ORIGINAL ARTICLE

Melatonin pretreatment alleviates the long-term synaptic toxicity and dysmyelination induced by neonatal Sevoflurane exposure via MT1 receptor-mediated Wnt signaling modulation

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Abstract
Sevoflurane (Sev) is one of the most widely used pediatric anesthetics. The major concern of neonatal repeated application of Sev is its potential long-term impairment of cognition and learning/memory, for which there still lacks effective treatment. At the cellular level, Sev exerts toxic effects in multiple aspects, making it difficult for effective interference. Melatonin is a pineal hormone regulated by and feedbacks to biological rhythm at physiological condition. Recent studies have revealed significant neuroprotective effects of exogenous melatonin or its agonists under various pathological conditions. Whether melatonin could prevent the long-term toxicity of Sev remains elusive. Here, we report that neonatal repeated Sev exposure up-regulated MT1 receptor in hippocampal neurons and oligodendrocytes. Pretreatment with melatonin significantly alleviated Sev-induced synaptic deficiency, dysmyelination, and long-term learning impairment. Both MT1-shRNA and MT1 knockout effectively blocked the protective effects of melatonin on synaptic development, myelination, and behavior performance. Interestingly, long-lasting suppression of Wnt signaling, instead of cAMP/PKA signaling, was observed in hippocampal neurons and oligodendrocytes after neonatal Sev exposure. Pharmacologically activating Wnt signaling rescued both the long-term synaptic deficits and dysmyelination induced by Sev. Further analysis showed that MT1 receptor co-expressed well with β-catenin and

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Axin2 and bound to β-catenin by its C-terminal. Melatonin pretreatment effectively rescued Sev-induced Wnt suppression. Wnt signaling inhibitor XAV939 significantly compromised the protective effects of melatonin. Taken together, our data demonstrated a beneficial effect of melatonin pretreatment on the long-term synaptic impairment and dysmyelination induced by neonatal Sev exposure, and a novel MT1 receptor-mediated interaction between melatonin and canonical Wnt signaling, indicating that melatonin may be clinically applied for improving the safety of pediatric Sev anesthesia.

KEYWORDS

melatonin, oligodendrocyte, Sevoflurane, synaptic toxicity, Wnt signaling

1 | INTRODUCTION

With the development of comfortable anesthesia, increasing clinical application of general anesthesia, particularly pediatric anesthesia, has been reported globally. In America, approximately millions of infants are subjected to general anesthesia every year.[1] However, the concerns on the safe use of general anesthetics in children are increasing because of the high sensitivity of neonatal central nerve system (CNS) to anesthetics. Recent studies in both rodents and primates have demonstrated notable long-term neural toxic effects of repeated exposure to anesthetics in neonatal,[2–6] as showed by signs of impairment of learning and cognition. FDA has warned that repeated general anesthesia would be harmful to brain development in children under the age of 3 years old.[7] Therefore, alleviating the potential toxic effects of neonatal anesthesia during clinical practice is urgently important.

Sevoflurane (Sev) is one of the most widely used pediatric anesthetics. Previous studies have reported that neonatal exposure of Sev could lead to neural toxicity in multiple aspects, for example, apoptosis induction, ROS stress, neurogenesis impairment, synaptic degeneration, and even inflammation.[8–13] Multiple mechanisms have been disclosed to account for these toxic effects. For example, MicroRNA-188-3p contributes to Sev-induced neural apoptosis[14] and Nectin-1/corticotrophin-releasing hormone receptor type 1 signaling contributes to Sev-induced spine loss.[15] Our recent study revealed that HIPK2/JNK signaling underlies the long-term synaptic toxicity of neonatal repeated Sev exposure.[16] Therefore, developing novel interferences which target multiple toxic aspects of Sev is important for the safe application of Sev in clinic.

Melatonin is a natural hormone produced by pineal gland of vertebrates under the regulation of the central circadian pacemaker in the suprachiasmatic nuclei of the hypothalamus and conveys information about day length to body physiology.[17,18] The intracellular signal of melatonin is mainly mediated by MT1/MT2 receptors and downstream cAMP/PKA signaling.[19] Later studies demonstrated that melatonin has a wide range of functions, mostly beneficial for the survival or for keeping the homeostasis of the organism with very few side effects.[20–22] Application of melatonin has been proved to be protective under multiple neuropathological conditions.[23,24] Multiple intracellular pathways, such as BDNF and Hippo signaling, have been demonstrated to be responsive to melatonin stimulation and mediate the effects of melatonin.[25–27] Therefore, it is of great interest to test whether melatonin could be protective for neonatal Sev anesthesia.

In the present study, we reported our data that Sev pretreatment had remarkable neuroprotective effects on the long-term learning/memory impairment, synaptic toxicity, and dysmyelination induced by repeated neonatal Sev exposure, possibly via modulating canonical Wnt/β-catenin signaling through MT1 receptor.

