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## Focal Cerebral Ischemia and Reperfusion Induce Brain Injury through $\alpha 2\delta$ -1–Bound NMDA Receptors

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### Abstract

**Background and Purpose:** Glutamate *N*-methyl-*D*-aspartate receptors (NMDARs) play a major role in the initiation of ischemic brain damage. However, NMDAR antagonists have no protective effects in stroke patients, possibly because they impair physiological functions of NMDARs.  $\alpha 2\delta$ -1 (encoded by *Cacna2d1*) is strongly expressed in many brain regions. We determined the contribution of  $\alpha 2\delta$ -1 to NMDAR hyperactivity and brain injury induced by ischemia and reperfusion.

**Methods:** Mice were subjected to 90 min of middle cerebral artery occlusion (MCAO) followed by 24 h of reperfusion. Neurological deficits, brain infarct volumes, and calpain/caspase-3 activity in brain tissues were measured. NMDAR activity of hippocampal CA1 neurons was measured in an *in vitro* ischemic model.

**Results:** MCAO increased  $\alpha 2\delta$ -1 protein glycosylation in the cerebral cortex, hippocampus, and striatum. Coimmunoprecipitation showed that ischemia rapidly enhanced the  $\alpha 2\delta$ -1–NMDAR physical interaction in the mouse brain tissue. Inhibiting  $\alpha 2\delta$ -1 with gabapentin, uncoupling the  $\alpha 2\delta$ -1–NMDAR interaction with an  $\alpha 2\delta$ -1 C-terminus–interfering peptide, or genetically ablating *Cacna2d1* had no effect on basal NMDAR currents but strikingly abolished oxygen-glucose deprivation-induced NMDAR hyperactivity in hippocampal CA1 neurons. Systemic treatment with gabapentin or  $\alpha 2\delta$ -1 C-terminus–interfering peptide or *Cacna2d1* genetic knockout reduced MCAO-induced infarct volumes, neurological deficit scores, and calpain/caspase-3 activation in brain tissues.

**Conclusions:**  $\alpha 2\delta$ -1 is essential for brain ischemia-induced neuronal NMDAR hyperactivity, and  $\alpha 2\delta$ -1–bound NMDARs mediate brain damage caused by cerebral ischemia. Targeting  $\alpha 2\delta$

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Disclosures  
None.

–1–bound NMDARs, without impairing physiological  $\alpha 2\delta$ –1–free NMDARs, may be a promising strategy for treating ischemic stroke.

## Keywords

neuroprotection; gabapentinoids; pregabalin; glycosylation; calpain; NMDA receptor

## Introduction

Glutamate is the major excitatory neurotransmitter in the central nervous system and plays an essential role in excitatory synaptic transmission and synaptic plasticity. After brain ischemia, however, glutamate accumulates rapidly at synapses, resulting in extensive stimulation of its *N*-methyl-*D*-aspartate receptors (NMDARs) that can eventually be toxic to neurons.<sup>1,2</sup> Excessive and prolonged activation of NMDARs induces a large amount of  $\text{Ca}^{2+}$  influx through the NMDAR channels. Intracellular  $\text{Ca}^{2+}$  overload can subsequently trigger several downstream lethal reactions including proteolysis of key cellular proteins, resulting in irreversible neuronal death in the brain.<sup>3–6</sup> Thus, NMDARs have long been considered a therapeutic target for ischemic stroke.

Despite abundant preclinical evidence that excessive NMDAR stimulation is a crucial initiating factor for brain damage during ischemic stroke,<sup>2,4</sup> NMDAR antagonists have failed to show neuroprotective efficacy in clinical trials or have produced intolerable adverse effects in stroke patients.<sup>7–10</sup> It has been recognized that blocking NMDARs totally may be detrimental because these receptors also have important physiological functions.<sup>11</sup> For example, the normal activity of NMDARs plays a pivotal role in the maintenance of cortical GABAergic interneurons.<sup>12</sup> However, little is known about how brain ischemia leads to neuronal NMDAR hyperactivity. Identifying the molecular mechanism leading to pathological NMDAR hyperactivity during brain ischemia is essential for developing targeted neuroprotective treatments for stroke patients.

$\alpha 2\delta$ –1 (encoded by *Cacna2d1*) has been considered a subunit of voltage-gated  $\text{Ca}^{2+}$  channels (VGCCs).  $\alpha 2\delta$ –1 is also a binding site of gabapentinoids, including gabapentin and pregabalin, which are clinically used to treat neuropathic pain. We discovered recently that  $\alpha 2\delta$ –1 is a novel interacting protein of NMDARs in the spinal cord and mediates nerve injury-induced synaptic NMDAR hyperactivity.<sup>13</sup>  $\alpha 2\delta$ –1 is strongly expressed in many brain regions, including the cerebral cortex and hippocampus, and is preferentially localized in excitatory neurons.<sup>14,15</sup> However, whether  $\alpha 2\delta$ –1–bound NMDARs play a role in brain injury caused by ischemia/reperfusion remains unknown. In this study, we used a mouse model of middle cerebral artery occlusion (MCAO) to determine the contribution of  $\alpha 2\delta$ –1 to ischemia/reperfusion-induced brain injury. We showed that  $\alpha 2\delta$ –1 is essential for ischemia-induced neuronal NMDAR hyperactivity and that uncoupling the  $\alpha 2\delta$ –1–NMDAR interaction protects against ischemia/reperfusion-induced brain injury without impairing physiological NMDAR activity. Thus,  $\alpha 2\delta$ –1–bound NMDARs may be targeted for treating ischemic stroke.

## Materials and Methods

This article adheres to the American Heart Association Journals implementation of the Transparency and Openness Promotion Guidelines. Details of Methods are available in the online-only Data Supplement. The data that support the findings of this study are available from the corresponding author on reasonable request.

### Animal model.

All procedures and protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center. Adult male wild-type (WT) and conventional *Cacna2d1* knockout (KO) mice (25–30 g, 8–10 weeks of age) were used in our study. We used the filament model with 90 min of MCAO in mice, as described previously.<sup>16,17</sup> The neurological deficits of mice subjected to MCAO were evaluated using a modified Longa test on a scale of 0–5, as reported previously.<sup>17,18</sup> The brain coronal sections were stained using 1% 2,3,5-triphenyltetrazolium chloride (TTC), and the stained sections were captured as digital images and quantified for infarct volume.

### Brain slice preparation and recording.

The tissues containing the hippocampus were sliced sagittally (300  $\mu\text{m}$  thick) using a vibratome. Oxygen-glucose deprivation (OGD) was induced by perfusing brain slices with aCSF saturated with 95%  $\text{N}_2$ /5%  $\text{CO}_2$  and by replacing the 11 mM glucose in aCSF with 11 mM sucrose.<sup>19</sup> NMDAR currents of hippocampal CA1 pyramidal neurons were recorded by puff application of 100  $\mu\text{M}$  NMDA directly to the recorded neuron.

### Western immunoblotting and coimmunoprecipitation.

These assays were performed using standard methods described in the Supplemental Information.

