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Arylalkylamine *N*-acetyltransferase (AANAT) is expressed in astrocytes and melatonin treatment maintains AANAT in the gerbil hippocampus induced by transient cerebral ischemia

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ABSTRACT

Melatonin is synthesized from serotonin by the action of arylalkylamine *N*-acetyltransferase (AANAT) and hydroxyindole-*O*-methyltransferase. In this study, we observed cellular changes of arylalkylamine *N*-acetyltransferase (EC 2.3.1.87; AANAT) in the hippocampal CA1 region at various time points after ischemia/reperfusion. In vehicle-treated sham group, AANAT immunoreaction was detected in pyramidal neurons of the CA1 region. AANAT immunoreactivity in the neurons was highest 2 days and disappeared from 4 days after ischemia/reperfusion. From 3 days after ischemia/reperfusion, AANAT immunoreaction was expressed in astrocytes in the strata oriens and radiatum of the CA1 region. AANAT protein and mRNA levels were significantly increased 2 days after ischemia/reperfusion, and markedly decreased from 5 days after ischemia/reperfusion. The repeated administration of melatonin (10 mg/kg, *i.p.*) 3 times (once a day) to gerbils before ischemia/reperfusion significantly reduced ischemia-induced hyperactivity as well as neuronal death compared to those in the vehicle-treated ischemia group. Melatonin treatment also maintained AANAT immunoreactivity and its protein levels in the CA1 region after ischemia/reperfusion. These results suggest that the reduction of AANAT in ischemic CA1 region is associated with delayed neuronal death following transient ischemia, and melatonin treatment shows neuroprotection with maintenance of AANAT levels in the ischemic CA1 region.

1. Introduction

Deprivation of blood glucose and oxygen in the brain results in neuronal damage in specific vulnerable regions such as the hippocampal CA1 region, neocortex and striatum [1,2]. Transient cerebral ischemia (ischemia/reperfusion) promotes the generation of reactive oxygen species (ROS) as by-products of mitochondrial electron transport chain, and increases of ROS can result in cell dysfunction and death [3–7].

Duan et al. [8] showed that melatonin and 6-hydroxymelatonin could be used as supplements in the treatment of neurological disorders involving oxidative stress using N2a cells which were cultured *in vitro* and deprived of glucose, serum and oxygen. It has been reported that melatonin (*N*-acetyl-5-methoxytryptamine), an indoleamine originally found in the pineal gland, is considered as an antioxidant or a free radical scavenger [9,10], and promotes the synthesis of glutathione, which is an antioxidant [11]. Melatonin is synthesized with sequential enzymatic reaction from serotonin by the action of arylalkylamine *N*-acetyltransferase (EC 2.3.1.87; AANAT) and hydroxyindole-*O*-methyltransferase (EC 2.1.1.4; HIOMT) [12–14]. AANAT produces *N*-acetylserotonin (NAS) from serotonin and there is an evidence for the neuronal presence of NAS in the mammalian brain [15]. In addition, significant AANAT mRNA expression has been found in the nervous system of Drosophila [16] and AANAT enzymatic activity has been found in the rat brain [17–19]. AANAT is heterogeneously distributed, with higher levels found in the spinal cord, olfactory bulb, cerebellum and hippocampus in rats [18], and AANAT is closely related to hippocampal functions in rodents [20–22].

Melatonin is a hormone that controls circadian rhythms and seasonal behavioral changes in vertebrates [23]. Although, melatonin exhibits both direct and indirect antioxidant effects [24–28], it prevents neuronal death induced by transient cerebral ischemia in gerbils [29]. It was reported that melatonin reduced DNA damage and

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the infarct volume induced by transient cerebral arteries occlusion in pinealectomized rats [30] and had neuroprotective action in focal cerebral ischemia [31]. In addition, melatonin regulates neuronal plasticity in the brain including the hippocampus [32].

No studies have been reported on changes in AANAT in the hippocampal CA1 region after transient forebrain ischemia. In the present study, therefore, we investigated changes in AANAT immunoreactivity, protein and mRNA levels in the hippocampal CA1 region at various time points after ischemia/reperfusion in gerbils. In addition, we also observed the effects of melatonin on ischemic damage and AANAT levels.

2. Materials and methods

2.1. Experimental animals

The progeny of male Mongolian gerbils (*Meriones unguiculatus*) were obtained from the Experimental Animal Center, Hallym University, Chuncheon, South Korea. Gerbils were used at 6 months (B.W., 65–75 g) of age. The animals were housed in a conventional state under adequate temperature (23 °C) and humidity (60%) control with a 12-h light/12-h dark cycle, and were provided with free access to food and water. The procedures for animal handling and care adhered to guidelines that are in compliance with the current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996), and they were approved by the Institutional Animal Care and Use Committee (IACUC) at Hallym's Medical Center. All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

2.2. Treatment with melatonin

To elucidate the protective effects of melatonin against ischemic damage, the animals were divided into 4 groups; 1) vehicle (2% ethanol)-treated sham group, 2) 10 mg/kg melatonin (Sigma)-treated sham group, 3) vehicle-treated ischemia group, and 4) melatonin-treated ischemia group. The vehicle or melatonin was intraperitoneally treated to animals 3 times for 3 days before ischemia/reperfusion: The 3rd treatment was at 30 min before ischemia/reperfusion.

2.3. Induction of transient cerebral ischemia

The animals were anesthetized with a mixture of 2.5% isoflurane (Baxtor, Deerfield, IL) in 33% oxygen and 67% nitrous oxide. Bilateral common carotid arteries were isolated and occluded using non-traumatic aneurysm clips. The complete interruption of blood flow was confirmed by observing the central artery in retinae using an ophthalmoscope. After 5 min of occlusion, the aneurysm clips were removed from the common carotid arteries. The body (rectal) temperature under free-regulating or normothermic (37 ± 0.5 °C) conditions was monitored with a rectal temperature probe (TR-100; Fine Science Tools, Foster City, CA) and maintained using a thermometric blanket before, during and after the surgery until the animals completely recovered from anesthesia. Thereafter, animals were kept on the thermal incubator (Mirae Medical Industry, Seoul, South Korea) to maintain the body temperature of animals until the animals were euthanized. Sham-operated animals were subjected to the same surgical procedures except that the common carotid arteries were not occluded.

2.4. Spontaneous motor activity

To elucidate the effect of melatonin against ischemia-induced hyperactivity, spontaneous motor activity was measured according to previous study [33,34]. For spontaneous motor activity, gerbils ($n = 7$ in each group) were individually placed in a Plexiglas cage (25 cm × 20 cm × 12 cm), located inside a soundproof chamber. Locomotor activity was also recorded with Photobeam Activity System-Home Cage (San Diego Instruments). Spontaneous motor activity was monitored during 24 h and, simultaneously, the number of times each animal reared and the time (in seconds) spent in grooming behavior were recorded. Each animal was observed continuously via a 4 × 8 photobeam. Scores were generated from live observations, and video sequences were used for subsequent re-analysis.

