Aripiprazole exerts a neuroprotective effect in mouse focal cerebral ischemia

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Abstract. Previous studies have demonstrated that aripiprazole (APZ), a third-generation atypical antipsychotic drug, exhibits anti-depressant and neuroprotective effects by promoting dopaminergic neuronal cell recovery in stroke. To investigate the neuroprotective effects of APZ, behavioral and histopathological experiments were performed in the current study a mouse model of middle cerebral artery occlusion (MCAO)-induced ischemia following administration of APZ. The subacute phase of ischemic assaults was divided into 3 periods, each with a duration of 5 days, according to the start of APZ (3 mg/kg) administration (1-5, 5-9 or 10-14 days following MCAO). The beneficial effects of APZ on motor behavior demonstrated in the cylinder, rotarod and wire suspension tests were greatest when APZ was administered 1-5 days following MCAO, with clear improvements in motor function compared with vehicle-treated mice. Histopathological analysis revealed that prominent atrophic changes occurred in the striatum of MCAO mice and that these changes were reduced following APZ treatment. APZ also attenuated dopaminergic neuronal injury in the striatum. Cell death and microglial activation were decreased and the expression of Ca^{2+}/calmodulin-dependent protein kinase II δ was enhanced following APZ treatment. These results indicate that the atypical antipsychotic drug, APZ, exhibits a neuroprotective effect in dopaminergic neuronal cells that may improve behavioral function following ischemic stroke.

Introduction

Aripiprazole (APZ) is a third-generation atypical antipsychotic drug that is a partial agonist of the dopaminergic D2 receptor (D2R) and the serotonin 5-HT1A and 5-HT7 receptors. APZ is used to treat schizophrenia (1-3) and acts as a dopamine-serotonin system stabilizer in adjunct therapy for major depressive disorder (3,4). Stroke is a neurological disease that induces sustained damage in the arteries in the brain and is often fatal. Studies have demonstrated that >30% of stroke survivors experience depression, including feelings of despair, anhedonia and anxiety (5,6).

APZ is widely used in combination with selective serotonin reuptake inhibitors as a treatment of major depressive disorder. Low-doses of APZ are also effective at treating patients with post-stroke emotional disorders and impaired cognitive function (3,7). Despite prospective clinical viewpoints, the mechanisms underlying the curative efficacy of APZ in post-stroke depression remain unclear. Previous studies have demonstrated that APZ exhibits benefits in patients with post-stroke depression, including the protection of primary lesions and secondary extrafocal sites following ischemic stroke (8,9).

Additionally, APZ decreases striatal kainate-induced lesion volumes in rodents by inducing a 5-HT1A-mediated protective effect (10). Dopaminergic D2Rs regulate inflammatory responses in the central nervous system and ameliorate neurological dysfunction by reducing microglia hyperactivity-related neuroinflammation (11). It has been demonstrated that dopamine D2/D3 receptor agonists exhibit protective effects against post-ischemic injury (12). However, the neuroprotective effects of APZ have only been demonstrated in a limited number of *in vitro* and *in vivo* studies (8-10,13).

The present study was designed based on the hypothesis that APZ inhibits the cell death and neuroinflammation caused by ischemic assaults, thus exerting a neuroprotective effect. To validate this hypothesis, the ability of APZ to induce motor-function behaviors associated with equilibrium and rotation asymmetry in a mouse model of middle cerebral artery occlusion (MCAO) was evaluated. To further assess the neuroprotective effects of APZ, histopathological analyses of brain sections were performed. The chronological sequence of events is fundamental to neuronal cell death and the neuroinflammatory response following ischemic insult (14). Therefore, in the current study, the subacute phase of ischemic stroke, characterized by marked apoptosis and inflammation, was divided into three periods according to the start of APZ treatment following MCAO.

