The Mammalian Interaural Time Difference Detection Circuit Is Differentially Controlled by GABA<sub>B</sub> Receptors during Development

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Throughout development GABA<sub>B</sub> receptors (GABA<sub>B</sub>Rs) are widely expressed in the mammalian brain. In mature auditory brainstem neurons, GABA<sub>B</sub>Rs are involved in the short-term regulation of the strength and dynamics of excitatory and inhibitory inputs, thus modulating sound analysis. During development, GABA<sub>B</sub>Rs also contribute to long-term changes in input strength. Using a combination of whole-cell patch-clamp recordings in acute brain slices and immunostainings in gerbils, we characterized developmental changes in GABA<sub>B</sub>R-mediated regulation of synaptic inputs to neurons in the medial superior olive (MSO), an auditory brainstem nucleus that analyzes interaural time differences (ITDs). Here, we show that, before hearing onset, GABA<sub>B</sub>R-mediated depression of transmitter release is much stronger for excitation than inhibition, whereas in mature animals GABA<sub>B</sub>Rs mainly control the inhibition. During the same developmental period, GABA<sub>B</sub>R immunoreactivity shifts from the dendritic to the somatic region of the MSO. Furthermore, only before hearing onset (postnatal day 12), stimulation of the fibers originating in the medial and the lateral nucleus of the trapezoid body (MNTB and LNTB) activates GABA<sub>B</sub>Rs on both the inhibitory and the excitatory inputs. After hearing onset, GAD65-positive endings devoid of glycine transporter reactivity suggest GABA release from sources other than the MNTB and LNTB. At this age, pharmacological increase of spontaneous synaptic release activates GABA<sub>B</sub>Rs only on the inhibitory inputs. This indicates not only a profound inhibitory effect of GABA<sub>B</sub>Rs on the major inputs to MSO neurons in neonatal animals but also a direct modulatory role of GABA<sub>B</sub>Rs for ITD analysis in the MSO of adult animals.

Introduction

Neurons in the medial superior olive (MSO), a nucleus in the mammalian auditory brainstem, analyze sound direction based on interaural time differences (ITDs) (Goldberg and Brown, 1969; Yin and Chan, 1990; Spitzer and Semple, 1995; Brand et al., 2002). This is achieved using a coincidence detection mechanism, which compares the relative arrival times of the two excitatory inputs deriving from the contralateral and the ipsilateral anteroventral cochlear nucleus (AVCN) (Cant and Casseday, 1986; Skottun, 1998). In addition, MSO neurons receive major inhibitory projections originating from the medial and the lateral nucleus of the trapezoid body (MNTB and LNTB) (Cant and Hyson, 1992; Kuwabara and Zook, 1992; Grothe and Sanes, 1993) (see Fig. 1A). These inhibitory inputs adjust the output signal of MSO neurons such that large changes in the discharge rate are occurring within the physiological relevant range of ITDs an animal experiences (Brand et al., 2002; Pecka et al., 2008). In adult animals, this inhibition is mediated by glycine (Helfert et al., 1989; Smith et al., 2000). Yet, in neonatal animals up to postnatal day 12 (P12), GABA also represents an important inhibitory transmitter, as MNTB fiber stimulation activates GABA<sub>A</sub> receptor-mediated currents in neurons of the medial and lateral superior olive (Kotak et al., 1998; Smith et al., 2000; Kullmann et al., 2002; Nabekura et al., 2004). In most brain regions, GABA not only induces a chloride current via GABA<sub>A</sub> receptors but also activates the metabotropic GABA<sub>B</sub> receptor (GABA<sub>B</sub>R). On the postsynaptic site, GABA<sub>B</sub>R activation triggers a direct inhibitory action via the activation of potassium channels (Pitler and Alger, 1994; Lüscher et al., 1997; Nicoll, 2004). Presynaptically situated GABA<sub>B</sub>Rs modulate the release probability of inhibitory and excitatory neurotransmitters by depressing Ca<sup>2+</sup> currents (Wojcik and Neff, 1984; Isaacs, 1998; Takahashi et al., 1998). Additionally, GABA<sub>B</sub>Rs can be indirectly involved in long-term plastic changes of synaptic efficacy (Kotak et al., 2001; Kamikubo et al., 2007). In the mature auditory brainstem, GABA<sub>B</sub>Rs primarily contribute to the dynamic regulation of transmitter release. We have previously shown that, in the lateral superior olive (LSO), GABA<sub>B</sub>R activation by retrogradely released GABA regulates the balance of excitation and inhibition and thereby adjusts the sensitivity of these neurons to interaural intensity differences (Magnusson et al., 2008). Furthermore, presynaptic GABA<sub>B</sub>Rs have been implicated in decreasing short-term synaptic depression and might thereby improve faithful synaptic transmission for the represen-
tation of sound structure (Brenowitz et al., 1998; Mapelli et al., 2009). Preliminary immunostainings have revealed that in the auditory brainstem GABA<sub>B</sub>Rs are not only present before hearing onset, when the MNTB releases both GABA and glycine, but also in adult animals, when the MNTB input has become glycinergic (Heise et al., 2005; Hilbig et al., 2007). The primary goal of this study was to determine whether the GABA<sub>B</sub>-mediated regulation of the main excitatory and inhibitory inputs to the MSO changes during development. Moreover, we wanted to show to what extent endogenous GABA<sub>B</sub>R activation in the MSO is altered during this developmental period.

Materials and Methods

Slice preparation. All experiments were performed in conformity with the rules set by the European Community Council Directive (86/609/EEC) and German animal welfare legislation.

Acute transverse brain slices (140–190 μm) of the auditory brainstem containing MSO, MNTB, and LNTB were obtained from male and female gerbils (Meriones unguiculatus) aged P8–P32. After decapitation under isoflurane anesthesia, the brainstem was carefully removed and placed in an ice-cold slice solution (see below). Slices were cut in the rostral direction from the level of the facial nerve, with a vibratome (VT1000S; Leica), and incubated at 32°C in oxygenated artificial CSF (aCSF) (see below) for 5 min, after which they were allowed to cool down to room temperature (22 ± 2°C). Recordings were obtained within 4–5 h after the preparation.

