Pre- and postsynaptic properties of glutamatergic transmission in the immature inhibitory MNTB-LSO pathway

Daniel T. Case1 and Deda C. Gillespie1,2
1Neuroscience Graduate Program and 2Department of Psychology, Neuroscience, and Behaviour, McMaster University, Hamilton, Ontario, Canada

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The lateral superior olive (LSO) is a binaural nucleus of the auditory brain stem whose primary output neurons, the “principal cells,” compute the interaural intensity differences necessary for azimuthal sound localization (Boudreau and Tsushima 1968; Caird and Klinke 1983). Principal cells of the tonotopically organized LSO integrate excitatory inputs that are themselves precisely tonotopically matched (Kandler 2004; Kotak and Sanes 1996; Sanes and Rubel 1988). The ipsilateral anteroventral cochlear nucleus (AVCN) provides the primary excitatory, glutamatergic, input (Cant and Casseday 1986; Wu and Kelly 1992), whereas the contralateral cochlear nucleus excites principal neurons of the sign-inverting medial nucleus of the trapezoid body (MNTB), which provides the primary inhibitory, glycinergic, input to the LSO (Bledsoe et al. 1990; Caspary and Finlayson 1991; Moore and Caspary 1983; Smith et al. 1991). As tonotopic precision of excitatory and inhibitory inputs to the LSO is achieved in the first few postnatal weeks in rats, the developing LSO offers a model system for understanding both how inhibitory maps are organized during development and how inhibitory and excitatory inputs are brought into register within a shared postsynaptic target.

The immature MNTB-LSO pathway exhibits activity-dependent synaptically plasticity in vitro (Chang et al. 2003; Kotak et al. 2001; Kotak and Sanes 2002), and major functional refinement that occurs before hearing onset in rats (Kim and Kandler 2003) is likely directed by patterned, spontaneous activity from the cochlea (Beutner and Moser 2001; Kros et al. 1998; Tritsch et al. 2007; Tritsch and Bergles 2010). During this period of refinement, GABA and glycine are coreleased at MNTB-LSO synapses (Korada and Schwartz 1999; Kotak et al. 1998; Nabekura et al. 2004), GABA and glycine exert depolarizing effects at principal neurons in the LSO (Ehrlich et al. 1999; Kakazu et al. 1999; Kandler and Frauf 1995), and immature MNTB terminals release glutamate onto LSO principal cells (Gillespie et al. 2005). The discovery that glutamate is released during a period characterized both by developmental refinement and by depolarizing GABA/glycine led to the hypothesis that the NMDA glutamate receptor subtype (NMDAR) might mediate developmental refinement in the inhibitory MNTB-LSO pathway. Under this hypothesis, release of depolarizing GABA/glycine can relieve Mg2+/H11001 block of NMDARs, allowing coreleased glutamate to activate NMDARs; influx of Ca2+ through open NMDARs would then provide the immature MNTB-LSO synapse access to a wide array of NMDAR-mediated mechanisms of plasticity (for review, see Malenka and Bear 2004). Consistent with this hypothesis, mice lacking glutamate release in the MNTB-LSO pathway exhibit perturbed developmental refinement in the LSO (Noh et al. 2010). Although NMDAR-dependent plasticity has yet to be demonstrated in this pathway, NMDARs have been shown to mediate plasticity at other inhibitory synapses where the glutamate source is still unknown (Gaiarsa et al. 2002; Marsden et al. 2007; McLean et al. 1996; Ouardouz and Sastry 2000; Wang et al. 2003).

In light of the hypothesized role for NMDARs in the developing inhibitory MNTB-LSO pathway, and in order to better understand the range of responses available to synaptic plasticity mechanisms, we examined glutamatergic transmission at immature MNTB-LSO synapses. Our results suggest that MNTB-LSO synapses may be able to switch between primarily GABA/glycinergic and mixed glutamate/GABA/glycinergic transmission, and that postsynaptic expression of pre- and postsynaptic properties of glutamatergic transmission would be consistent with the hypothesis that NMDARs mediate developmental refinement in the immature MNTB-LSO pathway.
GLUTAMATERGIC TRANSMISSION IN AN IMMATURE INHIBITORY PATHWAY

GluN2B-containing NMDARs in part defines a discrete temporal window corresponding to the period of major circuit refinement.

