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ORIGINAL ARTICLE

The 5α-Reductase Inhibitor Finasteride Exerts Neuroprotection Against Ischemic Brain Injury in Aged Male Rats

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Abstract

Progesterone (P4) exerts potent neuroprotection both in young and aged animal models of stroke. The neuroprotection is likely to be mediated by allopregnanolone (ALLO) metabolized from P4 by 5α-reductase, since the neuroprotection is attenuated by the 5α-reductase inhibitor finasteride, which was done only with young animals though. Thus, we do not know the contribution of ALLO to the P4-induced neuroprotection in aged animals. We examined effects of finasteride on the P4-induced neuroprotection in aged (16–18-month-old) male rats subjected to transient focal cerebral ischemia. Transient focal cerebral ischemia was induced by left middle cerebral artery occlusion (MCAO) and occlusion of the bilateral common carotid arteries. MCAO rats were given an 8 mg/kg P4 6 h after MCAO followed by the same treatment once a day for successive 3 days. Finasteride, a 5α-reductase inhibitor, at 20 mg/kg was intraperitoneally injected 30 min prior to the P4-injections. P4 markedly reduced neuronal damage 72 h after MCAO, and the P4-induced neuroprotection was apparently suppressed by finasteride in the aged animals. However, post-ischemic administration of finasteride alone (20 mg/kg) significantly prevented neuronal damage and the impairment of Rotarod performance after MCAO in aged male rats, but not in young ones. The androgen receptor antagonist flutamide markedly suppressed the neuroprotection of finasteride in the cerebral cortex, but not in the striatum, suggesting the androgen receptor-dependent mechanism of the finasteride-induced neuroprotection in the cerebral cortex. Our findings suggested, for the first time, the potential of finasteride as a therapeutic agent in post-ischemic treatment of strokes in aged population.

Keywords 5α-reductase inhibitor · Finasteride · Middle cerebral artery occlusion · Progesterone · Stroke

Introduction

Strokes are a major leading cause of death in many countries. Even though stroke patients survive, they have serious long-term disabilities that require social care support. The absolute number of individuals who have strokes is increasing every year worldwide [1]. Thrombolytic therapy with tissue plasminogen activator (tPA) is commonly used as the most effective pharmacological treatment currently available, but it only has a very narrow therapeutic time window of 4.5 h after the onset of a stroke, and barriers to its application include its severe side effects such as intracerebral hemorrhage [2, 3]. Therefore, there is an urgent need to develop safer and more effective therapeutic agents against strokes.

Our recent study demonstrated that post-ischemic treatment of transient cerebral ischemia with progesterone (P4) strongly prevented delayed neuronal cell death and learning/motility impairments in young male rats [4]. Another group has also shown potential of P4 as a potent therapeutic agent against strokes in aged male rats [5–7]. Importantly, P4 can exert powerful neuroprotection even though its administration is started 6 h after the onset of permanent focal cerebral ischemia, which is a wider time window than the 4.5 h of tPA [6]. However, the detailed molecular mechanisms of action of P4 require further investigations.

In the brain as well as peripheral tissues, P4 is rapidly converted to 5α-dihydroprogesterone (5α-DHP) by 5α-reductase and subsequently converted to allopregnanolone (ALLO) by 3α-hydroxysteroid dehydrogenase (3α-HSD). Our and other groups have reported that the P4-metabolite

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ALLO mediates neuroprotection of P4 because the neuroprotection is attenuated by the 5α-reductase inhibitor finasteride [4, 8–10]. ALLO is well known to be a positive modulator of $GABA_A$ receptors [11–13]. The potentiation of $GABA_A$ receptor function by ALLO can suppress the excitotoxicity after an ischemic event through inhibitory actions, and thus can result in neuroprotection against ischemic neuronal cell death. Although finasteride also blocks conversion of the other P4-metabolite testosterone into dihydrotestosterone by its inhibitory effect on 5α-reductase, high levels of dihydrotestosterone have been reported to exacerbate ischemic brain injuries in young male animals [14, 15]. Therefore, the P4-metabolite ALLO is highly likely to prevent the ischemic neuronal cell death by suppressing over-excitation of neurons through the activation of $GABA_A$ receptors.

However, as the inhibition of the P4-induced neuroprotection by finasteride was reported in experiments using young animals, we do not know the contribution of ALLO to the P4-induced neuroprotection in aged animals. Age-related changes in mRNA expressions of steroidogenic enzymes including 5α-reductase have been observed in the male rat brain [16, 17]. These changes can affect the metabolism of steroids within the brain, and thus, the contributions of P4-metabolites may be modified in the different ages of animals. Aged anima1s are better models of old human population who typically has a higher incidence of stroke than young one. Therefore, investigations in aged animals are needed to understand the effect of P4 and its mechanism of action in the treatment of strokes in aged human population. In the present study, we examined the effects of finasteride on the P4-induced neuroprotection in the striatum and cerebral cortex in an aged male rat model of stroke.

Materials and Methods

Animals and Housing Conditions

Young adult (3–5-month-old) and aged (16–18-month-old) male Sprague-Dawley rats were purchased from Chubu Kagaku Shizai (Nagoya, Japan) and housed in a light controlled room under a 12-h light/dark cycle starting at 9:00 AM, and maintained at a temperature of 25 °C. Animals had free access to food and water, and were handled at least six times over 1 week before surgery. Rats that had surgery were given agar chow instead of solid chow everyday. In all experiments, rats were randomly assigned in each group. Experimental procedures were approved by the Institutional Animal Care and Use Committee of Nagoya University and carried out in accordance with the guidelines established by the Nagoya University.

