Primary Afferent Plasticity following Partial Denervation of the Trigeminal Brainstem Nuclear Complex in the Postnatal Rat

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Although interaxonal competition is believed to be an essential component of the normal development of numerous mammalian neuronal populations, there is considerable debate regarding the role of competition in the development and maintenance of the somatic sensory system. The results of recent investigations suggest that trigeminal primary afferents may compete for target territory in the brainstem, but it is unclear whether these interactions continue after birth. The present study explored this important issue by examining the response of individual trigeminal primary afferent neurons to partial denervation of the trigeminal brainstem nuclear complex at early postnatal ages. We utilized intracellular recording and HRP injection techniques to label primary afferent central terminal arbors in rats that sustained electrocautery of mystacial vibrissae in rows A, C, and E on the day of birth. A total of 42 low-threshold trigeminal primary afferent neurons were labeled in subnucleus interpolaris. Twenty-eight of these afferents supplied undamaged B or D row vibrissae while 14 supplied lesioned vibrissae. Qualitative and quantitative analyses revealed that the arbors associated with undamaged afferents were enlarged (mean arbor area of $13512 \pm 790.67 \mu\text{m}^2$ vs normal area of $6130 \pm 214 \mu\text{m}^2$) and were oriented toward the adjacent (partially denervated) territory. There was no significant change in the size of the lesioned afferent arbor area. The perimeter of the lesioned afferent arbors was increased, however, suggesting that the arbor shape had changed. This was confirmed with a form factor calculation that indicated that the circularity of the arbors associated with lesioned vibrissae was significantly reduced. Thus, while the arbors of undamaged afferents were enlarged and oriented in the direction of the partially denervated territory, the lesioned afferent arbors were not enlarged but assumed a flattened/elongate morphology within their appropriate row. The lesion-induced increase in the size of the undamaged afferent arbors was not associated with an increase in the number of bouton-like fiber swellings. The density of boutons was only 25% the value seen in normal animals. Thus, while the area supplied by the undamaged afferent arbors increased, there was

no evidence that the absolute amount of terminal arbor was similarly increased (as would be the case if sprouting had occurred; see Renehan et al., 1989). We would therefore conclude that the undamaged afferents had undergone arbor expansion, but not sprouting. These data are consistent with prior suggestions that trigeminal primary afferents utilize some form of competitive interaction(s) to establish their final form and disposition. This competition would appear to continue into early postnatal periods. It appears, however, that undamaged trigeminal primary afferents are most likely to expand their central terminal arbors into adjacent partially denervated territory if the denervation is extensive and includes more than one row of mystacial vibrissae.

[Key words: development, competition, somatosensory cortex, sensory system, intracellular recording, reorganization]

There is a large body of evidence to support the contention that the formation of neuronal pathways, including those pathways associated with sensory systems, is dependent on both genetic and epigenetic processes (for reviews, see Dodd and Jessell, 1988; Jacobson, 1991). The initial stages of axon guidance are most likely under genetic control. The final disposition of axons, however, seems to be sensitive to environmental influences. Once vertebrate axons arrive in the vicinity of their final targets, they often form highly ordered projections within the target field. The mechanisms responsible for the development and maintenance of these patterned projections are not understood. Evidence obtained in the visual system indicates that pattern formation is preceded by a period of axon arbor proliferation (Ferster and LeVay, 1978; Stretavan and Shatz, 1986; Naegle et al., 1988; Callaway and Katz, 1990, 1991; Jhaveri et al., 1991; O'Leary, 1992), with the proliferating axons emitting collateral sprouts that may be widely distributed throughout the terminal region. Recent data suggest that many of these sprouts synapse on neurons in "inappropriate" territory (Campbell and Shatz, 1992). Under normal circumstances, however, these widespread projections are not maintained. Driven by an unknown signal (or multiple signals), the axons undergo a period of collateral elimination (see O'Leary, 1992, for review). Thus, the incorrect collaterals are "pruned" from the axon arbor, resulting in the appropriate patterned projection. What causes this selective elimination of axon terminals? Although a number of mechanisms have been proposed to account for the selective maintenance of a given population of axons (for review, see Fraser, 1989; Fraser and Perkel, 1990), it appears likely that some form of competitive interaction(s) is fundamental to this process.

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As discussed by Chiaia et al. (1992), interaxonal competition has been implicated in the development of numerous neuronal systems (for additional discussion, see Cotman et al., 1981; Jacobson, 1991). There is considerable debate regarding the role of competition in the development and maintenance of the somatic sensory system, however. Investigators have typically examined competitive interactions by removing one or more inputs to a given target, allowing the remaining input(s) to have a competitive advantage. Liu and Chambers pioneered this work by partially denervating the cat spinal cord (Liu and Chambers, 1958). They found that the distribution of the intraspinal dorsal root processes of an intervening (undamaged) dorsal root was markedly increased following this partial denervation paradigm. The authors concluded that this increase was the result of the outgrowth of new processes from the spared dorsal root axons (axonal sprouting). This work established axonal sprouting as a phenomenon relevant to plasticity in the CNS and suggested that the central processes of primary afferent neurons compete with each other for target territory. These observations have since been expanded by a number of groups, and there is now substantial evidence indicating that partial denervation of the spinal cord via the spared root preparation results in an increase in the density of the projection of the spared root in the segment of entry and adjacent segments (Goldberger and Murray, 1982; Strominger and Woolsey, 1987; McNeill et al., 1991; Besse et al., 1992; but see Devor and Claman, 1980; Rodin et al., 1983; Rodin and Kruger, 1984), an increase in the number of unmyelinated axons in the spared root (Hulsebosch and Coggeshall, 1981, 1983; Devor, 1983), and sprouting-related reactive reinnervation (Murray and Goldberger, 1986; Polistina et al., 1990).

