An Approach for Neuroprotective Therapies of Secondary Brain Damage after Excitotoxic Retinal Injury in Mice

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SUMMARY

Background: Many current therapeutic strategies for several eye diseases, such as glaucoma, retinal ischemia, and optic neuropathy, are focused on protection of the retinal ganglion cells (RGCs). In fact, loss of visual field, including irreversible blindness, is caused by RGC damage in these diseases. However, recent evidence suggests that the RGC damage extends to visual center in brain: the visual impairment induced by these diseases may result not only from RGC loss, but also from neuronal degeneration within the visual center in brain. **Objective:** To protect neurons within the visual center in the brain, as well as retinal treatment, for the prevention of visual disorder in these diseases. **Methods:** Once considered difficult to study the visual center in brain following RGCs loss, because obtaining the human samples that are suitable for the study may be difficult. In addition, the monkey, mainly used as glaucomatous model, is relatively high cost and needs to long experiment-span. Here, we focused on mice, because of their high degree of availability, relatively low cost, and amenability to experimental and genetic manipulation. **Conclusion:** In this review, we describe time-dependent alterations in the visual center in brain following RGCs loss, and whether some drugs prevent the neuronal damage of the visual center in the brain.

Introduction

There are 1.2–1.5 million retinal ganglion cells (RGCs) in the ganglion cell layer (GCL) in the human. They receive visual information from photoreceptors via the bipolar and amacrine cells, and deliver the visual signal through the axons of the optic nerve (ON) to the lateral geniculate nucleus (LGN) and the superior colliculus (SC) [1,2]. The majority of RGC terminals convey the visual information to the LGN [1], and the remaining RGCs target other areas, including SC for eye movement [2,3]. Thus, visual information entering the eye is processed in the retina and then transmitted via the ON axons to the visual center in brain.

RGC death is a common feature of several eye diseases, such as glaucoma, retinal ischemia, and optic neuropathy [4]. Currently, RGC is a potential therapeutic target for these diseases which present in the RGC death that may occur via a variety of mechanisms involving, for exam-

ple, reactive oxygen species [5], excitatory amino acids [6], and nitric oxide [7,8]. However, the initial loss of RGC does not lead to visual field loss in human eyes with glaucoma [9]. In fact, a loss of more than 50% of RGC has been reported to induce visual field loss in clinical study [9]. In addition, recent evidence suggests that glaucomatous damage extends from RGCs to visual center in brain including the LGN [10,11] and the SC [12]. These findings raise the possibility that the visual disorder may not only result from RGC loss, but also from neuronal degeneration in the visual center in brain.

Especially in glaucoma, it is considered difficult to study the visual center in brain following RGCs loss in human patients as some clinical diseases. Although such studies have been using primate models, primates are difficult to study in large numbers because of their limited availability, high cost, and difficulty handling. For the solution of these problems, focusing on mice may be beneficial because they are high degree of

Figure 1 Visual center in mice. Illustrations showing pathway from retina to the LGN and the SC (A). Coronal sections through the SC level (bregma -3.40 mm) (B) and the LGN level (bregma -2.30 mm) (C) [boxed areas are shown diagrammatically]. Representative photographs of hematoxylin

and eosin staining of sections obtained from normal retina and retina at 7 days after NMDA injection (D). 1: ganglion cell layer (GCL), 2: inner plexiform layer (IPL), 3: inner nuclear layer (INL), 4: outer plexiform layer (OPL), and 5: outer nuclear layer (ONL).

availability and relatively low cost [13,14]. Moreover, it is relatively easy to experimentally manipulate the genetic composition of mice that share many biological characteristics with human, and they are commonly used as model organisms for understanding disease processes and testing treatments. However, to our knowledge, previous investigation has not been made of time-dependent alterations in visual center in brain following RGC loss in mice. In addition, the possible pathophysiological mechanisms underlying neuronal cell death in the visual center in the brain following RGC loss remain uncertain. This review attempts to introduce time-dependent alterations in the visual center in brain following RGC loss and to determine whether memantine, an NMDA antagonist, and lomerizine, a Ca^{2+} channel blocker, prevent the neuronal damage using murine model. Hence, these findings may provide useful information concerning the pathological mechanisms of several disorders accompanied by RGC loss.

