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Gain adjustment of inhibitory synapses in the auditory system

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Abstract. A group of central auditory neurons residing in the lateral superior olivary nucleus (LSO) responds selectively to interaural level differences and may contribute to sound localization. In this simple circuit, ipsilateral sound increases firing of LSO neurons, whereas contralateral sound inhibits the firing rate via activation of the medial nucleus of the trapezoid body (MNTB). During development, individual MNTB fibers arborize within the LSO, but they undergo a restriction of their boutons that ultimately leads to mature topography. A critical issue is whether a distinct form of inhibitory synaptic plasticity contributes to MNTB synapse elimination within LSO. Whole-cell recording from LSO neurons in brain slices from developing gerbils show robust long-term depression (LTD) of the MNTB-evoked IPSP/Cs when the MNTB was activated at a low frequency (1 Hz). These inhibitory synapses also display mixed GABA/glycinergic transmission during development, as assessed physiologically and immunohistochemically (Kotak et al. 1998). While either glycine or $GABA_A$ receptors could independently display inhibitory LTD, focal delivery of GABA, but not glycine, at the postsynaptic-locus induces depression. Furthermore, the $GABA_B$ receptor antagonist, SCH-50911, prevents GABA or synaptically induced depression. Preliminary evidence also indicated strengthening of inhibitory transmission (LTP) by a distinct pattern of inhibitory activity. These data support the idea that GABA is crucial for the expression inhibitory LTD and that this plasticity may underlie the early refinement of inhibitory synaptic connections in the LSO.

Introduction

When the neuronal growth cone comes in contact with a likely postsynaptic partner, the process of synapse formation begins. At first, there are a series of molecular interactions that lead to the differentiation of growth cone into presynaptic terminal and elaboration of the postsynaptic site to include a cluster of neurotransmitter receptors. These changes can occur within minutes to hours, at least in cell culture systems. In fact, the growth cone itself can release neurotransmitter and the postsynaptic cell already expresses cognate receptors prior to contact. The strength (for example, the amplitude of an evoked synaptic potential) of neurotransmission can increase within a few minutes of contact (Young and Poo 1983; Evers et al. 1989). Our understanding of synapse formation is based on an elucidation of the anterograde and retrograde signaling that occurs as these two cells first come into contact with one another.

Once formed, synapses are often eliminated over the course of development. Furthermore, the stabilization or elimination of a synapse can fall under the influence of the synapse itself. This latter stage of development, often referred to as *activity-dependent plasticity*, has been the subject of intense study for many decades (Sanes et al. 2000). For many good reasons, those interested in synapse plasticity have focused on excitatory synapses. Developmental model systems include the vertebrate and fly neuromuscular junction, the frog retinotectal projection, the owl midbrain, and the mammalian thalamus and cortex. There has been overwhelming success in these studies in characterizing both the types of environmental stimulation that influence synapse development and the cellular mechanisms that support changes in synapse gain.

Inhibitory synapses contribute to neural processing in approximately equal numbers to excitatory connections. Despite this, surprisingly little is known about the plasticity of these synapses. As it turns out, most inhibitory synapses in the mammalian brain come from interneurons (Eccles 1969), making it difficult to stimulate them selectively, or subject them to experimental manipulation. In contrast, the central auditory system is rife with inhibitory projections, and we have made use of one such pathway in the gerbil (*Meriones unguiculatus*) to study the development and plasticity of inhibitory connections.

