Activation of Dopamine D2 Receptor Suppresses Neuroinflammation Through αB-Crystalline by Inhibition of NF-κB Nuclear Translocation in Experimental ICH Mice Model

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Background and Purpose—Inflammatory injury plays a critical role in intracerebral hemorrhage (ICH)—induced secondary brain injury. Recently, dopamine D2 receptor (DRD2) is identified as an important component controlling innate immunity and inflammatory response in central nervous system, and αB-crystallin (CRY AB) is a potent negative regulator on inflammatory pathways. Here, we sought to investigate the role of DRD2 on neuroinflammation after experimental ICH and the potential mechanism mediated by CRY AB.

Methods—Two hundred and twenty-four (224) male CD-1 mice were subjected to intrastriatal infusion of bacterial collagenase or autologous blood. Two DRD2 agonists quinpirole and ropinirole were administrated by daily intraperitoneal injection starting at 1 hour after ICH. DRD2 and CRY AB in vivo knockdown was performed 48 hours before ICH insult. Behavioral deficits and brain water content, Western blots, immunofluorescence staining, coimmunoprecipitation (Co-IP) assay, and proteome cytokine array were evaluated.

Results—Endogenous DRD2 and CRY AB expressions were increased after ICH. DRD2 knockdown aggravated the neurobehavioral deficits and the pronounced cytokine expressions. DRD2 activation by quinpirole and ropinirole ameliorated neurological outcome, brain edema, interleukin-1β, and monocyte chemoattractant protein-1 expression, as well as microglia/macrophages activation, in the perihematomal region. These effects were abolished by pretreatment with CRY AB siRNAs. Quinpirole enhanced cytoplasmic binding activity between CRY AB and NF-κB and decreased nuclear NF-κB expression. Similar therapeutic benefits were observed using autologous blood injection model and intranasal delivery of quinpirole.

Conclusions—DRD2 may have anti-inflammatory effects after ICH. DRD2 agonists inhibited neuroinflammation and attenuated brain injury after ICH, which is probably mediated by CRY AB and enhanced cytoplasmic binding activity with NF-κB. (Stroke. 2015;46:2637-2646. DOI: 10.1161/STROKEAHA.115.009792.)

Key Words: αB-crystalline ■ dopamine D2 receptor ■ intracerebral hemorrhage ■ neuroinflammation ■ NF-κB

Spontaneous intracerebral hemorrhage (ICH) is a fatal stroke subtype that accounts for ≈15% of all strokes with highest morbidity and mortality rates.1 Numerous studies have demonstrated that pronounced inflammatory reaction play an important role in the secondary brain injury after ICH, including microglial activation and neutrophil infiltration.2–4 After ICH, various stimuli could activate microglia and initiate inflammatory response, such as thrombin and glutamate, which activate microglia and subsequently release proinflammatory cytokines and chemokines to enhance neuroinflammation.2,5 Therefore, the strategy based on inhibition of microglia activation might provide therapeutic potential for ICH.6 Dopamine D2 receptor (DRD2), a member of the rhodopsin-like heptahelical receptor family, is an important target for anti-Parkinsonian drugs that ameliorate the motor deficits.7 Recent studies have demonstrated the presence of dopamine receptors in immune cells8 and in glial cells.9 Increasing evidence indicated a protective role for DRD2 agonists in regulating immune functions and inflammatory reaction, which is probably through inhibiting activated T cell proliferation10 and cytokines secretion.11 Additionally, GLC756, a novel mixed

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dopamine D1R antagonist and D2R agonist, was shown to inhibit the release of tumor necrosis factor-α from activated mast cells.12 Another specific DRD2 agonist, quinpirole, suppressed neuroinflammation and protected against brain injury in a mouse model of Parkinson’s disease.13 However, the expression and immune function of DRD2 in central nervous system after experimental stroke have not been systemically studied.

