Does Reorganization in the Cuneate Nucleus Following Neonatal Forelimb Amputation Influence Development of Anomalous Circuits Within the Somatosensory Cortex?

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INTRODUCTION

Numerous studies have documented the reorganization of the primary somatosensory cortex (S-I) that occurs in response to peripheral nerve damage or due to altered peripheral stimulation in either neonatal or adult mammals (for examples, see Aglioti et al. 1994; Calford and Tweedale 1991a; Diamond et al. 1993; Florence et al. 2000; Fox and Wong 2005; Halligan et al. 1993; Kaas and Collins 2003; Kelahan et al. 1981; Li and Waters 1996; Merzenich et al. 1983; Pascual-Leone and Torres 1993; Pearson et al. 2003; Pons et al. 1991; Rasmusson 1982; Rasmusson and Turnbull 1983; Recanzone et al. 1992). It is also clear that reorganization within the somatosensory pathways occurs at subcortical levels, such as the thalamus (Alloway and Aaron 1996; Florence et al. 2000; Garraghty and Kaas 1991; Jones and Pons 1998; Kiss et al. 1994; Nicoletis et al. 1991, 1993; Rasmusson 1996; Rhoades et al. 1987; Verley and Onnen 1981), brain stem (Chiaia et al. 2001; Jain et al. 2000; Kalaska and Pomerantz 1979; Northgrave and Rasmusson 1996; Panetsos et al. 1995; Pettit and Schwark 1993; Rasmusson and Northgrave 1997; Waite 1984), and spinal cord (Wu and Kaas 2002). There has been much controversy regarding the degree to which subcortical changes contribute to cortical reorganization (see Jones 2000; Wall et al. 2002).

In a series of reports, we have examined the consequences of neonatal forelimb removal on somatotopic organization in the brain stem, thalamus, and S-I. At each level, we have observed recording sites in the forelimb-stump representation that are also responsive to tactile stimulation of the hindlimb (Fig. 1A). These dual (stump and hindlimb) receptive field sites are referred to as split-Rfs. This abundant expression of hindlimb receptive fields at the level of the brain stem, i.e., 40% of neurons sampled in the cuneate nucleus (CN) (Lane et al. 1995), decreases as one ascends the somatosensory pathway to 19% in ventroposterolateral thalamic (VPL) neurons (Stojic et al. 1998) and only 2.2% of neurons in the S-I stump representation (Stojic et al. 2000)(Fig. 1B). However, treatment of the cortex of neonatally amputated rats with $\gamma$-aminobutyric acid (GABA) receptor blockers, bicuculline (GABA\textsubscript{A} receptor antagonist) and saclofen (GABA\textsubscript{B} receptor antagonist), reveals 44% of the individual cells recorded in the S-I stump representation responsive to hindlimb stimulation (Stojic et al. 2000). Electrolytic lesions and cobalt chloride silencing of discrete areas of the cortex were used to determine that this GABA blocked hindlimb input originates primarily from the S-I hindlimb representation and travels via a polysynaptic intracortical pathway to reach the S-I forelimb-stump representation (Stojic et al. 2001).

The developmental mechanisms resulting in an aberrant intracortical hindlimb projecting pathway(s) are unknown but may be viewed as possibly another example of lower level changes driving reorganization at higher levels and, in this case, resulting in topographic errors within S-I, which are subsequently suppressed by GABAergic mechanisms. As a first step in gaining an understanding of how these pathways develop, the specific question tested in this study is whether sciatic nerve sprouting into the CN is required to produce the normally suppressed hindlimb inputs in the S-I stump area of neonatally amputated rats? We have addressed this question by
reducing the sprouting of sciatic nerve fibers via application of neurotrophin-3 (NT-3) to the proximal ends of the lesioned brachial nerves. NT-3 is a ligand for the TrkB receptor (Lam-balle et al. 1991; Tessarollo et al. 1993), which has been shown to promote the survival of deafferented dorsal root ganglion (DRG) neurons when applied to a lesioned peripheral nerve (Eriksson et al. 1994). The rationale for this design is that promoting the survival of the primary afferent neurons and their terminations within the CN should reduce the likelihood of sciatic central axons sprouting into the CN. The effectiveness of the NT-3 treatment was assessed by tracing central projections of sciatic nerve fibers and by electrophysiologically mapping receptive field locations of the cuneate neurons. The experimental question whether cortical reorganization would be blocked or reduced if sciatic sprouting within CN was diminished was tested by multi-unit recording in the S-I of these animals.

