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Alteration of Immunoreactivity for SNARE Proteins in the Rat Hippocampus after Middle Cerebral Artery Occlusion

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Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) proteins, composed of two presynaptic membrane proteins [synaptosomal-associated protein of 25 kDa (SNAP-25) and syntaxin] and a presynaptic vesicular protein [vesicle-associated membrane protein (VAMP)], serve as a core of exocytotic fusion machinery, which can be affected by ischemia. Synaptic protein in core region, striatum and cortex has been shown to alter after focal ischemia, however, little is known in hippocampus. Hippocampus is remote from ischemic core, but it is one of the most vulnerable regions. Using immunohistochemistry, the present study was undertaken to investigate the alteration of expression of SNAP-25, syntaxin, and VAMP in the hippocampus of rats which were subjected to middle cerebral artery occlusion (MCAO) for 2h and allowed to reperfuse. At 2 weeks of reperfusion, the SNAP-25 and syntaxin immunoreactivity was increased in the stratum oriens of the CA1 and the stratum lucidum of the CA3 in the ipsilateral hippocampus. However, VAMP immunoreactivity didn't show significant change. These results demonstrate that the level of the presynatpic plasma membrane proteins (SNAP-25 and syntaxin) in the rat hippocampus is more sensitively affected by focal ischemia than that of the synaptic vesicle protein (VAMP).

Key Words: Middle cerebral artery occlusion, Hippocampus, SNAP25, Syntaxin, VAMP

INTRODUCTION

The brain is extremely sensitive to ischemia and reperfusion injury. However, the extent and the severity of cerebral ischemic damage differ, depending on the brain regions or ischemic types. For example, the hippocampus is one of the regions most vulnerable to a global type cerebral ischemic insult (Pulsinelli et al, 1982; Yang et al, 2000). In contrast, the hippocampus is spared from a direct ischemic insult in the middle cerebral artery occlusion (MCAO)-induced focal type ischemia, which resembles human thromboembolitic stroke. Although the hippocampus is known as an area remote from the ischemic core regions such as the striatum and the cortex, focal ischemia can lead to various neuropathological changes in the hippocampus.

In addition to morphological changes, focal ischemia produces functional changes that can be assessed by behavioral studies. There have been a number of studies to reveal learning and memory deficit in rats with a transient/permanent occlusion in laboratory tasks (Okada et al, 1995; Sakai et al, 1996). Since the hippocampus participates in learning/memory, the observed deficits can be attributable to hippocampal dysfunction resulting from MCAO (Kiyota et al, 1990; Eichenbaum et al, 1992; Wood et al, 1992; Jarrad, 1993).

The cellular and molecular mechanisms of the memory and learning depend on the synaptic function in the hippocampus, which is regulated by the exocytosis of neurotransmitter (Nicoll et al, 1988). At the nerve terminals, the neurotransmitter release is a highly regulated process requiring a Ca^{++}-triggered exocytotic fusion via specific interaction of the synaptic vesicle proteins with the presynaptic plasma membrane proteins (Nicoll et al, 1988; Sollner et al, 1993a; Shudhof, 1995; Zucker, 1996). The pivotal exocytotic fusion machinery is the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins. They are composed of two proteins at the target presynaptic membrane (t-SNAREs) [synaptosomal-associated protein of 25 kDa (SNAP-25) and syntaxin] and a protein at the vesicular membrane (v-SNARE) [synaptobrevin or vesicle-associated membrane protein (VAMP)] (Sollner et al, 1993b; Pevsner et al, 1994; Rothman, 1994; Sollner & Rothmann, 1994; Calakos & Scheller, 1996; Fon & Edward, 2001). The importance and functional involvement of the SNARE proteins in exocytosis have been demonstrated in studies which showed that the botulinum

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ABBREVIATIONS: MCAO, middle cerebral artery occlusion; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; t-SNAREs, target presynaptic membrane- soluble N-ethylmaleimide-sensitive factor attachment protein receptor; v-SNARE, vesicular membrane-soluble N-ethylmaleimide- sensitive factor attachment protein receptor; SNAP-25, synaptosomal-associated protein of 25 kDa; VAMP, vesicle-associated membrane protein; CA, cornus ammonis

and the tetanus toxin block the transmitter release by acting as zinc-dependent endopeptides that hydrolyse several SNARE proteins (Shiavo et al, 1992; Stroemer et al, 1992; Niemamm et al, 1994; Shiavo et al, 1994).

