Research Reports

LONG-TERM STATUS OF PYRAMIDAL CELL AXON COLLATERALS AND APICAL DENDRITIC SPINES IN DENERVATED CORTEX

L. T. RUTLEDGE, JOYCE DUNCAN AND NELL CANT

Department of Physiology, University of Michigan Medical School, Ann Arbor, Mich. 48104 (U.S.A.)

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INTRODUCTION

Some central nervous system neurons when deafferented and deefferented (denervated) undergo progressive degeneration leading to cellular death and resorption of neuronal debris. In others, in what appears to be compensatory new growth, axonal proliferation is seen[1,12]. Whether proliferation occurs depends upon such critical factors as neuronal age, type and location of neurons and probably whether deafferentation has been complete or partial. The latter must be especially important since presynaptic impingement upon a partially deafferented neuron likely determines whether cytoplasmic metabolic pathways remain functional.

A most fundamental problem relative to proliferation of axonal processes is whether the neoformations effect structural and functional contact with neighboring neurons. If such contacts are not established then the new growth is of little interest in the consideration of reorganization within the nervous system following injury. It is possible where proliferation has been observed, that rather than *additional* new growth, neurons were expressing a maintenance of protoplasmic mass, a feature inherent in the constitution of certain neuronal tissues[1].

In the cerebral cortex of neonatal cats (likely also at least in dogs), if a cut is made slightly below gray matter (cortex undercut), axon collateral proliferation of pyramidal cells may be observed within a few days[1,12]. One or more collaterals from the truncated axon grow recurrently in a branching manner among neighboring cellular processes giving the appearance of arciform or short axon neurons[12]. However, the changes may involve only thickening or hypertrophy of collateral trunks[1,16]. Nearly 70 years ago Cajal astutely observed that axonal proliferation toward nearby neurons might preserve the nervous impulse in the injured neuron and actually 'in-

crease the energy of the motor response'[1]. However, he cautioned that for such reorganization, as observed in young brains, to be effective, it would be necessary to prove that the changes endured indefinitely. It has been suggested, after morphological and excitability studies on young brains, that such axon collateral proliferation is a likely basis for the hyperexcitability and convulsant activity seen in the similarly traumatized adult brain[12,19]. These interpretations have been questioned when in denervated adult cortex substantial evidence of axon collateral proliferation was not found[15,16].

Since axonal proliferation apparently occurs in young brains following denervation, an important question is whether the proliferation represents a permanent reorganization. It remains to be determined also whether the proliferation results in structural and functional contacts with other neurons. A further unanswered question is to what extent the neonatal age at denervation determines the kinds of proliferation and whether there is a relationship to convulsive tendencies seen at brain maturity. Answers to these questions may help in understanding certain brain traumas and the extent to which stable compensatory reorganization is possible.

This report compares axon collateral and dendritic spine morphology and electrocortical seizure susceptibility, months after cerebral cortical denervation in kittens, with observations made in adult cats whose cortices were either intact or denervated.

METHODS

Studies were completed through all phases on 17 cats. A sterile undercutting surgical procedure[17] was done on 4-day-old kittens (N = 7), 40-day-old kittens (N = 3) and on adult cats (N = 4). Three adult cats comprised an intact group. Kittens with a postnatal age of 4 days were chosen since most maturation of cortical pyramidal cells occurs within the first two weeks or so[9,13]. Because of differences in cortical maturity at birth, even among litter mates[18] more animals were studied in this group (4-day kittens) than in the others. The developmental status of a 40-day-old kitten would be essentially that of a mature cortex[4,9,10,21], however, in the 40-day-old dog, evoked potentials are still not of a configuration seen in adult cortex[3,6].

After the undercutting, animals were left undisturbed for several months before a terminal acute experiment[17]. The intervening time varied as follows: 4-day group, average 250 days (range 144–287); 40-day group, average 153 days (range 105–222); and adult undercut (UC), average 89 days (range 64–126).

Details of the procedures for terminal acute experiments have been published[17]. Briefly, bilateral craniectomy to expose undercut and homotopic cortex was done under chloralose anesthesia. Electrocorticograms were made before and after electrical stimulation of the cortex at intensities strong enough to produce abnormal spiking and/or repetitive afterdischarges. Previous work indicated that the most reliable measure of hyperexcitability of undercut cortex was duration of afterdischarge to electrical stimulation, although qualitative changes such as spike configuration and amplitudes have proven valuable adjuncts to duration.