2.5. Tissue processing for histology

For the histological analysis, animals were anesthetized with sodium pentobarbital and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brains were removed and postfixed in the same fixative for 6 h. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter frozen tissues were serially sectioned on a cryostat (Leica, Wetzlar, Germany) into 30-μm coronal sections, and they were then collected into six-well plates containing PBS.

2.6. Immunohistochemistry for NeuN, AANAT, GFAP and Iba-1

To obtain the accurate data, the sections from 4 groups ($n = 7$ at each time point in each group) were used at designated times (30 min, 3, 6, 12 h, 1, 2, 3, 4, 5, 7 and 10 days) under the same conditions. The sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min and 10% normal goat serum in 0.05 M PBS for 30 min. They were then incubated with diluted mouse anti-Neuronal nuclei (NeuN, 1:100, Chemicon International, Temecula, CA) to detect the neurons, diluted rabbit anti-AANAT antibody (1:250, Abcam, Cambridge, UK), diluted rabbit anti-glial fibrillary acidic protein (GFAP, 1:1000, Chemicon International) to detect the astrocytes and diluted rabbit anti-ionized calcium-binding adapter molecule 1 (Iba-1) (diluted 1:500, Wako, Osaka, Japan) overnight at 4 °C and subsequently exposed to biotinylated goat anti-rabbit IgG and streptavidin peroxidase complex (diluted 1:200, Vector, Burlingame, CA). They were then visualized by reacting to 3,3′-diaminobenzidine tetrachloride (Sigma) in 0.1 M Tris–HCl buffer (pH 7.2) and mounted on gelatin-coated slides.

2.7. Double immunofluorescence for AANAT/GFAP or AANAT/OX-42

To confirm the glial type containing AANAT immunoreactivity, the sections 5 and 7 days after ischemic surgery were processed by double immunofluorescence staining. Double immunofluorescence staining was performed using rabbit anti-AANAT (1:50)/mouse anti-glial fibrillary acidic protein (GFAP) (1:200, Chemicon International, Temecula, CA) for astrocytes or mouse anti-OX-42 (1:25, AbD Serotec, Oxford, UK) for microglia. The sections were incubated in the mixture of antisera overnight at 4 °C. After washing 3 times for 10 min with PBS, they were

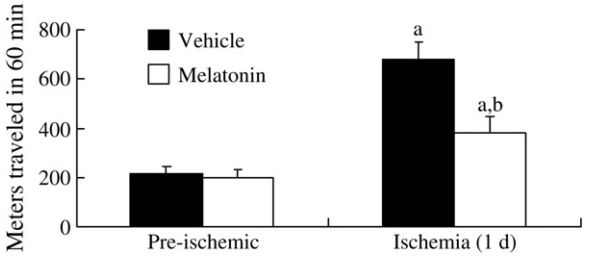

Fig. 1. Change in locomotor activity in vehicle- and melatonin-treated groups before and 1 day after ischemia/reperfusion. The spontaneous locomotor activity is evaluated in terms of entire distance (meters) traveled after ischemia/reperfusion ($n = 7$ per group; $^{a}P < 0.05$, significantly different from the pre-ischemic state, $^{b}P < 0.05$, significantly different from the vehicle-treated ischemia group). The bars indicate the means ± SEM.

then incubated in a mixture of both Cy3-conjugated goat anti-rabbit IgG (1:200; Jackson ImmunoResearch, West Grove, PA) and FITC-conjugated goat anti-mouse IgG (1:600; Jackson ImmunoResearch) for 2 h at room temperature and mounted with DAPI-contained medium (Vector). The immunoreactions were observed under the AxioM1 microscope attached HBO100, which is the microscope lamp power supply for fluorescence (Carl Zeiss).

2.8. Western blot analysis for AANAT

To obtain the accurate data for change in AANAT levels in the hippocampus after transient cerebral ischemia, animals of 4 groups ($n=5$ at each time point in each group) were used for western blot analysis at designated times (3, 6, 12 h, 2, 5 and 7 days) after the ischemic surgery. After sacrificing them and removing the brain, it was serially and

