Metabotropic glutamate receptors and glutamate transporters shape transmission at the developing retinogeniculate synapse

Jessica L. Hauser,* Eleanore B. Edson,† Bryan M. Hooks, and Chinefi Chen

Department of Neurology, F. M. Kirby Neurobiology Center, Children’s Hospital, Boston, Massachusetts; and Program in Neuroscience, Harvard Medical School, Boston, Massachusetts

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Metabotropic glutamate receptors and glutamate transporters shape transmission at the developing retinogeniculate synapse. J Neurophysiol 109: 113–123, 2013. First published October 17, 2012; doi:10.1152/jn.00897.2012.—Over the first few postnatal weeks, extensive remodeling occurs at the developing murine retinogeniculate synapse, the connection between retinal ganglion cells (RGCs) and the visual thalamus. Although numerous studies have described the role of activity in the refinement of this connection, little is known about the mechanisms that regulate glutamate concentration at and around the synapse over development. Here we show that interactions between glutamate transporters and metabotropic glutamate receptors (mGluRs) dynamically control the peak and time course of the excitatory postsynaptic current (EPSC) at the immature synapse. Inhibiting glutamate transporters by bath application of TBOA (L-threo-β-benzyloxyaspartic acid) prolonged the decay kinetics of both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor ( AMPAR) and N-methyl-D-aspartate receptor (NMDAR) currents at all ages. Moreover, at the immature synapse, TBOA-induced increases in glutamate concentration led to the activation of group II/III mGluRs and a subsequent reduction in neurotransmitter release at RGC terminals. Inhibition of this negative-feedback mechanism resulted in a small but significant increase in peak NMDAR EPSCs during basal stimulation and a substantial increase in the peak with coapplication of TBOA. Activation of mGluRs also shaped the synaptic response during high-frequency trains of stimulation that mimic spontaneous RGC activity. At the mature synapse, however, the group II mGluRs and the group III mGluR7-mediated response are downregulated. Our results suggest that transporters reduce spillover of glutamate, shielding NMDARs and mGluRs from the neurotransmitter. Furthermore, mechanisms of glutamate clearance and release interact dynamically to control the glutamate transient at the developing retinogeniculate synapse.

development; glutamate transporters; metabotropic glutamate receptors; synapses; visual system

FAST EXCITATORY NEUROTRANSMISSION in the central nervous system (CNS) is primarily mediated by the presynaptic release of glutamate and its clearance from the synaptic cleft. At the retinogeniculate synapse, glutamate is released from retinal ganglion cells (RGCs) onto thalamic relay neurons in the lateral geniculate nucleus (LGN). Over development, information is continuously relayed through this synapse to cortex (Akerman et al. 2002; Huttenlocher 1967; Krug et al. 2001; Moseley et al. 1988). Before eye opening, around postnatal day (P)12 in mice, information is encoded in correlated spontaneous retinal activity characterized by prolonged bursts of spik-
response with age, suggesting neurotransmission is tightly controlled throughout this developmental period.