The spinal cord can also be partially denervated by transecting a peripheral nerve in early postnatal animals. This manipulation results in a 30–50% reduction in the number of primary afferent neurons (Aldskogius et al., 1985; Cadusseau and Roger, 1985). Fitzgerald and colleagues have shown that the central terminals of neighboring intact primary afferents respond to this type of injury by sprouting into the denervated spinal cord territory (Fitzgerald, 1985; Fitzgerald and Vrbova, 1985; Fitzgerald et al., 1990). These data would seem to lend further support to the postulate that the central projections of somatic primary afferent neurons compete for target territory in the CNS. It is unclear whether this important assumption can be extended to include *brainstem* primary afferents, however. Unfortunately, the extant literature is complicated and at times contradictory. Investigators have employed two principal paradigms to eliminate a subpopulation of primary afferents and produce partial brainstem denervation. Some groups have transected the infra-orbital (IO) nerve, the sole afferent innervation of the mystacial vibrissae in the rodent, while others have damaged a subset of the mystacial vibrissae (leaving adjacent vibrissae intact). In each case, the studies used physiological and/or anatomical techniques to search for evidence that the central arbors of undamaged afferents had expanded into the denervated territory. Killackey and collaborators have shown that neonatal IO nerve transection results in a sizable deafferentation of the trigeminal brainstem nuclei (Killackey and Shinder, 1981; Erzurumlu and Killackey, 1983). Jacquin and Rhoades employed this paradigm to examine the central projections of undamaged trigeminal primary afferents in adult rats and hamsters (Jacquin and Rhoades, 1985). Using a variety of transganglionic and anterograde HRP labeling techniques, these investigators found no

evidence of abnormal projections from mandibular or undamaged ophthalmic-maxillary fibers in the rat. In the hamster, however, neonatal IO lesions were associated with substantial increases in mandibular terminal fields in the trigeminal brainstem nuclear complex. Jacquin and Rhoades suggested that the relative immaturity of the hamster trigeminal brainstem complex at birth might be linked to a greater growth potential of the undamaged primary afferents. Waite and Permentier (1991) and Jacquin et al. (1993) have recently employed the IO transection model to reexamine the interaction between the central terminal arbors of rat trigeminal primary afferents. These studies focused on the central projections of primary afferent neurons that innervate the postero-orbital and supraorbital sinus hairs. The postero-orbital follicle is supplied by the zygomaticofacial nerve, a branch of the maxillary division. The supraorbital vibrissa is supplied by a branch of the ophthalmic division. Waite and Permentier (1991) found that the terminal area corresponding to the central terminations of the postero-orbital afferents was approximately doubled in three of the four trigeminal brainstem subnuclei following the IO lesion in neonates (a smaller but significant increase was also noted when the lesion was performed on postnatal day 7). Jacquin et al. (1993) were able to show that the neonatal IO injury also results in an increase in the size of the cytochrome oxidase (CO) patches corresponding to the postero-orbital and supraorbital vibrissae. These data suggest that undamaged trigeminal primary afferents may indeed be capable of sprouting into denervated territory under certain circumstances. It is possible that interaxonal competition is most evident when the primary afferents are derived from the same division of the trigeminal nerve (in this case the maxillary). If so, selective vibrissa electrocautery experiments, which damage subsets of IO primary afferents (and result in the death of up to 62% of the injured neurons; Waite and Cragg, 1979), would seem a potentially fruitful model for examining axonal competition, plasticity, and sprouting. To examine this important issue with confidence, however, it is necessary to examine the response of *individual* neurons to the manipulation. This study, therefore, has employed the intracellular recording and labeling technique to investigate the response of individual trigeminal primary afferents to a partial denervation of the trigeminal brainstem nuclear complex.

Materials and Methods

All aspects of this study were reviewed and approved by the Institutional Animal Care and Use Committees of the Henry Ford Health Sciences Institute and the St. Louis University School of Medicine.

Neonatal lesions. Forty-two newborn Sprague–Dawley rats were anesthetized by hypothermia until totally unresponsive to noxious stimuli. The vibrissae in rows A (most dorsal row), C (middle), and E (most ventral) of the left and right mystacial pad were cauterized and removed using methods described by Woolsey and Wann (1976). Animals recovered without signs of distress and were able to feed immediately. The lesioned rats were allowed to survive for at least 60 d prior to use in the recording experiments.

Neurophysiology. The techniques used for neuronal recording and axon labeling have been described previously (Jacquin et al., 1986b; Renehan et al., 1989). Briefly, rats were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, i.p.) and administered atropine sulfate (1–2 mg/kg, i.p.). The animals were then tracheotomized, paralyzed with gallamine triethiodide (Flaxedil, 40 mg/kg, i.p.), ventilated with room air, and placed in a stereotaxic headholder. Anesthesia was maintained with regular supplemental doses of sodium pentobarbital. Heart rate and body temperature were monitored continuously. Stimulating electrodes were placed in the ophthalmic-maxillary portion of both trigeminal ganglia.

Beveled glass microelectrodes (tip diameter of 0.5–1.0 μm , resistance

of 70–120 M Ω) filled with 6.0% HRP in 0.05 M Tris and 0.3 M KCl (pH 8.3) were lowered into the left or right trigeminal spinal tract and advanced at 1.0 μ m intervals until a suitable unit was encountered. The micropipettes were connected to a Eutectics 400B intracellular pre-amplifier, the output of which was displayed and documented using conventional methods. Search stimuli were monophasic, 50 msec, rectangular 1.0–1.5 mA pulses delivered at 1 Hz. Trigeminal primary afferents were identified and characterized as described previously (Jacquin et al., 1986a,b).

Primary afferents were physiologically characterized while the electrode was extracellular, and then impaled. Entry into the cell was accompanied by a sudden increase in action potential amplitude and a drop in the resting membrane potential. The response properties of the neurons were quickly reassessed to ensure that the desired axon had been impaled. If a stable membrane shift of at least 15 mV was maintained, HRP was injected by passing 5–10 nA, 250 msec positive current pulses at 2 Hz for 5–10 min. Injection of HRP was discontinued when the peripheral evoked responses were no longer observed or if the resting potential rose.

In most cases, a maximum of two primary afferents were labeled on each side of the brainstem. In a limited number of cases we purposely labeled multiple physiologically characterized afferents to better demonstrate expansion of undamaged primary afferent central terminal arbors (see Results and Figs. 7, 8).