Ischemic insult results in the loss of neuronal cells and the depletion of both the pre- and post-synaptic sites. Under these circumstances, plastic modifications, which are believed to contribute to the reorganization of the neuronal network, may occur in the neurons that are resistant to an ischemic insult. Therefore, a modification of expression of the synaptic proteins, particularly the SNARE proteins, which are the key mediator of membrane fusion in neurotransmitter release after ischemia, is supposed.

However, there has been no study to show alteration of the SNARE proteins in non-ischemic and remote areas such as hippocampus following the MCAO. There are only some studies on the synaptic organization and synaptic proteins in the ischemic core region after the MCAO (Stroemer et al, 1992; Yam et al, 1998) or in the hippocampus after a global ischemic insult (Marti et al, 1998; Ishimaru et al, 2001). Therefore, this study was undertaken to investigate the level of the SNARE proteins, SNAP-25, syntaxin, and VAMP in rat hippocampus after the MCAO.

METHODS

Animal care and housing

Sprague-Dawley male rats (280~320 g) were kept under routine laboratory conditions at Animal Laboratory, College of Medicine, Ewha Womans University. Rats were housed in polyethylene cages with stainless steel tops on a 12 h light-dark cycle with access to food and water *ad libitum*. All rats received human care.

Induction of MCA occlusion

The right middle cerebral artery (MCA) was occluded for 2 h by the intraluminal-suture method (ZeaLonga et al, 1989). Briefly, after an intraperitoneal injection of atropine sulfate (0.5 mg/kg), anesthesia was induced with 3.5% halothane in a mixture of 70% nitrous oxide and a balance of oxygen. The rats were orally intubated, immobilized with intravenous pancuronium bromide (0.6 mg/kg), and mechanically ventilated. The right common carotid artery (CCA) was exposed through a midline neck incision and dissected free of the surrounding nerves; the occipital branches of the external carotid artery (ECA) were coagulated and the pterygopalatine artery was ligated. A 4 cm length of a 3-0 monofilament nylon suture was then inserted via the proximal ECA into the internal carotid artery (ICA) and MCA to a distance of 19-20 mm from the CCA bifurcation according to the animal's weight, which occluded the MCA. Prior to use, the tip of the suture was heat-blunted, and a 20 mm distal segment of the suture was coated with poly-L-lysine solution (0.1%w/v) and dried at 60°C for 1h; this coating procedure increases the reproducibility of the resulting infarct (Bclaycy et al, 1996). After suture placement, the neck incision was closed, and the animals were allowed to awaken from the anesthesia. After 2 h of MCAO, they were tested on a standardized neurobehavioral battery by a blinded investigator to confirm the presence of a neurological deficit. The battery consisted of a postural reflex test to examine the upper body posture, while the animal was suspended by the tail and the forelimb placing test to examine the sensori-motor integration in the forelimb placing responses to visual, tactile, and proprioceptive stimuli. After 2 h of MCAO, rats were reanesthetized and reperfusion was allowed by removing the intraluminal suture. After surgery, the soft tissues were replaced and the wound was sutured. The animals were maintained on a heated mat until they regained consciousness.

