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Alterations in [^{3}H]L-N^G-nitroarginine binding in brain after transient global or transient focal ischemia in gerbils and rats

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Abstract

We investigated the post-ischemic change in [^{3}H]L-N^G-nitroarginine binding as a marker of nitric oxide (NO) synthase in the animal brain after transient global ischemia or transient focal ischemia. Transient global ischemia in gerbils was induced for 10 min followed by 1 h to 7 days of recirculation. Transient focal ischemia in rats was induced for 45 min followed by 3 days of recirculation. Following transient global ischemia, [^{3}H]L-N^G-nitroarginine binding showed a significant increase in the striatum (17–18%) and hippocampal CA1 sector (24%) at 48 and 24 h after recirculation, respectively. The hippocampal CA3 sector also showed a significant elevation (32–40%) in [^{3}H]L-N^G-nitroarginine binding at 24 and 48 h after global ischemia. Furthermore, the dentate gyrus showed a significant increase (30–32%) in [^{3}H]L-N^G-nitroarginine binding at 5, 24 and 48 h after global ischemia. Thereafter, a significant reduction in [^{3}H]L-N^G-nitroarginine binding was observed only in the dentate gyrus 7 days after recirculation. In contrast, [^{3}H]L-N^G-nitroarginine binding was unchanged in the thalamus throughout the recirculation periods. Histological analysis revealed that transient global ischemia caused severe damage or cellular damage in the striatum and the hippocampal CA1 sector. The hippocampal CA3 sector and thalamus were mildly damaged, whereas the dentate gyrus was morphologically intact. Following transient focal ischemia, a marked elevation (50–52%) in [^{3}H]L-N^G-nitroarginine binding was found in the regions of the ipsilateral striatum in which severe infarction occurred. Our findings suggest that [^{3}H]L-N^G-nitroarginine binding increases in the striatum and hippocampus after transient global ischemia or transient focal ischemia. This increase in [^{3}H]L-N^G-nitroarginine binding may play a pivotal role not only in the pathogenesis of ischemic brain damage, but also in the restoration of injury areas after cerebral ischemia.

Keywords: Nitric oxide (NO); Nitric oxide (NO) synthase; Global ischemia, gerbil; Focal ischemia, rat; Autoradiography

1. Introduction

Nitric oxide (NO), which is endogenously produced by NO synthase, has been implicated in vascular relaxation, neuronal function and immunological responses (Moncada et al., 1991; Nathan, 1992). NO is synthesized by NO synthase during the conversion of L-arginine to L-citrulline. At least three distinct NO synthase enzymes have been identified, including neuronal, endothelial and inducible NO synthase (Curran et al., 1989; Bredt et al., 1991; Lamas et al., 1992). Several lines of evidence indicate that the formation of NO is increased in brain after focal or global cerebral ischemia (Malinski et al., 1993; Tominaga et al., 1993; Kumura et al., 1994; Zhang et al., 1994). Furthermore, recent studies demonstrate that the inhibitors of NO can prevent ischemic neuronal damage in several animal models (Nowicki et al., 1991; Buisson et al., 1992; Nagafuji et al., 1993; Pelligrino, 1993; Huang et al., 1996; Zhang et al., 1996). Therefore, NO may play an important role in the pathogenesis of ischemic neuronal damage.

NO synthase is expressed by a wide variety of neuronal phenotypes throughout the central nervous system (CNS) as previously shown by immunohistochemistry, in situ hybridization and histochemical staining for NADPH-diaphorase (Dawson et al., 1991a; Bredt et al., 1991; Hope et al., 1991; Vincent and Kimura, 1992). Also, regional localization of [^{3}H]L-N^G-nitroarginine binding as a marker of NO synthase has been studied in the brain, using quantitative autoradiography (Burazin and Gundlach, 1995;

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Kidd et al., 1995; Rao and Butterworth, 1996). However, little is known about the sequential changes in NO synthase during ischemic neuronal damage. In order to further elucidate the mechanisms of ischemic neuronal damage, we studied whether cerebral ischemia is associated with changes in NO synthase levels in brain by examining changes in [^{3}H]L-N^G-nitroarginine binding after transient global or focal ischemia.

