Review

Development and influence of inhibition in the lateral superior olivary nucleus

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Abstract

While studies of neuronal development and plasticity have focused on excitatory pathways, the inhibitory projection from the MNTB to the LSO provides a favorable model for studies of synaptic inhibition. This review covers recent studies from our laboratories indicating that inhibitory connections are quite dynamic during development. These findings suggest that there are two phases inhibitory transmission. During an initial depolarizing phase is growth and branching of pre- and postsynaptic elements in the LSO. During a second hyperpolarizing phase there is refinement of inhibitory afferent arborizations and the LSO dendrites that they innervate. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Neurotransmission and electrical activity influence a wide range of developmental events, including migration, growth cone motility, and the expression of voltage-gated and ligand-gated ion channels themselves. There is now a rich literature focusing on the influence of excitatory synaptic transmission during development, and the majority of this work was performed at the nerve–muscle junction or at retinal ganglion cell synapses in the midbrain (amphibians) or thalamus (mammals). These studies generally assume that alterations in the number, or the strength, of excitatory connections can explain how environmental stimulation influences brain development.

There is, in fact, excellent evidence that excitatory synapses are modified by experience, but there are many other types of synapses in the brain that may also be changing. We have chosen to examine the development and plasticity of inhibitory synapses which are estimated to form 30–50% of synaptic connections in the central nervous system. In adult animals, inhibitory terminals release glycine or γ-aminobutyric acid (GABA) and hyperpolarize postsynaptic neurons by gating open either Cl^- or K^+ channels. The changes in neuron excitability that accompany an environmental manipulation (e.g. visual deprivation) have usually been interpreted as either an enhancement or a depression of excitatory synapses. However, such changes could just as easily be due to an alteration of synaptic inhibition. The work described below shows how a well identified inhibitory projection develops, and how inhibitory synaptic transmission appears to influence neuronal maturation.

Motor neuron and retinal ganglion cell projections have been chosen as model systems for studies of excitatory synaptogenesis because they form homogeneous projections. Manipulations of the spinal motor nerve, or the optic nerve, lead to changes in excitatory synaptic transmission at the first postsynaptic target. The central auditory system has a large number of inhibitory projections, and we have chosen one such pathway in the rat and gerbil to explore the development and plasticity of inhibitory connections. The lateral superior olivary nucleus (LSO) receives excitatory projections driven by the ipsilateral ear and inhibitory

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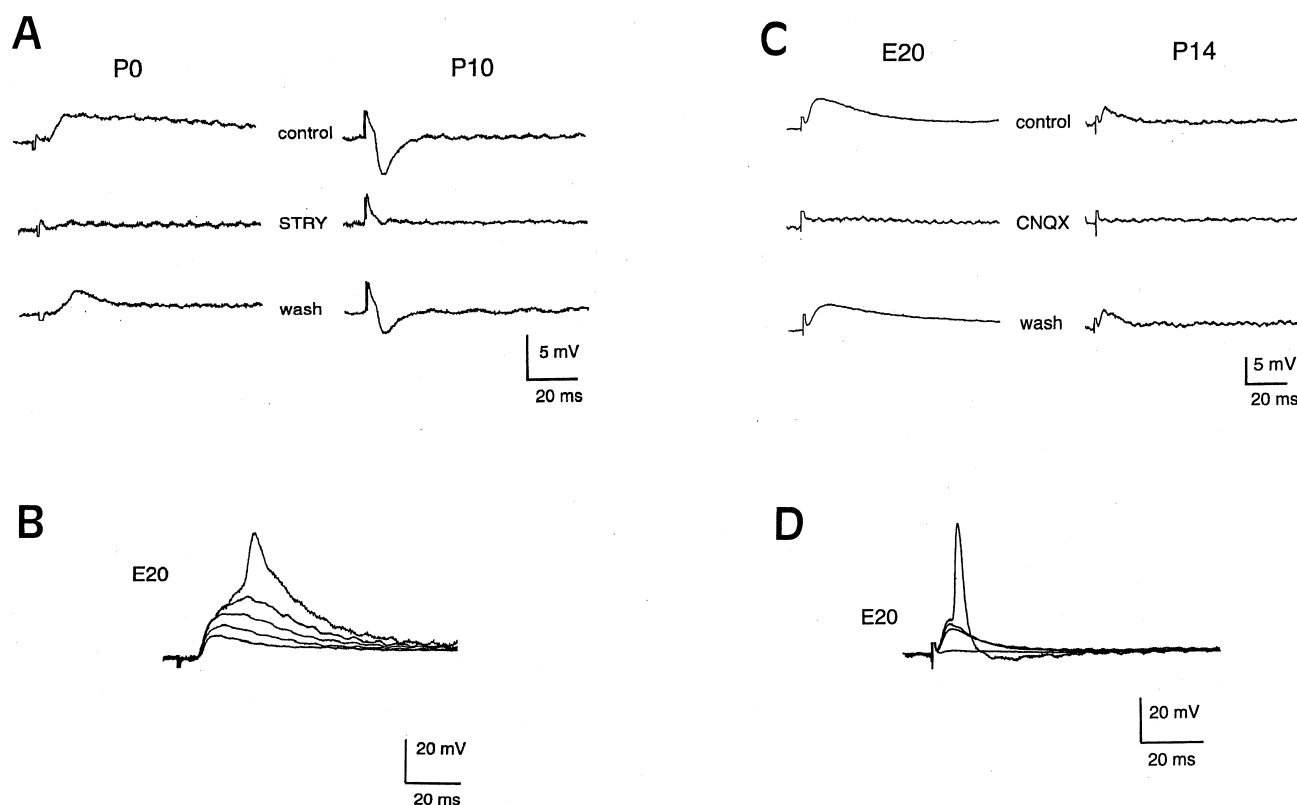
A

B

C

D

Fig. 1. Development of glycinergic (A,B) and glutamatergic (C,D) neurotransmission in the LSO of rats. Panel A shows postsynaptic potentials elicited after stimulation in the MNTB at P0 and P10. Responses were reversibly blocked by strychnine (STRY), demonstrating their glycinergic character. They are depolarizing at P0, yet hyperpolarizing at P10. Panel B shows growing amplitudes with increasing stimulus strength and the emergence of an action potential. Panel C shows postsynaptic potentials elicited after stimulation of the afferent pathway from the cochlear nucleus at E20 and P14. Responses were reversibly blocked by the non-NMDA receptor specific antagonist CNQX, demonstrating their gluta-matergic character. They are depolarizing in each case. Panel D shows growing amplitudes with increasing stimulus strength and the emergence of an action potential. (Modified from Kandler and Friauf, 1995a.)

