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Activity-dependent Refinement of Inhibitory Connections

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Abstract

Several lines of evidence suggest that excitatory synaptic transmission contributes to the maturation of precise neuronal connections. In the present study we determined whether the specific innervation pattern of single inhibitory arborizations was dependent upon neuronal activity during development. A homogeneous group of glycinergic inhibitory neurons in the central auditory system, the medial nucleus of the trapezoid body (MNTB), was functionally denervated in neonatal gerbils. The anatomical specificity of single MNTB terminal arborizations was subsequently measured along the tonotopic axis of a postsynaptic target, the lateral superior olive. Here we demonstrate that inhibitory terminal boutons spread a significantly greater distance along the frequency axis of the postsynaptic target following functional denervation. Although total arbor length remained unchanged, there was a significant increase in the number of branch points, suggesting *de novo* sprouting. The results indicate that normal inhibitory synaptic activity contributes to the developmental refinement of specific neuronal connections.

Introduction

Maintained excitatory synaptic transmission is necessary for the normal maturation of both synaptic strength and specificity in many neuronal systems (Hubel and Wiesel, 1965; Changeux and Danchin, 1976; Hubel *et al.*, 1977; Brown and Ironton, 1977; Sanes and Constantine-Paton, 1985; Innocenti *et al.*, 1985; Dubin *et al.*, 1986; Magchielse and Meeter, 1986; Nelson *et al.*, 1989; Lo and Poo, 1991). Selective manipulations of inhibitory synaptic activity during development have been less common because inhibitory neurons are usually intrinsic to the postsynaptic population (Eccles, 1969). Although it is not known whether developing inhibitory connections make use of an activity-mediated mechanism, they must also attain a high degree of synaptic specificity during the course of maturation.

There is a favourable location in the central auditory system to examine the development of inhibitory connections. Neurons within the lateral superior olive (LSO) are innervated predominantly by a glycinergic projection from the medial nucleus of the trapezoid body (MNTB) which is activated exclusively by the contralateral ear, and an excitatory projection from the ipsilateral cochlear nucleus (Browner and Webster, 1975; Warr, 1982; Moore and Caspary, 1983; Glendenning *et al.*, 1985; Spangler *et al.*, 1985; Cant and Casseday, 1986; Zook and DiCaprio, 1988; Sanes, 1990; Wu and Kelly, 1991). The MNTB boutonal endings are commonly found surrounding LSO neuronal somata (Zook and DiCaprio, 1988; Sanes and Siverls, 1991), consistent with the primary site for symmetric synaptic profiles (Cant, 1984).

Our understanding of the cellular mechanisms underlying the plasticity of excitatory connections has progressed rapidly. For example, when retinal afferent activity is disrupted during development the axonal projections form abnormal or enlarged arborizations in the postsynaptic target (Udin, 1983; Reh and Constantine-Paton, 1985; Stryker and Harris, 1986; Sretavan *et al.*, 1988; Schmidt, 1991). At glutamatergic synapses the mechanism for developmental plasticity probably involves the NMDA receptor (Cline *et al.*, 1987; Kleinschmidt *et al.*, 1987), analogous to a neural model of learning, long-term potentiation (Collingridge *et al.*, 1983; Bekkers and Stevens, 1990). In fact, the elimination of afferent connections that normally occurs during postnatal development also appears to be mediated by an NMDA-dependent mechanism (Rabacchi *et al.*, 1992).

We have previously shown that inhibitory terminal arborizations are refined along the LSO frequency axis during postnatal development (Sanes *et al.*, 1989; Sanes and Siverls, 1991). The distribution of boutonal endings from single complete MNTB arborizations retracted by ~30% during the third postnatal week. Moreover, we have recently demonstrated that inhibitory transmission does influence the maturation of postsynaptic dendritic form (Sanes and Chokshi, 1992; Sanes *et al.*, 1992), suggesting that inhibitory transmission contributes to neuronal development. In the present study we unilaterally ablated a cochlea in gerbils prior to the onset of sound-evoked responses, thus functionally denervating the contralateral MNTB. Our results indicate that individual inhibitory arbors failed to restrict their terminal fields when neural activity was decreased.