## Results

### $\alpha 2\delta$ -1 and its interaction with NMDARs are essential for the OGD-induced increase in neuronal NMDAR activity

Although increased NMDAR activity has been implicated in neuronal injury during cerebral ischemia, the underlying mechanisms remain unclear. We thus determined the potential role of  $\alpha 2\delta$ -1 in ischemia-induced NMDAR hyperactivity in the brain. Because MCAO causes severe neuronal damage, it is difficult to record NMDAR activity in and around the ischemic region. We therefore used hippocampal brain slices subjected to oxygen-glucose deprivation (OGD) to simulate cerebral ischemia. OGD increases NMDAR activity in hippocampal CA1 pyramidal neurons, which are highly susceptible to ischemic injury.<sup>19,20</sup> OGD induction for 5 min induced a large increase in the amplitude of inward NMDAR currents in WT mice (**Figure 1A and 1B**). Gabapentin binds primarily to  $\alpha 2\delta$ -1 and is a clinically used  $\alpha 2\delta$ -1 inhibitory ligand.<sup>21,22</sup> Treatment with gabapentin (30  $\mu\text{M}$  or 100  $\mu\text{M}$  for 60 min) had no effect on the basal puff NMDAR currents of hippocampal CA1 pyramidal neurons before OGD. Strikingly, the OGD-induced increase in the puff NMDAR currents was abolished in the presence of 30  $\mu\text{M}$  or 100  $\mu\text{M}$  gabapentin (**Figure 1A and 1B**).

$\alpha 2\delta$ -1 interacts with NMDARs predominantly through its C-terminal domain, and a 30-amino-acid peptide (VSGLNPSLWSIFGLQFILLWLVSRSRHYLW) mimicking the C-terminal domain of  $\alpha 2\delta$ -1 fused with Tat protein (YGRKKRRQRRR;  $\alpha 2\delta$ -1Tat peptide) interrupts the  $\alpha 2\delta$ -1-NMDAR interaction *in vivo*.<sup>13</sup> Treatment with  $\alpha 2\delta$ -1Tat peptide (1  $\mu$ M for 60 min) had no effect on the basal puff NMDAR currents of hippocampal CA1 pyramidal neurons from WT mice. However, OGD failed to increase NMDAR currents in neurons treated with  $\alpha 2\delta$ -1Tat peptide (**Figure 1A and 1B**). Similar treatment with a control peptide, which was a Tat-fused scrambled peptide (FGLGWQPWSLSFYLVWVSGLLSVLHLIRSN), did not alter the OGD-induced increase in the NMDAR currents (**Figure 1A and 1B**).

We used coimmunoprecipitation (co-IP) to determine whether  $\alpha 2\delta$ -1 physically interacts with GluN1, an obligatory subunit of NMDARs, in mouse brain tissues. Co-IP analysis showed that OGD increased the  $\alpha 2\delta$ -1-GluN1 protein complex level but had no effect on the  $\alpha 2\delta$ -1-Cav2.2 association in membrane extracts from brain tissues (**Figure 1C**). Treatment with  $\alpha 2\delta$ -1Tat peptide substantially reduced the  $\alpha 2\delta$ -1-GluN1 interaction but had no effect on the  $\alpha 2\delta$ -1-Cav2.2 association in brain tissues subjected to OGD (**Figure 1D**).

In addition, we used *Cacna2d1* KO mice to validate the critical role of  $\alpha 2\delta$ -1 in OGD-induced NMDAR hyperactivity of hippocampal CA1 pyramidal neurons. *Cacna2d1* KO mice do not show any evident neurological abnormalities although they exhibit reduced pain and reduced spinal NMDAR activity potentiated by nerve injury.<sup>13</sup> The basal NMDAR currents of CA1 neurons were similar in WT and *Cacna2d1* KO mice before OGD induction. In contrast to OGD in the WT mice, OGD failed to affect puff NMDAR currents in CA1 pyramidal neurons in *Cacna2d1* KO mice (**Figure 1A and 1B**). Collectively, these data indicate that  $\alpha 2\delta$ -1, by interacting with NMDARs, is essential for ischemia-induced neuronal NMDAR hyperactivity in the brain.

### Focal cerebral ischemia/reperfusion increases $\alpha 2\delta$ -1 protein glycosylation in the brain

To determine whether focal cerebral ischemia/reperfusion affects  $\alpha 2\delta$ -1 protein levels in brain tissues, we induced 90 min of MCAO followed by 24 h of reperfusion in mice to produce stable infarct volume and neurological impairment. Immunoblotting of the forebrain tissues detected two  $\alpha 2\delta$ -1 protein bands at 140 kDa and 110 kDa in sham control mice but a single 140 kDa band in MCAO mice (**Figure 2A**). The  $\alpha 2\delta$ -1 protein level at 140 kDa in the cerebral cortex, hippocampus, and striatum was significantly higher in the MCAO group than in the sham control group (**Figure 2A**).

Immunoblotting of brain tissues using *Cacna2d1* KO mice confirmed that both 110 kDa and 140 kDa protein bands in control mice are  $\alpha 2\delta$ -1 proteins (**Figure 2B**). Because  $\alpha 2\delta$ -1 is a highly glycosylated protein,<sup>22</sup> we determined whether the MCAO-induced  $\alpha 2\delta$ -1 protein band shift results from increased glycosylation. We treated cortical tissue samples from sham and MCAO mice with 500 units of PNGase F for 60 min.<sup>23</sup> In tissues treated with PNGase F, only a single 110 kDa protein band was detected in both sham and MCAO mice (**Figure 2B**). The GluN1 protein level in brain tissues was similar in WT and *Cacna2d1* KO

mice (**Figure 2C**). The  $\alpha 2\delta-1$  mRNA level did not differ significantly between sham control and MCAO groups (**Figure 2D**). These data suggest that cerebral ischemia/reperfusion increases  $\alpha 2\delta-1$  glycosylation in brain tissues.

### $\alpha 2\delta-1$ Contributes to MCAO-induced neurological deficits and brain infarct

We next used gabapentin to determine the contribution of  $\alpha 2\delta-1$  to brain injury and neurological deficits caused by 90 min of MCAO followed by 24 h of reperfusion in WT mice. We administered gabapentin intraperitoneally (i.p.) either before or after initiating MCAO in separate groups of mice (pretreatment and posttreatment groups). For pretreatment, a single dose of gabapentin (100 mg/kg, i.p.) was injected 15 min before MCAO. For posttreatment, two doses of gabapentin (100 mg/kg, i.p.) were injected: one at 30 min after MCAO and another at 6 h after reperfusion. Saline at the same volume as the gabapentin was administered i.p. in a vehicle group that also received MCAO. Both pretreatment and posttreatment with gabapentin significantly improved neurological deficit scores compared with vehicle-treated MCAO mice (**Figure 3A**). Furthermore, the MCAO-induced brain infarct volume was much smaller in both groups of mice treated with gabapentin than in mice treated with vehicle (**Figure 3B**). These data suggest that  $\alpha 2\delta-1$  contributes significantly to ischemia/reperfusion-induced brain damage and neurological deficits.