projections driven by the contralateral ear (Boudreau and Tsuchitani, 1970). The inhibitory projection arises from a homogeneous glycinergic nucleus, the medial nucleus of the trapezoid body (MNTB), and this pro-jection maps topographically along the LSO frequency axis. The excitatory projection emerges from the cochle-ar nucleus (CN), is glutamatergic, and its tonotopic projection is perfectly matched to the inhibitory projec-tion from the MNTB. This arrangement allows LSO neurons to respond selectively to differences in sound level at the two ears, referred to as interaural level dif-ferences (ILD). The postsynaptic neuron integrates ex-citatory and inhibitory inputs to produce a discharge rate that is correlated with azimuthal sound position. From a developmental perspective, it seems critical that the same numbers of excitatory and inhibitory afferents, each arising from the same tonotopic location, inner-vate a postsynaptic LSO neuron. This conformation would be expected to permit an LSO neuron to com-pute ILDs over a reasonably broad range of absolute sound levels. The following experiments explore how the LSO circuit forms. The major elements of this sys-

tem can be extracted as a brain slice preparation for acute recording or organotypic cultures. We are also able to selectively manipulate the inhibitory MNTB neurons in vivo, and independently assess the function-al consequences of such manipulations in vitro. Taken together, these experiments suggest that the inhibitory projection to LSO is a unique model system for studies of inhibitory development and plasticity.

2. The depolarizing stage of development

Many essential steps in the development of the LSO circuit take place before the onset of hearing (reviews: Sanes and Walsh, 1997; Friauf et al., 1997b; Friauf and Lohmann, 1999). The axonal input to LSO neurons from both the excitatory (CN) and inhibitory (MNTB) afferents is established prenatally. Evidence comes from anterograde tracing studies in rats and shows that the afferents originating from the CN have formed terminal branches in the LSO by embryonic day (E) 18, i.e. 4 days before birth (Kandler and Friauf,

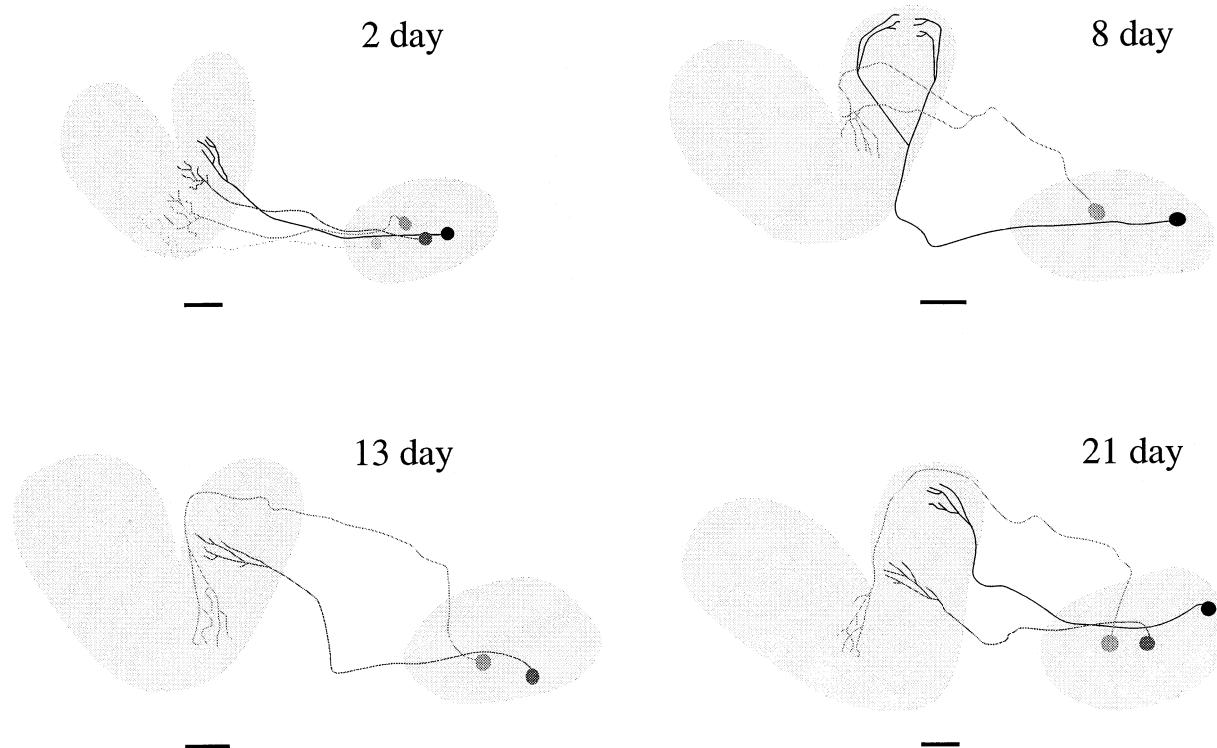## 2 day

8 day

13 day

21 day

Fig. 2. Topography of MNTB projections to the LSO during development. Each panel shows the reconstruction of 2–4 axonal projections from HRP-filled MNTB neurons into the gerbil LSO at a different postnatal age. In each case, the relative order of MNTB somata and the position of their terminal arbors within the LSO were well matched. Medially located MNTB somata projected to successively more dorsomedial loci within the LSO. Medial is to the right and ventral is to the bottom for all ages. Scale bars = 100 μm. (From Sanes and Siverls, 1991.)

1993). In gerbils, axons from the CN have established their pathways to the LSO and the MNTB by postnatal day (P) 0 (Kil et al., 1995). Electrophysiological in vitro studies in rats, in which postsynaptic responses in LSO neurons were evoked as early as E18 following electrical stimulation of the two afferent systems, have demonstrated that the synaptic inputs are functional during late fetal life (Fig. 1; Kandler and Friauf, 1995a). The postsynaptic responses were pharmacologically identified as glycinergic and glutamatergic, supporting the conclusion that they originated from MNTB and CN neurons, respectively. A morphological analysis of the MNTB–LSO projection has been performed by intracellular dye injections, and axon terminals from MNTB neurons were found in the gerbil LSO at P2, the earliest age examined (Fig. 2; Sanes and Siverls, 1991), further demonstrating the early establishment of the LSO circuit. The projections into the LSO are established with good precision, as demonstrated by the fact that topographically correct connections from the MNTB (Fig. 2) and the CN are present from the earliest age examined (Zook and DiCaprio, 1988; Sanes and Siverls, 1991; Kandler and Friauf, 1993; Kil et al., 1995). However, electrophysiological analyses of inhibitory and excitatory tonotopic maps indicate that they are not mature at P13–14. The final precision is gener-

ated some time after the onset of hearing (Sanes and Rubel, 1988).

