

# Transcortical Alterations in $\text{Na}^+ \text{-K}^+$ ATPase and Microtubule-Associated Proteins Immunoreactivity in the Rat Cortical Atrophy Model Induced by Hypoxic Ischemia

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## SUMMARY

To identify the chronological transcortical change in the contralateral hemisphere following ischemic insults, we investigated the changes in microtubule associated protein (MAP) and  $\text{Na}^+ \text{-K}^+$  ATPase expressions in the peri-infarct zone and contralateral hemisphere, including the hippocampus. Two days after hypoxic ischemia,  $\text{Na}^+ \text{-K}^+$  ATPase immunoreactivity was significantly enhanced in the contralateral cortex and was maintained up to 7 days after ischemia, whereas  $\text{Na}^+ \text{-K}^+$  ATPase immunoreactivity in the peri- and infarct zones was unaffected by hypoxic ischemia. In contrast, 2 to 7 days after ischemia, MAP1A and MAP2 immunoreactivity in the ipsi- and contralateral cortex significantly decreased, whereas in layer V, MAP1 immunoreactivity obviously accumulated in the neurons and their processes. In the hippocampus, 2 days after insults both MAP1A and MAP2 immunoreactivity was significantly reduced within the ipsi- and contralateral

hippocampus. In the contralateral hippocampus, however, the distribution of MAP2 immunoreactivity recovered to the sham level 7 days after ischemia, whereas MAP1A immunoreactive axons remained 2 months after ischemia. The results suggest that the unilateral elevation of  $\text{Na}^+ \text{-K}^+$  ATPase immunoreactivity reflects elevated neuronal activity. In addition, this asymmetric hyperexcitability might play an important role in the recovery or the reorganization of the brain, accompanied by transcortical changes in MAPs expression.

## KEYWORDS

transcortical change, diaschisis, rat, hippocampus, microtubule associated protein,  $\text{Na}^+ \text{-K}^+$  ATPase

## INTRODUCTION

Neurodegenerative diseases evoke certain morphological alterations at a lesion site; e.g., dendritic sprouting and axonal degeneration. Moreover, neuronal responses after injury can play an important role in the maintenance or reconstruction of brain functionality. In fact, such

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alterations increase behavioral recovery after various cortical lesions including cortical ablations, contusions, and focal ischemia in animals and after stroke in humans. Microtubule-associated proteins (MAPs) are generally used as morphological markers of neuronal plasticity because these proteins are abundant in both axons and dendrites, where they are thought to be involved in intracellular transport functions (Grafstein & Forman, 1980). MAPs are also responsible for the maintenance of mature neuronal morphology and for the initiation and stabilization of new fibers and synaptic contacts (Tucker et al., 1989; Hirokawa et al., 1996; Marsden et al., 1996).

In addition, one of the earliest events after brain damage, including cerebral ischemia, is a rapid decline in the activity of energy dependent sodium-potassium adenosine triphosphatase ( $\text{Na}^+ \text{-K}^+$  ATPase) (Lees, 1991). Several investigators (Stys et al., 1992; Tasker et al., 1992) have examined the role of tetrodotoxin-sensitive ion channels in hypoxic-ischemic neuronal damage and concluded that  $\text{Na}^+$  influx is an important initiating event leading to neuronal damage. The truth is that the state of membrane failure of the  $\text{Na}^+ \text{-K}^+$  transport mechanism leads to the net leakage of these ions, and to the accumulation of  $\text{K}^+$  in the extracellular space. Thus, at this point the cells take up  $\text{Na}^+$  and  $\text{Cl}^-$ , with osmotically obligated water. These events lead to rapid edema, resulting in cell death in the ischemic brain (Astrup, 1982; Siesjo, 1988).

On the other hand, one hypothesis is that cortical damage is accompanied by secondary changes in the contralateral brain regions, which contributes to initial non-specific behavioral depression. These remote effects, referred to as 'disachisis', are most commonly observed several hours after an insult and recover within the following month (Reinecke et al., 1999; Lagreze et al., 1987). With respect to etiology, brain edema or reactive plasticity causes the remote changes

(Witte et al., 2000). The functional importance of such alterations is presumably a prerequisite for restitution (Kotila & Waltimo, 1992; So et al., 1996). Unfortunately, few systemic studies have been done on the chronological transcortical change in the peri-infarct region or in the contralateral hemisphere following ischemic insults. In the present study, therefore, we investigated changes of both MAPs and  $\text{Na}^+ \text{-K}^+$  ATPase expressions to identify the chronic patterns of regional specific alterations in the peri-infarct zone and its contralateral hemisphere, including the hippocampus, following ischemic insults.

