Memantine Protects Against Secondary Neuronal Degeneration in Lateral Geniculate Nucleus and Superior Colliculus after Retinal Damage in Mice

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The purpose of this study, on mice, was to determine whether memantine, a glutamate-receptor antagonist of the *N*-methyl-D-aspartate (NMDA) subtype, protects against neuronal degeneration in the dorsal lateral geniculate nucleus (dLGN) and superior colliculus (SC) after the induction of retinal damage by intravitreal injection of NMDA.

NMDA (20 mM/2 μ l) 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: receiving a daily administration of memantine at 30 mg/kg/day, p.o. either from day 0 (administered at 1 h before NMDA injection) to day 90 (pretreated group) or from day 7 to day 90 (post-treated group).

The pretreated group exhibited significant suppression of the retinal damage induced by intravitreal injection of NMDA and significant prevention of transsynaptic neuronal degeneration in the dLGN and SC on the contralateral side. Although the mice of the post-treated group displayed no reversion of such retinal damage, they did exhibit protection against neuronal degeneration in the LGN and SC on the contralateral side. These data indicate that memantine can protect against transsynaptic neuronal degeneration in the murine brain (LGN and SC) even if treatment is begun after retinal ganglion cell (RGC) death has started.

Memantine protects against the secondary neuronal degeneration in brain regions in the visual pathway that occurs after retinal damage in mice.

Introduction

Memantine, 1-amino-3,5-dimethyladamantane, an *N*-methyl-D-aspartate (NMDA) antagonist, has therapeutic potential against several central nervous system (CNS) disorders [1]. In the United States and Europe, memantine has been used clinically for moderate to severe Alzheimer's disease [2] for a number of years. In addition, it has been reported that memantine shows evidence of therapeutic benefits in several animal models of glaucoma, the leading cause of blindness [3–5]. In glaucoma, the concentration of glutamate, the principal excitatory

neurotransmitter, has been reported to be increased in the vitreous body [6], although this finding was not confirmed by [7]. Be that as it may, it is known that the toxic effects of elevated glutamate levels are predominantly mediated by overstimulation of ionotropic receptors. Overstimulation of the class of these receptors that respond specifically to the glutamate analog NMDA has been shown to contribute to the death of retinal ganglion cells (RGC) [8,9].

The animal model employed in the present study (involving intravitreal injection of NMDA) shows decreasing cell number in the ganglion cell layer (GCL) [10].

Because of its high sensitivity, stability, and good reproducibility, this model has been widely used for investigating the mechanisms underlying neuronal cell death in the retina [11]. Our previous study on this model revealed that dramatic retinal damage occurred within 7 days after NMDA injection, and that subsequently neurons in the lateral geniculate nucleus (LGN), the major relay center between the eye and the visual cortex, were decreased in number at 90 days after the NMDA injection [12–14]. There is also recent evidence that the RGC death that occurs in glaucoma models leads to neuronal degeneration within the LGN [15,16]. Furthermore, while a loss of more than 50% of RGC has been reported to induce visual field loss, the initial loss of RGC in glaucoma patients does not lead to visual field loss [17]. These data suggest that the visual field loss induced by glaucoma may result not only from RGC loss, but also from neuronal degeneration within LGN [18].

Although it is generally believed that in rodents most axonal projections from the retina pass to SC, with only a small percentage of axons going to LGN, visual information entering the human eye is processed in the retina and transmitted via the optic nerve to LGN. This suggests that protection of neurons within LGN, as well as retinal treatment, could be effective for the prevention of eye diseases that cause blindness, such as glaucoma. Therefore, in this study we investigated these two pathways (retino-geniculate and retino-superior colliculus pathways) in mice. With the aim of determining whether memantine might protect against the secondary neuronal degeneration within the visual pathway (LGN and SC) that occurs after RGC damage.

Materials and Methods

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and they were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University.

