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Dopamine Attenuates Ketamine-Induced Neuronal Apoptosis in the Developing Rat Retina Independent of Early Synchronized Spontaneous Network Activity

Jing Dong[1] · Lingqi Gao[1] · Junde Han[1] · Junjie Zhang[2] · Jijian Zheng[3]

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Abstract Deprivation of spontaneous rhythmic electrical activity in early development by anesthesia administration, among other interventions, induces neuronal apoptosis. However, it is unclear whether enhancement of neuronal electrical activity attenuates neuronal apoptosis in either normal development or after anesthesia exposure. The present study investigated the effects of dopamine, an enhancer of spontaneous rhythmic electrical activity, on ketamine-induced neuronal apoptosis in the developing rat retina. TUNEL and immunohistochemical assays indicated that ketamine time- and dose-dependently aggravated physiological and ketamine-induced apoptosis and inhibited early-synchronized spontaneous network activity. Dopamine administration reversed ketamine-induced neuronal apoptosis, but did not reverse the inhibitory effects of ketamine on early synchronized spontaneous network activity despite enhancing it in controls. Blockade of D1, D2, and A2A receptors and inhibition of cAMP/PKA signaling partially antagonized the protective effect of dopamine against ketamine-induced apoptosis. Together, these data indicate that dopamine attenuates ketamine-induced neuronal apoptosis in the developing rat retina by activating the D1, D2, and A2A receptors, and upregulating cAMP/PKA signaling, rather than through modulation of early synchronized spontaneous network activity.

Keywords Ketamine · Retina · Apoptosis · Development · Early synchronized spontaneous network activity · Dopamine

Introduction

Although the risks associated with anesthetic neurotoxicity in early human brain development are still under debate, mounting evidence from in vitro and in vivo animal models suggests that exposure to general anesthetics during development can cause widespread neurotoxic insults that lead to adverse neurological outcomes later in life [1–6]. Ketamine, a once widely used pediatric anesthetic, is now known to cause substantial increases in neuronal apoptosis after prolonged and/or repeated neonatal exposure in a variety of animal species [7–10]. Ketamine-induced neuronal apoptosis in early development is thought to be mediated by non-competitive blockade of *N*-methyl-D-aspartate (NMDA) receptors [7, 8] and disturbances of early synchronized spontaneous network activity [11].

It is well established that early synchronized spontaneous network activity appears at very early developmental stages in various brain structures and in the retina, where it is referred to as retinal waves [12–14]. In electrophysiological recordings, early synchronized spontaneous network activity manifests as either periodic synchronized action potential discharges or synaptic inputs [11], while in calcium imaging recordings, it appears as periodic wave-like cytoplasmic calcium increases

Jing Dong, MD and Lingqi Gao, MD are First Authors.

✉ Junjie Zhang
deutschinese@163.com

✉ Jijian Zheng
zhengjijian626@sina.com

[1] Department of Anesthesiology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

[2] Department of Anesthesiology, Tong Ren Hospital, Shanghai Jiao Tong University School of Medicine, 1111 Xianxia Road, Shanghai 200050, China

[3] Department of Anesthesiology, Shanghai Children's Medical Center, Shanghai Jiao Tong University, 1678 Dongfang Road, Shanghai 200127, China

composed of initiation, propagation, and termination phases [14–17]. Several neurotransmitters, including acetylcholine, GABA, and glutamate, are known to modulate synchronized spontaneous network activity throughout development [14, 18] and play important roles in neuronal differentiation, migration, apoptosis, synaptogenesis, network formation, and connectivity, among other developmental processes [19–23]. Prolonged suppression of early synchronized network activity leads to increased neuronal apoptosis [11, 24]. By contrast, it is unclear whether enhancement of such activity can attenuate neuronal apoptosis during normal development or after ketamine administration.

Dopamine, a widely distributed neurotransmitter in the brain and retina and a frequently used vasoactive drug, has been shown to significantly enhance early synchronized spontaneous network activity [25]. We therefore speculated that dopamine might inhibit ketamine-induced neuronal apoptosis in early developmental stages by enhancing such activity. In the present study, we used the neonatal rat retina as a model to explore the possible effects of dopamine on ketamine-induced neuronal apoptosis and early synchronized spontaneous network activity in the developing rat retina.

Materials and Methods

Animals and Tissue Dissection

Sprague–Dawley rat pups aged 0, 3, 7, 14, and 21 postnatal (P) days were provided by the Experimental Animal Centre of Shanghai General Hospital, Shanghai, China. All pups were kept with their mother under a 12-h light/dark cycle, with food and water available ad libitum until experiments were commenced at P0, P3, P7, P14, and P21. All male and female rat pups were included in this study. All experimental procedures were reviewed and approved by the Animal Care Committee at Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine and were 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. Every effort was made to minimize the numbers and discomfort of animals during the experiments.

In this study, we used a whole layer retinal culture model that not only ensures the integrity of the neural network but also creates a simple network structure that is especially suitable for studies of synapse and network development [26–28]. Simultaneously, the basic experimental protocol was slightly modified from a previous report [29]. Briefly, all experimental rat pups were killed instantaneously by decapitation, except rats at P14 and P21, which were euthanized by carbon dioxide inhalation. The eyeballs were rapidly removed with fine scissors and transferred to an ice-cold (0–4 °C) bath of artificial cerebrospinal fluid (ACSF); ACSF composition in millimolar: NaCl 119, KCl 2.5, KH_2PO_4 1.0, $CaCl_2$ 2.5, $MgCl_2$ 1.3, $NaHCO_3$ 26.2, and D-Glucose 11. The bath was continuously bubbled with a 95 % O_2/5 % CO_2 gas mixture. A small incision was cut between the edges of cornea and sclera to facilitate delivery of ACSF and interventional drugs into the retina. Then, the eyeballs were incubated in ACSF (37 °C) bubbled with a 95 % O_2/5 % CO_2 gas mixture until the experiment was finished.