An interesting observation was made when the characteristics of the glycinergic projection from the MNTB to the LSO were analyzed. During fetal life and the first 7 days of postnatal life, activation of glycine receptors evokes depolarizing responses in the majority of LSO neurons, rather than inhibitory, hyperpolarizing responses (Kandler and Friauf, 1995a; Ehrlich et al., 1999). At perinatal ages, these depolarizing responses are powerful enough to elicit action potentials (Fig. 1) and, therefore, they were considered to be excitatory, rather than resulting in shunting inhibition (Ehrlich et al., 1999). Throughout the developmental period under consideration, glycine receptors on LSO neurons are coupled to Cl^- channels. However, at perinatal ages, an active Cl^- regulation mechanism maintains a high intracellular Cl^- concentration, and a relatively positive Cl^- equilibrium potential, which leads to the depolarizing glycine-evoked responses (Kakazu et al., 1999; Ehrlich et al., 1999). Thus, at early ages, both afferent pathways to the LSO elicit an excitatory response at the target neurons. It is conceivable that the action of the two components is synergistic, i.e. that they achieve an effect in LSO neurons of which each is individually incapable. At present, this has not been demonstrated,
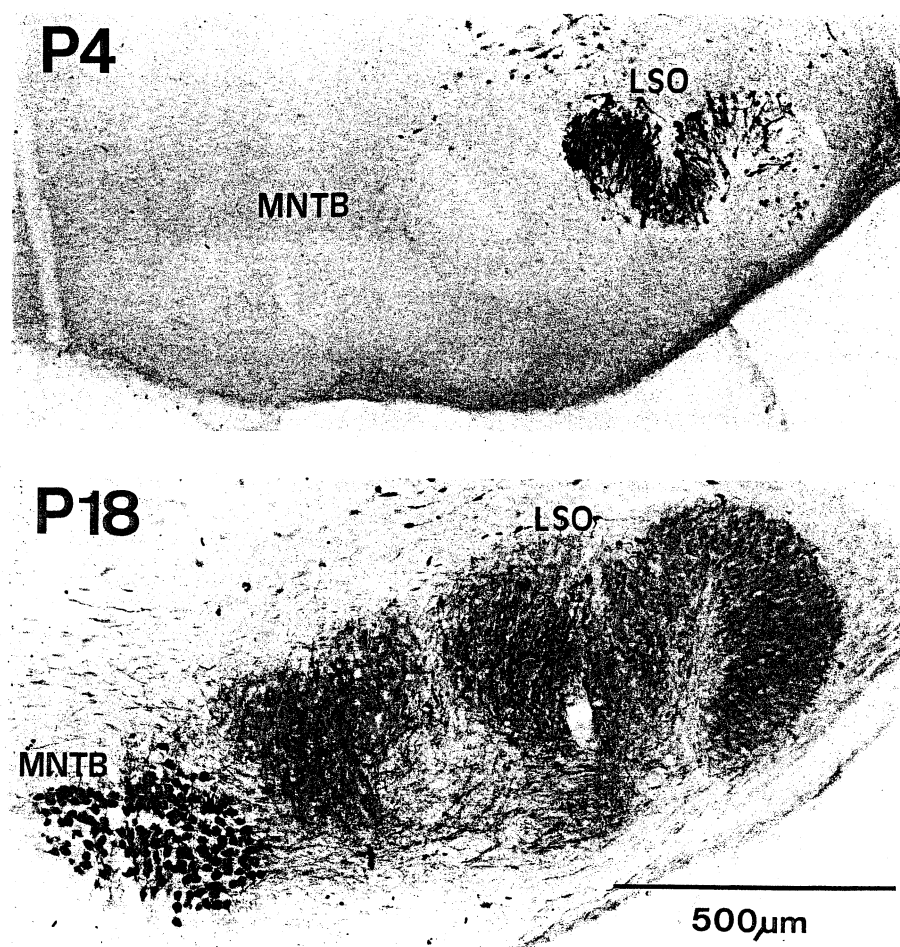
Fig. 3. Development of immunoreactivity to the calcium-binding molecule calbindin-D28k. At P4, rat LSO neurons display a strong cytoplasmic staining, whereas other nuclei in the superior olivary complex are virtually unstained. By P18, calbindin-D28k is strongly expressed in MNTB neurons, but no longer in the majority of LSO neurons (labeling in the LSO is in the neuropil and most probably located in axon terminals of MNTB neurons). (Modified from Friauf, 1993.)

but the observation that glycine and GABA induce a Ca^{2+} influx and an increase of the intracellular Ca^{2+} concentration in LSO neurons of neonatal rats and mice (Kullmann and Kandler, 1999) suggests that the 'inhibitory' inputs to the LSO per se may affect similar, Ca^{2+}-dependent signal transduction cascades during the depolarizing phase of glycine as those later activated by glutamatergic synapses (Johnston et al., 1992; Malenka and Nicoll, 1993).

Our present thinking is that the intracellular Ca^{2+} increase activates a mechanism that is involved in the activity-dependent maturation of the LSO circuit, particularly in the growth of dendritic arbors and/or the strengthening of synaptic connections. Ca^{2+} buffering in LSO neurons appears to be important during this period, as indicated by the transient expression of calbindin-D28k. The expression of this calcium-binding protein is strong during the first postnatal week in rats (Fig. 3, top; Friauf, 1993), i.e. exactly during the time of glycine- and GABA-induced Ca^{2+} influx.

Around P8, when glycine-induced responses convert into hyperpolarization, calbindin-D28k immunoreactivity begins to decline and decays rapidly between P10 and P18 (Fig. 3, bottom), virtually disappearing in LSO neurons of adult animals. A transient expression with a similar time course in the LSO is also observed with calretinin, another calcium-binding protein (Lohmann and Friauf, 1996).

