Neuronal Damage and Plasticity Identified by Microtubule-Associated Protein 2, Growth-Associated Protein 43, and Cyclin D1 Immunoreactivity After Focal Cerebral Ischemia in Rats

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Background and Purpose—An objective of therapeutic intervention after cerebral ischemia is to promote improved functional outcome. Improved outcome may be associated with a reduction of the volume of cerebral infarction and the promotion of cerebral plasticity. In the developing brain, neuronal growth is concomitant with expression of particular proteins, including microtubule-associated protein 2 (MAP-2), growth-associated protein 43 (GAP-43), and cyclin D1. In the present study we measured the expression of select proteins associated with neurite damage and plasticity (MAP-2 and GAP-43) as well as cell cycle (cyclin D1) after induction of focal cerebral ischemia in the rat.

Methods—Brains from rats (n=28) subjected to 2 hours of middle cerebral artery occlusion and 6 hours, 12 hours, and 2, 7, 14, 21, and 28 days (n=4 per time point) of reperfusion and control sham-operated (n=3) and normal (n=2) rats were processed by immunohistochemistry with antibodies raised against MAP-2, GAP-43, and cyclin D1. Double staining of these proteins for cellular colocalization was also performed.

Results—Loss of immunoreactivity of both MAP-2 and GAP-43 was observed in most damaged neurons in the ischemic core. In contrast, MAP-2, GAP-43, and cyclin D1 were selectively increased in morphologically intact or altered neurons localized to the ischemic core at an early stage (eg, 6 hours) of reperfusion and in the boundary zone to the ischemic core (penumbra) during longer reperfusion times.

Conclusions—The selective expressions of the neuronal structural proteins (MAP-2 in dendrites and GAP-43 in axons) and the cyclin D1 cell cycle protein in neurons observed in the boundary zone to the ischemic core are suggestive of compensatory and repair mechanisms in ischemia-damaged neurons after transient focal cerebral ischemia. (Stroke. 1998;29:1972-1981.)

Key Words: middle cerebral artery occlusion • neuronal plasticity • penumbra • proteins • rats
Continuity is essential for signals to be conducted along axons. A 43-kDa growth-associated protein (GAP-43) is a nervous tissue–specific protein. GAP-43 is synthesized at high levels during axonal outgrowth in neuronal development and regeneration. Axonal sprouting, a component of anatomic plasticity, can be identified by the elevated expression of GAP-43. Recent in vivo studies suggest that molecules within the damaged neurons affect their regenerative properties. Overexpression of GAP-43 in transgenic mice that constitutively express GAP-43 causes sprouting of CNS axons beyond the borders of their normal territory. Acute increase of expression of GAP-43 is found after cortical ischemia and in the substantia nigra after striatal ischemic injury in adult rats. These reports suggest that GAP-43 is a sensitive marker for the assessment of axonal damage and the regenerative response in the ischemic area of the mature CNS neurons.

In normal cells, cellular proliferation follows an orderly progression controlled by protein complexes that are composed of cyclins and cyclin-dependent kinases (cdks). Cyclins are a family of proteins that serve as the regulatory proteins for the cdk family members and are differentially synthesized and degraded at specific points during the cell cycle. Cyclin D1 is upregulated early in G1, subsequently peaks by mid G1, and usually decreases as cells approach S phase. Recent evidence indicates that cyclin D1 is necessary for nuclear relocation of the proliferating cell nuclear antigen protein and for DNA repair, as well as for initiation of DNA replication. Although neurons are terminally differentiated cells in the cortex and in the striatum in adult rats, cyclin D1 is selectively expressed in morphologically intact or altered neurons and oligodendrocytes localized to these ischemic tissues after focal cerebral ischemia, and cyclin D1 is highly expressed in the boundary zone to the ischemic core. Since cyclin proteins are associated with mitotic cells and development, the expression of these proteins lends credence to the concept that after stroke and brain injury neurons revert to an earlier stage of development, and that the expression of a cell cycle protein is suggestive of tissue plasticity.

In an effort to elucidate the role of these growth- and repair-associated proteins in cerebral ischemia, we measured their temporal profile of expression and colocalization from 6 hours to 28 days after 2 hours of middle cerebral artery occlusion (MCAO) in the rat. Our data demonstrate that these proteins are preferentially overexpressed in tissue at the outer boundary zone of the cerebral infarct, suggesting a compensatory role for these proteins in this region.

