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## Glycinergic and GABAergic calcium responses in the developing lateral superior olive

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### Abstract

The lateral superior olive (LSO), a binaural nucleus involved in sound localization, receives tonotopically organized inhibitory inputs from the medial nucleus of the trapezoid body (MNTB). During development, the tonotopic organization of this glycinergic/GABAergic MNTB–LSO pathway is established by activity-dependent axonal reorganization. However, the underlying mechanisms by which this reorganization takes place have remained largely unknown. As cytosolic calcium is one of the most important second messengers responsible for inducing synaptic plasticity and reorganization, we examined whether and how activity in the MNTB–LSO pathway changes the intracellular calcium concentration ( $[Ca^{2+}]_i$ ) in developing LSO neurons. By applying calcium imaging techniques to Fura-2-labelled slices from neonatal rats and mice, we found that glycine and GABA ( $\gamma$ -aminobutyric acid) affect  $[Ca^{2+}]_i$  in LSO neurons in an age-dependent manner; during the first postnatal week, the period at which glycine and GABA are depolarizing in the LSO, glycine and GABA always increased  $[Ca^{2+}]_i$ . However, in 2-week-old animals, the time around hearing onset when glycine and GABA are hyperpolarizing, glycine and GABA slightly decreased  $[Ca^{2+}]_i$ . Calcium responses could also be elicited by stimulation of afferent fibres from the MNTB, and these synaptic responses were mediated by glycine and GABA<sub>A</sub> receptors. Furthermore, GABA, which is a neurotransmitter only in the immature MNTB–LSO pathway, played a major role in generating MNTB-elicited  $Ca^{2+}$  responses. The direct link of glycinergic/GABAergic synaptic activity to intracellular calcium signalling during the period of inhibitory synaptic plasticity could be one of the mechanisms by which tonotopic MNTB–LSO connections become established.

### Keywords

caged glutamate; calcium imaging; inhibition; mouse; rat

### Introduction

Precise and fast processing of interaural time and intensity differences is required for sound localization. In mammals, interaural intensity differences are first processed in the lateral superior olive (LSO), a binaural auditory nucleus in the auditory brainstem (Boudreau &

Tsuchitani, 1968; Caird & Klinke, 1983; for review see Oertel, 1999). The LSO receives information from the ipsilateral ear via excitatory, glutamatergic axons from the cochlear nucleus and receives information from the contralateral ear via inhibitory, glycinergic axons from the medial nucleus of the trapezoid body (MNTB). Both afferent pathways terminate in the LSO in a strictly tonotopic manner and both pathways are spatially aligned such that single LSO neurons are excited and inhibited by axons encoding the same stimulus frequency.

The development of these highly organized connections has been the subject of a number of studies (for recent reviews see Cant, 1998; Sanes & Friauf, 2000). An interesting outcome of these studies was the finding that the precise tonotopic organization of the inhibitory MNTB–LSO pathway emerges gradually through axonal and dendritic reorganization (Sanes & Siverls, 1991; Sanes *et al.*, 1992; Rietzel & Friauf, 1998). Single cell analysis demonstrated that MNTB axons initially innervate relatively broad areas along the tonotopic axis of the LSO and subsequently become refined, terminating in narrow isofrequency bands. This reorganization occurs to a great extent before hearing onset and, thus, in the absence of sound-evoked neuronal activity. Nevertheless, axonal reorganization requires the presence of spontaneous activity, which is endogenously generated in the auditory system before the onset of hearing (Lippe, 1994; Kotak & Sanes, 1995; Kros *et al.*, 1998). Blocking or decreasing neuronal activity in the LSO prevents the refinement of LSO circuitry (Sanes *et al.*, 1992; Sanes & Takacs, 1993). While the importance of glycinergic/GABAergic synaptic activity for normal LSO development is well established, the mechanisms by which MNTB activity exerts its effects on the maturation of neuronal circuits in the LSO are still poorly understood.

Previous studies have demonstrated that during the period in which the LSO circuit becomes reorganized, synaptic transmission in the MNTB–LSO pathway is quantitatively and qualitatively different from that in mature animals. For example, while the MNTB–LSO pathway in mature animals is purely glycinergic (Casparly & Finlayson, 1991; Schwartz, 1992), the MNTB–LSO pathway in neonatal animals is both glycinergic and GABAergic (Kotak *et al.*, 1998; Korada & Schwartz, 1999). Furthermore, GABA ( $\gamma$ -aminobutyric acid) and glycine, which are hyperpolarizing in the LSO of mature animals, are depolarizing in the LSO of neonatal animals (Kandler & Friauf, 1995; Ehrlich *et al.*, 1999) similar to other neuronal systems (Obata *et al.*, 1978; Ben-Ari *et al.*, 1989). It has been suggested that this depolarizing action could increase the intracellular calcium concentration ( $[Ca^{2+}]_i$ ) in LSO neurons — a scenario which has been demonstrated previously in other developing systems (Yuste & Katz, 1991; Reichling *et al.*, 1994; Leinekugel *et al.*, 1995; Obrietan & van den Pol, 1995; Owens *et al.*, 1996). Such glycinergic/GABAergic  $Ca^{2+}$  responses could link MNTB–LSO synapses to calcium-regulated intracellular pathways, many of which play key roles in neuronal differentiation and synaptic plasticity (Bootman *et al.*, 2001).

In the present study we applied calcium imaging techniques to auditory brainstem slices of neonatal rats and mice in order to re-examine the idea that depolarizing glycine and GABA raise  $[Ca^{2+}]_i$  in immature LSO neurons during the period of synaptic refinement. Contrary to previous studies (Schmanns & Friauf, 1994), we found that pharmacological stimulation of glycine and GABA<sub>A</sub> receptors, as well as glycinergic and GABAergic synaptic activity,

increased  $[Ca^{2+}]_i$  in LSO neurons during the first postnatal week, when glycine and GABA are depolarizing (Kandler & Friauf, 1995), but not at later ages. Our results also revealed that GABA plays a prominent role in mediating MNTB-evoked  $Ca^{2+}$  responses, indicating that the transient GABAergic nature of the immature MNTB–LSO pathway is important for linking MNTB activity to  $Ca^{2+}$  responses in the developing LSO.

Some of the results have been published in abstract form (Kullmann & Kandler, 1999).

## Materials and methods

### Animals and slice preparation

Experiments were performed in slices prepared from neonatal rats (Sprague–Dawley, Charles River Laboratories, Wilmington, MA, USA) and mice (C57Bl/6 J, Jackson Laboratory, Bar Harbor, ME, USA). Data presented in this report were obtained from 21 mice aged between postnatal day (P) 1 and P5, six mice aged between P13 and P15, 19 rats P1–P5, and two rats P14. Experimental procedures were in accordance with USA National Institute of Health, guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Brainstem slices were prepared as described previously (Kandler & Friauf, 1995). In short, animals were anaesthetized by hypothermia (P1–P5) or by pentobarbital (P13–P15, 100 mg/kg, i.p.) and decapitated. The brain was removed quickly and transferred to cold (4–8 °C) artificial cerebrospinal fluid (ACSF, composition in mM: NaCl 124, NaHCO<sub>3</sub> 26, glucose 10, KCl 5, KH<sub>2</sub>PO<sub>4</sub> 1.25, MgSO<sub>4</sub> 1.3, CaCl<sub>2</sub> 2, pH = 7.4 when aerated with 95% O<sub>2</sub>/5% CO<sub>2</sub>). ACSF used for preparation, storage and staining of slices also contained 1 mM kynurenic acid. Coronal slices of the brainstem (200–300 μm thick) were cut on a vibrating microtome (DTK-1500E, Ted Pella, Redding, CA, USA). Slices that contained the superior olivary complex were kept either submerged in ACSF (for Fura-2 bulk labelling, discussed later) or were kept in an interface chamber (for Fura-2 AM pressure injections). Slices were allowed to recover for at least 1 h before being used for recordings.

### Fura-2 labelling

Calcium imaging of LSO neurons was performed using the calcium indicator Fura-2 (Grynkiewicz *et al.*, 1985). Slices from animals up to P5 were bulk labelled by submersion in ACSF, which contained 10 μM Fura-2 AM (Molecular Probes, Eugene, OR, USA), prepared from a stock solution of 1 mM Fura-2 AM in a 20% (w/v) solution of pluronic acid in dimethylsulphoxide (DMSO). Slices were stained for 1–3 h at 30–32 °C. In slices from P13–P15 animals, LSO neurons were labelled by injecting Fura-2 AM (100 μM Fura-2 AM, 1% DMSO in ACSF) into the slice using pressure application (Leinekugel *et al.*, 1995). This approach labelled many neurons simultaneously without changing the native intracellular chloride concentration, the main determinant for the polarity of glycinergic and GABAergic membrane potential responses in LSO neurons (Ehrlich *et al.*, 1999; Kakazu *et al.*, 1999).

