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DEMYELINATION, AND REMYELINATION BY SCHWANN CELLS AND OLIGODENDROCYTES AFTER KAINATE-INDUCED NEURONAL DEPLETION IN THE CENTRAL NERVOUS SYSTEM

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Abstract—Excitotoxins are thought to kill neurons while sparing afferent fibers and axons of passage. The validity of this classical conclusion has recently been questioned by the demonstration of axonal demyelination. In addition, axons are submitted to a profound alteration of their glial environment. This work was, therefore, undertaken to reassess axonoglial interactions over time after an excitotoxic lesion in the rat. Ultrastructural studies were carried out in the ventrobasal thalamus two days to 18 months after neuronal depletion by in situ injections of kainic acid. In some cases, lemniscal afferents were identified by using anterograde transport of wheatgerm agglutinin conjugated to horseradish peroxidase from the dorsal column nuclei.

Two and four days after kainate injection, numerous dying axons displaying typical signs of Wallerian degeneration were observed in a neuropile characterized by the loss of neuronal somata and dendrites, an increase in number of microglia/macrophages and the disappearance of astrocytes. Ten and 12 days after kainate injection, degenerating axons were no longer observed although myelin degeneration of otherwise unaltered axons was ongoing with an accumulation of myelin remnants in the neuropile. At 16 and 20 days, the demyelination process was apparently complete and axons of different diameters were sometimes packed together. One and two months after kainate injection, the axonal environment changed again: remyelination of large-caliber axons occurred at the same time as reactive astrocytes, oligodendrocytes and numerous Schwann cells appeared in the tissue. Schwann cell processes surrounded aggregates of axons of diverse calibers, ensheathed small ones and myelinated larger ones. Axons were also remyelinated by oligodendrocytes. Horseradish peroxidase–labeled lemniscal afferents could be myelinated by either of the two cell types. After three months, the neuropile exhibited an increase in number of hypertrophied astrocytes and the progressive loss of any other cellular or axonal element. At this stage, remaining Schwann cells were surrounded by a glia limitans formed by astrocytic processes.

These data indicate that although excitotoxins are sparing the axons, they are having a profound and complex effect on the axonal environment. Demyelination occurs over the first weeks, accompanying the loss of astrocytes and oligodendrocytes. Axonal ensheathment and remyelination takes place in a second period, associated with the reappearance of oligodendrocytes and recruitment of numerous Schwann cells, while reactive astrocytes appear in the tissue at a slightly later time.

Over the following months, astrocytes occupy a greater proportion of the neuron-depleted territory and other elements decrease in number. These successive stages in alteration of axonoglial interactions seem to evolve in parallel to the changes in density and terminal morphology that we described earlier for myelinated afferent fibers to the excitotoxic lesion.

Axons afferent to a CNS area subjected to an excitotoxic agent are not directly damaged, structurally or functionally, in sharp contrast to neuronal somata and dendrites that are rapidly destroyed. After a few weeks, however, axonopathy has been described in many cases, probably resulting in a dying-back process leading to a decrease in the number of axons that have been deprived of their targets (see discussion in Ref. 16). In parallel to these regressive events, we have observed structural changes that resemble attempts at axonal regeneration by myelinated fibers (lemniscal and corticothalamic afferents, thalamostriatal axons, cortico- and rubrospinal fibers) while unmyelinated afferents to the same areas, either monoaminergic or not, remained unaltered.

This distinction made between the fate of axons on the basis of their normal glial ensheathment suggested that excitotoxins may secondarily affect axons by interfering with axonoglial relationships. As a result of the excitotoxic lesion, profound glial and vascular changes are observed. Leakage of the blood–brain barrier occurs rapidly, together with activation of resident microglia. Recruitment and proliferation of microglia/macrophages lead to a major increase in the number of glial cells over the first weeks following neuronal loss while astrocytes are no longer observed. Later, the area is filled with