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Materials and methods

Animals. A total of 30 male C57BL/6 mice aged 6 weeks old (weight, 18-20 g) were purchased from DooYeol Biotech (Seoul, Korea). Mice were housed at 22˚C and 55±5% humidity under a 12-h light-dark cycle and were fed a commercial diet. Mice had *ad libitum* access to food and water. All experiments were approved by the Pusan National University Animal Care and Use Committee in accordance with the National Institutes of Health Guidelines (approval no. PNU-2016-1149). After 1 week the mice were randomly divided into 5 groups (n=6) as follows: A control group, a MCAO+vehicle group and three MCAO+APZ treatment groups according to the start of APZ treatment (1-5, 5-9 and 10-14 days following MCAO). All mice were sacrificed at 24 days following MCAO.

MCAO model. Mice were anesthetized with isoflurane (Choongwae Pharma Corp., Seoul, Korea) using a model VIP 3000 calibrated vaporizer (Midmark Corporation, Orchard Park, OH, USA). Isoflurane was induced at a concentration of 3% and maintained at a concentration of 2% in 80% N_2O and 20% O_2. Rectal temperatures were maintained at 36.5-37.5˚C. Isoflurane anesthesia was delivered using a facemask and a fibre optic probe was fixed to the portion of skull that covered the middle cerebral artery. Regional cerebral blood flow was then measured using the PeriFlux Laser Doppler System 5000 (Perimed, Stockholm, Sweden) and a left MCAO model was produced. A silicon-coated 7-0 monofilament was advanced through the internal carotid artery to occlude the middle cerebral artery for 30 min and subsequently withdrawn. Reperfusion was confirmed using the Laser Doppler System. The control group underwent isoflurane anesthesia, however no further procedures were performed.

Drug administration. APZ was donated by Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). The drug was orally administered using a probe once a day for 5 days. Treatment was initiated 1, 5 or 10 days following MCAO, depending on the group mice were in. APZ was dissolved in distilled water to obtain a concentration of 3 mg/kg. The vehicle group were administrated the same volume of distilled water from 1 day following MCAO for 5 days.

Behavioral experiments. To evaluate whether APZ had an effect on motor function, cylinder, rotarod and wire suspension tests were conducted in all groups once a week for 3 weeks following MCAO. The cylinder test was performed to evaluate forelimb use and rotation asymmetry (15). Each mouse was individually placed on the floor of a plastic cylinder (diameter, 9 cm; height, 15 cm). The number of times that mice used their front paws to touch the cylinder was recorded and repeated 20 times. The motor coordination and equilibrium of mice were measured using a rotarod apparatus (Panlab S.L.U, Barcelona, Spain). All mice were pre-trained with two trials per day for two days on a rotarod apparatus at a fixed speed (20 rpm). Mice were then placed on the rotating rod, with a cut-off time of 3 min (16). The experiment comprised of five trials per day, once a week over a 3-week period. The wire suspension test was performed to measure muscular strength and neuromuscular endurance of mice following MCAO (17). The grip capabilities of the mice were evaluated using a sustained horizontal bar. The time that mice spent hanging on the bar was recorded and the mean of five trials from each mouse was analyzed.

Determination of infarct volume. To measure ischemic damage, mice were fully anesthetized using 500 mg/kg chloral hydrate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and received an intraperitoneal perfusion of saline followed by 4% paraformaldehyde in PBS. Brains were removed, post-fixed in the same fixative for 24 h at 4˚C and immersed in 30% sucrose solution for 72 h at 4˚C for cryoprotection. Brain infarct sizes or atrophies were estimated by staining frozen sections of 30-μm thickness with 0.1% cresyl violet at room temperature for 2 min (Sigma-Aldrich; Merck KGaA) and mounting slides in mounting medium (cat. no. H-5000; Vector Laboratories, Inc., Burlingame, CA, USA). To measure infarct area, the contralateral and ipsilateral segmentum sizes of each section (including the striatum, corpus callosum, cortex and midbrain) images were captured at magnification x10 using a Stemi 305 light microscope (Carl Zeiss AG, Oberkochen, Germany) and quantified using i-solution full image analysis software (version 10.1; Image and Microscope Technology, Hackettstown, NJ, USA).

