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Age-dependent neuroprotection of retinal ganglion cells by tempol-C8 acyl ester in a rat NMDA toxicity model

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Abstract

Background: *The efficacy of tempol and its acyl derivative tempol-C8 as retinoprotective agents was compared in a rat model of NMDA-induced retinal ganglion cell (RGC) damage.*

Material and methods: *Tempol or tempol-C8 in different doses was administered intraperitoneally to 6 weeks old (pre-adolescent) and 9-10 weeks old (young adult) rats before and after an intravitreous NMDA injection. Retinal ganglion cell were retrogradely labeled with the fluorescent tracer hydroxystilbamidine and RGC counting was performed on retinal flatmounts.*

Results: *Intravitreal NMDA reduced RGC counts by about 90%, independently of age ($p < 0.001$). In pre-adolescent animals tempol-C8, but not tempol unmodified, showed a significant, dose-dependent RGC rescue effect, with peak activity at 5.8 µmol/kg ($p < 0.001$). In young adult animals, however, no neuroprotective effect was found for either tempol or tempol-C8.*

Conclusions: *In contrast to tempol itself, tempol-C8 acyl ester was neuroprotective in pre-adolescent rats in the NMDA-induced RGC damage model. Therefore, neuroprotection by tempol acyl esters seems to be superior to that of tempol under certain conditions.*

Key words: *tempol, tempol acyl ester, NMDA, ganglion cell, neuroprotection.*

Introduction

Retinal ganglion cell loss is a feature of several important chronic disorders of the eye, including glaucoma, ischemia, and diabetic retinopathy. Several pathways modifying the course of this selective neuronal cell death have been identified in the last decades. The exact mechanisms still remain unclear, but excitotoxicity appears to play an important role. Anti-excitotoxic drugs have been shown to act neuroprotectively in animal models of ocular hypertension and ischemia [3,9].

Antioxidant enzymes play a key role in defending cells against free radical toxicity; superoxide dismutases (SOD), for example, catalyze the decomposition (dismutation) of the superoxide anion into hydrogen peroxide. Superoxide anion dismutation is crucial to free radical defense and limits the gen-

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eration of highly reactive species such as hydroxyl radical ($OH^{\bullet}$) or peroxynitrite ($ONOO^{-}$). *In vitro* and *in vivo* experiments have demonstrated that antioxidant enzyme mimetics – small, non-protein molecules with catalytic properties similar to those of enzymes – can improve oxidative stress defenses. A well-known SOD mimetic in this respect is tempol (4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxyl), a stable nitroxide radical. Tempol is highly cell-permeable and easily crosses the blood brain barrier [14]. It has been shown previously both *in vitro* and *in vivo* to protect neuronal cells in models of brain trauma, ischemic stroke, and Parkinson's disease [1], all of which are neurodegenerative conditions that share many similarities with retinal neurodegenerative disorders such as glaucoma. In an *in vitro* model of retinal ganglion cell (RGC) damage via TNF-α and hypoxia, tempol significantly improved RGC survival after the onset of mitochondrial damage [21]. Tempol and its derivative tempol hydroxylamine also acted neuroprotectively in models of light damage [26]. Unfortunately, tempol is effective only in relatively high doses; some authors have reported effective doses of $\geq$ 100 mg/kg body weight in *in vivo* situations [18]. Such high doses in conscious animals have been reported to trigger serious side effects, including seizures, hypotension, and agitation [7].

Increased tempol effectiveness can be achieved by attaching an "address molecule" to tempol that will direct it to sites of free radical generation. An example of this might be mitochondria-targeted TEMPO conjugates with hemigramicidin as an "address molecule" [6]. However, free radicals can be generated not only in mitochondria but also in cell membranes, for example in the plasma respiratory chain called plasma membrane oxidoreductase (PMOR), which is also present in neuronal tissue [27]. We therefore conjectured that adding a lipophilic "address molecule" could increase both the amount and neuroprotective efficacy of tempol in membranes. Recently, we were able to show that one such tempol derivative, tempol-C8 acyl ester, provides RGC neuroprotection in lower doses than unmodified tempol in a rat partial optic nerve crush (PONC) model [22].

The purpose of the present study was to compare the efficacy of tempol and its acyl derivative tempol-C8 as retinoprotective agents in the excitotoxicity model of N-methyl-D-aspartate (NMDA)-induced RGC damage in pre-adolescent and young adult rats.

