

Long-Term Depression of Synaptic Inhibition Is Expressed Postsynaptically in the Developing Auditory System

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Chang, Eric H., Vibhakar C. Kotak, and Dan H. Sanes. Long-term depression of synaptic inhibition is expressed postsynaptically in the developing auditory system. *J Neurophysiol* 90: 1479–1488, 2003. First published May 21, 2003; 10.1152/jn.00386.2003. Inhibitory transmission is critically involved in the functional maturation of neural circuits within the brain. However, the mechanisms involved in its plasticity and development remain poorly understood. At an inhibitory synapse of the developing auditory brain stem, we used whole cell recordings to determine the site of induction and expression of long-term depression (LTD), a robust activity-dependent phenomenon that decreases inhibitory synaptic gain and is postulated to underlie synapse elimination. Recordings were obtained from lateral superior olivary (LSO) neurons, and hyperpolarizing inhibitory potentials were evoked by stimulation of the medial nucleus of the trapezoid body (MNTB). Both postsynaptic glycine and GABA_A receptors could independently display LTD when isolated pharmacologically. Focal application of GABA, but not glycine, on the postsynaptic LSO neuron was sufficient to induce depression of the amino acid-evoked response, or MNTB-evoked inhibitory postsynaptic potentials. This GABA-mediated depression, in the absence of MNTB stimulation, was blocked by a GABA_B receptor antagonist. To assess whether a change in neurotransmitter release is associated with the LTD, the polyvalent cation, ruthenium red, was used to increase the frequency of miniature inhibitory synaptic events. Consistent with a postsynaptic locus of expression, we found that the mean amplitude of miniature events decreased after LTD with no change in their frequency of occurrence. Furthermore, there was no change in the paired-pulse ratio or release kinetics of evoked inhibitory responses. Together, these results provide direct evidence that activity-dependent LTD of inhibition has a postsynaptic locus of induction and alteration, and that GABA but not glycine plays a pivotal role.

INTRODUCTION

Inhibitory synapses constitute $\leq 40\%$ of the connections in the brain, yet an understanding of their development and plasticity remains poor. This is because anatomically discrete inhibitory projections, amenable to selective manipulation, are rare (Eccles 1969). Here, we study an anatomically discrete inhibitory pathway in the auditory system from the medial nucleus of the trapezoid body (MNTB) to the lateral superior olive (LSO). This pathway can be directly and selectively manipulated in a brain slice preparation to gain insights on the development and plasticity of inhibitory transmission. Using this model, the influence of inhibitory activity on synaptic remodeling (Kandler and Friauf 1995; Kim and Kandler 2003;

Sanes 1993; Sanes and Takács 1993), consequences of the disruption of inhibitory activity on the efficacy of developing excitatory and inhibitory synapses (Kotak and Sanes 1997; Vale and Sanes 2000), and the developmental transformation of inhibitory transmitter phenotypes (Kotak et al. 1998) have all been investigated. LSO neurons integrate excitation driven by the ipsilateral ear and inhibition driven by the contralateral ear (by the MNTB). The MNTB afferents form a topographic projection within the LSO and the plasticity of this projection has been established (Kotak and Sanes 1996, 1997; Sanes 1990; Sanes and Hafidi 1996; Sanes and Siverls 1991; Sanes et al. 1992).

Observations from the LSO and other central auditory nuclei suggest that inhibitory connections are rearranged during normal maturation and that this process is activity-dependent (Gabriele et al. 2000a,b; Kapfer et al. 2002; Kim and Kandler 2003; Sanes and Siverls 1991; Sanes and Takács 1993). Axonal terminals from MNTB neurons undergo a process of synapse elimination during postnatal development, becoming restricted along the tonotopic axis of the LSO. There is both an early phase of refinement during the time when inhibitory postsynaptic potentials (IPSPs) are depolarizing (Kim et al. 2003), and a later phase of refinement when IPSPs are hyperpolarizing (Sanes and Siverls 1991). When the contralateral cochlea is removed before the onset of hearing, a process that functionally denervates the MNTB, the inhibitory terminals fail to become restricted (Sanes and Takács 1993). A similar phenomenon is present in the medial superior olivary nucleus (Kapfer et al. 2002). During this period of synapse elimination, an activity-dependent long-term depression (LTD) can be induced at the inhibitory synapses between the MNTB and the LSO (Kotak and Sanes 2000; Sanes and Siverls 1991). This LTD is age- and calcium-dependent, and is most prominent at the same time that MNTB terminals are undergoing the process of refinement (Sanes and Siverls 1991). Although GABA_B signaling has been implicated (Kotak and Sanes 2002; Kotak et al. 2001), the site(s) of LTD induction and expression have not been identified.

In excitatory synapses, depolarization of the postsynaptic membrane with the resulting calcium entry is critical to changes in synaptic strength. Indeed, many forms of inhibitory synaptic plasticity are heterosynaptic, depending on excitatory activity for induction (Caillard et al. 2000; Komatsu and Iwakiri 1993; Ouardouz and Sastry 2000). However, because in-

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hibitory synaptic activity by itself leads to chloride entry and membrane hyperpolarization, it seems likely that alternative mechanisms are required to mediate a homosynaptically induced change in synaptic strength. A simple possibility is that the depolarizing inhibitory synaptic potentials that occur in neonatal animals could recruit voltage-gated calcium channels or *N*-methyl-D-aspartate (NMDA)-coupled channels, to raise intracellular calcium (Ben-Ari et al. 1997; Connor et al. 1987; Obrietan and Van den Pol 1995). However, depolarizing IPSPs are no longer present beyond postnatal day 7 (P7), the age range in which we observe LTD of inhibitory transmission (Kandler and Friauf 1995; Kotak and Sanes 1996).

Another intriguing possibility for the signaling pathway is suggested by the observation that MNTB-evoked inhibition is primarily GABAergic at first, and gradually becomes glycinergic during postnatal wk 1 and 2 (Kotak et al. 1998). Whereas glycine receptors appear to be solely ionotropic, the presence of GABAergic transmission permits inhibitory terminals to communicate through a metabotropic pathway (Barthel et al. 1996; Tremblay et al. 1995; Zhang et al. 1997). In fact, LTD in the LSO can be blocked with a GABA_B receptor antagonist (Kotak et al. 2001). However, it was not established whether the GABA_B signaling took place at the presynaptic terminals or the postsynaptic neuron. In the present study, we show that inhibitory LTD is induced by a GABAergic mechanism and is expressed postsynaptically.

METHODS

MNTB-LSO slice preparation

Gerbils (*Meriones unguiculatus*) aged P8 to P11 were used to generate 300- μ m brain stem slices containing the LSO and the MNTB (Sanes 1993). This postnatal range was chosen because the magnitude of LTD is greatest at these ages (Kotak and Sanes 2000). The artificial cerebrospinal fluid (ACSF) contained (in mM): 125 NaCl, 4 KCl, 1.2 KH₂PO₄, 1.3 MgSO₄, 24 NaHCO₃, 15 glucose, 2.4 CaCl₂, and 0.4 L-ascorbic acid (pH = 7.3) when bubbled with 95% O₂-5% CO₂. ACSF was continuously superfused in the recording chamber at 4–5 ml/min at room temperature (22–24°C).