Preparation of the Transient Focal Cerebral Ischemia Model

Transient focal cerebral ischemia was induced by left middle cerebral artery occlusion (MCAO) and occlusion of the bilateral common carotid arteries (CCAs). MCAO was conducted as described previously with small modifications [18]. Rats were anesthetized using 5% isoflurane and maintained at 2–2.5% during surgery. Core body temperatures were monitored and maintained at 36.5 ± 1 °C using a warm pad during surgery. The incision area was swabbed with 70% ethanol to ensure a clean dry surface and then shaved. A ventral midline incision was made in the neck in order to expose both CCAs. Strings were placed loosely around each CCA to immediately pull them up and apply micro-clips just after MCAO. The left eye was covered by white petrolatum and a piece of a kimtowel to protect it from surgical lights. A vertical incision was made above the left zygomatic arch. The temporal and masseter muscles connected to the edge of the zygomatic arch were cut off, and a portion of the exposed zygomatic arch was then removed. The ligament connecting to the temporal and mandible bones and that of the temporal muscle connecting to the coronoid process were dissected, and the coronoid process and small portion of the notch of the mandible bone were removed. The deep layer of the temporal muscle was partially removed to expose the ventral surface of the skull. A burr hole was made 3 mm superior-rostral to the foramen ovale with a drill in order to expose the MCA. The dura was opened using a 26-gauge needle and the tips of a micro-forceps. A micro-clip (Unique Medical, TS-93026) was applied to the left MCA trunk just at the lateral border of the olfactory tract. The bilateral CCAs were occluded by micro-clips (Natsume, KN-353) within 5 min of MCAO. The interruptions of the flows of the left MCA and the bilateral CCAs were carefully confirmed visually after each applying of the clips. Successive both the left MCA and bilateral CCAs occlusions produced reductions of regional cerebral blood flow to approximately 20% of baseline when the probe of the laser blood flowmeter (FLO-C1, Omegawave, Japan) was positioned at 1 mm rostral and 2 mm dorsal to the clipping site of the left MCA. Surgical incisions were closed within 10 min after the CCAs had been occluded. Rats were returned to their home cages on the warm pad and allowed to recover from anesthesia. After 45 min of the three vessel occlusion, the micro-clips were removed from the left MCA and then the bilateral CCAs. The reperfusion of each artery was carefully confirmed visually. The burr hole was plugged using a gelatin-compressed sponge (Pfizer), and surgical incisions were closed again. Rats were again recovered from anesthesia on the warm pad. Sham-operated rats were treated identically, except that the left MCA and bilateral CCAs were not occluded.

Drug Administration

P4 (Sigma) or finasteride (Sigma) was dissolved in 30% 2-hydroxypropyl-β-cyclodextrin (HBC, Wako; vehicle) in saline. Flutamide (Sigma) was dissolved in 100% ethanol followed by mixed with sesame oil (the final concentration of ethanol: 5%). Rats were given a combined intraperitoneal and subcutaneous injection of 8 mg/kg P4 or its vehicle 6 h after MCAO, followed by subcutaneous injections 16 h later and then once daily for the next 2 days. To analyze effects of the 5α-reductase inhibitor on ischemic brain injury and P4-neuroprotection, the 5α-reductase inhibitor finasteride (20 mg/kg, the same dose as our recent study in young male rats [4]) was intraperitoneally injected at 30 min prior to the first (6 h after MCAO) and second P4-injections (22 h after MCAO), and successive subcutaneous injections of P4 were given without finasteride because our previous study has reported that finasteride prevented the neuroprotection by P4 injected at 1 h, but not 24 h after MCAO [4]. In the separate experiment to examine long-term outcomes in finasteride-treated rats after MAO, rats were given an intraperitoneal injection of 20 mg/kg finasteride 6 h after MCAO, followed by 16 h later and then once daily for the next 6 days. To analyze effects of the androgen receptor antagonist on finasteride-neuroprotection, flutamide (10 mg/kg) was given by subcutaneous injections 30 min prior to the administration of finasteride. This dose of flutamide has been used for inhibiting androgen-mediated action on experimental strokes in other previous studies [15, 19]. Drugs were prepared freshly on the day of the experiment.

Tissue Preparation

Brain samples were collected from male rats 7 days (for young), 72 h or 8 days (for aged) after MCAO. Rats were deeply anesthetized with a combination anesthetic agent (i.p.) containing 0.15 mg/kg medetomidine, 2 mg/kg midazolam, and 2.5 mg/kg butorphanol, and transcardially perfused with Lactated Ringer's solution (Otsuka Pharmaceutical) followed by 4% paraformaldehyde in PBS after MCAO. The brains were removed, post-fixed in 4% paraformaldehyde in PBS, and then cut at 8 mm from the olfactory bulb on a rat brain slicer (Muromachi kikai). Both the anterior and posterior brains were processed for paraffin embedding. One or four-series of coronal sections (5-μm-thick) were cut every 300 μm from the cut surface of the anterior brains using REM-710 (Yamato Koki Industrial Co., Ltd, Japan). The 6th and 10th sections of each anterior brain were used for the histological analysis. The deparaffinization of specimens was performed by washing twice with fresh Isopropenyl-1-methylcyclohexene (+)-Limonene (Wako) for 5 min or three times with fresh xylene (Wako) for 5 min each time followed by two washes with PBS, and then with graded ethanol and again with water.

