NCAM mimetic peptide P2 synergizes with bone marrow mesenchymal stem cells in promoting functional recovery after stroke

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Running headline: P2 synergizes BMSCs in treatment of stroke

Abstract

The neural cell adhesion molecule (NCAM) promotes neural development and regeneration. Whether NCAM mimetic peptides could synergize with bone marrow mesenchymal stem cells (BMSCs) in stroke treatment deserves investigation. We found that the NCAM mimetic peptide P2 promoted BMSC proliferation, migration, and neurotrophic factor expression, protected neurons from oxygen-glucose deprivation through ERK and PI3K/AKT activation and anti-apoptotic mechanisms *in vitro*. Following middle cerebral artery occlusion (MCAO) in rats, P2 alone or in combination with BMSCs inhibited neuronal apoptosis and induced the phosphorylation of ERK and AKT. P2 combined with BMSCs enhanced neurotrophic factor expression and BMSC proliferation in the ischemic boundary zone. Moreover, combined P2 and BMSC therapy induced nuclear translocation of factor erythroid 2-related factor, up-regulated heme oxygenase-1 expression, reduced infarct volume, and increased functional recovery as compared to monotreatments. Treatment with LY294002 (PI3K inhibitor) and PD98059 (ERK inhibitor) decreased the neuroprotective effects of P2 in oxygen-glucose deprivation neurons and in MCAO rats receiving P2 and BMSC therapy. Collectively, P2 is neuroprotective while P2 and BMSCs work synergistically to improve functional outcomes after ischemic stroke, which may be attributed to mechanisms involving

enhanced BMSC proliferation and neurotrophic factor release, anti-apoptosis, and PI3K/AKT and ERK pathways activation.

Keywords: Ischemic stroke, Bone marrow mesenchymal stem cells, Neural cell adhesion molecule, NCAM mimetic peptide, Middle cerebral artery occlusion

Introduction

Despite the massive personal, societal, and economic burden of stroke, effective restorative treatments are not yet available. Currently, the only validated therapeutic intervention for ischemic stroke is timely recanalization by thrombolysis or mechanical thrombectomy.[1] However, the number of patients benefitting from those interventions remains very low despite continues progress in the field.

Stem cell-based approaches have received much attention as potential treatments for a wide variety of diseases, including stroke.[2, 3] Preclinically, transplantation experiments using bone marrow mesenchymal stem cells (BMSCs) improve functional recovery by a broad spectrum of mechanisms.[4-7] Mesenchymal stem cell therapy appears to be clinically safe,[8] however, the efficacy of the treatment is less apparent.[9] A comprehensive analysis of several clinical studies showed that there was neither lower mortality nor improvement in National Institute of Health Stroke Scale scores after BMSC therapy in ischemic stroke patients.[3] This indicates that effect sizes of BMSC treatments in human patients might be considerably lower than in animal models. Enhancing the efficacy of BMSC treatments, for instance by designing synergistic combination therapies, is therefore a valuable approach. Indeed, a few studies combining BMSC administration with pharmacological agents have shown improved therapeutic effects.[10, 11]

The neural cell adhesion molecule (NCAM) plays a pivotal role in neural development, synaptic plasticity, and regeneration of damaged neural tissue.[12, 13] NCAM homophilic (NCAM-NCAM) and heterophilic (NCAM-other receptors) binding induce cellular reactions by activating a series of intracellular signaling cascades, most notably the Ras-mitogen-activated protein kinase (MAPK) pathway.[14] NCAM mimetic peptides such as C3, plannexin, FGL, P2, and others are functional fragments of NCAM. Preclinical studies indicate that NCAM mimetic peptides may be successfully used to treat neurological disorders including traumatic brain injury and Alzheimer's disease.[12, 15, 16] NCAM mimetics have displayed neuritogenic and neuroprotective capabilities, as well as synaptic modulation properties *in vitro* and *in vivo*.[17] P2, a 12-amino-acid peptide derived from the second immunoglobulin-like (Ig) module of NCAM, is a potent NCAM agonist. It is a particularly promising therapeutic candidate that is capable of promoting neuronal differentiation and survival.[18, 19] P2 was shown to activate the MAPK/extracellular signal regulated kinase (ERK) pathway, inducing neurite extension in primary rat hippocampal neurons.[19] There is also preliminary evidence that P2 exerts neuritogenic and neuroprotective effects in primary dopaminergic and cerebellar neurons through the phosphatidylinositol-3-hydroxykinase (PI3K)/protein kinase B (AKT) pathway.[18] Both MAPK and PI3K pathways can promote the activation and nuclear translocation of factor

erythroid 2-related factor (Nrf2)[20, 21] to increase the expression of downstream target heme oxygenase-1 (HO-1) to counteract oxidative damage.[22] However, it is currently unclear whether P2 may act synergistically with BMSCs to improve stroke recovery, and whether this may be mediated through ERK or PI3K/AKT pathways.

In the present study, we uncovered the role of P2 on BMSC proliferation, migration, and neurotrophic factor production as well as its neuroprotective effects on ischemia-challenged neurons. We also showed enhanced therapeutic effects of a combined P2-BMSC treatment for acute ischemic stroke in rats. We further demonstrated that these effects are potentially mediated by PI3K/AKT and ERK pathways.

Materials and Methods

Peptide

The P2 peptide, GRILARGEINFK, was synthesized as a dendrimer coupled to a lysine backbone[19] by Scilight Biotechnology LLC (Beijing, China). For subcutaneous injection, P2 was dissolved in normal saline (NS).

Animals

A total of 226 adult male Sprague–Dawley (SD) rats (8-10 weeks, weighing 200-250 g) and 10 newborn SD rats (1-3 days, for isolation of neurons) were enrolled (Additional file 1: Table S1 for an overview on animal assignment and treatment). Adult rats were subjected to sham-operation (n=23) or middle cerebral artery occlusion (MCAO, n=203). Rats were housed in a clean environment with adequate food and water, daily light hours of 8:00-20:00, temperature maintained at 24-26°C, and humidity maintained between 40%-60%. All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with a completely randomized (drawing lots) and blinded fashion according to protocols approved by the Institutional Animal Care and Use Committee of Dalian Municipal Central Hospital (No. 2017-031-05). Experiments and results are reported in compliance with the ARRIVE guidelines (Animal Research: Reporting in Vivo Experiments). Please refer to Additional file 2:

Figure S1 for the animal study flowchart.

Culture of primary cortical neurons

Primary cortical neurons were prepared as described previously.[23] In brief, newborn SD rats were sacrificed by excessive isoflurane inhalation. The heads were quickly cut off, brains were carefully taken out, and the meninges were removed. The cerebral cortex was cut into small pieces using micro-scissors. Chemical digestion was performed with trypsin and DNaseI, followed by repeated gentle pipetting with different sizes of pipettes until the tissues were separated into single cells. Neurons were collected by centrifugation and filtering with 40 μm sterile microfiltration membranes. Harvested neurons were cultured in Neurobasal A medium (Cat. 10888022, Gibco) supplemented with 2% B-27 (Cat. 17504044, Gibco), 2 mM glutamine (Cat. 25030081, Gibco) and 2 ng/ml fibroblast growth factor-2 (Cat. 233-FB-010, R&D Systems) on 10-cm disks or 96-well plates coated with poly-L-lysine (Cat. P-2636, Sigma).

Culture of BMSCs

Cryopreserved green fluorescent protein (GFP)-BMSCs (Cat. RASMX-01101) and BMSCs (Cat. RASMX-01001) from syngeneic SD rats were both purchased from Cyagen Biosciences. Cells were thawed and expanded in Dulbecco's modified Eagle's medium (DMEM)/F12 (Cat. 11320033, Gibco) supplemented with 10% fetal bovine serum (Cat.

16140071, Gibco) and 1% penicillin/streptomycin at 37°C under 5% CO_2. Cells were stored in liquid nitrogen for no more than one year and cultured for no more than eight passages before use. The cell number and viability were determined by incubating with Acridine Orange/Propidium Iodide Stain (Cat. F23001, Logos Biosystems) and counting with Luna™ Dual Fluorescence Cell Counter (Logos Biosystems). The vials of cells with more than 90% viability were used in further experiments.