RGC Axonal Projection in Mice

In the mouse brain, there are two sets of targets to which RGCs project axons, depending on whether the pathway is image forming or nonimage forming [15,16]. Image-forming RGCs send axons to the LGN (both dorsal and ventral), and the SC, with at least 70%, and possibly all, RGCs projecting to the SC (Figure 1) [17]. The LGN (especially dorsal LGN), positioned in the flow of information from eye to cortex, plays a key role in the murine brain's processing of the visual world. Most fundamental job of the LGN is to provide the neocortex with visual input. On the other hand, the SC, a major target of RGCs axon terminal, plays an important role in guiding the movements of the eye, ear, and head [3,18]. In the SC, there are three main types of sites: the superficial, intermediate, and deep layers. Because the superficial layer of the SC is innervated by RGCs axon, their input is almost exclusively related to vision. Although the

majority of RGCs send information to the visual centers in the brain, non-image-forming RGCs project to central circadian center, such as suprachiasmatic nucleus, and intergeniculate leaflet, as well as the olivary pretectal nucleus, which mediates pupil reflexes [16].

The use of murine models is beneficial to understand morphological changes and molecular mechanisms of RGC and visual center in brain longitudinally, and, as well as to explore neuroprotective treatments, including gene therapy [19]. In fact, murine models for several eye diseases including a variety of blinding disorders such as glaucoma, retinal ischemia, and optic neuropathy, are being developed at an increasing rate to investigate specific pathophysiological mechanisms. However, substantial differences do exist between the visual systems of mice and those of primates, including human. In fact, RGC projection to the LGN is comparably less in mice than in primates. Thus, although transgenic and knockout mice can be provide useful models with which to assess visual function, there is a need to maintain a comparative approach.

NMDA-Induced Retinal Damage in Murine Model

Recently, growing evidence suggests that excessive activation of glutamate receptors by abnormally high levels of glutamate released from injured RGCs is implicated in the RGC death process of a variety of acute and chronic eye diseases, such as glaucoma, retinal ischemia, and optic neuropathy [20–24]. Glutamate is the predominant neurotransmitter in the retina as well as in other regions of the central nervous system (CNS). It interacts with numerous receptor subtypes, which fall into two major classes: those coupled to G-proteins (metabotropic) and those connected directly to transmembrane channels (ionotropic). The toxic effects of elevated levels of glutamate are predominantly mediated by the overstimulation of ionotropic glutamate receptors (iGluRs) that are found on the retina and can be divided into *N*-methyl-D-aspartate (NMDA) (Figure 2A), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate (KA) receptors [25]. Among iGluRs, the role of the NMDA receptor (NMDAR) in cell death has been extensively studied; excessive doses of the glutamate analog NMDA lead to an overload of intracellular Ca^{2+} which in turn triggers formation of nitric oxide (NO) via neuronal NO synthase (nNOS), and finally induce apoptotic cell death of RGC [26–29]. Two other classes of iGluRs, which respond to the agonists AMPA and KA, respectively, can also mediate Ca^{2+} overload when overstimulated, but they are somewhat less permeable to this ion

Figure 2 The chemical structure of NMDA (A), memantine (B), and lomerizine (C).

than the NMDAR as the primary mediator of glutamate neurotoxicity [30]. Other studies, however, suggest that AMPA receptors also play an important role in the death of RGCs under a variety of damaging conditions [31–33]. Whatever the mechanism, elevated glutamate levels may play an important role in RGC death [34,35].

In mice, excitotoxin-treated retinal model by intravitreal injection of NMDA is widely used in many studies for investigating the mechanisms underlying neuronal cell death in the retina because of high sensitivity and stability, as well as good reproducibility [36]. In fact, RGC death and visual dysfunction occur after the NMDA injection [37–39]. Similarly, a single intravitreal injection of NMDA has been reported to damage the cells in GCL and the inner plexiform layer without affecting the other retinal layers in rats 7 days after the injection [40]. Hence, using this model is beneficial to investigate time-dependent alterations in the murine visual center in brain following RGC loss.