NeuN Staining

For retrieving antigens, samples were boiled in 10 mM citrate buffer (pH 6.0) for 10 min. Blocking was performed with 2% goat serum (Sigma) in PBS and then washed twice with PBS. The anti-NeuN monoclonal antibody (Millipore) diluted 1:300 with PBS. The samples were incubated with the antibody for 1 h at room temperature (RT) and then washed twice with PBS. Alexa Fluor 488 or 555 Goat anti-mouse IgG (lifetechnologies) diluted 1:500 (for Alexa Fluor 488) or 1:700 (Alexa Fluor 555) was used as a secondary antibody. Incubation with the secondary antibody was performed for 1 h at RT. Digital images were collected on BX-Z700 (Keyence). To calculate the number of NeuN-positive cells, we chose three 200 × ipsilateral striatal or cortical fields (500 μm-width × 1000 μm-height) per section and three 200 × contralateral striatal or cortical fields per section (Fig. 1a). The one counting field in the cerebral cortex was situated 1.3 mm lateral to the midline. This field was positioned in the region which can be considered as the border zone of infarct at 8 days after MCAO. We then positioned an adjacent field, and the other field was placed next to that. Three striatal fields in the contralateral side were situated close to the border between the striatum and cerebral cortex, and then three striatal fields in the corresponding ipsilateral side were positioned at the corresponding distance from the midline, respectively. In the striatum of some sections, a counting field in the ipsilateral region was reduced to fit within the striatal region when the field protruded from the striatum probably due to a reduction in the striatal volume by ischemic damage. NeuN-positive cells were automatically counted using a BX-Z analyzer. The ischemic neuronal damage was estimated as the number of NeuN-positive cells in the ipsilateral regions/the number of NeuN-positive cells in the corresponding contralateral regions × 100. The histological analysis was conducted by an investigator blinded to the treatment or vehicle groups.

Rotarod Test

The Rotarod test was used to assess the sensorimotor coordination and motor skill learning of rats after MCAO using an accelerating treadmill (Brain science idea), as described previously with some modifications [20]. Rats were tested for three trials (15 min apart) on each the 7th and 8th day after MCAO. The tests were performed between 15 PM and 18 PM. Animals were