**Materials and Methods**

**Animal Model**
A total of 33 male Wistar rats weighing 260 to 300 g were used. We induced transient MCAO using a method of intraluminal vascular occlusion modified in our laboratory. Briefly, rats (n = 28) were anesthetized with 3.5% halothane and maintained with 1.0% halothane in 70% N2O and 30% O2 with the use of a face mask. The rectal temperature was controlled at 37°C with a feedback-regulated water heating system. The right femoral artery and vein were cannulated for measuring blood gases pH, Po2, and PCO2 before ischemia and for monitoring blood pressure during the surgery, respectively. A length of 18.5 to 19.0 mm 4-0 surgical nylon suture, with its tip rounded by heating near a flame, was advanced from the external carotid artery into the lumen of the internal carotid artery until it blocked the origin of the middle cerebral arterial (MCA). Two hours after MCAO, rats were reanesthetized with halothane, and reperfusion was performed by withdrawal of the suture until the tip cleared the internal carotid artery. Experimental rats were then given an overdose of ketamine and xylazine and were killed at 6 hours, 12 hours, and 2, 7, 14, 21, and 28 days (n = 4 per time point) for immunohistochemistry. Three rats served as a sham-operated control population; a 15-mm-long nylon monofilament was inserted into the internal carotid artery for 2 hours, and the rats were killed at 48 hours. This length of nylon monofilament was too short to occlude the MCA. Two normal rats served as a control for detection of immunoreactive proteins.

**Morphological Analysis**
Rat brains (n = 33) were fixed by transcardial perfusion with heparinized saline, followed by perfusion and immersion in 10% buffered formalin phosphate. A standard block, corresponding to coronal coordinates interaural 7.6 to 9.6 mm, bregma −1.4 to −0.6 mm, was obtained with the use of a rodent brain matrix and was embedded in paraffin. A series of adjacent 6-μm-thick sections were obtained from paraffin tissues for morphological evaluation.

**Histological Assessment of Neuronal Damage**
To determine the neuronal damage in the cortex and striatum, brain sections were processed with hematoxylin and eosin (H&E) staining. Histological features used to identify the ischemic lesion included vacuolation (sponginess) of the neuropil, diffuse pallor of the eosinophilic background, and alterations in the shape and stainability of cellular perikarya. By light microscopy, distinct histological abnormalities defined reversible and irreversible injury. Morphological features defining the acute reversible ischemic cellular injury included scalloped/shrunken dark neurons and swollen neurons. Morphological abnormalities defining irreversible cellular injury were necrosis and apoptosis. We applied criteria developed by Farber et al and Trump et al, who outlined morphological features (light and electron microscopy) of necrotic cells. Necrotic injury included pyknotic nuclei exhibiting an intense eosinophilic cytoplasm (red neurons) and nuclei lacking cellular structures (ghost neurons). Apoptotic cells were identified as cells exhibiting protuberances on the cell surface with plasmalemma sealing to produce membrane-bounded apoptotic bodies of roughly spherical or ovoid shape.

**Single-Label Immunohistochemistry**
After they were deparaffinized, brain sections (n = 198 sections; n = 33 animals × 3 specific antibodies, both peroxidase and fluorescein staining) were put in boiled citrate buffer (pH 6) within a microwave oven (650 to 720 W). After they were blocked in normal bovine serum albumin, sections were treated with primary monoclonal antibodies mouse anti–MAP-2 (dilution 1:50, Boehringer Mannheim) and mouse anti–GAP-43 (dilution 1:50, Calbiochem), and a polyclonal antibody, rabbit anti–cyclin D1 (dilution 1:60, Santa Cruz Bio). Biotinylated secondary antibodies anti-mouse IgG (H + L) (Vector) and anti-rabbit IgG (H + L) (Vector) were bound to the first antibodies against MAP-2, GAP-43, and cyclin D1, respectively. The standard anti-peroxidase procedure of avidin-biotin complex with Vector kit was used. Then coronal sections stained with 3,3′-diaminobenzidine (DAB, Sigma) were also counterstained with hematoxylin for light microscopy. For the immunofluorescent procedure, fluorescein isothiocyanate–conjugated secondary antibodies anti-mouse IgG (H + L) and anti-rabbit IgG (H + L) (dilution 1:20, Calbiochem) were bound to the first antibodies against MAP-2, GAP-43, and cyclin D1. Fluorescent intensity was detected by immunofluorescence microscopy. To control for nonspecific immunoreactions, a number of representative sections were processed for each experiment from each animal and received identical staining preparation, except that the primary antibodies or the secondary antibody was omitted. These negative control sections were consistently devoid of immunolabeling. Finally, the immunostaining pattern between the lesioned versus the nonlesioned hemisphere was used as an internal control within the same animal.
Double-Label Immunoreactivity