At the conclusion of the electrophysiological experiment, brain tissues were

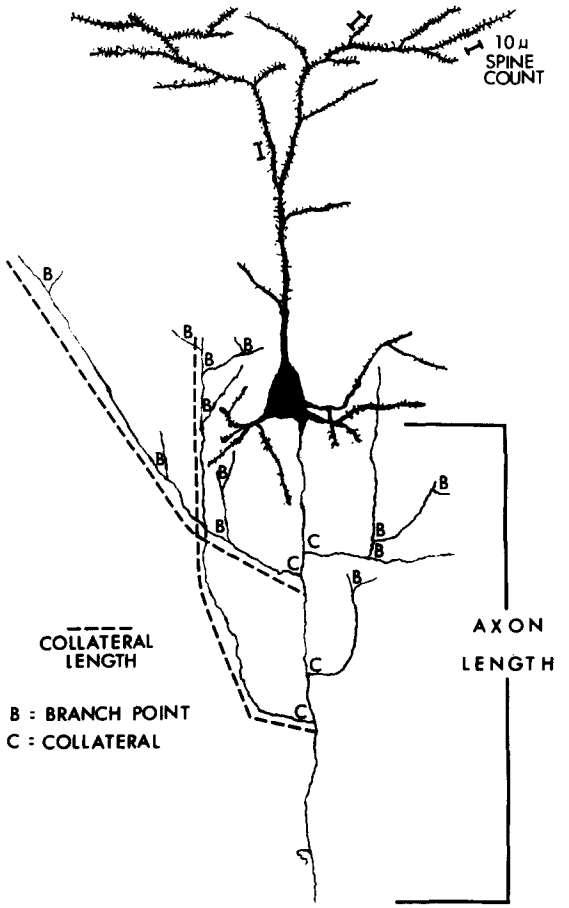

Fig. 1. Drawing of a cortical pyramidal cell showing where and how measures were made of number of collaterals, collateral lengths, collateral branch points and apical dendritic spines. There are 4 collaterals and 12 branch points. Spine counts were made in 10 μm sections in the upper part of the apical dendritic tree.

prepared for light microscopic study using a modification of the Golgi–Cox method[16]. For all experimental animals cortex from undercut and homotopic areas was processed.

Studies were made of selected cortical pyramidal cells as illustrated in Fig. 1. Cells were selected on the bases that the histological sections showed good staining of a pyramidal cell's dendrites, dendritic spines and axon, and the cell had to have an orientation with centripetally coursing axon of at least 200 μm length with at least one recurrent axon collateral of 100 μm length. Averages were made of two collateral lengths greater than 100 μm in 78% of the cases for the 4-day group, 92% for the 40-day group, 82% for the adult UC and 66% for the intact group. In the remaining percentages only one collateral was measured.

Most of the pyramidal cells studied were located in cortical layers II and III (Table I). Since axons when cut usually degenerate back to the first collateral[1] it was

TABLE I

NUMBER OF ANIMALS (N), CELLS AND HISTOLOGICAL SECTIONS STUDIED, AND CORTICAL LOCATION OF PARENT SOMA OF AXONS AND COLLATERALS

	N	*Cells*	*Sections*	*Layers*		
				II–III	*IV*	*V–VI*
4-Day	7	519	216	451	44	24
40-Day	3	218	68	184	25	9
Adult UC	4	220	90	187	26	7
Intact	3	111	67	107	3	1

difficult to find cells with long axons in layers V and VI. The very criterion that the primary axon had to be at least 200 μm in length eliminated most lower lying pyramidal cells, including fusiform and arciform, from quantitative study. However, qualitative axonal changes in some of these cells were important. They will be discussed below.

Another limitation was that it was rare to find large pyramidal cells (more than about 25 μm soma diameter) whose axons were stained for 200 μm, and thus our samples consisted mostly of pyramidal cells with small or small-medium somas. Cajal noted that large pyramidal cells were the first to disappear after cuts were made below cortex[1]. The total distribution of samples for measurements is shown in Table I.