BMSC proliferation analysis

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay was performed as previously described.[24] Briefly, BMSCs were seeded at 5×10^4 cells/ml in 96-well plates. After 24, 48 and 72 hours (h) of incubation with phosphate buffered saline (PBS) or different concentrations of P2 (ranging from 0.5 to 20 μM) at 37℃ in a serum free medium, 20 μl MTT (5 mg/ml, Cat. M2003, Sigma) was added to each well. Four hours later, the MTT solution was removed and 150 μl dimethylsulfoxide (DMSO, Cat. D2650, Sigma) was added to each well. Absorbance at 570 nm was measured using a microplate reader (Thermo).

In the molecular mechanism studies, 10 μM LY294002 (PI3K inhibitor, PI3KI; Cat.154447-36-6, MCE) or 10 μM PD98059 (ERK inhibitor, ERKI; Cat.167869-21-8, MCE) were added to the cells immediately after P2 incubation. The same volume of

142 DMSO (dissolution medium of the inhibitors) was applied to an additional group as a
143 control.

144 **Cell migration assay**

145 Cell migration was assessed using a Transwell Boyden chamber (Corning, USA). In
146 brief, BMSCs were harvested, counted, resuspended in DMEM/F12, and loaded (3×10^4
147 in 100 μl medium) into the upper compartment of each chamber. The lower compartment
148 was filled with 600 μl of DMEM/F12 supplemented with P2. The upper and lower
149 compartments were separated by a polycarbonate filter (pore diameter of 8 μm) coated
150 with 50 μg/ml collagen (Cat. C7661, Sigma). Control BMSCs were cultured under the
151 same conditions but without P2 supplementation. The cells were cultured at 37°C in an
152 incubator containing 95% air and 5% CO_2 for 24 h. A cotton-tipped swab was used to
153 remove the cells on the upper side of the filters, and the cells that migrated through the
154 filter pores were stained with 0.05% crystal violet in PBS for 30 minutes (min). Then, the
155 filters were washed with PBS. The stained Transwell membranes were cut, mounted onto
156 microscope slides, and visualized under a Nikon Eclipse Ti microscope (Nikon). Three
157 randomly selected non-overlapping fields were counted for the average numbers of
158 migrated cells using ImageJ software (Version 1.52p).

159 **Oxygen-glucose deprivation (OGD) and cell viability assay**

160 The culture media for primary neurons and BMSCs were discarded. Cells were washed
161 with PBS and incubated with glucose- and serum-free DMEM (Cat. 11966025, Gibco).
162 Then, the cells were transferred to the anaerobic incubator saturated with 94% N_2/5%
163 CO_2/1% O_2 at 37°C. Following incubation under OGD conditions for 2 h, the cells were
164 removed from the anaerobic incubator, rinsed with PBS, and fresh growth medium was
165 supplied.

166 Cell viability was evaluated by the cell counting kit-8 (CCK-8) assay (Cat. CK04,
167 Dojindo) according to manufacturer's instruction. Briefly, BMSCs (5×10^4 cells/ml) or
168 neurons (5×10^5 cells/ml) were plated into 96-well plates (100 μl/well), incubated at 37°C
169 for 24 h, and treated with 0-5 μM P2 for 24 h. Then, 10 μl CCK-8 solution were added
170 into each well followed by incubation at 37°C for 2 h. The absorbance value of each well
171 was recorded using a microplate reader (Thermo) at a wavelength of 450 nm.

172 **Annexin V/Propidium iodide assay for apoptosis assessment**

173 Neuronal apoptosis was examined by Annexin V (AV)-FITC and propidium iodide (PI,
174 Cat. KGA108, KeyGen Biotech) staining according to the manufacturer's protocol. After
175 OGD treatment, 1×10^6 neurons were collected and washed twice with cold PBS. The
176 samples were gently resuspended in 500 μl of binding buffer mixed with 5 μl of AV and
177 5 μl PI solutions, followed by incubation in the dark at room temperature for 15 min.

178 Samples were then analyzed using a FACSCalibur flow cytometer (Becton-Dickinson).
179 The apoptotic rate was calculated as the sum of early-stage apoptotic cells (AV positive)
180 and middle- to late-stage apoptotic cells (both AV and PI positive).

181 **Ischemic stroke model**

182 Ischemic stroke was induced by transient MCAO as described elsewhere.[25] Briefly, rats
183 were anesthetized by isoflurane (5% for induction, 2% for maintenance). Body
184 temperature was maintained at 37°C throughout surgery using a heating pad. The right
185 common carotid artery (CCA), external carotid artery (ECA), and internal carotid arteries
186 (ICA) were exposed and a 3.0 monofilament nylon suture with rounded tip (0.32±0.02
187 mm diameter) was inserted *via* ECA into the lumen of ICA until it blocked the origin of
188 the middle cerebral artery (≈18-20 mm). Two hours after MCAO, the filament was gently
189 pulled out and the ECA was closed by electro-coagulation leaving a long ECA stump for
190 subsequent cell infusion (MCAO rats). Sham-operated animals underwent the same
191 procedure but without filament insertion. Buprenorphine (0.03 mg/kg) was applied for
192 post-surgical pain relief every 12 hours for at least 3 days. Successful infarct induction
193 was confirmed by beam-walking test which is sensitive to motor dysfunctions.[26]

194 **Exploration of the therapeutic doses of P2 for *in vivo* experiment**

To determine P2 therapeutic dose *in vivo*, we started with a dose (5 mg/kg, subcutaneously injected for 15 d) reported in the literature[27] and then conducted a series of dose-descent trials. Briefly, 34 MCAO rats were included (4 dead and 2 model failure rats were excluded prior to group assignment). Rats were subcutaneously injected with different doses of P2 daily for 14 d beginning at 24 h after stroke induction, including 0 (NS, n=6), 0.5 mg/kg (n=6), 1 mg/kg (n=6), 2 mg/kg (n=6), and 5 mg/kg (n=10). The beam-walking test was used to evaluate the therapeutic effects at 1, 7, 14, 28 d after MCAO. The numbers of deaths in different groups were recorded.

BMSC transplantation and P2 injection

One hundred and thirty-five MCAO rats were included (17 dead and 11 model failure rats were excluded prior to group assignment). Twenty-four hours after surgery, MCAO rats were randomly assigned into NS (n=26), BMSC (n=26), P2 (n=25), BMSC+P2 (n=26), BMSC+P2+PD98059 (n=16), orBMSC+P2+LY294002 (n=16) groups. Since the risk of cell-evoked cerebral embolism is increased at high cell dose infusion,[28] a concentration of 5×10^5 GFP-BMSCs (in 0.5 ml NS), being safe according to the literature, was infused. Cells were slowly (0.17 ml/min) injected into the ICA (*via* the ECA stump following re-anesthesia using isoflurane) to the BMSC, BMSC+P2, BMSC+P2+PD98059, and BMSC+P2+LY294002 groups, respectively. Rats from sham-

operated (n=23), NS and P2 groups received 0.5 ml NS intra-arterially. Rats from P2, BMSC+P2, BMSC+P2+PD98059, and BMSC+P2+LY294002 groups were injected subcutaneously with P2 peptide (1 mg/kg at 2 mg/ml concentration) daily for 14 d beginning 24 h after MCAO. Rats in BMSC and BMSC+P2 groups were injected intraperitoneally with bromodeoxyuridine (BrdU, 50 mg/kg, Cat. 5002, Sigma) twice per day for 10 d starting from BMSC infusion. Rats in BMSC+P2+PD98059 or BMSC+P2+LY294002 groups were injected intraperitoneally with PD98059 (1 mg/kg) or LY294002 (10 mg/kg) at day 1, 5, 10, 14 after MCAO operation, respectively. The beam-walking and adhesive tape removal tests were performed to evaluate motor[29] and sensorimotor functional outcome[30] before and at 1, 7, and 14 d after MCAO.

Beam-walking test

Three days before surgery, all rats received daily training sessions. Each rat was tested twice in succession. Tests were videotaped and analyzed by calculating the slip ratio of the impaired (contralateral to lesioned hemisphere) forelimb and hindlimb. Steps onto the ledge were scored as a full slip, and a half slip was given if the limb touched the side of the beam. The slip ratio was calculated as: (full slips+1/2 half slips) / (total steps) ×100%.

Adhesive tape removal test

The adhesive tape removal test was conducted to evaluate the sensorimotor integration as previously described.[30] An adhesive tape was affixed to the bottom of the left forepaw (contralateral to the cerebral lesion) of each rat. The time until the animal removed the adhesive tape was measured. The test was stopped when the animal did not remove the adhesive tape at this time, and 180 s were recorded. Animals were pretrained before sham/MCAO surgery and tested before surgery as well as 1, 7 and 14 d after MCAO or sham operation. Each rat was tested three times per day.

