

Neuroprotection by acetoacetate and β -hydroxybutyrate against NMDA-induced RGC damage in rat—possible involvement of kynurenic acid

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Abstract

Purpose This study investigated the effects of systemically administered lithium acetoacetate (ACA) and sodium β -hydroxybutyrate (BHB) in a rat model of N-methyl-D-aspartate (NMDA)-induced damage of retinal ganglion cells (RGC). Additionally, the influence of ACA and BHB on kynurenic acid (KYNA) production was assessed in vitro in bovine retinal slices.

Methods Female adult Brown–Norway rats in groups of 5–8 animals were used. ACA and BHB were administered intraperitoneally once a day for 21 consecutive days, and phosphate buffered saline (PBS) was administered to

control animals. After 2 weeks, the animals received intraocular NMDA (2 μ l of a 10 mM solution in PBS) or intraocular PBS as a control. On day 19, retinal ganglion cells were labeled retrogradely with hydroxystilbamidine. Two days later, RGC density (cells per mm^2) was assessed on retinal flatmounts. Additionally, bovine retinal slices were incubated with NMDA and ACA or BHB at concentrations of 1.0 mM and 3.0 mM, and de novo KYNA production was measured using HPLC.

Results Intraperitoneal ACA (250 mg/kg) or BHB (291.2 mg/kg) significantly protected RGC against NMDA-induced neurodegeneration. De novo KYNA production in bovine retinal slices was lowered by NMDA. Both ACA and BHB at a concentration of 3.0 mM significantly reduced the effects of NMDA.

Conclusions ACA and BHB had a significant dose-dependent neuroprotective effect on RGC in a rat model of NMDA-induced RGC damage. Both ketone bodies also significantly attenuated NMDA-induced reduction of retinal KYNA production in vitro, suggesting that this mechanism may be essential for the neuroprotective effects of ACA and BHB in vivo. Our results imply that ketone bodies may represent an additional treatment option in chronic neurodegenerative disorders of the eye.

Keywords β -hydroxybutyrate · Acetoacetate · Retinal ganglion cells · Kynurenic acid · Neuroprotection

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Introduction

The anticonvulsant effects of ketogenic diets have been clinically used for decades in the treatment of epilepsy (see [1] for a review). In recent years, accumulating evidence

indicates a neuroprotective action of ketone bodies in various neurodegenerative disorders of the brain.

A ketogenic diet consists of a high fat content (80–90%) with little, but sufficient protein and insufficient carbohydrates for normal metabolism. Under a ketogenic diet, fat is metabolized in the liver into the ketone bodies acetoacetate (ACA), β -hydroxybutyrate (BHB) and acetone, thus providing an alternative energy source for glucose. The production of ketone bodies is especially important in brain metabolism, which under normal circumstances depends mainly on glucose. In contrast to fatty acids, ketone bodies can cross the blood-brain barrier—in the case of BHB and ACA with the help of monocarboxylate transporters [2].

In recent years, several studies have shown neuroprotective properties of the ketone bodies BHB and ACA in experimental models of neurodegenerative diseases of the brain, in particular Parkinson's disease or Alzheimer's disease but also in traumatic brain injury or cerebral hypoxia [3–8].

It is of importance that crucial pathways leading to deceleration of neuronal degeneration in chronic neurodegenerative diseases of other brain regions, i.e., Alzheimer's or Parkinson's disease, share similar characteristics with neurodegenerative diseases of the retina, e.g., glaucoma, diabetic retinopathy, ischemia, and secondary degeneration after optic nerve trauma. Glutamate-related excitotoxicity has been implicated as a key contributing factor in different neurodegenerative disorders of the brain (see [9] for a review) including glaucomatous retinal ganglion cell (RGC) loss or in retinal ischemia [10, 11]. Excitotoxic damage is thought to be mediated partly by overstimulation of N-methyl-D-aspartate (NMDA)-type glutamate receptors, leading to intracellular Ca^{2+} elevation and consecutively to mitochondrial dysfunction and an excess of free radical formation [12]. Increase of oxidative stress and nitric oxide derived (nitrosative) stress has been shown to play an important role in experimental models of glaucoma and other neurodegenerative diseases [13–15].

Notably, ketone bodies can exert effective neuroprotection in different models of neuronal excitotoxicity and ischemia [4, 16]. Although the underlying mechanisms are not fully understood, both an elevation of ATP production via Krebs' cycle, and mitochondrial membrane stabilization, seem to play an important role [17, 18]. Other highly interesting possibilities include enhancement of antioxidative mechanisms and modulation of endogenous neuroprotectants, in particular kynurenic acid (KYNA), an excitatory amino acid antagonist [19].