Drugs and Chemicals

After a 1-h post-dissection period for the prepared eyeballs, normal ACSF was replaced by ACSF with different concentrations of ketamine and/or dopamine with or without various antagonists or agonists depending on our experimental protocols. The following drugs were used: ketamine and dopamine (purchased from Gutian Pharmaceutical Company, China) and SCH 58261, SQ 22536, H-89, SCH 23390, Forskolin, and Raclopride (purchased from Sigma-Aldrich Company, USA). All drugs were dissolved in ACSF except for SCH 58261, which was first dissolved as a stock solution in DMSO, and then diluted to the working concentrations in ACSF with DMSO of less than 0.1 % concentration on the day of the experiment.

Immunohistochemistry and TUNEL Staining

After drug treatment, rat retinas were immediately detached from the eyeballs in ice-cold (0–4 °C) ACSF, and then fixed in 4 % paraformaldehyde (4 °C) for 24 h. After fixation, retinas were cut into 4–6 μm tissue slices for later processing by a paraffin-slicing machine (Leica-2135, German). After carrying out inactivation of endogenous peroxidases by incubation in 3 % H_2O_2 and heat-induced epitope retrieval by treatment with Tris/EDTA buffer (PH 9.0) in a pressure cooker, retinal tissue sections were incubated with rabbit anti-activated cleaved caspase-3 (AC3) antibody (#9661S, dilution, 1:300, Cell Signalling Technology, Danvers, MA, USA) and a horseradish peroxidase conjugated goat anti-rabbit IgG (PV-9001, ZSGB-BIO, Beijing, China) at 37 °C for 1 h. Then, AC3 immunoreacti12©vity was detected by a chromogenic reaction using 3,3'-diaminobenzidine (DAB) (ZLI-9017, ZSGB-BIO, Beijing, China) as a substrate. All sections were counterstained with haematoxylin to stain nuclei. Finally, retinal tissue sections were dehydrated and sealed by a coverslip for further microscopic examination.

For TUNEL assays of apoptosis, retinal tissue sections were deparaffinised and rehydrated and then treated with proteinase K (Roche Applied Science, Indianapolis, IN, USA) followed by 3 % H_2O_2. Next, the sections were incubated in

a terminal deoxynucleotidyl transferase (TdT) reaction mix (Roche Applied Science, Indianapolis, IN, USA) for 1 h at 37 °C and then incubated with DAPI for 5 min.

Apoptotic cells were counted in a double-blinded manner from randomly selected sampling areas (one slide from each pup was randomly selected, and a total three fields were used for analysis). Changes in the proportion of AC3- and TUNEL-positive neuronal cells are presented as the percentage of AC3- and TUNEL-positive cells to all retinal ganglion layer cells.

Patch-clamp Recording From Ganglion Cells in Rat Retinal Flat Mounts

Retinal flat mounts for patch-clamp recordings were prepared from SD rat pups aged P0 to P7, as described previously [30]. After the Müller cell endfeet and extracellular connective tissues on the top of retinal ganglion cell layer were removed mechanically with a large-tip patch pipette or a pair of fine forceps, a piece of prepared retina was mounted to a heated recording chamber with the sclera side down and held to the bottom of the recording chamber by a platinum ring with a nylon mesh. The retina was continuously superfused at 2–4 mL/min with ACSF in the recording chamber (35–37 °C). Whole-cell patch-clamp recordings on the ganglion cells were performed using Axopatch 700B (Axon Instruments, Inc., Union City, CA, USA) amplifiers under a 40× water immersion objective lens of an upright fluorescence microscope equipped with a fixed-stage (BX50WI, Olympus USA, NY, USA). All the recording data were low-pass filtered at 2 kHz and digitized at 10 kHz. pCLAMP 9 (Axon Instruments) and Origin 8 (MicroCal Software Inc., Northampton, MA) software were used to collect and analyse the recorded data. The voltage-gated currents were recorded without leak subtraction.

Statistical Analysis

All data are shown as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA) and Origin 8 (MicroCal Software Inc., Northampton, MA). For statistical analyses, unpaired Student's t test was used to analyse comparisons of continuous variables. Analysis of variance (ANOVA) followed by a Bonferroni post hoc tests was applied for multiple comparisons. When the samples did not follow a Gaussian distribution, a non-parametric Mann–Whitney U test and the Kruskal-Wallis with Dunn's multiple comparisons test were used. Sample sizes in all experiments were ≥5 retinas per group (the exact number of retinas in each group is presented in the figure legends). A p value of <0.05 was considered to be statistically significant.

Results

Ketamine Dose- and Time-dependently Aggravates Physiological Apoptosis in the Developing Rat Retina

During normal synaptogenesis, redundant rat retinal ganglion cells are eliminated through physiological apoptosis, which mainly occurs between P3 and P7. We therefore investigated apoptosis in ketamine-treated and control rat eyeball preparations over postnatal development (P0, P3, P7, P14, and P21) using immunohistochemical and TUNEL assays of apoptosis. We found that incubation with 150 μM ketamine (5 h) did not affect the percentages of retinal ganglion cells undergoing apoptosis at P0, P14, and P21, but did lead to significant increases in the percentages of retinal ganglion cells expressing markers of apoptosis at P3 and P7 (Fig. 1). Specifically, the percentages of AC3-positive cells after 150 μM ketamine intervention at P3 and P7 were increased from 1.7 ± 0.20 % to 2.9 ± 0.30 % ($n = 5$, $p = 0.004$) and 2.4 ± 0.3 % to 6.1 ± 0.27 % ($n = 5$, $p < 0.001$), respectively (Fig. 1a, c). TUNEL assays confirmed these results, as the percentages of TUNEL-positive retinal ganglion cells after 150 μM ketamine intervention at P3 and P7 increased from 6.1 ± 0.50 % to 17.6 ± 1.39 % ($n = 5$, $p < 0.001$) and 14.4 ± 1.38 % to 45.6 ± 1.76 % ($n = 5$, $p < 0.001$), respectively, (Fig. 1b, d).