Terminal-deoxynucleoitidyl transferase mediated dUTP nick end labeling (TUNEL) assay and immunofluorescence staining

On day 1 or 14 following BMSCs and/or P2 therapy (day 2 after sham/MCAO surgery), rats were deeply anesthetized with isoflurane and transcardially perfused with 5% sucrose, followed by 4% paraformaldehyde (PFA). After perfusion, brains were removed and post-fixed in 4% PFA for 24 h at 4℃. Then the brains were transferred to 30% sucrose at 4℃ for dehydration, followed by fast-frozen, and cryosectioned into 30-μm slices.

Antibody details are given in Additional file 1: Table S2. A TUNEL (Cat. KGA7062, KeyGen Biotech) assay was performed for histological evaluation of apoptotic neurons *in situ* (on post BMSC or P2 therapy day 1) according to the manufacturer's instructions. Sections were then incubated overnight at 4°C with anti-NeuN antibodies (a neuronal

248 marker) and incubated for another 2 h with Alexa-488 secondary antibodies at room
249 temperature. Tissues were then mounted with VECTASHIELD® Antifade Mounting
250 Medium with 4',6-diamidino-2-phenylindole (DAPI, Cat. H-1200-10, Vector
251 Laboratories). Five randomly selected areas from the ischemic boundary zone defined as
252 the area between the infarct core (with significantly reduced viable cells) and normal brain
253 tissues (with comparable numbers of viable cells to the contralateral brain regions, n=4
254 brains/group) were investigated under the microscope (Nikon Eclipse Ti, Nikon). Then,
255 TUNEL-positive or NeuN-positive cells were counted using ImageJ software. The
256 apoptosis rate was calculated as the percentage of TUNEL-positive neurons (%).
257 BMSC proliferation in MCAO rat brains was evaluated by immunofluorescence on
258 post-treatment day 14. Sections were blocked using 3% bovine serum albumin (BSA, Cat.
259 SRE0096, Sigma) in Tris-buffered saline (TBS) plus 0.1% Triton and incubated overnight
260 with primary antibodies including anti-BrdU (a marker for cell proliferation) and anti-
261 GFP at 4°C. Sections were washed three times with TBS and incubated for 2 h with either
262 goat anti-rabbit IgG (Alexa-488) or goat anti-mouse IgG (Alexa-594) secondary
263 antibodies at room temperature. Sections were then washed three times with TBS and
264 mounted. For quantification, five randomly selected areas from the ischemic boundary

zone (n=4 brains/group) were investigated under a microscope (Nikon Eclipse Ti, Nikon). Both BrdU- and GFP-positive cells were counted using ImageJ.

Nissl staining and infarct volume assessment

Nissl staining was performed to calculate the cerebral infarct area at day 1 and 14 after BMSC and/or P2 therapy. Brain slices were washed with distilled water and stained with Nissl solution (Cat. C0117, Beyotime Biotechnology) for 10 min at 37°C, then rinsed twice with distilled water, and immersed in 95% ethanol (two changes) for decoloration. Following exposure to xylene (2 times for 5 min each), the sections were mounted using DPX Mounting medium (Sigma-Aldrich) and observed under a microscope (Axio Scope A1, Zeiss). The infarct area was traced and analyzed using ImageJ. The infarct volumes were calculated by taking into consideration the inter-slice distance (480 μm) from five consecutive brain slices. Brain infarct volume was calculated as: (volume of contralateral - volume of non-ischemic ipsilateral)/2×volume of contralateral×100%.

Real-time quantitative polymerase chain reaction (RT-qPCR)

On day 14 following BMSCs and/or P2 therapy, rats were deeply anesthetized with isoflurane and brains were collected on ice. The RNAs from cultured BMSCs or rat brains were isolated using RNAiso Plus Kit (Cat. 9108, Takara Bio). RNA concentration and purity were evaluated by Nanodrop One (Thermo). The RNAs were converted to cDNA

by reverse transcription with the PrimeScript^{TM} RT Master Mix (Cat. RR036A, Takara Bio). The primers of neurotrophic factors and reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) were synthesized (Ruizhen Bio Inc.) and primer sequences are listed in Additional file 1: Table S3. RT-qPCR reaction was conducted according to manufacturer's instruction (Tli RNaseH Plus, Cat. RR820A, Takara Bio Inc.). Samples were amplified and quantified using the AB Applied Biosystems Viia7 DX device (Life Technologies). Each reaction was performed in triplicates and the melting curves were constructed to ensure that a single product was amplified. The threshold cycle (CT) was determined, and the relative gene expression was calculated as follows: fold change = $2^{-\Delta\Delta CT}$, where $\Delta CT = CT_{target} - CT_{housekeeping}$, and $\Delta\Delta CT = \Delta CT_{treated} - \Delta CT_{control}$.

Western blotting

Total proteins were extracted from cultured neurons, BMSCs, or rat brain tissues. Nuclear proteins were isolated with 5000 g centrifugation (5 min) using a Subcellular Protein Fractionation Kit (Cat. 87790, Thermo) according to the manufacturer's instructions. Proteins were separated in SDS-PAGE gels and transferred to PVDF membranes (Immobilon®-P, Merck). The membranes were blocked for 1 h with 3% BSA in TBS with 0.1% Tween-20 (TBST), and then incubated with primary antibodies (detailed information given in Additional file 1: Table S2) at 4°C overnight. Thereafter,

horseradish peroxidase-conjugated goat anti-rabbit/mouse secondary antibodies were applied at room temperature for 2 h before washing with TBST. The blots were then incubated in SuperSignal West Pico Chemiluminescent Substrate (Cat. 34577, Thermo) working solution according to the manufacturer's instructions and exposed for visualization. The data was adjusted to β-actin expression levels (integral optical density (IOD) value of the target protein/IOD of β-actin) using ImageJ software. Original (uncropped Western blots are provided in Additional file 3: Figures S3 to S8).

Statistical analysis

Since this is an exploratory study, we decided to keep group sizes relatively low (n=4-6 for *in vivo*, n=4-7 for *in vitro* experiments, please see Figure legends for details). Data distribution was examined by Shapiro-Wilk test. Normally distributed data are presented as mean±standard deviation (SD). Independent *t*-tests were used to analyze the difference between two groups, and the analysis of variance (ANOVA) test was used for multiple group comparisons followed by a post hoc test (least significant difference). Non-normally distributed data were presented as median (interquartile range, IQR), and Mann-Whitney or Kruskal-Wallis tests were used when comparing intergroup differences, respectively. All statistical analyses were performed using SPSS software package (Version 22.0, USA). $p<0.05$ was considered as statistically significant. All statistical tests

319 and results for multiple group comparisons are given in Additional file 4.

320

Results

Low concentrations of P2 promote proliferation and migration of BMSCs *in vitro*

Cultured BMSCs were supplemented with PBS or different concentrations of P2 for 24, 48, and 72 h before MTT assay (n=6/group). Compared to PBS treatment, 0.5-2 μM P2 treatment increased BMSC numbers after 24 h ($p<0.05$, Figure 1A). The proliferation-promoting effect of 1 μM P2 extended to 48 h and 72 h after treatment ($p<0.05$, Figure 1A). Higher concentrations of P2 (5, 10, and 20 μM) did not increase but inhibited BMSC proliferation after 48 h at 10 and 20 μM ($p<0.01$, Figure 1A). Moreover, 0.5 and 1 μM P2 promoted BMSC proliferation under OGD ($p<0.001$, n=6/group, Figure 1B). Based on these results, a concentration of 1 μM P2 was chosen for the following *in vitro* experiments.

The number of migrated BMSCs in a Transwell Boyden Chamber was increased in 1 μM P2-treated group than in PBS-treated group ($p<0.05$, n=6/group, Figure 1C), indicating that P2 promoted BMSC migration.