MATERIALS AND METHODS

All procedures adhered to Canadian Council on Animal Care guidelines and were approved by the Animal Research Ethics Board of McMaster University. Sprague-Dawley rats (Charles River Laboratories) aged postnatal days 0 to 12 (P0–12) were anesthetized with isoflurane and quickly decapitated, and the brains were removed into ice-cold artificial cerebrospinal fluid (ACSF, pH 7.2) containing (in mM) 125 NaCl, 1 K2SO4, 5 KCl, 1.25 KH2PO4, 10 dextrose, 26 NaHCO3, 2 CaCl2·2H2O, and 1 kynurenic acid. The brain stem was cut at 300 μm (Vibratome 3000 Series), and slices containing the MNTB and LSO were transferred to a humidified chamber, where they were allowed to recover for at least 45 min.

For recording, slices were transferred to a recording chamber at an upright microscope. Slices were kept at room temperature and were constantly perfused with ACSF superﬁsed with 95% O2-5% CO2. Kynurenic acid was not added to ACSF used in recordings. All of the ifenprodil pharmacology experiments, and ~60% of the developmental proﬁling experiments, were performed in Mg-free ACSF. To reduce noise through NMDARs, ~40% of the developmental recordings were performed in Mg-containing ACSF; no differences were seen between measurements obtained in Mg-free and Mg-containing ACSF. For experiments performed in Mg-containing ACSF, K2SO4 was replaced with MgSO4 (1.3 mM). For experiments performed in Mg-containing ACSF, the internal solution contained 5 mM QX-314 (Ascent Scientiﬁc) and control (no drug treatment) recordings were taken at −60 mV and +40 mV. After GABA(A) (GABAARs) and glycine (GlyRs) receptors were blocked pharmacologically, recordings were taken at −60 mV to observe AMPA receptor (AMPAR)-mediated currents and at +40 mV to observe NMDAR-mediated currents. Peak amplitudes have been converted to conductances, and charge transfers have been corrected to allow for differences in driving force [i.e., current normalized \( I_{-60\,\text{mV}} \times \left( \frac{\text{df}}{\text{d}V} \right)_{+40\,\text{mV}} \) before charge integrated].

Stimulating electrodes, 1- to 2-MΩ glass pipettes ﬁlled with ACSF, were placed in the MNTB or in the ﬁber tract at the lateral edge of the MNTB, and stimuli were delivered via Grass S88 with SIU or Master 8 with Iso-Flex. For whole cell recording, cells in the higher-frequency (medial and middle) limbs of the LSO were targeted by using DIC-IR to identify principal cells based on their bipolar morphology and their orientation relative to the mediolateral axis of the nucleus. Cells were targeted in the medial and middle limbs where developmental reﬁnement is best understood; the lateral limb contains cells with different response characteristics and protein expression (Friauf 1993). For all cells examined, a GABA/glycinergic response to MNTB stimulation was recorded before application of the GABAAR and GlyR antagonists picrotoxin and strychnine. Recordings were made at room temperature (20°C), and for any recording in which the onset of the glutamate component lagged the GABA/glycinergic component by ≥2 ms, the glutamate response was considered to result from a separate input and the glutamate recording was excluded from analysis. Electrodes for whole cell voltage clamp (8250 borosilicate glass, AM Systems) had resistances of 3–6 MΩ and were ﬁlled with a Cs-glucuronate solution (pH 7.2) containing (in mM) 64 Cs-glucuronate acid, 64 CsOH, 11 EGTA, 56 CsCl, 1 MgCl2-6H2O, 1 CaCl2, 10 HEPES, 0.3 GTP-Na, and 4 ATP-Mg-3.5H2O. In most cases, the internal solution also contained 0.5% biocytin for subsequent histological veriﬁcation of cell type. Some neurons were also recorded with K-glucuronate internal solution (in mM: 100 K-glucuronate, 20 KCl, 10 Na2-phosphocreatine, 10 HEPES, 0.3 GTP-Na, 4 ATP-Mg-3.5H2O, pH 7.3) to avoid blocking K+ channels possibly involved in metabotropic glutamate receptor (mGLUR) responses. Recordings (Axopatch 200B ampliﬁer, pCLAMP 9.2; Molecular Devices) were sampled at 10 kHz, ﬁltered at 5 kHz, and saved for off-line analysis with custom Matlab software. Recordings were compensated by a minimum of 80% with <10-µs lag and were discarded if series resistance changed by >15% from its initial value. Electrical stimulation intensity was set near a minimum intensity that reliably yielded responses.