MATERIALS AND METHODS

Slice preparation and extracellular solutions. Parasagittal slices (250 μm) that preserved optic tract and visual thalamus were prepared from P8–12 or P26–34 C57BL/6 or Black Swiss mice (Charles River, Wilmington, MA or Taconic Farms, Germantown, NY) of either sex, as described previously (Chen and Regehr 2000) and in accordance with federal guidelines and protocols approved by Children’s Hospital Boston. Slices were prepared in ice-cold choline-based dissection solution containing the following (in mM): 130 choline chloride, 26 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 7.0 MgCl₂, 0.5 CaCl₂, and 25 glucose. Slices were incubated in this solution at 32°C for 15–20 min, followed by an additional 10 min in artificial cerebral spinal fluid (aCSF) containing (in mM) 125 NaCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 1.0 MgCl₂, 2.0 CaCl₂, and 25 glucose (Sigma, St. Louis, MO). aCSF was adjusted to 310–315 mosmol/l. Slices were then transferred into the recording chamber superfused with aCSF at 2–3 ml/min. All experiments were performed at 35 ± 1°C, unless otherwise indicated. aCSF and choline dissection solutions were saturated with 95% O₂-5% CO₂. Slices were used for up to 5 h after preparation. Experiments were performed with aCSF containing the γ-aminobutyric acid type A (GABA_A) receptor antagonist bicuculline (20 μM) or picrotoxin (50 μM, Sigma), the GABA_B receptor antagonist 3-N-[1-(3,4-dichlorophenyl)ethyl]amino-2-(S)-hydroxypropyl-5-benzyl-1-phosphonic acid (CGP-55845; 2 μM), and the A1-adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 10 μM). To isolate the N-methyl-d-aspartate receptor (NMDAR) excitatory postsynaptic current (EPSC), we used 2,3-dihydro-6-nitro-7-sulfamoylbenzoi/lquinazoline-2,3-dione (NBQX; 5 μM) while holding the cell at +40 mV. To isolate the AMPA receptor (AMPA) EPSC, we included 3-[(R)-2-carboxyepiperazin-4-yl]propyl-1-phosphonic acid ([(R)-CPP]; 20 μM) and (-)-2-amino-5-phosphonopentanoic acid (n-AP5; 50–100 μM) in the bath while holding the cell at −70 mV. For some experiments, AMPAR desensitization was inhibited by including 6-chloro-3,4-dihydro-3-(5-norbornen-2-yl)-2H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide (cyclothiazide; 50 μM) in the bath. To inhibit glutamate transport, we added 10–50 μM DL-threo-β-benzyloxyaspartic acid (TBOA) to the superfusion solution. The following agents were also added depending on the experiment: the antagonist to mGluRs, 25–2-amino-2-(15S,2S-2-carboxycyclopent-1-yl)-3-(xanth-9-yl)propanoic acid ([LY341495]; 50 μM); agonists to group I, II, and III mGluRs, respectively, (S)-3,5-dihydroxyphenylglycine ([S]-DHPG; 1–25 μM), (2R,4R)-aminompropylidine-2,4-dicarboxylate (APDC; 1–30 μM), and (1S,2S)-amino-4-phosphonobutyllic acid (t-AP4; 10–500 μM); and the low-affinity NMDAR agonist t-(+)-(2S)-2-amino-5-phosphonopentanoic acid (t-AP5; 1 mM). Stock solutions of pharmacological agents were stored at −20°C and diluted according to the final concentrations immediately before experiments. All pharmacological agents were purchased from Tocris Bioscience (Ellisville, MO) unless otherwise indicated.

Electrophysiology. Whole cell recordings of thalamic relay neurons were acquired using glass patch pipettes (resistance 1.1–1.7 MΩ) filled with internal solution containing (in mM) 35 CsF, 100 CsCl, 10 EGTA, 10 HEPES, and 0.1 methoxyverapamil hydrochloride (L-type Ca²⁺ channel antagonist; Sigma) and adjusted to 290–300 mosmol/l, pH 7.3. This solution is designed to minimize postsynaptic contributions to synaptic transmission. Cesium blocks K⁺ channels and thus optimizes voltage clamp, whereas EGTA and fluoride inactivate many second messenger systems. Relay neurons were visualized using differential interference contrast optics (Olympus). Voltage-clamp recordings were made using an Axopatch 200B or Multiclamp 700A amplifier (Axon Instruments, Foster City, CA), filtered at 1 kHz, and digitized at 4–50 kHz with an ITC-16 interface (InstruTECH, Port Washington, NY). EPSCs were evoked by current pulses delivered to the intact optic tract by a pair of aCSF-filled glass micropipettes (Drummond Scientific, Broomall, PA), connected to a stimulus isolator (World Precision Instruments, Sarasota, FL), with intensities that ranged from 10 to 150 μA. The membrane potential of the relay neuron was clamped at either −70 or +40 mV (for AMPAR-mediated EPSCs and NMDAR-mediated EPSCs, respectively) during stimulation of the optic tract and was held at 0 mV between stimulation trials. For baseline recordings, optic tract was stimulated at a frequency of 0.025 Hz for NMDAR EPSCs and 0.05 Hz for AMPA EPSCs. To ensure consistent access resistance of the recording electrode throughout the entire experiment, we monitored the peak amplitude of a brief (10 ms) hyperpolarizing test pulse (−5 mV) given before the optic tract stimulation. Access resistances of relay neurons were <15 MΩ.

Analysis. Data acquisition and analysis was performed using custom software written in IgorPro (Wave-Metrics, Portland, OR), Prism (GraphPad Software, San Diego, CA), and Excel (Microsoft, Redmond, WA). EPSCs were analyzed as the average of 5–10 waves. The decay time course of the AMPAR EPSC is well fit with the double-exponential function f(t) = A1e(−t/τ1)+A2e(−t/τ2 swornoff) and is quantified as the weighted tau: τ = τf × A1/(A1 + A2) + [τslow × A2/(A1 + A2)]. At the immature synapse, the time constant of the slow component (τs) contributes to a significant component of the AMPAR waveform (Liu and Chen 2008) and is the component most sensitive to TBOA. For experiments involving trains of stimuli, stimulus sets were performed using randomized, interleaved trains at frequencies of 10, 20, and 50 Hz. AMPAR amplitudes following the first EPSC (EPSCs_1) were quantified as the difference between the peak EPSC and the baseline current immediately after the stimulus artifact. Data are summarized as means ± SE using the two-tailed paired t-test unless otherwise indicated.