Drugs and solutions. To minimize potentially damaging Ca<sup>2+</sup> influx into the neurons, a low-sodium, high-sucrose slice solution (containing the following (in mM): 85 NaCl, 2.5 KCl, 1.3 NaH<sub>2</sub>PO<sub>4</sub>, 2.5 NaHCO<sub>3</sub>, 75 sucrose, 25 glucose, 0.5 CaCl<sub>2</sub>, and 4 MgCl<sub>2</sub>) was used for the slicing procedure. The control aCSF, used for storage and recordings, was composed of the following (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 25 glucose, 2 CaCl<sub>2</sub>, and 1 MgCl<sub>2</sub>. These external solutions were bubbled continuously with carbogen gas (95% O<sub>2</sub>, 5% CO<sub>2</sub>), generating a pH of 7.4.

Whole-cell voltage-clamp recordings were performed using a Cs-based internal solution (containing the following (in mM): 70 Cs<sub>2</sub>SO<sub>4</sub>, 70 Cs<sub>Cl</sub>, 10 HEPES, 10 EGTA, 2 Na<sub>2</sub>-ATP, 2 Mg<sub>2</sub>-ATP, 0.3 Na<sub>2</sub>-GTP, 1 CaCl<sub>2</sub>, and adjusted to pH 7.3 with CsOH). Voltage-gated sodium and potassium currents were blocked by adding QX-314 (lidocaine N-ethyl bromide) (1 mM) and TEA-Cl (tetraethylammonium chloride) (5 mM) to the solution before usage. For whole-cell current-clamp recordings were performed from the MSO with a Multiclamp 700A amplifier (Molecular Devices). Borosilicate glass microelectrodes (GC150F-10; Harvard Apparatus) were pulled on a DMZ Universal Puller (Zeitz Instruments), yielding a final tip resistance of 2–3 MΩ. The series resistance ranged from 5 to 13 MΩ and was compensated by 70–80% for voltage-clamp recordings. Furthermore, the series resistance was monitored throughout the duration of these experiments and was not allowed to vary by >20%. For current-clamp experiments, the bridge balance was applied.

Experimental procedure. MSO principal neurons were optically identified through the bipolar fusiform shape of their somata with dendrites extending medially and laterally. In addition, only neurons with capacities >20 pF, as read from the compensation of the Multiclamp amplifier, were regarded as principal neurons and included in this study. Evoked synaptic responses were elicited with a glass microelectrode (tip opening, 1–2 μm) filled with NaCl (2 M), which was positioned in the ipsilateral MNTB or the ipsilateral LNTB fiber tract 100 to 150 μm away from the somatic region of the MSO. An analog isolated pulse generator (BSI 950; Dagan) at a rate of 0.2 Hz triggered a bipolar (+/-), paired stimulus pulse (from here on referred to as test pulse) with an interstimulus interval of 20 ms. The threshold stimulus strength was typically 20–80 V with pulse durations between 200 and 400 μs.

IPSCs were pharmacologically isolated by bath application of the AMPA receptor antagonist DNQX (10 μM). EPSCs were evoked in the presence of the glycine receptor antagonist strychnine (1 μM). In all experiments, ts-APV (50 μM) was applied to block NMDA receptors and SR95531 (10 μM) to block GABA<sub>A</sub> receptors. To determine GABA<sub>B</sub>-mediated effects, the corresponding agonist baclofen (1 μM) was applied to the perfusate. This baclofen concentration is slightly above the IC<sub>50</sub> values of the dose–response curve for the excitatory (IC<sub>50</sub> = 0.62 μM) and inhibitory (IC<sub>50</sub> = 0.2 μM) inputs as measured in P14 animals (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Some of the train stimulation experiments were performed under the presence of the synaptosomal GABA uptake blocker NO711 (preferred GAT-1 selectivity) to increase the residual GABA concentration during MNTB or LNTB stimulation. Dependent on the hypothesis, baseline conditions consisted in all experiments of 5 min with or without the 100 Hz train preceding the test pulse. In some experiments, 4-AP (2 mM) was used to raise spontaneous synaptic activity levels in the slice (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

Data analysis. The signals were filtered with a low-pass four-pole Bessel filter at 10 kHz, sampled at 20–50 kHz, and digitized using a Digidata 1322A interface (Molecular Devices). Traces were digitally filtered at 2–5 kHz. Stimulus generation, data acquisition, and off-line analysis of data were performed using the pClamp Software (version 10.2; Molecular Devices). The paired-pulse ratio (PPR) was calculated as the mean amplitude of the synaptic response evoked by the second stimulus over that evoked by the first one. The coefficient of variation (CV) was calculated as the ratio between the SD of synaptic current amplitude and the mean amplitude (Faber and Korn, 1991). All data shown in percentage reflect values normalized to baseline conditions. Figures, which display averaged evoked responses, consist of at least 13 traces. Stimulation artifacts have been deleted for clarity in figures that show averaged traces. Results are expressed as mean ± SEM. Significant differences are marked as follows: *p < 0.05, **p < 0.01, and ***p < 0.001. Values of p in this study were obtained by using Student’s two-tailed paired or unpaired t test.