To visualize the possible colocalization of MAP-2, GAP-43, or cyclin D1 in the same cells, dual immunohistochemistry staining was used. Each coronal section (n=99 sections; n=33 animals ×3 specific double staining, MAP-2/cyclin D1, GAP-43/cyclin D1, MAP-2/GAP-43) was initially treated with primary monoclonal antisera (MAP-2 and GAP-43) and then stained with DAB for brown color development, as described above. Subsequently, sections were washed, and the secondary primary polyclonal antibody against cyclin D1 was applied. Then FITC-conjugated anti-rabbit IgG (dilution 1:20, Calbiochem) was performed for double-label immunoreactivity identification (MAP-2/cyclin D1 and GAP-43/cyclin D1). Because MAP-2/GAP-43 double immunolabeling with primary antibodies from identical species (mouse) sometimes produces false labels because of cross-reactivity, we have taken advantage of the IgG F(ab\(^9\))\(_{2}\) fragment (BM 4702, Accurate) was used as the reagent secondary antibodies. Briefly, a biotinylated-conjugated affinity purified IgG F(ab\(^9\))\(_{2}\) fragment (BM 4702, Accurate) was used in the immunofluorescent method for the identification of second-labeled GAP-43 immunoreactivity. Although GAP-43 was initially considered to be neuron-specific, it has more recently been found in astrocytes.46,47 We also performed double staining with GAP-43/glial fibrillary acidic protein and GAP-43/B4 -isolectin from all rats subjected to 2 hours of MCAO and killed at 2 days. For astrocyte identification, brain sections were initially treated with pepsin for a polyclonal antibody, glial fibrillary acidic protein (Dakopatts), and then stained with DAB. Subsequently, the sections were treated with the second primary antibody, mouse anti–GAP-43. Then the FITC-conjugated secondary antibody staining was performed for double-label immunoreactivity. Microglial cells were identified by means of histochemistry with the B4 -isolectin (Sigma). Paraffin sections were incubated with isolecitin in PBS containing divalent cations and were stained with DAB. Mouse anti–GAP-43 was then applied to the same sections, and FITC-conjugated secondary antibody staining was performed for double-label identification. Negative control procedures included omitting either both primary antibodies or the secondary antibodies.

Results

The blood gases were within normal ranges for all animals and did not differ between experimental rats and sham control rats (data not shown). After MCAO, gross swelling at 48 hours, as suggested by compression of the ventricles, and marked pallor of the MCA territory of the ipsilateral hemisphere were clearly detected. Atrophy was evident from a granular brain surface and ventricular enlargement from 7 to 28 days (the end point), with concomitant narrowing of the cortex and the striatum. Cyst formation in the cortex and the striatum was present after 2 weeks.

Within 6-μm-thick coronal sections stained with H&E in each hemisphere of all normal rats and sham-operated rats as well as the contralateral hemisphere of ischemic rats, no cells were classified as necrotic; however, a few scattered apoptotic cells were found in each hemisphere. Scattered and focal damaged neurons were seen in the ischemic core at 6 hours after 2 hours of MCAO. With increasing length of survival, neuronal loss progressed to almost total destruction of the cortical layers and the striatum in the ischemic core. In the boundary zone to the ischemic core, mixed morphologically relatively intact, damaged, necrotic, and apoptotic cells were detected until 4 weeks after ischemia.

The immunoperoxidase or immunofluorescent staining patterns of MAP-2, GAP-43, and cyclin D1 were obtained from the ischemic brain. Figure 1 shows a representative temporal profile from MAP-2 immunoperoxidase single staining after MCAO. Figure 2 shows the cellular colocalization by means of double-labeled immunoreactivities of MAP-2, GAP-43, and cyclin D1.

MAP-2 and GAP-43 Immunoreactivities

In both hemispheres of control animals, neuronal MAP-2 immunoreactivity (Figures 1a and 2a) was characterized by uniform and smooth labeling of both soma and dendrites, and neuronal GAP-43 immunoreactivity was present in both cytoplasm and axon (Figure 2b). MAP-2 and GAP-43 immunoreactivities were colocalized in the soma of morphologically intact neurons in the nonischemic brain (Figure 2a and 2b). However, a reciprocal staining pattern of neurites was detected, ie, immunoreactivity of MAP-2 (arrows, Figure 2a) in some neurites without immunoreactivity of GAP-43 (arrows, Figure 2b); alternately, immunoreactivity of GAP-43 (arrowheads, Figure 2b) was detected in some neurites without immunoreactivity of MAP-2 (arrowheads, Figure 2a).

