Glutamatergic inputs and glutamate-releasing immature inhibitory inputs activate a shared postsynaptic receptor population in lateral superior olive

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Abstract

Principal cells of the lateral superior olive (LSO) compute interaural intensity differences by comparing converging excitatory and inhibitory inputs. The excitatory input carries information from the ipsilateral ear and the inhibitory input carries information from the contralateral ear. Throughout life, the excitatory input pathway releases glutamate. In adulthood, the inhibitory input pathway releases glycine. During a period of major developmental refinement in the LSO, however, synaptic terminals of the immature inhibitory input pathway release not only glycine, but also GABA and glutamate. To determine whether glutamate released by terminals in either pathway could spill over to activate postsynaptic NMDA receptors under the other pathway, we made whole-cell recordings from LSO principal cells in acute slices of neonatal rat brainstem bathed in the use-dependent NMDA receptor antagonist MK-801, and stimulated in the two opposing pathways. We found that during the first postnatal week glutamate spillover occurs bidirectionally from both immature excitatory terminals and immature inhibitory terminals. We further found that a population of postsynaptic NMDA receptors is shared: glutamate released from either pathway can diffuse to and activate these receptors. We suggest that these shared receptors contain the GluN2B subunit and are located extrasynaptically.

Introduction

In mature mammals, binaural integration of acoustic information is first carried out in the brainstem, in nuclei of the superior olivary complex. Large bipolar principal cells of the lateral superior olive (LSO) compute interaural intensity differences used for localizing high-frequency sounds (Boudreau and Tsuchitani, 1968; Caird and Klinke, 1983). Input to the LSO from the ipsilateral ear is glutamatergic and arises from the ipsilateral anteroventral cochlear nucleus (AVCN) (Cant and Casseday, 1986; Wu and Kelly, 1992). Input from the contralateral ear is glycinergic and arises from the medial nucleus of the trapezoid body (MNTB), which is itself activated by the contralateral AVCN (Moore and Caspary, 1983;

Bledsoe et al., 1990; Smith et al., 1991). Both inputs are tonotopically organized, and the inputs from the two sides converge in precise tonotopic register (for review, see (Tollin, 2009).

The inhibitory MNTB-LSO pathway undergoes major developmental refinement during approximately the first postnatal week in rats and mice (Kim and Kandler, 2003, 2010). During this period, immature MNTB terminals within the LSO express both the vesicular GABA transporter VGAT (aka vesicular inhibitory amino acid transporter VIAAT) and the vesicular glutamate transporter VGLUT3. These terminals release the three classical small amino acid neurotransmitters—GABA, glycine, and glutamate—and the co-release of these transmitters may mediate development refinement in the MNTB-LSO pathway (Nabekura et al., 2004) (Gillespie et al., 2005; Kandler et al., 2009; Noh et al., 2010).

Although little is known about the relative positions of AVCN and MNTB synapses on immature LSO principal cells, glutamate spillover from AVCN terminals onto presynaptic terminals from the MNTB has been reported (Nishimaki et al., 2007), suggesting that these synapses are physically close to one another. NMDARs are present in the AVCN-LSO pathway (Ene et al., 2003), and during the first postnatal week terminals from both the MNTB-LSO and AVCN-LSO pathways release glutamate onto functional postsynaptic GluN2B-containing NMDA receptors (NMDARs) (Case et al., 2011); Case and Gillespie, 2011). Throughout the nervous system, the GluN2B subunit is expressed at high levels at early ages before replacement by GluN2A (Monyer et al., 1994; Sheng et al., 1994; Stocca and Vicini, 1998) and NMDAR subunit composition may be associated with specific developmental events (Yoshimura et al., 2003; Liu et al., 2004; Philpot et al., 2007; Zhao and Constantine-Paton, 2007). An intriguing feature of GluN2B-containing NMDARs is that they appear disproportionately often at extrasynaptic sites, where they may mediate synaptic crosstalk when activated by glutamate spillover (Tovar and Westbrook, 1999; Scimemi et al., 2004).

During early postnatal life, the immature expression of glutamate transporters may lead to a larger role for diffusion than for re-uptake in restricting glutamate to the synapse (Thomas et al., 2011). As glutamate is released from both AVCN-LSO and MNTB-LSO pathways from birth through postnatal day 8 (P8), we asked whether glutamate might diffuse far enough in the immature LSO to cause measurable crosstalk between postsynaptic receptors under immature excitatory and inhibitory synapses in the LSO. Using whole-cell voltage clamp recordings from LSO principal cells in acute slices obtained from rats before P9, we asked whether stimulation of either pathway could activate postsynaptic NMDARs under the opposite pathway. To measure previous NMDAR activation, we bathed the slices in saturating concentrations of the use-dependent blocker MK-801; in the presence of MK-801, NMDARs are blocked upon activation, such that decrement in the NMDAR-mediated response amplitude indicates the previous activation of NMDARs (Huettner and Bean, 1988).

Experimental Procedures

Slice preparation

Sprague-Dawley rats born to animals bred on site or shipped pregnant (Charles-River Laboratories) were used; all procedures were performed in accord with Canadian Council on Animal Care guidelines and were previously approved by the Animal Review Ethics Board of McMaster University. Rat pups aged postnatal day 3 to 8 (P3-8) were deeply anaesthetized (isoflurane) and the brain was quickly removed and placed in ice-cold cerebrospinal fluid (ACSF) containing (in mM): NaCl, 124; MgSO₄, 1; KCl, 5; KH₂PO₄, 1.25; dextrose, 10; NaCO₃, 26; CaCl₂·2H₂O, 2; kynurenic acid, 1, oxygenated with 95% O₂/5% CO₂. Coronal sections (300 µm) were obtained at the vibratome and slices containing LSO, MNTB and AVCN pathway (lateral acoustic stria) were allowed to recover at room temperature (19–21°C) for 1 hour in a humidified, oxygenated, interface chamber with fresh ACSF. The slice was then placed in the recording chamber and continuously perfused in oxygenated, kynurenic acid-free, ACSF (2.5–5 ml·min). In cases where Mg⁺⁺-free ACSF was used, Mg⁺⁺ was replaced by K₂SO₄ for dissection, recovery, and recording. Principal cells of the LSO were visually identified under IR-DIC by morphology and position within the LSO.

Recording protocols

Whole-cell recordings (Axoclamp 700B, Axon Instruments) were made at room temperature or at physiological temperature (defined here as 34–36°C in bath, maintained with ThermoClamp inline heater, AutoMate Scientific). Recording electrodes (2–4 MΩ, P97 puller, Sutter Instruments) were filled with intracellular solution containing (in mM): D-gluconic acid, 64; CsOH, 64; EGTA 11; CsCl, 56; MgCl₂, 1; CaCl₂, 1; HEPES, 10; and QX-314, 5. Series resistance was compensated (80%) and the seal monitored throughout each experiment. Cells were held at –70mV (in Mg⁺⁺-free ACSF) or at +40mV (in normal ACSF), as indicated. Cells were discarded if input resistance was <150 MΩ or if access resistance exceeded 25 MΩ or changed by >15%. Recordings were sampled at 10 kHz and filtered at 5 kHz (pClamp, Digidata 1400, Axon Instruments).