RESULTS

Glutamate transporters shape the synaptic waveform at the immature retinogeniculate synapse.

To investigate the role of glutamate transporters at the immature retinogeniculate synapse, we tested the effects of the competitive nontransportable blocker of glutamate transporters, TBOA, on acute slices prepared from P8–12 mice. Whole cell recordings were made with patch electrodes in voltage-clamp mode from relay neurons in the LGN, and synaptic responses were evoked by stimulating optic tract fibers (Chen and Regehr 2000). Figure 1 illustrates the effects of bath application of TBOA (50 μM) while either NMDAR- or AMPAR-mediated EPSCs were recorded.

When we inhibited glutamate transport with TBOA, the NMDAR EPSC amplitude was reduced to 56 ± 4% of control (n = 5, P = 0.01) and the time course of decay was significantly prolonged [half-decay time (τ₁/₂g) 542.9 ± 156.9% of control, n = 5, P < 0.05; Fig. 1, A and C]. The remaining current in the presence of TBOA was blocked by NMDAR-specific antagonists (Fig. 1A, right). We found a similar effect of TBOA on the waveform of the AMPAR EPSC (Fig. 1, B and C). The peak amplitude of the AMPAR EPSC decreased to 54 ± 6% of control (n = 5, P = 0.001), and the time course of the current also slowed. The decay kinetics of the AMPAR EPSC at immature synapses is well described by a double-exponential relationship (Liu and Chen 2008). In the presence of TBOA, τs increased to 430 ± 82% of control (n = 5, P < 0.05) and the current could be completely blocked by AMPAR-specific antagonists (Fig. 1B, right). The average effects...
of TBOA on the peak EPSC and the decay time courses of both NMDAR and AMPAR currents are compared in Fig. 1C.

These results demonstrate that glutamate transporters are present at the immature retinogeniculate synapse and that they actively remove glutamate during synaptic transmission. Slowing of the EPSC decay kinetics implies that reduced glutamate clearance results in either extended activation of glutamatergic receptors in the synaptic cleft and/or spillover to receptors farther from the release sites. In addition, TBOA increased the relay neuron holding current (I_{hold}) elicited by a step depolarization from 0 to +40 mV to 200 ± 30% of control (n = 5, P < 0.05). The difference in I_{hold}, with and without TBOA, can be attributed to increased basal activation of NMDARs because it is reversed with bath application of NMDAR antagonists [100 μM DL-APV and 20 μM (R)-CPP; Fig. 1D]. Since NMDA antagonists do not block all of I_{hold} in control conditions, our results suggest that TBOA exposure increases ambient glutamate concentration by significantly more than 200%.

Biphasic synaptic response to glutamate transporter inhibition.

Although an increase in ambient glutamate or glutamate spillover could explain the slowing of NMDAR current decay kinetics and the change in I_{hold} in response to bath application of TBOA, it does not explain the decrease in EPSC peak amplitudes. However, we noticed in some experiments, such as in Fig. 1A, that there was often an initial transient increase followed by a reduction in the peak EPSC. Thus we asked whether the decrease in peak EPSC was a secondary effect of elevated glutamate concentrations. To test this possibility, we took advantage of the fact that the activity of glutamate receptors (both metabotropic and ionotropic) is more temperature-dependent than glutamate diffusion. We reasoned that we might be able to better appreciate the initial transient increase in NMDAR EPSC peak amplitude by recording at room temperature (RT) and by sampling more frequently. Figure 2A shows that at RT the time course of the peak EPSC amplitude following inhibition of glutamate transport consisted of two distinct phases. Immediately following the addition of TBOA, evoked NMDAR currents increased in amplitude and duration. Over the ensuing 2–5 min, however, the current decreased in amplitude while maintaining slow decay kinetics. These observations could be explained by a mechanism wherein an increase in glutamate spillover and/or ambient glutamate concentration leads to a decrease in neurotransmitter release. Activation of mGluRs has been shown to decrease the probability of release at a number of synapses in the CNS (Basksys and Malenka 1991; Maki et al. 1994; Min et al. 1998; Oliet et al. 2001; Renden et al. 2005; Scanziani et al. 1997; von Gersdorff et al. 1997). However, little is known about the role of mGluRs at the immature retinogeniculate synapse.

