SUPPLEMTARY MATERIALS

Focal Cerebral Ischemia and Reperfusion Induce Brain Injury through α2δ-1–Bound NMDA Receptors

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Materials and Methods

Animals. All procedures and protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center and were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Adult male wild-type (WT) and conventional *Cacna2d1* knockout (KO) mice (25-30 g, 8-10 weeks of age) were used in our study. *Cacna2d1* KO mice and WT littermates were generated as described previously [1]. Two breeding pairs of *Cacna2d1*$^{+/-}$ mice (C57BL/6 genetic background) were purchased from Medical Research Council Harwell Institute (Oxfordshire, UK), and *Cacna2d1*$^{-/-}$ mice and *Cacna2d1*$^{+/+}$ WT littermates were obtained by breeding the heterozygous mice. All animals were housed in a controlled environment (24 ± 2 ℃, 12 h light and dark cycles) and received standard laboratory food and filtered clean water *ad libitum*. A total 136 mice was used for this study, and 21 mice with a neurological deficit score of 4 or 5 after MCAO were excluded from the study (~17% attrition rate).

Middle cerebral artery occlusion. We used MCAO in mice as an ischemic stroke model, as described previously [2, 3]. Briefly, mice were anesthetized with isoflurane (1.5% in a mixture of 70% NO_2 and 30% O_2). A nylon filament 0.16-mm in diameter (tip diameter, 0.22 mm; Cinontech, Beijing, China) was inserted from the right common carotid artery into the right internal carotid artery and then gently advanced 10 mm to occlude the origin of the right middle cerebral artery. After 90 min of MCAO, the occluding filament was withdrawn for cerebral reperfusion. In the sham-operated group, mice were subjected to similar surgical procedures without MCAO. During the surgery, the body temperature of mice was kept at 37 ± 1 ℃ using a heating pad. After recovery from anesthesia, mice were returned to the animal housing facility until further experiments.

Gabapentin was purchased from Tocris Bioscience (Minneapolis, MN). α2δ-1Tat peptide and scrambled control peptide were synthesized by Bio Basic Inc. (Markham, Ontario, Canada) and validated by liquid chromatography and mass spectrometry. In the mouse models of brain ischemia [4, 5] and neuropathic pain [6, 7], the gabapentin dose is typically used at 100-300 mg/kg for systemic administration. The ability of Tat-fused peptides to enter the brain after systemic injection is well demonstrated [8-10].

Neurological deficit assessment. The neurological deficits of mice subjected to MCAO were evaluated using a modified Longa test on a scale of 0 – 5, as reported previously [3, 11]. The scoring was as follows: 0, no observable neurological deficits; 1, failure to extend left forepaw completely (a mild focal neurological deficit); 2, circling to the left (a moderate focal neurological deficit); 3, falling to the left (a severe focal neurological deficit); 4, not walking spontaneously with a depressed level of consciousness; 5, death due to brain ischemia/reperfusion.

Quantification of brain infarct volume. With mice under anesthesia with 2-3% isoflurane, the forebrains of the mice were removed rapidly, frozen at –20 ℃ for 15 min, and sectioned into four coronal slices (2 mm-thick). The coronal sections were stained using 1% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, St Louis, MO) at 37 ℃ for 20 min. Then the stained sections were captured as digital images and analyzed using Image-J software (National Institutes of Health, Bethesda, MD). The TTC-stained viable brain tissue was dark red, whereas the infarcted tissue was unstained. The corrected infarct volume (%) was calculated using the following formula: (contralateral hemispheric volume – ipsilateral hemispheric non-infarcted volume)/contralateral hemispheric volume × 2 [12].

Brain slice preparation and electrophysiological recording. Brain tissues were removed rapidly from mice anesthetized with 2-3% isoflurane. The tissues containing the hippocampus were sliced sagittally (300 μm thick) using a vibratome (Leica, Wetzlar, Germany) in ice-cold artificial cerebrospinal fluid (aCSF) bubbled continuously with 95% O_2/5% CO_2. The aCSF solution contained (in mM) 126.0 NaCl, 3.0 KCl, 1.2 NaH_2PO_4, 11.0 glucose, 26.0 $NaHCO_3$, 1.5 $MgCl_2$, and 2.4 $CaCl_2$. The brain slices were incubated in a chamber with the aCSF at 33 ℃ for at least 60 min before being used for recording.