Whole cell electrophysiology and pharmacological manipulations

Whole cell current-clamp and voltage-clamp recordings were obtained as described previously (Kotak et al. 1998). Recording electrodes were fabricated from borosilicate glass microcapillaries (1.5 mm OD), with a tip resistance of 5–10 M Ω when filled with the internal solution. All recordings were performed in the presence of kynurenic acid (4 mM; pH adjusted to 7.3) to block ionotropic glutamate receptors (Moore et al. 1998). Current-clamp recordings were used during paired-pulse stimulation to obtain inhibitory responses in the absence of cesium, which affects the potassium/chloride ion cotransporter (Kakazu et al. 1999). Voltage-clamp recordings were used in all other experiments to obtain the largest signal before inducing LTD. This permitted a greater sensitivity to differences in the levels of depression displayed by either GABA or glycine receptors (Kotak et al. 1998). For current-clamp experiments, the internal solution contained (in mM): 135 potassium gluconate, 0.6 EGTA, 10 HEPES, 2 MgCl₂, 5 KCl, 2 ATP, 0.3 GTP (pH = 7.2). For voltage-clamp recordings, the internal solution contained (in mM): 127.5 cesium gluconate, 0.6 EGTA, 10 HEPES, 2 MgCl₂, 5 KCl, 2 ATP, 0.3 GTP, and 5 QX-314. With the exception of the ruthenium red (RR) experiments, all LSO neurons were held at –55 mV during voltage-

clamp recordings to keep conditions close to resting membrane potentials as recorded in age-matched animals (Kotak and Sanes 1995; Sanes 1993). To maximize the sensitivity of the assay in the RR experiments, LSO neurons were held at 0 mV, which increased the driving force of inhibitory postsynaptic currents (IPSCs) (Kotak and Sanes 2000).

In experiments measuring MNTB-evoked responses, a bipolar stimulating electrode was placed on the MNTB and a 200- μ s pulse was delivered by an isolated biphasic stimulator in constant current mode (Axon Instruments). LTD was induced by single shocks given at low-frequency stimulation (LFS: 1 Hz, 15 min) of the MNTB, as described previously (Kotak and Sanes 2000). Paired-pulse stimulation of the MNTB was delivered by the same bipolar stimulating electrode with an interpulse interval (IPI) of 200, 250, or 300 ms for the entire experiment. These IPIs were chosen to minimize temporal summation during the long (>100 ms) decay kinetics of IPSPs as observed at this stage of development. To measure the magnitude of depression in the paired-pulse experiments, the amplitude of the three IPSPs immediately before LFS were compared with the amplitude of the last three IPSPs in the experiment, taken 50–60 min after LFS. The paired-pulse ratio (PPR) is defined as the amplitude of the second evoked response divided by the amplitude of the first response (P2/P1).

To isolate glycinergic synaptic responses, GABA_A receptors were blocked with 20 μ M bicuculline (BIC; Sigma). To isolate GABAergic synaptic responses, glycine receptors were blocked with 1 μ M strychnine (SN; Sigma). To block GABA_B receptors, 10 μ M SCH-50911 (Sigma) was added to the bath perfusate throughout the experiment. In the miniature IPSC (mIPSC) experiments, 10 μ M RR (Sigma) was bath-applied for the duration of the experiment. To directly activate postsynaptic receptors, a second pipette containing GABA or glycine (tip diameter = 2–4 μ m) was positioned about 200–300 μ m from the somatic recording site. Typically, a 50-psi pressure pulse was applied by a picospritzer (General Valve) to deliver one of the amino acids through the pipette. In the first set of experiments, the effect of brief pulses of agonist (4–5 pulses delivered over the course of 1 min) was assessed by recording MNTB-evoked IPSPs before and after the treatment. For these MNTB-evoked responses, a 1-s duration pulse was used for the focal delivery of GABA (10 mM) or glycine (10 mM). In the second set of experiments, the effect of brief (50 ms) focal delivery of the transmitter (1 mM every 2 min) was assessed by measuring the amplitude of GABA- or glycine-evoked hyperpolarizations without any MNTB stimulation for 60 min (total number of pulses = 30). Measurements of mIPSCs were made by recording 10 s of continuous spontaneous activity without any stimulation, once per minute for 5 min. Pre-LFS traces were taken in the 5 min immediately before LFS, whereas post-LFS traces were taken from 55 to 60 min after the LFS ended.

Data were sampled at 10 kHz and stored on a Macintosh G4 running a custom-designed *Igor* macro (Wavemetrics, v4.0.5, see http://www.cns.nyu.edu/~sanes/slice_software; Kotak et al. 2001). The *Slice* macro controls the stimulus isolation units and patch clamp amplifier by an ITC-18 Computer Interface (Instrutech) using an *Igor* external operation command (XOP version 2.6; Instrutech). *Slice* makes use of all four available DAC channels. Stimuli to afferent pathways are delivered by DAC0 and DAC1. Current and voltage commands to the cell are produced by DAC2 (step command) and DAC3 (tonic command). For this study, only DAC0 was used to stimulate the pathway from the MNTB to the LSO. All data, including miniature synaptic events, were analyzed off-line using a second *IGOR* macro called *Slice Analysis*. The analyses include peak amplitude, latency to rise (time between stimulus artifact and trace values that first exceed 2 SDs of the prestimulus mean), the duration (time difference between the latency to fall and the latency at which trace values no longer exceed 2 SDs of the prestimulus mean), and time to peak (time difference between stimulus artifact and value of trace at maximum amplitude). All averaged values are given as means \pm SE

and were compared statistically with the paired Student's *t*-test. The statistical criterion of significance was $P < 0.05$.

RESULTS

GABAergic and glycinergic receptors can express depression

MNTB-evoked inhibitory responses are mediated by GABA and glycine during postnatal wk 1 and 2, but the relative contribution of GABA_A and glycine receptors to inhibitory depression has not yet been investigated. The present recordings were obtained at P7–P11, when inhibitory transmission is about 50% GABAergic and 50% glycinergic (Kotak et al. 1998). To determine the relative contribution of each receptor to LTD, we recorded IPSCs in the presence of the glycine receptor antagonist SN, or the GABA_A receptor antagonist BIC, during the entire course of an experiment. The isolation of either GABAergic or glycinergic currents was assessed by comparing the durations of pre-LFS IPSCs. The mean duration of GABAergic IPSCs measured in the presence of SN was 318 ± 48 ms ($n = 8$), whereas glycinergic IPSC duration measured in the presence of BIC was 98 ± 13 ms ($n = 10$). As expected from the known response kinetics of these two neurotransmitters, glycinergic currents were significantly shorter in duration than GABAergic currents ($P < 0.005$).