Strychnine (Sigma) and picrotoxin (Tocris) were added to the ACSF perfusate to block GlyRs and GABAARs; D-2-amino-5-phosphovaleric acid (D-APV) (Ascent Scientiﬁc, Tocris) and CNQX (Tocris) were added to block NMDARs and AMPARs. Charge transfer measurements were taken after any necessary corrections for driving force. The selective GluN2B-containing NMDAR antagonist ifenprodil (Ascent) was used to evaluate NMDAR subunit composition across age. The presence of GluA2-lacking AMPARs was assessed both by rectification and by application of the selective GluA2-lacking AMPAR antagonist IEM 1460 (Tocris). For AMPAR rectiﬁcation recordings the internal solution contained 0.1 mM spermine (Acros Organics). In release probability experiments cyclothiazide (Ascent) was included in the perfusate to prevent AMPAR desensitization. Drug concentrations used in the perfusate were (in mM): 5–10 strychnine, 50 picrotoxin, 50 D-APV, 5 CNQX, 3–100 ifenprodil, 100 IEM 1460, and 100 cyclothiazide.

Rectiﬁcation of AMPARs was assessed by taking recordings at −60 mV, −40 mV, +40 mV, and +60 mV. The rectiﬁcation index is deﬁned here as \( RI = \left( I_{+40\,\text{mV}} - I_{-40\,\text{mV}} \right) / \left( I_{+60\,\text{mV}} - I_{-60\,\text{mV}} \right) \). Average reversal potential for AMPARs was 7 mV. Probability of release was examined by delivering 10 pulses of electrical stimulation at 20 or 50 Hz. Peak current amplitudes resulting from the second to tenth pulses were divided by the peak current amplitude resulting from the ﬁrst pulse \( \left( I_1 / I_2 \right) \) to determine paired-pulse ratios (PPRs); peak current was measured relative to current immediately before the stimulus for each pulse.

RESULTS

We used whole cell voltage clamp in acute brain stem slices of neonatal rat pups to characterize the glutamatergic response to MNTB stimulation at immature LSO principal neurons receiving GABA/glycine- and glutamate-releasing inputs. Because strychnine washout is nearly impossible to achieve, we recorded from a single cell per slice (e.g., Fig. 1A), to ensure that all cells analyzed for a glutamatergic response received dual inputs. As shown in Fig. 1, A and B, after block of GABAARs, glyRs, and NMDARs many of the residual, unfiltered, AMPAR-mediated currents were small relative to baseline. These residual component were classiﬁed as AMPAR components if 1) the current amplitude was ≥3 standard deviations above background noise, 2) the current decay time constant was on the order of microseconds (not 10s or 100s of ms), and 3) the current disappeared after application of the AMPAR antagonist CNQX (Fig. 1B). Recording from 113 cells (P0–P12), we found, consistent with previous results (Gillespie et al. 2005), that glutamatergic responses declined after the ﬁrst postnatal week (Fig. 1C) and that the glutamatergic component lagged the GABA/glycinergic component [onset latencies, in ms, for GABA/gly: P0–P4: 2.5 ± 0.1 (n = 50), P5–P8: 2.3 ± 0.1 (n = 46), P9–P12: 2.7 ± 0.1 (n = 15); for glu: P0–P4: 3.4 ± 0.1, P5–P8: 3.2 ± 0.1, P9–P12: 3.6 ± 0.1; mean % difference in onset latency calculated from these means is within 5% of mean of individual cell % differences, for each age]. To ask whether we might in some of these experiments have stimulated (hypothetical) glutamatergic ﬁbers of passage rather than ﬁbers from GABA/glycinergic MNTB neurons, in an additional six cells (P3–P7) we puffed
glutamate directly in the MNTB while recording from an LSO principal cell. In all (5/5) slices aged P3–P5 an NMDAR-mediated component could be clearly observed before the addition of strychnine/picrotoxin (Fig. 1D), and in all cases onset latency for the glutamate component lagged that for the GABA/glycine component.

NMDA receptors are the main contributors to MNTB-elicited glutamatergic responses in LSO neurons. The proposed role of glutamate release in inhibitory synaptic refinement assumes that postsynaptic NMDARs are present at MNTB-LSO synapses during the period of refinement, and so we first asked what proportion of cells exhibited NMDAR and/or AMPAR components. Figure 2A shows receptor subtypes for 67 cells that exhibited glutamatergic responses to MNTB-LSO stimulation, and in which the NMDA and AMPA components could be clearly separated. Surprisingly, in nearly one-third (7 of 22) of cells recorded before P3 the glutamatergic response was mediated solely by AMPARs. By contrast, from P3 onward every glutamatergic response exhibited an NMDAR-mediated component. After P3, not only were the NMDAR-mediated responses more numerous than the AMPAR-mediated responses but they were also larger in magnitude, such that NMDARs contributed on average over half the peak conductance of the glutamatergic responses (Fig. 2B). The proportional magnitude of the NMDAR component was noticeably largest between P3 and P9, with the estimated mean contribution larger than 75% at each age tested during this period (see Table 1; Kruskal-Wallis test for NMDA current contribution, $P = 0.0124$; post hoc Mann-Whitney P1 vs. P3, $P = 0.0129$; post hoc Mann-Whitney P9 vs. P10, $P = 0.0047$).