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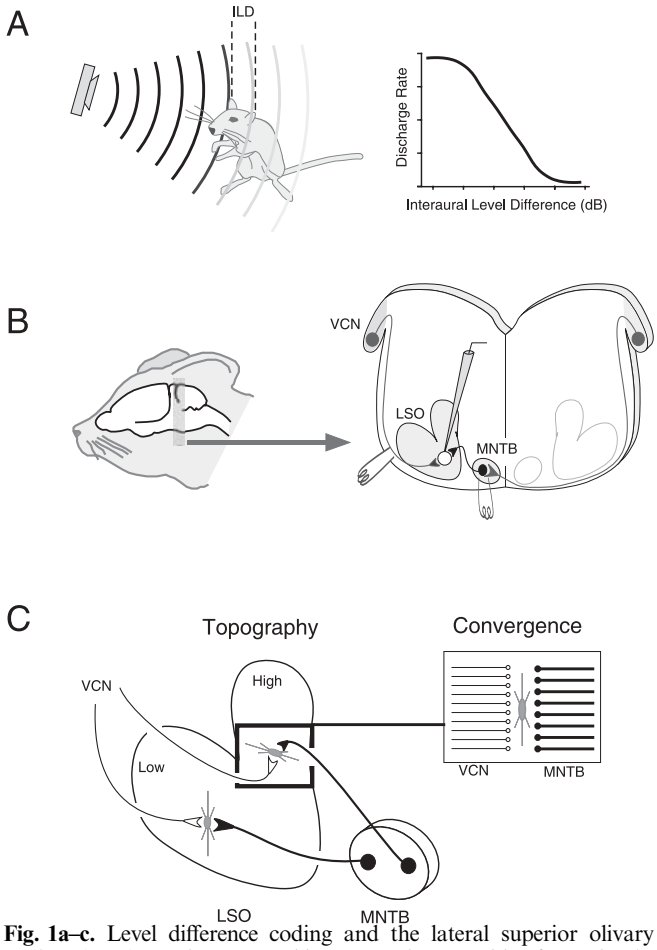

Fig. 1a–c. Level difference coding and the lateral superior olivary nucleus (LSO). **a** When a sound is presented to one side of an animal's head, the two ears receive a difference in sound level. The head attenuates sound level for frequencies with a wavelength that is smaller than the head diameter. Neurons in the LSO respond to interaural level differences; discharge rates are high when sound level is relatively greater on the ipsilateral side and low when sound level is relatively greater on the contralateral side. **b** A brain slice through the auditory brain stem contains the LSO and its afferent pathways from the ipsilateral and contralateral ears. The ipsilateral pathway emerges from the ventral cochlear nucleus (VCN), which sends a glutamatergic projection to the LSO. The contralateral pathway begins in the contralateral VCN, which sends a glutamatergic projection to the medial nucleus of the trapezoid body (MNTB). The MNTB sends a glycinergic projection to the LSO. **c** The VCN and MNTB projections are well matched for stimulus frequency (topography). High sound frequencies activate neurons in the dorsal and medial region of LSO, while low sound frequencies activate neurons in the ventral and lateral region. In addition, each LSO neuron receives about the same number of axonal contacts from VCN and MNTB neurons (convergence).

The inhibitory neurons that we study participate in a simple brain stem circuit that computes interaural level differences (ILD), a cue that we (and gerbils) use to locate a sound along the horizontal axis (Heffner and Heffner 1988). As shown in Fig. 1a, sound originating on one side of an animal's head produces a larger intensity at the proximal ear, whereas intensity is decreased at the distal ear because the head acts as a baffle (when the sound frequency wavelength is smaller than the width of the head). It follows that a sound at the midline would produce equal intensities at each ear. Thus, interaural level difference is well correlated with the azimuthal position of sound.

The first group of mammalian central auditory neurons to respond selectively to ILD are in the lateral superior olivary nucleus (LSO). When the sound is ipsilateral to the LSO, the excitatory pathway is maximally activated, whereas the inhibitory pathway is minimally activated (Fig. 1a). In this case, the postsynaptic LSO neuron produces a large discharge rate. When the sound is contralateral to the LSO, the inhibitory pathway is maximally activated, whereas the excitatory pathway is minimally activated, and the postsynaptic neuron produces a small rate of discharge. Thus, the overall discharge pattern of LSO neurons is a reliable signature for ILD.

LSO neurons receive an excitatory projection driven by the ipsilateral ear and an inhibitory projections driven by the contralateral ear (Boudreau and Tsuchitani 1970). A major advantage of the LSO circuit is that its inhibitory projection is anatomically independent. As shown in Fig. 1b, the inhibitory neurons are found in the medial nucleus of the trapezoid body (MNTB), and they project a short distance to the LSO. This permits one to stimulate the inhibitory pathway selectively in vivo with sound to the contralateral ear or in vitro with direct electrical stimulation of the MNTB. Each LSO neuron also receives a projection from the cochlear nucleus (CN). Both the ipsilateral and the contralateral afferent projections form topographic maps of frequency in the LSO, with low frequencies represented ventrolaterally and high frequencies represented dorsomedially (Sanes et al. 1989). A general treatment of the anatomy in the superior olivary complex may be found in Schwartz (1992).