P2 promoted BMSC proliferation through ERK and PI3K/AKT pathways

MAPK/ERK and PI3K/AKT pathways are involved in the neuroprotective and neuritogenic effects of P2 on primary neurons.[18, 19] We therefore examined whether these pathways also mediate the effects of P2 on BMSC proliferation. The levels of

phosphorylated ERK (p-ERK, Thr202/Tyr204) in P2-treated BMSCs were increased compared to the controls (p<0.001), and this effect was inhibited by PD98059 (ERK inhibitor, p<0.001, n=6/group, Figure 1D-E). There were no differences in phosphorylated P38 (p-P38, Thr180/Tyr182) and phosphorylated JNK (p-JNK, T183/T221) levels between P2- and PBS-treated BMSCs (n=6/group, Figure 1D-E). Total protein levels of ERK, P38, and JNK were also comparable between the two groups. Furthermore, level of phosphorylated AKT (p-AKT, Ser473, p<0.001) in the P2-treated group was increased compared to the PBS group (n=6/group). LY294002 (PI3K inhibitor) blocked P2-induced upregulation of p-AKT (p<0.001, Figure 1D-E). The total protein level of AKT was not changed by P2- or PI3K inhibitor-treatment.

MTT assays showed that the promoting effect of P2 on BMSC proliferation was eliminated by ERK inhibitor (p<0.001) and PI3K inhibitor (p<0.001), but not the control DMSO solvent (n=6/group, Figure 1F). These observations indicate that P2 promotes BMSC proliferation through activation of MAPK/ERK and PI3K/AKT pathways.

P2 enhances neurotrophic factor expression of BMSCs *in vitro*

Since neurotrophic effects are believed to be one of the principal mechanisms of BMSC therapy for stroke,[4, 31] we further investigated whether P2 promotes neurotrophic factor expression by BMSCs *in vitro*. P2 (1 μM) significantly increased the mRNA levels of

BDNF (p<0.01), GDNF (p<0.05), IGF-1 (p<0.01), and NGF (p<0.01), but not of VEGF in BMSCs when compared to PBS treatment (n=6/group, Figure 2A). The protein levels of BDNF (p<0.01) and IGF-1 (p<0.05) in BMSCs were increased after 24 h of P2 incubation, while GDNF, VEGF, and NGF protein levels were unchanged (n=6/group, Figure 2A).

P2-mediated neuroprotection under OGD through anti-apoptotic pathways

To investigate whether P2 could exert neuroprotective effects under hypoxic/ischemic stress, primary cortical neurons were cultured and exposed to OGD in the presence of different concentrations of P2. CCK8 assays showed that OGD induced considerable neuronal cell death (p<0.001), while P2 mitigated neuronal OGD damage at concentrations of 0.5 (p<0.001), 1 (p<0.001) and 2 μM (p<0.01), but not 5 μM (n=7/group, Figure 2B). This neuroprotective effect was diminished by ERK inhibitor or PI3K inhibitor (p<0.001, n=6/group, Figure 2C).

To further investigate the neuroprotective mechanisms of P2, we conducted apoptosis screening by flow cytometry and Western blotting. Neuronal apoptosis rates were increased under OGD condition as compared to normal culture conditions (p<0.001). Apoptosis was reduced in the presence of 1 μM P2 (p<0.001, n=4/group, Figure 2D). Western blotting of neuron lysates showed that the generation of activated fragments of

caspase-3 (cleaved caspase-3, CC3, $p<0.001$) and caspase-9 (cleaved caspase-9, CC9, $p<0.01$), as well as the pro-apoptotic protein Bax ($p<0.05$) were all increased in OGD-treated neurons. The neuronal expression of anti-apoptotic protein Bcl-2 was decreased under OGD ($p<0.001$). In contrast, the protein levels of CC3 ($p<0.001$), CC9 ($p<0.01$), and Bax ($p<0.05$) were all decreased, while Bcl-2 ($p<0.01$) was increased after 1 μM P2 treatment compared to PBS treatment under OGD. Moreover, ERK and PI3K inhibitorsblocked the regulatory effects of P2 on the expression of these apoptosis-related proteins ($p<0.05$, n=6/group, Figure 2E-F).

P2 is neuroprotective after MCAO

Compared to NS treatment, 1 mg/kg P2 promoted functional recovering of MCAO rats at 28 d after surgery ($p<0.05$, n=6/group, Additional file 2: Figure S2), while no effect was found at doses of 0.5 and 2 mg/kg. A concentration 5 mg/kg P2 even resulted in harmful effects, inducing a mortality rate of 60% (6 death out of 10). Thus, 1 mg/kg P2 was chosen for the following *in vivo* experiments.

Since our *in vitro* data indicated that P2 protected neurons from apoptosis under OGD, we performed TUNEL staining to confirm this effect *in vivo* (n=6/group, Figure 3). There were few apoptotic neurons in the sham-operation group. MCAO increased neuronal apoptosis ($TUNEL^+NeuN^+$) in the ischemic boundary zone ($p<0.001$), while the number

of apoptotic neurons in that area of P2-treated MCAO rats was reduced (p<0.05). P2 combined with intraarterial BMSC infusion reduced neuronal apoptosis when compared to NS-treated (p<0.001) or BMSC-treated (p<0.05) MCAO rats. Nevertheless, this anti-apoptosis effect was decreased by both PD98059 and LY294002 (p<0.001).

P2 promotes BMSC proliferation in MCAO rat brains

To investigate whether P2 promotes the proliferation of BMSCs *in vivo*, BrdU was repetitively injected to label intraarterially administered, proliferating GFP-BMSCs after subcutaneous administration of P2 or NS in MCAO rats. GFP-BMSCs were found to distribute mostly in the ischemic boundary zone 14 d after transplantation. The number of $BrdU^+$ GFP-BMSCs in the P2 group was higher than in the NS group (p<0.05), suggesting that P2 increases BMSC proliferation in MCAO rat brains after intraarterial infusion (n=4/group, Figure 4A).

P2 synergizes with BMSCs in enhancing neurotrophic factor expression *in vivo*

Using RT-qPCR and WB, we investigated whether subcutaneous administration of P2 promotes neurotrophic factor expression in rat brains after MCAO and whether P2 could synergize with BMSCs on this effect. Compared to sham-operated rats, BMSCs increased the mRNA levels of IGF-1 (p<0.05), P2 slightly increased the mRNA levels of GDNF (p<0.05), and P2 in combination with BMSCs increased the mRNA levels of GDNF

411 (p<0.05) and IGF-1 (p<0.001). Moreover, P2 in combination with BMSCs increased the
412 mRNA expression of VEGF as compared to BMSC treatment alone (p<0.01) and
413 increased the mRNA expression of IGF-1 as compared to vehicle-treated MCAO rats
414 (p<0.05, n=5/group, Figure 4B). BMSC or P2 treatment alone increased the protein levels
415 of BDNF and IGF-1 (p<0.05). Moreover, the protein levels of BDNF, GDNF, and IGF-1
416 (but not VEGF) were higher after P2/BMSC combination treatment compared to levels
417 in sham (all p<0.001) or vehicle-treated MCAO rats (p<0.001, n=5/group, Figure 4C-D).

418 **P2 synergizes with BMSCs in reducing the infarct volume after MCAO**

419 Nissl staining was used to assess the infarct volume at 1 d and 14 d after P2 and/or
420 BMSC therapy (Figure 5A). There was no difference in infarct volume between NS-,
421 BMSC-, P2- or P2 combined BMSC-treated MCAO rats 1 d after intervention (n=6/group,
422 Figure 5B). P2 in combination with BMSCs reduced the infarct volume and tissue loss in
423 the ipsilateral hemisphere of MCAO rat brains 14 d after treatment in comparison to NS
424 treatment (p<0.01, n=6/group, Figure 5C). Moreover, the infarct volume of P2 combined
425 BMSC-treated MCAO rats was smaller than BMSC treated MCAO rats (p<0.05).

426 **P2 synergizes with BMSCs in promoting the functional recovery after MCAO**

427 Fore- and hindlimb motor function was assessed with the beam-walking test
428 (n=6/group, Figure 6A). No inter-group differences were noticed prior to ischemia. There

was an apparent fore- and hindlimb (contralateral to the lesion site) dysfunction after MCAO on postoperative day 1, 7, and 14 in all groups. Fewer fore- and hindlimb slips were observed in BMSC-treated ($p<0.05$) rats or rats treated with P2 in combination with BMS ($p<0.001$) rats with MCAO on postoperative day 14. Moreover, the combined treatment of P2 and BMSCs resulted in better hindlimb functional recovery compared to BMSC or P2 treatment alone ($p<0.05$). However, PD98059 or LY294002 administration impeded the limb functional recovery as compared to the combined therapy group ($p\leq0.001$).