The importance of Ca^{2+} influx and an optimal intracellular Ca^{2+} concentration range during the formation of the neonatal LSO circuit has been directly demonstrated in organotypic slice culture experiments (Fig. 4). Such experiments revealed that an activity-dependent Ca^{2+} influx through voltage-operated L-type Ca^{2+} channels is necessary for the cytoarchitecture of the LSO, the neuronal morphology, the specific topography of the MNTB–LSO projection, and the electrical membrane properties of MNTB neurons (Lohmann et al., 1998; Löhrke et al., 1998). The integrity of the circuit was obtained by K^+-induced depolarization (25 mM;
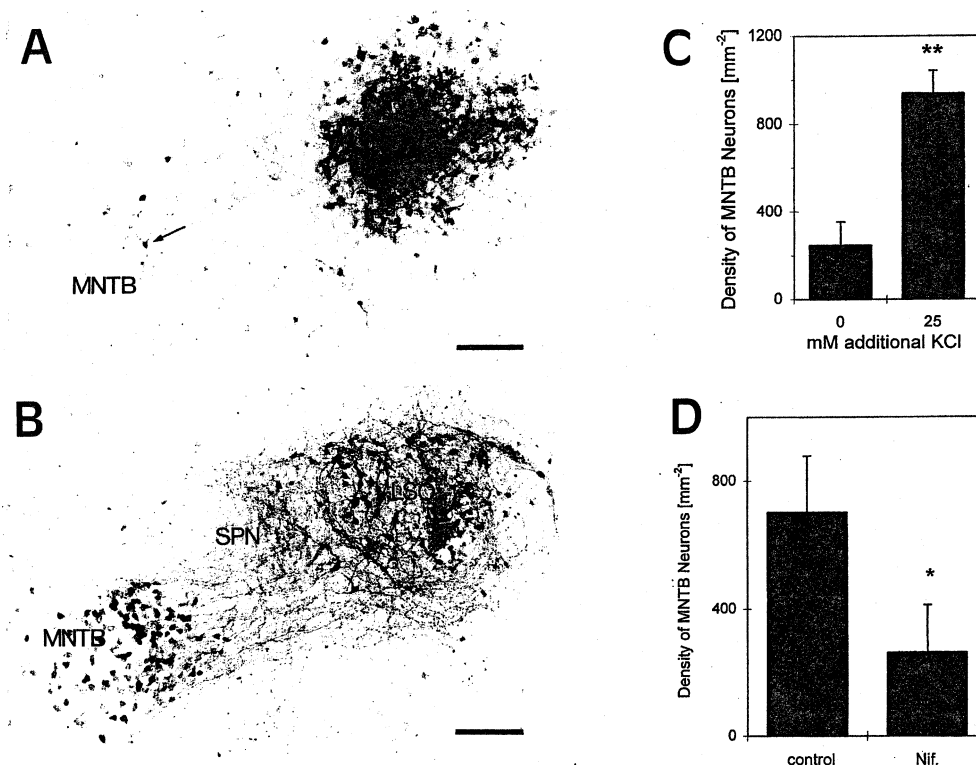
Fig. 4. Effect of depolarizing activity and Ca^{2+} influx on the organotypicity of slice cultures in rats. The MNTB–LSO projection was identified by retrograde transport following biocytin application into the LSO. Panel A shows loss of normal features when cultures were grown in normal serum. Panel B shows presence of normal features when 25 mM KCl was added to the serum in order to depolarize the cells. Panel C shows the beneficial effect of 25 mM KCl on the MNTB–LSO projection (**$P < 0.01$). Panel D shows that the beneficial effect is mediated through voltage-operated L-type Ca^{2+} channels, since it could be blocked by nifedipine (Nif.; *$P < 0.05$). (Modified from Lohmann et al., 1998.)

Lohmann et al., 1998), and very similar effects were achieved when neuronal activity was increased by application of the Na^+ channel agonist veratridine (Sanes and Hafidi, 1996). If the extracellular K^+ concentration was raised above 40 mM, the integrity was no longer obtained, implying that the Ca^{2+} influx needs to be within an optimal range to be beneficial. Recent preliminary data have provided evidence that only during the initial 4 days of incubation of the organotypic cultures, the K^+-induced depolarization is of crucial importance (Piechotta and Friauf, 1999). Since incubation starts at P4, this indicates that Ca^{2+} influx into LSO neurons may no longer be essential at the time when the hyperpolarizing stage has been reached. Collectively, the above results are in line with the idea that the depolarizing stage of glycinergic transmission has a trophic function on LSO neurons.

The depolarizing stage of glycine correlates with the period of dendritic growth in the LSO. In experiments employing intracellular dye injections into LSO neurons of rats between P4 and P36, we found a drastic decline in the number of dendritic endpoints until about P20 (Fig. 5; more than 80% of the endpoints are lost). As will be outlined below, the decline is associated with the

hyperpolarizing stage of glycine, and this period of regression results in a refinement of dendritic trees that is influenced by glycinergic transmission. Experiments in which glycinergic transmission was blocked or interrupted during the depolarizing stage have not been done so far. These experiments will clarify whether depolarizing glycinergic activity enhances dendritic growth, and provide further evidence for a causal relationship between the polarity of glycine-evoked responses and a particular stage of dendritic morphogenesis. It is interesting to note that there is a parallel expansion of the presynaptic MNTB arborizations within the LSO, suggesting that exuberant contacts are formed during the first postnatal week, but are eliminated during a later stage of development (Zook and DiCaprio, 1988; Sanes and Siverls, 1991).

At the molecular level, age-related changes in the glycinergic input to the LSO are also observed. The synaptic machinery involved in glycinergic neurotransmission is modified in several ways. First, the subunit composition of the glycine receptors, pentameric molecules composed of ligand-binding α subunits and assembly-mediating β subunits with a stoichiometry of 3:2 (Langosch et al., 1988), is developmentally regu-
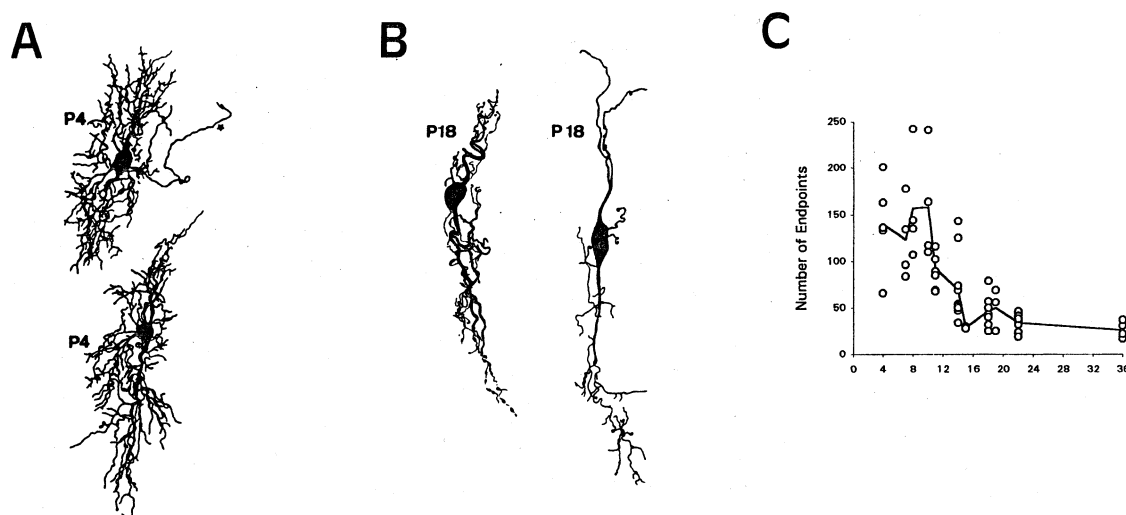
Fig. 5. Development of dendritic morphology in the rat LSO. Representative neurons shown at P4 (A) and P18 (B), demonstrating dramatic changes in the complexity of the dendritic arbors as illustrated by the number of dendritic endpoints. Many more dendritic endpoints are present at P4 than at P18. Panel C shows that the number of dendritic endpoints increases until about P10, after which a rapid decline is observed until about P20. (Modified from Rietzel and Friauf, 1998.)

