AN ELECTRON MICROSCOPIC STUDY OF THE FORMATION OF CORTICORUBRAL SYNAPSES IN THE CAT

YASUHIKO SAITO

Department of Biophysical Engineering
Faculty of Engineering Science
Osaka University

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GENERAL INTRODUCTION

The vertebrate central nervous system (CNS) is composed of complex but orderly connected neuronal circuits, on which proper functions of the CNS depends. The tips of axons called axonal growth cones play a key role in the establishment of neuronal connections. The growth cones select precise pathways and grow along the pathway in a highly stereotyped manner to find their appropriate targets. In the target, they often localize to specific regions of the target and form synapses. At a later stage of the development, the area of axons termination within the target is further restricted. Thus the process of neuronal circuit formation is often divided into four steps, (1) axon pathfinding, (2) target selection, (3) synapse formation, and (4) refinement of connections (Cowan et al., 1984; Goodman and Shatz,1993; Tessier-Lavigne and Goodman, 1996; Katz and Shatz, 1996).

Recent progress of molecular biological techniques has enabled to identify many molecules implicated in the mechanisms of the axon pathfinding, leading to the understanding of axon guidance mechanisms to the targets (Goodman, 1996; Tessier-Lavigne and Goodman, 1996). These mechanisms appear to include 1) contact attraction and 2) contact repulsion as short-range cues, and 3) chemoattraction and 4) chemorepulsion as long-range cues (Dodd and Jessell, 1988; Goodman, 1996; Tessier-Lavigne and Goodman, 1996). Molecular mechanisms of target recognition by the growth cones have also been studied extensively and several important molecules implicated in the target recognition have been identified. This includes neurotrophins which appear to regulate the axonal introduction into the target region (ElShamy et al., 1996; Tessier-Lavigne and Goodman, 1996). The molecules involved in the

topographically organized neuronal connections have also been revealed using the retino-tectal system (Udin and Fawcett, 1990; Goodman and Shatz, 1993; Tessier-Lavigne and Goodman, 1996).

The third step, synapse formation, may be the most complex process and the mechanisms for synapse formation is relatively poorly understood. In the neuromuscular junction (NMJ), some molecules that may be involved in the formation of the junction have been identified (Bennett and Pettigrew, 1976; Hall and Sanes, 1993; Sanes, 1997). These includes, nerve-derived signaling molecules such as ARIA/neuregulin and calcitonin gene-related peptide (CGRP) that stimulates the synthesis of acetylcholine (Ach) receptors (Falls et al., 1993; New and Mudge, 1986), and agrin that stimulates the clustering of Ach receptors (McMahan, 1990).

In the NMJ, the initial synapse formation does not occur at a particular point along the myotube membrane (Bennett and Pettigrew, 1976; Frank and Fischbach, 1979; Hall and Sanes, 1993). This means that the entire myotube membrane is equally receptive for the initial synapse formation. The equal susceptibility to innervation together with the feature of its growth has led us to an idea that target muscles may play a passive role in the process of the synapse formation. The central synapses, however, have features distinct from NMJ: axon terminals from various sources converge on a single neuron and the postsynaptic cells displays a complex three-dimensional structures allowing different kinds of afferent axons to terminate on different regions of the target neurons (Gottlieb and Cowan, 1972; Bayer, 1980; Kimmel, 1982; Bayer and Altman, 1987; Kimmel et al., 1990; Jacobson, 1991). Thus, it seems important to examine the process of synaptogenesis in central synapses.

In chapter 1 of this thesis, I carried out an electron microscopic study to

analyze the synaptogenesis in the red nucleus of the cat. The reason why I chose this synapse will be given in chapter 2 in detail. I observed biocytin-labeled corticorubral terminals of cats during the period of synaptogenesis. In chapter 2, it is aimed to analyze the sites of synapses quantitatively to determine whether the afferent axons preferentially form synapses on spine-like dendritic protrusions (SLDPs) *in vivo*. Moreover, HRP-labeled rubrospinal neurons were observed to examine whether postsynaptic SLDPs originated from the neurons.

This thesis also contribute to the further understanding of the last step, the refinement of the neuronal connections (Shatz, 1990a, b; Goodman and Shatz, 1993; Katz and Shatz, 1996). Although it has been proposed that the activity-dependent mechanisms operates (Shatz, 1990a, b; Goodman and Shatz, 1993; Katz and Shatz, 1996), it remains unclear whether axon form synapses with targets in inappropriate areas. Moreover, although several models have been proposed to explain the contribution of the activity to modify neuronal connections (e.g. Cowan et al., 1984; Constantin-Paton et al., 1990), it has not been known whether the refinement accompanying with structural changes can be explained simply by an activity-dependent manner. Therefore, it is important it is necessary to investigate whether the afferent axons which project to the inappropriate regions form synapses on the target neurons and how they change in morphology during their development. In chapter 3 of this thesis, I investigated whether the axons form synapses on inappropriate targets. To obtain an answer, I observed biocytin- or PHA-L-labeled aberrant (crossed) corticorubral axons of neonatal cats using an electron microscope. To gain insight into the mechanism of the refinement, I also investigated the morphological change of individual crossed corticorubral axons labeled with biocytin or PHA-L during

development and after early unilateral lesions of the sensorimotor cortex in chapter 4.

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BACKGROUND OF CORTICORUBRAL SYSTEM IN CATS

This thesis is concerned with the synapse formation of the corticorubral (CR) system in cats. The study was performed to analyze quantitatively the sites of synapse formation *in vivo* using an electron microscope.

The red nucleus (RN) is located in the mesencephalon at the level of the oculomotor nerve, rostral to the decussation of the brachium conjunctivum and is important in processing information from sensorimotor cortex and from the deep cerebellar nuclei in mammals (Allen and Tsukahara, 1974; Houk, 1991). Appearance of the RN may be related to the development of limb phylogenetically (ten Donkelaar, 1988). The nucleus is divided into two parts from the cell size and the pattern of the axonal projections : the magnocellular part which is in the caudal part of the RN and composed of medium- to large-sized neurons projecting to the caudal brainstem and spinal cord, and the parvocellular part which is in the rostral position and composed of small- to medium-sized neurons projecting to the inferior olive and the thalamus (Massion, 1967). The RN receives afferents from two major sources : the cerebral cortex and the deep cerebellar nuclei (Massion, 1967; Allen and Tsukahara, 1974).

The synaptic organization of the ferine CR system has been extensively studied (Tsukahara and Kosaka, 1968; Pizzini et al., 1975; Tsukahara et al., 1975; Murakami et al., 1982, 1983; Katsumaru et al., 1984; Murakami et al., 1986). Anatomical as well as physiological studies demonstrated that there is a topographic connection in the CR system: the neurons located in the lateral part of the pericuruciate cortex project to the medio-dorsal area of the RN which contains neurons projecting to the upper spinal segments , whereas those in the lateral part to the ventro-lateral area which contains

neurons projecting to the lower spinal segments (Pompeiano and Brodal, 1957; Rinvik and Walberg, 1963; Mabuchi and Kusama, 1966; Padel et al., 1973; Jeneskog and Padel, 1983). There are at least two pathways from pyramidal neurons to rubrospinal (RS) cells in the RN; direct pathway and indirect one via the inhibitory interneurons in the RN (Tsukahara et al., 1968). At the cellular level, the CR fibers terminate on the dendritic membrane remote from the soma of RS cells (Tsukahara and Kosaka, 1968; Tsukahara et al., 1975; Murakami et al., 1982). The CR input whose putative neurotransmitters may be glutamate and aspartate (Nieoullon et al., 1988) is excitatory (Tsukahara et al., 1967), mediated by NMDA and non-NMDA receptors (Davis et al., 1986; Harris and Davis, 1992).

The development of the CR projection has been studied (Higashi et al. ,1990; Murakami et al., 1991a,b; Murakami et al., 1993; Song et al., 1993; 1995). Although the CR projection is ipsilateral in adult cats, it is bilateral during the early development (Murakami and Higashi, 1988; Higashi et al. ,1990; Murakami et al., 1993). Axonal profiles at this stage infrequently bifurcated both in ipsilateral and contralateral sides and the projection are not in topographic order apparently (Higashi et al. ,1990). After the later stage of development (about the 2nd week after birth), adult-like topography are discernible only in the ipsilateral projection (Higashi et al. ,1990).

Although there has been no morphological study describing the period of the CR synaptogenesis, Song et al. (1993) demonstrated that excitatory postsynaptic potentials were induced in rubrospinal cells of fetal cats by stimulation of the ipsilateral pericuruciate cortex, suggesting that CR synapses have been already formed prenatally. Higashi et al. (1990), however, reported that CR fibers of newborn cats were simple in morphology with few branches. During one month, they showed extensively

elaborated arbors. This suggests that extensive synaptogenesis occurs postnatally (see Saches et al., 1986).

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Chapter 1

Developing corticorubral axons of the cat form synapses on filopodial dendritic protrusions

ABSTRACT

Developing neurons transiently grow numerous spine- or filopodium-like dendtitic protrusions (SLDPs). Electron microscopy on identified input and intracellualr staining of postsynaptic cells were performed to gain insight into their significance. Newborn kitten-corticorubral axons, labelled with biocytin, commonly made synapses on SLDP, often multiply invaginated by the SLDPs. Correspondingly, intracellularly labeled kitten rubrospinal cells had numerous SLDPs. Taking into account that corticorubral synapses are largely formed on dendritic shafts in adult cats, it is likely that the SLDPs play some important role in the development of corticorubral synapses. We hypothesize that rubrospinal cells elongate SLDPs searching for corticorubral axons to form synapses.

INTRODUCTION

Neurons of the vertebrate undergo remarkable changes in morphology during development. A commonly observed phenomenon is the loss of spine- or filopodium-like protrusions (SLDPs) which are numerous in certain stages of develpment (Morest, 1969; Lund et al., 1977; Garey and Saini, 1981; Phelps et al., 1983; Ramoa, 1988), but the reason for this transience remain unknown. The notion that overproduction of axons and synapses occurs in development (Rakic et al., 1986; LaMantia and Rakic, 1990) raises the possibility that they are necessary as the site of transient input

termination; this idea is supported by observations of synaptic contacts on small dendritic protrusions during pre- and postnatal development of mammals (Vaughn et al., 1977; Phelps and Adinolfi, 1982; Bhide et al., 1988). Another possibility could be that the dendrites elongate spines to reach and form synapses with growing axons nearby. While studies of identified inputs are important to examine these possibilities, only a few electron microscopic studies have been carried out on identified synapses in developing brains. In the present study we labeled the corticorubral fibers in neonatal cats with biocytin and observed the synapses in the red nucleus with an electron microscope. The corticorubral projection is of particular interest, since (1) the sites of input termination on soma-dendritic membranes have been extensively studied anatomically (Murakami et al., 1982) as well as physiologically (Tsukahara and Kosaka, 1968; Tsukahara et al., 1975a; Tsukahara et al., 1975b) and, (2) the existence and the period of two types of transient projections from the cortex were demonstrated (Higashi et al., 1990).

MATERIALS AND METHODS

Kittens aged from postnatal day (PND) 3-4 (n=3) received unilateral biocytin injection into the pericruciate cortex; biocytin (Sigma, 5% in Tris buffer) was pressure-injected using a glass micropipette connected to a Hamilton syringe in a total of 1 μl, and the injection was made into 2-4 loci of the cortex, under anesthesia with Nembutal (25 mg/ kg, i.p.). After a survival of 3 days, the kittens deeply anesthetized with

Nembutal were transcardially perfused with 1 % paraformaldehyde and 1 % glutaraldehyde followed by 6 % glutaraldehyde. After leaving the brain in situ for 1 h, the red nucleus-containing region was blocked and cut near horizontally with a Microslicer (Dosaka EM) at 50 – 70 μm. Then the sections were incubated in avidin-biotin peroxidase complex (Vector Lab) and reached with diaminobenzidine tetrahydrochloride. They were further fixed in osmium tetroxide, stained *en bloc* with 1.5 % uranyl acetate, dehydrated and flat embedded in Epon (TAAB). Following observations with a light microscope (Olympus BH2), appropriate area in the ipsilateral red nucleus were selected from the 50 – 70 μm thick sections, attached to a cylindrical Epon block and further cut into 5 μm thick sections. The small piece of the section was coverslipped with Epon and observed under the light microscope with an x 100 objective. Appropriate stained profiles were selected and photographed, and then ultrathin sections were cut serially or semiserially, followed by observations with an electron microscope (Jeol 1200EX).

Three additional animals with corresponding ages (PND 3-8) were subjected to intracellular biocytin injection to reveal the morphology of red nucleus cells at this developmental stage. The experimental procedures followed Song et al. (1993) but with some modifications: kittens anesthetized with Nembutal and immobilized with gallamine triethiodide, were artificially ventilated. Cells were identified by antidromic responses elicited from the spinal cord before the biocytin injection.

RESULTS

At the light microscopic level, corticorubral axons showed swellings along their course. Electron microscopy revealed that they usually corresponded to synapses, contacting in most instances postsynaptic profiles that were very small in size (< 1 μm)(see ref. Murakami et al., 1991). We noticed that the swelling often looked fenestrated under the light microscope, including an unstained or lightly stained portion. Electron microscopic observation demonstrated that such lightly stained portion corresponds to an invagination by a dendritic profile (Fig. 1 A1-A4, B); examination of neighboring sections, in many cases, revealed that the invagination arises from a SLDP from a shaft of the dendrites. Occationally, a single axonal bouton had multiple invaginations and synaptic specialization. Only a small proportion (about 10 %) of the labeled synapses were formed on dendritic shafts and not on the somatic membrane, although these sites were only partially occupied by synaptic boutons.

To ascertain that the rubrospinal cells at this developmental stage have SLDPs, intracellular staining was carried out. As shown in Fig. 2 numerous protrusions with various shapes and length were observed emerging from the dendrites of stained cells. They were more numerous on the distal portion of dendrites (Fig. 2C) and, near the end of dendrites, abundance of protrusions formed bush-like structures (Fig. 2D). They were barely observed on the somatic membrane.

DISCUSSION

Corticorubral axons appear to form synapses preferentially on protrusion emerging from dendritic profiles, presumably of rubrospinal neurons. The presence of numerous SLDPs on intracelluarly stained rubrospinal neurons supports this view, although part of the synapses could be on inhibitory interneurons (Katsumaru et al., 1984). At the stage of development in which the present observations were made, the corticorubral projections are bilateral and diffuse (Higashi et al., 1990). This might indicate the presence of excess synapses. However, the observation that individual axons remain relatively simple in morphology and lack terminal arborizations at this stage (Higashi et al., 1990), suggests that the period of synaptogenesis of corticorubral projection peaks at later stages of development (see ref. Sachs et al., 1986). Furthermore, partial covering of the dendritic shafts by synaptic boutons indicates that synaptic sites are available on dendritic shafts. These observations argue against the possibility that SLDPs are necessary to receive excess synapses. Previous electron microscopic studies indicated that the corticorubral synapses in adult cats mostly reside on dendritic shafts (Murakami et al., 1982; Murakami et al., 1984), a fact that is in accordance with the poverty of dendritic spines on HRP-filled red nucleus cells of adult cats (Wilson et al., 1987). Thus, the site of synapses in adult cats contrasts with that in neonates. Taken together, it would be interesting to hypothesize that, during development, dendritic protrusions grow, searching for axons nearby to form synapses; thereafter, they disappear due, for example, to retraction of SLDPs , eventually establishing synapses on dendritic shafts. Although the possibility that synapses on SLDPs are transient cannot be excluded, the intimate relation between the pre- and

postsynaptic profiles as discussed below suggests that this is unlikely.

The synapses on the dendritic protrusions often exhibited invaginated structures. Since such structures have not been observed in adult red nucleus, they may be related to some developmental event. Dendritic invagination was also observed in the retinogeniculate axons of developing hamster and was suggested to be concerned in the induction of branching by retinal axons (Bhide et al., 1988). In light of above speculation, we would rather suggest that the presence of dendritic invagination represents ongoing interactions for recognizing each other. Further studies are needed to disclose its significance.

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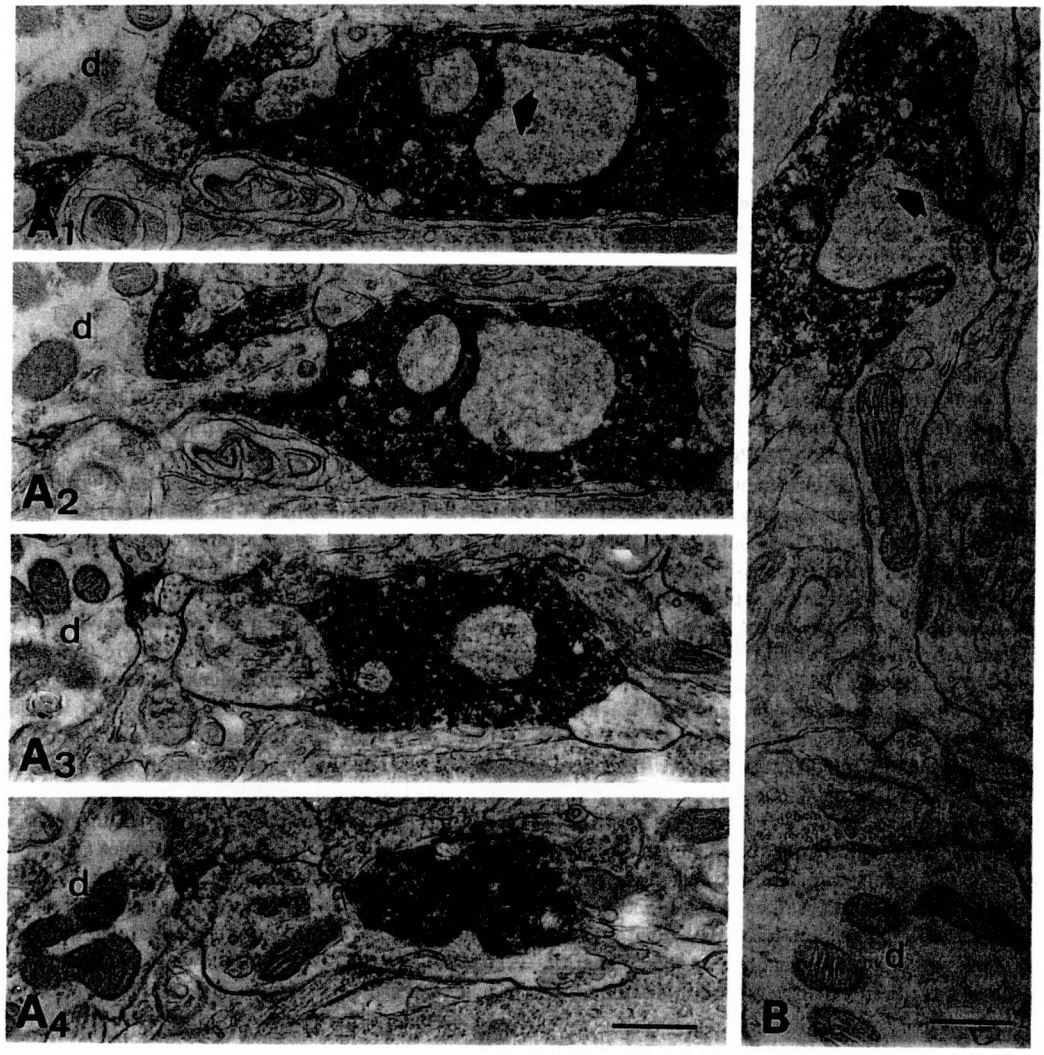
FIGURE LEGENDS