METHODS

All protocols described here were developed in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Toledo, College of Medicine.

Neonatal forelimb amputation and NT-3 treatment

Neonatal forelimb removals were performed on newborn (postnatal day 0) rats as previously described (Lane et al. 1995). Briefly, pups were anesthetized by hypothermia until immobile. The left forelimb was amputated at the mid-humerus region and the brachial artery was sealed by electrocautery. A 3 mm² piece of Gelfoam sterile sponge (Pharmacia and Upjohn) soaked with 10 μg of NT-3 (Bachem) in sterile saline or saline alone (control animals) was placed over the proximal ends of the lesioned brachial nerves. A long-acting local anesthetic (0.7% Bupivacaine) was infiltrated into the wound, and the skin was closed with cyanoacrylate adhesive. The pups were rewarmed and returned to their mothers. On postnatal day 4 (P4), the pups were anesthetized with isoflurane, an incision made on the medial side of the stump, new sponges soaked with NT-3 or saline alone were used to replace the originals, and the skin closed as in the preceding text. After awakening, the animals were returned to their mothers. The animals were analyzed in subsequent electrophysiological or tract-tracing experiments at ≥60 days after surgical manipulation.

Labeling of sciatic nerve fibers

Anterograde labeling of the sciatic nerve by means of horseradish peroxidase (HRP) transport was used to visualize projections of sciatic afferents into the CN ipsilateral to the forelimb amputation as was previously described (Lane et al. 1995; Rhoades et al. 1993). Prior to surgery, rats were treated with buprenorphine (0.04 mg/kg)
and anesthetized with an intraperitoneal (ip) injection of a combination of ketamine (60 mg/kg) and xylazine (15 mg/kg). The sciatic nerve was exposed, and a 20 μl cocktail of 2.5% wheat germ agglutinin, 0.25% cholera toxin-conjugated HRP, and 15% type VI HRP in 2% DMSO was injected into the nerve with a glass microinjection pipette (tip diameter: 50 μm). Bupivacaine was applied to the incision and the wound closed with 11-mm surgical clips. After 3–4 days, rats were killed by exposure to a lethal dose of CO2 and perfused transcardially with heparinized saline followed by a solution containing 1% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer (pH 7.4). The brain stem was removed, cut into 50-μm coronal sections, and stained according to the protocol of Mesulam (1978).

Recording from the dorsal column nuclei

Multi-unit recordings were used to provide physiological evidence of the extent and form of brain stem reorganization after neonatal amputation with or without NT-3 treatment. Rats were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) and placed in a stereotaxic head holder. The skin, muscle, and dura overlying the brain stem were reflected. Occasionally the inferior margin of the occipital bone was also removed to better expose the rostral portion of the brain stem. The dorsal surface of the brain stem was photographed to record the placement of microelectrodes and the surface covered with warm silicone to prevent drying. Mapping of unit clusters was accomplished using varnish-coated tungsten microelectrodes (Z = 0.9–1.3 MΩ). Cutaneous receptive fields were defined with tactile stimuli delivered to the skin and hairs of the stump, trunk, and hindlimb with brushes and blunt probes. Electrode penetrations spaced ~200–400 μm apart were made in a roughly rectangular array across the left side of the brain stem. Recordings were made at the surface and at 200-μm intervals deeper until units unresponsive to tactile stimulation were noted. Attention was paid to determine the lateral and medial borders for the brain stem representation of the skin overlying the stump. Several electrode tracts in each experiment were marked with electrolytic lesions. At the end of each mapping experiment, the animal was exposed to a lethal dose of CO2 and perfused in the manner described in the preceding text. Brain stems were removed and histologically processed as described in the preceding text except that the sections were stained with cresyl violet.