### $\alpha 2\delta-1$ Ablation attenuates MCAO-induced neurological deficits and brain injury

To validate the important role of  $\alpha 2\delta-1$  in MCAO-induced brain damage, we induced 90 min of MCAO followed by 24 h of reperfusion in *Cacna2d1* KO mice. The MCAO-induced neurological deficit score ( $1.36 \pm 0.81$  vs.  $2.50 \pm 0.76$ ,  $p = 0.0064$ ) and brain infarct volume ( $11.54 \pm 3.07\%$  vs.  $30.41 \pm 5.08\%$ ,  $p < 0.0001$ ) were much lower in *Cacna2d1* KO mice than in WT mice (**Figure 3C and 3D**). These findings provide unequivocal evidence for the critical role of  $\alpha 2\delta-1$  in ischemia/reperfusion-induced brain injury and neurological impairment.

### $\alpha 2\delta-1$ -bound NMDARs contribute to MCAO-induced neurological deficits and brain infarct

Using mouse brain tissues, co-IP analysis showed that the  $\alpha 2\delta-1$  protein was precipitated by an anti-GluN1 antibody, but not by an irrelevant IgG (**Figure 4A**). To determine whether  $\alpha 2\delta-1$ Tat peptide reduces the  $\alpha 2\delta-1$ -NMDAR interaction in the mouse tissue, we conducted additional co-IP assays using mouse brain slices treated with  $\alpha 2\delta-1$ Tat peptide or a Tat-fused control peptide (1  $\mu$ M for 60 min). Treatment with  $\alpha 2\delta-1$ Tat peptide caused a large reduction in the  $\alpha 2\delta-1$ -GluN1 protein complex in the brain tissues (**Figure 4B**). Furthermore, 15 min of MCAO followed by 2 h of reperfusion significantly increased the  $\alpha 2\delta-1$ -GluN1 complex level in mouse brain tissues (**Figure 4C**).

We then determined whether the  $\alpha 2\delta-1$ -NMDAR interaction is involved in brain injury and neurological deficits caused by MCAO. In this protocol, we treated MCAO-subjected mice with two injections of  $\alpha 2\delta-1$ Tat peptide or control peptide (both 200  $\mu$ g/kg, i.p.): one at 15 min before MCAO and another immediately before reperfusion. Treatment with  $\alpha 2\delta-1$ Tat peptide significantly reduced the neurological deficit score increased by MCAO compared with that in control peptide-treated mice (**Figure 4D**). Furthermore, the brain infarct volume

was much less in mice treated with  $\alpha 2\delta$ -1Tat peptide than in mice treated with the control peptide (**Figure 4D**). These results indicate that  $\alpha 2\delta$ -1-bound NMDARs play a major role in ischemia/reperfusion-induced brain injury and associated neurological deficits.

### **$\alpha 2\delta$ -1 and $\alpha 2\delta$ -1-bound NMDARs mediate MCAO-induced calpain activation in brain tissues**

Cerebral ischemia increases calpain activity, and calpain inhibition is neuroprotective against ischemia-induced brain injury.<sup>24</sup> Spectrin is highly sensitive to proteolysis by calpain, and the stable  $\alpha$ II-spectrin breakdown product (spectrin BD, 150 kDa) is commonly used as a biochemical assay for calpain activation. Immunoblotting analysis showed that compared with the sham control group, 90 min of MCAO followed by 24 h of reperfusion caused a large increase in the spectrin BD level in the cerebral cortex, hippocampus, and striatum in WT mice subjected to MCAO (**Figure 5A and 5B**). Systemic administration of gabapentin in WT mice subjected to MCAO (posttreatment protocol) and *Cacna2d1* KO mice subjected to MCAO significantly reduced the spectrin BD level in all three regions compared with that in vehicle-treated WT mice subjected to MCAO (**Figure 5A and 5B**).

Also, compared with the MCAO group treated with control peptide,  $\alpha 2\delta$ -1Tat peptide treatment (two injections of 200  $\mu$ g/kg, i.p.: one at 15 min before MCAO and another immediately before reperfusion) significantly reduced the spectrin BD level in the cortex, hippocampus, and striatum in WT mice (**Figure 5C and 5D**). Together, these data suggest that  $\alpha 2\delta$ -1, through its interaction with NMDARs, mediates ischemia/reperfusion-induced calpain activation in brain tissues.

### **$\alpha 2\delta$ -1 and $\alpha 2\delta$ -1-bound NMDARs contribute to MCAO-induced caspase-3 activation in brain tissues**

Increased caspase-3 activity usually denotes the cleavage of procaspase-3 to active caspase-3, and inhibition of caspase-3 can protect against ischemic neuronal death.<sup>25</sup> Immunoblotting analysis showed that 90 min of MCAO followed by 24 h of reperfusion substantially increased the cleaved caspase-3 level in the cerebral cortex, hippocampus, and striatum in vehicle-treated WT mice compared with WT sham control mice (**Figure 6A and 6B**). Although systemic treatment with gabapentin in WT mice and the use of *Cacna2d1* KO mice significantly attenuated the MCAO-induced increase in the cleaved caspase-3 level in the cerebral cortex and hippocampus, gabapentin and *Cacna2d1* KO each only slightly attenuated the MCAO-induced increase in the cleaved caspase-3 level in the striatum (**Figure 6A and 6B**).

In addition, systemic treatment with  $\alpha 2\delta$ -1Tat peptide in WT MCAO mice significantly reduced the cleaved caspase-3 level in the cerebral cortex and hippocampus, but not in the striatum, compared with control peptide-treated WT MCAO mice (**Figure 6C and 6D**). These results suggest that  $\alpha 2\delta$ -1-bound NMDARs contribute to ischemia/reperfusion-induced caspase-3 activation in the cortex and hippocampus.

## Discussion

A salient finding of our study is that  $\alpha 2\delta - 1$  is essential for ischemia-induced neuronal NMDAR hyperactivity. Although  $\alpha 2\delta - 1$  is commonly considered a VGCC subunit, quantitative proteomic analysis indicates that  $\alpha 2\delta - 1$  has a weak interaction with VGCCs in the brain tissues.<sup>26</sup> Also, VGCC currents in brain neurons are similar in WT and *Cacna2d1* KO mice.<sup>27</sup> In the present study, we found that  $\alpha 2\delta - 1$  physically interacted with NMDARs in the mouse brain and that ischemia increased the glycosylated form of  $\alpha 2\delta - 1$  proteins and the  $\alpha 2\delta - 1$ -NMDAR association. Because the hippocampal CA1 area is highly vulnerable to ischemic brain damage via glutamate-mediated excitotoxicity, we used hippocampal slices subjected to OGD to simulate ischemia to identify the molecular mechanism responsible for ischemia-induced NMDAR hyperactivity in CA1 neurons. We showed that inhibiting  $\alpha 2\delta - 1$  with gabapentin, interrupting the  $\alpha 2\delta - 1$ -NMDAR interaction with  $\alpha 2\delta - 1$ Tat peptide, or ablating  $\alpha 2\delta - 1$  completely blocked OGD-induced NMDAR hyperactivity in hippocampal neurons. In addition, presynaptic  $\alpha 2\delta - 1$ -NMDAR complexes may be involved in increased glutamate release during brain ischemia. The OGD-induced NMDAR hyperactivity may involve a rapid activity-dependent  $\alpha 2\delta - 1$ -NMDAR interaction and mobilization to the neuronal membrane. Importantly, gabapentin,  $\alpha 2\delta - 1$ Tat peptide, or  $\alpha 2\delta - 1$  ablation did not affect basal NMDAR activity. In a heterologous expression system, gabapentin inhibits NMDAR activity only when  $\alpha 2\delta - 1$  is coexpressed.<sup>13</sup> Thus, our findings suggest that  $\alpha 2\delta - 1$ -bound NMDARs are distinctively involved in brain ischemia-induced abnormal NMDAR hyperactivity.