Immunohistochemistry

At different times of reperfusion after MCAO (24 h, 3 days, 1 week, 2 weeks), the rats were anesthetized with diethyl ether and perfused transcardially, first with heparinised saline, followed by 4 % paraformaldehyde in 0.1 M phosphate buffer saline (PBS, pH 7.4). Paraffin-embedded sections, 5 um thick, were prepared. After the coronal sections were microwaved in sodium citrate buffer, pH 6.0, for 5 min, they were stained using the avidin-biotin-peroxidase method (Vectastain Elite ABC kit: Vector Laboratories, Burlingame, CA, USA). The sections were washed several times in PBS and incubated overnight in a humidified chamber at 4°C with the primary antibody diluted in PBS containing 10% normal horse serum. The primary antibodies and their dilution factors were anti-mouse SNAP-25 antibody (1 : 100, Oncogen research product, Boston, MA, USA) and anti-mouse syntaxin antibody (1 : 100, Sigma, ST. Louis, Mo, USA), and anti-mouse synaptobrevin antibody (1 : 100, Chemicon, Temecula, CA). Subsequently, they were incubated with biotinylated horse anti-mouse IgG/anti-rabbit IgG (H+L) (Vector Laboratories, Burlingame, CA, USA) at 1 : 200 dilution, and followed by incubation with ABC at 1 : 200 dilution for 1 h each. The peroxidase activity was visualized with 0.05% diaminobenzidine (DAB) and 0.01% hydrogen peroxide. The negative controls contained no primary antibody, but included all other procedures.

RESULTS

Cresyl violet staining in hippocampus after MCAO

Cresyl violet staining was performed on the brain sections reperfused for 24 h, 3 days, 1 week, and 2 weeks in order to evaluate the degree of ischemic damage in the hippocampus after MCAO.

The pyramidal cells in the contralateral hippocampus of 24 h (Fig. 1A, C, D), 3 days, 1 week, and 2 weeks (Fig. 1G) showed round and pale stained nuclei. At 24 h of reperfusion, the pyramidal cells in the cornus ammonis (CA)1 and the CA3 of the ipsilateral hippocampus were slightly swollen and began to die (Fig. 1B, E, F). At 3 days and 1 week of reperfusion, the pyramidal cell death proceeded (data not shown) and most of the pyramidal cells disappeared in these regions at 2 weeks of reperfusion (Fig. 1H, arrow). The granular cells in the dentate gyrus still survived at 2 weeks of reperfusion after MCAO (Fig. 1 insert in H). There were no abnormal findings in the contralateral hippocampus until 2 weeks.