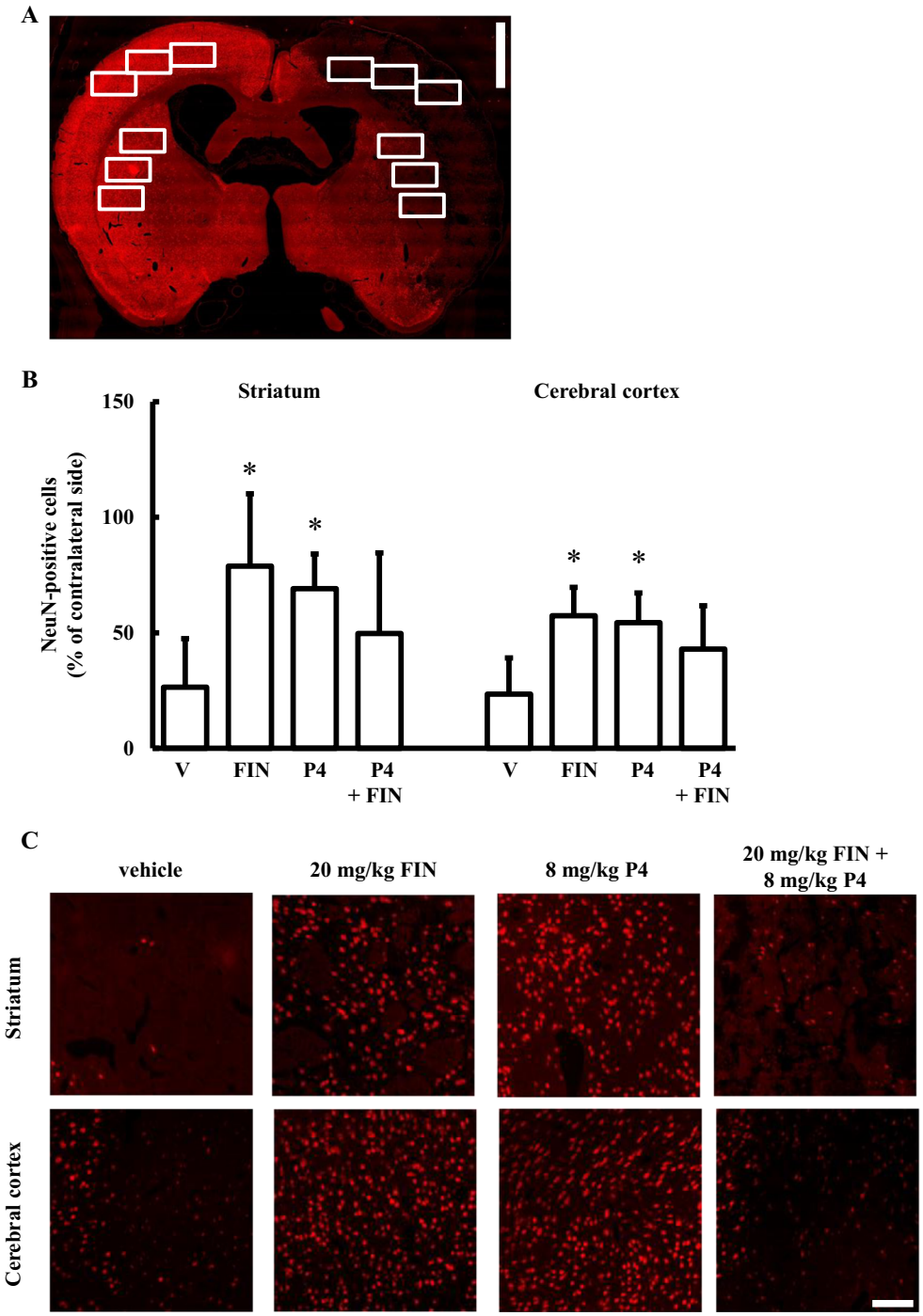

Fig. 1 Effects of finasteride on ischemic neuronal damage and the P4-induced neuroprotection in the striatum and cerebral cortex in aged male rats. **a** A coronal section of rat brain with NeuN staining 8 days after MCAO. Boxed areas (500 μm-width × 1000 μm-height) indicate striatal and cortical regions to calculate the number of NeuN (red)-positive cells. Scale bar = 1500 μm. **b** The ratio of NeuN-positive cells in the ipsilateral areas to the corresponding contralateral areas of the striatum and cerebral cortex 72 h after MCAO in aged male rats given injections of 20 mg/kg finasteride (FIN), 8 mg/kg P4 with (P4 + FIN) or without 20 mg/kg finasteride (P4), or its vehicle (V). *$P < 0.05$ compared to V. **c** Representative ipsilateral NeuN staining 72 h after MCAO in the striatum and cerebral cortex of aged male rats given injections of 20 mg/kg finasteride, 8 mg/kg P4 with or without 20 mg/kg finasteride, or its vehicle. Cortical images were obtained from the fields situated 1.3 mm lateral to the midline of brain sections from a rat of each group. Striatal images were obtained from the fields close to the border between the striatum and cerebral cortex. Scale bar = 100 μm

habituated in the experimental room for at least 1 h prior to the start of the tests. In Rotarod training sessions, animals were habituated to the Rotarod and trained to remain on the rotating drum (constant speed 2 rpm). If animals fell from the rod, they were placed on it again until they were able to stay for 60 s. After a 5-min rest, the animals were placed on the Rotarod, and the rotational speed was set to accelerate from 2 to 20 rpm over 300 s. The latency time to fall (time on the rod) was recorded with a cutoff time of 300 s.

Data Analysis

Data are expressed as the mean ± S.D. Differences in histological analysis were estimated by a two-tailed independent samples *t* test (equal or unequal variance) or two-tailed multiple *t* tests with Bonferroni corrections. Differences in Rotarod test were analyzed by repeated measures analysis of variance (ANOVA) followed by two-tailed paired or unpaired multiple *t* tests with Bonferroni corrections. A *P* value of less than 0.05 was considered significant.

Results

The 5α-Reductase Inhibitor Finasteride Suppressed P4-Induced Neuroprotection Against Ischemic Stroke in Aged Male Rats

The steroidogenic enzyme 5α-reductase is needed to convert P4 to ALLO, and two distinct isozymes of 5α-reductase type 1 and type 2 are found in the rat brain [21, 22]. Finasteride reversibly inhibits the activity of the rat type 1 and irreversibly inhibits the rat type 2 of 5α-reductase, and an intraperitoneal injection of 10 mg/kg finasteride can almost completely deplete ALLO in the rat brain [23, 24]. Our and other groups have reported that finasteride attenuated the P4-induced neuroprotection in young animals, suggesting that the P4-metabolite ALLO mediates the neuroprotection of P4 [4, 9, 10].

To estimate contributions of ALLO metabolized from P4 to the P4-induced neuroprotection in aged animals, we examined the effects of finasteride on ischemic neuronal damage 72 h after MCAO and the P4-induced neuroprotection in aged male rats. Post-ischemic administration of 8 mg/kg P4 showed a significant reduction in neuronal damage in the striatum (26.4 ± 21.1% in vehicle-treated rats, $n = 6$; 69.1 ± 15.0% in P4-treated rats, $n = 6$, $P < 0.05$ compared with vehicle-treated rats, two-tailed multiple t tests with Bonferroni correction, Fig. 1b) and cerebral cortex (23.4 ± 15.7% in vehicle-treated rats, $n = 6$; 54.4 ± 12.8% in P4 and finasteride-treated rats, $n = 6$, $P < 0.05$ compared with vehicle-treated rats, two-tailed multiple t tests with Bonferroni correction, Fig. 1b), while its administration with finasteride partially but not significantly prevented neuronal damage in the striatum (49.7 ± 34.8% in P4 and finasteride-treated rats, $n = 6$, $P > 0.05$ compared with vehicle-treated rats, two-tailed multiple t tests with Bonferroni correction, Fig. 1b) and cerebral cortex (43.0 ± 18.8% in P4 and finasteride-treated rats, $n = 6$, $P > 0.05$ compared with vehicle-treated rats, two-tailed multiple t tests with Bonferroni correction, Fig. 1b) 72 h after MCAO in aged male rats, indicating a partial suppressive effect of finasteride on the P4-induced neuroprotection. However, post-ischemic administration of finasteride alone showed a significant decrease in neuronal damage in the striatum (78.8 ± 31.3% in finasteride-treated rats, $n = 6$, $P < 0.05$ compared with vehicle-treated rats, two-tailed multiple t tests with Bonferroni correction, Fig. 1b) and cerebral cortex (57.4 ± 12.2% in finasteride-treated rats, $n = 6$, $P < 0.05$ compared with vehicle-treated rats, two-tailed multiple t tests with Bonferroni correction, Fig. 1b) 72 h after MCAO in aged male rats. Taken together, finasteride apparently showed a partial suppressive effect on the P4-induced neuroprotection, but this may not necessarily suggest the contribution of ALLO to the P4-inducd neuroprotection in the aged animals.