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Abbreviations: BDHC, benzidine di-hydrochloride; HRP, horseradish peroxidase; PNS, peripheral nervous system.
reactive astrocytes that progressively form the so-called glial scar. Two recent observations have suggested that these changes may alter axonoglial interactions. Coffey et al. have observed that an injection of ibotenic acid into the septum provoked a massive loss of immunocytochemical markers of myelin within days, indicating a demyelination of axons passing through the area. This observation was in keeping with previous suggestions of a rapid demyelination process after kainate injection. Analysis of the fine structure of the thalamic neuropile, one month after kainate injection, has additionally revealed the presence of Schwann cells. The abnormal location of these peripheral nervous system (PNS) cells in a CNS area deep in the brain, and their involvement in axonal ensheathment and myelination are clear signs of remyelination and, therefore, of the previous occurrence of axonal demyelination.

Since it is well established that pathological changes of myelin ensheathment of central axons is responsible not only for functional but also for structural axonal alteration (see References and Discussion in Ref. 49), excitotoxins may have a secondary deleterious effect on afferent systems to a neuron-depleted area.

The present study has therefore been undertaken to precisely analyse the changes in axonoglial relationships over time after an excitotoxic lesion.

**EXPERIMENTAL PROCEDURES**

Twenty-seven female Sprague-Dawley albino rats (200 g at the beginning of the experiments, Charles River, France) were used. They were anesthetized with chloral hydrate (400 mg/kg, i.p.) and received a slow-pressure injection of 5 nmol of kainic acid (in 0.15 ml of saline, over 12 min). The injection was aimed at the lateral border of the rostral thalamus as previously described, in order to obtain a large area of total neuronal depletion in the thalamic reticular and ventrobasal nuclei.

Following various survival times (two, four, 10, 12, 16 and 20 days, one, two, three, six, 12 and 18 months), 24 animals were prepared directly for standard electron microscopy. Rats were perfused with warm heparinized phosphate-buffered saline (0.1 M, pH 7.4) followed by cold fixative containing 2% glutaraldehyde, 2% paraformaldehyde and 4% sucrose in the same buffer. The brain was postfixed for 2 h. The diencephalon was trimmed out and cut serially in coronal sections (50 μm thick) on a Vibratome (Oxford Inst.). Sections were fixed with osmium (phosphate-buffered solution as described above) and to the zona incerta, respectively. At the ultrastructural level, the identification of the axons was easily made when axons were surrounded by layers of myelin. In other cases, for unmyelinated or totally demyelinated axons, we relied upon the commonly accepted criteria. Axons, in the CNS neuropile, are characterized as profiles having smooth contours and a cylindrical shape, display a clear cytoplasm that contains neurofilaments, arrays of microtubules and a few mitochondria and that, mostly, contains no ribosomes. Analysis of the neuropile between two and 18 months after kainate injection allowed us to characterize four different periods according to specific features of axonoglial injections: (i) a few days during which Wallerian degeneration predominated; (ii) the second week, characterized by profuse demyelination of axons; (iii) the third week in which the neuropile was devoid of myelinated axons; and (iv) subsequent periods in which large axons were remyelinated.

**RESULTS**

In embedded sections, the area of neuronal loss that was to be trimmed out for our study was conspicuous because of its colorless aspect related to a decreased osmication. When compared to adjacent Nissl-stained sections, this area comprised a large part of the ventralbasal thalamus but never extended to the large fiber tracts around it, the internal capsule and medial lemniscus, that were as well stained as on the opposite unlesioned side. Preservation of the osmication of these white matter areas was observed even when the kainate lesion had extended in the gray matter on both sides of the tracts, i.e. to the striatum and to the zona incerta, respectively. At the ultrastructural level, the identification of the axons was easily made when axons were surrounded by layers of myelin. In other cases, for unmyelinated or totally demyelinated axons, we relied upon the commonly accepted criteria. Axons, in the CNS neuropile, are characterized as profiles having smooth contours and a cylindrical shape, display a clear cytoplasm that contains neurofilaments, arrays of microtubules and a few mitochondria and that, mostly, contains no ribosomes. Analysis of the neuropile between two and 18 months after kainate injection allowed us to characterize four different periods according to specific features of axonoglial injections: (i) a few days during which Wallerian degeneration predominated; (ii) the second week, characterized by profuse demyelination of axons; (iii) the third week in which the neuropile was devoid of myelinated axons; and (iv) subsequent periods in which large axons were remyelinated.