At 6 hours after onset of ischemia, a beaded MAP-2 immunostaining pattern appeared at the distorted neuronal dendrites, and/or MAP-2 immunostaining was concentrated in the neuronal somata of the cortex (Figures 1b and 2c) and striatum of the ischemic core, in contrast to smooth dendro-somatic MAP-2 immunostaining in normal neurons (Figures 1a and 2a). Disrupted axons along with rapid loss of GAP-43 immunoreactivity and/or GAP-43 immunoreactivity was focally concentrated in soma and cone-shaped axon hillock of some neurons in the cortex (Figure 2d) and the striatum, in contrast to smooth axonal and somatic GAP-43 immunostaining in normal neurons (Figure 2b).

At 2 to 28 days after MCAO, in the ischemic core, a pronounced loss of MAP-2 (left side, Figure 1c, 1e, 1g, 1i) and GAP-43 (left side, Figure 2f and 2j) immunoreactivities was observed from dendrites, axons, and soma of neurons in the cortices. An obvious loss of MAP-2 immunostaining in neurons of the striatum is also shown in Figure 1g and 1h. Interestingly, the accumulation of MAP-2 immunostaining in the soma and the dendrites was observed in both inner (Figures 1d, 1h, 1j, 2e, and 2i) and outer boundary zones (Figures 1f, 2g, and 2k). However, a selective increase of GAP-43 immunoreactivity was only observed in the outer boundary zone (right side, Figure 2f and 2j; Figure 2h and 2l). In the outer boundary zone to the ischemic core, similar to that in nonischemic tissue, a reciprocal staining pattern was present in neurons, ie, increased immunoreactivity of MAP-2 (arrows, Figure 2e, 2g, 2i, and 2k) in some neurites without immunoreactivity of GAP-43 (arrows, Figure 2f, 2h, 2j, and 2l); alternately, immunoreactivity of GAP-43 (arrowheads, Figure 2f, 2h, 2j, and 2l) was detected in some neurites without immunoreactivity of MAP-2 (arrowheads, Figure 2e, 2g, 2i, and 2k). Moreover, the distribution pattern of MAP-2 and GAP-43 immunoreactivities of parallel cortical neurons and radial fibers appeared extended in the outer boundary zone at 28 days (Figure 2k and 2l) compared with 2 days (Figure 2g and 2h) after MCAO. Two days after operation, GAP-43 immunoreactivity was present in few scattered
astrocytes and microglia in both inner and outer boundary zones (data not shown). These data suggest that GAP-43 is a multifunctional protein involved in the synthesis of membranes associated with the various types of cellular processes.

Cyclin D1 Immunoreactivity

With the use of dual immunohistochemistry staining of MAP-2/cyclin D1 and GAP-43/cyclin D1, weak cyclin D1 immunoreactivity was primarily localized to the cytoplasm of neurons in the cortex and the striatum of the control normal
and sham-operated brains and the contralateral hemispheres of ischemic rats (far right side, Figure 2m). At 6 hours after MCAO, cyclin D1 immunoreactivity decreased in some neurons of focal ischemic areas; however, cyclin D1 immunoreactivity increased in nuclei of scattered neurons within the lesion. At 2 to 28 days after ischemia, the expression of cyclin D1 immunoreactivity decreased in most neurons throughout the ischemic core. Within the ischemic core, a pronounced increase of cyclin D1 immunoreactivity was found in the nuclei that did not colocalize with neuronal marker of MAP-2 or GAP-43 immunoreactivity especially at 2 days after 2 hours of MCAO, suggesting that they were not