Oxygen-glucose deprivation (OGD) was induced by perfusing brain slices with aCSF saturated with 95% N_2/5% CO_2 and by replacing the 11 mM glucose in aCSF with 11 mM sucrose [13]. NMDAR currents of pyramidal neurons in the hippocampal CA1 region were recorded by puff application of 100 μM NMDA directly to the recorded neuron at a holding potential of –60 mV. Puff application of NMDA was performed using a Pressure System (4 p.s.i., 15 ms; Toohey Company, Fairfield, NJ). The puff electrode was placed about 150 μm away from the neuron being recorded. To minimize the magnesium block of NMDARs, the NMDAR currents were recorded in Mg^{2+}-free aCSF solution [14, 15]. The pipette internal solution contained (in mM) 110.0 Cs_2SO_4, 2.0 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 10.0 HEPES, 2.0 MgATP and 0.3 Na_2GTP (pH adjusted to 7.25 with 1.0 M CsOH [280-300 mosM]). Lidocaine N-ethyl bromide (QX-314, 10.0 mM) was added into the internal solution to block voltage-gated sodium channels. Signals were recorded using an amplifier (MultiClamp 700B; Axon Instruments Inc., Union City, CA), filtered at 1 to 2 kHz, and digitized at 10 kHz. In tissue slice studies, 100 μM of gabapentin is commonly used [14, 16, 17].

Western immunoblotting and deglycosylation of α2δ-1 protein. For immunoblotting analysis, the forebrains of sham control and MCAO mice were removed quickly with mice under deep anesthesia with isoflurane. The cerebral cortex, hippocampus, and striatum were dissected separately and homogenized in cold RIPA lysis buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich). After centrifugation (13,000 × g for 20 min at 4 ℃), the supernatants of the samples were collected. Equal amounts (40 μg) of proteins were separated by electrophoresis using 4–20% gradient sodium dodecyl sulfate polyacrylamide gel (Bio-Rad, Hercules, CA), transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA), and incubated in blocking solution (5% nonfat dry milk) for 1 h. The following primary antibodies were used: rabbit anti-α2δ-1 (1:500; #ACC-015, Alomone Labs, Jerusalem, Israel), rabbit anti-

GluN1 (1:1000; G8913, Sigma-Aldrich), mouse anti-Spectrin α chain (1:500; #MAB1622, Millipore Corporation), rabbit anti-cleaved caspase-3 (Asp175) (1:1,000; #9661, Cell Signaling Technology, Danvers, MA), and rabbit anti-GAPDH (1:2,000; #5174, Cell Signaling Technology). The following secondary antibodies were used: anti-rabbit IgG (1:5,000; #111-036-003, Jackson ImmunoResearch, West Grove, PA), and anti-mouse IgG (1:10,000; #115-036-062, Jackson ImmunoResearch). The protein bands were detected with an enhanced chemiluminescence kit (Thermo Fisher Scientific, Waltham, MA), visualized and quantified using an Odyssey Fc Imager (LI-COR, Lincoln, NE), and normalized using the GAPDH band on the same gel.

Cortical tissues from control and MCAO mice were isolated and solubilized in an IP lysis buffer (50 mM Tris [pH, 7.4], 250 mM NaCl, 10% glycerol, 0.5% NP-40, 20 mM NaF, 1 mM Na_3VO_4, 10 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, and 2 mM benzamide) supplemented with a protease inhibitor cocktail. The lysates (20 μg of proteins) were first incubated under denaturing conditions (0.5% SDS and 40 mM DTT) and then treated with 500 units of peptide-N-glycosidase F (PNGase F; New England Biolab, Ipswich, MA) for 60 min at 37 ℃ according to the manufacturer's instructions. Proteins were added to 2 × Laemmli buffer (Sigma-Aldrich), resolved by SDS/PAGE, and analyzed by Western blotting.

Quantitative PCR analysis. The forebrains of sham control and MCAO mice were removed quickly after the mice were deeply anesthetized with isoflurane. Total RNA from the cerebral cortex, hippocampus, and striatum were extracted using TRIsure (Bioline, Taunton, MA). After treatment with RNase-free DNase (QIAGEN, Hilden, Germany), 1 μg RNA was used for reverse transcription with a RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific). Two μl of 5-times diluted cDNA was added to a 20 μl reaction volume with SYBR Green PCR Mix (Thermo Fisher Scientific). Real-time PCR was performed using a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA). The thermal cycling conditions were: 1 cycle at 95 ℃ for 10 min; 40 cycles at 95℃ for 15 s and 60 ℃ for 60 s. The following primers were used: mouse *Cacna2d1* forward, GGGTGGACAAGATGCAAGAAGAC; mouse *Cacna2d1* reverse, TGGCGTGCATTGTTGGGCTC; mouse *Tuba1a* forward, CCACTACACCATTGGCAAGGA GA; and mouse *Tuba1a* reverse, GGAGGTGAAGCCAGAGCCAGT. Relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to the *Tuba1a* level in the same sample.