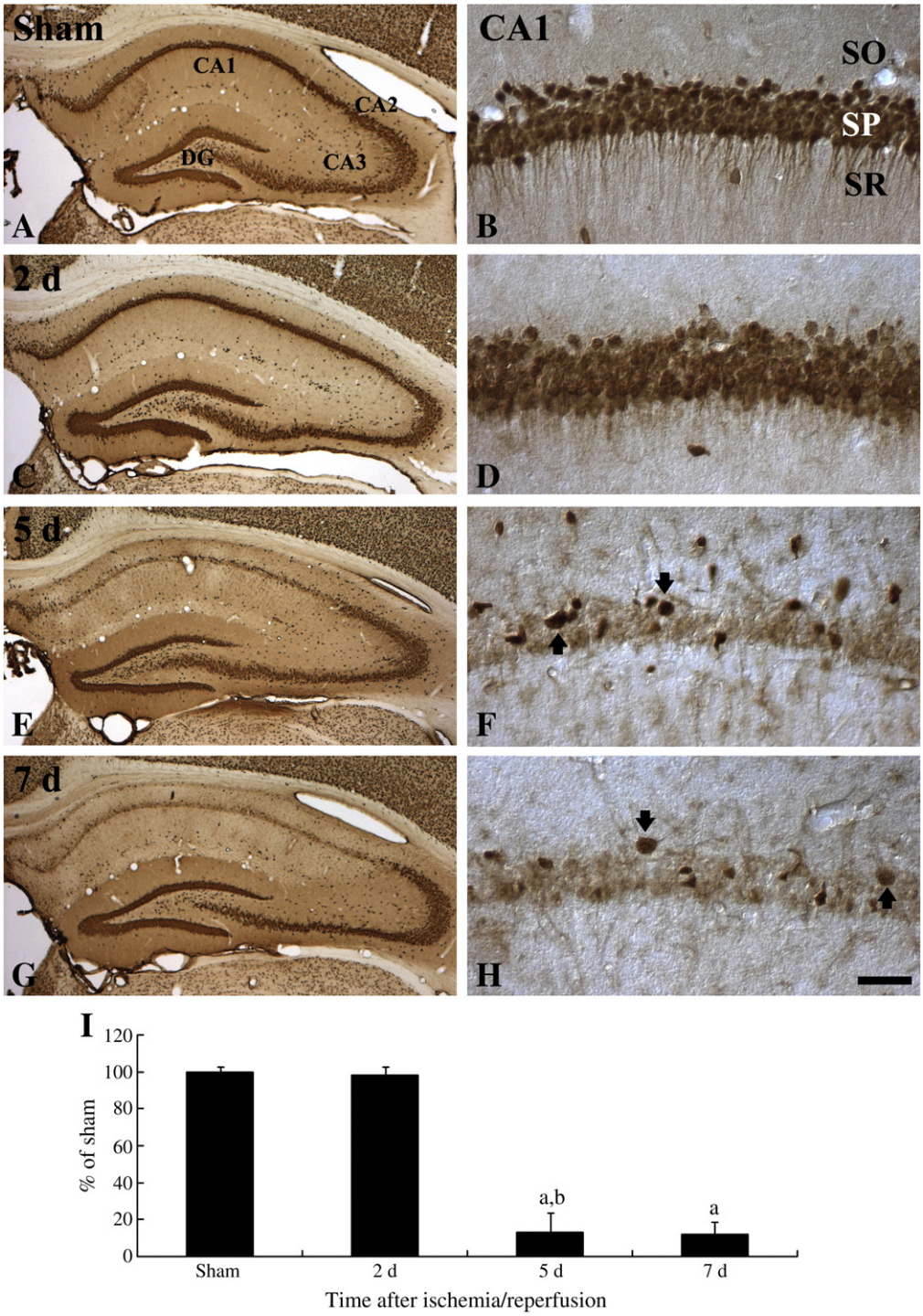

Fig. 2. NeuN immunohistochemistry in the CA1 region in sham- (A and B) and ischemia-operated (C–H) groups at 2 (C and D), 5 (E and F) and 7 (G and H) days post-ischemia. Five to 7 days after I/R, a few NeuN-immunoreactive pyramidal cells are detected; NeuN immunoreaction is expressed in non-pyramidal cells (arrows). SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. Scale bar = 800 μm (A, C, E and G), 50 μm (B, D, F and H). I: Relative analysis in the number of $NeuN^+$ neurons 2, 4 and 7 days after I/R ($^aP<0.05$, significantly different from the sham groups, $^bP<0.05$, significantly different from the pre-adjacent group). The bars indicate the means ± SEM.

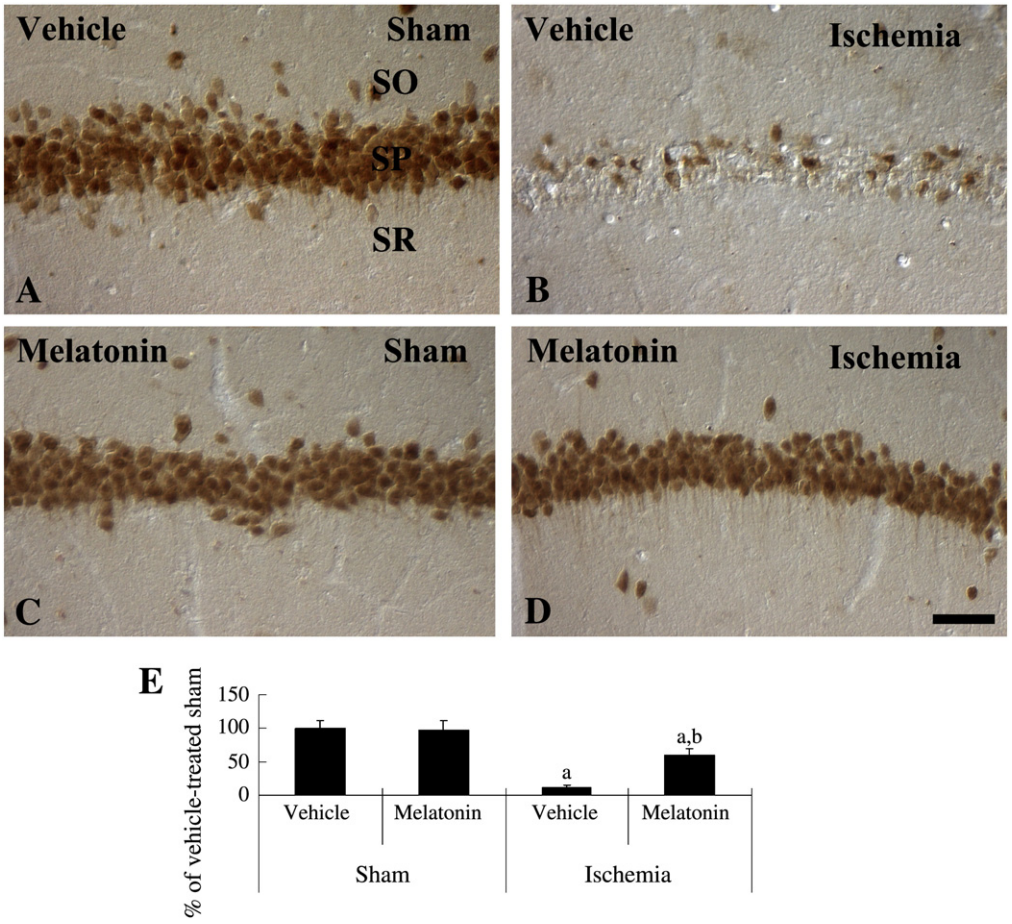

Fig. 3. NeuN immunohistochemistry in the CA1 region 5 days after I/R in vehicle-treated sham (A), vehicle-treated ischemia (B) and melatonin-treated sham (C), melatonin-treated ischemia (D) groups. In the vehicle-treated ischemia group, a few neurons are stained with NeuN. In the melatonin-treated sham group, $NeuN^+$ neurons are similar to those in the vehicle-treated sham group. In the melatonin-treated ischemia group, $NeuN^+$ neurons are abundant in the CA1 region. SO; stratum oriens, SP; stratum pyramidale, SR; stratum radiatum. Scale bar = 50 μm. E: Relative numeric analysis of $NeuN^+$ pyramidal neurons in the vehicle-treated sham, 10 mg/kg melatonin-treated sham, vehicle-treated ischemia and melatonin-treated ischemia groups 5 days after I/R ($n = 7$ per group; $^aP<0.05$, significantly different from the vehicle-treated sham group, $^bP<0.05$, significantly different from the vehicle-treated ischemia group). The bars indicate the means ± SEM.

transversely cut into a thickness of 400 μm on a vibratome (Leica), and the hippocampal CA1 region was then dissected with a surgical blade. The tissues were homogenized in 50 mM PBS (pH 7.4) containing 0.1 mM ethylene glycol bis (2-aminoethyl ether)-N,N,N′,N′ tetraacetic acid (EGTA) (pH 8.0), 0.2% Nonidet P-40, 10 mM ethylendiamine tetraacetic acid (EDTA) (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). After centrifugation, the protein level in the supernatants was determined using a Micro BCA protein assay kit with bovine serum albumin as a standard (Pierce Chemical, Rockford, IL). Aliquots containing 50 μg of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots were then loaded onto a 5% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose membranes (Pall Crop, East Hills, NY). To reduce background staining, the membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with rabbit anti-AANAT antiserum (1:250), peroxidase-conjugated goat anti-rabbit IgG (Sigma) and an ECL kit (Pierce Chemical).