Time-Dependent Alterations in the Visual Center after NMDA Injection

In murine model, unilateral intravitreal injection of NMDA (40 nmol/eye) revealed that dramatic retinal

damage occurred within 7 days after NMDA injection, and subsequently neurons in the visual center in brain (LGN and SC) at 90 days after the NMDA injection [41–44]. In this model, intravitreal injection of NMDA induced neuronal damage that was detected, in turn, in RGC, the optic tract, in neurons contralateral LGN, and SC the maximal extent of the neuronal damage in these tissues (versus control, sham-treated mice) being about 70%, 60%, 18%, and 30.8%, respectively [43,44]. This suggests that the retinal damage induced by intravitreal injection of NMDA in mice lead to neuronal loss in the LGN and the SC connected to that particular retina. Such neuronal loss in visual center in brain may be mediated by NMDAR expressed in the LGN and the SC neurons [45], because the response of such neurons to afferent stimulation depends on the NMDAR [46–48]. Both a reduction in the visual stimuli and lack of anterograde-transported neurotrophins from RGC may be caused by RGC loss induced by intravitreal injection of NMDA. In this condition, following intracellular Ca^{2+} concentration elevations in the astrocytic processes glutamate may be rapidly released [49]. Such glutamate acts on NMDAR located presynaptically and/or postsynaptically [49,50]. As a result of this, overstimulation of NMDAR leads to an overload of intracellular Ca^{2+} . Such elevations in Ca^{2+} elicit various cytotoxic biochemical reactions including the activation of NO formation through NOS activation [30,51]. In fact, NO via NOS1 causes DNA fragmentation and activates poly ADP-ribose polymerase which in turn may take part in the pathologic processes of neuronal degeneration in the LGN of glaucomatous rats [52,53]. Thus, these studies indicate that neurotoxicity through the NMDA– Ca^{2+} –NOS/NO pathway may involve in the mechanism of neuronal death in visual center in brain following RGC loss.

Furthermore, a reduction in the visual stimuli transmitted from RGC and a depletion of neurotrophins accompanying by a dysfunction of anterograde transport from RGC may lead to “Wallerian degeneration,” in a process where the part of the axon separated from the neuron’s cell nucleus will degenerate [54–58]. During the period of neuronal degeneration, increased activated astrocytes and expressions of brain-derived neurotrophic factor (BDNF), a member of the family of neurotrophic factors, were observed in the LGN and SC. Furthermore, the presence of glial fibrillary acidic protein (GFAP)-positive astroglial cells exhibiting BDNF immunoreactivity in the LGN and the SC following NMDA injection [43,44]. BDNF is produced by activated astroglial cells. Thus, functional changes in astroglial cells are important for the neuronal repair process in the damaged CNS [59]. Reportedly, BDNF has a neuroprotective effect in various neuronal injury models [60–62], promotes neu-

roregeneration [63]. In fact, tropomyosin receptor kinase B (TrkB) expressed in the LGN neurons [64] and protects cortical neurons through the extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) pathways [65,66]. In contrast, BDNF binding to p75 neurotrophin receptor has been shown to stimulate cell death [67,68]. Therefore, it cannot rule out the possibility that neuronal cell death in the LGN and the SC after NMDA injection is triggered by the increased expressions of BDNF. Further studies will be needed to clarify the precise roles performed by BDNF in GFAP positive astroglial cells during the processes leading to the LGN and the SC damage following NMDA injection.

Protective Effects of Memantine in LGN and SC

Memantine, 1-amino-3,5-dimethyladamantane, an NMDA antagonist, has therapeutic potential against several CNS disorders (Figure 2B) [69]. In the United States and Europe, memantine has been used clinically for moderate to severe Alzheimer’s disease for a number of years [70]. In addition, it has been reported that memantine shows evidence of therapeutic benefits in several animal models of glaucoma, the leading cause of blindness [71–73].

