Metabotropic glutamate receptors in the lateral superior olive activate TRP-like channels: Age and experience-dependent regulation.

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Running head: mGluRs and TRP-like channels in developing LSO neurons

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Abstract

The lateral superior olive (LSO) is the primary auditory nucleus for processing of interaural sound level differences, which is one of the major cues for sound localization. During development, survival and maturation of LSO neurons critically depend on synaptic activity and intracellular calcium signaling. Before hearing onset, glutamatergic synaptic inputs from the cochlear nucleus (CN) to the LSO activate group I metabotropic glutamate receptors (mGluRs) which leads to calcium release from intracellular stores and large calcium influx from the extracellular milieu. Here, we investigated the nature of the mGluR-activated membrane channel that mediates the influx of extracellular calcium. Using Fura-2 calcium imaging in brainstem slices of neonatal and juvenile mice, we found that this calcium channel is blocked by Ni\(^{2+}\), La\(^{3+}\) and 2-aminoethoxydiphenylborane (2-APB), known antagonists of transient receptor potential (TRP) channels. During postnatal development, the contribution of extracellular calcium influx to mGluR-mediated Ca\(^{2+}\) responses gradually decreased and was almost abolished by the end of the third postnatal week. Over this period, the contribution of Ca\(^{2+}\) release from internal stores remained unchanged. The developmental decrease of TRP-like channel mediated calcium influx was significantly less in congenitally deaf waltzer mice suggesting that early auditory experience is necessary for the normal age-dependent downregulation of functional TRP channels.

Keywords: Calcium, auditory, sound localization, waltzer, cadherin 23
Introduction

The lateral superior olive (LSO) is a binaural auditory brainstem nucleus involved in the processing of interaural sound level differences. LSO neurons receive excitatory, glutamatergic inputs from the ipsilateral cochlea via the anteroventral cochlear nucleus (AVCN) and inhibitory, glycinergic, inputs from the contralateral cochlea via the medial nucleus of the trapezoid body (MNTB) (Boudreau and Tsuchitani 1968; Boudreau and Tsuchitani 1968; (Oertel) 1999; (Tollin) 2003). Both inputs are tonotopically organized and aligned, which enables LSO neurons to process binaural inputs in a frequency-specific manner (Sanes and Rubel 1988; (Tollin) 2003).

During development, the LSO circuit undergoes a number of morphological and functional changes which include growth and refinement of dendritic arbors (Sanes et al. 1992; Rietzel and Friauf 1998), functional and structural refinement of the MNTB-LSO pathway (Sanes and Siverls 1991; Sanes and Takacs 1993; Kim and Kandler 2003; Kandler and Gillespie 2005), and a switch in neurotransmitter phenotype (Gillespie et al. 2005; Kotak et al. 1998; Nabekura et al. 2004). Many of these processes occur before hearing onset but nevertheless depend on neuronal activity and intracellular Ca$^{2+}$ signaling (Lohmann et al. 1998; Friauf and Lohmann 1999; Sanes and Takacs 1993; Sanes and Siverls 1991; Kotak and Sanes 2000).

Before hearing onset, spontaneous neuronal activity is present in the form of high-frequency bursts at various levels in the auditory pathway (Jones et al. 2001; Kros et al. 1998; Gummer and Mark 1994; (Lippe) 1994; Romand and Ehret 1990; Kotak and Sanes 1995; Glowatzki and Fuchs 2000). In the pre-hearing mouse, activation of glutamatergic cochlear nucleus (CN) inputs onto LSO neurons elicits postsynaptic Ca$^{2+}$ responses that are mediated by ionotropic and metabotropic glutamate receptors (mGlRs). The recruitment of specific types of glutamate receptors depends on the spatial and temporal patterns of synaptic activation (Ene et
al. 2003). Burst-like synaptic activity activates group I and II mGluRs which in turn generate postsynaptic Ca^{2+} responses with a typical biphasic profile. The initial phase of mGluR-elicited Ca^{2+} responses in immature LSO neurons are mediated by Ca^{2+} release from intracellular stores while the later and prolonged phase is mediated by an influx of extracellular Ca^{2+} through membrane Ca^{2+} channels.

The identity of mGluR-activated membrane Ca^{2+} channels in LSO neurons is unclear but their dependence on intracellular Ca^{2+} release suggests that they belong to the family of transient receptor potential (TRP) channels (Clapham 2003, Montell 2005) which can be activated by mGluRs (Gee et al. 2003; Kim et al. 2003; Tozzi et al. 2003; Bengtson et al. 2004). In this study, we investigated the developmental changes of mGluR-mediated Ca^{2+} responses in mouse LSO neurons and characterized the pharmacological properties of the mGluR-activated Ca^{2+} channel. We found that the pharmacological profile of mGluR-activated Ca^{2+} channels is consistent with the characteristics of a TRPC channel. During the first three postnatal week, group I mGluR-evoked Ca^{2+} responses decreased, with the most pronounced changes occurring around the onset of hearing when mGluRs ceased to trigger extracellular Ca^{2+} influx. These developmental changes were delayed in congenitally deaf waltzer mice suggesting that developmental downregulation of TRP-like Ca^{2+} responses is influenced by normal auditory experience.
Material and methods

Animals. Experiments were performed in C57BL/6J and homozygote waltzer Cdh23 mice (C57BL/6J-Cdh23<sup>-2J</sup>) of both genders (Jackson Laboratory, Bar Harbor, ME, Charles River, Wilmington, MA), aged between postnatal day 0 (P0 - the day of birth) and P20. Waltzer homozygotes were identified by their rollover behavior deficits (Wada et al. 2001). Heterozygote mice were obtained by breeding homozygote/wild-type pairs. All experimental procedures were in accordance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