Immunohistochemistry. The 30-μm-thick brain sections were frozen at -25˚C and then incubated in blocking buffer [1X PBS/5% normal goat serum (cat. no. s-1000; Vector Laboratories Inc.)/0.3% Triton X-100] for 1 h at room temperature. Sections were incubated with the following primary antibodies for 48 h in PBS at 4˚C: Neuronal nuclei (NeuN; cat. no. MAB377; 1:500; EMD Millipore, Billerica, MA, USA), tyrosine hydroxylase (TH; cat. no. AB152; 1:500; EMD Millipore), dopamine D2R (cat. no. AB5084p; 1:100; EMD Millipore), Ca^{2+}/calmodulin-dependent protein kinase IIδ (CaMKIIδ; cat. no. ab181052; 1:100; Abcam, Cambridge, UK), ionized calcium binding adaptor molecule 1 (Iba1; cat. no. 019-19741; 1:500; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and cluster of differentiation 68 (CD68; cat. no. MCA1957; 1:500; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Slides were then washed with PBS and sections were incubated with the fluorescein-conjugated goat-anti-rabbit (cat. no. A11008; 1:500) or Texas red-conjugated goat-anti-mouse (cat. no. A11005; 1:500), goat-anti-rat (cat. no. A11007; 1:500) and DAPI (cat. no. H3570; 1:10,000) (all Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 2 h at room temperature in the dark and then washed with PBS three times. Subsequently, slides were mounted in mounting medium (Vector Laboratories, Inc.) and captured at magnifications x25 and x400 using a fluorescence microscope (Carl Zeiss Imager M1; Carl Zeiss AG, Oberkochen, Germany) and confocal microscope (Carl Zeiss observer Z1; Carl Zeiss AG).

Measurement of apoptotic cells. Apoptotic cells were identified using staining, with a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and TH. The TUNEL assay was performed using a TUNEL assay kit (DeadEnd™ Fluorometric TUNEL System; Promega Corporation, Madison, WI, USA) following the manufacturer's protocol. Slides were mounted in mounting medium (Vector

Laboratories, Inc.). The number of TUNEL/TH-positive cells were counted. Quantitative blind analysis was performed by counting the number of apoptotic cells using a fluorescence microscope. Data are presented as the mean number of apoptotic cells from all brain tissue samples as counted in one field of the striatum at magnification x200.

Data analyses. All data are expressed as mean ± standard error of the mean and analyzed using the Sigma statistical program version 11.2 (Systat Software Inc., San Jose, CA, USA). Data were analyzed using one-way analysis of variance for repeated measures and Tukey's post hoc test of least significant difference. $P<0.05$ was considered to indicate statistically significance.

Results

Effect of APZ on motor-function behaviors. Various symptoms, including loss of balance and arm weakness or numbness, were observed during behavioral experiments conducted following MCAO. The number of times a mouse touched the cylinder with both paws during the cylinder test was significantly higher in all APZ-treated groups compared with the vehicle group at 2 weeks. However, no significant differences were observed between any of the treatment groups and the vehicle group at 3 weeks. At week 1 only the groups treated with APZ between 1-5 and 10-14 days following MCAO demonstrated a significant difference compared with the vehicle group (Fig. 1A). Mice treated with APZ 1-5 days following MCAO attained a significantly higher time in the rotarod test compared with the vehicle group at 2 and 3 weeks, indicating that immediate APZ administration post-MCAO increases motor coordination and balance performance (Fig. 1B). Gripping time in the wire suspension test was also significantly increased at 3 weeks in the group treated with APZ between 1-5 days following MCAO (Fig. 1C). These results suggest that treatment with APZ reverses motor dysfunction and that APZ treatment is most effective when administered immediately following stroke induction.