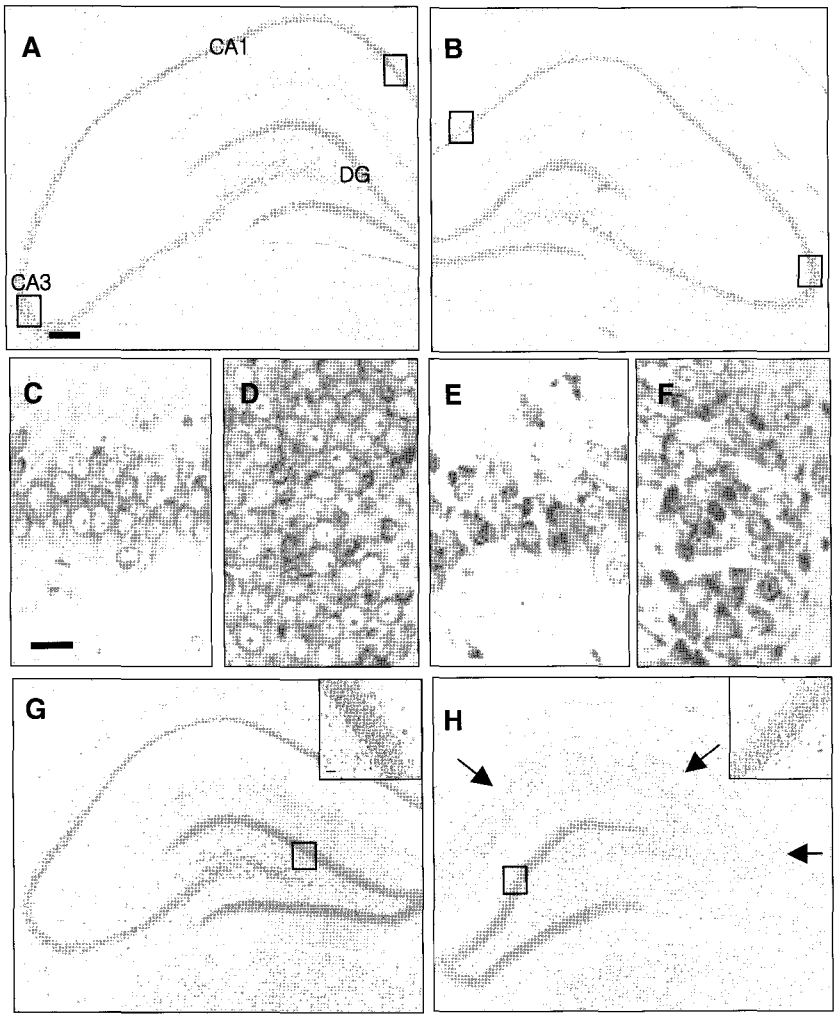

Fig. 1. Morphological aspects in the rat hippocampus during reperfusion after middle cerebral artery occlusion. Cresyl violet staining was performed on the brain sections at 24 h and 2 weeks of reperfusion. With cresyl violet staining, the extent of brain cell damage was examined by optical microscopy. At 24 h of reperfusion, pyramidal neuron death in ipsilateral hippocampus began in the CA1 (E) and the CA3 (F). C~F are high magnification view of the boxed area signed in the A and the B. C, E and D, F is the CA1 and the CA3 of the contralateral (C, D) and the ipsilateral hippocampus (E, F), respectively. At 2 weeks of reperfusion, most of the pyramidal cells disappeared in the CA1 and the CA3 of the ipsilateral hippocampus (H). The small insert in G and H is the high magnification of boxed area signed in the dentate gyrus. Granular cells in the dentate gyrus survived at 24 hr (A, B) and 2 week (G, H) of reperfusion in contralateral (A, G) and ipsilateral hippocampus (B, H). DG: dentate gyrus. Scale bars=500 μm in A, B, G, and H, 50 μm in C-F and insert of G and H.

Change of SNAP-25 immunoreactivity in hippocampus after MCAO

No immunoreactivity was detected in the cell bodies of the pyramidal cells and the granular cells of the dentate gyrus. The SNAP-25 immunoreactivity in the contralateral hippocampus was moderate in the stratum oriens and the stratum radiatum of the CA1 and CA3, and the stratum lucidum of the CA3 as well as the hilus of the dentate gyrus. However, it was weak in the molecular layer of the dentate gyrus (Fig. 2A, B, C). Until 1 week of reperfusion, there was no significant difference in the SNAP-25 immunoreactivity between the contralateral and the ipsilateral hippocampus (data not shown). At 2 weeks of reperfusion, there was a strong increase in the SNAP-25 immunoreactivity in the stratum lucidum of the CA3 and the stratum oriens of the CA1 and CA3. Moreover, the immunoreacitivity was slightly increased in the inner zone of the molecular layer of the dentate gyrus in the ipsilateral hippocampus (Fig. 2D, E, F). In contrast, the SNAP-25 immunoreactivity in the stratum radiatum of the CA1 decreased in the ipsilateral hippocampus.