The GABAergic IPSCs recorded in the presence of SN exhibited a $53 \pm 7\%$ decline in amplitude after LFS (Fig. 1A). Thus GABA_A receptors in isolation could undergo induction and expression of LTD, without activation of glycine receptors. In separate experiments, glycinergic IPSCs recorded in the presence of BIC displayed a decrease in amplitude of $65 \pm 6\%$ (Fig. 1B). Therefore both GABA_A receptors and glycine receptors in isolation were able to induce and express the LTD.

Focal delivery of GABA depresses MNTB-evoked IPSPs

To explore whether the locus of induction was postsynaptic, we examined the effect of delivering GABA or glycine pulses through a small pipette positioned upstream, about 200–300 μ m from the recording site. In the first set of experiments, we monitored the effect of 4 to 5 pulses of GABA (10 mM; delivered over a 1-min period) on MNTB-evoked IPSPs. MNTB-evoked IPSPs were significantly depressed from 8.7 ± 0.9 to 4.7 ± 1.1 mV after GABA delivery ($P = 0.02$, $n = 5$). In another set of experiments, an equivalent number of glycine pulses did not significantly alter MNTB-evoked IPSP amplitudes (before treatment: 8.8 ± 1.1 mV vs. 15 min after treatment: 8.0 ± 1.5 mV; $P = 0.6$, $n = 7$). Thus GABA application was sufficient to induce depression of evoked inhibitory responses, whereas glycine application was not (Fig. 2). Because the requirement for GABA_A receptor activation was ruled out by the finding that LTD occurred in the presence of BIC (Fig. 1B), these findings are consistent with the idea that GABA_B receptor activation is necessary for the induction of LTD (Kotak et al. 2001).

Focal delivery of GABA depresses GABA_A receptor function

To examine the effect of GABA or glycine exposure in the absence of presynaptic transmission, the effects of neurotransmitter pulses on the recorded LSO neuron were assessed in the absence of MNTB stimulation. GABA or glycine (1 mM, 50

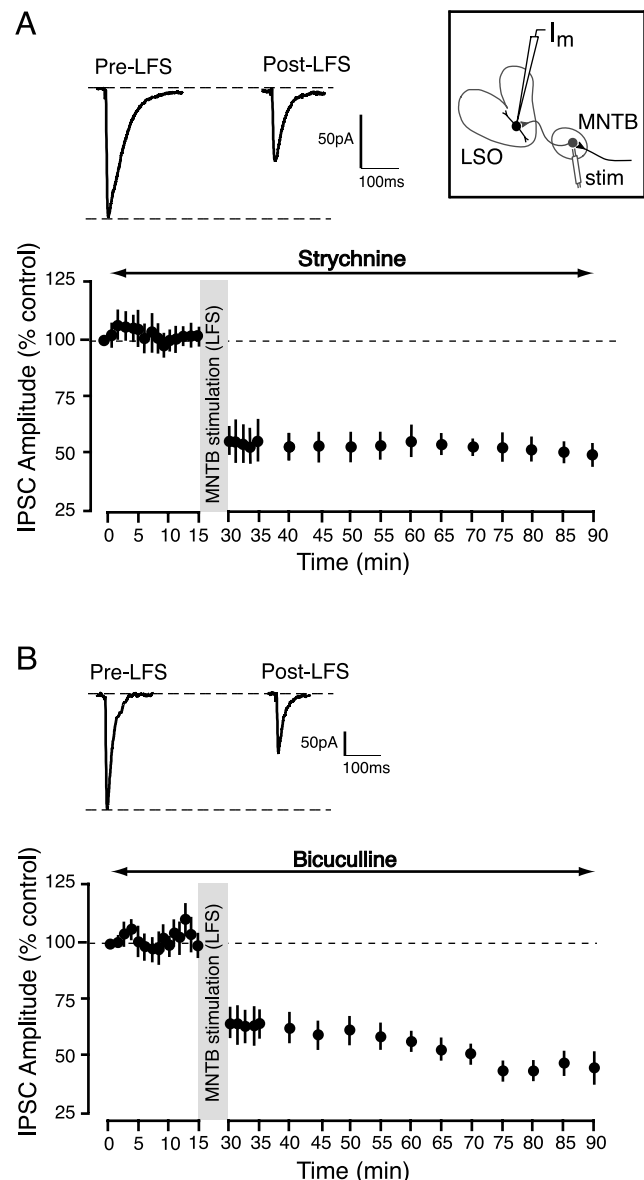


FIG. 1. Long-term depression (LTD) of glycinergic and GABAergic inhibitory postsynaptic currents (IPSCs). A: LTD of GABAergic IPSCs for all recorded lateral superior olivary (LSO) neurons at postnatal days P8–P11 in continuous presence of 1μ M strychnine ($n = 8$). Means (\pm SE) of IPSCs show $53 \pm 6.6\%$ depression in amplitude by 60 min post-low-frequency stimulation (LFS). Top: sample recordings of evoked GABAergic IPSCs at beginning of experiment and 60 min post-LFS. Average duration of GABAergic IPSCs, 318 ± 47.7 ms. Boxed inset: recording configuration. B: LTD of glycinergic IPSCs for all recorded LSO neurons at P8–P11 in continuous presence of 20μ M bicuculline (BIC, $n = 10$). Means (\pm SE) of IPSCs show $65 \pm 5.5\%$ depression in amplitude by 60 min post-LFS. Top: sample recordings of evoked glycinergic IPSCs at beginning of experiment and 60 min post-LFS. Average duration of glycinergic IPSCs was 98 ± 13.2 ms. Holding potential was -55 mV for all recordings. MNTB, medial nucleus of trapezoid body.

ms) were delivered focally from a separate pipette onto recorded LSO neurons once every 2 min, and the transmitter-evoked responses were analyzed (Fig. 3). During the first 10 min of pulses, GABA-evoked hyperpolarizations had an amplitude of 10.2 ± 0.8 mV and a duration of 7.4 ± 0.7 s. At 50–60 min after the initial GABA application, the amplitude of GABA-elicited hyperpolarizations had decreased significantly to an amplitude of 6.5 ± 0.8 mV ($P = 0.01$, $n = 8$). The