Slice preparation. Animals between P0 to P7 were anesthetized by hypothermia and animals older than P7 were anesthetized using isofluorane. Animals were quickly decapitated, the brains removed, and placed into cold (4-8°C) artificial cerebrospinal fluid with kynurenic acid (ACSF with KA, 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, kynurenic acid 1, pH 7.4 when aerated with 95% O<sub>2</sub>/5% CO<sub>2</sub>). Transversal, 200-300 µm thick slices of the brainstem were cut with a vibrotome (DTK-1500E, Ted Pella, Redding, CA) and slices containing the LSO were used for Fura-2 labeling.

Fura-2 labeling. Slices from P0-P7 animals were labeled with Fura-2 AM using bulk-labeling, as described previously (Ene et al., 2003). In slices from animals older than P7 LSO neurons labeled poorly or not at all using the bulk-labeling method, most likely due to poor penetration of Fura-2AM through the extracellular matrix. Therefore, slices from P9-P20 were labeled using a novel spin-labeling procedure. Slices were placed on filter paper (12 µm pores; Corning Incorporated Life Sciences, Acton, MA) in an interface-type chamber and after 15-30 minutes were transferred into a microcentrifuge tube equipped with a 10 kDa cutoff molecular filter (Amicon, Milipore Corporation, Bedford, MA). Slices were covered with 100 µM Fura-
2AM and aerated with 95% O₂/5% CO₂. The microcentrifuge tube was centrifuged for 15-20 min at approximately 430g (IEC Clinical Centrifuge, International Equipment Company, USA) forcing the Fura-2AM solution to pass through the slice. Slices were then removed from the microcentrifuge tube, immediately washed with fresh aerated ACSF, and kept in an interface chamber in the dark at room temperature (22 - 25 °C) until used for Ca²⁺ imaging.

**Calcium imaging.** Ca²⁺ imaging was performed using an inverted epifluorescence microscope (Nikon Eclipse TE200) equipped with 10x and 20x air objectives (NA: 0.5 and 0.75 respectively) as previously described (Ene et al., 2003). Slices were continuously superfused with oxygenated ACSF at 30 - 32 °C (perfusion rate 2 - 3 ml/min) containing 100 μM Trolox to minimize photodamage (Scheenen et al. 1996). Fluorescence images were acquired every 5-10 s with a 12 bit, cooled, interline-transfer CCD camera (IMAGO, T.I.L.L. Photonics, Martinsried, Germany) using alternating excitation (duration 20-50 ms) at 340 nm and 380 nm (Polychrome II, T.I.L.L. Photonics).

In some experiments, Fura-2 fluorescence was converted to intracellular Ca²⁺ concentrations ([Ca²⁺]ᵢ) as previously described (Ene et al. 2003) using the equation: 

\[ [Ca^{2+}]_i = K_d \beta (R - R_{min})/(R_{max} - R), \]

where \( R_{min} \) is the fluorescence ratio of Ca²⁺ free Fura - 2, \( R_{max} \) is the ratio of Ca²⁺ bound Fura - 2, \( \beta \) is the ratio of the fluorescence intensity of Ca²⁺ free Fura - 2 at 380 nm to the fluorescence intensity of Ca²⁺-bound Fura - 2 at 380 nm (Grynkiewicz et al. 1985). The Ca²⁺ \( K_d \) value for Fura-2 was taken as 224. \( R_{min} \) and \( R_{max} \) were determined by incubating the slices first in Ca²⁺ - free ACSF (Ca²⁺ was replaced with equimolar concentration of Mg²⁺) with 2 mM EGTA and 4 μM Ca²⁺ ionophore Br-A23187 (Alomone Labs, Jerusalem, Israel). CaCl₂ (10 mM ) was then added to determine \( R_{max} \). The imaging system was calibrated repeatedly over the


course of this study and $R_{\text{min}}$ varied from 0.25 to 0.31, $R_{\text{max}}$ varied from 1.22 to 1.8, and $\beta$ varied from 2.3 to 3.7.

**Drug application.** Drugs from concentrated stock solutions were dissolved in ACSF and delivered via bath application. Group I mGluRs were stimulated using the specific agonist (S)-3,5-dihydroxyphenylglycine (DHPG 20-50 $\mu$M). All experiments were performed in the presence of antagonists of ionotropic GluRs, glycine receptors, and GABA$_A$ receptors. Ionotropic GluRs were blocked by 6-Cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX, 20 $\mu$M, Tocris, Ballwin, MO) and DL-2-Amino-5-phosphonopentanoic acid (DL-APV, 100 $\mu$M, Tocris, Ballwin, MO). Glycine receptors were blocked by strychnine (10 $\mu$M; Sigma, St. Louis, MO) and GABA$_A$ receptors were blocked by Bicuculline (10 $\mu$M; Tocris, Ballwin, MO). Tetrodotoxin (TTX 1 $\mu$M; Alomone Labs Jerusalem, Israel) was added to block glutamate release due to spontaneous action potentials. For TRP channel antagonists we used Ni$^{2+}$, La$^{3+}$ (Sigma, St. Louis, MO) and 2-APB (Calbiochem, San Diego, CA).