In the present study, we tested the effects of the systemically applied ketone bodies ACA and BHB on the survival of RGC in a rat model with NMDA-induced excitotoxic RGC damage. We also evaluated the effects of ACA and BHB on de novo production of KYNA in bovine

retinas. To our knowledge, this is the first time that effects of ketone bodies in a retinal neurodegeneration model have been systematically studied.

Material and methods

Animals

Adult female Brown–Norway rats (Charles River, Sulzfeld, Germany) with a body weight (BW) of 150–200 g were used. The animals were kept under a 12-hour light–dark cycle with food and water ad libitum. All experiments were performed in compliance with the ARVO (Association for Research in Vision and Ophthalmology) statement for the Use of Animals in Ophthalmic and Vision research.

Ketone bodies—intraperitoneal injections

The ketone bodies lithium acetoacetate (ACA) and sodium β -hydroxybutyrate (BHB) (both Sigma-Aldrich, Munich, Germany) were dissolved in 0.1 M phosphate buffered saline (PBS, pH 7.4) to a concentration of 10% w/v as a stock solution.

Intraperitoneal injections were performed once a day for 21 consecutive days on four treatment groups and one control group ($n=5-8$): (1) ACA (62.5 mg/kg BW), (2) ACA (250 mg/kg BW), (3) BHB (72.8 mg/kg BW), (4) BHB (291.2 mg/kg BW), and (5) PBS alone.

NMDA injections

On day 14, animals received intravitreal injections of NMDA versus PBS. Rats were anesthetized with an intraperitoneal injection of chloral hydrate (7%, 6 ml/kg BW). Single injections of 10 mM NMDA (Sigma-Aldrich, Munich, Germany) in 0.1 M PBS (pH 7.4) were administered intravitreally with a heat-pulled glass capillary connected to a microsyringe (Drummond Scientific Co., Broomall, PA, USA) under direct observation through a microscope. The capillary was inserted approximately 1 mm posterior to the corneal limbus at the temporal inferior side (OD: eight o'clock position, OS: four o'clock position). Animals with visible lens damage were excluded from the experiments and not used thereafter. A single injection of 2 μl NMDA was given. In each case, the contralateral eye served as a control eye and was injected with PBS.

Quantification of retinal ganglion cells

On day 19, retrograde labeling of RGC was performed. Animals were anaesthetized with chloral hydrate, and 7 μl

of the fluorescent tracer hydroxystilbamidine methanesulfonate (Fluorogold, Molecular Probes, Eugene, OR, USA) was injected stereotaxically into each superior colliculus. Two days later, the number of RGC (cells per mm^2) was assessed. The animals were sacrificed with CO_2 ; the eyes were enucleated, and the retinas dissected, flat-mounted on cellulose nitrate filters (pore size 60 μm ; Sartorius, Long Island, NY) and fixed in 2% paraformaldehyde for 30 min. Labeled cells were defined as surviving. Visualization of RGC was performed on the same day by fluorescence microscopy. Images obtained using a digital imaging system (ImagePro 3.0, Media Cybernetics Inc., Silver Spring, MD, USA) connected to a microscope were coded and analyzed in a masked fashion. Three areas of 0.04 mm^2 each per retinal quadrant at three different eccentricities of one-sixth, one-half, and five-sixths of the retinal radius were counted, corresponding to 0.7 mm, 2.0 mm, and 3.3 mm distance from the optic nerve head at the 45°, 135°, 225°, and 315° meridian [20]. Labeled cells were thereby counted in 12 distinct areas of 62,500 μm^2 each in each retina. The data are presented as the mean RGC count per mm^2 of retina \pm SEM, unless stated otherwise.