Measurements were made of number of collaterals, length of two longest collaterals (one in a small percentage of the cases, see above), number of collateral branch points and number of dendritic spines per 10 μm dendritic length (Fig. 1).

Dendritic spine counts, as a measure of the loss of presynaptic terminals[5], were made from 10 μm lengths of apical dendritic twigs located in the crown of the midmarginal gyrus and no deeper than layer II. No determination of cell soma location was made although undoubtedly a large majority were in layers II and III. Spines were counted in a single representative 10 μm section from a cell. No more than twenty 10 μm lengths were taken from each histological section but at least 120 lengths were counted from each experimental animal. Ten μm lengths were measured with a calibrated eyepiece at $\times$ 450. In an unpublished preliminary study a high level of interobserver reliability was found using this method. Number of measurements and histological sections studied were: 960-62 (4-day), 420-30 (40-day), 480-36 (adult UC) and 420-30 (intact).

RESULTS

Quantitative morphological changes

Fig. 2a is a summary of measurements on numbers of axon collaterals. Both the 4-day and 40-day groups had significantly more collaterals than intact or adult UC. In contrast, the adult UC was not significantly different from intact ($P > 0.1$).

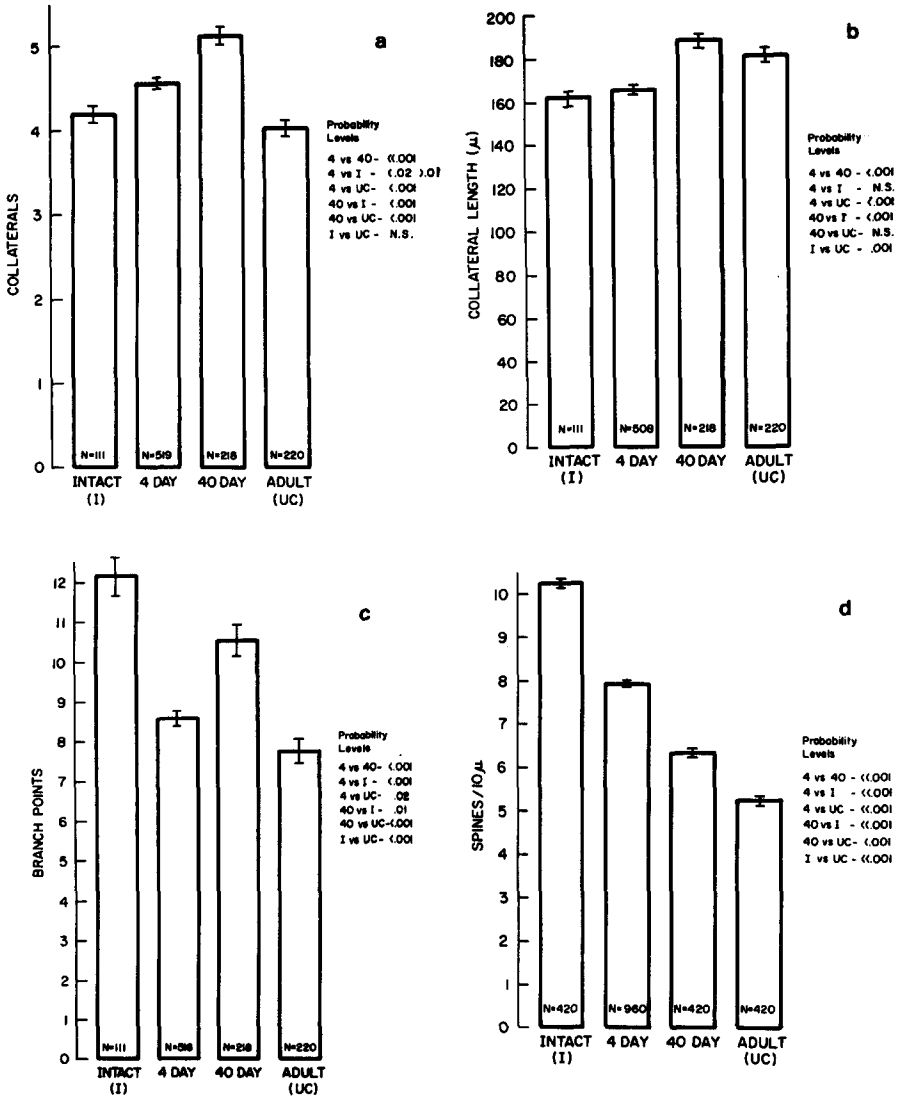

Fig. 2. Summary of quantitative measures of number of collaterals, length of collaterals, number of collateral branch points and counts of dendritic spines. Computer program used for computing *F*-statistic to ascertain equality of variance in compared groups and *t*-statistic to test differences between means. Probability levels of significance of mean differences shown for every two-group comparison. N.S. = not significant. Vertical bars are mean values of the number of measures, each measure from an individual cell studied (in a, b, c), as indicated at bottom of each bar, *e.g.*, N = 111, N = 519, etc. Plus or minus one standard error of the mean shown at top of each bar. a, Number of axon collaterals, recurrent type, more than 25 μm in length, 'intact' cells studied, 111, '4-day undercut', 519, etc. Both 4-day and 40-day groups had significantly more collaterals than did the intact ($0.01 < P < 0.02$ and $P < 0.001$). The adult UC group was not different from the intact. b, Collateral lengths, averaged