Sensorimotor function was assessed with the adhesive tape removal test (n=6/group, Figure 6A). There were no inter-group differences on baseline or 1 d performance after operation. Compared with sham-operated rats, MCAO rats needed more time to remove the adhesive tapes on postoperative days 1 ($p<0.01$), 7 ($p<0.05$) and 14 ($p<0.01$). Furthermore, there was no tape removal time difference between P2 or BMSC-treated alone and NS-treated MCAO rats at any observation timepoints after therapy, while the time in P2 combined BMSCs-treated MCAO rats was significantly shorter than the vehicle-treated ($p<0.01$) or BMSC-treated ($p<0.05$) MCAO rats on postoperative day 14, which indicated a better sensorimotor functional recovery under combination treatment

446 regimen. Similarly, this sensorimotor function recovery was hindered by both ERK
447 inhibitor and PI3K inhibitor ($p<0.001$).

448 **P2 activates ERK and AKT pathways in MCAO rat brains**

449 Since the *in vitro* experiments showed that P2 increased BMSC proliferation through
450 ERK and PI3K/AKT pathways, we further examined whether these pathways are also
451 involved in the therapeutic effects of P2 *in vivo*. Western blotting showed that the cerebral
452 protein levels of p-ERK as well as p-AKT in P2- and P2 in combination with BMSC-
453 treated MCAO rats were higher than in NS- or BMSC-treated MCAO rats ($p<0.01$,
454 n=5/group, Figure 6B), while there was no difference in the total protein levels of ERK
455 and AKT between these groups. The combined therapy also increased the protein levels
456 of Nrf2 in the nucleus and upregulated the expression of HO-1 in MCAO rats. After
457 intraperitoneal injection of PD98059 or LY294002, the levels of phosphorylated ERK and
458 AKT in MCAO rats receiving P2 in combination of BMSC treatment were decreased
459 ($p\leq0.001$, n=6/group, Figure 6C), which indicated the successful inhibition of the
460 activation of these two proteins in the brain tissues. In addition, treatment with LY294002
461 or PD98059 attenuated Nrf2 nucleus translocation and HO-1expression in response to P2
462 combined with BMSC treatment ($p\leq0.001$, n=5-6/group, Figure 6D). These data
463 suggested that ERK and AKT phosphorylation contributed to the activation of Nrf2/HO-

464 1 pathway, which may be the molecular mechanisms of P2 in combination with BMSC

465 therapy.

Discussion

We investigated the effects of the NCAM mimetic peptide P2 on cultured BMSCs and neurons as well as possible synergistic effects of a combined P2 and BMSC treatment after ischemic stroke in rats. We found that P2 promoted BMSC proliferation, migration, and neurotrophic factor expression *in vitro*. P2 achieved neuroprotection through anti-apoptotic mechanisms. The cytoprotective effects of P2 depended on ERK and PI3K/AKT pathways. Combined P2 and BMSC treatment was superior to monotreatment in key readouts after ischemic stroke including increased neurotrophic factor secretion and functional recovery, as well as reduced infarct volume. These results suggest synergistic effects of the combination approach.

P2 represents the natural cis-binding site for the first NCAM Ig module[12] and induces NCAM activation on cell membranes, thereby initiating a number of intracellular signaling events leading to the stimulation of neurite outgrowth and promotion of neuronal survival *in vitro*.[18] Our study added evidence for beneficial P2 effects after ischemic stroke both in the absence and presence of BMSCs. This is consistent with a previous study showing that P2 promoted motor and cognitive function recovery, modulated emotional behavior,[27, 32] and promoted neuroanatomical remodeling in the neocortex after traumatic brain injury.[27] P2 can pass blood-brain barrier after systemic

484 administration.[27] It targets polysialylated-NCAM molecules expressed on immature
485 neurons[33] and modulates their decision on migration or differentiation. This neurogenesis-
486 activating role probably contributes to the beneficial effects of P2 in the damaged central
487 nervous system. Other probable cyto-/neuroprotective mechanisms of P2 include anti-
488 oxidative stress,[27] stimulation of neuronal survival by activating the MAPK pathway and
489 increasing intracellular Ca^{2+}, and anti-apoptosis through the PI3K/AKT pathway.[18, 19, 34]

490 In our study, the neuroprotective effect of P2 on neurons was investigated by exposing
491 cultured cortical neurons to OGD causing metabolic stress and inducing apoptosis.
492 Cortical neurons were chosen because they are one of the most commonly used neuronal
493 cell types for *in vitro* experiments. Robust protocols for isolation, characterization and
494 cultivation have been established, and using cortical neurons also allows comparability
495 to other studies. Moreover, the 2-hour MCAO protocol used in our study reliably
496 produces combined striatal and cortical lesions that lead to motor, sensory, and cognitive
497 dysfunctions. We found that P2 inhibited neuronal apoptosis by inhibition of pro- and
498 activation of anti-apoptotic proteins. Caspase-3 is considered to be the major effector
499 caspase in mammalian cells,[35] while DNA fragmentation appears downstream from
500 caspase-3 activation.[36] Our data shows that P2 reduced the caspase-3 activity *in vitro* and
501 DNA fragmentation (TUNEL) *in vivo*. Caspase-3 can also be expressed in other cell

502 populations after hypoxia and ischemia and does not necessarily indicate apoptosis.[37, 38]
503 However, we also found that P2 inhibited neuronal apoptosis by decreasing the expression
504 of cleaved caspase-9, pro-apoptotic Bax, and by increasing the expression of anti-
505 apoptotic Bcl-2 upstream of caspase-3.

506 Neuroprotective and neuritogenic effects of P2 on primary dopaminergic and cerebellar
507 neurons depend on PI3K/AKT pathways.[18] We found that AKT was phosphorylated, and
508 thus activated after P2 treatment both *in vitro* and *in vivo*. The blockage of P2-dependent
509 BMSC proliferation and anti-neuronal apoptosis functions by a PI3K inhibitor confirmed
510 that the PI3K/AKT pathway is an important downstream effector pathway of P2. ERK,
511 but no other MAPK family members (JNK and P38), were activated by P2, suggesting
512 that ERK plays a central role in mediating P2 treatment effects. Indeed, ERK inhibition
513 repressed promotion of BMSC proliferation, anti-apoptotic protein expression, and
514 inhibition of pro-apoptotic protein expression by P2. Therefore, both PI3K/AKT and ERK
515 pathways are involved in the regulatory function of P2 on BMSCs. Moreover, we
516 demonstrated that the anti-neuronal apoptosis and neurological recovery promoting
517 effects of the combined P2 and BMSC therapy in MCAO rats were attenuated by injection
518 of PI3K and ERK inhibitors, suggesting that the PI3K/AKT and ERK pathways are

519 important downstream effectors of the combined therapy *in vivo*. Therapeutic effects may
520 be achieved through Nrf2 and HO-1.

521 Although intravenous transplantation is the most commonly used approach for stem
522 cell therapy in both preclinical and clinical trials,[39-41] it is not the optimal strategy for
523 intracerebral lesions. Limited numbers of BMSCs could enter the brain after intravenous
524 transplantation due to pulmonary passage filtering and cell relocation to internal organs.[41,
525 42] Intra-arterial cell delivery has been postulated to enhance the homing efficiency to the
526 target organ, which may also be associated with additional therapeutic benefits,
527 particularly in the brain.[43, 44] However, the risk of microembolism should be considered.[45,
528 46] Cell dose and infusion velocity define the safety of intra-arterial administration, and
529 both lower cell doses and infusion velocity (≤0.2 ml/min) were proven safe. Thus,
530 administration of 5×10^5 BMSCs in 0.5 ml NS at an infusion velocity of 0.17 ml/min was
531 chosen for *in vivo* experiments in this study, and was proven to be effective in previous
532 studies.[28, 47] Safety of intraarterial infusion might be improved by increasing vessel
533 clearance of BMSCs by bioengineering approaches[48] what could allow administration of
534 even larger BMSC numbers in future studies.

