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**Original Paper**

# Pituitary Adenylate Cyclase Activating Polypeptide Elicits Neuroprotection Against Acute Ischemic Neuronal Cell Death Associated with NMDA Receptors

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**Key Words**
PACAP • OGD/R • NMDAR • Acute ischemic stroke • Neuroprotection

**Abstract**
***Background/Aims:*** The endogenous neurotrophic peptides pituitary adenylate cyclase-activating polypeptides (PACAP-27/38) protect against stroke, but the molecular mechanism remains unknown. ***Methods:*** Primary rat neural cells were exposed to PACAP-27 or PACAP-38 before induction of experimental acute ischemic stroke via oxygen-glucose deprivation-reperfusion (OGD/R) injury. To reveal PACAP's role in neuroprotection, we employed fluorescent live/dead cell viability and caspase 3 assays, optical densitometry of mitochondrial dehydrogenase and cell growth, glutathione disulfide luciferase activity, ELISA for high mobility group box1 extracellular concentration, ATP bioluminescence, Western blot analysis of PACAP, NMDA subunits, apoptosis regulator Bcl-2, social interaction hormone oxytocin, and trophic factor BDNF, and immunocytochemical analysis of PACAP. ***Results:*** Both PACAP-27 and PACAP-38 (PACAP-27/38) increased cell viability, decreased oxidative stress-induced cell damage, maintained mitochondrial activity, prevented the release of high mobility group box1, and reduced cytochrome c/caspase 3-induced apoptosis. PACAP-27/38 increased the protein expression levels of BDNF, Bcl-2, oxytocin, and precursor PACAP. N-methyl-D-aspartate receptor (NMDAR)-induced excitotoxicity contributes to the cell death associated with stroke. PACAP-27/38 modulated the protein expression levels of NMDAR subunits. PACAP-27/38 increased the protein expression levels of the GluN1 subunit, and decreased that of the GluN2B and GluN2D subunits. PACAP-27, but not PACAP-38, increased the expression level of the GluN2C subunit. ***Conclusion:*** This study provides evidence that PACAP regulated NMDAR subunits, affording neuroprotection after OGD/R injury.



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## Introduction

Despite advances in the management and care of stroke, ischemic-reperfusion injury is still a major cause of mortality and morbidity [1, 2]. Disease progression with stroke primarily involves a primary insult characterized by an infracted core, and subsequent formation of an ischemic penumbra, which over a subacute period remains as salvageable neuronal tissue, thereby amenable to therapeutic intervention [2, 3]. Secondary cell death processes, including oxidative stress, can further exacerbate cell death in the penumbra limiting neuro-restoration. Oxidative stress has been implicated in the pathogenesis of many central nervous system (CNS) disorders, including Alzheimer's disease [4] and Parkinson's disease [5].

Pituitary adenylate cyclase-activating polypeptide (PACAP), the first isolated hypothalamic neuropeptide [6], is a highly conserved member of the VIP/secretin/glucagon peptide family. PACAP exerts neurotrophic and neuromodulating effects, and exists as two biologically active forms: PACAP-38 and PACAP-27, which is produced by proteolytic cleavage of PACAP-38 [7]. PACAP-38 is the predominant form found in the brain, and PACAP-27 constitutes a minority, approximately 10%, of total brain PACAP content [8]. Both PACAP-27 and PACAP-38 (PACAP-27/38) exhibit anti-inflammatory [9, 10] and neuroprotective properties mediated through the PAC1 receptor (PAC1R), which is associated with the G-protein coupled signaling pathway via adenylate cyclase [11]. However, the mechanisms underlying PACAP-induced neuroprotection against stroke are still elusive.

The *N*-methyl-D-aspartate receptor (NMDAR) is a glutamate-gated ion channel that is critically involved in physiological and pathological functions in the CNS [1]. NMDAR is activated by allosteric modulation and is composed of seven subunits: the GluN1 subunit, four distinct GluN2 subunits (GluN2A, GluN2B, GluN2C, and GluN2D), and a pair of GluN3 subunits (GluN3A and GluN3B). NMDARs function as heterotetrameric assemblies that typically associate two GluN1 subunits with the same two GluN2 subunits or a mixture of GluN2 (for example, 2GluN1/2GluN2A or 2GluN1/GluN2A/GluN2B). In the CNS, particularly cortex and hippocampus, GluN2A and GluN2B are the predominant subunits. The GluN2A subunit-containing NMDAR in the synaptic cleft activates pro-survival signaling pathways. During ischemic conditions, extracellular glutamate concentrations increase, and stimulation of the GluN2B-containing NMDAR in the extrasynaptic sites triggers excitotoxic neuronal cell death [1]. PACAP is upregulated in an NMDAR-dependent manner in cortical neurons after focal ischemia [12]. The equilibrium shift of NMDAR subunit expression pattern is a key control point for determination of receptor diversity of the neuronal plasma membrane. Conversely, an imbalance between inhibitory and excitatory neurotransmission after an ischemic insult creates an excessive secretion of NMDA-relevant molecules, selectively limiting the tracking of NMDARs. The subsequent reperfusion after ischemia exacerbates neuronal functions and causes massive brain injuries when oxygen-saturated and nutrient-rich blood suddenly returns to the lesion after a period of ischemia. Such pathological conditions are closely resembled by the oxygen-glucose deprivation/reperfusion (OGD/R) paradigm, making it suitable as an ischemic-stroke model *in vitro*.

In this study, we demonstrated that pretreatment of primary rat neuronal cells (PRNCs) with PACAP-27 or PACAP-38 resulted in robust neuroprotective effects under OGD/R conditions. We also showed that PACAP-27/38 shifted the expression patterns of NMDAR subunits in these cells. These observations provide evidence that PACAP-27/38 modulated NMDAR-mediated neuroprotective effects against acute ischemia-induced neuronal cell death.