in most cases from the two longest, each over 100 μm. Both the 40-day and adult UC groups had maintained collateral lengths longer than did the intact group ($P < 0.001$). c, Number of collateral branch points in cells studied. All experimental groups had significantly fewer branch points than did the intact group. d, Number of dendritic spines per 10 μm sections of apical dendrites. Samples from all groups were large, see text. All experimental groups had significantly fewer spines than did the intact group. The adult UC group had fewer than the 40-day group and the latter had fewer spines than did the 4-day group.

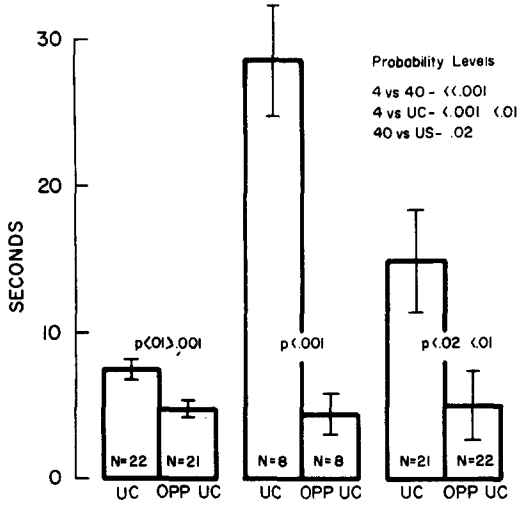

Fig. 3. Averaged durations in seconds of afterdischarges to surface electrical stimulation determined in terminal acute experiments. Probability levels of significance from computer program (see caption for Fig. 2). Plus or minus one standard error of the mean at the top of each bar. N = number of separate observations of afterdischarges. UC = undercut cortex, OPP UC = homotopic cortex opposite to undercut. Durations of afterdischarges on the undercut side were compared with those in the homotopic area of the opposite hemisphere. In all groups the undercut cortex gave significantly longer afterdischarges than did the opposite cortex. In the between-groups comparisons the 40-day group had the longest afterdischarges followed by the adult UC and the 4-day group.

On the average, then, deefferented immature pyramidal cells (4-day) showed a persistent compensatory tendency to produce additional collaterals. Furthermore, the same tendency was expressed in the relatively mature cortex (40-day).

Although number of collaterals increased in cortex denervated before complete maturity a similar 'proliferative change' was not reflected in collateral lengths for both young groups. In Fig. 2b, where averaged collateral lengths are compared, only the 40-day group of the two young groups showed a persistent increase. Apparently pyramidal cells in the cortex of the neonatal brain do not have growth capacity to maintain both the appearance of new collaterals and their increased length through maturation to adulthood. The state of brain maturity seems to make a difference, since denervation of pyramidal cells in the adult results in no new collaterals but it does produce an increase in collateral length and these new lengths apparently persist (Fig. 2b).

