Original Article
The stress peptide PACAP-38 protects neurons against ketamine-induced apoptosis in developing rat retina

Lingqi Gao[1], Junde Han[1], Yingtian Wang[1], Jing Dong[1], Jijian Zheng[2]

[1]Department of Anesthesiology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, P. R. China; [2]Department of Anesthesiology, Shanghai Children's Medical Center, Shanghai Jiao Tong University School of Medicine, Shanghai, P. R. China

Abstract: Early exposure to general anesthetics can cause widespread neuronal apoptosis and long-term neurocognitive deficits. However, the combined effects of general anesthetics and various noxious stimulations on animal neuronal survival are controversial. This study aimed to assess the effects and mechanisms of pituitary adenylate cyclase-activating peptide-38 (PACAP-38, a master stress peptide produced by noxious stimulation) on ketamine-induced neuronal apoptosis. Whole-mount retinas isolated from postnatal day 7 Sprague Dawley rats were cultured and incubated with 150 μM ketamine for 5 hours in the presence or absence of PACAP-38. Then, immunohistochemistry detecting active caspase-3 and TUNEL assay were used to evaluate neuronal apoptosis in the retinal ganglion cell layer. Interestingly, 10^{-8} M PACAP-38 significantly reduced ketamine-induced neuronal apoptosis in the retinal ganglion cell layer with mean ratios of caspase-3 positive cells decreasing from 6.5±0.7% to 3.2±0.2% (P<0.001) and TUNEL-positive cells from 31.3±4.4% to 16.9±3.0% (P=0.004), respectively. PACAP6-38, a PACAP-38 antagonist, abolished the anti-apoptotic effect of PACAP-38 at 10^{-7} M. Further assessments showed that PACAP's anti-apoptotic effects could be partly antagonized by adenylate cyclase (AC) and protein kinase A (PKA) inhibitors, respectively, and mostly counteracted by extracellular signal-regulated kinase (ERK1/2) inhibitor. In summary, our study demonstrated PACAP-38 could protect neurons from ketamine-induced apoptosis in early developmental rat retina. This anti-apoptotic effect PACAP-38 is partly dependent on the cAMP/PKA signaling pathway, and mainly ascribed to ERK1/2.

Keywords: PACPA, ketamine, anesthetic, neuronal apoptosis

Introduction

Preclinical studies have clearly shown that long time or repetitive exposure of general anesthetics to neonatal animals can lead to widespread neuronal apoptosis, neurocognitive deficits, and neurodegenerative disorders; however, whether general anesthetics also cause similar changes during the early developmental stage in human brain cells remains elusive [1-5]. Contrasting with most animal experiments, in which anesthesia is the sole interference factor, clinical studies confront considerably more complex confounding factors, including patient's general conditions, various diseases and surgical stimulations, and balance between noxious stimulation and depth of anesthesia [6]. It remains controversial whether different noxious stimulations could affect anesthesia-induced neuronal apoptosis [7-9].

Multiple clinical and animal studies have convincingly shown that noxious stimulation in early life can cause various abnormalities in long-term pain perception and behavior, with neuronal generation depending on the types and degrees of noxious stimulations [10, 11]. In addition to inflammatory cytokines, a number of neurotransmitters and stress peptides are also involved in noxious stimulation induced developmental abnormalities [12].

Among the stress peptides, pituitary adenylate cyclase-activating peptide (PACAP) is a highly conserved pleiotropic neuropeptide widely expressed in the nervous system; it functions as a neurotransmitter, neuromodulator, and/or neurotrophic factor to regulate neuroendocrine stress response at multi-levels, especially under prolonged or traumatic stress [13-16]. After noxious stimulation, the level of PACAP-like

immunoreactivity significantly increases in the brain, retina, and other tissues [15, 17]. PACAP molecules exist in two forms, including PACAP-27 and -38, with PACAP-38 showing a more potent protective effect compared with PACAP-27 [18]. As a neurotrophic factor, PACAP protects neurons from a variety of noxious stimulations such as ischemia, oxidative stress, and hypoxia [19, 20], and promotes the survival of central and peripheral neurons as well as pluripotent stem cell differentiation [21]. However, whether PACAP also protects neurons from general anesthesia induced apoptosis during early development remains unclear.

In rats, postnatal day 7 is considered the most vulnerable period to general anesthetics, since synaptic genesis is prevalent in the developing central nervous system [22, 23]. Retina, an extension of the central nervous system, is composed of three neuronal layers. It provides an excellent model to assess neuronal degenerations [24]. Therefore, the whole-mount retina culture model of P7 animals was used to assess the effects and mechanisms of PACAP-38 on ketamine-induced neuronal apoptosis in developing rat retina.

Materials and methods

Animals

Animal procedures were approved by the Animal Care Committee at Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, and conducted in strict accordance with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication No. 85-23, revised in 1996) and ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Postnatal day 7 Sprague Dawley (SD) rats were provided by Experimental Animal Center, Shanghai General Hospital, Shanghai (China) and lived with their mother under a 12-h light/dark cycle, with food and water available *ad libitum* to their mother until experiment start. Male and female rat pups were included in this study. Every effort was made to minimize animal number and discomfort during the experiments.