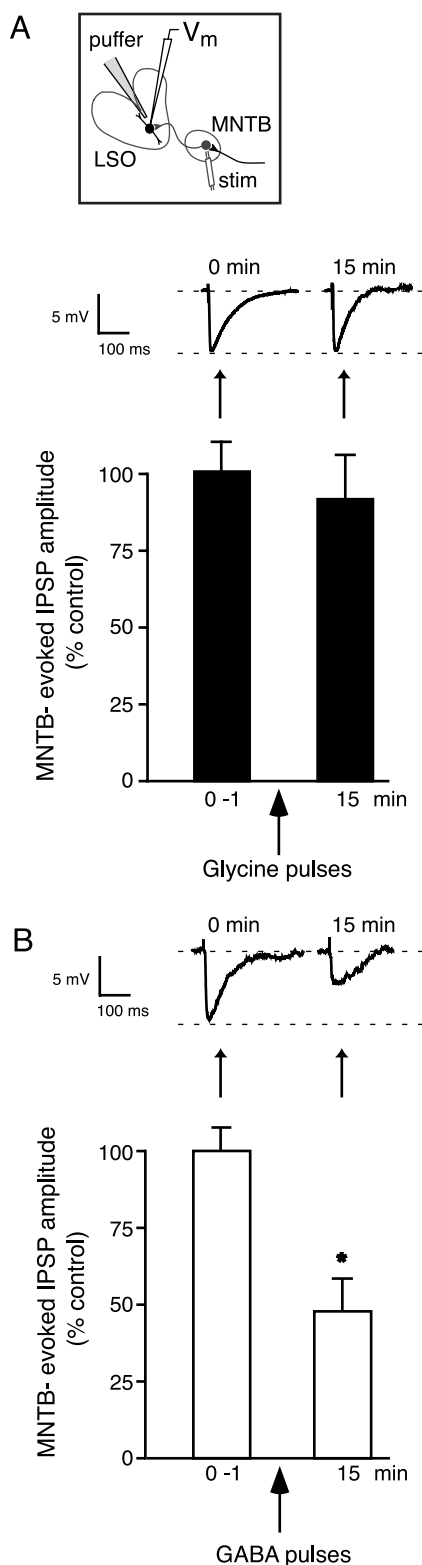


FIG. 2. Focal delivery of GABA (10 mM) at recording site depresses MNTB-evoked IPSPs. *A*: MNTB-evoked inhibitory postsynaptic potential (IPSP) amplitudes recorded before agonist delivery do not change 15 min after delivery of glycine pulses ($P = 0.6$, $n = 7$). *Top*: sample recordings of MNTB-evoked IPSPs before and 15 min after delivery of glycine pulses. *Boxed inset*: recording configuration. *B*: delivery of GABA pulses has significant depressive effect on MNTB-evoked IPSP amplitudes ($P = 0.02$, $n = 5$). *Top*: sample recordings of MNTB-evoked IPSPs before and 15 min after delivery of GABA pulses.

duration of GABA hyperpolarizations also decreased significantly to 3.4 ± 0.7 s ($P < 0.01$, $n = 8$). Separate experiments were performed to examine how quickly GABA puffs induced depression. Amplitudes of GABA hyperpolarizations for the first 4 min were 8.6 ± 0.4 mV, which decreased significantly to 7.1 ± 0.3 mV by 14–18 min ($P = 0.04$, $n = 4$). Similarly, the duration of GABA hyperpolarizations decreased significantly during this same time period (0–4 min: 10.9 ± 0.7 s; 14–18 min: 7.1 ± 0.3 s; $P = 0.01$, $n = 4$). To ensure that the depression of GABA hyperpolarizations did not reflect a general decay in the response over time, puff experiments were performed in the absence of any stimulation between the initial (0–4 min) and final (54–60 min) time points. Under these conditions, no significant decline was found in the amplitude (0–4 min: 9.3 ± 0.9 mV; 54–60 min: 9.2 ± 0.8 mV; $P > 0.9$, $n = 3$) or durations (0–4 min: 5.7 ± 2.6 s; 54–60 min: 5.3 ± 2.7 mV; $P > 0.91$, $n = 3$) of GABA hyperpolarizations.

To test for the involvement of GABA_B receptors in this agonist-induced depression paradigm, we puffed GABA in the presence of a GABA_B antagonist, SCH-50911 (10 μ M). In the presence of SCH-50911, GABA-evoked hyperpolarizations had a mean amplitude of 7.5 ± 0.2 mV during the first 10 min. After 50 min of GABA application, the mean hyperpolarization amplitude of GABA-evoked responses did not change (50–60 min: 7.4 ± 0.1 mV; $P = 0.5$, $n = 5$). Therefore blocking GABA_B receptors prevents the induction of inhibitory agonist-induced depression (Fig. 3*B*).

In contrast to the effects of GABA, glycine-evoked hyperpolarizations remained similar to their initial amplitude and duration 50 min after the initial glycine pulse (amplitude during first 10 min: 12.5 ± 0.3 mV; duration = 1.0 ± 0.3 s; amplitude 50–60 min later: 11.8 ± 0.3 mV; duration = 0.9 ± 0.2 s; $P = 0.6$, $n = 8$). Thus repetitive focal delivery of GABA is sufficient to induce a postsynaptic change in the GABA-gated ionotropic response, provided that GABA_B receptors are available for activation. This effect was not observed for glycine despite the fact that it evoked a strong membrane hyperpolarization.

No evidence for presynaptic alterations

Evidence for a postsynaptic locus of LTD induction and expression does not exclude presynaptic involvement. Hence, we used a variety of methods to test whether there was a presynaptic site for changes related to LTD. First, we chose to use a paired-pulse stimulation paradigm as an indirect measure of release properties. The paradigm is widely accepted as an index of presynaptic function. For central synapses, neurotransmitter release probability across all release sites is inversely related to the amount of facilitation/depression observed after paired-pulse stimulation (Dobrunz and Stevens 1997; Manabe et al. 1993). In addition, we also characterized a variety of release kinetic parameters, such as the latency and time-to-peak (TTP) of inhibitory responses.

To examine whether presynaptic release properties are changed during LTD, paired-pulse stimuli were delivered before and after LFS. Because paired-pulse stimuli were used throughout the entire recording session (except during the 15-min LFS treatment period), the total amount of MNTB stimulation is twice as much as that in the original report of this LTD. Nevertheless, the characteristics of the LTD were similar

to previous results. The average magnitude of IPSP depression was $36 \pm 8\%$ (Fig. 4A), in accordance with the data obtained under similar current-clamp experimental conditions (Kotak and Sanes 2000).

Paired-pulse stimuli were delivered to the MNTB while performing whole cell current-clamp recordings from LSO neurons, and the ratio of evoked IPSP amplitudes was examined. In principle, a high probability of release is associated with a paired-pulse depression (PPD) because the large quantal content released on the first pulse depletes the vesicle pool available for release when the second action potential arrives at the terminal. A low probability of release is associated with paired-pulse facilitation (PPF), with more neurotransmitter released on the second pulse, attributed to residual calcium in the terminals. Therefore any manipulation that alters the probability of release or the number of release sites would result in a change in the paired-pulse ratio (PPR). No change in the PPR would suggest that postsynaptic changes are responsible for expression of the LTD.