**Data analysis.** Series of 340 nm and 380 nm pair images were low-pass filtered (Gaussian 3x3 kernel) and background subtracted using the program Tillvision (T.I.L.L. Photonics) as described previously (Ene et al. 2003). Ca$^{2+}$ responses were monitored and measured from the soma of neurons. Cells with high resting [Ca$^{2+}$]$_i$ (>250nM; indicating unhealthy cells; (Zirpel and Rubel 1996)), cells in which the Fura-2 signal was saturated ([Ca$^{2+}$]$_i$ >1500 nM; as Fura-2 does not faithfully report [Ca$^{2+}$]$_i$ above 1.5-2 $\mu$M), and cells in which [Ca$^{2+}$]$_i$ did not return to baseline after stimulation were excluded from analysis. Changes in [Ca$^{2+}$]$_i$ that exceeded 2 standard deviations (SD) of the baseline and changes in $\Delta R/R > 0.1$ were considered as responses. Excel (Microsoft, Redmond, WA, USA), Origin (OriginLab Corporation, Northampton, MA, USA) and Matlab (MathWorks, Natick, MA, USA) were used for data analysis.
Responses elicited by the specific group I mGluR agonist DHPG (50 µM, 90s) were quantified and categorized using the following parameters: peak amplitude, plateau amplitude, duration, and area under the curve. Traces were aligned to the onset of the response which was considered time 0. Baseline [Ca^{2+}] was determined as the average of 5 data points before response onset (in the window -50s to 0s). Peak amplitude was measured in the time window 0s to 40s. Plateau amplitude was determined by averaging 3-5 data points in the window 80s to 100s. Response duration was measured from the onset of the response until the response returned to baseline. Based on these measurements, responses were classified into three response types (Table 1, Fig. 2): peak and plateau (pp), peak small plateau (psp) and peak no plateau (pnp). In pp responses the peak response was followed by a clear plateau (peak amplitude 236 ± 50 nM; plateau amplitude 107 ± 22 nM, n=287 cells), with a duration that always exceeded the 90s duration of drug application (182 ± 13 s, n=287 cells). In pnp responses the plateau phase was completely missing, the peak amplitude was small (60 ± 8 nM, n=177 cells), and the duration was always shorter than the duration of drug application (70 ± 3 s, n=177 cells). The intermediate group of psp responses was characterized by a gradually decreasing plateau phase which always exceeded the duration of drug application (141 ± 15s, n=124 cells).

**Statistical analysis.** Statistical significance was analyzed using paired t-tests, ANOVA followed by Student Newman-Keuls post-hoc test, Fisher’s exact test, linear (Pearson) correlation test, Mann-Whitney non-parametric statistical test, Kolmogorow Smirnov test and chi-square test. Values of p<0.05 were considered significant. Throughout the text, values are expressed as mean ± SEM.
Results

Group I mGluR-elicited Ca$^{2+}$ responses were analyzed in 738 LSO neurons from 51 C57Bl6 mice aged between P0-19, in 60 LSO neurons from 6 heterozygote waltzer mice, and in 156 LSO neurons from 14 waltzer Cdh23 homozygote mice aged between P11-20. All cells included in the analysis responded to KCl-evoked depolarizations (60 mM, 30 s) with peak amplitudes ranging from around 100 nM to Fura-2 saturating responses (>1.5μM).

Spin-labeling of LSO neurons in older slices

In order to overcome the poor labeling with Fura-2 AM of slices from animals older than P7 we developed a new method, spin-labeling, which uses centrifugation to force Fura-2 AM into the slice. To evaluate whether this new method affects Ca$^{2+}$ responses of LSO neurons, the two labeling methods were compared in slices from P0-5 animals. At this age, both methods produced a similar number of cells loaded with Fura-2 (bulk-labeling: n = 7.39 ± 0.92 cells/100 μm$^2$, N= 7 slices; spin-labeling: n=7.98 ± 0.37 cells/100μm$^2$, N=3 slices; t-test p>0.05) and a similar somatic florescence intensity at 360 nm, the isosbestic point of Fura-2, measured with identical settings (bulk-labeling: 338 ± 67 AU, N= 7 slices; spin-labeling: 272 ± 108 AU, N=3 slices; t-test p>0.05; Fig. 1A). Resting somatic Ca$^{2+}$ concentrations were not significantly different (bulk-labeling: 94.2 ± 47.6 nM, n=96 cells, N=4 slices; spin-labeling: 109.2 ± 57.6 nM, n=70 cells, N=3 slices; t-test p>0.05). Finally, Ca$^{2+}$ responses elicited by bath application of the selective group I mGluR agonist (S)-3,5-dihydroxyphenylglycine (DHPG, 50 μM, 90s) and by KCl depolarization (60mM, 30s) were undistinguishable (Fig. 1B). These results suggest that spin-labeling, as compared to conventional bulk-labeling, does not change the basic Ca$^{2+}$ response properties of LSO neurons.
Developmental changes of group I mGluR-mediated Ca$^{2+}$ responses

In LSO neurons from neonatal mice (P0-5), Ca$^{2+}$ responses elicited by DHPG (50 µM, 90s) consisted of an initial peak which was followed by a large plateau phase that always exceeded the duration of the agonist application (Fig. 2). During postnatal development, these Ca$^{2+}$ responses gradually changed their profile, amplitude and duration (Fig. 2 and Table 1). At P9-12, around hearing onset (~P10-12, (Song et al. 2006; Shnerson and Pujol 1981), peak and plateau amplitudes decreased and response durations had become shorter. By the end of the third postnatal week, plateau phases were absent in almost all responses. Response amplitudes, durations, and areas were significantly larger in the P0-5 group compared to all older age groups (Mann-Whitney Test p<0.05) and were significantly smaller in the P17-19 group compared to all younger age groups (Mann-Whitney Test p<0.05; Fig. 2B and Table 1, supplemental figure 2). Plateau amplitudes and response durations also significantly decreased from P9-12 to P13-16 (Mann-Whitney Test p<0.05; Fig. 2B and Table 1).