Materials and methods

Gerbils (*Meriones unguiculatus*) aged 7 days postnatal were anaesthetized with hypothermia such that respiration and the cardiac cycle ceased and a nociceptive response was absent. The right cochlea was surgically removed and a piece of gel foam inserted in the cochlear cavity, as described previously (Sanes *et al.*, 1992). Animals were allowed to

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recover, returned to the nest and reared for an additional 12–16 days in the same conditions as normal animals.

A brain slice preparation through the ventral auditory brain stem was produced (Sanes, 1990; Sanes and Siverls, 1991) and the MNTB was visualized in the plane of section. Neurons of the MNTB were selectively impaled under visual inspection and horseradish peroxidase (HRP) was iontophoresed (Sanes and Siverls 1991). Following a 3–6 h period to allow for transport, the tissue was fixed, resectioned at 100 μm and processed with diaminobenzidine and $CoCl_2$ (Adams, 1981).

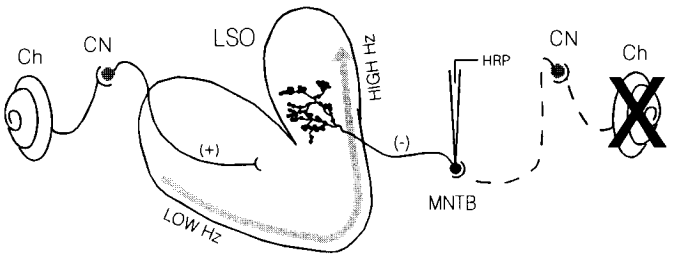

FIG. 1. A schematic of the experimental preparation showing that the LSO is innervated by excitatory afferents driven by the ipsilateral cochlea and by inhibitory afferents (MNTB) driven by the contralateral cochlea. Therefore, contralateral cochlear ablation effectively eliminates driven inhibitory transmission within the LSO. Single MNTB neurons are filled selectively with HRP and the specificity of their arborization is assessed by the distance that they spread along the tonotopic axis, which runs in the transverse plane of section, indicated by the shaded arrow within the LSO (Sanes *et al.*, 1989). Ch = cochlea; CN = cochlear nucleus; LSO = lateral superior olive; MNTB = medial nucleus of the trapezoid body.

Only complete MNTB terminal arborizations within the LSO, without cut ends or beading, were used in this study. Arbors were visualized on a video monitor (Zeiss Standard 16; Nikon 60× PlanApo; N.A. = 1.4; Dage 68) and traced in three dimensions with a mouse-driven cursor using the Cellmate (Biomate Inc.) data acquisition system (Sanes and Siverls, 1991).

The analysis software generated values for the total arbor length, the number of branch points, the number of boutonal endings and the cross-sectional area of the LSO. The distance that boutons spread across the tonotopic axis was measured from calibrated two-dimensional plots (e.g. transverse orientation) of the boutonal pattern within the LSO nucleus boarders. The analysis of arbor width was performed with reference to the LSO frequency axis (Sanes *et al.*, 1989).

An analysis of variance was first performed with group (e.g. normal, denervated and immature) and topographic position (e.g. high frequency or low frequency region) as categorical variables to determine whether