**Demyelination process**

Two and four days after kainate injection, the neuropile was characterized by the presence of cellular debris and numerous microglia/macrophagic cells. In contrast, astrocytes were noticeably less numerous than in the intact tissue (Fig. 1). Dendrites and...
Fig. 1. Four days after kainate injection, the neuropile is characterized by the presence of degenerating dendrites exhibiting remnants of synaptic organizations (D) and axons (A1, A4) whose cytoplasm is abnormally either electron-lucent or electron-dense and contains altered mitochondria. All axons in this picture exhibit alteration of myelin sheaths although axonopathy is not conspicuous in some of them (A2, A3). Note the absence of astrocytic endfeet around the capillary. Scale bar = 1 μm.

neuronal somata displaying a normal morphology were totally absent and membranes surrounded apparently empty spaces that sometimes contained a few organelles that exhibited various signs of degeneration. The identification of these structures as formerly dendritic or somatic could be made because of the persistence of postsynaptic densities (Fig. 1). Axonal profiles could still be clearly

Fig. 2. Ten days after kainate injection. (a) The neuropile contains cellular and myelin debris together with numerous macrophages (M). Degenerating axons are not conspicuous at this stage although demyelination is clearly continuing. (b) At higher magnification, inner layers of myelin appear dismantled before outer ones. (c) A large axon, totally demyelinated, is surrounded by myelin debris that is sometimes still attached to its membrane. Scale bar in c = 2 μm for a, 0.5 μm for b and 1.5 μm for c.
identified. Many of them exhibited signs of Wallerian degeneration with morphologically altered mitochondria—enlarged with widened cristae—and an increase of the axoplasmic electron density. Other axons, however, either myelinated or not, did not display these degenerative features and looked healthy. One striking characteristic of the tissue, however, was the widespread abnormality of myelin sheaths surrounding the normal and degenerating axons (Fig. 1). Myelin sheaths were dismantled and the lamellae appeared loosely arranged around the axons, sometimes there was better preservation of the outermost lamellae when the axons looked normal (See Figs 1, 2a, b).

Ten and twelve days after kainate injection, the alteration of the neuropile had progressed with the almost total disappearance of any structures reminiscent of dendrites or somata. Small numbers of synaptic boutons were still attached to an electron-dense postsynaptic membrane and formed what have been called “moustache” contacts. Macrophages, often loaded with debris, were observed mostly close to the blood vessels (Fig. 3a). The neuropile was characterized by the near disappearance of myelin, except for a few remnants (Fig. 3b). Axons appeared, therefore, systematically unmyelinated, whatever their caliber. In some cases (as in Fig. 3), axons were packed together in large numbers. Some unidentified cells extended long processes between axons in these clusters (Fig. 3b).

Remyelination process

One and two months after the lesion had been induced, the general features of the neuropile changed again, due to the reappearance of numerous myelinated axons and to the presence of astrocytes.
Fig. 4. Areas containing Schwann cells in one- (b), two- (a, c) and six- (d) month-old kainate lesions. (a–c) In the earlier periods, Schwann cells are numerous and interspersed with macrophages (M) (a, b), or with axons myelinated by oligodendrocytes (stars, c). (d) At later stages, Schwann cells are few in number and appear surrounded by astroglial processes, containing gliofilaments (g), that are bordered by a continuous basal lamina (arrowheads). Note the presence of collagen fibrils (arrows) around the Schwann cell. Scale bar in d = 3.3 μm for a, 2 μm for b, 1 μm for c and 0.6 μm for d.
oligodendrocytes and numerous Schwann cells. Schwann cell myelin was differentiated from oligoden-
drocytic myelination on the basis of three main morphological criteria:46 (i) a thin rim of cytoplasm
around the entire perimeter of the ensheathed axon; (ii) the presence of the nucleus and cytoplasm in the
sheath surrounding the axon; and (iii) a basal lamina
and collagen fibrils around the Schwann cells. Many
areas in the tissue appeared to contain numerous
Schwann cells, some of which myelinated axons
(Fig. 4a, b). Many clusters of Schwann cells were close
to blood vessels (Fig. 4a), but this was not always the
case (Fig. 4b). Axons myelinated by oligodendrocytes
were also often grouped together (Fig. 4c). There was,
however, no strict delineation of territories between
Schwann cells and oligodendrocytes and adjacent
axons could be myelinated by either one (Fig. 4c).