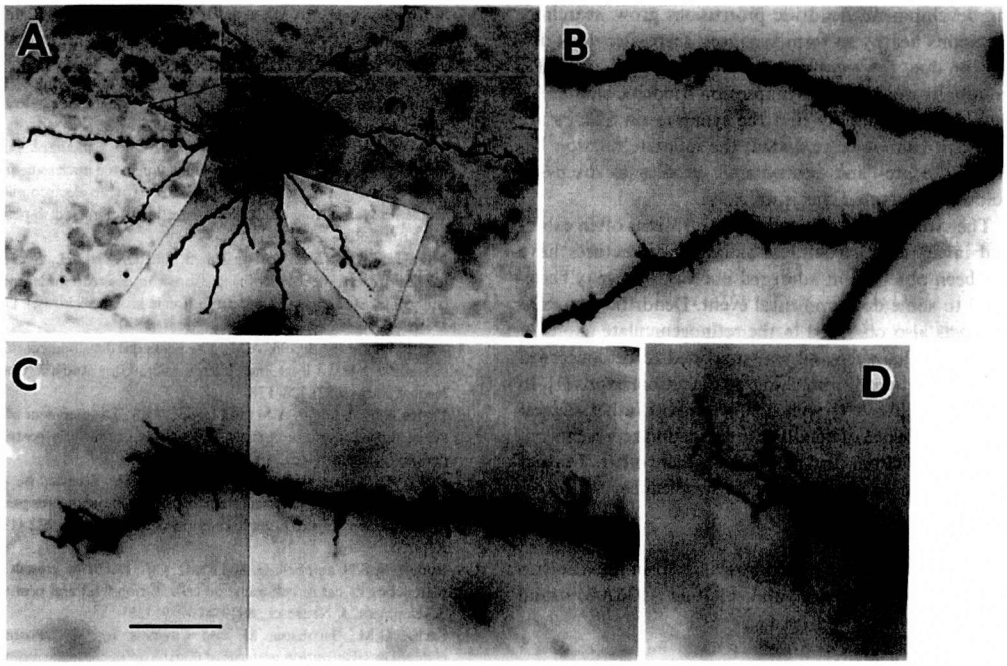
Fig. 1 Electron micrographs of corticorubral synapses in newborn (PND6) kittens. The corticorubral axons were stained with biocytin. In a series of electron micrograph shown in A1-A4, the synaptic bouton has multiple indentations, which are found to be on a part of a spike-like protrusion from a dendritic shaft (d), by examining neighboring

sections. In B the synapse is obviously formed on a part of lengthy spiny profile, which also emerges from a dendritic shaft to the bottom (d). Their continuity was confirmed by an examination of serial sections (not shown). Arrow point to synaptic specializations. Bar = 0.5 μm.

Fig. 2 Photomicrographs of an intracellularly stained rubrospinal neuron of a newborn (PND3) kitten. Following identification by antidromic responses from the spinal cord, the cell was injected with biocytin. A: a photomicrograph of a biocytin-stained rubrospinal neuron. B-D: higher power photomicrographs of proximal (B) and distal (C) dendrites, and dendritic ending (D). Note the presence of numerous filopodia-like protrusions, particularly on distal portion of dendrites. Bar = 100 μm in A and 20 μm in B-D.



Chapter 1, Fig.1



Chapter 1, Fig.2

Chapter 2

Preferential Termination of Corticorubral Axons on Spine-like Dendritic Protrusions in Developing Cat

ABSTRACT

The formation of synaptic contacts is a crucial event during neural development and is thought to be achieved by complex interactions between incoming axons and the neurons in the target. We have focused on spine-like dendritic protrusions (SLDPs), which are transient pleomorphic protrusive structures seen in developing brains. Although the functional significance of SLDPs remains unknown, accumulating *in vitro* evidence suggests that the SLDP plays an important role in synaptogenetic interactions with axons. As a test of this idea, the present study was performed to examine whether the SLDPs are the preferential sites of synapse formation *in vivo*.

The ultrastructure of biocytin-labeled corticorubral (CR) terminals was examined in serial thin sections during the period of synaptogenesis in newborn cats. We found that a major proportion (86%) of the CR synapses was formed on SLDPs. The presynaptic terminals were often invaginated by fine processes extending from the tips of SLDPs. Synaptic structures presumably of cortical origin were also found on SLDPs of HRP-labeled rubrospinal cells, suggesting that SLDPs postsynaptic to labeled CR terminals originate at least in part from rubrospinal cells. Taken together, these results indicate that SLDPs may represent preferred sites of synapse formation and support the notion that SLDPs play a role in synaptogenic interactions during brain development.

INTRODUCTION

During brain development, growth cones navigate through a complex environment to reach their target. Recent studies have revealed various kinds of interactions during growth cone navigation (for review, see Dodd and Jessel, 1988; Goodman and Shatz, 1993; Goodman, 1996), but relatively little is known about what interactions occur within the final target. It is presumed that a cascade of complex events must take place at the target, because not only the presynaptic axons but also the postsynaptic cells must be continuously growing and remodeling (for review, see Jacobson, 1991). Among these events, one of the most important is the interaction associated with synaptogenesis. In this context, dendritic filopodium- or spine-like dendritic protrusions (SLDPs) as well as dendritic growth cones have attracted considerable attention. SLDPs emanate from the dendritic shaft and show pleomorphic morphological features, including filiform structures distinct from the dendritic spines in adults (e.g., Morest, 1969; Scheibel et al., 1973). They also transiently increase in number at an early stage of development (e.g., Morest, 1969; Scheibel et al., 1973; Lund et al., 1977; Boothe et al., 1979; Garey and Saini, 1981; Hammer et al., 1981; Phelps et al., 1983; Dvergsten et al., 1986; Ramoa et al., 1988). These *in vivo* findings suggest that SLDPs are continuously remodeled in development.

Time-lapse studies of developing hippocampal neurons *in vitro* have demonstrated that SLDPs are rapidly remodeled by protrusive activity (Dailey and Smith, 1996; Ziv and Smith, 1996). The *in vitro* observation that SLDPs initiated contacts with axons, leading to the formation of presynaptic bouton-like structures raised the possibility that SLDPs actively initiate synaptogenetic contacts with axons

(Dailey and Smith, 1996; Ziv and Smith, 1996). Consistent with this idea are the finding that synapses occur on filopodial dendritic protrusions in developing brains (Saito et al., 1992).

If the notion that SLDPs play a crucial role in the formation of synapses on the dendrite is correct, SLDPs should be sites of termination for incoming axons. In addition, a specialized synaptic morphology might occur between incoming axons and SLDPs. These notions can be tested in the most straightforward manner by quantitative electron microscopy of identified synaptic profiles.

The present study was performed to analyze quantitatively whether incoming axons form synaptic contacts on SLDPs in a specific manner. To achieve this aim, we examined biocytin-labeled corticorubral (CR) synapses of newborn cats in three-dimensional reconstructions of serial thin sections. We have chosen the CR system of the cat, because there are abundant data about the synaptic organization of the feline red nucleus (RN) (Tsukahara and Kosaka, 1968; Nakamura and Mizuno, 1971; Pizzini et al., 1975; Tsukahara et al., 1975; Nakamura et al., 1978; Murakami et al., 1982; Murakami et al., 1983; Katsumaru et al., 1984; Murakami et al., 1986) and the development of its afferents (Higashi et al. ,1990; Kosar et al., 1985; Murakami and Higashi, 1988; Murakami et al., 1991a,b; Murakami et al., 1993; Song et al., 1995a; Tsukahara et al., 1983; Villablanca et al., 1982); particularly, it is well established that CR fibers in the adult cat rubrospinal cells terminate on the dendritic membrane remote from the soma (Tsukahara and Kosaka, 1968; Tsukahara et al., 1975; Murakami et al., 1982).

In the present study, we show that a major proportion of the CR synapses in newborn cats is formed on SLDPs, presumably originating from rubrospinal cells. These results suggest that SLDPs of rubrospinal cells represent preferred sites of

synapse formation for cortical inputs.

MATERIALS and METHODS

Labeling of corticorubral fibers and rubrospinal cells

Seven kittens at 2 - 5 postnatal days were obtained from a breeding colony of Aburahi Labs (Shionogi and Co., Ltd., Shiga, Japan). Newborn kittens were used because extensive synaptogenesis of the CR inputs appears to occur during the first postnatal month (Higashi et al., 1990; Song et al., 1995a). For injection of biocytin, glass micropipette with a tip diameter of 40-50 μm was connected to a Hamilton syringe with a polyethylene tube. Biocytin (Sigma, St. Louis, MO; 5% in Tris buffer) was pressure-injected into the sensorimotor cortex under anesthesia with sodium pentobarbital (Nembutal; 20 mg/kg, i.p.). The unilateral injection totaling 0.5 - 1.5 μl in each animal was made at one to three loci of the pericruciate cortex; biocytin was injected at two depths (1.5 and 1.0 mm from the pial surface) per locus, 0.25 μl per depth.

The procedure for intracellular staining in newborn cats was detailed previously (Song et al., 1995b). HRP was intracellularly injected into physiologically identified rubrospinal cells by iontophoresis with a glass microelectrode filled with 5-8% HRP (grade II; Toyobo, Osaka, Japan). The injection was made by passing 550-800 msec positive current pulses of 2.0-5.0 nA followed by 40-60 msec negative pulses of 0.5-1.0 nA at 0.8-1.5 s intervals for 10-45 min. One to three rubrospinal (RS) cells were injected in each of four animals that had also been injected with biocytin.

Histological procedures

Immediately after the HRP injection (or 3 d after the biocytin injection in animals that had not received an HRP injection), the kittens were transcardially perfused with a mixture of 1 % paraformaldehyde and 1 % glutaraldehyde followed by 6 % glutaraldehyde under deep anesthesia with Nembutal (> 35mg/kg, i.p.). This was followed by a postwash with 50 mM PBS (pH 7.4) in the HRP-injected animals. The brain was then dissected and kept in 6 % glutaraldehyde or PBS overnight. A brain block including the RN was trimmed and cut horizontally into sections with a Microslicer (Dosaka EM, Kyoto, Japan) at 50 - 80 μm.

Sections containing the RN were first incubated with 0.05 % 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 30 min followed by reaction with a mixture of DAB and 0.015 % H_2O_2 for 30 min at room temperature. Then the sections were processed according to the gold-substituted silver peroxidase intensification method (van den Pol and Gorse, 1986) with a slight modification. In brief, the sections were incubated in thioglycolic acid for 4 hr and then reacted for silver intensification for 8 min. This procedure produced fine granular reaction products which permitted the discrimination of the HRP-injected cells from the biocytin-incorporated CR fibers which had not undergone the intensification procedure.

To visualize the CR fibers, the sections were permeabilized with 0.05 % Triton X-100 in 50 mM Tris-buffered saline (pH 7.6) and processed using the avidin-biotin peroxidase complex method (Vector Laboratories, Burlingame, CA). The sections were post-fixed in 2% OsO_4, stained *en bloc* with 1.5 % uranyl acetate, dehydrated, infiltrated in propylene oxide, and then flat-embedded in Epon (TAAB, Berkshire, England).

Electron microscopy

Fibers were sampled from the magnocellular region of the RN (RMG), which was easily discerned by its darker appearance under the light microscope (Fig. 1B). Epon blocks (500 x 500 μm) including biocytin-labeled fibers were trimmed out from the RMG ipsilateral to the injection site. One block near the center of the RMG or two blocks each from the medial and the lateral parts of the RMG were picked out from three to six sections. In total, four to six blocks were thus obtained from each animal. The block was attached to the top of a cylindrical Epon block and further cut into 5 μm sections. The 5 μm sections were coverslipped with Epon. After detailed light microscopic observations, sections containing labeled fibers with several axonal swellings were arbitrarily chosen and photographed. The 5 μm-thick sections were reattached to other cylindrical Epon blocks. Blocks were trimmed to center the CR fibers that had been chosen in the preceding light microscopic observation and then rephotographed for electron microscopic analysis. They were then cut into ultrathin sections with an ultramicrotome (Reichert-Jung, Ultracut E), collected on Formvar-coated single-slot grids and observed with an electron microscope (1200EX, Jeol, Tokyo, Japan). The light micrographs were referred to to find the corresponding profiles under the electron microscope. To examine whether the selection of axonal swellings described above caused a sampling bias, one 50 μm-thick Epon block containing biocytin-labeled fibers was directly cut into ultrathin sections and collected on single-slot grids for electron microscopy.

HRP-labeled RS cells were drawn using a drawing tube attached to a light microscope (BH2; Olympus, Tokyo, Japan) and reconstructed from 50-80 μm-thick serial sections. The sections were then cut into 5 - 8 μm sections and processed as

described above.

Identification of synapses

Profiles with parallel membranes at putative presynaptic and postsynaptic terminals and dense material in the synaptic cleft were regarded as synapses if they further satisfied one or both of the following criteria (see Vaughn, 1989): (1) synaptic vesicles accumulating at the presynaptic membrane specialization, and (2) postsynaptic membrane specialization and thickening. The presence of synapses was confirmed by observing at least three consecutive sections.

Synapses on HRP-labeled dendrites were often obscured by dense DAB reaction product and, therefore, did not satisfy the latter criterion. However, parallel membranes between the presynaptic and postsynaptic plasma membranes with dense material in the cleft and an accumulation of synaptic vesicles towards the presynaptic membrane could be recognized. Approximately 10 % of the CR synapses did not exhibit an obvious postsynaptic membrane specialization. It is likely that these represent primordial synapses (Hayes and Roberts, 1973; Hinds and Hinds, 1976; Juraska and Fifkova, 1979; Blue and Parnavelas, 1983; Kunkel et al., 1987; Vaughn, 1989). These synapses were, therefore, included in the analysis, although they did not satisfy the latter criterion.

Any postsynaptic profiles containing microtubules were regarded as dendritic shafts, and dendritic protrusions lacking microtubules and with cytoplasmic features described below (see Results) were regarded as SLDPs.

Three-dimensional reconstruction from serial sections

The outlines of the presynaptic and the postsynaptic profiles were traced on sheets of semitransparent paper overlaid on electron micrographs. The tracings were then captured through a video camera onto a hard disk using an image processing device (Olympus TVIP-5100), and reconstructed and edited using a three-dimensional reconstruction program (TRI programs; Ratoc System, Tokyo, Japan).

Quantitative analysis

Three kittens injected with biocytin but not HRP were used for quantitative analysis of synaptic loci on the soma-dendritic membrane of RN cells. Biocytin-labeled fibers in 5 μm-thick Epon blocks were cut into serial thin sections. In total, 41 blocks were analyzed and the total length of the axons analyzed was 4.8 mm. A single presynaptic bouton sometimes formed synapses with multiple postsynaptic profiles; in such cases, the number of synapses was counted as 1 irrespective of the number of active zones formed by the presynaptic bouton.

To estimate the length of SLDPs, their outlines were traced from electron micrographs of serial sections onto sheets of semitransparent paper, which were then superimposed to obtain a two-dimensional projection of the dendritic protrusions. The distance from the dendritic shaft to the ending of the protrusion was then measured to give its length. SLDPs on the HRP-labeled RS cells extending from dendrites < 100 μm from the soma were chosen for analysis, because most CR synapses terminated on SLDPs in this region (see Fig. 8). The diameter of the parent dendritic shaft, D, was estimated from the equation, $D = 2\sqrt{(A/\pi)}$, where A is the cross-sectional area of the dendritic shaft. When the dendrites were cut parallel to their longitudinal axis, the diameter was defined as the maximum length perpendicular to the axis.

Three HRP-labeled RS cells from three kittens were used to estimate the relation between the diameter of the dendritic shaft and the distance from the soma of HRP-labeled RS cells. Five to seven dendrites were selected arbitrarily from each cell and reconstructed with a 60 x objective with the aid of a Neuron Tracing System (Eutectic Electronics, Inc., Raleigh, NC). The ratio of the dendritic surface area occupied by the SLDPs and the shafts was estimated from two dendritic fragments of HRP-labeled RS cell dendrites 2 ~ μm in diameter (see Fig. 8). From a series of electron micrographs taken from serial sections of the dendritic fragments, the circumferences of the SLDP cross-sections and of the dendritic shafts were measured. Then the ratios between the total lengths of the circumferences of the SLDP cross sections and those of the dendritic shafts were calculated for each dendritic segment. These ratios should approximate the ratios of the surface area of SLDP to that of dendritic shafts. The total length of the dendrites analyzed was 33 μm.

All surgery and procedures on animals followed the guidelines of animal experiments approved by the Committee of Osaka University on Animal Research.

RESULTS

Light microscopic appearance of CR fibers

Injection of biocytin was restricted to the sensorimotor cortex as shown in Fig. 1A. Labeled fibers coursed through the internal capsule and extended through the cerebral peduncle. In the RMG region, numerous labeled fibers were observed in contrast to surrounding regions (Fig. 1B,C). These fibers were generally thin (< 0.1 μm) and infrequently bifurcated (Fig. 1C) and often ended in growth cones. Short side branches were occasionally seen (data not shown). Axonal swellings, 0.1 - 1 μm in diameter were observed along the course of the fibers (Fig. 1D, arrowheads), occasionally exhibiting a lighter region in their centers (Fig. 1E).

Synaptic structure of CR axons

Electron microscopic observations of labeled fibers with various light microscopic morphological features revealed that synapses were mostly associated with axonal swellings. As shown in the electron micrographs of Figs. 2 and 3, CR fibers often formed synapses on SLDPs extending from a dendritic shaft (Fig. 2). These SLDPs often protruded into the labeled axonal swellings and were sometimes encapsulated by the swellings (Fig. 3A-D); such invaginated structures were found to correspond to axonal swellings with a lighter central region under the light microscope (see Fig. 1E). Three-dimensional reconstruction of CR synapses revealed that dendritic protrusions sometimes branched in a complicated manner (see Fig. 3E). Unlabeled axon terminals were also found to be invaginated by dendritic protrusions (data not shown).

The cytoplasm of SLDPs usually contained vesicular structures (Figs 2, 3, arrowheads). The SLDPs were frequently associated with smooth endoplasmic reticulum (Fig. 2A,B, small arrows), occasionally with multivesicular bodies, but the spine apparatus (Peters et al., 1991), was rarely observed. Mitochondria were occasionally found, most of them being restricted to the necks or proximal parts of the protrusions (see Fig. 3). Polyribosome-like granules were also observed in the heads or tips of the protrusions (data not shown).