Histochemistry. Animals were given a lethal dose of sodium pentobarbital at the conclusion of the experiment and perfused through the heart with a 0.1 M sodium phosphate buffer (pH 7.3) rinse containing 0.9% NaCl, 40 mg/liter xylocaine, and 20,000 U/liter heparin, followed by 1 liter of fixative containing 2.5% glutaraldehyde, 1.0% paraformaldehyde, and 4.0% sucrose in 0.1 M phosphate buffer (4°C, pH 7.3). The brainstem and spinal cord were stored overnight in 0.1 M phosphate buffer containing 4.0% sucrose. A modified version of Graham and Karnovsky's and Adam's cobalt-intensified diaminobenzidine (DAB) reaction was used to visualize HRP reaction product (Graham and Karnovsky, 1966; Adams, 1977, 1981) in 100- μ m-thick coronal sections.

The extent of the vibrissa lesions was verified using two methods: (1) cytochrome oxidase (CO) histochemistry of the barrelfield cortex and (2) determination of the number of axons supplying the cauterized and adjacent nonlesioned vibrissae. CO staining was performed using a modification of the method described by Wong-Riley (1979). Tangential 50 μ m sections of the cortex were collected in sodium phosphate buffer. Sections were incubated in 0.05 M Tris buffer for 5 min, 0.5% CoCl₂ in Tris buffer for 10 min, and then transferred to a cytochrome solution containing 13.2 gm of sucrose, 180 mg of DAB, 90 mg of cytochrome C (Sigma), and 300 mg of catalase in 300 ml of 0.1 M sodium phosphate buffer (pH 7.4). The sections remained in the cytochrome solution for 10–20 min, and were rinsed in sodium phosphate buffer, plated on glass slides, and allowed to air dry.

The vibrissae innervated by injected primary afferents, together with the surrounding four vibrissae, were removed from the mystacial pad and postfixed in 1.5% osmium tetroxide and 1.5% potassium ferricyanide for 2 hr. After the postfixation the vibrissae were dehydrated in a graded series of alcohols followed by propylene oxide and embedded in an Epon-Araldite mixture. As described previously (Klein et al., 1988), each vibrissa was oriented to facilitate sectioning the main vibrissa nerve as it entered the hair follicle. The base of the vibrissae, including the main vibrissa nerve, was sectioned at 1 μ m and the sections stained with toluidine blue. Myelinated axons were counted with the light microscope using a 40 $\times$ objective. Each vibrissa nerve was counted three times and the average of these counts was used for subsequent analyses.

Row widths were assessed in nucleus principalis in an attempt to relate changes in arbor morphology to changes in the size of whisker-related patches in the trigeminal brainstem nuclear complex. *This region was chosen for analysis because whisker-related row borders are difficult to discern in adult interpolaris.* Our DAB protocol produced a staining pattern in principalis that is very similar to that obtained with CO staining techniques. We determined whether the neonatal ACE injury affected this staining pattern by measuring the width of the vibrissa-associated rows. A 10 $\times$ objective and camera lucida apparatus were used to trace the outline of the DAB patches in nucleus principalis (left and right sides) of eight experimental and eight normal control animals. Each of the eight experimental rats contained one or more HRP-labeled fibers in subnucleus interpolaris (SpVi). We restricted our analysis to

those sections that exhibited well-defined rows with clear boundaries. Each row was measured 100 μ m from the inner boundary of the spinal trigeminal tract. The row width measures obtained for the left and right side were pooled and analyzed using single-factor analysis of variance (ANOVA) with Bonferroni correction. Potential differences between pairs were analyzed using two-tailed independent *t* tests.

Axon reconstruction. Axons were judged to be successfully injected only if they were well labeled throughout SpVi. All recovered axons were drawn in their entirety at a total magnification of 260 $\times$ using a Nikon Optiphot microscope and drawing tube. Quantitative analyses of labeled axons were performed as described by Jacquin et al. (1988). The total number of boutons per collateral was determined by one investigator (W.E.R.) via direct examination of sections at 400 $\times$. Arbor areas were computed from axon reconstructions by connecting the outermost boutons of each collateral with a graphics tablet cursor. The area and perimeter were calculated using a commercially available analysis program (SIGMASCAN, Jandel Scientific). The form factor (FF), a measure of circularity, was computed using the formula $FF = 4\pi(\text{area})/(\text{perimeter})^2$. To gain an estimate of bouton density, the average number of boutons per collateral was divided by the average arbor area. The data obtained in the present study were compared to those obtained in our previous investigations of primary afferent morphology in normal (Jacquin et al., 1988) and IO-lesioned animals (Renehan et al., 1989). Data were compared by using single-factor ANOVA with Bonferroni correction. Two-tailed, independent *t* tests were used to evaluate differences between pairs.

Possible correlations between the extent of peripheral damage and the size of the central terminal arbors of axons supplying undamaged vibrissae were determined by performing a least-squares linear regression analysis. Arbor area and perimeter were compared to the average number of axons supplying the lesioned vibrissae adjacent to the vibrissa innervated by the labeled afferent.

Results

Electrocautery of the vibrissae in rows A, C, and E on the day of birth (ACE electrocautery) resulted in distinct changes in the CO staining of somatosensory cortex. As shown in Figure 1, the vibrissa damage was associated with abnormal patterns of segmentation. Each of the lesioned rows was represented by a fused band of diffuse staining. Rows B and D, however, exhibited the punctate labeling associated with the normal rodent barrelfield cortex (cf. Woolsey and Van der Loos, 1970; Woolsey and Wann, 1976). These changes were consistent with those seen by previous investigators following vibrissa damage in the neonatal rat or mouse (Belford and Killackey, 1980; Durham and Woolsey, 1984; Bates and Killackey, 1985).