2. Materials and methods

2.1. Transient global ischemia

Male adult Mongolian gerbils weighing 60–80 g were in this study. The animals were anesthetized with 2% halothane in a mixture of 30% oxygen and 70% nitrous oxide. Bilateral common carotid arteries were gently exposed and the arteries were occluded with aneurysm clips for 10 min, and then the gerbils were allowed to survive for 1, 5, 24 and 48 h and 7 days after transient global ischemia, as described previously (Araki et al., 1992b, 1995). Sham-operated animals were treated in the same manner, except for the clipping of the bilateral common carotid arteries. Body temperature was maintained at 37–38°C with a heating pad equipped with a thermostat until the gerbils began to move. Each group contained five to eight animals. All experiments were performed in accordance with the Guidelines for Animal Experiments for the Tohoku University School of Medicine.

2.2. Transient focal ischemia

Male adult Wistar rats weighing 220–250 g were used in this study. The animals were anesthetized with 2% halothane in a mixture of 30% oxygen and 70% nitrous oxide. Focal cerebral ischemia was induced by means of middle cerebral artery occlusion, as described previously (Abe et al., 1988; Araki et al., 1992a; Nagasawa et al., 1992). In brief, the animals were anesthetized with 2% halothane in a mixture of 30% oxygen and 70% nitrous oxide. The right common carotid artery was gently exposed, and was then occluded with a silicone rubber-coated nylon surgical thread introduced from the bifurcation of the internal carotid artery immediately after ligation of the ipsilateral common and external carotid arteries. The cylinder was made of 4-0 nylon surgical thread, 16 mm long. This cylinder was coated with silicone (Xantopren, Bayer Dental) mixed with a hardener (Elastomer activator) to increase the thickness of the distal 5 mm to 0.25–0.30 mm. After introduction of the embolus, the internal carotid artery was ligated just distal to the point of insertion. The embolus extended from the bifurcation of the internal carotid artery to the proximal portion of the anterior cerebral artery. The origin of the right middle cerebral artery and that of the right posterior communicating artery were occluded by the silicone rubber cylinder. Following surgery, anesthesia was discontinued and all rats exhibited neurological deficits characterized by left hemiparesis with upper extremity dominance and right Horner's syndrome, as described previously (Nagasawa et al., 1992). After 45 min of middle cerebral artery occlusion, recirculation was achieved by pulling the thread out of the internal carotid artery under the same anesthetic conditions as during surgery. Body temperature was maintained at 37–38°C with a heating pad equipped with a thermostat until the animals began to move. The rats were allowed to survive for 3 days after recirculation. Sham-operated rats were treated in the same manner, except for the occlusion of middle cerebral artery. Each group contained five to eight rats. All experiments were performed in accordance with the Guidelines for Animal Experiments for the Tohoku University School of Medicine.

2.3. Histology

The gerbils and rats were lightly anesthetized with ether and then killed by decapitation at different times as described above. The brains were removed quickly and frozen in powered dry-ice: coronal sections, 12 μm thick, were cut on a cryostat at −20°C and thaw-mounted onto gelatin-coated slides. The sections were stored at −80°C until assay. Adjacent frozen sections were stained with Cresyl violet and anatomic structures were verified by comparing theses stained sections with the gerbil brain atlas of Loskota et al. (1974) and the rat brain atlas of Paxinos and Watson (1982) using a light microscope.

2.4. [^{3}H]L-N^G-nitroarginine autoradiography

Autoradiographic localization of NOS was detected according to the method of Burazin and Gundlach (1995) with minor modifications. Brain sections were preincubated for 15 min at room temperature in 50 mM potassium phosphate buffer (pH 6.8) containing 1 mM EGTA. The sections were then incubated with 20 nM [^{3}H]L-N^G-nitroarginine (specific activity 34.60 Ci/mmol, NEN) in 50 mM sodium phosphate buffer (pH 6.8) containing 10 μM $CaCl_2$ for 120 min at room temperature. After incubation, the sections were washed at 4°C twice for 5 min in the buffer, briefly rinsed in distilled water at 4°C. Non-specific binding was determined using 100 μM L-N^G-nitroarginine (Sigma). The sections were quickly dried under a cold air stream and were exposed to Hyperfilm-^{3}H (Amersham) for 4 months in X-ray cassettes with a set of [^{3}H]microscales (Amersham). The optical density of the brain areas was measured with a computer-associated image analyzer, as described previously (Araki et al., 1992b,