Stimulating electrodes (1 MΩ patch electrodes containing ACSF) were placed in the MNTB and in the lateral acoustic stria. After verifying glutamatergic responses to MNTB stimulation, stimulus strength was adjusted to the lowest intensity that produced a reliable response, and 5–10 traces were recorded without drugs in the bath. Strychnine (10µM), CNQX (5µM) and picrotoxin (50µM) were added to isolate the NMDA component, and baseline responses were collected in response to alternating stimulation (0.2 Hz; Master 8 stimulator with Iso-Flex SIU) in the MNTB and the lateral acoustic stria. Path 1 was assigned to MNTB if the MNTB response was very small, but otherwise was assigned to AVCN or MNTB randomly (AVCN was Path 1 42% of the time). DL-TBOA (50µM) was used in a subset of experiments. Drugs were obtained from Tocris, Ascent Scientific, and Sigma.

Sequential Paradigm S—Once the baseline responses were collected, stimulation was discontinued and the use-dependent blocker MK-801 (10 µM) was added to the bath for 10

minutes to allow saturation of the slice. After 10 minutes, 50 stimuli (0.2 Hz) were delivered to Path 1 (P_1), followed by 50 stimuli at 0.2 Hz to Path 2 (P_2). Time from onset of MK-801 incubation to first stimulus in P_2 was slightly under 15 minutes.

Interleaved Paradigm—After baseline collection, MK-801 was added to the bath and stimulation ceased for 15 minutes. Stimulation of P_1 and P_2 was interleaved at 0.2 Hz.

Minimal stimulation experiments—Older (P5-8) slices were used to maximize the likelihood of stimulating single fibers whose responses could be easily distinguished from noise. For each pathway, a stimulus strength was found that produced a failure rate of ~40%, and then the experiment was performed. The stimulus was unchanged for the duration of the experiment (baseline, initial MK-801 incubation, and test period).

Paired recordings—An LSO principal cell was identified and patched, and the microscope was re-centered at the MNTB. During visualization of the MNTB, small groups of candidate presynaptic neurons were identified by monitoring whole-cell responses at the LSO principal neuron while pressure-ejecting glutamate in the MNTB. Candidate presynaptic neurons were then stimulated in loose patch until one produced a postsynaptic response in the LSO.

Analysis was performed offline using Origin (OriginLab), MiniAnalysis (Synaptosoft), and custom-written Matlab (MathWorks) programs. Non-parametric (Wilcoxon and Mann Whitney) statistical tests were used to assess significance at alpha levels of 0.05 (significant) and 0.01 (highly significant). Rise-times (10–90%) were calculated from average response. Population statistics are reported as mean $\pm$ S.E.M.

Results

Glutamate spillover at room temperature

As glutamate release at MNTB-LSO synapses is most prevalent before postnatal day 9 (P9), we focused on slices from animals age P3-8. Little is known about the subcellular position of synapses in the immature LSO, and we asked whether synapses under the two pathways were sufficiently close to be within each other's glutamate diffusion radius at room temperature. To test for the possibility of spillover, we located LSO principal cells that showed a mixed GABA/glycine/glutamate response to MNTB stimulation (Fig. 1Aa, 'No drug' condition), pharmacologically isolated NMDAR-currents, and recorded Baseline responses to stimulation in AVCN-LSO and MNTB-LSO pathways (Fig. 1Aa, 'Baseline'). We then applied MK-801 using the sequential protocol *S* (see Methods), stimulating one pathway > 50 times (which fully decremented the response), and then stimulating the opposite pathway (Fig. 1Aa–b, 'Test'). The logic of this experiment was that glutamate spillover resulting from stimulation of the first path (P_1) would activate—and in the presence of MK-801 block—NMDARs in the silent path (P_2), thereby reducing the response for the first Test (T_1) in P_2 relative to baseline. Alternatively, if no glutamate spilled over, no NMDARs would be blocked in P_2 and the first Test response amplitude (T_1) for P_2 (denoted $S.P_2.T_1$) would be unchanged. Initially, simply to determine whether synapses under the two pathways were sufficiently close to one another to be within each other's glutamate diffusion

radius, we recorded at room temperature, under the assumption that low temperatures would depress activity of any functional glutamate re-uptake transporters (Bergles and Jahr, 1998).

For each pathway, a decrease in response amplitude to the first Test trial after MK-801 incubation (T_1) was taken to reflect NMDAR activation in the period between Baseline and Test. Although T_1 responses were reduced in both pathways, the reduction was larger in P_2 than in P_1 (EPSC amplitude [as % Baseline] for $S.P_1.T_1$ $84.9 \pm 3.6\%$; for $S.P_2.T_1$ $35.7 \pm 3.7\%$; $P=0.002$, $W=55$, $N=10$, Wilcoxon, Fig. 1B). As in the example shown here, the MNTB was stimulated first ($P_1 = \text{MNTB}$) in a subset of experiments (red symbols in Fig. 1B), whereas the AVCN-LSO pathway was stimulated first in other instances (green symbols). The reduced amplitude for the first trial in P_2 is consistent with glutamate spilling over from P_1 to activate and block NMDARs under P_2 . However, P_2 also experienced a longer MK-801 exposure before testing than did P_1 , and so these results might alternatively have occurred if non-specific activity in the slice during the longer MK-801 exposure had allowed NMDAR activation and block, independent of spillover from P_1 . We therefore modified the experiment to use an interleaved stimulus protocol *I* (Fig. 1Ca–b), in which the two pathways experienced nearly identical periods of MK-801 exposure (slightly longer than the P_2 exposure in protocol *S*) and Test stimuli were interleaved between pathways.

Results using protocol *I* also pointed to glutamate spillover between synapses under P_1 and P_2 . In fact, a single previous stimulation in P_1 was sufficient to reduce the response to the first Test in P_2 (Fig. 1D; EPSC as % baseline for $I.P_1.T_1$ as above; $I.P_2.T_1$: $52.6 \pm 7.9\%$; $P=0.0298$, $W=32$, $N=8$, Wilcoxon). To compare non-specific NMDAR activation in shorter and longer incubation conditions, we used the T_1 EPSC for P_1 . $P_1.T_1$ amplitudes did not differ for long (*I*) or short (*S*) MK-801 incubations (Fig. 1E; EPSC as % baseline for $S.P_1.T_1$ as above; $I.P_1.T_1$: $79.3 \pm 3.9\%$, $N=8$, $P=0.303$, $U=28$, Mann-Whitney), showing that the larger decrement in P_2 ($S.P_2.T_1$) than in P_1 ($S.P_1.T_1$) for the sequential protocol had resulted not merely from non-specific activity in the slice, but rather from previous stimulation of the opposite pathway. The multiple stimuli in P_1 under protocol *S* reduced the response in P_2 more effectively than did a single previous stimulus in P_1 under protocol *I* (Fig. 1F; $S.P_2.T_1$ as above; $I.P_2.T_1$ as above; $P=0.0414$, $U=20$, Mann-Whitney). Finally, block of NMDARs under P_1 was accelerated by interleaved stimulation (Fig. 1G; $S.P_1$: $\tau = 6.7 \pm 1.3$ trials, $N=10$; $I.P_1$: $\tau = 3.3 \pm 0.5$ trials, $N=8$, $P=0.0273$, $U=18$, Mann-Whitney), as expected if glutamate spillover from and to both pathways was causing reciprocal block of NMDARs under both pathways. Block of NMDARs in P_2 under protocols *S* and *I*, regardless of whether P_2 was the MNTB- or AVCN-LSO pathway, indicated that glutamate spillover had occurred bi-directionally, from presynaptic terminals and toward postsynaptic receptors serving both pathways.