To test for the presence of mGluRs, we examined the effects of TBOA in the presence of an mGluR antagonist, LY-341495 (LY) (Kingston et al. 1998). LY is a very selective antagonist to group II mGluRs at low concentrations; however, at higher concentrations (50 μM), it has measureable effects on all

Fig. 1. Effects of N-β-threo-3-benzylloxyaspartic acid (TBOA) at the immature retinogeniculate synapse. Excitatory postsynaptic currents (EPSCs) were measured before and during bath application of 50 μM TBOA. A and B: traces from representative experiments are shown for N-methyl-D-aspartate receptor (NMDAR)-mediated (holding potential V_h = +40 mV; A, left) and AMPA receptor (AMPAR)-mediated (AMPAR)-mediated EPSCs (V_h = −70 mV; B, left) before (control; black line) and during bath application of 50 μM TBOA (gray line). Graphs show time course of NMDAR (A, right) and AMPAR EPSC amplitudes (B, right) before and during bath application of 50 μM TBOA, followed by their respective antagonists, 3-(R)-2-carboxypiperazine-4-ylphosphonic acid (CPP) and 2-amino-5-phosphono-pentanoic acid (APV; A, right) or 2,3-dihydro-6-nitro-7-sulfamoylbenzof[quinazoline-2,3-dione (NBQX; B, right). C: summary graphs show the mean normalized amplitudes (± SE) of both NMDAR and AMPAR EPSCs (left) and the effects of TBOA on EPSC decay (T_{1/2} or T_5) shown as a percentage of control (right). *P < 0.01; **P < 0.001; #P < 0.05. D: effects of TBOA on the holding current (I_{hold}) in response to a +40-mV step. Representative traces (left) and time course (right) are shown before (control; thin line) and during bath application of TBOA (gray line) and NMDAR antagonists (dotted line). Recordings were performed at 35 ± 1°C.
Recordings in mGluR groups (Linden et al. 2009). In the following experiments we used a lower concentration of TBOA, since we found that bath application of 50 μM TBOA often caused unstable recordings attributable to excessive changes in $I_{\text{hold}}$. As with 50 μM TBOA, 10 μM TBOA effectively reduced the NMDAR peak EPSC (to 75 ± 5% of control, $n = 7$, $P < 0.05$; data not shown); thus the previously described effect on the peak current is still present even with reduced TBOA concentration. In the presence of 50 μM LY we found that the inhibitory effect of TBOA on EPSC amplitude was prevented. Instead, there was a lasting increase in peak EPSC to nearly 150% of control (147 ± 6.8% of control; $n = 6$, $P < 0.01$; Fig. 2, B and D). Despite the fivefold reduction in TBOA concentration, we still observed a doubling in NMDAR EPSC $T_{1/2}$ that was reversed with washout of the transporter inhibitor (Fig. 2, B and D). We also found smaller changes in $I_{\text{hold}}$ in the presence of 10 μM TBOA compared with 50 μM TBOA, which were reversible (TBOA: 147.8 ± 12.6% of control $n = 6$, $P = 0.01$; wash: 112.9 ± 17.7% of control $n = 5$, $P = 0.4$; data not shown).

These results suggest that TBOA mediates the reduction in peak EPSC through accumulation of glutamate and subsequent activation of mGluRs. Application of LY alone had a small but significant effect on the NMDAR EPSC amplitude, but not $T_{1/2}$, at baseline stimulation frequencies (peak increased to 116 ± 5% of control, $n = 6$, $P < 0.05$; 0.025-Hz stimulation, Fig. 2, C and D). Our baseline stimulation of the optic tract (0.025 Hz) is at a much lower frequency than the reported mean RGC firing rates of 0.3–0.45 Hz at ages P9–13 in mice (Demas et al. 2003; Torborg and Feller 2005). Thus we interpret these data to indicate that mGluRs can be activated by glutamate spillover and/or ambient glutamate and inhibit neurotransmitter release during physiological levels of RGC activity.