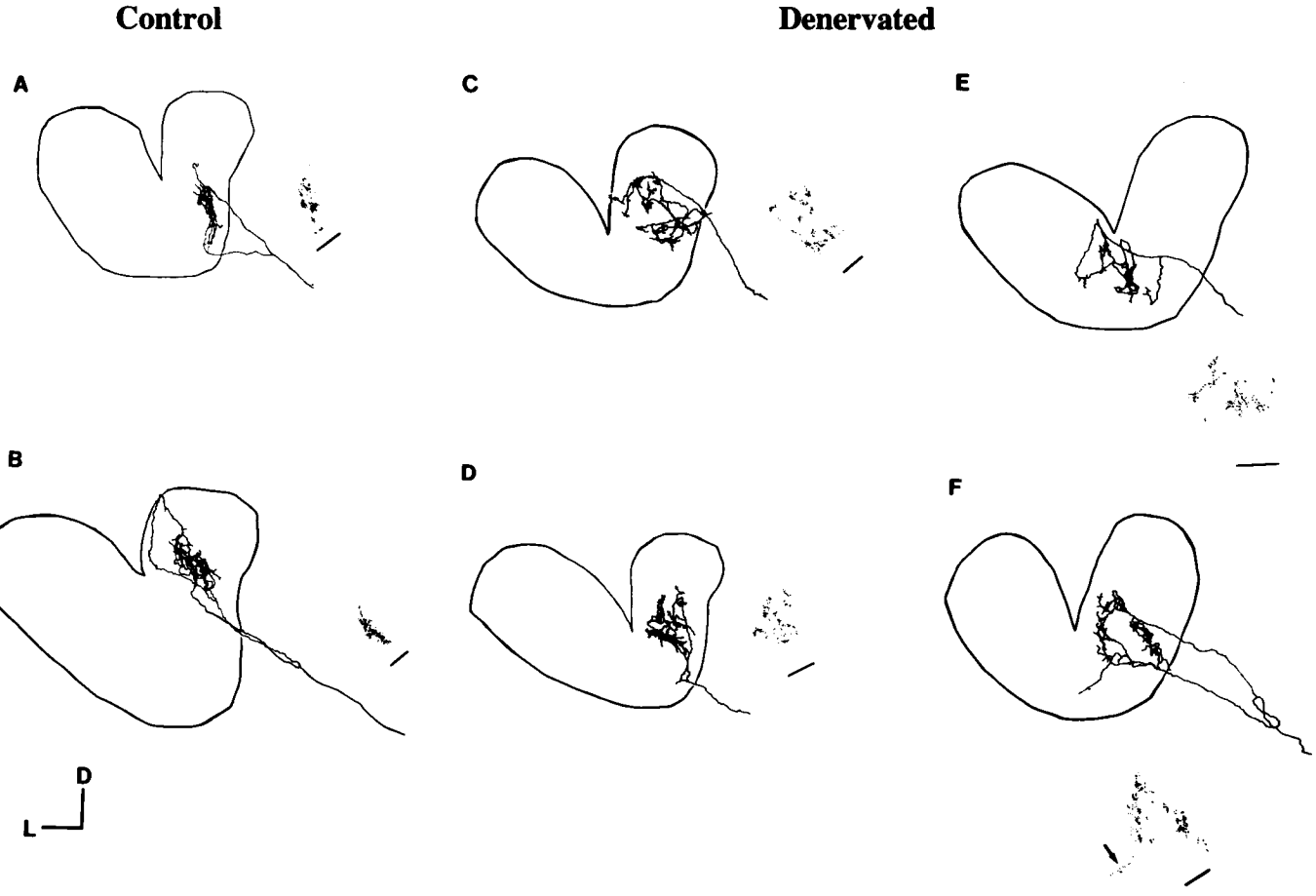

FIG. 2. Representative MNTB arborizations and boutonal distributions within the LSO of control (a and b) and denervated (c–f) animals. The control arbors (a and b) form extremely narrow termination regions within the LSO, presumably along isofrequency contours [see Sanes and Siverls (1991) for additional examples from control animals]. In both cases, this circumscribed terminal region was established from two converging afferent branches. The experimental arbors (c–f) were spread out much more along the tonotopic axis of the LSO. Terminal bouton patterns also were more diffuse, significantly obscuring an isofrequency contour. When the boutonal spread for each arbor was measured, we did not include small patches of boutons which would have increased the values considerably (f, arrow).