αB-crystallin (CRYAB) is known as a small heat-shock protein with anti-inflammatory properties.14–16 Although CRYAB is constitutively expressed in the lens of the eye and muscles, numerous studies indicate that it can be induced in both neurodegenerative disorders and in acute brain injury, including multiple scleroses,17 Parkinson’s disease,13 and ischemic stroke.18 Recently, CRYAB has been shown to play a crucial role in DRD2-mediated anti-inflammation effect.13 In the present study, we sought to investigate the potential role and mechanisms of activation of dopamine D2 receptor in neuroinflammation after ICH and explore the potential therapeutic utility of DRD2 agonists in collagenase and autologous blood-induced ICH models.

Materials and Methods

Animals

All animal procedures for this study were approved by the institutional animal care and use committee at Loma Linda University. Eight-week-old male CD1 mice (weight=30 g; Charles River, Wilmington, MA) were housed in a 12-hour light/dark cycle at a controlled temperature and humidity with unlimited access to food and water.

ICH Model

The general procedures for inducing ICH by intrastriatal injection of either bacterial collagenase (0.075 U dissolved in 0.5 μL of PBS) or autologous arterial blood (30 μL) into the basal ganglia were performed as described in previous publications.4,19

Experimental Design

The experiment was designed as follows (Figure I in the online-only Data Supplement).

Experiment I

To determine the time course of DRD2 and CRYAB after ICH, Western blot analysis for DRD2 expression was performed in ipsilateral/right hemisphere of each group at 6 and 12 hours and on days 1, 3, 5, and 7 after ICH insult, n=5 each time point (Figure I in the online-only Data Supplement).

Experiment II

Negative control siRNA (si-NC) or DRD2 siRNA was intracerebroventricularly injected 48 hours before ICH modeling and then followed by quinpirole (5 mg/kg) treatment. Modified Garcia test, forelimb placement test, and brain water content test were performed at 24 hours after ICH, n=6/8 each group. The relative data in sham, ICH+vehicle, and ICH+quinpirole (5 mg/kg) groups were shared with Experiment III. Western blots of ipsilateral hemisphere were conducted at 24 hours after ICH in all groups, n=5 each group (Figure I in the online-only Data Supplement).

Experiment III

For outcome evaluation, mice were randomly divided into 5 groups: sham, ICH+vehicle (sterile saline), ICH+quinpirole (1 and 5 mg/kg), and ICH+ropinirole (5 mg/kg). For treatment, 2 D2 receptor agonist quinpirole (1 or 5 mg/kg) and ropinirole (5 mg/kg) were administered by daily intraperitoneal injection starting at 1 hour after ICH, n=6/8 each group. Neurobehavioral functions (modified Garcia test and forelimb placement test) and brain edema were evaluated at 24 and 72 hours after ICH. In sham, ICH+vehicle, and ICH+quinpirole (5 mg/kg) groups, double immunohistochemistry staining of ionized calcium-binding adaptor molecule 1 (IBA-1) and glial fibrillary acidic protein (GFAP) was also performed, n=4 each group (Figure I in the online-only Data Supplement).

Experiment IV

si-NC or CRYAB siRNA (si-CRYAB) was intracerebroventricularly injected 48 hours before ICH modeling and then followed by quinpirole (5 mg/kg) treatment (Figure I in the online-only Data Supplement).

In addition, intranasal delivery of quinpirole (3 and 15 mg/kg) 1 hour after ICH insult was performed to investigate the clinical translational delivery of quinpirole. Modified Garcia test, forelimb placement test, and brain water content test were performed at 24 hours after ICH, n=6/8 each group. The relative data in sham, ICH+vehicle, and ICH+quinpirole (5 mg/kg) groups, double immunohistochemistry staining of ionized calcium-binding adaptor molecule 1 (IBA-1) and glial fibrillary acidic protein (GFAP) was also performed, n=4 each group (Figure I in the online-only Data Supplement).

Experiment V and VI

The therapeutic benefits of DRD2 agonists were tested in the autologous blood injection ICH model, followed by quinpirole (5 mg/kg) treatment (Figure I in the online-only Data Supplement).