Effect of APZ on atrophic changes and dopaminergic neuronal injury in the brain. Histological analysis of brain sections revealed that severe atrophic changes in the vehicle group only occurred in the striatum, which was the primary lesion site of MCAO (Fig. 2). These atrophic changes were countered by treating mice with APZ 1-5 days following MCAO (Fig. 2). Brain sections were then stained with NeuN to verify neuronal cell survival in the striatum. The mean integrated optical density (IOD) of NeuN expression was significantly increased in the striatum of mice treated with APZ 1-5 days following MCAO (Fig. 3). APZ treatment also increased the number of TH-positive dopaminergic cells in the striatum of the same group, although this increase was not significant (Fig. 3). These results indicate that APZ may have a neuroprotective effect on dopaminergic neuronal cells, protecting them from damage caused by ischemic assaults.

Effect of APZ on cell death and activation of microglia. To evaluate apoptosis, TUNEL and TH double staining was performed. Staining indicated that the vehicle group exhibited a significantly increased number of apoptotic cells compared with the control group (Fig. 4A and B). However, significantly fewer TUNEL/TH-positive cells were detected in the mice treated with APZ 1-5 days following MCAO compared with the vehicle group (Fig. 4B). To better understand the activation of microglia in damaged striatum, Iba1 and CD68 double staining was performed. The number of activated microglia exhibiting Iba1/CD68 double-positive expression were decreased in mice treated with APZ 1-5 days following MCAO compared with the vehicle group (Fig. 4A and C). To investigate the neuroprotective effects of the dopamine receptor, brain sections were stained using the CaMKIIδ antibody, which regulates Ca^{2+}-mediated neuronal activities in the brain. The number of CaMKIIδ-positive cells in the striatum was decreased following MCAO, but increased following APZ treatment administered 1-5 days following MCAO (Fig. 4C). These results indicate that APZ may reduce the dopaminergic neuronal cell death and microglial activation caused by ischemic assaults in the striatum, while enhancing CaMKIIδ expression.

Discussion

The present study evaluated the neuroprotective effects of APZ, an atypical antipsychotic drug, in a mouse model of ischemic stroke. The results indicated that APZ induces the functional recovery of neurological deficit caused by ischemia and reduces the atrophic changes in the striatum of the brain. Additionally, APZ treatment reduced dopaminergic neuronal cell injury and neuroinflammation in the striatum, while enhancing CaMKIIδ expression, indicating that APZ enhances neuroprotection.

Atypical antipsychotics are associated with a lower risk of all-cause mortality and extrapyramidal symptoms. However, certain atypical antipsychotics induce a higher risk of stroke compared with conventional antipsychotics (18). In previous studies, APZ treatment has been associated with a lower risk of cardiovascular morbidity and mortality (19), while inducing positive effects following multiple episodes of schizophrenia (4,20,21). Therefore, the present study hypothesized that APZ treatment following ischemic assaults may enhance functional recovery via neuroprotection.

APZ exhibits antidepressant effects, which makes it particularly useful for treating complex post-stroke emotional disorders (7,8). APZ has also been demonstrated to recover dopaminergic neuronal cells that serve beneficial roles in protecting against neurodegeneration (8). Additionally, certain antipsychotics including APZ, may slow the neurodegenerative changes that occur in patients with schizophrenia for whom such treatment may be useful (13). Thus, APZ may enhance functional recovery following stroke by protecting neuronal cells.

The present study identified the effect of APZ on behavioral function. Motor function test results were improved following treatment with APZ, particularly when administered 1-5 days following MCAO. When stroke occurs, it causes brain atrophy and the loss of brain cells (22). The degradation of motor function and asymmetry may occur due to the atrophic changes that occur in various regions of the brain. Therefore, the atrophic changes occurring in the brain cortex, corpus callosum,