lated: $\alpha 1$ subunits, whose expression is characteristic for 'adult'-type glycine receptors and a high antagonist (strychnine)-binding affinity, become detectable at P0 in the rat LSO and their expression pattern increases quickly during the first postnatal week (Friauf et al., 1997a). The type of α subunit that is present before birth is not known; in situ hybridization studies have shown that mRNA for $\alpha 2$ subunits, which normally precede $\alpha 1$ subunits in the developing central nervous system and are characterized by a low strychnine sensitivity of only about 25% of the adult isoform (Becker et al., 1988), is expressed at a constantly low level between P0 and P28 in the LSO (Piechotta et al., 1998). Because of the low expression level, it is possible that $\alpha 2$ subunits do not contribute significantly to the receptor stoichiometry and $\alpha 3$ (Kuhse et al., 1990) or $\alpha 4$ (Matzenbach et al., 1994) glycine receptor subunits, usually forming rare glycine receptor isoforms, may instead be components of glycine receptors in the LSO before and shortly after birth. As the open kinetics of glycine receptors are determined by the type of α subunits (Takahashi et al., 1992), the subunit switching is likely to be responsible for developmental changes in glycine receptor function.

A second major change occurs in the expression of GLYT2 glycine transporter molecules, which are involved in the re-uptake of glycine and in the termination of synaptic action (López-Corcuera et al., 1998). These proteins are detectable at P0 and display an increasing immunoreactivity in the LSO between P0 and P10 (Friauf et al., 1999). The GLYT2 molecules appear to be located on axon terminals of MNTB neurons, and it is conceivable that they contribute to the age-related

shortening of glycine-induced responses that is seen in LSO neurons (Sanes, 1993).

Concerning the maturation of excitatory input to the LSO, our knowledge about the early period, which ends after the first postnatal week, is much less than that about the period thereafter. The reason for that is that experimental manipulations have either been performed in animals aged $\geq$P7 or, if earlier, the effects were analyzed at later ages. Because of this, it is not possible to unequivocally attribute the observed effects to the depolarizing stage. The data show that glutamatergic input to the LSO is already functional in fetuses and able to elicit action potentials (Fig. 1C,D). Before and shortly after birth, the glutamatergic excitatory postsynaptic potentials (EPSPs) are still of long duration (>50 ms) and display a shallow rising slope (Sanes, 1993; Kandler and Friauf, 1995a). The glutamatergic EPSPs are mediated through both AMPA/kainate as well as NMDA receptors. While changes in the kinetics and the glutamate receptor isoforms occur during development, it is not yet known whether these changes are specifically associated with the depolarizing stage.

The maturation of the glutamatergic input to the LSO appears to be under the control of intrinsic neuromodulators, such as neuropeptides. Somatostatin immunoreactivity is heavy in ventral cochlear nucleus (VCN) neurons during the first postnatal week in the rat and at the same time, a dense network of somatostatin-positive fibers is present in the LSO and other target nuclei of VCN neurons, indicating that the somatostatinergic innervation of LSO neurons comes from VCN neurons (Kungel and Friauf, 1995). The

peak immunoreactivity is reached at P7, followed by a decline to very low levels in adults. Most likely, somatostatin acts via sst2-type receptors in the LSO, because this receptor type has been demonstrated by receptor binding and in situ hybridization (Thoss et al., 1996) as well as by immunohistochemistry (Friauf and Schindler, 1999). Depletion of somatostatin during neonatal development results in an impaired dendritic morphology of LSO neurons, demonstrating a trophic effect of somatostatin (Kungel et al., 1997). Although the relevance of the coincidence between a high somatostatin level and the depolarizing stage is not known, these results emphasize that synaptic inhibition provides just one of many influences during development. There is yet to be a satisfying model of synaptic influence that incorporates both excitatory and inhibitory transmission, let alone modulatory agents such as somatostatin.

Fig. 7. Development of level difference coding. ILD functions were recorded from single neurons in the gerbil LSO near the onset of sound-evoked responses (P13–16) and in adults. The functions from young animals are shallower and are somewhat irregular in shape. Negative values denote greater intensity to the ipsilateral ear. Adult curves have a much larger dynamic range. (From Sanes and Rubel, 1988.)

3. The hyperpolarizing stage of development

After the first postnatal week, the characteristics of inhibitory synaptic transmission change in two major ways. First, the MNTB- or glycine-evoked responses in LSO neurons become hyperpolarizing (Kandler and Friauf, 1995a). As discussed above, this dramatic change in function is apparently due to the maturation of the chloride homeostatic mechanism (Hablitz et al., 1989; Kakazu et al., 1999). Second, the kinetics become much faster (Sanes, 1993; Kandler and Friauf, 1995a). In the gerbil LSO, the maximum duration of hyperpolarizing inhibitory postsynaptic potentials (IPSPs) decreases from 62 ms in P1–7 neurons to 8 ms after P17 (Sanes, 1993). Fig. 6 shows examples of IPSPs recorded from neurons at five different postnatal ages. The rising slope of IPSPs increases from 6.5 to 9 mV/ms during the same period. These kinetic changes probably derive from several maturational events, including the transition from the 'neonatal' to the 'adult' glycine receptor isoform (Friauf et al., 1997a), the maturation of a glycine uptake mechanism (Friauf et al., 1999), and changes in passive membrane properties (Sanes, 1993; Kandler and Friauf, 1995b).
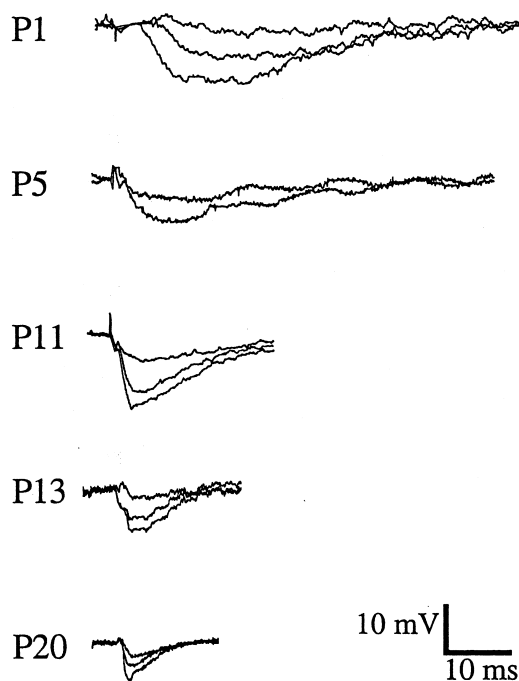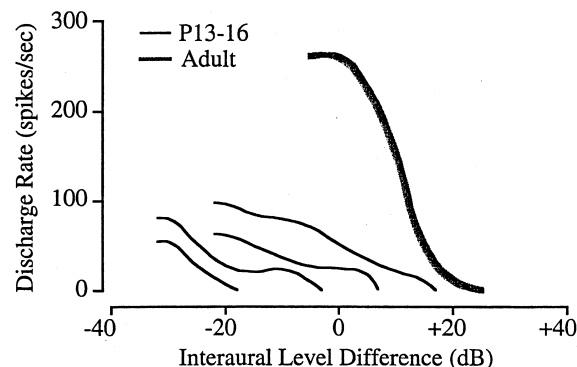