Table 1
Sequential changes in [^{3}H]L-N^G-nitroarginine binding in the gerbil brain after transient global ischemia

	Sham-operated	Recirculation period				
		1 h	5 h	24 h	48 h	7 days
Striatum						
Lateral	11.2 ± 0.7	12.3 ± 1.5	11.9 ± 0.8	12.6 ± 1.5	13.2 ± 1.2[a]	12.5 ± 1.3
Medial	11.3 ± 1.2	12.6 ± 1.7	12.4 ± 0.9	12.1 ± 1.2	13.2 ± 0.8[a]	12.0 ± 1.4
Hippocampus						
CA1 sector	8.8 ± 0.9	8.9 ± 0.8	9.0 ± 1.8	10.9 ± 1.7[a]	9.8 ± 1.4	8.9 ± 2.1
CA3 sector	11.4 ± 1.1	11.8 ± 0.5	12.6 ± 2.3	15.1 ± 2.7[b]	15.5 ± 1.4[b]	9.7 ± 1.4
Dentate gyrus	19.5 ± 2.1	22.3 ± 2.3	25.8 ± 2.9[b]	25.6 ± 4.4[b]	25.3 ± 3.3[a]	16.7 ± 2.1[a]
Thalamus	6.9 ± 0.5	7.2 ± 1.5	6.7 ± 1.9	8.5 ± 1.3	7.0 ± 0.7	7.2 ± 1.7

Optical density was converted to fmol/mg tissue using [^{3}H]microscales.
Values are expressed as means ± S.D.
[a] $P < 0.05$, [b] $P < 0.01$ vs. sham-operated group (Williams multiple comparison test).
Striatum (lateral): dorsolateral part of the striatum.
Striatum (medial): ventromedial part of the striatum.
$n = 5–8$ animals.

1995). The relationship between optical density and radioactivity was obtained with reference to [^{3}H]microscales co-exposed with the tissue sections using a third-order polynomial function. Optical density was converted to fmol/mg tissue. Binding assays were performed in duplicate, as described previously (Araki et al., 1992b, 1995).

2.5. Analysis

Specific binding was determined by subtracting the value of non-specific binding from that of total binding. Under our experimental conditions, specific binding was observed to be 90–95% of the total binding. All values for