In addition, intranasal delivery of quinpirole (3 and 15 mg/kg) 1 hour after ICH insult was performed to investigate the clinical translational delivery of quinpirole. Modified Garcia test, forelimb placement test, and brain water content test were performed at 24 hours after ICH, n=6/7 each group.

Intranasal Administration of Quinpirole

Intranasal administration was performed as previously reported20: mice were placed on their backs and administered either saline, quinpirole (3 mg/kg), or quinpirole (15 mg/kg) as nose drops (5 μL/drop) over a period of 20 mins, alternating drops every 2 minutes between the left and right nares. For studies of neuroprotection, the total volume delivered was 50 μL 1 hour after initiating ICH, starting at a dose of 3 mg/kg, which is similar to the previously identified protective dose of dopamine by intranasal administration (3 mg/kg).

In Vivo RNAi

Both for DRD2 and CRYAB in vivo RNAi, a set of 3 different Ambion In Vivo siRNA mixture were applied by intracerebral injection as previously described.9 The mouse DRD2–siRNA and CRYAB–siRNA (0.1 nmol, stealth siRNA; Life Technologies) and negative control (0.1 nmol) siRNA were delivered into the ipsilateral ventricle after diluting with Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol.
Neurobehavioral Function Assessment

Neurobehavioral functions were evaluated by a 21-point score system named Garcia test and another 10-point score system named forelimb placement test as previously reported. Two trained investigators who were blinded to animal groups performed the test, and the mean score of the subscales was the final score of each mouse. In blood injection model, the sensorimotor Garcia test has been modified with a maximum neurological score at 12 (healthy animal).

Measurement of Brain Water Content

The brain water content was measured as previously reported.

Hematoma Volume

Hematoma volume was analyzed by hemoglobin assay 24 hours after ICH operation. Ipsilateral hemispheres were homogenized for 60 s in 3 mL of distilled water. The homogenized specimens were centrifuged (30 minutes, 12,000 rcf), and 80 μL of Drabkin’s reagent (Sigma-Aldrich, St Louis, MO) was added into 20 μL of supernatant in the 96-well microplate. After 15 minutes incubation, the hemoglobin absorbance was measured spectrophotometrically at a wavelength of 540 nm and quantified using a standard curve. Results were presented as microliters of blood.

Proteome Profiler Mouse Cytokine Array

Protein expression analysis of sham or DRD2 RNAi group or negative control group (si-NC) was performed 24 hours after ICH insult using the Proteome Profiler Antibody Arrays—Mouse Cytokine Antibody Array, Panel A (R&D Systems, Catalog No. ARY006)—according to the manufacturer’s instructions as previously reported. The antibody array detects the relative levels of 40 different cytokines and chemokines in a single sample.

Western Blotting

Western Blotting was performed as previously described. Nuclear proteins were extracted by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL) following the instruction manual. Primary antibodies used in this study were anti-DRD2, anti-CRYAB (Santa Cruz Biotechnology, Santa Cruz, CA), anti-interleukin (IL)-1β, and anti-MCP-1 (monocyte chemoattractant protein-1), (Abcam, Cambridge, MA). β-actin was used as an internal loading control. The secondary antibodies were all from Santa Cruz Biotechnology; ECL Plus kit (Amersham Bioscience, Arlington Heights, IL).

Co-Immunoprecipitation Assay

A Pierce Co-IP Kit (Thermo Scientific) was used for examination of the change in association between CRYAB and NF-κB p65 in the ipsilateral hemisphere 24 hours after ICH. A general procedure was followed using the manufacturer’s guidelines as previously described. Protein extracts were precipitated by an anti-CRYAB or an anti-NF-κB p65 antibody, and then the precipitated protein was evaluated by Western blot using
anti-NF-κB p65 or an anti-CRY AB antibody. IB assay for CRY AB or NF-κB p65 was used as a loading control.

