

# Potential Neuroprotective Effects of an LSD1 Inhibitor in Retinal Ganglion Cells via p38 MAPK Activity

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**PURPOSE.** The epigenetic mechanisms associated with ocular neurodegenerative diseases remain unclear. The present study aimed to determine the role of lysine-specific demethylase 1 (LSD1), which represses transcription by removing the methyl group from methylated lysine 4 of histone H3, in retinal ganglion cell (RGC) survival, and to investigate the details of the neuroprotective mechanism of tranilcypromine, a major LSD1 inhibitor.

**METHODS.** The authors evaluated whether tranilcypromine contributes to neuronal survival following stress-induced damage using primary cultured rat RGCs and in vivo N-methyl-D-aspartate (NMDA)-induced excitotoxicity. Additionally, the molecules associated with tranilcypromine treatment were assessed by microarray and immunoblot analysis.

**RESULTS.** Tranilcypromine significantly suppressed neuronal cell death following glutamate neurotoxicity and oxidative stress. Microarray and immunoblot analyses revealed that p38 mitogen-activated protein kinase (MAPK) $\gamma$  was a key molecule involved in the neuroprotective mechanisms induced by tranilcypromine because the significant suppression of p38 MAPK $\gamma$  by glutamate was reversed by tranilcypromine. Moreover, although pharmacologic inhibition of the phosphorylation of the total p38 MAPKs interfered with neuroprotective effects of tranilcypromine, the specific inhibition of p38 MAPK $\alpha$  and p38 MAPK $\beta$  did not influence RGC survival. This suggests that the non-p38 MAPK $\alpha/\beta$  isoforms have important roles in neuronal survival by tranilcypromine. Additionally, the intravitreal administration of tranilcypromine significantly saved RGC numbers in an in vivo glaucoma model employing NMDA-induced excitotoxicity.

**CONCLUSIONS.** These findings indicate that tranilcypromine-induced transcriptional and epigenetic regulation modulated RGC survival via the promotion of p38 MAPK $\gamma$  activity. Therefore, pharmacologic treatments that suppress LSD1 activity may be a novel therapeutic strategy that can be used to treat neurodegenerative diseases.

Keywords: tranilcypromine, neuroprotection, survival, glaucoma, epigenetic drug

Glaucoma is a major optic neuropathy characterized by the significant loss of retinal ganglion cells (RGCs)<sup>1</sup> and is a leading cause of blindness around the world.<sup>2-4</sup> Retinal ganglion cell death directly causes visual field deficits, and the primary risk factor associated with progressive damage to the visual field is elevated intraocular pressure (IOP).<sup>5-7</sup> However, RGCs may suffer damage even in normal-tension glaucoma patients in which IOP is within the normal range (10–21 mm Hg).<sup>8</sup>

Recent epidemiologic studies have demonstrated that normal-tension glaucoma is more common than high-tension glaucoma among primary open-angle glaucoma patients, particularly in Asian populations.<sup>9-12</sup> The causes of normal-tension glaucoma have yet to be completely elucidated; however, a variety of factors, including reduced blood flow in the optic nerve,<sup>13</sup> genetic variables,<sup>14,15</sup> and an enlarged gap between cerebrospinal fluid pressure and IOP,<sup>16</sup> may be involved in the pathophysiology of this disease. Evidence-based treatments for patients with glaucoma, including normal-

tension patients, target reductions in IOP via ophthalmic solutions, laser therapy, or surgery.<sup>17</sup> However, the therapeutic efficacies of these treatments are often limited and may be clinically insufficient even though they lower IOP. Thus, novel treatment strategies for glaucoma, such as the neuroprotection of RGCs, are urgently required.

Except for alterations in the original DNA sequence via DNA methylation, the modulation of histone, or noncoding RNA, epigenetic changes influence gene expression and function.<sup>18</sup> It is well known that histone is specifically modified by a variety of mechanisms, including the acetylation of histone N-terminal tails, methylation, phosphorylation, ubiquitination, and adenosine diphosphate (ADP) ribosylation.<sup>19</sup> Histone modification involves switches that alter chromatin structure or form binding platforms that allow for downstream effector proteins to induce transcriptional activation or repression.<sup>20</sup>

Several recent reports have suggested that multiple epigenetic factors play important roles in the survival and pathogenesis of RGCs in models of glaucoma. For example,



damage to RGCs via crushing of the optic nerve leads to changes in the activity of histone deacetylases (HDACs), which are a key factor in the deacetylation of histone.<sup>21</sup> However, these authors also reported that the inhibition of retinal HDAC activity by trichostatin A preserved the expression of representative RGC-specific genes and attenuated cell loss following optic nerve damage. Valproic acid, which is another popular HDAC inhibitor, also exerts neuroprotective effects and induces axonal regeneration following optic nerve crushing via regulation of the transcription factor cyclic adenosine monophosphate (cAMP) response element binding protein (CREB).<sup>22</sup> Additionally, valproic acid prevents RGC death in N-methyl-D-aspartate (NMDA)-induced glaucoma model and GLAST-knockout glaucoma model mice, via stimulation of neuronal TrkB receptor signaling.<sup>23,24</sup> Furthermore, the genetic ablation of *Hdac3* in RGCs results in a significant amelioration of nuclear atrophy as well as significant suppression of cell death during the acute phase after optic nerve injury.<sup>21,25</sup> These findings imply that the transcriptional downregulation and initiation of cell death mechanisms in neurodegenerative diseases, including glaucoma, may be linked with epigenetic processes.

Recent investigations of the relationship between histone acetylation/deacetylation and neuronal survival have revealed that HDAC inhibitors exert neuroprotective effects against neurodegeneration.<sup>21–25</sup> However, the role that histone methylation plays in ocular neurodegenerative diseases, including glaucoma, remains unclear. Thus, the present study aimed to investigate the relationship between histone methylation and the function of lysine-specific demethylase 1 (LSD1), which demethylates histone H3 at lysine 4 (H3K4) or H3K9 via a flavin-dependent oxidation reaction and induces transcriptional repression in general<sup>26,27</sup> during neuronal survival and neurodegeneration in glaucoma. The present findings demonstrated the importance of LSD1 activity in RGC survival as well as the neuroprotective effects of tranlycypromine, which is a major LSD1 inhibitor. Furthermore, this study identified and clarified possible molecular targets involved in the tranlycypromine-mediated protection of RGCs. Taken together, these findings suggest that LSD1 is a key regulator of neuronal cell survival and a potential molecular target for the treatment of neurodegenerative diseases.

## METHODS

### Reagents and Animals

The present study utilized tranlycypromine, which is also known as 2-phenylcyclopropylamine hydrochloride (Sigma-Aldrich Corp., St Louis, MO, USA), and S2101, which is also known as LSD1 Inhibitor II (no. 489477; Merck Millipore, Billerica, MA, USA). The isolation of RGCs was performed using 2-day-old Sprague-Dawley (SD) rats (Kyudo Co., Ltd., Kumamoto, Japan), and 8-week-old male Slc:SD rats (Japan SLC, Inc., Shizuoka, Japan) were used for optic nerve crush experiments. All experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all experimental procedures were approved by the Animal Care Committee of Kumamoto University.

### Primary Cultures of RGCs

Primary cultured RGCs were purified using a two-step immunopanning method, as previously described,<sup>28</sup> with minor modifications.<sup>29–31</sup> Briefly, the retinas were digested with papain (16.5 units/mL) and triturated with rabbit anti-rat

macrophage antiserum (Accurate Chemical, Westbury, NY, USA). The cell suspension was first incubated on a panning plate (150-mm petri dish; BD Falcon, Franklin Lakes, NJ, USA) coated with goat anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA, USA). The nonadherent cells were incubated on a second panning plate (100-mm petri dish; Thermo Fisher Scientific) coated with goat anti-mouse IgM $\mu$  (Thermo Fisher Scientific) and mouse anti-Thy1.1 antibodies secreted from T11D7e2 cells (American Type Culture Collection, Manassas, VA, USA). Next, the plate was washed with phosphate-buffered saline (PBS), and the adherent RGCs were released by treatment with 0.125% trypsin (Sigma-Aldrich Corp.).