## Materials and Methods

*Cell culture and oxygen-glucose deprivation-reperfusion (OGD/R) progression*

Primary rat neural cells (PRNCs; consisted of 40% neurons and 60% astrocytes) were obtained from BrainBits (E18 rat cortex). As described elsewhere [13], cells ($4 \times 10^4$ cells/well) were suspended in 200 µl Neural Medium (NbActive 4, BrainBits) containing 2 mM *l*-glutamine and 2% B27 in the absence of antibiotics and grown in poly-*l*-lysine-coated 96-well plates at 37°C in humidified atmosphere containing 95% $O_2$ and 5% $CO_2$ in 40% of the neuron and 60% astrocyte cell population (determined immunocytochemically using vesicular glutamate tensmate-1) [13]. After 3 days in culture, PRNCs were exposed to 0.2 nM ~ 1 µM PACAP-27 (GeneScript, RP10334-0.5), 0.2 nM ~ 1 µM PACAP-38 (GeneScript, RP10335-0.5), 300 nM PACAP-27 + 1 µg/ml anti PACAP sheep polyclonal antibody (R and D Systems Cat# AF6380, RRID: AB_10717691), 300 nM PACAP-38 + 1 µg/ml anti PACAP sheep polyclonal antibody, 1 µg/ml anti PACAP sheep polyclonal antibody alone, and the absence of reagents (control) for 4 days at 37°C. After 7 days in culture (Fig. 1), RPNCs were exposed to OGD as described previously [13]. The cells were initially exposed to OGD medium (glucose-free Dulbecco's Modified Eagle Medium), then placed in an anaerobic chamber containing 95% $N_2$ and 5% $CO_2$ for 15 min at 37°C (preincubation), for 90 min at 37°C (culture medium pH 6.7 ~ 6.8; mimicking the acidic environment of ischemic brain *in vivo*). OGD was terminated by adding 5 mM glucose to medium and cell cultures were re-introduced to the regular $CO_2$ incubator at 37°C for 2 h (reperfusion treatment). Control cells were incubated in the same buffer containing 5 mM glucose at 37°C in a regular 95% $O_2$ and 5% $CO_2$ incubator.

*Measurement of cell viability*

Measurement of cell viability was performed using fluorescent live/dead cell assay and trypan blue exclusion method [13, 14]. In accordance with OGD/R procedure, the cells were incubated with 2 µM Calcein-AM and 4 µM EthD-1 (Invitrogen, L3224) for 45 min at room temperature (RT) in the dark. After washing once with phosphate buffer saline (PBS), the green fluorescence of the live cells was measured by the Gemini EX plate reader (Ex/Em = 490/520; Molecular Devices). In addition, trypan blue (Gibco, 15250) exclusion method was conducted and mean viable cell counts were calculated in 16 randomly selected areas (1 $mm^2$, n = 10) to reveal the cell viability. Briefly, within 5 min after adding trypan blue, we digitally captured under microscope (200×) 10 pictures (approximately 100 cells/picture) for each condition, then randomly selected 5 pictures, and counted the number of cells for each individual treatment condition. Normalized cell viability was calculated from the following equation: viable cells (%) = [1.00 – (Number of blue cells / Number of total cells)] × 100. To precisely calibrate the cell viability, the values were standardized from fluorescence intensity and trypan blue data.

*Measurement of mitochondrial activity and membrane potential*

Following cell culture, reduction of 3-(4, 5-dimethyl-2-thiazoyl)-2, 5-diphenyltetrazolium bromide (MTT; Roche, 11465007001) by mitochondrial dehydrogenases was used as a measure of mitochondrial activity as previously described [13]. The optical density of solubilized purple formazan was measured at 570 nm on the EnSpire plate reader (ParkinElmer). For the measurement of membrane potential, PRNCs

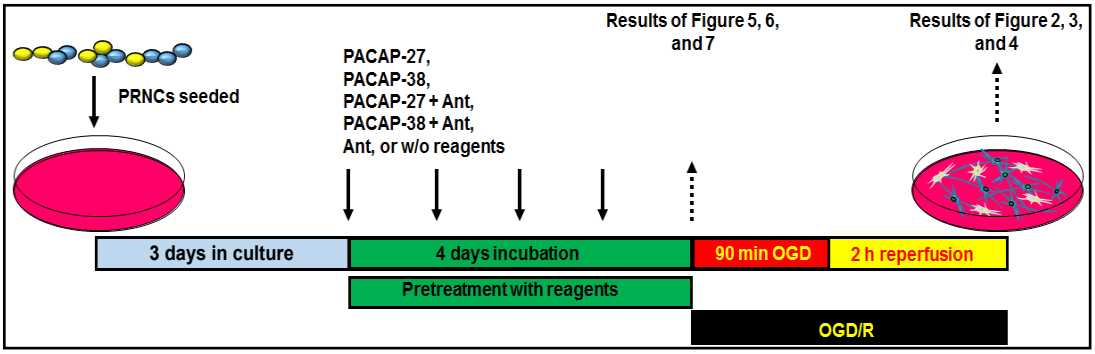


**Fig. 1.** Experimental design. PRNCs; primary rat neural cells. OGD; oxygen and glycose deprivation. Ant; Anti PACAP antibody.

were incubated with 25 nM tetramethylrhodamine methyl ester perchlorate (Sigma-Aldrich, T5428) for 45 min and the fluorescence intensity was measured by the Max Gemini EM plate reader.

*Measurement of glutathione disulfide (GSSH) activity*

Cells were treated with oxidized glutathione lysis reagent (Promega, V6611), and GSSG activity was measured by luciferase activity on the Max Gemini EM plate reader.