2 | MATERIALS AND METHODS

2.1 | Animals

All the C57BL/6J male mice were bought from the animal center of the Fourth Military Medical University. MT1 receptor knockout mice were bought from Cyagen Biosciences (Stock No. KOCMP-17773-Mtnr1a-B6N). All animal experiments were carried out under protocols approved by the Animal Care and Use Committee of Fourth Military Medical University and according to "Policies on the Use of Animals and Humans in Neuroscience Research" revised and approved by the Society for

Neuroscience in 1995. 515 mice (at least 6 mice per group for each experiment) in total were used in the present study.

2.2 | Experimental design and mice treatments

Neonatal C57BL/6J mice at P6 were assigned to the following groups and treated. (1) Control group. Mice were treated with 60% oxygen (balanced with nitrogen) for 2 h/day for 3 consecutive days as described,[5,28] and DMSO were injected (i.p.) as control treatment. (2) Sev group. Mice were treated with 3% Sev (Cat #: H20110142) +60% oxygen (balanced with nitrogen) for 2 h/day for 3 consecutive days. (3) Sev + mel (melatonin, Sigma-Aldrich M5250) group. Mice were treated with melatonin (10 mg/kg) at 0.5 h before each anesthesia. (4) Sev + LiCl group. Mice were treated with LiCl (141.3 mg/kg, Sigma-Aldrich L4408) at 1h before each anesthesia. (5) Sev + mel + Lentivirus containing empty vector (EV) group. (6) Sev + mel + Lentivirus expressing shMT1 group. For MT1 receptor knockout mice, littermate control and MT1 receptor knockout mice were grouped as follows: (1) Sev treatment; (2) Sev + melatonin treatment. All treatments were carried out at 8:00–10:00 AM of the day. To prevent hypothermia, a warming blanket was used during anesthesia.

2.3 | Immunohistochemistry

For immunohistochemistry, mice were perfused intracardially with 4% paraformaldehyde phosphate buffer. Serial coronal sections were prepared and blocked by PBS containing 3% BSA and 0.3% Triton-X100 and then incubated with primary antibodies overnight at room temperature as the following: rabbit anti-Axin2 (Abcam, ab109307, 1:200), rat anti-MBP (Merck Millipore, MAB386, 1:500), mouse anti-MT1 receptor (Santa cruz, sc-390328, 1:50), mouse anti-NeuN (Abcam, ab104224, 1:600), rabbit anti-NG2, (Abcam, ab255811, 1:150), rabbit anti-GFAP (Abcam, ab207165, 1:500), mouse anti-Neurofliment (Abcam, ab7795, 1:200), rabbit anti-Olig2(Abcam, ab109186, 1:200), mouse anti-CC1(Sigma-Aldrich, OP80, 1:100), mouse anti-Flag (Sigma-Aldrich, F1804, 1:100), Rabbit anti-β-catenin (Abcam, ab16051, 1:200), Rabbit anti-vglut1 (Synaptic Systems Cat. No.135308, 1:200). After washing with PBS, corresponding secondary antibodies conjugated with Alexa Fluro 488 (donkey anti-rabbit, donkey anti-mouse, Jackson ImmunoResearch, 1:500, AB_2313584, AB_2338845) or Alexa Fluro 594 (donkey anti-rat, donkey anti-mouse,

donkey anti-rabbit, Jackson ImmunoResearch, Jackson 1:500, RRID:AB_2338372, AB_2338871, AB_2338059) were incubated with the sections for 2–4 h at room temperature protected from light. After washing with PBS, sections were counterstained with Hoechst33324 (1:1000, Sigma) for 20 min.

2.4 | Golgi staining

Mice were perfused with PBS at 8 weeks of age. Brains were dissected and immersed in Golgi staining solution which contained 50% potassium dichromate (MP 021563389), 5% mercuric chloride (Sigma-Aldrich M1136), and 5% potassium chromate (Sigma-Aldrich 529508) and protected from light for 7 days. When performing Golgi staining, brain sections were washed with distilled water, dehydrated with ethanol, and then treated with ammonia (3:1). The sections were subsequently washed and incubated in 5% sodium thiosulfate for 10 min and then dehydrated with degraded ethanol and clarified with xylene. In the end, the sections were observed under bright field of Olympus FV1000. The images were taken by z-stack scanning with the excitation wavelength of 405 nm, and the virtual color was converted into green color. For dendrites branching, Sholl analysis was conducted. For spine density, dendrites of pyramidal neurons in CA region were reconstructed and analyzed by using IMARIS.