Postsynaptic glutamate receptors activated by glutamate release at MNTB-LSO synapses are ionotropic; AMPARs contain GluA2. The substantial subset of glutamatergic responses mediated solely by AMPARs appears to run counter to the hypothesis that NMDARs mediate plasticity at MNTB-LSO synapses before hearing onset. However, Ca$^{2+}$-permeable GluA2-lacking AMPARs (CP-AMPARs) have also been implicated in synaptic plasticity (for review, see Cull-Candy et al. 2006), and so we asked whether CP-AMPARs could have mediated the AMPAR responses observed at MNTB-LSO synapses. For 17 slices, we added spermine to the intracellular solution, isolated the AMPAR-mediated response pharmacologically, and examined the AMPAR responses for the inward rectification characteristic of CP-AMPARs (Fig. 3A; Burnashev et al. 1992; Muller et al. 1992). We found neither evidence for significant rectification nor a change in rectification indices between early and later ages (Fig. 3B; mean RI = 1.02 ± 0.09, n = 18). To further test for the presence of CP-AMPARs, we isolated AMPAR-mediated responses pharmacologically and applied IEM 1460, an antagonist of GluA2-lacking (and hence calcium permeable) AMPARs. No effect of IEM 1460 was observed (Fig. 3C; mean residual current =
To further investigate the non-NMDAR-mediated glutamatergic response we asked whether there was any relationship between amplitude of the AMPAR response and maturation of the inhibitory pathway. AMPAR currents were larger, relative to GABA/glycine currents, at earlier ages than at later ages (Fig. 4A). Peak GABA/glycine current increased between P0 and P8 (Fig. 4B), as has been previously reported (Kim and Kandler 2003). The decline in AMPAR amplitude relative to GABA/glycine receptor amplitude during the first postnatal week resulted largely from this maturational strengthening of the GABA/glycine inputs, while AMPAR responses remained small (Fig. 4C).

Changes in NMDAR current duration define a window between P2/3 and P8/9. The specific subunit composition of NMDARs may influence the plasticity available to a synapse (Liu et al. 2004), and in many areas NMDAR subtype composition changes across development (Monyer et al. 1994; Sheng et al. 1994). We therefore attempted to ascertain whether NMDAR subunit composition at MNTB-LSO synapses changes before hearing onset. In 87 cells, we isolated NMDAR-mediated responses pharmacologically and then assessed charge transfer and used subunit-specific pharmacology to look for evidence of a change in subunit composition. As seen in representative traces (Fig. 5A), average charge transfer for the NMDAR current increased over the first few days and dropped sharply at P9 (Fig. 5B), recalling the pattern observed for the relative proportion of total glutamatergic current carried by NMDAR (Fig. 2B) (see Table 1; Kruskal-Wallis, P = 2.2 × 10^{-4}; post hoc Mann-Whitney P2 vs. P3, P = 0.0125; post hoc Mann-Whitney P8 vs. P9, P = 0.0043). Additionally, when charge transfer was normalized to peak response to estimate relative NMDAR excitatory PSC (EPSC) duration, a sharp drop was observed between P8 and P9 (Fig. 5C) (see Table 1; Kruskal-Wallis, P = 1.9 × 10^{-4}, post hoc Mann-Whitney P8 vs. P9, P = 0.0087). These findings are consistent with a