*Measurement of extracellular HMGB1 concentration*

After OGD/R treatment, the culture medium was centrifuged at 3,000 g, 4°C for 15 min, and the supernatant was processed for HMGB1 releasing using ELISA kit (BlueGene, E02H0009) and the absorbance at 450 nm was measured on the EnSpire plate reader.

*Measurement of cell growth and ATP content*

Following cell culture, the cleavage of the tetrazolium salt, WST-1 (4-[3(4-lodophenyl)-2-(4-nirtophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate; Roche, 05015944011) formazan was used as a measure of cell growth. The optical density was measured at 450 nm on the EnSpire plate reader. The measurement of ATP content was analyzed using ATP bioluminescence assay kit (Roche, 11699709001) according to the manufacturer instructions. Luciferase reagent was added to the samples, and the luminescence was measured by the Max Gemini EM plate reader.

*Measurement of caspase 3 activity*

The caspase 3 activity was measured by caspase 3 assay kit (Abcam, ab39383) according to the manufacturer instructions. Cell lysate (30 μg) was processed on DEVD-AFC substrate, and incubated at 37°C for 2h. The fluorescence of the caspase 3 activity was measured by the Max Gemini EM plate reader (Ex/Em = 400/505).

*Western blot analysis*

PRNCs were treated with CelLytic MT mammalian lysis reagent (Sigma-Aldrich, C3228) with protease inhibitor cocktail (Sigma-Aldrich, I3786). The lysate was centrifuged at 3,000 g, 4°C for 15 min, and the supernatant was stored at -80°C until analysis. Protein samples (4 ~ 35 μg/lane) were processed on 4~14% Tris-Glycine SDS-PAGE gel and then transferred onto a nitrocellulose membrane (Bio-Rad, 162-0112) at 30 V, 4°C for 14h. The nitrocellulose membranes were treated with PBS containing 0.1% Tween-20 (PBST) and 3% non-fat milk (Bio-Rad, 170-6404) for 45 min at RT. Membranes were then incubated with the primary antibodies, anti Bcl-2 rabbit polyclonal (1/500, Abcam Cat# ab115807, RRID: AB_10933855), anti oxytocin rabbit polyclonal (1/1,000, Abcam Cat# ab67457, RRID: AB_1952293), anti BDNF rabbit monoclonal (1/1,000, Abcam, ab1083919), anti PACAP goat polyclonal (1/1,000, Abcam, ab223668), anti GAPDH mouse polyclonal (1/5,000, Abcam Cat# ab8245, RRID: AB_2107448), anti β-tubulin rabbit polyclonal (1/30,000, Abcam Cat# ab6046, RRID: AB_2210370), anti caspase 3 rabbit polyclonal (1/100, Abcam Cat# ab4051, RRID: AB_304243), anti cleaved caspase 3 rabbit polyclonal (1/2,000, Sigma-Aldrich Cat# C8487, RRID: AB_476884), anti GluN1 rabbit polyclonal (1/1,000, Abcam Cat# ab109182, RRID: AB_10862307), and GluN2A rabbit polyclonal (1/1,000, Abcam, ab169873), anti GluN2B rabbit polyclonal (1/1,000, Abcam, ab565783), anti GluN2C rabbit polyclonal (1/1,000, Abcam Cat# ab110, RRID: AB_297655), anti GluN2D rabbit polyclonal (1/1,000, Thermo Fisher Scientific Cat# PA5-39190, RRID: AB_2555782), anti GluN3A rabbit polyclonal (1/1,000, Antibodies-Online Cat# ABIN571959, RRID: AB_10787799) and anti GluN3B (1/1,000, Antibodies, ABX135999) at 4°C for 14h. After washing with PBST, the nitrocellulose membrane was incubated with donkey anti mouse IRDye800®CW secondary antibody (1/5,000, LI-COR Biosciences Cat# 926-32212, RRID: AB_621847), or donkey anti-rabbit IRDye800®CW secondary antibody (1/5,000, LI-COR Biosciences Cat# 926-32213, RRID: AB_621848) for 90 min at RT in the dark. Immunoreactive detection using near-infrared fluorescence was performed according to the protocol of Odyssey® Infrared Imaging System (LI-COR®).

*Immunocytochemistry analysis*

PRNCs ($8 \times 10^4$ cell/well) were cultured in 400 µl Neural medium containing 2 mM *l*-glutamine and 2% B27 in the absence of antibiotics in poly-*l*-lysine 8-chamber (BD Bioscience, 354632) for 3 days, then the cells were exposed to 300 nM PACAP-27, or 300 nM PACAP-38, or the absence of reagents (CONT) for 4 days and fixed in 4% paraformaldehyde [13]. The cells were washed 5 times for 10 min in PBST. Then they were blocked by 5% normal goat serum (Invitrogen, 50062Z) in PBST for 1 h at RT. Primary antibodies included anti cytochrome c rabbit monoclonal antibody (1/1,000, Abcam Cat# ab76237, RRID: AB_1523454) and anti ATP synthase β chain (mitochondria) (1/200, Cell Signaling Technology, 05-709). The cells were incubated overnight at 4°C in primary antibody with 5% normal goat serum. The cells were washed 5 times for 10 min in PBST and then soaked in 5% normal goat serum in PBST containing corresponding secondary antibodies goat anti rabbit IgG-Alexa 488 (green; 1/1,000, Invitrogen, A11034), and goat anti-mouse IgG-Alexa 594 (red; 1/1,000, Invitrogen, Cat# A11032, RRID: AB_141672) for 90 min in the dark. Immunofluorescent images were visualized using a confocal microscope (FV1000, Olympus). Control experiments were performed with the omission of the primary antibodies yielding negative results.