**Immunofluorescence Staining**
Immunofluorescence staining for brain was performed on fixed frozen section as previously described. Coronal brain sections (10 μm) were obtained with the help of cryostat (Leica CM3050S-3-1-1, Bannockburn, IL) and permeabilized with 0.3% Triton X-100 in PBS for 30 minutes. Sections were blocked with 5% donkey serum for 1 hour and incubated at 4°C overnight with primary antibodies: anti-Iba-1 (Abcam, Cambridge, MA) and anti-DRD2 and anti-GFAP (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with appropriate fluorescence-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) for 2 hours at room temperature. Activated microglia/macrophages were counted in 3 different fields immediately adjacent to the hematoma in at least 3 sections/animal using a magnification of ×400 over a microscopic field of 0.01 mm², and data were expressed as cells/mm². Four mice/group per time point were used for quantification by an observer blinded to the experimental treatment.

**Statistical Analysis**
Analysis was performed using GraphPad Prism software, and all data were expressed as mean±standard error of the mean. Student’s *t* test and 1-way analysis of variance) followed by Scheffe post hoc test were used to compare differences between 2 and ≥3 groups, respectively. Two-way analysis of variance was performed to analyze the effects of multiple comparisons followed by Student–Newman–Keuls test. Statistical significance was defined as *P*<0.05. We used the Kruskal–Wallis 1-way analysis of variance on ranks, followed by the Steel–Dwass multiple comparison tests.

**Results**

**Endogenous Dopamine D2 Receptor Was Upregulated After ICH Injury**
We first investigated whether DRD2 alterations would respond to brain injury after ICH. As shown in Figure 1, DRD2 level (Figure 1A) was significantly increased as early as 24 hours after ICH, with a peak around 72 hours (*P*<0.05), and remained at high levels till day 7 when compared with sham group (*P*<0.05; Figure 1A). Accordingly, CRY AB was also significantly increased from day 1 and reached peak at day 3 (*P*<0.05; Figure 1B). Furthermore, the DRD2 immunoreactivity increased on neurons (D2R/NeuN) (Figure 1B), the resident immune cells, microglia (D2R/Iba-1) (Figure 1C), and astrocytes (D2R/GFAP) (Figure 1D) in the perihemorrhage area 24 hours after ICH.

**DRD2 In Vivo Knockdown Aggravated Neurobehavioral Deficits and Pronounced Inflammatory Response After ICH**
To identify the physiological function of endogenous DRD2 after ICH injury, the in vivo RNAi for DRD2 were performed, and the silencing efficacy was confirmed by Western blot (Figure IIA in the online-only Data Supplement). The animals
pretreated with si-DRD2s developed more severe sensorimotor deficits on modified Garcia test and forelimb placement test compared with the animals in the negative control group (\(P<0.05\) respectively, Figure 2A and 2B). Based on the proteome profiler mouse cytokine array analysis, we obtained a list of 13 upregulated target proteins in ICH groups compared with sham group (Figure 2C and 2D). Of these candidates, significantly increased cytokines (IL-1\(\alpha\), IL-1\(\beta\), IL-16, and tumor necrosis factor-\(\alpha\)) and chemokines (ICAM-1, CXCL9, CXCL10, CXCL11, M-CSF, TIMP-1, and MCP-1) were observed in si-DRD2 groups compared with negative control group (Figure 2D). Of these candidate cytokines, we chose IL-1\(\beta\) and MCP-1 for the following study.

**Dopamine D2 Receptor Agonists Treatment Improved Neurobehavioral Outcomes and Reduced Brain Water Content Both at 24 and 72 Hours After ICH**