Another important finding of our study is that  $\alpha 2\delta - 1$ -bound NMDARs play a critical role in brain injury caused by ischemia/reperfusion. We found that pretreatment or posttreatment with gabapentin attenuated both the neurological deficits and brain infarct volumes caused by MCAO. Furthermore, these neuroprotective effects were validated in mice devoid of  $\alpha 2\delta - 1$  in our study. It has been reported that gabapentinoids produce neuroprotective effects in rodent models of cerebral ischemia.<sup>28,29</sup> The neuroprotective effects of gabapentinoids were previously assumed to be mediated by VGCC inhibition. However, gabapentinoids have little effect on VGCC activity.<sup>13,30,31</sup> We showed that  $\alpha 2\delta - 1$ Tat peptide, targeting the C-terminal domain of  $\alpha 2\delta - 1$ , disrupted the  $\alpha 2\delta - 1$ -NMDAR interaction and that  $\alpha 2\delta - 1$ Tat peptide treatment effectively decreased the MCAO-induced neurological deficits and brain infarct volumes. In addition to promoting surface trafficking of NMDARs,  $\alpha 2\delta - 1$  can also reduce the  $Mg^{2+}$  block of GluN2A-containing NMDARs.<sup>13</sup> Thus, treatment with gabapentinoids or  $\alpha 2\delta - 1$ Tat peptide not only attenuates the number of  $\alpha 2\delta - 1$ -bound NMDARs on the membrane surface but also restores the  $Mg^{2+}$  block of NMDAR channels to limit  $Ca^{2+}$  influx.

Our study provides new information about the downstream signaling mechanism involved in the neuroprotective effects of inhibiting  $\alpha 2\delta - 1$ -bound NMDARs during cerebral ischemia/reperfusion. There is a direct link between the early calcium-mediated calpain activation after cerebral ischemia and the subsequent caspase-3 activation.<sup>32</sup> We found that gabapentin,  $\alpha 2\delta - 1$ Tat peptide, or  $\alpha 2\delta - 1$  ablation largely normalized the increased calpain and caspase-3 activity in brain tissues induced by ischemia/reperfusion. Interestingly, we found that these interventions had little effect on the increased caspase-3 activity in the ischemic

striatum. A limitation of our study is that many of the biochemical measurements were made at 24 h after MCAO when the majority of the neurons are dead and their proteins are undergoing degradation. Because striatal infarction is mostly necrotic and occurs rapidly after MCAO induction,<sup>33,34</sup> this may explain why inhibition of  $\alpha 2\delta$ -1-bound NMDARs failed to reverse the increased caspase-3 activity in this location. Our findings suggest that  $\alpha 2\delta$ -1-bound NMDARs play a critical role in the increased calpain/caspase-3 activity in ischemic brain tissues.

Because  $\alpha 2\delta$ -1 is mainly expressed in excitatory neurons,<sup>14</sup> gabapentinoids and  $\alpha 2\delta$ -1Tat peptide may selectively inhibit  $\alpha 2\delta$ -1-bound NMDARs expressed in excitatory neurons and spare the NMDARs present in inhibitory neurons. Such a preferential effect on  $\alpha 2\delta$ -1-bound NMDARs by gabapentinoids and  $\alpha 2\delta$ -1Tat peptide may be highly desirable for treating ischemic stroke since this effect does not interfere with basal  $\alpha 2\delta$ -1-free NMDAR function, which may be an endogenous physiological mechanism for neuronal survival in ischemic brain tissues.<sup>11,35</sup> Gabapentinoids bind to both  $\alpha 2\delta$ -1 and  $\alpha 2\delta$ -2 in the central nervous system.<sup>22</sup> The fact that  $\alpha 2\delta$ -2 is predominantly expressed in inhibitory neurons<sup>14</sup> may account for the adverse central nervous system effects of gabapentinoids. Because the amino acid sequence of  $\alpha 2\delta$ -1Tat peptide is unique to  $\alpha 2\delta$ -1, it is conceivable that  $\alpha 2\delta$ -1 C-terminus-interfering peptides or drugs specifically targeting  $\alpha 2\delta$ -1 may have fewer adverse effects than gabapentinoids. Thus,  $\alpha 2\delta$ -1 C-terminus-interfering peptides or inhibitors could circumvent the negative consequences associated with NMDAR antagonists and could be developed as neuroprotective agents for treating ischemic stroke. Preemptive treatment with gabapentinoids or  $\alpha 2\delta$ -1 competing peptides/inhibitors may also offer neuroprotection in patients undergoing major neurological and cardiac surgeries.