2.9. AANAT mRNA analysis by reverse transcriptase-PCR (RT-PCR)

To examine changes in AANAT mRNA levels in the hippocampus after transient cerebral ischemia, at designated times (3, 12 h, 1, 2, 4, 5 and 7 days) after the surgery, animals of 4 groups ($n = 5$ at each time point in each group) were used for the reverse-transcription polymerase chain reaction (RT-PCR) study. Total RNA was extracted from gerbil hippocampal CA1 region with Trizol reagent (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's protocol. The nucleic acid concentration was determined spectrophotometrically and then, RNA samples were stored at −70 °C until being used.

Before analysis of the RNA samples by RT-PCR, DNA contamination of the RNA samples was removed by treatment with DNase I at 37 °C for 30 min with (GIBCO-BRL) in the presence of RNasin. The first-strand cDNA synthesized in a 20 μl reaction which contained 5 μg total RNA, 20 units RNasin (Promega, Madison, WI), 1 mM dNTP (GIBCO-BRL), 15 units avian myeloblastosis (AMV) reverse transcriptase (Promega), oligo dT 17 as primers, 5 mM $MgCl_2$ (GIBCO-BRL). The oligonucleotide primers were as follows: AANAT (accession number : AF004110AF004110), 5′-TCCTGTGGAGATACCTTCACCA-′3 (forward) and 5′-CACAGTTCAGAAGGCAAGAGGT-3′ (reverse); β-actin, 5′-GACCT-GACTGACTACCTCAT-3′ (forward) and 5′-TCGTCATACTCCTGCTTGCT-3′ (reverse). Multitarget PCRs were performed by coamplifying β-actin as the internal standard. The reaction mixture for NBC contained 2 μl of the RT reaction product, and the reaction was carried out for 30 cycles, using a 94 °C, 30-s denaturing step; a 57 °C, 30-s annealing step; and a 72 °C, 3-min extension step. The experiment was repeated multiple times, with the relative difference in RNA quantity being reproducibly observed over three experiments.

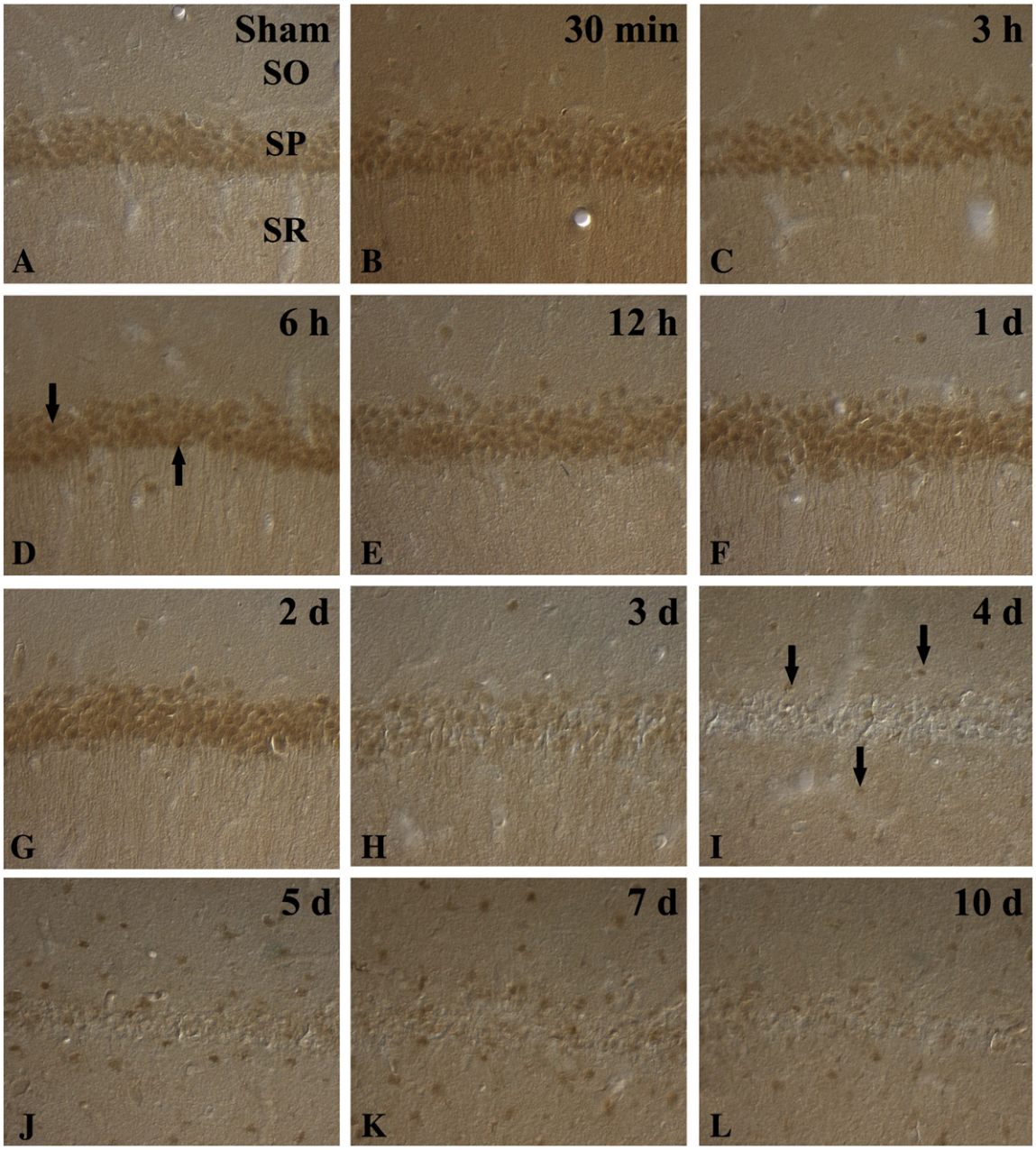

Fig. 4. AANAT immunohistochemistry in the CA1 region in sham- (A) and ischemia-operated (B–L) groups. AANAT immunoreactivity is increased in the stratum pyramidale (SP) 6 h (arrows) and 2 days after I/R, and is decreased from 3 days after I/R. Four to 10 days after I/R, AANAT immunoreaction is expressed in non-pyramidal cells (arrows) in the strata oriens (SO) and radiatum (SR). Scale bar = 50 μm.

2.10. Data analysis

All measurements were performed in order to ensure objectivity in blind conditions, by two observers for each experiment, carrying out the measures of control and experimental samples at the same moment in the same day.

The studied tissue sections were selected according to anatomical landmarks corresponding to AP −1.4 to −2.0 mm of the gerbil brain atlas [35]. To evaluate the neuroprotective effects of melatonin against ischemic damage, NeuN-immunoreactive neurons were counted at the center of the CA1 region using an image analyzing system equipped with a computer-based CCD camera (software: Optimas 6.5, CyberMetrics, Scottsdale, AZ). Ten sections were selected with 30-μm interval, and cell counts were obtained by averaging the counts from each animal: A ratio of the count was calibrated as %.

In order to quantitatively analyze AANAT immunoreactivity, semi-quantification of the immunostaining intensities was evaluated with digital image analysis software (MetaMorph 4.01, Universal Imaging

Table 1
The time-course levels of AANAT immunoreactivity in cells in the gerbil hippocampal CA1 region following transient ischemia.