Coimmunoprecipitation (co-IP). To determine the effect of OGD and α2δ-1Tat peptide on the α2δ-1 interaction with GluN1 and Cav2.2, forebrain tissues were rapidly removed from mice under isoflurane anesthesia and were cut into slices 600 μm thick using a vibratome (Leica). Brain slices were incubated in the aCSF containing 1 μM α2δ-1Tat peptide or 1 μM control peptide for 30 min. The brain slices were dissected and homogenized in ice-cold hypotonic buffer (20 mM Tris [pH 7.4], 1 mM $CaCl_2$, and 1 mM $MgCl_2$) containing 1% protease inhibitor cocktail (Sigma-Aldrich) for membrane preparation. The nuclei and unbroken cells were removed by centrifugation at 1000 × g for 10 min. The supernatant was centrifuged for 30 min at 21,000 × g. The pellets were re-suspended and solubilized in IP lysis buffer and 1% protease inhibitor cocktail. Then the soluble fraction was incubated with Protein G agarose beads (#16-266, Millipore Corporation) prebound to 2 μg of mouse anti-α2δ-1 antibody (1:1,000; #sc-271697, Santa Cruz Biotechnology, Dallas, TX) at 4 ℃ overnight. Protein A/G beads prebound to mouse IgG were used as controls. The protein-bead complex was then washed five times, collected by centrifugation, and then immunoblotted. The following antibodies were selected for immunoblotting: rabbit anti-α2δ-1 antibody (1:500, #ACC-015, Alomone Labs), rabbit anti-GluN1 (1:1,000; #G8913, Sigma-Aldrich), and rabbit anti-Cav2.2 (1:200, #681505, Millipore Corporation).

Data collection and statistical analysis. The neurological deficit score data were presented in box-and-whisker plots, which show the minimum, 25th percentile, median, 75th percentile, and maximum values. All other data were expressed as means ± SEM. Although no statistical methods were used to predetermine sample sizes for the studies, our sample sizes were similar to those generally used in the field. Data collection was randomized (1:1 allocation), and the investigators assessing behavior and measuring brain infarct volume were blinded to the experimental treatments. For proper exclusion of data points, the exclusion criteria were established before data collection. Mice with a neurological deficit score of 4 or 5 after MCAO were excluded from analysis. In electrophysiological recording experiments, only one neuron was recorded in each brain slice, and at least three mice were used in each group. We monitored cell capacitance, input resistance, series resistance, and baseline holding current; we excluded cells if the recording indicated a rundown condition. The amplitude of NMDAR currents was analyzed using Clampfit 9.2 software (Molecular Devices). Two-tailed Student's *t* tests were used to compare two groups, and one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's post hoc test was used to determine the differences between more than two groups. We used non-parametric tests (Mann-Whitney test and Kruskal-Wallis test) to compare neurological deficit scores between groups. Statistical analysis was performed using Prism 7 software (GraphPad Software Inc., La Jolla, CA). $P < 0.05$ was considered to be statistically significant.

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Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

Methodological and Reporting Aspects	Description of Procedures
Experimental groups and study timeline	☑ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. ☑ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated. ☑ An overall study timeline is provided.
Inclusion and exclusion criteria	☑ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.
Randomization	☑ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. ☑ Type and methods of randomization have been described. ☑ Methods used for allocation concealment have been reported.
Blinding	☑ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. ☑ Blinding procedures have been described with regard to masking of group assignment during outcome assessment.
Sample size and power calculations	☑ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.
Data reporting and statistical methods	☑ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. ☑ Baseline data on assessed outcome(s) for all experimental groups have been reported. ☑ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. ☑ Statistical methods used have been reported. ☑ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.
Experimental details, ethics, and funding statements	☑ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. ☑ Different sex animals have been used. If not, the reason/justification is provided. ☑ Statements on approval by ethics boards and ethical conduct of studies have been provided. ☑ Statements on funding and conflicts of interests have been provided.