535 Our study has several limitations. First, the sample size in this exploratory study is
536 small. Small sample sizes severely hinder interpretation of neutral results, but the

statistically significant benefits seen in our investigations indicate a remarkable effect size of the treatment approaches. Nevertheless, larger, and adequately powered confirmative studies are imperative to verify these large effects. Such confirmative studies should also investigate the therapeutic impact in mixed-sex and comorbid cohorts. Second, the overall surveillance time of 14 d falls short of the recommended 3- or 4-week follow ups.[49] On the other hand, the chosen time point is a reasonable tradeoff between the investigation of processes such as apoptosis predominantly occurring in acute and sub-acute stages after stroke and a sufficiently long surveillance time. Application of the therapeutic regimen in a model simulating late recanalization also meets current recommendations in the field[50, 51] and should contribute to the external validity of our study. Third, the applied behavioral tests are robust, but not highly sensitive, in particular at late time points.[26] The fact that they clearly indicated therapeutic effects and even revealed evidence for superiority of the combination approach again suggests relatively large effect sizes. Nevertheless, further confirmative studies should include additional tests assessing fine motor function, being applied over a longer time. Fourth, future studies should include a scrambled amino acid peptide of P2 as a control that would be unlikely to have beneficial therapeutic effects.

554 Other NCAM mimetic peptides, including FGL and C3, also activate MAPK and
555 PI3K/AKT pathways,[52-54] but the high rate of adverse events when using FGL[55] and the
556 NCAM-antagonistic effects of C3 (interference with cell adhesion and inhibition of
557 transmitter release) may make safe and efficient use of these peptides complicated,
558 potentially hindering their widespread application in combination treatment.[54] A big
559 advantage of P2 is its easy applicability and excellent bioavailability.[27] Its plasticity-
560 promoting effects might also be beneficial for approaches including neurorehabilitation
561 which is considered an important element of future restorative therapies in stroke.[56]
562 Clinical translation of the combination approach might be further facilitated by the fact
563 that BMSC treatments were already shown to be safe. Translation should, however, be
564 based on experimental results suggesting which stroke patient populations may benefit
565 most from the approach to specifically address these patients into clinical trials.[57]

566 In conclusion, we demonstrate that the combination treatment of P2 and BMSCs has
567 the potential to enhance BMSC proliferation and migration, to increase the expression of
568 multiple trophic factors and to ameliorate neurological deficits after stroke, and that these
569 effects were mostly superior to either P2 or BMSC monotreatment. P2 provides protective
570 effects probably through the activation of PI3K/AKT and ERK pathways (Figure 7).

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577 **Author contributions**

578 All authors contributed to the study conception and design. XYL: experiment
579 conduction, data analysis, data interpretation, and manuscript writing. XSL and MXC:
580 experiment conduction, data analysis and interpretation, critical review of the manuscript;
581 HMQ: immunohistochemical staining, data analysis and interpretation, critical review of
582 the manuscript; CYC: animal experiment conduction, data analysis and interpretation,
583 critical review of the manuscript; JB: data interpretation, critical review of the manuscript;
584 SL: data interpretation, manuscript writing, financial support. All authors have read and
585 approved the final version of the manuscript.

586 **Declaration of conflicting interests**

587 The authors declare that there is no conflict of interest.

588 **Availability of data and materials**

589 The datasets used and analyzed during the current study are available from the

corresponding author (Shen Li, lishen@mail.ccmu.edu.cn) on reasonable request.

Supplemental files

Additional file 1. Table S1. Animal assignments and measurements. Table S2. The antibodies for Western blotting and immunofluorescence staining. Table S3. The primer sequences for RT-qPCR.

Additional file 2. Figure S1. Flowchart of the *in vivo* study. Figure S2. P2 (1 mg/kg) promotes functional recovery after MCAO.

Additional file 3. Full unedited gels/blots for Western blotting.

Additional file 4. Outcome parameters of multiple group comparisons.

599 # References

600 1. Saver JL, Goyal M, van der Lugt A, et al. Time to Treatment With Endovascular Thrombectomy and
601 Outcomes From Ischemic Stroke: A Meta-analysis. *Jama* 2016; 316: 1279-1288.
602 2. Lan X, Sun Z, Chu C, et al. Dental Pulp Stem Cells: An Attractive Alternative for Cell Therapy in Ischemic
603 Stroke. *Front Neurol* 2019; 10: 824.
604 3. Wang Q, Duan F, Wang MX, et al. Effect of stem cell-based therapy for ischemic stroke treatment: A
605 meta-analysis. *Clin Neurol Neurosurg* 2016; 146: 1-11.
606 4. Wakabayashi K, Nagai A, Sheikh AM, et al. Transplantation of human mesenchymal stem cells
607 promotes functional improvement and increased expression of neurotrophic factors in a rat focal cerebral
608 ischemia model. *J Neurosci Res* 2010; 88: 1017-1025.
609 5. Shen LH, Li Y, Chen J, et al. Intracarotid transplantation of bone marrow stromal cells increases axon-
610 myelin remodeling after stroke. *Neuroscience* 2006; 137: 393-399.
611 6. Scheibe F, Ladhoff J, Huck J, et al. Immune effects of mesenchymal stromal cells in experimental
612 stroke. *J Cereb Blood Flow Metab* 2012; 32: 1578-1588.
613 7. Borlongan CV, Glover LE, Tajiri N, et al. The great migration of bone marrow-derived stem cells toward
614 the ischemic brain: therapeutic implications for stroke and other neurological disorders. *Prog Neurobiol*
615 2011; 95: 213-228.
616 8. Lalu MM, Montroy J, Dowlatshahi D, et al. From the Lab to Patients: a Systematic Review and Meta-
617 Analysis of Mesenchymal Stem Cell Therapy for Stroke. *Transl Stroke Res* 2020; 11: 345-364.
618 9. Cui LL, Golubczyk D, Tolppanen AM, et al. Cell therapy for ischemic stroke: Are differences in
619 preclinical and clinical study design responsible for the translational loss of efficacy? *Ann Neurol* 2019; 86:
620 5-16.
621 10. Zhang Q, Zhao Y, Xu Y, et al. Sodium ferulate and n-butylidenephthalate combined with bone marrow
622 stromal cells (BMSCs) improve the therapeutic effects of angiogenesis and neurogenesis after rat focal
623 cerebral ischemia. *J Transl Med* 2016; 14: 223.
624 11. Cui X, Chopp M, Zacharek A, et al. Chemokine, vascular and therapeutic effects of combination
625 Simvastatin and BMSC treatment of stroke. *Neurobiol Dis* 2009; 36: 35-41.
626 12. Chu C, Gao Y, Lan X, et al. NCAM Mimetic Peptides: Potential Therapeutic Target for Neurological
627 Disorders. *Neurochem Res* 2018; 43: 1714-1722.
628 13. Huang R, Yuan DJ, Li S, et al. NCAM regulates temporal specification of neural progenitor cells via
629 profilin2 during corticogenesis. *J Cell Biol* 2020; 219: e201902164.
630 14. Schmid RS, Graff RD, Schaller MD, et al. NCAM stimulates the Ras-MAPK pathway and CREB
631 phosphorylation in neuronal cells. *J Neurobiol* 1999; 38: 542-558.
632 15. Pedersen MV, Helweg-Larsen RB, Nielsen FC, et al. The synthetic NCAM-derived peptide, FGL,