Localization of CR synapses

To examine the localization of CR synapses on the soma-dendritic membrane of RN cells, a quantitative analysis was performed. In total, 76 synapses were analyzed from sets of serial sections cut from 5 μm-thick sections in three kittens injected with biocytin but not HRP. In all of the three kittens, most of the synapses were found on SLDPs (Fig. 4A-C). Moreover, a similar result was obtained from a block that was directly cut into thin sections (Fig. 4D), suggesting that selection of axonal swelling-rich 5 μm sections (see Materials and Methods) did not affect the result. Eighty-six percent of the CR synapses analyzed in newborn kittens occurred on SLDPs. Of these CR synaptic endings on SLDPs, ~ 35 % (36 of 103 synapses) were invaginated by SLDPs, forming complex synaptic structures. The length of SLDPs on which CR synapses were formed ranged from 0.28 to 3.87 μm with a mean of 1.10 μm (n = 31). Together, these findings indicate that most CR fibers in newborn cats form synapses on SLDPs.

Dendritic protrusions on RS cells

Next we analyzed the morphology of RS cell dendrites and the synapses on these dendrites to determine whether SLDPs that bear CR synapses originate from RS cells. HRP-filled RS cells in newborn cats elongated dendrites, up to 500 μm in length, infrequently ending in growth cone-like bulbous endings (data not shown). As shown in Fig. 5, numerous SLDPs, up to 2 μm in length, emanated from these dendritic shafts extending from RS cells. The protrusions were notable on proximal dendrites and those somewhat remote from the soma; they varied in both length and shape; some exhibited filiform shapes whereas others had bulbous heads. Electron microscopic observation confirmed that synapses were formed on RS cell SLDPs (Fig. 6). As shown in the high-power electron micrographs of Fig. 6, C and D, synapses often occurred on HRP-labeled SLDPs (Fig. 6C, D, arrows) which in some cases branched in a complicated manner. Synapses were also found on SLDPs extending into presynaptic axonal terminals (Fig. 7). The length of the SLDPs that received synaptic contacts ranged from 0.42 to 2.71 μm, with a mean of 1.09 μm ($n = 24$). These values are not statistically different from the length of the unlabeled SLDPs which were postsynaptic to CR terminals (Mann-Whitney U-test, $p > 0.1$), suggesting that CR axons terminate on the SLDPs of RS cells.

We then compared the surface areas of SLDPs and the dendritic shafts to assess the possible contribution of the surface area to the specific termination of synapses on SLDPs. From a series of electron micrographs taken from serial sections of the dendrites, the ratio of the dendritic surface area occupied by the SLDPs to that of the shafts was estimated. Two fragments of HRP-labeled RS cell dendrites with a diameter of ~ 2 μm were chosen for this analysis, because these corresponded to the diameters of dendrites on which most CR synapses were formed (see Fig 8). The

ratios of the total length of the circumference of SLDPs to that of the shafts for the two dendrites analyzed were 1.69 and 2.16, which are far less than the ratio of synapses on SLDPs and dendritic shafts (86:14 ≈6.1).

Location of SLDPs on which CR synapses are formed

To assess how far the SLDPs are from the soma, the dendritic diameter of HRP-labeled cells was plotted against the distance from the soma. As shown in Fig. 8, *left panel*, the diameter of dendrites decreases as the distance from the soma increases. In addition, by analyzing serial thin sections of CR fibers, we estimated the diameter of parent dendrites of SLDPs that received CR synapses. The diameter ranged from 0.50 to 5.73 μm with a mean of 2.15 μm (n = 41) (histogram in Fig. 8). A comparison of the diameter of the dendrites from which SLDPs bearing CR synapses emanated (histogram in Fig. 8) with that of HRP-labeled cells indicated that CR fibers formed synapses on SLDPs originating from the proximal dendrites (< 100 μm from the soma).

Synapses on somatic membrane

We also examined synapses on the somatic membrane of HRP-labeled RS cells to compare the synaptic organization (Fig. 9). As shown in Fig. 9A, the perimeter of the soma was rugged in contour and synaptic terminals often penetrated into or were engulfed by the soma (Fig. 9B,C). Although there appeared to be protrusive structures on the somatic membrane, most of them were not associated with synapses, implying that they resulted from synaptic bouton penetration into the somatic membrane. Taking into account of the present results that CR synapses were virtually absent on the somatic membrane, these results raise the possibility

that SLDP's play a role in the specific termination of CR axons on the dendritic membrane.

DISCUSSION

The present analysis of serially reconstructed CR synapses has revealed that CR synapses in neonatal cats are formed preferentially on SLDPs of the RN neurons. Such protrusions often invaginated into CR terminals, forming complex synaptic structures.

The origin of postsynaptic dendritic protrusions

Although CR fibers terminate on inhibitory interneurons (Katsumaru et al., 1984) in addition to RS cells in adult cats, it is highly likely that the SLDPs postsynaptic to CR fibers included RS cells. In fact, in two cases, we could follow the postsynaptic profiles in serial sections from the SLDPs back to the somata, the diameters of which were > 20 μm. The fact that the diameter of interneurons is < 20 μm even in the adult cat (Katsumaru et al., 1984) suggests that these postsynaptic profiles belong to RS cells. Furthermore, the finding that HRP-labeled RS cells had numerous SLDPs with comparable lengths (Figs. 5, 6) is consistent with the view that RS cells are the target of CR fibers. Moreover, our preliminary analysis of the relation of SLDP length with the difference between the length of SLDP head and that of the neck or the ratio between them indicated that there is no difference in morphology between SLDPs postsynaptic to labeled CR fibers and the SLDPs of labeled RS cells. Together, these findings indicate that SLDPs postsynaptic to labeled CR axons included those of RS cells.

Localization of CR synapses on dendritic protrusions

Sérial reconstruction of synapses in the present study unequivocally demonstrated that most of the CR synapses were formed on SLDPs directly emanating from dendritic trunks. This is consistent with the light microscopic observation that dendrites ending in growth cones occurred only rarely. Together, these findings indicate the CR axons terminate on filopodial extensions protruding from the dendritic shaft.

The result that most CR synapses were formed on SLDPs (Fig. 4) indicates that SLDPs may represent the preferred synaptic sites for CR axons. The presence of numerous SLDPs on the dendritic surface (Figs. 5, 6), however, raises the possibility that the localization of CR synapses on SLDPs simply resulted from the larger surface area of the SLDPs compared with that of the dendritic shafts. The estimated ratio between the dendritic surface areas occupied by the SLDPs and the shafts (1.69 and 2.16), however, was much smaller than that of the number of CR synapses on the SLDPs compared with those on the shafts (6.1; Fig. 4). Thus, the preferential localization of CR synapses on SLDPs can only partially be explained by the larger surface area of the SLDP compared with the dendritic shaft, and raises the possibility that there are some other mechanisms that promote the preferential termination of CR axons on the SLDPs.

Role of SLDPs in synaptogenesis

It is likely that the SLDP represents a dynamic feature of the dendrite, because 1) vesicular structures in SLDPs that were associated with smooth endoplasmic reticulum (Figs. 2, 3), were often observed in the growing tips of axons (Peters et al., 1991); 2) invaginated synapses were rarely observed in adult cat RN

(Murakami et al., 1982); 3) spine-like profiles on large-sized RN neurons appear to decrease in number during the first month of postnatal development (Sadun and Pappas, 1978). The idea that SLDPs dynamically change their structures gains further support from recent time-lapse studies of dissociated neurons and of slice preparations of the hippocampus, which demonstrated that SLDPs are indeed rapidly remodeled (Cooper and Smith, 1992; Dailey and Smith, 1996; Ziv and Smith, 1996).

These findings together with the preferential termination of CR synaptic endings on SLDPs raise the possibility that SLDPs play some role in ongoing synaptogenetic interactions between presynaptic and postsynaptic elements by dynamically changing their structures. Our previous finding that CR axons elaborate arbor during the first month of postnatal development (Higashi et al., 1990), whereas SLDPs decrease in number in the same period (Sadun and Papas, 1978) may be taken to indicate that SLDPs disappear after synaptogenic interactions with CR axons (see below). Moreover, the notion that SLDPs play a role in synaptogenetic interactions is consistent with observations of hippocampal neurons in dissociated culture, in which filopodial extension occurs from the dendritic membrane (Cooper and Smith, 1992; Ziv and Smith, 1996), and the dendritic filopodia seem to initiate physical contact with nearby axons (Cooper and Smith, 1992; Ziv and Smith, 1996). It was also shown that the presumed synaptic site, as indicated by a fluorescent dye that labels synaptic vesicles, occurred in association with dendritic filopodia (Ziv and Smith, 1996), which is consistent with the present results.

What could be the mechanism that allows CR axons to terminate preferentially on SLDPs? One possibility is that SLDPs attract incoming axons to

form synapses (Wong et al., 1992; Papa et al., 1995). In fact, chemoattraction of axons has been shown to play an important role in the guidance of the commissural axons of the spinal cord (Tessier-Lavigne et al., 1988; Kennedy et al., 1994) and the hindbrain (Shirasaki et al., 1995; Tamada et al., 1995) during development. On the other hand, a number of studies have indicated that afferents regulate dendritic morphology (e.g., Rakic, 1975; Kimmel et al., 1977; Caceres and Steward, 1983; Baptista et al., 1994), leading to the hypothesis that incoming axons induce the growth of SLDPs from dendrites (see Vaughn, 1989). Another possibility is that SLDPs dynamically change their structure (see below), thereby increasing the chance of the incoming CR fibers encountering SLDPs. Elucidation of the role of SLDPs, however, awaits further studies.

The present findings raise another intriguing possibility that SLDPs contribute to the establishment of synaptic site specificity. Rubrospinal neurons also receive inputs from the interpositus nucleus of the cerebellum (Toyama et al., 1970; Tsukahara et al., 1975) and, in the adult cat, the cerebellar inputs impinge on the somatic membrane of RN cells. These inputs arrive in the RN before embryonic day 35 (Song et al., 1995a), far earlier than the entry of cortical inputs and it is likely that the dendrites of RN cells are only poorly developed at this stage of development. Subsequently, the dendrites may elongate and SLDPs develop. The presence of numerous SLDPs at the developmental stage when CR axons arrive at the RN could facilitate the dendritic termination of CR axons, whereas the virtual absence of filopodial extensions, associated synapses on the somatic membrane might provide little chance for somatic termination (but see Povlischock, 1974).

The fate of synapses on SLDPs

In hippocampal preparations of developing neurons of the rat, motile filopodia decreased in number over extended culture periods, whereas dendritic spines concomitantly increased in number, leading to the speculation that dendritic filopodia may be withdrawn into the dendritic shaft or may evolve into spines after synapse formation (Dailey and Smith, 1996; Ziv and Smith, 1996; see also Hammer et al., 1981 and Saito et al., 1992). RS cells in adult cats have spines on their dendrites at distances in excess of 300 μm from the soma (Wilson et al., 1987) and our preliminary electron microscopic study of the adult cat revealed the presence of CR synapses on dendritic spines (Y. Saito and F. Murakami, unpublished observations). These findings suggest that at least a certain proportion of SLDPs transforms into mature spines. However, SLDPs on RN cells decreases in number with maturity of the dendrite (Sadun and Papas, 1978; C. J. Wilson, F. Murakami and Y. Saito, unpublished observation), indicating that retraction of SLDPs on RS cells would also occur. The presence of SLDPs without synapses (data not shown, see also Papa et al., 1995) implies that those that failed to make synapses eventually retract.

In conclusion, in the present *in vivo* study, we demonstrated what cannot be shown with *in vitro* techniques: Preferential termination of synapses on spine-like dendritic protrusions. Taken together with previous *in vitro* studies that demonstrated dynamical features of SLDPs, our results suggest that SLDPs may commonly play an active role in *synaptogenic* interactions.

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FIGURE LEGENDS

Fig. 1 Identification of corticorubral fibers.

A, Injection site of biocytin. A sagittal section of the sensorimotor cortex. The *arrow* indicates the cruciate sulcus. r, rostral; d, dorsal B: Low-magnification photomicrograph of a horizontal section of the RN. Asterisks indicate the oculomotor nerve. c, caudal; l, lateral C, High-magnification photomicrograph of the area of the RN outlined by the rectangle in B. Many biocytin-labeled fibers can be seen. D, E, Higher magnification photomicrographs of biocytin-labeled fibers. Axonal swellings are seen along the fibers (arrowheads). Some of the axonal swellings were fenestrated as shown in E. Scale bars : A, 500 μm; B, 200 μm; C, 50 μm; D, 4 μm; E, 2 μm.

Fig. 2 Biocytin-labeled CR synapse on an SLDP. A, B, Electron micrographs from semiserial sections of a CR fiber. The CR fiber formed synapses (black arrow) on an SLDP emanating from a dendritic shaft (asterisk). SLDPs often contained vesicular structures (arrowheads) and smooth endoplasmic reticulum (small arrows). C: Low-magnification photomicrograph showing a filiform process emerging from a dendritic shaft (asterisk). Scale bars: A, B, 0.5 μm; C, 1 μm.

Fig. 3 Biocytin-labeled CR axon terminal invaginated by a SLDP. A-D, Selected-serial electron micrographs of a CR fiber. A, Low-magnification electron micrograph of a synapse-bearing dendritic protrusion. B-D, High-magnification electron micrographs of serial sections showing an SLDP encapsulated by a CR synaptic ending. The asterisk shows the dendritic shaft. Arrows point to a synapse. Note that many

vesicles can be seen within the SLDP (arrowheads). A single CR terminal rarely contacted both the SLDP and the dendrite. E, Three-dimensional reconstruction of CR axon terminal. Profiles shown in tan and green represent the dendrite and the axon terminal, respectively. Other protrusions except for the one shown here were omitted for clarity. The protrusion has multiple branches and invaginates into the synaptic terminal. The left and the center panels show the side view and the right panel is top view of the invaginated CR terminal. Scale bars: A, 1 μm; B-D, 0.5 μm.

Fig. 4 Localization of CR synapses on SLDPs. White and shaded bars represent the number of synapses on SLDPs and dendritic shafts, respectively. A-C, Data from individual kittens. A 50 μm-thick Epon block was cut into 5 μm-thick sections and the areas with abundant axonal swellings were selected for thin sectioning. The lengths of the CR fibers analyzed in A, B and C are 1.1, 1.4, and 2.3 mm, respectively.
D, Graph from a 50 μm-thick block directly cut into serial thin sections. In each case most of the synapses are found on SLDPs.

Fig. 5 SLDPs emanating from a dendrite of an HRP-labeled RS cell. A, Drawing of an RS cell in a newborn kitten. The cell was reconstructed from 50 μm-thick horizontal sections. The arrow shows the axon. B, C, Photomicrographs of the dendritic areas indicated by the two squares and letters in panel A. The photomicrographs in B and C correspond to the areas outlined by b and c, respectively. Note that numerous protrusions extend from the dendritic shaft. Scale bars: A, 50 μm; B, C, 5 μm.

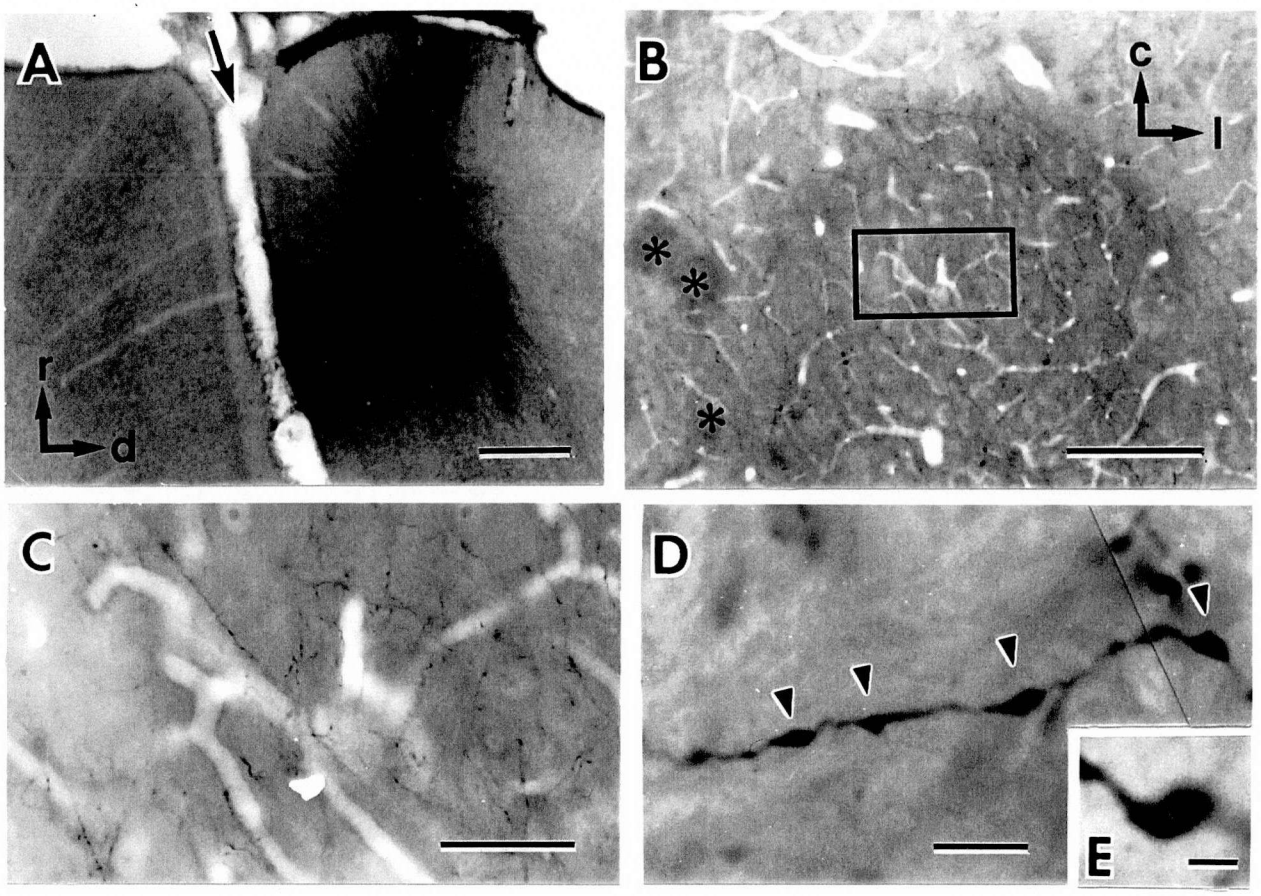
Fig. 6 Synapses on SLDPs extending from an HRP-labeled RS cell dendrite. A, Photomicrograph of an RS cell dendrite. B, Low-magnification electron micrograph of the dendrite shown in A. Arrowheads point to labeled SLDPs. C, D, SLDPs emanating from the dendritic shaft form synapses (arrows) with unidentified endings. High-magnification electron micrographs of selected serial sections from the two areas outlined by rectangles and letters in B. C and D correspond to the areas labeled by c and d in B, respectively. Granular profiles in HRP-stained dendrites are reaction products caused by the intensification procedure (see Materials and Methods). Scale bars: A , 5 μm; B, 2 μm,; C, 0.5 μm; D, 0.25 μm.

Fig. 7 SLDPs of an HRP-labeled RS cell invaginating into an axon terminal. Asterisks show the dendritic shaft from which SLDPs emanate. The black arrow shows the synaptic terminal. A-H, Electron micrographs of selected serial sections. I: Three-dimensional reconstruction of the presynaptic axon terminal and the SLDP. See legend of Figure 3 for detail. Scale bar, 0.25 μm.