### Retrograde labelling

In some experiments, rhodamine-labelled beads (40 nL, Red Retrobeads, Lumafuor, Naples, FL, USA) were injected bilaterally into the inferior colliculus of P1 rats in order to

unequivocally identify Fura-2-labelled cells as neurons. Injected rats were allowed to survive for 2 days and brainstem slices (300  $\mu\text{m}$ ) were prepared at P3, as described earlier. Beads were visualized with 548 nm excitation (monochromator, Polychrome II, TILL Photonics, Martinsried, Germany) and a 590-long pass emission filter (Chroma Technology Corp., Brattleboro, VT, USA).

### Electrical stimulation

The MNTB–LSO pathway was stimulated with bipolar electrodes (two tungsten microelectrodes at 100–200  $\mu\text{m}$  distance; FHC, Bowdoinham, ME, USA) placed in the ipsilateral MNTB. Most electrical stimulation experiments were performed in the presence of the potassium channel antagonist tetraethylammonium (TEA; 10–20 mM) in order to increase response amplitudes and reliability, while using minimal stimulation currents. The effects of TEA on LSO neurons were characterized using gramicidin-perforated patch-clamp recordings (Wang *et al.*, 1994) in current clamp mode (Fig. 1). In these preliminary studies, amplitudes of MNTB-evoked postsynaptic potentials were increased to  $244.5 \pm 29.1\%$  (mean  $\pm$  SEM,  $n = 3$ ), which is within the range that we also achieved by simply increasing the stimulation strength. The effects of TEA on the decay times of depolarizing postsynaptic potentials were minor and did not result in multi-peaked postsynaptic potentials indicative of repetitive presynaptic action potentials, which would result in prolonged and repetitive activation of glycine or GABA<sub>A</sub> receptors.

### Focal photolysis of caged glutamate

For photolytic cleavage of caged glutamate, 150–200  $\mu\text{M}$  p-hydroxyphenacyl-glutamate (Givens *et al.*, 1997) was added to the ACSF. Focal photolytic cleavage was achieved by guiding the light from a 100-watt mercury arc lamp to the MNTB, using fused silica optical fibres (core diameter, 50  $\mu\text{m}$ ; Kandler *et al.*, 1998). The duration of the light pulses (50–100 ms) was regulated by a shutter (Vincent Associates, Rochester, NY, USA), located between the arc lamp and the optical fibre.

### Calcium imaging

Individual slices were placed in a submerged-type recording chamber mounted on an upright epifluorescence microscope (BX50WI, Olympus America, Melville, NY, USA). Slices were superfused continuously with oxygenated ACSF (30–32  $^{\circ}\text{C}$ , perfusion rate 2–4 mL/min, chamber volume 2 mL). Fura-2-loaded neurons were alternately excited with light at 340 nm and 380 nm (bandwidth 15 nm, duration 10–100 ms) using a computer-controlled monochromator (Polychrome II, TILL Photonics, Martinsried, Germany). Fluorescence emission was filtered (510/40 nm bandpass filter, Chroma Technology Corp.) and was detected with a CCD camera (IMAGO, TILL Photonics). Image pairs at 340 and 380 nm were acquired every 0.5–10 s using 10 $\times$  (NA 0.3) or 20 $\times$  (NA 0.5) water-immersion objectives (Olympus America). In photolysis experiments, only single wavelength excitation (380 nm) was used due to absorption of 340 nm light by p-hydroxyphenacyl-glutamate.

## Data analysis

Digital images were low pass filtered with a Gaussian  $3 \times 3$  kernel and background fluorescence was subtracted using the program Tillvision (TILL Photonics).  $\text{Ca}^{2+}$  responses were analysed by calculating the ratio of images obtained with 340 nm excitation over images obtained with 380 nm excitation (340 : 380 ratio). Due to large variations of  $R_{\min}$  and  $R_{\max}$  (Grynkiewicz *et al.*, 1985) in our preparation and setup, we opted to express the changes in  $[\text{Ca}^{2+}]_i$  in the form of the 340 : 380 ratio which, over physiological concentrations, is linearly related to changes in  $[\text{Ca}^{2+}]_i$ . For photolysis experiments, in which only 380 nm excitation was used, changes in intracellular calcium are expressed as  $F/F \times 100$  using the average of five images before photolysis as a baseline.

Fluorescence measurements were performed from the cell soma of LSO neurons (see e.g. Fig. 2). Cells with high resting 340 : 380 ratios ( $10 \times$  objective  $> 0.5$ ,  $20 \times$  objective  $> 1.2$ ), indicating high levels of baseline calcium concentrations, and cells that did not return to baseline after stimulation were excluded from analysis.

Statistical significance was analysed using Student's *t*-test and ANOVA followed by Dunnett's *post hoc* test as indicated in the text. Throughout the text, values are expressed as arithmetic mean  $\pm$  SEM.

## Chemicals

Tetrodotoxin (TTX) was purchased from Alomone Laboratories (Jerusalem, Israel), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), DL-amino-5-phosphonovalerate (APV), bicuculline methiodide and SKF 97541 from Tocris (Ballwin, MO, USA) and (+)- $\alpha$ -methyl-4-carboxyphenylglycine (MCPG) from RBI (Natick, MA, USA). If not stated otherwise, all other chemicals used in this study were from Sigma (St Louis, MO, USA).

## Results

Incubation of slices from P1–P5 animals in Fura-2 resulted in numerous labelled cells throughout the LSO (Fig. 2A and B). At higher magnification (Fig. 2C), most of these labelled cells depicted morphological features, such as a bipolar shape, that are reminiscent of immature LSO neurons (Henkel & Brunso-Bechtold, 1991; Rietzel & Friauf, 1988). To verify the neuronal nature of Fura-2 labelled cells, rhodamine beads were injected into the inferior colliculus of neonatal rats (P1,  $n = 2$ ) and slices from these animals labelled with fura-2 at P3 (Fig. 2D–E). Cells that were double-labelled with beads and fura-2 responded to application of GABA (2 mM) indicating that our recordings were indeed from neurons.

We first examined the effects of direct stimulation of glycine and GABA receptors on the  $[\text{Ca}^{2+}]_i$  of developing LSO neurons. In these experiments, glycine and GABA were bath applied in the presence of the sodium channel blocker TTX (0.5–1  $\mu\text{M}$ ) to prevent synaptically evoked responses. No differences were observed between mice and rats, and results from both species will be presented together for the rest of this paper.

## Calcium responses in neonatal animals (P1–P5)

In newborn animals, bath application of glycine (2 mM) or GABA (2 mM) consistently elicited a transient increase in the  $[Ca^{2+}]_i$  of LSO neurons (Fig. 3). In preliminary experiments, 2 mM glycine and GABA were found to be saturating concentrations and these concentrations were used in all subsequent experiments. These  $Ca^{2+}$  responses were sensitive to antagonists for glycine receptors and GABA<sub>A</sub> receptors (Fig. 4). The glycine receptor antagonist strychnine (25  $\mu$ M) completely blocked glycine-induced  $Ca^{2+}$  responses in 76% of cells (25 out of 33, two slices) and reduced the response in the remaining 24% (eight cells) to  $51.4 \pm 7.4\%$  (Fig. 4A and B). Consistent with previous studies (Kandler & Friauf, 1995; Ehrlich *et al.*, 1998; Lo *et al.*, 1998), a high strychnine concentration was necessary to block glycinergic responses in neonatal LSO neurons, reflecting the fact that glycine receptors in immature LSO neurons contain the 'neonatal'  $\alpha 2$  subunit that results in reduced strychnine sensitivity (Kuhse *et al.*, 1995; Friauf *et al.*, 1997). The GABA<sub>A</sub> receptor antagonist bicuculline (100  $\mu$ M) completely blocked GABA-induced  $Ca^{2+}$  responses in 95% of cells (56 out of 59, two slices) and reduced the response in the remaining three cells to  $46.7 \pm 15.8\%$  (Fig. 4C and D). This indicates that GABAergic  $Ca^{2+}$  responses in the LSO are mediated by GABA<sub>A</sub> receptors without any significant involvement of GABA<sub>B</sub> receptors. Consistent with this, application of the selective GABA<sub>B</sub> receptor agonist SKF 97541 (10  $\mu$ M) did not elicit  $Ca^{2+}$  responses in 68 GABA-responding neurons.