Cell type	Time after ischemia/reperfusion											
	Sham	30 min	3 h	6 h	12 h	1 d	2 d	3 d	4 d	5 d	7 d	10 d
Pyramidal neurons	+	+	+	+	+	+	+	±	±	−	−	−
				+			+					
Glial cells	−	−	−	−	−	−	−	−	+	+	+	+
										+	+	

The levels of immunoreactivity were defined as four grades, negative (−), weakly positive (±), moderate (+) and strong (++).

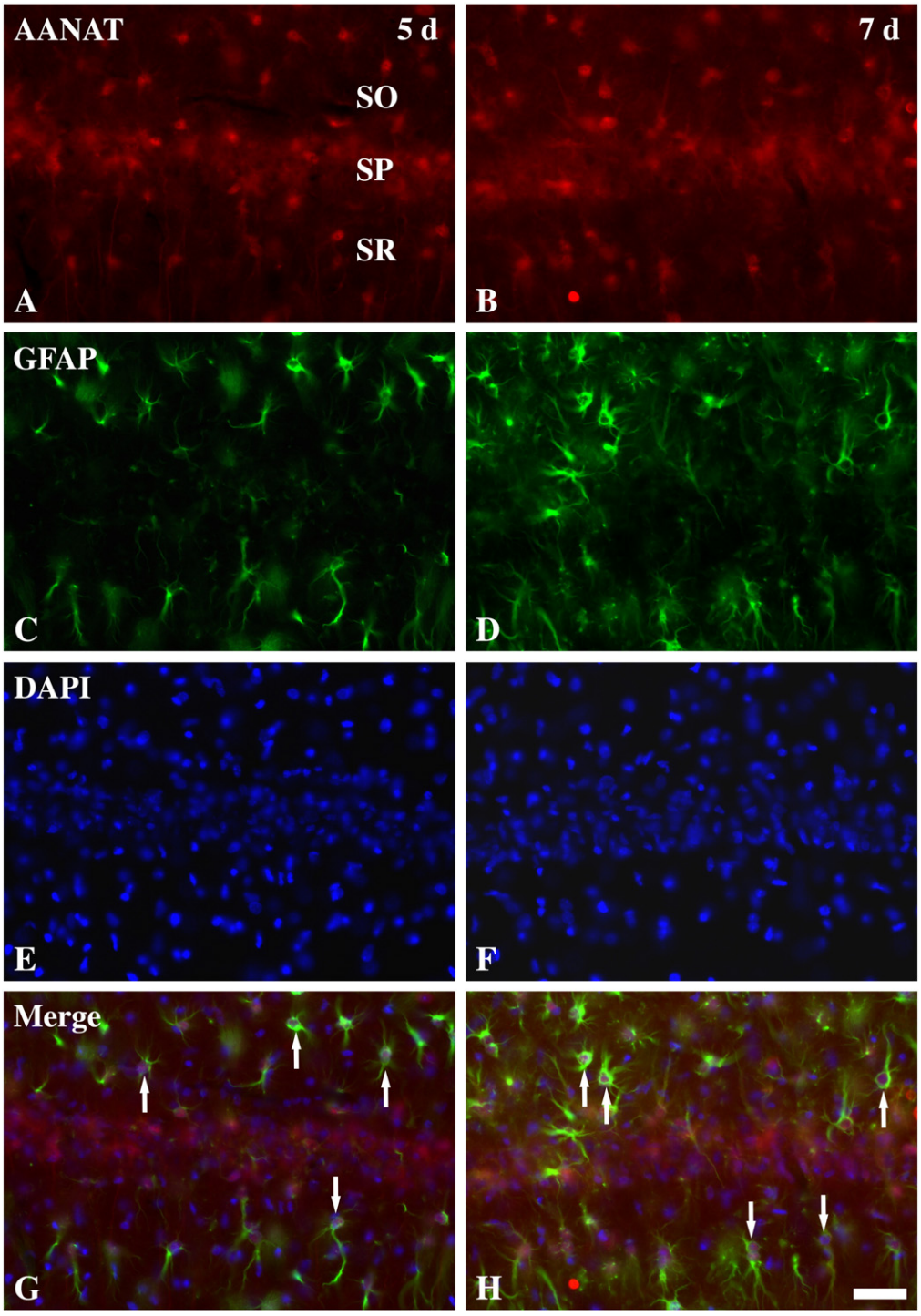

Fig. 5. Double immunofluorescence staining for AANAT (red, A and B), GFAP (green, C and D), DAPI (blue, E and F) and merged images (G and H) in the hippocampal CA1 region 5 days (A, C, E and G) and 7 days (B, D, F and H) after I/R. AANAT immunoreaction is colabeled within GFAP immunoreactive astrocytes (arrows) which are distributed mainly in the strata oriens (SO) and radiatum (SR) of the CA1 region. SP, stratum pyramidale. Bar = 50 μm.

Corp.). The mean intensity of immunostaining in each immunoreactive structures was measured by a 0–255 gray scale system (white to dark signal corresponded from 255 to 0). Based on this approach, the level of immunoreactivity was scaled as −, ±, + or ++, representing no staining (gray scale value: ≥200), weakly positive (gray scale value: 150–199), moderate (gray scale value: 100–149), or strong (gray scale value: ≤99), respectively.

The results of western blot and RT-PCR analyses were scanned, and the quantification of the analyses was done using Scion Image software (Scion Corp., Frederick, MD), which was used to count relative optical density (ROD): A ratio of the ROD was calibrated as%.

2.11. Statistical analysis

The data shown here represent the means ± SEM. Differences among the means were statistically analyzed by Student *t*-test in order to elucidate the neuroprotective effects of the vehicle- and melatonin-treated groups against ischemic damage. In addition, differences of the

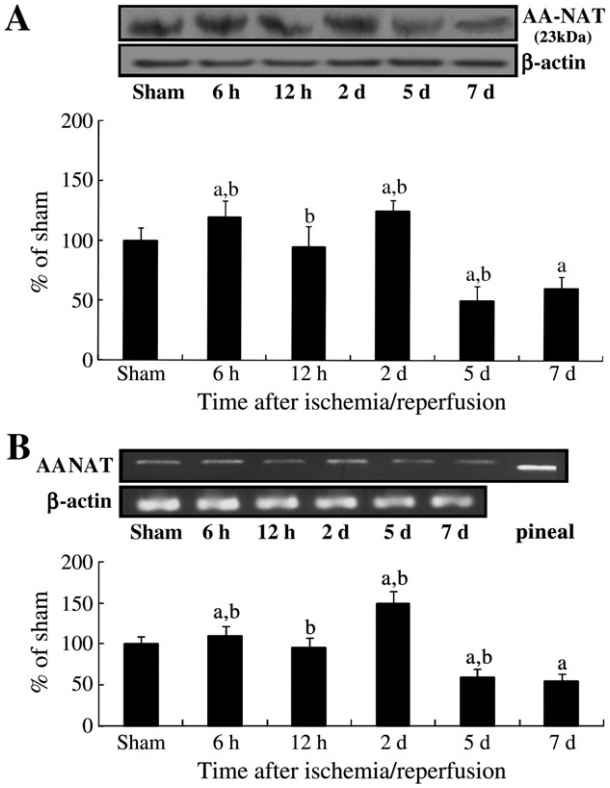

Fig. 6. Western blot (A) and RT-PCR (B) analysis of AANAT in the hippocampus derived from sham- and ischemia-operated groups. The pineal gland is used for positive control in RT-PCR analysis. Relative optical density as% values of immunoblot band is also represented ($n = 10$ per group; $^{a}P<0.05$, significantly different from the sham-operated group, $^{b}P<0.05$, significantly different from the pre-adjacent group). The bars indicate the means ± SEM.

mean ROD among the groups were statistically analyzed by analysis of variance (ANOVA) followed by Tukey's multiple range method in order to elucidate ischemia-related differences among experimental groups. Statistical significance was considered at $P<0.05$.