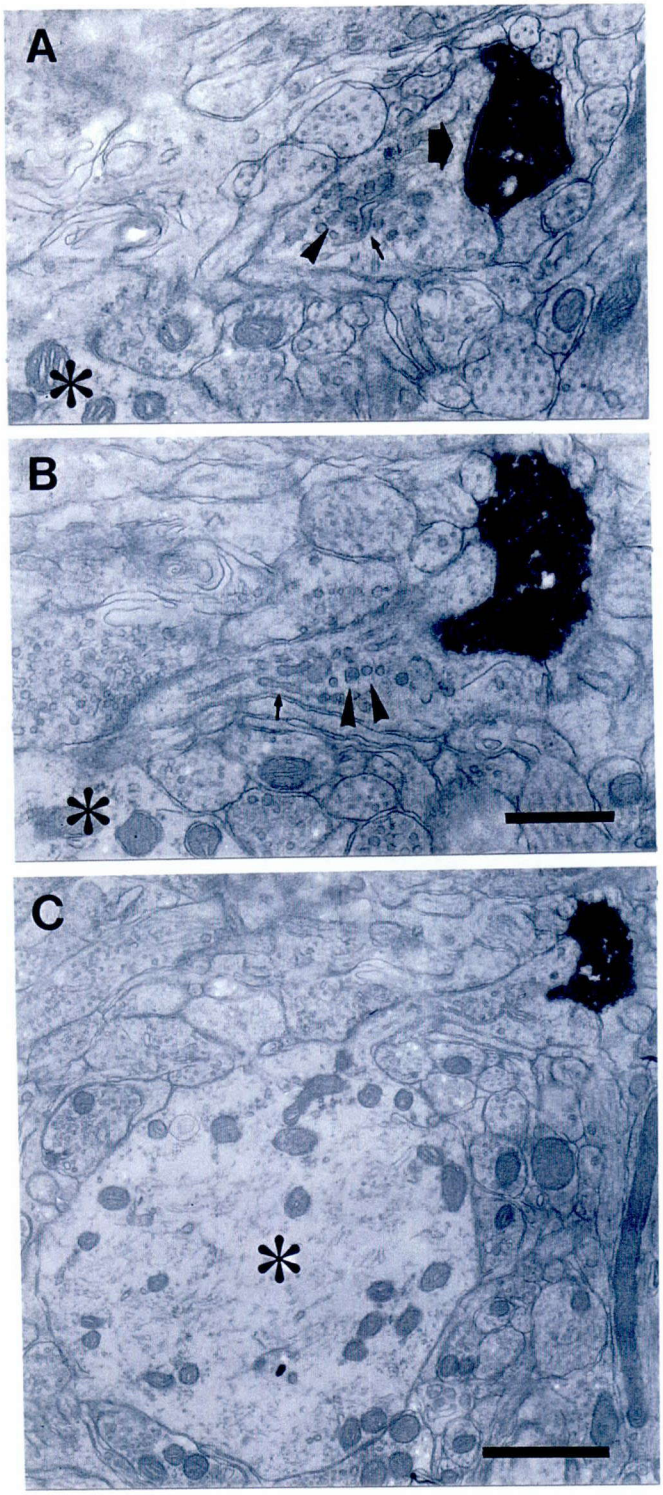
Fig. 8 CR synaptic sites on the soma-dendritic membrane of RS cells. The graph to the left shows the diameter of HRP-labeled RS cell dendrites (n = 17) plotted against the distance from the soma. The histogram on the right shows the distribution of the diameter of the dendrites from which synapse-bearing SLDPs emanated (n = 41). Comparison of the two graphs demonstrates that most of the synapses were located on dendritic regions < 100 μm away from the soma.

Fig. 9 Synapses on the somatic membrane of RS cells. A, Electron micrograph of an

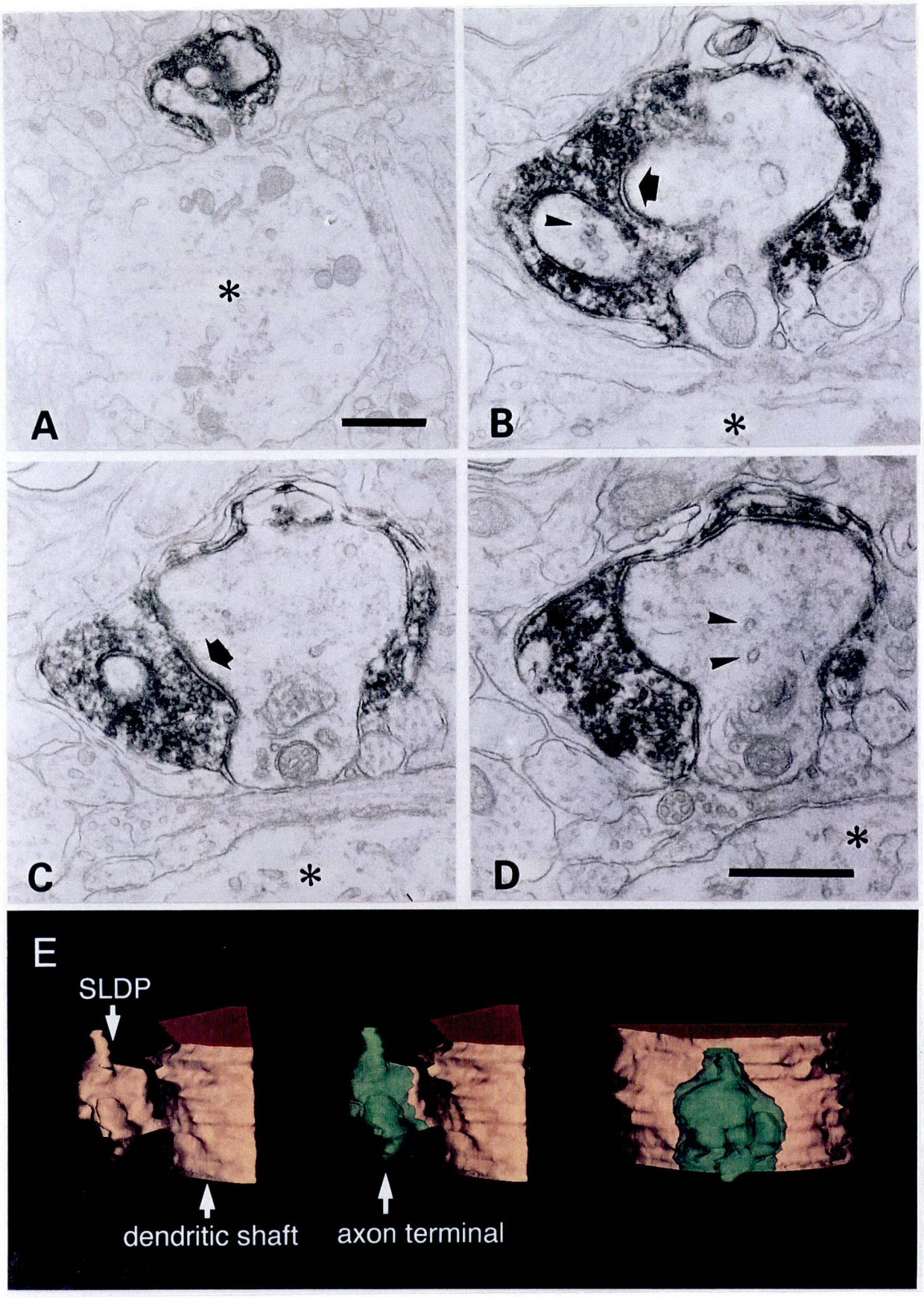
HRP-filled RS cell. B, Higher magnification of the area outlined by the rectangle in A. Note the presence of inclusions of synaptic endings in the soma. C, Synapses formed by terminals included in the soma (arrows). Similar inclusions were observed in nonstained soma, indicating that these are not artifacts of the HRP injection (not shown). Scale bars: A, 5 μm, B, 1 μm, C, 0.5 μm.



Chapter 2 Fig.1



Chapter 2 Fig.2



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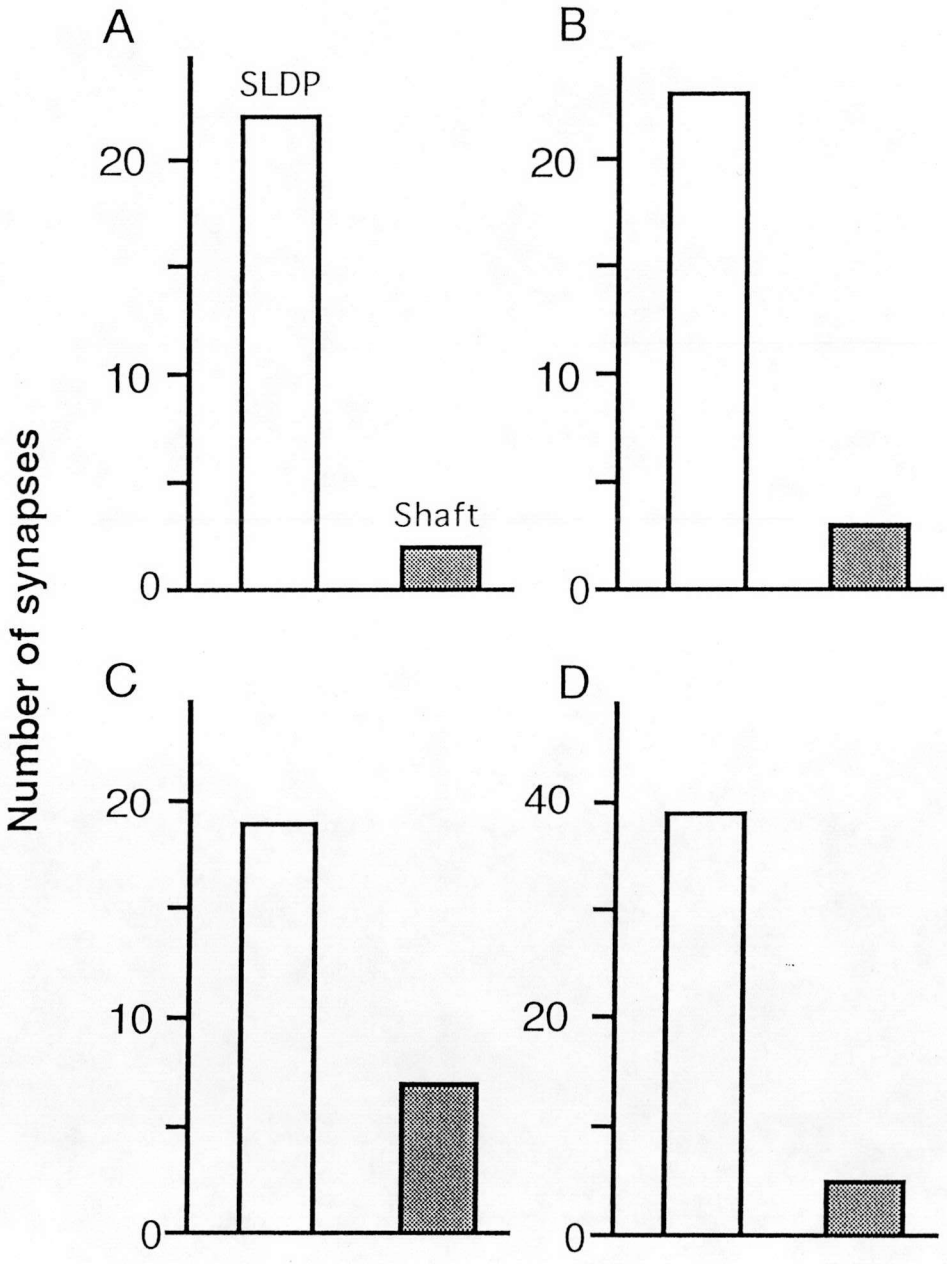

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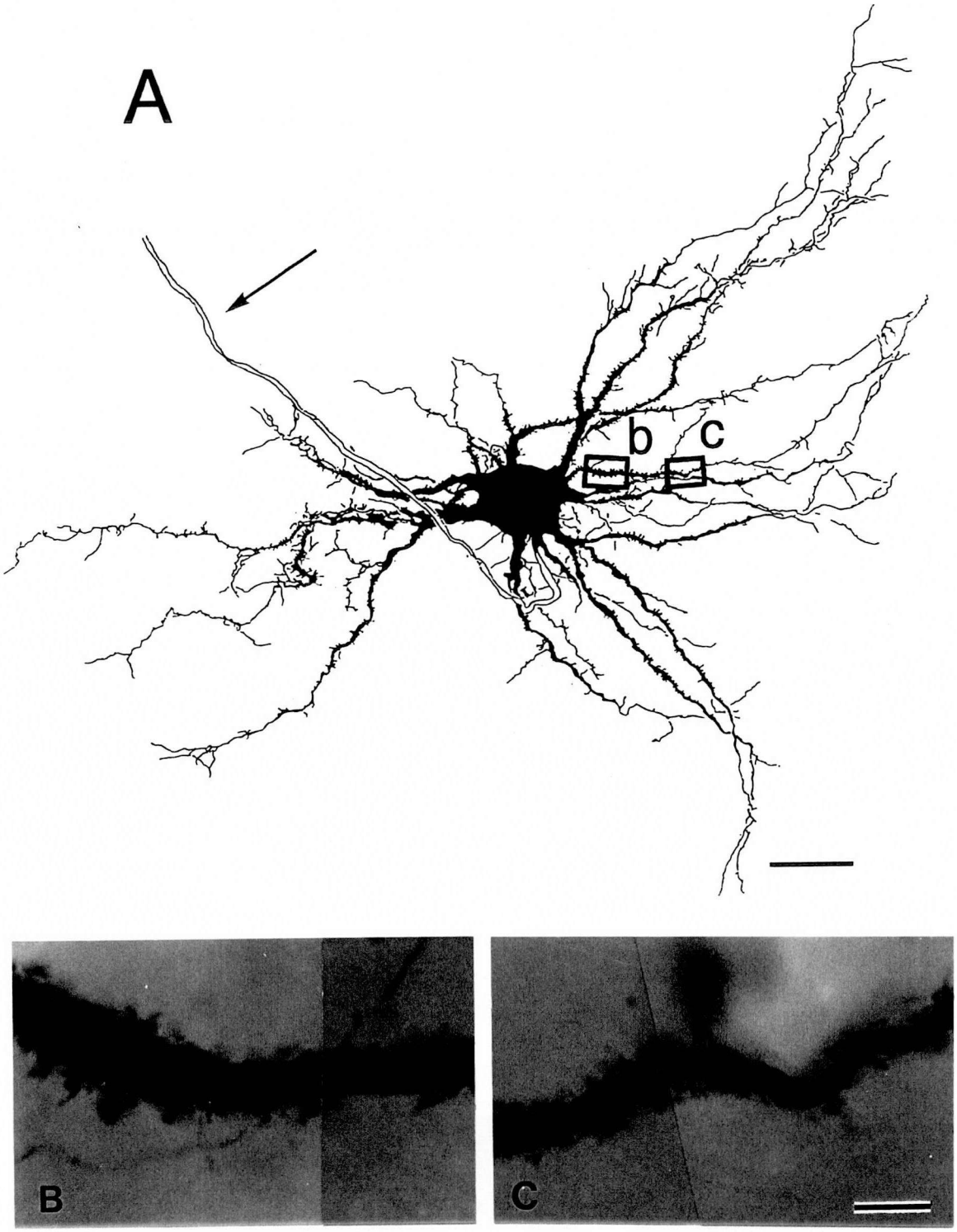

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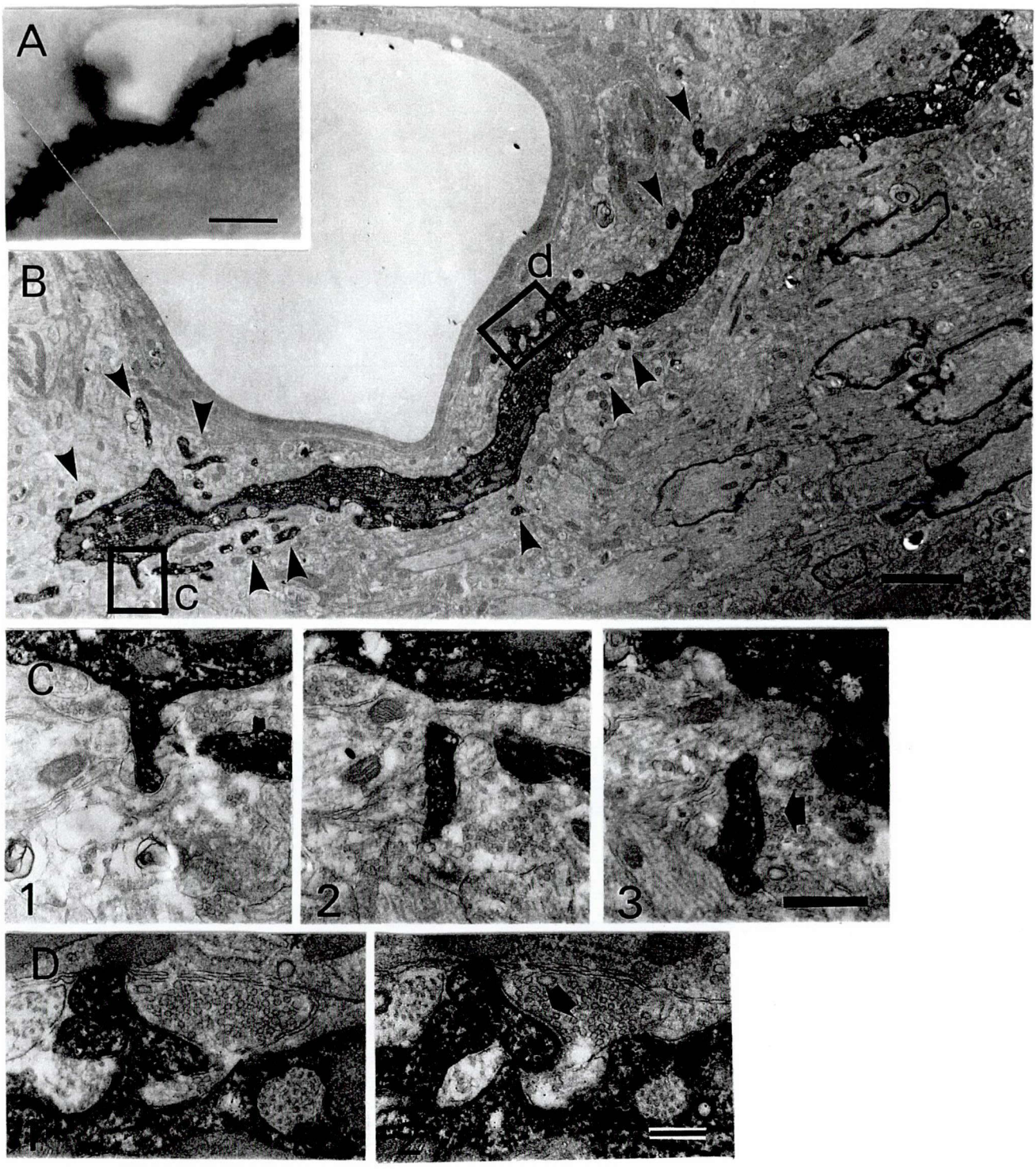