## EXPERIMENTAL

### Hypoxic ischemia

The animals used in this study were the progeny of male Sprague-Dawley rats (aged 4 wk) obtained from the Experimental Animal Center, Hallym University, Chunchon, South Korea. The animals were provided with a commercial diet and water *ad libitum* under controlled temperature, humidity, and lighting conditions ( $22 \pm 4^\circ\text{C}$ ,  $55 \pm 5\%$  and a 12:12 light/dark cycle). The procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

Focal hypoxic ischemia was induced using the procedure of Levine (1960) as modified by Rice et al. (1981). Briefly, the right carotid artery of animals was ligated, after which the animals were exposed for 1.5 h to hypoxic conditions (8%  $\text{O}_2$ , 92%  $\text{N}_2\text{O}$ ,  $30^\circ\text{C}$  temperature, 60% humidity). Two control groups were used for this experiment; a sham-operated group, which was subjected to the same surgical procedures but without hypoxia-

ischemia ( $n = 5$ ), and the other control group was exposed to the hypoxic condition only ( $n = 5$ ).

### Preparation of animals and tissue

After a period of 2 days, 7 days, and 2 months ( $n = 10$ , respectively), animals were perfused transcardially with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 0.1 M PB (pH 7.4), after deep anesthesia (i.p.) had been induced with Ketamine. The brains were removed and postfixed for 4 h in the same fixative. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter, the tissues were frozen and sectioned with a cryostat at 30  $\mu\text{m}$ , and consecutive sections were collected in 6-well plates containing PBS.

### Immunohistochemistry

The sections were first incubated for 30 min with 3% bovine serum albumin in PBS at room temperature. Some sections were then incubated overnight in mouse anti-MAP1A (Chemicon, USA), MAP2 (Boehringer Mannheim, USA), or anti- $\text{Na}^+-\text{K}^+$  ATPase  $\alpha 1$  subunit antibodies ( $\alpha 6\text{F}$ , Developmental Studies Hybridoma Bank, USA), diluted 1:200 to 1:400 in PBS containing 0.3% triton X-100 and 2% normal horse serum at room temperature. The sections were washed with PBS three times for 10 min each, incubated sequentially, in horse anti-goat IgG (Vector, USA) and streptavidin (Vector, USA), diluted 1:200 in the same solution used for the primary antiserum. Between the incubations, the tissues were washed with PBS three times for 10 min each. The sections were visualized with 3,3'-diaminobenzidine (DAB) in 0.1 M Tris buffer and mounted on gelatin-coated slides. The immunoreactivity was observed under the Axioscope microscope (Carl Zeiss, Germany). To establish the specificity of the immunostaining, a negative control test was carried out with pre-

immune serum instead of the primary antibody. The control for immunohistochemistry resulted in the absence of immunoreactivity in any structure.

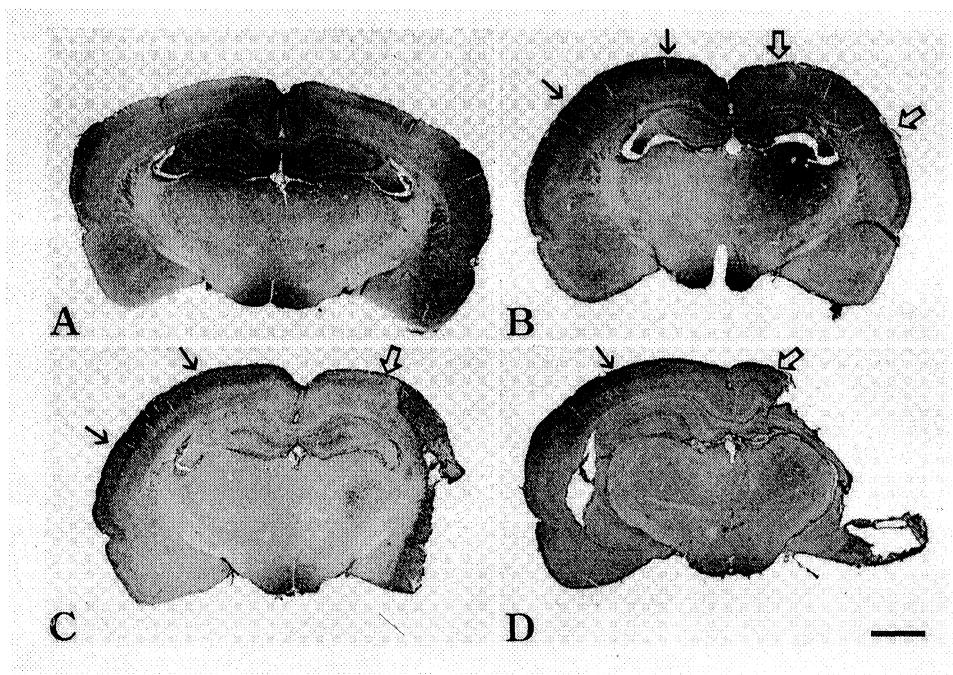
### RESULTS

In the present study, preparations from the control animals did not show any morphological change in the brain tissue (data not shown). The operated animals had ipsilaterally extensive damage to the cortex, including the hippocampus, similar to that described previously by Romijn et al. (1992, 1993), although the contralateral hemisphere had no lesions.