633 modulates the transcriptional response to traumatic brain injury. *Neurosci Lett* 2008; 437: 148-153.
634 16. Corbett NJ, Gabbott PL, Klementiev B, et al. Amyloid-beta induced CA1 pyramidal cell loss in young
635 adult rats is alleviated by systemic treatment with FGL, a neural cell adhesion molecule-derived mimetic
636 peptide. *PLoS One* 2013; 8: e71479.
637 17. Berezin V and Bock E. NCAM mimetic peptides: an update. *Adv Exp Med Biol* 2010; 663: 337-353.
638 18. Pedersen MV, Køhler LB, Ditlevsen DK, et al. Neuritogenic and survival-promoting effects of the P2
639 peptide derived from a homophilic binding site in the neural cell adhesion molecule. *J Neurosci Res* 2004;
640 75: 55-65.
641 19. Soroka V, Kiryushko D, Novitskaya V, et al. Induction of neuronal differentiation by a peptide
642 corresponding to the homophilic binding site of the second Ig module of the neural cell adhesion molecule.
643 *J Biol Chem* 2002; 277: 24676-24683.
644 20. Nakaso K, Yano H, Fukuhara Y, et al. PI3K is a key molecule in the Nrf2-mediated regulation of
645 antioxidative proteins by hemin in human neuroblastoma cells. *FEBS Lett* 2003; 546: 181-184.
646 21. Wang HQ, Xu YX and Zhu CQ. Upregulation of heme oxygenase-1 by acteoside through ERK and PI3
647 K/Akt pathway confer neuroprotection against beta-amyloid-induced neurotoxicity. *Neurotox Res* 2012;
648 21: 368-378.
649 22. Takahashi T, Morita K, Akagi R, et al. Heme oxygenase-1: a novel therapeutic target in oxidative tissue
650 injuries. *Curr Med Chem* 2004; 11: 1545-1561.
651 23. Andreyeva A, Leshchyns'ka I, Knepper M, et al. CHL1 is a selective organizer of the presynaptic
652 machinery chaperoning the SNARE complex. *PLoS One* 2010; 5: e12018.
653 24. Lan X, Sun H, Liu J, et al. Effects of garlic oil on pancreatic cancer cells. *Asian Pac J Cancer Prev* 2013;
654 14: 5905-5910.
655 25. Mitkari B, Nitzsche F, Kerkelä E, et al. Human bone marrow mesenchymal stem/stromal cells produce
656 efficient localization in the brain and enhanced angiogenesis after intra-arterial delivery in rats with
657 cerebral ischemia, but this is not translated to behavioral recovery. *Behav Brain Res* 2014; 259: 50-59.
658 26. Balkaya MG, Trueman RC, Boltze J, et al. Behavioral outcome measures to improve experimental
659 stroke research. *Behav Brain Res* 2018; 352: 161-171.
660 27. Klementiev B, Novikova T, Korshunova I, et al. The NCAM-derived P2 peptide facilitates recovery of
661 cognitive and motor function and ameliorates neuropathology following traumatic brain injury. *Eur J*
662 *Neurosci* 2008; 27: 2885-2896.
663 28. Cui LL, Kerkelä E, Bakreen A, et al. The cerebral embolism evoked by intra-arterial delivery of
664 allogeneic bone marrow mesenchymal stem cells in rats is related to cell dose and infusion velocity. *Stem*
665 *Cell Res Ther* 2015; 6: 11.
666 29. Lipsanen A, Hiltunen M and Jolkkonen J. Chronic ibuprofen treatment does not affect the secondary
667 pathology in the thalamus or improve behavioral outcome in middle cerebral artery occlusion rats.

668 *Pharmacol Biochem Behav* 2011; 99: 468-474.
669 30. Albertsmeier M, Teschendorf P, Popp E, et al. Evaluation of a tape removal test to assess neurological
670 deficit after cardiac arrest in rats. *Resuscitation* 2007; 74: 552-558.
671 31. Janowski M, Wagner DC and Boltze J. Stem Cell-Based Tissue Replacement After Stroke: Factual
672 Necessity or Notorious Fiction? *Stroke* 2015; 46: 2354-2363.
673 32. Rizhova L, Klementiev B, Cambon K, et al. Effects of P2, a peptide derived from a homophilic binding
674 site in the neural cell adhesion molecule on learning and memory in rats. *Neuroscience* 2007; 149: 931-
675 942.
676 33. von Bohlen Und Halbach O. Immunohistological markers for staging neurogenesis in adult
677 hippocampus. *Cell Tissue Res* 2007; 329: 409-420.
678 34. Kiryushko D, Korshunova I, Berezin V, et al. Neural cell adhesion molecule induces intracellular
679 signaling via multiple mechanisms of Ca2+ homeostasis. *Mol Biol Cell* 2006; 17: 2278-2286.
680 35. Bredesen DE. Apoptosis: overview and signal transduction pathways. *J Neurotrauma* 2000; 17: 801-
681 810.
682 36. D'Mello SR, Aglieco F, Roberts MR, et al. A DEVD-inhibited caspase other than CPP32 is involved in
683 the commitment of cerebellar granule neurons to apoptosis induced by K+ deprivation. *J Neurochem* 1998;
684 70: 1809-1818.
685 37. Riegelsberger UM, Deten A, Pösel C, et al. Intravenous human umbilical cord blood transplantation
686 for stroke: impact on infarct volume and caspase-3-dependent cell death in spontaneously hypertensive
687 rats. *Exp Neurol* 2011; 227: 218-223.
688 38. Wagner DC, Riegelsberger UM, Michalk S, et al. Cleaved caspase-3 expression after experimental
689 stroke exhibits different phenotypes and is predominantly non-apoptotic. *Brain Res* 2011; 1381: 237-242.
690 39. Bhasin A, Srivastava MV, Mohanty S, et al. Stem cell therapy: a clinical trial of stroke. *Clin Neurol*
691 *Neurosurg* 2013; 115: 1003-1008.
692 40. Lee JS, Hong JM, Moon GJ, et al. A long-term follow-up study of intravenous autologous mesenchymal
693 stem cell transplantation in patients with ischemic stroke. *Stem Cells* 2010; 28: 1099-1106.
694 41. Goldmacher GV, Nasser R, Lee DY, et al. Tracking transplanted bone marrow stem cells and their
695 effects in the rat MCAO stroke model. *PLoS One* 2013; 8: e60049.
696 42. Rosado-de-Castro PH, Schmidt Fda R, Battistella V, et al. Biodistribution of bone marrow
697 mononuclear cells after intra-arterial or intravenous transplantation in subacute stroke patients. *Regen*
698 *Med* 2013; 8: 145-155.
699 43. Lundberg J, Södersten E, Sundström E, et al. Targeted intra-arterial transplantation of stem cells to
700 the injured CNS is more effective than intravenous administration: engraftment is dependent on cell type
701 and adhesion molecule expression. *Cell Transplant* 2012; 21: 333-343.
702 44. Misra V, Ritchie MM, Stone LL, et al. Stem cell therapy in ischemic stroke: role of IV and intra-arterial

703 therapy. *Neurology* 2012; 79: S207-S212.
704 45. Li L, Jiang Q, Ding G, et al. Effects of administration route on migration and distribution of neural
705 progenitor cells transplanted into rats with focal cerebral ischemia, an MRI study. *J Cereb Blood Flow*
706 *Metab* 2010; 30: 653-662.
707 46. Walczak P, Zhang J, Gilad AA, et al. Dual-modality monitoring of targeted intraarterial delivery of
708 mesenchymal stem cells after transient ischemia. *Stroke* 2008; 39: 1569-1574.
709 47. Janowski M, Lyczek A, Engels C, et al. Cell size and velocity of injection are major determinants of the
710 safety of intracarotid stem cell transplantation. *J Cereb Blood Flow Metab* 2013; 33: 921-927.
711 48. Cui LL, Nitzsche F, Pryazhnikov E, et al. Integrin α4 Overexpression on Rat Mesenchymal Stem Cells
712 Enhances Transmigration and Reduces Cerebral Embolism After Intracarotid Injection. *Stroke* 2017; 48:
713 2895-2900.
714 49. Fisher M, Feuerstein G, Howells DW, et al. Update of the stroke therapy academic industry roundtable
715 preclinical recommendations. *Stroke* 2009; 40: 2244-2250.
716 50. Savitz SI, Baron JC and Fisher M. Stroke Treatment Academic Industry Roundtable X: Brain
717 Cytoprotection Therapies in the Reperfusion Era. *Stroke* 2019; 50: 1026–1031.
718 51. Lyden P, Buchan A, Boltze J, et al. Top Priorities for Cerebroprotective Studies-A Paradigm Shift:
719 Report From STAIR XI. *Stroke* 2021; 52: 3063-3071.
720 52. Neiiendam JL, Køhler LB, Christensen C, et al. An NCAM-derived FGF-receptor agonist, the FGL-
721 peptide, induces neurite outgrowth and neuronal survival in primary rat neurons. *J Neurochem* 2004; 91:
722 920-935.
723 53. Kiselyov VV, Skladchikova G, Hinsby AM, et al. Structural basis for a direct interaction between FGFR1
724 and NCAM and evidence for a regulatory role of ATP. *Structure* 2003; 11: 691-701.
725 54. Berezin V and Bock E. NCAM mimetic peptides: Pharmacological and therapeutic potential. *J Mol*
726 *Neurosci* 2004; 22: 33-39.
727 55. Xu R, Feyeux M, Julien S, et al. Screening of bioactive peptides using an embryonic stem cell-based
728 neurodifferentiation assay. *Aaps j* 2014; 16: 400-412.
729 56. Modo MM, Jolkkonen J, Zille M, et al. Future of Animal Modeling for Poststroke Tissue Repair. *Stroke*
730 2018; 49: 1099-1106.
731 57. Boltze J, Modo MM, Mays RW, et al. Stem Cells as an Emerging Paradigm in Stroke 4: Advancing and
732 Accelerating Preclinical Research. *Stroke* 2019; 50: 3299-3306.
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734