Animal Subjects

Male adult C57BL/6J mice weighing 20–32 g (Clea Japan Inc., Fujimiya, Japan) were kept under lighting conditions of 12 h light and 12 h dark. They were anesthetized with 3.0% isoflurane (Merck, Osaka, Japan) and maintained with 1.5% isoflurane in 70% N₂O and 30% O₂ via an animal general anesthesia apparatus (Soft Lander; Sin-ei Industry Co. Ltd., Saitama, Japan). Retinal damage was induced (on day 0) by the intravitreal injection (2 μ l/eye) of NMDA (Sigma-Aldrich, St. Louis, MO) dis-

solved at 20 mM in 0.01 M phosphate-buffered saline (PBS) at pH 7.4. This was injected into the vitreous body of the left eye under the above anesthesia. One drop of levofloxacin ophthalmic solution (Santen Pharmaceuticals Co. Ltd., Osaka, Japan) was applied topically to the treated eye immediately after the intravitreal injection. Mice that received intravitreal NMDA were also treated either with memantine (30 mg/kg, p.o.) (Merz Pharmaceuticals GmbH., Frankfurt, Germany) or with the vehicle for memantine (distilled water, p.o.) by oral gavage for the periods detailed below (memantine or vehicle administration was started at 1 h before NMDA injection). For simplicity the latter (NMDA + vehicle-treated) group is referred to hereafter as the “vehicle-treated group.” Mice receiving an intravitreal injection of saline instead of NMDA were prepared for sham-treated group. Mice were euthanized either on day 7 (Fig. 3) or on day 90 (Figs. 4–8). For most experiments (i.e., those illustrated by Figs. 4–8), four groups of mice were evaluated and compared: (i) sham-treated group, (ii) vehicle-treated group [mice receiving an intravitreal injection of NMDA and also (from day 0 to day 90; p.o.) the vehicle for memantine], (iii) pretreated group (mice receiving an intravitreal injection of NMDA and also a daily administration of memantine at 30 mg/kg, p.o. from day 0 to day 90), and (iv) post-treated group (mice receiving an intravitreal injection of NMDA and also a daily administration of memantine at 30 mg/kg, p.o. from day 7 to day 90) (Fig. 1).

For the experiment illustrated by Figure 3, only three groups of mice were employed: (i) sham-treated group (mice receiving an intravitreal injection of saline instead of NMDA), (ii) vehicle-treated group [mice receiving an intravitreal injection of NMDA and also (from day 0 to day 7; p.o.) the vehicle for memantine], (iii) memantine-treated group (mice receiving an intravitreal injection of NMDA and also a daily administration of memantine at 30 mg/kg, p.o. from day 0 to day 7).

Tissue Processing

At the end of their assigned survival period, mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) (Nembutal; Dainippon, Osaka, Japan), then perfused with 2% (w/v) paraformaldehyde solution in 0.01 M PBS. The brains were removed after 15 min perfusion at 4°C, immersed in the same fixative solution for 24 h, soaked in 25% (w/v) sucrose for 1 day, then frozen in embedding compound (Tissue-Tek; Sakura Finetechnical Co. Ltd., Tokyo, Japan). Serial coronal sections through the levels of the LGN (bregma $-2.10 \sim -2.40$ mm) and superior colliculus (SC) (bregma $-3.20 \sim -3.50$ mm) were cut at 50- μ m thickness, then stained with cresyl violet. Each eye was enucleated at the time of brain

Figure 1. Schedule for memantine administration in the experiments illustrated by Figs. 4–8. Mice were euthanized at 90 days after NMDA intravitreal injection. Four groups of mice were evaluated and compared: (i) sham-treated group (mice received an intravitreal injection of saline instead of NMDA), (ii) vehicle-treated group (mice receiving an intravitreal injection of NMDA and also a daily oral administration of the vehicle for

memantine from day 0 to day 90), (iii) pretreated group (mice intravitreally injected with NMDA who also received a daily administration of memantine at 30 mg/kg, p.o. from day 0 to day 90), and (iv) post-treated group (mice intravitreally injected with NMDA who also received a daily administration of memantine at 30 mg/kg, p.o. from day 7 to day 90).

removal, and 4% paraformaldehyde solution was injected into the vitreous body. The eye was then kept immersed for at least 24 h in the same fixative solution at 4°C. Six paraffin-embedded sections (thickness, 5 μ m) cut through the optic disc of each eye were prepared in a standard manner, then stained with hematoxylin and eosin.