Although both glycine and GABA were able to increase  $[Ca^{2+}]_i$ , GABA appeared to be more effective than glycine. First, GABA induced responses in about twice as many cells as glycine did: 71% of the LSO neurons (163 out of 230, seven slices) responded to GABA, whereas only 34% of the neurons (94 out of 280, seven slices) responded to glycine (Fig. 5A). Second, the amplitudes of GABAergic calcium changes were about twice as large as the amplitudes of glycinergic responses (peak 340 : 380 ratio change relative to baseline: GABA,  $41.8 \pm 1.4\%$ ,  $n = 163$  vs. glycine,  $22.6 \pm 1.7\%$ ,  $n = 94$ ;  $P = 0.001$ ,  $t$ -test) (Figs 3 and 5B). These differences are not attributable to the presence of two distinct cell populations, one GABA-responsive and one glycine-responsive, because these differences were usually also observed in the same cells. When GABA and glycine were applied to the same slice, 8.2% of cells (six out of 73, three slices) responded to glycine only, 37% (27 cells) responded to both glycine and GABA, and 54.8% (40 cells) responded to GABA only. Thus, the majority of glycine-responding cells (82%, 27 out of 33) also responded to GABA and more than half of neonatal LSO neurons were purely GABA-responsive. Third, GABA increased  $[Ca^{2+}]_i$  much more rapidly than glycine (see traces in Figs 3, 6 and 7). A quantitative comparison of the responses in dual-responding cells revealed that the rise times (time from onset to peak) of GABAergic  $Ca^{2+}$  responses were about half as long as those for glycinergic responses (GABA  $31 \pm 3$  s vs. glycine  $64 \pm 5$  s,  $P = 0.001$ , paired  $t$ -test; Fig. 5C).

Another effect of GABA was that it could elicit long lasting, irregular oscillating  $Ca^{2+}$ -responses. Figure 6 illustrates some of these recurrent oscillations in six LSO neurons. Application of a 1 min GABA pulse caused an initial calcium response that was followed by repetitive calcium changes which outlasted the presence of GABA for several minutes (up to 7 min observed). These oscillations showed no temporal correlation between cells. Such

GABA-induced calcium oscillations were observed in 22 cells from 5 out of 12 slices (4/9 from mouse; 1/3 from rat). In contrast, Ca<sup>2+</sup>-oscillations never occurred following application of glycine. Although we did not further explore the underlying mechanism responsible for these slow calcium oscillations, the fact that they occurred in the presence of TTX argues against the possibility that they were generated by neuronal network activity. In this respect, GABAergic Ca<sup>2+</sup>-oscillations in immature LSO neurons are similar to glutamatergic or acetylcholinergic Ca<sup>2+</sup>-oscillations that have been described previously in the immature neocortex (Yuste and Katz, 1991; Flint *et al.*, 1999).

### Calcium responses are mediated by voltage-gated calcium channels

The above experiments were performed during the period in which glycine and GABA are depolarizing in the LSO. The observed calcium responses could, thus, have been generated by activation of voltage-gated Ca<sup>2+</sup> channels (VGCCs) and the resulting influx of extracellular calcium. To test this possibility, glycine and GABA were applied in the absence of extracellular Ca<sup>2+</sup> (nominally free Ca<sup>2+</sup>-ACSF with 0.15 mM EGTA). Under this condition Ca<sup>2+</sup> responses were completely abolished (glycine,  $n = 19$  cells; GABA,  $n = 43$  cells; three slices; Fig. 7A). In addition, in the presence of the calcium channel antagonist Ni<sup>2+</sup> (2 mM, five slices), glycinergic Ca<sup>2+</sup> responses were blocked in 71% of cells (45 out of 63) and reduced in 29% of cells (18 cells) by  $52.0 \pm 5.8\%$ . Similarly, Ni<sup>2+</sup> abolished GABAergic Ca<sup>2+</sup> responses in 83% of cells (57 out of 69) and reduced the responses in the remaining 17% of cells (12 cells) by  $43.0 \pm 4.2\%$  (Fig. 7B). Nifedipine (20  $\mu$ M), a specific L-type calcium channels inhibitor (Bean & Mintz, 1994), reduced glycine-evoked calcium responses by  $66.4 \pm 3.7\%$  ( $n = 43$  cells in five slices, rats P1–P2) and reduced GABA-evoked responses by  $71.6 \pm 1.6\%$  ( $n = 87$  cells in three slices, rat P1). These results demonstrate that glycine and GABA increase [Ca<sup>2+</sup>]<sub>i</sub> in developing LSO neurons primarily by activation of L-type calcium channels and an influx of extracellular calcium.

### Calcium responses in animals after hearing onset (P13–P15)

Although glycine and GABA are only depolarizing during the first postnatal week in the LSO, prolonged activation of glycine and GABA receptors (Backus *et al.*, 1998) or repeated activation of dendritic GABA receptors can cause the breakdown of the transmembrane chloride concentration gradient and produce depolarizations (Staley *et al.*, 1995). In the next experiments we therefore tested whether glycinergic and GABAergic increases in [Ca<sup>2+</sup>]<sub>i</sub> are restricted to the developmental period during which glycine and GABA are depolarizing, or whether glycine and GABA also increase [Ca<sup>2+</sup>]<sub>i</sub> after these neurotransmitters have become primarily hyperpolarizing.

In these experiments, LSO neurons were labelled in slices from 2-week-old animals by injecting Fura-2 AM directly into the extracellular space (Leinekugel *et al.*, 1995), labelling about 10–30 cells per injection site. Because these cells were exposed to higher DMSO concentrations than cells labelled by bulk staining, responses to glutamate or membrane depolarizations with 60 mM KCl were examined at the end of each experiment in order to assess the viability of labelled neurons. In contrast to neonatal slices, in P13–P15 slices ( $n = 8$ ) neither glycine (2 mM) nor GABA (2 mM) increased [Ca<sup>2+</sup>]<sub>i</sub>, but instead consistently elicited a small *decrease* in [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 8). Selective stimulation of GABA<sub>A</sub> receptors with

muscimol (100  $\mu\text{M}$ ) or stimulation of GABA<sub>B</sub> receptors with SKF 97541 (10  $\mu\text{M}$ ) also decreased  $[\text{Ca}^{2+}]_i$ . This indicates that in 2-week-old animals, GABA-induced decreases of  $[\text{Ca}^{2+}]_i$  can be attributed to the activation of both GABA<sub>A</sub> and GABA<sub>B</sub> receptors. The results obtained from eight animals are summarized in Fig. 8B. On average, glycine reduced the 340 : 380 ratio by  $2.9 \pm 0.3\%$  ( $n = 36$ ), GABA by  $5.5 \pm 0.3\%$  ( $n = 83$ ), muscimol (100  $\mu\text{M}$ ) by  $3.0 \pm 0.4\%$  ( $n = 20$ ) and SKF 97541 (10  $\mu\text{M}$ ) by  $4.5 \pm 0.2\%$  ( $n = 44$ ). Together, these results demonstrate that even strong and prolonged activation of glycine or GABA receptors (90 s of 2 mM glycine or GABA) does not increase  $[\text{Ca}^{2+}]_i$  in LSO neurons after hearing onset when glycine and GABA have become primarily hyperpolarizing.

### Synaptically elicited calcium responses in neonatal LSO neurons

Next, we examined whether  $\text{Ca}^{2+}$ -responses can also be elicited by selectively stimulating glycine and GABA receptors that are activated by MNTB–LSO synapses in neonatal animals. Single electrical shocks or stimulus trains (five stimuli at 20 Hz) delivered to the ipsilateral MNTB elicited  $\text{Ca}^{2+}$ -responses in LSO neurons (Fig. 9A). However, with these conditions, responses had small amplitudes and were unreliable; i.e., for a given neuron, they only occurred in about one third of the trials. Detection of these weak responses also required relatively long illumination times (100–500 ms) in order to obtain reasonable signal-to-noise ratios, which in turn increased the possibility of inducing phototoxicity. The small amplitudes and low probability of synaptic  $\text{Ca}^{2+}$ -responses made it difficult to perform pharmacological experiments, which require reliable responses and repetitive illumination. To circumvent these problems, we performed pharmacological experiments in the presence of the  $\text{K}^+$ -channel blocker tetraethylammonium (TEA, 10–20 mM), which strongly increased response amplitudes but hardly increased the frequency of spontaneously occurring calcium transients (Fig. 9A).