Pharmacology

The following drugs were used: Ketamine (Gutian Pharmaceutical Company, China), PACAP-38 and PACAP6-38 (Enzo Life Sciences, USA), adenylate cyclase (AC) inhibitor SQ 22536 and protein kinase A (PKA) inhibitor H-89 (Sigma-Aldrich Company, USA), extracellular signal-regulated kinase (ERK1/2) inhibitor U0126 (Beyotime Company, China). All drugs were prepared as concentrated stock solutions, stored at -20°C, and diluted at 1:1000 in artificial cerebrospinal fluid (ACSF) on the experimental day.

Methods

The experimental protocol was slightly modified from previous methods [25]. Briefly, experimental rat pups were euthanized by over-exposure to CO_2, and instantaneously decapitated. Freshly enucleated eyes were immersed in cold ACSF (in mM: 119 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 1.0 KH_2PO_4, 2.5 $CaCl_2$, 26.2 $NaHCO_3$, and 11 D-glucose) bubbled with 95% O_2 and 5% CO_2. Then, the eyeballs were transferred to ACSF-containing incubation chambers in the presence of different drugs for 5 hours at 37°C. Based on a previous study [26], PACAP-38 at 10^{-8} M was first assessed. In order to facilitate the diffusion of PACAP-38 and other drugs into eyeballs, a small incision was made between the edges of cornea and sclera using ophthalmology scissors. After drug intervention, the eyeballs were rapidly transferred into ice-cold (0-4°C) ACSF. The cornea, iris, lens, and vitreous humor were removed from eyes using scissors under a stereomicroscope. Then, the detached retinas were fixed in 4% paraformaldehyde at 4°C for 24 hours. After fixation, retina specimens were dehydrated with an ethanol gradient, paraffin embedded, and sectioned at 4-6 µM on a paraffin slicing machine (Leica-2135, German) for activated caspase-3 and TUNEL detection.

Cleaved Caspase-3 staining

Cleaved Caspase-3 detection in the retinal ganglion cell layer was performed according to the manufacturer's recommendations. First, endogenous peroxidase was inactivated by 3% of hydrogen peroxide, and heat-induced epitope retrieval was carried out in Tris/EDTA buffer (pH 9.0) in a pressure cooker; then, retinal tissue sections were successively incubated with rabbit anti-cleaved caspases-3 antibody (#9661s, 1/300, Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C, and horseradish peroxi-
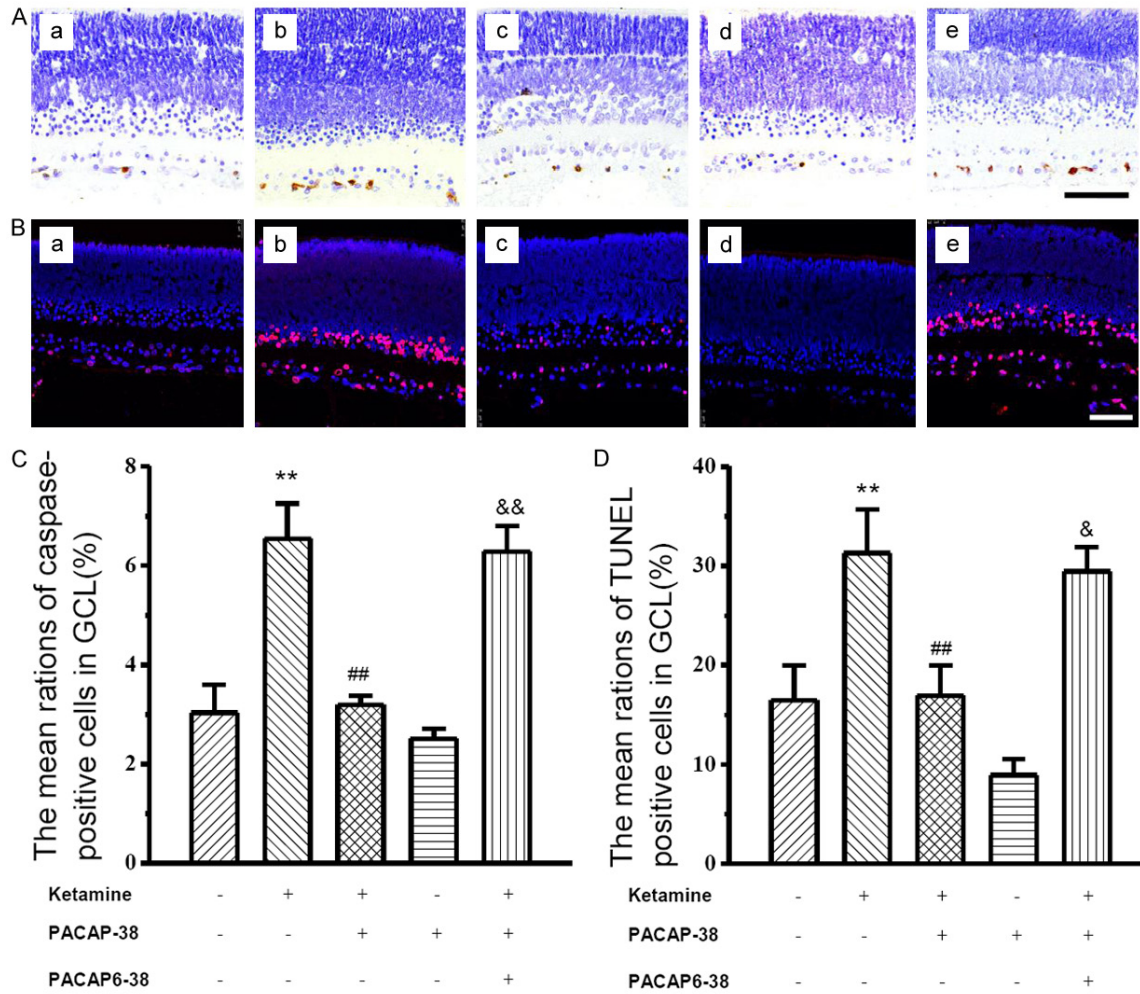
Figure 1. PACAP-38 alleviates ketamine-induced neuronal apoptosis in early retinal development. (A and B) representative photomicrograph of apoptotic cells expression in the retinal ganglion cell layer using Caspase-3 immunohistochemistry and TUNEL labeling (brown color in A and red fluorescence in B). a: Control; b: Ketamine 150 μM; c: Ketamine 150 μM + PACAP-38 10^{-8} M; d: PACAP-38 10^{-8} M; e: Ketamine + PACAP-38 10^{-8} M + PACAP6-38 10^{-7} M. (n=5 retinas per subgroup). Scale bar, 50 μm. (C and D) Quantification of the rations of Caspase-3-positive and TUNEL-positive cells present in the retinal cells layer (GCL). The results are expressed as mean ± SEM. *$P<0.05$, **$P<0.01$ versus with control group; #$P<0.05$, ##$P<0.01$ versus with ketamine group; &$P<0.05$, &&$P<0.01$ versus with ketamine + PACAP-38 group.