### $\text{Na}^+-\text{K}^+$ ATPase immunoreactivity

$\text{Na}^+-\text{K}^+$  ATPase immunoreactivity in the cortices of control animals showed symmetrical distribution, and this pattern was similarly observed in the hippocampus (Fig. 1A). 2 days after hypoxic ischemia,  $\text{Na}^+-\text{K}^+$  ATPase immunoreactivity was significantly enhanced in the contralateral cerebral cortex, whereas  $\text{Na}^+-\text{K}^+$  ATPase immunoreactivity in the peri- and infarct zones was unaltered. In contrast to the cerebral cortex,  $\text{Na}^+-\text{K}^+$  ATPase immunoreactivity was increased in the lentiform nucleus ipsilateral to the infarct lesion (Fig. 1B). Seven days after hypoxic ischemia, elevated  $\text{Na}^+-\text{K}^+$  ATPase immunoreactivity was maintained in the contralateral cortex, and its immunoreactivity was unchanged in the peri-infarct zone. In the infarct zone of the cerebral cortex, however, non-specific  $\text{Na}^+-\text{K}^+$  ATPase immunoreactivity occurred because this region was degenerated (Fig. 1C). Two months after ischemic insult, the  $\text{Na}^+-\text{K}^+$  ATPase immunoreactivity was unchanged in the peri-infarct zone.

$\text{Na}^+-\text{K}^+$  ATPase immunoreactivity in the contralateral cortex, however, was significantly



**Fig. 1:**  $\text{Na}^+\text{-K}^+$  ATPase immunoreactivity in rat cortices following hypoxic ischemia. In the control animal, a symmetrical distribution of  $\text{Na}^+\text{-K}^+$  ATPase immunoreactivity is observed in the brain (A). Two days after hypoxic ischemia (B),  $\text{Na}^+\text{-K}^+$  ATPase immunoreactivity is significantly enhanced in the contralateral cerebral cortex (arrows), whereas  $\text{Na}^+\text{-K}^+$  ATPase immunoreactivity in peri- and infarct zones is unaltered (open arrows). Seven days after hypoxic ischemia (C), elevated  $\text{Na}^+\text{-K}^+$  ATPase immunoreactivity in the contralateral cortex is maintained (arrows), and its immunoreactivity is unchanged in the peri-infarct zone (open arrow). Two months after ischemic insult (D), the  $\text{Na}^+\text{-K}^+$  ATPase immunoreactivity is unchanged in the peri-infarct zone (open arrow).  $\text{Na}^+\text{-K}^+$  ATPase immunoreactivity in the contralateral cortex, however, is significantly decreased (arrow). Bar = 1,500  $\mu\text{m}$ .