Glutamate spillover onto postsynaptic receptors at physiological temperature

Little is known about glutamate uptake in the developing auditory brainstem; nevertheless, we assumed that if glutamate reuptake transporters were functional before P9 they would be largely inactivated at room temperature. We next asked whether glutamate spillover might exist at physiologically relevant temperatures. Although responses at elevated temperatures (34–36°C) decayed more rapidly (Fig. 2A), spillover persisted (Fig. 2B). The similar

magnitudes found at room temperature and elevated temperature (Fig. 2C) could reflect immature function of excitatory amino acid transporters in the first postnatal week. All subsequent experiments were performed at physiological temperatures. Finally, as a control for possible differences (e.g., release probability, receptor open probability) between the two pathways that might bias our results, in an additional 12 slices we acquired Baseline EPSCs in a single pathway, incubated in MK-801 for 10 minutes, and then began stimulating in the continued presence of MK-801. This provided a measurement analogous to P_1T_1 , and a decay time constant, for each pathway. We found no differences between pathways for EPSC amplitude (EPSC as % Baseline: MNTB 97.2 ± 6.9 , N=6; AVCN 97.7 ± 4.0 , N=6; $P=0.6991$, U=15, Mann Whitney) or for decay time (time constant τ : MNTB 5.3 ± 0.9 ms; N=6; AVCN 6.7 ± 0.8 ; N=6; $P=0.3358$, U=11.5, Mann Whitney).

Our results to this point might also have occurred if glutamate or GABA spillover to presynaptic terminals had reduced NMDAR activation through modulation of release. To assess whether presynaptic modulation could explain our results, we used protocol **I** without adding MK-801 to the perfusate, but with a 10-minute ‘incubation’ period analogous to that with MK-801, and then compared the mean amplitude of the last 20 ‘Test’ EPSCs with the mean baseline EPSC amplitude (Fig. 2E). In the absence of MK-801, the NMDAR-mediated response was not significantly decremented either pathway (Fig. 2F). As Test EPSCs were reduced much more in the presence than in the absence of MK-801, we conclude that the apparent glutamate spillover could not be caused by purely presynaptic effects.

Glutamate spillover in physiological conditions

Activity of certain glutamate transporters is voltage-sensitive (Wadiche et al., 1995). To determine whether apparent glutamate spillover was affected by depolarization, for a small additional subset of slices, we bathed the slice in normal Mg^{++} -ACSF and performed the crosstalk experiments (sequential protocol) by stepping to depolarized potentials to measure NMDA currents (Fig. 3A). In 4 such experiments, P_2T_1 was significantly reduced (Fig. 3B), to a similar degree as in the experiments conducted near normal resting potential. To more explicitly test the function of glutamate transporters in this immature circuit, in 5 additional cells we first established a baseline NMDA EPSC in normal Mg^{++} -ACSF and then applied the glutamate transporter antagonist TBOA (50 μ M). The lack of effect of TBOA on EPSC amplitude, rise time, or decay time (Table 1) suggests that glutamate transporters are largely non-functional at this age. After applying TBOA, we performed the crosstalk experiment as above (ie., stepping to +40mV). In these experiments, the Test EPSC in P_2 was reduced more than in P_1 (% Baseline for P_1T_1 : 96.7 ± 1.4 ; P_2T_1 : 39.6 ± 5.2 ; $P=0.0079$, N=5, Mann Whitney), and P_2T_1 was reduced to a similar degree as in the experiments of Figure 3B ($P = 1.000$, U=10.0, Mann Whitney).

In all of the experiments reported here, stimulus strength was chosen with the aim of stimulating the smallest feasible number of fibers, preferably a single fiber. To ask more specifically whether spillover could occur at physiological stimulus strengths, we performed two additional experiments. In order to stimulate a single MNTB cell with absolute confidence we searched for synaptically connected MNTB and LSO principal cells. In two slices (P3) we were able to obtain paired recordings (Fig. 3C), and we then performed the

sequential protocol *S* using direct stimulation at the soma of the presynaptic MNTB neuron and AVCN pathway stimulation in the acoustic stria. In both of these experiments, in which P_1 was the MNTB-LSO pathway, P_2T_1 was reduced relative to baseline (by 22.1% and 34.4%). Similar experiments could not be performed in the AVCN pathway, as the geometry of the slice precludes paired AVCN-LSO recordings. However, in order to test both pathways with presumed single-fiber stimulation, in an additional 8 slices we established minimal stimulation in both pathways and then performed the sequential protocol (Fig. 3D). For each pathway, the bimodal EPSC histogram constructed from the Baseline responses (Fig. 3E) was used to identify failures, and all values at or below the mean failure value + 2SD were discarded. Baseline and Test EPSC amplitudes were measured from the remaining responses. In a majority of these experiments (7/8), the Test EPSC histograms for P_2 were shifted leftward relative to P_1 (Fig. 3E), and in all cells T_1 was smaller in P_2 than in P_1 . (Fig. 3F; EPSC amplitude as % Baseline: P_1T_1 98.7 ± 9.6 ; P_2T_1 60.0 ± 3.3 ; $P=0.0078$, $W=36$, $N=8$, Wilcoxon). Although the reductions in P_2T_1 amplitude were smaller (~22–40%) for these physiological stimulation strengths than for the previous experiments, they demonstrate that spillover occurs for single-fiber stimulation.

Glutamate spillover cannot be explained by stimulation of overlapping fibers

The major input to the LSO is thought to course directly through the ventral acoustic stria on the one (excitatory) side and through the MNTB on the other (inhibitory) side of the LSO. We considered the possibility that if the circuitry were more complex, such that collaterals from one pathway overlapped with the opposite pathway, we might have inadvertently excited both pathways while nominally exciting a single pathway, giving rise to an apparent but entirely artifactual spillover. Although such hypothetical circuitry has not been reported, we performed two additional experiments to determine whether a similar scenario could have produced our results. In the first experiment, we asked whether we could induce short-term plasticity that would transfer from one pathway to the other. Accordingly, we delivered trains of 10 stimuli at 20 Hz to measure paired-pulse ratios (PPRs) of the AMPAR EPSCs in each pathway separately (Fig. 4A, top traces), and then we delivered the same pulse trains preceded immediately by 10 stimuli to the opposite pathway (Fig. 4A, bottom traces). In the presence of cyclothiazide (100 μ M) to prevent AMPAR desensitization, there was no apparent effect of previous stimulation in the opposite pathway (Fig. 4B; PPR for MNTB alone 0.76 ± 0.09 , for MNTB preceded by AVCN 0.77 ± 0.06 ; PPR for AVCN alone 0.73 ± 0.05 , for AVCN preceded by MNTB 0.77 ± 0.05). These population data suggest that in general we were not stimulating both pathways with a single electrode, but in order to estimate as cautiously as possible the independence of our stimulated pathways, we also performed multiple t-tests without correction, testing for each cell the PPR for MNTB alone vs that for MNTB preceded by AVCN stimulation and the PPR for AVCN alone vs that for AVCN preceded by MNTB stimulation. This analysis yielded nominally significant p-values for 3 of 22 t-tests ($P=0.05$, $P=0.033$, and $P=0.002$), based on which we suggest that inadvertent stimulation of both pathways during nominal stimulation of a single pathway might have occurred at most 15% of the time, or in too few cases to explain our results. Finally, in a subset of these experiments, after having first verified the independence of our stimuli by determining for both pathways that prior stimulation in the opposite pathway caused no change in PPR, we performed the spillover experiments (*S* protocol) by stepping