dase-conjugated goat anti-rabbit IgG (PV-90-01, ZSGB-BIO, Beijing, China) at 37°C for 1 hour. Finally, caspase-3 immunoreactivity was detected by chromogenic reaction using 3, 3'-diaminobenzidine (DAB) (ZLI-9017, ZSGB-BIO, Beijing, China) oxidization. Following hematoxylin (blue) counterstaining, images were acquired under a microscope at 100 × magnification.

TUNEL assay

According to instructions of the TUNEL kit (Roche Applied Science, Indianapolis, IN, USA),

retinal tissue slides were deparaffinized and rehydrated. This was followed by treatment with proteinase K (Roche Applied Science, Indianapolis, IN, USA) to facilitate the exposure of antigen binding sites, and incubation with 3% hydrogen peroxide for endogenous peroxidase inactivation. After the preliminary preparation, retinal slices were incubated in a terminal deoxynucleotidyl transferase (TdT) reaction mix at 37°C for 1 hour, followed by DAPI for 5 min. TUNEL-positive cells were visualized by confocal fluorescence microscopy and imaged with a digital camera (Leica TCS SP8; Leica, Germany). Five images of randomly selected
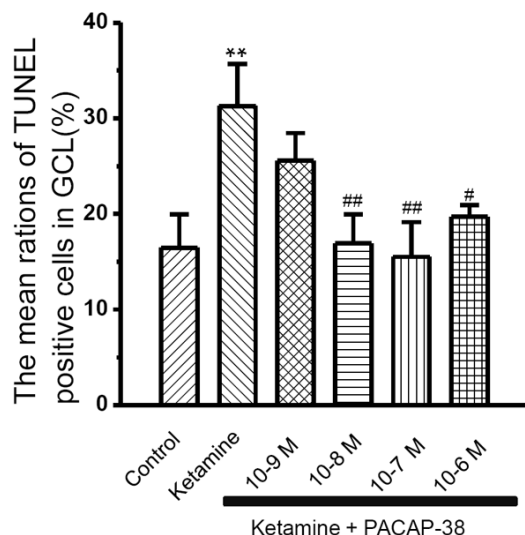
Figure 2. Effects of PACAP-38 with different concentrations on ketamine-induced retinal apoptosis. The whole-mount retinas were treated with ketamine for 5 hours in the absence or presence of different concentrations of PACAP-38 (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M), The neuronal apoptosis was detected by TUNEL assay. The results are expressed as mean ± SEM (n=5 retinas per subgroup). #P<0.05, ##P<0.01 versus ketamine group.

high power fields were captured in each retinal specimen.

Statistical analysis

Mean ratios of cleaved caspase-3 and TUNEL positive neurons in the retinal ganglion cell layer were calculated by the Image-Pro Plus software (Media Cybemetics Company, USA). Statistical analyses were performed using Origin version 8.0 (Origin, OriginLab Corporation, USA). Data were tested for normal distribution using the Shapiro-Wilk test, and for variance homogeneity with the Levene test. Differences among groups were assessed by one-way ANOVA followed by LSD post hoc test. Statistical comparisons were performed using the Mann-Whitney test for very small sample size with non-normal distributed data. Data are mean ± standard error of the mean (SEM). All differences were considered statistically significant at P<0.05.

Results

PACAP-38 alleviates ketamine-induced neuronal apoptosis in early retinal development

Both immunohistochemistry and TUNEL assays showed that PACAP-38 significantly alleviated

ketamine-induced neuronal apoptosis in early retinal development (**Figure 1**). 10^{-8} M PACAP-38 reduced mean ratios of caspase-3 positive cells from 6.5±0.7% to 3.2±0.2% (P<0.001) (**Figure 1A** and **1C**); meanwhile, TUNEL-positive cells were decreased from 31.3±4.4% to 16.9±3.0% (P=0.004) (**Figure 1B** and **1D**). In addition, the PACAP receptor antagonist PACAP6-38 (10^{-7} M) almost blocked the neuroprotective effect of PACAP-38. Mean ratios of caspase-3 and TUNEL-positive cells increased from 3.2±0.2% to 6.3±0.5% (P=0.001) (**Figure 1A** and **1C**), and from 16.9±3.0% to 29.4±2.5% (P=0.01) (**Figure 1B** and **1D**), respectively, after co-incubation with 10^{-7} M PACAP6-38. Mean ratios of TUNEL-positive cells in the ganglion cell layer after treatment with different concentrations of PACAP-38 are shown in **Figure 2**.