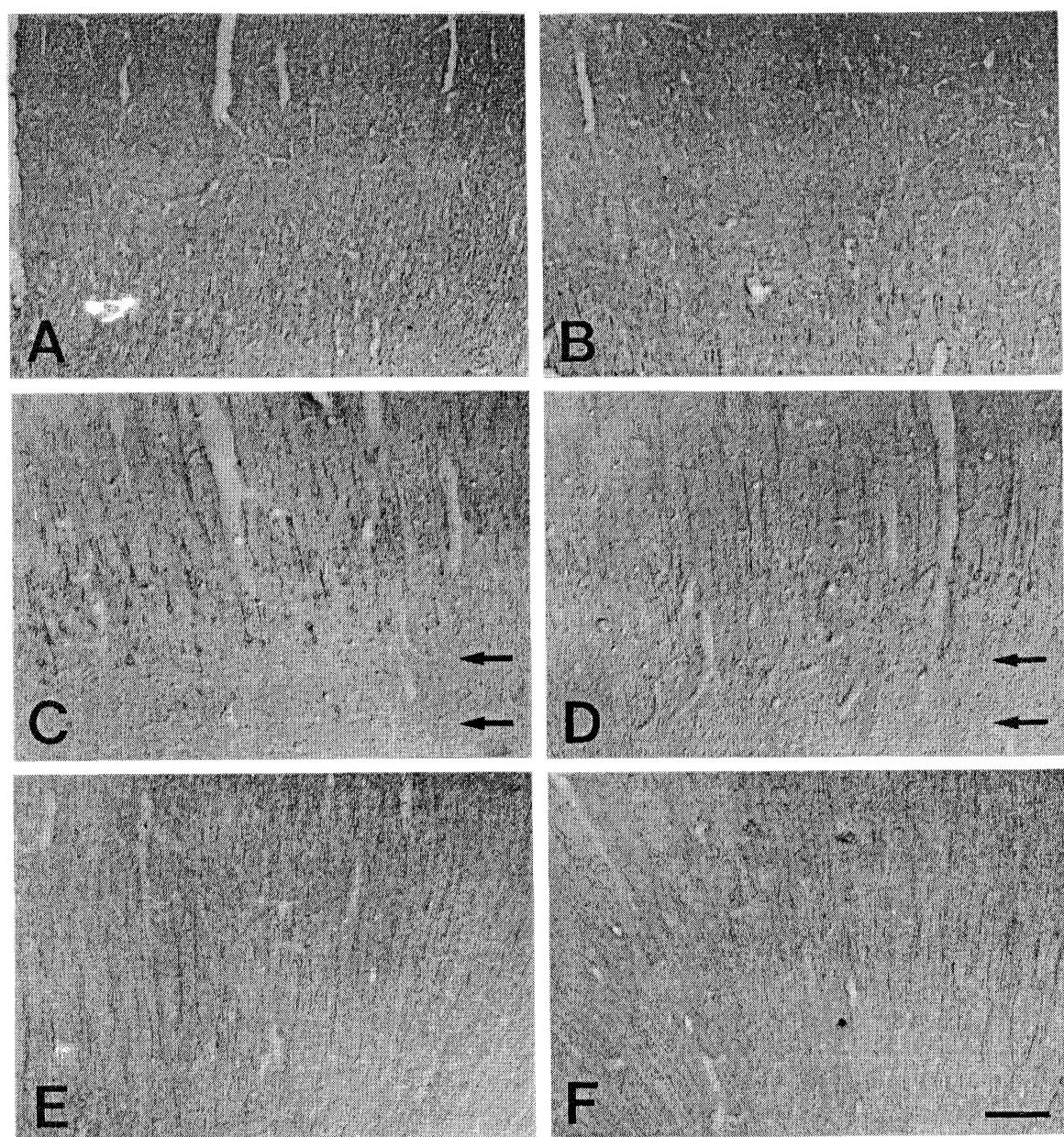
decreased as compared with that observed 7 days after hypoxic ischemia. Both the infarct zone and the ipsilateral hippocampus had completely disappeared at this stage, due to atrophy of the ischemic lesion (Fig. 1D).

#### MAP1A immunoreactivity

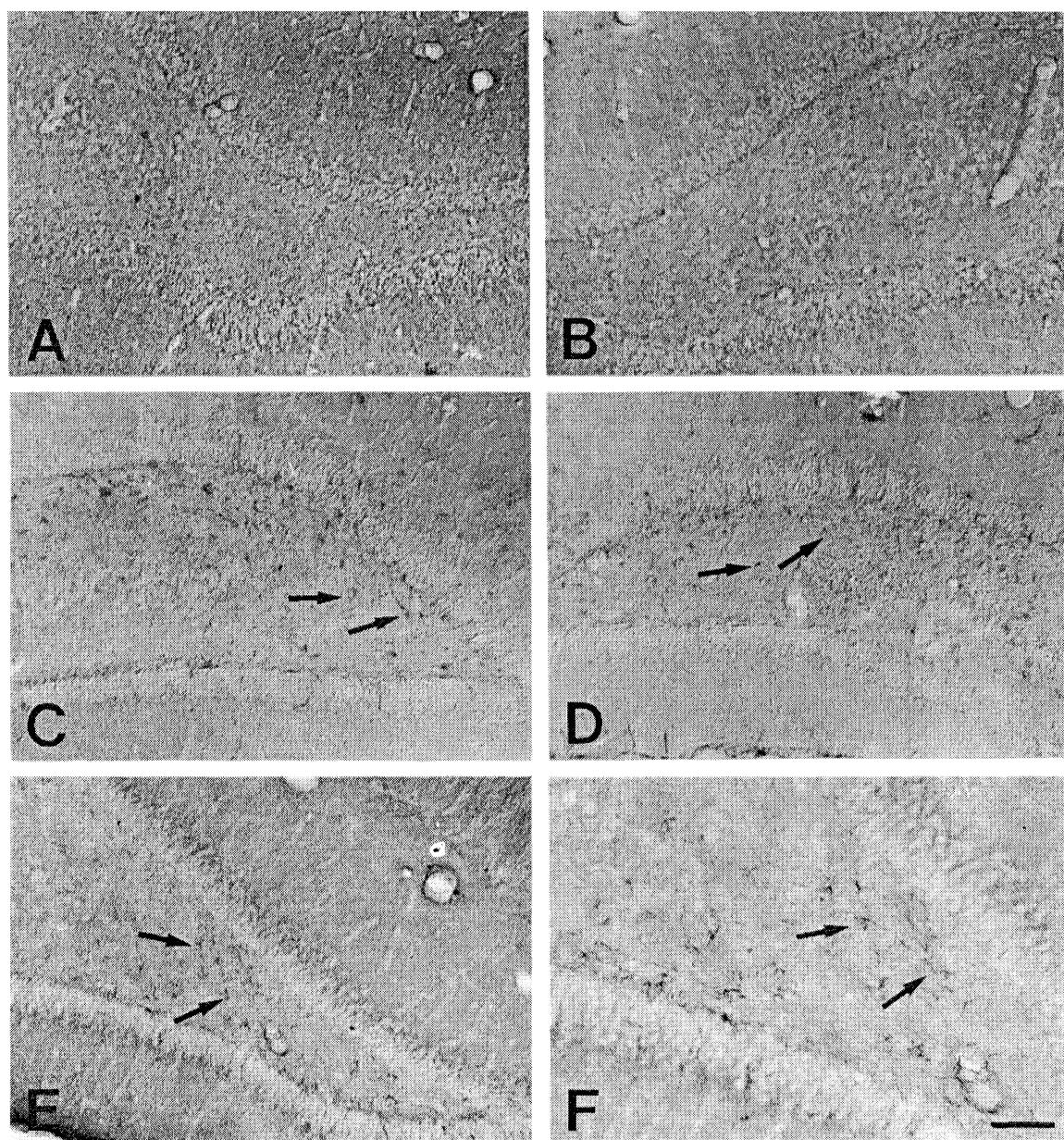
Unlike  $\text{Na}^+\text{-K}^+$  ATPase immunoreactivity, MAP1A immunoreactivity was simultaneously altered in the cerebral cortex and the hippocampus. Compared with the sham cortex (Figs. 2A, 2B), 2 days after ischemia, MAP1A immunoreactivity significantly decreased, but in layer V, MAP1A