A number of previous investigators have used succinic dehydrogenase or CO staining techniques to search for injury-induced changes in brainstem organization (e.g., Waite and De Permentier, 1991; Chiaia et al., 1992). Although we were unable to use these techniques with tissue that had been reacted with DAB to demonstrate labeled neurons, the DAB histochemistry does produce a CO-like staining pattern in nucleus principalis (Fig. 2; a similar pattern is not visible in SpVi). Figure 2 illustrates the DAB background staining pattern in two normal adults and in three of the adult rats subjected to ACE electrocautery. Note that the background staining is patchy, that five rows of patches can be discerned, and that the major portion of the terminal arbor of an individually labeled primary afferent neuron tends to be restricted to the patch corresponding to the appropriate whisker. We found that the neonatal ACE lesion altered the width of the vibrissa-related rows in this nucleus. The widths of undamaged rows B and D were 110.0 (SE = 3.5) and 112.8 (SE = 4.8) μ m, respectively (Fig. 3). The widths of the adjacent A, C, and E rows were determined to be 104.0 (SE = 6.6), 85.2 (SE = 4.3), and 90.1 (SE = 4.5) μ m. These measures of mean row width were statistically different (ANOVA, *F* =

Figure 1. Tangential section through right somatosensory cortex of a rat that sustained electrocautery of all mystacial vibrissae in rows A, C, and E on the day of birth. CO histochemistry has been used to demonstrate the cortical barrels corresponding to the vibrissae on the face. The orientation of the photomicrograph is indicated in the *upper right corner* (medial, *M*; rostral, *R*). Whereas the barrels in rows B and D are consistent with those seen in normal animals, rows A, C, and E are represented by fused bands of diffuse staining.

7.56, $p < 0.0001$). Post hoc analyses indicated that the *widths of rows C and E were significantly less than their neighbors* (Bonferroni $p < 0.01$). The width of row A, however, was not significantly reduced. In *normal* adult rats, the widths of rows C and E were found to be 97.2 (SE = 5.1) and 111.0 (SE = 8.6) μm , respectively. These values were *also* significantly different than the measurements obtained in the lesioned animals ($t =$

2.09, $p = 0.043$ for C row comparison and $t = 2.82$, $p = 0.008$ for E row comparison). There were no statistically significant differences between the widths of rows A, B, or D in the lesioned and normal animals. Thus, while the ACE lesion has no effect on the widths of undamaged rows based on DAB staining patterns, there does appear to be a reduction in the widths of the C and E row representations (relative to both the adjacent un-

Figure 2. DAB histochemical staining patterns in transverse sections through trigeminal nucleus principalis from two normal adults (*A–C*) and from three adults subjected to ACE electrocautery, bilaterally, at birth. In *A* and *B*, terminal arbors from individually labeled B1 and E5 whisker primary afferents (*arrows*) are shown against the background of the whisker-related staining patterns. Regions bound by the letters A–E denote

those areas representing the A-E row whiskers. Note that the background staining is patchy, that five rows of patches can be discerned, and that the terminal arbors are primarily located within patches corresponding to the appropriate whiskers. *C* is a higher magnification view of *B*, with the E1-4 and D1-5 patches indicated. In *D-I*, the left and right nuclei are shown for three cases used in the present study, where whisker primary afferent morphologies were quantified in SpVi. Note that regions devoted to individual rows of whiskers can be discerned, and these rows are lettered. Scale bar in *A* applies also to *B*; scale bar in *D* applies also to *E-I*.

Figure 3. Summary of row width measurements in nucleus principalis of eight animals used in the intracellular labeling studies. Data for the left and right sides were pooled. Each column in the histogram illustrates the mean width and SE (in micrometers). The widths of rows C and E were found to be statistically different than the widths of their neighbors (rows B and D).

damaged rows and the values obtained in normal animals). We currently have no conclusive explanation for our failure to demonstrate a change in the width of the A row staining pattern. It is possible, however, that this observation is related to the relatively poorly defined medial boundary of the A row representation (see Fig. 2). The indistinct border between this region

and the adjacent ophthalmic and maxillary guard hair territory may have caused us to misinterpret the width of row A.

In addition to the central changes discussed above, electrocautery also reduced the number of axons supplying the lesioned vibrissae in adult animals. The vibrissae supplied by the labeled afferents ("target" vibrissae) were innervated by 157.2 (SE = 7.4) myelinated axons (in the main vibrissa nerve). The main vibrissa nerves supplying the undamaged follicles adjacent to the "target" whiskers contained an average of 150.9 (SE = 8.9) myelinated axons (Fig. 4). The adjacent *lesioned* vibrissae, however, were supplied by only 98.3 myelinated axons (SE = 6.9). There was no significant difference between the number of axons supplying the target and adjacent undamaged vibrissae. In contrast, the differences between these values and the number of axons innervating the lesioned vibrissae were significant ($t = 4.67$ and $t = 5.31$; $p < 0.001$).

Morphology of undamaged primary afferents in SpVi

Forty-two low-threshold primary afferent neurons supplying mystacial vibrissae were successfully injected with HRP and subjected to qualitative and quantitative analysis. Twenty-eight of these afferents responded to deflection of vibrissae in row B or D. Figure 5 presents a series of camera lucida depictions of one such afferent. This low-threshold neuron exhibited a rapidly adapting response to deflection of the D2 vibrissa in any direction. The afferent was capable of following electrical stimulation of the trigeminal ganglion up to 500 Hz at a latency of 0.3 msec. These response properties would be consistent with the response of low-threshold vibrissa afferents in normal animals (Jacquin et al., 1986a,b, 1988) and are representative of the responses exhibited by all of the undamaged afferents.

As illustrated in Figure 6, the vibrissa supplied by this primary afferent received a normal complement of myelinated axons. The main vibrissa nerve supplying the D2 whisker contained 179 myelinated axons. The adjacent undamaged D1 and D3

Figure 4. Histogram illustrating the number of myelinated axons innervating the vibrissae supplied by HRP-labeled undamaged primary afferents (*Target Vibrissae*) as well as the number of axons entering the adjacent lesioned and adjacent undamaged vibrissae.

Figure 5. Camera lucida reconstruction of the central terminal arbors of a primary afferent sensitive to deflection of the hair shaft associated with the D2 vibrissa. The panels show the parent axon (*open arrow, panel 0.0*) and collaterals in serial coronal sections through subnucleus interpolaris. Each panel represents one coronal section, staged relative to the interpolaris–caudalis border. In each case, the panel is oriented such that lateral is to the left and dorsal is toward the top. Boutons are shown as *dots*, but their number is only an approximation of the actual bouton count (determined from direct inspection of sections at higher magnification). *TrV*, trigeminal spinal tract; *sct*, spinocerebellar tract.

vibrissae were supplied by 180 and 166 axons, respectively. The main vibrissa nerves innervating the adjacent lesioned vibrissae contained only 91 (C2) and 124 (E2) axons. These data clearly show that the neonatal lesion reduced the number of axons in

the C row and E row vibrissae adjacent to the D2 vibrissa supplied by the labeled primary afferent (though the relatively large number of axons supplying the E2 whisker suggest that the lesion did not cause as much damage to this follicle).