The isolated RGCs were suspended in a medium containing 1 mM glutamine, 5  $\mu$ g/mL insulin, 60  $\mu$ g/mL *N*-acetylcysteine, 62 ng/mL progesterone, 16  $\mu$ g/mL putrescine, 40 ng/mL sodium selenite, 0.1 mg/mL bovine serum albumin (BSA), 40 ng/mL triiodothyronine, 0.1 mg/mL transferrin, 1 mM sodium pyruvate, 2% B27 supplement (no. 17504-044; Invitrogen, Carlsbad, CA, USA), 10  $\mu$ M forskolin, 50 ng/mL brain-derived neurotrophic factor (BDNF; PeproTech, Rocky Hill, NJ, USA), 50 ng/mL ciliary neurotrophic factor (CNTF; PeproTech), and 50 ng/mL basic fibroblast growth factor (bFGF; PeproTech) in Neurobasal medium (Thermo Fisher Scientific). Finally, 96-well culture plates were coated with poly-D-lysine (Sigma-Aldrich Corp.) and laminin (Sigma-Aldrich Corp.), and the RGCs were plated at a density of 5000 cells/well and cultured for at least 10 days prior to the experimental procedures.

### Immunoblotting

The immunoblotting procedures were performed as previously described.<sup>29–31</sup> Briefly, the proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes, and probed with primary and peroxidase-conjugated secondary antibodies. The immunoreactive proteins were visualized with SuperSignal West Pico, Dura, or Femto (Thermo Fisher Scientific) using the following primary antibodies: mouse anti- $\beta$ -actin (no. A5441; Sigma-Aldrich Corp.), rabbit anti-LSD1 (C69G12, no. 2184; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-phospho-p38MAPK ([Thr180/Tyr182][D3F9], no. 4511; Cell Signaling Technology), rabbit anti-p38MAPK (no. 9212; Cell Signaling Technology), rabbit anti-p38 MAPK $\gamma$  (no. 2307; Cell Signaling Technology), rabbit anti-phospho-Akt (Ser473, no. 9271; Cell Signaling Technology), rabbit anti-Akt (no. 9272; Cell Signaling Technology), and rabbit anti-cleaved caspase 3 (Asp175, no. 9661; Cell Signaling Technology).

### Apoptosis of RGCs

The evaluation of RGC apoptosis was performed as previously described.<sup>31</sup> Briefly, the primary cultured RGCs were washed twice (15-minute incubation at 37°C) with Hanks' balanced salt solution (HBSS; Invitrogen) containing 2.4 mM CaCl<sub>2</sub> and 20 mM HEPES without magnesium; the magnesium was omitted from the washing solution to avoid blocking the NMDA receptor.<sup>32</sup> Subsequently, the RGCs were incubated in 300  $\mu$ M glutamate and 10  $\mu$ M glycine, which is a coactivator of the NMDA receptor, in HBSS containing 2.4 mM CaCl<sub>2</sub> and 20 mM HEPES without magnesium for 2 hours at 37°C. After treatment with glutamate, the RGCs were cultured in the same medium without any neurotrophic factors such as forskolin, BDNF, CNTF, or bFGF for 22 hours at 37°C. Oxidative stress-induced cell death was achieved by the addition of 50  $\mu$ M hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) with trophic additives containing B27 supplement AO depleted of antioxidants (Invitrogen) for 30 minutes and then incubating the cells for 24 hours. Tranlycypromine

promine (100  $\mu$ M) and wortmannin (100 nM, no. 681675; Merck Millipore) were simultaneously administered with the glutamate or H<sub>2</sub>O<sub>2</sub>, while S2101, BIRB796 (10  $\mu$ M, no. S1574; Selleck Chemicals, Houston, TX, USA), and SB203580 (10  $\mu$ M, no. 199-16551; Wako, Osaka, Japan) were added 24 hours before the induction of apoptosis. Subsequently, the treated RGCs were incubated for 24 hours prior to the detection of apoptosis.

Apoptosis was detected by incubating the RGCs with 1.0  $\mu$ g/mL Hoechst 33342 (Dojindo, Kumamoto, Japan) for 15 minutes. The fluorescent images were observed using an IX71 fluorescence microscope (Olympus, Tokyo, Japan), and at least six images/well were obtained from the 96-well plates. As previously described,<sup>30,31</sup> the fragmented or shrunken nuclei stained with Hoechst dye were counted as apoptotic neurons and the round/smooth nuclei were considered to be healthy neurons. For each condition, more than 200 neurons were counted using MetaMorph imaging software (Molecular Devices, Sunnyvale, CA, USA) to minimize measurement biases.

### Lsd1 RNA Silencing

Either nonsilencing small interfering RNA (siRNA; 1  $\mu$ M Accell nontargeting siRNA no. 1; Thermo Fisher Scientific) or *Lsd1*-specific siRNA (E-105863-00-0010; Accell Rat *Kdm1a* [Gene ID: 500569] siRNA SMARTpool; Thermo Fisher Scientific) was added to the culture medium according to the manufacturer's instructions and then incubated with the RGCs for 6 days. The knockdown effect of the *Lsd1*-specific siRNA was confirmed using an immunoblotting technique.

### Gene Expression Microarrays

The total RNA was amplified, labeled, and hybridized using a Rat GE 4x44K v3 Microarray Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. All hybridized microarrays were scanned with an Agilent scanner, and the signals of all the probes were calculated using Feature Extraction Software (Agilent Technologies). Employing procedures recommended by Agilent, the raw signal intensities and flags for each probe were calculated from the hybridization intensities and spot information. The present study utilized probes that registered P flags in at least one sample and then calculated intensity based on Z-scores<sup>33</sup> and ratios (non-log-scaled fold change) from the normalized signal intensities of each probe in order to compare the control and experimental samples to identify up- and downregulated genes. Upregulated genes were defined based on Z-scores  $\geq 2.0$  and ratios  $\geq 1.5$ -fold, while downregulated genes were defined based on Z-scores  $\leq -2.0$  and ratios  $\leq 0.66$ . The results were generated from comparisons of control versus glutamate stimulated and of glutamate stimulated versus glutamate stimulated plus tranlycypromine. To determine the significant enrichment of pathways, tools and data provided by the Database for Annotation, Visualization, and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov/home.jsp>; in the public domain)<sup>34</sup> were used, and a Kyoto Encyclopedia of Genes and Genomes (KEGG)<sup>35</sup> pathway annotation analysis was carried out.

### NMDA-Induced Retinal Damage In Vivo

Intravitreal injections of NMDA (Sigma-Aldrich Corp.) were performed in the same fashion as previously described.<sup>36</sup> Briefly, the rats were anesthetized with an intraperitoneal injection of a 1:1 mixture of xylazine hydrochloride (4 mg/kg; Bayer HealthCare, Leverkusen, Germany) and ketamine hydro-

chloride (10 mg/kg; Daiichi Sankyo Propharma Co., Ltd., Tokyo, Japan). Then, the pupil was dilated with phenylephrine hydrochloride and tropicamide eye drops (Santen Pharmaceutical Co., Ltd., Osaka, Japan), and 20 nmol NMDA with or without tranlycypromine was injected into the vitreous cavity. To assess the inhibitory effect of mitogen-activated protein kinase (MAPK), 100 nmol BIRB796 was intravitreally injected at the same time of NMDA injection. The injections were performed under a microscope using a 33-gauge needle connected to a microsyringe (Ito Corporation, Shizuoka, Japan); the needle was inserted approximately 1.0 mm behind the corneal limbus. Next, either PBS (vehicle control) or 500 mM tranlycypromine (1000 nmol) mixed with 10 mM NMDA (20 nmol) in a total volume of 2.0  $\mu$ L was injected into the vitreous cavity.

### In Vivo Morphometric Analyses

A morphometric analysis to assess the protective effects of tranlycypromine was conducted as previously described.<sup>36</sup> Briefly, 7 days after the NMDA injection, the rats were euthanized by suffocation with carbon dioxide (CO<sub>2</sub>); the eyes were enucleated and fixed in 4% paraformaldehyde overnight at 4°C, and then dehydrated and embedded in paraffin ( $n = 5-6$  in each condition). Transverse sections (4  $\mu$ m thick) were prepared from the optic discs, stained with hematoxylin and eosin, and then subjected to the morphometric analysis. The thickness of the inner plexiform layer (IPL) at 1.0 to 1.5 mm from the optic disc was measured, and data from a minimum of three sections for each eye were averaged using a microscope (BX51; Olympus).