As shown in Fig. 2, AC3 immunohistochemistry and TUNEL assay results all showed that ketamine-induced retinal apoptosis was dose- and time-dependent. After 0.5 h of ketamine treatment at P7, no significant effects on the percentage of retinal ganglion cells undergoing apoptosis were found when the concentration of ketamine was below 1000 μM. However, 2 h of exposure to ketamine at doses of 100, 150, or 1000 μM significantly increased the percentage of apoptotic cells. After 4 and 5 h of ketamine exposure, all of the tested concentrations, except 10 μM, significantly increased the percentages of retinal ganglion cells undergoing apoptosis (Fig. 2). These results suggest that ketamine-induced retinal ganglion cell apoptosis in early development might reflect an aggravation of physiological apoptosis in a time- and dose-dependent manner.

Effects of Ketamine on Early Synchronized Spontaneous Network Activity

To investigate the relationship between early synchronized spontaneous network activity and ketamine-induced apoptosis in early retinal development, we performed patch-clamp recordings from rat retinal ganglion cells. We found that 150 μM ketamine completely blocked early synchronized spontaneous network activity in P0, P3, and P7 rat retina ($n = 6$ retinas per each group, Fig. 3).

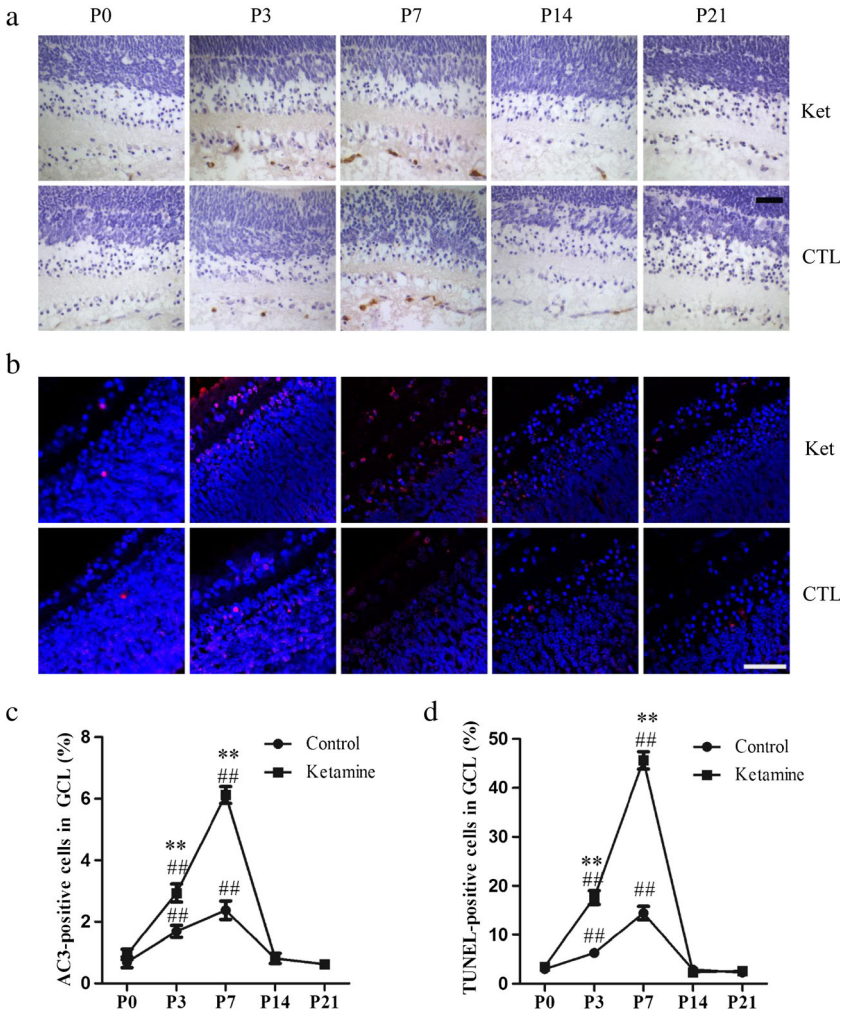

Fig. 1 Effects of ketamine on neuronal apoptosis in early developing rat retina. Postnatal day 0, 3, 7, 14, and 21 (P0, P3, P7, P14, and P21) rat retinas were exposed to 150 μM ketamine for 5 h ($n = 5$ retinas per group). **a** Representative photomicrograph of Caspase-3 (AC3) positive expressions (*brown color*) in rat retina, scale bar, 50 μm. **b** Representative photomicrograph of TUNEL positive staining (*red color*) in rat retina, scale bar, 25 μm. **c** Percentages of AC3-positive cells in the GCL. **d** Percentages of TUNEL-positive cells in the GCL. *Ket* ketamine, *CTL* control, *GCL* ganglion cell layer. Physiological- and ketamine-induced apoptosis peaks occurred in P3 and P7 rat retina, especially in P7. $*p < 0.05$, $**p < 0.01$ when compared between groups, $\#p < 0.05$, $\#\#p < 0.01$ when compared within groups

Effects of Dopamine on Early Synchronized Spontaneous Network Activity and Ketamine-Induced Neuronal Apoptosis in the Developing Rat Retina