735 **Titles and legends to figures**

736 **Figure 1. P2 promotes proliferation and migration of BMSCs through ERK and**
737 **PI3K/AKT pathways *in vitro*.** (A) BMSC proliferation under different concentrations of
738 P2 at 24, 48, and 72 h. (B) BMSC proliferation after treatment with different P2
739 concentrations after 2 h of OGD. (C) Migration of BMSCs in the absence or presence of
740 1 μM P2 was visualized with 0.05% crystal violet staining and evaluated using a
741 Transwell assay. Scale bar: 50 μm. (D) Protein levels in the MAPK pathway including p-
742 ERK, ERK, p-P38, P38, p-JNK, JNK, and PI3K/AKT in BMSCs were detected by
743 Western blotting. β-actin served as a control. (E) Quantitative analysis of p-ERK, ERK,
744 p-P38, P38, p-JNK, JNK, p-AKT, and AKT. Data are presented as relative expression (P2
745 or P2+inhibitor group/PBS group). (F) BMSC proliferation under ERK and PI3K
746 inhibitors was examined by MTT assay. N=6/group for all experiments. *p<0.05,
747 **p<0.01 versus PBS group. $^{\#\#}p$<0.01 versus P2 group.

748

749 **Figure 2**. **P2 enhances neurotrophic factor expression by BMSCs and reduces**
750 **neuronal cell death through anti-apoptosis pathways.** (A) RT-qPCR and Western
751 blotting showed elevated mRNA and protein levels of BDNF and IGF-1 in BMSCs.
752 GAPDH and β-actin served as controls. Protein quantification results are presented as

753 relative expression (P2 group/PBS group). N=6/group. *p<0.05, **p<0.01 versus PBS
754 group. (B) CCK8 assay of neurons cultured under normal conditions (control), OGD, or
755 OGD with different concentrations of P2. *p<0.05, **p<0.01 versus control (non-OGD)
756 group, $^{\#\#}p$<0.01 versus OGD+PBS (0 μM) group. N=7/group. (C) ERK inhibitor (ERKI)
757 and PI3K inhibitor (PI3KI) nullified the neuroprotective effects of P2 under OGD
758 condition. N=6/group. **p<0.01 versus OGD+PBS group, $^{\#\#}p$<0.01 versus OGD+P2 (1
759 μM) group. (D) Flow cytometry was performed to evaluate the numbers of apoptotic
760 neurons under normal (control), OGD, and OGD+1 μM P2 conditions. The apoptosis rate
761 was calculated as the sum of quadrant 2 (Q2, middle- to late-stage apoptotic cells) and
762 quadrant 3 (Q3, early-stage apoptotic cells). N=4/group. **p<0.01 versus control (non-
763 OGD) group, $^{\#\#}p$<0.01 versus OGD+PBS group. (E) The expression of cleaved caspase-
764 3 (CC3), cleaved caspase-9 (CC9), Bax, and Bcl-2 in neurons in the presence or absence
765 of ERKI and PI3KI were analyzed by Western blotting. (F) Quantitative analysis of CC3,
766 CC9, Bax, and Bcl-2 expression. N=6/group. *p<0.05, **p<0.01 versus control (non-
767 OGD) group; $^{\#}p$<0.05, $^{\#\#}p$<0.01 versus OGD+P2 group.
768
769 **Figure 3**. **P2 and P2 in combination with BMSCs decrease the number of apoptotic**
770 **neurons after MCAO.** (A) Representative images of neurons (NeuN, green), apoptotic

771 cell (TUNEL, red), and nuclei (DAPI, blue) immunostaining in sham-operated rats, as
772 well as in vehicle (NS)-, P2-, BMSC-, P2+BMSC-, P2+BMSC+ERK inhibitor (ERKI,
773 PD98059), and P2+BMSC+PI3K inhibitor (PI3KI, LY294002)-treated rats after MCAO.
774 Scale bars: 50 μm. (B) Quantitative analysis of TUNEL-positive neurons in rat brains.
775 Neuronal apoptosis was calculated as the percentage of $TUNEL^{+}NeuN^{+}$ cells in all $NeuN^{+}$
776 cells. N=6 rats/group. *p<0.05 or **p<0.01 versus MCAO+NS group, $^{\#}p$<0.05 or
777 $^{\#\#}p$<0.01 versus MCAO+P2+BMSC group. M: MCAO; B: BMSC.
778
779 **Figure 4. P2 promotes BMSC proliferation and synergizes with BMSCs in**
780 **upregulating neurotrophic factors expression *in vivo*.** (A) Immunofluorescence
781 staining for GFP-positive BMSCs (green) and a proliferation marker (BrdU, red) in the
782 ischemic boundary zone of BMSC- or P2+BMSC-treated MCAO rat brains. Scale bar:
783 50 μm. Quantification of proliferating BMSCs in BMSC- or P2+BMSC-treated MCAO
784 rat brains. N=4 rats/group. *p<0.05 versus MCAO+BMSC group. (B) The mRNA levels
785 of BDNF, GDNF, NGF, VEGF, and IGF-1 in rat brain tissues were evaluated using RT-
786 qPCR. GAPDH served as a control. N=5/group. $^{\&}p$<0.05 or $^{\&\&}p$<0.01 versus SHAM
787 group, *p<0.05 versus MCAO+NS group, $^{\#\#}p$<0.01 versus MCAO+BMSC group. (C)
788 The protein levels of BDNF, GDNF, IGF-1, VEGF and NGF in rat brains were examined

using Western blotting. (D) Quantitative analysis of neurotrophic protein levels in rat brains after MCAO. Data are presented as relative expression (each group/SHAM group). N=5/group. $^{\&}p<0.05$ or $^{\&\&}p<0.01$ versus SHAM group, $^{*}p<0.05$ or $^{**}p<0.01$ versus MCAO+NS group, $^{\#}p<0.05$ or $^{\#\#}p<0.01$ versus MCAO+BMSC group.

Figure 5. P2 synergizes with BMSCs in reducing infarct volume after MCAO. (A) Representative images of the Nissl-stained brain sections in SHAM, MCAO+NS, MCAO+BMSC, MCAO+P2, MCAO+P2+BMSC rats. Quantitative analysis of the infarction volume at 1 d (B) and 14 d (C) after P2 and/or BMSC treatment. N=6/group. $^{**}p<0.01$ versus MCAO+NS group, $^{\#}p<0.05$ versus MCAO+P2+BMSC group.

Figure 6. **P2 synergizes with BMSCs in promoting functional recovery and increases the activation of ERK, AKT, Nrf2, and HO-1 post-MCAO.** (A) Forelimb and hindlimb slip ratios in the beam-walking test as well as tape removal time in adhesive tape removal test. N=6/group. $^{*}p<0.05$ or $^{**}p<0.01$ versus MCAO+NS group, $^{\#}p<0.05$ or $^{\#\#}p<0.01$ versus MCAO+P2+BMSC group. (B) Representative Western blots and quantification of p-ERK, ERK, p-AKT, and AKT in rat brains. $^{**}p<0.01$ versus MCAO+NS group, $^{\#\#}p<0.01$ versus MCAO+BMSC group. N=5/group. (C) Representative Western blots and

807 quantification of p-ERK, ERK, p-AKT, and AKT in rat brains with ERK inhibitor or PI3K
808 inhibitor. N=6/group. *p<0.05 or **p<0.01 versus MCAO+NS group, $^{\#\#}p$<0.01 versus
809 MCAO+P2+BMSC group. (D) Representative Western blots and quantification of HO-1
810 and nuclear Nrf2 in rat brains. Lamin B served as the loading control of nuclear protein.
811 N=5-6/group. *p<0.05 or **p<0.01 versus MCAO+NS group, $^{\#\#}p$<0.01 versus
812 MCAO+P2+BMSC group.
813
814 **Figure 7**. **P2 and BMSCs exert synergistic neuroprotective effects.** The combination
815 treatment of P2 and BMSCs reduces brain infarct volume, ameliorates neurological
816 deficits after stroke, and these effects are mostly superior to either P2 or BMSC
817 monotreatment. Both PI3K/AKT and MAPK/ERK pathways are involved in the
818 regulatory function of P2 on BMSC and neurocyte behavior, and Nrf2 and HO-1 are
819 potential downstream effectors.