### Immunohistochemistry

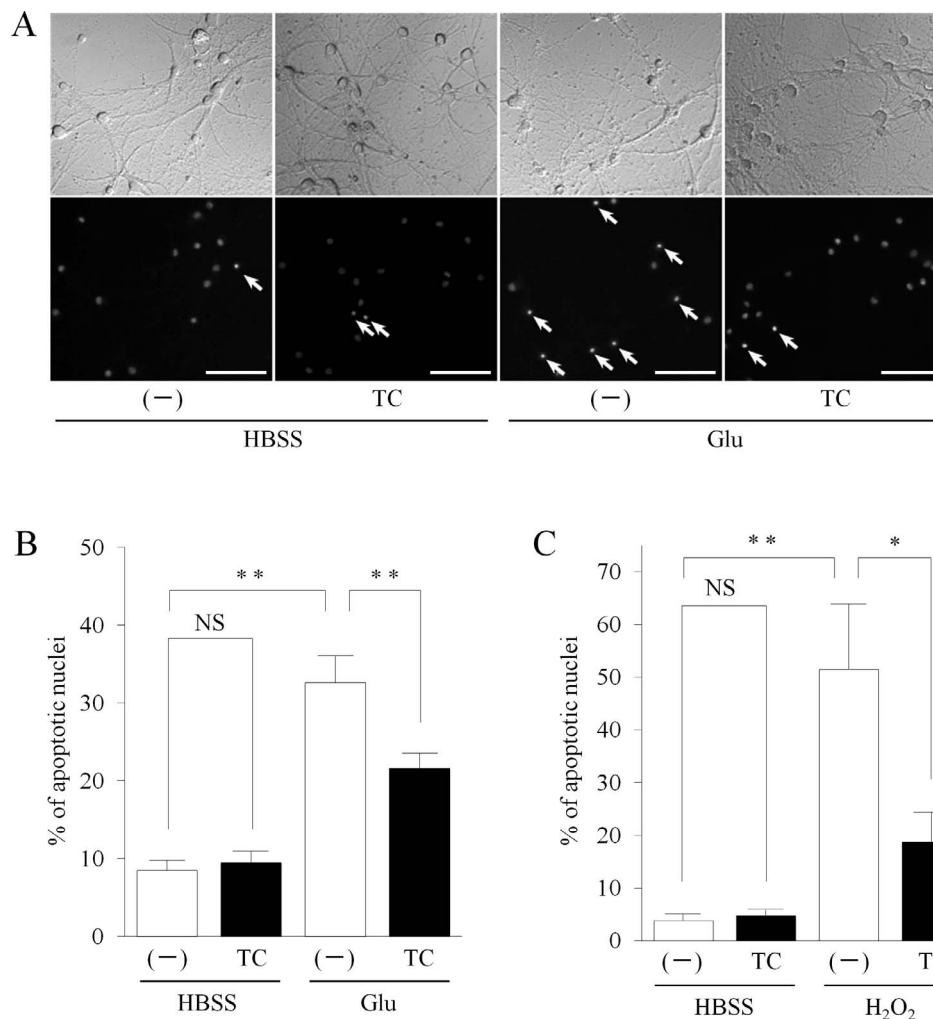
Seven days after the NMDA injection, frozen 14- $\mu$ m-thick sections were fixed with 4% paraformaldehyde. Following rinses in PBS, samples were permeabilized with 0.02% Triton X-100 in antibody buffer (150 mM NaCl, 50 mM Tris base, 1% BSA, 100 mM L-Lysine, 0.04% Na azide, pH 7.4) and blocked by Image-iT FX Signal Enhancer (Thermo Fisher Scientific) to reduce nonspecific binding. Samples were incubated overnight at 4°C in antibody buffer containing anti-p38 MAPK $\gamma$  primary antibody (R&D Systems, Minneapolis, MN, USA), washed with PBS, incubated in antibody buffer containing secondary antibody using Alexa Fluor 488 anti-rabbit IgG (no. A-11008, Thermo Fisher Scientific) and 4',6-diamidino-2-phenylindole (DAPI) for 1 hour at room temperature, and washed with PBS. Samples were mounted using Vectashield mounting medium (no. H-1200; Vector Laboratories, Burlingame, CA, USA), and examined using fluorescence microscopy (BZ-X710; Keyence, Tokyo, Japan).

### In Vivo Expression Levels of Caspase 3

The levels of cleaved caspase 3 were evaluated with an immunoblot analysis. Briefly, the retinas ( $n = 4$  in each group) were removed 7 days after the NMDA injection and then immediately homogenized using a lysis buffer containing 50 mM Tris-buffered saline (TBS; pH 7.0-7.6), 0.1% sodium deoxycholate, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1% Triton X-100 with complete protease inhibitor cocktail (no. 11836153001; Roche, Basel, Switzerland). Standard immunoblotting was performed as described above.

### Retrograde Labeling to Detect the Survival of RGCs

Three days after the NMDA injections, retrograde labeling of RGCs with FluoroGold (FG), in a manner similar to that described previously,<sup>37,38</sup> was performed to assess whether



**FIGURE 1.** Tranlycypromine (TC) attenuates glutamate (Glu) neurotoxicity- and oxidative stress-induced apoptosis. Fragmented or shrunken nuclei detected by Hoechst staining 24 hours after Glu or oxidative stress under conditions with or without TC. (**A, B**) Photo images (*top*) and fluorescence images (*bottom*) of retinal ganglion cells (RGCs) after Glu stimulation. TC significantly suppressed Glu-induced apoptotic cells (indicated by *arrows*) compared with Glu stimulation without TC. (**C**) TC also significantly promoted RGC survival following oxidative stress. All data are represented as means  $\pm$  standard error (SE). Tukey-Kramer test ( $n = 6-9$ ). \* $P < 0.05$ , \*\* $P < 0.01$ . NS, not significant. *Scale bars:* 50  $\mu\text{m}$ .

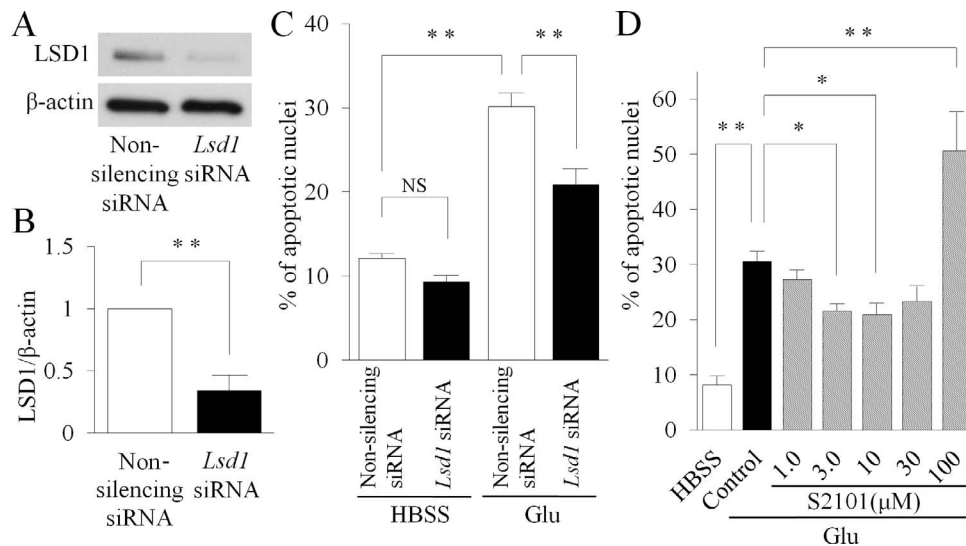
tranlycypromine influenced the survival of RGCs. Briefly, after each animal was deeply anesthetized with an intraperitoneal injection of a 1:1 mixture of xylazine hydrochloride (4 mg/kg; Bayer HealthCare) and ketamine hydrochloride (10 mg/kg; Daiichi Sankyo Propharma Co., Ltd.), its head fur was shaved and the skin was incised along the midline to expose the skull and sagittal, coronal, and transverse sutures. Along the bilateral 2-mm diameter, a craniotomy was performed 0.5 mm posterolateral to the sagittal and transverse sutures, and the cerebral content over the superior colliculus was carefully removed with a vacuum pump. Next, a small piece of sterile sponge that was presoaked in 6  $\mu\text{L}$  6% FG solution was left on the surface of the superior colliculus and the skin wound was sutured and closed.