Table 1. Properties of NMDAR-mediated neurotransmission as a function of age

<table>
<thead>
<tr>
<th>Age</th>
<th>% NMDA Conductance</th>
<th>NMDA Charge Transfer, pC</th>
<th>NMDA CT/Peak, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>67 ± 33 (3)</td>
<td>28.3 ± 1.0 (2)</td>
<td>1,330 ± 215.7 (2)</td>
</tr>
<tr>
<td>P1</td>
<td>34 ± 12 (11)</td>
<td>59.2 ± 20.4 (5)</td>
<td>1,355 ± 251.0 (5)</td>
</tr>
<tr>
<td>P2</td>
<td>62 ± 14 (8)</td>
<td>78.6 ± 14.1 (8)</td>
<td>1,437 ± 167.3 (8)</td>
</tr>
<tr>
<td>P3</td>
<td>81 ± 3 (12)</td>
<td>193.4 ± 34.9 (13)</td>
<td>1,637 ± 112.3 (13)</td>
</tr>
<tr>
<td>P4</td>
<td>83 ± 5 (7)</td>
<td>106.9 ± 18.2 (12)</td>
<td>1,603 ± 196.0 (12)</td>
</tr>
<tr>
<td>P5</td>
<td>85 ± 4 (12)</td>
<td>83.9 ± 16.7 (15)</td>
<td>1,063 ± 109.5 (15)</td>
</tr>
<tr>
<td>P6</td>
<td>81 ± 5 (9)</td>
<td>150.5 ± 38.4 (12)</td>
<td>1,512 ± 139.2 (12)</td>
</tr>
<tr>
<td>P7</td>
<td>91 ± 2 (4)</td>
<td>132.5 ± 27.7 (5)</td>
<td>960 ± 227.5 (5)</td>
</tr>
<tr>
<td>P8</td>
<td>88 ± 3 (3)</td>
<td>244.3 ± 107.1 (5)</td>
<td>1,382 ± 133.5 (5)</td>
</tr>
<tr>
<td>P9</td>
<td>85 ± 5 (7)</td>
<td>16.6 ± 8.2 (6)</td>
<td>529 ± 120.8 (6)</td>
</tr>
<tr>
<td>P10-P12</td>
<td>57 ± 6 (6)</td>
<td>18.1 ± 7.4 (4)</td>
<td>464 ± 76.1 (4)</td>
</tr>
</tbody>
</table>

Values are means ± SE for % NMDA receptor (NMDAR) conductance calculated as (NMDA/AMPA + GlyR) × 100; NMDAR charge transfer (CT), and NMDAR charge transfer normalized to peak (NMDAR CT/Peak) as a function of age before hearing onset. Number of values for each measurement are in parentheses. P, postnatal day.
change in NMDAR subunit composition, in addition to the
decline in NMDAR activation, around P8/9.

In many systems, a decrease in NMDAR EPSC duration
is caused by a developmental switch from expression of
GluN2B- to GluN2A-containing NMDARs. To ascertain
whether GluN2B-enriched postsynaptic membranes might
account for the longer NMDAR EPSCs seen in the first
postnatal week, in slices aged P0 – P10 we tested an addi-
tional 17 cells for sensitivity to ifenprodil (10 μM), an
antagonist that preferentially blocks NMDARs containing
the GluN2B subunit. In all cells tested from P0 to P8,
addition of ifenprodil to the perfusate caused a reduction in
charge transfer (Fig. 5D) and in peak current amplitude
(data not shown), whereas at P9 – P10 ifenprodil had little
effect [% reduction: P0 – P2, 35 ± 7 (n = 4); P3–P4, 37 ±
12 (n = 4); P5–P8, 34 ± 14 (n = 5); P9–P10, 4 ± 11 (n =
4); 1-way ANOVA, P = 0.1952]. Dose-response curves for
ifenprodil were also similar for all ages between P0 and P8
(Fig. 5E). In four additional slices from animals < 8 days
old, the selective GluN2B antagonist Ro25-6981 (3 μM)
was added; Ro25-6981 reduced peak current and charge
transfer by 34% and 38% (data not shown). These results are
consistent with the expression of GluN2B-containing
NMDARs at MNTB-LSO synapses in the first postnatal
week, and they suggest that the large charge transfer seen
in the first postnatal week results from the relatively long
decay kinetics conferred by the GluN2B subunit. Finally, we asked
whether the size of the NMDAR-mediated response was
related to the size of the GABA/glycine response. For these
experiments, as in all others, the minimum stimulus intensity that
reliably elicited a response was first determined, and this stimulus
intensity was then used throughout the experiment in order to
ensure that the same single fiber (or small subset of fibers) was
stimulated when evaluating both GABA/glycine receptor and the
NMDAR-mediated responses. Neither peak NMDAR current (to
reflect total NMDAR contribution; Fig. 6A) nor charge transfer

Fig. 4. AMPAR-mediated responses to MNTB stimulation P0–P12. For cells in which an AMPAR component was measured AMPAR-mediated peak current ($I_{\text{AMP}}$) is larger, relative to GABA/glycine peak current ($I_{\text{GABA}}$), at the earliest ages than at later ages (A); GABA/glycine receptor-mediated currents increase between P0 and P8 (B); and AMPAR-mediated currents change little in peak amplitude over the first 2 postnatal weeks (C). For clarity, 2 outlying points at P8 (5,615 and 5,235 pA) are not included in B.