Histological Analysis of Mouse Retina

Retinal damage was evaluated as previously described [11], three of the six sections stained with hematoxylin and eosin from each eye being used for the morphometric analysis. Light-microscope photographs were taken using a digital camera (Coolpix 4500, Nikon) and the cell counts in the GCL and the thickness of the inner plexiform layer (IPL) at a distance between 375 and 625 μ m from the optic disc were measured on the images in a masked fashion by a single observer (S.N.). Data from three sections (selected randomly from the six sections) were averaged for each eye, and the values obtained were used to evaluate the GCL cell count and the IPL thickness.

Neuron Numbers in dLGN and SC

To assess the protective effects of memantine against neuronal loss in dorsal lateral geniculate nucleus (dLGN) and SC, sections stained with cresyl violet were used for neu-

ronal cell counting: four sections for dLGN (L1: bregma -2.10 mm, L2: -2.20 mm, L3: -2.30 mm, and L4: -2.40 mm) and four for SC (S1: bregma -3.20 mm, S2: -3.30 mm, S3: -3.40 mm, and S4: -3.50 mm), respectively, in each mouse (Fig. 2A). The area of each field used for this cell counting was 0.047 mm² of dLGN (Fig. 2B and D) and 0.047 mm² of the superficial layer of SC (Fig. 2C and E). Cell counts were carried out under a microscope at 400 $\times$ magnification in a masked fashion by a single observer (S.N.). Care was taken to count only neurons with clearly visible nuclei and cytoplasm.

Volume of dLGN

Measurements of the surface area of dLGN were made in four equally spaced sections (interval, 100 μ m) from section L1 to section L4 (i.e., the sections counting the bulk of dLGN). These area measurements were carried out under a microscope at 100 $\times$ magnification in a masked fashion by a single observer (S.N.), recorded as images using a digital camera (Nikon Coolpix 4500), and then quantitated using Image J. Cavalieri's estimator of volume was used to calculate the volume (V_T) of dLGN (bregma $-1.70 \sim -2.90$ mm) using the following formula:

$$V_T = \sum a_i \times s_f$$

where a_i is the cross-sectional area of the dLGN in the i th profile and s_f is the mean distance between sections

Figure 2. Illustrations showing (A) serial coronal sections through levels of LGN (bregma $-2.10 \sim -2.40$ mm) and SC (bregma $-3.20 \sim -3.50$ mm) in mice. Coronal sections through levels of LGN (bregma -2.30 mm) (B) and SC (bregma -3.40 mm) (C) in mice [boxed areas are shown diagrammatically in (D) and (E), respectively].

(section thickness multiplied by the inverse of the periodicity of sections in the series) [19].

Statistical Analysis

Data are presented as means $\pm$ S.E.M. Statistical comparisons (one-way ANOVA followed by a Student's *t*-test) were made using STAT VIEW version 5.0 (SAS Institute, Inc., Cary, NC, USA). A value of $P < 0.05$ was considered to indicate statistical significance.

Results

Protective Effect of Memantine Against Retinal Damage at 7 Days after NMDA Injection

Intravitreal injection of NMDA at 40 nmol/eye decreased both the cell count in GCL (32.7 ± 3.1 cells/mm, $n =$

8) and the thickness of IPL ($13.2 \pm 0.9 \mu\text{m}$, $n = 8$) in the retina (Fig. 3B, D, and E) versus those in the sham-treated retina (135.0 ± 9.2 cells/mm for GCL and $36.8 \pm 2.3 \mu\text{m}$ for IPL, $n = 8$; $P < 0.05$ in each case) (Fig. 3A, D, and E). The memantine-treated group (mice receiving an intravitreal injection of NMDA who also received a daily administration of memantine at 30 mg/kg, p.o. from day 0 to day 7) displayed significant suppression of both of these decreases (54.5 ± 6.3 cells/mm for GCL and $19.8 \pm 1.2 \mu\text{m}$ for IPL, $n = 8$) (Fig. 3C, D, and E).