Immunohistochemistry. Animals of different ages (P7, P18, P19, P30) were deeply anesthetized with isoflurane and then perfused with 0.9% Ringer’s solution (5 min) followed by 4% paraformaldehyde (PFA) (30 min). Brains were removed and postfixed in PFA overnight at 4°C. The tissue was then sectioned at 40–60 μm using a vibratome. Immunohistochemistry was applied to free-floating sections. After extensive rinsing in PBS, sections were exposed to a blocking solution (BS) containing 1% bovine serum albumin, 0.5% Triton X-100, and 0.1% saponin in PBS (30 min). Subsequently, a double-immunofluorescence labeling was performed with the following primary antibody combinations: guinea pig anti-GABA<sub>B</sub>R1 (1:2000; Millipore Bioscience Research Reagents); chicken anti-microtubule-associated protein 2 (MAP2) (1:2000; Neurotech) and guinea pig anti-glycine transporter 2 (GlyT2) (1:1000; Millipore)/mouse anti-glutamate decarboxylase 65 (GAD65) (1:500; Millipore Bioscience Research Reagents) in PBS, containing the same BS.
GABABRs (Fig. 1A). Neurons in the MSO show strong immunoreactivity against GABA<sub>B</sub>Rs (Fig. 1B, left). At higher magnification, this staining appears punctuated with a distribution over the entire cell body and along the proximal dendrites (Fig. 1B, right), which suggests a functional role of GABA<sub>B</sub>Rs in MSO neurons. Using whole-cell voltage-clamp recordings from MSO neurons in acute brain slices, we investigated whether pharmacological activation of GABA<sub>B</sub>Rs modulates the excitatory and inhibitory inputs to MSO neurons. These experiments, as well as the immunolabeling in Figure 1B, were performed in 19-d-old animals, an age when the properties of the excitatory and inhibitory inputs to the MSO are considered to be adult-like (Magnusson et al., 2005; Scott et al., 2005; Sonntag et al., 2009). In adult animals, MSO neurons receive two excitatory glutamatergic inputs from the ipsilateral and contralateral AVCN and two inhibitory glycinergic inputs from the ipsilateral MNTB and LNTB (Fig. 1A). IPSCs and EPSCs were evoked by either stimulation of the ipsilateral or contralateral fiber tract to MSO neurons. Since the excitatory and inhibitory inputs from each side run in the same fiber bundle (Cant and Hyson, 1992; Kuwabara and Zook, 1992; Grothe and Sanes, 1993; Smith et al., 1993), fiber stimulation usually resulted in a mixed excitatory/inhibitory response. For this reason, EPSCs and IPSCs had to be isolated pharmacologically by antagonizing either the excitatory or the inhibitory inputs, respectively (see Materials and Methods). To find out whether GABA<sub>B</sub>R activation regulates MSO inputs, GABA<sub>B</sub>Rs were pharmacologically activated by bath application of baclofen (1 μM), a concentration that lies within the dynamic part of the dose–response curve for excitatory and inhibitory currents (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Both excitatory and inhibitory inputs projecting to the MSO profoundly decreased in amplitude on activation of GABA<sub>B</sub>Rs by baclofen application (Fig. 1C–E) (MNTB: control, −6.9 ± 1.2 nA; baclofen, −2.8 ± 0.5 nA; n = 7; LNTB: control, −1.7 ± 0.5 nA; baclofen, −0.8 ± 0.2 nA; n = 5; contra AVCN: control, −3.1 ± 0.6 nA; baclofen, −1.9 ± 0.6 nA; n = 7; ipsi AVCN: control, −3.3 ± 1.0 nA; baclofen, −2.1 ± 0.6 nA; n = 4). However, the relative decrease of current amplitude by baclofen application was strongest for MNTB inputs, whereas the excitatory inputs were significantly less affected (MNTB: 59.4 ± 3.5%, n = 7; contra AVCN: 42.2 ± 5.8%, n = 7; LNTB: 45.8 ± 9.2%, n = 5; ipsi AVCN: 35.3 ± 2.9%, n = 4; MNTB–contra AVCN: p ≤ 0.05; MNTB–ipsi AVCN: p ≤ 0.001) (Fig. 1F). On the contrary, the time course (decay time) of both the inhibitory or excitatory currents was not changed by pharmacological activation of GABA<sub>B</sub>Rs with baclofen (1 μM) (data not shown).

**Results**

GABA<sub>B</sub> receptors modulate all four major inputs to MSO neurons

Neurons in the MSO show strong immunoreactivity against GABA<sub>B</sub>Rs (Fig. 1B, left). At higher magnification, this staining...
functional changes. Most importantly, these inputs change from a mixed GABA/glutameric to a pure glycinergic transmitter phenotype (Kotak et al., 1998; Smith et al., 2000; Kullmann et al., 2002; Nabekura et al., 2004). In addition, this input switches from being depolarizing to hyperpolarizing caused by a significant decrease in the postsynaptic chloride concentration (Kandler and Friauf, 1995; Kakazu et al., 1999; Löhrie et al., 2005). Our next goal was to determine whether GABA\(_{\beta}\)Rs might be involved in these functional changes. Therefore, we quantified GABA\(_{\beta}\)R-induced input modulation in MSO neurons considerably before hearing onset (P9), shortly after hearing onset (P14), at a more mature stage (P19), and from mature animals (P32) by application of baclofen (1 \(\mu\)M). In all age groups tested, baclofen depressed the inhibitory inputs evoked by MNTB fiber stimulation. Interestingly, this GABA\(_{\beta}\)R-mediated effect on the inhibitory inputs remained approximately constant during all developmental stages tested (P9: 48.2 \(\pm\) 4.5\%, \(n = 7\); P14 contra AVCN: 47.4 \(\pm\) 2.8\%, \(n = 6\); \(p \leq 0.001\)) (Fig. 2A). In contrast, GABA\(_{\beta}\)R-mediated depression of the excitatory, glutamergic inputs decreased significantly after hearing onset (P9 contra AVCN: 78.1 \(\pm\) 2.7\%, \(n = 5\); P14 contra AVCN: 47.4 \(\pm\) 2.8\%, \(n = 6\); \(p \leq 0.001\)) (Fig. 2B). This decrease of GABA\(_{\beta}\)R-mediated regulation of the excitatory inputs continued up to P32 when input properties are generally considered to be mature (P19 contra AVCN: 42.2 \(\pm\) 5.7\%, \(n = 7\); P32 contra AVCN: 19.6 \(\pm\) 2.5\%, \(n = 4\); \(p \leq 0.05\)) (Fig. 2B). Similar developmental changes of GABA\(_{\beta}\)R-mediated modulation of inputs were observed for the projections from the LNTB (P9: 47.2 \(\pm\) 4.2\%, \(n = 4\); P19: 45.8 \(\pm\) 9.2\%, \(n = 5\)) and the ipsilateral AVCN (P9: 71.8 \(\pm\) 4.9\%, \(n = 5\); P19: 35.3 \(\pm\) 2.9\%, \(n = 4\); \(p \leq 0.001\)).

Together, these data suggest that, before hearing onset, GABA\(_{\beta}\)Rs more strongly regulate the excitation, whereas in the matured animal, GABA\(_{\beta}\)Rs seem to be predominantly located on the presynaptic inputs at the soma, which are mostly glycinergic and thus inhibitory (Kapfer et al., 2002).