Recording from the S-I

Multi-unit recordings of the S-I cortex were used to determine the borders of the forelimb-stump and hindlimb representations in amputated rats. Rats were initially anesthetized and prepared for recordings as previously described (Lane et al. 1995, 1997, 1999). A state of light anesthesia was maintained by administering urethane (1 g/kg ip) as needed. The cisterna magna was opened to drain cerebral spinal fluid, and a craniotomy was performed over the cerebral cortex contralateral to the amputated forelimb. The dura was reflected, and the surface of the cortex photographed to provide a map on which the locations of recording sites were recorded. Unit clusters were recorded in the same fashion as in the brain stem except that the penetrations were spaced ~300 μm apart and the activity was recorded at a depth of ~700 μm (within layer IV). Tactile stimuli to the stump, whisker pad, lower jaw, trunk, and hindlimb were delivered separately by lightly tapping the body surface with a cropped artist’s brush. The S-I forelimb-stump representations were mapped, and each site within the representation was classified as either stump or stump + hindlimb. After the initial S-I mapping, 30 μl of a solution containing 50 μM bicuculline methiodide and 50 μM saclofen (Research Biochemicals International) was applied to the pial surface to produce GABA receptor blockade (GRB), as previously described (Lane et al. 1997). After ~15 min, when spontaneous activity had increased at least twofold in

![FIG. 2. The influence of neurotrophin-3 (NT-3) treatment on sprouting of horseradish peroxidase (HRP)-labeled sciatic nerve fibers into deafferented cuneate nuclei of amputated rats. Polarized dark-field photomicrographs of the dorsal column nuclei of adult rats that have their sciatic nerves labeled with HRP. A: normal rat with extensive labeling of the gracile nucleus (GN) and an absence of labeled fibers in the CN. The borders of each CN are outlined with white dots. B and C: brain stems of 2 rats that sustained neonatal forelimb amputation (FLX). Additionally, the cut brachial nerves of the rat shown in C were treated with saline-soaked gelfoam as a control. Both B and C show similar levels of sprouting of labeled sciatic fibers into the CN. D–F: brain stem sections of 3 rats that were treated with NT-3 after amputation. D and E show minimal amount of sciatic nerve fibers that approach the medial side of the CN, whereas F shows a few labeled fibers in the CN. The vertical and horizontal orientation arrows in A indicate dorsal and lateral, respectively, for each image. Bar = 500 μm.](http://jn.physiology.org/issue/99/i2/688.html)
layer IV indicating that GRB was in effect, all of the recording sites within the forelimb-stump representation were remapped and reclassified as described in the preceding text. The S-I mapping and characterization of the stump receptive fields preceded the mapping and characterization of the receptive fields in the CN for four of the NT-3-treated and all six of the saline-treated animals.

Data analysis

All cuneate and S-I forelimb-stump recording sites were classified as responsive to cutaneous stimulation of the stump only or both stump and hindlimb. For the S-I sites, this was done before and during GRB. During the recording sessions, the experimenters were blinded to whether a given animal had been treated with NT-3 or saline. In the main analysis, the between-groups (saline vs. NT-3, pre- and post-GRB) differences in percentages of hindlimb responsive sites were compared at a level for significance of \( P < 0.05 \) using the Fisher’s exact test. The correlation between the expression of hindlimb responsive sites in the CN and the S-I of the same animal was assessed by linear regression analysis with \( P < 0.05 \) for significance.