to +40mV (Fig. 4C). All cells for which stimulus independence was previously verified showed evidence for spillover (Fig. 4D; P_1T_I 96.5 ± 4.9 , P_2T_I 49.0 ± 6.2 ; $P=0.0143$, $U=0$, $N=4$, Mann Whitney). These experiments cannot rule out the existence of circuitous collaterals, but they show 1) that spillover persists for inputs that have been rigorously determined to be independent, and 2) that overlapping input fibers, if they exist, are too rare to explain the majority of our results.

AVCN and MNTB pathways share NMDA receptors

In a new set of slices, we asked whether simultaneous stimulation of both pathways could occlude the single-pathway response. In normal Mg^{++} -containing ACSF, we collected AMPAR-mediated responses to stimulation of each pathway individually and to simultaneous stimulation of the two pathways (Fig. 5A), then added CNQX and collected NMDAR-mediated responses for the same stimulation conditions (Fig. 5B). The AMPAR-mediated response to joint stimulation of the two pathways was on average equal to the sum of the individual responses (Fig. 5C), whereas the NMDAR-mediated response to joint stimulation was on average smaller than the summed response (Fig. 5D). These results are consistent with independent stimulation of the two pathways and with the existence of a shared population of NMDA, but not AMPA, receptors.

Sharing of NMDARs but not AMPARs might occur if the NMDARs were located extrasynaptically and AMPARs within the synapse. Under the assumption that differences in rise-times could point to differences in receptor location relative to release site (Rusakov and Kullmann, 1998), we measured 10–90% rise-times in 20 cells. We found that mean rise-times for the NMDAR response were larger in the MNTB-LSO pathway than in the AVCN-LSO pathway (Fig. 6A; MNTB 8.3 ± 7.0 ms; AVCN 6.3 ± 4.2 ms; $P=0.0338$, $U=117$, $N=20$, Mann Whitney). Overall, we saw a wider range of rise times in the MNTB pathway, which was reflected in the larger coefficients of variation for rise-times in the MNTB than in the AVCN pathway (Fig. 6A; MNTB 0.35 ± 0.03 ; AVCN 0.22 ± 0.01 ; $P=0.0106$, $U=105$, Mann Whitney). To ask whether receptor subtypes might differentially contribute to the differences in rise times, in an additional group of 9 cells we isolated the NMDA component pharmacologically and then applied the subunit-selective antagonist Ro-25 ($3 \mu M$) to block GluN2B-containing NMDARs. Blocking GluN2B subunits had similar effects on the EPSC amplitude in both pathways (Fig. 6C; EPSC amplitude as % baseline in MNTB 57.4 ± 5.7 ; in AVCN 50.3 ± 5.3 ; $P=0.3401$, $U=29$, $N=9$, Mann Whitney), but very different effects on EPSC rise times in the two pathways. Whereas Ro-25 significantly shortened rise times in the MNTB pathway (Fig. 6D; Ctrl 9.1 ± 1.1 ms; Ro-25 5.0 ± 0.5 ms; $P=0.0078$, $W=43$, Wilcoxon), it had no effect on rise times in the AVCN pathway (Fig. 6E; Ctrl 7.1 ± 0.4 ms; Ro-25 6.9 ± 0.4 ms; $P=0.4961$, $W=13$, Wilcoxon). Thus, while glutamate release from either pathway activates a similar proportion of GluN2B-containing NMDARs, the receptors may be at different locations relative to the presynaptic terminals.

Discussion

In rodent auditory brainstem, expression of VGLUT3 allows glutamate release from immature inhibitory terminals during the first postnatal week, and before P9 a majority of

LSO principal neurons exhibit a glutamatergic response to stimulation of the MNTB pathway (Gillespie et al., 2005), as well of course to stimulation of the AVCN pathway (Ene et al., 2003). Investigating the possibility of glutamate spillover in the LSO, we found that stimulation of either pathway resulted in the activation of NMDARs serving the opposite pathway. We further found different summation for NMDAR and AMPAR components, and we saw that NMDAR response times differed between AVCN and MNTB pathways. Based on these results, we suggest that at least during the first postnatal week, the period of major glutamate release from MNTB, a population of postsynaptic NMDARs is shared by both AVCN and MNTB pathways.

As in other areas (Thomas et al., 2011), it appears that glutamate re-uptake mechanisms in the LSO do not become functionally mature until well after local circuits are established. The excitatory amino acid transporters are modulated by low temperature and by membrane depolarization (Wadiche et al., 1995), yet we found that spillover was similar at physiological and room temperature, at resting and depolarized membrane potentials, and in the presence and absence of glutamate uptake antagonists. Furthermore, though it was of smaller magnitude, the spillover effect persisted even for presumed single-fiber stimulation. Together, these findings suggest that glutamate re-uptake in the LSO is not fully functional until at least after P8.

We have examined several caveats that might have explained our results through actions other than a shared postsynaptic population of NMDARs. In particular, modulation of transmitter release in auditory brainstem can be mediated by presynaptic GABA, glycine, and metabotropic glutamate receptors (Kotak et al., 2001; Turecek and Trussell, 2001; Nishimaki et al., 2007). As we have blocked only GABA_A, glycine, and AMPA receptors, substantial presynaptic modulation may still be present in our preparation. When we perform our crosstalk protocol in the absence of MK-801, however, we see no evidence for reduction in EPSC amplitude, even after relatively long recording periods. Thus, though presynaptic modulation may be present in our experimental context, it cannot account for our spillover findings.

Glutamate release activates GluN2B-containing NMDARs in both MNTB-LSO and AVCN-LSO pathways during the first postnatal week (Ene et al., 2003; Gillespie et al., 2005; Case et al., 2011). Although glutamate spillover in the LSO might have been predicted from the presence of GluN2B subunits, from the prominent role of GluN2B-containing NMDARs in mediating synaptic crosstalk (Scimemi et al., 2004), and from the occurrence of glutamate crosstalk at other immature synapses (Carter and Regehr, 2000; Thomas et al., 2011), crosstalk of any kind between nominally inhibitory and excitatory synapses is unusual, and it was surprising to find evidence for spillover in every case in which the MNTB input released glutamate. Given the magnitude of the effect, it is possible that glutamate release from glial cells or from (unknown) recurrent glutamatergic inputs may have amplified the apparent spillover by activating additional NMDARs.