*Data analysis*

Data were evaluated using one-way analysis of variance (ANOVA) followed by post hoc compromised t-tests (GraphPad Prism 6® software, RRID: SCR_002798). Statistical significance was preset at $P < 0.05$. Data are represented as means ± SD from quintuplicates of each treatment condition.

## Results

*PACAP-27/38 exerts neuroprotection against OGD/R*

As shown in Fig. 1, PRNCs were exposed to the OGD/R *in vitro* model of acute ischemic stroke [13, 15]. Under OGD/R conditions, PRNC viability was significantly decreased compared to control, which did not undergo OGD/R (Fig. 2A, CONT *vs* w/o, $P < 0.001$). Cell viability was significantly increased in a dose-dependent manner with PACAP-27 and PACAP-38 administration; the estimated $EC_{50}$ values for cell viability were 35.7 ± 1.68 nM and 49.9 ± 1.28 nM, respectively (Fig. 2A). The maximal neuroprotection of both PACAP-27 and PACAP-38 treatment was reached at 300 nM (Fig. 2A). Following acute ischemic stroke experiments *in vitro*, PRNCs were cultured with 300 nM PACAP-27 (PACAP-27), 300 nM PACAP-38 (PACAP-38), or in the absence of reagent (w/o) for 4 days at 37°C before OGD (Fig. 1). To confirm that protection of cell viability under OGD/R depends on PACAP-27/38 treatment, we compared cell viability in the presence of the PACAP antibody (PACAP-27 + Ant and PACAP-38 + Ant; Fig. 2B) with PACAP-27 or PACAP-38 alone. Pretreatment with PACAP-27 and PACAP-38 significantly increased cell viability ($F_{(2, 14)} = 48.33$, $P < 0.0001$ and $F_{(2, 19)} = 30.38$, $P < 0.0001$, respectively; Fig. 2B), and efficacy was abolished in the presence of PACAP antibody (Fig. 2B, *g vs g*, and *h vs h*). PACAP-27/38 recovered mitochondrial activity ($F_{(2, 23)} = 64.74$, $P < 0.0001$ and $F_{(2, 22)} = 88.18$, $P < 0.0001$, respectively; Fig. 2C) and mitochondrial membrane potential ($F_{(2, 35)} = 28.13$, $P < 0.0001$ and $F_{(2, 35)} = 29.63$, $P < 0.0001$, respectively; Fig. 2D), decreased glutathione disulfide (GSSG) activity ($F_{(2, 21)} = 69.04$, $P < 0.0001$, and $F_{(2, 21)} = 69.97$, $P < 0.0001$, respectively; Fig. 2E), and prevented the secretion of high mobility group box1 (HMGB1), a mediator of ischemic progression [15, 16] from PRNCs (Fig. 2F). These results indicate that PACAP-27/38 elicited neuroprotection against OGD/R.

*PACAP-27/38 acts on the mitochondrial apoptotic pathway*

In ischemic conditions, reactive oxygen species (ROS) activate the mitochondrial apoptotic signaling pathway, which intermediates caspase 3 activation [17]. PACAP-27/38 administration significantly reduced the expression level of cleavage caspase 3 (17 kDa, Fig. 3A, B) and its activity (Fig. 3C), although caspase 3 expression levels were similar between experimental groups (35 kDa, Fig. 3A, B). As caspase 3 is downstream of cytochrome c-mediated apoptotic signaling, we sought to determine whether PACAP-27/38 prohibited the release of cytochrome c from mitochondria into the soluble cytoplasm (Fig. 4). In