Finasteride Prevented Ischemic Neuronal Damage and Motor Impairments After Ischemic Strokes in Aged Male Rats

Unexpectedly, finasteride alone showed a significant reduction in neuronal damage 72 h after MCAO in aged male rats. We thus further evaluated long-term histological and functional outcomes in the finasteride-treated rats 1 week after MCAO.

Rotarod test was performed to access motor coordination and skill learning on each the 7th and 8th day after MCAO. On the 1st trial of Rotarod test, vehicle-treated MCAO rats showed a significant reduction in the time on the rod compared to sham-operated rats ($F_{(2,\ 15)} = 17.2$, $P < 0.05$; 86.8 ± 8.6 s % in sham-operated rats, $n = 6$; 55.2 ± 10.4 s in vehicle-treated MCAO rats, $n = 6$, $P < 0.05$ compared with sham-operated rats, two-tailed unpaired multiple t tests with Bonferroni corrections, Fig. 2), and post-ischemic administration of finasteride significantly prevented the impairment of the Rotarod performance after MCAO (82.5 ± 17.9 s in finasteride-treated MCAO rats, $n = 6$, $P < 0.05$ compared with vehicle-treated MCAO rats, two-tailed unpaired multiple t tests with Bonferroni corrections, Fig. 2). The sham-operated and finasteride-treated MCAO rats showed significant improvements of the Rotarod performance on the 6th trial ($F_{(5,\ 75)} = 5.6$, $P < 0.05$; 150.3 ± 46.1 s, 205.2 ± 67.5 s, 153.3 ± 40.3 s, 181.0 ± 49.6 s, 218.5 ± 48.5 s in sham-operated rats; 138.2 ± 48.0 s, 153.3 ± 17.0 s, 157.0 ± 52.3 s, 198.2 ± 52.3 s, 214.2 ± 39.1 s in finasteride-treated MCAO rats, on the 2nd, 3rd, 4th, 5th, and 6th trial, $n = 6$, $P < 0.05$ compared with the 1st trial of the corresponding group, two-tailed paired multiple t tests with Bonferroni corrections, Fig. 2), whereas vehicle-treated MCAO rats did not (73.0 ± 18.5 s, 79.7 ± 17.1 s, 60.2 ± 15.7 s, 79.8 ± 23.0 s, 90.5 ± 17.8 s on the 2nd, 3rd, 4th, 5th, and 6th trial, $n = 6$, $P > 0.05$ compared with the 1st trial, two-tailed paired multiple t tests with Bonferroni corrections, Fig. 2). The Rotarod performances of finasteride-treated MCAO rats were significantly higher than those of vehicle-treated MCAO rats on every trials ($P < 0.05$, two-tailed unpaired multiple t tests with Bonferroni corrections, Fig. 2).

Consistent with the results in Rotarod test, post-ischemic administration of finasteride significantly reduced neuronal damage in the striatum (23.9 ± 11.0% in vehicle-treated rats, $n = 6$; 75.1 ± 23.4% in finasteride-treated rats, $n = 6$, $P < 0.05$ compared with vehicle-treated rats, independent samples equal variance t test, Fig. 3a) and cerebral cortex (11.7 ± 9.0% in vehicle-treated rats, $n = 6$; 59.3 ± 30.0% in finasteride-treated rats, $n = 6$, $P < 0.05$ compared with vehicle-treated rats, independent samples unequal variance t test, Fig. 3a) 8 days after MCAO in aged male rats. In young male rats (3–5-month-old), the same administration way of finasteride did not prevent neuronal damage in the striatum

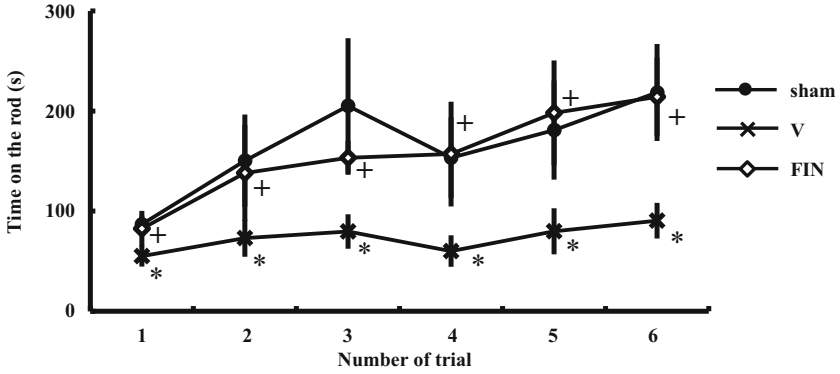

Fig. 2 Effects of finasteride on Rotarod performance after MCAO in aged male rats. A graph shows time on the rod on each trial of the 7th and 8th day after MCAO in aged male rats given injections of 20 mg/kg finasteride (FIN) or its vehicle (V), or sham-operated aged male rats (sham). *$P < 0.05$ compared to sham; $^{+}P < 0.05$ compared to V