In the first postnatal week, all Ca$^{2+}$ responses elicited by DHPG showed a prominent plateau and were categorized as pp responses ( n=222/222 cells, Fig. 2C). With increasing age, the frequency of pp responses decreased, from 100% in at P0-5 to 24% at P9-12, (n=46/178 cells), 11% at P13-16 (n=15/132 cells), and to 7% at P17-19 (n=4/54 cells) (Fig. 2C). Responses with a peak and small plateau (psp responses) were found in all older age groups. The relative occurrence of responses that completely lacked a plateau (pnp responses) was age dependent as well, steadily increasing from 32% at P9-12 (n=59/178 cells), to 57% at P13-16 (n=75/132), and to 80% at P17-19 (n=43/54 cells).
In summary, during the first three weeks of postnatal development, group I mGluR-elicited Ca\(^{2+}\) responses gradually lose the sustained plateau phase which, in neonatal mice, is caused by an influx of extracellular Ca\(^{2+}\) through a channel not sensitive to VGCC blockers (Ene et al. 2003). Next we used pharmacological tools to investigate the sources of [Ca\(^{2+}\)], triggered by group I mGluRs in developing LSO neurons in more detail.

**Contribution of extracellular Ca\(^{2+}\) to group I mGluR-elicited responses decreases during development**

To determine the age dependency of mGluR-elicited influx of extracellular Ca\(^{2+}\) and its contribution to the three types of responses (pp, psp, pnp) we compared mGluRs-elicited Ca\(^{2+}\) responses in standard and nominally Ca\(^{2+}\)-free ACSF (Fig. 3). Regardless of age, eliminating extracellular Ca\(^{2+}\) significantly reduced both the peak and the plateau phase of responses of the pp and psp type (P0-5 pp response: peak reduced to 32 ± 2 % and area to 13 ± 1 %, n=82; P9-12 psp response: peak reduced to 23 ± 4 % and area to 17 ± 4 %, n=14; P13-16 psp response: peak reduced to 44 ± 10 % and area to 29 ± 6 %, n=22). For pnp responses, however, removal of extracellular Ca\(^{2+}\) had no significant effect on response amplitudes or areas (P13-16 pnp response: peak reduced to 63 ± 11 % and area to 80.9 ± 12.7 %, n=19; P17-18 pnp response: peak reduced to 71 ± 12 % and area to 88 ± 14 %, n=16). Thus, in pp and psp responses extracellular Ca\(^{2+}\) influx accounted for 60 - 85%, while in pnp responses, extracellular Ca\(^{2+}\) contributed little (10 - 25%, statistically not significant). Finally, the hypothesis that mGluRs activate a membrane ion channel is supported by electrophysiological whole-cell patch clamp recordings from neonatal LSO neurons demonstrating that DHPG elicits long-lasting membrane depolarizations (Suppl. Material).
In summary, during LSO development, the contribution of external Ca\(^{2+}\) influx to group I mGluR-elicited responses diminished progressively until it was absent in three week-old mice, while the contribution of Ca\(^{2+}\) release from internal stores was present throughout the investigated period. We next investigated the pharmacological characteristics of the channel that mediates mGluR-elicited influx of extracellular Ca\(^{2+}\).

**MGlur-evoked extracellular Ca\(^{2+}\) influx is blocked by Ni\(^{2+}\)**

In neonatal animals, short application of Ni\(^{2+}\) (2 mM for 2 min) during the plateau phase transiently abolished the response, indicating that the plateau is mediated by a Ni\(^{2+}\) sensitive channel (Fig. 4 Ai). Application of Ni\(^{2+}\) during the peak substantially decreased the peak amplitude to \(14 \pm 2\%\) of its initial value (n = 10 cells, P5), indicating the contribution of Ni\(^{2+}\) sensitive Ca\(^{2+}\) channels to the peak as well (Fig. 4 Aii). In cells with pp and psp responses, DHPG application in the presence of Ni\(^{2+}\) only elicited small peak responses without plateaus (Fig. 4Aiii-vi). In contrast, in cells with pnp responses, Ni\(^{2+}\) had no effect (pnp responses, P13-16: n=38; P17-18: n=23) (Fig. 4Avii, 4Aviii). This is consistent with our finding that extracellular Ca\(^{2+}\) contributes little, if any, to pnp responses (Fig. 3B). As a positive control, in cells with pnp responses, Ni\(^{2+}\) significantly reduced subsequent Ca\(^{2+}\) responses elicited by KCl depolarization (60 mM, 30s) (66 ± 7% reduction at P13-16, n=38, and 80 ± 2% reduction at P17-19, n=23; t-test p<0.05).