Fig. 3. Glutamate released at MNTB-LSO synapses does not activate Ca$^{2+}$-permeable AMPARs. A: example rectification measurements for 2 cells with relatively extreme rectification index (RI) measurements, showing raw traces for high (Ac, P1 cell) and low (Ab, P3 cell) RIs and summary measurements with linear fits (Ac). Note that although the P3 cell had an RI value below 1.0, significant current was passed at positive membrane potentials. B: RIs are distributed around 1 between P1 and P8; n = 17. Filled circles correspond to the traces shown in Ac and Ab. g$_{\text{g40}}$ g$_{\text{g60}}$ conductance at +40 mV and −60 mV. C: no consistent change was seen in peak AMPAR current ($I_{\text{AMP}}$) after application of IEM 1460 (n = 13).

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(to reflect GluN2B subunit contribution; Fig. 6B) was significantly correlated with the size of the initial GABA/glycine response (NMDA $I_{\text{peak}}$ vs. GABA/gly $I_{\text{peak}}$: Pearson’s $R = 0.30$; NMDA charge transfer vs. GABA/gly $I_{\text{peak}}$: Pearson’s $R = 0.26$). Thus, although NMDARs passed substantial charge during the first postnatal week, degree of NMDAR activation was correlated not with strength of GABA/glycine synapses but with postnatal age (Fig. 6C).

Release probabilities differ for GABA/glycine and for glutamate. An open question surrounding cotransmission of GABA/glycine and glutamate has been whether the two neurotransmitters are released together or whether they could be released independently of one another. To examine this question, we measured release probabilities for GABA/glycine and for glutamate in the same cell by recording responses to 20- and 50-Hz stimulus trains delivered to the MNTB (Fig. 7A). To avoid artifact due to possible incomplete washoff of AMPAR antagonists, GABA/glycinergic responses were measured first without any pharmacological antagonists in the perfusate. Strychnine, picrotoxin, and APV were then applied to isolate AMPAR-mediated currents, and pulse trains were delivered again to determine the release probability for glutamate. Release probabilities for both GABA/glycine and glutamate remained fairly stable during the first postnatal week (Fig. 7,

Fig. 6. Peak NMDAR-mediated current and charge transfer as a function of peak GABA/glycine current. A and B: neither NMDAR-mediated peak current ($I_{\text{NMDA,peak}}$) nor charge transfer ($Q_{\text{NMDA}}$) correlates strongly with the size of GABA/glycinergic inputs. C: NMDAR-mediated charge transfer ($Q_{\text{NMDA}}$) is high relative to GABA/glycinergic receptor-mediated peak current between P2 and P6. For clarity, extreme outliers have been omitted from A (NMDA $pA$ vs. GABA/gly $pA$: $287 \times 424$, $122 \times 1,584$, $363 \times 2,802$, $52 \times 3,280$, $96 \times 5,615$), B (NMDA $pC$ vs. GABA/gly $pA$: $392 \times 424$, $98 \times 1,584$, $34 \times 1,911$, $432 \times 2,802$, $48 \times 3,280$, $160 \times 5,615$), and C (1,635 at P3).
B–E), but at all ages paired-pulse depression was greater for GABA/glycinergic transmission than for glutamatergic transmission (Fig. 7, B–E), consistent with the release of glutamate and GABA/glycine from distinct vesicle populations. As activation of presynaptic receptors can modulate release, and to control for the possibility that Ca\(^{2+}\)/\(H_1\) entry into the postsynaptic cell might have affected GABA/glycine currents, we recorded from an additional three cells in the presence of the GABAB receptor antagonist CGP 52432 (10 \(M\)) and the NMDAR antagonist D-APV (50 \(M\)) throughout. Although the PPRs increased slightly (GABA/gly: \(-10\%\) at 20 Hz, \(<1\%\) at 50 Hz; glu: \(-5\%\) at 20 and 50 Hz), GABA/glycine and glutamate still exhibited distinctly different release probabilities (data not shown).

**DISCUSSION**

Here we have examined glutamatergic neurotransmission at a glutamate-releasing immature inhibitory synapse. We find that NMDARs are the major contributors to glutamatergic responses at immature MNTB-LSO synapses and that activation of GluN2B-containing NMDARs defines a temporal period whose closure correlates with the known decline in major functional refinement in this pathway. Functional refinement of the MNTB-LSO pathway, characterized by a decrease in input area and an increase in strength of individual inputs, is thought to be driven by spontaneous activity generated in the cochlea and is complete in rats by about P9 (Kandler 2004; Kim and Kandler 2003; Tritsch et al. 2007; Tritsch and Bergles 2010). Although recent evidence suggests that glutamate release is necessary for normal functional refinement in this pathway (Noh et al. 2010), glutamate receptors can play many roles in synaptic plasticity at inhibitory and excitatory synapses; these results identify specific receptors and timepoints that may be especially revealing for further studies of refinement in the MNTB-LSO circuit.