MNTB-evoked synaptic  $\text{Ca}^{2+}$  responses were analysed in 68 cells (P1–P4). Application of ionotropic and metabotropic glutamate receptor antagonists (10  $\mu\text{M}$  CNQX, 100  $\mu\text{M}$  APV, 250  $\mu\text{M}$  MCPG) abolished responses in 26 cells (38%), consistent with previous electrophysiological findings indicating that electrical stimulation of the MNTB can elicit glutamatergic responses in LSO neurons (Wu & Kelly, 1991; Kotak *et al.*, 1998). In the continued presence of glutamate receptor antagonists, bicuculline and strychnine (10  $\mu\text{M}$  each) abolished responses in the remaining 88% of cells (37 of 42) (Fig. 9B). Responses in the remaining five cells were not affected by glutamate and glycine/GABA antagonists, suggesting that these responses were the result of direct electrical activation.

Recently it has been shown that in addition to glycine, which is the only neurotransmitter in the adult MNTB–LSO pathway (Finlayson & Caspary, 1989; Bledsoe *et al.*, 1990; Glendenning *et al.*, 1991), GABA is used transiently as an additional neurotransmitter in the developing MNTB–LSO pathway (Kotak *et al.*, 1998; Korada & Schwartz, 1999). We therefore examined whether, and to what degree, synaptically released GABA contributes to MNTB-evoked  $\text{Ca}^{2+}$  responses (Fig. 10; Table 1). Blocking glycine receptors with strychnine (10  $\mu\text{M}$ ) completely abolished synaptic  $\text{Ca}^{2+}$  responses in 67% of neurons (10 out of 15; three slices, P2–P3) and reduced responses in 13% of neurons (average reduction 50%, two out of 15 cells). Responses in 20% of the neurons ( $n = 3$ ) were insensitive to



strychnine. All strychnine-insensitive  $\text{Ca}^{2+}$  responses ( $n = 5$  cells) were blocked by additional application of bicuculline ( $10 \mu\text{M}$ ). When bicuculline was applied prior to strychnine, it abolished synaptic responses in 31% of neurons (10 out of 32 neurons), reduced responses in 53% of the neurons (average reduction,  $52.6 \pm 1.8\%$ ;  $n = 17$ ) and had no effect on 16% of the neurons ( $n = 5$ ). Subsequent addition of strychnine abolished all bicuculline-insensitive responses. These results indicate that for MNTB-elicited responses, glycine partially mediates responses in 80% of LSO neurons, is necessary in 67% of neurons and is solely responsible for eliciting  $\text{Ca}^{2+}$  responses in 20% of neurons. Our results also highlight a strong contribution of GABA in eliciting  $\text{Ca}^{2+}$  responses, as GABA is not only involved in mediating synaptic  $\text{Ca}^{2+}$  responses in 84% of neurons, but is also necessary for  $\text{Ca}^{2+}$  responses in 31% of LSO neurons.

The strong contribution of GABA in mediating MNTB-evoked  $\text{Ca}^{2+}$  responses contradicts previous electrophysiological studies in rats and mice that reported no or only a weak contribution of GABA to contralateral synaptic transmission to the LSO (Wu & Kelly, 1992; Kandler & Friauf, 1995; Ehrlich *et al.*, 1998). This discrepancy could be explained if our electrical stimulation activated other GABAergic afferent pathways that run in close vicinity to the MNTB (Moore & Moore, 1987; Adams & Mugnaini, 1990; Warr & Beck, 1996). In order to address this possibility, we stimulated MNTB neurons using focal photolysis of caged glutamate (p-hydroxyphenacyl-glutamate,  $150\text{--}200 \mu\text{M}$  (Givens *et al.*, 1997)). This approach allowed us to excite MNTB neurons selectively by completely restricting the excited area to the boundaries of the MNTB and thus avoiding the accidental stimulation of periolivary neurons (Fig. 11A and B). Pure MNTB-evoked  $\text{Ca}^{2+}$  responses were examined in 42 LSO neurons. Application of bicuculline ( $20 \mu\text{M}$ ) completely abolished MNTB-evoked  $\text{Ca}^{2+}$  responses in 19% of cells ( $n = 8$ ), reduced the responses in 29% of cells ( $n = 12$ ; reduction to  $39 \pm 5\%$  of control) and did not affect responses in 52% of cells ( $n = 22$ ; reduction to  $99 \pm 1\%$ ; Fig. 11C). The effect of strychnine ( $10 \mu\text{M}$ ) was tested in 21 cells, which had bicuculline-insensitive responses. In 42% of these cells ( $n = 9$ ) bicuculline-resistant responses were completely abolished by strychnine, indicating that these responses were purely glycinergic. In another 12 cells, in which bicuculline partly decreased response amplitudes, additional application of strychnine eliminated all responses (data not shown), indicating that MNTB-evoked responses were mediated by both glycine and GABA. Upon washout of bicuculline and strychnine, responses fully recovered (amplitude after washout  $102.6 \pm 3.1\%$  compared with control). In summary, these experiments indicate that glycine participates in 81% of MNTB-evoked  $\text{Ca}^{2+}$  responses in LSO neurons and GABA participates in at least 48% of responses confirming our results, which were obtained with electrical stimulation.

## Discussion

In this study we characterized the effects of the inhibitory neurotransmitters glycine and GABA on  $[\text{Ca}^{2+}]_i$  in developing LSO neurons. Our results demonstrate that during the first postnatal week, the period during which these neurotransmitters are depolarizing (Kandler & Friauf, 1995; Ehrlich *et al.*, 1999), glycine and GABA increase  $[\text{Ca}^{2+}]_i$ . Around hearing onset (P12–P14), when GABA and glycine have become hyperpolarizing, they slightly decrease  $[\text{Ca}^{2+}]_i$ . In addition, brief stimulation of the MNTB–LSO pathway also increased

[Ca<sup>2+</sup>]<sub>i</sub>, indicating that glycinergic/GABAergic synaptic activity is directly coupled to postsynaptic [Ca<sup>2+</sup>]<sub>i</sub> in neonatal LSO neurons.

GABAergic and glycinergic Ca<sup>2+</sup> responses have been described previously in a variety of developing neuronal systems (e.g. Yuste & Katz, 1991; Reichling *et al.*, 1994; Leinekugel *et al.*, 1995; Obrietan & van den Pol, 1995; Owens *et al.*, 1996). In the developing auditory system, an increase in [Ca<sup>2+</sup>]<sub>i</sub> by GABA or glycine has been demonstrated in the nucleus magnocellularis of the chick (Lachica *et al.*, 1998) and the inferior colliculus of rodents (Lo *et al.*, 1998; Frech *et al.*, 1999). However, such glycinergic and GABAergic calcium responses appeared to be absent in the immature LSO (Schmanns & Friauf, 1994). Results of the present study are in obvious contrast to this previous finding. The reason for this discrepancy is not clear, but differences in the imaging techniques used in both studies might account for the different findings. For example, the present study used a CCD camera to measure responses from individual neurons, while the previous study used a photomultiplier tube to measure the average response of a large number of cells. Due to this spatial averaging, minor calcium changes in a small number of individual cells might have remained undetected.

Ca<sup>2+</sup> responses in the immature LSO differ in several aspects from those reported in the avian nucleus magnocellularis (Lachica *et al.*, 1998) and the inferior colliculus (Lo *et al.*, 1998; Frech *et al.*, 1999). For example, in the avian nucleus magnocellularis, GABAergic Ca<sup>2+</sup> responses are mediated by a metabotropic, G-protein-linked GABA receptor and by release of calcium from intracellular stores. In the inferior colliculus, glycinergic and GABAergic Ca<sup>2+</sup> responses occur only with prolonged pharmacological stimulation, a condition that can result in a breakdown of the transmembrane chloride gradient and produce depolarizations caused by the efflux of bicarbonate (Staley *et al.*, 1995; Frech *et al.*, 1999). In agreement with this, brief activation of glycinergic or GABAergic synapses in the inferior colliculus produces hyperpolarizing responses, which actually decrease the amplitude of glutamate-evoked Ca<sup>2+</sup> responses (Lo *et al.*, 1998). This scenario is distinct from the situation in the neonatal LSO, in which glycinergic or GABAergic synapses consistently increase [Ca<sup>2+</sup>]<sub>i</sub> and in which even prolonged activation of glycine and GABA<sub>A</sub> receptors does not generate calcium responses once inhibitory synapses have become hyperpolarizing.