After the surgery, the rats were kept warm and allowed to recover on their own. Then, 7 days after the NMDA injection, the animals were euthanized, intracardially perfused with PBS to achieve exsanguination, and then perfused with 4% paraformaldehyde. After the perfusion fixation, the eyes were enucleated and fixed with 4% paraformaldehyde overnight at 4°C. Subsequently, the retinas were removed from the sclera and divided into four quadrants (superior, inferior, nasal, and temporal) and mounted on slides. Each quadrant was

subdivided into three areas (central, middle, and peripheral) that were 1.0, 2.0, and 3.0 mm from the optic nerve head, respectively. Retinal ganglion cell number was assessed at the point of 1 week ( $n = 7$  in each group), 2 weeks ( $n = 5-7$  eyes in each group), and 4 weeks ( $n = 3$  in each group) after NMDA intravitreal injection. A total of 12 fields per retina were analyzed by counting the number of FG-labeled RGCs with an all-in-one fluorescence microscope (BZ-X710; Keyence) in a masked fashion.

### Optic Nerve Crush to Detect Neuroprotective Dose of Tranlycypromine

Eight-week-old male rats were anesthetized by inhalation of 2% isoflurane. After incision of conjunctiva, the optic nerve was bluntly exposed under a microscope. The optic nerve was clamped 1 to 2 mm distal to the globe for 30 seconds with Micro Crip (RS-542; Roboz Surgical Instrument Co., Inc., Gaithersburg, MD, USA), and target reagent was injected intravitreally (5.0  $\mu\text{L}/\text{eye}$ ) by a Hamilton microsyringe fitted with a 33-gauge needle ( $n = 6$  in each condition). For collecting retinas, rats were deeply anaesthetized and euthanized 3 days after optic nerve injury. Total RNA was purified with RNeasy



**FIGURE 2.** Inhibition of lysine-specific demethylase 1 (LSD1) protects RGCs from apoptosis. (A, B) Western blot analyses and densitometry showing that *Lsd1*-specific siRNA significantly suppressed the expression of LSD1. Bar graph representing these proportions relative to control ( $n = 4$  in each group; Student's *t*-test). (C) *Lsd1* siRNA significantly suppressed apoptosis after glutamate (Glu) overload ( $n = 4-6$  in each group; Tukey-Kramer test). (D) The LSD1-specific pharmacologic inhibitor S2101 also significantly inhibited glutamate (Glu)-induced apoptosis compared with control. All data are represented as means  $\pm$  SE. Tukey-Kramer test ( $n = 4-6$  in each group). \* $P < 0.05$ , \*\* $P < 0.01$ . NS, not significant.

Plus Universal Mini Kit (Qiagen, Valencia, CA, USA), subjected to reverse transcription (PrimeScript RT Master Mix; Takara Bio, Inc., Shiga, Japan), and the resulting cDNA used as the template for a quantitative-PCR reaction (QuantiFast SYBR GreenPCR Kit; Qiagen) performed with Nefl and Gapdh primers. Nefl gene expression was corrected based on Gapdh cDNA.

### Statistical Analysis

All data are presented as means  $\pm$  standard errors. All statistical analyses were performed with either Tukey-Kramer tests or Student's *t*-tests, and the microarray experiments were analyzed with Fisher's exact tests (modified as EASE scores [adjusted Fisher exact probability]). All data analyses were performed using JMP v. 8.0 software (SAS Institute, Inc., Cary, NC, USA), and a confidence level of 95% was considered to indicate statistical significance ( $P < 0.05$ ).

## RESULTS

### Neuroprotective Effects of Tranlycypromine

Glutamate is a major excitatory neurotransmitter, but it can also act as a neurotoxin during the course of central nervous system (CNS) diseases, such as brain ischemia or injury, multiple sclerosis, Alzheimer's disease, Parkinson's disease, and glaucoma.<sup>39,40</sup> In the present study, glutamate was used to induce the caspase-dependent apoptosis of rat primary cultured RGCs via the NMDA receptor. Hoechst staining revealed that 32.6% of the RGCs that experienced glutamate overload for 2 hours contained shrunken or fragmented nuclei, which are morphologic markers of apoptosis. However, tranlycypromine significantly protected RGCs from glutamate-induced neuronal cell death (Figs. 1A, 1B).

To determine whether neuronal death induced by other modalities could be prevented by tranlycypromine, the present study exposed RGCs to oxidative stress caused by exposure to  $H_2O_2$  (50  $\mu$ M) for 30 minutes. B27 supplements without antioxidants rather than the standard B27 supplements used in

other experiments were employed in the present study to ensure minimal antioxidant content in the culture medium. There were no significant differences in neuronal survival following the use of either formulation of the B27 supplement (>90%). Upon the exposure of RGCs to  $H_2O_2$  for 30 minutes, 51.4% of the RGCs exhibited apoptotic nuclei; tranlycypromine significantly attenuated neuronal death by 18.6% (Fig. 1C). Thus, tranlycypromine protected RGCs from glutamate neurotoxicity-induced apoptosis as well as apoptosis induced by oxidative stress.

### The Inhibition of LSD1 Protects RGCs From Apoptosis

The present study also aimed to determine whether tranlycypromine would have neuroprotective actions via its anti-LSD1 activity. Tranlycypromine possesses dual pharmacologic effects as an LSD1 inhibitor and a monoamine oxidase (MAO) inhibitor, which may be responsible for its antiapoptotic and neuroprotective activities.<sup>41</sup> Thus, the present study investigated neuronal survival after *Lsd1* knockdown.

First, the knockdown effect of the *Lsd1*-specific siRNA was validated with an immunoblotting procedure. Densitometry of the immunoblots revealed that *Lsd1* (*Kdm1a*)-specific siRNA significantly suppressed LSD1 expression by 30.4% compared with the nonsilencing siRNA control (Figs. 2A, 2B). Next, the apoptosis rates of the *Lsd1*-knockdown RGCs were evaluated after glutamate-stimulated neural stress. In RGCs transfected with nonsilencing siRNA, 30.1% of the cells suffered from glutamate-induced apoptosis, but the number of apoptotic RGCs significantly decreased in cells transfected with *Lsd1*-specific siRNA (Fig. 2C). Similarly, the pharmacologic inhibition of LSD1 with S2101, which is structurally related to tranlycypromine but has a higher affinity for LSD1 despite a substantially weaker effect on MAOs,<sup>42</sup> promoted cell survival following glutamate-induced stress (Fig. 2D). At 100  $\mu$ M, S2101 showed a high rate of apoptotic nuclei in RGCs, and this cytotoxicity of S2101 would be a secondary and adverse effect. Taken together, these findings indicate that the suppression of LSD1 activity may contribute to neuroprotection following apoptotic stress.

**TABLE 1.** KEGG Pathway Functional Classification for Downstream Involved in Tranilcypromine

Term	P Value
rno04666: Fc-gamma R-mediated phagocytosis	0.010
rno04722: Neurotrophin signaling pathway	0.026
rno00230: Purine metabolism	0.044
rno04914: Progesterone-mediated oocyte maturation	0.074
rno04144: Endocytosis	0.084

KEGG, Kyoto Encyclopedia of Genes and Genomes. EASE score, modified Fisher's exact *P* value < 0.05.

### Target Pathways and Genes Involved in the Tranilcypromine-Induced Survival of RGCs

Next, the present study investigated the signaling pathways involved in the neuroprotective effects of tranilcypromine. To identify the gene expression changes induced by tranilcypromine in RGCs, a microarray analysis evaluating 26,930 gene expression changes was carried out. Because previous studies have shown that LSD1 suppresses gene transcription via a demethylation reaction of histone H3 lysine 4,<sup>26,27</sup> it was hypothesized that the genes whose expression is enhanced by tranilcypromine and simultaneously decreased by glutamate administration could be promising candidates as gene targets for the regulation of neuronal survival. The microarray data were reported as *Z*-scores and ratios and revealed that 110 genes satisfied these criteria.