RESULTS

Influence of NT-3 on the reorganization of sciatic nerve projections to the dorsal column nuclei

In normal adult rats, ipsilateral injection of HRP into the sciatic nerve produces abundant and dense labeling of the

FIG. 3. The influence of NT-3 treatment on the expression of hindlimb receptive fields within the CN of amputated rats. A: map of the receptive fields identified on the affected side of an amputated rat the cut brachial nerves of which were treated with NT-3-soaked gelfoam. B: receptive field map of a neonatally amputated control rat the brachial plexus of which was treated with saline-soaked gelfoam. Note, the larger number of split-Rfs sites (yellow triangles) detected in B vs. A. Bar = 1 mm.
nucleus gracilis (Fig. 2A). No labeled fibers were observed in the CN of normal animals. In contrast, labeled fibers extend into the CN of rats that sustained neonatal forelimb removal (Fig. 2B). To a similar extent, labeled fibers in the CN were also observed in all the rats that sustained neonatal forelimb removal followed by treatment of their severed brachial nerves with control saline sponges (Fig. 2C). In contrast, the neonatally amputated rats that were treated with NT-3 sponges displayed either minimal (Fig. 2, D and E) to a few labeled sciatic fibers in the CN (Fig. 2F).

Influence of NT-3 on the functional reorganization in the CN

Multi-unit electrophysiological recordings were used to define and map the CN of NT-3- and saline-treated, forelimb-amputated rats. Figure 3 shows images of the dorsal surfaces of the brain stems and the numbered recording sites that displayed stump receptive fields. The relative number of recording sites in each row that are both stump and hindlimb responsive (i.e., split-Rfs, yellow triangles) shows relatively few such sites detected in the NT-3-treated animal compared with the saline-treated animal (Fig. 3, A and B, respectively). The percentage of these split-Rf sites in the NT-3 (n = 6 animals generating a total of 429 stump-responsive sites) and the saline (n = 6 animals generating a total of 404 stump-responsive sites) treated groups was 6.3 ± 1.9 and 30.5 ± 4.0, respectively (means ± SE; Fig. 4). This 79% decrease in the occurrence of split-Rf in the brain stems of the NT-3 group relative to that of the saline group was significant (P < 0.05).

Influence of NT-3 on the functional reorganization in the S-I cortex

Examples of the S-I forelimb-stump representations recorded from animals that sustained neonatal forelimb amputations and were treated with NT-3 or saline are shown in Fig. 5. Prior to GRB, the organization of the maps in the two rats is quite similar (Figs. 5, A and B) with few sites showing the ability to respond to both stump and hindlimb stimulation (yellow triangles). Figure 5A’ and B’ show the stump representations from the same animals during application of bicuculline and suclofen. For both rats, GRB produced a large increase in the number of split-Rf sites.

Across both experimental groups, prior to GRB, a large majority of sites within the stump representation were responsive to cutaneous stimulation of the stump and nonresponsive to stimulation of the hindlimb (384 of 389 sites in the NT-3 group, n = 6, and 344 of 347 sites in the saline group, n = 6). Several (1–3) sites located near the hindlimb border in two NT-3-treated and two saline-treated animals displayed split-Rfs. During GRB, 34.2 ± 3.9% of the previously stump-exclusive 384 recording sites in the NT-3-treated rats could also be activated by stimulation of the hindlimb (Fig. 6A). For the saline control animals, 31.5 ± 1.7% of the 344 stump recording sites revealed hindlimb RFs during GRB (Fig. 6B).

Statistical comparison of results from these groups revealed no significant difference in the expression of hindlimb responsive sites within the S-I forelimb-stump representation during GRB (P > 0.05).