Qualitative observation of the myelin sheaths
formed by oligodendrocytes or Schwann cells showed
no specific characteristics (Fig. 5a, b). Quantification
demonstrated that the new sheaths of myelin formed
by oligodendrocytes in the lesion were especially
thin since the ratio between axon and axon + myelin
diameters was 0.874 ± 0.043 (n = 27) as compared
to 0.687 ± 0.08 (n = 22) in the intact thalamus
(P < 0.001). There was no statistically significant
difference, in contrast, between the ratios calculated
for oligodendrocytes in the intact thalamus and
Schwann cells in the lesion (0.731 ± 0.097, n = 20,
P > 0.1) whereas the difference was significant be-
tween oligodendrocytes in the lesion and Schwann
cells (P < 0.001).

In the lesioned thalamus, as well as in the intact
nucleus, unmyelinated axons ensheathed by profiles
that could be identified as oligodendroglial were not
observed. In contrast, Schwann cells ensheathed many
of them according to arrangements that seemed to
depend upon the diameter of the axons. Large axons
were myelinated by Schwann cells according to a
one-to-one relationship (Fig. 6a and see also
Fig. 4a, b). Medium-caliber axons were often, but not
always, isolated in a separate trough of a Schwann
cell, even though the same cell ensheathed other
unmyelinated axons (Fig. 6b, c). In some cases,
medium-caliber axons were surrounded by several
interdigitating processes of the same Schwann cell
(Fig. 6c), suggesting the appearance of a mesaxon
and the potential start of a myelination process. Smaller
axons were not isolated but, quite the contrary,
ensheathed together by one cytoplasmic process
(Fig. 6b). Morphological features atypical for the
CNS also appeared at the level of nodes of Ranvier,
in particular when the nodes were surrounded by
cytoplasmic processes of the two Schwann cells that
myelinated adjacent axonal segments (Fig. 6d).

In the animals in which lemniscal axons had been
anterogradely labeled from the dorsal column nuclei,
crystals of BDHC reaction product were observed
not only in axons myelinated by oligodendrocytes
(Fig. 7a) but also in axons myelinated (Fig. 7b) or
simply ensheathed, without myelination, by Schwann
cells (Fig. 7c). Limitation of the technique prevented
a strict quantification of the different types of
ensheathment to be made but there did not seem to
be a particular preference of lemniscal fibers to be
associated with either oligodendrocytes or Schwann
cells.

At later stages, no further modification of ax-
onoglial relationships occurred and the overall
features of the neuropile progressively evolved toward
a loss of all elements except for hypertrophied astro-
cytes containing bundles of gliofilaments that formed
a dense network. Remaining Schwann cells myelinating
axons were totally surrounded by astrogial pro-
cesses that were lined up with a basal lamina on their
side facing the PNS element (Fig. 4d).
DISCUSSION

This study confirms and extends previous results that had indicated profound alteration of axonoglial interactions in an area excitotoxically depleted of neurons. Two major issues are raised by these results. First, the demyelination process leads, within two weeks, to an almost complete disappearance of myelin sheaths. This suggests that the excitotoxic lesion provides conditions that initiate demyelination which may be related to the death of oligodendrocytes. Second, remyelination and axonal ensheathment are carried out by Schwann cells and oligodendrocytes that are recruited into the lesion area.
Demyelination of unlesioned axons accompanies massive neuronal loss