KYNA production—in vitro

Assessment of de novo KYNA production was performed using the method of Turski et al. [21]. Adult bovine eyes were obtained from a local slaughterhouse up to 2 hours post mortem ($n=5-7$). Briefly, their retinas were cut in slices with a McIlwain tissue chopper and put on culture wells containing 1 ml oxygenated Krebs-Ringer buffer, pH 7.4. Following 10 min of preincubation at 37°C, the tissues were incubated with 25 μM L-kynurenine, 1–3 mM NMDA (leading to reduction of KYNA production by 40–60%, see also [22]) and 1.0 mM and 3.0 mM ACA or BHB for 2 hours. ACA and BHB were added to the incubation media 15 min before NMDA, and NMDA 15 min before L-kynurenine. Afterwards, the media were separated from the tissue and applied to the cation-exchange columns (Dowex 50⁺W). KYNA was eluted with 2 ml water, and the eluate was subjected to high-performance liquid chromatography (HPLC). KYNA was detected fluorimetrically according to the method of Shibata [23]. KYNA production was measured in pmol/well.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Analysis of variance (ANOVA) was performed followed by Bonferroni's mul-

tipole comparison post-hoc test. Differences were regarded significant when $p < 0.05$. Unless stated otherwise, the data are presented as means with respective standard errors.

Results

Effect of NMDA on RGC

When injected intravitreally, NMDA reduced RGC numbers from $2,364 \pm 37$ cells/ mm^2 (mean \pm SEM; $n=6$; PBS injected controls) to 217 ± 6 cells/ mm^2 ($n=6$) (Figs. 1 and 2).

Effect of NMDA on RGC in ACA treated animals

RGC density in retinas collected from NMDA-injected eyes after 21 days of treatment intraperitoneally with ACA 62.5 and 250 mg/kg was 190 ± 14 cells/ mm^2 ($n=4$) and 322 ± 25 cells/ mm^2 ($n=5$) respectively. The mean cell count in rats treated with 250 mg/kg ACA was significantly higher ($p < 0.001$) than in the PBS-injected controls (Figs. 1 and 2).

Effect of NMDA on RGC in BHB treated animals

RGC density in retinas collected from NMDA-injected eyes after 21 days of treatment with intraperitoneally injected BHB 72.8 and 291.2 mg/kg was 178 ± 10 cells/ mm^2 ($n=5$) and 307 ± 17 cells/ mm^2 ($n=5$) respectively. The mean cell count in eyes treated with BHB in a dose of 291.2 mg/kg was significantly higher ($p < 0.01$) than in the PBS-injected controls (Figs. 1 and 2).

Effect of ACA and BHB on RGC in control animals

Neither ACA nor BHB treatment affected RGC density in PBS-injected eyes. RGC density in retinas collected from PBS-injected eyes was 2328 ± 60 cells/ mm^2 ($n=5$) for ACA-treated rats and 2310 ± 51 cells/ mm^2 ($n=5$) for BHB-treated rats.

Effect of ACA on KYNA production in bovine retinal slices

NMDA decreased de novo production of KYNA to 41.2% ($p < 0.001$) of that in controls. ACA 3.0 mM significantly countered this: KYNA production after NMDA remained at 75.3% of that in controls ($p < 0.001$). However, ACA 1.0 mM was ineffective in this respect. Incubation of retinal slices with ACA 1.0 or 3.0 mM without NMDA did not change KYNA production (Fig. 3).