Figure 6. Photomicrographs of the main vibrissa nerves innervating D2 and the adjacent D1, D3, C2, and E2 vibrissae. The D2 vibrissa was supplied by the afferent reconstructed in Figure 5. The C2 and E2 follicles were cauterized on the day of birth and contain fewer myelinated axons than the D1, D2, and D3 vibrissae.

Subjective examination of the axon arbors illustrated in Figure 5 suggests that the follicle lesions may have induced moderate arbor expansion. The arbors seen 0.0, 0.4, and 0.6 mm rostral to the interpolaris–caudalis border appear to be particularly widespread. One of the principal aims of this investigation was to determine if neonatal peripheral lesions might induce the central terminal arbors of undamaged primary afferents to invade the adjacent “denervated” brainstem territory. While our conclusions regarding this question are based on quantitative measurements of terminal arbors in animals where a max-

imum of two afferents were labeled on in each nucleus, we found that this phenomenon could be effectively visualized by injecting multiple primary afferents in a given animal. Examples of this approach are illustrated in Figures 7 and 8. Figure 7 depicts portions of the central terminal arbors of two undamaged mystacial afferents. One afferent responded to movement of the B3 whisker, and the other was sensitive to D2. Each of the afferents, in addition to terminating in the brainstem regions appropriate to their peripheral innervation, possessed collaterals that supplied portions of SpVi normally innervated by C row afferents.

Figure 7. Photomicrographs illustrating portions of two coronal sections through interpolaris, showing parent axons (*open arrows*) and central terminal arbors of two afferents. The afferent on the left supplied the B3 vibrissa, and the afferent on the right supplied D2. In *A* the B3 arbor can be seen to send a collateral across the C row territory to terminate adjacent to the D2 arbor. Similarly, *B* shows the D2 arbor approaching the B3 terminal area.

Figure 8. Serial reconstructions of central terminal arbors of four low-threshold primary afferents. The afferents supplied the undamaged B1, B3, D1, and D3 vibrissae. All conventions (including orientation) and abbreviations are as in Figure 5. Note that a number of the sections contained arbors that appeared to be oriented toward the partially denervated brainstem territory.

Figure 8 presents camera lucida reconstructions of the central terminal arbors of primary afferents supplying the B1, B3, D1, and D3 vibrissae in another neonatally lesioned rat. A number of the panels in Figure 8 are particularly instructive. For example, in the first panel (0.0 mm caudal to interparietal–caudalis border), note the position of the arbors associated with the B1 and D1 vibrissae. Each of these arbors is directed toward (and enters) the adjacent C row territory. Presumably, this territory was partially denervated by the neonatal vibrissa follicle lesions. Similar examples of arbors oriented toward the region normally occupied by C row afferents can be seen in panels 0.1, 0.2, and 0.8.

In contrast, the central terminal arbors of primary afferents supplying *lesioned* afferents appeared to respect their appropriate boundaries. Figure 9 illustrates the central terminal arbors of a primary afferent that supplied an A1 vibrissa in an animal that sustained the ACE lesion on the day of birth (primary afferents supplying lesioned vibrissa typically exhibited receptive fields restricted to one vibrissa). In each section the terminal arbor is confined to a circumscribed portion of SpVi (note that the scale in this figure is different from those used in Figs. 5 and 8).

These qualitative observations suggest the possibility that the central terminal arbors associated with undamaged vibrissa afferents have expanded to innervate adjacent brainstem territory denervated by the neonatal cautery lesions. In contrast, the primary afferents supplying lesioned vibrissae appear to respect their row boundaries, though they may be slightly elongated. To gain further evidence for this conclusion, we performed the quantitative analyses presented below.

Quantitative analysis of labeled primary afferents

The data from the 42 labeled primary afferents were used for analysis of arbor area, arbor perimeter, number of collaterals, number of boutons, boutons per collateral, and bouton density. Figure 10 compares the area and perimeter of the arbors labeled in the present study with data obtained in previous investigations of vibrissa primary afferent morphology in normal animals (Jacquin et al., 1988) and animals subjected to complete transection of the IO nerve (Renehan et al., 1989). In normal animals, the central terminal arbors of vibrissa primary afferents occupy approximately $6130 \mu\text{m}^2$ (SE = 214). In ACE electrocauterized animals, the average terminal arbor area of primary afferents supplying undamaged B or D row vibrissae was $13512.2 \mu\text{m}^2$ (SE = 790.67). The area of the arbors corresponding to surviving afferents that supplied the *lesioned* follicles (ACE lesioned) was only $7342.9 \mu\text{m}^2$ (SE = 643.9), however. The difference between the ACE undamaged and the normal populations was clearly significant ($T = 9.52$, $df = 159$, $p < 0.001$). The difference between the areas of the arbors of the ACE undamaged and ACE lesioned animals was also statistically significant ($T = 5.02$, $df = 106$, $p < 0.001$). The ACE lesioned and the normal groups were not statistically significant. Similarly, there was no significant difference between the areas of the ACE undamaged arbors and the arbors associated with primary afferents that were transected on the day of birth ($12940 \pm 1107.9 \mu\text{m}^2$). As would be expected from the above data, the ACE lesion also significantly increased the *perimeter* of the central terminal arbors of the afferents supplying undamaged follicles ($T = 8.65$, $df = 132$, $p < 0.001$). The average vibrissa primary afferent central terminal arbor perimeter in normal animals is $379.0 \mu\text{m}$

(SE = 9.07). We found that the ACE lesion increased this value to $657.14 \mu\text{m}$ (SE = 35.2) for the ACE undamaged group. Curiously, however, the peripheral damage also increased the perimeter of the arbors belonging to afferents that supplied the lesioned vibrissae ($T = 8.41$, $df = 129$, $p < 0.001$). Since this result could occur if the arbor shape had been altered, we used Form Factor analysis to explore the possibility that the arbors had become more elongate. Indeed, as illustrated in Figure 11, the Form Factor (and thus the circularity) of arbors corresponding to primary afferents that supplied lesioned vibrissae was significantly reduced, as compared to normal ($T = 12.87$, $df = 129$, $p < 0.01$). There was also a significant, though smaller, reduction in the circularity of the ACE undamaged arbors ($T = 6.40$, $df = 133$, $p < 0.01$). Each of these results would appear to corroborate the qualitative observations noted above. *The evidence indicates that the arbors of ACE undamaged afferents are enlarged and oriented in the direction of the adjacent, partially denervated rows. The arbors of ACE lesioned afferents, however, are not enlarged but have assumed a more flattened/elongate morphology.* It is interesting to contrast these results with those obtained when the entire IO nerve was transected. In these IO transection animals, the arbor area and perimeter is increased, but the Form Factor is not altered. As we have described previously (Renehan et al., 1989), transection of the IO nerve causes the arbors to expand in all directions, with no preferred orientation.