Two DRD2 agonists quinpirole (1 or 5 mg/kg) and ropinirole (5 mg/kg) were administered by daily intraperitoneal injection starting at 1 hour after ICH. At 24 hours after surgery, mice subjected to ICH presented a significantly worse Garcia test performance than the sham group (Figure 3A). Moreover, the brain water content was significantly increased in the ICH group at both 24 and 72 hours after ICH compared with the sham group (Figure 3B). The microglia activation was significantly increased in the ICH group compared with the sham group at both 24 and 72 hours after ICH (Figure 3D). These results suggest that dopamine D2 receptor agonists may have neuroprotective effects on ICH.
test performance ($P<0.05$; Figure 3A), as well as increased perihematomal brain water content in the ipsilateral basal ganglia (ipsi-BG; $P<0.05$ versus sham; Figure 3B); however, in both quinpirole 1 mg/kg and 5 mg/kg groups, the Garcia test score were significantly improved ($P<0.05$, respectively, versus vehicle; Figure 3A), and perihematomal brain edema were reduced ($P<0.05$, respectively, versus vehicle; Figure 3B). Another DRD2 agonist ropinirole (5 mg/kg) also significantly improved the Garcia test performance ($P<0.05$ versus vehicle; Figure 3A) and reduced brain water content in ipsi-BG ($P<0.05$ versus vehicle; Figure 3B). Overall, high dosage of quinpirole (5 mg/kg) seems more effective among the pharmacological treatments, and it was used for all following experiments.

We also evaluated neurobehavioral function and brain edema at the delayed stage (72 hours) after ICH using the quinpirole 5 mg/kg. The results demonstrated that quinpirole 5 mg/kg significantly ameliorated neurofunctional deficits and reduced perihematomal brain water content in the ipsi-BG when compared with the vehicle group ($P<0.05$, Figure 3A and 3B).

**Quinpirole Suppressed Microglia Activation and Macrophage Infiltration Both at 24 and 72 Hours After ICH**

To study the potential role of DRD2 in neuroinflammation, we visualized microglia/macrophages recruited to the ICH using Iba-1, a microglia/macrophage marker. Iba-1+ cells showed large cell bodies and short processes in 24 hours after ICH, indicating the transformation toward an activated state. Additionally, activated microglia was observed to be increased slightly and accumulated in the border zone surrounding the hematoma 24 hours after ICH, and more strong intensity of the Iba-1+ cells was observed at 72 hours after ICH (Figure 3C). However, infusion of quinpirole 5 mg/kg led to a dramatic reduction of activated microglia/macrophages, as evidenced by a decreased quantification of Iba-1+ cells in...
the perihematomal region, compared with the vehicle group both at 24 and 72 hours after ICH (P<0.05, 12 fields/group; Figure 3D).

**CRYAB In Vivo Knockdown Abolished the Protective Effect of Quinpirole on Neuroinflammation Inhibition After ICH**

CRYAB in vivo knockdown was performed to investigate the potential role of CRYAB in the protective effects of DRD2 activation; the knockdown efficiency was validated by Western blot (Figure IIB in the online-only Data Supplement). Pretreatment with si-CRYABs sufficiently abolished the protective effect of quinpirole (5 mg/kg) as shown in modified Garcia test (Figure 4A) and brain water content test (Figure 4B) when compared with quinpirole treatment group with or without control siRNAs.

Furthermore, Western blot results showed that the expression of IL-1β and MCP-1 was dramatically increased in ICH group compared with sham. On the contrary, quinpirole 5 mg/kg treatment attenuated the expression of IL-1β and MCP-1 compared with the vehicle group (P<0.05, respectively, Figure 4C and 4D), which was abolished by pretreatment with si-CRYABs, whereas scrambled siRNA did not show those effects (Figure 4C and 4D).

Administration of quinpirole 5 mg/kg and ropinirole (5 mg/kg) did not affect the hemorrhage volume at 24 hours after ICH (P=0.9898; Figure III in the online-only Data Supplement).

**More Cytoplasmic CRYAB Co-Immunoprecipitated With NF-κB p65 in Quinpirole-Treated Mice 24 Hours After ICH**

Co-IP was performed to investigate the physical interaction between CRYAB and NF-κB p65 subunits in the ipsilateral hemisphere after ICH. On Co-IP using CRYAB antibody, more NF-κB p65 were detected in quinpirole group, whereas
si-CRYABs abolished this effect ($P<0.05$, versus vehicle; Figure 5A). On the contrary, Co-IP using NF-κB p65 antibody showed an increase in the CRYAB intensity ($P<0.05$, versus vehicle; Figure 5B), which confirmed the enhanced binding activity between CRYAB and NF-κB p65 after quinpirole treatment (Figure 5A and 5B). However, no change in IkB-a expression (the negative regulator of NF-κB) was seen between vehicle and quinpirole groups (Figure IV in the online-only Data Supplement).