Fig. 6. Development of inhibitory synaptic transmission in gerbils. Representative IPSPs were recorded intracellularly from single LSO neurons at P1 to P20. At P1, the synaptic potentials evoked by a single stimulus pulse to the MNTB (gray arrow) are extremely long-lasting. (Note: These IPSPs are hyperpolarizing whereas those recorded in P1 rats are usually depolarizing. In fact, depolarizing IPSPs are observed in gerbils before P7 (Kotak and Sanes, unpublished observations), and hyperpolarizing IPSPs or glycine-evoked potentials are observed in rat before P7; Ehrlich et al., in press; Kakazu et al., 1999). Electrical stimuli were delivered at increasing voltage, and they evoked IPSPs of increasing size, presumably due to the recruitment of MNTB afferent to the recorded neuron. The duration of synaptic potentials decreases by eight-fold during the first 3 weeks of postnatal development. (From Sanes, 1993.)

More recently, an unexpected transformation in the inhibitory system was discovered in the gerbil LSO. A voltage clamp analysis of MNTB-evoked synaptic currents showed that inhibition is primarily GABAergic during the first postnatal week, and becomes primarily glycinergic by about P14 (Kotak et al., 1998). This phenomenon does not appear to be as profound in the rat, although it has been observed in developing mouse tissue (Kotak and Sanes, unpublished observations). This change is highly significant: the GABAergic, bicuculline-sensitive inhibitory postsynaptic current (IPSC) component decreases from 115 pA at P3–5 to 15
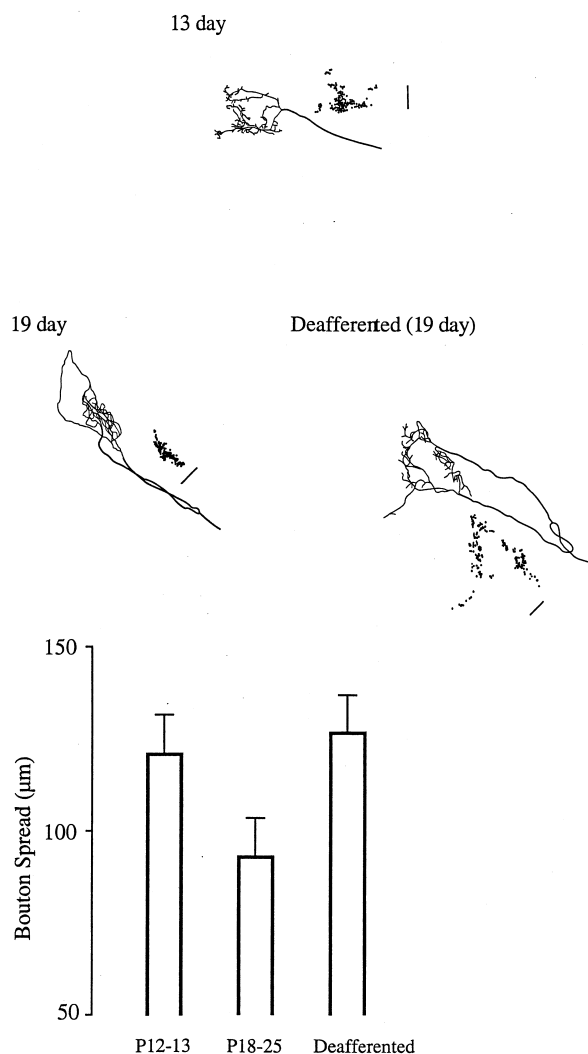
Fig. 8. Development and plasticity of MNTB arbors. Reconstructions of representative HRP-filled MNTB neurons of gerbils are shown near the onset of hearing (P13) and about one week later (P19). The pattern of terminal boutons from each arbor is shown to the right (bar = 100 μm). When the boutonal patterns were analyzed at P12–13 and P18–25, they were found to become more restricted across the LSO tonotopic axis (bottom). Following contralateral cochlea ablation, this refinement failed to occur. A representative MNTB arbor and bouton pattern is shown for a deafferented MNTB fiber (Deafferented). (From Sanes and Takács, 1993.)

pA at P12–16, and there is a complementary increase in the glycinergic, strychnine-sensitive component. Furthermore, this transition is correlated with the expression pattern of gephyrin, a glycine receptor-associated protein, and the β2/3 GABA$_A$ receptor subunit (it should be noted that, in some areas of the brain, gephyrin is associated with GABA receptors; Sassoé-Pognetto et al., 1995; Craig et al., 1996; Giustetto et al., 1998; Essrich et al., 1998). This transition seems to occur primarily in the medial (high frequency) limb of the LSO, the target of the majority of MNTB axons (Sanes and Siverls, 1991). There is an important caveat

to these studies: paired MNTB–LSO recordings have not yet been performed, and it remains possible that an undescribed GABAergic pathway transiently innervates the LSO and somehow suppresses the glycinergic component from the MNTB.

By the time animals experience airborne sound, inhibitory synapses are certainly acting to decrease excitatory-evoked responses. This has been shown by recording ILD function in vivo in animals as young as P13 (Sanes and Rubel, 1988). Ipsilateral stimulation evokes action potentials in single LSO neurons, and contralateral stimulation decreases this discharge rate in an intensity-dependent manner. However, as shown in Fig. 7, the shape of these ILD functions is not as linear in many neurons from young animals. In addition, LSO neurons become more sensitive to intensity differences corresponding to contralateral loci (e.g. contralateral intensity greater than ipsilateral intensity) as maturation progresses. This suggests that the relative strength of contralateral inhibition may be changing during postnatal development. In fact, glycine receptor expression is at first uniform within the gerbil LSO, but assumes an inhomogeneous distribution pattern during the first three postnatal weeks (Sanes and Wooten, 1987). These functional observations indicate that synaptic inhibition may be somewhat dynamic during this early period of development.

Whereas the depolarizing stage of inhibition (above) appears to be associated with the elaboration of pre- and postsynaptic processes, the hyperpolarization stage is correlated with a period of refinement. A comparison between single MNTB arborizations from a P13 and a P19 animal is shown in Fig. 8. In general, the projections from neonatal animals were accurate and occupied a fairly discrete region of the tonotopic axis within the LSO (Sanes and Siverls, 1991). The anatomical specificity of these arborizations along the tonotopic axis was quantified by measuring the distance that the presynaptic boutons (i.e. enlargements of the arbor thought to be the site of synaptic transmission) spread across the frequency axis. This is shown as a pattern of dots next to each arbor in Fig. 8. The boutons become restricted along the tonotopic axis during the third postnatal week, decreasing from 123 μm at P12–13 to 93 μm at P18–25. We have speculated that the less refined arbors affect those auditory percepts involving frequency selectivity.