after ischemia, accumulated MAP1A immunoreactive neurons disappeared from the peri-infarct zone and the contralateral cortex, but immunoreactivity was maintained in axons (Figs. 2E, 2F).

MAP1A immunoreactivity also decreased in the hippocampus compared with the sham (Figs. 3A, 3B). Like in the cerebral cortex, MAP1A immunoreactivity accumulated in some hilar neurons in both sides of the hippocampus 2 days after insult and was maintained 7 days after hypoxic ischemia (Figs. 3C, 3D). Interestingly, MAP1A immunoreactive axons remained in the contralateral hippocampus 2 months after ischemia (Figs. 3E, 3F).



**Fig. 2:** MAP1A immunoreactivity in the rat cerebral cortex. In the sham cortex, MAP1A immunoreactivity is similarly detected both in contralateral (A) and ipsilateral (B) cortices to infarct region. Two days after ischemia (C and D), MAP1A immunoreactivity in layer V is had obviously accumulated in neurons and their axons, although MAP1A immunoreactivity was significantly decreased (arrows indicate layer VI). Two months after ischemia, accumulated MAP1A immunoreactive neurons disappeared from the contralateral cortex (E) and the peri-infarct zone (F), in contrast, MAP1A immunoreactivity is maintained in axons. Bar = 100  $\mu\text{m}$ .



**Fig. 3:** MAP1A immunoreactivity in the rat hippocampus. In the sham cortex, MAP1A immunoreactivity is similarly detected both in the contralateral (A) and ipsilateral (B) hippocampi to infarct region. Two days after insults (C and D), MAP1A immunoreactivity had accumulated in some hilar neurons in both sides of the hippocampus (arrows). Two months after ischemia, MAP1A immunoreactive axons (arrows) remained in the contralateral hippocampus (E and F). Bar = 100  $\mu$ m (A-E); 50  $\mu$ m (F).

### MAP2 immunoreactivity

Contrary to MAP1A immunoreactivity, the distributions of MAP2 immunoreactivity was simultaneously altered in the cerebral cortex, but not in the hippocampus. Compared with the sham cortex (Figs. 4A, 4B), MAP2 immunoreactivity significantly decreased, particularly in layers IV and V, thus MAP2 immunoreactive processes were scattered in these layers. In layer VI, however, MAP2 immunoreactivity was unaltered (Figs. 4C, 4D). Similar MAP2 immunoreactive patterns were detected in the peri-infarct zone and in the contralateral cortex until 7 days after hypoxic ischemia. Two months after ischemia, MAP2 immunoreactivity in these regions recovered to the sham levels (Figs. 4E, 4F).

Compared with the sham (Figs. 5A, 5B), 2 days after insult, MAP2 immunoreactivity in the contralateral hippocampus significantly decreased. In the ipsilateral hippocampus, MAP2 immunoreactivity in the dentate hilar region also declined (Figs. 5C, 5D). Seven days after hypoxic ischemia, in the contralateral hippocampus, the immunodensity and the distribution of MAP2 immunoreactivity recovered to the sham level. In the ipsilateral hippocampus, however, MAP2 immunoreactivity was detected in the sprouting dendrites of the remaining hilar neurons and in the granule cells (Figs. 5E, 5F).