Before LFS was applied, there was a consistently small PPD (pre-LFS PPR = 0.94 ± 0.02 , $n = 8$). As shown in Fig. 4B, the PPRs did not change after induction of LTD (post-LFS PPR = 0.95 ± 0.02 ; $P = 0.6$, $n = 8$), indicating that the release properties of the presynaptic terminals did not change after depression. An additional set of measures were taken to confirm this result. A shift in the latency histogram is generally interpreted as a change in the kinetics of transmitter release. More specifically, the latency histogram is a sampling of the first release events after a stimulating pulse (Waldeck et al. 2000). We measured the latency of release for both pulse 1 and pulse 2 before and after LFS. The pre-LFS latency histograms of both pulse 1 and pulse 2 are not different from their post-LFS counterparts at any time point measured (Fig. 4C). A second kinetic parameter is the TTP. The TTP for pulse 1 before LFS was 15 ± 0.47 ms, whereas after LFS the TTP was 15 ± 0.49 ms. The TTP for pulse 2 before LFS was 15 ± 0.5 ms, and after LFS the TTP was 14 ± 0.44 ms. For both pulses, the TTP did not change after induction of LTD (pulse 1: $P = 0.9$, $n = 8$; pulse 2: $P = 0.9$, $n = 8$). Taken together, the lack of change in the PPRs, latencies, and TTPs suggest that the mechanisms underlying LTD of inhibitory synapses do not include a presynaptic element.

Decline in mIPSC amplitudes after LTD induction

The results from indirect measures of presynaptic release all suggest that the changes involved in this LTD occur postsynaptically. However, the most convincing evidence would arise from an analysis of miniature synaptic events. Under our

experimental conditions, spontaneous mIPSCs occur at a very low frequency at this MNTB–LSO synapse, with average rates of ≤ 10 events/min (Fitzgerald and Sanes 1999). Therefore to examine whether mIPSC size decreases during LTD, we required a manipulation to reliably increase the frequency at

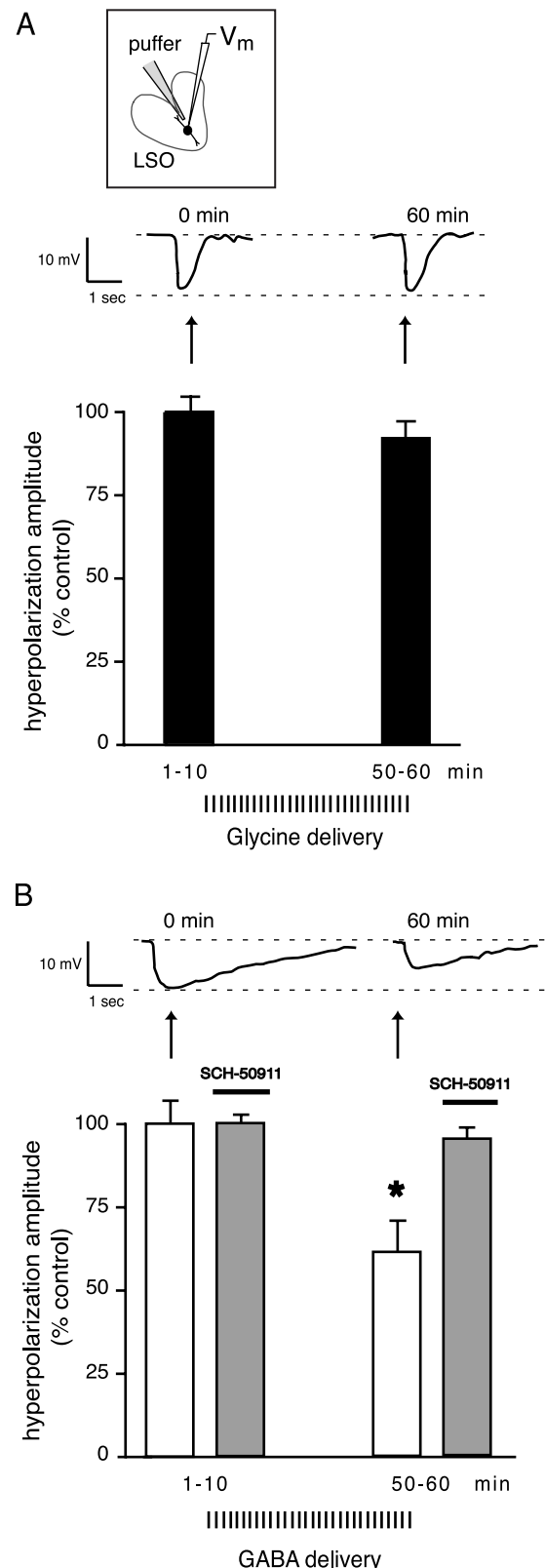


FIG. 3. Focal delivery of GABA depresses agonist-evoked responses by $GABA_B$ receptor. MNTB was not stimulated in this set of experiments. **A**: glycine-evoked hyperpolarizations do not change after delivery of glycine pulses ($P = 0.7$, $n = 8$). Bar graph: changes (%control \pm SE) of agonist-evoked hyperpolarization amplitudes relative to baseline control period before focal application began. Each vertical line beneath bar graphs denotes individual delivery of transmitter (1 mM, 50 ms) by focal delivery pipette positioned about 200–300 μ m from recording site. Boxed inset: recording configuration. **B**: GABA-evoked hyperpolarization amplitudes become significantly depressed after delivery of GABA pulses (white bars; $P = 0.01$, $n = 8$). Presence of $GABA_B$ receptor antagonist, SCH-50911 (10 μ M), prevented any significant decline in GABA-evoked hyperpolarization amplitudes over same amount of time (gray bars, traces not shown).