Contrary to what might be expected from the changes in axon collaterals above, new growth is not expressed as an increase in collateral branching in any of the 3 experimental groups (Fig. 2c). The findings on collateral branch points are actually the reverse of those for number and length of collaterals since there is a decrease of collateral terminal branching in all groups compared with intact. In the 40-day group there is a significantly smaller loss than in either of the other two experimental groups.

All groups, compared with intact, showed a significant loss of dendritic spines (Fig. 2d). The findings are clear and emphasize an increasing vulnerability of synaptic

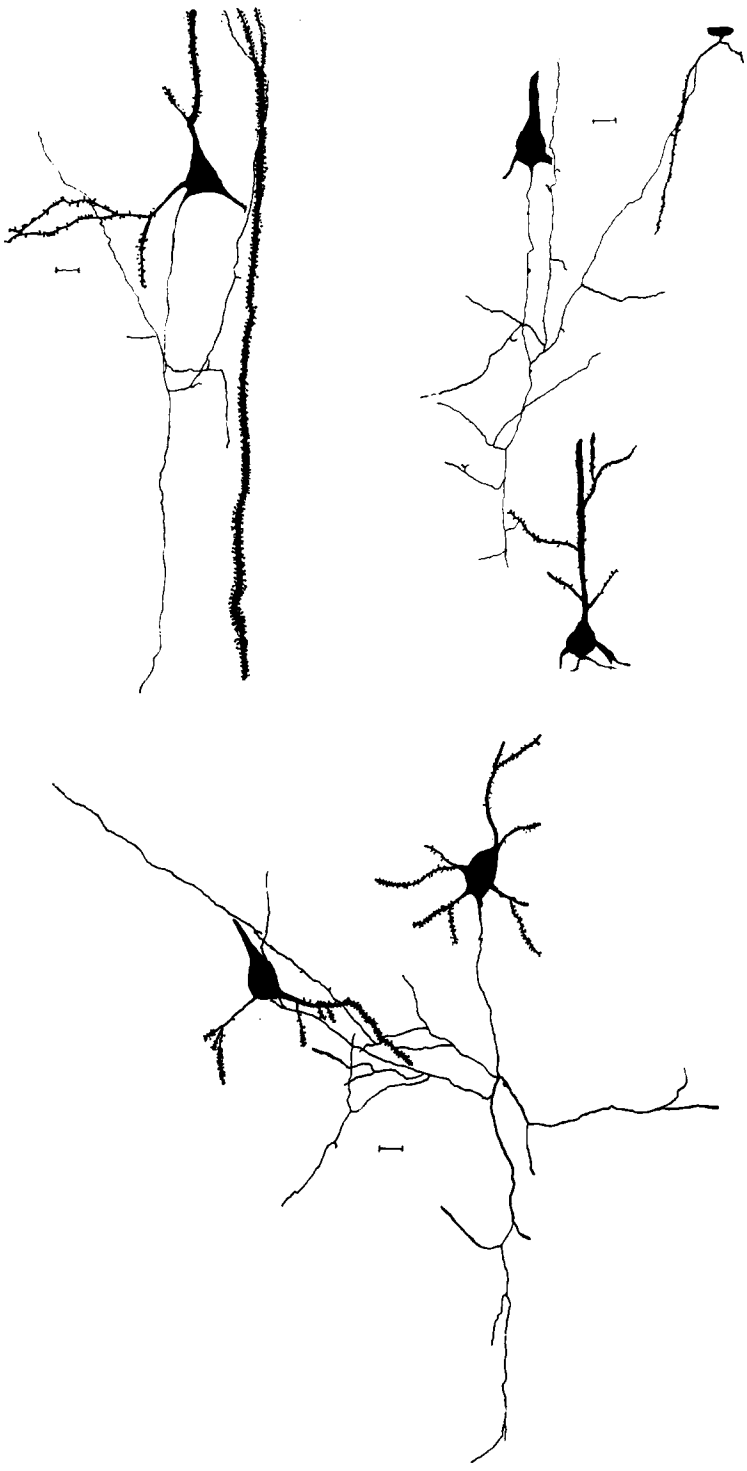

Fig. 4. Apparently terminal recurrent axons, selected from 4-day and 40-day groups. Upper left and upper right: cells from cortical layers II, III, 4-day group. Lower: two cells from upper part cortical layer IV, 40-day group. Scale drawings, length bars = 10 μm.