GABA and glycine can increase [Ca<sup>2+</sup>]<sub>i</sub> by several mechanisms, such as activation of VGCCs (Connor *et al.*, 1987; Yuste & Katz, 1991; Reichling *et al.*, 1994; Owens *et al.*, 1996; Lo *et al.*, 1998; Frech *et al.*, 1999), removal of the magnesium block of NMDA receptors (Staley *et al.*, 1995; Leinekugel *et al.*, 1997) or activation of G-protein-coupled metabotropic GABA receptors (Lachica *et al.*, 1998). In the LSO, the primary mechanism by which GABA and glycine increase [Ca<sup>2+</sup>]<sub>i</sub> seems to be the activation of VGCCs, and mainly the L-type channels. First, blockage of glycine and GABA<sub>A</sub> receptors abolished Ca<sup>2+</sup> responses, whereas activation of metabotropic GABA receptors failed to have an effect on [Ca<sup>2+</sup>]<sub>i</sub>. Second, an increase in [Ca<sup>2+</sup>]<sub>i</sub> was observed only during the period when GABA and glycine are depolarizing and third, responses were abolished if extracellular calcium was removed or greatly reduced, or if VGCC were blocked by nickel or nifedipine. Although VGCCs seem to be the main source of calcium influx, our results do not exclude the

possibility that calcium-induced calcium release from internal stores, a mechanism which is present in the avian cochlear nucleus (Kato & Rubel, 1999), amplifies the initial calcium influx through VGCC. In the LSO of 2-week-old animals, glycine and GABA slightly, yet consistently, decreased  $[Ca^{2+}]_i$ . This could be due to glycinergic and GABAergic hyperpolarizations that are present in the LSO at this age and which could inactivate L-type channels that are spontaneously open at the resting membrane potential (Magee *et al.*, 1996).

### GABAergic $Ca^{2+}$ responses in the neonatal LSO

Our results revealed that GABA partly or completely mediated MNTB-evoked  $Ca^{2+}$  responses in over 80% of LSO neurons in newborn rats and mice. This is consistent with recent anatomical and electrophysiological studies that demonstrated the transient use of GABA in the immature MNTB–LSO pathway of gerbils (Kotak *et al.*, 1998; Korada & Schwartz, 1999). However, the extent by which GABA contributes to  $Ca^{2+}$  responses in rats and mice is significantly higher than what was expected from electrophysiological recordings in these species (Kandler & Friauf, 1995; Ehrlich *et al.*, 1998). It is unlikely that the GABAergic  $Ca^{2+}$  responses in the present study were the result of activation of periolivary GABAergic neurons which project to the LSO (Moore & Moore, 1987; Adams & Mugnaini, 1990; Warr & Beck, 1996) because specific stimulation of MNTB neurons with focal photolysis of glutamate still elicited prominent GABAergic responses. Thus, while GABA plays only a minor role in eliciting MNTB-evoked membrane potential responses in the rat LSO, it has a substantial role in mediating MNTB-evoked  $Ca^{2+}$  responses. One possible explanation for this apparent discrepancy could lie in the nonlinear relationship between membrane potential and activation of VGCCs. This possibility is supported by the observation that bicuculline completely abolished  $Ca^{2+}$  responses in over 30% of all LSO neurons, despite the fact that only about 16% of responses were purely GABAergic.

In addition to enhancing MNTB-evoked  $Ca^{2+}$  responses, GABA, when applied externally, could also generate longer-lasting calcium oscillations which exceeded the presence of GABA by several minutes. These calcium oscillations are somewhat reminiscent of glutamate-induced calcium oscillations that have been described in other neuronal systems and which are caused by oscillatory activity of intracellular second messenger pathways (Yuste & Katz, 1991; Kawabata *et al.*, 1996; Gu & Spitzer, 1997; Flint *et al.*, 1999). The long duration and TTX-insensitivity of the oscillations in the LSO suggest that GABA can activate similar pathways in immature LSO neurons. While the exact nature of these pathways is currently unclear, our data indicate a specific requirement of GABA<sub>A</sub> receptor activation because strong increases in  $[Ca^{2+}]_i$  *per se*, as seen after glycine application, were not sufficient to trigger oscillations. As calcium oscillations can activate distinct sets of signalling pathways and regulate the expression of specific genes (Gu & Spitzer, 1995; Bootman *et al.*, 2001), it will be interesting to determine the age or conditions under which synaptic activity can induce GABAergic calcium oscillations in the immature LSO.

The functional role of the transient expression of GABA in the immature MNTB–LSO pathway is currently unknown. It has been suggested that the additional release of GABA provides a negative feedback by activating presynaptic GABA<sub>B</sub> receptors on glycinergic

MNTB terminals (Kotak *et al.*, 1998). The present study indicates that GABA also plays an important role in amplifying or mediating postsynaptic  $\text{Ca}^{2+}$  responses. The question remains as to why this effect could not be accomplished by simply increasing the amount of glycine released. A possible explanation could lie in the different time-course of glycinergic and GABAergic postsynaptic currents. Previous studies at spinal interneuron–motoneuron synapses, which corelease glycine and GABA, have demonstrated slower decay times for GABAergic components than for glycinergic components (Jonas *et al.*, 1998; Gao *et al.*, 2001). If such differences also exist in the LSO, GABA would be much more effective in depolarizing LSO neurons and activating VGCCs than glycine.

### Functional implications of glycinergic and GABAergic calcium responses in the developing LSO

Numerous studies have demonstrated that spontaneous synaptic activity before hearing onset is necessary for the normal development of the LSO (for review see Sanes & Friauf, 2000). Decreasing or abolishing spontaneous activity in the MNTB or blocking glycinergic transmission in the LSO interferes with the establishment of the precise tonotopic organization of the MNTB–LSO pathway and results in dendritic hypertrophy (Sanes *et al.*, 1992; Sanes & Takacs, 1993), abnormal glycinergic and glutamatergic synaptic transmission, and altered membrane properties (Kotak & Sanes, 1996). The chain of intracellular events by which glycinergic and GABAergic synaptic activities exert their effect on the developing LSO is still largely unknown, but most likely involves calcium-dependent processes (Kirsch & Betz, 1998; Liu & Kaczmarek, 1998; Lohmann *et al.*, 1998; Parks, 1999; Kotak & Sanes 2000). Results of the present study provide a first step in deciphering these mechanisms by demonstrating a direct link between glycinergic/GABAergic synaptic activity and postsynaptic calcium signalling. One possible reason for the paradoxical depolarizing action of immature inhibitory MNTB–LSO synapses could thus be to provide this link during the period of activity-dependent remodelling of sound localization circuits. Finally, in addition to directly increase  $[\text{Ca}^{2+}]_i$ , glycinergic/GABAergic depolarizations could also enhance glutamatergic neurotransmission by removing the magnesium block from NMDA receptors, as has been shown in immature hippocampal neurons (for review see Ben-Ari *et al.*, 1997). Such synergistic action of glycinergic/GABAergic and glutamatergic activity could provide a mechanism for encoding temporally correlated activity in converging inhibitory and excitatory inputs to LSO neurons.

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### Abbreviations

ACSF	artificial cerebrospinal fluid
APV	amino-5-phosphonovalerate

<b>CNQX</b>	6-cyano-7-nitroquinoxaline-2,3-dione
<b>DMSO</b>	dimethylsulphoxide
<b>EGTA</b>	ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid
<b>GABA</b>	$\gamma$ -aminobutyric acid
<b>LSO</b>	lateral superior olive
<b>MCPG</b>	$\alpha$ -methyl-4-carboxyphenylglycine
<b>MNTB</b>	medial nucleus of the trapezoid body
<b>P</b>	postnatal day
<b>TEA</b>	tetraethylammonium
<b>TTX</b>	tetrodotoxin
<b>VGCC</b>	voltagegated calcium channel

