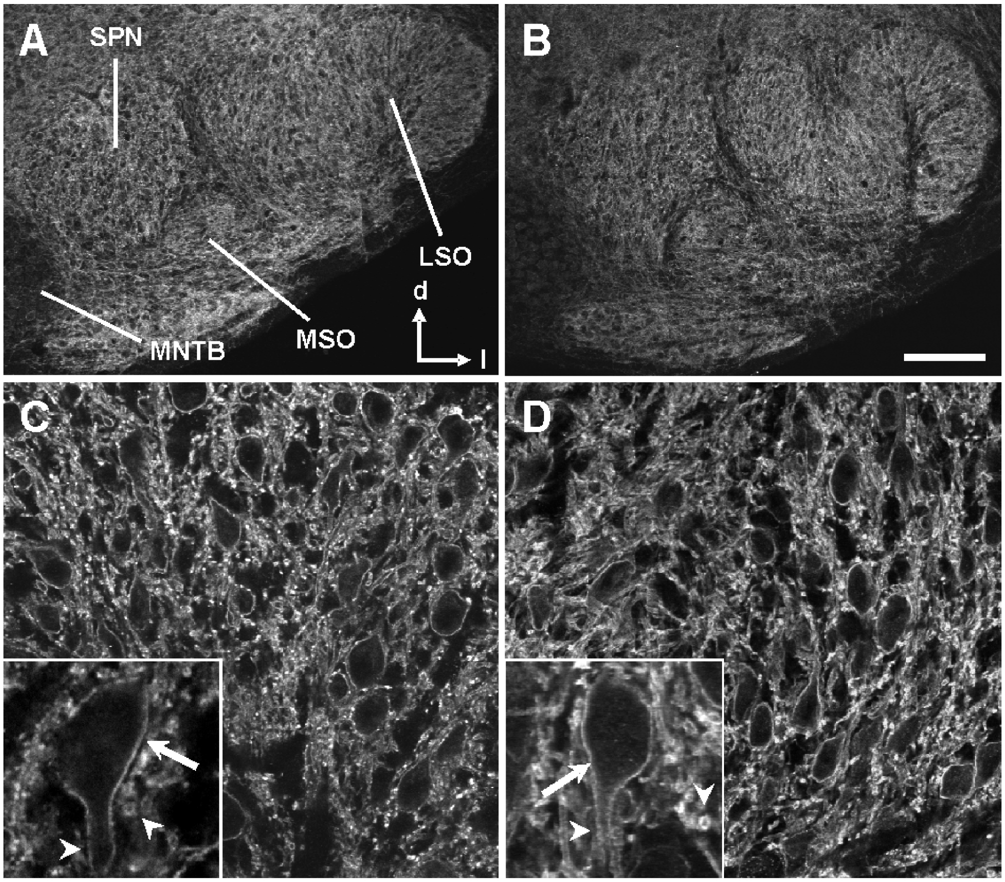

FIG. 4. The amount and distribution of KCC2 protein in the LSO is not affected by hypothyroidism. (A,B) Overview of the superior olivary complex of a control (A) and a hypothyroid (B) rat at P12, illustrating KCC2 immunofluorescent signals in the LSO, MNTB (medial nucleus of the trapezoid body), MSO (medial superior olive), and SPN (superior paraolivary nucleus). None of the nuclei in the hypothyroid rats displayed a change of the KCC2 immunosignal. LSO, MSO, and SPN showed strong signals which were evenly distributed, and MNTB showed a relatively weak signal. (C,D) Part of the LSO at higher magnification. Again, we observed no difference between control (C) and hypothyroid rats (D), i.e., KCC2 immunoreactivity was localized to the plasma membrane of somata (arrows in insets) and dendrites (arrowheads in insets). Dorsal is up and lateral to the right; bar in B equals 200 µm (A,B), 50 µm (C,D), and 25 µm (insets).