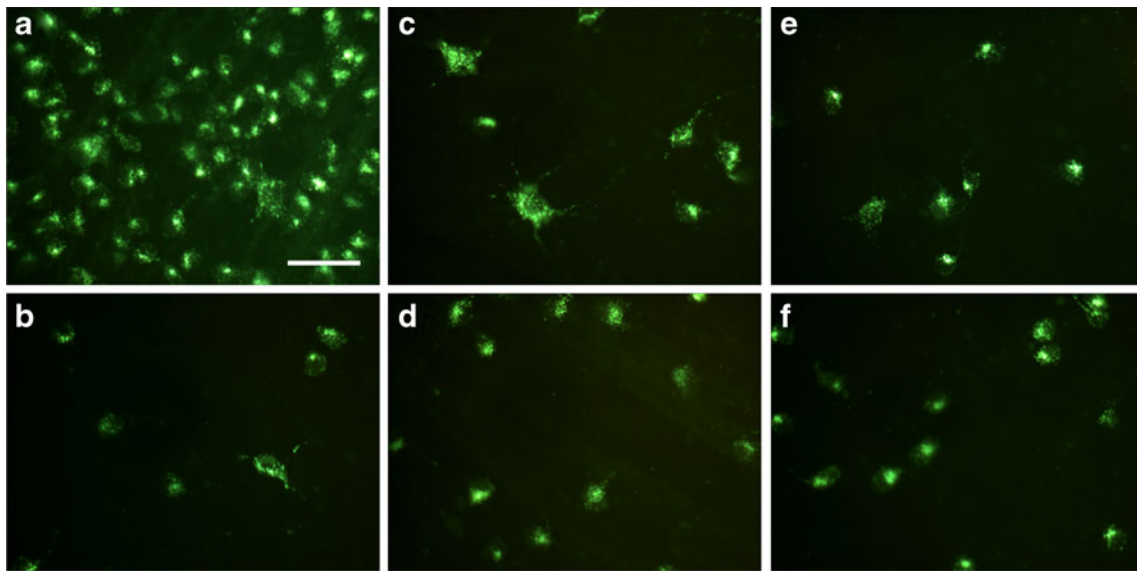


Fig. 1 Retinal flatmounts of rat retinas. RGCs were backlabeled with the fluorescent marker hydroxystilbamidine. **a** Control, no NMDA. **b-f** Seven days after intraocular injection of NMDA in groups intraperitoneally treated with **(b)** PBS as control, **(c)** ACA 62.5 mg/

kg, **(d)** ACA 250 mg/kg, **(e)** BHB 72.8 mg/kg and **(f)** BHB 291.2 mg/kg. Retinal area of images **a-f**: 2 mm from the optic nerve head, nasal inferior or superior quadrants. Scale bar, 50 μ m

Effect of BHB on KYNA production in bovine retinal slices

NMDA decreased de novo production of KYNA to 60.3% ($p < 0.01$) of that in controls, but BHB 3.0 mM provided complete protection against NMDA-induced inhibition of KYNA production ($p < 0.001$). BHB 1.0 mM was ineffective in this respect. Incubation of retinal slices with BHB 1.0 or 3.0 mM without NMDA did not change KYNA production (Fig. 4).

Discussion

Ketogenic diets have been used for over 80 years in the treatment of epilepsy. Recently, neuroprotective properties of ketone bodies have been found in diverse models of such neurodegenerative diseases of the brain as Parkinson's or Alzheimer's disease, and in models of brain trauma, hypoxia or ischemia [3–8]. To date, however, similar studies regarding retinal degeneration have not been performed.

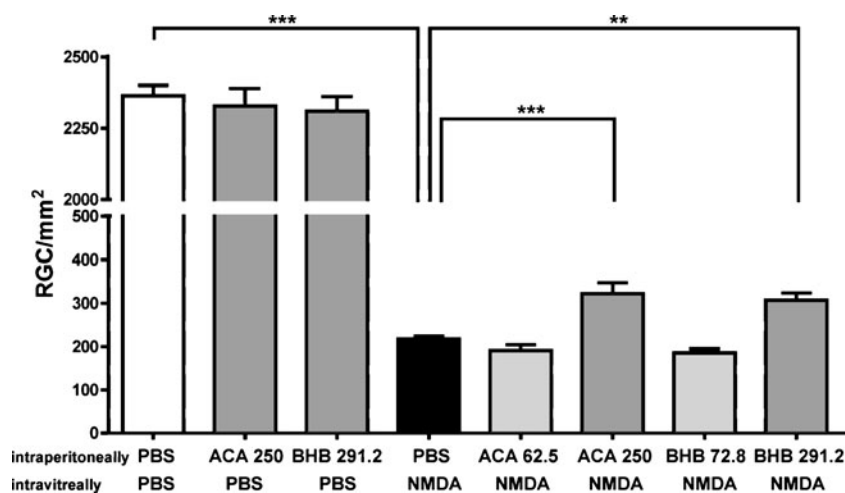


Fig. 2 RGC numbers 7 days after intraocular NMDA injections in systemically ACA- and BHB-treated animals, PBS served as control ($n = 6$). ACA was used in the following doses: 250 mg/kg in PBS-treated eyes ($n = 5$); 62.5 mg/kg ($n = 4$) or 250 mg/kg ($n = 5$) in

NMDA-treated eyes. BHB was used in the following doses: 291.2 mg/kg in PBS-treated eyes ($n = 5$); 72.8 mg/kg ($n = 5$) or 291.2 mg/kg ($n = 5$) in NMDA-treated eyes. ** $p < 0.01$; *** $p < 0.001$