2.5 | Electron microscopic study

Animals were perfusion fixed with a mixture of 4% paraformaldehyde containing 1% glutaraldehyde. Tissue sections of 50 μm were prepared with a vibratome and further fixed with 1% osmium tetroxide, dehydrated with graded ethanol, replaced with propylene oxide, and flat-embedded in Epon 812. Ultrathin sections (70–80 nm) were prepared on an LKB Nova Ultratome (Bromma). After being counterstained with uranyl acetate and lead citrate, the sections were examined under a JEM-1230 electron microscope (JEM). The thickness of myelin and diameter of axons were measured manually under electron microscopy. The myelin g-ratio was calculated as the ratio of the inner to the outer radius of the myelin sheath for a circular axon cross section as described.[29,30]

2.6 | Western blotting

Hippocampal tissues were homogenized in RIPA lysis buffer containing proteinase inhibitors cocktail. Protein

concentration was measured by BCA assay. Protein samples were separated by 10%–15% gel. After SDS-PAGE, protein was transferred to PVDF membrane. Membranes were blocked with TBS containing 5% non-fat milk and 0.1% Tween20 and then incubated with primary antibodies overnight at 4°C as the following: mouse anti-β-actin (sigma, MABT825, 1:10 000), rat anti-MBP (Merck Millipore, MAB386, 1:500), mouse anti-MT1 receptor (Santa cruz, sc-390328, 1:500), rabbit anti-MT2 receptor (Abcam, ab203346, 1:1000), rabbit anti-PKA(BioVision, #3115, 1:1000), rabbit anti-β-Catenin(CST, #8480, 1:1000), Axin2 (Abcam, ab32197, 1:1000), rabbit anti-p-GSK3β(T216) (Genetex, GTX79063 1:1000), rabbit anti-GSK3β (CST, #12456, 1:1000), PDGFRα(Santa cruz, sc-398206), rabbit anti-Olig2 (Proteintech, 1:1000), rabbit anti-NG2 (Proteintech, 55027-1-AP, 1:1000), goat anti-Sox10 (Santa cruz, sc-374170, 1:500). Mouse anti-GluR1(CST, #13185, 1:500), mouse anti-GluR2(CST, #13607, 1:500), rabbit anti-Homer (Abcam, ab97593, 1:1000), rabbit anti-PSD-95(Abcam, ab76115, 1:1000), mouse anti-Flag (Sigma-Aldrich, F1804, 1:1000), Rabbit anti-SFRP1(Abcam, ab267466, 1:1000), rabbit anti-vglut1(Cell Signaling Technology, #12331, 1:1000), rabbit anti-p-PKA (Thr197) (CST, #5661, 1:1000), rabbit anti-p-CREB(Ser133) (CST, #9198, 1:1000), rabbit anti-CREB (genetex rabbit 1:1000 GTX112846), mouse anti-Neurofilament (abcam, ab7795, 1:1000), rabbit anti-5-HT1A (Genetex, GTX104703, 1:1000), rabbit anti-5HT2C (genetex, GTX100329, 1:1000), rabbit anti-phosphorylated-β-Catenin (CST, #9561, 1:1000). After washing with TBST, membranes were incubated with HRP-conjugated anti-rabbit, HRP-conjugated anti-mouse, and HRP-conjugated anti-goat or anti-rat IgG (1:5000; Proteintech, Cat#:SA00001-2, Cat:#SA00001-1, Cat:#SA00001-4, Cat#:SA00001-15) for 1 h at room temperature. Bands were visualized with an ECL kit (Thermo, Cat#:32106). Images were analyzed by Image J.

2.7 | MT1 receptor shRNA preparation and treatment

For preparing lentivirus expressing MT1 receptor shRNA (shMT1), targeting sequence (CAGCCAA-GATGTTCTTT GTGG. gene ID: 17773) was cloned into LV3 vector. The virus was packaged by 293T cells (1×10^9 TU/ml, Genepharma, Co.), and the efficiency of gene silencing was testified by Western blotting. At P0/P1, 500 nl lentivirus expressing MT1 receptor shRNA or empty vector (EV) which express GFP was injected into bilateral hippocampus, as described.[31] The injection sites were from Lambda: 2 mm rostral, 0.7 mm lateral, and 1.8 mm ventral. The injection was verified by GFP expression. At P6-8,

mice were treated with Sev. The expression of synaptic proteins and oligodendrocyte markers in mice treated with shMT1 or EV was analyzed in adult.

2.8 | Protein interaction between MT1 receptor and β-catenin

Protein interaction between MT1 receptor and β-catenin was evaluated in hippocampal tissue and 293T cells transfected with plasmids expressing the C-terminal of MT1 receptor (MT1-C, 299–353 aa). Both hippocampal tissue and 293T cell homogenate were prepared using mild NP-40 (Nonidet P-40) buffer (PL004, Fei Yang Bio) with PMSF and subjected to immunoprecipitation after removing the insoluble material by centrifugation. For affinity enrichment, supernatants were incubated with anti-MT1 receptor antibody (Santa cruz, sc-390328, 1:50) or anti-Flag antibody (CST, #14793, 1:100) at 4°C overnight. Protein A/G agarose beads were added to each antibody conjugated sample, and samples were incubated at 4 °C for 4 h for immunoprecipitation. Subsequently, beads were washed five times with cold lysis buffer. Immunoprecipitates were finally eluted from the beads by adding 50 μl of SDS-PAGE sample buffer and verified by Western blotting of MT1 receptor (Santa cruz, sc-390328, 1:500) or Flag (CST, #14793, 1:500). Subsequently, Western blotting of β-catenin (CST, #8480, 1:1000) was performed to test protein interaction. To eliminate nonspecific interaction, a normal mouse IgG antibody (CST, #3420) was used as a negative control.