Refinement of an inhibitory projection

As with other areas of the nervous system, the connections to the LSO are the result of accurate outgrowth and innervation mechanisms. Individual MNTB fibers initially terminate with great accuracy within the LSO (Sanes and Siverls 1991). However, there is evidence that this accuracy is not entirely adult-like from the outset. There is a significant improvement in the matching of excitatory and inhibitory sound frequencies between P13–14 (when gerbils first respond to airborne sound) and adulthood (Sanes and Rubel 1988). Thus, it is of interest to determine the mechanisms that lead to mature topography (Fig. 1c). Functional estimates of the number of excitatory and inhibitory terminals per LSO neuron suggest that convergence declines during development (Sanes 1993). Single MNTB terminal arborizations in the LSO become physically restricted during development (Sanes and Siverls 1991). At P12–13, MNTB terminals contained an average of 244 boutons, and these boutons spread 121 μm across the LSO frequency axis. By P18–25 the average number of boutons have decreased to 158, and these spread over 93 μm. There is also an earlier period of refinement in the projection from MNTB to LSO (Kim and Kandler

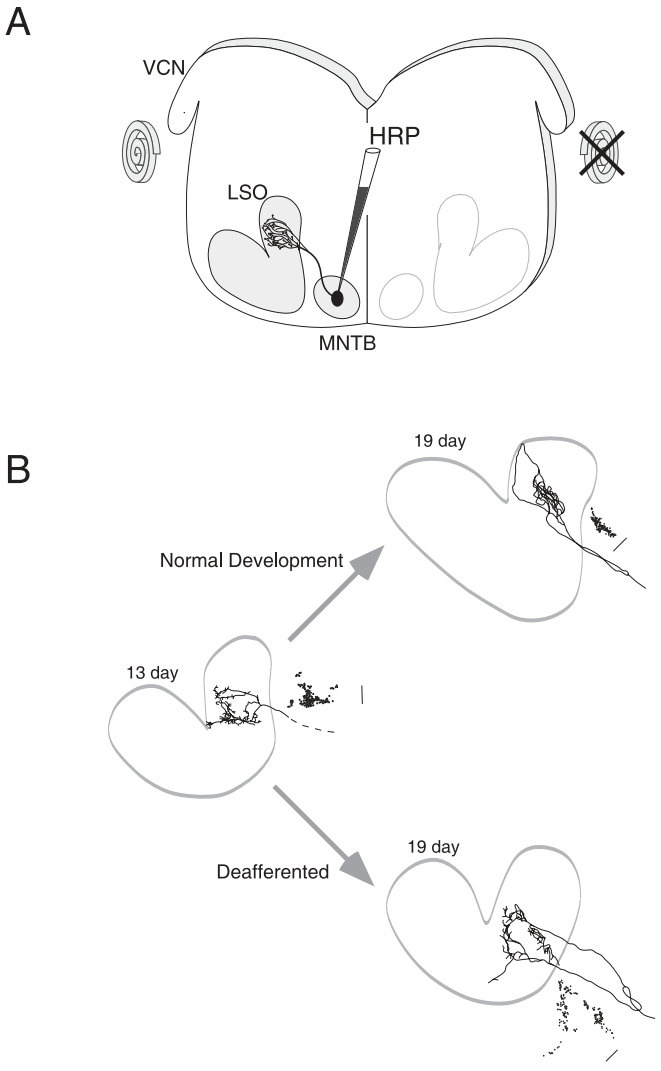

Fig. 2a,b. Activity-dependent development of MNTB arbors within the LSO. **a** Single MNTB neurons can be filled with an anatomical tracer (HRP), and the arborizations with the LSO can be drawn and quantified with a morphometry system. Labeling was performed in normal animals and following contralateral cochlea removal, which deafferents the MNTB. **b** Single MNTB arborizations are shown for a control P19 neuron, and a deafferented P19 neuron. The *dot pattern* next to each arbor represents the pattern of synaptic boutons produced by that arbor. The *scale bar* is 100 μm for each example. The spread of MNTB arbors across the LSO frequency axis (see Fig. 1) declines by about 30% during normal development. This refinement is not observed in MNTB neurons that do not receive an input from the contralateral ear

2003). Therefore, it is possible that inhibitory and excitatory terminals in LSO undergo small changes in connectivity such that an appropriate convergence is obtained (Fig. 1c).