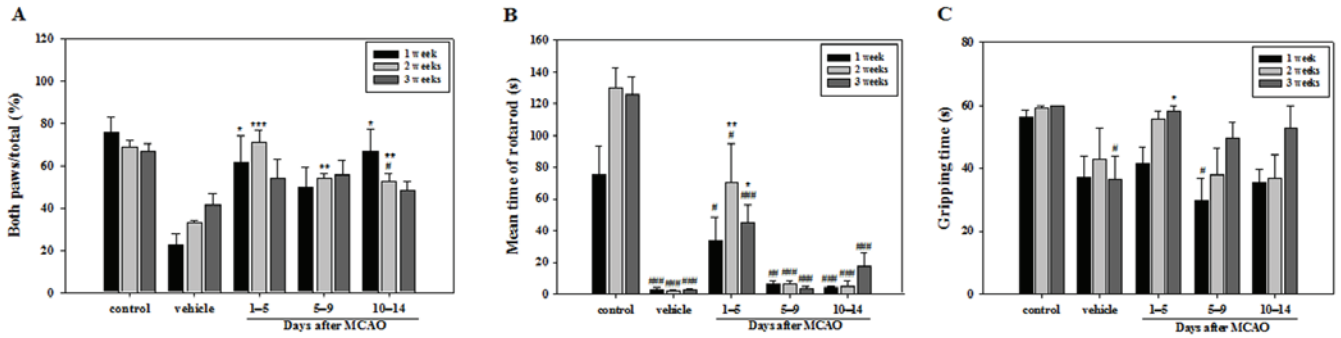

Figure 1. Behavioral test evaluating the motor dysfunction induced by ischemic stroke. (A) Cylinder test; (B) rotarod test and (C) wire suspension test. Data are expressed as the mean ± standard error of the mean. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. control group; *P<0.05, **P<0.01 and ***P<0.001 vs. vehicle group. MCAO, middle cerebral artery occlusion.