We next assessed the effects of dopamine administration on ketamine-induced apoptosis. In accordance with the previous reports, we found that forskolin (an activator of adenylate cyclase) and dopamine significantly enhanced early synchronized spontaneous network activity in both P3 and P7 retina ($n = 6$ retinas per group) (Fig. 3b). In addition, AC3 immunohistochemistry and TUNEL assays showed that dopamine dose dependently attenuated ketamine-induced retinal ganglion cell apoptosis at P7 (Fig. 4). The minimal effective concentration of dopamine against 150 μM ketamine-induced retinal apoptosis in P7 rats was 1.0 μM; at this dose, the percentage of AC3-positive retinal ganglion neurons was reduced from 5.5 ± 0.24 % to 3.3 ± 0.27 % ($p = 0.039$) (Fig. 4a, c) and the percentage of TUNEL- positive retinal neurons was reduced from 52.3 ± 1.65 % to 32.0 ± 1.82 % ($p < 0.001$) (Fig. 4b, d). Both 10 and 100 μM of dopamine completely reversed ketamine-induced apoptosis (Fig. 4). Dopamine had no statistically significant effects on reducing the neuronal apoptosis in normal developmental retina (data were not shown). We next conducted electrophysiology to determine if dopamine or forskolin affected spontaneous network activity. We found that neither 10 μM dopamine nor 1 μM forskolin reversed the inhibition of early synchronized spontaneous network activity by 150 μM ketamine (Fig. 3c), indicating that protection against ketamine-induced retinal ganglion cell apoptosis by dopamine is likely not mediated by enhancement of spontaneous network activity.

Roles of Dopamine D1, D2, and Adenosine A2A Receptors in Dopamine-Mediated Protection Against Ketamine-Induced Neuronal Apoptosis in the Developing Retina

To dissect the molecular mechanisms of dopamine-mediated protection against ketamine-induced retinal apoptosis, we explored the effects of the adenosine A2A receptor and dopamine D1 and D2 receptors and on ketamine-induced retinal apoptosis. All three receptors were examined, because they

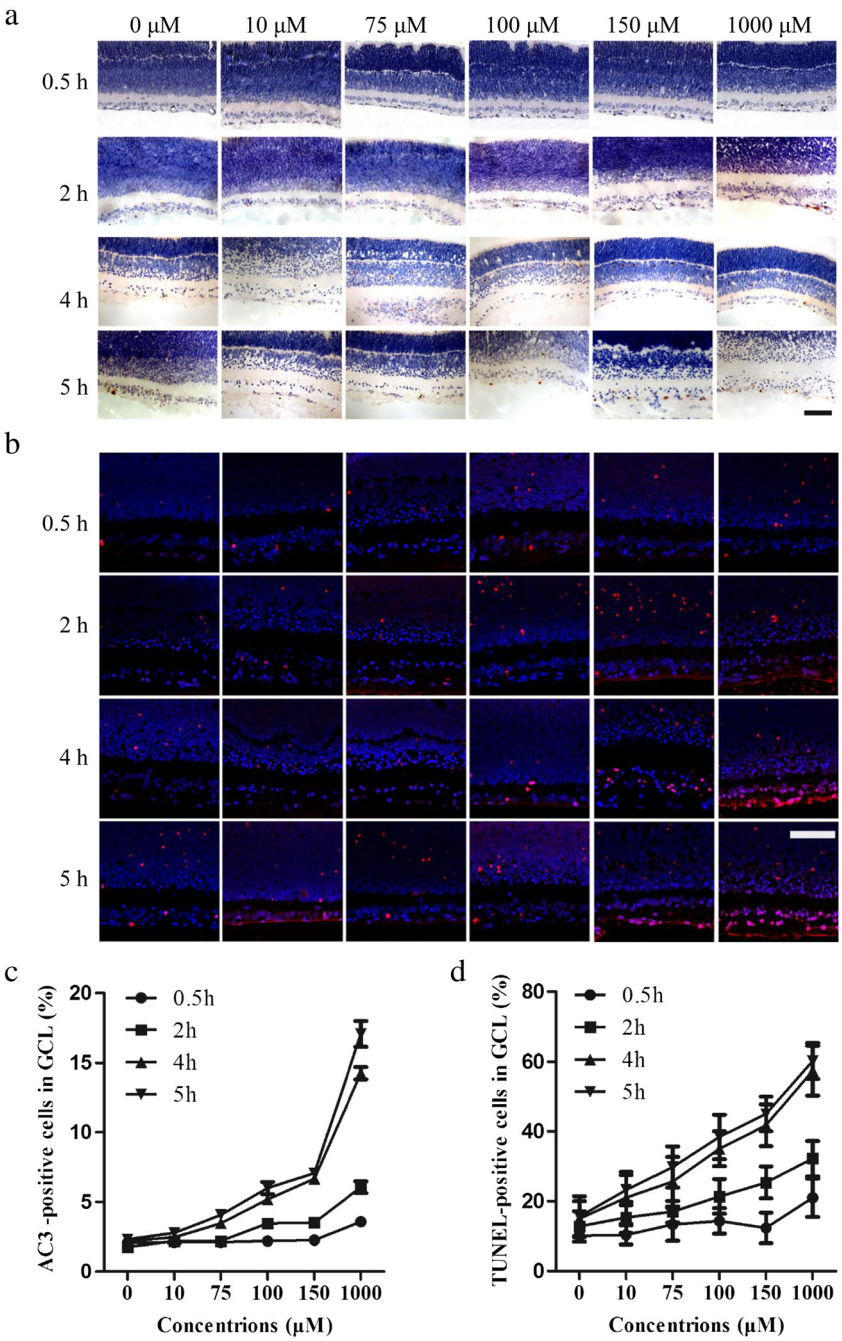

Fig. 2 Concentration- and duration-dependent effects of ketamine on neuro-apoptosis in P7 rat retina. Rat retinas were exposed to different concentrations of ketamine (0, 10, 75, 100, 150, and 1000 μM) for different time (0.5, 2, 4, and 5 h). $n = 5$ retinas per group. **a** Representative photomicrograph of Caspase-3 (AC3) positive expressions (*brown color*) in rat retina, scale bar, 50 μm. **b** Representative photomicrograph of TUNEL positive staining (*red color*) in rat retina, scale bar, 25 μm. **c** Percentages of AC3-positive cells in the GCL. **d** Percentages of TUNEL-positive cells in the GCL. *GCL* ganglion cell layer