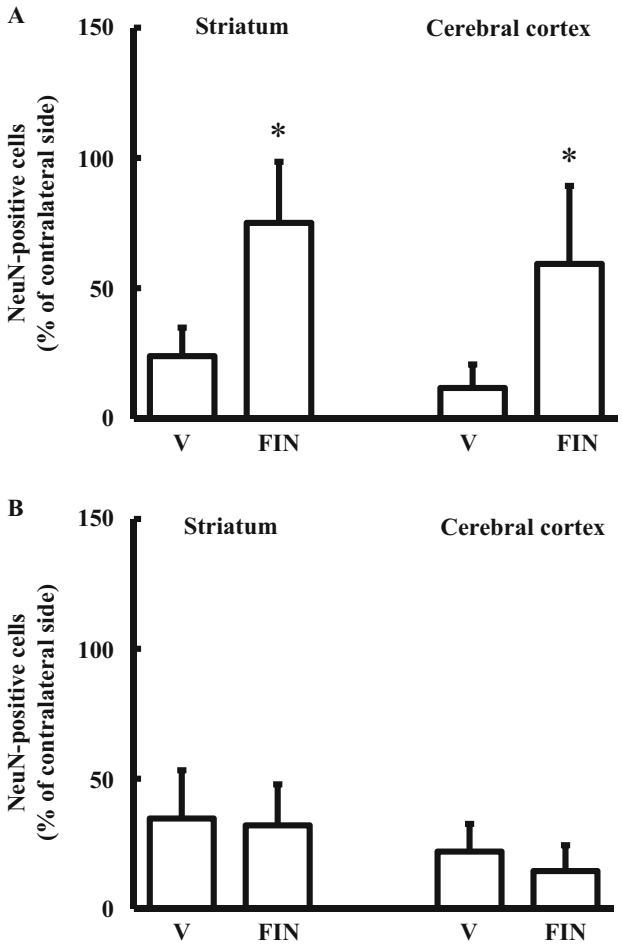

Fig. 3 Effects of finasteride on ischemic neuronal damage in the striatum and cerebral cortex in young and aged male rats. **a** The ratio of NeuN-positive cells in the ipsilateral areas to the corresponding contralateral areas of the striatum and cerebral cortex 8 days after MCAO in aged male rats given injections of 20 mg/kg finasteride (FIN) or its vehicle (V). *$P < 0.05$ compared to V. **b** The ratio of NeuN-positive cells in the ipsilateral areas to the corresponding contralateral areas of the striatum and cerebral cortex 7 days after MCAO in young male rats given injections of 20 mg/kg finasteride (FIN) or its vehicle (V). Finasteride was intraperitoneally injected 6 h after MCAO, followed by 16 h later and then once daily for 5 days

(34.6 ± 18.6% in vehicle-treated rats, $n = 6$; 32.0 ± 15.8% in finasteride-treated rats, $n = 6$, $P > 0.05$ compared with vehicle-treated rats, independent samples equal variance t test, Fig. 3b) and cerebral cortex (21.9 ± 10.7% in vehicle-treated rats, $n = 6$; 14.4 ± 9.9% in finasteride-treated rats, $n = 6$, $P > 0.05$ compared with vehicle-treated rats, independent samples equal variance t test, Fig. 3b) 7 days after MCAO. These results suggested that post-ischemic administration of finasteride alone exerted an age-dependent neuroprotective effect against ischemic neuronal damage in the striatum and cerebral cortex.

Androgens Mediated the Finasteride-Induced Neuroprotection Against Ischemic Strokes in the Cerebral Cortex

Androgens exert both beneficial and adverse effects depending on their doses on ischemic brain injury in male animals, and low levels of testosterone reduces infarct size in castrated young male animals [15, 19]. Finasteride blocks the conversion of testosterone into dihydrotestosterone and concomitantly could lead to accumulation of testosterone. Interestingly, testosterone exacerbates ischemic brain damage in young male animals, whereas it alleviates in aged ones [25]. Testosterone thus may be a possible mediator of the finasteride-induced neuroprotection in aged male rats.

To test this hypothesis, we examined effects of the selective androgen receptor antagonist flutamide on the finasteride-induced neuroprotection in experimental strokes. Post-ischemic administration of finasteride alone produced significant reductions in neuronal damage both in the striatum and cerebral cortex 8 days after MCAO compared to vehicle-treated MCAO rats (striatum: 23.0 ± 12.0% in vehicle-treated rats, $n = 6$; 76.4 ± 23.9% in finasteride-treated rats, $n = 6$, $P < 0.05$ compared with vehicle-treated rats, cerebral cortex: 11.9 ± 10.1% in vehicle-treated rats, $n = 6$; 43.9 ± 20.3% in finasteride-treated rats, $n = 6$, $P < 0.05$ compared with vehicle-treated rats, independent samples equal variance t test, Fig. 4). Flutamide significantly suppressed the finasteride-induced neuroprotection