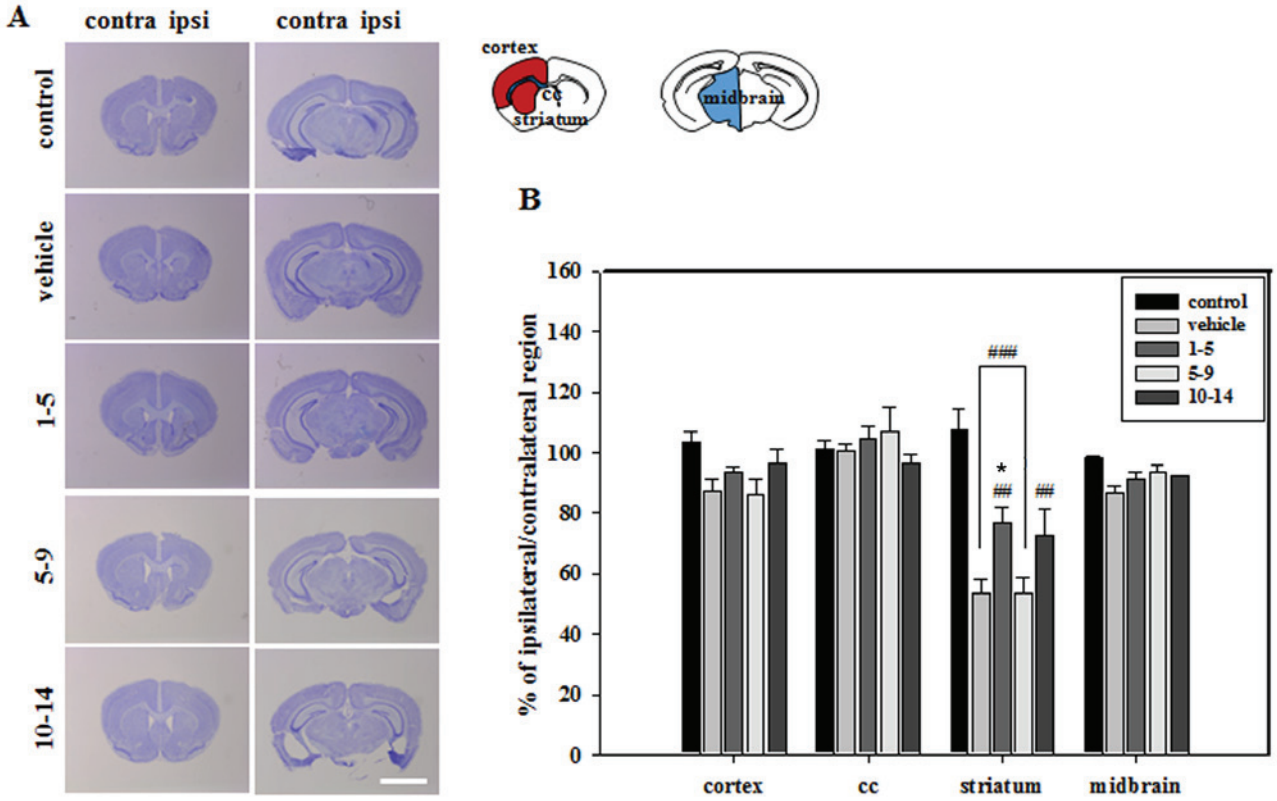

Figure 2. Comparison of ipsilateral/contralateral atrophy volume in each region of the brain. (A) Cresyl violet staining examining the regions of cortex, corpus callosum, striatum and midbrain. (B) Histological analysis of atrophic volume in each region of the brain. $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. control group; *P=0.055 in APZ treatment 1-5 days following MCAO vs. the vehicle group, however this was not significant. Scale bar=2 mm. MCAO, middle cerebral artery occlusion; contra, contralateral; ipsi, ipsilateral; cc, corpus callosum.

striatum and midbrain were analyzed in the present study. Severe atrophic changes in the striatum, the primary lesion site of MCAO, were alleviated with APZ treatment administered 1-5 days following MCAO. This result was similar to that of a previous study, which demonstrated that APZ decreases striatal kainate-induced lesion volumes in rodents (10).

APZ exerts antioxidant effects, meaning that it is highly effective at preventing the cell death that occurs as a result of oxidative stress (23). It has also been demonstated that APZ treatment enhances neurite extension and inhibits cell death in cultured dopaminergic neurons (24). Another dopamine D2/D3 receptor agonist, pramipexole, exhibits protective effects against post-ischemic damage (12). Dopamine is an important neurotransmitter that maintains and controls attention and body movement (11). Therefore, APZ treatment may preserve the survival of dopaminergic neurons. In the present study, the survival of dopaminergic neurons in the striatum was analyzed. Many neuronal cells in the striatum demonstrated NeuN and TH immunoreactions in APZ treated mice compared with vehicle-treated mice, suggesting that APZ exerts a strong protective effect on dopaminergic neuronal cells. However, the results of D2R IOD did not vary with dopamine levels and its variation was very small (data not shown).

Abundant apoptotic cells were detected in the pre-infarction area of mice following ischemic stroke. However,