In a small fraction of cells (P13-16, n=7 cells, N=3 slices), Ni\(^{2+}\) potentiated the amplitude of pnp responses about 4 fold (data not shown). It is possible that these cells represent a different population of LSO neurons (Ollo and Schwartz 1979), perhaps expressing different channel types.
DHPG-mediated Ca$^{2+}$ responses are sensitive to 2-APB

In several neuron types, mGluRs can activate Ca$^{2+}$ permeable TRP channels (Kim et al. 2003; Tozzi et al. 2003; Bengtson et al. 2004). Because in LSO neurons, DHPG-elicited extracellular Ca$^{2+}$ influx is independent of voltage gated Ca$^{2+}$ channels (Ene et al. 2003) but is blocked by Ni$^{2+}$, an unspecific blocker of TRP channels (Parekh et al. 1997), LSO neurons might express mGluR-activated TRP channels. To test this possibility, we used 2-Aminoethoxydiphenyl borate (2-APB) which, in addition to its well described function as a membrane permeable IP$_3$ receptor (IP$_3$R) inhibitor, also can act from the extracellular site to block TRP channels (Maruyama et al. 1997; Ma et al. 2001; Bootman et al. 2002).

At concentrations of 2 - 5 $\mu$M, 2-APB increased DHPG-elicited Ca$^{2+}$ responses in LSO neurons (Fig. 5), consistent with what has been reported in other neuronal and non-neuronal cells (Prakriya and Lewis 2001; Bootman et al. 2002; Tozzi et al. 2003). At concentrations of 10-50 $\mu$M, 2-APB reduced both peak and plateau amplitudes with the effect being more pronounced on the plateau phase, consistent with the hypothesis that TRP channels contribute more to the plateau phase than to the peak. Finally, at concentrations above 50 $\mu$M, 2-APB abolished the response completely, most likely by inhibiting intracellular IP$_3$ receptors and blocking Ca$^{2+}$ release from intracellular stores which is necessary to active extracellular Ca$^{2+}$ influx (Ene et al. 2003). Washout of 50 $\mu$M 2-APB for 30 minutes restored the peak component to 87 $\pm$ 3%, while the plateau recovered only to 16 $\pm$ 2% (n=65 cells). 2-APB did not reduce KCl-evoked Ca$^{2+}$ responses (Fig. 5D) consistent with its reported lack of inhibiting VGCCs (Maruyama et al. 1997; Bootman et al. 2002) and further supporting our previous finding that mGluR-elicited influx of extracellular Ca$^{2+}$ is independent of VGCCs (Ene et al. 2003).
Taken together, the sensitivity of DHPG-elicited Ca\textsuperscript{2+} response plateaus to Ni\textsuperscript{2+} and 2-APB and its insensitivity to specific VGCC blockers suggests that the plateau component is mediated by Ca\textsuperscript{2+} influx through a TRP-like channel.

**DHPG-mediated responses are sensitive to La\textsuperscript{3+}**

Most members of the TRPC channel family are either inhibited (TRPC3, TRPC6 and TRPC7; (Zhu et al. 1998; Inoue et al. 2001; Okada et al. 1999) or potentiated (TRPC4 and TRPC5; (Schaefer et al. 2000; Strubing et al. 2001) by micromolar concentrations of La\textsuperscript{3+}. In neonatal LSO neurons (P0-5), La\textsuperscript{3+} reduced DHPG-responses in a dose-dependent manner and completely abolished them at 1mM (Fig. 6A). La\textsuperscript{3+} at 1 mM also completely abolished KCl responses (n=49 cells, data not shown), consistent with the known effect of La\textsuperscript{3+} on VGCCs (Ozawa et al. 1989). In P9-P12 animals, La\textsuperscript{3+} potentiated responses at 0.1 mM and 0.5 mM but had no effect at 1mM (Fig. 6 B,C). Together, sensitivity of DHPG-evoked responses to La\textsuperscript{3+} provides additional support that mGluR-evoked Ca\textsuperscript{2+} responses involve the activation of TRPC channels. The age-dependent transition from inhibition to potentiation might be due to an age-dependent expression of different TRP channels in LSO neurons.

**Developmental downregulation of mGluR-mediated Ca\textsuperscript{2+} responses is impaired in deaf animals.**

The downregulation of group I mGluR-elicited Ca\textsuperscript{2+} responses and the disappearance of the plateau phase after hearing onset raises the question of whether these changes depend on auditory-evoked neuronal activity. To explore this idea, we compared DHPG-elicited Ca\textsuperscript{2+} responses from hearing wild-type C57/Bl mice (wt) to responses from age-matched deaf waltzer
mice. In these experiments, we assessed hearing ability by the presence of a startle response to hand clapping. All wt mice older than P11 but none of the waltzer mice showed a clear startle response. LSO neurons from deaf waltzer mice showed DHPG-elicited Ca$^{2+}$ responses of the pp, psp, and pnp types, (Fig. 7A). Waltzer LSO neurons also showed an age-dependent decrease in peak amplitudes, areas, and duration of responses (P11-16: n=107 cells, P17-20: 58 cells; Mann-Whitney test p<0.05). However, compared to age-matched wt mice, these changes were significantly different in waltzer mice (Fig. 7B). In both the P11-P16 group and P17-20 group, peak amplitudes were larger in waltzer mice than in aged-matched wt-mice (P11-16: waltzer n=107, wt n=68; P17-20: Waltzer n=58, wt n=72; Kolmogorow Smirnov test p<0.05). This difference may be due to a developmental delay because peak amplitudes from P17-20 waltzer mice were not significantly different from those from P11-16 wt-mice (Kolmogorow Smirnov test p>0.05; Fig. 7Bi). Similarly, response durations were significantly larger in waltzer mice compared to aged-matched wt-mice (Kolmogorow Smirnov test p<0.01, Fig. 7Bii). However, response durations in P17-20 waltzer mice were shorter than P11-16 wt-mice (Kolmogorow Smirnov test p<0.01).