**Activation of group II/III mGluRs regulates neurotransmitter release at the immature retinogeniculate synapse.**

We next sought to identify the class of mGluRs responsible for the decrease in EPSC amplitude seen with application of TBOA. Our finding that both AMPAR and NMDAR peak currents decreased to a similar extent in the presence of TBOA favors a presynaptic mechanism. Moreover, our experimental conditions were designed to minimize known postsynaptic effects of mGluR signaling (see MATERIALS AND METHODS). Thus we examined the effects of specific mGluR agonists on synaptic strength and the paired-pulse response (PPR) to address whether the probability of release was affected. At the retinogeniculate synapse, both pre- and postsynaptic mechanisms have been shown to contribute to PPR (Budisanto et al. 2012; Chen et al. 2002). To accurately monitor a presynaptic process without contamination of postsynaptic AMPAR desensitization, we added 50 μM cyclothiazide (CTZ) to the bath. CTZ prevents desensitization of AMPARs and does not alter release probability at the retinogeniculate synapse (Chen and Regehr 2000). Figure 3, A–C, shows the effects of bath application of agonists to different classes of mGluRs on pairs of stimuli separated by an interstimulus interval of 50 ms. The time courses of the peak amplitude of the first (EPSC$_1$) and second EPSCs (EPSC$_2$) are plotted before and during agonist application (top right). The PPR values, calculated as EPSC$_2$/EPSC$_1$, are shown at bottom right. Application of group II (APDC) and group III (t-AP4) agonists led to a sustained reduction in EPSC$_1$ amplitude (Fig. 3D). For example, 30 μM APDC inhibited the peak EPSC to 73 ± 4% of control ($n = 5$, **
suggest that mGluR7 is also present at the immature retinogeniculate synapse. Bath application of 10 µM L-AP4 further reduced synaptic strength to 48.9 ± 4% of control (n = 5, P < 0.01; compared with 50 µM L-AP4: P < 0.05, unpaired t-test) and increased PPR to 196 ± 31% of control (n = 5, P < 0.01; Fig. 3, B and D). These data suggest that mGluR7 is also present at the immature retinogeniculate synapse.

In contrast to the group II and III agonists, the group I agonist DHPG (≥10 µM) did not significantly alter the average peak EPSC amplitude (110 ± 16% of control, n = 4, P = 0.7) or the PPR (110 ± 6% of control, n = 4, P = 0.4) (Fig. 3, C and D). The dose-dependent relationship of agonists to the three groups of mGluR are compared in Fig. 3D. These results demonstrate that agonists for group II and III, but not group I, mGluRs lead to a sustained reduction in release probability at the immature retinogeniculate synapse.

**Role of glutamate transporters at the mature retinogeniculate synapse.** The functional properties of the rodent retinogeniculate synapse remodel dramatically over the first 3–4 postnatal weeks (Chen and Regehr 2000; Hooks and Chen 2006; Jaubert-Miazza et al. 2005). To test whether the role of glutamate transporters also changes over development at this visual synapse, we examined the effects of TBOA in LGN slices prepared from P27–34 mice. Bath application of 10 µM TBOA significantly alters the waveform of the mature NMDAR EPSC. In contrast to the immature synapse, where we found a decrease in NMDAR EPSC amplitude without LY (Fig. 1), TBOA caused a reversible increase in the EPSC amplitude to 163 ± 20.2% of control (n = 6, P < 0.05; Fig. 4A). The TBOA-mediated effects on EPSC kinetics and I_{hold}, however, were similar to those of the immature synapse, with a significant and reversible increase in the T_{1/2} (to 185 ± 23.9% of control, n = 6, P < 0.05; Fig. 4A) and I_{hold} (to 119 ± 3.9% of control, n = 6, P < 0.01; Fig. 4C).

We also found a small but significant decrease in the mature AMPAR EPSC in the presence of 10 µM TBOA (to 94.2 ± 0.8% of control, n = 5, P < 0.01) and a slowing of the time constant of decay, τ (to 174 ± 23.2% of control, n = 5, P < 0.05; Fig. 4B). This reduction in peak amplitude is due to AMPAR desensitization, because in the presence of 50 µM

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**Fig. 3. mGluR agonists modulate synaptic currents at the immature synapse.** Representative traces (left) and time course (right) of AMPAR EPSC responses to pairs of pulses before (thin line) and during (thick line) bath application of the group II mGluR agonist (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (APDC; 30 µM; A); group III agonist 1- (+) -2-amino-4- phosphonobutyric acid (i-AP4; 500 µM; B); and group I agonist (S)-3,5-dihydroxyphenylglycine (DHPG; 25 µM; C). Peak amplitude of first (EPSC1; circles) and paired-pulse ratio (PPR; triangles) are plotted. D: summary data of peak EPSCs (top) and PPR (bottom) shown as a percentage of control in the presence of various concentrations of mGluR agonists. #P < 0.05; *P < 0.01. Recordings were performed at 25 ± 1°C.
Fig. 4. Effects of TBOA on EPSCs at the mature retinogeniculate synapse. A: representative traces (left) and time course (middle) of NMDAR EPSCs recorded before (black line), during (dark gray line), and after (dashed gray line) bath application of 10 μM TBOA; summary data (means ± SE) shown as a percentage of control (right). B: representative traces (left) and time course (middle) of AMPAR EPSCs recorded before (black line), during (dark gray line), and after (dashed gray line) application of TBOA, followed by receptor antagonist; summary data (means ± SE) shown as a percentage of control (right). C: I_{hold} in response to a +40-mV step. Average traces (left) and time course (middle) are shown before (black line), during (dark gray line), and after (dashed gray line) application of TBOA; summary I_{hold} data (means ± SE) shown as a percentage of control. Recordings were performed at 35 ± 1°C. #P < 0.05; *P < 0.01. **P < 0.001.