Additionally, a KEGG pathway annotation analysis was performed using DAVID. This analysis revealed the significant detection of three enriched KEGG pathway terms for functional classification that were associated with the 110 gene candidates identified in the present study; these terms were involved in Fc-gamma R-mediated phagocytosis, the neurotrophin signaling pathway, and purine metabolism (Table 1). The details of the expression profiles are listed in Table 2. Next, the analyses focused on two neurotrophin signaling molecules, v-akt murine thymoma viral oncogene homolog 1

(Akt1) and mitogen-activated protein kinase 12 (p38 MAPK $\gamma$ ), because the phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR and MAPK pathways are key signaling pathways for cell survival and antiapoptotic activity.<sup>43-46</sup>

### Tranilcypromine Enhances RGC Survival via P38 MAPK Activity

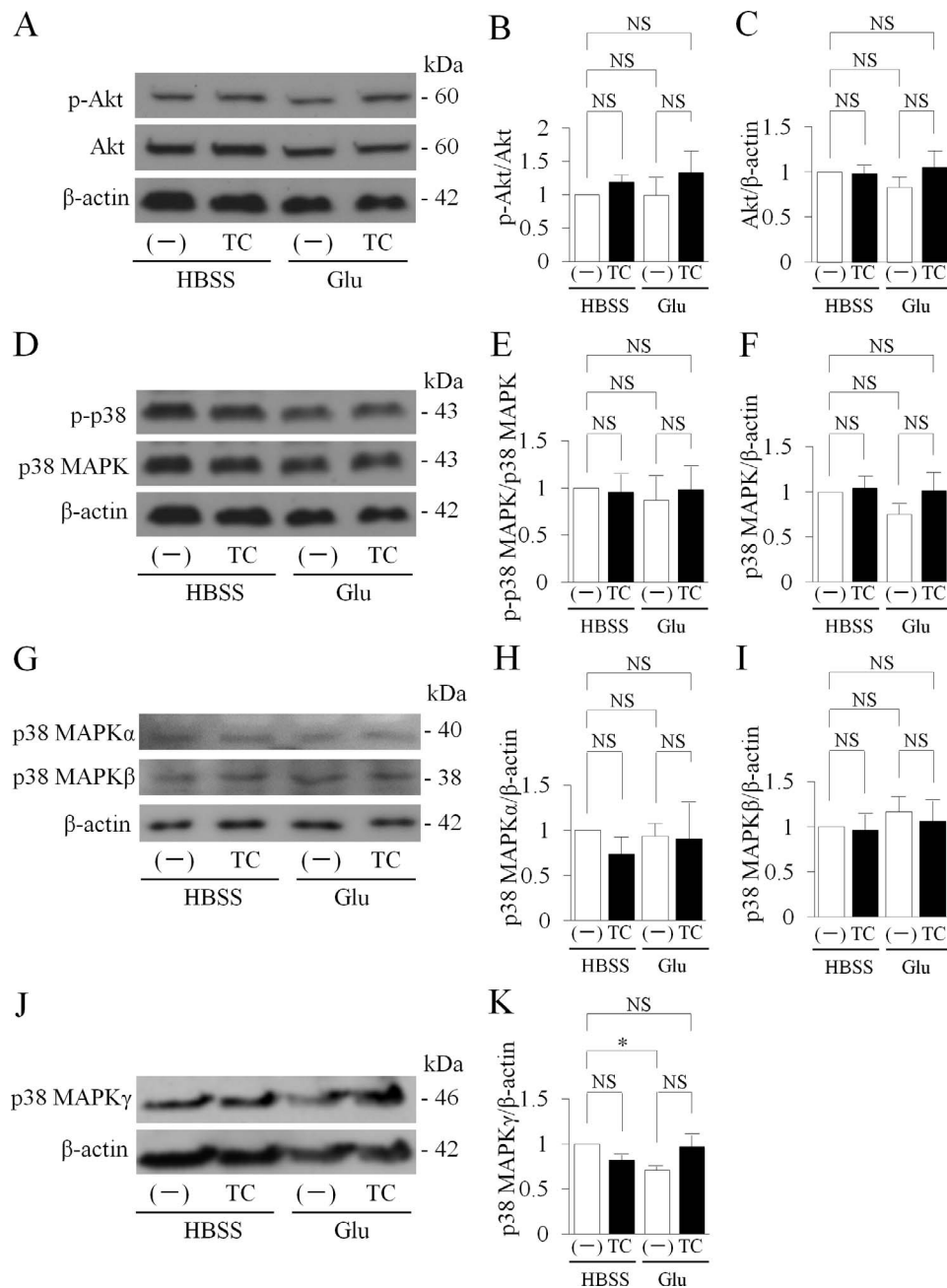
The present study also investigated changes in the activity of two candidate pathways involved in RGC survival as well as the expression and phosphorylation statuses of Akt and total p38 MAPKs using immunoblot analyses. The ratios of total Akt/ $\beta$ -actin and phospho-Akt/total Akt were unchanged following the administration of tranilcypromine in both the control and glutamate-induced stress conditions (Figs. 3A-C). Similarly, the total p38 MAPK expression and phosphor/p38 MAPK ratio did not show any statistical changes following the application of either tranilcypromine or glutamate stress (Figs. 3D-F). On the other hand, the expression of p38 MAPK $\gamma$ , which is one of four p38 MAPK subtypes, was significantly suppressed by glutamate administration; this suppression was ameliorated by tranilcypromine (Figs. 3J, 3K). Additionally, microarray data and immunoblot analysis showed that the expression of p38 MAPK $\alpha$  or p38 MAPK $\beta$  was not affected by tranilcypromine (Figs. 3G-I; Supplementary Table S1). These findings suggest that the neuroprotective effects of tranilcypromine might be profoundly and specifically affected by p38 MAPK $\gamma$  rather than the total p38 MAPKs.

To elucidate whether p38 MAPK $\gamma$  or Akt protects RGCs from glutamate overload, the cell survival rates after the pharmacologic inhibition of Akt or p38 MAPK $\gamma$  were assessed using wortmannin as a potent and selective inhibitor of PI3K, and BIRB796 and SB203580 as specific inhibitors of p38 MAPK. Wortmannin had no effect on RGC survival in either the glutamate stress or glutamate with tranilcypromine condition (Fig. 4A). Although there were no significant effects of SB203580, the p38 MAPK inhibitor BIRB796, which inhibits all four p38 MAPK subtypes,<sup>47</sup> clearly blocked the neuroprotective effects of tranilcypromine against glutamate-induced

**TABLE 2.** Candidate Genes Associated With Tranilcypromine-Induced Neuroprotection

GenBank Accession	Gene Symbol	Gene Name	Control vs. Glu		Glu vs. Glu + TC	
			Z-Score	Ratio	Z-Score	Ratio
Fc-gamma R-mediated phagocytosis						
NM_031146	arpc1a	actin-related protein 2/3 complex, subunit 1A	-2.47	0.50	2.06	2.06
XM_001067293	LOC688430	cofilin 1, nonmuscle; similar to cofilin-1, cofilin, nonmuscle isoform	-2.11	0.61	3.80	2.89
AF503609	PPAP2A	phosphatidic acid phosphatase type 2A	-2.62	0.11	2.76	9.52
NM_033230	akt1	v-akt murine thymoma viral oncogene homolog 1	-3.01	0.43	2.12	2.11
Neurotrophin signaling pathway						
NM_053409	MAGED1	melanoma antigen, family D, 1	-2.45	0.56	2.12	1.81
NM_021746	MAPK12	mitogen-activated protein kinase 12	-2.10	0.56	2.84	2.71
NM_031767	Sort1	sortilin 1	-2.40	0.51	2.15	2.13
NM_033230	akt1	v-akt murine thymoma viral oncogene homolog 1	-3.01	0.43	2.12	2.11
Purine metabolism						
NM_053396	ADCY7	adenylate cyclase 7	-2.43	0.13	2.55	8.01
NM_022710	pde1b	phosphodiesterase 1B, calmodulin dependent	-2.18	0.43	2.32	2.93
NM_001014259	POLR3K	polymerase (RNA) III, DNA directed, polypeptide K, 12.3 kDa	-2.05	0.61	2.38	1.94
NM_001105791	PFAS	Similar to phosphoribosylformylglycinamide synthase; phosphoribosylformylglycinamide synthase	-2.40	0.40	2.13	2.68

Glu, glutamate; TC, tranilcypromine.