The refinement of inhibitory MNTB arbors with the LSO may depend, in part, on their activity. When the contralateral cochlea is ablated at P7, single MNTB terminal arbors fail to attain the normal level of anatomical specificity, as compared to controls (Fig. 2). Terminal boutons spread the same distance across the tonotopic axis as found in control P12–13 animals (Sanes and Takács 1993). Even though the manipulation functionally denervates MNTB cell bodies, the arbors do not display signs of atrophy. Both the total axonal length within the LSO borders and the number of boutons per MNTB fiber were similar in control and ablated animals. Thus, inhibitory MNTB arbors do not go through their normal period of anatomical refinement when denervated, suggesting that inhibitory activity also plays a role in the maintenance or stabilization of inhibitory synaptic contacts. A complementary phenomenon has recently been described in a second target nucleus of the MNTB, the medial superior olivary nucleus (MSO). Terminals from the MNTB are eliminated from MSO dendrites during early development. This process is prevented by unilateral cochlear ablation and diminished by rearing animals in white noise (Kapfer et al. 2002).

Question

One important question arising from these studies is whether the physical elimination of inhibitory synapses is associated with a weakening in the strength of inhibitory transmission. If some synapses do become weakened during development, then one might expect other inhibitory connections to become strengthened and stabilized.

Techniques

To address these issues, we examined whether the gain of evoked inhibitory synaptic potentials (or currents) could be altered in an activity-dependent fashion using a slice preparation of the auditory brain stem. Inhibitory connections from the MNTB to the LSO are preserved in these slices (Fig. 1b), and the afferents can be selectively activated with current pulses while whole-cell recordings are obtained from individual LSO neurons (Kotak and Sanes 1995; Kotak et al. 1998). A full description of the techniques can be found in our published reports. Briefly, gerbils (*M. unguiculatus*) aged postnatal days 8–19 (P8–P19) were used to generate 300-μm brain stem slices containing the LSO and the MNTB (Sanes 1993). The slices were placed in oxygenated artificial cerebrospinal fluid (ACSF) in the recording chamber at room temperature (22–24 °C). Whole-cell current-clamp and voltage-clamp recordings were obtained as described previously (Kotak et al. 1998). Most recordings were performed in the presence of 4 mM kynurenic acid to block ionotropic glutamate receptors. In experiments measuring MNTB-evoked responses, a bipolar stimulating electrode was placed on the MNTB and a 200-μs pulse was delivered via an isolated biphasic stimulator in constant current mode. 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). To directly activate postsynaptic receptors, a second pipette containing GABA or glycine (tip diameter = 2–4 μm) was positioned approximately 200–300 μm from the somatic

recording site. Typically, a 50 psi pressure pulse was applied to deliver one of the amino acids through the pipette. Data were sampled at 10 kHz and stored on a Macintosh G4 running a custom designed *Igor* macro (Wavemetrics). All data were analyzed offline using a second IGOR macro.

Experiments

In the first set of experiments performed in slices from normal developing gerbil pups, we discovered that LSO neurons undergo a profound long-term depression (LTD) of MNTB-evoked inhibitory postsynaptic currents (IPSCs) by over 50% when the MNTB was stimulated at a low frequency (1 Hz). Figure 3 shows that low-frequency stimulation of MNTB for only 15 min (gray bar: LFS) produced a robust and long-lasting depression of IPSCs, as compared with the 15-min pre-LFS control period. The LTD persisted for as long as we were able to record from the LSO neurons (up to 4 h).

If inhibitory LTD were a developmental mechanism, then we would expect it to decline with age. In fact, this form of synaptic plasticity does decline dramatically by postnatal days 17–19, about 1 week following hearing onset (Fig. 3). We have not yet determined whether LTD has vanished in mature animals due to the limitations of the brain slice preparation; it is extremely difficult to obtain whole-cell recordings from LSO neurons as the glial environment and extracellular matrix mature.

Inhibitory LTD in developing LSO occurs while MNTB synapses are becoming reconfigured, implying that the two phenomena are causally related (Kotak and Sanes 2000). Demonstrating a linkage between these structural and functional phenomena may be feasible in a long-term brain slice preparation or co-culture preparations of MNTB and LSO neurons. However, it will be necessary to identify a candidate manipulation that can block inhibitory LTD. This consideration led us to study the mechanism of inhibitory LTD induction and maintenance (below).