CTZ, bath application of 10 μM TBOA had no significant effect on peak amplitude (105.5 ± 2.9% of control, n = 5, P = 0.12; data not shown). However, in the presence of CTZ, EPSC decay kinetics still increased in response to transporter inhibition (τ: 231 ± 3.8% of control, n = 5, P < 0.05; data not shown). AMPAR desensitization can occur at the mature retinogeniculate synapse as a result of glutamate spillover and/or increased ambient glutamate concentration (Budisantoso et al. 2012; Chen et al. 2002).

Downregulation of mGluRs at the mature retinogeniculate synapse.

The difference between the synaptic responses to TBOA at the mature and immature retinogeniculate synapse suggests a reduction over development in the negative feedback loop mediated through mGluRs. To confirm that there is a change in the role of mGluRs at the mature synapse, we tested for the presence of mGluRs by using specific agonists, as we did for the immature synapse. Figure 5A shows that bath application of 50 μM L-AP4 resulted in a small but persistent decrease in peak AMPAR EPSC (to 84.7 ± 5.6% of control, n = 5, P = 0.05), whereas PPR increased to 122.8 ± 5% of control (n = 5, P < 0.05). This suggests that group III mGluRs are present at the mature synapse. However, increasing the concentration of L-AP4 to 500 μM did not elicit further inhibition, as it did at the immature synapse (see Fig. 3). This is consistent with a loss of mGluR7 (500 μM L-AP4: EPSC1 amplitude, 82.7 ± 3.6% of control, n = 6, P < 0.05 and PPR, 122.4 ± 9% of control, n = 6, P < 0.05; but compared with 50 μM L-AP4: EPSC1 amplitude, P = 0.78, unpaired t-test; PPR, P = 0.97; Fig. 5, B and C). A summary of the dose dependence of L-AP4 on the mature AMPAR EPSC and PPR is shown in Fig. 5B. Thus, although some group III mGluRs remain at the synapse over development, activation of these receptors results in a smaller effect on synaptic strength and release probability in mature synapses compared with the immature synapses (compare Figs. 3 and 5).

In contrast to group III mGluRs, agonists of group I (10 μM DHPG) and group II (30 μM APDC) did not significantly alter the peak EPSC amplitude or the PPR at the mature retinogeniculate synapse (Fig. 5C). These results demonstrate that the role of group II mGluRs in mediating release probability is lost over development at the retinogeniculate synapse. Taken together, our data suggest that the mGluR negative feedback mechanism that is present at the immature retinogeniculate synapse is downregulated with age.

Activation of mGluRs during physiologically relevant stimulus trains.

Our findings demonstrate that inhibition of glutamate transporter activity can activate a group II/III mGluR-mediated reduction in release probability at the immature synapse. The
Because the AMPAR current is relatively small compared with Kerschensteiner and Wong (2008; Torborg and Feller 2005).

Activity that has been shown to occur during this development to prevent excess glutamate accumulation and excitotoxicity. Our data demonstrate that the mGluR-mediated feedback is downregulated at the immature synapse.

Indeed, our results are consistent with this prediction. We found a significant increase in the strength of the EPSC1 and a decrease in PPR following relief of mGluR activation (Fig. 6). EPSC1 amplitude of each train increased to $126 \pm 8\%$ of control $(n = 6, P < 0.05)$. Figure 6 (left) shows average traces in response to 5 stimuli at either 10 (A), 20 (B), or 50 Hz (C) before (black) and during (gray) bath application of 50 $\mu$M LY. A summary plot of the peak amplitudes of the subsequent EPSCs relative to EPSC1 is shown at right. The degree of synaptic depression in response to trains of stimuli was enhanced with higher frequencies in control conditions. Bath application of the mGluR antagonist reduced the peak amplitudes of subsequent EPSCs following EPSC1. Synaptic depression was significantly enhanced in the presence of LY $(P < 0.002$ for all frequencies tested, 1-way ANOVA).