converge on common signaling pathways. Both AC3 immunohistochemistry and TUNEL assays showed that 100 nM SCH 58261, a highly selective adenosine competitive antagonist of the A2A receptor, significantly reduced the protective effects of 10 μM dopamine against 150 μM ketamine-induced retinal ganglion cell apoptosis (Fig. 5); the percentage of AC3-positive neurons was increased from 2.3 ± 0.35 % to 4.3 ± 0.25 % ($p < 0.001$) (Fig. 5a, c) and the percentage of TUNEL-positive neurons was increased from 16.7 ± 1.07 % to 34.8 ± 0.91 % ($p < 0.001$) (Fig. 5b, d). Similarly, both SCH 23390 (a dopamine D1 receptor antagonist) and raclopride (a dopamine D2 receptor antagonist) significantly, but not completely, reduced the protective effects of 10 μM dopamine against 150 μM ketamine-induced retinal ganglion cell apoptosis ($p < 0.01$ for both groups) (Fig. 6).

Effects of cAMP/PKA Signaling Pathway in Dopamine-Mediated Protection Against Ketamine-Induced Neuronal Apoptosis

We further explored the role of cAMP/PKA signaling pathway in the protective effects of dopamine against ketamine-

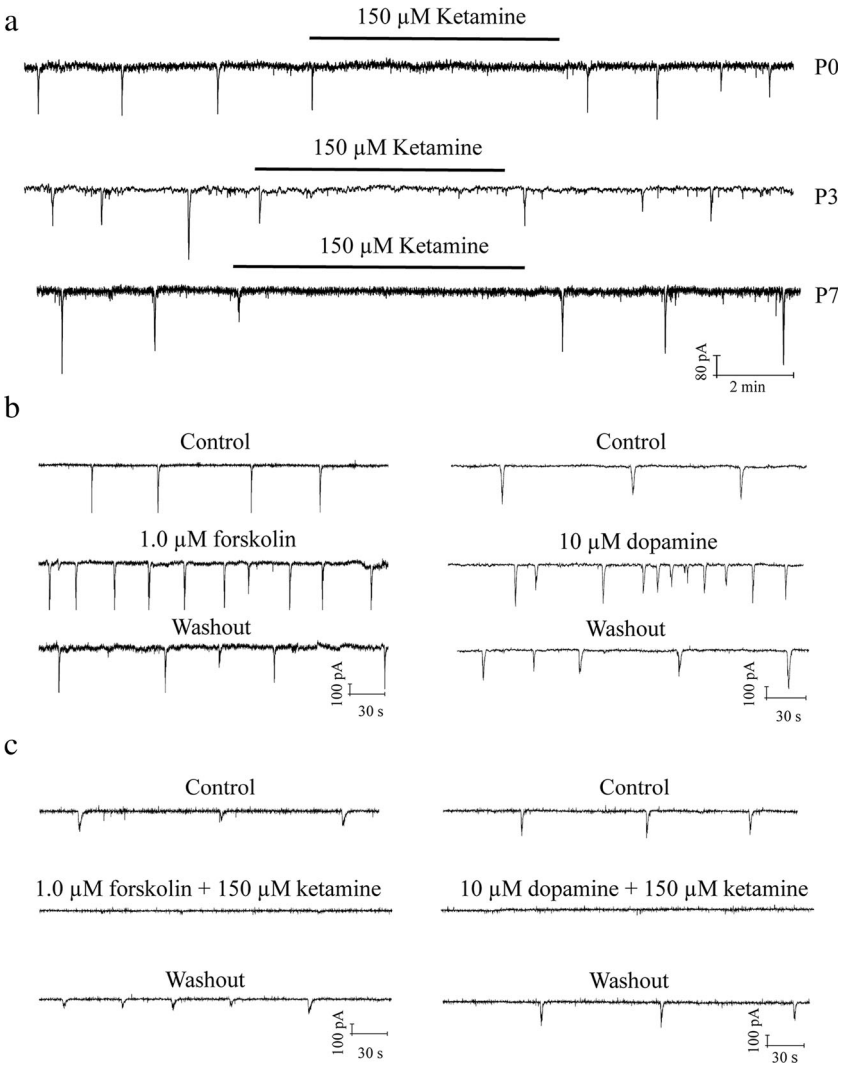

Fig. 3 Effects of ketamine and dopamine on early synchronized spontaneous network activity of rat retina. **a** Early synchronized spontaneous network activity was blocked by 150 μM ketamine in P0, P3, and P7 rat retinas ($n = 6$ retinas per group). **b** 1.0 μM forskolin (left) and 10 μM dopamine (right) enhanced early synchronized spontaneous network activity. **c** 1.0 μM forskolin (left) or 10 μM dopamine (right) did not affect the inhibition of early synchronized spontaneous network activity induced by 150 μM ketamine

induced retinal ganglion cell apoptosis. In both AC3 immunohistochemistry and TUNEL assays, the cell-permeable cAMP inhibitor SQ22536 (100 μM) and H-89 (1 μM), a PKA inhibitor, significantly reduced the protective effects of 10 μM dopamine against 150 μM ketamine-induced retinal ganglion cell apoptosis ($p < 0.01$ for two both, Fig. 7). These data suggest that the cAMP/PKA signaling pathway played a key role in dopamine-mediated protection against ketamine-induced apoptosis in the retina.

Discussion

We found that ketamine time- and dose-dependently aggravated physiological apoptosis in the early developing rat retina. Exogenous application of dopamine significantly enhanced early synchronized spontaneous network activity in controls and attenuated ketamine-induced apoptosis, but did not affect ketamine-induced inhibition of early synchronized spontaneous network activity. Furthermore, we found that blockade of D1, D2, and A2A receptors and inhibition of the cAMP/PKA signaling pathway partially antagonized the protective effect of dopamine against ketamine-induced apoptosis.