2.9 | Primary neuron culture and treatment

Hippocampal neurons were isolated from E15-17 mice. Briefly, a cesarean section was performed and the embryos were taken out carefully. The brains were dissected and put into a dish containing D-hanks buffer. We carefully dissected out the hippocampus, removed meninges, and cut the tissue into size of 1 mm³ and digested the tissue with 0.125% trypsin for 15 min. After trituration, cells were centrifuged for at 800 rpm for 5 min and then cultured in serum-free B27/neurobasal medium.

For sevoflurane exposure, sevoflurane was delivered from an anesthesia machine to a sealed plastic box in a 37°C incubator. An anesthetic gas monitor (Drager) was used to continuously monitor the concentrations of carbon dioxide, oxygen, and sevoflurane. At 2 days in vitro, neurons were treated with sevoflurane (21% O2, 5% CO2, and 4.1% sevoflurane) for six hours. At 14 days in vitro, neurons were collected for experiments.
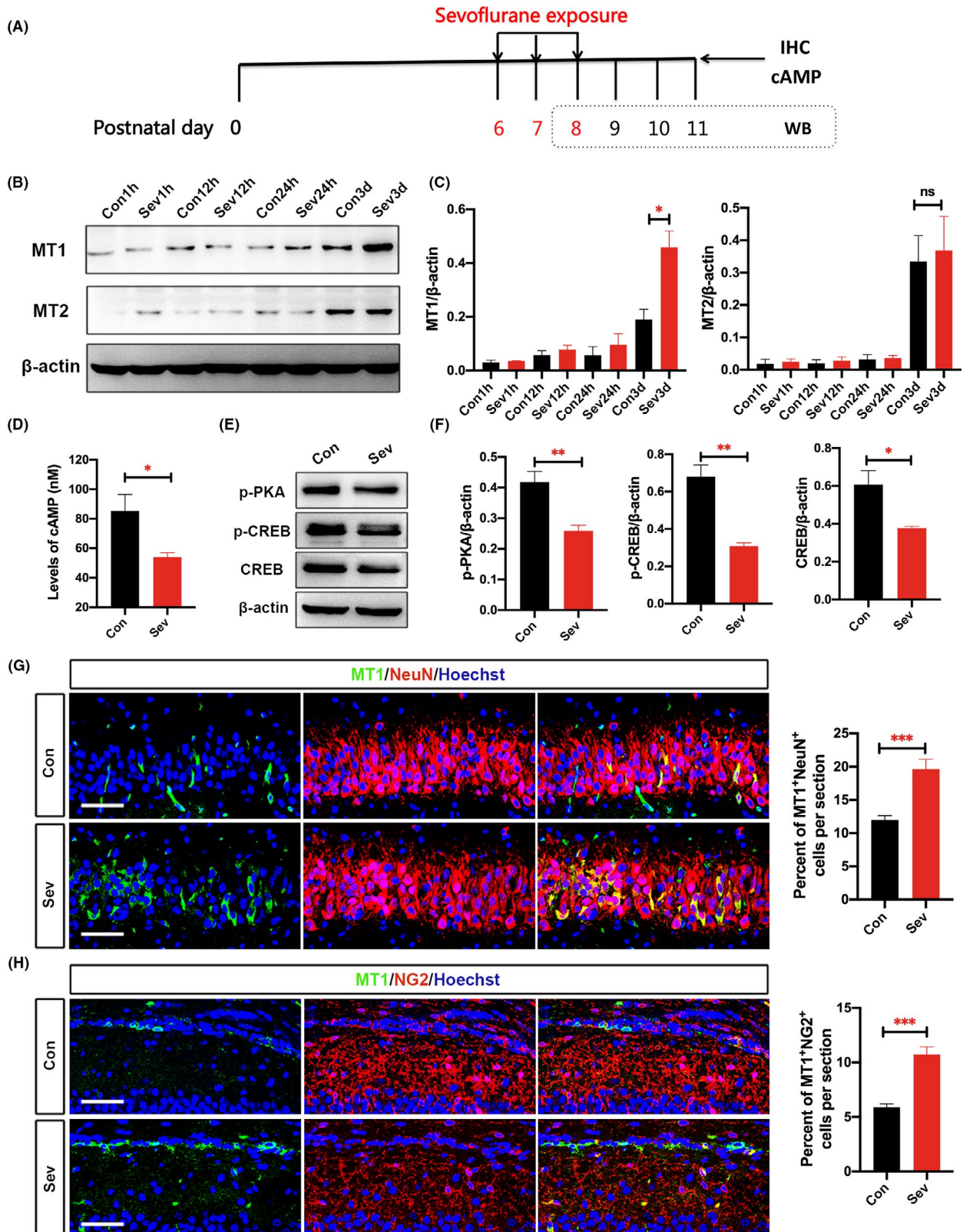
FIGURE 1 Acute effects of repeated neonatal Sev exposure on melatonin signaling in hippocampus. (A) Experimental design. (B, C) Western blotting and quantification of MT1 and MT2 receptors in the hippocampus of control and Sev-treated mice. MT1, but not MT2 receptor was up-regulated in Sev-treated mice at 3 dps. (D) Levels of cAMP in hippocampus of control and Sev-treated mice at 3 dps. (E, F) Western blotting and quantification of p-PKA, p-CREB, and CREB in the hippocampus of control and Sev-treated mice at 3dps. (G, H) Double immunostaining of MT1/NeuN and MT1/NG2 in the hippocampus of control and Sev-treated mice. Notice the expression of MT1 by neurons and oligodendrocyte precursors. $N = 6$ mice per group. Con, control. Sev, Sevoflurane. *$p < .05$. **$p < .01$. ***$p < .001$. One-way ANOVA (C). Student' t test (D, F–H). Bars = 50 µm