### DISCUSSION

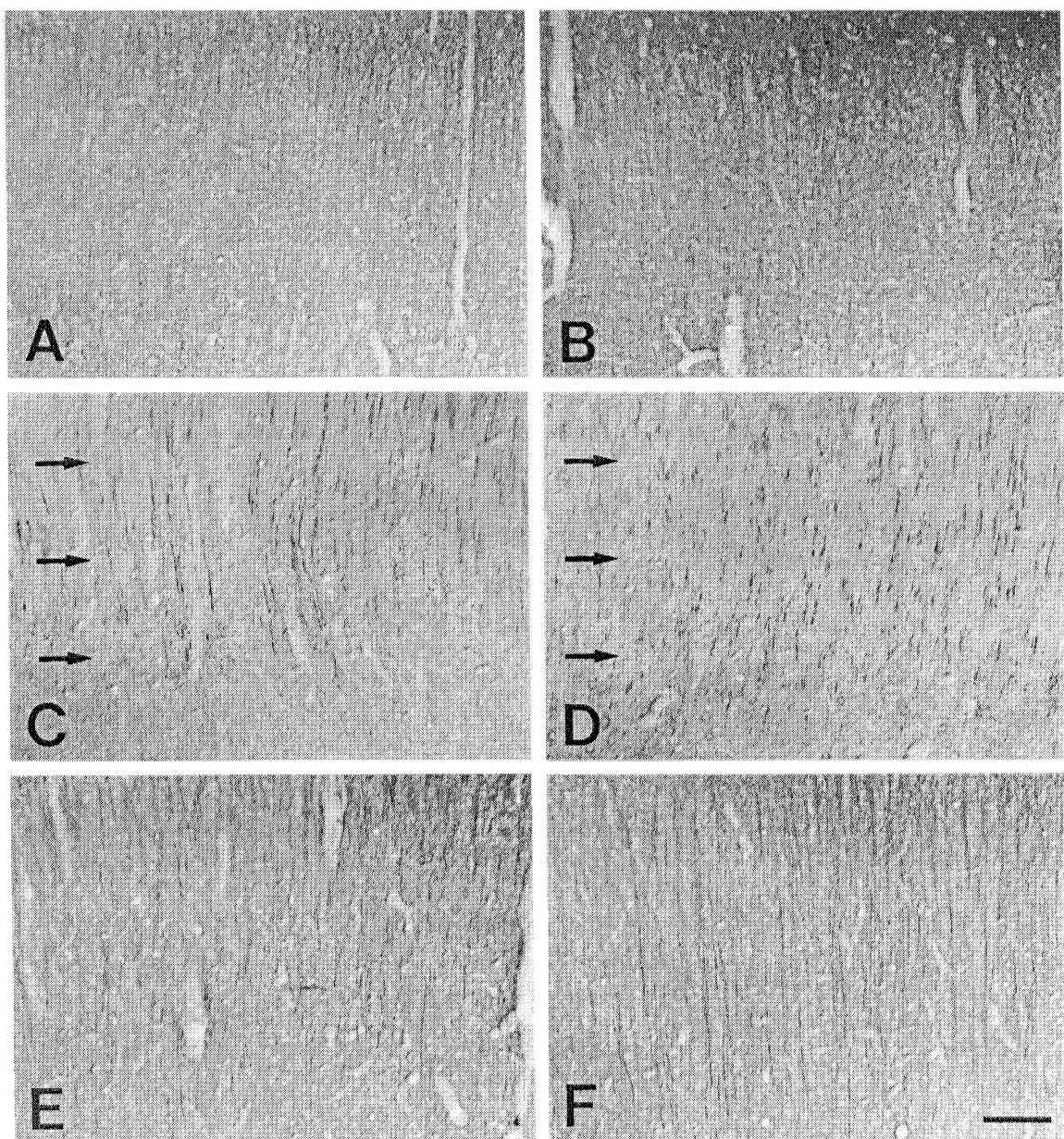
Within a few hours after ischemia, a progressive net uptake of water from the blood-brain barrier results in an increase of total tissue water (Huang et al., 1999; Hatashita et al., 1990). This brain edema is remarkably prolonged after 24 to 72 h (Forsting et al., 1994; Rosenberg, 1999), during which it produces massive secondary damage by compressing the contralateral brain and

other remote brain areas and induces an associated secondary ischemia caused by the compression of the vessels. This secondary contralateral edema returns almost to baseline 2 weeks after the injury (for review see Witte et al., 2000).