In summary, our study provides substantial new evidence that  $\alpha 2\delta$ -1-bound NMDARs play a major role in brain damage caused by ischemia/reperfusion. This new information advances our understanding of the molecular mechanism of neuronal NMDAR hyperactivity during ischemic stroke.  $\alpha 2\delta$ -1-bound NMDARs are a promising target for the development of novel neuroprotective therapies that could have greater therapeutic windows than general NMDAR antagonists.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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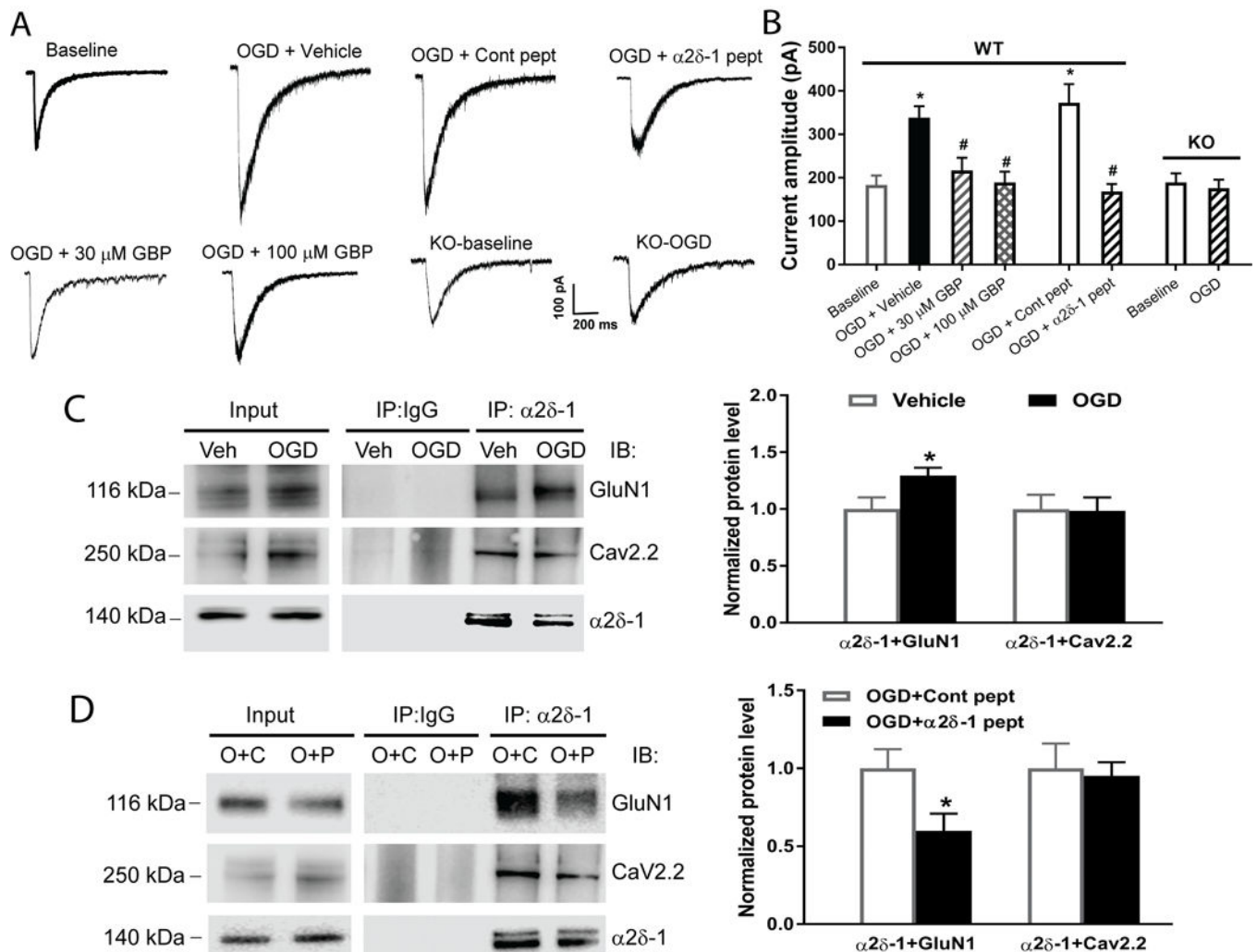
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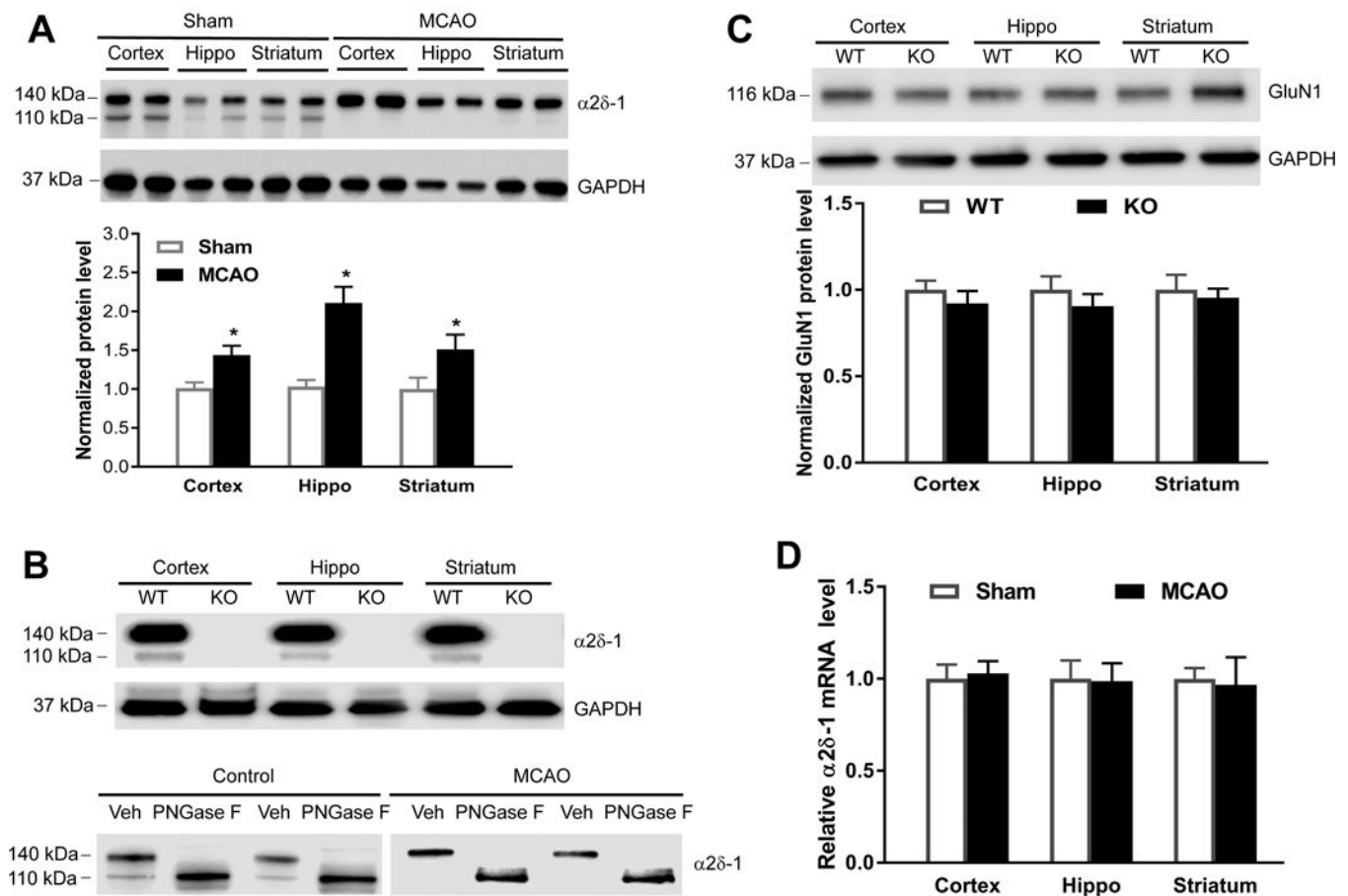
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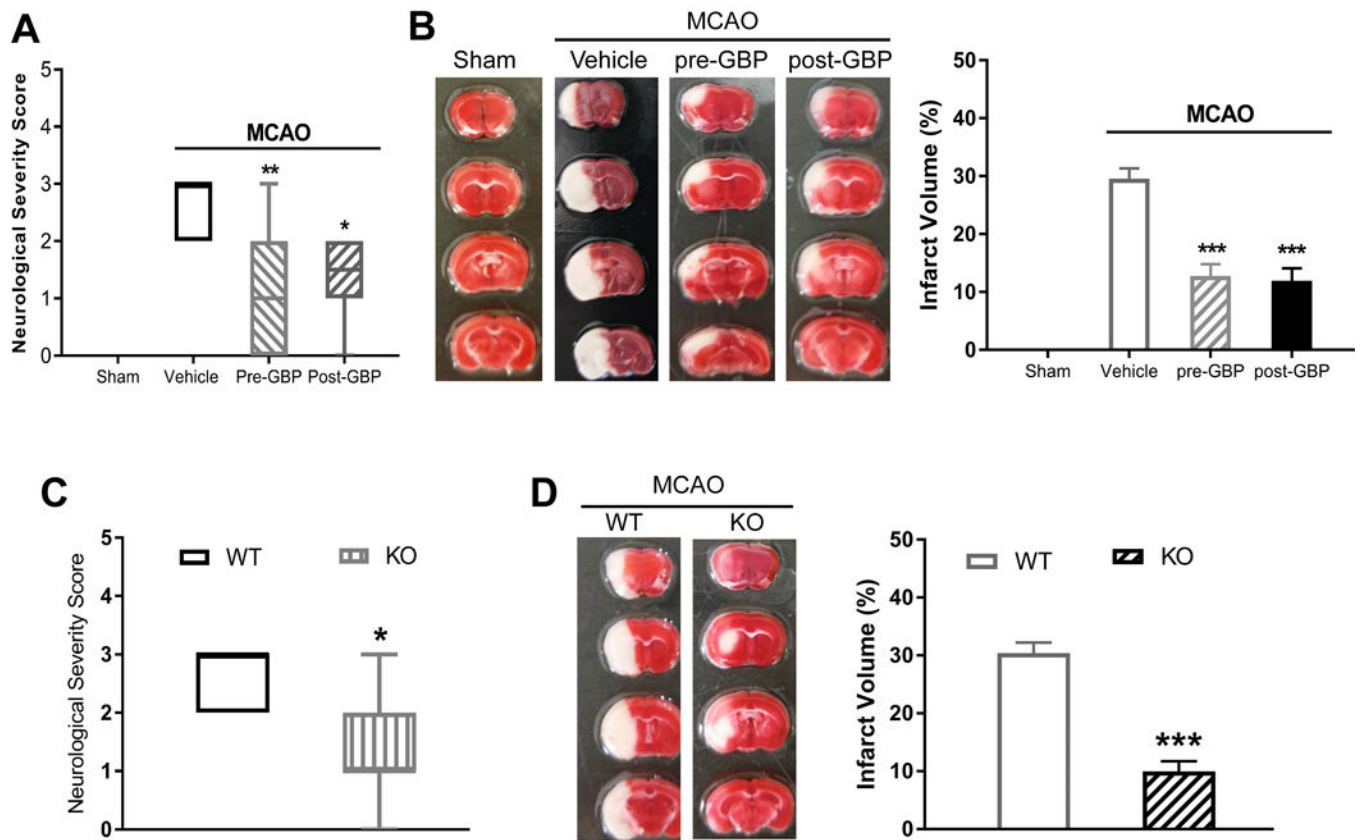
**Figure 1.  $\alpha 2\delta -1$  and its interaction with NMDARs are essential for OGD-induced potentiation of NMDAR activity in hippocampal CA1 neurons.**