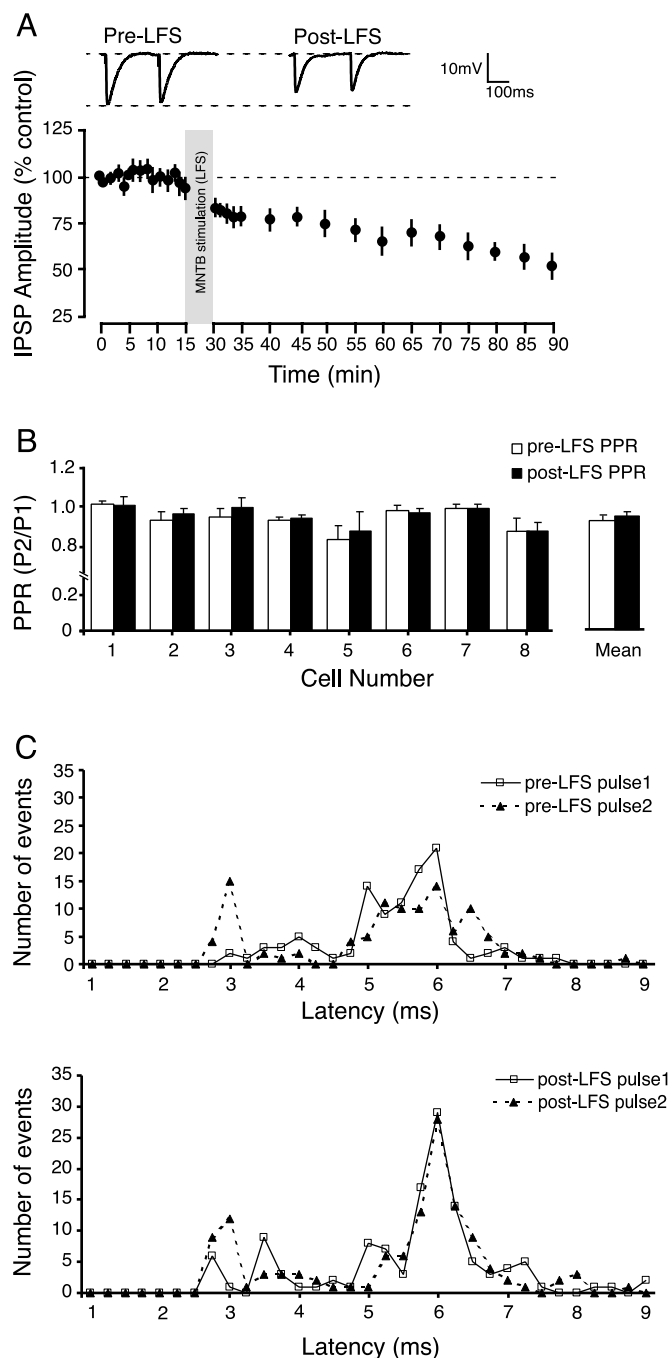


FIG. 4. LTD of responses is not associated with changes in paired-pulse ratios (PPRs) or latency of transmitter release. **A**: LTD of IPSPs for all recorded LSO neurons at P8–P11, $n = 8$. Means (\pm SE) of IPSPs show $36 \pm 7.7\%$ depression in amplitude by 60 min post-LFS. **Top**: sample paired-pulse recordings at beginning of experiment and 60 min post-LFS. **B**: PPRs for each individual recorded cell shown before LFS (white bars) and after LFS (black bars) with standard error bars. Pre-LFS value is average across all IPSPs recorded during 15 min of baseline before LFS. Post-LFS value is average across all IPSPs recorded after LFS (60 min). **C**: **top**: latency to onset for IPSPs before LFS for pulse 1 (solid line) and pulse 2 (dotted line). **Bottom**: latency to onset for IPSPs after LFS; pulse 1 (solid line) and pulse 2 (dotted line). The two histograms essentially overlap with no significant difference between any time points.

which mIPSCs occur without affecting the phenomenon of interest. The polyvalent cation RR was chosen because it has been successfully used in other inhibitory systems to increase

mIPSC frequency and amplitude (Lim et al. 2003; Sciancalepore et al. 1998). RR evokes quantal secretion by a calcium-independent mechanism, possibly by binding to external sites on the presynaptic membranes (Sciancalepore et al. 1998; Trudeau et al. 1996). Importantly, the calcium independence of RR's action allowed us to reliably measure mIPSCs without altering calcium or potassium levels in the bath perfusate. There is no evidence that IPSC amplitude declines during prolonged periods of RR exposure.

To examine whether mIPSC amplitude changed during LTD, $10 \mu\text{M}$ RR was added to the bath perfusate after a stable whole cell recording was obtained. LSO neurons were voltage-clamped to 0 mV to maximize the amplitude of mIPSCs, which are outward at this holding potential (Fig. 5A). The kinetics of RR action were rapid, with a dramatic increase of spontaneous mIPSCs within 1 min of reaching the recording chamber. Baseline MNTB-evoked IPSCs were taken after RR-facilitated mIPSCs had reached a steady frequency (typically 5 min after the addition of RR to the perfusate). After acquisition of baseline-evoked responses and pre-LFS mIPSC traces, LTD was induced with the standard LFS protocol. By 60 min after LFS, a $40 \pm 5\%$ depression in MNTB-evoked IPSCs was observed (data not shown). LTD also caused a significant decline in the mean amplitude of mIPSCs without changing the frequency at which they occurred (Fig. 5C). As illustrated in the amplitude distribution histogram and the cumulative amplitude distributions (Fig. 5B), there was a highly significant leftward shift after LTD induction, indicating a decline in mIPSC amplitudes ($P < 0.0001$, $n = 5$). The frequency of mIPSCs in RR was 6.8 ± 2.3 Hz before LFS and did not change after LTD induction (post-LFS: 6.7 ± 2.1 Hz; $P > 0.9$, $n = 5$).

DISCUSSION

Our studies of inhibitory developmental plasticity have been conducted on a distinct nucleus of inhibitory projection neurons in the auditory brain stem, the MNTB, that innervates the LSO.

These synapses display an age-dependent LTD (Kotak and Sanes 2000), which may support the elimination of MNTB connections within the LSO. Through selective manipulation of this projection, it has been shown that inhibitory synapses influence the anatomical specificity of dendritic and axonal arborizations (Sanes and Hafidi 1996; Sanes and Takács 1993; Sanes et al. 1992). Normal developmental observations from this pathway led to the discovery that there is a developmental transition from GABAergic to glycinergic transmission (Kotak et al. 1998). The prevalence of GABAergic transmission in the LSO during neonatal development suggests a unique role in the maturation of these synapses, and raises two major questions: Is the locus of LTD pre- or postsynaptic? Does one of the inhibitory neurotransmitters induce LTD?

Assessment of pre- and postsynaptic roles

There are three compelling arguments favoring a postsynaptic locus for inhibitory LTD. First, a decline in the magnitude of postsynaptic hyperpolarizations occurs after application of GABA, but not glycine, in the absence of presynaptic stimulation. Furthermore, this effect requires GABA_B receptor acti-

vation (Fig. 3). Recordings from LSO indicate that synaptic potentials are stable in the absence of stimulation (Kotak and Sanes 2000; Kotak et al. 2001). Second, the effect of LTD on the mean amplitude, but not the frequency, of spontaneous mIPSCs provided compelling confirmation of the indirect mea-

asures (TTP, latency) that the site of change in this LTD is postsynaptic (Fig. 5). In addition, the lack of any significant alteration in the PPRs (Fig. 4B) corroborates that release properties of the presynaptic terminal population did not change during LTD (Schulz et al. 1994; Schweizer et al. 1998; Waldeck et al. 2000). Third, postsynaptic buffering of intracellular calcium (Kotak and Sanes 2000), postsynaptic blockade of tyrosine kinase receptors (Kotak et al. 2001), or postsynaptic blockade of protein kinase A, CAMKII, or protein kinase C decreases or blocks this LTD (Kotak and Sanes 2002).

The induction signal most likely involves GABA binding to the postsynaptic GABA_B receptor. GABA_A-mediated inhibitory potentials decline with age, although there is a concomitant increase of glycine-receptor-mediated transmission (Kotak et al. 1998). Because LTD is observed in the presence of either SN or BIC (Fig. 1, A and B), neither GABA_A nor glycine receptor activation is essential to induce synaptic depression. However, it remains possible that one of these ionotropic receptors must be activated along with the GABA_B receptor to trigger the depression. In fact, a selective GABA_B receptor antagonist is able to completely block activity-dependent inhibitory LTD (Kotak et al. 2001). The ability of the same GABA_B antagonist to block agonist-evoked depression in the complete absence of presynaptic stimulation (Fig. 3B) strongly supports the hypothesis that postsynaptic activation of GABA_B receptor is necessary to initiate LTD. Collectively, these findings argue for induction by a metabotropic GABAergic signal acting postsynaptically.