Material and methods

Animals

Brown Norway rats (Charles River, Wilmington, MA), body weight 100-120 g (6 weeks of age – "pre-adolescent") and 190-210 g (9-10 weeks of age – "young adult") were housed under a 12-hour light-dark cycle. The treatment of the animals was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved and monitored by the respective authorities (Regierungspräsidium Tübingen).

Intravitreal injection

The rats were anesthetized with an intraperitoneal injection of chloral hydrate (7%, 6 ml/kg body weight). Local anesthesia in the form of eye drops (oxybuprocaine) was also applied. 2 µl of NMDA solution (10 mM in 0.2 phosphate buffered saline [PBS], pH 7.2; Sigma-Aldrich, Steinheim, Germany) were intravitreally injected into the posterior side of the globe, 1 mm behind the limbus, with a heat-pulled glass capillary connected to a microsyringe (Drummond Scientific, Broomall, PA) under direct observation through the microscope. The eye volume was estimated to be about 20 µl, leading to a final intraocular NMDA concentration of 1 mM. Antibiotic eye drops (ofloxacin) were applied directly after the injection. Any rat that exhibited postoperative complications such as lens damage, retinal hemorrhage, retinal detachment, or vitreous hemorrhage was excluded from the study. Contralateral eyes served as control eyes and were injected with PBS (NMDA vehicle).

Treatment

Either tempol (Sigma-Aldrich, Steinheim, Germany) in doses of 116 and 290 µmol/kg (20 and 50 mg/kg bw) or tempol-C8 (synthesized by M. Wozniak as described previously [22]) in doses of 0.58, 2.9, 5.8, 29, and 58 µmol/kg (molar equivalents of tempol 0.1, 0.5, 1, 5, and 10 mg/kg bw) was administered intraperitoneally twice before NMDA injection (24 h and 30 min), and then once daily for 6 consecutive days. Control rats were treated with tempol vehicle (5% ethanol in PBS, pH 7.2).

Quantification of retinal ganglion cells

Retrograde labeling of retinal ganglion cells was performed as described previously [25]. Briefly, 5 days after NMDA injection the animals were anesthetized

as described above, and 7 µl of the fluorescent tracer hydroxystilbamidine methane sulfonate (Molecular Probes, Eugene, OR) was injected stereotaxically into each superior colliculus. Two days after labeling (i.e. 7 days after NMDA injection), the animals were sacrificed with CO_2, the eyes were enucleated, and the retinas were dissected, flat-mounted on cellulose nitrate filters (pore size 60 µm; Sartorius, Long Island, NY), and fixed in 2% PFA for 30 min. Visualization of RGC was performed immediately by fluorescence microscopy. Images obtained using a digital imaging system (Image-Pro 3.0, Media Cybernetics Inc., Silver Spring, MD) connected to a microscope were coded and analyzed in a masked fashion by an observer who was unaware of the treatment scheme. Counts taken in 12 distinct areas of 62,500 µm^2 each per retina were added and expressed as cell density (cells per square millimeter; mean ± SE), as described in detail previously [23].

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA, USA). Kruskal-Wallis non-parametric ANOVA was used followed by Dunn's multiple 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

NMDA vehicle-treated animals

The average density of RGC in vehicle (PBS)-treated animals was 2342.1 ± 78.0. There was no significant difference between pre-adolescent and young adult rats ($p > 0.05$). Neither tempol nor tempol-C8 treatment significantly affected RGC numbers in vehicle-treated animals.

NMDA-injected animals

NMDA injection reduced RGC counts by around 90%, i.e. to 278.1 ± 12.1 ($n = 8$, $p < 0.001$). There was no significant difference between pre-adolescent and young adult rats ($p > 0.05$).

Tempol-treated animals

Tempol treatment exerted no significant protective effect against NMDA-induced RGC loss in any dose tested (up to 50 mg/kg BW) ($p > 0.05$). There were no significant differences between the pre-adolescent and young adult rat groups ($p > 0.05$) (Fig. 1).

Tempol-C8-treated animals

Tempol acyl ester tempol-C8 partially rescued RGC against NMDA-induced cell loss, but only in pre-adolescent animals. The most effective dose was 5.8 µmol/kg BW (i.e. equivalent of 1 mg of tempol/kg BW). Tempol-C8 treatment increased RGC numbers after NMDA to a level 63% higher than that of untreated control animals (452.8 ± 16.4, $p < 0.001$). Other concentrations which were tested did not increase RGC counts significantly (336.8 ± 20.8; 354.4 ± 28.7 and 261.2 ± 16.1 for 2.9, 29 and 58 µmol/kg doses respectively, $p > 0.05$ for all groups vs. controls). Tempol-C8 did not result in any significant differences in RGC counts in the young adult animals after NMDA compared to the control animals (322.9 ± 15.7; 318.4 ± 9.8; 330.1 ± 27.5; 232.9 ± 4.3 for 0.58, 2.9, 5.8 and 29 µmol/kg doses respectively, $p > 0.05$ for all groups) (Fig. 2).