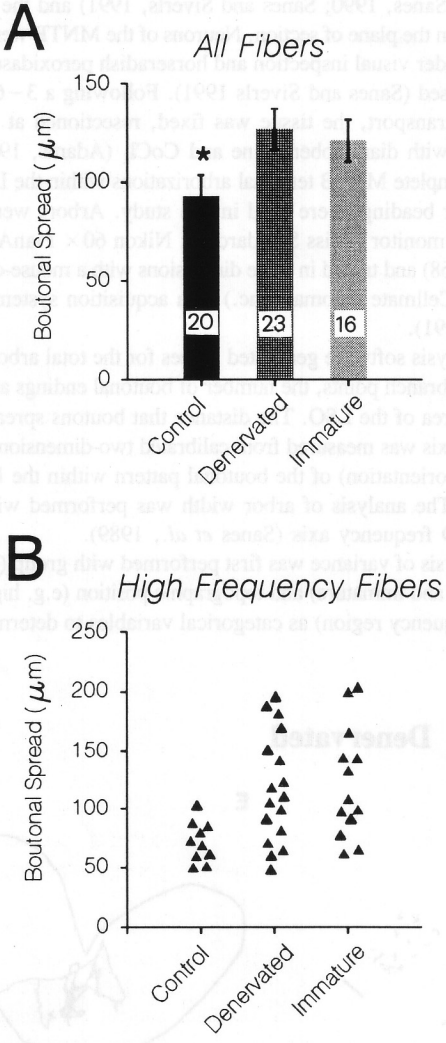

FIG. 3. The distance that inhibitory terminal boutons spread along the tonotopic axis of the LSO was measured (denervated) and compared with previously generated data from normal age-matched animals (control) and from normal animals at the time of ear canal opening (immature) (Sanes and Siverls, 1991). (A) Statistical analysis of all fibres revealed that boutons were significantly more spread in denervated animals than in controls. No significant difference existed between fibres from denervated and immature animals. The values are given as the mean and standard error, and the number of fibres is denoted within each bar. (* = significant at $P < 0.05$.) (B) When only high frequency fibres were considered (i.e. those innervating the region >50% from the apical-most projection region), the difference between denervated and control fibres was even greater (see text for statistical analyses). The scatter plot indicates that the bouton spread was nearly identical in denervated and immature animals.

or not there was a main effect. Only the subsequent pairwise comparisons are shown below.

Results

Contralateral cochlear ablation results in the functional denervation of the contralateral neurons (Fig. 1). Therefore, inhibitory transmission is selectively disrupted. As shown in Figure 2, MNTB arbors from denervated animals formed diffuse projections in the LSO compared with arbors from control animals. The boutonal distributions, shown adjacent to each arbor, also were more spread out in denervated animals than in controls. In normal MNTB projections the terminal boutons always formed compact, elongated patterns that were well-oriented along the presumptive isofrequency contours of the LSO (Fig. 2A and B). These isofrequency contours are perpendicular to the LSO tonotopic axis schematized in Figure 1 (Sanes *et al.*, 1989; Sanes and Siverls, 1991). In experimental MNTB arbors it was often difficult to discern any orientation (see Fig. 2C–F), and some of these arbors appeared to innervate a larger area of the LSO than controls.

The spread of MNTB terminal boutons along the LSO tonotopic axis was quantified and compared with previously published values from normal animals of the same age and with immature animals 12–13 days postnatal (Sanes and Siverls, 1991). For experimental arbors, our measure was intended to yield a conservative estimate of anatomical specificity because: (i) the smallest dimension across the bouton distribution of denervated fibres was used regardless of the known orientation of the isofrequency contours; and (ii) very small patches of boutons that were found at some distance from the primary zone of innervation were not included in the measure of width (see arrow in Fig. 2F). As shown in Figure 3(A), the MNTB arbor boutons following contralateral cochlear ablation were significantly more spread out than controls (127 ± 10 μm versus 93 ± 11 μm; x ± SEM; $t = 2.26$; df = 41; $P < 0.05$). In fact, the spread of denervated fibres was nearly identical to that of 12–13 day animals (Sanes and Siverls, 1991), at an age when sound first evokes a neural response.

Since previous studies have shown that there is a 4-fold greater density of glycine receptors in the high frequency limb of the gerbil LSO than in the low frequency limb (Sanes and Wooten, 1987; Sanes *et al.*, 1987), and that MNTB arbors within the high frequency region are normally more refined (Sanes and Siverls 1991), we examined high frequency arbors separately. As shown in Figure 3(B), the difference between denervated and control arbors in the high frequency region was even more pronounced (119 ± 11 versus 72 ± 5; $t = 3.05$; df = 25; $P < 0.005$). The scatter plot in Figure 3(B) again illustrates the similarity between denervated and immature arbor dimensions.