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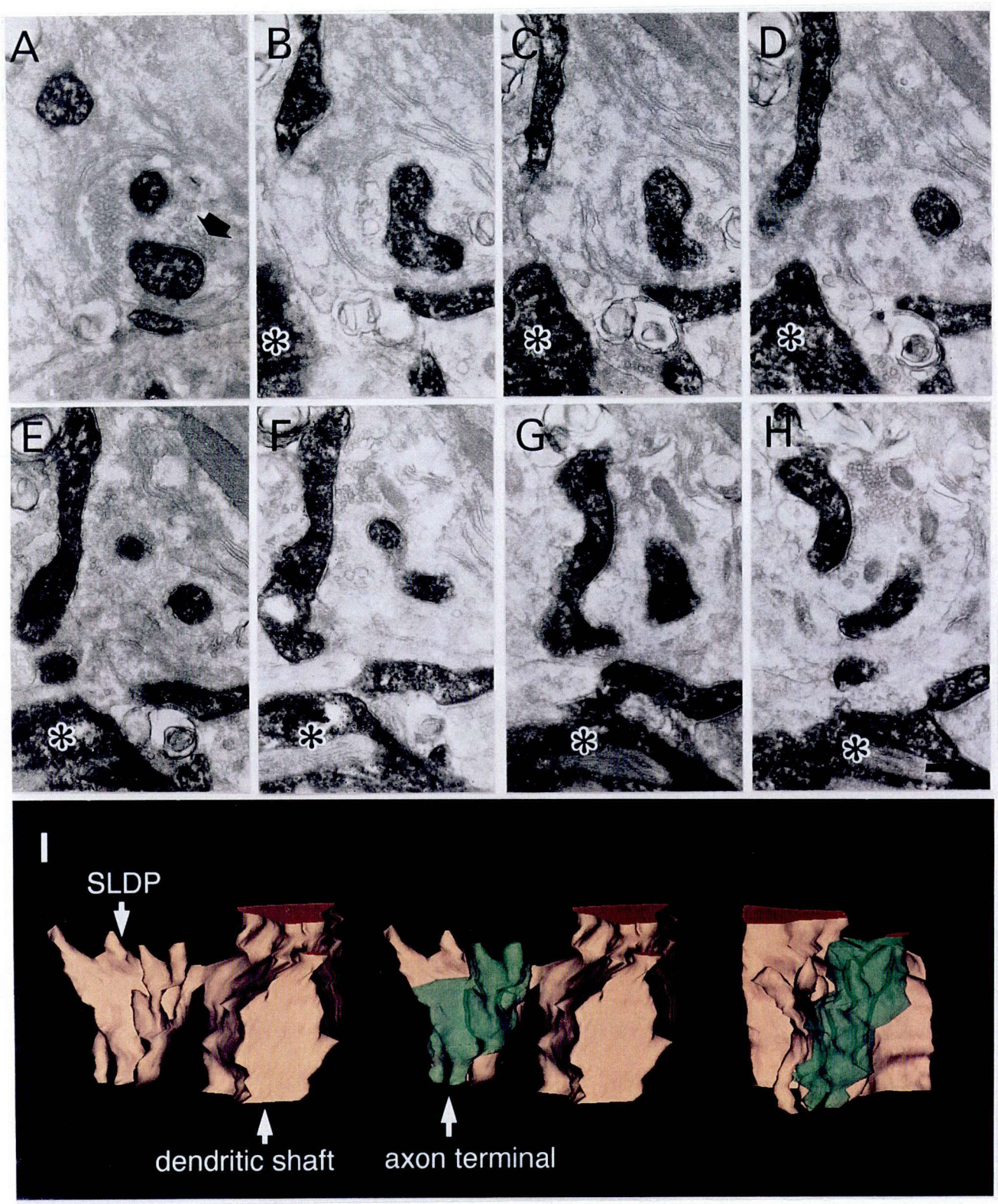

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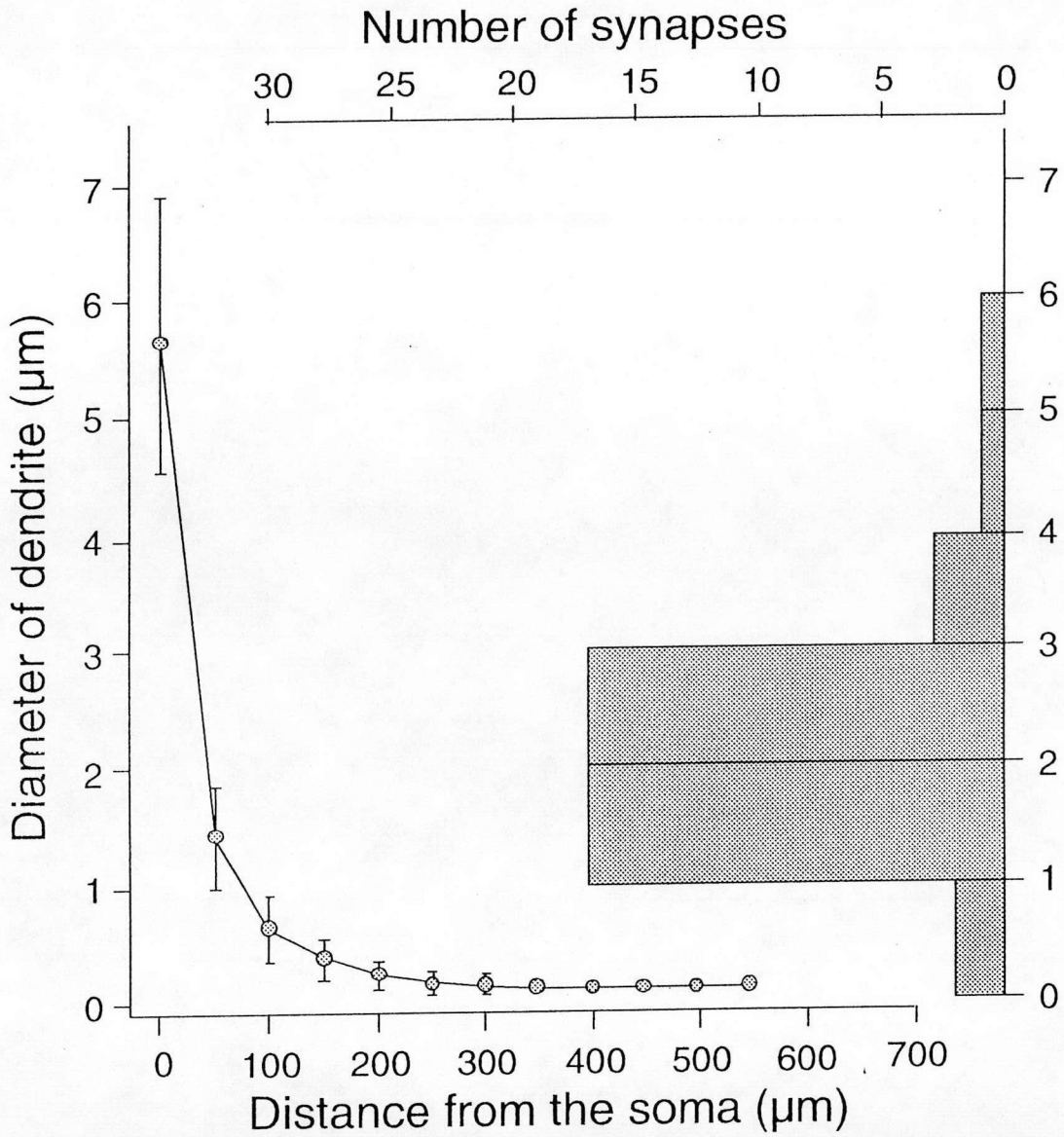

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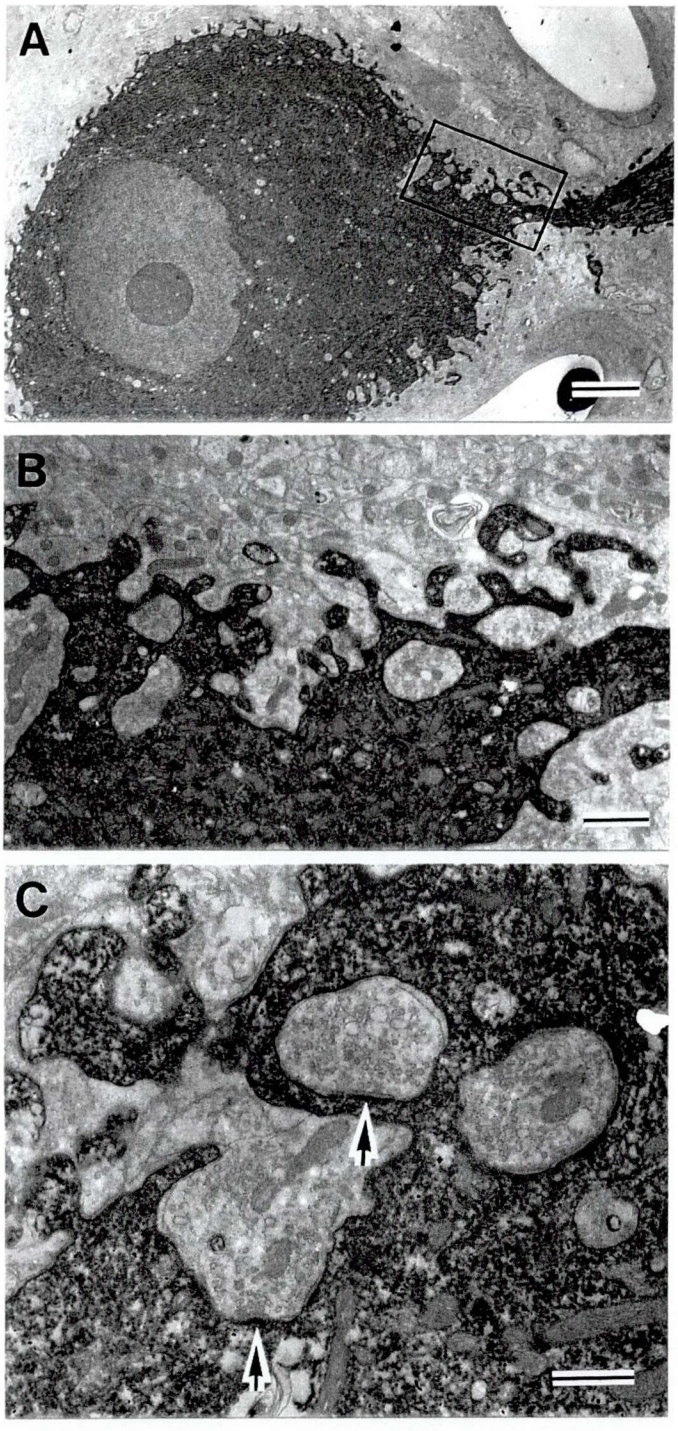

Chapter 2 Fig.9

Chapter 3

Synapses formed by ectopic corticofugal axons: an electron microscopic study of crossed corticorubral projections in kittens

ABSTRACT

The corticorubral projection in newborn kittens are bilateral, while the projections are unilateral in adults. We addressed the question whether or not the crossed corticorubral projection in kitten forms synaptic contacts in the red nucleus. The neurons in the sensorimotor cortex of the kitten were labeled by *Phaseolus vulgaris*-leucoagglutinin or biocytin. Electron microscopic observations revealed that corticofugal axons form synapses both in the contralateral and the ipsilateral red nucleus; most of them were on small postsynaptic profiles, possibly dendritic spines or distal dendrites.

INTRODUCTION

Neuronal connections in higher vertebrates undergo remarkable refinement during development (Purves and Lichtman, 1985). This is thought to be largely attained by cell death, and elaboration and elimination of axon collaterals (Cowan et al., 1984). Although the mechanisms underlying these changes remain unclarified, evidence has been accumulating that activity-dependent neuronal interactions operate as a mechanism for the refinement (Constantine-Paton et al., 1990; Hebb, 1949). It appears to be well accepted that synapses on inappropriate targets fail to stabilize and eventually disappear due to insufficient correlated activity of pre- and postsynaptic neuronal elements (Brown et al., 1990; Changeux and Danchin, 1976; Stent, 1973).

However, it has not been well established whether or not axons ending in inappropriate areas make synaptic connections in development. Corticorubral projections in the cat provide an excellent substrate for studying this issue; the projections undergo lateralization and topographic refinement during postnatal development (Higashi et al., 1990) and can be easily traced by injecting a limited amount of tracers into the sersori-motor cortex (Higashi et al., 1990; Murakami and Higashi. 1988). The present study was performed to elucidate whether or not crossed (ectopic) corticorubral axons in kittens make synaptic contacts with the target cells.

MATERIALS AND METHODS

Kittens aged from postnatal day (PND) 2 to 7 (n=5) were anesthetized with Nembutal (25 mg/ kg, i.p.) and either *Phaseolus vulgaris*-leucoagglutinin (PHA-L)(Vector Labs) or biocytin (Sigma) was injected unilaterally into the lateral part of the anterior and posterior sigmoid gyrus. Biocytin (10% in Tris buffer) was pressure-injected using a Hamilton syringe into three loci of the cortex in a total of 1 μl. The methods of PHA-L injection were previously described (Murakami and Higashi, 1988). After appropriate survival (8-15 days for PHA-L-injected animals and 3-4 days for biocytin-injected animals), the kittens were transcardially perfused either with phosphate-buffered saline followed by 2% paraformaldehide (PA) and 2.5% glutaraldehyde (GA), or 1% PA and 1% GA followed by 6% GA, under deep anaesthesia with Nembtal. After leaving the brain *in situ* for 1h, the red nucleus-

containing region was blocked and cut near horizontally with a Microslicer (Doska EM) at 50-70 μm. Then the sections were processed as detailed previously (Higashi et al., 1990). In brief, they were incubated in avidin-biotin peroxidase complex (Vector Labs), with or without (for a biocytin-injected animal) pretreatment by anti-PHA-L serum (Vector Labs), and reacted with diaminobenzidine tetrahydrochloride. They were further fixed in osmium tetroxide, stained *en bloc* with 1.5 % uranyl acetate, dehydrate and flat embedded in Epon (TAAB). The sections were observed and photographed with a light microscope (Olympus BH2) and then ultrathin sections were cut and examined with an electron microscope (Jeol 1200EX). Diameter of postsynaptic profiles (D) were estimated from electron micrographs based on the following equation: $D = 2 \sqrt{A / \pi}$ where A is the cross-sectional area of the postsynaptic profiles. This parameter was introduced since the postsynaptic profiles were often irregular in shape (see below).

RESULT

At the light microscopic level, crossed as well as uncrossed corticorubral axons exhibited simple morphology with infrequent branching in agreement with our previous observation (Higashi et al., 1990). They were undulated in contour in most part. No difference was noted between PHA-L- and biocytin-labeled axons. Electron microscopy, which was performed with 3 of the animals, demonstrated that labeled axons in the contralateral red nucleus exhibited contacts with typical synaptic

morphology. Shown in Fig. 1 are electron micrographs of the corticorubral synapses in the red nucleus contralateral to the injections; Fig. 1A-C are from a PND 7 animal and D and E are from a PND 17 animal. In each panel it is possible to discern synaptic cleft and postsynaptic density. In some instances, it was possible to recognize accumulation of synaptic vesicles at the presynaptic membrane (e.g. Fig. 1C). It was noted that most of the postsynaptic profiles were irregular in contour and small in the area of cross-section, suggesting that the synapses are formed on distal dendrites or dendritic spines. In the example shown in Fig. 1D, examination of neighboring sections revealed that the synapse was formed with a spine-like protrusion. Synapses formed by uncrossed corticorubral axons were also examined with an electron microscope, but no difference in morphology was noted between the synapses in the red nuclei on both sides. Interestingly, the synapses on both sides showed similar localization on soma-dendritic membrane of red nucleus cells. This is illustrated in Fig. 2, in which the number of the synapses is plotted against diameter of the postsynaptic profiles. The corticorubral synapses in the contralateral red nucleus (Fig. 2A) as well as the ipsilateral red nucleus (Fig. 2B) were mostly formed on postsynaptic profiles with very small cross sectional areas.

DISCUSSION

The present study unequivocally demonstrated that crossed corticorubral axons in kittens make synaptic contacts with the target cells. Considering that the presently

utilized tracers clearly label corticorubral neurons along their trajectories (Murakami and Higashi, 1988; Higashi et al., 1990; Murakami et al, 1991), there is no doubt that the labeled profiles are axonal endings of corticorubral cells. Since there appears to be no crossed corticorubral axons in adult cats (Rinvik and Walberg, 1963; Fujito et al., 1983, but see ref. Fisher et al., 1988), the synapses formed by the crossed axons may be transitory.

Shatz and Kirkwood recorded extracellular unit activity of the cells in the lateral geniculate nucleus by stimulating the optic nerves in isolated diencephalon preparation of fetal cats (Shatz and Kirkwood, 1984). The cells responded to stimulation of the optic nerves on the both sides, good evidence suggesting that optic nerve axons make functional synaptic contacts with inappropriate targets if the responses were indeed monosynaptic as the author suggested (analyzing extracellular unit response, however, generally provides insufficient information for determining monosynaptic nature of the responses). The present study provides more convincing evidence for the presence of the transitory ectopic synapses than their studies in that postsynaptic structure, i.e. the red nucleus, can be clearly identified as an isolated structure even in newborn kittens (Song and Murakami, 1990). The synapses formed by crossed corticorubral axons appear to be functional, since stimulation of the sensorimotor cortex elicited excitatory postsynaptic potentials, contralaterally as well as ipsilaterally, in red nucleus neurons of the kitten (Murakami et al., 1991). This is in accord with the view that ectopic projections are eliminated by an activity-dependent mechanism (Stent, 1973).

The present results indicate that crossed corticorubral axons form synapses on red nucleus neurons, although they fail to form complex terminal arbors (Higashi et al.,

1990). The transitory crossed axons are peculiar in that they are not somototopically arranged (Murakami et al, 1991) unlike the uncrossed ones (Higashi et al., 1990). It is quite interesting that they nevertheless appear to form synapses on distal dendrites or dendritic spines like uncrossed ones do. This situation might be important for synapse elimination, since it allow the synapses from both sides of the cortex to interact locally without initiating an action potential at the cell body of the postsynaptic neuron.