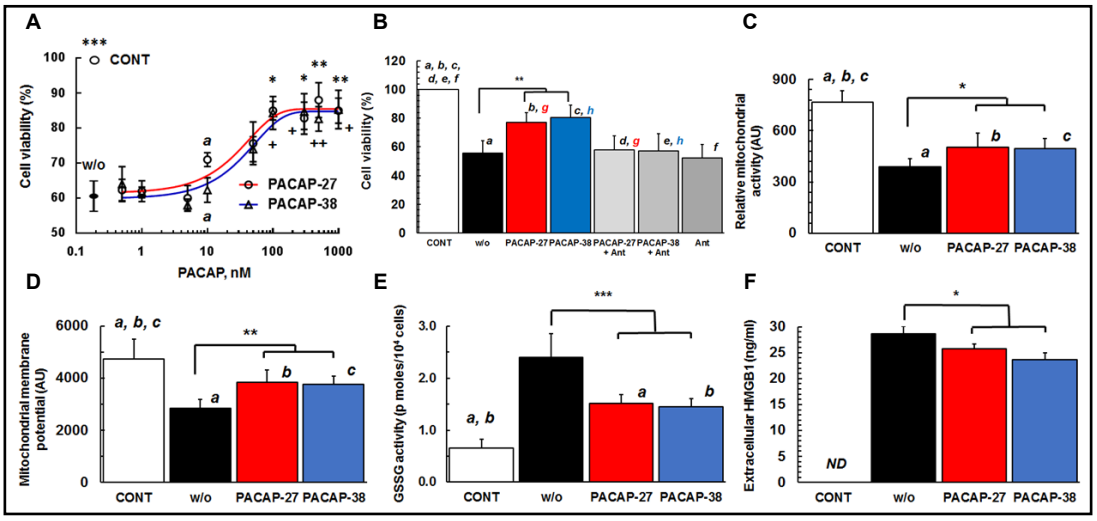


**Fig. 2.** PACAP-27 and PACAP-38 confer neuroprotective effects and attenuate the oxidative stress against OGD/R. (A) Dose-response effects on cell viability based on PACAP-27 and PACAP-38 concentration. The maximal neuroprotection of both PACAP-27 and PACAP-38 was reached at 300 nM. Following incubation of PRNCs in the absence of reagents (w/o), 300 nM PACAP-27 (PACAP-27), and 300 nM PACAP-38 (PACAP-38) for 4 days at 37°C, PRNCs were performed with OGD/R treatment. Control PRNCs (CONT) were not performed with the OGD/R. a versus (vs) a P<0.05. (B) Anti PACAP antibody significantly reduced both PACAP-27 and PACAP-38 neuroprotective effects. Following incubation of PRNCs in the absence of reagents (w/o), 300 nM PACAP-27, 300 nM PACAP-38, 300 nM PACAP-27 + 1 μg/ml anti PACAP antibody (PACAP-27 + Ant), 300 nM PCAP-38 + 1 μg/ml anti PACAP antibody (PACAP-38 + Ant), and 1 μg/ml anti PACAP antibody (Ant) only for 4 days at 37°C, PRNCs were performed with OGD/R treatment. a vs a P<0.001, b vs b P<0.001, c vs c P<0.001, d vs d P<0.001, e vs e P<0.001, f vs f P<0.001. (C) Mitochondrial activity by MTT assay. a vs a P<0.001, b vs b P<0.001, and c vs c P<0.001. (D) Mitochondrial membrane potential by TMRM assay. a vs a P<0.001, b vs b P<0.001, and c vs c P<0.001. (E) GSSG activity by GSH/GSSG-Glo assay. a vs a P<0.001, b vs b P<0.001, and c vs c P<0.001. (F) Extracellular HMGB1 levels by HMGB1 ELISA assay. a vs a P<0.001, b vs b P<0.001, and c vs c P<0.001. (C, D, E, F). *P<0.05, **P<0.01, ***P<0.001, ⁺P<0.05, and ⁺⁺P<0.01 vs w/o. Experiments were conducted in triplicate, with n = 3 ~ 5 per treatment condition in each run.

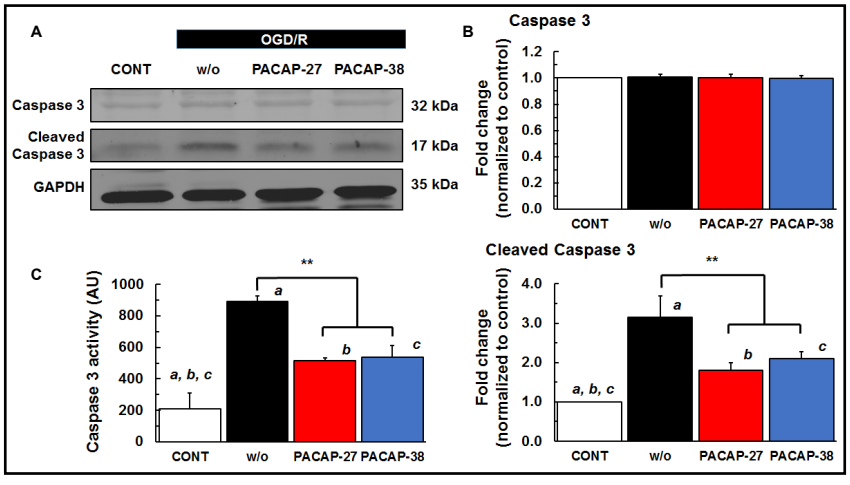


**Fig. 3.** PACAP-27 and PACAP-38 decreased both caspase 3-cleavage and -activity. Following incubation of PRNCs in the absence of reagents (w/o), 300 nM PACAP-27, and 300 nM PACAP-38 for 4 days at 37°C, PRNCs were performed with OGD/R treatment. Control (CONT) was not performed with the OGD/R treatment. (A) Western blot analysis. (B) Relative quantification of caspase 3 (top) and cleave caspase 3 (bottom). a vs a P<0.01, b vs b P<0.05, and c vs c P<0.05. (C) Caspase 3 activity. a vs a P<0.001, b vs b P<0.01, c vs c P<0.01, and **P<0.01 vs w/o. Experiments were independently conducted 3 ~ 4 times.

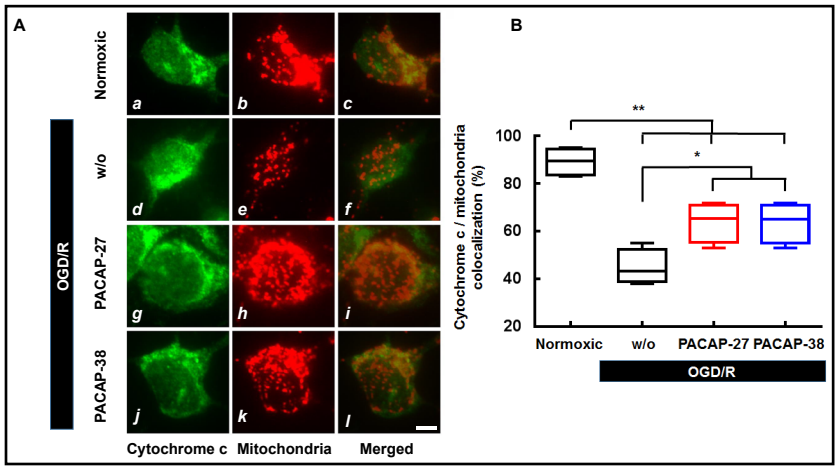


**Fig. 4.** PACAP-27 and PACAP-38 decrease the release of cytochrome c from mitochondria, which turns on the caspase 3-induced apoptotic signal pathway. Following incubation of PRNCs in the absence of reagents (w/o), 300 nM PACAP-27, and 300 nM PACAP-38 for 4 days at 37°C, PRNCs were performed with the OGD/R treatment. Control (CONT) was not performed with the OGD/R treatment. (A) Immunocytochemistry analysis. Cytochrome c (a, d, g, and j) and mitochondria (b, e, h, and k) staining reveals an apparent maintained within the mitochondria under OGD/R condition when treated with 300 nM PACAP-27 and PACAP-38 compared to without PACAP treatment (w/o). Cytochrome c and mitochondria double-positive cells are shown in panels. Green: cytochrome c, Red: mitochondria, scale bar = 5 μm. (B) Colocalization of cytochrome c and mitochondria was quantified independently 4 times. * P<0.01 and ** P<0.01.

normoxic conditions, cytochrome c was retained within the mitochondria (Fig. 4A (*a* ~ *c*), B). Under the OGD/R condition, cytochrome c was released into the cytoplasmic area (Fig. 4A (*d* ~ *f*), B). When the cells were treated with PACAP-27/38, cytochrome c remained within the mitochondria (Fig. 4A (*g* ~ *l*), B).