One of the issues we wished to examine was whether the size of the central terminal arbors of undamaged afferents was correlated with the extent of the peripheral damage. We performed this analysis by comparing the area of the undamaged afferent's terminal arbor with the average number of axons supplying the lesioned vibrissae adjacent to the whisker supplied by this afferent. A least-squares linear regression analysis failed to demonstrate a significant correlation between the extent of the damage and the size of the arbor (correlation coefficient, $r = -0.29$; $p = 0.2976$). This result is probably due to the fact that the lesions and their effect on arbor area were relatively uniform (note the small SEs in Fig. 10).

The lesion-induced increase in arbor area was also not associated with an increase in the number of bouton-like fiber swellings. We have previously shown that normal trigeminal primary afferents exhibit an average of 124.3 (SE = 15.1) boutons per arbor collateral in SpVi. In the present study, low-threshold afferents supplying undamaged vibrissae had an average of 102.3 (SE = 12.9) boutons per collateral (Fig. 12). Afferents supplying lesioned vibrissae manifested 99.9 (SE = 5.15) boutons per collateral. There were no significant differences between these populations. There was a significant difference, however, between the number of boutons per collateral in the IO transection group (42 ± 8.3) and these two groups ($T = 3.2$, $df = 21$, $p = 0.004$ for comparison between ACE lesioned and IO transection groups). We obtained an approximation of the synaptic bouton density by dividing the average number of boutons per collateral by the average arbor area. As Figure 12 illustrates, the density of boutons in the ACE Undamaged animals was only 25% the density seen in normal animals. Since the number of boutons per collateral was essentially unchanged, this difference is largely due to the increase in arbor area. The bouton density in the ACE lesioned group was slightly less than normal, and this probably reflects the small (but insignificant) decrease in boutons per collateral and small (also insignificant) increase in arbor area.

Figure 9. Brainstem terminations of an A1 vibrissa afferent. The follicle supplied by this afferent was cauterized on the day of birth. All conventions (including orientation) and abbreviations are as in Figure 5.

Discussion

This study has focused on the response of undamaged mystacial vibrissa afferents to electrocautery of neighboring vibrissae on the day of birth. Previous investigations have shown that this manipulation destroys the peripheral terminals of the primary afferents supplying the lesioned vibrissae and results in the death

of up to 62% of the injured neurons (Waite and Cragg, 1979). This model therefore allows us to examine the response of trigeminal primary afferents to partial denervation of the trigeminal brainstem nuclear complex. We found that the central terminal arbors of undamaged primary afferents are significantly larger than normal following damage to the vibrissae in rows A, C, and E of the mystacial pad (ACE electrocautery). Exam-

Figure 10. Area (top) and perimeter (bottom) of terminal arbors associated with trigeminal primary afferents supplying normal vibrissae (from Jacquin et al., 1988) and vibrissae in lesioned animals. The *IO Transection* values were obtained from animals that sustained a complete transection of the IO nerve on the day of birth (from Renehan et al., 1989). The *ACE Undamaged* group refers to primary afferents that supplied B or D row vibrissae in ACE electrocauterized animals. Values for afferents that supplied the lesioned vibrissae in the ACE animals are in the *ACE Lesioned* category. The area of the ACE undamaged arbors was significantly greater than the area of the normal and ACE lesioned arbors. The ACE lesioned and normal groups were not statistically different. The perimeter of the ACE undamaged arbors was significantly greater than the normal or IO transection arbor perimeters but not statistically different than the ACE lesioned value. The ACE lesioned perimeter was also significantly greater than the perimeter of the normal or IO transection arbors.

ination of individual HRP-labeled axon arbors suggests that the arbors have expanded into the territory normally occupied by the lesioned afferents. There was no increase in the number of boutons per collateral, and the bouton density was only 25% the density seen in normal animals. Thus, while the area supplied by the undamaged afferent arbors increased, there was no evidence that the absolute amount of terminal arbor was similarly increased (as would be the case if sprouting had occurred; see Renehan et al., 1989). We would therefore conclude that the undamaged afferents had undergone arbor expansion, but not sprouting.

Arbor expansion versus maintenance of immature configuration

We must consider at least two possible mechanisms that may be responsible for the lesion-induced increase in arbor area. First, it is possible that the undamaged primary afferent neurons have responded to the partial brainstem denervation by actively expanding their central projections. Second, it is also possible that arbor extent has actually *not* increased, but has instead maintained a configuration that normally would be reduced during development. As noted in the introductory remarks, ev-

Figure 11. Form Factor (a measure of circularity) of central terminal arbors. See Figure 10 for explanation of the four groups.