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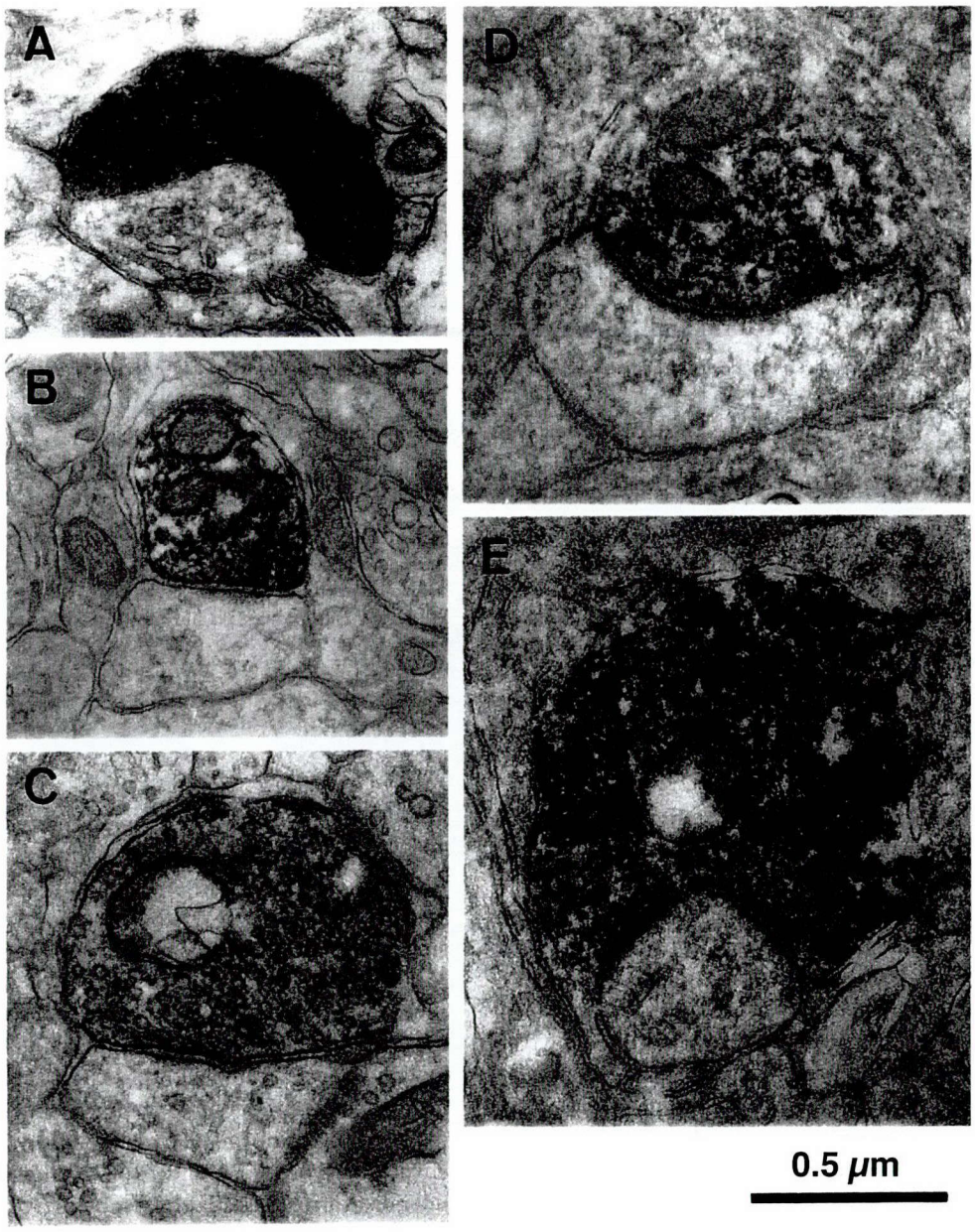
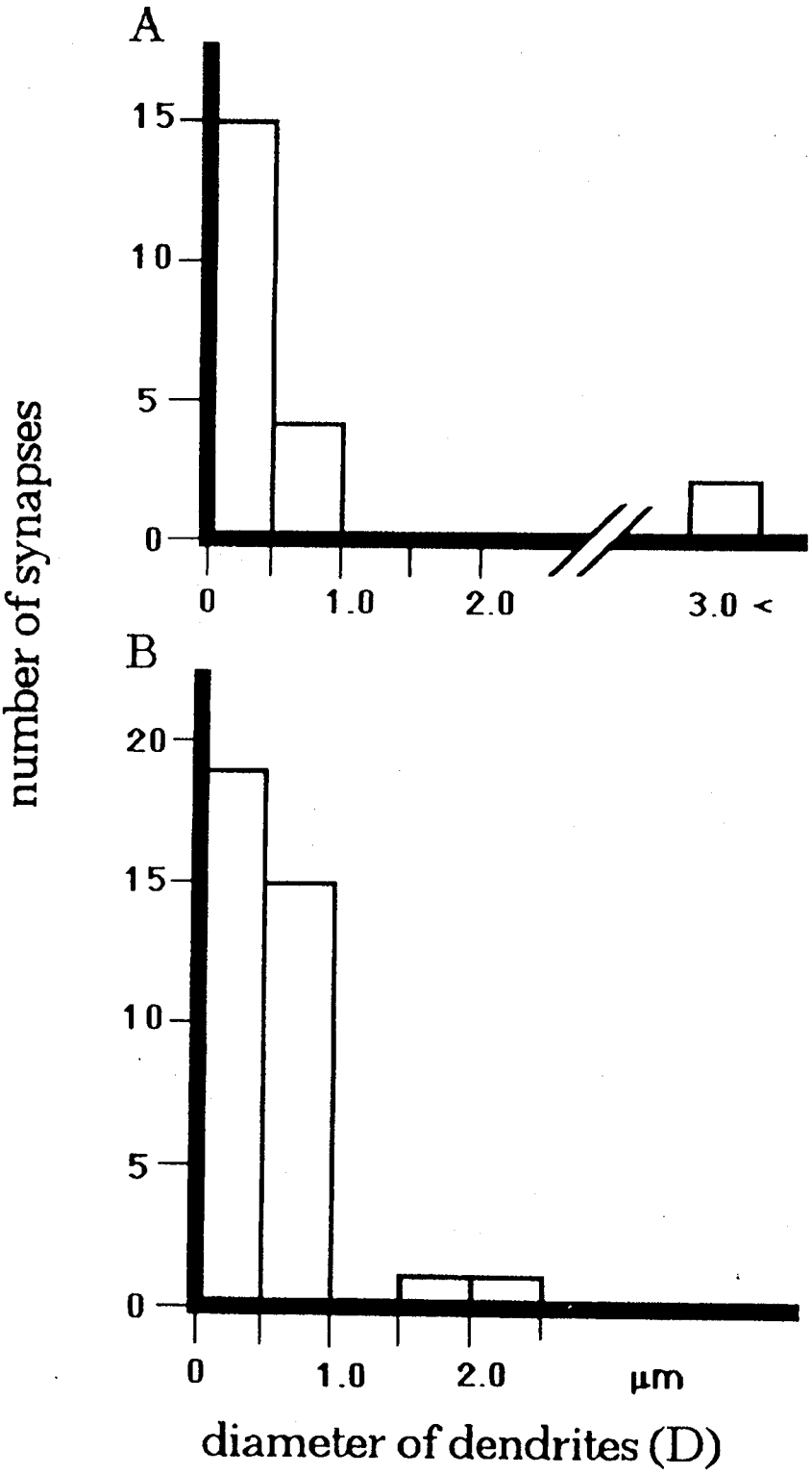
FIGURE LEGENDS

Fig. 1 Electron micrographs of synapses formed by crossed corticorubral axons in the kitten. A-C: synapses observed in the red nucleus of a PND 7 kitten. Axons were labeled with biocytin. D, E: synapses observed in a PND 17 animal. Axons were labeled with PHA-L. Note that every axonal ending forms a synapse with small postsynaptic profiles.

Fig. 2 The relationship between the number of synapses and the size of postsynaptic profiles. A: synapses in the corticorubral red nucleus. B: those in the ipsilateral red nucleus. The diameters of postsynaptic profiles were calculated according to the equation: $D = 2 \sqrt{A / \pi}$. Ordinate: number of synapses; abscissa: diameter of postsynaptic profiles (dendrites). Synapses formed with profiles with unclear contour, such as traversely sectioned dendrites, are not included.



Chapter 3, Fig.1



Chapter 3, Fig.2

Chapter 4

Morphology of individual axons in crossed corticorubral projections in developing cats and effects of partial denervation

ABSTRACT

Ordered neuronal connections in mature brains are thought to be sculpted from initially diffuse projections by elimination of inappropriate projections and strengthening of appropriate ones. Although evidence suggests that neuronal activity plays a role in these processes, the mechanism behind the modification of neuronal connections remains obscure. To gain insight into the mechanisms of axonal elimination and projection strengthening, we examined the morphology of individual axons that were to be eliminated as well as the consequences of partial denervation. While corticorubral projections in adult cats are thought to be uncrossed, early in postnatal development and after early unilateral lesions to the sensorimotor cortex, however, a significant amount of crossed corticorubral projections occurs. We examined the morphology of individual corticorubral axons in fetal cats and kittens from embryonic day 59 to postnatal day 48 and those that had received early unilateral lesions to the cortex, by serial reconstruction of *Phaseolus-vulgaris*-leucoagglutinin- or biocytin-labeled axons. For about 2 weeks during pre- and postnatal development, crossed axons remained simple in morphology, with few branches. Thereafter, they showed an increase in branch number, but then began to show fewer branches again. Axons and their collaterals were found in nonrestricted areas of the red nucleus (RN) throughout the period of observation, indicating that axons can sit at an inappropriate target for weeks but fail to ramify. In contrast, crossed corticorubral axons in kittens with cortical lesions showed terminal-arbor-like structures in the RN region that are in mirror symmetry to topographically appropriate areas in the ipsilateral RN, although some showed simple morphology without arbors. These complicated forms of

morphology of individual axons during development and after partial denervation may not be explained by a simple activity-dependent mechanism.

INTRODUCTION

Highly ordered and elaborate neuronal projections in higher vertebrates emerge from initially diffuse projections (Lund, 1978; Purves and Lichtman, 1985; Goodman and Shatz, 1993). At least three cellular mechanisms have been suggested to contribute to the formation of neuronal projections: selective death of neurons, elimination of axons collaterals (Cowan et al., 1984; Fawcett, 1988; O'leary and Koester, 1993) and elaboration of axonal arbors (reviewed in Murakami et al., 1992). Although the factors regulating these phenomena are not well understood, it has been hypothesized that retraction and growth of synapses are regulated by a mechanism related to neuronal activity: synapse coactive with their target cells are strengthened or stabilized, while those that are not are weakened and eventually eliminated (Brown et al., 1990; Changeux and Danchin, 1976; Hebb, 1949; Stent, 1973). Much effort has been applied to studying the role of such an activity-dependent mechanism in elimination and growth of axons in neural development and plasticity. The finding that blockade of neuronal activity during development disrupts elaboration of neuronal connections (Reh and Constantin-Paton, 1985; Shatz and Stryker, 1988; for reviews, see Garraghty et al., 1988; Shatz, 1990) supports a role for neural activity. Several models have been proposed to explain how neuronal activity leads to elimination and proliferation of synapses (Cowan et al., 1984). While knowledge of the morphology of individual axons that undergo such development changes, particularly how such developmental change proceeds and how elimination of axons takes place, would provide insight into the underlying mechanisms, there have been few studies addressed to these issues.

Corticorubral projections of the cat are an excellent model in which to examine the morphology of individual axons that undergo such developmental changes. The projections are thought to be uncrossed in adult cats; however, during early development and after early unilateral lesions to the sensorimotor cortex, a substantial amount of crossed corticorubral projections is observed (Villablanca et al., 1982; Tsukahara et al., 1983; Kosar et al., 1985; Leonard and Goldberger, 1987; Higashi et al., 1990; Murakami et al., 1991a, 1993; Song et al, 1995, 1996). The projections can be easily and clearly visualized in kittens by injecting anterograde tracers such as biocytin and *Phaseolus-vulgaris*-leucoagglutinin (PHA-L) into the sensorimotor cortex. In the present study, we examined the morphology of crossed corticorubral axons in kittens of various development stages by injection of limited amount of the tracers. We also examined the axons in kittens that had received early unilateral lesions to the sensorimotor cortex, since an activity-dependent mechanism has also been suggested to underlie lesion-induced changes of neuronal projection.

MATERIALS AND METHODS

Three fetal cats of embryonic days 59-63, 10 normal kittens of postnatal days (PND) 5-48 and 2 kittens that had undergone unilateral ablation of the sensorimotor cortex (see Murakami and Higashi, 1988)(Fig. 1A) at PND 23 or 24 were used. Cats were mated in Aburahi Laboratories, Shionogi & Co., Ltd. Mating was permitted for 24 h, and the time of mating was recorded as embryonic day 0. Kittens were injected with PHA-L (Vector Laboratories) or biocytin (Sigma) into lateral sigmoid gyrus, sometimes covering the lateral portion of the anterior and posterior sigmoid gyrus of the sensorimotor cortex (Fig. 1A, hatched area) that has been shown to project to the red nucleus (RN)(Murakami and Higashi, 1988; Higashi et al.,1990), under anesthesia with Nembutal (35 mg / kg, i.p.). A total of 1 μl of biocytin, 5-10 % in phosphate buffer, was pressure-injected with a glass pipette attached to a 1-μl Hamilton syringe into three loci of the cortex. PHA-L was iontophoresed with 2- to 5-μA positive current pulses (7.5 sec on and 7.5 sec off) for 10 or 15 min with a glass pipette tipped at 6 – 20 μm. After an appropriate recovery period (8–15 days for PHA-L-injected animals and 3-4 days for biocytin-injected animals), the kittens were transcardially perfused under deep anesthesia with Nembutal, either with phosphate-buffered saline followed by 2% paraformaldehyde and 2.5 % glutaraldehyde or 1 % paraformaldehyde and 1 % glutaraldehyde followed by 6 % glutaraldehyde. The method of surgery and tracer injection into fetal cats is detailed elsewhere (Song et al., 1995, 1996). In brief, the fetus head was exposed by aseptic cesarotomy in a mother cat anesthetized with a mixture of nitrous oxide and oxygen (1:2) containing 1 % halothane, and 5 % biocytin was then pressure-injected into the lateral sigmoid gyrus. The surgical site was sutured

and the animal was allowed to recover.

Animal ages at the time of fixation ranged between embryonic day 59 and PND 48 for normal animals, while the 2 animals that had received lesions were PND 52 and 67. Brains were postfixed for at least 4 h and the part of the brainstem which contains the RN and cerebral cortices was then dissected out. The cerebral cortices were stored until they sank in Tris-buffered saline containing 30 % sucrose. They were then sectioned parasagittally at 70 μm using a freezing microtome. The RN-containing brain block was sectioned near-horizontally at 50 μm with a Microslicer (Dosaka EM, Kyoto, Japan). The PHA-L-labeled axons were visualized immunohistochemically as described previously (Murakami and Higashi, 1988; Higashi et al., 1990; Murakami et al., 1991a). In brief, the brain sections were incubated in avidin-biotin peroxidase complex (Vector), with anti-PHA-L serum (Vector) for non-biocytin-injected animals, and reacted with diaminobenzidine tetrahydrochloride. Randomly selected axons were reconstructed from serial sections using a drawing tube attached to a light microscope. Most reconstructions were performed at x200, but higher magnifications (x400 and x1,000) were employed when necessary. Although sections were not counterstained, it was possible to discern the RN border due to its brownish color.

The number of sections used in the reconstruction of individual axons ranged from 3 to 11. In the RN, axons termination was easy to discern when axons ended in the middle of sections. In many instances they ended in a growth-cone-like structure characterized by triangular-shaped swellings and a rugged contour, sometimes associated with filopodial protrusions (Murakami and Higashi, 1988; Higashi et al., 1990; Murakami et al., 1991a); occasionally, axonal endings exhibited bulbous

swellings. Axons sometimes appeared to fade away midway through a section. When an axon extended beyond a section surface, neighboring sections were observed, and by referring to the position of blood vessels and other axonal fragments, axon continuity was examined.

RESULTS

The appearance of PHA-L- and biocytin-stained corticorubral axons was similar to that described previously (Higashi et al., 1990; Murakami et al., 1991a. b), although the size of the injection site was smaller than that in previous studies (Fig. 1B). A total of 40 axons was reconstructed from normal animals and other 10 axons from animals that had received unilateral lesions to the cortex. Animal ages and number of axons reconstructed from each animal are shown in table 1. Staining of a moderate number of axons in the RN of these animals permitted reconstruction of individual axons.

Course and Branching Patterns of Axons

Normal animals. The axons from fetal cats were of simple morphology, generally lacking or displaying only a single branching point in the RN, as illustrated in figure 2, although 1 axon had 5 branching points (not shown). Two thirds of axonal endings showed growth-cone-like structures. Axons tended to follow a straight path.

Figure 3 shows examples of axons reconstructed from a PND 5 animal (upper panel) and one of PND 8 (lower panel). In both cases, the axons are remarkably straight and do not follow a convoluted path. Although they occasionally changed growth direction or bifurcated, the axons continued growing in a straight line over long distances before branching. The number of branching points observed in the RN ranged from 0 to 5 with a mean of 2.3. It should be noted that the axons do not grow around any particular loci within the RN, and some even appear as though they are just passing through the nucleus. Most may probably not be axons *en passant*, however, as

the number of axons observed in areas surrounding the RN was very low compared with that in the RN. Since injections were made into the lateral sigmoid gyrus in these kittens, the location of the topographically appropriate region was expected to be dorsomedial in the RN (Higashi et al., 1990). Few axons, however, had collaterals in this region.

At later stages of development, axons with a somewhat more complex morphology appeared (Fig. 4). Although the axons generally tended to branch more frequently (mean number of branching points = 6) than those from earlier stages of development, no arbor-like structures, characterized by short collaterals concentrated at a restricted region of the axons (Murakami et al., 1991a), were observed. From these examples, it can be noted that axonal branching does not occur in any particular region of the RN. Uncrossed projections begin to show obvious topographic order at this stage (Higashi et al., 1990). Crossed axons did not display branches in topographically inappropriate regions at these stages of development (Higashi et al., 1990; Murakami et al., 1991a), however.

At PND 28 and later, very few axons could be labeled, presumably reflecting elimination of crossed projections. Axons were less complex in morphology than those observed earlier in development (Fig. 5). Although they appeared similar to those observed at PND 5-8 in that they tended to show less frequent branching, axons at later stages of development followed convoluted paths, unlike the latter.

Our previous double-labeling experiment demonstrated that individual corticorubral axons bilaterally innervate the RN (Murakami et al., 1993). To confirm this, we attempted to follow crossed axons to the other side of the RN. Three axons each from one PND 8 and one PND 48 animal were successfully followed. Four of the

6 axons examined were found to innervate the RN bilaterally, as exemplified in Fig. 6. This axon gave off several collaterals in the RN ipsilateral to the injection before reaching the contralateral RN, where it terminated after bifurcation.

Animals with Cortical Lesions. As reported earlier (Villablanca et al., 1982; Kosar et al., 1985; Murakami et al., 1991a), unilateral lesions to the sensorimotor cortex induce enlargement of crossed corticorubral projections, which is associated with the appearance of axonal arbor and topographic order. Although the increase in branching frequency in lesioned animals caused some difficulty in the reconstruction of individual axons, 10 axons were nevertheless successfully reconstructed in the present study. Examples of such axons are shown in Fig. 7. As expected from a previous study (Murakami et al., 1991a), axons with terminal-arbor-like structures were observed (Fig. 7, left and middle panels). It is interesting that individual axons almost completely lack branches along their entire length with the exception of the area of terminal arbors. Axonal arbors were found in areas that are in mirror symmetry to topographically appropriate areas in the ipsilateral projections (dorsomedial part of the RN, corresponding to the injections into the lateral sigmoid gyrus of the cortex)(Higashi et al., 1990; Murakami et al., 1991a). Axons devoid of such structures were also found (n = 3; Fig. 7, right panel).