These results demonstrate that group II/III mGluR are activated during physiological RGC firing patterns and reduce release probability at the immature retinogeniculate synapse.

**DISCUSSION**

Developmental regulation of synaptic glutamate concentration. We examined mechanisms regulating glutamate release and clearance at the developing retinogeniculate synapse. First, we have shown that inhibiting glutamate transporters changes the shape of synaptic currents. This confirms an active role for transporters in removal of glutamate. Second, our experiments unveiled the presence of a negative feedback mechanism present at the immature synapse. When glutamate accumulates, both group II and III mGluRs are activated, resulting in a sustained reduction in neurotransmitter release. Moreover, our results demonstrate that these mGluRs are activated during presynaptic frequencies that mimic immature RGC firing patterns.

The mGluR-mediated negative feedback loop may play an important role during periods of high-frequency presynaptic activity. This mechanism could be advantageous early in development to prevent excess glutamate accumulation during correlated bursts of presynaptic activity. The inhibitory network is not fully matured in the LGN until after eye opening (P12–14) (Bickford et al.); thus the activation of group II/III mGluRs may be a major mechanism preventing excessive glutamate accumulation and excitotoxicity. Our data demonstrate that the mGluR-mediated feedback is downregulated at the mature synapse. This suggests that once connections between retina and thalamus have refined and stabilized, an mGluR type of autoregulation may no longer be needed to control glutamate release.
Glutamate transporters at the retinogeniculate synapse.

Glutamate clearance from the synaptic cleft is controlled by diffusion and by its active removal and buffering by transporters. In addition to removing synaptically released glutamate, transporters also maintain extracellular concentrations of the neurotransmitter (Herman and Jahr 2007). Consistent with previous studies (Tzingounis and Wadiche 2007), we have shown that glutamate transporters maintain ambient levels of extracellular glutamate, shape the synaptic waveform at the retinogeniculate synapse over development, and shield mGluRs from excessive activation at the immature synapse. However, what makes this visual synapse distinct from other CNS synapses is the sensitivity of both NMDAR and AMPAR currents to transporter inhibition.

At both the immature synapse when mGluRs are inhibited, and at the mature synapse, a low concentration of TBOA (10 µM) results in a robust increase in the NMDAR EPSC peak and a doubling of the decay kinetics (see Figs. 2 and 4). The degree of increase of the peak current is striking compared with the Schaffer collateral (SC)-CA1 synapse. At this hippocampal connection, TBOA did not alter the amplitude of the NMDAR EPSC at immature ages and had a small effect at older ages (Christie and Jahr 2006; Diamond 2005; Thomas et al. 2011). Here, at the retinogeniculate synapse, the peak of the NMDAR but not AMPAR EPSC increases in the presence of TBOA. This can be explained by the higher affinity of NMDARs for glutamate and the slower kinetics of the channel compared with the AMPAR. The increase in NMDAR EPSC peak is likely influenced by the extent of glutamate spillover and the activation of nearby extrasynaptic NMDARs. Moreover, our results also showed a significant increase in AMPAR EPSC decay kinetics throughout development consistent with spillover to neighboring synapses (Budisantoso et al. 2012). In contrast to our study, TBOA does not alter AMPAR kinetics at the SC-CA1 synapse (Christie and Jahr 2006). In addition, 200 µM TBOA has modest or no effects on AMPAR kinetics at the mossy fiber-granule cell synapse in the cerebellum and the calyx of Held, respectively (DiGregorio et al. 2002; Renden et al. 2005). Thus our findings suggest currents at this visual synapse rely heavily on glutamate transporters to shape the synaptic response. Interestingly, the effects of TBOA on this sensory synapse are similar to the effects seen on the primary afferent synapses in the mature spinal cord (Napier et al. 2012; Nie and Weng 2009).

It is still unclear which glutamate transporters are present at the immature retinogeniculate synapse. Five excitatory amino acid transporters (EAATs) comprise the family of glutamate transporters in the brain. EAAT1, EAAT2, and EAAT3, also known as GLAST, GLT-1, and EAAC1 in the rodent, are expressed throughout the nervous system (Arriza et al. 1997; Danbolt 2001; Fairman et al. 1995; Kanai and Hediger 1992; Pines et al. 1992; Storck et al. 1992). Given the IC50 values of dl-TBOA to GLAST, GLT-1, and EAAC1 (70, 6, and 6 µM, respectively), our results showing a dramatic effect of 10 µM TBOA suggest that GLT-1 and/or EAAC1 play a role in shaping the synaptic transient at the retinogeniculate synapse (Lebrun et al. 1997; Shimamoto et al. 2000).
consistent with a previous report that localized both GLT-1 and GLAST to nearby glia at the mature synapse (Budisantoso et al. 2012). However, it remains unknown whether there are changes in transporter expression or their subcellular localization over development. It will be interesting in future studies to further investigate the distinct locations and roles of specific glutamate transporter subtypes at the immature synapse.