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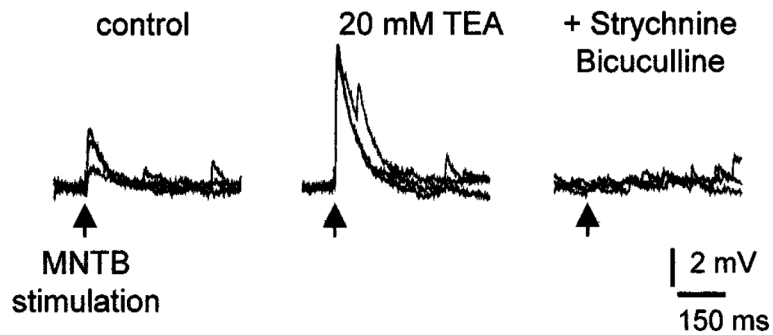
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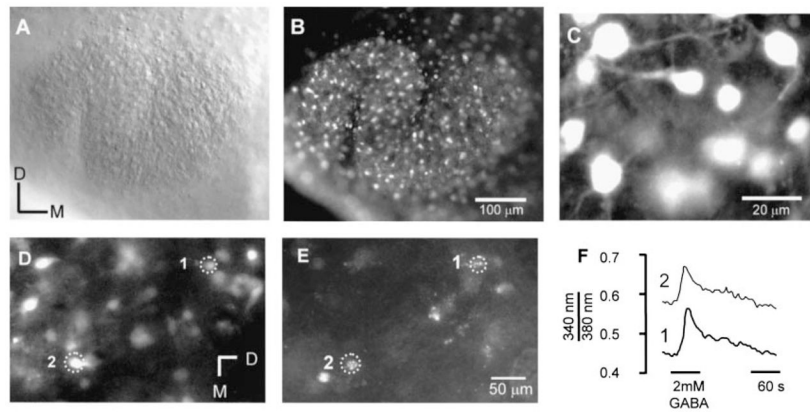
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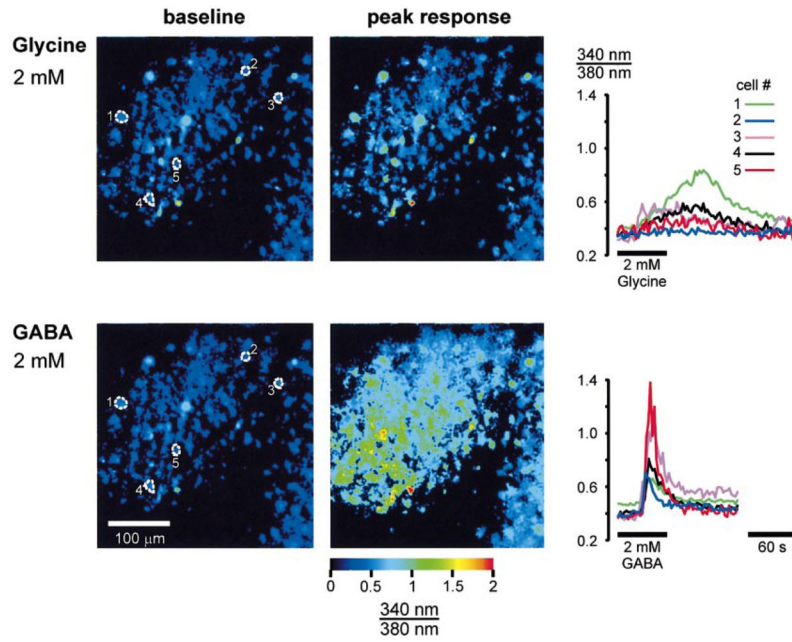




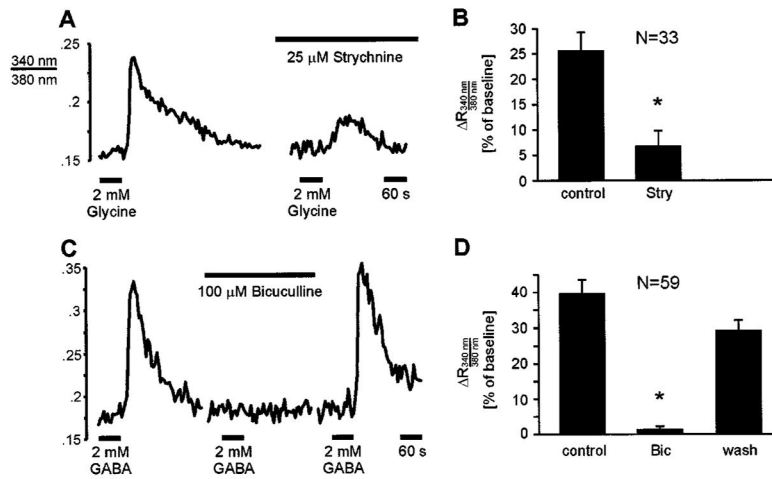
**Fig. 1.** Effect of the potassium channel blocker TEA on medial nucleus of the trapezoid body- (MNTB-) elicited, depolarizing postsynaptic potentials (PSPs) in immature lateral superior olive neurons. TEA (20 mM) more than doubled the amplitude of subthreshold PSPs without significantly changing their decay time (control,  $\tau = 74 \pm 23$  ms; TEA,  $\tau = 82 \pm 3$  ms). Depolarizing PSPs were abolished by blocking glycine receptors and GABA<sub>A</sub> receptors with strychnine and bicuculline (10  $\mu$ M). Recordings were performed in perforated patch clamp mode and slice was prepared from a P3 rat.



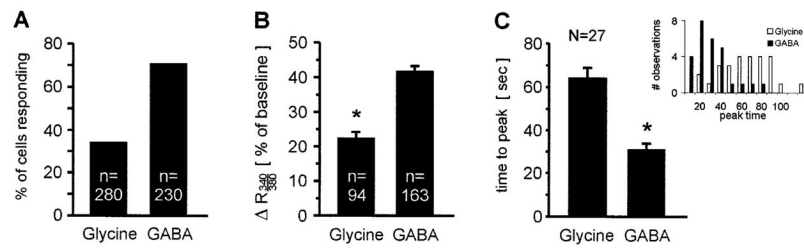
**Fig. 2.** Fura-2 staining of lateral superior olive (LSO) neurons in brainstem slices. (A) Brightfield image of the LSO from a P4 rat. (B) Fluorescence image with 380 nm excitation of the same LSO shown in A. Numerous cells along the extent of the LSO are labelled. (C) Higher magnification reveals that most labelled cells have a typical neuron-like appearance. (D–F) GABA elicited calcium responses in neurons retrogradely labelled from the inferior colliculus. (D) Fura-2-labelled cells in a P3 rat. (E) In the same slice some cells are retrogradely labelled with rhodamine beads, identifying them as inferior colliculus-projecting neurons. (F). Response of two inferior colliculus-projecting neurons to application of 2 mM GABA. D, dorsal, M, medial.



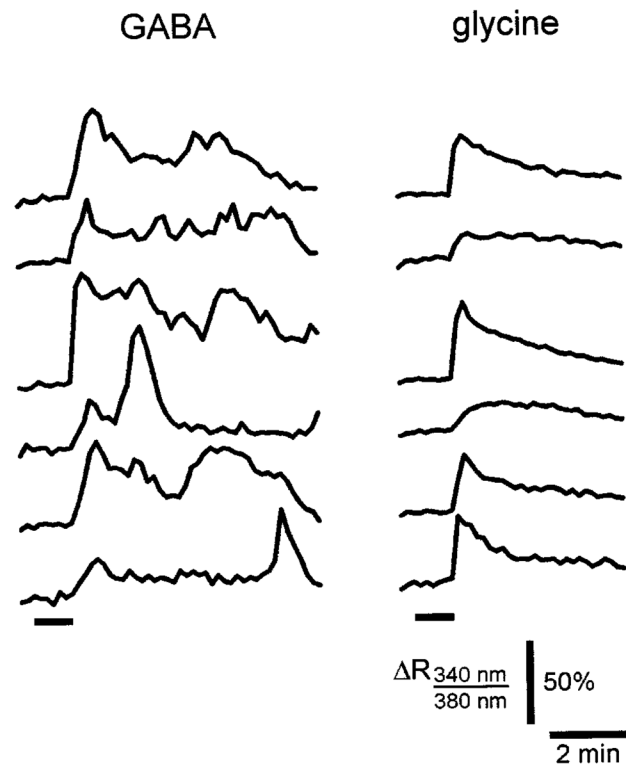
**Fig. 3.** Glycine and GABA-elicited calcium responses in immature lateral superior olive (LSO) neurons. Top row: 340 : 380 ratio images before (baseline) and at the peak of the response following bath application of 2 mM glycine. The graph on the right illustrates the time-course of the response of the five cells outlined in the baseline image. Bottom row: response of the same LSO to bath application of 2 mM GABA. Notice that many more cells respond to GABA than to glycine. GABAergic responses also have a more rapid onset and higher amplitudes. The slice was prepared from a P3 rat. Dorsal is up and medial is to the right in all panels.