DISCUSSION

The results of the present study are summarized in Fig. 8. During the 2 perinatal weeks, crossed corticorubral axons are simple in morphology, displaying few collaterals. Thereafter they transiently increase in collateral number, demonstrate no preference for any specific region of the RN and fail to form arbors. Unilateral lesions to the sensorimotor cortex, however, result in a loss of collaterals in the RN concurrent with the formation of arbor in topographically appropriate areas, i.e. ones that are in mirror symmetry to topographically appropriate regions in the ipsilateral projections.

Methodological Consideration

In the present study, corticorubral axons were labeled by extracellular application of PHA-L or biocytin. We selected intensely stained axons and carefully followed both them and their branches to their respective terminals. In some cases, however, axons faded away midway through a section. Some potential for error did exist since axons which left the RN were not pursued further. Any such axons which later returned to the nucleus would therefore render the reconstruction incomplete. In most cases, however, we were able to follow axons to their endings with certainty, since such axons terminated with characteristic morphology, i.e. growth-cone-like or bulbous swellings. Thus, although they may be partially incomplete, the axons reconstructions prepared in the present study are probably representative of corticorubral axon morphology.

Assal and Innocenti (1993) observed transient axons in the cat visual cortex. They found that about one third of the endings of transient intra-areal axons were

growth cones, although bulbous enlargements were also frequently observed as was the case for the crossed corticorubral axons. Such bulbous enlargement are probably retraction bulbs (Riley, 1977; O'Brien et al., 1978; Balice-Gordon, 1993).

Postnatal Development of Individual Axons

Our earlier observation of axonal fragment in kittens suggested that crossed corticorubral axons remain simple in morphology and unbranched during postnatal development, while uncrossed ones elaborate terminal arbors (Higashi et al., 1990). The present results confirm this observation. In addition, they indicate that although crossed corticorubral axons remain simple in morphology, the axons do develop to a certain extent; while axons were more or less branchless from embryonic day 59 till PND 5-8, a substantial degree of axonal bifurcation was observed at PND 14-20. This may serve to indicate that crossed axons do not retract immediately upon arrival even at an 'inappropriate' target, but rather increase branch number. Another possible interpretation is that a dynamic state exists between the degree of exiting–axon retraction and the degree of the entering-axon branching, so that entering axons would form more complex branches at PND 14-20.

There is evidence suggesting that uncrossed axons outnumber crossed ones beginning at early stages of innervation (Song et al., 1996). Furthermore, electrophysiological (Song et al., 1993) as well as electron-microscopic (Murakami et al., 1991b) studies indicate that crossed corticorubral axons form synapses early in postnatal development. This might suggest that an activity-dependent competition operates between synapses from both sides of the cortex (Hebb, 1949; Stent, 1973; Garraghty et al., 1988; Constantin-Paton et al., 1990; Shatz, 1990). This theory alone,

however, does not explain why crossed axons do show a certain degree of development. It would therefore seem necessary to assume that, for example, axons possess a strong intrinsic growth capacity which allows them to grow branches around 2 weeks postnatally. An activity-dependent mechanism, if present, might start to operate or overcome such intrinsic growth capacity of axons only after formation of a certain number of synapses.

Previously, based on the observation of axonal fragments, we reported that crossed corticorubral fibers, unlike uncrossed ones, lack topographic order and distribute diffusely in the RN (Murakami et al., 1991a). The present observations of individual corticorubral axons verified this interpretation. At no stage of development did crossed corticorubral axons appear to prefer any loci in the RN, i.e. axons did not preferentially ramify in any particular region of the RN, although we made restricted injection. This may serve to indicate that crossed corticorubral axons lack the mechanisms necessary to locate a topographically appropriate target. Taking into account that a major proportion of the axons projecting to the contralateral RN are collaterals from ipsilaterally projecting axons (Murakami et al., 1993), these results may suggest that the molecular characteristics of axons change when they cross the midline, as appears to be the case with ventral commissural axons of the spinal cord, which cease TAG-1 expression when they grow along the floor plate (Dodd et al., 1988; Shirasaki et al., 1995). Alternatively, the number of synapses formed by crossed corticorubral axons might be too low for functioning of an activity-dependent mechanism which could also serve in topographic fine tuning.

There are few reports on morphological studies of transitory axons. Sretavan and Shatz (1986) examined the prenatal development of retinogeniculate axons by

injecting horseradish peroxidase into the optic tract. They found that axons form short side branches along the whole of their path in the lateral geniculate nucleus but that these disappear as development proceeds concomitantly with elaboration of axonal arbors in 'appropriate' layers of the lateral geniculate nucleus. The side branches in 'inappropriate' layers appeared to be very short (< 100 μm) and infrequently bifurcated. Similar elimination of short side branches appears to occur in the development of the murine retinocollicular system (Sachs et al., 1986). Retinofugal axons in hamsters also show similar developmental changes (Bhide and Frost, 1991), projecting unbranched or poorly branched collaterals to multiple targets and thereafter elaborating terminal arbors within definitive targets concurrent with elimination of other collaterals. In all these experiments, collaterals that are to be eliminated appear to remain unbranched (or poorly branched) during development, similar to crossed corticorubral fibers.

Post-Lesion Changes in Axonal Branching Patterns

Changes in axon morphology after partial denervation have been extensively studied in visual systems. In accordance with the present results, Garraghty et al. (1988) showed that in monocularly deprived cats, retinogeniculate axons form arbors in layers in which they are normally not present.

Axons in the denervated RN developed arbors not seen in normal development. Additionally, unlike those in normal animals, axonal branchings were rarely seen along the axon course except at the site of arborization. This suggests that lesions to the cortex facilitate elimination of axonal branches in topographically inappropriate areas concurrently with formation of axonal arbors in topographically appropriate areas. In addition, the observation that some crossed axons in normal animals lack branches in

topographically appropriate areas may suggest that, following denervation, crossed axons begin to grow and form arbors upon arrival at topographically appropriate regions. Alternatively, axons that lack branches in topographically inappropriate areas may retract, while those that have branches in topographically appropriate areas survive and form arbors; axons that lack complex arbors in lesioned animals (Fig.7, rightmost panel) may represent those that in the course of retraction.

A possible explanation for the lesion-induced changes in axonal morphology might be that denervation causes up-regulation of factor(s) concurrently with changes in the expression of certain surface molecules on the crossed branch of the corticorubral axons, allowing them to recognize presumptive positional markers in the RN. A recent study reported the occurrence of ciliary neurotrophic factor mRNA up-regulation following injury (Ip et al., 1993); ciliary neurotrophic factor has been reported not only to support survival of neurons but also to induce axonal sprouting from endplates (Gurney et al., 1992). Furthermore, there is evidence that denervation induces reexpression of guidance cue(s) in the target (Wizenmann et al., 1993). Such changes in the denervated target might explain the present results.

Denervation-induced changes in activity-dependent competitive interaction between inputs from both sides of the cortex might also cause axon ramification. The formation of axonal arbors in topographically appropriate areas might be explained by this mechanism if crossed axons were topographically ordered in normal animals at the time of lesioning. This was not the case, however. An alternative possibility is involvement of heterosynaptic interactions between cortical input and other topographically organized input. Input from the contralateral nucleus interpositus and the spinal cord is topographically organized in adult cats (Condé, 1988; Padel et al.,

1988). If it is also topographically organized in kittens, depolarization of the RN cells as a result of the activity of such input, when synchronized with the activity of corticorubral input, might serve to reinforce synapses in topographically appropriate areas. This theory requires the retraction of axons that lack branches in topographically appropriate regions of the RN in normal cats.

In any case, axon growth seems to play a major role in the appearance of topographic order in crossed corticorubral projections. In accordance with this view, evidence has recently been supplied demonstrating that growth and axonal arbor formation play an important role in the formation of the ocular dominance column (Antonini and Stryker, 1993).

In conclusion, our observation of the morphology of individual corticorubral axons suggest that development and retraction of axons transiently projecting to a wrong side of the brain follow a rather complex process and may not be explained by simple either the activity-dependent mechanism proposed by Hebb (1949) or its modified version. Although such a mechanism could operate for denervation-induced changes of projections, it appears to be too simple to explain the lesion-induced changes of corticorubral projections. Other mechanisms or additional mechanisms may be required to explain the changes in morphology of crossed corticorubral axons in normal development and after partial denervation.

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FIGURE LEGENDS

Fig. 1 Lesion and injection sites of the cerebral cortex. A, Drawing of the cat cerebral cortex showing the site of lesions (shaded area) and tracer injection (dotted area). as = Anterior sigmoid gyrus; ps = posterior sigmoid gyrus; ls = lateral sigmoid gyrus. B, Light micrograph of a sagittal section of the pericruciate cortex showing a representative injection site of PHA-L (arrow). cr = Cruciate sulcus. Bar = 1 mm.

Fig. 2 Crossed corticorubral axons in fetal cats as reconstructed from serial sections. E = Embryonic day. Axons were labeled with biocytin injected into the pericruciate sulcus. The dotted line indicates the approximate border of the RN. The line was drawn using the middle of the sections employed for reconstruction. Note that axons are simple in form and rarely display branches. Axon branches indicated by asterisks represent extensions outside the RN.

Fig. 3 Crossed corticorubral axons in kittens as reconstructed from serial sections. The RN was sectioned nearly horizontally. The axons follow a rather direct path and changes in direction of growth occur abruptly. The frequency of branching is low. Same representation as figure 2.

Fig. 4 Crossed corticorubral axons in kittens as reconstructed from serial sections in kittens of PND 14 and PND 20. Although displaying simple branching patterns, the axons are more complex than those of earlier stages. Same representation as figure 2.

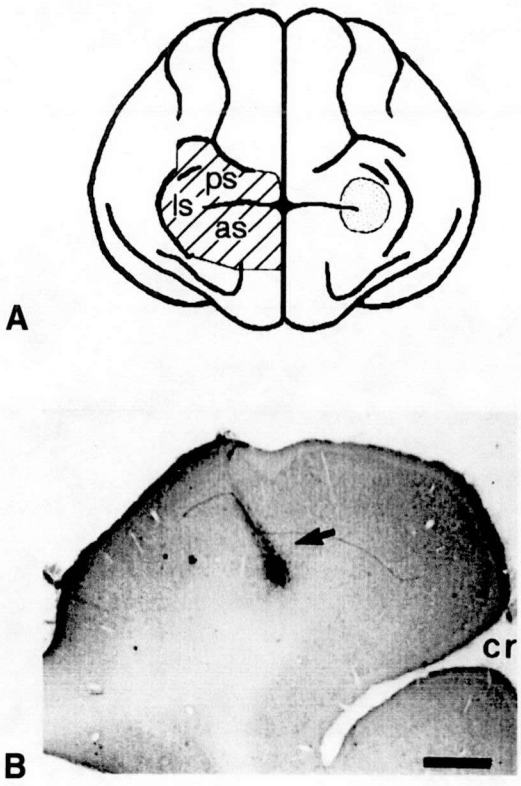
Fig. 5 Crossed corticorubral axons in kittens as reconstructed from serial sections in kittens of PND 28 and PND 48. Note the simple patterns of innervation. Though as simple as those of PND 5 – 8, they appear more convoluted. Same representation as figure 2.

Fig. 6 Bilaterally innervating corticorubral axon in a normal cat. The axon was reconstructed from 13 serial sections that were sectioned nearly horizontally. The axon enters the contralateral RN (right side) after emitting collaterals in the ipsilateral nucleus (left side). The dotted line indicates the approximate border of the RN. PND 9. 3N = Oculomotor nerve. The upper side is rostral. Bar = 500 μm

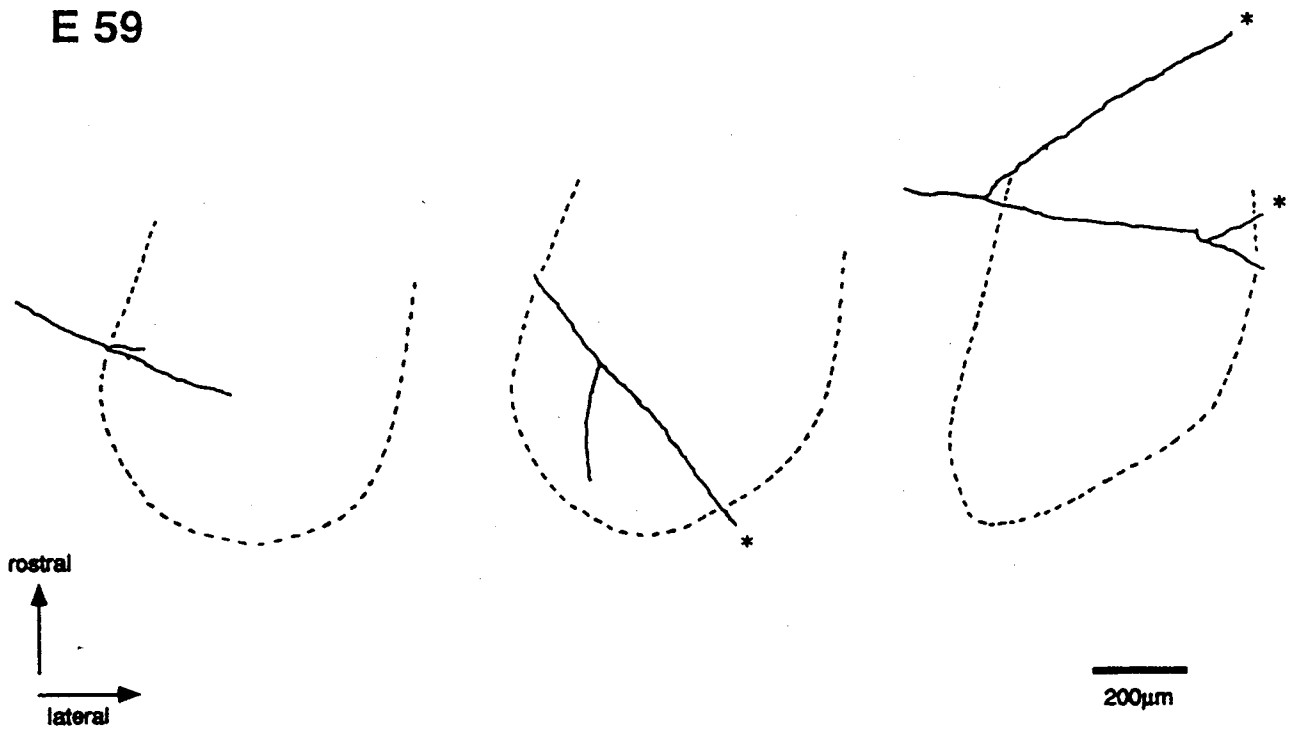
Fig. 7 Crossed corticorubral axons reconstructed from serial sections from a cat that underwent unilateral lesions to the sensorimotor cortex. Terminal arbors are observed in 2 axons, while the rightmost axon lacks arbors. The border of the RN (dotted line) was drawn at the dorsoventral plane of the arbor formation.

Fig. 8 Schematic diagrams summarizing the present results. Continuous lines represent corticorubral axons while dotted lines indicate the border of the RN. E = Embryonic day; PN = Postnatally.

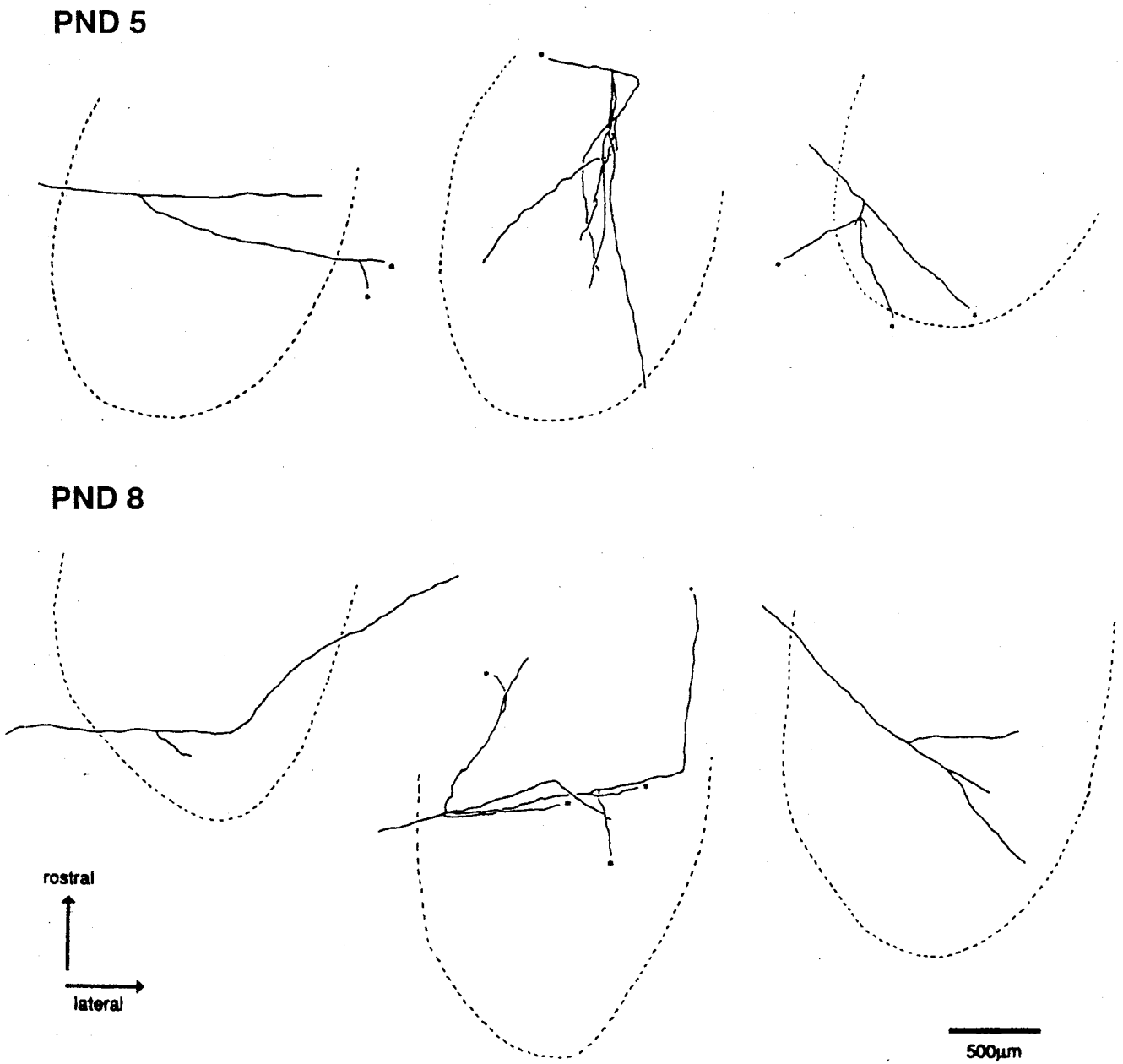
Table 1. Summary of experimental animals used in the present study