Previous studies have demonstrated that early synchronized spontaneous network activity promotes neuronal survival in the neonatal mouse cerebral cortex by activating inotropic glutamate receptors and high-threshold calcium channels, as well as by promoting gap junction coupling [11, 31]. Our study also showed that ketamine, a non-competitive inotropic glutamate receptor subtype (NMDA receptor) blocker, inhibited synchronized spontaneous network activity and caused a significant increase of neuronal apoptosis in the developing rat retina. However, our results additionally showed that dopamine significantly increased early synchronized spontaneous network activity in controls and attenuated ketamine-induced apoptosis without antagonizing ketamine-induced inhibition of synchronized spontaneous network

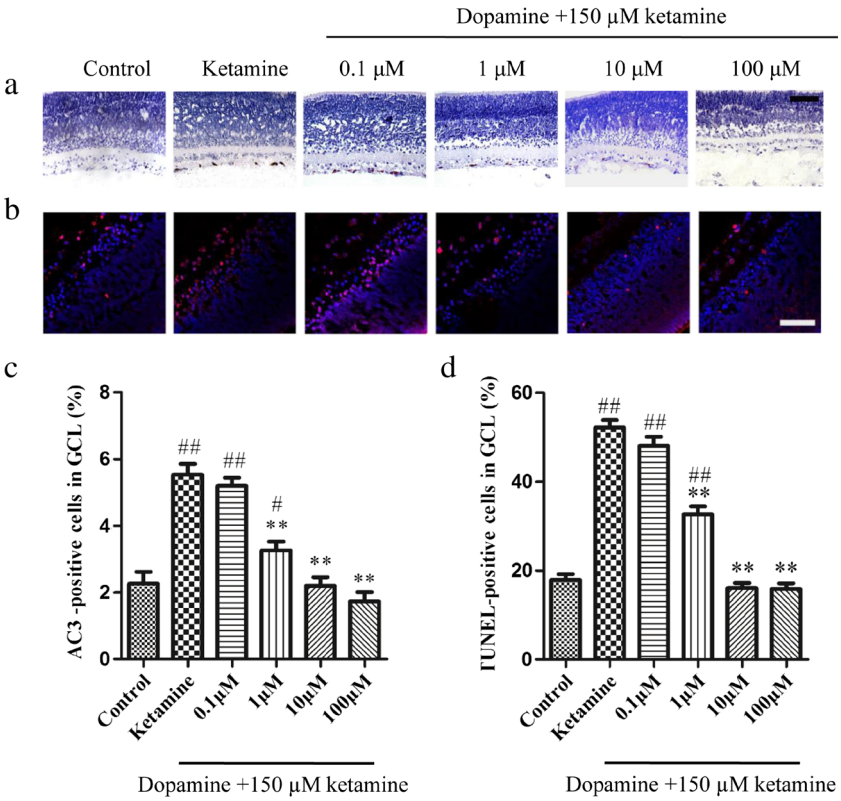

Fig. 4 Effects of dopamine on ketamine-induced neuronal apoptosis in P7 rat retina. Rat retinas were treated with 150 μM ketamine for 5 h in the absence or presence of dopamine (0.1, 1, 10, and 100 μM). $n = 5$ retinas per group. **a** Representative photomicrograph of Caspase-3 (AC3) positive expressions (*brown color*) in rat retina, scale bar, 50 μm. **b** Representative photomicrograph of TUNEL positive staining (*red color*) in rat retina, scale bar, 25 μm. **c** Percentages of AC3-positive cells in the GCL. **d** Percentages of TUNEL-positive cells in the GCL. *GCL* ganglion cell layer. $*p < 0.05$, $**p < 0.01$ compared to ketamine group, $\#p < 0.05$, $\#\#p < 0.01$ compared to control group