Our studies of LTD induction began with a rather unexpected observation. Inhibitory transmission within the LSO has long been thought of as exclusively glycinergic in adult animals (Moore and Caspary 1983; Sanes et al. 1987; Wenthold et al. 1987, 1990). Thus, we assumed that this held true for neonatal and juvenile animals as well. To our surprise, we found that MNTB terminals mediate primarily GABAergic transmission to the LSO during the first postnatal week, before sound-evoked responses are present (Kotak et al. 1998). Whole-cell voltage-clamp recordings were obtained from LSO neurons, and the pharmacology of MNTB-evoked IPSCs was characterized at different postnatal ages. GABAergic transmission was blocked with bicuculline, and glycinergic transmission was blocked with strychnine (SN). Thus, we could record the percentage of an evoked IPSC that was blocked by BIC or SN. We found a switch from GABAergic to glycinergic transmission

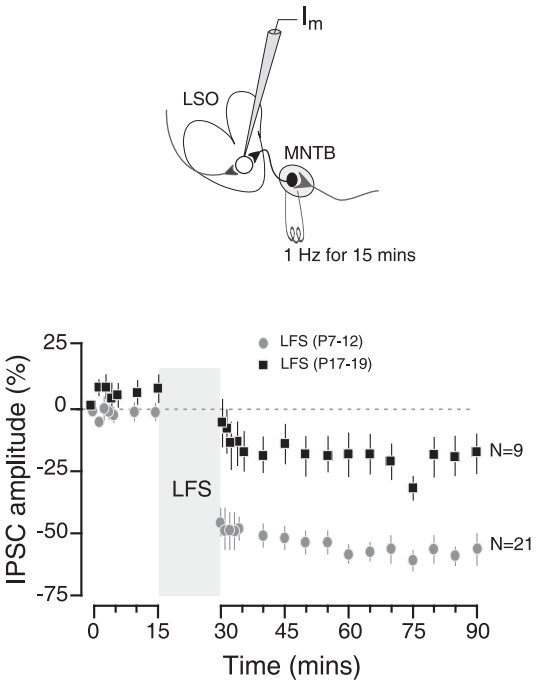

Fig. 3. Long-term depression of the inhibitory synapses in LSO. A schematic shows that stimulation protocol. The MNTB was activated at 1 Hz for 15 min (LFS) while recordings were made from the LSO neuron in a brain slice preparation (*top*). MNTB-evoked IPSCs were obtained for 15 min prior to LFS and for 60 min thereafter. The graph compares summary data from two age groups. At P7–12, the mean IPSC amplitude declined by more than 50% following LFS (*gray circles*), and this depression was significant. In contrast, the magnitude of synaptic depression at P17–19 was only about 20% below control values (*black squares*). Age-matched control neurons that did not receive any LFS or intermediary MNTB stimulation did not get depressed (Kotak and Sanes 2000).

during the first two postnatal weeks (Fig. 4). GABAergic transmission contributed 78% of the IPSC amplitude at postnatal (P) days 3–5 and declined to only 12% by P12–16. There was a commensurate increase in the glycinergic component. These results have been confirmed anatomically using immunohistochemical staining against GABA, glycine, the glycine receptor achoring protein (gephyrin), and a $GABA_A$ receptor subunit (Kotak et al. 1998; Korada and Schwartz 1999). Collectively, these data demonstrated a remarkable transformation of the inhibitory synapses within the LSO. Co-release of these two neurotransmitters has now been demonstrated more directly in other systems (Jonas et al. 1998; O'Brien and Berger 1999; Dumoulin et al. 2001).

This study suggested the interesting possibility that GABA is a developmental signal. Therefore, we decided to test the hypothesis that GABA release is necessary for the induction of inhibitory LTD in the LSO. It is not immediately clear why the GABAergic system would provide a different sort of signal than the glycinergic system. One possibility is that GABA activates a set of G-protein coupled receptors ($GABA_B$ receptors) and is able to activate a second messenger system within the postsynaptic neuron. In contrast, the ionotropic glycine and $GABA_A$ receptors both gate chloride channels.