At all developmental stages, GABA\(_{\beta}\)Rs control transmitter release probability on the excitatory and inhibitory inputs to MSO principal neurons

In most cases, modulation of input strength by GABA\(_{\beta}\)R activation is achieved either by presynaptic changes in release probability or by postsynaptic activation of \(K^+\)-currents. Since in the previous experiments postsynaptic potassium channels were blocked pharmacologically (see Materials and Methods), the above-described decrease in synaptic strength by baclofen application is likely to be induced via activation of presynaptically situated GABA\(_{\beta}\)Rs. To test this hypothesis, we analyzed the PPRs of evoked IPSCs and EPSCs before and during pharmacological activation of GABA\(_{\beta}\)Rs. For both excitation and inhibition, baclofen significantly increased the PPR before (P9) and after hearing onset (P19), most likely reflecting a reduction in transmitter

**Figure 2.** Physiological efficiency and anatomical distribution of GABA\(_{\beta}\)Rs changes during maturation for excitatory but not inhibitory inputs. **A,** Average decrease of normalized IPSC amplitudes by baclofen (1 \(\mu\)M) for different age groups (MNTB fiber stimulation). **B,** Average decrease of normalized EPSC amplitudes by baclofen (1 \(\mu\)M) for different age groups (contralateral AVCN fiber stimulation). **C,** Top, Antibody labeling in the MSO against GABA\(_{\beta}\)R1 (red) and MAP2 (green) at different developmental stages. Bottom, Isolated immunofluorescence of GABA\(_{\beta}\)R1. Scale bars, 50 \(\mu\)M. The asterisks represent \(p\) values obtained by Student's two-tailed unpaired t test. Error bars indicate SEM.
release probability at the given presynaptic input (Fig. 3A). We also analyzed the CV, an additional measurement to estimate changes in presynaptic release probability, for all major MSO inputs. Current amplitudes exhibited a stronger fluctuation in peak sizes after application of baclofen (Fig. 3B). To visualize this effect, we show a Gaussian function fitted to the IPSC amplitude distribution evoked by MNTB stimulation. As for the PPR, baclofen application increased the CV for excitatory and inhibitory miniature postsynaptic currents induced by application of baclofen (1 μM). The asterisks represent p values obtained by Student’s two-tailed paired t test. Error bars indicate SEM.

**Figure 3.** GABABRs are located presynaptically at inhibitory and excitatory inputs to the MSO before and after hearing onset. A, Left, PPR of IPSCs and EPSCs decreased during exposure to baclofen (examples display averaged evoked responses of P19 neurons). Right, Summary and statistics of baclofen (1 μM)-induced changes in PPR for all stimulation conditions in animals before (P9) and after (P19) hearing onset. B, Left, Top, Single IPSCs (gray) illustrate the scatter around the mean (black) under control conditions and during baclofen application in a P19 animal. Left bottom, Distribution of normalized IPSC amplitudes under control conditions and during baclofen application. Gaussian fit for the variance of IPSC amplitudes around the mean became broader during baclofen exposure (gray). Right, Quantification of frequency changes of inhibitory and excitatory miniature postsynaptic currents induced by application of baclofen (1 μM)-induced changes in PPR for all stimulation conditions in animals before (P9) and after (P19) hearing onset. C, Left, Example raw traces of mEPSCs under control conditions (black) and during baclofen exposure (gray). Right, Quantification of frequency changes of inhibitory and excitatory miniature postsynaptic currents induced by application of baclofen (1 μM). CTR, Control; BAC, baclofen. The asterisks represent p values obtained by Student’s two-tailed paired t test. Error bars indicate SEM.

**GABA<sub>B</sub> receptor activation has a postsynaptic effect in MSO neurons only before hearing onset**

GABA<sub>B</sub>Rs not only control transmitter release at the presynaptic terminal but also change the conductance of postsynaptic ion channels, thereby exerting inhibition and altering the integrative properties of neurons. In the majority of neurons, postsynaptic GABA<sub>B</sub>Rs are directly coupled to G-protein inwardly rectifying K<sup>+</sup> channels (GIRK channels). Activation of postsynaptic GABA<sub>B</sub>Rs leads to an opening of the GIRK channels and induces an increase in K<sup>+</sup> current. We tested whether pharmacological activation of GABA<sub>B</sub>Rs activates a K<sup>+</sup> current in MSO neurons, by analyzing the currents evoked by a hyperpolarizing voltage ramp (duration, 900 ms; voltage change, 0.14 mV/ms) before and during baclofen application (100 μM). In prehearing animals (P9), the hyperpolarizing current ramp induced a net inward current that, to a large part, consists of the hyperpolarization activated current (I<sub>h</sub>) (Fig. 4A,B). At this age, the average amplitude of pharmacologically isolated I<sub>h</sub> was 815 ± 91 pA (n = 11) (for −50 to −110 voltage steps) (U. Koch and B. Hassfurth, unpublished observation). Activation of GABA<sub>B</sub>Rs with baclofen (100 μM) in all neurons consistently and with a rapid onset changed the holding current to more positive values and increased the current evoked by the hyperpolarizing current ramp (Fig. 4B,C). This is consistent with an activation of a K<sup>+</sup> conductance, presumably a GIRK conductance. In more mature animals (P18), the hyperpolarizing voltage ramp activated even larger I<sub>h</sub> currents (Khurana et al., 2008). To avoid that the large I<sub>h</sub> obscured activation of GABA<sub>B</sub> R currents, I<sub>h</sub> was partially blocked by the specific I<sub>h</sub> antagonist ZD7288 (20 μM), which does not block baclofen-induced GIRK channel activation (Svoboda and Lupica, 1998; Takigawa and Alzheimer, 2002). At this developmental stage, no rapid change in holding current or evoked current was observed during baclofen application (Fig. 4D,E). On average, there was a slow but small increase in holding current and a decrease in evoked current, which is mostly consistent with a slow and
gradual inactivation of the remaining $I_h$ (Fig. 4F). We conclude that, only before hearing onset, activation of postsynaptic GABA_B Rs induces a change in postsynaptic conductances, most likely an activation of GIRK. Several days after hearing onset, GABA_B R activation does not alter postsynaptic conductances. However, as indicated by the intracellular GABA_B R immunoreactivity, these receptors might be involved in long-term changes of synaptic efficacy (Kotak et al., 2001; Yevenes et al., 2003).