(A) Melatonin i.p. 10mg/kg — Sevoflurane exposure — Postnatal day 0, 6, 7, 8, 65-70 — Golgi staining, WB, patch clamp

(B) Con+Vehicle | Sev+Vehicle | Sev+mel

(C) Intersection number vs Distance from soma (um) — Con +Vehicle, Sev+Vehicle, Sev+mel

(D) Total dendrite length (um) — Apical, Basal

(E) Spine density/10um — Apical, Basal — Con+Vehicle, Sev+Vehicle, Sev+mel

(F) GluR1, GluR2, Homer, PSD95, β-actin — Con+Vehicle, Sev+Vehicle, Sev+mel

(G) GluR1/β-actin, GluR2/β-actin, Homer/β-actin, PSD95/β-actin

(H) Con+Vehicle, Sev+Vehicle, Sev+mel

(I) sEPSC Frequency (Hz)

(J) sEPSC Amplitude (pA)

(K) Con+Vehicle, Sev+Vehicle, Sev+mel

(L) EPSC amplitude (pA) vs Stim. intensity (mA) — Con+Vehicle, Sev+Vehicle, Sev+mel

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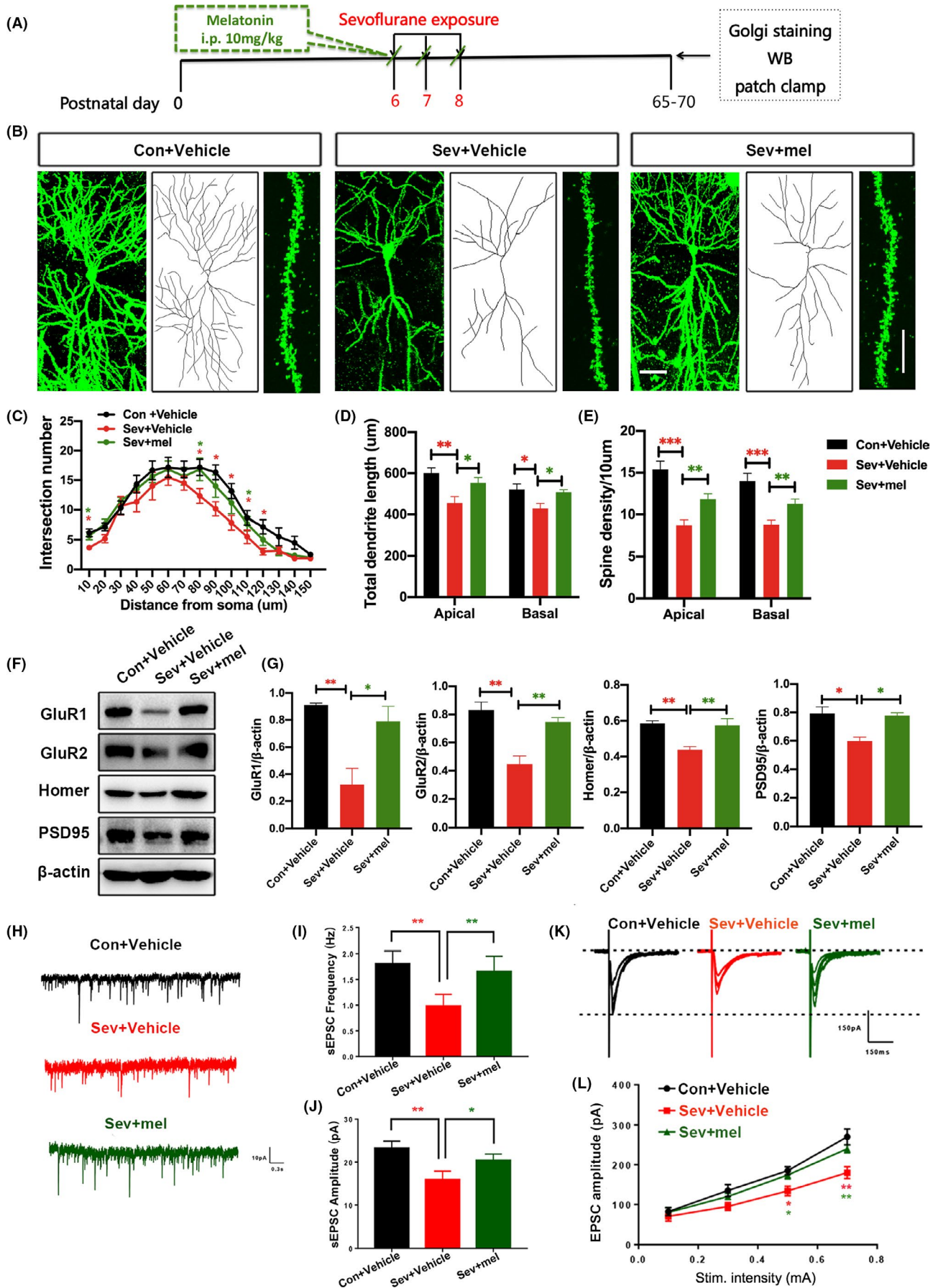Clinical Aspects of Melatonin