idence obtained in the visual system suggests that many topographic projections are established by the refinement of an initially widespread axon arbor (O'Leary, 1992). For example, hamster geniculate fibers have reached the future subcortical white matter below area 17 by the day of birth, but display only a crude topography across the mediolateral axis of the cortex (Naegele et al., 1988). Axons enter the cortical plate between postnatal days 3 and 5, often extending multiple collateral branches. This coarse topography becomes rapidly refined in the first 2 postnatal weeks, however. During this period, correctly positioned arbors are retained, but the incorrect branches are eliminated. This sequence of axonal arborization followed by collateral pruning has been documented in the superior colliculus (Sachs and Schneider, 1984; Sachs et al., 1986), visual cortex (Naegele et al., 1988; Callaway and Katz, 1990, 1991; O'Leary, 1992), cochlear nucleus (Jackson and Parks, 1982), LGN (Stretavan and Shatz, 1986), brainstem (O'Leary et al., 1990), and spinal cord (O'Leary et al., 1990). Is it possible that the widespread arbors documented in the present study represent the maintenance of arbors that have failed to be eliminated? To answer this question we need to know the status of individual vibrissa afferent arbors in the normal perinatal rat. Unfortunately, these data are not directly available. The results of recent CO experiments do provide *indirect* evidence that vibrissa primary afferents have attained a mature configuration by the day of birth. Chiaia and colleagues have used CO to evaluate the staining patterns in prenatal and postnatal rats (Chiaia et al., 1992). They were able to demonstrate that a vibrissa-related segmentation pattern appeared between embryonic day 19 (E19) and E20. Both Chiaia et al. (1992) and Bates and Killackey (1985) have presented evidence that suggests that the vibrissa pattern seen with CO closely matches the distribution of primary afferent axon terminals demonstrated by anterograde transport

of HRP (see next section, however). The data obtained by Chiaia et al. would suggest, therefore, that the morphology of individual trigeminal primary afferent central terminal arbors is mature (or close to mature) at birth. If true, this would further suggest that the enlarged arbors noted in the present study are the result of *lesion-induced arbor expansion*, and *not* the maintenance of an immature configuration.

This discussion has concentrated on the response of undamaged trigeminal primary afferent neurons to a neonatal lesion that partially denervates the trigeminal brainstem nuclear complex. It is important to consider, however, that this manipulation also partially denervates the periphery. The number of myelinated axons innervating the lesioned vibrissae was reduced by approximately 35%. Thus, either the peripheral or central denervation could serve as the critical stimulus for arbor expansion. There is evidence that undamaged somatic primary afferents will sprout into adjacent denervated skin under certain circumstances (Nixon et al., 1984; Jackson and Diamond, 1984; Diamond et al., 1987) and neonatal IO nerve transections have been shown to induce the development of abnormal peripheral projections by undamaged neurons (Jacquin et al., 1986a; Rhoades et al., 1987; Chiaia et al., 1988). Chiaia et al. (1992) have shown, however, that peripheral sprouting does *not* occur when the damage is restricted to a portion of the mystacial pad (as opposed to complete section of a peripheral nerve). It would therefore appear unlikely that our results are related to expansion of the *peripheral* projections of undamaged primary afferents.

Factors influencing the ability to demonstrate arbor expansion

Some of our observations would seem to be inconsistent with one of the results reported by Chiaia et al. (1992). These investigators used electrocautery to ablate subsets of the mystacial

Figure 12. Average boutons per collateral and bouton density measurements. The boutons per collateral values obtained for the *ACE Undamaged* and *ACE Lesioned* categories were not significantly different from normal. The density of the boutons associated with the *ACE undamaged* arbors was only 25% the density seen in normal animals.

vibrissae on E15–E20 and the day of birth. They found that cautery of vibrissae on E15–E18, but *not* at later ages, resulted in significant increases in the cross-sectional area of the brainstem patches that remained in ipsilateral SpVi. This group was thus unable to demonstrate an increase in the brainstem representation of single vibrissae following a neonatal follicle lesion. Our intracellular labeling studies, however, *did* provide evidence for axon arbor expansion following neonatal follicle injury. There are at least three possible explanations for this apparent discrepancy, including differences in (1) survival time, (2) analysis methodology, and (3) lesion paradigm. Chiaia et al. (1992) killed

their lesioned animals on postnatal days 5–7 (CO staining is most robust in the early postnatal period). For animals receiving follicle lesions on the day of birth, therefore, the survival period would have been between 5 and 7 d. In our study, animals were used in recording experiments and killed *between 60 and 120 d* following the neonatal follicle damage. There is evidence that some aspects of lesion-induced somatosensory reorganization require many weeks to develop (e.g., Liu and Chambers, 1958; Rasmusson, 1982; Lisney, 1983; Cusick et al., 1990; Dunn-Meynell et al., 1992). Although this issue is the subject of considerable debate, it has been suggested that acute changes reflect

the potentiation and/or modification of existing synapses, while chronic effects may be due to axonal reorganization (for discussion, see Wilson et al., 1987; Kaas, 1991). It is possible that an inadequate survival period may have prevented Chiaia et al. (1992), as well as some other investigators, from finding evidence of anatomic rearrangement following vibrissa damage. For example, Bates and Killackey (1985) and Belford and Killackey (1980) employed survival periods of 11 d or less and failed to find evidence of arbor enlargement following neonatal follicle damage. There is evidence, however, that the length of the survival period is not *the* critical factor. Durham and Woolsey extended the survival period to 100 d and still failed to see compensatory changes in the vibrissa representation (Durham and Woolsey, 1984). In contrast, Belford and Killackey (1979) and Bates et al. (1982) waited only 8 d or less and *did* see row enlargement. Another element that may have prevented some previous investigators from demonstrating altered brainstem projections following vibrissa injury may be the choice of staining technique. Metabolic staining methods, such as CO or succinic dehydrogenase histochemistry, do not directly stain axon terminals. In fact, CO histochemistry has been shown to be especially effective in demonstrating the mitochondria in cell somata and dendrites (Wong-Riley, 1989; Karmy et al., 1991). Therefore, these assays may not be sensitive enough to identify changes in the disposition of primary afferent arbors. Our own row width data would seem to support this conclusion. Although we did find a decrease in the width of the rows corresponding to the *lesioned* vibrissae, we were unable to demonstrate a change in the width of the B and D rows in principalis. Our intracellular labeling studies, however, clearly show that the arbors of individual trigeminal primary afferents supplying undamaged vibrissae are enlarged following the neonatal injury. It would appear that the DAB staining pattern may be capable of demonstrating the reduction in brainstem cell number that accompanies neonatal IO transection, but does not accurately illustrate the primary afferent reorganization that also occurs. It is possible, therefore, that the staining techniques employed by Chiaia and collaborators were not sufficiently sensitive to detect primary afferent plasticity. On the other hand, when Belford and Killackey (1979) removed the vibrissae of rows A, B, D, and E and cauterized their follicles at birth, they found that the succinic dehydrogenase representation of the intact row C *was enlarged* in comparison with the contralateral C-row patches. This expansion was *not* seen when only row C was ablated, however (Belford and Killackey, 1980). Subsequent studies by Bates and Killackey (1985) and Durham and Woolsey (1984) confirmed the failure of intact primary afferent terminals to invade brainstem territory denervated by single row damage (Bates and Killackey, 1985). Bates and colleagues, however, *did* note an enlargement of the D row representation following C and E row cauterization (Bates et al., 1982). In addition, it appeared that the D row expansion occurred primarily in the direction of the E row territory. The authors postulated that the explanation for this observation may be linked to the lack of a fasciculation boundary between the axons of the IO nerve supplying rows D and E. These data would seem to provide evidence that metabolic staining techniques can provide evidence of trigeminal primary afferent reorganization following neonatal follicle lesions, *if certain conditions are met*. It appears that the *type* of injury is especially important. For example, Jacquin et al. (1993) have recently shown that the brainstem CO patches corresponding to the supraorbital and postero-orbital whiskers