Protective Effect of Memantine Against Retinal Damage at 90 Days after NMDA Injection

The pretreated group displayed a significantly greater cell number in GCL (37.7 ± 2.3 cells/mm, $n = 8$) than the vehicle-treated group (28.1 ± 1.2 cells/mm, $n = 7$)

Figure 3. Protective effect of memantine against retinal damage, as assessed at 7 days after NMDA intravitreal injection. Representative photographs of retinas from sham-treated (A), vehicle-treated (B), and memantine (30 mg/kg/day p.o.)-treated (C) groups. Vertical bars show

thickness of IPL. Horizontal bar represents 20 μm. Arrows indicate live cells within GCL. Cells number in GCL (D) and thickness of IPL (E) were measured. Each value represents the mean ± S.E.M. for 8 eyes. * $P < 0.05$, ** $P < 0.01$ versus vehicle (Student's *t*-test).

(Fig. 4B, C, and E). On the other hand, in the thickness of IPL there was no significant difference between the vehicle-treated group ($17.1 \pm 0.9 \mu\text{m}$, $n = 7$) and the pretreated group ($18.5 \pm 0.7 \mu\text{m}$, $n = 12$), although the latter group tended to show a suppressed decrease in IPL thickness versus the vehicle-treated group (Fig. 4B, C, and F). The post-treated group showed no significant difference from the vehicle-treated group in either of the NMDA-induced decreases (25.3 ± 1.7 cells/mm for GCL and $14.7 \pm 0.4 \mu\text{m}$ for IPL, $n = 12$) (Fig. 4B, D–F).

Neuron Numbers in dLGN

In the contralateral dLGN, the numbers of neurons in the vehicle-treated group were 60.5 ± 2.5 ($n = 8$), 61.5 ± 3.0 ($n = 8$), 62.8 ± 4.8 ($n = 8$), and 65.5 ± 1.7 ($n = 8$) in sections L1, L2, L3, and L4, respectively. Each of these counts was smaller than the corresponding one for contralateral dLGN neurons in the sham-treated group (94.3 ± 3.4 , 98.9 ± 5.0 , 103.0 ± 3.5 , and 93.1 ± 3.9 ,

respectively, each $n = 8$; $P < 0.05$ in each case) (Fig. 5A, B, and E). The pretreated group (78.8 ± 4.3 for L1, 80.6 ± 4.0 for L2, 83.3 ± 4.4 for L3, and 74.5 ± 1.8 for L4, each $n = 8$) and the post-treated group (70.6 ± 2.9 for L1, 76.3 ± 3.8 for L2, and 78.1 ± 3.7 for L3, each $n = 8$) exhibited significantly greater contralateral dLGN neuron numbers than the vehicle-treated group (Fig. 5B–E). In contrast, in the ipsilateral dLGN there were no significant differences in numbers of neurons among the four groups (sham-, vehicle-, pre-, and post-treated mice) (Fig. 5F).

Volume of dLGN

The volume of the contralateral dLGN was smaller in the vehicle-treated group ($0.124 \pm 0.003 \text{ mm}^3$, $n = 6$) than in the sham-treated group ($0.153 \pm 0.007 \text{ mm}^3$, $n = 6$; $P < 0.05$) (Fig. 6A, B, and E). The pretreated group ($0.137 \pm 0.002 \text{ mm}^3$, $n = 6$) and the post-treated group ($0.136 \pm 0.002 \text{ mm}^3$, $n = 8$) exhibited significantly greater contralateral dLGN volumes than the vehicle-treated group (Fig. 6B–E). In contrast, in the ipsilateral

Figure 4. Protective effect of memantine against retinal damage (assessed on day 90). Representative photographs of retinas from sham-treated (A), vehicle-treated (B), pretreated (C), and post-treated (D) groups. Vertical bars show thickness of IPL. Horizontal bar represents

20 μm. Arrows indicate live cells within GCL. Cells number in GCL (E) and thickness of IPL (F) were measured. Each value represents the mean ± S.E.M. for 7–12 eyes. ** $P < 0.01$ versus vehicle (Student's *t*-test).

dLGN there were no significant differences in volume among the four groups (sham-, vehicle-, pre-, and post-treated mice) (Fig. 6E).