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FIGURE 2 Long-term effects of melatonin pretreatment on the synaptic deficits induced by neonatal Sev exposure. (A) Experimental design. (B) Representative images of Golgi staining in adult control mice, Sev-treated mice, and Sev-melatonin-treated mice. (C–E) Quantification of Sholl analysis of Golgi staining. Notice the recovery of dendritic length, dendritic branches, and spine density in Sev-melatonin-treated mice. (F, G) Western blotting and quantification of GluR1, GluR2, Homer, and PSD-95. Notice the decrease in GluR1, GluR2, Homer, and PSD-95 in Sev-treated mice and recover of these proteins in Sev-melatonin-treated mice. (H–J) Patch-clamp recording of sEPSC and quantification. Notice the decrease in frequency and amplitude of sEPSC in Sev-treated mice, and increase in frequency and amplitude of sEPSC in melatonin-pretreated mice. (K, L) Patch-clamp recording of AMPA receptor-mediated EPSC and quantification. Notice the effects of Sev treatment and Sev-melatonin treatment on the amplitude of AMPA receptor-mediated EPSC. $N = 6$ mice per group in B–G. $N = 7$ neurons per group in H–L. Con, control. Sev, Sevoflurane. mel, melatonin. *$p < .05$. **$p < .01$. ***$p < .001$. One-way ANOVA. Bars = 20 μm (B, left panel), 10 μm (B, right panel)

2.10 | Morris water maze test

Water maze was made by a round container with the diameter of 120 cm and the wall of 40 cm. The water maze was divided into 4 quadrants. The objective platform was placed 1.5 cm under water. For training, mice were put into water randomly in one quadrant facing the wall. The time when mice found the platform was recorded as escaping time. The mice which could not find the platform within 60 s were guided to the platform for learning. Mice were trained 4 times per day for 4 days. One hour later after training in the fourth day and in the fifth day, mice were let swim freely in the maze. The total time mice stayed in the platform quadrant and the crossing times in the platform quadrant were recorded. The swimming traces and the escaping behavior were recorded using the software SMART 3.0.

2.11 | Novel object recognition test

Day 1, mice were put into the open field (40 × 40 × 35 cm) for 10 min free exploration. Day 2, mice were first put into the same open field with two identical Lego blocks for 10 min exploration. One hour later, one Lego block was replaced with a novel block which had different shape, and the mice were put back for 5 min exploration. Day 3, mice were tested exactly the same as Day 2 except that the novel block was replaced by another one the mice never met before. The ratio of exploration time on novel object and old object was calculated.

2.12 | Fear Conditioned memory

All mice were pre-exposed to the startle chambers (SanDiego Instruments) 3 days before training. During cued fear training, mice received five paired conditioned stimulus (CS) tones (30 s, 6 or 12 kHz, 90 db) and unconditioned stimulus (US) shock (500 ms, 1.0 mA) trials with a 5-min interval. Startle response to the shocks and

percentage of time spent freezing to the tones were measured by Xmaze software (XinRuan informatics Co.).

2.13 | Patch-clamp recording

Brain was removed from mice (6–8 weeks old) and kept in pre-oxygenated ice-cold Krebs solution. Transverse slices (200–300 μm) were cut on a vibrating microslicer. The slices were perfused with Kreb's solution that was saturated with 95% O_2 and 5% CO_2 at 36 ± 1°C for at least 1–3 h prior to experiment. The Kreb's solution contains (in mM): NaCl 117, KCl 3.6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11. The whole-cell patch-clamp recordings were made from the pyramidal neurons of the CA1 region in voltage-clamp mode. Patch pipettes were fabricated from thin-walled, borosilicate, glass-capillary tubing (1.5 mm o.d., World Precision Instruments). After establishing the whole-cell configuration, neurons were held at the potential of −70 mV to record sEPSCs. The internal solution contains (in mM) potassium gluconate 135, KCl 5, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, HEPES 5, and ATP 5. Membrane currents were amplified with an Axopatch 700B amplifier (Axon Instruments) in voltage-clamp mode. Signals were filtered at 2 kHz and digitized at 5 kHz. Data were stored with a personal computer using pCLAMP 10 software and analyzed with Mini Analysis (Synaptosoft Inc.).