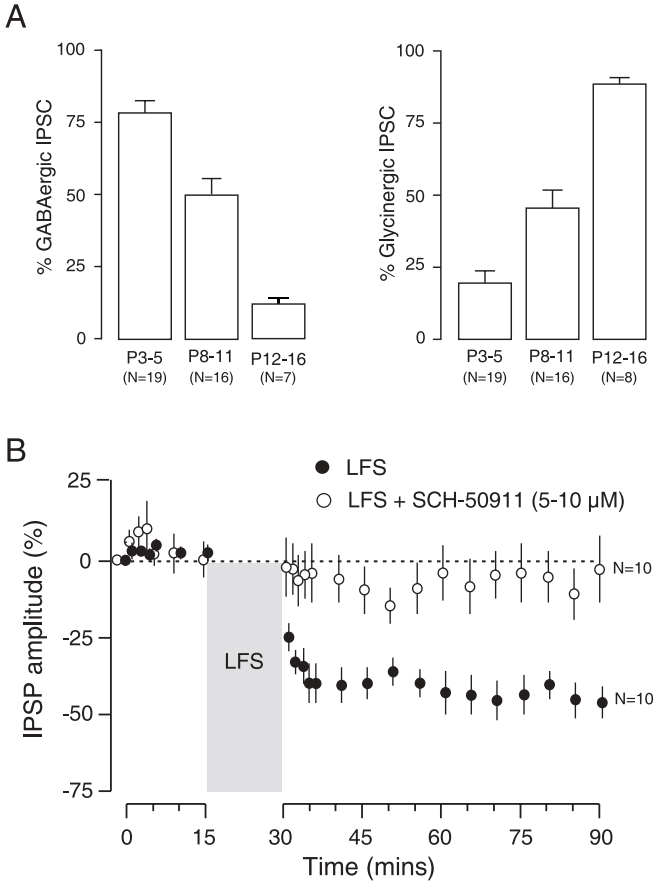

Fig. 4. Early presence of GABAergic transmission and its role in LTD. **a** The normalized percent GABAergic IPSC component (i.e., blocked by the $GABA_A$ receptor antagonist bicuculline) predominant in early postnatal life (P3–5) was present at an approximately 50% level at P8–11 and was only about 10% in older animals (P12–P16). There was an almost equal and opposite increase in the glycinergic IPSC component (i.e., blocked by the glycine receptor antagonist strychnine) recorded from the same group of neurons. Means + SEM are shown. **b** Long-lasting depression of inhibitory transmission was mediated by $GABA_B$ receptors. MNTB-evoked maximum IPSPs were recorded in the absence and presence of the $GABA_B$ receptor antagonist SCH-50911. In control neurons, synaptic depression was robust (43%) at 50–60 min following LFS (*filled circles*). Age-matched neurons treated with SCH-50911 (*open circles*) displayed an insignificant change in IPSP amplitude following LFS (mean ± SEM).

To test the hypothesis that a $GABA_B$-receptor-mediated metabotropic pathway could initiate a cellular process to scale down inhibitory synaptic gain, whole-cell recordings were made from LSO neurons in the presence of the $GABA_B$ receptor antagonist SCH-50911. This manipulation led to the near total blockade of inhibitory LTD. In separate experiments, the $GABA_B$ receptor agonist, baclofen, was found to depress evoked inhibition, mimicking the activity-dependent depression (Kotak et al. 2001).

While these experiments showed that GABA was an important instigator of LTD, they could not distinguish whether the mechanism occurred at the pre- or postsynaptic membrane. Therefore, we designed a set of experiments in which it was possible to assign the site of LTD induction and maintenance to the postsynaptic membrane. First, we demonstrated that either glycine or $GABA_A$ receptors were able to independently display LTD following MNTB stimulation (i.e., when either $GABA_A$ or glycine receptors were blocked pharmacologically).

We next designed a specific test of whether GABA or glycine could cause depression independent of MNTB activation. A micropipette, containing either GABA or glycine, was positioned in close proximity to the recorded LSO neuron within the LSO (Fig. 5a). Using a brief pulse of pressure to the pipette, we delivered focal pulses of either amino acid directly upon the postsynaptic LSO neuron. We found that GABA, but *not* glycine, was sufficient to trigger depression of the puff-evoked hyperpolarizations (Fig. 5). Although glycine pulses did produce postsynaptic hyperpolarizations, the size of these potentials remained stable (Chang et al. 2003). Additionally, the GABA-induced depression could be blocked by the $GABA_B$ receptor antagonist, SCH-50911. Together with our previous data that the $GABA_B$ receptor blocker disrupted synaptically induced LTD (Kotak et al. 2001), these observations lend credibility to the notion that GABA plays a pivotal role in the induction and maintenance of inhibitory LTD (Kotak et al. 2001; Chang et al. 2003).