Quinpirole Attenuated NF-κB Nuclear Translocation via Enhanced Cytoplasmic Binding Activity With CRYAB

The nucleic expression of NF-κB p65 was significantly increased at 24 hours after ICH (Figure 5D). Quinpirole preserved the expression levels of NF-κB p65 in cytoplasmic fraction and decreased the nuclear portion of NF-κB p65 levels when compared with the vehicle groups (Figures 5C and 5D). si-CRYABs pretreatment abolished the reduced nuclear transportation compared with the quinpirole group; the scrambled siRNAs did not show those effects (Figure 5C and 5D).

Quinpirole Treatment Reduced the Behavior Deficits and Brain Edema 24 Hours After the Autologous Blood Injection ICH Model

Except testing the therapeutic benefits of DRD2 agonists in the collagenase-induced ICH model, DRD2 agonists were tested also in the autologous blood injection ICH model. The results showed that quinpirole (5 mg/kg)-treated mice performed markedly better in the modified Garcia test compared with the animals in the vehicle group ($P<0.05$; Figure 6A). In addition, quinpirole (5 mg/kg) treatment groups had significantly reduced brain edema accumulations in the ipsi-BG compared with the vehicle group 24 hours after blood injection ICH model ($P<0.05$; Figure 6B).

Intranasal Delivery of Quinpirole Ameliorated Neurological Deficits and Brain Edema at 24 Hours After ICH

To investigate the clinical translational treatment with quinpirole, 2 doses of quinpirole (3 and 15 mg/kg) were administered by intranasal delivery 1 hour after ICH insult. Both quinpirole 3 and 15 mg/kg treatments significantly improved neurobehavioral scores in the Garcia test and forelimb place ment test 24 hours after ICH when compared with the vehicle group ($P<0.05$, respectively; Figure 6C). For brain water content, there was also a significant reduction in the ipsi-BG in both quinpirole 3 and 15 mg/kg groups ($P<0.05$, respectively, versus vehicle; Figure 6D).

Discussion

ICH is a devastating clinical event without effective therapies. Emerging evidence suggests that inflammatory mechanisms are involved in the progression of ICH-induced brain injury. This study demonstrated that DRD2 and its essential downstream protein CRYAB were upregulated in the injured hemisphere after ICH. Exogenous DRD2 agonists alleviated neurological impairment and reduced microglia activation and inflammatory cytokines (IL-1β and MCP-1) production, which were associated with enhanced cytoplasmic binding...
activity between CRYAB and NF-κB and decreased NF-κB nuclear translocation. Additionally, we assessed the therapeutic potential and efficiency of quinpirole by intranasal drug delivery. Taking together, these observations suggested that DRD2 may be involved in controlling innate immunity in the central nervous system. DRD2 agonists may have the potential to reduce neuroinflammation after ICH.

There are studies which indicated a protective role for DRD2 agonists in regulating immune functions and inflammatory reaction. Upregulation of DRD2 was observed in the preinfarction area after ischemic stroke. Most recently, it was reported that the DRD2−/− mice exhibited remarkable inflammatory response with pronounced microglia activation and aberrant inflammatory mediators in Parkinson’s disease. In our study, we observed that DRD2 was upregulated, and its immunoreactivity was increased in microglia in the peri-hemorrhage area after ICH. By DRD2 in vivo knockdown, we observed aggravated neurobehavioral deficits and pronounced edema and cytokines expression, supporting the hypothesis that CRY AB may contribute to the suppression of microglia hyperactivity–related neuroinflammation. Consistent with our results, another DRD2 agonist bromocriptine was also reported to suppress glial inflammation in a model of amyotrophic lateral sclerosis.