**Before hearing onset, MNTB fiber stimulation activates presynaptic GABA_A Rs**

During early prehearing postnatal stages, LSO and MSO neurons receive a mixed GABAergic and glycinergic inhibition from inputs originating from the MNTB, whereas after hearing onset inhibitory transmission from the MNTB is predominantly glycinergic (Kotak et al., 1998; Smith et al., 2000). Hence, we next asked the question whether in young animals (P8) GABA release from MNTB fibers activates GABA_A Rs and thereby modulates the glycinergic and/or glutamatergic inputs to MSO neurons. If, at this age, MNTB neurons indeed release GABA together with glycine from their terminals, this should activate GABA_A Rs located presynaptically on the same synapse and thereby decrease the release probability of the glycinergic transmission. This was tested by analyzing the amplitude of the glycinergic inhibitory currents in response to a test stimulus 200 ms after a high-frequency train stimulation of MNTB fibers (100 Hz, 200 ms) (Sodickson and Bean, 1996). If GABA_A Rs were activated during this high-frequency stimulation, the GABA_B R antagonist SCH50911 (10 μM) should block the GABA_A R-induced decrease in transmitter release probability of the glycinergic input. Surprisingly, pharmacological blockade of GABA_A Rs did not induce a significant change in the amplitude of the inhibitory current at this developmental stage (7.8 ± 5.6%; n = 5) (Fig. 4A, E). It is possible that, in P8 animals, the concentration of released GABA is not sufficient to activate the presynaptic GABA_A Rs and thereby modulates the glycinergic and/or glutamatergic inputs. Before hearing onset, MNTB fibers also terminate on the dendrites of MSO neurons (Kapfer et al., 2002; Werth et al., 2008), which receive the majority of excitatory inputs from the ipsilateral and contralateral AVCN (Stotler, 1953; Clark, 1969; Russell and Moore, 1999). Therefore, GABA released from MNTB fibers should potentially also activate GABA_A Rs located on the excitatory presynaptic terminals, which at this developmental stage show high sensitivity to very low concentrations of baclofen (Fig. 2B). Indeed, stimulating the fibers of the trapezoid body as in the previous experiment in combination with the application of the GABA uptake blocker NO711 resulted in a decrease of the excitatory presynaptic terminals, which at this developmental stage show high sensitivity to very low concentrations of baclofen (Fig. 5B, E). This decrease in IPSC amplitude was completely abolished by the GABA_B R antagonist SCH50911 (10 μM) (−1.8 ± 4.7%; n = 4) (Fig. 5C, E) and differed significantly from application of NO711 alone (p = 0.01).

Before hearing onset, MNTB fibers also terminate on the dendrites of MSO neurons (Kapfer et al., 2002; Werth et al., 2008), which receive the majority of excitatory inputs from the ipsilateral and contralateral AVCN (Stotler, 1953; Clark, 1969; Russell and Moore, 1999). Therefore, GABA released from MNTB fibers should potentially also activate GABA_A Rs located on the excitatory presynaptic terminals, which at this developmental stage show high sensitivity to very low concentrations of baclofen (Fig. 2B). Indeed, stimulating the fibers of the trapezoid body as in the previous experiment in combination with the application of the GABA uptake blocker NO711 resulted in a decrease of the exci-
after hearing onset (P8), high-frequency MNTB fiber stimulation activates GABA \(_R\)s at the excitatory and inhibitory MSO inputs, but only under the prerequisite of a GABA uptake inhibitor. A. Example traces of averaged IPSCs evoked by MNTB fiber stimulation (stimulation protocol: high-frequency stimulation (100 Hz, 200 ms); 200 ms gap; test pulse with interstimulus interval of 20 ms) under control conditions and during GABA \(_R\) blockade with SCH50911. B. Example traces of averaged IPSCs evoked by MNTB fiber stimulation (see A) under control conditions and during bath application of the GABA uptake inhibitor NO711. NO711 application decreased the response to the test pulse after high-frequency MNTB fiber stimulation. C. Blocking GABA \(_R\)s abolished the NO711-induced amplitude change after high-frequency MNTB fiber stimulation. D. Example traces of averaged EPSCs evoked by AVCN fiber stimulation (stimulation protocol; see A) under control conditions and during bath application of the GABA uptake inhibitor NO711. NO711 application decreased also excitatory currents after high-frequency fiber stimulation. E. Quantification of the GABA \(_R\)-mediated decrease in IPSC and EPSC amplitudes induced by high-frequency fiber stimulation during application of the GABA \(_R\) blocker and/or GABA uptake inhibitor (all at P8). The asterisks under columns represent values obtained by Student’s two-tailed paired t-test; the asterisks between columns represent values obtained by Student’s two-tailed unpaired t-test. Error bars indicate SEM.

**Figure 5.** Before hearing onset (P8), high-frequency MNTB fiber stimulation activates GABA \(_R\)s at the excitatory and inhibitory MSO inputs, but only under the prerequisite of a GABA uptake inhibitor. A. Example traces of averaged IPSCs evoked by MNTB fiber stimulation (stimulation protocol: high-frequency stimulation (100 Hz, 200 ms); 200 ms gap; test pulse with interstimulus interval of 20 ms) under control conditions and during GABA \(_R\) blockade with SCH50911. B. Example traces of averaged IPSCs evoked by MNTB fiber stimulation (see A) under control conditions and during bath application of the GABA uptake inhibitor NO711. NO711 application decreased the response to the test pulse after high-frequency MNTB fiber stimulation. C. Blocking GABA \(_R\)s abolished the NO711-induced amplitude change after high-frequency MNTB fiber stimulation. D. Example traces of averaged EPSCs evoked by AVCN fiber stimulation (stimulation protocol; see A) under control conditions and during bath application of the GABA uptake inhibitor NO711. NO711 application decreased also excitatory currents after high-frequency fiber stimulation. E. Quantification of the GABA \(_R\)-mediated decrease in IPSC and EPSC amplitudes induced by high-frequency fiber stimulation during application of the GABA \(_R\) blocker and/or GABA uptake inhibitor (all at P8). The asterisks under columns represent values obtained by Student’s two-tailed paired t-test; the asterisks between columns represent values obtained by Student’s two-tailed unpaired t-test. Error bars indicate SEM.

GABA to induce GABA \(_R\)-mediated control of MSO inputs.