The focal pipette experiments show that a dramatic decline in inhibitory synaptic gain can be initiated postsynaptically without presynaptic (MNTB) involvement. The postsynaptic theory is consistent with our previous data that inhibitory LTD can be blocked by intracellular manipulations exclusively to the recorded postsynaptic neuron. These include buffering of calcium (Kotak and Sanes 2000) or blockade of several kinases that have been implicated in excitatory synaptic plasticity, including calcium calmodulin protein kinase II, protein kinase A, and protein kinase C (Kotak and Sanes 2002b). A direct link between the activation of postsynaptic $GABA_B$ receptors and mobilization of intracellular calcium or activation of specific second messenger pathways remains to be shown.

The majority of our research has focused on the mechanism that leads to a decrease of inhibitory synaptic gain. However, it is likely that many synapses increase their strength during development, as they become stabilized. In fact, we have recently identified a form of plasticity that increases the amplitude-evoked inhibitory synaptic events within the LSO. In this case, extremely low levels of MNTB activation produces inhibitory long-term potentiation in the LSO (Kotak and Sanes 2002a). This finding is consistent with a 12-fold increase in the MNTB-evoked synaptic conductance measured in the rat LSO during development (Kim and Kandler 2003).

Discussion

There are two issues that will require critical examination in the future. First, what role – if any – does inhibitory LTD play during development? We have argued for the hypothesis that inhibitory LTD is