CRYAB is a heat-shock protein that exerts cell protection under several stress-related conditions with antineurotoxic and prosurvival properties. Recently, studies demonstrated that CRYAB is a potent negative regulator on inflammatory pathways in both neurodegenerative disorders and acute brain injury models. In our study, pretreatment with CRYAB siRNAs eliminated the protective effects of quinpirole in brain edema and cytokines expression, supporting the hypothesis that CRYAB may contribute to the suppression of neuroinflammation by DRD2 agonists in experimental ICH model. However, molecular mechanism that how CRYAB regulates inflammatory response has not been well understood. NF-κB plays a critical role in the inflammatory response by nuclear translocation and by regulating the transcription of inflammatory genes. CRYAB has been indicated to modulate NF-κB signaling. However, it is still controversial whether CRYAB negatively or positively regulates NF-κB activity. Several studies reported that CRYAB enhanced NF-κB activity. Another study showed that CRYAB-dependent NF-κB activation protected myoblasts from tumor necrosis factor-α–induced cytotoxicity. In addition, another study indicated that CRYAB probably suppresses the inflammatory role of NF-κB in astrocytes during autoimmune demyelination, with the enhancement NF-κB p65 DNA binding activity in Cryab−/− mice. Consistent with this report, we elucidated the mechanism that CRYAB negatively modulated NF-κB activity by directly binding with cytoplasmic NF-κB p65 by using Co-IP method. In addition, we also found enhanced cytoplasmic binding activity between CRYAB and NF-κB p65 in quinpirole-treated group, which reduced its nuclear transportation and the transcription of proinflammatory cytokines. These observations may help to explain how DRD2 activation could suppress neuroinflammation after ICH injury.

One important issue is how DRD2 agonists suppressed the microglia activation? Previous studies and our own results demonstrated that DRD2 was expressed on several cell types, not only microglia but also neuron and astrocyte. In this study, we demonstrated that the dopamine agonists directly activated DRD2 on microglia and suppressed the CRYAB/NF-κB inflammatory pathway, which initiates the microglia activation. However, we could not exclude a potential indirect suppression on microglia activation by DRD2 through preventing neuron apoptosis or the activation of astrocytes. Further studies are needed to verify this important issue.

In conclusion, the present study demonstrated that DRD2 elevation occurred after ICH in brain tissues, and exogenous DRD2 agonists inhibited neuroinflammation and improved neurological outcome, which was probably mediated by CRYAB and enhanced cytoplasmic binding activity with NF-κB. This novel observation indicated an endogenous physiology role of glial DRD2 on microglia innate immunity and neuroinflammation after ICH. DRD2 agonists have potentials to reduce neuroinflammation and decrease secondary brain injury after ICH. It should be noted that previous studies indicated that DRD2 agonists have antiapoptosis property and may be involved in neurogenesis and angiogenesis. Therefore, further studies are needed to elucidate other mechanisms involved in the protective effect of dopamine stimulants.

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Disclosures
None.

References
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Supplementary Figure I. Experimental design and animal group classification. cICH = collagenase induced Intracerebral hemorrhage; bICH = blood induced Intracerebral hemorrhage; WB = Western blotting; DRD2 = dopamine D2 receptor; CRYAB = αB-crystalline; QUIN = Quinpirole; ROPi = Ropinirole.
Supplementary Figure II. Protein expression of DRD2 and CRYAB after in vivo knockdown. Representative bands and quantitative analysis of DRD2 (A) and CRYAB (B) expressions in ipsilateral (Ipsi) and contralateral (Contra) hemisphere of brain specimen at 24 hours after ICH. Relative densities of each protein have been normalized against the sham group. Si-NC indicates negative control siRNAs; si-DRD2, DRD2 siRNA mixtures; si-CRYAB, CRYAB siRNA mixtures; n=5, *: vs si-NC, P<0.05.
Supplementary Figure III. The hemorrhage volume between vehicle and treatment group. n=5 each group, ns indicate no significant difference, one-way ANOVA.
Supplementary Figure IV. The expression of IκB-α after CRYAB knock down 24 hours post ICH. Western blot bands and quantitative analyses of IκB-α from ipsilateral hemisphere after ICH. n=5, ns indicate no significant difference, one-way ANOVA.