*Biological activity readouts across treatments*

The following experiments were performed with PRNCs cultured with 300 nM PACAP-27 (PACAP-27), 300 nM PACAP-38 (PACAP-38), and in the absence of reagent (CONT) for 4 days at 37 °C (Fig. 1). PACAP-27/38 is related to cell growth pathways [18] and anti-inflammatory signaling [9, 10], suggesting its potential application in the treatment of neuronal diseases [19]. However, PACAP-27/38 administration did not alter cell growth (Fig. 5A) or ATP content (Fig. 5B) of PRNCs compared with control treatment (CONT). Next, because peripheral PACAP-27/38 participates in glucose metabolism and modulation of ROS production [20], we examined the effects of PACAP-27 and PACAP-38 on mitochondrial membrane potential and GSSG activity. PACAP-27/38 administration did not change the mitochondrial membrane potential (Fig. 5C) and GSSG activity (Fig. 5D) in comparison with control values (CONT).

*PACAP-27/38 increases neurotrophic factor expression*

PACAP-related neuroprotective effects are due to inhibition of apoptosis and reduction of oxidative stress [9]. We therefore assessed whether PACAP-27 and PACAP-38 modulated expression of neurotrophic factors. PACAP-27/38 administration significantly increased the protein expression levels of prototype brain-derived neurotrophic factor BDNF (proBDNF), mature BDNF (mBDNF), antiapoptotic protein Bcl-2 (B-cell lymphoma 2), oxytocin, and precursor PACAP, although the PAC1R expression levels were similar to control levels (Fig. 6).

*PACAP-27/38 shifts the NMDAR subunit expression patterns*

PACAP promotes survival of rat cortical neurons against glutamate-induced neuronal cell death [21] and modulates the components of NMDARs specific to glutamate [22, 23]. We investigated whether PACAP-27 and PACAP-38 altered the expression patterns of NMDAR subunits (Fig. 7). PACAP-27/38 administration significantly increased GluN1 subunit

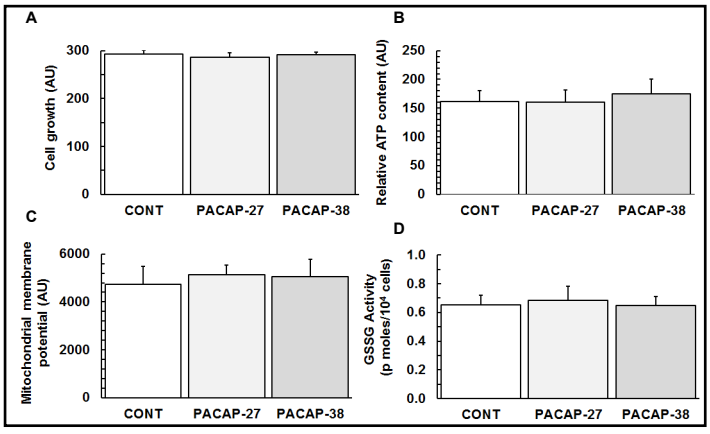


**Fig. 5.** Effects of PACAP-27 and PACAP-38 treatment on biological activity before OGD. Following incubation of PRNCs in the absence of reagents (control; CONT), 300 nM PACAP-27, and 300 nM PACAP-38 for 4 days at 37°C, the biological activity was measured. (A) PRNCs' cell growth. PACAP-27 and PACAP-38 had no significant effects on cell growth. (B) ATP contents. (C) Mitochondria membrane potential. (D) GSSG activity. GSSG is a biomarker of oxidative stress. Experiments were conducted in triplicate, with n = 5 per treatment condition in each run.

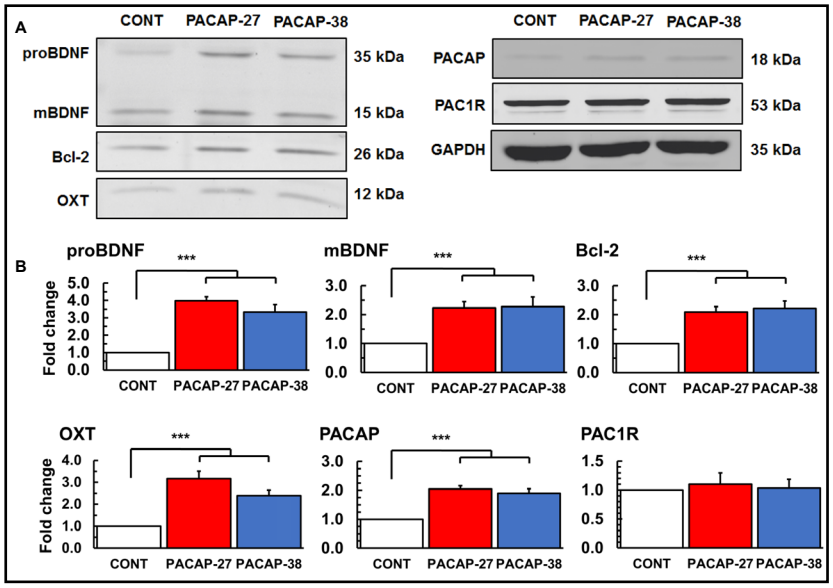


**Fig. 6.** PACAP-27 and PACAP-38 enhanced neuroprotective factor expression. Following incubation of PRNCs in the absence of reagents (control; CONT), 300 nM PACAP-27, and 300 nM PACAP-38 for 4 days at 37°C, Western blot was performed. (A) Western blot analysis. (B) Relative quantification of protein expression levels. ***P<0.001. Experiments were independently conducted 3 ~ 4 times.

expression levels, but decreased GluN2B and GluN2D subunits. Interestingly, PACAP-27, but not PACAP-38, significantly increased GluN2C subunit expression levels.