The LNTB–MSO projection has no GABAergic component after hearing onset

Previous anatomical studies provide evidence that the ipsilateral inhibitory input to the MSO, the LNTB, comprises GABAergic neurons also in adult animals (Roberts and Ribak, 1987; Helfert et al., 1989; Spirou et al., 1998). Thus, we attempted to find out whether the LNTB could serve as a GABA source for the activation of GABA \(_R\)s even in the presence of GABA uptake blockers.

**Presynaptic GABA \(_R\)s are not activated by retrograde GABA release in the MSO**

We have previously demonstrated that neurons in the LSO retrogradely release GABA on spiking activity (Magnusson et al., 2008). This GABA activates presynaptic GABA \(_R\)s on the respective inputs, thereby modulating transmitter release. Since neu-
rons in the LSO and the MSO receive similar inputs, we tested whether this scenario might also hold for MSO principal cells. As in the LSO, this was tested by inducing high-frequency spiking activity (100–300 Hz; 500 ms) in MSO principal cells by short current step injections well above spiking threshold (1.5–2.5 nA) (supplemental Fig. 3A, available at www.jneurosci.org as supplemental material). If spiking activity retrogradely released GABA and thereby activated presynaptic GABA \(_\text{B} \)Rs, the amplitude of the postsynaptic potentials induced by fiber stimulation should decrease and the PPR should increase. However, neither the amplitude nor the PPR of EPSPs or IPSPs were changed by the preceding high-frequency spiking activity of the MSO neurons (supplemental Fig. 3B, C, available at www.jneurosci.org as supplemental material). This indicates that, unlike in the LSO, spiking activity of MSO neurons does not activate presynaptic GABA \(_\text{B} \)Rs by presumed retrograde release of GABA. This is also consistent with the lack of GABA and GAD immunoreactivity in neurons of the MSO (Roberts and Ribak, 1987).

**Anatomical evidence for other GABAergic input to MSO neurons**

In the previous experiments, we found that, after hearing onset, despite clear evidence for pharmacological activation of presynaptic GABA \(_\text{B} \)Rs in adult animals, neither the MNTB nor the LNTB input seems to release enough GABA to activate these receptors. We also did not observe GABA \(_\text{B} \)R activation by retrogradely released GABA from MSO principal neurons. To identify possible other sources of GABA on MSO neurons, we performed antibody stainings on paraformaldehyde-fixed brainstem sections containing the MSO (P18) against the GABA-synthesizing enzyme GAD65. To evaluate the distribution of GAD65-positive inputs, the dendrites of MSO neurons were visualized with a MAP2 antibody. Confocal microscopy at low magnification showed widely distributed GAD65 staining along the somata and dendrites of MSO neurons, indicating the presence of GABAergic inputs at the soma and the dendrites. We also tested whether possible GABAergic inputs colocalized with the presynaptic endings deriving from the MNTB or LNTB by immunostaining against GlyT2 (Fig. 7A). In contrast to the GAD65 distribution, the GlyT2 staining was very focused and dense only on the soma. At higher magnification, it became apparent that only little colocalization of GAD65- and GlyT2-positive synapses could be detected. Most of the GAD65-associated staining was not in close proximity to GlyT2-positive terminal endings. This indicates that MSO neurons at P18 receive GABAergic projections mainly from other sources than the glycineric MNTB or LNTB neurons.

**Raising spontaneous activity levels induces GABA \(_\text{B} \)Rs activation even later during development**

We next asked the question whether these GABAergic terminals could serve as a possible source for GABA release that activates the GABA \(_\text{B} \)Rs located on the MSO inputs. To test this, spontaneous synaptic transmitter release was increased by applying 4-AP (2 mM) to the perfusate. 4-AP is a nonselective blocker of low-threshold potassium channels (\(K_{\text{L}}\)) and has been shown to depolarize neurons, thereby lowering the action potential threshold and increasing the spontaneous firing rate in, for example, the hippocampus and mouse inner hair cells (Avoli et al., 1996; Marcotti et al., 2003). In the present experiment, 4-AP elevated the spontaneous frequency of IPSCs in MSO neurons (55.8 ± 2.9 Hz; \(n = 23\)) to values that resemble in vivo-like spontaneous firing frequencies of MNTB neurons (Kopp-Scheinpflug et al., 2003, 2008; Hermann et al., 2007; McLaughlin et al., 2008). The influence of possible GABA release through elevated spontaneous release was now tested by application of the GABA \(_\text{B} \)R inhibitors SCH50911 (10 \(\mu\)M) or CGP55845 (2 \(\mu\)M). The results from these recordings were pooled since the data displayed no differences among drugs. MNTB fibers were stimulated to investigate GABA \(_\text{B} \)R-induced changes of MNTB input currents. As depicted in Figure 7B, IPSC amplitudes significantly increased when GABA \(_\text{B} \)R activity was blocked compared with baseline conditions (4-AP alone). This implies that, under baseline conditions, when spontaneous activity is raised by 4-AP, presynaptic GABA \(_\text{B} \)Rs were endogenously activated, which resulted in a reduction of IPSC amplitudes. Application of the GABA \(_\text{B} \)R antagonists then abolished this GABA \(_\text{B} \)R-mediated depression of the current, resulting in an increase of current amplitude (22.3 ± 4.2%; \(n = 16\); \(p = 0.01\)) (Fig. 7C). Since neither 4-AP (-6.9 ± 7.6%; \(n = 8\)) nor GABA \(_\text{B} \)R inhibitors alone (-3.8 ± 5.9%; \(n = 4\)) increased peak current amplitudes (Fig. 7C), this effect could be ascribed directly to the
activation of GABA<sub>B</sub>Rs. No difference was observed between P14 and P18 gerbils, which indicates that this mechanism persists in more mature animals (P14: 21.3 ± 5.0%, n = 9; P18: 23.5 ± 7.8%, n = 7) (Fig. 7D). Interestingly, excitatory input currents did not display an increase in amplitude during GABA<sub>B</sub>R blockade which suggests that the GABA released by spontaneous activity did not activate the GABA<sub>B</sub>Rs on the excitatory inputs (Fig. 7E) (P14–P18: 5.6 ± 6.6%, n = 9) (Fig. 7C). This is in line with the observed downregulation of GABA<sub>B</sub>Rs on the dendrites later during development. Together, these results show that, even considerably beyond hearing onset, GABA is released onto MSO neurons, which selectively modulates the amplitude of the inhibitory inputs. The origin and thus the activation pattern of this GABAergic projection needs to be determined in future studies.