Our results from joint pathway stimulation corroborate the pulse-train experiments, and are consistent with the activation of separate populations of presynaptic terminals (hence the linear summation of AMPAR responses) but shared populations of postsynaptic NMDARs

(hence the sublinear summation). Although an extrasynaptic NMDAR population is consistent with the longer response latencies for glutamate than for GABA/gly components in the MNTB pathway (Gillespie et al., 2005), an alternative explanation is that the differing glutamate affinities of NMDARs and AMPARs (Patneau and Mayer, 1990) yield an apparent occlusion for NMDARs but not for AMPARs. This alternative would require that a single action potential in the AVCN pathway cause saturation of the NMDARs, but this is not what has been seen (Ene et al., 2003). Additionally, our findings on rise times support the idea of spillover onto shared receptors located nearer AVCN than MNTB terminals. A better understanding of these events will require learning more about the myriad factors that regulate spillover, including relative synapse location, the glutamate diffusion constant in the extracellular space, vesicular content, release site, geometry of the extracellular space, and expression of functional glutamate reuptake transporters (for review, see (Rusakov et al., 1999), all of which are as yet uncharacterized in the immature LSO.

One explanation for the greater apparent rise times and variabilities in the MNTB-LSO response could be that the MNTB-LSO synapses were more distal and therefore less well-clamped than the AVCN-LSO synapses. Although the subcellular distribution of synapses on immature LSO neurons is unknown, MNTB-LSO synapses in the adult are thought to be perisomatic (Schwartz, 1992) and likely proximal to AVCN-LSO synapses, and thus any rise-time differences due to clamping artifact would likely be in the opposite direction from those seen here. While these differences might arise from distinct release properties in the two pathways, we speculate that the differences in rise time means and variabilities reflect a fixed, relatively small distance between AVCN terminals and their associated NMDARs versus a more variable, and on average larger, distance between MNTB terminals and NMDARs, such as might result if glutamate were released ectopically in the MNTB-LSO pathway (Matsui and Jahr, 2003) or if NMDARs were more closely associated with AVCN than with MNTB synapses. These experiments are necessarily indirect, but we note that our results with Ro-25 may be consistent with a variant of the second model. Based on the similar reductions in EPSC amplitude, we conclude that glutamate released from either pathway activates a similar proportion of GluN2B-containing NMDARs. Based on the striking asymmetry in rise time, however, we suggest that these GluN2B-containing NMDARs may be at different distances from release sites in the two pathways. As rise-times for the AVCN pathway do not change in Ro-25, glutamate released from AVCN terminals may activate a mixed population of NMDARs all equidistant from (and relatively near to) the AVCN release site. By contrast, glutamate release from MNTB terminals may activate two populations of NMDARs, one near the release site that does not contain GluN2B and one far from the release site (and presumably near AVCN terminals) that is enriched in GluN2B.

As we are at the early stages of understanding development in the AVCN-LSO pathway (Case et al., 2011), refinement in the MNTB-LSO pathway (Kim and Kandler, 2010), and glutamate release in the MNTB-LSO pathway (Gillespie et al., 2005; Noh et al., 2010), any statement about the function of glutamate spillover must be speculative. For example, glutamate spillover may signal glial cells as well as the postsynaptic neuron (Matsui et al., 2005). It is tempting to suggest that glutamate spillover and postsynaptic GluN2B-containing NMDARs mediate activity-dependent tonotopic registration of the two pathways,

but several factors render this scenario unlikely. Fine scale activity-dependent topographic refinement is generally understood to rely on nearest-neighbor correlations in activity (Ruthazer and Cline, 2004; Cang et al., 2005; Butts et al., 2007; Huberman et al., 2008). Glutamate release—and therefore crosstalk—declines after P8 (Gillespie et al., 2005); until P12 the two cochleae exhibit spontaneous activity that is presumably uncorrelated (Tritsch and Bergles, 2010; Tritsch et al., 2010); and soon afterward, the cochlear place-code may shift (Mills and Rubel, 1998). Diverse correlations that may be imposed by reciprocal connections between the cochlear nuclei (Shore et al., 2003; Ingham et al., 2006; Bledsoe et al., 2009) probably do not preserve the precise relationships in activity that would be required for registration of the two pathways. Together, these factors argue against glutamate crosstalk as a mediator of tonotopic registration.

Glutamate spillover may however function less as a way to link synapses, and more as a general signal converging onto a shared postsynaptic mechanism. In this model, extrasynaptic NMDARs could monitor overall activity from both input pathways, possibly mediating plasticity in one or both input pathways. The period of greatest glutamate release in the MNTB-LSO pathway coincides with the major period of functional synapse elimination in both pathways (Kim and Kandler, 2003; Gillespie et al., 2005; Case et al., 2011), and an intriguing possibility is that glutamate spillover could initiate heterosynaptic depression through activation of postsynaptic NMDARs or possibly through presynaptic mGluRs (Vogt and Nicoll, 1999; Liu et al., 2004; Harvey-Girard et al., 2010). To our knowledge, no one has yet attempted to develop a preparation for examining plasticity in the LSO in the presence of glutamatergic transmission, and it will be of great interest to discover whether glutamate spillover operates within a local diffusion neighborhood to mediate synapse elimination in one or both pathways.

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References

- Bergles DE, Jahr CE. Glial contribution to glutamate uptake at Schaffer collateral-commissural synapses in the hippocampus. *J Neurosci.* 1998; 18:7709–7716. [PubMed: 9742141]
- Bledsoe SC Jr, Snead CR, Helfert RH, Prasad V, Wenthold RJ, Altschuler RA. Immunocytochemical and lesion studies support the hypothesis that the projection from the medial nucleus of the trapezoid body to the lateral superior olive is glycinergic. *Brain Res.* 1990; 517:189–194. [PubMed: 2375987]
- Bledsoe SC Jr, Koehler S, Tucci DL, Zhou J, Le Prell C, Shore SE. Ventral cochlear nucleus responses to contralateral sound are mediated by commissural and olivocochlear pathways. *J Neurophysiol.* 2009; 102:886–900. [PubMed: 19458143]
- Boudreau JC, Tsuchitani C. Binaural interaction in the cat superior olive S segment. *J Neurophysiol.* 1968; 31:442–454. [PubMed: 5687764]
- Butts DA, Kanold PO, Shatz CJ. A burst-based “Hebbian” learning rule at retinogeniculate synapses links retinal waves to activity-dependent refinement. *PLoS Biol.* 2007; 5:e61. [PubMed: 17341130]
- Caird D, Klinke R. Processing of binaural stimuli by cat superior olivary complex neurons. *Exp Brain Res.* 1983; 52:385–399. [PubMed: 6653700]