Chapter 4, Fig.1



Chapter 4, Fig.2



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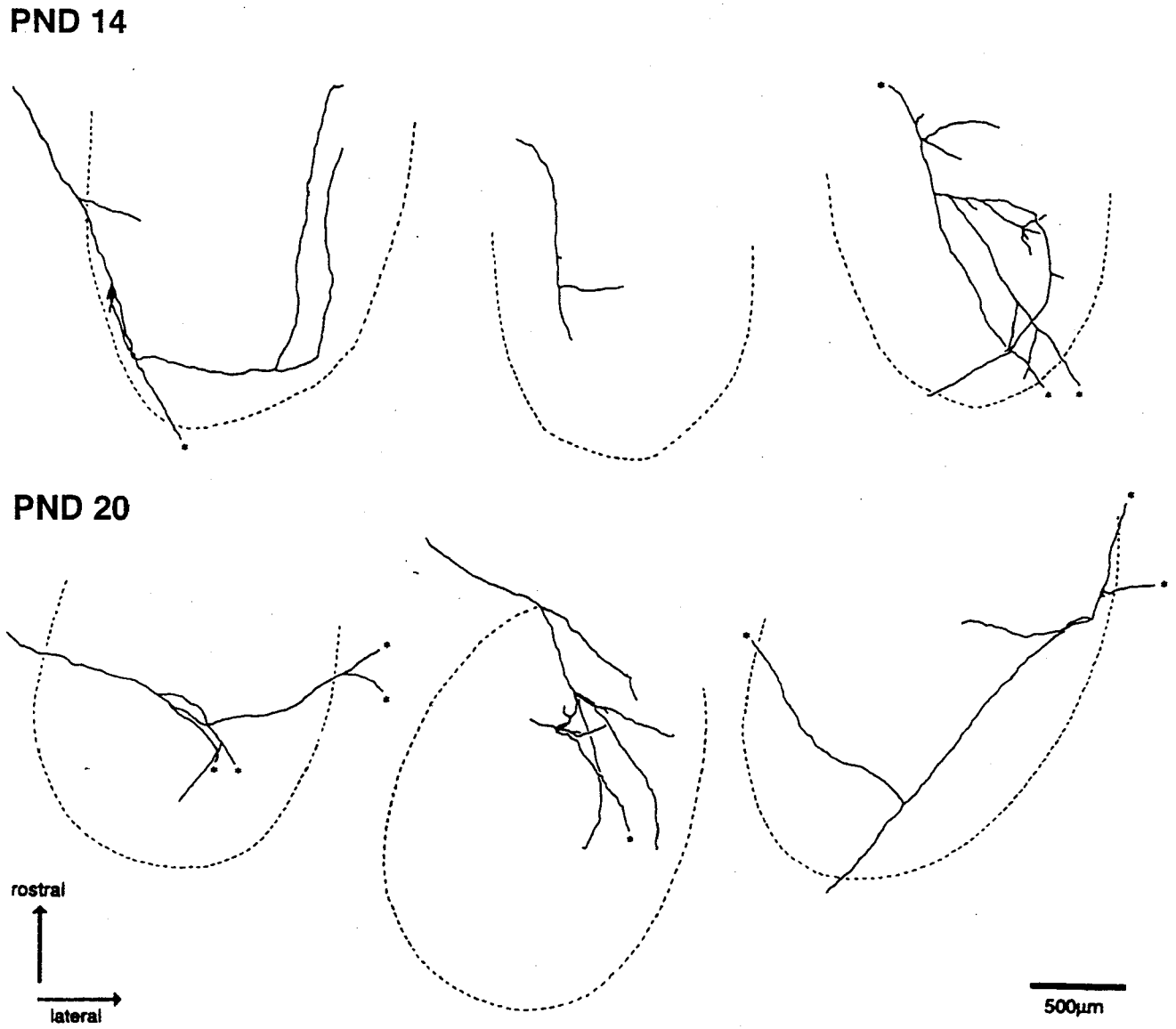

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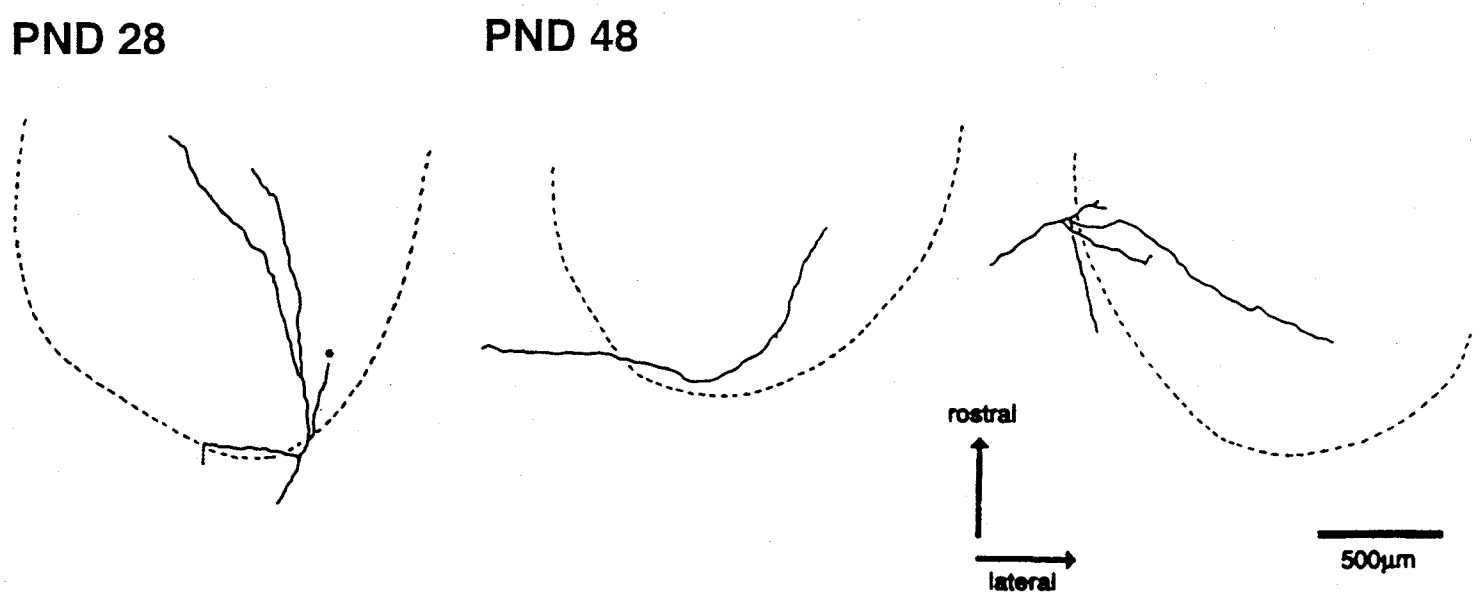
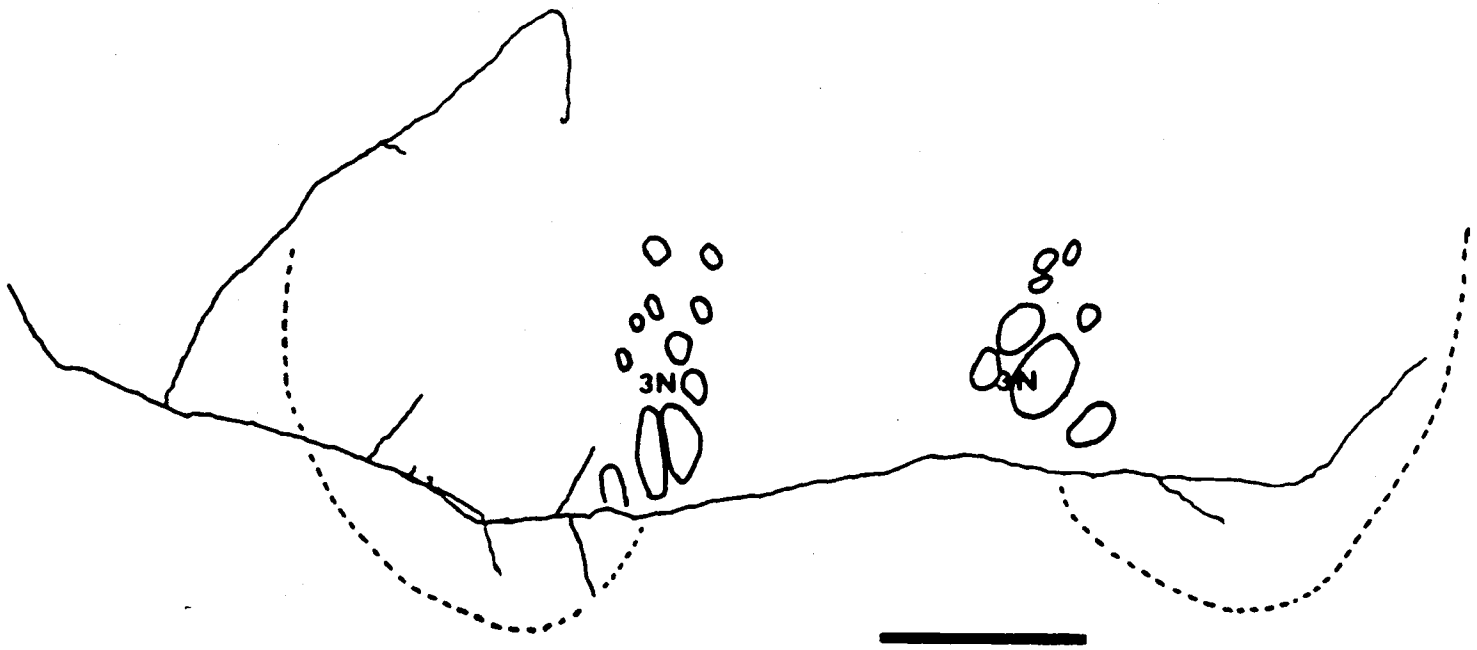
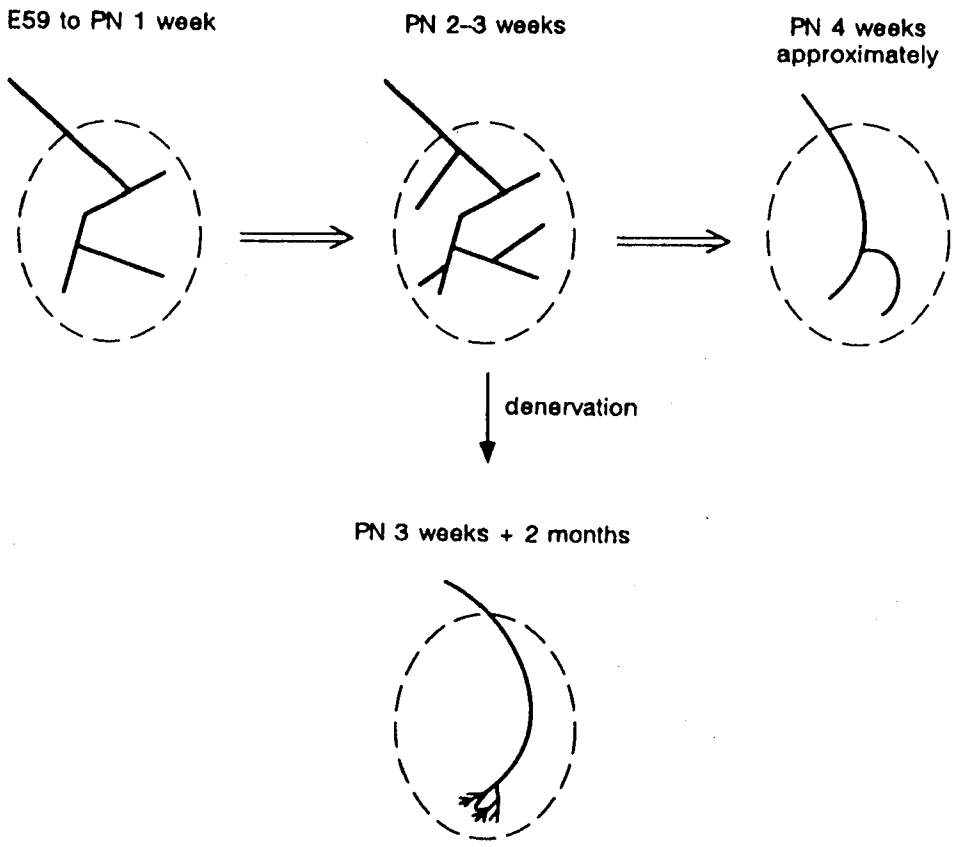


Chapter 4, Fig.5



Chapter 4, Fig.6

Chapter 4, Fig.7



Chapter 4, Fig.8

Table 1. Summary of experimental animals used in the present study

A. Intact kittens

	Animal No.										
	1	2	3	4	5	6	7	8	9	10	11
PND	59[1]	59[1]	63[1]	5	8	14	20	20	24	28	48
Reconstructed axons, n	3	4	1	8	7	6	1	5	1	1	3

[1] Embryonic day.

B. Kittens with cortical lesions

	Animal 1	Animal 2
PND at the time of lesion	23	24
PND at the time of observation	52	67
Reconstructed axons, n	5	5

GENERAL DISCUSSION

The study on development of the nervous system supplies a blueprint of the neuronal connections which is often difficult to reveal from the study on mature nervous system. Moreover, it is possible that the mechanisms underlying neural development is shared by neural plasticity that is likely to underlie learning and memory (Kandel and O'Dell, 1992). Therefore, studying the development of the nervous system may provide a crucial clue for understanding of functional principles of the brain.

Synapse formation in the CNS

The mechanisms of synapse formation have been intensively studied using vertebrate skeletal neuromuscular junction (NMJ). Interneuronal synapses resemble the NMJ at morphological (Peters et al., 1991) and molecular levels (Hall and Sanes, 1993) except for some differences, e.g. the absence of basal lamina and junctional fold, and different neurotransmitters, suggesting that the processes in interneuronal synapses formation are similar to those in the NMJ formation (Hall and Sanes, 1993). In the NMJ, the entire myotube membrane is equally receptive for formation of synapses, suggesting that target muscles may play a passive role in the process of the synapse formation. (Bennett and Pettigrew, 1976; Frank and Fischbach, 1979; Hall and Sanes, 1993). Therefore, the presynaptic axons which show dynamic behaviors to search for targets have been thought to be key elements in the synapse formation. However, in the CNS, the target neurons as well as the afferent axons change in morphology dramatically during synaptogenesis (Jacobson, 1991), leading to an intriguing possibility that the target neurons also contribute the synapse formation. In chapter 1

and 2 of this thesis, I showed that CR axons of newborn cats form synapses preferentially on SLDPs of target cells, rubrospinal neurons. The finding that SLDPs invaginate into axons which were rarely observed in adult cats (Murakami et al. 1982), together with the preferential termination of CR endings on SLDPs, suggests that SLDPs contribute to ongoing synaptogenetic interactions. Although there have been the findings that dendritic growth cones may interact with afferent axons (Vaughn, 1989; Kimmel et al., 1990), there has not been the idea that SLDPs interact with afferent axons to form synapses until recently (Cooper and Smith, 1992; Saito et al., 1992; Dailey and Smith, 1996; Ziv and Smith, 1996). My study as well as previous *in vitro* studies (Dailey and Smith, 1996; Ziv and Smith, 1996) provides the basis for further understanding of the synapse formation in the CNS.

Previous anatomical studies in the CNS have shown that early synapse formation occurs on dendritic shafts (Cotman et al., 1973; Hinds and Hinds, 1976; Miller and Peters, 1981; Steward, 1987; Harris et al., 1989). Since I did not observe CR synapses in fetal cats, the possibility that initial CR synapses are formed on dendritic shafts of RS cells can not be ruled out . However, the present study showed that additional synapse formation which are formed dramatically at postnatal stage occur mainly on SLDPs. The finding that SLDPs are postsynaptic sites is consistent with an *in vitro* study of developing hippocampal neurons (Ziv and Smith, 1996). If it is true that early synapses are formed on dendritic shafts and that the later synapse formation occurs on SLDPs, the following process of synapse formation in the CNS might be considered. At the stage that early afferent axons reach their target neurons, the target neurons do not have elaborate dendrites. The target neurons extend protrusions from the somata (e.g. see Altman, 1972) as well as the short dendrites which have not SLDPs. At the

time, the entire membrane of dendrites including dendritic growth cones can behave like SLDPs which interact with incoming axons to form synapses (see Vaughn et al., 1974; Vaughn, 1989). Therefore, most of early synapses can be observed on dendritic shafts. As development proceeds, membrane of dendritic shafts fails to interact with axons, but then SLDPs emanate from dendritic shafts and interact with axons instead of shafts. Although I do not know whether some molecular factors control such process, development or maturation of postsynaptic neurons might be important to form synapses and to determine sites of synapses (see Fletcher et al., 1994) .

Crossed CR synapses and refinement of neuronal connections

The refinement of the immature exuberant circuits into the organized connection are thought to be dependent on neuronal activity (Shatz, 1990a, b; Goodman and Shatz, 1993; Katz and Shatz, 1996). This notion has been supported by the findings that blockade of neuronal activity by sensory deprivation or infusion of tetrodotoxin during development disrupted the refinement of neuronal connections (Hubel and Wiesel, 1970; Stryker and Harris, 1986; Shatz and Stryker, 1988; Constantin-Paton et al., 1990; Antonini and Stryker, 1993). The mechanism could be explained by Hebb's coincident rule or its modified version that synapses coactive with their target cells (appropriate connections) are strengthened and stabilized whereas those are not (inappropriate connections) are weakened and eventually eliminated (Hebb, 1949; Stent, 1973; Changeux and Danchin; 1976; Brown, 1990). In chapter 3, I showed CR axons of neonatal cats formed synapses both in the ipsilateral and contralateral RN. Taken together with the electrophysiological study (Song et al., 1993), this suggests that the afferent axons which project to the inappropriate regions and eventually eliminated

form functional synapses with the targets. Therefore, it is possible that the activity-dependent competition between crossed and uncrossed cortical inputs occurs on RS cells. However, since RS cells receive inputs from other sources such as the deep cerebellar nuclei, the dorsal column nuclei and the pretectal area (Massion, 1967, Fujito et al., 1983), the involvement of other systems has to be considered. Moreover, crossed CR axons form arbors only at topographically appropriate regions after unilateral cortical lesion, suggesting other or additional mechanisms may be necessary to explain the refinement of CR system (chapter 4). Since axonal growth, arborization, and synapse formation as well as elimination of misdirected branches and synapses are important in refinement of the immature exuberant circuits into the organized connections (Antonini and Stryker, 1993; Katz and Shatz, 1995), there may be some mechanisms controlling the axonal growth, arborization, and synapse formation.

To gain further insight into this issue, it is necessary to investigate how crossed CR axons form arbors at appropriate regions after unilateral lesions. Moreover, it is interesting to test whether blockade of activity by injection of TTX into one side of cortical hemisphere induces the arborization of CR axons on the other side such as the case in unilateral lesion.

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