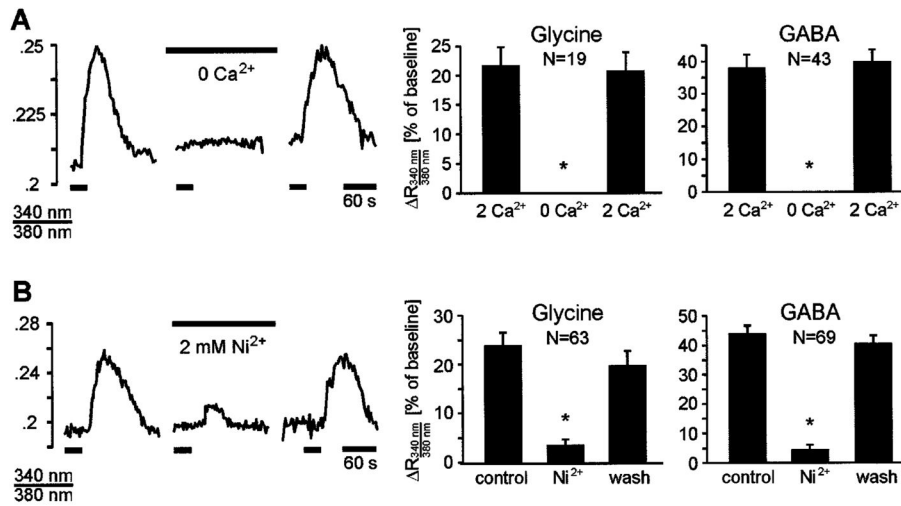
**Fig. 4.** Glycine- and GABA-elicited  $Ca^{2+}$  responses are mediated by glycine and  $GABA_A$  receptors. (A) Examples of glycine-elicited  $Ca^{2+}$  responses of a lateral superior olive neuron from a P3 rat. Responses are strongly reduced by the glycine receptor antagonist strychnine. (B) Summary of the effects of strychnine. Asterisk indicates value significantly different from controls ( $P < 0.001$ , paired  $t$ -test). (C) The  $GABA_A$  receptor antagonist bicuculline blocks GABA-elicited  $Ca^{2+}$  responses (mouse P2). (D) Summary of the effects of bicuculline. Asterisk indicates value significantly different from controls ( $P < 0.01$ ; ANOVA followed by Dunnett's test).

**Fig. 5.**

Comparison of glycine and GABA-elicited calcium responses. (A) Glycine (2 mM) elicits responses in 34% of monitored lateral superior olive neurons, whereas GABA (2 mM) elicits responses in 71% of neurons. (B) GABA-evoked peak responses are almost twice as large as glycine-evoked responses ( $P = 0.001$ , unpaired  $t$ -test). (C) The mean time from response onset to peak is about twice as long for glycine- (64 s) than for GABA-elicited responses (31 s;  $P < 0.001$ , paired  $t$ -test). Inset shows distribution histograms of peak times for glycine (open bars) and GABA (closed bars).

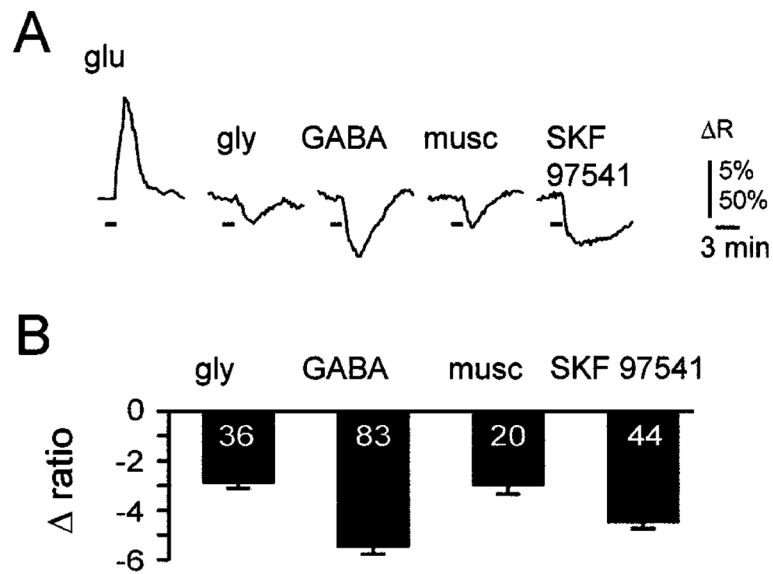


**Fig. 6.** GABA-elicited calcium oscillations. Left column: examples of responses of six lateral superior olive neurons from a P2 mouse. Neurons respond to a single GABA pulse (1 min, indicated by the bar) with longlasting, multi-peaked responses that persist for several minutes after the washout of GABA. Right column: glycine-elicited calcium responses with similar amplitudes illustrating that prolonged increase in  $[Ca^{2+}]_i$  is insufficient to elicit oscillations.



**Fig. 7.**

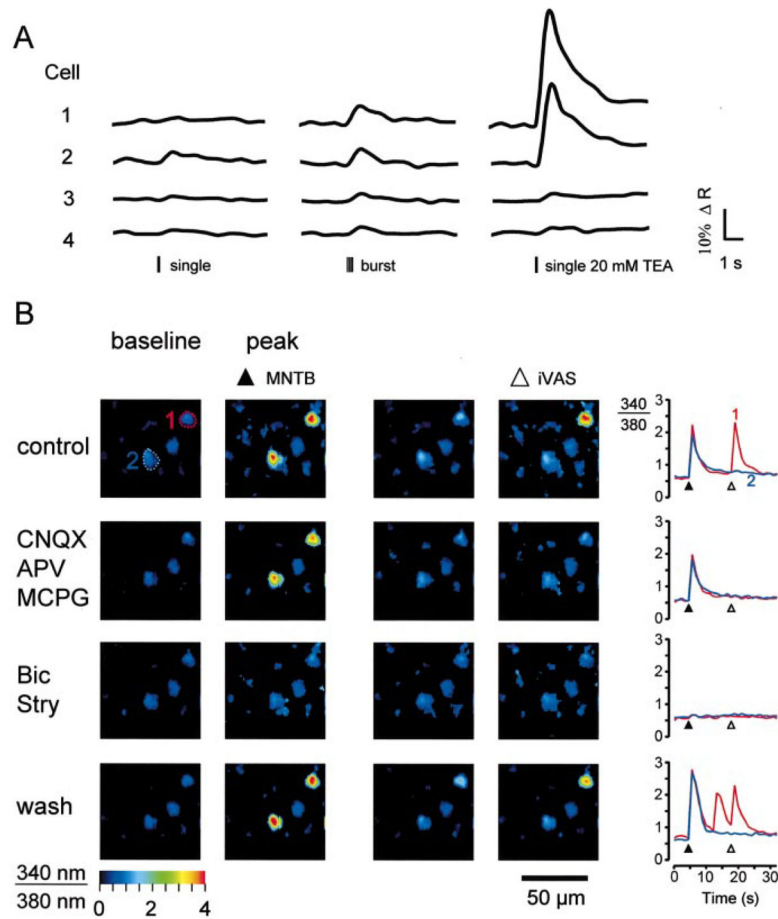
Glycine- and GABA-evoked calcium responses are mediated by influx of extracellular calcium through voltage-gated calcium channels. (A) Glycinergic and GABAergic  $\text{Ca}^{2+}$  responses are abolished in calcium-free solution. Traces show glycine-evoked  $\text{Ca}^{2+}$  responses in a P2 mouse. (B) Glycinergic and GABAergic  $\text{Ca}^{2+}$  responses are blocked by the voltage-gated  $\text{Ca}^{2+}$ -channel antagonist nickel (2 mM). Traces show glycine-evoked  $\text{Ca}^{2+}$  responses in a P2 rat. Asterisks in bar graphs indicate significantly different from controls ( $P < 0.01$ ; ANOVA followed by Dunnett's test).