The arborizations of LSO dendrites also become more restricted during postnatal development (Sanes et al., 1992a; Rietzel and Friauf, 1998). The number of branch points decreases significantly from P12 to P21. In the gerbil, this change is more profound in the high frequency region. Second, the dendritic arbors decrease their arbor span along the frequency axis by about 40–50% during the third postnatal week. Again,
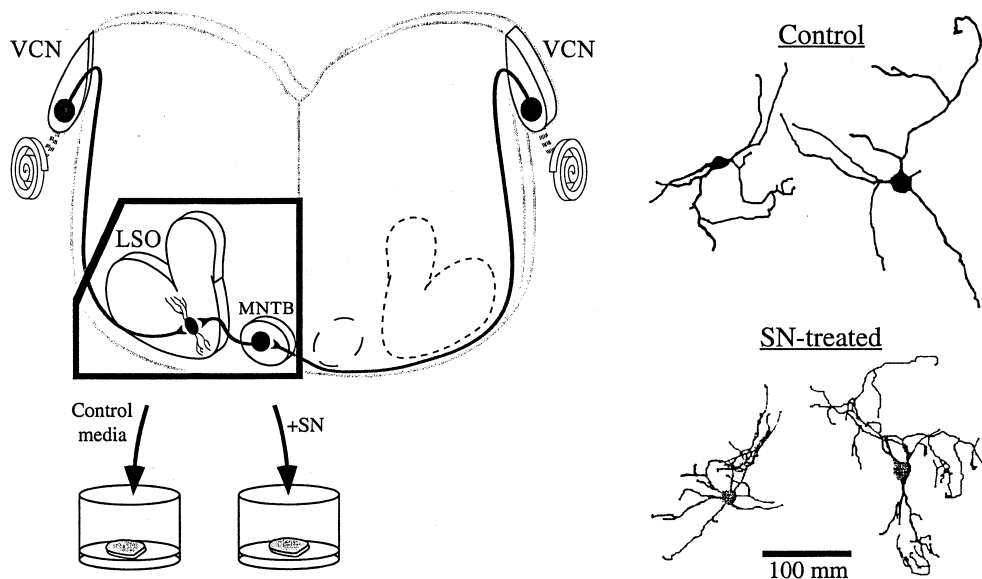
Fig. 9. Effect of glycine receptor blockade in vitro. Organotypic cultures containing the LSO and MNTB were obtained from P6–8 gerbils grown for about 1 week in normal media or SN-containing media (left). LSO neurons were filled with biocytin (right), and it was found that the SN-treated neurons displayed about three times as many branches compared to controls. (From Sanes and Hafidi, 1996).

this event is more obvious in the medial (high frequency) limb of the gerbil LSO. Interestingly, this dendritic refinement is well-correlated with a refinement of frequency tuning in the gerbil (Sanes et al., 1992a). Therefore, both LSO dendritic arborizations and MNTB afferent arborizations undergo a concomitant modification of morphology.

4. Effects of disrupting inhibitory innervation to LSO

A principal reason for choosing the MNTB projection to the LSO as a model for inhibitory development is its unique geometry, which allows for selective inactivation of this pathway. The influence of inhibitory synaptic transmission on the development or maintenance of neural elements was explored with series of experimental manipulations designed to decrease inhibitory transmission within the gerbil LSO during the period when dendrites and axonal arbors are reaching a mature phenotype. In one set of experiments, the right cochlea was surgically removed at P7, leading to the functional deafferentation of afferents from the MNTB to the left LSO (Sanes et al., 1992b; Aponte et al., 1996). A second set of experiments used strychnine-containing continuous release pellets, implanted at P3, to attenuate the level of glycinergic transmission, albeit throughout the nervous system (Sanes and Chokshi, 1992). Both manipulations occur before the onset of sound-evoked activity, and the effect on LSO dendrites is compared with age-matched controls. The effects of both manipulations are nearly identical. LSO dendrites have more branches and are more spread out

along the tonotopic axis of LSO compared to controls. Interestingly, this effect seems to be most prominent in the medial (high frequency) limb of the LSO, the same area that receives the major fraction of MNTB axons and contains the highest density of glycine receptors (Sanes and Wooten, 1987; Sanes and Siverls, 1991).

Both in vivo manipulations produce changes that make interpretation difficult: cochlear ablation induces sprouting in the superior olivary complex (Kitzes et al., 1995; Russell and Moore, 1995), and strychnine treatment blocks glycinergic synapses throughout the nervous system. A more direct test of the idea that inhibitory synapses influence dendrite growth was performed in an organotypic culture of the MNTB and the LSO. As shown in Fig. 9, these cultures were grown in normal media or in strychnine-containing media. Consistent with in vivo observations, strychnine-treated cultures contained LSO dendrites with a significantly greater number of branches compared to controls (Sanes and Hafidi, 1996). Taken together, the in vivo and in vitro findings suggest that spontaneous or evoked inhibitory transmission does influence the normal maturation of dendrite structure within the LSO.

Since MNTB afferent arborizations within the LSO undergo a period of refinement during development (Fig. 8), the effect of deafferentation was assessed (Sanes and Takács, 1993). Following contralateral cochlear ablation at P7, single horseradish peroxidase (HRP)-filled MNTB arborizations fail to attain the normal level of anatomical specificity within the LSO by P19 (Fig. 8). On average, the arbors are found to be more spread out across the tonotopic axis, similar to that found in younger animals. However, the arbors do
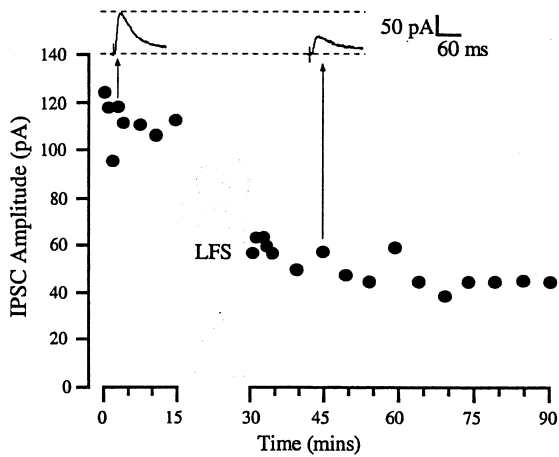
Fig. 10. Long-term inhibitory synaptic depression in the gerbil LSO. A whole cell voltage clamp recording ($V_{hold} = 0$ mV) was obtained in a P10 neuron in the presence of 5 mM kynurenic acid (to block all ionotropic glutamatergic synapses). MNTB-evoked IPSCs were recorded for 15 min to establish a baseline, and 1 Hz stimulation was then delivered to the MNTB pathway for 15 min. The stimulated synapses were depressed by this treatment and remained so for the duration of the recording. Nearly identical observations were made in 21 other LSO neurons using the same paradigm. (From Kotak and Sanes, unpublished observations).

not display signs of atrophy in that the total axonal length and the number of boutons per fiber are similar in control and ablated animals.