We also investigated DHPG-elicited Ca$^{2+}$ responses in heterozygote waltzer mice which can hear but have elevated compound action potential thresholds and are more prone to noise induced hearing loss (Holme and Steel, 2004). The duration of DHPG-elicited Ca$^{2+}$ responses in LSO neurons from hearing (startle positive) heterozygote mice (N= 4 animals) was the same as in neurons from hearing wild-type mice (N=11 animals) (P11-16: het mice n=44, wt n=68; P17-20: het mice n=16, wt n=72; Kolmogorow Smirnov test p>0.1) (Fig. 8A, B). AT P11-P16, responses were significantly shorter in heterozygote mice compared to homozygote waltzer mice (N=6 animals; n= 107 cells, Kolmogorow Smirnov test p<0.005). In heterozygote mice,
response durations in startle-negative P12-13 mice were significantly shorter compared to startle-positive P14-16 mice (Fig. 8 C, D).

Similar to wt-mice, in waltzer mice we also observed a developmental decrease in the percentage of DHPG-responses of the pp and psp type and a developmental increase in the percentage of pnp responses (Fig. 9; Fisher's Exact Test $p<0.05$). However, in waltzer mice, more cells showed pp and psp responses at P11-16 (Fig. 9A; waltzer n=80 cells, wt n=32 cells; Fisher's Exact Test $p<0.05$).

Taken together, in deaf waltzer mice the age-dependent changes of mGluR-mediated Ca$^{2+}$ responses were significantly smaller, suggesting that auditory experience is necessary for the normal time course of the functional downregulation of TRP-like channel(s). In support of this hypothesis, in P16 waltzer mice, DHPG-elicited responses were strongly diminished by the TRP channel antagonist Ni$^{2+}$ (peak amplitude reduced by 80.5 ± 4.7%; area reduced by 82.4 ± 4.2%; n= 21 cell with responses in Ni$^{2+}$).

Discussion

This study provides a developmental picture of the Ca$^{2+}$ entry pathways underlying group I mGluR-mediated Ca$^{2+}$ responses in developing LSO neurons. In neonatal mice, these Ca$^{2+}$ responses were characterized by a large transient peak followed by a sustained plateau. We identified two Ca$^{2+}$ sources that contributed to these responses: release of Ca$^{2+}$ from internal stores and influx of Ca$^{2+}$ from the extracellular milieu. The extracellular component was sensitive to the TRP channel blockers Ni$^{2+}$, 2-APB, and La$^{3+}$ suggesting that it was mediated by a TRP-like Ca$^{2+}$ channel. With increasing age, the contribution of extracellular Ca$^{2+}$ influx to
group I mGluR-mediated Ca\textsuperscript{2+} responses diminished and was mostly absent after hearing onset, while the contribution of internal stores was unchanged. Finally, the developmental downregulation of mGluR-elicited extracellular Ca\textsuperscript{2+} influx was delayed in deaf waltzer mice suggesting that age-dependent decrease in the activation and/or expression of TRP channels is influenced by auditory experience.

**Developing LSO neurons express functional TRP-like channels**

Our results suggest that LSO neurons in pre-hearing mice express Ca\textsuperscript{2+} permeable, TRP-like channels which are activated by group I mGluRs. This is supported by the following observations. *First*, the typical biphasic peak-plateau profile of mGluRs-elicited Ca\textsuperscript{2+} responses in LSO neurons resembles the response profiles that have been observed in a number of other neuronal and non-neuronal cell types which express store operated Ca\textsuperscript{2+} channels or TRP channels (Moller et al. 1997; Sosa et al. 2002; Hu et al. 1994; Boulay et al. 1997; Ramsey et al. 2006). *Second*, the plateau phase of mGluR-elicted Ca\textsuperscript{2+} responses was mediated entirely by influx of extracellular Ca\textsuperscript{2+} (Ene et al. 2003). *Third*, extracellular Ca\textsuperscript{2+} influx was insensitive to specific blockers of voltage-gate Ca\textsuperscript{2+} channels (Ene et al. 2003) but was sensitive to blockers of TRP channels such as Ni\textsuperscript{2+}, 2-APB, and La\textsuperscript{3+} (Fig. 4-6). Fourth, activation of mGluRs by the specific agonist DHPG resulted in depolarizations (Suppl. Material) similar to the activation of TRP channels in other neuronal systems (Kim et al. 2003. Tempia et al. 2001).

TRP channels can be activated by a variety of intracellular messengers depending on the exact TRP channel and cell type. In LSO neurons, Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores is required for coupling mGluR activation to the opening of TRP-like channels; based on the observation that depletion of intracellular Ca\textsuperscript{2+} stores with thapsigargine abolished all mGluR-
elicited Ca\textsuperscript{2+} responses (Ene et al. 2003). However, Ca\textsuperscript{2+} does not appear to be the only signal responsible for activation of TRP-like channels because influx of Ca\textsuperscript{2+} through VGCC while intracellular Ca\textsuperscript{2+} stores were depleted by thapsigargine did not trigger long-lasting Ca\textsuperscript{2+} plateaus. Further studies are needed to identify what additional components of the group I mGluR activated intracellular cascade are the relevant stimuli that, together with Ca\textsuperscript{2+}, activate the opening of TRP-like channels in LSO neurons.