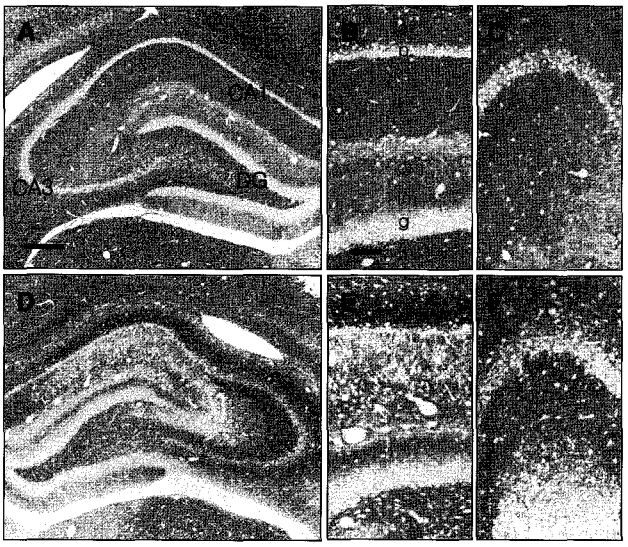

Fig. 2. Immunoreactivity for synaptosomal-associated protein of 25 kDa (SNAP-25) in the rat hippocampus at 2 weeks of reperfusion. In the ipsilateral hippocampus (D, E, F), an increase in the SNAP25 immunoreactivity was observed in the stratum oriens of CA1 (E), the stratum lucidum of the CA3 (F), and the inner zone of the molecular layer of the dentate gyrus (E), compared to the contralateral hippocampus (A, B, C). B, E and C, F are high magnification images of the CA1 and the CA3, respectively. The pyramidal cells and the granular cells of the dentate gyrus were not stained. o:stratum oriens, p: pyramidal cell layer, r: stratum radiatum, om: outer zone of the molecular layer of the dentate gyrus, im: inner zone of the molecular layer of the dentate gyrus, g:granular cell layer of the dentate gyrus, h: hilus of the dentate gyrus, l: stratum lucidum of the CA3. Scale bars=500 μm in A and D, 200 μm in B, C, E, and F.

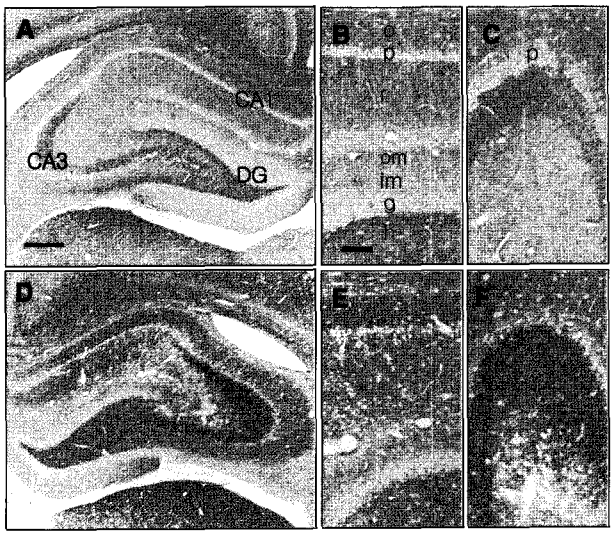

Fig. 3. Immunoreactivity for syntaxin in the rat hippocampus at 2 weeks of reperfusion. In the ipsilateral side (D, E, F), there was an increase in the syntaxin immunoreactivity in the stratum oriens and the stratum radiatum of the CA1, the stratum lucidum of the CA3, and the hilus of the dentate gyrus. High-power photomicrographs show syntaxin immunoreactivity of the CA1 (B, E) and the CA3 (C, F) in the contralateral (A, B, C) and ipsilateral hippocampus (D, E, F). The pyramidal cells and the granular cells of the dentate gyrus were not stained. o: stratum oriens, p: pyramidal cell layer, r: stratum radiatum, om: outer zone of the molecular layer of the dentate gyrus, im: inner zone of the molecular layer of the dentate gyrus, g: granular layer of the dentate gyrus, h: hilus of the dentate gyrus, l: stratum lucidum of the CA3. Scale bars=500 μm in A and D, 200 μm in B, C, E, and F.

Change of syntaxin immunoreactivity in hippocampus after MCAO

In the contralateral hippocampus of all groups, the syntaxin immunoreactivity was moderate in the stratum oriens and the stratum radiatum of the CA1 and the CA3, and was slightly strong in the stratum lucidum of the CA3 and the hilus of the dentate gyrus (Fig. 3A, B, C). The cell bodies in the pyramidal cells and the granular cells of the dentate gyrus were not stained. Until 1 week of reperfusion, the syntaxin immunoreacitivity in the ipsilateral hippocampus was similar to that of the contralateral hippocampus (data not shown). At 2 weeks of reperfusion, the level of syntaxin immunoreactivity was increased in the stratum radiatum and the stratum oriens of the CA1 and the CA3, and the hilus of the dentate gyrus in the ipsilateral hippocampus. In addition, it was strongly increased in the stratum lucidum of the CA3, and the stained area of the CA3 was expanded (Fig. 3D, E, F).