(A and B) Representative recording traces (A) and quantification (B) of puff NMDA-elicited NMDAR currents show the effects of gabapentin (GBP, 30  $\mu$ M for 60 min,  $n = 10$  neurons; 100  $\mu$ M for 60 min,  $n = 12$  neurons), vehicle ( $n = 11$  neurons),  $\alpha 2\delta -1$ Tat peptide (1  $\mu$ M for 60 min,  $n = 10$  neurons), control peptide (cont peptide; 1  $\mu$ M for 60 min,  $n = 10$  neurons), and *Cacna2d1* KO ( $n = 12$  neurons for baseline and OGD groups) on NMDAR activity in hippocampal CA1 neurons subjected to 5 min of OGD. (C) Representative gel images and quantification of co-IP show the effect of OGD on the  $\alpha 2\delta -1$ –GluN1 or  $\alpha 2\delta -1$ –Cav2.2 association in mouse brain tissues ( $n = 6$  mice per group). (D) Original gel images and quantification of co-IP show the effect of  $\alpha 2\delta -1$ Tat peptide (P) and control peptide (C; both 1  $\mu$ M for 30 min) on the  $\alpha 2\delta -1$ –GluN1 or  $\alpha 2\delta -1$ –Cav2.2 association in mouse brain slices subjected to OGD (O,  $n = 6$  mice per group). Data are shown as means  $\pm$  SEM. \* $p < 0.05$  compared with the baseline current amplitude before OGD or with the vehicle or control peptide-treated group. # $p < 0.05$  compared with the OGD+vehicle or OGD+control peptide group.