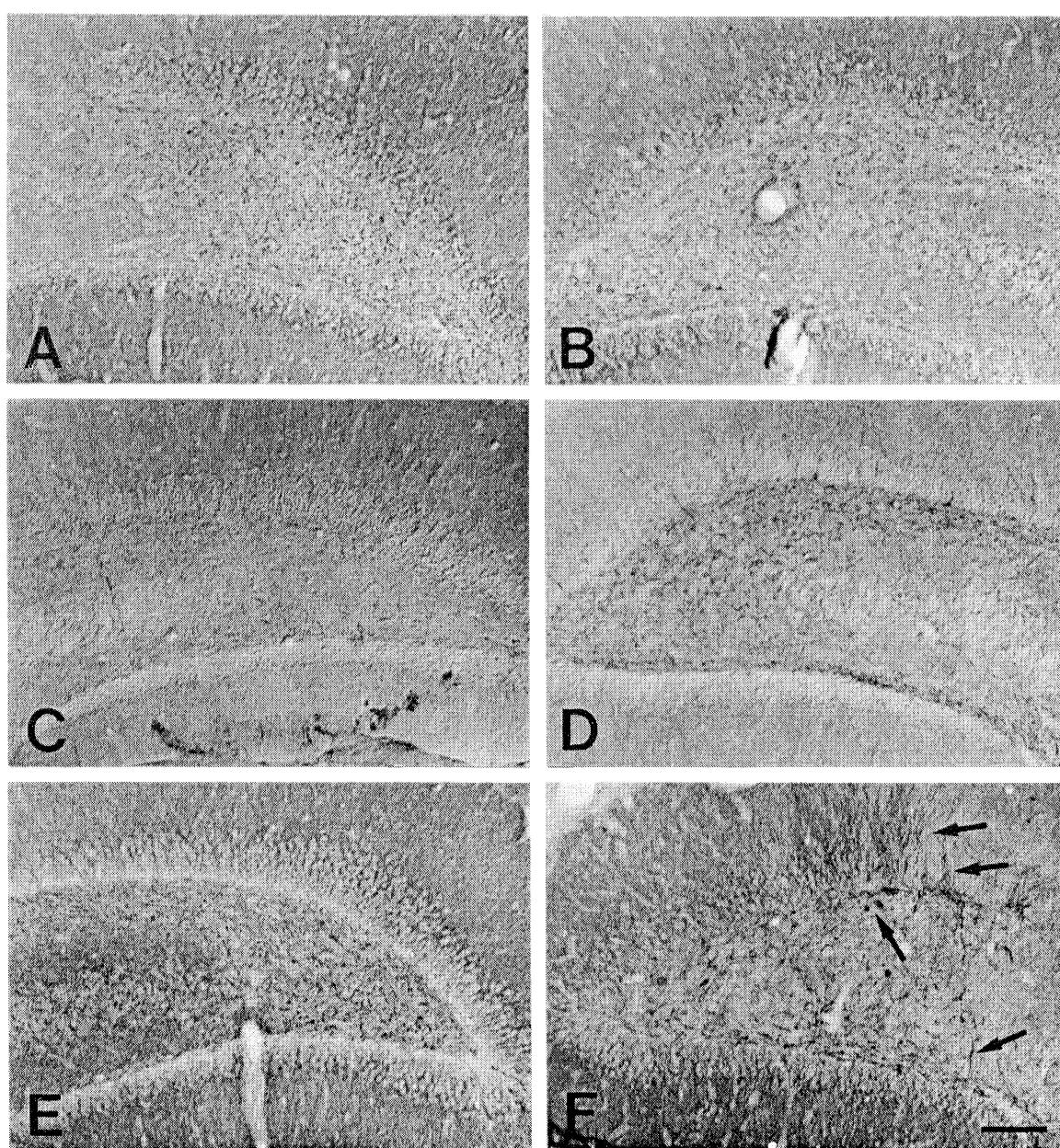
In the present study, 2 days after hypoxic ischemia, ATPase  $\text{Na}^+ \text{-K}^+$  immunoreactivity was significantly enhanced in the contralateral cortex and was maintained for 7 days after ischemia, whereas  $\text{Na}^+ \text{-K}^+$  ATPase immunoreactivity in the peri- and infarct zones was unaltered. The results of this experiment indicate that the up-regulation of  $\text{Na}^+ \text{-K}^+$  ATPase immunoreactivity may play an important role in the recovery of the contralateral cortical lesion because  $\text{Na}^+ \text{-K}^+$  ATPase maintains neuronal volume by regulating osmolarity (Astrup, 1982; Siesjo, 1988). Therefore, this unilateral (contralateral) elevation of  $\text{Na}^+ \text{-K}^+$  ATPase immunoreactivity may be a compensatory response that prevents cytotoxic edema. The asymmetric increase of  $\text{Na}^+ \text{-K}^+$  ATPase immunoreactivity also suggests that neuronal activity in the contralateral cortex may be elevated because hyperexcitability in the contralateral cortex is induced by the decreased GABAergic inhibition (Buchkremer-Ratzmann & Witte, 1996; Redecker et al., 2000; Witte et al., 2000).