Figure 2. The cortices from a nonischemic control rat (a, b) as well as representative rats subjected to 2 hours of MCAO and killed at 6 hours (c, d), 2 days (e through h, m through o), and 28 days (i through l) are double stained for MAP-2/GAP-43 (a through l) and GAP-43/cyclin D1 (m through o) and single stained for cyclin D1 (m) immunoreactivities. a, c, e, g, i, k, MAP-2; b, d, f, h, j, l, n, GAP-43; m, o, cyclin D1. a, b, In nonischemic neurons, MAP-2 and GAP-43 immunoreactivities are colocalized in soma of most neurons. However, a reciprocal staining pattern of neuronal neurites is seen, ie, immunoreactivity of MAP-2 (arrows, a) in some neurites without immunoreactivity of GAP-43 (arrows, b); alternately, immunoreactivity of GAP-43 (arrowheads, b) was detected in some neurites without immunoreactivity of MAP-2 (arrowheads, a), suggesting that MAP-2 (a) is expressed in both dendrites and soma (arrows) and GAP-43 (b) is expressed in both axons and soma of neurons (arrowheads). c,d, At 6 hours after 2 hours of MCAO, focal loss of MAP-2 (c) and GAP-43 (d) immunoreactivities is detected in cone-shaped axon hillock of some neurons in the ischemic core. e through h, At 48 hours after 2 hours of MCAO, MAP-2 immunoreactivity is disintegrated in the ischemic core (left side, e), and MAP-2 immunoreactivity is expressed in soma and dendrites in the boundary zone to the ischemic core, both inner (middle) and outer (right side). GAP-43 immunoreactivity is lost in the ischemic core (left side, f) and in the inner boundary zone (middle); however, it is expressed in the soma and axons of neurons in the outer boundary zone (right side, f, h), which may or may not colocalize with MAP-2 immunoreactivity. g, h, High-power magnification shows MAP-2 and GAP-43 immunoreactivity in the soma of the same cells, and MAP-2 (arrows) and GAP-43 (arrowhead) proteins in different neurites. i through l, At 28 days after 2 hours of MCAO, MAP-2 and GAP-43 immunoreactivities decrease in the ischemic core (left side, i, j), and MAP-2 is expressed in both the inner and the outer zones (i, k). GAP-43 is more strongly expressed in the outer boundary zone than in the inner boundary zone (j, l). m, weak cyclin D1 immunoreactivity is present in the neuronal cytoplasm in the normal brain (far right side, m) at 48 hours after 2 hours of MCAO. In the ischemic core, the expression of cyclin D1 immunoreactivity is lost in most neurons, as suggested by an absence of cyclin D1 colocalization with MAP-2 (e) and/or GAP-43 (f). However, cyclin D1 is expressed primarily in inflammatory cells in the ischemic core (left side, m). In the boundary zones, cyclin D1 is strongly expressed in nuclei of many cells, including morphologically relative intact neurons. Cyclin D1 immunoreactivity (o, curved arrows) colocalizes with MAP-2 and GAP-43 immunoreactivities in most neurons. Magnification is as follows: a through d, g, and h, ×320; e, f, i, j, ×110; k, l, ×240; m, ×46; n, o, ×700.
neurons but most likely inflammatory cells (identified by anatomic distribution and morphology). Colocalizations of MAP-2/cyclin D1 and GAP-43 (Figure 2n)/cyclin D1 (Figure 2o) immunoreactivities were observed in the nucleus, cytoplasm, and neurites of some neurons but not in all cells in the boundary zones from 2 to 28 days after MCAO. Comparisons of MAP-2/cyclin D1 and GAP-43/cyclin D1 expression with H&E staining in adjacent sections indicated that the expressions of MAP-2/cyclin D1 and GAP-43/cyclin D1 were localized to the morphologically relatively intact or scalloped/shrunken dark neurons but not to red or ghost neurons. Cyclin D1 immunoreactivity was observed in many nonneuronal cells in the ischemic core and within neurons in the boundary zones, suggesting that MCAO evokes a complex cyclin D1 reactivity in different cell types and different anatomic distributions.

**Discussion**

To ascertain the expression and the temporal profile of select proteins associated with neurite damage and neuronal plasticity after transient focal cerebral ischemia, we subjected Wistar rats to a filament-induced 2-hour MCAO and allowed recovery for 6 hours, 12 hours, and 2, 7, 14, 21, and 28 days. The data obtained from immunohistochemical methods provide evidence that in the adult nonischemic brain, MAP-2, GAP-43, and cyclin D1 are uniformly expressed in the neuronal cytoplasm. In normal neurites, a reciprocal immunostaining pattern between MAP-2 and GAP-43 was detected, i.e., MAP-2 is expressed in the dendrite and GAP-43 is expressed in the axon. In the ischemic core at 6 hours after MCAO, disruption of neurites was evident by the beaded appearance of MAP-2 immunoreactivity and disintegration of scattered and focal axons with loss of GAP-43 immunoreactivity. Cyclin D1 immunoreactivity decreased in focal neurons; however, cyclin D1 immunoreactivity increased in some nuclei of scattered neurons, suggesting heterogeneous changes early after ischemia. In most neurons in the ischemic core from day 2 after MCAO onward, a distinct loss of MAP-2, GAP-43, and cyclin D1 was evident. In the boundary zone (penumbra) to the ischemic core, relatively intact, damaged, and dead neurons were observed in the transition from normal tissue to the outer and inner boundary zones and the ischemic core, respectively. In the penumbra, especially in the outer boundary zone, neurons showed increased labeling of the MAP-2, GAP-43, and cyclin D1 immunoreactivities from day 2 and maintained higher than normal levels of these proteins through at least day 28 (end point). These 3 proteins are associated with cell development, neurite sprouting, and cell cycle and therefore may represent molecular correlates of enhanced cerebral plasticity.