Discussion

In this study intravitreous NMDA led to a massive RGC loss after one week, in accordance with previous studies of our group and others [10,22,24]. Against this background we tested the neuroprotective effects of the free radical scavenger tempol and its acyl ester tempol-C8 in a rat NMDA-induced RGC damage model. Both compounds were shown previously to provide neuroprotection to RGC in a rat PONC model [22,24]. Li *et al*. have suggested that RGC death after NMDA displays signs of apoptosis similar to that of RGC death after PONC [10]. It was, therefore, reasonable to assume that these two models may share similarities concerning the nature of RGC damage, although with differences in the onset and course. However, both models have their limitations and exhibit different aspects of glaucomatous degeneration in the form of mechanical or intrinsic activation of neurodegeneration. PONC simulates particularly a mechanical onset of optic nerve damage in glaucomatous neuropathy, while the NMDA toxicity model described here reflects more the excitotoxic aspect of glaucoma, whose significance is still under discussion [17,20]. Nevertheless, both models produce selective and pronounced loss of RGC within a relatively short time (days-weeks).

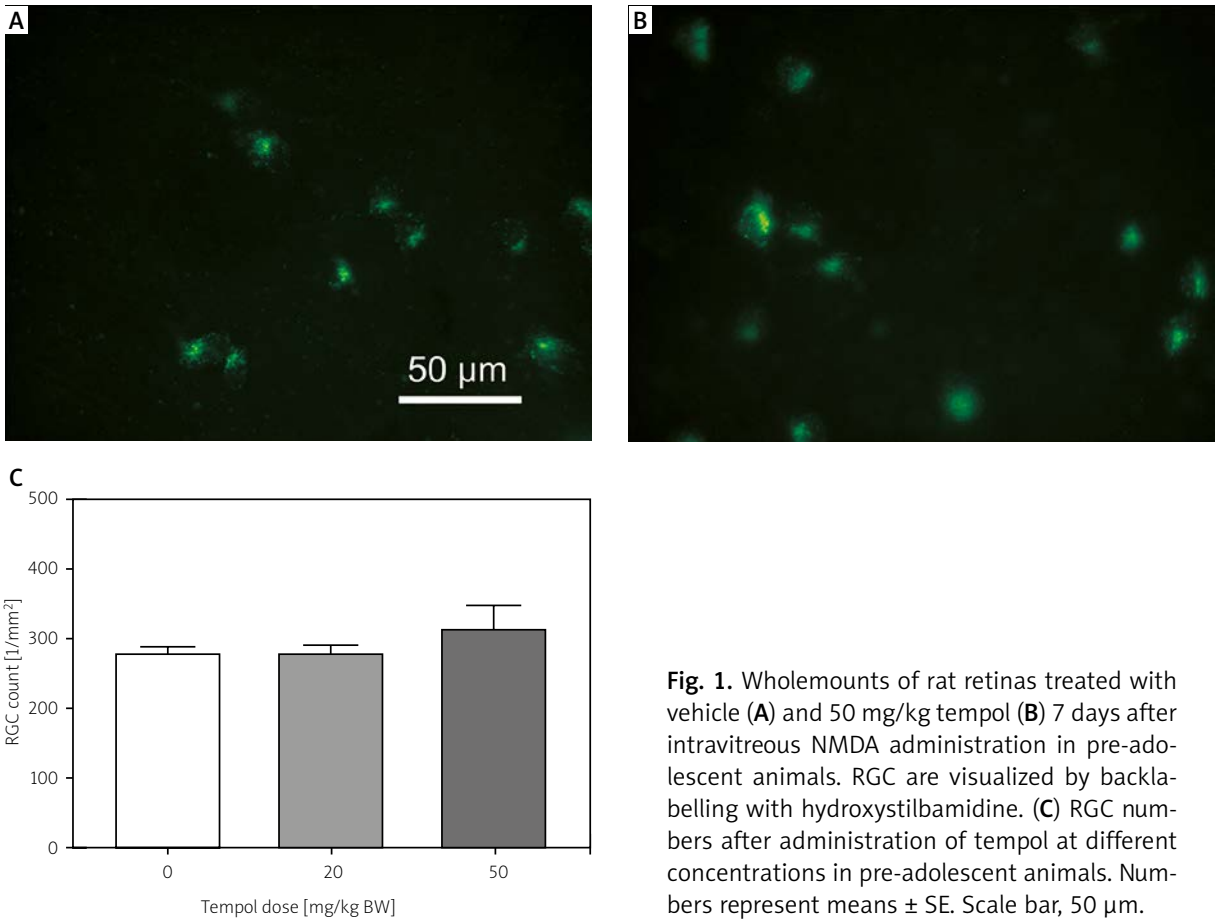

Fig. 1. Wholemounts of rat retinas treated with vehicle (**A**) and 50 mg/kg tempol (**B**) 7 days after intravitreous NMDA administration in pre-adolescent animals. RGC are visualized by backlabelling with hydroxystilbamidine. (**C**) RGC numbers after administration of tempol at different concentrations in pre-adolescent animals. Numbers represent means ± SE. Scale bar, 50 µm.