Correlation between brain stem and cortical reorganization in individual animals

Figure 7 shows the relationships of the percent hindlimb-responsive sites in the CN with that of the S-I stump representation during GRB for individual animals. Four of the NT-3-treated and six of the saline-treated animals had both their brain stem and cortical reorganization assessed. As noted in the preceding text, there was minimal evidence of functional reorganization in the CN of any of the NT-3-rats and differing degrees of CN reorganization in saline-treated controls. However, the reorganization apparent within S-I following GRB held between 25 and 38% within and across groups over widely differing levels of CN alteration. No statistically significant brain stem-cortex correlation was found within the NT-3 group, the saline group, or the combined group. This lack of correlation is illustrated by the horizontal regression line in Fig. 7.
DISCUSSION

The results described in the preceding section indicate that sprouting of sciatic nerve fibers was inhibited and the occurrence of hindlimb-receptive fields in the CN of neonatally amputated rats was significantly reduced by treating the cut nerves with NT-3 at the time of and at 4 days post-amputation. In contrast to the significant effect this treatment had on structural and functional reorganization at the brain stem, the level of expression of HL receptive fields in the S-I stump representation was unaffected by the NT-3 treatment, suggesting that the cortical reorganization occurs independent of the anatomical and physiological changes in the CN. The lack of correlation between the relative expression of hindlimb-receptive fields in the CN with that in the S-I stump representation of the same animals further supports this conclusion. Before exploring the implications of these findings, several technical limitations of our approach must be acknowledged.

Technical limitations

The present study is limited by the fact that we assume that the only significant influence of the application of NT-3 to the lesioned brachial nerves is to promote the survival of the associated dorsal root ganglia cells and that this enhanced survival is an effective barrier to the sprouting of sciatic fibers into the deafferented CN. This effect was expected based on the results of Eriksson et al. (1994) and White et al. (1996). A similar study conducted by Calia et al. (1998) found that NT-3 applied to the cut infraorbital nerve fibers of neonatal rats after peripheral deafferentation helped preserve the acetylcholinesterase and serotonin S-I barrel staining patterns presumably by sustaining the deafferented trigeminal ganglion neurons during a critical period of development.

Other reported influences of NT-3 include mitogenic and differentiation activities of neuronal precursor cells (Korschning 1993). However, it is not apparent that these potential effects...
would influence our results. Several studies (Lessmann 1998; Schuman 1999) suggest that NT-3 application may act to enhance the activity of excitatory synapses in the CN. As discussed in a later section, it is anticipated that the influence of enhanced brain stem activity would be to reduce the expression of hindlimb receptive fields in the S-I stump area, which did not occur. Although the observed reduced sprouting and establishment of fewer hindlimb receptive fields in the CN supports the rescue of axotomized primary afferent neurons, it is likely that the exogenous NT-3 acts via several mechanisms to achieve this. The Trk C receptor has been shown to be present only on a subset representing 25% of chick lumbar DRG neurons (Hory-Lee et al. 1993). Hence assuming no significant species differences, only partial rescue would be expected via the application of NT-3. However, several studies have found that either exogenously applied NT-3 or NGF will rescue virtually all the rat DRG neurons supplying a lesioned nerve (Eriksson et al. 1994; Otto et al. 1987; Rich et al. 1987). One explanation for this phenomena proposed by Eriksson et al. (1994) is that the action of the neurotrophin on one subset of DRG neurons allows this subset to promote the survival of other subsets via release of endogenous neurotrophic factors within the ganglion. Alternatively, the NT-3 may also act via Trk A and B receptors present on other DRG subpopulations (Soppet et al. 1991; White et al. 1996) or the p75 low-affinity receptor (Chao and Hempstead 1995). A single-functional epitope that binds to Trk A, Trk B, and p75 and is distinct from that recognized by TrkC has been identified on the NT-3 protein (Ryden and Ibanez 1996). Hence NT-3 may promote survival of DRG subsets expressing these other receptors. Other technical limitations in this study relate to the use of multi-unit versus single-unit electrophysiological recording and the application manner for GRB have been discussed previously (Lane et al. 1997).