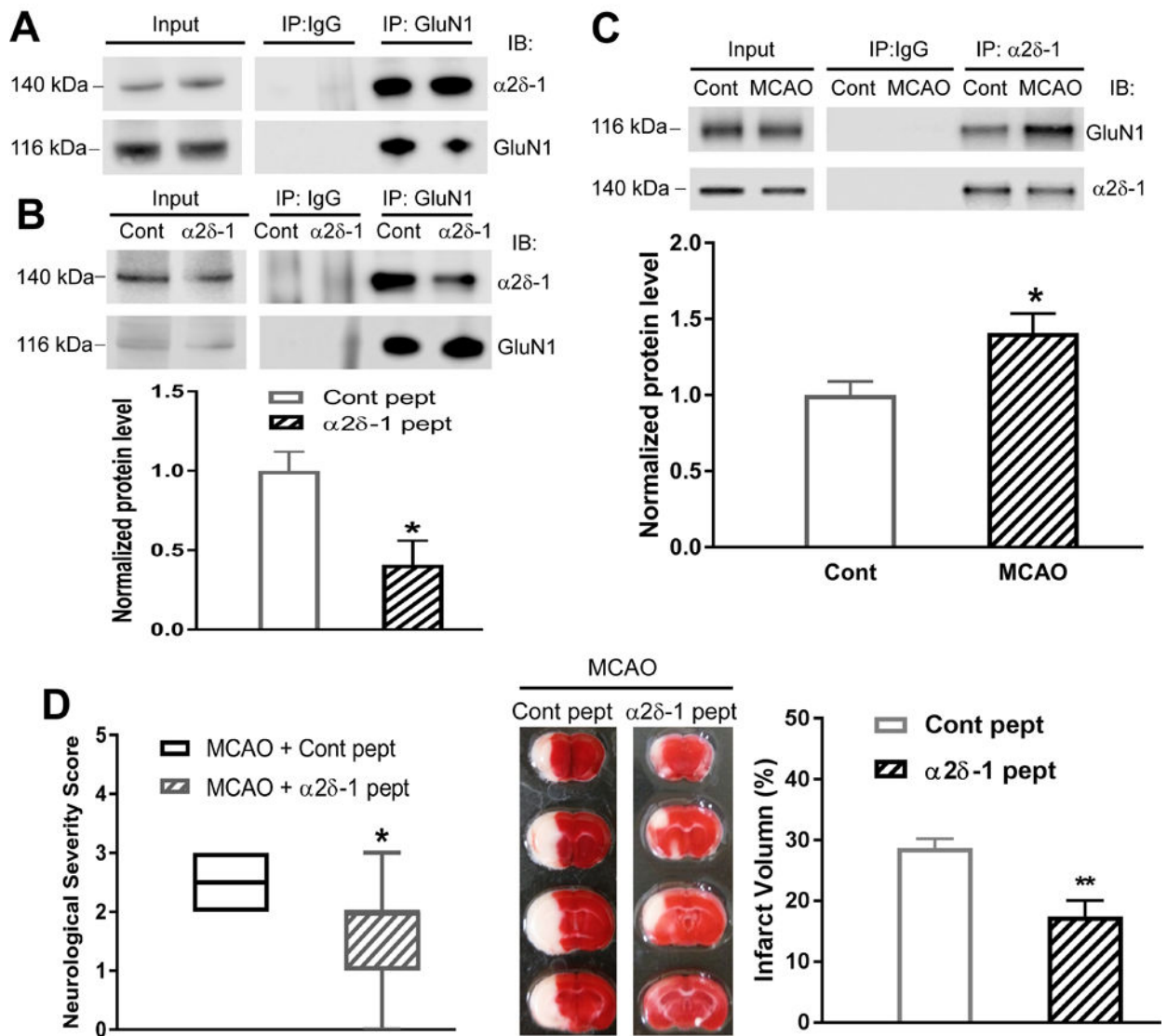


**Figure 2. Focal cerebral ischemia increases the protein level and glycosylation of α2δ-1.**

(A) Representative gel images and quantification of α2δ-1 protein levels in the cerebral cortex, hippocampus (Hippo), and striatum from sham control mice and mice subjected to 90 min of MCAO followed by 24 h of reperfusion (n = 8 mice per group). (B) Upper: Representative gel images of α2δ-1 protein bands in different regions of brain tissues from wild-type (WT) and *Cacna2d1* knockout (KO) mice; Lower: α2δ-1 protein bands detected in 2 sets of cortical tissue lysates from control and MCAO mice treated with PNGase F or vehicle (Veh). (C) Original gel images and quantification of GluN1 protein levels in brain tissues from WT and *Cacna2d1* KO mice (n = 8 mice per group). (D) Quantification of α2δ-1 mRNA levels in the cerebral cortex, hippocampus (Hippo), and striatum in MCAO and sham control mice (n = 6 mice per group). Data are shown as means ± SEM. \**p* < 0.05 compared with the sham group in the corresponding brain region.

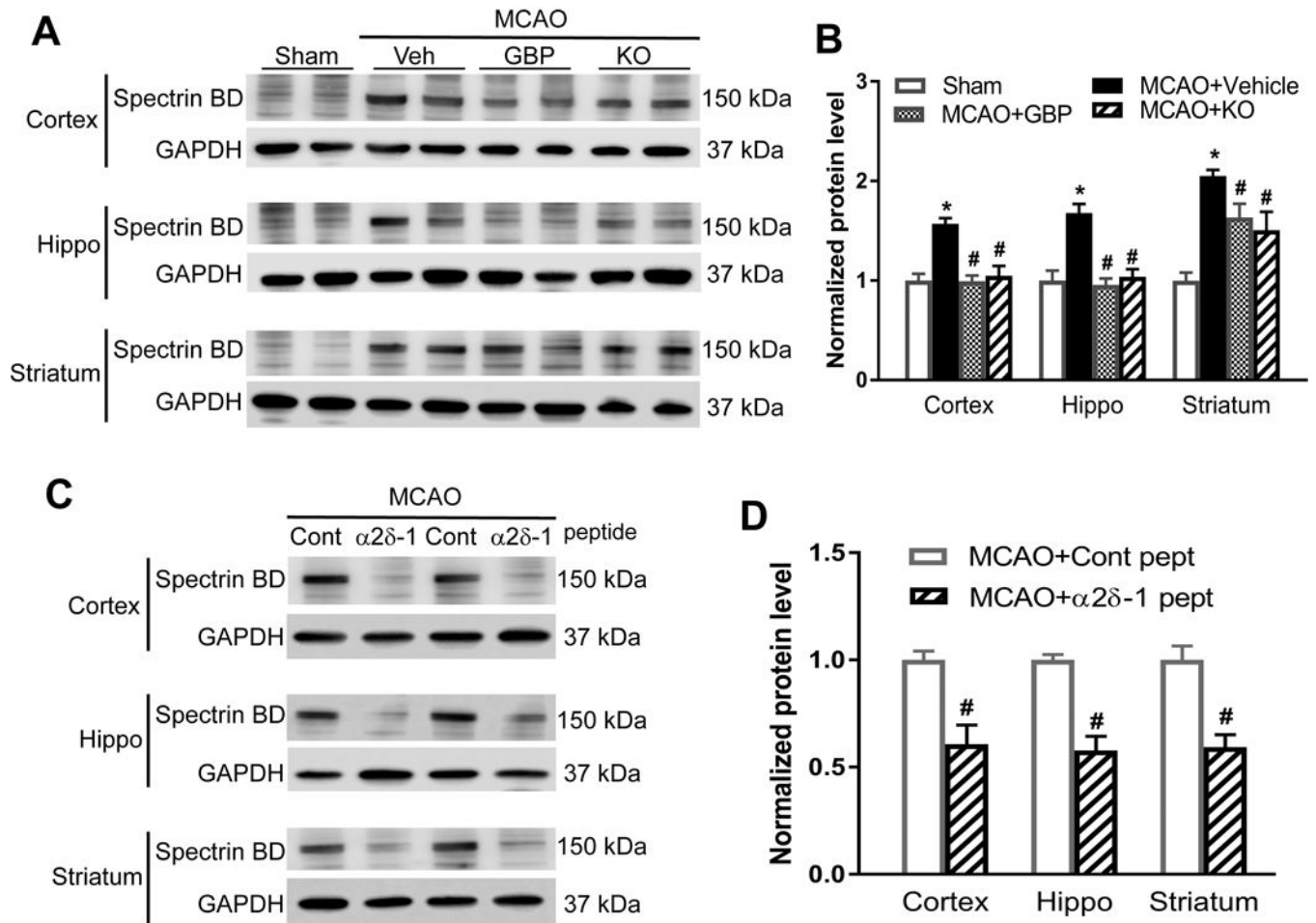


**Figure 3.  $\alpha 2\delta$ -1 critically contributes to brain injury and neurological deficit caused by MCAO.** (A) Neurological deficit scores shown in a box-whisker plot in sham control mice (n = 8 mice) and MCAO mice treated with vehicle (n = 9 mice), gabapentin before MCAO (pre-GBP, n = 10 mice), or gabapentin after MCAO (post-GBP, n = 8 mice). (B) Representative TTC staining and quantification of brain infarct volume in sham control mice (n = 8 mice) and MCAO mice treated with vehicle (n = 9 mice), gabapentin before MCAO (n = 10 mice), or gabapentin after MCAO (n = 8 mice). (C) Neurological deficit scores shown in a box-whisker plot in *Cacna2d1* knockout (KO) mice and wild-type (WT) mice subjected to MCAO and reperfusion (n = 8 mice per group). (D) Representative TTC staining and quantification of brain infarct volume in *Cacna2d1* KO and WT mice (n = 8 mice per group). Data are shown as means  $\pm$  SEM (neurological deficit score data shown as median  $\pm$  min-max). \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 each compared with the vehicle MCAO or WT control group.