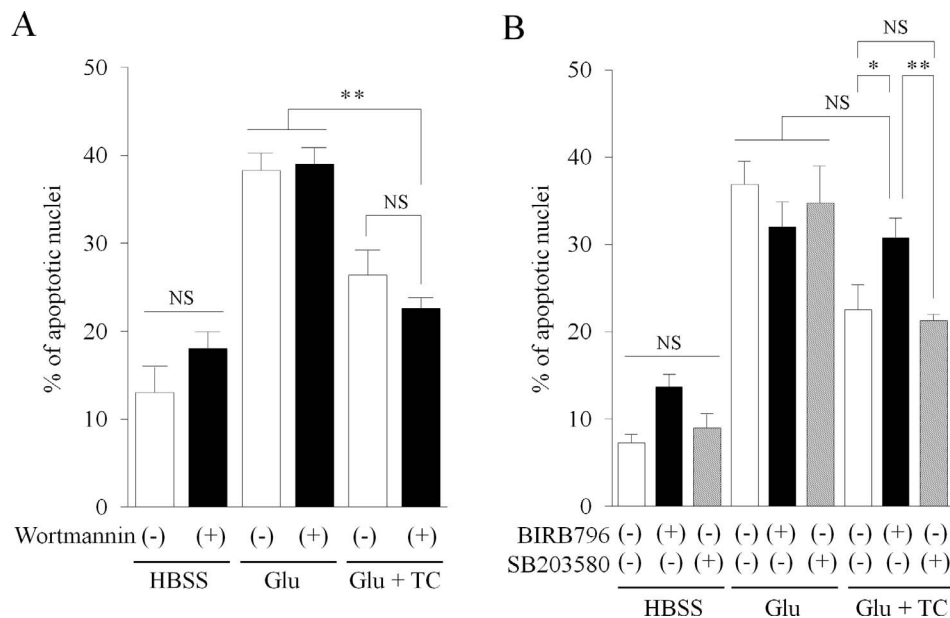
**FIGURE 3.** Tranlycypromine (TC) promotes mitogen-activated protein kinase 12 (p38 MAPK $\gamma$ ) expression under conditions of glutamate (Glu)-induced stress. (A–C) Western blot analyses and densitometry showing that Glu had no effect on the expression of Akt. TC did not influence Akt expression or the phosphorylation of Akt. (D–F) Glu had no effect on the expression of total p38 MAPK or its phosphorylation status. TC did not change total p38 MAPK expression or its phosphorylation status. (G–I) The expression of p38 MAPK $\alpha$  or p38 MAPK $\beta$  was not affected by TC. (J, K) However, TC significantly promoted the expression of p38 MAPK $\gamma$ . All data are represented as means  $\pm$  SE. Tukey-Kramer test ( $n = 5-7$  for each experiment, except p38 MAPK $\alpha/\beta$  experiments [ $n = 3$ ]). \* $P < 0.05$ . NS, not significant.

RGC death (Fig. 4B). SB203580 is a typical inhibitor of p38 MAPK $\alpha$  and p38 MAPK $\beta$  but not p38 MAPK $\gamma$  or p38 MAPK $\delta$ .<sup>48,49</sup> Thus, the present pharmacologic results supported the proposed hypothesis that tranlycypromine contributes to RGC survival via alterations of p38 MAPK $\gamma$  activity.

### Tranlycypromine Promotes the In Vivo Survival of RGCs

Finally, the present study investigated whether tranlycypromine regulates in vivo RGC survival under conditions of stress.

First, morphologic changes in the retinas after tranlycypromine administration were assessed using an in vivo rat model of NMDA-induced retinal damage. Intravitreal injections of NMDA significantly reduced IPL thickness compared with intravitreal injections of PBS. However, the NMDA-injected retinas that received treatment with tranlycypromine maintained IPL thickness to a degree similar to that of the control retinas (Fig. 5). Second, because the expression levels of the caspase family generally reflect apoptotic activity,<sup>50,51</sup> the present study aimed to determine the level of NMDA-induced caspase 3 cleavage, which is an indicator of caspase 3 activation,



**FIGURE 4.** Tranlycypromine (TC) contributes to RGC survival via p38MAPK $\gamma$  activity. RGC apoptosis was evaluated based on morphologic changes of the nuclei. **(A)** Wortmannin had no effect on the survival ratio in either the glutamate (Glu)-stressed or Glu with TC (Glu + TC) conditions. **(B)** Similarly, SB203580, which is a p38 $\alpha$ /p38 $\beta$ -specific inhibitor, did not have an effect in the Glu + TC condition, but the protective effects of TC on RGCs were attenuated by the p38MAPK inhibitor BIRB796. This suggests that TC may protect neurons via alterations in p38MAPK isoforms, except for p38 $\alpha$  and p38 $\beta$ . All data are represented as means  $\pm$  SE. Student's *t*-test ( $n = 4$  in each group). \* $P < 0.05$ , \*\* $P < 0.01$ . NS, not significant.

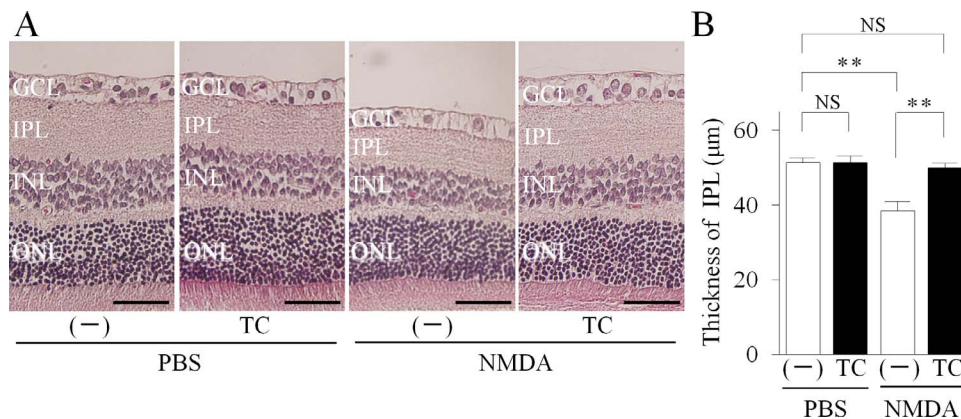
following the administration of tranlycypromine using immunoblot analyses. In the retinas treated with NMDA and vehicle, there was a significant increase in the levels of cleaved caspase 3 at 18 hours after the NMDA injection; this activity was significantly suppressed in the tranlycypromine-treated retinas (Fig. 6). Taken together, these findings indicate that the intravitreal tranlycypromine injections exerted neuroprotective effects within intracellular apoptotic signaling pathways and suppressed morphologic changes in the retina.

Additionally, we investigated whether tranlycypromine showed modification effects of p38 MAPK $\gamma$  expression in vivo similar to those in vitro. Seven days after NMDA-induced retinal neurotoxicity, p38 MAPK $\gamma$  expression was significantly suppressed in the RGC layers. However, this suppression was recovered by tranlycypromine and its effect was canceled by

BIRB796 (Fig. 6C), suggesting that tranlycypromine might similarly show an in vivo antiapoptotic effect via p38 MAPK $\gamma$  activity.