Three additional measures of MNTB arbor morphology revealed that they did not show signs of degeneration, even though the manipulation caused a functional denervation of their cell bodies. Figure 4(A) illustrates that total axonal length within the LSO borders did not differ between denervated and control groups (2246 ± 199 μm versus 2382 ± 242 μm; $t = 0.43$; df = 42; $P > 0.05$). As shown in Figure 4(B), the number of boutons per fibre did not differ either (151 ± 13 versus 158 ± 13; $t = 0.38$; df = 41; $P > 0.05$). The maximum cross-sectional area of the LSO was not significantly different between experimental and control animals (298 688 ± 8460 μm^2 versus 322 877 ± 10 281 μm^2; $t = 1.8$; df = 25; $P > 0.05$).

Figure 4(C) shows that the number of branch points per fibre, however, was significantly greater in the experimental group than in the control group (48 ± 7 versus 26 ± 3; $t = 3.13$; df = 42; $P < 0.005$). The mean experimental value also exceeded the number of branch points found for immature arbors (30 ± 4; $t = 2.27$; df = 41; $P < 0.05$) at 12–13 days postnatal (Sanes and Siverls, 1991).

Discussion

The present results demonstrate that single inhibitory arborizations failed to refine their tonotopic projection when normal activity was disrupted by contralateral cochlear ablation (Fig. 1). We have recently shown that inhibitory transmission also influences the development of LSO dendrites. Either contralateral cochlear ablation or sublethal doses of strychnine result in a hypertrophic response during development (Sanes and Chokshi, 1992; Sanes *et al.*, 1992). Therefore, glycinergic transmission may subserve more than one role during neural development.