- Cang J, Renteria RC, Kaneko M, Liu X, Copenhagen DR, Stryker MP. Development of precise maps in visual cortex requires patterned spontaneous activity in the retina. *Neuron*. 2005; 48:797–809. [PubMed: 16337917]
- Cant NB, Casseday JH. Projections from the anteroventral cochlear nucleus to the lateral and medial superior olivary nuclei. *J Comp Neurol*. 1986; 247:457–476. [PubMed: 3722446]
- Carter AG, Regehr WG. Prolonged synaptic currents and glutamate spillover at the parallel fiber to stellate cell synapse. *J Neurosci*. 2000; 20:4423–4434. [PubMed: 10844011]
- Case DT, Gillespie DC. Pre- and post-synaptic properties of glutamatergic transmission in the immature inhibitory MNTB-LSO pathway. *J Neurophysiol*. 2011; 106:2570–2579. [PubMed: 21832038]
- Case DT, Zhao X, Gillespie DC. Functional refinement in the projection from ventral cochlear nucleus to lateral superior olive precedes hearing onset in rat. *PLoS One*. 2011; 6:e20756. [PubMed: 21694776]
- Ene FA, Kullmann PH, Gillespie DC, Kandler K. Glutamatergic calcium responses in the developing lateral superior olive: receptor types and their specific activation by synaptic activity patterns. *J Neurophysiol*. 2003; 90:2581–2591. [PubMed: 12853437]
- Gillespie DC, Kim G, Kandler K. Inhibitory synapses in the developing auditory system are glutamatergic. *Nat Neurosci*. 2005; 8:332–338. [PubMed: 15746915]
- Harvey-Girard E, Lewis J, Maler L. Burst-induced anti-Hebbian depression acts through short-term synaptic dynamics to cancel redundant sensory signals. *J Neurosci*. 2010; 30:6152–6169. [PubMed: 20427673]
- Huberman AD, Feller MB, Chapman B. Mechanisms underlying development of visual maps and receptive fields. *Annu Rev Neurosci*. 2008; 31:479–509. [PubMed: 18558864]
- Huettnner JE, Bean BP. Block of N-methyl-D-aspartate-activated current by the anticonvulsant MK-801: selective binding to open channels. *Proc Natl Acad Sci U S A*. 1988; 85:1307–1311. [PubMed: 2448800]
- Ingham NJ, Bleack S, Winter IM. Contralateral inhibitory and excitatory frequency response maps in the mammalian cochlear nucleus. *Eur J Neurosci*. 2006; 24:2515–2529. [PubMed: 17100840]
- Kandler K, Clause A, Noh J. Tonotopic reorganization of developing auditory brainstem circuits. *Nat Neurosci*. 2009; 12:711–717. [PubMed: 19471270]
- Kim G, Kandler K. Elimination and strengthening of glycinergic/GABAergic connections during tonotopic map formation. *Nat Neurosci*. 2003; 6:282–290. [PubMed: 12577063]
- Kim G, Kandler K. Synaptic changes underlying the strengthening of GABA/glycinergic connections in the developing lateral superior olive. *Neuroscience*. 2010; 171:924–933. [PubMed: 20888399]
- Kotak VC, DiMattina C, Sanes DH. GABA(B) and Trk receptor signaling mediates long-lasting inhibitory synaptic depression. *J Neurophysiol*. 2001; 86:536–540. [PubMed: 11431532]
- Liu L, Wong TP, Pozza MF, Lingenhoehl K, Wang Y, Sheng M, Auberson YP, Wang YT. Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. *Science*. 2004; 304:1021–1024. [PubMed: 15143284]
- Matsui K, Jahr CE. Ectopic release of synaptic vesicles. *Neuron*. 2003; 40:1173–1183. [PubMed: 14687551]
- Matsui K, Jahr CE, Rubio ME. High-concentration rapid transients of glutamate mediate neural-glial communication via ectopic release. *J Neurosci*. 2005; 25:7538–7547. [PubMed: 16107641]
- Mills DM, Rubel EW. Development of the base of the cochlea: place code shift in the gerbil. *Hear Res*. 1998; 122:82–96. [PubMed: 9714577]
- Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron*. 1994; 12:529–540. [PubMed: 7512349]
- Moore MJ, Caspary DM. Strychnine blocks binaural inhibition in lateral superior olivary neurons. *J Neurosci*. 1983; 3:237–242. [PubMed: 6822858]
- Nabekura J, Katsurabayashi S, Kakazu Y, Shibata S, Matsubara A, Jinno S, Mizoguchi Y, Sasaki A, Ishibashi H. Developmental switch from GABA to glycine release in single central synaptic terminals. *Nat Neurosci*. 2004; 7:17–23. [PubMed: 14699415]

- Nishimaki T, Jang IS, Ishibashi H, Yamaguchi J, Nabekura J. Reduction of metabotropic glutamate receptor-mediated heterosynaptic inhibition of developing MNTB-LSO inhibitory synapses. *Eur J Neurosci.* 2007; 26:323–330. [PubMed: 17623021]
- Noh J, Seal RP, Garver JA, Edwards RH, Kandler K. Glutamate co-release at GABA/glycinergic synapses is crucial for the refinement of an inhibitory map. *Nat Neurosci.* 2010; 13:232–238. [PubMed: 20081852]
- Patneau DK, Mayer ML. Structure-activity relationships for amino acid transmitter candidates acting at N-methyl-D-aspartate and quisqualate receptors. *J Neurosci.* 1990; 10:2385–2399. [PubMed: 2165523]
- Philpot BD, Cho KK, Bear MF. Obligatory role of NR2A for metaplasticity in visual cortex. *Neuron.* 2007; 53:495–502. [PubMed: 17296552]
- Rusakov DA, Kullmann DM. Extrasynaptic glutamate diffusion in the hippocampus: ultrastructural constraints, uptake, and receptor activation. *J Neurosci.* 1998; 18:3158–3170. [PubMed: 9547224]
- Rusakov DA, Kullmann DM, Stewart MG. Hippocampal synapses: do they talk to their neighbours? *Trends Neurosci.* 1999; 22:382–388. [PubMed: 10441295]
- Ruthazer ES, Cline HT. Insights into activity-dependent map formation from the retinotectal system: a middle-of-the-brain perspective. *J Neurobiol.* 2004; 59:134–146. [PubMed: 15007832]
- Schwartz, IR. The superior olivary complex and lateral lemniscal nuclei. In: Webster, DB, Popper, AN., Fay, RR., editors. *The mammalian auditory pathway: neuroanatomy.* New York: Springer-Verlag; 1992. p. 117-167.
- Scimemi A, Fine A, Kullmann DM, Rusakov DA. NR2B-containing receptors mediate cross talk among hippocampal synapses. *J Neurosci.* 2004; 24:4767–4777. [PubMed: 15152037]
- Sheng M, Cummings J, Roldan LA, Jan YN, Jan LY. Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature.* 1994; 368:144–147. [PubMed: 8139656]
- Shore SE, Sumner CJ, Bledsoe SC, Lu J. Effects of contralateral sound stimulation on unit activity of ventral cochlear nucleus neurons. *Exp Brain Res.* 2003; 153:427–435. [PubMed: 12961054]
- Smith PH, Joris PX, Carney LH, Yin TC. Projections of physiologically characterized globular bushy cell axons from the cochlear nucleus of the cat. *J Comp Neurol.* 1991; 304:387–407. [PubMed: 2022755]
- Stocca G, Vicini S. Increased contribution of NR2A subunit to synaptic NMDA receptors in developing rat cortical neurons. *J Physiol.* 1998; 507 (Pt 1):13–24. [PubMed: 9490809]
- Thomas CG, Tian H, Diamond JS. The relative roles of diffusion and uptake in clearing synaptically released glutamate change during early postnatal development. *J Neurosci.* 2011; 31:4743–4754. [PubMed: 21430173]
- Tollin DJ. The lateral superior olive: a functional role in sound source localization. *Neuroscientist.* 2009; 9:127–143.
- Tovar KR, Westbrook GL. The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. *J Neurosci.* 1999; 19:4180–4188. [PubMed: 10234045]
- Tritsch NX, Bergles DE. Developmental regulation of spontaneous activity in the Mammalian cochlea. *J Neurosci.* 2010; 30:1539–1550. [PubMed: 20107081]
- Tritsch NX, Rodriguez-Contreras A, Crins TT, Wang HC, Borst JG, Bergles DE. Calcium action potentials in hair cells pattern auditory neuron activity before hearing onset. *Nat Neurosci.* 2010; 13:1050–1052. [PubMed: 20676105]
- Turecek R, Trussell LO. Presynaptic glycine receptors enhance transmitter release at a mammalian central synapse. *Nature.* 2001; 411:587–590. [PubMed: 11385573]
- Vogt KE, Nicoll RA. Glutamate and gamma-aminobutyric acid mediate a heterosynaptic depression at mossy fiber synapses in the hippocampus. *Proc Natl Acad Sci U S A.* 1999; 96:1118–1122. [PubMed: 9927703]
- Wadiche JI, Amara SG, Kavanaugh MP. Ion fluxes associated with excitatory amino acid transport. *Neuron.* 1995; 15:721–728. [PubMed: 7546750]
- Wu SH, Kelly JB. Synaptic pharmacology of the superior olivary complex studied in mouse brain slice. *J Neurosci.* 1992; 12:3084–3097. [PubMed: 1494947]