are enlarged within 1.5–3.0 d following complete transection of the IO nerve on the day of birth. With the exception of the study by Chiaia et al. (1992), every investigation that failed to demonstrate brainstem primary afferent reorganization following vibrissa injury restricted the damage to approximately one row of vibrissae. In each of the studies in which evidence of primary afferent arbor enlargement *was* seen (including our arbor morphometry study), the investigators lesioned *multiple* rows or the whole IO nerve. These data indicate that undamaged trigeminal primary afferents are most likely to expand their central terminal arbors into adjacent territory if the denervation is extensive. This logical conclusion suggests that capacious denervation provides the best opportunity for undamaged primary afferents to exert a competitive advantage over surviving lesioned afferents.

Competitive interactions

The denervation-induced increase in the size of undamaged trigeminal primary afferent arbors suggests that primary afferents may compete for terminal space in the brainstem. This observation is by no means revolutionary—competitive interactions have long been implicated in the development of the auditory and visual systems (e.g., Hubel et al., 1977; Purves and Lichtman, 1983; Lichtman and Balice-Gordon, 1990; Rubel et al., 1990). An impressive body of literature now supports the hypothesis that segregation of inputs in the visual system is achieved via an activity-mediated competitive process that may involve the formation and elimination of synapses (see Shatz, 1990, for review). In the case of the mammalian visual system, where eye-specific layers in the lateral geniculate form during a period in which vision is not possible, this segregation and pattern formation appears to be driven by the spontaneous activity of retinal ganglion cells. If this spontaneous activity is blocked with TTX, the developing ganglion cells fail to segregate into the appropriate layers and the size of individual axon arbors is dramatically increased (Shatz, 1990). The role of activity in the development of the *trigeminal* system is less clear, however. Henderson and colleagues have recently demonstrated that complete blockade of IO nerve activity during the early postnatal period has no effect on CO staining patterns in the trigeminal brainstem complex, thalamus, or cortex (Henderson et al., 1992). Similarly, IO nerve activity does not appear to alter the size of the CO patches corresponding to nonmystacial vibrissae (although these patches *are* enlarged when the nerve is transected; Jacquin et al., 1993). As noted by Henderson and colleagues, a potentially critical difference between the developing visual and trigeminal systems is that immature trigeminal ganglion neurons, in contrast to immature retinal ganglion cells, rarely exhibit spontaneous activity (Chiaia et al., 1990). It must be stressed, however, that the failure of TTX to alter CO staining patterns does not preclude a role for activity-dependent competition in some aspects of trigeminal development. For example, Fox has shown that removal of all whiskers but one (the D1), beginning on the day of birth, causes the area of cortex responding to this spared whisker to be enlarged, *although the CO pattern is unchanged* (Fox, 1992). Fox has argued that his results are the result of a failure of thalamocortical afferents to segregate as they would in the presence of normal activity in the pathways associated with the deprived vibrissae. A related study by Simons and Land has demonstrated that chronic whisker trimming from birth also alters cortical layer IV receptive fields without affecting pattern formation in barrel cortex (Simons and

Land, 1987). The effects of chronic whisker trimming are not restricted to somatosensory cortex. In two recent investigations, Jacquin and collaborators have shown that this manipulation alters the morphology of primary afferent central terminal arbors in SpVi (Woerner et al., 1986) and the receptive field properties of SpVi second-order neurons (Jacquin and Hobart, 1991). Again, these changes were not accompanied by alterations in CO staining characteristics. It would appear that postnatal manipulations that alter trigeminal primary afferent activity can indeed alter the segregation of inputs to the trigeminal brainstem complex and somatosensory cortex, but do not necessarily affect the appearance of vibrissa-related patterns as demonstrated by metabolic staining techniques.

As the discussion above suggests, it is difficult to explain all aspects of input segregation and pattern formation during development of the trigeminal system by employing activity-dependent interactions alone. It is possible that other factors, acting independently or in concert with activity, may be important in the generation of topographic neuronal connections. For example, Purves has suggested that the innervation of a particular target is regulated by trophic interactions between the pre- and postsynaptic neurons (Purves, 1988). Trophic interactions are generally thought to involve a specific molecular message that passes between the cells. The presence of trophic factors was originally proposed as an explanation for the developmental regulation of neuronal numbers (e.g., Oppenheim, 1981, 1985) but Purves maintains that the arrangement of axonal and dendritic arborizations is *also* subject to trophic regulation (Purves et al., 1988). Presumably, therefore, axon terminals would compete for a limited amount of trophic support. Although relatively little attention has been devoted to the role of trophic factors in trigeminal development, it is important that we not underestimate their potential contribution. In fact, the importance of considering multiple constraints when attempting to prove or disprove the involvement of a given mechanism in the patterning of sensory projections was recently highlighted in an elegant computer model of the development of the retinotectal projection (Fraser and Perkel, 1990). Fraser and Perkel have developed a simulation in which optic nerve fibers are proposed to be guided to their target sites by one or more affinity or competition cues (including activity-dependent competition). A major lesson provided by the simulations is that a system in which multiple redundant interactions operate can appear to behave paradoxically if viewed from an "either/or" perspective. Thus, caution must be exercised when one employs a paradigm that alters (or *appears* to alter) one constraint in a system that depends on multiple interactions for the development of normal topography.

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