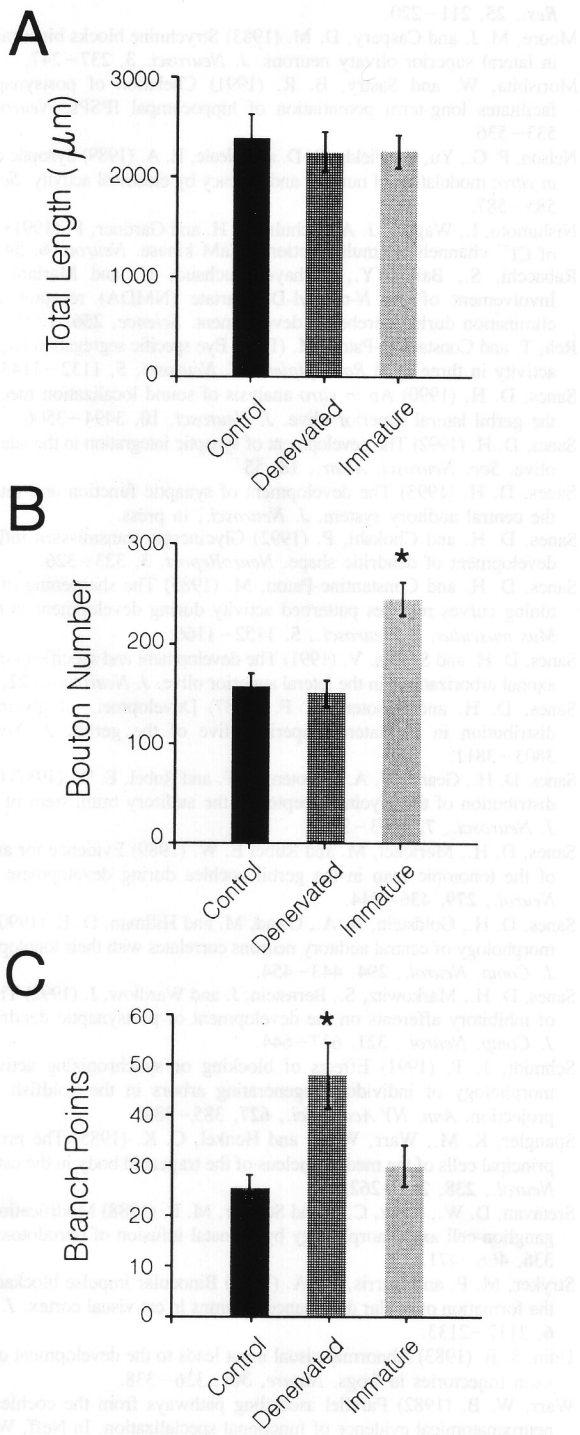

FIG. 4. The mean MNTB terminal arbor length, bouton number and number of branch points were measured (denervated) and compared with previously generated data from normal age-matched animals (control) and from normal animals at the time of ear canal opening (immature) (Sanes and Siverls, 1991). (A) There was no significant difference in terminal length between the three groups. (B) The experimental arbors obtained the control number of terminal boutons, which was significantly less than in the immature group. (C) The denervated arbors had a significantly greater number of branch points than those in either the control or the immature group. The values are given as the mean and standard error (* = significant at $P < 0.05$).

It is possible that functional denervation prevents the MNTB arbors from refining their terminal fields, as normally occurs between 12 and 25 days postnatal (Sanes and Siverls, 1991). This would be consistent with the finding that the boutonal spread of experimental arbors was identical to that observed at 12−13 days postnatal (Fig. 3). However, the decline in bouton number that is normally seen during development (Sanes and Siverls, 1991) was also observed for experimental fibres (Fig. 4B). In addition, there was a significantly greater number of branch points in experimental versus immature arbors, indicating that new processes could be added following functional denervation (Fig. 4C). It is possible to generate more widely dispersed boutons and a greater number of branch points without resorting to an increase in total arbor length. If the branch angle is increased for bouton-bearing daughter branches, then spread of boutons also increases. Furthermore, if a process re-branches but retains the length of the original daughter branch, then total arbor length will not necessarily increase. Therefore, it is likely that attenuated inhibitory synaptic transmission permitted dynamic changes in the MNTB arborization but interfered with the mechanism(s) responsible for a precise innervation pattern.

The effect of functional denervation was more conspicuous when only the MNTB fibres projecting to the high frequency region of the LSO (e.g. medial limb) was considered. We have previously shown that this region of the gerbil LSO receives the majority of the glycinergic innervation from the MNTB (Sanes and Wooten, 1987; Sanes and Siverls, 1991). The trophic influence of inhibitory transmission is also more pronounced for neurons in the high frequency region of the LSO (Sanes and Chokshi, 1992; Sanes *et al.*, 1992). Nevertheless, it is apparent that bouton width values from denervated arbors overlapped those of control arbors. It should be noted that the present manipulation is not as severe as tetradotoxin-rearing in that spontaneous synaptic transmission may continue.

In the present experiment the expanded inhibitory arborizations could also have resulted from a decrease in competing MNTB terminal arbors. It is known that unilateral ablation of a cochlea in gerbils leads to cell loss in the ipsilateral ventral cochlear nucleus (Hashisaki and Rubel, 1989), and unilateral atresia in mice leads to a decrease in contralateral MNTB soma size (but not LSO soma size) (Webster, 1983). However, there was no increase in total MNTB arbor length following functional denervation, as would be expected if compensatory sprouting had occurred.

Our results do not reveal a cellular mechanism for the activity-mediated refinement of inhibitory arbors. We recently have shown, however, that electrical stimulation of the MNTB in neonatal animals yields large and prolonged IPSPs in LSO neurons which themselves can elicit a rebound depolarization or action potential (Sanes, 1992, 1993). This suggests that inhibitory synapses may take advantage of ionic mechanisms, such as Ca^{2+} entry (Llano *et al.*, 1991; Morishita and Sastry, 1991; Nishimoto *et al.*, 1991; Kano *et al.*, 1992), normally associated with excitatory synaptic plasticity. Consistent with this notion is the recent observation that glycinergic synapses in the vertebrate brain stem exhibit long-term potentiation (Korn *et al.*, 1992).

The present study suggests that the aberrant sensory coding properties which often result from periods of decreased or abnormal neural activity during development should be interpreted as arising from changes in both excitatory and inhibitory connections. We have not ruled out the possibility that functional modifications at inhibitory synapses could also arise from such manipulations, independent of the anatomical consequences that we have observed.

Acknowledgements

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Abbreviations

HRP	horseradish peroxidase
LSO	lateral superior olive
MNTB	medial nucleus of the trapezoid body

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