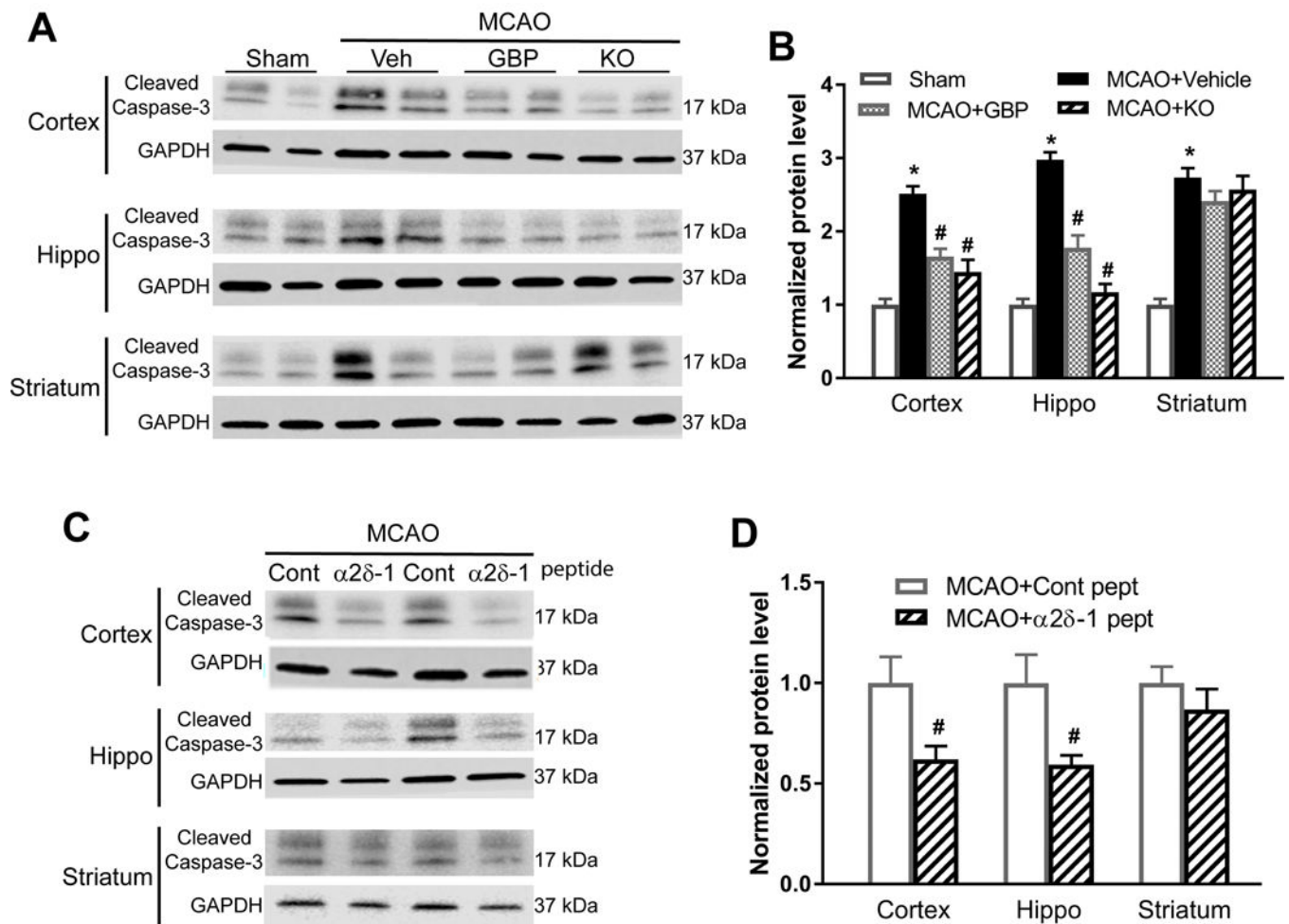
**Figure 4. α2δ-1-bound NMDARs contribute to brain injury and neurological deficit caused by MCAO in the brain.**

(A) Original co-IP gel images show the interaction between α2δ-1 and GluN1 in two samples from mouse brain tissues. (B) Effect of α2δ-1Tat peptide (1 μM for 60 min) on the α2δ-1-GluN1 protein complex in mouse brain tissues (n = 6 mice per group). (C) Representative gel images and quantification of co-IP analysis show the effect of 15 min of MCAO followed by 2 h of reperfusion on the α2δ-1-GluN1 protein complex level in mouse brain tissues (Cont, sham control; n = 6 samples from 12 mice per group). (D) Neurological severity scores shown in a box-whisker plot (left) and representative TTC staining and quantification (right) of brain infarct volume in MCAO mice treated with α2δ-1Tat peptide or control peptide (both 200 μg/kg, i.p.) at 15 min before MCAO and immediately before reperfusion (n = 8 mice per group). Data are shown as means ± SEM (neurological deficit score data shown as median ± min-max). \* $p < 0.05$ , \*\* $p < 0.01$  compared with the control peptide-treated or sham control group.



**Figure 5. Gabapentin,  $\alpha 2\delta$ -1Tat peptide, or  $\alpha 2\delta$ -1 genetic deletion reduces MCAO-induced calpain activity in brain tissues.**

(A and B) Original gel images (A) and quantification (B) of spectrin BD levels in the cerebral cortex, hippocampus (Hippo), and striatum from sham control mice, MCAO mice treated with vehicle (Veh) or gabapentin (GBP), and *Cacna2d1* knockout (KO) MCAO mice (n = 8 mice per group). (C and D) Original blotting images (C, two pairs of samples) and quantification (D) of spectrin BD levels in the cerebral cortex, hippocampus, and striatum from MCAO mice treated with  $\alpha 2\delta$ -1Tat peptide or control peptide (n = 8 mice per group). Data are shown as means  $\pm$  SEM. \* $p < 0.05$  compared with the sham group. # $p < 0.05$  compared with MCAO+vehicle group or control peptide group in the same brain region.



**Figure 6. Gabapentin,  $\alpha 2\delta$ -1Tat peptide, or  $\alpha 2\delta$ -1 ablation suppresses MCAO-induced apoptotic signaling in brain tissues.**

(A and B) Representative blotting images (A) and quantification (B) of cleaved caspase-3 protein levels in the cerebral cortex, hippocampus (Hippo), and striatum from WT sham control mice, WT MCAO mice treated with vehicle (Veh) or gabapentin (GBP), and *Cacna2d1* knockout (KO) MCAO mice ( $n = 8$  mice per group). (C and D) Original gel images (C, two pairs of samples) and quantification (D) of cleaved caspase-3 protein levels in the cerebral cortex, hippocampus, and striatum from MCAO mice treated with  $\alpha 2\delta$ -1Tat peptide or control peptide ( $n = 8$  mice per group). Data are shown as means  $\pm$  SEM.  $*p < 0.05$  compared with the sham group.  $\#p < 0.05$  compared with MCAO+vehicle group or control peptide group in the same brain region.