Fig. 5. Scale drawings of 4 layer VI pyramidal cells with bifurcating axons running parallel to location of old subcortical 'fiber paths', 4-day group. Lined sections = approximate location of nearest edge of subcortical 'paths', see Fig. 6. Length marks = 10 μm.

contacts as the brain matures. As seen with the light microscope, denervation of adult cortex results in about a 50% loss of spines on pyramidal cell apical dendrites, compared with intact cortex. The observed losses are undoubtedly conservative[20].

In Fig. 3 the averaged durations of afterdischarges are compared in the two hemispheres for animals in each group. For the side opposite to the undercut, durations of afterdischarges are not different between the groups. The 40-day group had by far the longest afterdischarges to surface electrical stimulation, but the adult UC group had longer afterdischarges than the 4-day group.

Qualitative morphological changes

There were differences in axon collateral morphology which were not reflected in the quantitative measures. It was noted that in histological sections from the 4-day animals it was easier to follow terminal axon collateral branches as they apparently impinged upon basal or apical dendrites. It was less easy to find similar instances among material from the 40-day animals and rare to do so in adult UC.

Fig. 4 illustrates axon collateral distribution for some recurrent branches in the 4-day and 40-day material. Although an assertion about terminals seen in the light

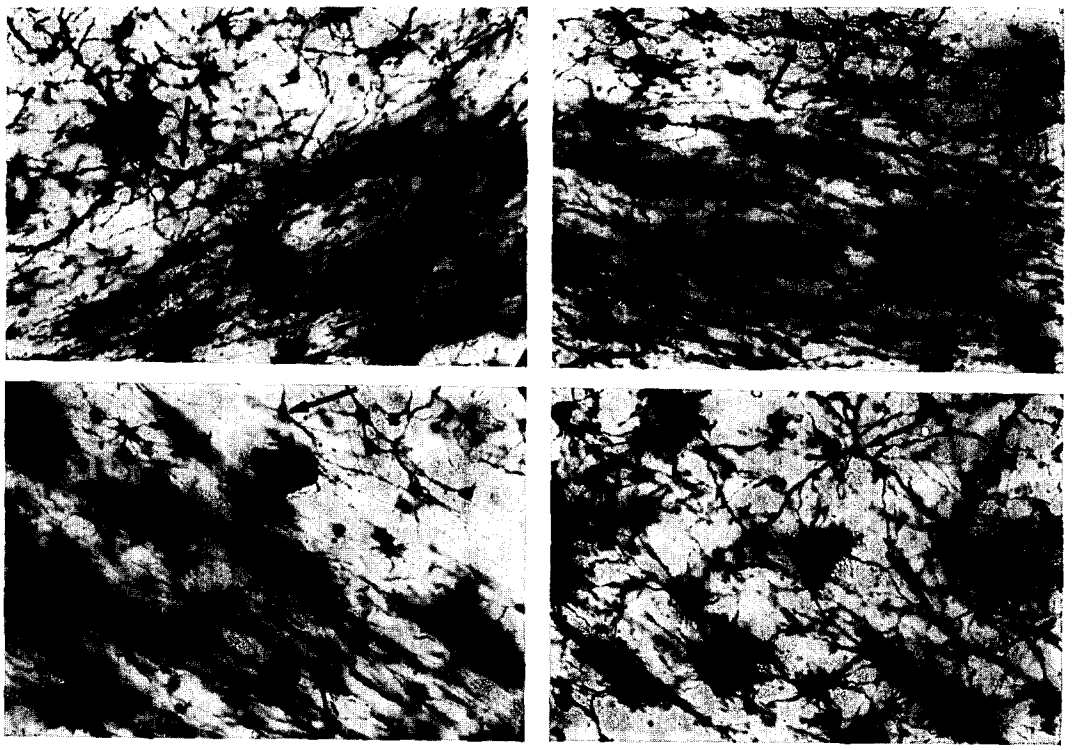

Fig. 6. Actual locations of cell somas corresponding to the 4 in Fig. 5. Fiber 'paths', characterized by presence of large fibrous astrocytes, are in the lower part of each photomicrograph. The cell-'fiber path' orientations are slightly different from those in Fig. 5. Somas indicated by arrows. Modification of Golgi–Cox method. × 200.

microscope actually making contact with elements of neighboring neurons should not be made, it can be appreciated from Fig. 4 that a small collateral branch, interwoven in the same focal plane about a dendrite, likely makes some synaptic contact. Some projections were convincing, such as the parallel ones in Fig. 4 apparently contacting several spines. This type has been recently described[2].