It should be noted that the GABAergic signal does not have to act exclusively to initiate LTD. MNTB neurons also express brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), whereas LSO neurons express the cognate receptors, TrkB and TrkC (Hafidi 1999; Hafidi et al. 1996). Although there is no direct evidence that these neurotrophins are released by MNTB neurons, we previously reported that they can produce inhibitory synaptic depression (Kotak et al. 2001).

What is the postsynaptic locus of change?

The elimination of inhibitory synapses during development may be initiated through the modification and/or partial loss of postsynaptic GABA_A and glycine receptors (Fig. 1, A and B). Modification of receptor function is well established for both excitatory and inhibitory terminals. The bidirectional alteration in AMPA receptor conductance that accompanies long-term potentiation (LTP) or LTD in hippocampal CA1 neurons is mediated by a process of phosphorylation and dephosphorylation (Castellani et al. 2001; Lee et al. 2000). Similar mechanisms are known to operate at GABA_A receptors (Chen et al. 1990; Melis et al. 2002; Poisbeau et al. 1999; Xi et al. 1997), and glycine receptors (Caraiscos et al. 2002; Mascia et al. 1998). An alteration in receptor number is also associated with

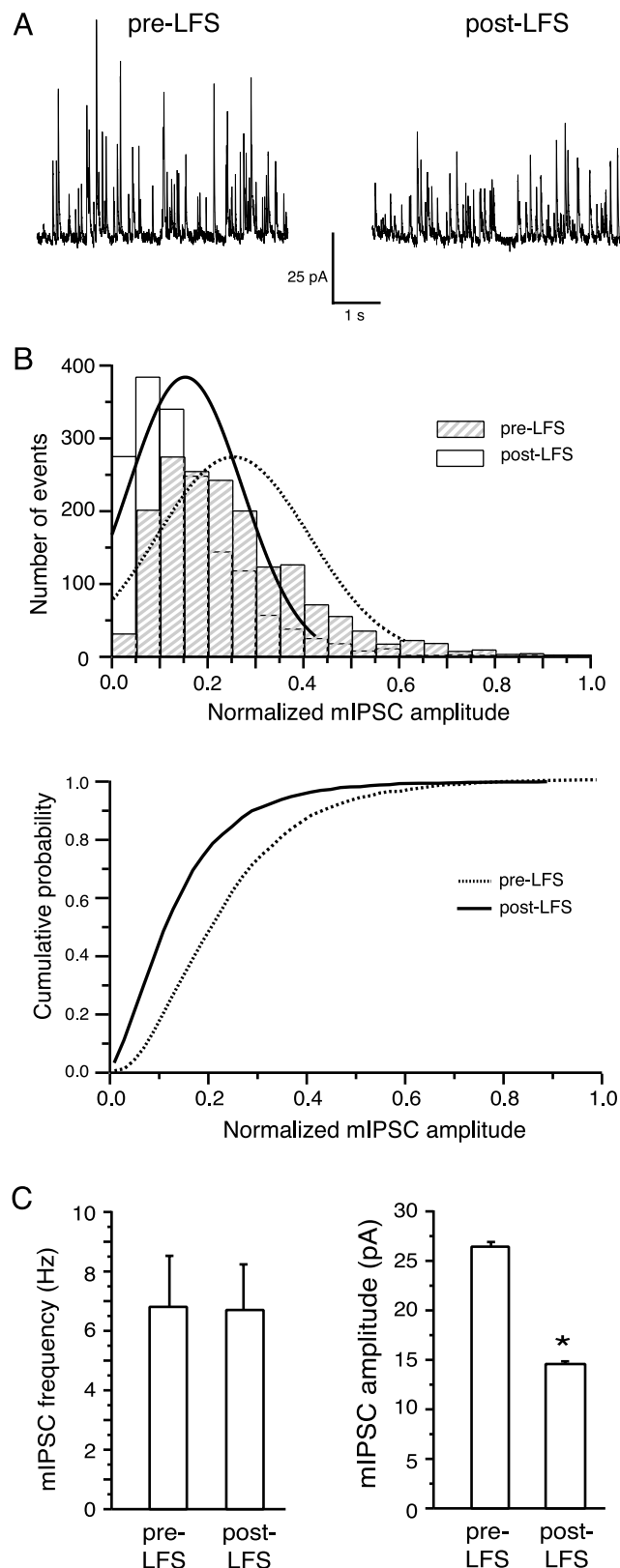


FIG. 5. LTD is associated with decline in amplitudes of mIPSCs. A: sample traces showing mIPSCs in ruthenium red (RR) both before and after LTD induction. B: amplitude frequency histogram fit with normal curves reveals leftward shift in mIPSC amplitude distribution after LFS (left). Cumulative amplitude distributions comparing events before (dotted line) and after (solid line) LFS also illustrate shift to lower amplitudes (right). Two cumulative distributions are statistically different ($P < 0.0001$). C: average rate of mIPSCs in RR did not change as a result of LTD ($P > 0.9$, $n = 5$), whereas average amplitude of mIPSCs decreased significantly ($P < 0.0001$, $n = 5$).

functional changes in several systems. For example, the activity-dependent reduction in mIPSC amplitude in neocortical cultures is associated with decreased staining for GABA_A receptors (Kilman et al. 2002). A similar reduction of inhibitory synaptic strength is produced by BDNF in hippocampal neurons, and this is associated with a decrease in GABA_A receptor staining (Brunig et al. 2001). Conversely, the insertion of postsynaptic GABA_A receptors into membranes is the basis for the potentiation of evoked IPSCs in kindled hippocampal granule cells (Nusser et al. 1998) and for increased mIPSC amplitudes in transfected HEK 293 cells (Wan et al. 1997). In hippocampal neurons, the disruption of clathrin-dependent GABA_A receptor endocytosis causes an increase in mIPSC amplitude (Kittler et al. 2000).

Thus a withdrawal of GABA_A or glycine receptors from the LSO synaptic membrane could contribute to the depression of evoked IPSCs. Although there is no direct evidence for this mechanism, it should be noted that GABA_A and glycine receptor localization is quite dynamic during the normal maturation of LSO neurons. There is a reduction of GABA_A receptors and an increase in gephyrin from P4 through P14 (Korada et al. 1999; Kotak et al. 1998). The anatomical distribution of glycine receptors is altered dramatically within the LSO during the postnatal wk 1–3 (Sanes and Wooten 1987). Finally, a second auditory brain stem nucleus that receives inhibitory input from the MNTB also displays a reduction of glycine receptors and glycinergic boutons during development, and this elimination depends on normal innervation (Kapfer et al. 2002). Thus GABA_A and glycine receptor expression is known to be dynamic during the period when inhibitory synapses are eliminated in the auditory brain stem, and circumstantial evidence from other systems suggests that receptor loss could account for the LTD that we observe.