Neuron Numbers in SC

In the contralateral SC, the numbers of neurons in the vehicle-treated group were 48.8 ± 1.8 ($n = 8$), 50.1 ± 2.9 ($n = 8$), 51.8 ± 2.7 ($n = 8$), and 50.4 ± 3.2 ($n = 8$) in sections S1, S2, S3, and S4, respectively. Each of these counts was smaller than the corresponding one for contralateral SC neurons in the sham-treated group (85.3 ± 2.3 , 85.4 ± 2.5 , 82.5 ± 3.7 , and 84.6 ± 1.5 , respectively, each $n = 8$; $P < 0.05$ in each case) (Fig. 7A, B, and E). The pretreated group (64.9 ± 2.4 for S1, 63.5 ± 2.7 for S2, 63.4 ± 3.5 for S3, and 64.6 ± 4.3 for S4, each $n = 8$) and the post-treated group (63.6 ± 1.8 for S1, 61.8 ± 3.1 for S2, 60.1 ± 2.7 for S3, and 63.9 ± 4.2 for S4, each n

$= 8$) exhibited significantly greater contralateral SC neuron numbers than the vehicle-treated group (Fig. 7B–E). In contrast, in the ipsilateral SC there were no significant differences in the numbers of neurons among the four groups (sham-, vehicle-, pre-, and post-treated mice) (Fig. 7F).

Discussion

The purpose of this study was to determine whether memantine exerts protective effects against secondary neuronal degeneration in the dLGN and SC following retinal damage in mice. In this study, we introduce the first murine model for evaluation of the effects of drugs on the changes in brain areas that occur following intravitreal injection of NMDA.

First, we had to choose an appropriate concentration of memantine for the evaluation of its neuroprotective

Figure 5. Protective effect of memantine against neuronal loss in dLGN (assessed on day 90). Representative photographs from sham-treated group (A), vehicle-treated group (B), pretreated group (C), and post-treated group (D). Neuronal cell number within dLGN was measured on the contralateral (E) and ipsilateral (F) sides. Coronal sections through level

of dLGN (L1: bregma -2.10 mm, L2: -2.20 mm, L3: -2.30 mm, and L4: -2.40 mm). In each field, the tissue area of dLGN examined was 0.047 mm². Horizontal bar represents 20 μ m. Arrows (in A–D) indicate neuronal cells. Each value represents the mean $\pm$ S.E.M. for eight brains. * $P < 0.05$, ** $P < 0.01$ versus vehicle (Student's *t*-test).

effects within the murine brain. Previous preclinical and clinical studies have indicated that the therapeutic plasma concentration of memantine is between 0.5 and 1 μ M [20, 21]. 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 μ M in C57BL/6J mice [22].

In this study, we found that mice receiving memantine from day 0 displayed significantly attenuated retinal damage at both 7 and 90 days after an intravitreal injection of NMDA (Figs. 3 and 4). An effect of memantine has been demonstrated on similar the retinal injury induced by glutamate toxicity in rats [23]. Memantine has previously been shown to suppress spontaneous RGC degeneration in a normal tension glaucoma model involving mice deficient in the glutamate/aspartate transporter (GLAST) [24]. Taken together, these data suggest that systemic administration of memantine may act on NMDA receptors located in the retina and thereby protect RGC from glutamate excitotoxicity [25]. On the other hand, our post-treated group did not show reversion of NMDA-induced retinal damage (Fig. 4). Since dramatic retinal damage has occurred by day 7 in the present model [10, 14;

present study], 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, we examined the neuroprotective effects of memantine within the LGN and SC after retinal damage. Neuronal loss was evident in the contralateral dLGN (number of neurons and volume) and in the contralateral SC (number of neurons) on day 90 after intravitreal NMDA injection (Figs. 5–7). Such neuronal loss may result from a reduction in the visual stimuli transmitted from RGCs and from a depletion of neurotrophins associated with a dysfunction of anterograde transport from RGCs [26]. These affects may lead to “Wallerian degeneration,” a process in which the part of the axon separated from the neuron’s cell nucleus will degenerate [27]. In this study, our findings indicate that in addition to the pretreated group, the post-treated group also exhibited evidence of protective effects of memantine against neuronal degeneration in the brain (LGN and SC), although the latter group displayed no significant difference in GCL cell number versus the vehicle-treated group (Figs. 4–8). These findings suggest that memantine protected against