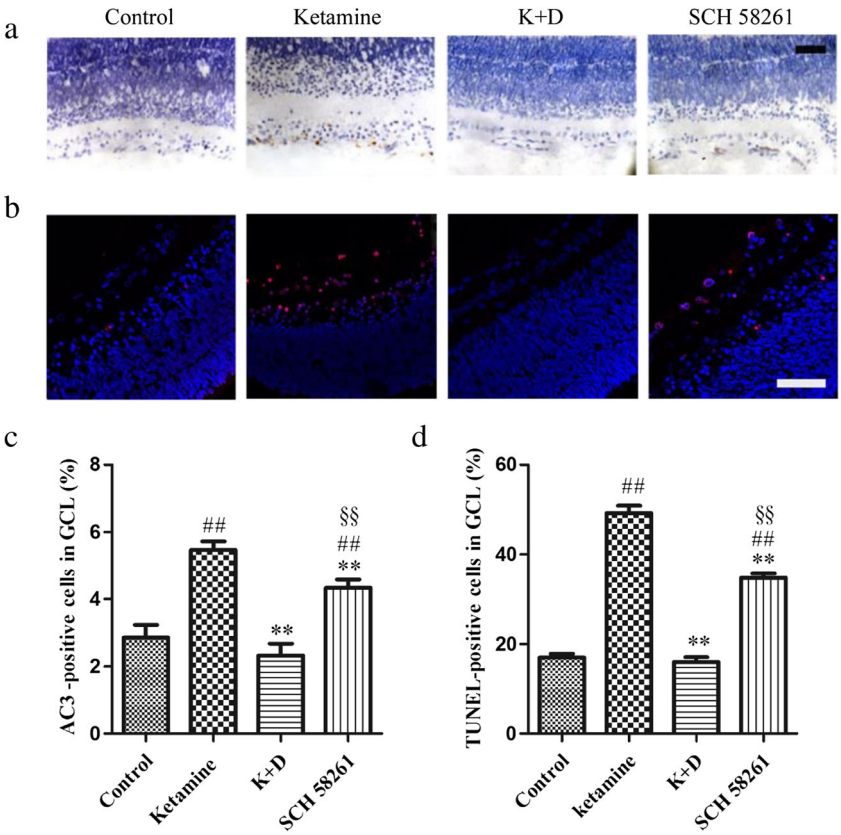

Fig. 5 Effects of adenosine A2A receptor in dopamine-mediated protection against ketamine-induced neuronal apoptosis in P7 rat retina. Rat retinas were treated with 150 μM ketamine, 150 μM ketamine + 10 μM dopamine, and 150 μM ketamine + 10 μM dopamine + 100 nM SCH 58261 (a highly selective adenosine A2A receptors competitive antagonist) for 5 h. **a** Representative photomicrograph of Caspase-3 (AC3) positive expressions (*brown color*) in rat retina, scale bar, 50 μm. **b** Representative photomicrograph of TUNEL positive staining (*red color*) in rat retina, scale bar, 25 μm. **c** Percentages of AC3-positive cells in the GCL. **d** Percentages of TUNEL-positive cells in the GCL. *GCL* ganglion cell layer. $*p < 0.05$, $**p < 0.01$ compared to ketamine group, $\#p < 0.05$, $\#\#p < 0.01$ compared to control group. $\S p < 0.05$, $\S\S p < 0.01$ compared to K + D group

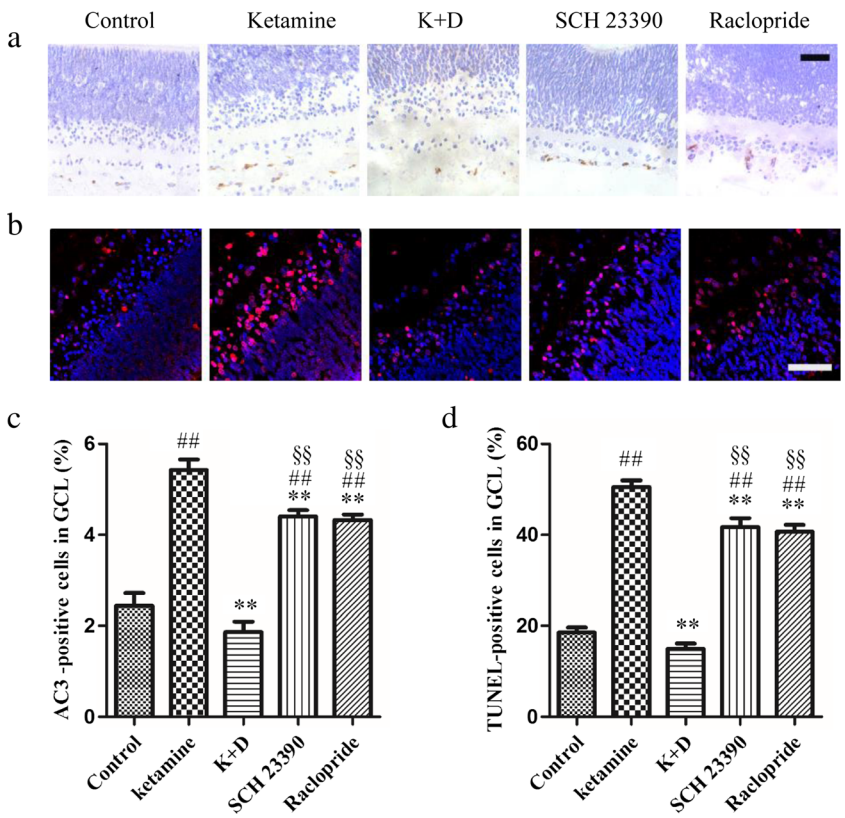

Fig. 6 Effects of dopamine D1, D2 receptors in dopamine-mediated protection against ketamine-induced neuronal apoptosis in P7 rat retina. The retinas were treated with 150 μM ketamine + 10 μM dopamine, 150 μM ketamine + 10 μM dopamine + 10 μM SCH 23390 (a highly selective dopamine D1 receptors antagonist), and 150 μM ketamine + 10 μM dopamine + 40 μM raclopride (a highly selective dopamine D2 receptors antagonist) for 5 h. $n = 5$ retinas per group. **a** Representative photomicrograph of Caspase-3 (AC3) positive expressions (*brown color*) in rat retina, scale bar, 50 μm. **b** Representative photomicrograph of TUNEL positive staining (*red color*) in rat retina, scale bar, 25 μm. **c** Percentages of AC3-positive cells in the GCL. **d** Percentages of TUNEL-positive cells in the GCL. *GCL* ganglion cell layer; K + D: 150 μM ketamine + 10 μM dopamine. $*p < 0.05$, $**p < 0.01$ compared to ketamine group, $\#p < 0.05$, $\#\#p < 0.01$ compared to control group. $\S p < 0.05$, $\S\S p < 0.01$ compared to K + D group

activity. This suggests that dopamine protects against ketamine-induced neurotoxicity through a mechanism that acts independently of network activity. Indeed, ketamine and dopamine modulate network activity through distinct processes. Ketamine inhibits the excitatory drive of synchronized spontaneous network activity by blocking NMDA receptors in the P7 rat retina, an age when retinal networks are transitioning from fast cholinergic signaling to fast glutamatergic signaling as the principle neurotransmitter systems [14, 32]. By contrast, dopamine is believed to up-regulate early synchronized spontaneous network activity through increasing intracellular cAMP via activation of A2 receptors [25, 33]. When excitatory drive is blocked, early synchronized spontaneous network activity cannot be initiated. Thus, taken together, these data suggest that dopamine is unlikely to attenuate ketamine-induced neuronal apoptosis by enhancing early synchronized spontaneous network activity, as it acts as downstream of glutamatergic and cholinergic synaptic transmission.