**Protein Expression in the Ischemic Core**

Several studies have reported decreases in MAP-2 in gerbil brain after ischemia.12,13 In gerbils, as early as 3 minutes after occlusion of the right common carotid artery MAP-2 was reduced in the subiculum-CA1 region of the ipsilateral hippocampus. With longer periods of ischemia, the loss of MAP-2 increased and spread to other areas of the hippocampus and to the cortex and other brain regions.13 Transient 5-minute ischemia also caused a decrease of MAP-2 measured after 1 hour, followed by greater losses after longer periods of time.12 Permanent MCAO induced a progressive loss in MAP-2 immunostaining in most neurons within the central ischemic core from 1 hour to 4 hours; however, some cells exhibited a marked increase in staining, with more dense soma and tortuous cellular processes, particularly at the 4-hour time point.17 These results indicate that MAP-2 is sensitive to ischemia and an early marker of ischemia-induced neuronal damage.

Ischemic neurons have a remarkable potential for compensatory sprouting accompanied by an increased expression of the GAP-43 in the necocortex after permanent occlusion of the MCA and ipsilateral common carotid artery.56 These data support the possibility of induction of plasticity after transient cerebral ischemic injury in brain. In the period after 2 hours of MCAO, tissue located in the ischemic core is not salvaged.46 Our present study indicates that at early stages (6 hours) of cerebral ischemia, MAP-2 and GAP-43 immunoreactivities are present in disrupted dendrites and axons and concentrate in the neuronal somata of scattered and focal neurons in the ischemic core; this is similar to the situation 1 to 4 hours after permanent MCAO.17 Both MAP-2 and GAP-43 are early and sensitive markers of neuronal damage after ischemia. Moreover, at an early stage after stroke, our data also show an increase of cyclin D1 immunoreactivity in the nuclei of scattered neurons in both the cortex and the striatum of the ischemic core, which suggests that focal neuronal death may provide a stimulus for damaged neurons to synthesize proteins. Therefore, axonal and dendritic alterations accompanying an increase of cyclin D1 protein synthesis in the nuclei of terminally differentiated neurons may suggest a tendency toward survival. After longer durations of reperfusion, most neurons located in the ischemic core die, as detected by H&E staining, and MAP-2, GAP-43, and cyclin D1 immunoreactivities are lost. Loss of the immunoreactivity for MAP-2, GAP-43, and cyclin D1 coincided with the progression of disintegration of dendrites and axons, as well as somata. We speculate that at an early stage after MCAO, viable ischemic dark neurons identified by H&E staining may show a potential for compensatory restructuring, accompanied by an increased expression and nuclear translocalization of cyclin D1.

**Protein Expression in the Penumbral Area**

The boundary zone (penumbra) is ischemically threatened tissue adjacent to the ischemic core. Penumbral areas were originally defined as those having a reduction in cerebral blood flow sufficiently severe to extinguish spontaneous or evoked electric potentials yet sufficiently mild to allow maintenance of membrane potentials and gross cellular ion homeostasis.45,50 It may be advantageous to adopt a wider definition of the ischemic penumbra and let it denote ischemic areas that can be salvaged by pharmacological agents or by relatively prompt reperfusion.54 The changes we observe in the boundary zones in the late postischemic phase are localized to penumbra, which is considered that zone of ischemically threatened tissue adjacent to the core zone of an evolving focal ischemic infarction.51 We do not know
whether our observation of MAP-2, GAP-43, and cyclin D1 expression is specific for transient ischemia with reperfusion. Protein expression in the boundary zone may be more affected by the severe edema secondary to transient ischemia than by reduced edema associated with permanent ischemia.