The cAMP/PKA signaling pathway partly contributes to PACAP-38's effect against ketamine-induced neuronal apoptosis in the developing rat retina

To explore the role of cAMP/PKA signaling in PACAP-38's effect against ketamine induced retinal apoptosis at P7, the cell-permeable AC inhibitor SQ22536 or selective PKA inhibitor H-89 was co-incubated with ketamine and PACAP-38. SQ22536 (100 μM) and H-89 (1.0 μM) significantly reduced but not completely counteracted the alleviation effects of 10^{-8} M PACAP-38 on neuronal apoptosis induced by 150 μM ketamine, with mean ratios of caspase-3 positive cells in the retinal ganglion cell layer increasing from 3.3%±0.2 to 5.3±0.2% (P=0.001), or 6.0±0.4% (P=0.0004), respectively (**Figure 3A** and **3C**); meanwhile, mean ratios of TUNEL-positive cells were increased from 16.9±2.9% to 26.9±1.0% (P=0.05) or 27.9±2.7% (P=0.02), respectively (**Figure 3B** and **3D**), indicating that cAMP/PKA signaling was partly involved in PACAP-38's effects on ketamine-induced neuronal apoptosis in P7 rats.

ERK1/2 mainly contributes to PACAP-38's effect against ketamine-induced neuronal apoptosis in developing rat retina

Next, we assessed whether ERK1/2, downstream of cAMP/PKA signaling, contributed to the alleviation effects of PACAP-38 on ketamine-induced retinal apoptosis in P7 rats. The ERK1/2 inhibitor U0126 (50 μM) almost completely abolished the alleviation effects of 10^{-8}
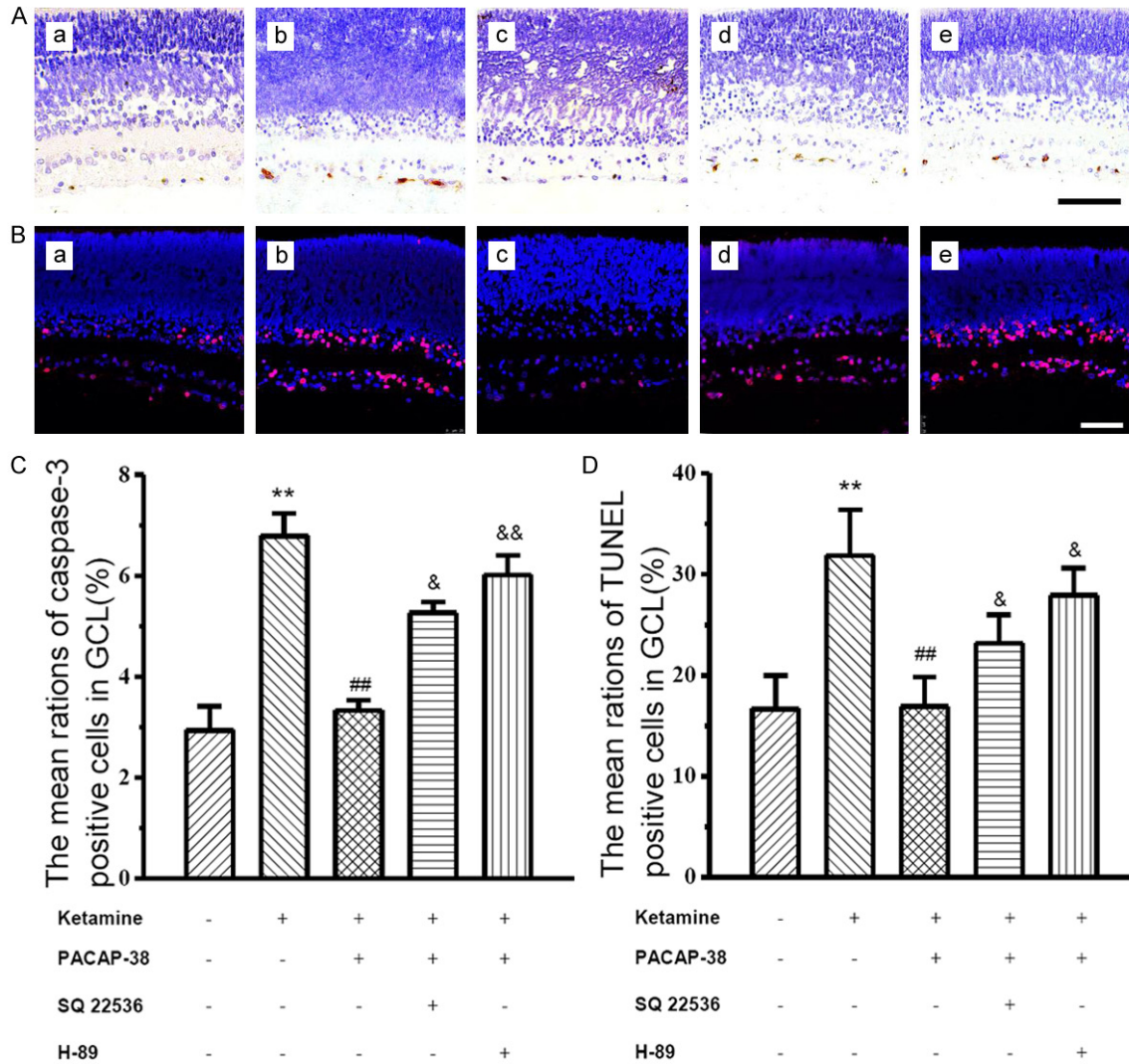
Figure 3. The cAMP/PKA Signaling Pathway partly contributes to PACAP-38's effect against ketamine-induced neuronal apoptosis in the developing rat retina. (A and B) Representative photomicrograph of apoptotic cells expression in the retinal ganglion cell layer using Caspase-3 immunohistochemistry and TUNEL labeling (brown color in A and red fluorescence in B). a: Control; b: Ketamine 150 μM; c: Ketamine 150 μM + PACAP-38 10^{-7} M; d: Ketamine 150 μM + PACAP-38 10^{-7} M + SQ22536 100 μM; e: Ketamine 150 μM + PACAP-38 10^{-7} M + H-89 10 μM. (n=5 retinas per subgroup). Scale bar, 50 μm. (C and D) Quantification of the rations of Caspase-3-positive and TUNEL-positive cells present in the retinal ganglion cell layer. The results are expressed as mean ± SEM. *P<0.05, **P<0.01 versus with control group; #P<0.05, ##P<0.01 versus with ketamine group; &P<0.05, &&P< 0.01 versus with ketamine + PACAP group.