Surprisingly, and in contrast to PONC, tempol in the doses used did not show any protection toward RGC in the NMDA toxicity model. This stands partly in contrast with a previous study of El-Remessy *et al*. [5], who found that intravitreal injection of tempol (0.4 mg/eye) diminished TUNEL staining in the ganglion cell layer and the inner nuclear layer (ganglion cell layer – GCL, inner nuclear layer – INL) when coadministered with 200 nmol of NMDA per eye (i.e. 2 µl of 100 mM NMDA). Tempol also diminished malondialdehyde and nitrotyrosine staining. However, there were significant differences in the experimental settings. The model utilized by El-Remessy *et al*. used a 10-times higher concentration of NMDA than was the case in our experiments. Furthermore, El-Remessy *et al*. administered tempol intravitreally and at the same time as NMDA, which includes the possibility of direct interaction. The intravitreal concentration of tempol in the eye might be even 1000-fold higher than in our experiments after intraperitoneal tempol administration. A much higher concentration of systemic tempol might have had a neuroprotective effect in our model as well, but we did not test it because we expected prohibitive systemic side effects [7].

We found that tempol-C8, a tempol acyl ester previously shown to be more effective than tempol in a rat model of PONC, was also effective in the NMDA toxicity model. However, in our experiments tempol-C8 was effective only in pre-adolescent animals, despite the fact that NMDA-induced damage to RGC did not depend on animal age. We suppose that the resistance of older animals to tempol-C8 may mirror different mechanisms engaged in the excitotoxic damage evoked by NMDA in the pre-adolescent and young adult rats.

Dramatic age-related changes of vulnerability to excitotoxicity have been observed in cultured hippocampal neurons within 35 days after birth [13]. In cortical slices glutamate-induced reactive oxygen species (ROS) formation and lactate dehydrogenase release (a marker of cell death) were significantly higher in 80-day-old rats than in 21-day-old rats [8].