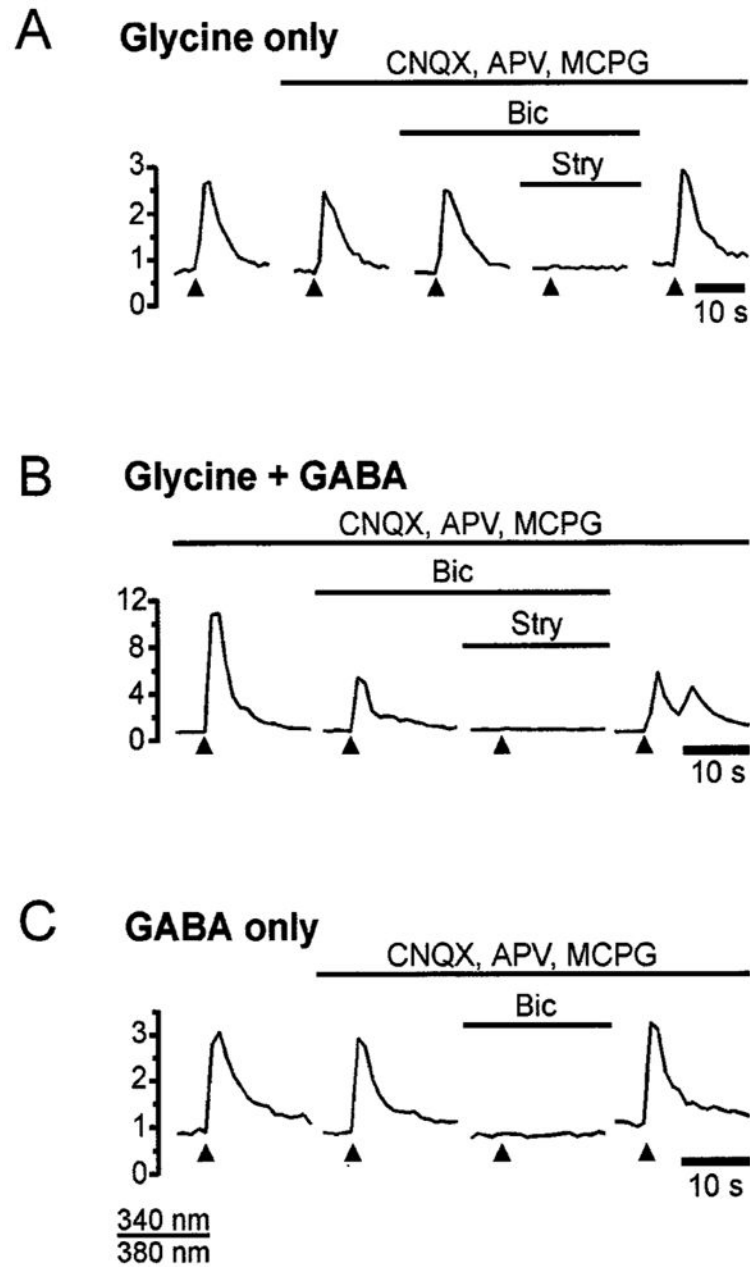
**Fig. 8.**

Glycine and GABA slightly decrease  $[Ca^{2+}]_i$  in 2-week-old animals. (A) Averaged responses of 20 cells from a P14 rat slice following applications of glutamate, glycine or GABA receptor agonists. Horizontal bars indicate agonist applications. Vertical scale bar represents changes in 340 : 380 ratio and is 50% for glutamate response and 5% for all others. (B) Average peak responses from lateral superior olive neurons in slices from eight P13 to P15 animals. Numbers within the bars indicate the number of cells.

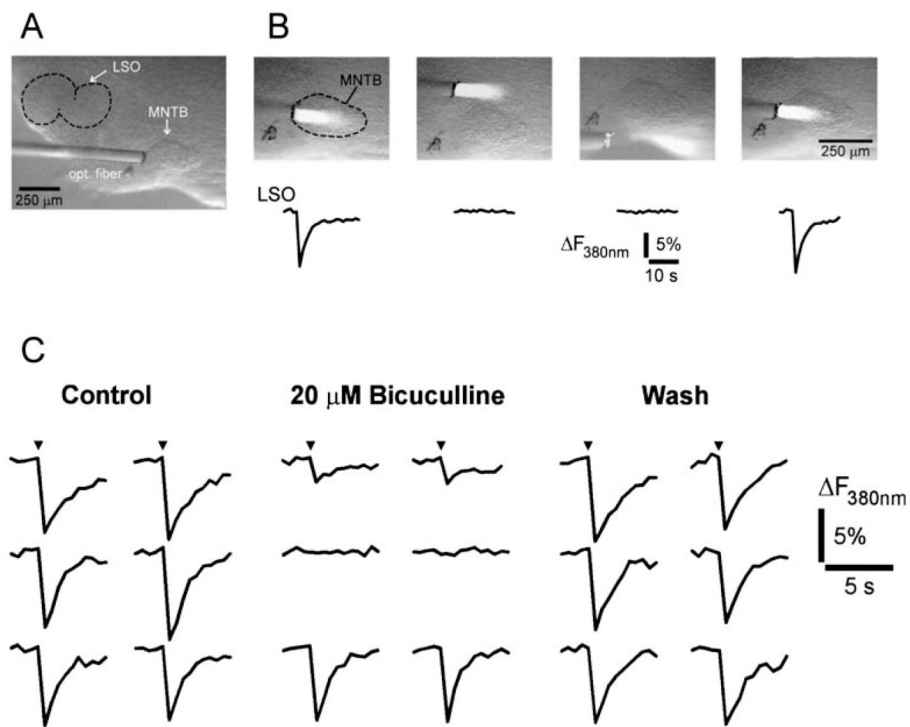




**Fig. 9.** Medial nucleus of the trapezoid body- (MNTB-) evoked synaptic Ca<sup>2+</sup> responses in neonatal lateral superior olive (LSO) neurons. (A) Left column: responses in four LSO neurons to single electrical shocks applied to the ipsilateral MNTB. Middle column: responses to five shocks delivered at 20 Hz. Right column: responses to a single shock in the presence of 20 mM TEA. (B) Pharmacology of synaptic Ca<sup>2+</sup> responses of two neurons in the medial limb of the LSO (P4 mouse). Both cells responded to stimulation of the MNTB and one cell responded to stimulation of the ipsilateral ventral acoustic stria (iVAS). Blocking ionotropic and metabotropic glutamate receptors with CNQX (10 μM), APV (100 μM), and MCPG (250 μM) abolished iVAS-evoked responses. Blocking glycine and GABA receptors with strychnine and bicuculline (10 μM each) reversibly abolished MNTB-evoked responses. Pseudocolour images were calculated from images just before stimulation (baseline) and at peak of responses.

**Fig. 10.**

Medial nucleus of the trapezoid body- (MNTB-) elicited  $\text{Ca}^{2+}$  responses are mediated by glycine, GABA or both. (A) Example of responses mediated only by glycine. The response is unaffected by glutamate antagonists or bicuculline, but is abolished by strychnine. (B) Example of responses mediated by glycine and GABA. The response is reduced by bicuculline and blocked by the addition of strychnine. (C) Example of responses mediated only by GABA. The response is completely abolished by bicuculline. Bicuculline and strychnine were applied at 10  $\mu\text{M}$ , CNQX at 10  $\mu\text{M}$ , APV at 100  $\mu\text{M}$  and MCPG at 250  $\mu\text{M}$ . Slices in A and B are from a P2 rat and slice in C from a P1 rat.



**Fig. 11.** Selective stimulation of medial nucleus of the trapezoid body (MNTB) neurons elicit GABAergic Ca<sup>2+</sup> responses in the lateral superior olive (LSO). (A) Overview of the superior olivary complex in a slice (P3 rat) in brightfield illumination to illustrate experimental approach. A thin optical fibre is used to deliver UV light focally to the MNTB. (B) Responses in the LSO are elicited only from neurons within the boundaries of the MNTB. Top row: area of UV-illumination (presumed site of glutamate release). Bottom row: corresponding responses in an LSO neuron (ΔF<sub>380nm</sub> with 380 nm excitation). (C) Pure MNTB-evoked Ca<sup>2+</sup> responses in three neurons. Responses of the upper neurons are partly or completely blocked by bicuculline. Responses in the bottom neuron are unaffected by bicuculline. Ca<sup>2+</sup> responses are expressed as fluorescence changes with 380 nm excitation. Glutamate was uncaged in the centre of the MNTB (150 μM p-hydroxyphenacyl-glutamate; 50 ms flash duration indicated by the arrowhead). Slice was prepared from a P3 rat.

**Table 1**

Effect of strychnine and bicuculline on medial nucleus of the trapezoid body- (MNTB-) evoked calcium responses

	<b>Percentages (and numbers, <i>n</i>) of cells with:</b>			
	<b>Complete block % (<i>n</i>)</b>	<b>Reduced block % (<i>n</i>)</b>	<b>No effect % (<i>n</i>)</b>	<b>Totals % (<i>n</i>)</b>
Strychnine	67 (10)	13 (2)	20 (3)	100 (15)
Bicuculline	31 (10)	53 (17)	16 (5)	100 (32)

*n* = number of cells.