## Discussion

The present study revealed a novel molecular mechanism underlying PACAP-mediated neuroprotection against acute ischemic stroke in a cell culture paradigm. We found that PACAP-elicited neuroprotection is associated with repression of apoptotic signaling induced by mitochondrial damage, prevention of HMGB1 secretion, enhancement of neurotrophic factor levels, and reconstitution of NMDAR subunit components in PRNCs.

Ischemic injury is exacerbated by aberrant ROS accumulation, generated primarily by damaged mitochondria [17], which leads to apoptosis and necrosis. During OGD/R, cell viability, mitochondrial activity, and mitochondrial membrane potential were decreased, and GSSG activity and extracellular HMGB1 levels were increased (Fig. 2). Under OGD/R conditions, pretreatment with PACAP-27 and PACAP-38 increased cell viability, mitochondrial activity, and mitochondrial membrane potential, while decreasing GSSG activity and

preventing HMGB1 secretion from the cell (Fig. 2). We showed that PACAP-27/38 confers neuroprotection against OGD/R (Fig. 2), and that PACAP-27 ameliorates cell death more than PACAP-38 under OGD/R ($EC_{50}$ values, Fig. 2A, *a* vs *a*). PACAP contributes to neuronal stem cell proliferation [24], energy balance [25], and differentiation [26, 27]. Cell growth of PRNCs was not affected by PACAP-27/38 (Fig. 5A). We predicted that PACAP-27/38 would affect GSSG activity, mitochondrial membrane potential, and ATP contents in PRNCs, increasing neuroprotection before OGD, but no significances were detected across treatment conditions (Fig. 5B-D), which suggest that PACAP-27/38 treatment-related neuroprotection against acute ischemic stroke was distinct from cell proliferation and mitochondrial maintenance signal pathways in PRNCs.

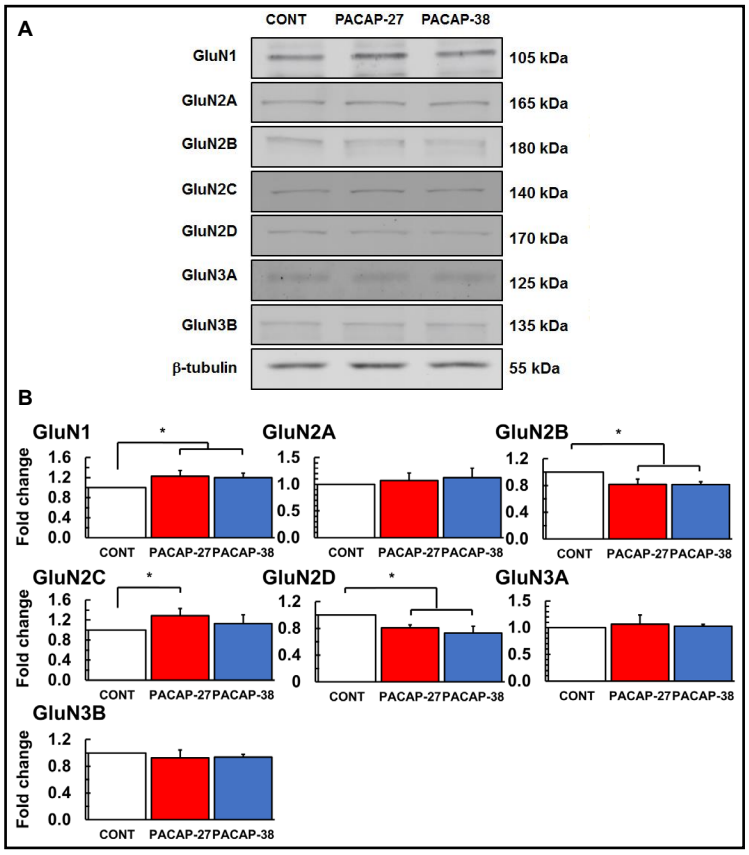


**Fig. 7.** PACAP-27 and PACAP-38 modulated the expression levels of NMDAR subunits. Following incubation of PRNCs in the absence of reagents (control; CONT), 300 nM PACAP-27, and 300 nM PACAP-38 for 4 days at 37°C, Western blot was performed. (A) Western blot analysis. (B) Relative quantification of protein expression levels. *P<0.05. Experiments were independently conducted 3 ~ 4 times.