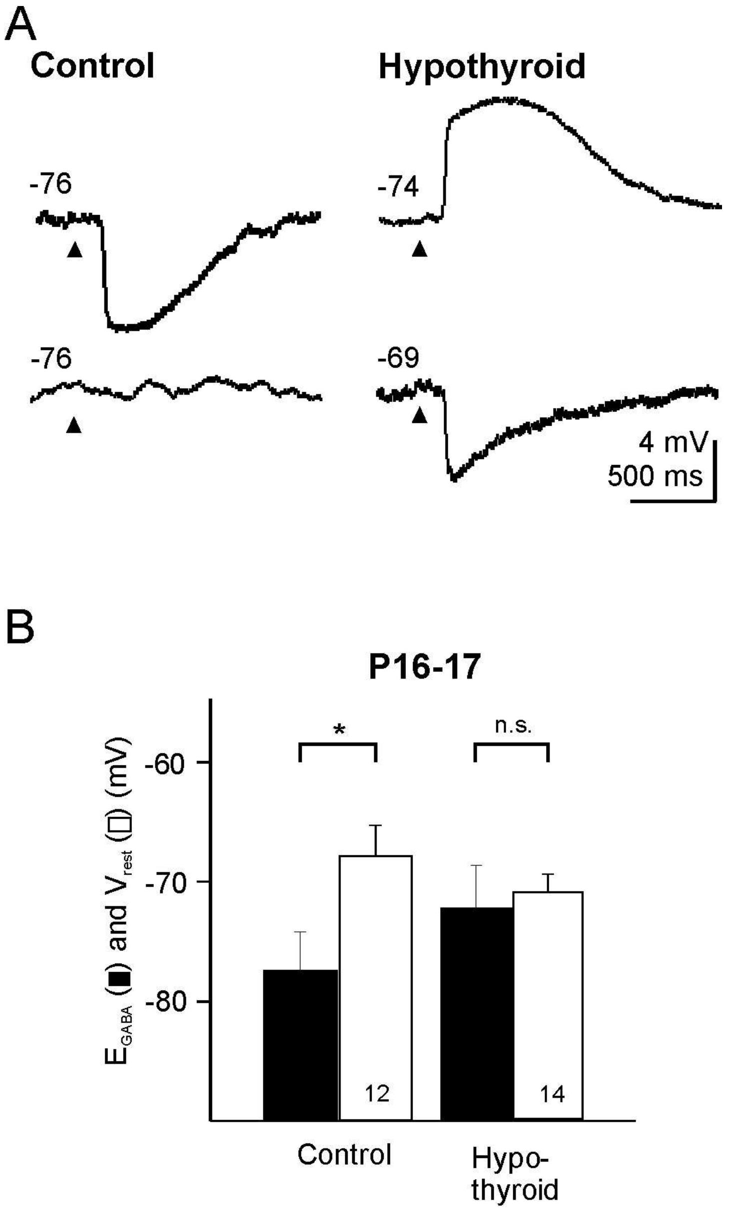

FIG. 5. Hypothyroidism affects Cl- homeostasis in hippocampal neurons. (A) Exemplary gramicidin perforated-patch recordings obtained from four neurons at P16 in the current-clamp mode. In control animals, GABA applications (triangles; 1 mM for 100 ms) typically induced hyperpolarizations (upper trace; n = 9/12); in three cases, no voltage changes were observed (lower trace). By contrast, in the hypothyroid group, depolarizations (upper trace; n = 6/14) as well as hyperpolarizations (lower trace, n = 7/14) were present. Values at the beginning of each trace depict Vrest in mV. (B) Quantitative analysis of EGABA and Vrest in hippocampal neurons of control and hypothyroid rats at P16-17. Whereas EGABA was significantly more negative than Vrest in the controls (p = 0.03), there was no difference in the hypothyroid animals (p = 0.7), demonstrating that TH deficiency leads to impaired Cl- regulation.

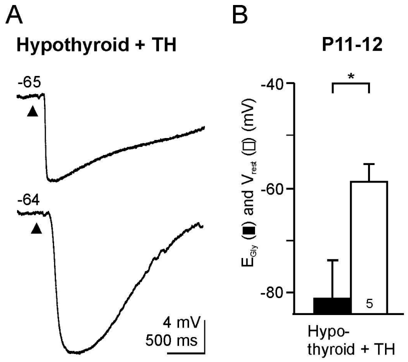

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Figure 6

FIG. 6. Normal EGly is restored upon TH substitution in LSO neurons of P11-12 hypothyroid rats. (A) Exemplary gramicidin perforated-patch recordings in the current-clamp mode obtained from two neurons of hypothyroid rats which received TH to substitute for the MMI-induced hormone deficiency. Both neurons displayed hyperpolarizations in response to glycine application (triangles; 1 mM for 100 ms). Values at the beginning of each trace depict Vrest in mV. (B) Quantitative analysis of EGly and Vrest in five LSO neurons of hypothyroid plus TH-substituted rats at P11-12. EGABA was significantly more negative than Vrest ($p = 0.02$), demonstrating that effective Cl- extrusion was reconstituted by TH substitution.