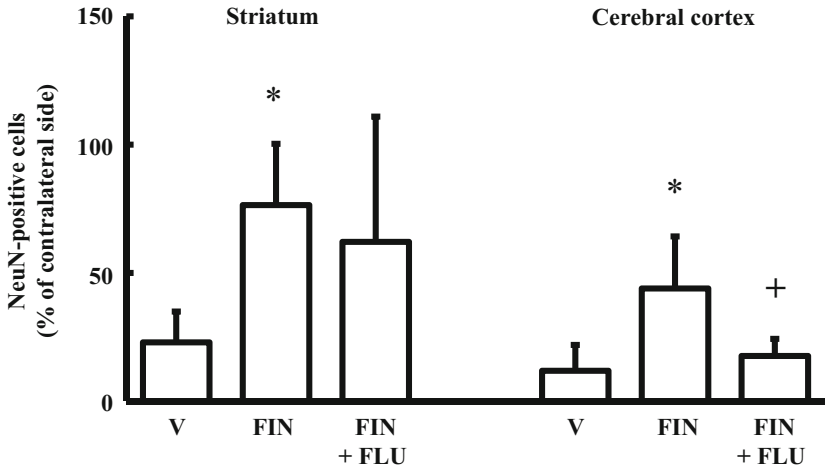

Fig. 4 Effects of flutamide on the neuroprotection of finasteride against ischemic neuronal damage in the striatum and cerebral cortex in aged male rats. The ratio of NeuN-positive cells in the ipsilateral areas to the corresponding contralateral areas of the striatum and cerebral cortex 8 days after MCAO in aged male rats given injections of 20 mg/kg finasteride (FIN), FIN and 10 mg/kg flutamide (FLU) or its vehicle (V). $^{*}P < 0.05$ compared to V, $^{+}P < 0.05$ compared to FIN

in the cerebral cortex, but not reliably in the striatum 8 days after MCAO (striatum: 62.1 ± 48.8% in finasteride and flutamide-treated rats, $n = 6$, $P > 0.05$ compared with finasteride-treated rats, cerebral cortex: 17.6 ± 6.7% in finasteride and flutamide-treated rats, $n = 6$, $P < 0.05$ compared with finasteride-treated rats, independent samples unequal variance t test, Fig. 4). These results suggested that the neuroprotection of finasteride in the cerebral cortex was mainly mediated via androgen receptors, while other mechanisms were also involved in the neuroprotection in the striatum.

Discussion

In the present study, we found, for the first time, that 6-h post-ischemic administration of the 5α-reductase inhibitor finasteride protected neurons in the striatum and cerebral cortex and prevented the impairment of Rotarod performance after focal cerebral ischemia in aged male rats, but not young ones. As the therapeutic effective time window of finasteride was wider (6 h) than the 4.5 h of tPA, this finding indicates a possibility that the 5α-reductase inhibitors are novel potent therapeutic agents against strokes by preventing delayed neuronal cell death in the penumbra of transient or permanent brain ischemia, at least, in aged or old human population.

Several histological and immunohistochemical methods have been used to assess ischemic brain damage/infarct volume and effects of candidate therapeutic agents in experimental focal cerebral ischemia, and every method has advantages and disadvantages [26–30]. NeuN, neuronal nuclear antigen, is commonly used as a specific marker of mature neuronal cells, and a loss of NeuN is indicative of a neuronal cell loss or an injured neuron [29, 31]. Quantitative analysis of NeuN-positive cells has been used to assess ischemic neuronal damage in specific fields of different anatomical regions, and data from NeuN staining are well correlated with those from other histological and immunohistochemical methods such as TTC staining [29, 32, 33]. In the present study, we therefore used NeuN staining to assess specifically neuronal damage in specific fields (the ischemic core or the border zone of infarct) of the infarct regions in the striatum and cerebral cortex after MCAO.

Our recent study has shown in young male rats that the neuroprotective effect of P4 against transient focal cerebral ischemia is mediated by the P4-metabolite ALLO because the neuroprotection was attenuated by the 5α-reductase inhibitor finasteride [4]. In the present study, we intended to examine whether the mechanisms of P4-induced neuroprotection in aged male rats are identical to those in young male rats. For this purpose, we tested effects of finasteride on P4-induced neuroprotection in aged male rats. Finasteride apparently showed a partial suppressive effect on the P4-induced neuroprotection, indicating a possibility that ALLO partially contributed to the P4-induced neuroprotection in the aged animals. However, surprisingly, post-ischemic administration of finasteride alone exerted a neuroprotective effect against focal cerebral ischemia in aged male rats. Our present study thus failed to accurately evaluate contributions of P4-matabolite ALLO to the P4-induced neuroprotection in aged male rats. The fact that finasteride exerted a neuroprotective effect against ischemic neuronal damage may indicate that ALLO is not essential for P4-induced neuroprotection against ischemic neuronal damage in aged animals. Rather, increased brain levels of P4 itself or following metabolites except for ALLO may be essential for the P4- or finasteride-induced neuroprotection against focal cerebral ischemia in aged male rats. However, our results do not exclude a neuroprotective effect of ALLO itself against ischemic neuronal damage in aged male rats. As ALLO is more effective than P4 in reducing ischemic brain damage, direct injections of ALLO can exert neuroprotection against stroke via distinct mechanisms in aged animals [34–36]. Interestingly, combined injections of P4 and finasteride showed less effectiveness of neuroprotection at 72 h after MCAO, suggesting the antagonistic effect of

P4 and finasteride. Preceding studies have demonstrated that optimal doses of P4 exert neuroprotection against strokes, while excessive doses of P4 are less effective or ineffective [6, 37]. The antagonistic action in aged male rats may be therefore due to an excessive level of P4 caused by the administration of P4 under the inhibition of 5α-reductase. Along this hypothesis, we could not exclude a possibility that the inhibition of the P4-induced neuroprotection by finasteride in young male rats is resulted from an excessive level of P4 under the inhibition of 5α-reductase but not from inhibiting the production of ALLO. However, at least, finasteride does not increase the brain level of P4 in young male rats [24]. In addition, finasteride alone should affect ischemic neuronal damage if finasteride markedly increases brain levels of endogenous P4 or its metabolites except for ALLO, which could exert some effects similar to the administrated P4, but it had no significant effect on the neuronal damage after MCAO in young male rats (Fig. 3b).