In mice, memantine can protect against transsynaptic neuronal loss in the visual center in brain even if treatment is begun after RGC death has ended [43]. Briefly, NMDA (40 nmol/eye) was injected into the vitreous body of the left eye in mice (day 0). To evaluate the neuroprotective effect of memantine, mice were assigned to one of two memantine-treated groups (Figure 3A): receiving a daily administration of memantine at 30 mg/kg/day, *p.o.* either (1) from day 0 to day 90 (pretreated group) or (2) from day 7 to day 90 (posttreated group). Previous pre-clinical and clinical studies have indicated that the therapeutic plasma concentration of memantine is between 0.5 and 1 μM [74,75]. Therefore, to mimic clinically relevant conditions, an oral dose of 30 mg/kg was selected on the basis of a pilot study that showed that this produced a steady state plasma level of 1.14 μM in mice [76]. Mice receiving memantine from day 0 displayed significantly attenuated retinal damage at both 7 and 90 days after an intravitreal injection of NMDA (Figure 3B). Memantine has previously been shown to suppress spontaneous RGC degeneration in a normal tension glaucoma model involving mice deficient in the glutamate/aspartate transporter [77]. Taken together, these data suggest that systemic administration of memantine may act on NMDAR located in the retina and thereby protect RGC from glutamate excitotoxicity [78]. On the other hand, posttreated

Figure 3 Schedule for memantine administration (A). Summary of RGC, dLGN, and SC, neuron survival in vehicle-treated, pretreated, and posttreated groups compared with that in the sham-treated group (B). ** $P < 0.01$ versus vehicle (Student's *t*-test).

group did not show reversion of NMDA-induced retinal damage (Figure 3B). Because dramatic retinal damage has occurred by day 7 in this model [43,79], daily administration of memantine from day 7 may be too late to rescue RGC to a significant degree through a blockage of excessive glutamate-receptor activation. Next, the neuroprotective effects of memantine within the LGN and SC after retinal damage were examined. In this study, in addition to the pretreated group, the posttreated group also exhibited evidence of protective effects of memantine against neuronal degeneration in the brain (LGN and SC), although the latter group displayed no reversion of NMDA-induced retinal damage (Figure 3B). These findings suggest that memantine protected against secondary neuronal degeneration within the LGN and SC after retinal damage [80].

Such cerebroprotection after retinal damage may allow retention of a compensatory action within the brain that serves to prevent visual field loss after retinal damage. Possibly, the neuroprotective mechanisms by which memantine acts within the brain may involve it acting on the NMDAR subtype expressed by the relay neurons within the LGN [45,81], and it may rescue neurons by blocking the excessive glutamate receptor activation that contributes to the pathobiology of glaucomatous neural

degeneration [30]. In fact, in an experimental ocular hypertension model involving glaucomatous monkeys, memantine has been found to be effective against ON-fiber loss and neuron shrinkage within the LGN by preventing the transsynaptic degeneration involved in glutamate excitotoxicity [71,73].

In addition, memantine specifically upregulates the expressions of mRNA and protein of BDNF, and its specific Trk B, which are implicated in cell survival [82,83]. Thus, the neuroprotective effects of memantine may be related to enhanced expressions of endogenous neurotrophic factors within the visual center in brain in a mechanism distinct from antagonism of NMDAR [84]. Memantine may enhance these cell survival signals, and this may be the mechanism by which it protected neuronal cells in the visual center in brain.

Protective Effects of Lomerizine in LGN and SC

Lomerizine, 1-[bis(4-fluorophenyl)methyl]-4-(2,3,4-trimethoxybenzyl)-piperazine dihydrochloride, is a Ca^{2+} channel blocker, which was developed as a potential agent for the selective improvement of the ocular or cerebrovascular circulation with minimal adverse

Figure 4 Schedule for lomerizine administration (**A**). Summary of RGC, dLGN, and, SC, neuron survival in vehicle-treated, and lomerizine-treated groups compared with that in the sham-treated group (**B**). ** $P < 0.01$ versus vehicle (Student's *t*-test).

cardiovascular effects (Figure 2C) [85,86]. Lomerizine selectively relaxes smooth muscle cells by inhibiting Ca^{2+} influx, and it can reduce tone and increase blood flow in cerebral vessels [87]. In Japan, lomerizine is used clinically for the prophylaxis of migraine, an inherited or acquired clinical syndrome consisting of moderate-to-severe pulsatile headache [88]. Migraine has traditionally been considered to be a cause of changes in the caliber of blood vessels in the head.