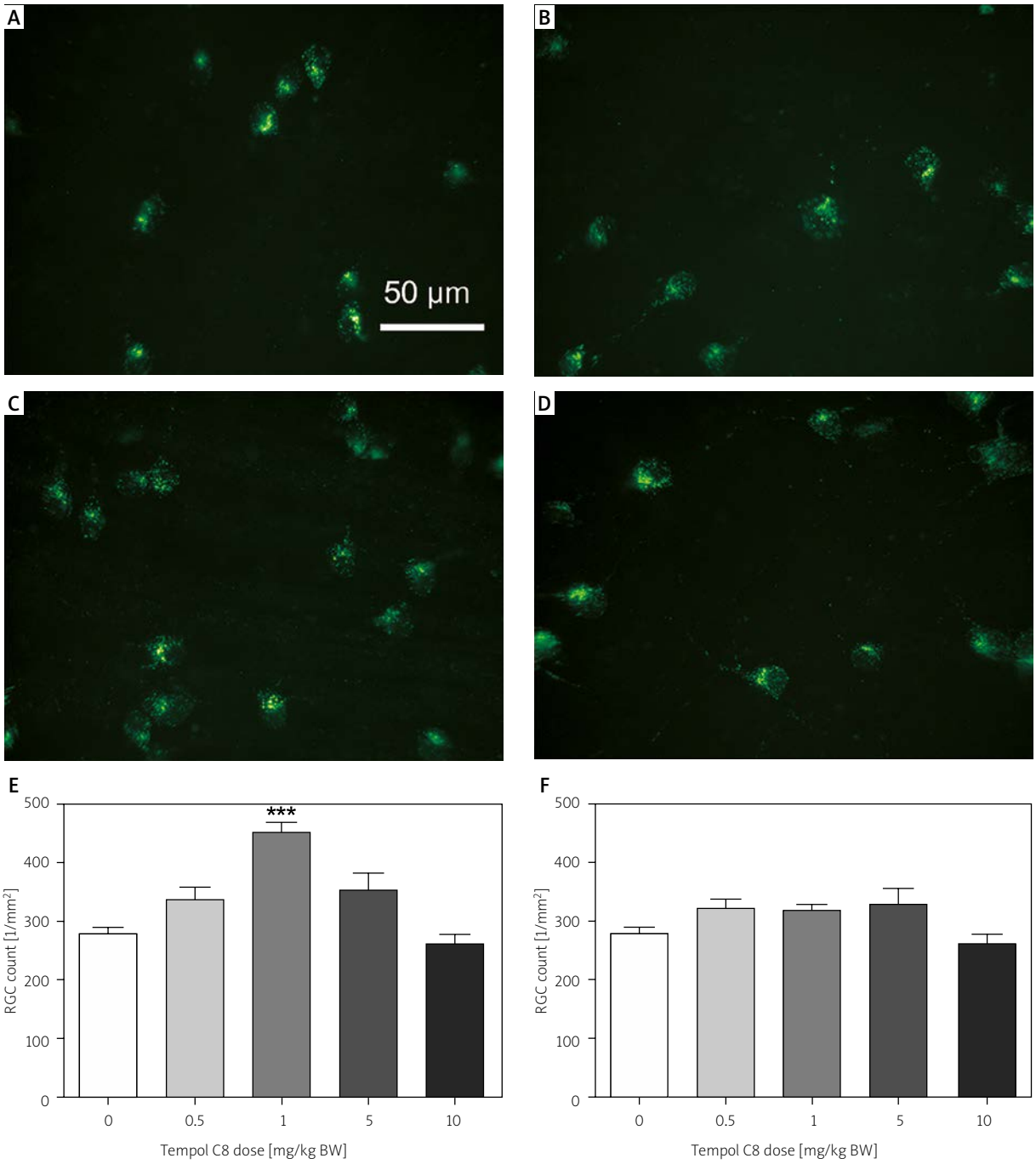

Fig. 2. Upper panel: Wholemounts of rat retinas treated with vehicle (**A, B**) or tempol-C8 1 mg/kg BW (**C, D**) in pre-adolescent (**A, C**) or young adult (**B, D**) animals. Lower panel: RGC numbers after administration of tempol-C8 at different concentrations in pre-adolescent (**E**) and young adult (**F**) animals. Tempol-C8 concentrations are given in molar equivalents of tempol. Numbers represent means ± SE. ***$p < 0.001$, Scale bar, 50 µm.

It is also well established that various receptors contribute to glutamate toxicity, depending on the developmental stage, as has been shown in cortical slices [19] and in RGC [2].

The age-dependent changes in excitotoxic insult vulnerability may be calcium-dependent [8]. Glutamate-induced calcium currents are similar in young and adult neurons from rat cortical slices [8] and

in isolated mouse RGC [11]. However, Mann *et al*. reported a dramatic difference in calcium buffering capacities between early postnatal and adult RGC. The difference seems to appear at the level of the mitochondria [13]. These differences are possibly due to the existence of cytosolic and mitochondrial nitric oxide synthase (NOS). Mitochondrial NOS mediates decreased vulnerability of young neurons to NMDA, and cytosolic NOS contributes to NMDA toxicity in mature neurons [11]. It may be of relevance that male rats display signs of sexual maturation between 40 and 60 days of age [4]; therefore our pre-adolescent rats presumably were sexually immature.

In our study, tempol-C8 was effective only in one of the tested doses, equivalent to tempol 1 mg/kg BW. It is known that SOD and its mimetics including tempol exert pro-oxidative effects in high doses (10^{-4}-10^{-2} M) [15]. Inefficacy of higher tempol doses may be analogous to the previously described decrease of SOD cytoprotective activity at higher levels of the enzyme [16].

In summary, we have shown that lipophilic tempol-C8 is a potent neuroprotectant in NMDA-induced RGC damage in rats, effective at a relatively low concentration, at which unmodified tempol is ineffective. However, the neuroprotective effect of tempol-C8 was found only in 6 weeks old, presumably sexually immature, rats. Whereas the issue of age dependency of free radical toxic effects on retinal ganglion cells needs further research, these results underscore the necessity of using older animals in experiments that model diseases with age as a significant risk factor.

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Disclosure

Authors report no conflict of interest.

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