**Discussion**

The data presented here show that GABA<sub>B</sub>Rs differentially modulate the excitatory and inhibitory inputs of MSO neurons at all developmental stages. Around hearing onset, the GABA<sub>B</sub>R-mediated depression of the excitatory inputs greatly decreases, whereas the GABA<sub>B</sub>R-mediated depression of the inhibitory inputs remains constant throughout the same period. This developmental decay in GABA<sub>B</sub>R-induced EPSC depression is paralleled by a progressive loss of GABA<sub>B</sub>R expression in dendrites of MSO neurons after hearing onset. During the same developmental period, we also observe a loss of postsynaptic GABA<sub>B</sub> receptor-induced K<sup>+</sup> current increase. Furthermore, we provide evidence that the source of GABA for GABA<sub>B</sub>R activation changes during development. Before hearing onset, MNTB and LNTB fiber stimulation activates presynaptic GABA<sub>B</sub>Rs on the excitatory and inhibitory inputs to MSO neurons. After hearing onset, GAD65-positive endings often lack GlyT2 reactivity, suggesting GABA sources other than the MNTB or the LNTB.

**Developmental changes in GABA<sub>B</sub>Rs distribution and function**

We have found that, throughout all developmental stages, GABA<sub>B</sub>Rs regulate transmitter release at the major inhibitory and excitatory inputs to MSO neurons. Yet the relative effect of GABA<sub>B</sub>Rs on excitation and inhibition changes during the first postnatal weeks. Regulation of transmitter release by presynaptic GABA<sub>B</sub>Rs is a widespread mechanism in the brain and has been described in several auditory re-
gions, such as the auditory brainstem (Isaacson, 1998; Takahashi et al., 1998; Lim et al., 2000; Yamauchi et al., 2000; Magnussen et al., 2008; Tang et al., 2009), midbrain (Sun et al., 2006), and the auditory cortex (Wehr and Zador, 2005). Furthermore, GABA<sub>B</sub>Rs have been implicated both in the regulation of circuit formation during development (Behar et al., 2000; Represa and Ben-Ari, 2005) and in the acute modulation of network properties in the mature animal (Scanziani, 2000; Magnussen et al., 2008; Oswald et al., 2009; Pan et al., 2009). Consistent with our data, anatomical and physiological studies in other brain structures suggest that presynaptic GABA<sub>B</sub>Rs are present and functional on excitatory and inhibitory inputs during early development and in adult animals (Gaiarsa et al., 1995; Varela et al., 1997; López-Bendito et al., 2002; Kirmse and Kirischuk, 2006). However, there is little information on changes in GABA<sub>B</sub>R function during development. Here, we show that GABA<sub>B</sub>R-mediated depression of the excitatory inputs declined significantly right after hearing onset with an additional decline over several weeks. In contrast, we observed that GABA<sub>B</sub>R-mediated depression of inhibition remains constant during development similar to the CA3 region of the hippocampus (Caillard et al., 1998). In contrast, activation of K<sup>-</sup>-currents by postsynaptic GABA<sub>B</sub> receptors, which is abundant in hippocampal and cortical neurons in adult animals (Buonomano and Merzenich, 1998; Scanziani, 2000; Chen and Johnston, 2005; Oswald et al., 2009), disappears after hearing onset in neurons of the MSO. Thus, the integrative properties of mature MSO neurons seem to be unaffected by postsynaptic GABA<sub>B</sub>Rs, as previously observed for neurons in the lateral superior olive (Magnussen et al., 2008).

An important question arising from this is how developmental changes in GABA<sub>B</sub>R distribution are regulated. One possibility is that the decline in GABA<sub>B</sub>R number is genetically determined. However, it is more likely that overall activity levels regulate the availability of GABA<sub>B</sub>Rs. Indeed, excess excitation and augmented glutamate levels can downregulate GABA<sub>B</sub>R expression and increase internalization of the receptor protein (Buhl et al., 1996; Haas et al., 1996; Vargas et al., 2008), which in both cases would result in an altered GABA<sub>B</sub>R efficacy as observed for excitatory MSO inputs.

**MNTB and LNTB fiber stimulation activates GABA<sub>B</sub>Rs in the MSO only before hearing onset**

Our data indicate that, before hearing onset, high-frequency firing of MNTB neurons activates GABA<sub>B</sub>Rs on both the excitatory and inhibitory inputs to MSO neurons. Similarly, previous physiological data show that, before hearing onset, MNTB fiber stimulation also activates postsynaptic GABA<sub>B</sub> receptors on MSO neurons (Smith et al., 2000). This suggests that indeed GABA released from the MNTB fiber terminals activates presynaptic GABA<sub>B</sub>Rs. The fact that at the MNTB–LSO projection GABA and glycine are released from the same synaptic terminals or vesicles (Nabekura et al., 2004) indicates that GABA<sub>B</sub>-mediated regulation of glycine and GABA transmitter release is autosynaptic and should therefore be similar for both transmitter types. Consequently, during prolonged firing of MNTB neurons, GABA release should decrease and GABA<sub>B</sub>R activation of the inputs should decline in a self-regulating process.

As GABA is released from inhibitory MNTB terminals and diffuses to excitatory presynaptic sites, GABA<sub>B</sub>R-mediated depression of excitation is most likely heterosynaptic. Heterosynaptic activation of GABA<sub>B</sub>Rs has been shown to occur at many different sites in the brain (Lim et al., 2000; Mitchell and Silver, 2000; Lei and McBain, 2003; Guetg et al., 2009). In the MSO, the relatively large distance between the release sites and the excitatory presynaptic endings should result in much lower concentrations of GABA at the excitatory compared with the inhibitory terminals (Kapfer et al., 2002). Why then is the amplitude of GABA<sub>B</sub>-mediated depression similar for inhibition and excitation? One possible explanation is a differential sensitivity of the receptors to GABA, which might be the underlying cause for the larger effect of baclofen on excitation than inhibition observed before hearing onset. Such a mechanism operates in the hippocampus, in which heterosynaptic GABA<sub>B</sub>Rs located on the excitatory inputs are indeed more efficient in downregulating transmitter release compared with the GABA<sub>B</sub>Rs on the inhibitory inputs because of a different subunit composition (Guetg et al., 2009).