Figure 6. Protective effect of memantine against shrinkage of dLGN volume (assessed on day 90). Representative photographs from sham-treated group (A), vehicle-treated group (B), pretreated group (C), and post-treated group (D). The contralateral and ipsilateral dLGN volumes were measured (E). Horizontal bar represents 100 μ m. Each value represents the mean $\pm$ S.E.M. for six to eight brains. * $P < 0.05$, ** $P < 0.01$ versus vehicle (Student's *t*-test).

secondary neuronal degeneration within the LGN and SC after retinal damage. 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 NMDA-receptor subtype expressed by the relay neurons within the LGN [28, 29], and it may rescue neurons by blocking the excessive glutamate-receptor activation that contributes to the pathobiology of glaucomatous neural degeneration [8]. In fact, in an experimental ocular hypertension model involving glaucomatous monkeys, memantine has been found to be effective against optic nerve-fiber loss and neuron-shrinkage within the LGN by preventing the transsynaptic degeneration involved in glutamate excitotoxicity [3,5].

In addition, in a mechanism distinct from NMDA-receptor antagonism, memantine specifically upregulate the mRNA and protein expressions of brain-derived neurotrophic factor (BDNF) and its specific tropomyosin receptor kinase B (Trk B), which are implicated in cell sur-

vival [30, 31]. Thus, the neuroprotective effects of memantine may be related to enhanced expressions of endogenous growth factors within the LGN [32]. In fact, Trk B is expressed in LGN neurons [33] and it protects cortical neurons through the extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) pathways [34, 35]. Memantine may enhance these cell-survival signals, and this may be the mechanism by which it protected neuronal cells in the LGN and SC in the present study.

Using mice is easy to long-term administration of memantine and further studies using transgenic mice may be an effective way of elucidating the mechanisms underlying neuronal degeneration and protection in brain after retinal damage. Therefore, mice were used in the present study. Since the protective mechanism by which memantine acts in the murine LGN and SC was not determined in this study, further studies will be needed to clarify the precise mechanism. In the meantime, we propose that in addition to therapies aimed at rescuing RGCs directly, a neuroprotective strategy aimed at rescuing neurons within the brain (LGN and SC) after RGC damage

Figure 7. Protective effect of memantine against neuronal loss within SC (assessed on day 90). Representative photographs from sham-treated group (A), vehicle-treated group (B), pretreated group (C), and post-treated group (D). Neuronal cell number within SC was measured on the contralateral (E) and ipsilateral (F) sides. Coronal sections through level of

SC (S1: bregma -3.20 mm, S2: -3.30 mm, S3: -3.40 mm, and S4: -3.50 mm). In each field, the tissue area of SC examined was 0.047 mm^2 . Horizontal bar represents $20 \mu\text{m}$. Arrows (in A–D) indicate neuronal cells. Each value represents the mean $\pm$ S.E.M. for eight brains. $**P < 0.01$ versus vehicle (Student's *t*-test).

Figure 8. Summary of derived from data shown in Figs. 4, 5, and 7. For RGC, dLGN, and SC, neuron survival in vehicle-treated, pre-treated, and post-treated groups was compared with that in the sham-treated (control) group. Data from four sections (L1–L4 and S1–S4, respectively) were averaged for each brain, and these were used to evaluate survival neurons within the LGN and SC. $**P < 0.01$ versus vehicle (Student's *t*-test).

may be of therapeutic benefit in preventing or reducing visual field loss, possibly by permitting a compensatory action to remain in operation within the brain.

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Conflict of Interest

The authors have no conflict of interest.

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