Demyelination of axons of passage or afferent fibers has been reported in only a few studies of excitotoxic lesions.\textsuperscript{12,13,39} An indirect confirmation of such a process was given by our observation that numerous Schwann cells ensheath and myelinate axons in the one-month-old kainate-lesioned thalamus.\textsuperscript{18} The results of the present study strongly support the existence of such a demyelinating process. Direct evidence of demyelination of surviving axons was difficult to obtain in short-term lesions because many myelinated axons were undergoing Wallerian degeneration. However, as early as ten days after the kainate injection demyelination was apparent with otherwise healthy axons surrounded by debris of myelin lamellae. The existence of this process was further supported by the observation of grouped unmyelinated axons of all calibers at a later stage. Large (greater than 0.5 \mu m in diameter), apparently intact, axons were devoid of a myelin sheath which is uncommon in the intact CNS but has been described in demyelinating experimental lesions.\textsuperscript{55} In addition, large axons myelinated by oligodendrocytes in long-term lesions displayed a myelin sheath much thinner than in intact tissue. Such thin myelin sheaths are typical of remyelination,\textsuperscript{5,28,41,48} and thus indirectly demonstrate a prior demyelination.

Demyelination of afferent fibers, and probably of fibers of passage in the neuron-depleted area could be the result of direct injury to the oligodendrocytes by kainate, comparable to that observed with specific gliotoxic agents such as lyssolecithin.\textsuperscript{4,25} However, even though oligodendrocytes have glutamate receptors,\textsuperscript{35} kainate is not toxic for them when applied for 1 min on acute brain slices (Kettenmann, personal communication). In addition, there is no demyelination in the internal capsule, a structure exposed to kainate injected at the border of the internal capsule, and sometimes spreads across it to the striatum. It is interesting to mention that, in sharp contrast to what occurs in the thalamic nucleus, there is no major recruitment or proliferation of microglia/macrophages in the internal capsule,\textsuperscript{35} where the leakage of the blood–brain barrier is weak (unpublished observations) and reactive astrocytes are present in large numbers\textsuperscript{20} in the first weeks after kainate injection. This indicates that glial changes in the white matter in which kainate is infused are quite different from

Fig. 7. Lemniscal afferents anterogradely labeled using wheatgerm agglutinin–HRP in the two-month-old kainate lesion. These identified CNS axons are ensheathed either by oligodendrocytes (a) or by Schwann cells that may (b) or may not (c) myelinate them. Arrows point to crystals of BDHC reaction product. Scale bar in c = 0.3 \mu m for a and 0.8 \mu m for b and c.
those observed in areas in which it induces neuronal degeneration, suggesting that those observed in the thalamus are not related to a direct injury of oligodendrocytes by kainate. Alternatively, the leakage of the blood–brain barrier and the macrophagic invasion that are observed in the thalamic lesion may lead to a progressive accumulation of oligodendroglial substances and, therefore, to a long-lasting demyelination process. Several recent in vitro studies suggest that blood-borne complement factors as well as cytokines produced by activated macrophages may be toxic to oligodendrocytes and CNS myelin. Thus, mature oligodendrocytes would be under attack as complement factors leak through the blood–brain barrier, or when the brain parenchyma contains activated macrophages. This hypothesis is supported by the demonstration that demyelination can be blocked shortly after the lesion by irradiation that decreases inflammatory response and reduces leakage of the blood–brain barrier. If one or both mechanisms are involved, it is most likely that the nature of the agent leading to neuronal death plays little role in the additional demyelination since any neuronal death of consequence will induce both a profuse leakage of the blood–brain barrier and an inflammatory response.