Which TRP channels are responsible for group I mGluR-mediated Ca\textsuperscript{2+} responses in LSO neurons? All members of the TRPC channel family (TRPC 1-7) are expressed in the brain (Riccio et al. 2002; Minke and Cook 2002), though their expression patterns have not been investigated specifically for the LSO. TRPC channels are activated by an increase in [Ca\textsuperscript{2+}]i and/or diacylglycerol (Hofmann et al. 1999; Kim et al. 2003; Zitt et al. 1997; Schaefer et al. 2000; Okada et al. 1998; Boulay et al. 1997) and it has been suggested that the TRPC3 channel mediates group I mGluR-mediated Ca\textsuperscript{2+} responses in retinal amacrine cells (Sosa et al. 2002). TRPC3 also seems to be a good candidate for LSO neurons because TRPC3 is strongly expressed in the embryonic and early postnatal brainstem, including in auditory brainstem nuclei (Li et al. 1999), and because TRPC3 expression levels decrease after P10, which corresponds to the time when group I mGluR-mediated Ca\textsuperscript{2+} responses in LSO neurons also decrease. Finally, DHPG-evoked responses in neonatal LSO neurons were inhibited by millimolar concentrations of La\textsuperscript{3+} (Fig. 6), concentrations which are in the range of TRPC3 sensitivity to La\textsuperscript{3+} (Zhu et al. 1998).

Developmental decrease in the contribution of TRP-like channels to mGluR-mediated Ca\textsuperscript{2+} responses and the role of sensory-evoked activity
During the first three postnatal weeks, group I mGluR-mediated Ca\(^{2+}\) responses became progressively smaller in amplitude, shorter in duration, and the plateau component disappeared (Fig. 2). These changes are consistent with the idea that the decrease in mGluR-elicited Ca\(^{2+}\) responses resulted, in large part, from a developmental downregulation of TRP-like channels or their functional uncoupling from mGluR activation. In support of this, both Ni\(^{2+}\), which reduced the peak amplitude and abolished the Ca\(^{2+}\) plateau by blocking the influx of extracellular Ca\(^{2+}\) in neonatal LSO neurons, and removal of extracellular Ca\(^{2+}\) (Fig. 3) had no effect on responses during the third postnatal week, when most responses were small and short and were mediated primarily by Ca\(^{2+}\) release from internal stores (Fig 4. A, B).

The contribution of extracellular Ca\(^{2+}\) influx to mGluR-mediated Ca\(^{2+}\) responses declined at, and shortly after, hearing onset (~P12, (Song et al. 2006) raising the possibility that sensory activity plays a role in the functional downregulation of TRP-like channels. We tested this hypothesis by investigating mGluR-mediated Ca\(^{2+}\) responses in developing waltzer mice. Waltzer mice have a spontaneous mutation in the gene for Cadherin 23 which causes a disorganization of stereocilia bundles of cochlear hair cells resulting in deafness (Di Palma et al. 2001; Holme and Steel 2002). In waltzer mice, the changes in group I mGluR-mediated Ca\(^{2+}\) responses appeared to be significantly delayed as compared to heterozygote and wild type control mice (Fig. 7, 8). Notably, more LSO neurons in waltzer mice than in age-matched control mice displayed responses with a long Ca\(^{2+}\) plateau and fewer neurons displayed peak-only responses. These results support the idea that auditory experience is important for the developmental downregulation of TRP-like channel expression or their developmental uncoupling from mGluR activation.
It should be noted that our results cannot exclude the possibility that spontaneous activity before hearing onset also contributes to the downregulation of TRP-like responses. Further studies which investigate mGluR-mediate Ca$^{2+}$ responses in pre-hearing waltzer mice and characterize spontaneous activity patterns \textit{in vivo} are necessary to conclusively address this possibility. Another issue to consider is that cadherin 23 is not only expressed in hair cells but in the brain as well (Di Palma, 2001; Rzadzinska et al., 2005) although it is currently unknown whether Cadherin 23 is expressed in the LSO or in any other auditory nuclei. Anatomical studies investigating cadherin expression in developing LSO neurons are required to address the question whether impairment in the developmental decrease in TRP-like channels activation by mGluRs in waltzer mice is influenced by potential changes of cadherin 23 in the LSO. Nevertheless, our conclusion that auditory experience is an important contributing factor to the downregulation of TRP activation by mGluRs is supported by our results from heterozygote waltzer mice. Heterozygotes, after hearing onset, as indicated by the presence of an acoustic startle response, showed mGluR-mediate Ca$^{2+}$ responses similar to age-matched wild type mice and in P12-P16 heterozygotes response durations became significantly shorter right after hearing onset (Fig. 8 C,D).

\textbf{Functional implications}

Similar to other neuronal types, in neonatal LSO neurons mGluRs are activated preferentially by high frequency stimulation of glutamatergic inputs (Ene et al. 2003). Spontaneous high-frequency bursts of neuronal activity exist in a variety of auditory nuclei before hearing onset (Gummer and Mark 1994; Kros et al. 1998; (Lippe) 1994; Kotak and Sanes 1995; Leao et al. 2006; Jones et al. 2001; Durham et al. 1989) making it likely that TRP-like
channels in LSO neurons are activated by spontaneous burst-like activity \textit{in vivo}. In addition to LSO neurons, opening of TRP channels by mGluR activation also seems to occur in other auditory brainstem neurons. For example, in neonatal rat MNTB neurons, DHPG elicits inward currents (Kushmerick et al. 2004) and in preliminary studies we observed long lasting Ca$^{2+}$ responses with typical peak-plateau profiles following DHPG application in the MNTB.