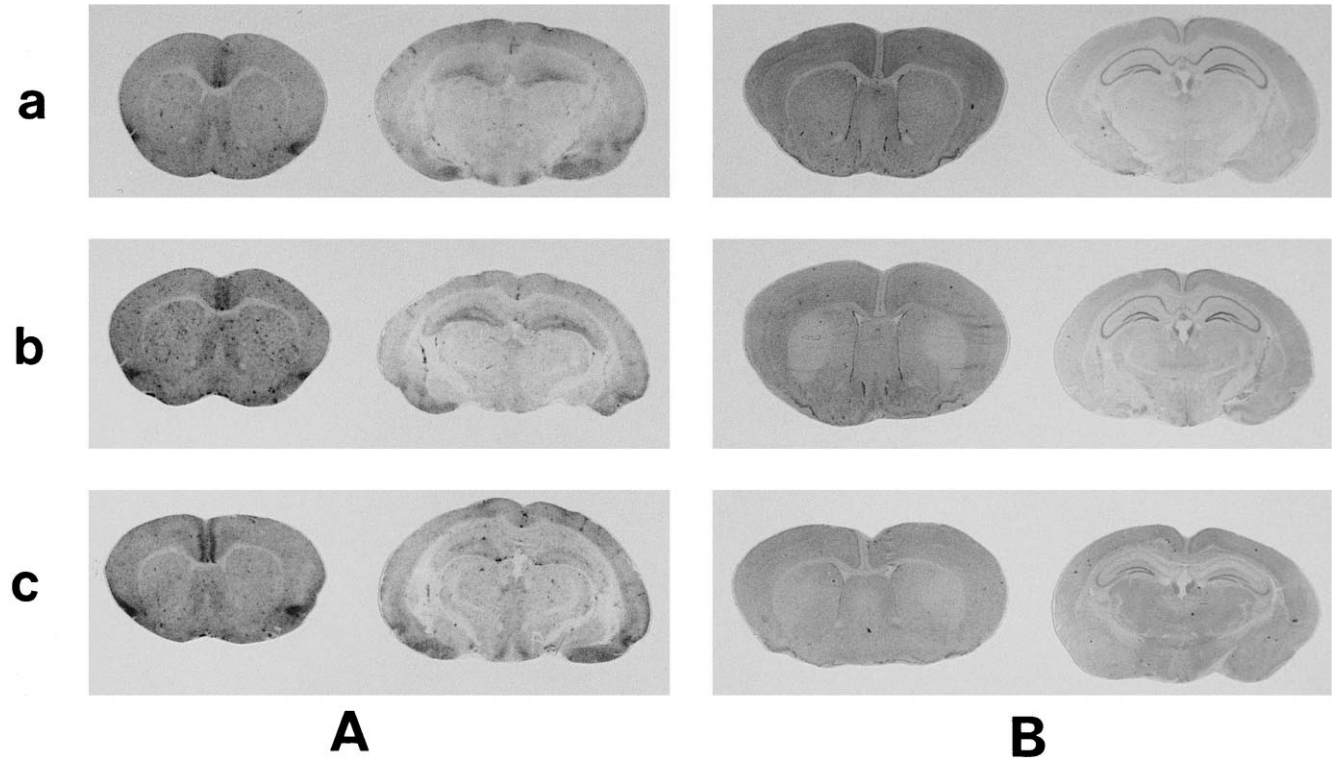

Fig. 1. Representative autoradiograms of [^{3}H]L-N^G-nitroarginine binding (A) and histological photographs with Cresyl violet staining (B) in the gerbil brain after transient global ischemia. Left column: striatum. Right column: hippocampus. (a) Sham-operated, (b) 48 h after ischemia, (c) 7 days after ischemia. [^{3}H]L-N^G-nitroarginine binding was evident in the striatum and hippocampus (A, a). A significant increase in [^{3}H]L-N^G-nitroarginine binding was noted in the striatum, hippocampal CA3 sector and dentate gyrus 48 h after ischemia (A, b). At 7 days after ischemia, [^{3}H]L-N^G-nitroarginine binding in most of regions returned to sham-operated levels (A, c). Histological analysis showed no neuronal damage throughout the brain of sham-operated gerbils (B, a). At 48 h after ischemia, ischemic neuronal damage was noted in the striatum, whereas the hippocampus showed no neuronal damage (B, b). At 7 days after ischemia, severe neuronal damage was observed in the striatum and hippocampus. However, the dentate gyrus was intact (B, c).

Table 2
Regional changes in [^{3}H]L-N^G-nitroarginine binding in the rat brain 3 days after focal ischemia in rats

	Sham-operated (5)	Ischemia (8)
Contralateral side		
Cerebral cortex		
FrPaM	14.8 ± 2.1	14.4 ± 1.5
FrPaSS	14.5 ± 1.8	13.3 ± 1.4
Striatum		
Lateral	13.8 ± 1.7	14.1 ± 2.1
Medial	13.4 ± 1.9	13.7 ± 1.6
Ipsilateral side		
Cerebral cortex		
FrPaM	15.8 ± 2.0	17.8 ± 3.4
FrPaSS	15.4 ± 1.9	17.2 ± 3.2
Striatum		
Lateral	15.1 ± 2.4	22.6 ± 6.6[a]
Medial	13.9 ± 1.9	21.1 ± 7.0[a]

Optical density was converted to fmol/mg tissue using [^{3}H]microscales. Values are expressed as means ± S.D.
Cerebral cortex (FrPaM); frontoparietal cortex, motor areas, supplied by anterior cerebral artery.
Cerebral cortex (FrPaSS); frontoparietal cortex, somatosensory areas, supplied by middle cerebral artery.
Striatum (lateral), dorsolateral part of the striatum.
Striatum (medial); ventromedial part of the striatum.
[a] $P < 0.05$ vs. sham-operated group (Student's *t*-test).
Figures in parentheses indicate the number of animals.

specific binding were expressed as the means ± S.D. Statistical significance was evaluated using an analysis of variance (one-way ANOVA) followed by a two-tailed Williams multiple comparison test or a two-tailed Student's *t*-test.