M PACAP-38 on 150 μM ketamine-induced retinal apoptosis: mean ratios of caspase-3 and TUNEL-positive cells were increased from 3.3± 0.3% to 7.3±0.3% (P<0.001) (**Figure 4A** and **4C**), and from 16.5±2.8% to 35.6±5.2% (P= 0.0039) (**Figure 4B** and **4D**), respectively.

Discussion

The data presented here showed that PACAP-38 significantly alleviated ketamine-induced apoptosis in early retinal development. This anti-apoptotic effect was partly dependent on the cAMP/PKA signaling pathway. Meanwhile, ERK1/2 mainly contributed to PACAP-38's effects against ketamine-induced retinal apoptosis in the developing rat retina.

Ketamine was used in this study at 150 μM. Its concentration in human plasma can reach 38-108 μM after intravenous administration at 1~2 mg/kg [27], indicating that ketamine levels
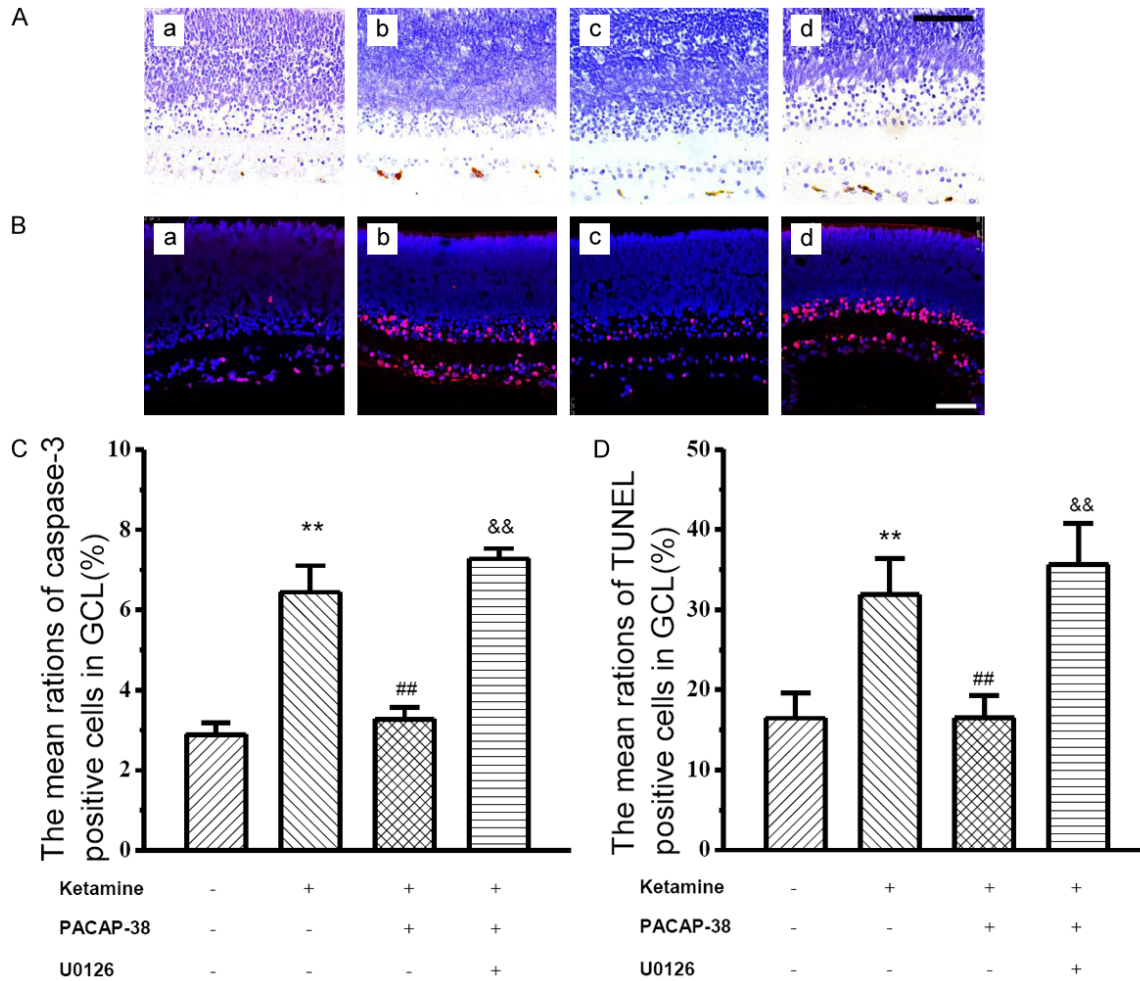
Figure 4. ERK1/2 mainly contributes to PACAP-38's effect against ketamine-induced neuronal apoptosis in developing rat retina. (A and B) Representative photomicrograph of apoptotic cells expression in the retinal ganglion cell layer using Caspase-3 immunohistochemistry and TUNEL labeling (brown color in A and red fluorescence in B). a: Control; b: Ketamine 150 µM; c: Ketamine 150 µM + PACAP-38 10^{-7} M; d: Ketamine 150 µM + PACAP-38 10^{-7} M + U0126 50 µM. (n=5 retinas per subgroup). Scale bar, 50 µm. (C and D) Quantification of the rations of Caspase-3-positive and TUNEL-positive cells present in the retinal ganglion cell layer. The results are expressed as mean ± SEM. $*P<0.05$, $**P<0.01$ versus with control group; $\#P<0.05$, $\#\#P<0.01$ versus with ketamine group; $\&P<0.05$, $\&\&P<0.01$ versus with ketamine + PACAP-38 group.