A dramatic morphological feature, seen nearly exclusively in material from 4-day animals, is illustrated in Fig. 5. Pyramidal cells, including the fusiform type in layers V and VI, were occasionally noted to have axons which bifurcated, without recurrence, and to project their aberrant growth in gray matter parallel to where the subcortical fiber bundles would have been. There is no doubt that such growth is new, abnormal and aberrant, and is maintained through maturity to adulthood. Such bifurcation without recurrence has not been seen in material from intact or adult UC, and only rarely in 40-day animals and then not as extensive as that shown in Fig. 5. The accompanying Fig. 6 contains low power photomicrographs showing where the somas of the cells in Fig. 5 were located relative to the old fiber paths. The outstanding descriptive feature of the immediate subcortical old white matter location is an area heavily invaded by large fibrous astrocytes. Astrocytic processes seemed to have a tendency to stream in the directions of the normally present neuronal axons. In every instance where bifurcating axons of the types seen in Fig. 5 were found they were always near the border of a glial barrier as shown in Fig. 6.

DISCUSSION

Proliferation of axon collaterals, if persistent, ought to be describable months after isolation by the measures used here, number of axon collaterals, collateral length and number of branch points. The number of collaterals actually increased in the 4- and 40-day groups as compared with those in intact cortex. There must be sufficient growth potential in the kitten brain through the 40th neonatal day, but not in the adult brain, to result in a permanent increase in the averaged number of collaterals.

A persistent increase in averaged collateral length was found in the 40-day group and the adult UC. It is puzzling why a collateral length increase, assuming that it must have occurred initially, did not persist into adulthood in the 4-day group. One possible explanation is that in the neonate after denervation the newly grown axonal terminals fail to develop synaptic contacts, perhaps because receptor sites are not available or ready, and without trophic influences the nonfunctional terminals then regress to eventually stabilized shorter lengths. For the adult UC once the adult configuration of the cortical neuropil is established trophic influences exist but synaptic sites are occupied and newly increased collateral lengths find no specific synaptic sites available. This should produce in the adult undercut slab a somewhat random directionality of recurrent collaterals. The increased collateral length in the adult UC was not observed in the previous study[16], although it may have escaped measurement since the two longest collaterals in that sample of 100 cells were measured regardless of their length (beyond 25 μm), whereas a more rigorous criterion that one collateral

had to be at least 100 μm in length, was used in the present sample of 220 cells.

Axon collateral branching should be a sensitive measure of proliferation and this is apparently the case a few days after undercutting cortex in the neonate[1,12]. Assuming increased branching occurred initially then there must have been a loss of branch points after several months since all experimental groups had fewer branches than did intact animals. This would seem to be a strong argument against maintained or persistent proliferation. The fact that the 40-day animals showed significantly more collateral branching than did the other two experimental groups may be related to the greater tendency for these animals to generate longer afterdischarges.

The concentration of spines upon apical dendrites has proven to be a reliable measure for distinguishing adult undercut cortex from intact[15]. Neocortical neurons of the cat at birth and shortly thereafter have incompletely developed apical dendrites and a paucity of dendritic spines[11,13]. Further, electron microscopy of rat cortex showed incomplete synaptic development even up to 14 days[7]. These authors point out that the cat at birth is approximately equal to a 7–10-day rat in synaptic morphogenesis and electrogenesis. It is reasonable to suppose that the effects of denervation upon dendritic spines would be especially important in the young animals and should reflect alterations of development and extent of persistence of synaptic contacts. The basic finding from our data is that dendritic spines are increasingly vulnerable to the effects of denervation as the brain matures. All experimental groups had significantly fewer spines than did the intact group. The maximum loss was 50% in the adult UC, but this is excessively conservative as proven by electron microscopy[20]. It would appear that there is no basis for assuming that axon collateral proliferation, however measured, results in any maintenance of synaptic spine contacts. It was not possible to study soma contacts with the Golgi method and light microscopy.