In recent studies, the steroidogenic enzyme 5α-reductase is highlighted as one of potential therapeutic targets against several brain disorders [38–40]. Two separate groups have reported neuroprotective effects of the 5α-reductase inhibitors in animal models of brain diseases. The one group has reported that finasteride improves motor and electroencephalogram changes in the young male rat model of hepatic encephalopathy [41, 42]. Another study has shown that dutasteride, which is a more potent 5α-reductase type 1 and 2 inhibitor than finasteride, exerts a neuroprotective effect in the young male mouse model of Parkinson's disease, but finasteride does not [43]. This difference in the finasteride-effect may be due to the differences in the mechanisms of the 5α-reductase inhibitor-induced neuroprotection against different diseases or to its higher dose in our study (20 mg/kg) than that in their study (12.5 mg/kg).

Two limitations of this study may need to be considered for the feasibility of post-stroke treatment with finasteride in humans. The one arises from the lack of data on effects of finasteride in female rats. Although there is no concrete evidence to suggest neuroprotective effects via 5α-reductase or androgen receptors in experimental strokes in females, at least, 5α-reductase and androgen receptors are also expressed in the cerebral cortex of young female rats [21, 44–46], and one study has reported the neuroprotective effects of androgens in the dentate gyrus of adrenalectomized female rats [47]. The other limitation is testing in the present study only a single dose (20 mg/kg) and administration period (8-day) of finasteride to evaluate the histological and functional outcomes 8 days after MCAO. This dose of finasteride is higher than the typical clinical doses in patients with benign prostatic hyperplasia (5 mg/day) or androgenic alopecia (1 mg/day) [48–50]. Finasteride, even at such clinical doses, has several adverse effects such as sexual dysfunction and depressive symptoms [48, 50–53]. The increased risk of high-grade prostate cancer and hepatic dysfunction may be potential adverse effects of finasteride [54–57]. However, its effective administration period in the post-stroke treatment may be much shorter than that in the treatment of benign prostatic hyperplasia or androgenic alopecia (≧ 3-month) because the enlargement of cerebral ischemic lesion volumes in humans is observed in the several days following strokes [58]. Thus, its serious adverse effects could be avoided or minimized in the post-stroke treatment. Further preclinical studies are needed to examine the optimal dose and administration period of finasteride in post-ischemic treatment in aged animals of both sexes.

We tried here to identify molecular mechanisms responsible for the finasteride-induced neuroprotection in aged male rats, but its mechanisms of action were more likely to be complicated. Our data suggested that the finasteride-induced neuroprotection in the cerebral cortex was mainly androgen receptor-dependent, whereas that in the striatum was more likely to be also mediated by other mechanisms. Similar to our results, Mladenović et al. have reported that brain sub-regional different effects of finasteride on brain oxidative stress and acetylcholinesterase activity in the rat model of hepatic encephalopathy [42]. In addition, different effects of castration or androgens have been observed between the cerebral cortex and striatum in experimental strokes [15]. These brain sub-regional different effects of finasteride or androgens may be due to different expression levels of sex steroidogenic enzymes and/or sex steroid receptors between brain sub-regions [17, 59]. Therefore, measurements of neuroactive steroid levels within each brain sub-region may be needed rather than those in whole brain or circulation to precisely understand action of finasteride in each brain sub-region. The inhibition of 5α-reductase by finasteride can affect brain levels of the other steroids such as P4 or estrogens which exert neuroprotective effects in experimental strokes [4–7, 36, 60–62]. These steroids may contribute to the neuroprotection of finasteride in the striatum. Other possible mechanisms in the striatum may be the effects of finasteride on antioxidant mechanisms and the mitochondrial permeability transition pore [35, 42, 63].

The present study showed the age-dependent neuroprotective effect of finasteride on ischemic brain injury. It may arise from age-related changes in the brain mRNA levels of steroidogenic enzymes in addition to decreases in circulating hormone levels with aging. Munemoto et al. have suggested a moderate decrease in androgen synthesis with aging, while estrogen synthesis from androgens by aromatase is unlikely to change with aging in the cerebral cortex of male rats [17, 25]. The previous literatures have suggested that optimal levels of androgens are neuroprotective, but its excessive levels are deleterious in experimental strokes [15, 25]. Therefore, finasteride may induce optimal increased levels of testosterone under the decreased synthesis of androgens in the cerebral cortex of aged male rats. On the other hand, as our data

suggested different mechanisms of the finasteride-induced neuroprotection between the striatum and cerebral cortex, other steroidogenic systems and steroid receptors may be also considered in the striatum.

As brain ischemia causes complicated pathological responses, multiple-target agents are considered to be more promising for treatment of strokes [64]. Finasteride may work as a promising agent in post-stroke treatment through its multiple-target actions as described above. Further separate studies are needed to fully understand the complicated age-dependent mechanisms of the finasteride-induced neuroprotection. In addition, due to these diverse effects of finasteride, our previous conclusion that ALLO mediates neuroprotection of P4 against strokes in the young animals may need to be reconsidered. The studies using P4 receptor knockout mice have shown the necessity for P4 receptors in the neuroprotective effect of P4 against experimental strokes, although finasteride also attenuated the P4-induced neuroprotection in the wild-type young male mice [36, 60].

The ALLO-mediated neuroprotection of P4 has been also reported in animal models of epilepsy [9, 10, 65]. However, these results came from experiments using young animals with finasteride. As found in our present study, effects of the 5α-reductase inhibitors might be different between different ages of epileptic animals. Our findings here indicate that P4-induced neuroprotection or effects of 5α-reductase inhibitors should be investigated with extreme caution for their age-dependent mechanisms of action.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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