3. Results

3.1. Transient global ischemia

3.1.1. [^{3}H]L-N^G-nitroarginine autoradiography

In sham-operated gerbils, [^{3}H]L-N^G-nitroarginine binding was found in the striatum and hippocampus. In animals subjected to ischemia, [^{3}H]L-N^G-nitroarginine binding was unchanged in the striatum up to 24 h after recirculation. At 48 h after ischemia, the striatum showed a significant elevation (17–18%) in [^{3}H]L-N^G-nitroarginine binding. Thereafter, the striatum showed no significant change in [^{3}H]L-N^G-nitroarginine binding. In the hippocampus, no significant change in [^{3}H]L-N^G-nitroarginine binding was found in the hippocampal CA1 sector and hippocampal CA3 sector up to 5 h after ischemia. Thereafter, [^{3}H]L-N^G-nitroarginine binding increased in the hippocampal CA1 sector (24%) and hippocampal CA3 sector (33–40%) 24 or 48 h after ischemia. At 7 days after ischemia, the hippocampal CA1 sector and hippocampal CA3 sector returned to sham-operated levels. In the dentate gyrus, no significant change in [^{3}H]L-N^G-nitroarginine binding was observed 1 h after ischemia. Thereafter, the dentate gyrus showed a significant increase (30–32%) in [^{3}H]L-N^G-nitroarginine binding 5, 24 and 48 h after ischemia. At 7 days after ischemia, a significant decline (14%) in [^{3}H]L-N^G-nitroarginine binding was observed in this region. In contrast, [^{3}H]L-N^G-nitroarginine binding was unchanged in the thalamus during recirculation periods (Table 1 and Fig. 1).

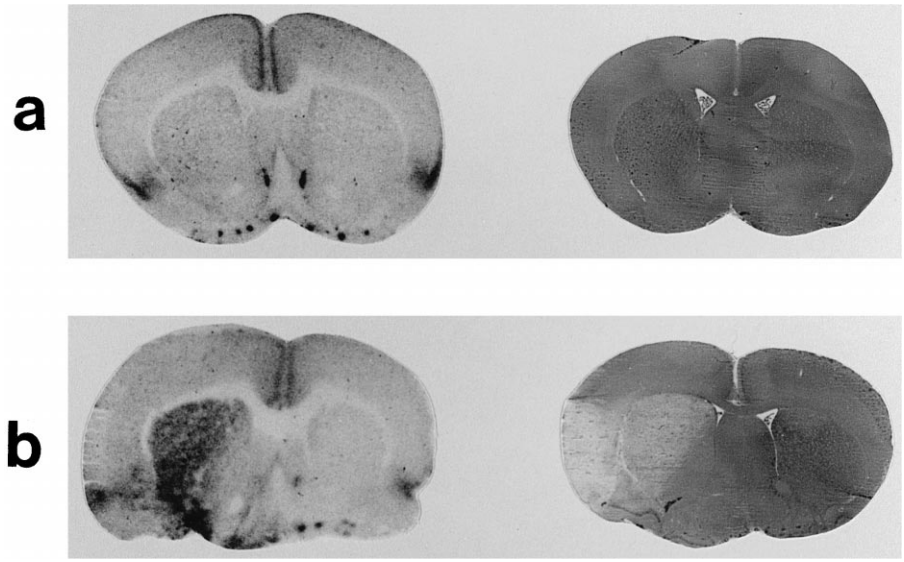

Fig. 2. Representative autoradiograms of [^{3}H]L-N^G-nitroarginine binding and histological photographs with Cresyl violet staining in the rat brain after transient focal ischemia. Left column: [^{3}H]L-N^G-niroarginine binding. Right column: Cresyl violet staining. (a) Sham-operated, (b) 3 days after focal ischemia. The cerebral cortex and striatum were morphologically intact. [^{3}H]L-N^G-niroarginine binding was evident in the cerebral cortex and striatum (a). At 3 days after focal ischemia, severe infarction was noted in the ipsilateral striatum and cerebral cortex and a significant increase in [^{3}H]L-N^G-niroarginine binding was observed in the ipsilateral striatum (b).