Change of VAMP immunoreactivity in hippocampus after MCAO

Basically, there was no change in the immunoreactivity of VAMP between the contralateral and the ipsilateral hippocampus in all groups. The immunoreactivity in the stratum oriens and the stratum radiatum of the CA1 and the CA3 and the molecular layer of the dentate gyrus was very weak. However, the immunoreactivity was slightly strong in the hilus of the dentate gyrus and the stratum lucidum of CA3 (Fig. 4).

DISCUSSION

To determine the degree of neuronal damage, cresyl violet staining was performed after MCAO. The loss of neurons began in the CA1 and the CA3 area of the ipsilateral hippocampus at 24 h of reperfusion after the MCAO, and significant neuronal loss in this region was observed at 2 weeks of reperfusion. After ischemia, most of the damaged cells in the ischemic core underwent rapid and passive cell destruction, while the cells in the region surrounding the ischemic core underwent slow progressive cell death. After MCAO for 2 h, the degeneration of the neurons in the CA1 and the CA3 area was observed from 24 h to 2 weeks of reperfusion. This phenomenon is called delayed neuronal death (Pulsinelli et al, 1982). In the case of the global ischemia model, the hippocampus is the most vulnerable to an ischemic insult. Within the hippocampus, the neurons in the CA1 and the hilus area are sensitive to ischemia, whereas the hippocampal CA3 neurons and granular cells of the dentate gyrus are largely resistant, suggesting selective vulnerability (Schmidt-Kastner & Freund, 1991; Kirino & Sano, 1984; Yang et al, 2000). However, at 2 weeks of reperfusion after MCAO in this study, most of the

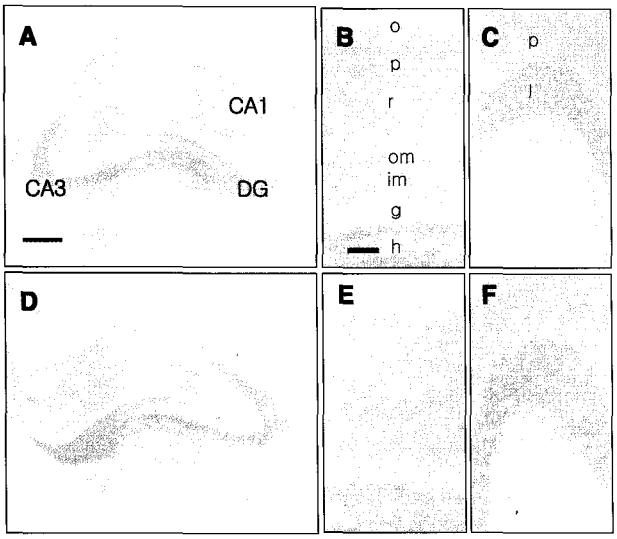

Fig. 4. *Immunoreacitvity for vesicle-associated membrane protein* (VAMP) in rat hippocampus at 2 weeks of reperfusion. A slightly strong VAMP immunoreactivity in the hilus of the dentate gyrus and the stratum lucidum of the CA3 persisted until 2 weeks of reperfusion. Basically, VAMP immunoreactivity was not different between the contralateral (A, B, C) and the ipsilateral hippocampus (D, E, F). B, E and C, F show high magnification images of the CA1 and the CA3, respectively. o: stratum oriens, p: pyramidal cell layer, r: stratum radiatum, om: outer zone of the molecular layer of the dentate gyrus, im: inner zone of the molecular layer of the dentate gyrus, g: granular layer of the dentate gyrus, h: hilus of the dentate gyrus, l: stratum lucidum of the CA3. Scale bars=500 μm in A and D, 200 μm in B, C, E, and F.

CA1 and CA3 neurons were destroyed. This may be due to involve a difference in the method that induced ischemia in animals, global ischemia or focal ischemia. Focal ischemia may lead to more widespread cell death in the hippocampus than global ischemia. Thus, the ischemic type in animal model can alter the specific region affected by the ischemic injury. However, the granular cells of the dentate gyrus are still very resistant to damage caused by the two ischemic types.