Our data demonstrate that MAP-2 and GAP-43 are not only early markers of neuronal damage but also may be associated with restructuring deformed or damaged neurons in the penumbra. Overexpression of MAP-2, GAP-43, and cyclin D1 is associated with morphologically intact neurons in the penumbral area after MCAO. Although no direct morphological evidence of sprouting was obtained in this in vivo study, substantially longer axons and dendrites were found in a series of coronal sections of tissue from 2 to 28 days, suggesting that neurite regrowth may be present in the penumbral areas. The pattern of MAP-2 and GAP-43 immunoreactivities of cortical neurons in parallel and radial fibers was longer at 28 days (Figure 2k and 2l) than at 2 days (Figure 2g and 2h), which may suggest that dendrites and axons extend from 2 to 28 days during reperfusion after ischemia. The regrowth of dendrites and axons is suggested by well-shaped and connected neurites, rich in MAP-2 and GAP-43 immunostaining. The parallel neurites in the penumbra, especially in the outer boundary zone, suggest that viable neurons derived from this region may potentially traverse complex structures, e.g., glial cells and vessels, to approach their targets. Each neuron is unique, and its singularity resides in its specific position in the nervous system. Position that is attributed to its peculiar synaptic connections with other neurons and, either directly or indirectly, with the periphery. The patterns of these connections are reflected by the geometry and location of neurons. Thus, the parallel form of neurites observed in the penumbra suggests that neurons may potentially function in the ischemia-damaged tissue.

Our data indicate that MAP-2 immunoreactivity decreased in the ischemic core from day 2 after MCAO and are in agreement with observations that MAP-2 significantly decreased in gerbil forebrain after ischemia and in rat after MCAO. The loss of MAP-2 immunoreactivity in the ischemic core surpasses the increased synthesis of these proteins in the narrow penumbral areas after ischemia and thus may explain the observed reduction of MAP-2 immunoreactivity in the ischemic regions measured by Western blotting analysis. Nevertheless, the overexpression of MAP-2, GAP-43, and cyclin D1 immunoreactivities to at least 28 days after MCAO in the penumbral region suggests an active ongoing process of neuronal repair.

Comparison of MAP-2, GAP-43, and Cyclin D1 Immunoreactivities

Genes dictate development of the major cytoarchitectonic plan that constitutes the CNS. During formation, this plan may become disturbed for a variety of reasons, but once development is completed, the principal wiring diagram remains fixed. In adult brain, MAP-2 is highly concentrated in dendrites and is virtually absent in axons, and the MAP-2 immunoreactivity in neuronal perikarya is less intense. GAP-43 is synthesized at high levels during axonal outgrowth in neuronal development and regeneration. Our data obtained from MAP-2 and GAP-43 double immunohistochemistry suggest that the disintegration and reduction of GAP-43 in the axons occur as early as the beading MAP-2 expression in dendrites, at 6 hours after MCAO. Breakdown of neuronal dendrites and axons in the rat brain, as indicated by a loss of immunoreactivity of MAP-2 and GAP-43, suggests that both proteins may participate as early pathogenic events after the ischemic insult. Longer durations of reperfusion after 2 hours of MCAO result in a differential anatomic distribution of protein expression in dendrites and axons. In the penumbra, loss of MAP-2 immunoreactivity may not occur in the inner penumbral boundary zone after MCAO. In contrast, GAP-43 immunoreactivity declines in this region, suggesting that axonal degradation is more severe than dendritic degeneration. The increase of MAP-2 immunoreactivity is observed in both outer and inner boundary zones (Figure 2e and 2i), whereas the increase of GAP-43 immunoreactivity is restricted to the outer penumbral boundary zone (Figure 2f and 2j) from day 2 onward after MCAO. A loss of protein expression in the ischemic core and an increased protein synthesis rate in the boundary zone suggest that viable neurons show a potential for compensatory sprouting.

The distribution patterns of MAP-2 and GAP-43 expression within cortical neurons parallel to radial fibers may suggest a basis for functional recovery. These data support the hypothesis that sprouting of both dendrites and axons is the intrinsic property in the CNS after cerebral ischemia. Data on MAP-2 and GAP-43 immunoreactivities may enhance our understanding of neuronal degeneration as well as neurite regrowth in mature neurons of the adult brain. In the ischemic core, the distribution of cyclin D1 immunoreactivity (Figure 2m) differed from MAP-2 (Figure 2e and 2i) and GAP-43 (Figure 2f and 2j) immunoreactivities. Cyclin D1 was expressed in the nuclei of scattered nonneuronal cells; however, both MAP-2 and GAP-43 lost immunoreactivity in all cells. Our data showed that within the ischemic core, decreased neuronal cyclin D1 immunoreactivity accompanied overexpression of nonneuronal cyclin D1 immunoreactivity. In the penumbral boundary zones, cyclin D1 was localized to nuclei of morphologically intact cells, and expression persisted through at least 28 days after MCAO. Cyclin D1 does not consistently colocalize with the two neuronal markers of MAP-2 and/or GAP-43, which suggests that cyclin D1 is expressed in neurons and nonneuronal cells in the penumbral boundary zone at 2 to 28 days after MCAO. The present study is consistent with and extends our previous data that cell cycle proteins (cyclin A, D1, cdk2, cdk4) are upregulated in morphologically relatively intact cells at 2 days after 2 hours of MCAO. The selective expression of cell cycle proteins observed in ischemic damaged tissue suggests a role for these proteins in cell survival after transient focal cerebral ischemia.