3.1.2. Histopathology

Sham-operated gerbils showed no neuronal damage throughout the brain. Gerbils subjected to ischemia also showed no neuronal damage in the brain 1–5 h after recirculation. After 24 and 48 h, severe neuronal damage was noted only in neurons of the dorsolateral striatum. At 7 days after ischemia, the neurons of the hippocampal CA1 sector and dorsolateral striatum were markedly affected. Mild neuronal damage was observed in the neurons of the hippocampal CA3 sector and thalamus. However, the neurons of ventromedial striatum and dentate gyrus were morphologically intact (Fig. 1).

3.2. Transient focal ischemia

3.2.1. [^{3}H]L-N^G-nitroarginine autoradiography

In sham-operated rats, [^{3}H]L-N^G-nitroarginine binding was observed in the cerebral cortex and striatum. In animals subjected to ischemia, [^{3}H]L-N^G-nitroarginine binding increased significantly (50–52%) in the ipsilateral striatum supplied by the occluded middle cerebral artery. In the ipsilateral cerebral cortex supplied by the occluded middle cerebral artery, however, [^{3}H]L-N^G-nitroarginine binding showed a mild but insignificant increase (12%). The ipsilateral cerebral cortex supplied by the anterior cerebral artery also showed a mild increase (13%). On the other hand, [^{3}H]L-N^G-nitroarginine binding was unchanged in both the contralateral cerebral cortex and striatum (Table 2 and Fig. 2).

3.2.2. Histopathology

Sham-operated rats showed no ischemic changes in the cerebral cortex and striatum. In animals subjected to focal ischemia, in contrast, ischemic damage was observed within the territory of the occluded middle cerebral artery, i.e., in the ipsilateral cerebral cortex and striatum. The ipsilateral cerebral cortex supplied by the anterior cerebral artery showed a mild ischemic change. In the contralateral cerebral cortex and striatum, no histological changes were observed (Fig. 2).

4. Discussion

It is well known that transient global ischemia causes degeneration of specific neuronal populations that lie in the hippocampus and striatum. In particular, most of the neurons in the hippocampal CA1 sector are destroyed after even brief global ischemia. However, glial cells are particularly resistant to transient global ischemia (Kirino, 1982; Pulsinelli et al., 1982). Animal models of focal cerebral ischemia with middle cerebral artery occlusion reproduce the pattern of brain damage observed in many human stroke patients. Several lines of evidence demonstrate that focal ischemia which causes nearly complete ischemia in the center of the vascular territory induces energy failure and lactic acidosis, and results in infarction of brain cells, including neurons, glial cells and capillary blood vessels with severe brain edema (Hakim, 1986; Abe et al., 1988; Nedergaard, 1988). We also reported that middle cerebral artery occlusion in rats can cause a severe reduction of local cerebral blood flow and protein synthesis and severe brain edema in the center of occluded middle cerebral artery territory (Araki et al., 1992a). Thus, in contrast to transient global ischemia, when selective neuronal damage occurs, focal ischemia causes extensive damage to the neurons, glial cells and capillary blood vessels.

Recently, much attention has been focused on the role of NO in the pathogenesis of ischemic neuronal damage. In cultured cortical neurons, NO has been reported to mediate in the neurotoxicity of *N*-methyl-D-aspartate (NMDA) (Dawson et al., 1991b), while, in vivo, inhibition of NO synthase decreases ischemic brain damage (Nowicki et al., 1991; Buisson et al., 1992). Based on these observations, it is suggested that NO is neurotoxic and mediates tissue injury in cerebral ischemia. However, results of other experimental studies suggest that NO may ameliorate ischemic brain damage (Yamamoto et al., 1992; Zhang and Iadecola, 1993). Thus, there are conflicting results regarding the possible role of NO or NO synthase in cerebral ischemia (Iadecola et al., 1994).