Comparison to induction of other forms of inhibitory synaptic plasticity

LTP or LTD at inhibitory synapses can be induced homosynaptically or heterosynaptically. For example, inhibitory LTP can be induced homosynaptically in goldfish Mauthner (M) neurons by activation of presynaptic inhibitory interneurons after sound stimuli in vivo or electrical stimulation of these interneurons in vitro. Such inhibitory potentiation decreases sound-evoked firing of M-cells and suppresses the M-cell-mediated escape behavior (Oda et al. 1998). Similarly, in layer V neurons of developing visual cortex, an age-dependent inhibitory LTP can be induced by activation of layer IV in the presence of ionotropic glutamate receptor blockers (Komatsu 1994), suggesting a homosynaptic mechanism. Glycine receptors do exist in these areas, but there are very few instances where their plasticity (Caraiscos et al. 2002) and their role in development (Flint et al. 1998) have been examined.

Under conditions when NMDA receptors are activated while GABA_A receptors are blocked, the same conditioning stimulus elicits inhibitory LTD (Komatsu and Iwakiri 1993), suggesting a heterosynaptic mechanism. In P2–P4 CA3 neurons, LTD displayed by GABA_A receptors is induced by depolarizing GABAergic potentials that remove the magnesium block of NMDA receptors, thus allowing calcium influx (Caillard et al. 1999). Excitatory terminals can also elicit calcium influxes by voltage-dependent calcium channels in Purkinje cells, and pro-

duce an enhancement of inhibitory synaptic gain, called *rebound potentiation* (Kano et al. 1992). Because the MNTB-evoked IPSPs in the LSO can be depolarizing during postnatal wk 1 (Kandler and Friauf 1995), excitatory synapses may facilitate the plasticity of inhibitory synapses; however, this remains to be explored. At the developing MNTB–LSO synapse, we have a unique situation where GABAergic and glycinergic transmission exist concurrently (Kotak and Sanes 1998). This allows for an assessment of the relative roles these ions and their receptors play in inhibitory plasticity at the same central synapse.

Another issue is whether plasticity is induced and expressed by pre- or postsynaptic mechanisms. Several studies demonstrate a postsynaptic locus of induction and GABA_B receptor involvement in inhibitory plasticity. The NMDA receptor-mediated inhibitory LTP in deep cerebellar nuclei (DCN) has a postsynaptic locus of induction because postsynaptic depolarization is sufficient to induce LTP and dialyzing the postsynaptic neuron with BAPTA blocks the phenomenon (Ouardouz and Sastry 2000). Inhibitory LTD in the DCN does not require activation of NMDA receptors, nor GABA receptors (Morishita and Sastry 1996). Furthermore, although LTP in the DCN results in an increased response to the application GABA_A agonist, it does not require the activation of GABA_A receptors for induction. Similarly, postsynaptic GABA_A receptor participation is not essential for the induction of inhibitory LTD, whereas postsynaptic GABA_B receptors are necessary for inhibitory LTP in layer V visual cortex (Komatsu 1996). Our data are consistent with both of these observations about GABA_A (Fig. 1) and GABA_B receptor involvement (Kotak et al. 2001). In the Purkinje cells, rebound potentiation of inhibitory synapses is prevented by inhibitory synaptic activity. The ability of inhibitory synapses to prevent LTP depends on postsynaptic activation of GABA_B receptors (Kawaguchi and Hirano 2000).

Against this background, characteristics of inhibitory LTD in the developing LSO share certain features with these other forms of inhibitory plasticity. The magnitude of LTD in the LSO is enhanced when the postsynaptic neuron is depolarized and attenuated by postsynaptic BAPTA treatment, suggesting the involvement of calcium (Kotak and Sanes 2000). Moreover, the requirement of GABA_B receptor activation in LSO (Kotak et al. 2001) is consistent with the involvement of this receptor in inhibitory plasticity in other brain regions. Our findings that LTD results from the decreased sensitivity of postsynaptic GABA_A receptors (Fig. 3) is similar to observations made in DCN neurons (Ouardouz and Sastry 2000). Furthermore, presynaptic involvement was tested and excluded as a major factor for LTD expression in the present study (Figs. 4 and 5).

Developmental mechanisms for synapse refinement

LSO neurons compute interaural level differences within a limited frequency band and over a reasonably broad range of absolute sound levels. Therefore it is essential that excitatory and inhibitory terminals are well matched for tonotopy and strength. This fine-tuning would seem to require a mechanism whereby inhibitory terminals compete with excitatory terminals for postsynaptic space, and the present study may provide insight into this mechanism.

Central auditory neuron responses to sound frequency or sound location are modified by the sound environment during postnatal development (DeBello et al. 2001; Hyson and Johnson 1999; Iyengar and Bottjer 2002; Knudsen et al. 1984; Lee et al. 2001; Moore and Irvine 1981; Poon and Chen 1992; Sanes and Constantine-Paton 1985; Silverman and Clopton 1977; Wilmington et al. 1994; Zhang et al. 2001). The strength of inhibitory synaptic connections appears to play an important role during this plasticity (Kapfer et al. 2002; Sanes and Takács 1993; Zheng and Knudsen 2001).

At the neuromuscular junction, polyneuronal innervation is reduced during postnatal wk 2, and this process is influenced by synaptic activity. Synapse elimination can be delayed or advanced with manipulations to activity in vivo (Busetto et al. 2000; Keller-Peck et al. 2001; O'Brien et al. 1978; Thompson et al. 1979). Furthermore, activation of one set of motor axons in vivo can produce depression in an unstimulated set of afferents (Ridge and Betz 1984). The neuromuscular synapse also displays heterosynaptic depression when assessed in vitro (Cash et al. 1996; Dan and Poo 1992; Lo and Poo 1991). The elimination of neuromuscular synapses is accompanied by the loss of acetylcholine receptors (AChR) and a reduction in quantal content and quantal efficacy (Balice-Gordon and Lichtman 1993; Colman et al. 1997). Because activity-dependent depression also leads to the loss of surface AChRs (Li et al. 2001), it is proposed that synapse elimination is initiated by the removal of postsynaptic receptors (Lichtman and Colman 2000).

Our studies of inhibitory synapse development and plasticity are consistent with the general model of excitatory synapse refinement at the neuromuscular junction, discussed above. Cochlear activity has been implicated in this refinement (Kapfer et al. 2002; Sanes and Takács 1993), and low levels of activity can dramatically affect inhibitory synaptic gain. Thus the LTD of inhibitory MNTB–LSO synapses is induced by a developmentally regulated neurotransmitter, GABA, operating at a postsynaptic locus.

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DISCLOSURES

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