One unexpected result of our measurements was that, even several days before hearing onset, MNTB fiber stimulation only activated presynaptic GABA<sub>B</sub>Rs in the presence of the GABA uptake blocker NO711. In general, physiological activation of GABA<sub>B</sub>Rs usually requires strong stimulation intensities suggesting that the pooling of synaptically released GABA is required for their activation (Isaacson, 1998; Scanziani, 2000). This is consistent with ultrastructural data showing that most GABA<sub>B</sub>Rs are located perisynaptically or extrasynaptically (Fritschy et al., 2004; López-Bendito et al., 2004; Luján and Shigemoto, 2006). Indeed, in many studies using acute brain slice preparations GABA uptake blockers were required to activate presynaptic GABA<sub>B</sub>Rs (Mougnot et al., 1998; Mitchell and Silver, 2000; Lei and McBain, 2003). This also highlights the important role of GABA uptake mechanisms for the regulation of GABA concentration. An alternative mechanism for the lack of effect with low GABA concentrations might be a masking of the change in release probability by an increased readily releasable pool as observed for metabotropic glutamate receptor (mGluR) activation in the MNTB (Billups et al., 2005). In this case, mGluR effects became apparent through a decrease in recovery time constants. However, in our experiments, train stimulation without NO711 did not seem to change the recovery time constant, when we analyzed PSC amplitudes 200 ms after the termination of the train, a time interval usually well within the range of recovery from synaptic depression.

**Endogenous GABA<sub>B</sub>R activation in the MSO after hearing onset**

All previous studies so far had focused on the developmental reduction in the activation of ionotropic GABA<sub>A</sub> receptors by MNTB fiber activity (Kotak et al., 1998; Smith et al., 2000). Our study indicates that presynaptic GABA<sub>B</sub>Rs cannot be activated by either MNTB or LNTB fiber stimulation after hearing onset. Furthermore, unlike in the LSO, spiking activity of MSO neurons does not retrogradely activate GABA<sub>B</sub>Rs on the presynaptic terminals. Under which physiological conditions are the GABA<sub>B</sub>Rs in the MSO then activated? In agreement with previous anatomical studies (Roberts and Ribak, 1987; Helfert et al., 1989; Adams and Mugnaini, 1990), our antibody labeling against the GABA-synthesizing enzyme GAD65 revealed a large number of GABAergic fibers contacting the soma and the proximal dendrites. We therefore propose that GABA released from these GAD65-positive terminals mediates the GABA<sub>B</sub>-induced depression of inhibitory inputs. But where do these GAD65-positive fibers originate, and under which physiological circumstances are they activated? Several possibilities have been discussed in the literature, including the ventral nucleus of the trapezoid body, the superior parvocellular nucleus, and descending fibers from the inferior colliculus (Roberts and Ribak, 1987). The
experimental evidence for any of these projections is sparse (Schwartz and Wittebort, 1976; Kiss and Majorossy, 1983), and future experiments are required to determine the origin of the GABAergic terminals to MSO neurons in adult animals.

**Possible functional significance of GABA_βRs in the MSO before and after hearing onset**

The observed shift in the effect of GABA_βR-mediated depression of excitation and inhibition suggests a change of GABA_βR function before and after hearing onset. Before hearing onset, we found a profound depression of the excitatory inputs by GABA_βR activation, which indicates a block of activity by GABA_βR activation. At this age, also inhibitory inputs are affected by GABA_βR activity. The functional interpretation of the GABA_βR-mediated depression of inhibitory inputs before hearing onset is more complex. In neonatal animals, the chloride reversal is positive to the resting membrane potential, which results in a depolarization of neurons during activation of the GABA/glycinergic inputs (Kandler and Friäuf, 1995; Kakazu et al., 1999; Löhrke et al., 2005). Hence, during this period, the main inputs to the MSO are excitatory. Accordingly, GABA_βRs might be the main source of inhibition in the MSO by decreasing transmitter release from both the glutamatergic excitatory and the depolarizing GABA/glycinergic inputs during periods of high spontaneous spiking activity. This scenario has previously been suggested for the neonatal hippocampal network (Gaiarsa et al., 1995; McLean et al., 1996) and could represent an effective mechanism to prevent overexcitation and apoptosis of neurons in the neonatal brain. In this case, the GABA_βR-mediated inhibition of excitation should remain elevated in deafened animals in which the chloride reversal potential remains depolarizing in neurons of the LSO (Shibata et al., 2004).

Our findings indicate that the function of presynaptic GABA_βR changes after hearing onset. First, at this developmental stage, GABA_βR activation mostly modulates the strength of the inhibitory inputs to the MSO. Second, whereas in neonatal animals GABA is released during high-frequency firing of MNTB and LNTB neurons, our data suggest that, after hearing onset, fibers other than the MNTB or LNTB release GABA. Whether these fibers are driven by sound or whether they are associated with an attention-driven descending system is unclear at the moment, but in both cases activation of these fibers should result in a tonic downregulation of the inhibitory inputs to the MSO. We do know from previous experiments that the inhibitory inputs from the MNTB and LNTB to the MSO modulate ITD processing by shifting the steepest slope of the ITD function into the physiologically relevant range (Brand et al., 2002; Pecka et al., 2008). Activation of GABA_βRs on the excitatory and inhibitory inputs to the MSO could fine-tune ITD analysis in several ways. A reduction in EPSC amplitude could sharpen the coincidence detection window in response to binaural stimuli (Kuba et al., 2002). However, the main effect of GABA_βRs in adult animals clearly lies in the downregulation of the inhibitory inputs. Given the presumptive role of inhibition for ITD coding, a reduction in IPSC amplitude would move the peak of the ITD function closer to the midline and thereby decrease spike frequency changes within the physiologically relevant range. Moreover, for both the excitatory and the inhibitory inputs, constituent GABA_βR activation attenuates short-term synaptic depression (Brenowitz et al., 1998) and might be one of the underlying causes for the observed lack of synaptic short-term depression in the in vivo preparation, as observed in MNTB neurons (Lorteije et al., 2009). In general, GABA_βR activation on the inputs to the MSO would provide a mechanism to dynamically adjust ITD analysis in less than a second.

**References**


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