**AMPA receptors in the MNTB-LSO pathway.** In contrast to the trend from NMDAR-containing “silent synapses” to AMPAR-containing synapses elsewhere (Isaac et al. 1997; Liao et al. 1995), about one-third of MNTB-LSO synapses before P2–P3 lacked NMDARs and were enriched for AMPARs. If Ca\(^{2+}\) influx is important for MNTB-LSO refinement, why would much of the early glutamatergic current be carried by AMPARs? The Ca\(^{2+}\)-permeable GluA2-lacking AMPARs that have been implicated in synaptic plasticity elsewhere (Lei and McBain 2002; Mahanty and Sah 1998) appear to be absent from this synapse. In fact, Ca\(^{2+}\) influx may not be needed until about P3, after which the mean MNTB input area declines steeply (Kim and Kandler 2003) and NMDARs reliably mediate most of the glutamatergic response.

Do AMPARs play a role in the MNTB-LSO pathway? Possibly, the small AMPAR currents we observed reflect an immature system in which AMPARs destined for AVCN-LSO synapses are inappropriately inserted near MNTB terminals. A scenario in which AMPAR activation unblocks NMDARs very locally as the chloride reversal potential becomes more negative with age is unlikely both because of the typically small size of the AMPAR responses and because these responses were most numerous at ages when GABA and glycine are frankly excitatory (Ehrlich et al. 1999; Kandler and Friau 1995; Kullmann and Kandler 2004).

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**Fig. 7.** Glutamate release probability differs from GABA/glycine release probability at MNTB-LSO synapses. A: representative LSO response to 10-pulse trains delivered to the MNTB at 20 Hz (P2 slice). GABA\(_R/glyR\)-mediated responses are shown on *left* and strychnine/picrotoxin/APV-isolated AMPAR-mediated responses for the same cell on *right*. *Inset*: absolute current amplitude of the first (F) and last (L) pulses for the GABA/glycine receptor-mediated (G/G) and AMPAR-mediated (AMPA) responses. B–E: average pulse train responses for P0–P3 (B and D) and P5–P7 (C and E) cells stimulated at 20 Hz (B and C) or 50 Hz (D and E); 6 cells/age group. Current amplitudes are normalized to average first peak amplitude. At all ages and at both 20 and 50 Hz, release probability for GABA/glycine is higher than that for glutamate.
We suggest that AMPAR-mediated responses reflect glutamate spillover to receptors under AVCN synapses and that this spillover declines with the maturation of functional diffusion barriers. Such spillover would also likely activate extrasynaptic NMDARs, a possibility corroborated by the longer glutamate latencies and by the prominent early GluN2B component in the NMDAR-mediated response (Rumbaugh and Vicini 1999).

**NMDA receptors in the MNTB-LSO pathway.** Fractional contribution of NMDARs to peak glutamatergic current defined a temporal window in the MNTB-LSO pathway: NMDAR-mediated currents were seen in most of the glutamatergic responses recorded here and, notably, were present in 100% of glutamatergic responses recorded after P2. The absence of NMDAR-mediated currents in some of the responses before P3 and the increased contribution of NMDARs to peak current suggest a developmental change in NMDAR expression or insertion in the MNTB-LSO pathway. In fact, the increased fractional contribution through NMDARs at P2/3 defined the onset of a temporal window from about P3 to about P9. Although the onset of this window at P3 was not previously predicted, the closure of the window at P9 matched the end of the period of functional refinement in the MNTB-LSO pathway, as would be expected if specific NMDARs mediate developmental plasticity at MNTB-LSO synapses and if these NMDARs are expressed at MNTB-LSO synapses during a period of heightened plasticity.

The P3–P9 window was delineated also in the isolated NMDAR response by larger amounts of charge transferred. This change in current duration strongly suggests a change in subunit composition for the NMDAR population. As the GluN2A and GluN2B subunits interact with distinct synaptic proteins and confer different Mg$^{2+}$ sensitivity and decay kinetics on the NMDAR channels, the relative proportions of these subunits can set several parameters of plasticity (Crair and Malenka 1995; Hrabetova et al. 2000; Liu et al. 2004; Monyer et al. 1992; Philipot et al. 2007). We did not attempt to obtain many recordings after P10, because of the steadily decreasing probability of glutamate release after the first postnatal week, but note the sharp reduction in ifenprodil sensitivity between P7/8 and P9/10 and suggest that functional GluN2B expression likely remains low after P10. In the glutamatergic VCN-LSO pathway, NMDAR responses also lose sensitivity to ifenprodil and exhibit shorter decay kinetics after P8 (Case et al. 2011). This developmental shortening of NMDAR EPSCs could result from a subunit substitution of GluN2A for GluN2B (Flint et al. 1997; Quinlan et al. 1999).