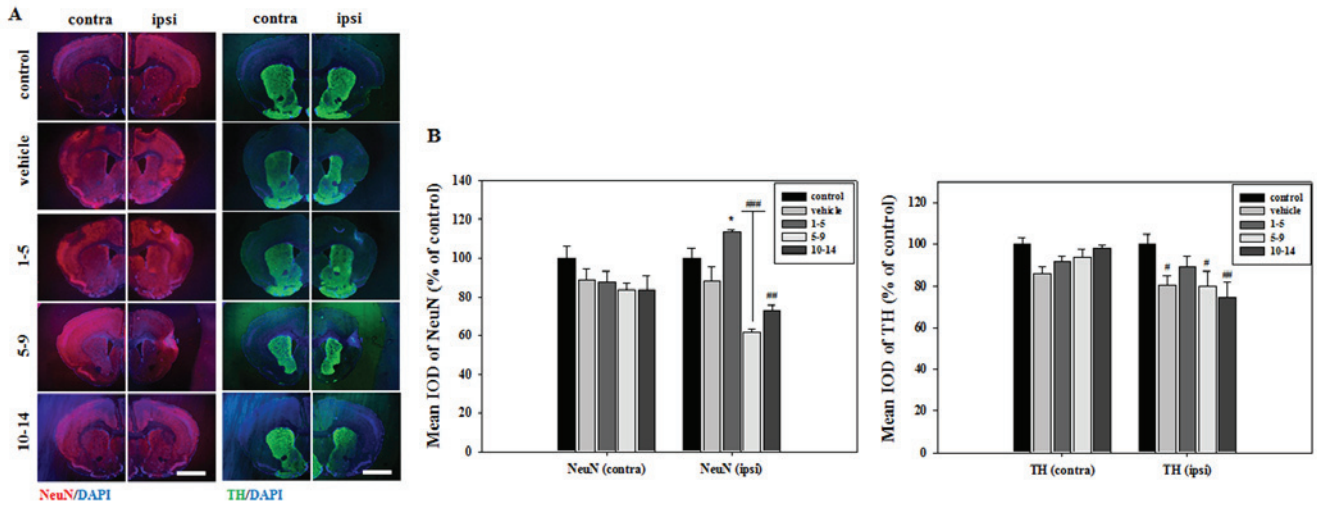

Figure 3. NeuN and TH staining examining dopaminergic neuronal cell survival in the striatum. (A) Photomicrograph for NeuN (red) and TH (green) counter-stained with DAPI (blue). (B) Histogram for mean IOD of NeuN and TH. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. control group; *P<0.05 vs. vehicle group. Scale bar=500 μm. NeuN, neuronal nuclei; TH, tyrosine hydroxylase; IOD, mean integrated optical density; contra, contralateral; ipsi, ipsilateral.