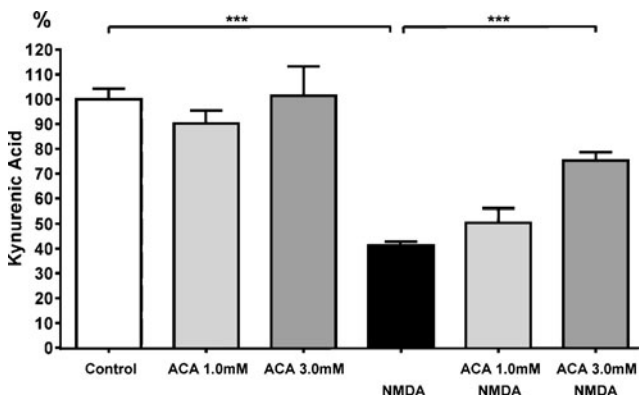


Fig. 3 Effect of ACA on de novo KYNA production in bovine retinal slices. Data is given as percentage of control. Control slices ($n = 6$) were incubated with $25 \mu\text{M}$ L-kynurenine alone. NMDA treated slices ($n = 6$) were incubated with $25 \mu\text{M}$ L-kynurenine and 3.0 mM NMDA. ACA was used at concentrations of 1.0 and 3.0 mM alone ($n = 8$ and $n = 6$ respectively) or in combination with 3.0 mM NMDA ($n = 5$ and $n = 6$ respectively). $100\% = 8.0 \text{ pmol}$. *** $p < 0.001$

Excitotoxicity, which contributes to free radical formation and neuronal cell death, is an important factor in diverse chronic neurodegenerative diseases in the brain and also in the retina [4]. Excitatory neurotoxicity is mediated in part by overactivation of the NMDA-type glutamate receptor [12]. In the present study, an excitotoxic rat model of NMDA-induced RGC damage [24] was used to evaluate the neuroprotective properties of systemic application of the ketone bodies ACA and BHB in the retina.

In rats, a single intravitreal NMDA injection reduced the RGC number to around 10% of that in PBS-injected controls. This RGC loss is comparable to that found in other studies using NMDA-induced retinal degeneration [25, 26].

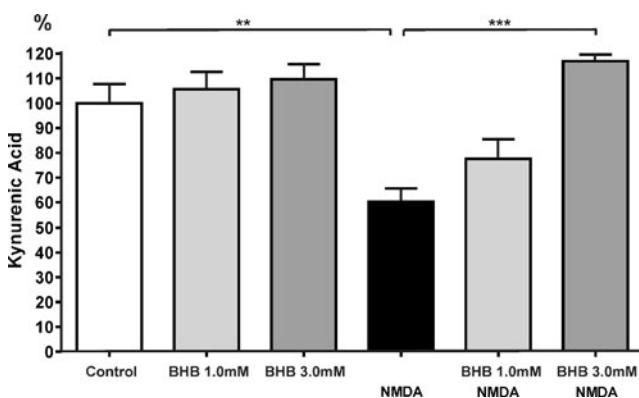


Fig. 4 Effect of BHB on de novo KYNA production in bovine retinal slices. Data is given as percentage of positive control. Control slices ($n = 6$) were incubated with $25 \mu\text{M}$ L-kynurenine alone. NMDA-treated slices ($n = 6$) were incubated with $25 \mu\text{M}$ L-kynurenine and 1.0 mM NMDA. BHB was used at concentrations of 1.0 and 3.0 mM alone ($n = 6$ for both groups) or in combination with 1.0 mM NMDA ($n = 6$ for both groups). $100\% = 3.1 \text{ pmol}$. ** $p < 0.01$; *** $p < 0.001$

However, ACA and BHB ameliorated the cell damage induced by NMDA, and resulted in a relative RGC increase of 48.4% and 41.5% respectively when administered intraperitoneally for 21 days.

It is possible that the lithium fraction in the lithium salt of ACA leads to neuroprotective effects, as shown previously in a rat model of partial optic nerve crush. However, the amount of lithium in doses of ACA as high as 250 mg/kg is less than that which showed a significant neuroprotective effect in previous studies of the retina [27]. Furthermore, Massieu et al. demonstrated a protective effect of the lithium salt of ACA (250 mg/kg i.p.) but not of the lithium fraction alone in a model of glutamate-mediated neuronal damage in the hippocampus [4]. Although an additive effect of lithium and ACA cannot be excluded, a substantial modification of ACA's effects by the lithium fraction seems unlikely. Moreover, the neuroprotective effect of BHB against RGC death was comparable to that of ACA, even though BHB is not a lithium compound.