3. Results

3.1. Spontaneous motor activity

To elucidate the effect of melatonin on ischemia-induced hyperactivity in gerbils, locomotor activity was checked before and 1 day after ischemia/reperfusion (Fig. 1). In the vehicle-treated ischemia group, the locomotor activity was significantly increased 1 day after ischemia/reperfusion by 3.2 folds compared to that in the pre-ischemic state. In the melatonin-treated sham group, locomotor activity in pre-ischemic state was similar to that in the vehicle-treated sham group. One day after ischemia/reperfusion, locomotor activity was significantly increased by 1.9 folds compared to that in the pre-ischemic state. However, the locomotor activity was significantly lower in the melatonin-treated ischemia group compared to that in the vehicle-treated ischemia group 1 day after ischemia/reperfusion.

3.2. Delayed neuronal death

In this study, we examined delayed neuronal death in the hippocampal CA1 region using NeuN immunohistochemistry (Fig. 2). In the vehicle-treated sham group, NeuN-immunoreactive ($^{+}$) neurons were abundant in the CA1 region (Fig. 2A). There were no significant changes in NeuN^{+} neurons in the CA1 region by 2 days after ischemia/reperfusion (Fig. 2C and D). Five days after ischemia/reperfusion, NeuN^{+} neurons were significantly decreased in the CA1 region (Fig. 2E, F and I). Delayed neuronal death occurred in pyramidal neurons of the CA1 region, not in CA2/3 region: Seven days after ischemia/reperfusion, delayed neuronal death was found only in the CA1 region (Fig. 2G, H and I).

3.3. Effects of melatonin on neuronal survival

In both the vehicle- and melatonin-treated sham groups, NeuN^{+} neurons were abundantly detected in the CA1 region (Fig. 3A and C). The number of NeuN^{+} neurons was similar between two groups (Fig. 3E). In the vehicle-treated ischemia group 5 days after ischemic/reperfusion, a few NeuN positive neurons were found: Only 11.2% of neurons were detected compared to the vehicle-treated sham group (Fig. 3B and E). In the melatonin-treated ischemia group, NeuN^{+} neurons were abundant in the CA1 region 5 days after ischemia/reperfusion (Fig. 3D). In this group, the number of NeuN^{+} neurons was 59.9% compared to the vehicle-treated sham group (Fig. 3E).

3.4. Change in AANAT immunoreactivity

In the vehicle-treated sham group, AANAT immunoreaction was detected in somata of pyramidal neurons and their dendrites in the CA1 region (Fig. 4A). AANAT immunoreactivity was changed after ischemia/reperfusion (Table 1, Fig. 4B–L). AANAT immunoreactivity in pyramidal neurons was increased 6 h and 2 days after ischemia/reperfusion (Table 1, Fig. 4D and G). Three and 4 days after ischemia/reperfusion, AANAT immunoreactivity in pyramidal neurons was markedly decreased (Table 1, Fig. 4H and I). Thereafter AANAT immunoreaction in pyramidal neurons was not detected (Table 1, Fig. 4J–L). On the other hand, AANAT immunoreaction was expressed in glial cells from 4 days post-ischemia and AANAT immunoreaction in glial cells was increased in the strata oriens and radiatum with time after ischemia/reperfusion (Table 1, Fig. 4I–L).

3.5. Colocalization of AANAT/GFAP

From 4 days after ischemia/reperfusion, AANAT immunoreaction was detected in non-pyramidal cells in the strata radiatum and oriens of the CA1 region. We performed double immunofluorescence staining for AANAT/GFAP or AANAT/OX-42 in the CA1 region 5 and 7 days after ischemia/reperfusion to identify glial type. Many AANAT^{+} cells were colocalized with GFAP^{+} astrocytes (Fig. 5). However, in this study, AANAT immunoreaction was not found in OX-42^{+} microglia (data not shown).

3.6. Changes in AANAT protein and mRNA levels

We found that AANAT protein levels in the CA1 region were changed after ischemia/reperfusion (Fig. 6A). AANAT protein levels were increased 6 h and 2 days after ischemia/reperfusion. From 5 days after ischemia/reperfusion, AANAT protein levels were significantly decreased.

We also found that change pattern in total AANAT mRNA levels was similar to that in AANAT protein levels in the hippocampus after ischemia/reperfusion (Fig. 6B). AANAT mRNA level was highest 2 days after ischemia/reperfusion. Thereafter, AANAT mRNA levels in the ischemic CA1 region were decreased with time after ischemia/reperfusion.

3.7. Effects of melatonin on AANAT immunoreactivity and its protein levels

In the vehicle-treated sham group, AANAT immunoreaction was observed in pyramidal neurons and their dendrites in the CA1 region (Fig. 7A). Five days after ischemia/reperfusion of the vehicle-treated ischemia group, a few AANAT^{+} glial cells were detected in the CA1 region (Fig. 7B). In this group, AANAT protein level was much lower