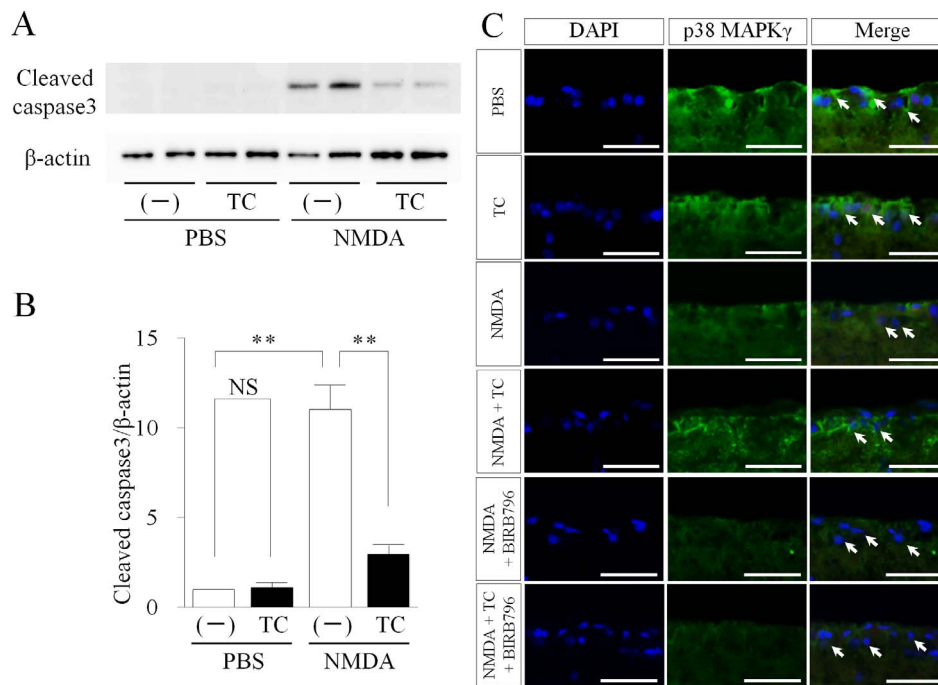
Next, the effective concentration of tranlycypromine for retinal neuroprotection was assessed. Three days after optic nerve crush, the light neurofilament (NF-L) gene expression after tranlycypromine treatment increased dose-dependently, and 1000-nmol injection of tranlycypromine showed an increase in NF-L expression similar to BDNF (5.0  $\mu$ g/eye) positive control (Supplementary Fig. S1).

Finally, the present study assessed the degree to which tranlycypromine protects RGCs from NMDA-induced retinal neurotoxicity. The actual numbers of RGCs that were labeled in a retrograde manner by FG were counted in the retinas of sham-treated, tranlycypromine-treated, NMDA-treated, and



**FIGURE 5.** Morphologic protection of retinal cells from N-methyl-D-aspartate (NMDA)-induced stress by intravitreal injections of tranlycypromine (TC). **(A)** Light microscopic images of retinal section samples showing a thick inner plexiform layer (IPL) after TC treatment, even in the NMDA-stressed condition. **(B)** IPL thickness following NMDA administration was significantly thinner than that after the administration of phosphate-buffered saline (PBS). However, TC injections fully recovered the NMDA-induced retinal damage. All data are represented as means  $\pm$  SE. Tukey-Kramer test ( $n = 5$ -6 for each). \*\* $P < 0.01$ . NS, not significant. Scale bars: 50  $\mu$ m. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.





**FIGURE 6.** Tranlycypromine (TC) suppresses caspase 3 activity and recovers p38 MAPK $\gamma$  expression in the retina after NMDA-induced injury. (**A**, **B**) Western blot analyses and densitometry showing the significant inhibition of the apoptotic signal cleaved caspase 3 by TC. (**C**) Whereas p38 MAPK $\gamma$  expression was significantly suppressed by NMDA in the retinal ganglion cell layer (indicated by *arrows*), this suppression was ameliorated by TC. Also, BIRB796 inhibited TC-induced p38 $\gamma$  recovery. All data are represented as means  $\pm$  SE. Tukey-Kramer test ( $n = 4$  for each).  $**P < 0.01$ . NS, not significant. Scale bars: 50  $\mu$ m.

NMDA- and tranlycypromine-treated animals. At 7 days after the intravitreal injections of NMDA, the numbers of RGCs in the NMDA-treated group decreased by 21.5% and 29.9% in the middle and peripheral regions of the retina, respectively. In contrast, the numbers of RGCs in the NMDA- and tranlycypromine-treated groups significantly increased by 49.9% and 53.3% in the middle and peripheral regions, respectively (Figs. 7A–C). Also, single injection of tranlycypromine enhanced RGC survival for at least 2 weeks in the middle and peripheral regions of the retina (Figs. 7D, 7E). Thus, intravitreal tranlycypromine treatment enhanced RGC survival after retinal injury via the attenuation of NMDA neurotoxicity.

## DISCUSSION

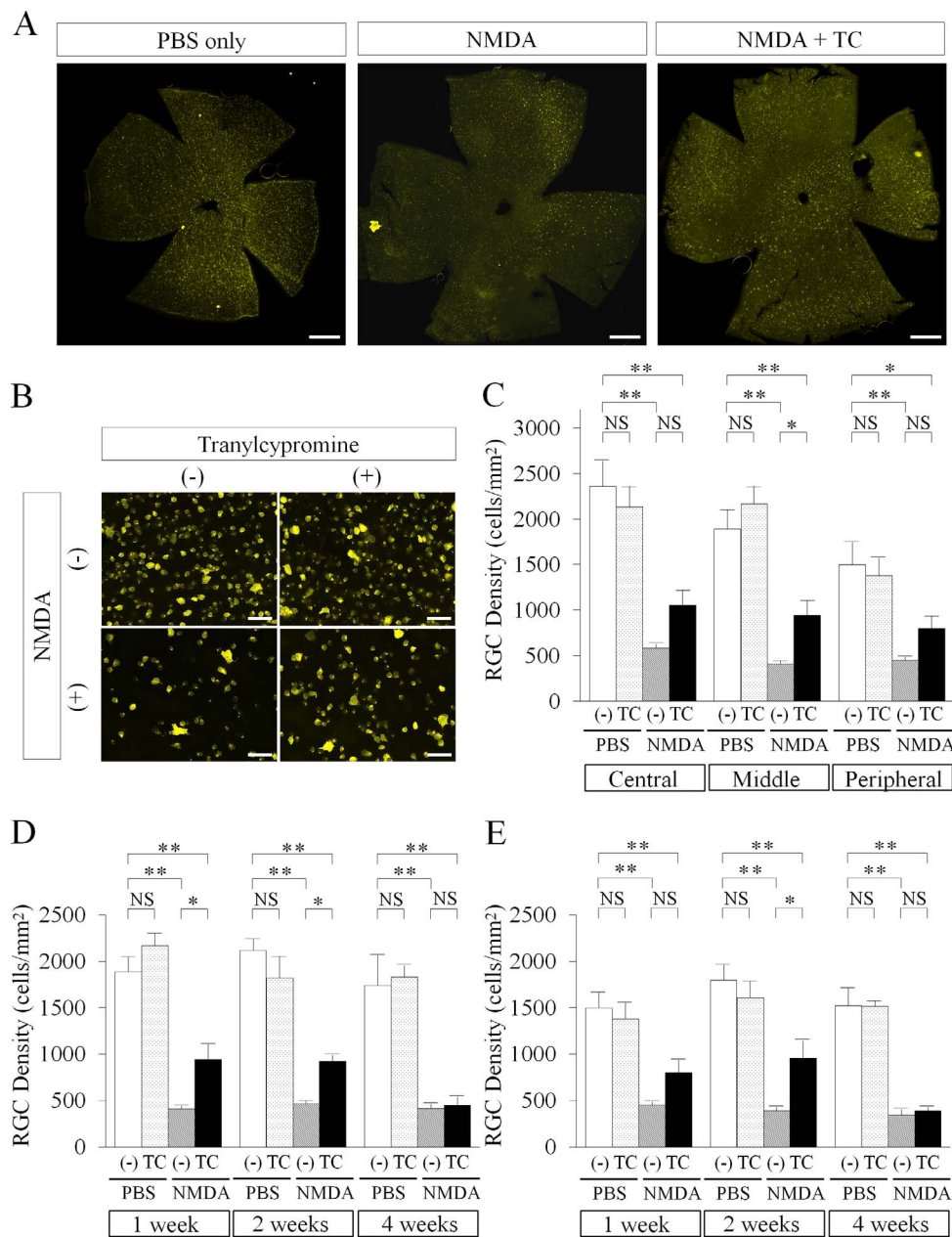
The findings of the present study demonstrated that a novel neuroprotective drug, tranlycypromine, exerted suppressive activity against LSD1. Lysine-specific demethylase 1 is a well-known member of the flavin-containing amine oxidase family that represses transcription by removing the methyl group from monomethylated and dimethylated lysine 4 of histone H3.<sup>26,27</sup> To our knowledge, this is the first study to report that tranlycypromine suppressed neuronal cell death induced by glutamate or oxidative stress via the inhibition of LSD1. Furthermore, the present study demonstrated that the intravitreal administration of tranlycypromine significantly protected RGCs in an in vivo rat model of NMDA-induced excitotoxicity that led to a significant loss of RGCs. Thus, the transcriptional and epigenetic regulation induced by tranlycypromine might be a potential therapeutic target for the treatment of neurodegenerative diseases.