Since inhibitory transmission is implicated in the early rearrangement of synaptic contacts, it is possible that inhibitory synapses undergo a change in functional strength that depends on pre- and postsynaptic activity. In a recent set of experiments, inhibitory synapses within the gerbil LSO were found to undergo long-term depression when stimulated at a low frequency while the LSO neuron was depolarized (Kotak and Sanes, 2000). As shown in Fig. 10, the effect of this stimulus protocol is to decrease the size of MNTB-evoked inhibitory postsynaptic currents by about 50%. Similar findings have been obtained in 21 recordings from P8–12 animals. Furthermore, the effect is age-dependent. When recordings are made from P17–19 neurons, the level of depression is only about 20%. These results suggest that inhibitory terminal refinement may be initiated with a mechanism of synaptic depression that leads to the elimination of inappropriate connections.

Decreased inhibition also affects the functional properties of the LSO circuit. Whole-cell recordings were made from LSO neurons, and the stimulus-evoked IPSPs or EPSPs were analyzed following contralateral cochlear ablation or strychnine (SN) rearing (Kotak and Sanes, 1996). The percentage of LSO neurons exhibiting MNTB-evoked IPSPs is reduced significantly in both ablated and SN-treated animals (only one of 11 SN-treated neurons had an MNTB-evoked IPSP). In those neurons that do have MNTB-evoked IPSPs, the amplitude is significantly reduced, and this decrease is accompanied by a 8 mV depolarization in the IPSP reversal potential. More surprisingly, the unmanipulated ipsilateral pathway is altered: ipsilaterally evoked EPSPs are of much longer duration in experimental animals, and these long duration EPSPs are significantly shortened by AP5, an NMDA receptor antagonist. A substantial amount of NMDA receptor mRNA is found in LSO neurons, even in the adult (Sato et al., 1999). Thus, inhibitory transmission may influence the strength of excitatory transmission by regulating the translation of NMDA receptor protein.

There is little indication that the density of glycine receptors in LSO changes following neonatal cochlea ablation, either ipsi- or contralateral to the ablation (Koch and Sanes, 1998). Unilateral cochlear ablations have also been performed in adult animals, and it is interesting to compare these findings to observations made during development. When a single cochlea is ablated in young adult guinea pigs, glycine receptor expression is modified in each LSO, as measured with [3H]strychnine labeling. Receptor expression declines in the ipsilateral LSO, but increases in the contralateral LSO (Suneja et al., 1998).

5. Summary

Studies of developmental plasticity typically address the modifications that occur at excitatory synaptic contacts. This is understandable because much of the early evidence for plasticity derived from single neuron coding properties that were obtained with extracellular recording of action potentials, particularly in the visual pathway. Since action potentials depend absolutely on the presence of excitatory synaptic input, the contribution of inhibitory synapses is less obvious. In addition, the most informative model system has been the mammalian nerve–muscle junction, which is exclusively excitatory, and this permits one to ignore the influence of synaptic inhibition.

We believe that the inhibitory projection from the MNTB to the LSO provides a favorable model for studies of synaptic inhibition for several reasons (see Section 1). The studies from our laboratories have shown that there are major functional and structural changes in this pathway during normal development. MNTB-evoked inhibitory responses are, at first, depolarizing, and gradually become hyperpolarizing in the second postnatal week of development. At the same time, inhibitory synaptic potentials are becoming more rapid, and the postsynaptic glycine receptor is undergoing changes in isoform and distribution. In the gerbil, there is even a profound change in transmitter phenotype, from GABAergic to glycinergic.

The early depolarizing stage of development appears to be closely linked to the growth phase for LSO dendrites and MNTB arbors. We speculate that MNTB-evoked depolarizations lead to postsynaptic Ca^{2+} entry (Kullmann and Kandler, 1999) and this signal supports dendrite outgrowth. In vitro experiments have previously implicated GABAergic signaling in process outgrowth, although it is not certain that the effect arises from depolarization (Michler-Stuke and Wolff, 1987; Spoerri, 1988; Mattson and Kater, 1989; Behar et al., 1996), synaptogenesis (Corner and Ramakers, 1992; Redburn, 1992), or $GABA_A$ receptor expression (Frieder and Grimm, 1985; Hablitz et al., 1989; Montpied et al., 1991; Barbin et al., 1993; Kim et al., 1993; Liu et al., 1997; Poulter et al., 1997).

A second stage of development, during which inhibitory synaptic potentials are hyperpolarizing, is associated with a refinement phase for LSO dendrites and MNTB arbors. Beginning at about P12, dendritic arbors and axonal terminals begin to decrease the number of branches. Although the changes are on the order of tens of micrometers, this refinement leads to a more precise alignment along the LSO tonotopic axis. Experimental work, in which inhibitory activity is decreased during development, generally results in failure of dendrites and axons to complete this anatomical refinement. Decreased inhibitory transmission also leads to profound changes in LSO functional properties. There is an apparent depolarizing shift in the IPSP reversal potential and the maintenance of NMDA receptors.

Having established many of the landmark events during the development of the MNTB–LSO projection, the system should now prove quite useful in discovering the cellular mechanisms that mediate inhibitory synapse development. Our recent research suggests two interesting possibilities. First, the depolarizing stage of development may recruit postsynaptic signaling pathways (e.g. Ca^{2+}-mediated) that have been implicated in excitatory synapse development. It will be interesting to learn whether inhibitory terminals do regulate postsynaptic process outgrowth by depolarizing the postsynaptic membrane. It will also be interesting to learn whether GABA is, in fact, co-released with glycine early in development, and whether GABA acts as a developmental signal. Second, the hyperpolarizing stage of development may contribute to a number of maturational events. Although the signals responsible remain to be elucidated, we have recently found that developing inhibitory synapses undergo a profound activity-dependent depression when stimulated at low frequency levels. Thus, it will be interesting to learn whether this physiological change is responsible for the normal refinement of MNTB terminals.

Our results suggest that inhibitory connections are quite dynamic during development, and that this issue can be pursued successfully in a simple auditory circuit. Since most, if not all, brain circuits receive inhibitory projections, it will be fascinating to learn how these contacts are modified by use, how they interact with excitatory synapses during development, and how their modification affects sensory perception and motor function.

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