The immunoreactivity of SNAP-25 and syntaxin at 2 weeks of reperfusion was markedly increased in the stratum lucidum of the CA3 area and the stratum oriens around the base dendrite of the CA1 and CA3 pyramidal cells in the ipsilateral hippocampus. This increased immunoreactivity might have been due either to an increase in the number of nerve endings immunostained by these antibodies or to an increase in the level of these proteins themselves localized in the same nerve terminals. While almost all the CA1 and the CA3 pyramidal cells died at 2 weeks of reperfusion, the loss of CA3 pyramidal cells, which is the postsynaptic target of mossy fibers from the granular neurons of the dentate gyrus might trigger rearrangement in the presynaptic mossy fiber nerve terminal, resulting in an alteration in the SNAP-25 and syntaxin immunoreactivity. This is in agreement with the results, showing an increased SNAP-25 immunoreactivity in the mossy fibers following a global ischemia (Marti et al, 1998; Ishimaru et al, 2001).

In addition, the SNAP-25 and syntaxin immunoreactivity were increased in the inner zone of the molecular layer of the dentate gyrus. The molecular layer is occupied mainly by the terminal axonal arbors from the various polymorphic cells such as mossy cells, as well as the dendrites from granule cells and basket cells (Tamamaki, 1999). Mossy fiber aberrant sprouting in the CA3 area, which is a compensatory response to ischemic damage, may affect the polymorphic cells that connect to the dendrites of the granular cells in the molecular layer of the dentate gyrus. The perforant path is the major cortical input to the hippocampus and terminates in the outer two-thirds of the molecular layer of the dentate gyrus (Steward, 1976; Steward & Scoville, 1976). This increase in immunoreactivity in the inner zone of the molecular layer of the dentate gyrus may represent a sprouting of the new afferents of the perforant path input and an expansion of the terminal field in the inner half of the molecular layer. The intact cell bodies that originated from the outside of the ischemic area might produce synaptic proteins, which are transported along the axons to the points of damage. The result described in this study is the first evidence of an increase of SNAP-25 and syntaxin in mossy fiber field and molecular layer of dentate gyrus in hippocampus after focal ischemia by MCAO. These findings suggest that the massive neuronal degeneration in the hippocampus during the reperfusion after MCAO stimulates undamaged neurons to sprout and establish new synaptic connections, supporting the hypothesis of neuronal remodeling and plasticity after a MCAO injury. The axonal and dendritic sprouting and alterations in the number of synapses are accompanied by increase of the proteins involved in neurite sprouting and in the synaptic vesicle population in this region.

In contrast, a decrease in the SNAP-25 immunoreactivity was also found in the stratum radiatum around the apical dendrite of the CA1 pyramidal cells in the ipsilateral hippocampus. The stratum radiatum can be defined as the region where the Schaffer collateral fibers, which are a major CA3 afferent projection to the CA1 pyramidal cells (Swanson et al, 1978), are located. A decrease in the SNAP-25 level in the stratum radiatum might be explained by structural changes in the presynaptic terminal and denervation of the Schaffer collateral connection resulting from death or the damage of the CA3 pyramidal cells.

The VAMP expression level was not altered. VAMP is a major protein on the vesicular membrane and a target of the tetanus toxin, which plays an important role in synaptic transmission (Shiavo et al, 1992; Sollner et al, 1993). VAMP also negatively regulates neurite outgrowth (Martinez-Arca et al, 2000). Marzur et al. (2001) reported that, after mild hypoxia, the VAMP immunoreactivity in the hippocampus was not changed. There are a few reports on the role of VAMP, but their function is still unclear.

This study demonstrated the differential expression between two groups of synaptic proteins in the rat hippocampus after MCAO. The synaptic vesicle protein, VAMP, did not show any changes, while the presynaptic plasma membrane proteins, SNAP-25 and syntaxin, were significantly changed in the selected regions at 2 weeks of reperfusion. This suggests that the presynaptic membrane proteins, SNAP25 and syntaxin, are more sensitive to the focal ischemic process than the synaptic vesicle protein, VAMP. These differences might be due to dissimilar localization and association with these proteins in the presynaptic terminal. In addition, the selective modification

of the t-SNARE proteins (SNAP-25, syntaxin) might result in synaptic dysfunction, and an alteration in the synaptic plasticity might play a role in the pathophysiology of an ischemic injury.

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