Instead, our results suggest that dopamine attenuates ketamine-induced neurotoxicity via the activation of dopamine receptor-mediated regulation of adenylate cyclase activity. Dopamine receptors are mainly classified into D1-like (D1 and D5) and D2-like (D2, D3, and D4) families. D1-like receptors are primarily coupled with Gs/olf proteins that activate adenylate cyclase and increase the intracellular levels of cAMP. D2-like receptors are mainly coupled with Gi/o proteins and reduce adenylate cyclase and cAMP levels [34]. In our study, blockade of D1, D2, and adenosine A2 receptors all partially antagonized the protection that dopamine conveyed against ketamine-induced apoptosis. Furthermore, inhibition of cAMP and the downstream PKA signaling pathways partially antagonized the protective effects of dopamine against ketamine-induced apoptosis at levels similar to those observed for blockade of D1, D2, and adenosine A2 receptors. Together, these findings indicate that dopamine-attenuated ketamine-induced retinal apoptosis through D1, D2, and A2A receptors and the cAMP/PKA signaling pathway. Additional experiments showed that co-application of either D1 and D2 receptor antagonists or D1, D2, and adenosine A2 receptor antagonists-attenuated ketamine-induced apoptosis to a similar degree as observed for

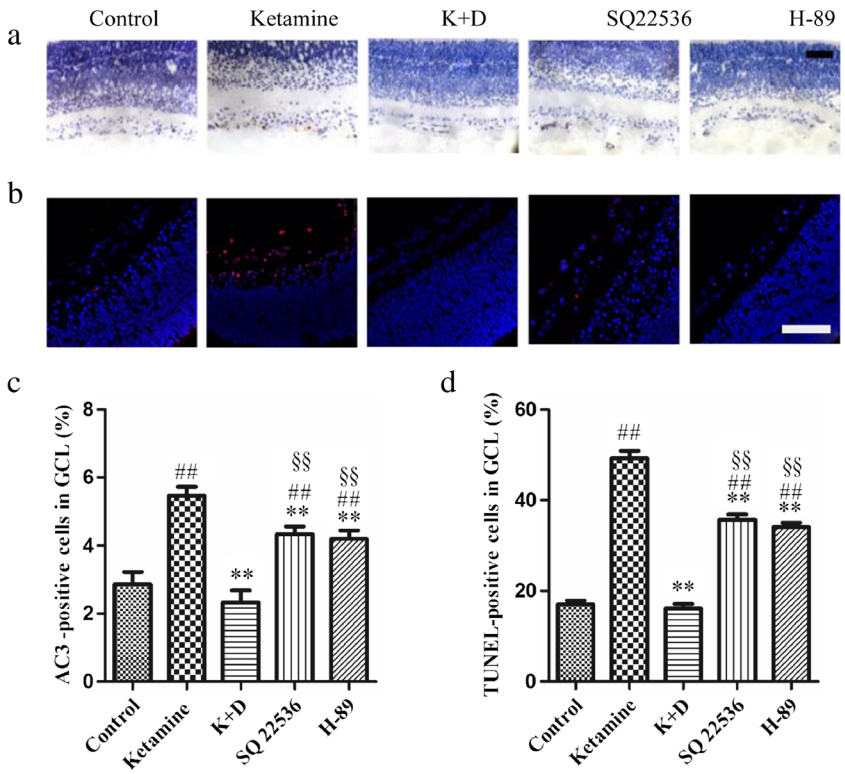

Fig. 7 Effects of cAMP/PKA signaling pathway in dopamine against ketamine-induced neuronal apoptosis in P7 rat retina. Rat retinas were treated with 150 μM ketamine, 150 μM ketamine + 10 μM dopamine, 150 μM ketamine + 10 μM dopamine + 100 μM SQ22536 (the adenylate cyclase inhibitor) and 150 μM ketamine + 10 μM dopamine + 1 μM H-89 (the PKA inhibitor) for 5 h. **a** Representative photomicrograph of Caspase-3 (AC3) positive expressions (*brown color*) in rat retina, scale bar, 50 μm. **b** Representative photomicrograph of TUNEL positive staining (*red color*) in rat retina, scale bar, 25 μm. **c** Percentages of AC3-positive cells in the GCL. **d** Percentages of TUNEL-positive cells in the GCL. *GCL* ganglion cell layer; K + D 150 μM ketamine + 10 μM dopamine. $*p < 0.05$, $**p < 0.01$ compared to ketamine group, $\#p < 0.05$, $\#\#p < 0.01$ compared to control group. $\S p < 0.05$, $\S\S p < 0.01$ compared to K + D group

each antagonist alone (data not shown). However, whether D1, D2, or A2 receptors acted alone or as components of heteroreceptor complexes remains to be elucidated, as the co-administration of D1, D2, or A2 receptors antagonists did not completely antagonize ketamine-induced apoptosis.

Our electrophysiological results showed that ketamine not only inhibited early synchronized spontaneous network activity in the rat retina at postnatal day 7, when activity is mainly driven by the glutamatergic system, but also inhibited activity at postnatal day 0 and 3, when activity is driven by cholinergic interactions. This suggests that blockade of cholinergic receptors might also contribute to ketamine-induced neuronal apoptosis in the developing rat retina. This is in line with previous observations that cholinergic neurons are more resistant to anesthetic exposure than glutamatergic, GABAergic, and dopaminergic neurons in neonatal rats [35, 36].

There are several limitations in our study. We did not determine the levels of cAMP and the activity of PKA, nor did we investigate the dynamic characteristics of early synchronized spontaneous network activity. The roles of dopamine heteroreceptor complexes and other signaling pathways in dopamine against ketamine-induced neuronal apoptosis during early development require further exploration.

In conclusion, ketamine time- and dose-dependently aggravated physiological apoptosis and inhibited early synchronized spontaneous network activity in the developing rat retina. Dopamine exerted protective effects on ketamine-induced retinal apoptosis by activating D1, D2, and A2A receptors, and upregulating the cAMP/PKA signaling pathway, rather than through the modulation of synchronized spontaneous network activity.

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Author Contributions Jing Dong, Junjie Zhang, Jijian Zheng conceived and designed the project. Jing Dong, Lingqing Gao, Junde Han performed all the experiments and prepared the figures. Jing Dong and Jijian Zheng wrote the manuscript. All authors have reviewed the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they no conflict of interests.

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