2.14 | Statistical analysis

All behavior analyses and statistics were performed by an investigator who was blinded to experimental design. No sample calculation was performed. At least 8 mice were included in each group for behavior assay. Each behavior test was conducted using distinct groups of animals. Data were presented as the mean ± standard error. Normal distribution was assessed by Shapiro-Wilk test. Statistical comparisons were made using Student's t test
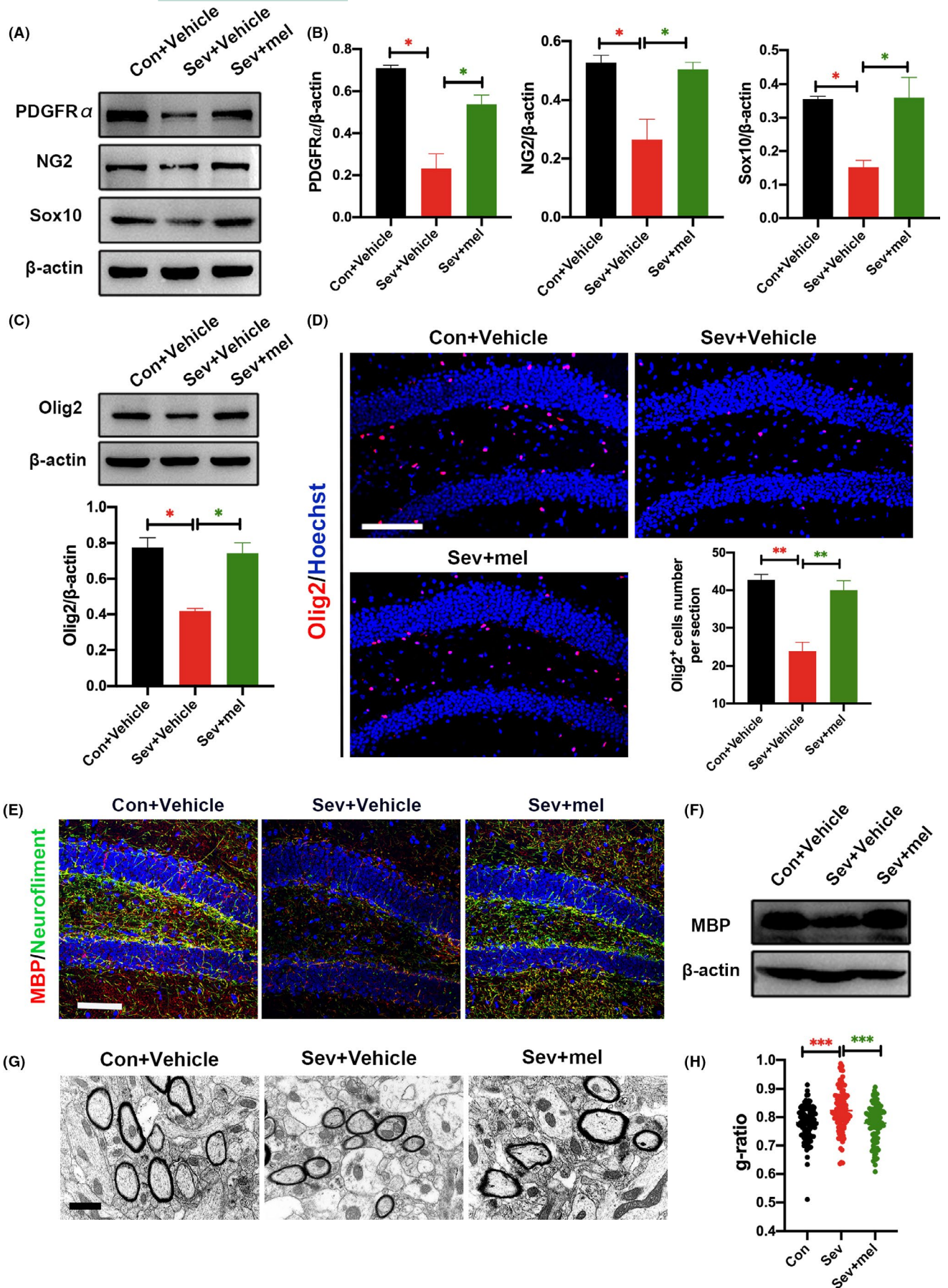
FIGURE 3 Protective effects of melatonin pretreatment on dysmyelination induced by neonatal Sev exposure. (A, B) Western blotting and quantification of PDGFRα, NG2, and Sox10 in the hippocampus of adult control, Sev-treated, and Sev-melatonin-treated mice. Notice the decrease in PDGFRα, NG2, and Sox10 in Sev-treated mice and restoration of these proteins in melatonin-pretreated mice. (C, D) Western blotting and immunostaining of Olig2 in the hippocampus of adult control, Sev-treated, and Sev-melatonin-treated mice. (E, F) Immunostaining and Western blotting of MBP in the hippocampus of adult control, Sev-treated, and Sev-melatonin-treated mice. Melatonin pretreatment restored the expression of Olig2 and MBP. (G, H) EM images of myelin and the g-ration in the hippocampus of adult control, Sev-treated, and Sev-melatonin-treated mice. Notice the decrease in myelin thickness in Sev-treated mice and the recovery of myelin thickness in melatonin-pretreated mice. $N = 6$ mice per group. Con, control. Sev, Sevoflurane. mel, melatonin. *$p < .05$. **$p < .01$. ***$p < .001$. One-way ANOVA. Bars = 100 μm (D), 50 μm (E), 1 μm (G)