- Yoshimura Y, Ohmura T, Komatsu Y. Two forms of synaptic plasticity with distinct dependence on age, experience, and NMDA receptor subtype in rat visual cortex. *J Neurosci.* 2003; 23:6557–6566. [PubMed: 12878697]
- Zhao JP, Constantine-Paton M. NR2A^{-/-} mice lack long-term potentiation but retain NMDA receptor and L-type Ca²⁺ channel-dependent long-term depression in the juvenile superior colliculus. *J Neurosci.* 2007; 27:13649–13654. [PubMed: 18077676]

Figure 1. Glutamate spillover between AVCN and MNTB inputs to LSO

A: Sequential protocol *S*. After an LSO principal cell with a glutamatergic response in both pathways was located, the NMDAR-mediated component was pharmacologically isolated, and then the experiment began. **Aa:** Representative raw (gray) and average (black) current traces (P3 cell), showing the mixed ('no drug') GABA/glycine/glutamate traces in MNTB and the glutamate traces in AVCN before the beginning of the experiment, the Baseline NMDAR responses after application of picrotoxin (Ptx), strychnine (Str), and CNQX, and the decrementing Test responses in the presence of MK-801. **Ab:** Schematic showing the spillover experiment on pharmacologically isolated NMDAR-mediated response, for the traces shown above. Baseline NMDAR responses ("Bsl") were obtained in both pathways (interleaved), MK-801 perfusion began (gray area), and Test responses to P_1 (MNTB-LSO in this instance) and then to P_2 (AVCN-LSO in this instance) were collected. **B:** The amplitude of the first Test EPSC (T_1) in MK-801 was reduced more in P_2 than in P_1 for all 10 cells recorded in this manner (T_1 EPSC amplitude as % of Baseline: P_1 $84.9 \pm 3.6\%$, P_2 $35.7 \pm 3.7\%$; $P=0.002$, $W=55$, $N=10$, Wilcoxon). Each pair of adjoining symbols represents one cell, red indicates that MNTB was the first pathway stimulated (P_1 = MNTB-LSO) and green that AVCN was the first pathway stimulated (P_1 = AVCN-LSO). **C:** Interleaved protocol *I*. **Ca:** Representative raw (gray) and average (black) current traces (P3 cell) for Baseline EPSCs and for Test EPSCs after the further addition of MK-801. **Cb:** Schematic of protocol *I*, for the traces shown above. Both Baseline ("Bsl") and Test responses were collected in an interleaved manner, and the period of MK-801 exposure before T_1 was nearly identical for the two pathways. **D:** T_1 amplitude in MK-801 was reduced more in P_2 than in P_1 for all cells recorded using protocol *I* (T_1 EPSC amplitude, % Baseline: P_1 $79.3 \pm 3.9\%$, P_2 $35.7 \pm 3.7\%$; $P=0.002$, $W=55$, $N=10$, Wilcoxon).

P_2 52.6 ± 7.9 %; $P=0.0298$, $W=32$, $N=8$, Wilcoxon). **E:** Responses to first test in P_I ($P_I T_I$) were similar for 10- (*S*) and 15-minute (*I*) MK-801 incubations ($P = 0.303$, $U=28$, Mann-Whitney). Note: data re-plotted from Figures 1B and D. **F:** Responses to first test in P_2 ($P_2 T_I$) were reduced by a larger amount in the sequential protocol (*S*) than in the interleaved protocol (*I*) ($P=0.0414$, $U=20$, Mann Whitney). Note: data re-plotted from Figures 1B and D. **G:** Response amplitude in P_I decremented more quickly when stimuli were interspersed with stimuli to opposite pathway (*I*), than when P_I alone was stimulated (time-constant τ : *S* 6.7 ± 1.3 trials, *I* 3.3 ± 0.5 trials; $P=0.0273$, $U=18$, Mann Whitney). All experiments in 0 Mg⁺⁺ ACSF, at room temperature.

Figure 2.

Glutamate spillover occurs at physiologically relevant temperatures. **A:** Representative raw (gray) and average (black) traces for Baseline and Test responses at physiologically relevant temperatures (34–36°C), in sequential protocol (P_7 slice), 0 Mg^{++} ACSF, $-70mV$ holding potential. **B:** Test EPSCs were reduced significantly in P_2 relative to P_1 (P_1T_1 $85.6 \pm 3.4\%$; P_2T_1 $33.5 \pm 3.3\%$; $P=0.0010$, $W=66$, $N=11$, Wilcoxon). Each pair of points corresponds to a single neuron, with red and green indicating P_1 as above. **C:** Under Protocol *S*, T_1 amplitudes in P_2 were equally decremented at room temperature and at physiological temperatures ($P=0.7513$, $U=50$, Mann Whitney). **D:** T_1 amplitudes in P_1 did not differ significantly between pathways (MNTB: $97.2 \pm 6.9\%$, $N=6$; AVCN 97.7 ± 4.0 , $N=6$; $P=0.6991$, $U=15$, Mann Whitney). **Ea:** Raw (gray) and average (black) traces for interleaved protocol shown in **Eb**, performed with no MK-801 in the perfusate (P_8 slice). Gray symbols indicate the values used for measuring Baseline and Test amplitudes (last 20 test stimuli). **F:** In the absence of MK-801, Test responses (last 20 stimuli) were not substantially

decremented relative to baseline in either the AVCN or the MNTB pathway (MNTB 94.1 ± 8.0 ; AVCN 83.6 ± 6.0 ; $N=9$, $P=0.4894$, $U=32$, Mann Whitney). All experiments in 0 Mg^{++} ACSF, at physiological temperature.