In our experiments we have detected expression and colocalization of MAP-2, GAP-43, and cyclin D1 immunoreactivities as indices of neuronal remodeling at various times after transient focal cerebral ischemia in adult rat brain. The synaptic arrangements of a given neuron can change rather dramatically during life for physiological as well as patho-

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physiological reasons. Other proteins associated with neuronal modeling should also be studied, eg, synaptophysin, synapsin, and spectrin. Finding ways to upregulate levels of neurite regrowth may be useful not only with a view to favoring axonal and dendritic elongation but also with enhanced cell-cell adhesion, which may reduce neuronal death in the CNS. When this approach is combined with strategies for modifying the environment of injured neurons, we may expect progress toward the goal of efficiently restoring axonal and dendritic elongation in the damaged CNS. Neurological scores improve over time, which may relate MAP-2, GAP-43, and cyclin D1 to functional changes and restoration in the adult CNS. In light of the potential clinical relevance of these observations, further studies are needed to elucidate pathways involved and to identify treatments that may enhance sprouting. Promoting expression of cytoskeletal proteins and cell cycle proteins may provide a basis for the design of new therapeutic strategies for treatment of brain and spinal cord injuries, as well as for neurodegenerative diseases.

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The authors address the important point that improved outcome after cerebral ischemia may be associated with cerebral plasticity. An emerging concept in neurobiology is that the adult brain retains a capacity for plasticity and functional reorganization throughout the lifespan. Intracellular recordings of cortical neurons in primates have demonstrated that cortical representation areas (“cortical maps”) are modified by loss of sensory input such as peripheral nerve block and amputation, by training and experience, and also in response to focal brain lesions. Some changes occur very rapidly, and others may take weeks and months to evolve. An important observation is that postlesion plasticity is activity dependent and can be influenced by training. As indicated in the present study, some postischemic cellular changes are presumably induced directly by the lesion. Whether or not such changes are related to postlesion functional improvement is an interesting question that remains to be answered.

To what extent poststroke compensatory mechanisms involve activation in the contralateral hemisphere is debated. Neuroimaging studies indicate altered poststroke activation patterns for sensory and motor functions, usually including activation of the intact hemisphere. However, there is some disagreement as to the correlation between activation pattern and functional outcome. Unilateral damage to the forelimb representation area of the sensorimotor cortex in adult rats has been reported to increase dendritic arborization of pyramidal neurons of the contralateral cortex. Restriction of movement of the intact limb blocked dendritic growth and aggravated functional deficits. Studies with different design have failed to confirm the observation of increased dendritic arborization in corticospinal motor neurons.

Electrophysiological studies in monkeys have shown that tissue surrounding a small cortical lesion in part of the hand representation area undergoes a further territorial loss in the functional representation of the hand, perhaps because of nonuse or disruption of local intrinsic cortical circuitry. However, retraining for hand use prevented these changes and induced functional reorganization in the peri-infarcted area normally responsive to other parts of the hand. A recent extensive study on somatosensory cortex plasticity before and after a cortical microlesion confirmed the presence of extensive multifocal reorganization in the same hemisphere, with no changes occurring in the opposite intact hemisphere. Some data suggest that training that is too extensive directly after a cortical lesion may lead to larger lesions and less recovery, an observation that obviously is of potential clinical concern and needs to be further investigated. Postoperative housing of rats in an activity-stimulating environment with no specific training does not induce larger lesions.

Glutamate, the main excitatory neurotransmitter, plays a crucial role in synaptic plasticity. Pharmacological modulation of the glutamate (stimulating) and γ-aminobutyric acid (inhibitory) systems is likely to influence the process. Other substances, including acetylcholine, monoamines, hormones, and growth factors, are likely to be able to act as modifiers.
The possible influence of commonly used drugs on postischemic recovery is an important issue.19 Twenty-five years ago, Alf Brodal, a Norwegian professor of anatomy, in an article discussing his own functional improvement after a stroke,20 wrote, “It seems in most instances that one must resort to the assumption that intact fibers ‘take over’ for the damaged ones.” Although many questions remain to be answered, today we have some ideas about how intact fibers may compensate for damaged ones.

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Neuronal Damage and Plasticity Identified by Microtubule-Associated Protein 2, Growth-Associated Protein 43, and Cyclin D1 Immunoreactivity After Focal Cerebral Ischemia in Rats

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