Autoregulation of neurotransmitter release by mGluRs over development.

We found that transporters at the immature retinogeniculate synapse prevented excessive glutamate binding to group II/III mGluRs. Consistent with reports from other synapses, activation of these metabotropic receptors reduced synaptic strength and increased PPR (Baskys and Malenka 1991; Conn and Pin 1997; Maki et al. 1994; Min et al. 1998; Oliet et al. 2001; Renden et al. 2005; Scanziani et al. 1997; von Gersdorff et al. 1997). The presence of these receptors was unexpected, because studies in the mature visual thalamus had shown that group II or III mGluRs regulate neurotransmitter release at corticothalamic projections but not at the retinogeniculate synapse (Alexander and Godwin 2005, 2006; Turner and Salt 1999). However, consistent with these previous studies at the mature synapse, we found that the group II/III mGluR-mediated responses are downregulated with age. Notably, the loss of mGluR function has been described over development at another sensory synapse, the calyx of Held. At this brain stem synapse, some presynaptic group II/III mGluRs are present in early development and then downregulate with age, although there is no evidence of presynaptic mGluR7-mediated function at this synapse (Renden et al. 2005). Interestingly, the spiral ganglion neurons in the cochlear hair cells that drive presynaptic input to the calyx of Held were recently shown to exhibit wavelike spontaneous activity, with prolonged bursts of spikes reaching 100 Hz, during a prehearing developmental period (Tritsch and Bergles 2010; Tritsch et al. 2010). The similarities in the two sensory synapses support the idea that mGluRs play an important role in regulating glutamate release during this period of activity-dependent synapse remodeling.

The rapid effects of group II/III mGluR agonists on release probability suggest that the receptors are located on the presynaptic RGC terminals as opposed to a neighboring cell. To date, no high-resolution immunoelectron microscopy study of the developing LGN has localized mGluRs. However, in another sensory nucleus of the developing thalamus, the ventral posterior nucleus of the somatosensory system, the group II class mGluR2/3 has been localized to 3% of presynaptic axon terminals of asymmetric synapses (Liu et al. 1998). This electron microscopy (EM) study also noted dense labeling of mGluR2 in glial processes near synapses. Our study cannot completely rule out that mGluR activation in glia could, through an indirect pathway, lead to the reduction of vesicular release at RGC axon terminals. An example of one such indirect pathway involves astrocyte-mediated accumulation of adenosine that binds to presynaptic A1 receptors and reduces release probability (Dittman and Regehr 1996; Pan et al. 1995; Pascual et al. 2005; Scanziani et al. 1992; Zhang and Schmidt 1999). However, we included the A1 receptor blocker DPCPX in all of our experiments. Thus, were astrocytes involved, a substance other than adenosine would be responsible for pre-synaptic modulation of release. Moreover, mGluR2 expression in astrocytes does not decrease over development in the thalamus (Liu et al. 1998). Thus we favor a model where mGluRs are transiently expressed in RGC terminals.

Our results demonstrate a dynamic range of mGluR-mediated regulation of glutamate release at the immature retinogeniculate synapse (see Figs. 1, 2, and 6). The negative feedback response appears to scale with the degree of glutamate accumulation and spillover. With low-frequency stimulation we see a small but significant mGluR-mediated effect, whereas a substantial decrease in synaptic strength is seen when transporters are inhibited. This could be explained by the subcellular localization of the different mGluRs and by their distinct affinities for glutamate. For example, EM studies in the hippocampus show immunoreactivity for the high-affinity group II mGluR2/3 to axon terminals outside of the synaptic cleft (Shigemoto et al. 1997; Tamaru et al. 2001). In contrast, low-affinity group III mGluR7 (Kd = 1 mM glutamate) is found in presynaptic active zones (Brandstatter et al. 1996; Conn and Pin 1997; Shigemoto et al. 1997). Interestingly, it has been shown that mGluR7s are activated during periods of robust presynaptic activity (Pelkey et al. 2005, 2007). Thus the varied location and glutamate affinities of different classes of mGluRs could contribute to a diverse repertoire of synaptic responses. Given the dynamic synaptic environment at the immature retinogeniculate synapse, it is possible that activation of mGluRs may play an important role in the process of developmental synaptic refinement.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