Schwann cells are recruited in the CNS

In demyelinating lesions produced in the spinal cord, Schwann cells have been observed mostly in superficial regions, close to the dorsal root entry zones, suggesting that they may have penetrated the CNS neuropile due to a leakage of the CNS–PNS barrier. Such an origin is highly unlikely in our experimental conditions, given the distance separating the dorsal thalamus from the CNS–PNS border. A more likely explanation comes from the demonstration that Schwann cells are normally present in the wall of small arteries, associated with innervation of the vessels. Using tritiated thymidine, Harrison and Pollard have demonstrated that Schwann cells located in perivascular spaces, rather than those in the dorsal roots, proliferate following a demyelinating lesion of the spinal cord. Schwann cells penetrating the experimentally-demyelinated spinal cord have been observed in clusters surrounding capillaries, possibly in close proximity to a suitable extracellular matrix. A similar observation was made in our study. This suggests that, in the neuron-depleted area, capillary walls may act as the substrate along which Schwann cells can migrate out of their original location in the perivascular walls of small arteries. The extent of this phenomenon is probably increased by the reactive angiogenesis that takes place after kainate lesion.

Astrocytes, bordered by a basal lamina, form a glia limitans wherever the CNS neuropile is in contact with peripheral elements. It has been clearly established that damage to the glial limiting membrane is an important prerequisite to Schwann cell invasion of the CNS in demyelinating lesions of the spinal cord and the retina. The results obtained in the present study are in agreement with those summarized by Blakemore concerning the remyelination by Schwann cells of spinal cord demyelinated axons. It has been observed in the present ultrastructural study that kainate injection is followed first by swelling of astrocytic processes leading to a disruption of the tight layer formed around capillaries by astrocytic endfeet. This is in keeping with our previous light microscopic analysis that showed that reactive astrocytes are not present in the neuron-depleted area during the first weeks following kainate injection. These results clearly underline the transient failure of astrocytes to oppose the invasion of Schwann cells. Astrocytes, however, rapidly increase in number after one month, becoming the prevailing elements after three months. In the kainate lesion, as in other experimental conditions in which a breach appears in the glia limitans, astrocytes tend to repair it, thereafter excluding Schwann cells, forming, in the excitotoxic lesion, a tight glia limitans around every Schwann cell.

Several studies have demonstrated that Schwann cells do not myelinate central axons unless these axons are demyelinated or in a growth process. The demyelination process observed in the neuron-depleted area provides a clear opportunity for Schwann cells to identify available axons. It is interesting to mention, however, that large (probably demyelinated) axons are not the only targets of Schwann cells. Numerous small axons are additionally ensheathed. The axonoglial relationships observed in the neuron-depleted thalamus are, in that sense, demonstrative of the fact that Schwann cells are not just replacing missing elements but acting with CNS axons as they would have done, had they been ensheathing PNS axons.

Structural alteration and possible growth of axons as a consequence of altered axonoglial relationships

In the excitotoxic lesioned thalamus, terminals of lemniscal fibers exhibit structural changes and possible growth. Time-course analysis of these changes has revealed that large varicosities formed over the first three weeks. Over the following months, thin processes grew out of these varicosities and extended into the neuron-depleted area, suggesting attempts at growth and regeneration. The fact that altered afferents only arise from myelinated axons and that the formation of terminal varicosities is concurrent with the period of demyelination observed here, suggest that there may be a relationship between these axonal changes and the alteration of axonoglial interactions.

The remyelination of central axons by Schwann cells may aid both axonal survival as well as the growth of the thin processes out of the varicosities. Schwann cells indeed produce neurotrophic...
factors which promote survival and growth of both peripheral and central axons. It has been proposed that target-deprived fibers rapidly retract from areas of neuronal depletion due to the loss of maintenance factors released by target cells. Following the same reasoning, the observation of a long-term survival of target-deprived axons suggests some recovery of trophic influences in the target zone. The release of trophic factors in an excitotocically lesioned area has been demonstrated biochemically and Schwann cells appear to be good candidates for the production of some of these factors. When Schwann cells are involved in the demyelination-remyelination process, it may lead to local changes that sustain survival and regenerative attempts. The subsequent action of astrocytes, systematically confining Schwann cells, would then appear to be quite a negative process that induces a complete sterilization of the neuron-depleted area which, in turn, could result in an extension of the neuronal loss to areas containing the neurons of origin of the then retracting axons.

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