The data presented here indicate that the neuroprotective effects of tranlycypromine are related to the modulation of LSD1 activity. Previous studies have shown that LSD1 influences cell proliferation, is prosurvival,<sup>52,53</sup> aids in neurite

morphogenesis,<sup>54</sup> and promotes neural differentiation and development.<sup>55</sup> It has also been shown that the inhibition of LSD1 by tranlycypromine modulates neural stem cell proliferation in the mouse brain<sup>53</sup> and changes zebrafish lateral line neuromast development.<sup>56</sup> Interestingly, the present findings indicate that tranlycypromine also significantly benefited cell survival in the mammalian CNS.

Tranlycypromine is a potent dual-action drug that inhibits the activities of LSD1 and MAOs.<sup>57,58</sup> Monoamine oxidases catalyze the oxidative deamination of dietary amines and monoamine neurotransmitters and, in turn, produce the by-products of MAO-related reactions, including 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL) and H<sub>2</sub>O<sub>2</sub>. These potentially neurotoxic metabolites lead to the apoptosis of neuronal cells and are considered to be possible causes of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease.<sup>59,60</sup> Therefore, inactivation of MAO is thought to be a good strategy for the protection of neuronal cells in vitro and in vivo.<sup>61,62</sup> In fact, MAO inhibitors such as l-deprenyl (selegiline) and (R)-N-(prop-2-ynyl)-2,3-dihydro-1H-inden-1-amine (rasagiline) have become archived pharmacologic therapeutic options for human neuropsychiatric diseases such as Parkinson's disease,<sup>63–65</sup> treatment-resistant depression,<sup>66</sup> and anxiety disorders.<sup>67</sup>

To clarify whether LSD1 and/or MAO were important factors in the neuroprotective effects provided by tranlycypromine, neuronal survival was investigated in *Lsd1*-knockdown samples using *Lsd1*-specific siRNA or via pharmacologic inhibition with S2101, which has a potent affinity for LSD1 despite a substantially weaker affinity for MAOs than tranlycypromine.<sup>42</sup> Both experimental conditions revealed that the suppression of LSD1 significantly diminished the protective effects of tranlycypromine on RGCs, which implies that LSD1 is more important for neuroprotection than MAO. To the best of



**FIGURE 7.** Tranlycypromine (TC) has significant neuroprotective effects against NMDA-induced apoptosis in vivo. FluoroGold-retrograde labeling of RGCs. (A, B) Flat-mount samples showing significant RGC loss after intravitreal NMDA injections. However, the RGCs were rescued by TC treatment. (C) Actual RGC numbers showing statistically greater numbers following TC treatment in the midregion of the retina (D, E) Single injection of TC enhanced RGC survival for at least 2 weeks in the middle region (D) and in the peripheral region (E). All data are represented as means  $\pm$  SE. Tukey-Kramer test. \* $P < 0.05$ , \*\* $P < 0.01$ . NS, not significant. Scale bars: 1.0 mm (A); 50  $\mu$ m (B).

our knowledge, this is the first study to demonstrate that the inhibition of LSD1 promotes neuroprotection.

However, the manner in which LSD1 regulates neuronal survival remains unclear. Because LSD1 is an important molecule involved in the epigenetic regulation of gene transcription,<sup>26,27</sup> the modulation of downstream gene expression was assessed in the present study. Exhaustive microarray analyses revealed that p38 MAPK $\gamma$  is one of the key players involved in the neuroprotection afforded by LSD1. Mitogen-activated protein kinases such as extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), c-jun N-terminal kinase (JNK), and p38 are well-known factors associated with the survival, proliferation, and differentiation of neuronal<sup>68,69</sup> and nonneuronal<sup>70,71</sup> cells. Recently, Jiang et al.<sup>72</sup> reported that the

inhibition of p38 MAPK had a neuroprotective effect via crosstalk with nuclear factor kappa-light-chain enhancer of activated B cells (NF- $\kappa$ B) p65 in a rat model of retinal ischemia/reperfusion injury. Interestingly, the p38 MAPK family consists of four major isoforms, p38 $\alpha$ , p38 $\beta$ , p38 $\gamma$ , and p38 $\delta$ , which are encoded by independent genes and are considered to have isoform-specific functions as well as functional redundancies.<sup>73</sup>

Using cells within the CNS, several studies have demonstrated that the p38 MAPK $\alpha$  isoform is a relatively more important contributor to stressor-induced proinflammatory cytokine production and neurotoxicity compared with the p38 $\beta$  isoform.<sup>74–76</sup> Several studies have reported that SB203580, a typical inhibitor of p38 MAPK $\alpha$  and p38 MAPK $\beta$ , rescues RGCs from NMDA and optic nerve injury, suggesting

the involvement of p38 MAPK $\alpha$  and p38 MAPK $\beta$  in RGC survival.<sup>77,78</sup> However, the roles of the other p38 isoforms in neuronal cells have yet to be elucidated. Whereas other studies have found that the p38 $\alpha$  isoform leads to neuronal death,<sup>76</sup> the present data demonstrated that an increased level of phosphorylated p38 $\gamma$  (and/or p38 $\delta$ ) enhanced the survival of primary RGCs. Interestingly, Ferrari et al.<sup>79</sup> recently identified a similar phenomenon showing that the survival effects of p38 $\gamma$  are opposite to those of p38 $\alpha$  in vascular endothelial cells. P38 $\alpha$  mediates apoptotic signaling from inducers of endothelial cell apoptosis, such as transforming growth factor- $\beta$  (TGF- $\beta$ ), but p38 $\gamma$  induces survival signaling.<sup>79</sup> Further studies are needed to define the relative importance of neuronal p38 $\gamma$  and p38 $\delta$  in excitotoxicity-stimulated RGCs.

Finally, the present study investigated whether tranlycypromine would regulate RGC survival in vivo. Intravitreal injections of tranlycypromine not only sustained the thickness of the IPL but also reduced RGC loss after overloading NMDA receptors in the rat retina. Thus, the in vivo targeting of LSD1 activity by tranlycypromine may protect RGCs following retinal damage. Clinical usefulness of systemic tranlycypromine in the treatment of mood and anxiety disorders was seriously limited because of adverse effects such as orthostatic hypotension, sleep disturbances, and nervousness/agitation, and the potential risk of hypertensive crisis and liver toxicity caused by interactions with tyramine-rich foods and sympathomimetic and serotonergic substances.<sup>80,81</sup> However, ocular topical administration including intravitreal injection may minimize the risk of systemic adverse events. Taken together, these in vitro and in vivo data strongly support that epigenetic regulation of transcriptional modulators affecting the signaling pathways critical for regulating neuronal survival may be a hopeful therapeutic target for neurodegenerative eye diseases.

In conclusion, the present study found that tranlycypromine, which is a typical LSD1 inhibitor, exerted significant neuroprotective effects on RGCs by promoting neuronal expression of the p38 MAPK $\gamma$  isoform, which might be one of main players in the enhancement of neuronal survival. Additionally, the intravitreal administration of tranlycypromine led to the neuroprotection of RGCs in a rat model of NMDA-induced excitotoxicity stress, which suggests that suppression of LSD1 may be a good therapeutic target for the treatment of neurodegenerative diseases, such as glaucoma.

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