Bcl-2 suppresses the release of cytochrome c from the mitochondria into the cytosol, and prevents the activation of caspase 9 and subsequent cleavage of caspase 3. BDNF plays a critical role in learning and memory, and is essential for synaptic function, plasticity, and neuronal survival. Oxytocin protects against acute ischemia-induced inflammation and oxidative stress, and is associated with GABA (γ-aminobutyric acid) signaling transduction in PRNCs [15]. PACAP-27/38 elevated the expression levels of Bcl-2, BDNF, and oxytocin before OGD (Fig. 6), although we could not detect BDNF and oxytocin levels in culture medium (data not shown). These observations indicated that PACAP-27/38 enhanced neuroprotection against acute ischemic stroke via increased expression of neurotrophic factors [28]. Additionally, delivering PACAP38 intranasally in Huntington's disease mouse models mitigated disease-related deficits by restoring cognitive function and PAC1R protein levels, and increasing expression of BDNF and proteins associated with synaptic plasticity [29]. PACAP increases its own expression in an autocrine positive feedback loop [30]. Our results confirmed that PACAP-27/38 increased the expression level of precursor PACAP (Fig. 6). PAC1R plays a pivotal role in the transcriptional regulation of BDNF. However, PAC1R expression levels were not changed by administration of PACAP-27/38 (Fig. 6). These data indicated that PAC1R-mediated signal transduction was attributed to neurotrophic factors' expression, and that the PAC1R expression mechanism independently contributed to differential function of the PACAP-27/38 in PRNCs.

Neuronal cell death after stroke is mediated by the activation of extrasynaptic GluN2B-containing NMDARs which inhibit the CREB (cAMP response element-binding protein) and Erk1/2 (extracellular signal-regulated kinase 1 and 2) pathways in addition to directly promoting apoptosis signaling induced by caspase 3 activation [31, 32]. GluN2B subunit-containing NMDARs mediate brain damage in stroke through intracellular signaling cascades [31]. Our observations suggested that PACAP-27/38 attenuated cytochrome c/caspase 3 death signaling (Fig. 3 and 4) due in part to decreased expression levels of the GluN2B subunit (Fig. 7). During ischemia/reperfusion, excessive glutamate accumulates in the synaptic cleft and extracellular space of neuronal cells, leading to neuronal cell death [33]. The GluN2D subunit has the highest affinity for glutamate ($EC_{50}$: 0.4 μM) compared to the other GluN2 subunits ($EC_{50}$: 1 ~ 4 μM) [34]. The NMDARs containing a combination of the GluN1 and GluN2A subunits have the lowest sensitivity to glutamate [35]. PACAP-27/38 robustly increased the GluN1 subunit expression and slightly enhanced the GluN2A subunit expression (Fig. 7). It is conceivable that under pretreatment of PRNCs with PACAP-27/38, an increase in GluN1/GluN2A subunits and decrease in GluN2B/GluN2D subunits may synergistically amplify neuroprotection before OGD.

The NMDAR is a cation channel that binds the neurotransmitter glutamate, and is constitutively deactivated by $Mg^{2+}$ at resting membrane potential; the "$Mg^{2+}$ block" [36]. Opening the NMDAR channel requires both glutamate binding to GluN2 subunits and membrane depolarization, which are necessary to remove the $Mg^{2+}$ block. NMDARs containing GluN2C are more resistant to the $Mg^{2+}$ block than those containing GluN2A and GluN2B [34, 37]. A GluN2C knockout model demonstrates more vulnerability to neuronal cell damage following ischemia, suggesting that GluN2C has a stronger neuroprotective effect than other GluN2 subunits [38]. BDNF signaling upregulates *GluN2C* gene expression [39]. BDNF has neuroprotective properties, and can rescue neurons from NMDAR blockade-induced neuronal cell death [40]. PACAP-27/38 increased the expression levels of both proBDNF and mBDNF (Fig. 6). Interestingly, PACAP-27, but not PACAP-38, significantly increased the expression level of GluN2C (Fig. 7), indicating that PACAP-27 may trigger an alternative signaling pathway promoting neuroprotection, which is emerging as a key factor in neurodegenerative diseases [41, 42].

That PACAP is altered in the present stroke model advances the notion of PACAP's involvement in aging. Of note, aging is major co-morbidity factor of stroke [43, 44]. An impaired neurovascular unit has been implicated as a key pathology of stroke [45, 46], and aging appears to dampen PACAP expression in the vasculature of mice, rendering the blood supply to the brain vulnerable to stroke [47, 48]. In other aging-related neurological disorders, such as Alzheimer's disease, pre-senile systemic amyloidosis displayed an accelerated trajectory in PACAP knockout mice, supporting the concept that PACAP may serve a neuroprotective role in age-induced degenerative processes [49], likely via sequestration of β-amyloid toxicity [50]. Moreover, PACAP-knockout mice exhibit accelerated retinal aging [51]. Such aging-mediated PACAP alteration seen in mice is also observed in aged non-human primates with cognitive impairments [52], suggesting that a deficient PACAP expression pathologically manifests as learning and memory dysfunction. Altogether, in tandem with the present data, PACAP may play a pivotal role in harnessing healthy aging, possibly by nurturing the homeostasis of the neurovascular unit and promoting neuroprotective effects.

A major contributor to neuronal cell death in stroke is excitotoxicity [1, 53, 54], which results in glutamatergic synapse dysfunction, particularly NMDAR signaling transduction [55]. Of note, inhibiting NMDARs with antagonists such as kynurenic acid, the synthetic analog of kyneuric acid, and MK-801 precluded migraine-related overexpression of PACAP-38 and presumably excessive glutamate release in the trigeminal nucleus caudalis of migraine model rats, suggesting a connection between NMDARs and PACAP expression [56]. While NMDAR antagonists can potentially mitigate glutamate-generated hyperexcitation in migraines and other neuronal maladies [56], they have also been associated with adverse effects such as addiction in rats [57, 58]. Our data demonstrated that PACAP-27/38 exerted neuroprotection against acute ischemic stroke by shifting the expression of NMDAR subunits. Pilot *in vitro*

data reveal that PACAP treatment also reduced levels of OGD/R-induced inflammation, including TNF-α expression. Although differences of physiological function and properties between PACAP-27 and PACAP-38 in PRNCs remain unexplored, our study highlights a close interaction between PACAP-27/38 and NMDARs that should advance our understanding of stroke pathology and its treatment.

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## Disclosure Statement

The authors have no conflicts of interest to declare.

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