The data from these experiments offer no solid basis for the hypothesis that convulsive, epileptic or afterdischarge activity in denervated cortex can be explained by persistent axon collateral proliferation. Data on the two most important measures, collateral branching and synaptic contacts as determined by concentration of spines upon apical dendrites, showed significant decreases for all experimental groups as compared with intact material. It is true that no spine counts were made on basal dendrites and synaptic contacts upon somas could not be evaluated, but it is suggested that collateral branching alone should reflect proliferation. There were significant differences between the groups so the measurements were likely reliable.

The 40-day animals gave by far the longest afterdischarges and had more and longer collaterals than the other groups. Were it not for the fact that the 4-day animals could not sustain afterdischarges, it could be argued that there was a relationship between number of collaterals and duration of afterdischarge since both the 4- and 40-day animals showed increases over the intact. Thus, on only one measure, length of collaterals, is there an association with duration of afterdischarge. Certainly this weak evidence would be less than supportive of the hypothesis that convulsive tendencies are associated with axon collateral proliferation. The present findings are consistent with most of our previous results and interpretations[15,16].

The unusual bifurcating axons, which ran parallel to what were subcortical

fiber pathways and seen especially in the 4-day animals, did not present a picture of terminals approximating neighboring pyramidal cell dendrites or somas, as observed by Cajal in short term undercut slabs in immature brains[1]. This is further evidence against effective reorganization of proliferative elements. The distinct impression, in looking at the cells with bifurcating, nonrecurrent axons (Fig. 5), was that the abnormal new growth expressed by these cells reflected an attempt of the axons to circumvent a barrier set up by the glial concentration in what was or would have been fiber pathways. To the authors' knowledge this is the first demonstration that a pure glial barrier apparently can prevent axonal regeneration when the potential for such regeneration is present.

The general problem of regeneration and growth in the central nervous system as related to the present studies is quite different conceptually from the few observations which have described sprouting following denervation. For example, it was found that sprouting occurred from intact axons and this growth was apparently related to an increased level of spasticity in spinal cats[8]. Windle[22] has emphasized the importance of brain immaturity in promoting additional growth from intact cells, but this was not to say that deefferented axons in the immature brain had greater regenerative capability. Rather, uninjured cells were able to express their growth potential into the injured area (spinal cord). In agreement with Windle[22] the present results confirm that immature neurons are as vulnerable to destructive effects of denervation as are nearly mature neurons or those in adult cortex.

The data from these experiments would seem to answer Cajal's question and skepticism about the persistence of axon collateral proliferation observed in the neonate or immature brain[1]. There was little evidence of persistence of proliferation through maturity, rather there were significant decreases in measures used to evaluate proliferation. There was also no evidence that growth of any new neuronal elements effected functional reorganization, although other perhaps more sensitive measures, should be used to evaluate this point. Finally, the data offer little support for the hypothesis that axon collateral proliferation in denervated cortex is related to or associated with an increased convulsive tendency after the brain has reached maturity.

SUMMARY

Cortices of animals in 3 groups were denervated by undercutting at 4 or 40 days of age or at adulthood. Months later electrocortical seizures were studied in terminal experiments and cortical tissues prepared for light microscopic study. Four measures, assumed to be descriptive of or related to axon collateral proliferation, were made: number of axon collaterals, length of collaterals, number of collateral branches and number of apical dendritic spines. Data from adult intact cortex served as controls.

Increases in number of collaterals and collateral length were observed, the best evidence being in the 40-day animals. However, since collateral branching decreased, the data do not support the concept of proliferation as described in young brains.

If dendritic spines are valid indicators of synaptic contacts then the observed

significant loss of spines argues against the maintenance of functional connections, if indeed they were established, following denervation at any age.

There were no obvious relationships between morphological changes and duration of afterdischarges.

A persistent alteration was frequently seen in cortex which had been undercut at 4 days of age. Lower lying pyramidal cell axons bifurcated with nonrecurrent spread parallel to the glia infiltrated site of the old fiber paths. This glial barrier appeared to have prevented centripetal axon growth.

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