in our study were close to clinically relevant amounts. It has been reported that developing rat brain exposed to ketamine at 10 µM for 48 hours displays wide spread neuron apoptosis [28]. In this study, Treatment of whole-mount rat retina with 150 µM ketamine for 5 hours also significantly increased neuronal apoptosis. In addition, our previous study showed that ketamine-induced neuronal apoptosis was time-dependent (1~5 h, data not show).

Physiological PACAP concentrations (10^{-10} to 10^{-8} M) protect neurons from apoptosis [29, 30]. As shown above, 10^{-8} M PACAP significantly reduced ketamine-induced neuronal apopto-

sis in early developing rat retina. In addition, PACAP's protective effects peaked at 10^{-7} M before decreasing at 10^{-6} M. In contrast, Shoge et al. showed that PACAP attenuates glutamate-induced neurotoxicity in retinal neurons in a dose-dependent manner, with maximum protective effect observed at 10^{-6} M [31]. The divergence between both studies may be due to different expression levels of PACAP receptors (mainly PAC1 receptor), which display remarkable changes during retina development [32].

It is widely accepted that PACAP-38 increases intracellular cAMP levels through activation of

PAC1 receptor, and induces the cAMP/PKA signaling pathway. However, it remains unclear whether this pathway is also involved in the neuroprotective effect of PACAP-38 against ketamine [31, 33, 34]. We found that PACAP-38's effect on ketamine-induced apoptosis was partly antagonized by AC and PKA inhibitors, respectively, in agreement with Shoge et al. [31] and Silveira et al. [35], but contrasted the findings by Monika et al. that the AC inhibitor does not block PACAP's anti-apoptotic effect, with neither phospho-PKA nor cAMP overtly induced in response to PACAP [36]. This discrepancy might be due to age difference since Monika et al. assessed younger rats (P1). In addition, they applied PACAP-38 at a low physiological concentration, which may not suffice to activate PAC1 receptors.

Previous data showed that PACAP could activate ERK1/2 and its downstream target, cAMP-responsive element (CREB), which is important in neuronal survival [37]. Here, the ERK1/2 inhibitor U0126 almost completely blocked the anti-apoptotic effect of PACAP-38. ERK1/2 is also crucial in mediating apoptosis in human retinal pigment epithelial cells; indeed, elevated ERK1/2 levels and decreased expression of the pro-apoptotic molecules JNK and p38 MAPK were observed after PACAP treatment [38].

The whole-mount rat retina culture is an ideal experimental model to study anesthetic neurotoxicity because it helps eliminate interfering factors, e.g. anoxia in *in vivo* studies. In addition, it is closer to *in situ* conditions in the central nervous system, thanks to its intact organizational structure.

A few limitations of this study should be mentioned. The effect of PACAP-38 was not evaluated below 10^{-9} M. At very low concentrations (sub-picomolar level) PACAP was reported to show some protective effect, involving the IP3/PLC pathway [39]. In addition, the effect of PACAP-38 pre-treatment on ketamine-induced neuron apoptosis was not assessed. It has been reported that PACAP-38 has long-lasting neuroprotective effect, with 1 and 6 hour pre-treatment displaying similar strength [40]. Therefore, it is possible that no difference exists between the effects of PACAP-38 pre-treatment and during co-treatment, on ketamine-induced neuron apoptosis.