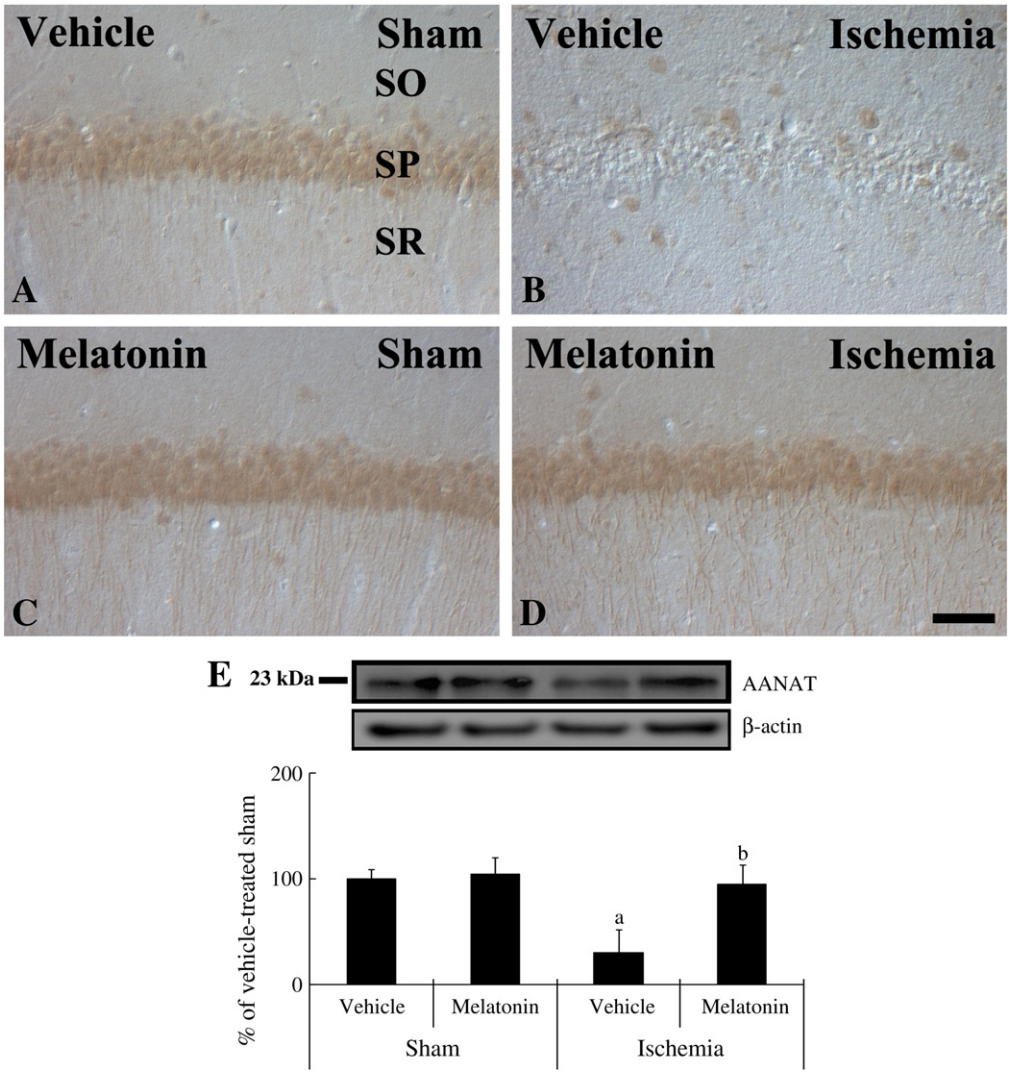

Fig. 7. A–D: AANAT immunohistochemistry in the CA1 region 5 days after I/R in vehicle-treated sham (A), vehicle-treated ischemia (B), melatonin-treated sham (C) and melatonin-treated ischemia (D) groups. In the vehicle-treated ischemia group, AANAT immunoreactivity is observed in a few glial cells in the ischemic CA1 region. However, in the melatonin-treated sham and melatonin-treated sham groups, AANAT immunoreactivity in the stratum pyramidale (SP) is similar to that in the vehicle-treated ischemia group. SO; stratum oriens, SP; stratum pyramidale, SR; stratum radiatum. Scale bar = 50 μm. E: Western blot analysis of AANAT in the hippocampus derived from vehicle-treated sham, melatonin-treated sham, vehicle-treated ischemia and melatonin-treated ischemia groups. Relative optical density as % values of immunoblot band is also represented ($n = 5$ per group; $^{a}P<0.05$, significantly different from the vehicle-treated sham group, $^{b}P<0.05$, significantly different from the vehicle-treated ischemia group). The bars indicate the means ± SEM.

than that in the vehicle-treated sham group (Fig. 7E). In both the melatonin-treated sham and ischemia groups, AANAT immunoreactivity was similar to the vehicle-treated sham group (Fig. 7C and D). In these groups, AANAT protein levels were also similar to the vehicle-treated sham group (Fig. 7E).

3.8. Effects of melatonin on glial activation

In the vehicle-treated sham group, GFAP^{+} astrocytes with thread-like processes and small cytoplasm were detected in the CA1 region (Fig. 8A). In the vehicle-treated ischemia group, the cytoplasm of GFAP^{+} astrocytes was hypertrophied and their processes were thickened (Fig. 8B). In the melatonin-treated sham group, many GFAP^{+} astrocytes showed the hypertrophy of cytoplasm and thickened processes, showing that many astrocytes were activated (Fig. 8B). In the melatonin-treated sham group, GFAP^{+} astrocytes were similar to those in the vehicle-treated sham group (Fig. 8C). In the melatonin-treated ischemia group, a few GFAP^{+} astrocytes were activated (Fig. 8D).

In the vehicle-treated sham group, Iba-1^{+} microglia had small cytoplasm (Fig. 8E). In the vehicle-treated ischemia group, many Iba-1^{+} microglia were located in the stratum pyramidale with dense and round cytoplasm: Overall Iba-1^{+} microglia showed the hypertrophy of cytoplasm and processes (Fig. 8F). In the melatonin-treated sham group, Iba-1 immunoreactivity in microglia was lower than that in the vehicle-treated sham group (Fig. 8G). In the melatonin-treated ischemia group, some Iba-1^{+} microglia had hypertrophied cytoplasm (Fig. 8H).

4. Discussion

In the present study, we examined neuronal damage in CA1 pyramidal neurons in the gerbil hippocampus using NeuN immunohistochemistry staining: The CA1 pyramidal neurons showed neuronal death 4 days post-ischemia. This result is coincident with many studies on gerbils [36–38].

We observed the neuroprotective effects of melatonin against ischemic damage. The administration of melatonin for 3 days significantly reduced the ischemia-related hyperactivity 1 day after ischemia/reperfusion. Spontaneous motor activity is a good method for evaluating the neuroprotection against ischemic damage in a gerbil ischemic model [39,40]. The melatonin also reduced the ischemia-related neuronal