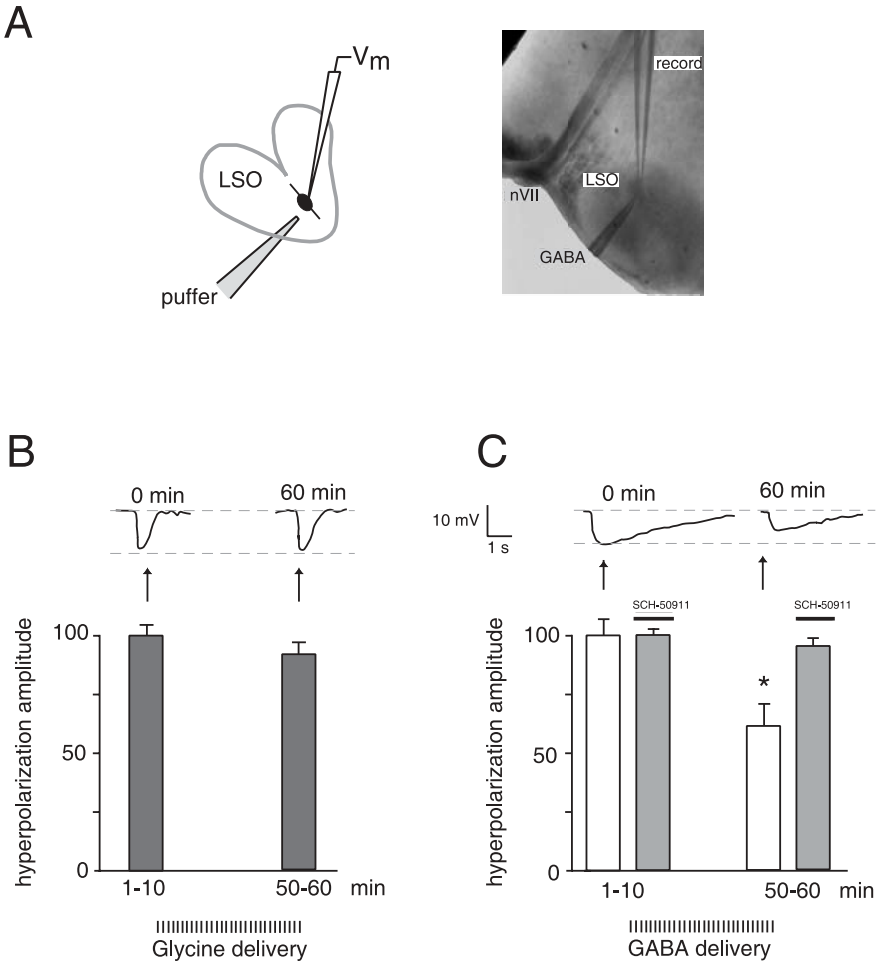

Fig. 5a–c. Inhibitory synaptic LTD is induced and expressed at the postsynaptic LSO neuron. **a** A schematic (*left*) and image (*right*) show the experimental configuration. A puffer pipette was positioned near the surface of the recorded LSO neuron. The MNTB was not stimulated in this set of experiments. **b** Glycine-evoked hyperpolarizations do not change following delivery of glycine pulses. The *bar graph* shows changes (% control ± SEM) of the agonist-evoked hyperpolarization amplitudes relative to a baseline control period before focal application began. Each *vertical line* underneath the *bar graphs* denotes an individual delivery of the transmitter (1 mM, 50 ms) by a focal delivery pipette positioned approximately 200–300 μm from the recording site. **c** GABA-evoked hyperpolarization amplitudes become significantly depressed following delivery of GABA pulses (*white bars*). The presence of a $GABA_B$ receptor antagonist, SCH-50911 (10 μM), prevented any significant decline in GABA-evoked hyperpolarization amplitudes over the same amount of time (*gray bars*)

responsible for the anatomical refinement of MNTB terminals within the LSO. Activity-dependent elimination of inhibitory connections is consistent with reports from several laboratories suggesting that synaptic depression is associated with the decrease of polyneuronal innervation at the developing nerve–muscle junction. For example, direct stimulation of one set of motor axons can accelerate synapse elimination and cause a weakening of unstimulated terminals (O'Brien et al. 1978; Ridge and Betz 1984; Balice-Gordon and Lichtman 1994; Lo and Poo 1994). However, a key test of this structure–function linkage will require an experimental strategy in which LTD and elimination of inhibitory boutons are demonstrated concurrently and then blocked by the same pharmacological or genetic manipulation. Our finding that $GABA_B$ receptor blockade can prevent the induction of LTD provides us with a valuable tool.

A small step could be taken by determining whether inhibitory LTD is present during the time when other inhibitory terminals are becoming refined. Thus, MNTB terminals are eliminated from MSO dendrites during development, and there is a model that accounts for this behavior through an activity-mediated process (Kapfer et al. 2002; reference to other chapters in this issue). It would be interesting to determine whether MSO neurons display inhibitory LTD during this phase of inhibitory synapse elimination.

The theoretical importance of $GABA_B$ receptors to developmental plasticity has been addressed above. This leaves open another fascinating question: What is the significance of $GABA_A$ receptor expression and function in early life? The chloride equilibrium potential in the neonatal rodent LSO is quite positive and favors membrane depolarization by either glycine or $GABA_A$ receptors. Thus, synaptic inhibition can depolarize the

LSO neuron and open voltage-gated calcium channels (for review see Sanes and Friauf 2000). Perhaps $GABA_A$ receptors provide a more efficient means for the developing inhibitory synapses to elicit postsynaptic calcium entry. It is also possible that phosphorylation-dependent modulation of $GABA_A$ receptors is distinct from that of glycine receptors (for references see Chang et al. 2003), and it may be advantageous to use these receptors for both short- and long-term changes in inhibitory synaptic function.

Many developmental mechanisms seem to persist into adulthood and take on new applications. For example, trophic factors support neuron survival in the embryo but contribute to synaptic plasticity in adults. Therefore, we should consider the possibility that inhibitory LTD (and LTP) contribute to synaptic gain adjustment without the actual physical loss or addition of connections.

It is certainly true that in vivo electrophysiology experiments performed in the LSO have not suggested that the strength of inhibition changes during the recording period (Boudreau and Tsuchitani 1970; Sanes and Rubel 1988). However, it is not clear that either the stimulus protocol or the analytic approach would have detected a change in gain. The recordings usually last for about 0.5–2 h, during which time the LSO neuron is exposed to a battery of sound stimuli to one or both ears. A more focused approach would first characterize the ILD coding properties of an LSO neuron and then impose a continuous stimulus protocol designed to alter inhibitory strength. For example, persistent stimulation of the contralateral pathway in the absence of ipsilateral stimulation might lead to down-regulation of contralateral inhibition. There have been many recent experimental and modeling studies that address this concept in vitro (Abbott and Nelson 2000), but its application to in vivo coding properties remains to be studied in simple systems such as the LSO.

Our experiments are quite explicit about the ability of inhibitory synapses to change their gain in response to activity patterns. Moreover, our studies have demonstrated the locus of change and some of the cellular mechanisms that support long-term inhibitory synaptic depression. Our discussion has focused on the biological impact of this mechanism, and we have concluded that it could support the early refinement of inhibitory synaptic connection and/or the adjustment of synaptic gain in mature animals.

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