Figure 3.

Glutamate spillover occurs in normal Mg^{++} concentrations, and at physiologically relevant stimulation intensities. **A:** Example cell (P_5), Baseline and Test responses in Mg^{++} -containing ACSF, at holding potential of +40mV. **B:** For 4 cells, in normal Mg^{++} -ACSF, using sequential protocol, T_1 EPSC is reduced more in P_2 than in P_1 ($P_1 T_1$ 92.0 ± 1.5 ; $P_2 T_1$ 42.9 ± 9.7 ; $P=0.0286$, $U=1$, $N=4$, Mann Whitney). **C:** Recording from synaptically connected MNTB-LSO cell pair, before addition of strychnine, picrotoxin and CNQX; P_3 slice. Current injection at MNTB cell (*top*) elicits an action potential that produces a postsynaptic potential in LSO principal cell (*bottom*). **D:** Representative minimal stimulation experiment, sequential protocol performed after establishing minimal stimulation in both pathways. P_8 slice, recordings in 0 Mg^{++} ACSF, at -70mV. **Da:** 50 Baseline responses were collected in each pathway, MK-801 was applied for 10 minutes, and then stimulation began again in P_1 . **Db:** Raw (gray) and average (black) responses for the cell in **a**. **E:** EPSC amplitude histograms for Baseline and Test periods in Path 1 (top) and Path 2 (bottom), for the cell shown in **C**. **F:** Population data from minimal stimulation experiments to ensure that single fibers were stimulated in both pathways. Solid horizontal line indicates mean failure 'amplitude' and dashed horizontal lines indicate mean ± 2

standard deviations, for each pathway. (EPSC amplitude, as % Baseline: P_1T_1 98.7 ± 9.6 ; P_2T_1 60.0 ± 3.3 ; $P=0.0078$, $W=36$, $N=8$, Wilcoxon). All experiments at physiological temperature; **AB** in Mg^{++} -containing ACSF; **C–F** in $0\text{ }Mg^{++}$ ACSF.

Figure 4.

Decrement in P_2 is not due to inadvertent prior stimulation of P_2 . **A:** Example cell (P6), response to 10-pulse train delivered to MNTB-LSO, in the presence of picrotoxin, strychnine, and cyclothiazide (Ctz), holding potential $-70mV$ (upper left). Paired-pulse ratio (PPR) for MNTB pathway alone (Response 2/Response 1) for this cell: 0.50 ± 0.03 . Upper right: Response to 10-pulse train delivered to AVCN-LSO (PPR for AVCN pathway alone 0.48 ± 0.02). Lower left: Response to 10-pulse MNTB-LSO train preceded by a 10-pulse train to the AVCN-LSO pathway. PPR for MNTB stimulation preceded by AVCN stimulation (Response 12/Response 11) for this cell is 0.50 ± 0.05 . Lower right: Response to 10-pulse AVCN-LSO train preceded by a 10-pulse train to the MNTB-LSO pathway (PPR = 0.51 ± 0.02). **Ba:** PPRs for MNTB alone do not differ from PPRs measured immediately after AVCN trains, (MNTB alone 0.76 ± 0.09 ; AVCN>>MNTB 0.77 ± 0.06 ; $P=1.0$, $U=32$, $N=8$, Mann Whitney). **Bb:** PPRs for AVCN alone do not differ from PPRs preconditioned by MNTB trains (AVCN alone 0.74 ± 0.06 ; MNTB>>AVCN 0.76 ± 0.06 , $P=0.6991$, $U=15$, Mann Whitney). **C:** Baseline and Test EPSCs for cell shown in A. **D:** Test EPSCs (T_1) were reduced more in P_2 than in P_1 ($P_1 T_1$ $96.5 \pm 4.9\%$, $P_2 T_1$ $49.1 \pm 6.2\%$; $P=0.0143$, $U=0.0$, Mann Whitney). All experiments in Mg^{++} -containing ACSF, at physiological temperature.

Figure 5.

AVCN-LSO and MNTB-LSO pathways share NMDA, but not AMPA, receptors. **A: Left:** AMPAR-mediated response recorded at -70mV for representative cell (P5), showing individual responses to AVCN-LSO and MNTB stimulation, and response to joint stimulation of both pathways. **Right:** For the same cell, with CNQX in the bath, joint stimulation yields an NMDAR-mediated response only slightly larger than the sum of responses in each pathway. **B: Left:** For 11 cells, joint response amplitude (to stimulation of both pathways) equals summed amplitudes from stimulation of AVCN-LSO and MNTB individually (MNTB $32.3 \pm 2.0\%$, AVCN $69.2 \pm 2.9\%$; joint $102.2 \pm 3.8\%$). **Right:** for the same neurons, the NMDAR-mediated response to joint stimulation is smaller than the sum of the individual responses (MNTB $32.1 \pm 3.2\%$, AVCN $67.7 \pm 3.5\%$; joint $73.6 \pm 3.9\%$). All experiments in Mg^{++} -containing ACSF, at physiological temperature.

Figure 6.

NMDAR-mediated EPSC exhibits a slower, more variable rise time in MNTB than in AVCN pathway. **A:** Rise times for isolated NMDAR-mediated response in MNTB-LSO and AVCN-LSO pathways, measured as the time (in ms) between 10% and 90% of peak response. (rise-time for MNTB: 8.3 ± 7.0 ms; AVCN 6.3 ± 4.2 ms, $P=0.0338$, $U=117$, Mann Whitney, $N=20$) **B:** Coefficients of variation of rise times, for cells shown in **A** (MNTB 0.35 ± 0.03 ; AVCN 0.22 ± 0.01 ; $P = 0.0106$, $U=105$, Mann Whitney) **C:** Representative traces from a P5 cell, held at +40mV. After application of strychnine, picrotoxin and CNQX to isolate the NMDAR-mediated EPSC, the further application of Ro-25 reduces response amplitude equally in both pathways (for this cell, EPSC amplitude as % Ctrl: MNTB 44.8 ± 4.5 ; AVCN 37.5 ± 3.4). **D:** In MNTB-LSO pathway, rise times are shortened by application of Ro-25 (Bsl 9.1 ± 1.1 ms, Ro-25 5.0 ± 0.5 ms; $P=0.0078$, $W=43$, $N=9$, Wilcoxon). **E:** Rise times in the AVCN-LSO pathway are unaffected by Ro-25 (Bsl 7.1 ± 0.4 ms, Ro-25 6.9 ± 0.4 ms; $P=0.4961$, $W=13$, $N=9$, Wilcoxon). All experiments at physiological temperature.

Table 1

	MNTB			AVCN		
	Bsl	TBOA	P value [/]	Bsl	TBOA	P value [/]
Amplitude	64.4±13.3	58.6±9.5	0.8413	86.3±7.2	80.1±8.1	0.5476
Rise time	10.3±0.8	10.1±0.6	0.6905	8.4±0.8	8.5±0.5	0.9132
Decay time	31.9±6.9	29.5±6.4	0.6905	38.1±3.4	42.2±4.8	0.6905

[/]Mann Whitney test