In summary, the data presented here demonstrated that PACAP-38, acting through PAC1 receptor, significantly protects neurons against ketamine-induced apoptosis in the early developing rat retina. The anti-apoptotic effect of PACAP-38 is partly dependent on cAMP/PKA signaling, and can be mainly ascribed to of ERK1/2.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jijian Zheng, Department of Anesthesiology, Shanghai Children's Medical Center, Shanghai Jiao Tong University School of Medicine, 1678 Dongfang Road, Pudong, Shanghai 200127, China. Tel: +86-21-38626161; E-mail: zhengjijian626@sina.com

References

[1] Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K, Tenkova TI, Stefovska V, Turski L and Olney JW. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 1999; 283: 70-74.

[2] Jevtovic-Todorovic V, Absalom AR, Blomgren K, Brambrink A, Crosby G, Culley DJ, Fiskum G, Giffard RG, Herold KF, Loepke AW, Ma D, Orser BA, Planel E, Slikker W Jr, Soriano SG, Stratmann G, Vutskits L, Xie Z and Hemmings HC Jr. Anaesthetic neurotoxicity and neuroplasticity: an expert group report and statement based on the BJA Salzburg Seminar. Br J Anaesth 2013; 111: 143-151.

[3] Mazoit JX, Roulleau P and Baujard C. Isoflurane-induced neuroapoptosis in the neonatal rhesus macaque brain: isoflurane or ischemia-reperfusion? Anesthesiology 2010; 113: 1245; author reply 1245-1246.

[4] Bittigau P, Sifringer M, Genz K, Reith E, Pospischil D, Govindarajalu S, Dzietko M, Pesditschek S, Mai I, Dikranian K, Olney JW and Ikonomidou C. Antiepileptic drugs and

apoptotic neurodegeneration in the developing brain. Proc Natl Acad Sci U S A 2002; 99: 15089-15094.

[5] Todd MM. Anesthetic Neurotoxicity: The Collision between Laboratory Neuroscience and Clinical Medicine. Anesthesiology 2004; 101: 272-273.

[6] Sanders RD, Hassell J, Davidson AJ, Robertson NJ and Ma D. Impact of anaesthetics and surgery on neurodevelopment: an update. Br J Anaesth 2013; 110 Suppl 1: i53-72.

[7] Shu Y, Zhou Z, Wan Y, Sanders RD, Li M, Pac-Soo CK, Maze M and Ma D. Nociceptive stimuli enhance anesthetic-induced neuroapoptosis in the rat developing brain. Neurobiol Dis 2012; 45: 743-750.

[8] Liu JR, Liu Q, Li J, Baek C, Han XH, Athiraman U and Soriano SG. Noxious stimulation attenuates ketamine-induced neuroapoptosis in the developing rat brain. Anesthesiology 2012; 117: 64-71.

[9] Shih J, May LD, Gonzalez HE, Lee EW, Alvi RS, Sall JW, Rau V, Bickler PE, Lalchandani GR, Yusupova M, Woodward E, Kang H, Wilk AJ, Carlston CM, Mendoza MV, Guggenheim JN, Schaefer M, Rowe AM and Stratmann G. Delayed environmental enrichment reverses sevoflurane-induced memory impairment in rats. Anesthesiology 2012; 116: 586-602.

[10] Johnston CC, Walker CD and Boyer K. Animal models of long-term consequences of early exposure to repetitive pain. Clin Perinatol 2002; 29: 395-414.

[11] Sternberg WF, Scorr L, Smith LD, Ridgway CG and Stout M. Long-term effects of neonatal surgery on adulthood pain behavior. Pain 2005; 113: 347-353.

[12] Miller DB and O'Callaghan JP. Neuroendocrine aspects of the response to stress. Metabolism 2002; 51: 5-10.

[13] Nakamachi T, Farkas J, Watanabe J, Ohtaki H, Dohi K, Arata S and Shioda S. Role of PACAP in neural stem/progenitor cell and astrocyte -from neural development to neural repair. Curr Pharm Des 2011; 17: 973-984.

[14] Stroth N, Holighaus Y, Ait-Ali D and Eiden LE. PACAP: a master regulator of neuroendocrine stress circuits and the cellular stress response. Ann N Y Acad Sci 2011; 1220: 49-59.

[15] Hammack SE and May V. Pituitary Adenylate Cyclase Activating Polypeptide in Stress-Related Disorders: Data Convergence from Animal and Human Studies. Biol Psychiatry 2015; 78: 167-177.

[16] Holighaus Y, Mustafa T and Eiden LE. PAC1hop, null and hip receptors mediate differential signaling through cyclic AMP and calcium leading to splice variant-specific gene induction in neural cells. Peptides 2011; 32: 1647-1655.

[17] Wang ZY, Alm P and Håkanson R. Distribution and effects of pituitary adenylate cyclase-activating peptide in the rabbit eye. Neuroscience 1995; 69: 297-308.

[18] Tabuchi A, Koizumi M, Nakatsubo J, Yaguchi T and Tsuda M. Involvement of endogenous PACAP expression in the activity-dependent survival of mouse cerebellar granule cells. Neurosci Res 2001; 39: 85-93.

[19] Botia B, Jolivel V, Burel D, Le Joncour V, Roy V, Naassila M, Benard M, Fournier A, Vaudry H and Vaudry D. Neuroprotective effects of PACAP against ethanol-induced toxicity in the developing rat cerebellum. Neurotox Res 2011; 19: 423-434.

[20] Masmoudi-Kouki O, Douiri S, Hamdi Y, Kaddour H, Bahdoudi S, Vaudry D, Basille M, Leprince J, Fournier A, Vaudry H, Tonon MC and Amri M. Pituitary adenylate cyclase-activating polypeptide protects astroglial cells against oxidative stress-induced apoptosis. J Neurochem 2011; 117: 403-411.