Subcortical reorganization

Peripheral nerve lesions produce losses of 30–49% of first-order neurons (Aldskogius and Risling 1981; Jacquin et al. 1986; Savy et al. 1981) and 20% of second-order cells (Waite 1984). Although we did not examine dorsal root ganglia or CN cell numbers, NT-3 treatment of the proximal stump of a lesioned sciatic nerve of a neonatal rat has been shown to provide almost complete rescue of the DRG neurons (Eriksson et al. 1994). In the current study, NT-3 treatment significantly reduced the percentage of cuneate recording sites that were hindlimb responsive. The averaged value of 6.3% of cuneate recording sites that express hindlimb responsiveness in the NT-3 animals can be compared with an observation in an earlier study in which the receptive fields of individual CN neurons of normal (non-amputated) rats were assessed (Lane et al. 1995). In that study, all 48 cuneate neurons responded to forelimb stimulation only. Although NT-3 treatment significantly reduced the level of cuneate sprouting as well as expression of hindlimb receptive fields by CN neurons, it appears likely that the minimal sprouting and associated receptive field alterations in these animals are still more than are present in normal intact animals. Note, however, that one NT-3-treated animal (Fig. 4A) that did not have any detected hindlimb fields in the CN displayed a degree of cortical reorganization in the S-I stump representation that was comparable to that of the control animals (Figs. 6 and 7).

Although thalamic reorganization was not investigated in this study, it is reasonable to assume that, based on the minimal brain stem reorganization in NT-3-treated animals, the expres-
sion of hindlimb receptive fields in the forelimb-stump area of the ventroposterior lateral nucleus (VPL) would be significantly lower in these animals than has been shown in amputated rats that received no other treatments (Stojic et al. 1998). While this study focused on relating the reorganization of the brain stem/dorsal columns (fine touch and proprioception pathway) with that of the S-I cortex, other ascending pathways including the spinothalamic and reticulothalamic tracks of the anterolateral system carry nociceptive, thermal, and crude touch information to the thalamus where it can be relayed to the cortex. The possibility that the non-dorsal column pathways could influence reorganization in the thalamus and S-I is supported by studies showing that nociceptive inputs can regulate the expression of tactile receptive fields in the cortex and thalamus (Calford and Tweedale 1991b; Greek et al. 2003; Katz et al. 1999; Kiani et al. 2004). What influence the NT-3 treatment has on nondorsal column pathways is unknown.

Cortical reorganization

In a previous study we investigated the development of the polysynaptic intracortical pathway connecting the S-I hindlimb and forelimb-stump representations (Stojic et al. 2001). The results suggested that neonatal amputation of a forelimb acts to strengthen or amplify a circuit that is normally present between these two representations. If this strengthening is not related to brain stem sprouting, what causes the S-I reorganization? One candidate is the reduced activity supplying the S-I forelimb-stump representation during the postnatal period of cortical development allows the hindlimb circuits to be relatively strengthened. Peripherally driven activity has been shown to play a critical and developmentally sensitive role in the development of the visual system (see Berardi et al. 2003 for review), auditory system (Illing 2004 for review), and somatosensory system (Erzurumlu and Kind 2001; Fox et al. 1996; Jensen and Killackey 1987; Kaas 2002). Correspondingly, the S-I hindlimb to stump cortical circuit does not develop after forelimb amputation in adult rats (Pluto et al. 2003). Components of the intracortical hindlimb-stump circuit (see Pluto et al. 2005 for a description of this circuit) that mature during the first postnatal weeks and that may be influenced by reduced activity include the network of excitatory and inhibitory intracortical neurons (Daw et al. 2007; De Lima et al. 2007 et al.; Jiao et al. 2006) as well as the intercortical fibers connecting the two contralateral S-I regions (Olavarria and Safaeian 2006). Reduced afferent activity has been observed to weaken the expression of GABAergic intracortical neurons (for examples, see Hendry and Jones 1988; Levy et al. 2002 and Rosier et al. 1995). The ability of reduced afferent activity to influence the distribution of intracortical fibers in the somatosensory cortex has been noted by Koralek and Killackey (1990) and Remple et al. (2004). Hence future experiments will focus on examining the role of peripheral neural activity during neonatal development in mediating the reorganization of the excitatory and inhibitory components that produce the anomalous hindlimb receptive fields in the forelimb-stump area of the somatosensory cortex.

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