Two other studies found similar results for ACA but not for BHB in vivo, whereas the effective dose of intraperitoneal ACA after glutamate-mediated neuronal damage in the hippocampus was the same as in our study [4, 28], BHB at 332 mg/kg failed to achieve a neuroprotective effect [28]. In contrast, another study on glutamate-mediated neuronal damage showed reduced levels of lipoperoxidation after a single intraperitoneal injection of 500 mg/kg BHB [29], and Suzuki et al. demonstrated prolonged survival times after intravenous application of 30 mg/kg BHB in a rat model of brain anoxia [6]. In all these studies, however, treatment times were shorter than in our study.

Plasma levels of ACA and BHB in rats have been shown to be increased from 0.06 mM (controls) to 0.6 mM 10 minutes after a single injection of 500 mg/kg ACA or BHB [29]. However, in vitro considerably higher concentrations (5 mM ACA and 4 mM BHB) were the most effective against glutamate toxicity in a mouse hippocampal cell line [16], in good agreement with our in vitro results in bovine retinal tissue.

How ketone bodies confer their neuroprotective effect on neuronal cells remains unclear, and several theories are currently under discussion. An important underlying mechanism may be an increase in neuronal stability due to enhanced ATP production [30, 31]. In accordance with this theory, Bough et al. found an increased number of mitochondrial profiles in rats fed a ketogenic diet, suggesting a stimulation of mitochondrial biogenesis and a higher phosphocreatine/creatine ratio in the hippocampus and higher available energy reserves [32]. Such an increased energy level of the neurons might ensure them a better supply of the Na/K-ATPase, leading to enhanced membrane stability, and thus providing an improved resistance to metabolic stress.

Other studies have found protective effects of ketogenic diet or ketone bodies against excitotoxic neuronal degeneration, and proposed that oxidative stress modulation rather than glutamate receptor modulation may be important for this effect [4, 16, 28, 33]. Direct interaction of ketone bodies with glutamate receptors has been shown only for L-BHB (voltage-dependent blockade of NMDA receptors), whereas ACA or D-BHB were not able to reduce NMDA, AMPA or Kainate receptor-induced currents [34, 35]. Furthermore, even though ACA has been demonstrated to have a protective effect in hippocampal cells, this protective effect was also found in cells which lacked glutamate receptors [16]. Such neuroprotective effects are probably due indirectly to an enhancement of cellular antioxidant capacity. Several studies describe an influence of ketone bodies on different antioxidative mechanisms of neuronal cells. Veech et al. showed that BHB is able to oxidize co-enzyme Q and reduce NADP⁺, thus reducing free radical formation [36]. In addition, the reduction of mitochondrial free radical formation resulting from an increase the NAD⁺/NADH ratio, along with the scavenging capacities of ACA and BHB for diverse reactive oxygen species (ROS), may contribute to neuroprotective effects, even when increase in oxidative stress is increased by excitotoxicity [29, 37].

Other factors which possibly play a role here include upregulation of glutathione peroxidase activity, an enzyme that can prevent lipid peroxidation, and upregulation of calbindin [33, 38, 39].

In addition to an indirect influence on glutamate-induced neurotoxicity via the antioxidant effects of ketogenic diet or ketone bodies, indirect glutamate receptor modulation via reversal of the NMDA-inhibiting effect on the production of KYNA, an endogenous NMDA receptor modulator, has been postulated, and may play an additional role in preventing glutamate-induced neurotoxicity [19, 40]. In our study, NMDA-induced reduction of de novo KYNA production in ACA- and BHB-incubated bovine retinas in vitro was significantly attenuated. This effect was much more pronounced than the increase in RGC numbers after treatment with ketone bodies in vivo; therefore, a sole survival effect of KYNA-producing cells in the in vitro setting seems rather unlikely. The in vivo situation, however, is not directly comparable to the in vitro setting; further studies have to show in detail how ketone bodies modify KYNA production in the context of excitotoxicity. Modulation of NMDA receptors via alteration of KYNA-levels is especially of therapeutic interest. Since NMDA receptors are essential for neuronal transmission, a full blockade of NMDA receptors would not be suitable for clinical application; rather, it would be preferable to prevent receptor overstimulation while reducing free radical formation [41, 42]. Ketone bodies therefore seem to offer

interesting features for treatment or prevention of cellular damage in different neuronal degenerations.

This study showed for the first time that systemically applied ketone bodies exert a partial neuroprotective action against NMDA-induced RGC damage in rats. Moreover, ACA and BHB were found to attenuate NMDA-induced inhibition of KYNA production in retinas in vitro. We believe that the use of ketone bodies or ketogenic diets should be considered and further evaluated in the quest for effective therapeutic approaches in the treatment of retinal degenerations.

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