In the development of several eye diseases, such as glaucoma, an insufficient blood flow leads to RGC death [89,90]. Following both clinical and experimental studies, there have been many reports of Ca^{2+} channel blockers being beneficial therapeutic agents for glaucoma [91–94]. In fact, several investigators have reported beneficial effects of Ca^{2+} channel blockers against RGC death [95–97], whereas systemic administration of Ca^{2+} channel blockers reportedly retards the progression of visual field loss, at least temporarily, in the subset of glaucoma patients with normal tension glaucoma [91,98,99]. Although the mechanisms underlying these effects are not fully understood, lomerizine has been reported to (1) increase blood flow in ocular neuronal tissue (the retina and optic nerve head [ONH]), without changing systemic blood pressure [100]; and (2) have beneficial effects against the RGC

death induced by ischemia/reperfusion injury in the rat retina [101]. These findings indicate that the protective effects of lomerizine against retinal ischemic injury may be partly due to improvements in the ocular circulation.

In mice, systemic administration of lomerizine (for 90 days, 30 mg/kg, p.o.) attenuated both the retinal and brain (dLGN and SC) damage in unilateral intravitreal injection of NMDA (40 nmol/eye) model (Figure 4) [102]. Within the visual center in the brain, neuronal loss was evident in the LGN (number of neurons and volume) and the SC (number of neurons) at 90 days after intravitreal NMDA injection (Figure 4B) [102]. Furthermore, daily administration of lomerizine (from 7 days to 90 days after NMDA injection) protected against transsynaptic neuronal degeneration in some regions of LGN, but not total [102].

Presumably, these neuronal cell-death mechanisms partly involve the NMDAR subtype expressed by the relay neurons within the LGN [27,28], and NMDAR activation and subsequent excessive Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCCs) induces neuronal death [45,81]. In previous *in vitro* studies, lomerizine (0.1–10 μM) reduced glutamate-induced neurotoxicity in rat hippocampal primary cell and retinal cell culture by blocking excess Ca^{2+} entry through VDCCs

[101,103–105]. *In vivo* studies, brain concentration ($C_{\max}$) of lomerizine (2 mg/kg, p.o.) in rats was about 0.43 μM [106]. Therefore, the brain concentration of lomerizine (30 mg/kg, p.o.) in mice is about 6.5 μM . The concentration of lomerizine exhibited protective effects on RGC death after ON injury and cerebral ischemic neuronal damage in the rat [94,107]. Taken together, these data suggest that lomerizine may block the influx of Ca^{2+} via VDCCs located both in RGC and within the brain and, by this means, may protect RGC and the neurons of the visual center in brain.

Anterograde and retrograde axonal transport processes are essential for the delivery of both extracellular components and intracellular components between the nerve terminals and the cell bodies of neurons. Disturbances of such transport can deprive the somata of various essential substances, eventually leading to the death of the neuron. It has been demonstrated that impairments of axonal transport can be induced by numerous pathological conditions, including transient ischemia, acute intraocular pressure elevation, and glaucoma [108–110]. A certain minimal level of Ca^{2+} is necessary to maintain both anterograde and retrograde transport [111,112], and an impairment of Ca^{2+} homeostasis is thought to play a pivotal role in triggering neuronal vulnerability in animal models [113]. Indeed, an elevation of the Ca^{2+} concentration inhibits both the anterograde and the retrograde transport of proteins [114]. Taken together, lomerizine may rescue RGCs and neurons within the visual center in brain by maintaining or improving axonal transport.

Conclusions

This review presented the time-dependent alterations in the visual center in brain following RGCs loss induced by NMDA injection. Using mice is easy to long-term experiment and further studies using transgenic mice may be an effective way of elucidating the mechanisms underlying neuronal degeneration and protection in visual center. Furthermore, long-term oral repeated administration of memantine and lomerizine partially protects neurons within LGN and SC against the secondary degeneration induced by retinal damage in mice. The protective mechanism may involve a suppression of NMDAR/ Ca^{2+} -mediated neurotoxicity and/or an increase in blood flow in the brain (via a blockage of Ca^{2+} channels in neuronal cells and endothelial cells). A neuroprotective strategy aimed at rescuing neurons within the visual center in the brain, in addition to therapies aimed at rescuing RGCs directly, may be of therapeutic benefit in preventing or reducing visual field loss after RGC damage.

Conflict of Interest

The authors have no conflict of interest.

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