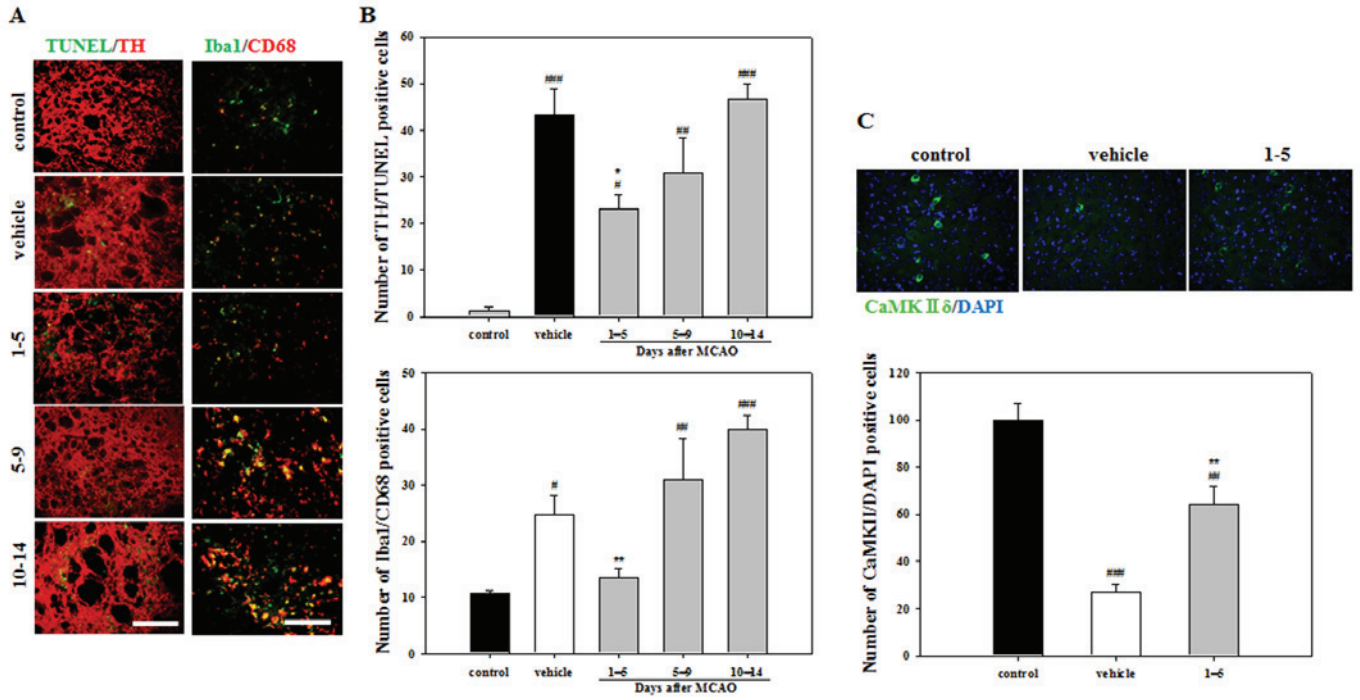

Figure 4. Histopathological analysis of apoptosis and neuroinflammation in the striatum. (A) Photomicrograph for apoptosis marker TUNEL (green) with TH (red) and activated microglia marker CD68 (red) and Iba1 (green) in the striatum. (B) Histogram for the number of TUNEL/TH and Iba1/CD68 double-positive cells. (C) Photomicrograph and histogram for the number of CaMKIIδ/DAPI double-positive cells in the striatum. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. control group; *P<0.05 and **P<0.01 vs. vehicle group. Scale bar=100 μm. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; TH, tyrosine hydroxylase; CaMKIIδ, Ca^{2+}/calmodulin-dependent protein kinase II δ; MCAO, middle cerebral artery occlusion; contra, contralateral; ipsi, ipsilateral; CD 68, cluster of differentiation 68; Iba1, ionized calcium binding adaptor molecule 1.

stroke-induced apoptosis was reduced during APZ treatment administered 1-5 days following MCAO. The chronic stimulation of dopamine D2R by APZ activates CaMKIIδ3, which regulates the transcription of the neurotrophin brain-derived neurotrophic factor (BDNF) by activating various nuclear proteins, including cyclic adenosine 3',5'-monophosphate response element-binding protein (24). CaMKIIδ staining in APZ-treated groups revealed that the number of CaMKIIδ-immunopositive cells were increased compared with the vehicle-treated group, indicating that the increase in BDNF expression induced by CaMKIIδ is involved in the neuroprotective effect of APZ.

Dopamine D2R agonists, including quinpirole and ropinirole, regulate the inflammatory response by alleviating microglia hyperactivity-induced neuroinflammation, thus attenuating brain injury following intracerebral hemorrhage (11,25). It has been demonstrated that DRD2$^{-/-}$ mice exhibit pronounced microglial activation as part of the inflammatory response that

occurs in Parkinson's disease (26). Cerebral ischemia induces the expression of dopamine D2R on activated resident microglia in the brain, which is thought to modulate microglia function during neuroinflammation (27). APZ induces anti-inflammatory effects that occur as following the inhibition of microglial activation (28). Therefore, CD68 and Iba1 double-staining was performed in the present study to evaluate the neuroinflammatory response following treatment with APZ. APZ treatment reduced Iba1/CD68 double-positive cell numbers, indicating that microglial activation was inhibited following stroke.

In conclusion, the present study demonstrated that treatment of ischemic mice with APZ ameliorated various behavioral characteristics of motor dysfunction by inhibiting dopaminergic neuronal cell injury and neuroinflammation. This neuroprotective effect may occur via dopamine D2R stimulation, which may in turn, activate CaMKII. Further studies are required to confirm this hypothesis; other potential mechanisms of APZ action, which may involve agonist and antagonistic activity at serotonin receptors were not assessed. However, the results of the present study provide evidence of APZ-mediated neuroprotection and a novel therapeutic insight into the overall pathogenesis of ischemic stroke.

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