The present study showed that transient global ischemia in gerbils caused a significant elevation in [^{3}H]L-N^G-nitroarginine binding in the hippocampal CA1 and CA3 sectors at 24 and 48 h after recirculation. However, no significant changes in [^{3}H]L-N^G-nitroarginine binding were found in the hippocampal CA1 and CA3 sectors where severe neuronal damage was observed at 7 days after recirculation. In contrast, the dentate gyrus, which was resistant to global ischemia, showed a significant elevation in [^{3}H]L-N^G-nitroarginine binding at 5, 24 and 48 h after recirculation. Thereafter, the dentate gyrus showed a significant reduction in [^{3}H]L-N^G-nitroarginine binding at 7 days after global ischemia. On the other hand, the thalamus showed no significant changes in [^{3}H]L-N^G-nitroarginine binding throughout the recirculation periods. Therefore, these results demonstrate that the post-ischemic elevation in [^{3}H]L-N^G-nitroarginine binding precedes neuronal damage in the hippocampus after global ischemia. In contrast, the striatum showed no significant changes in [^{3}H]L-N^G-nitroarginine binding, even when ischemic neuronal damage was evident at 24 h after recirculation. At 48 h after global ischemia, however, a transient increase in [^{3}H]L-N^G-nitroarginine binding was seen in the striatum. Thereafter, no conspicuous changes in [^{3}H]L-N^G-nitroarginine binding were observed in the striatum. The present findings suggest that the post-ischemic increase in [^{3}H]L-N^G-nitroarginine binding does not precede the neuronal damage in the striatum after global ischemia. Transient focal ischemia in rats also caused a significant increase in [^{3}H]L-N^G-nitroarginine binding in the ipsilateral striatum where severe infarction was noted. Furthermore,

the ipsilateral cerebral cortex showed a tendency to an increase in [^{3}H]L-N^G-nitroarginine binding, although no significant change in the binding was observed in this region. These results suggest that transient focal ischemia as well as transient global ischemia can cause a post-ischemic elevation in [^{3}H]L-N^G-nitroarginine binding in the striatum. Furthermore, our findings suggest that the post-ischemic elevation in [^{3}H]L-N^G-nitroarginine binding may play a role in the pathogenesis of ischemic hippocampal damage. In the striatum, in contrast, the post-ischemic elevation in [^{3}H]L-N^G-nitroarginine binding may play some role in the restoration of injury areas after cerebral ischemia. Furthermore, the post-ischemic elevation in [^{3}H]L-N^G-nitroarginine binding may reflect a possible up-regulation of NO synthase.

Several experimental studies have demonstrated that NO synthase activity increases during and/or after cerebral ischemia (Kader et al., 1993; Kumura et al., 1994; Hara et al., 1997). In rats, NO synthase mRNA is known to increase after permanent focal ischemia (Zhang et al., 1994). In the same study, NO synthase immunoreactivity and NADPH-diaphorase-positive neurons increased in the ischemic areas following ischemia. Results of a recent study also suggested that the increase in NO synthase mRNA began at 12 h, reached a peak at 2 days and returned to the sham-operated levels after focal cerebral ischemia in rats (Iadecola et al., 1995). These observations were, at least in part, consistent with our present results. In addition, many NO synthase isoforms have been described in a variety of cell types (Förstermann et al., 1991). In the present study, however, we could not exactly determine the cell type in which the increase of [^{3}H]L-N^G-nitroarginine binding was observed in the striatum and hippocampus after cerebral ischemia Therefore, the precise mechanisms responsible for our findings should be investigated in further studies.

5. Conclusion

The present study demonstrated that [^{3}H]L-N^G-nitroarginine binding increases in the striatum and hippocampus after transient global ischemia or transient focal ischemia. This increase in [^{3}H]L-N^G-nitroarginine binding may play a pivotal role, not only in the pathogenesis of ischemic brain damage, but also in the restoration of injury areas after cerebral ischemia.

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