The developmental profile of glutamatergic transmission—in which NMDAR-mediated charge transfer rises between P3 and P9—could be suggestive of an NMDAR-mediated sensitive period, in light of several correlated events: 1) the close of major functional refinement occurs around P9 (Kim and Kandler 2003); 2) GABA/glycine are depolarizing before about P9 (Ehrlich et al. 1999; Kandler and Friaul 1995); 3) normalized MNTB input area to a given LSO cell changes before about P9 (Ehrlich et al. 1999; Kandler and Friauf 1995); (Kim and Kandler 2003); and 4) spontaneous activity begins to increase in the cochlea around P3 (Tritsch and Bergles 2010). In light of these events, we speculate that the MNTB-LSO pathway may be another example of an inhibitory circuit that experiences a sensitive period (Maffei et al. 2010), perhaps a window that opens around P3 to permit plasticity driven by spontaneous cochlear activity and then closes around P9.

If NMDARs are necessary for synaptic strengthening, one could ask whether the size of NMDAR EPSCs is inversely correlated with size of developing GABA/glycine synapses. We saw no correlation between size of the two response types, although NMDARs containing different subunit types could be differentially distributed on subpopulations undergoing strengthening or weakening. Studies in the gerbil slice preparation have linked long-term depression at the MNTB-LSO synapse to several mechanisms that do not specifically involve NMDARs (Kotak and Sanes 2000, 2002; Kotak et al. 2001). Long-term potentiation has not yet been reported in the MNTB-LSO pathway, and, in fact, we still do not know whether NMDAR activation mediates Hebbian developmental refinement in the MNTB-LSO pathway. To our knowledge, no one has yet attempted to induce synaptic plasticity in this pathway in the absence of NMDAR antagonists, and it will be important to determine whether NMDAR-mediated plasticity occurs in the MNTB-LSO pathway.

**Release probability at GABA/glycine/glutamatergic MNTB-LSO synapses.** Are GABA, glycine, and glutamate released together at MNTB terminals? If so, they might be expected to occupy the same set of presynaptic vesicles, characterized by coexpression of vesicular transporters for glutamate and GABA/glycine. Such copackaging is possible, as other pairs of distinct transporters can be expressed in the same vesicles (Gras et al. 2008; Herzog et al. 2006). Our results show different PPRs for GABA/glycine and for glutamate-mediated neurotransmission. A minor caveat is that GABA/glycine PPRs were obtained without block of glutamate receptors, whereas glutamate PPRs were obtained in the presence of GABA/glycine antagonists. The different treatments arose from our requirement of obtaining PPRs for both neurotransmitter types from the same slices, the near impossibility of washing off strychnine within a typical recording period, the small size of most of the AMPAR-mediated currents (Fig. 7A, inset), and our consequent concern that incomplete washoff of AMPAR antagonists might compromise the AMPAR recordings. However, given the relative amplitudes for AMPAR-mediated currents and GABA/glycine receptor-mediated currents, AMPARs are unlikely to contribute significantly to differences in apparent GABA/glycine current. Furthermore, if the AMPAR-mediated component with its smaller PPR affected the GABA/glycine PPR, it would make GABA/glycine paired-pulse depression appear smaller than reality; this would have caused us to underestimate the differences in PPRs between the two neurotransmitter systems.

One interpretation for the differences in PPR for GABA/glycine and for glutamate is that GABA/glycine and glutamate are released from different vesicles at MNTB terminals. GABA/glycine and glutamate may occupy distinct vesicle populations, possibly distinguished by their proximity to the synaptic cleft or by their association with different forms of the calcium sensor synaptotagmin (Cooper and Gillespie 2011; Fox and Sanes 2007; Xu et al. 2007), which could confer distinct release probabilities on the two vesicle populations. Alternatively, differences in vesicle filling properties could give rise to the differing paired-pulse depression (Aubrey et al. 2007). Regardless of the cause, these distinct release probabilities might allow the presynaptic terminal—depending on
patterns of neural activity—-to switch between single (GABA/ 
glycine) and dual (GABA/glycine + glutamate) release. A key
implication of such a switch is that if dual release indeed 
mediates synaptic plasticity, the temporal statistics of the 
incoming spike train would determine whether the synapse
operated in a normal transmission mode or in a plasticity-
inducing mode.

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