MAPs are a family of cytoskeletal proteins that are responsible for maintaining mature neuronal morphology and for initiating and stabilizing new fibers and intracellular transport functions (Grafstein & Forman, 1980; Solomon, 1980; Tucker et al., 1989). Ischemic lesions also cause functional and structural changes throughout the brain during the initial phase, and during post-lesional degeneration and regeneration. Bidmon et al. (1997) reported that the distribution pattern and staining intensity of MAP2 in the contralateral cortex appeared normal, although MAP2 immunoreactivity in the perilesional cortex increased.

In the present study, however, 2-7 days after ischemia MAP1A and MAP2 immunoreactivity in



**Fig. 4:** MAP2 immunoreactivity in the rat cortical coronal section. In the sham cortex, MAP2 immunoreactivity is similarly detected both in contralateral (A) and ipsilateral (B) cortices to infarct region. Two days after insult (C and D), MAP2 immunoreactivity significantly decreased in layers IV and V (arrows indicate layers IV and V). Two months after ischemia, MAP2 immunoreactivity in these regions recovered to the sham level (E and F). Bar = 100  $\mu$ m.



**Fig. 5:** MAP2 immunoreactivity in the rat hippocampus. In the sham cortex, MAP2 immunoreactivity is similarly detected both in contralateral (A) and ipsilateral (B) hippocampi to infarct region. Two days after insult, MAP2 immunoreactivity in the contralateral hippocampus significantly decreased (C). In the ipsilateral hippocampus, MAP2 immunoreactivity in the hilar region also declined (D). Seven days after ischemia, in the contralateral hippocampus (E), the immunodensity and the distribution of MAP2 immunoreactivity recovered to the sham level, in contrast MAP2 immunoreactivity in the ipsilateral hippocampus (F) was detected in sprouting dendrites of remained hilar neurons and in granule cells (arrows). Bar = 100  $\mu\text{m}$ .

in the ipsi- and contralateral cortices significantly decreased, whereas in layer V, MAP1A immunoreactivity had obviously accumulated in neurons and their processes. This distribution pattern indicates that a time-dependent dramatic growth of neuronal processes, followed by hypoxic ischemia, occurs in the homotopic cortex of the opposite hemisphere. These findings are consistent with those of a previous study, which reported that the widespread degeneration of nerve fibers extending to most of ipsi- and contralateral cortical areas that showed a change of GABAergic inhibition (Witte et al., 2000). Therefore, this result suggests that both MAP1A and MAP2 expressions may be altered in the perilesional cortex, as well as in the contralateral cortex. Furthermore, regarding the elevated  $\text{Na}^+/\text{K}^+$  ATPase immunoreactivity in the contralateral cortex observed in the present study, Jones and colleagues (Jones & Schallert, 1994; Jones et al., 1999) reported that hyperactivity in the contralateral cortex can induce dendritic sprouting or axonal regeneration.

Unexpectedly, 2 days after insult, MAP2 immunoreactivity was significantly decreased within the ipsi- and contralateral hippocampi. This result suggests that remote changes in MAP2 expression following hypoxic ischemia may be evoked within both hippocampi, as was the case in the cerebral cortex. Seven days after ischemia, however, the immunodensity and the distribution of MAP2 immunoreactivity in the contralateral hippocampus recovered to the sham level. In addition, MAP2 immunoreactivity in the ipsilateral hippocampus was detected in sprouting dendrites remaining hilar neurons and in granule cells. With respect to the role of MAP2 in the adult brain, it is viewed that this increased MAP2 immunoreactivity may indicate neuronal plasticity and dendritic sprouting in both hippocampi. The fact is that MAP2 expression is known to increase in the rat brain during remodeling after brain lesions (Johnson & Jope, 1992; Pollard et al., 1994;

Stewart et al., 1994; Burnham et al., 1995; Pei et al., 1998). Therefore, the altered MAP2 immunoreactivity may be related to dendritic structural changes of the hilar neurons in response to synaptic activity. In addition, our finding suggests that remote changes after insult may not be restricted to the cerebral cortex but may be associated with various brain regions.

In contrast to MAP2, MAP1A immunoreactivity accumulated in some hilar neurons in both sides of the hippocampus 2 days after insult, and MAP1A immunoreactive axons remained in the contralateral hippocampus 2 months after hypoxic ischemia. In view of the fact that the MAP1 family plays an important role in the maintenance of axonal morphology and axon-like neurite outgrowth (Tucker, 1990), our results suggest that axonal reorganization may be also evoked in both hippocampi, and that this time-dependant alteration may be long lasting compared with the dendritic sprouting.

## CONCLUSIONS

In conclusion, unilateral (contralateral) elevation of  $\text{Na}^+/\text{K}^+$  ATPase immunoreactivity can prevent cytotoxic edema and reflects the elevated neuronal activity in the contralateral cortex. In addition, this asymmetric hyperexcitability in the cortex may play an important role in the recovery and reorganization of the brain, accompanied by remote changes in MAPs expressions that are not restricted to the cerebral cortex.

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