[21] Scharf E, May V, Braas KM, Shutz KC and Mao-Draayer Y. Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) regulate murine neural progenitor cell survival, proliferation, and differentiation. J Mol Neurosci 2008; 36: 79-88.

[22] Sanchez V, Feinstein SD, Lunardi N, Joksovic PM, Boscolo A, Todorovic SM and Jevtovic-Todorovic V. General Anesthesia Causes Long-term Impairment of Mitochondrial Morphogenesis and Synaptic Transmission in Developing Rat Brain. Anesthesiology 2011; 115: 992-1002.

[23] Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW and Wozniak DF. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. J Neurosci 2003; 23: 876-882.

[24] Ogilvie JM, Speck JD, Lett JM and Fleming TT. A reliable method for organ culture of neonatal mouse retina with long-term survival. J Neurosci Methods 1999; 87: 57-65.

[25] Xin H, Yannazzo JA, Duncan RS, Gregg EV, Singh M and Koulen P. A novel organotypic culture model of the postnatal mouse retina allows the study of glutamate-mediated excitotoxicity. J Neurosci Methods 2007; 159: 35-42.

[26] Bhave SV and Hoffman PL. Phosphatidylinositol 3'-OH kinase and protein kinase A pathways mediate the anti-apoptotic effect of pituitary adenylyl cyclase-activating polypeptide in cultured cerebellar granule neurons: modulation by ethanol. J Neurochem 2004; 88: 359-369.

[27] Domino EF, Zsigmond EK, Domino LE, Domino KE, Kothary SP and Domino SE. Plasma levels

of ketamine and two of its metabolites in surgical patients using a gas chromatographic mass fragmentographic assay. Anesth Analg 1982; 61: 87-92.

[28] Wang C, Sadovova N, Fu X, Schmued L, Scallet A, Hanig J and Slikker W. The role of the N-methyl-D-aspartate receptor in ketamine-induced apoptosis in rat forebrain culture. Neuroscience 2005; 132: 967-977.

[29] Tanaka J, Koshimura K, Murakami Y, Sohmiya M, Yanaihara N and Kato Y. Neuronal protection from apoptosis by pituitary adenylate cyclase-activating polypeptide. Regul Pept 1997; 72: 1-8.

[30] Ohno F, Watanabe J, Sekihara H, Hirabayashi T, Arata S, Kikuyama S, Shioda S, Nakaya K and Nakajo S. Pituitary adenylate cyclase-activating polypeptide promotes differentiation of mouse neural stem cells into astrocytes. Regul Pept 2005; 126: 115-122.

[31] Shoge K, Mishima HK, Saitoh T, Ishihara K, Tamura Y, Shiomi H and Sasa M. Attenuation by PACAP of glutamate-induced neurotoxicity in cultured retinal neurons. Brain Res 1999; 839: 66-73.

[32] Lakk M, Szabo B, Volgyi B, Gabriel R and Denes V. Development-related splicing regulates pituitary adenylate cyclase-activating polypeptide (PACAP) receptors in the retina. Invest Ophthalmol Vis Sci 2012; 53: 7825-7832.

[33] Han P and Lucero MT. Pituitary adenylate cyclase activating polypeptide reduces A-type K+ currents and caspase activity in cultured adult mouse olfactory neurons. Neuroscience 2005; 134: 745-756.

[34] Kanekar S, Gandham M and Lucero MT. PACAP protects against TNFα-induced cell death in olfactory epithelium and olfactory placodal cell lines. Mol Cell Neurosci 2010; 45: 345-354.

[35] Silveira MS, Costa MR, Bozza M and Linden R. Pituitary adenylyl cyclase-activating polypeptide prevents induced cell death in retinal tissue through activation of cyclic AMP-dependent protein kinase. J Biol Chem 2002; 277: 16075-16080.

[36] Lakk M, Denes V and Gabriel R. Pituitary Adenylate Cyclase-Activating Polypeptide Receptors Signal via Phospholipase C Pathway to Block Apoptosis in Newborn Rat Retina. Neurochem Res 2015; 40: 1402-1409.

[37] Racz B, Tamas A, Kiss P, Toth G, Gasz B, Borsiczky B, Ferencz A, Gallyas F Jr, Roth E and Reglodi D. Involvement of ERK and CREB signaling pathways in the protective effect of PACAP in monosodium glutamate-induced retinal lesion. Ann N Y Acad Sci 2006; 1070: 507-511.

[38] Fabian E, Reglodi D, Mester L, Szabo A, Szabadfi K, Tamas A, Toth G and Kovacs K. Effects of PACAP on intracellular signaling pathways in human retinal pigment epithelial cells exposed to oxidative stress. J Mol Neurosci 2012; 48: 493-500.

[39] Nakamachi T, Li M, Shioda S and Arimura A. Signaling involved in pituitary adenylate cyclase-activating polypeptide-stimulated ADNP expression. Peptides 2006; 27: 1859-1864.

[40] Vaudry D, Gonzalez BJ, Basille M, Anouar Y, Fournier A and Vaudry H. Pituitary adenylate cyclase-activating polypeptide stimulates both c-fos gene expression and cell survival in rat cerebellar granule neurons through activation of the protein kinase A pathway. Neuroscience 1998; 84: 801-812.