Spontaneous activity before hearing onset is important for numerous developmental processes including neuronal survival of auditory brainstem neurons (Sie and Rubel 1992; Zirpel and Rubel 1996; Sie and Rubel 1992) and various other aspects of auditory brainstem circuits (Kitzes et al. 1995; Sanes and Takacs 1993; Kotak and Sanes 1997; Russell and Moore 1995; Gabriele et al. 2000). Intracellular Ca$^{2+}$ plays a central role in linking neuronal activity to the survival of auditory brainstem neurons (Zirpel et al. 1995; Lachica et al. 1995; Lohmann et al. 1998; Lohmann et al. 1998) and has been implicated in activity-depended refinement of LSO circuitry (Kotak and Sanes 2000). TRP-like channels, by greatly amplifying the initial mGluR-elicited Ca$^{2+}$ response (Fig. 3, 4), may provide an important function in translating burst-like synaptic activity into functional LSO circuitry, perhaps by influencing growth cone behavior (Li et al. 2005; Wang and Poo 2005), regulating neurite length (Greka et al. 2003), or by adjusting synaptic strength (Baba et al. 2003). In the LSO, TRP-like channels are activated preferentially before hearing onset which is the period of major developmental changes that include dendritic and synaptic refinement (Sanes and Takacs 1993; Kim and Kandler 2003; Rietzel and Friauf 1998; Sanes et al. 1992), activity-dependent plasticity (Kotak and Sanes 2000), and a switch of neurotransmitter phenotype (Kotak et al. 1998; Nabekura et al. 2004; Gillespie et al. 2005).
It will be interesting to find out whether and to what degree mGluR activation and TRP channels are involved in mediating any or which of these events and, therefore, contribute to the development of normal sound localization and hearing.

While activation of TRP channels by mGluRs might play an important role in the cellular detection and encoding of spontaneous bursts of spontaneous activity, auditory experience-dependent downregulation of TRP channel activation might protect LSO neurons from reaching toxic levels of [Ca$^{2+}$]$_i$ after hearing onset when glutamatergic inputs to LSO neurons fire at high rates and when LSO neurons express Ca$^{2+}$ permeable AMPA receptors (Caicedo et al. 1998).

Finally, TRP-channels might plausibly be the mechanism responsible for long-lasting depolarizations that occur in LSO neurons of neonatal gerbils following tetanic stimulation of glutamatergic cochlea nucleus afferents (Kotak and Sanes 1995). These long-lasting depolarizations occur during the first two postnatal weeks, require mGluR receptors activation, and are sensitive to Ni$^{2+}$, properties which parallel mGluR-mediated TRP-channel activation.

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Figure legend

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**Figure 5. Effect of 2-APB on group I mGluR-mediated Ca$^{2+}$ responses.** **A.** Effect of increasing concentrations of 2-APB on DHPG-elicited Ca$^{2+}$ responses in a P10 LSO neuron **B.** Summary data. **C.** Effect of 2-APB on KCl-elicited (60 mM) Ca$^{2+}$ responses. Data are from the same cells as in B.

**Figure 6. Effect of La$^{3+}$ on group I mGluR-mediated Ca$^{2+}$ responses.** **A.** Dose-dependent effect of La$^{3+}$ on peak and area of DHPG-evoked (50mM, 90s) responses at P0-5. **B,C.** In LSO
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**Figure 7.** Group I mGluR-mediated Ca\(^{2+}\) responses in deaf waltzer mice. **A.** Examples of DHPG-elicited Ca\(^{2+}\) responses in wild-type and waltzer mice. **B.** Cumulative distribution plots of peak (i) and duration (ii) of DHPG-elicited responses of LSO neurons from wild-type (wt, black lines, P11-16: n=68 cells, P17-20: n=72 cells) and waltzer mice (wz, grey lines, P11-16: n=107 cells, P17-20: n=58 cells). P-values for Kolmogorov Smirnov test.

**Figure 8.** Durations of group I mGluR-mediated Ca\(^{2+}\) responses in waltzer, heterozygote, and wild-type mice. **A.** Cumulative distribution of DHPG-elicited Ca\(^{2+}\) response durations in P11 to P16 LSO neurons from homozygote waltzer (wz, grey line, n=107 cells), hearing heterozygote (het, dotted line, n=44 cells), and hearing wild-type mice (wt, black line, n=68 cells). The durations in heterozygote mice were significantly shorter compared to homozygote mice (p<0.005) and were not different to wild-type mice (p>0.1). **B.** Same as (A) but for postnatal day 17-20. The durations in heterozygote mice were not significantly different from wild-type or homozygote mice (wz: n=58 cells; het: n=16 cells; wt: n=72 cells). **C.** Response durations in heterozygote mice with a startle response (black line; P14-16, n=44 cells) and mice lacking a startle response (grey line; P12-13, n=29 cells). Durations in mice lacking a startle response were significantly longer than in mice with a startle response (p<0.001). **D.** Examples of DHPG-elicited Ca\(^{2+}\)-responses from heterozygote mice with and without startle response. Scale bars, 0.02 \(\Delta R/R\) (upper traces), 0.01 \(\Delta R/R\) (lower traces), 50 s. P-values for Kolmogorov Smirnov test.
Fig. 9. Developmental changes in response types in wild-type and waltzer mice.

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Table 1. Parameters describing mGluR-elicited calcium responses in developing LSO neurons.

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<td>215±8</td>
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N= total number of cells tested; n= number of cells in a response group; Data are mean ± SEM
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