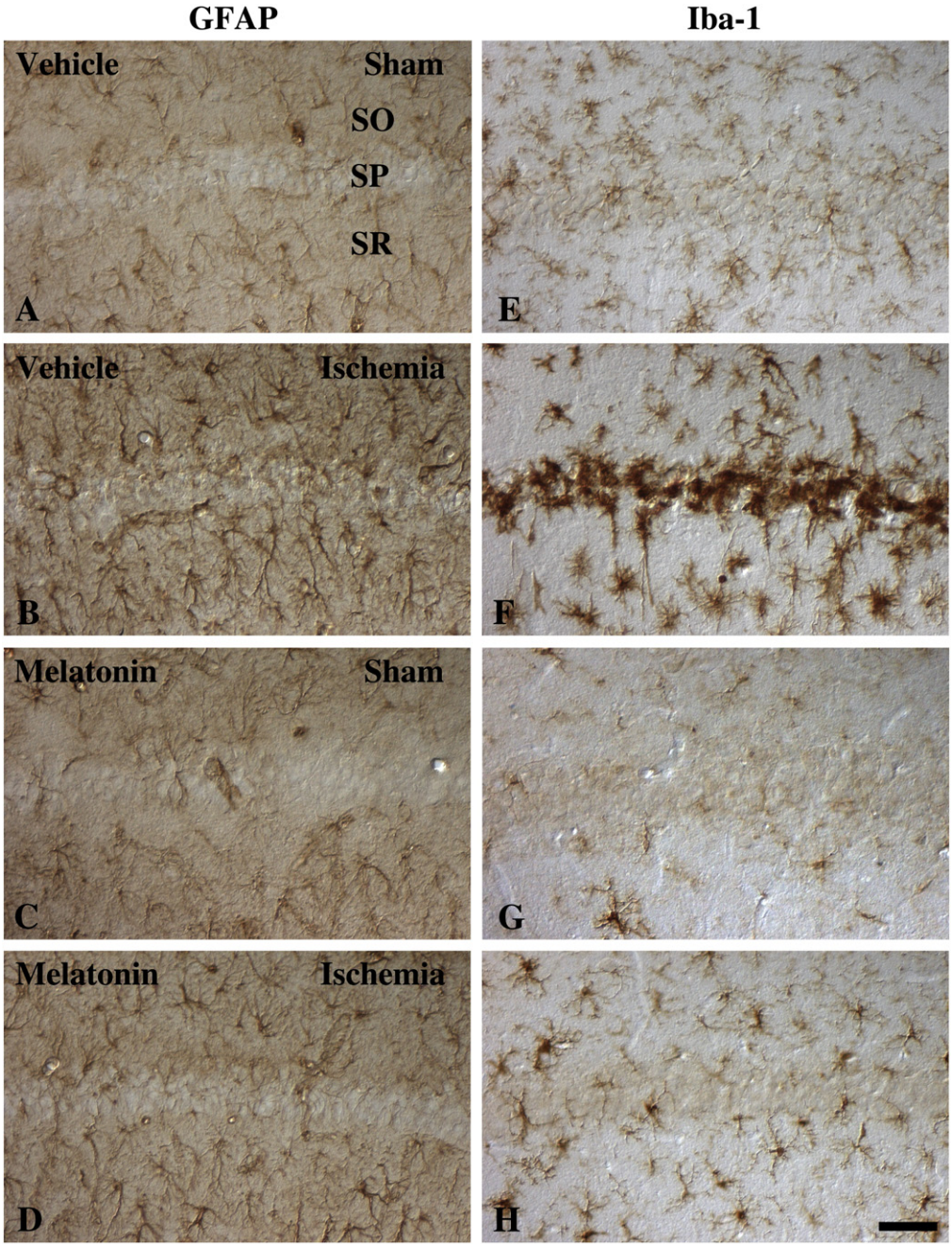

Fig. 8. GFAP and Iba-1 immunohistochemistry in the CA1 region 5 days after I/R in vehicle-treated sham (A and E), vehicle-treated ischemia (B and F), melatonin-treated sham (C and G) and melatonin-treated ischemia (D and H) groups. The cytoplasm of GFAP and Iba-1$^+$ structures in the vehicle-treated ischemia group is hypertrophied and the processes are also thickened 5 days after I/R. In the melatonin-treated ischemia group, many GFAP and Iba-1$^+$ structures are similar to those in the vehicle-treated sham. SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. Scale bar = 50 μm.

death in the hippocampus 5 days after ischemia/reperfusion. This result was supported by previous studies that melatonin protects brain damage from the ischemic damage in rats [30,31,41–44] and gerbils [29].

ROS are involved in pathophysiology of ischemia/reperfusion injury in several organs. It is known that melatonin is synthesized with sequential enzymatic reaction from serotonin by the action of AANAT and HIOMT [12–14]. CNS AANAT was heterogeneously distributed, with higher levels found in the spinal cord, olfactory bulb, cerebellum and hippocampus of the rat [18], and that AANAT was closely related to the hippocampal functions in rodents [20–22].

AANAT expression in the hippocampus is not related with circadian rhythm [22,45]. It has been reported that the content of hippocampal AANAT mRNA is not affected by the circadian rhythm [22] although a greater than 150-fold nocturnal increase in AANAT mRNA content has been described in the rat pineal gland [45].

In this study, we observed changes in AANAT immunoreactivity, protein and mRNA levels in the CA1 region at various time points after ischemia/reperfusion. AANAT immunoreactivity and protein level in CA1 pyramidal neurons were significantly increased 2 days post-ischemia and very low from 5 days after ischemia/reperfusion. The change pattern of AANAT mRNA level was similar to AANAT immunoreactivity and its protein level after ischemia/reperfusion. These changes in AANAT expression in the CA1 pyramidal neurons means that AANAT can be involved in neuronal damage following an ischemic insult. Because AANAT produces NAS from serotonin [15],

serotonin may be related with the delayed neuronal death in the ischemic CA1 region. This result is supported by a previous study that the treatment of fluoxetin, a selective serotonin reuptake inhibitor, reduced the neuronal damage of hippocampal CA1 pyramidal neurons induced by ischemia/reperfusion in gerbils [34]. Administration of antidepressant such as fluoxetin to rats for 21 days increased AANAT mRNA in the hippocampus by 5 folds [20]. In addition, AANAT mutant mice exhibit prolonged immobility in the test of forced swimming [21].

On the other hand, we found that AANAT immunoreaction was expressed in astrocytes in the strata oriens and radiatum of the CA1 region from 4 days after ischemia/reperfusion. This is the first study to demonstrate AANAT was expressed in astrocytes in the CA1 region with the delayed neuronal death after transient forebrain ischemia. We could not explain why AANAT immunoreaction was expressed in astrocytes from 4 days post-ischemia. Pei and Cheung [46] reported that melatonin treatment protected cultured neuronal cells but not astrocytes against oxygen/glucose deprivation-induced cell death in a dose-dependent manner.

Although there are some reports about the neuroprotective effects of melatonin, the mechanism of melatonin was not fully elucidated. In this study, we observed that melatonin treatment significantly reduced the activation of astrocytes and microglia induced by ischemia/reperfusion. In addition, melatonin maintained AANAT immunoreactivity in the stratum pyramidale of the CA1 region and protein levels in CA1 homogenates 5 days after ischemia/reperfusion. These effects may be associated with the neuroprotective roles of melatonin or AANAT against ischemic damage. It was reported that melatonin levels in plasma, as well as AANAT activity, were lower in diabetic than in nondiabetic rats and humans [47], suggesting that they provide protection against ROS.

In conclusion, AANAT is changed mainly in pyramidal neurons, which is very vulnerable to an ischemic insult, early time after ischemia/reperfusion and expressed in astrocytes in the CA1 region of the gerbil later after ischemia/reperfusion. These results suggesting that the reduction of AANAT in neurons can be involved in delayed neuronal death of CA1 pyramidal neurons after ischemia/reperfusion and that melatonin treatment maintains AANAT levels in the ischemic CA1 region.

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