or one-way ANOVA with Student Newman-Keuls post hoc analysis. p value <.05 was considered as statistical significant.

3 | RESULTS

3.1 | Repeated neonatal Sev exposure induces response of melatonin signaling in hippocampal neurons and oligodendrocytes

We first examined the response of melatonin signaling to Sev exposure by evaluating the expression of melatonin receptors and their intracellular signaling in hippocampus. Neonatal mice were exposed to 3% Sev for 3 consecutive days (P6-P8). The levels of melatonin receptors MT1 and MT2 were examined after Sev exposure (Figure 1A). Both MT1 and MT2 increased gradually in control mice from P8 to P11 (Figure 1B,C). Interestingly, in comparison with that in control group, the levels of MT1, but not MT2, significantly increased in experimental group at 3 day post the last Sev treatment (dps, Figure 1B,C). Next, we examined the levels of cAMP, PKA, and CREB, key downstream signaling components of MT1.[19] Significant decrease in p-PKA, cAMP, and p-CREB was observed at 3 dps (Figure 1D–F), consisting with previous observation that Sev treatment induced acute inhibition of cAMP signaling,[32] and also indicating the activation of MT1 signaling. Double immunostaining of MT1 with different neural cell markers showed that, under normal condition, approximately 78% MT1-positive cells in the hippocampus were NeuN-positive and approximately 20% were NG2-positive (Figure S1A,B). Very few astrocytes or other cells express MT1. In mice treated with Sev, significantly more MT1/NeuN- and MT1-NG2-positive cells were observed as compared with that in control mice (Figure 1G,H). To verify the specificity of the anti-MT1 antibody, antigen absorption assay was conducted. 40 μg/ml MT1 peptide (sc-390328P, Santa Cruz biotechnology) almost completely eliminated the immunoreactivity of MT1 in hippocampus (Figure S1C). These data indicated that

Sev treatment induces a delayed response of MT1 signaling in the hippocampal neurons and oligodendrocytes.

3.2 | Melatonin pretreatment alleviates the long-term toxic effects of repeated neonatal Sev exposure

Our previous study has demonstrated a long-term synaptic toxicity of Sev.[16] We tested whether melatonin could rescue this toxic effect of Sev. Considering that in clinic, preoperative melatonin has been adopted as sedative,[33] we administered melatonin at 10 mg/kg as this dosage had been used in rats without affecting diurnal rhythms and been adopted in mice studies.[34–36] Melatonin was given at 30 min before each Sev exposure, and the long-term effects were evaluated in adult mice (Figure 2A). Golgi staining showed that melatonin pretreatment significantly increased the branching of dendrites in pyramidal neurons as compared with Sev-treated mice (Figure 2B,C). In melatonin-pretreated mice, the total length of dendrites recovered to a similar level of control mice (Figure 2D). In addition, both the apical and basal spine density significantly increased in melatonin-pretreated mice, as compared with that in mice treated with Sev alone (Figure 2E). The expression of AMPA receptors (GluR1, GluR2) and synaptic skeleton proteins (Homer, PSD-95) decreased significantly in Sev-treated mice, and this reduction was all restored in melatonin-pretreated group (Figure 2F,G). Time-matched studies revealed that the lowering of GluR1 and PSD95 appeared from 14 dps, which was reversed by melatonin (Figure S2A). Furthermore, patch-clamp recording showed that Sev treatment resulted in significant reduction in both the frequency and amplitude of spontaneous excitatory post synaptic currents (sEPSC) in the hippocampal neurons (Figure 2H–J), which was restored by melatonin pretreatment. In addition, melatonin pretreatment also restored the amplitude of AMPA receptor-mediated EPSCs to a level even similar to normal control (Figure 2K,L). These data indicated that melatonin pretreatment has remarkable long-term
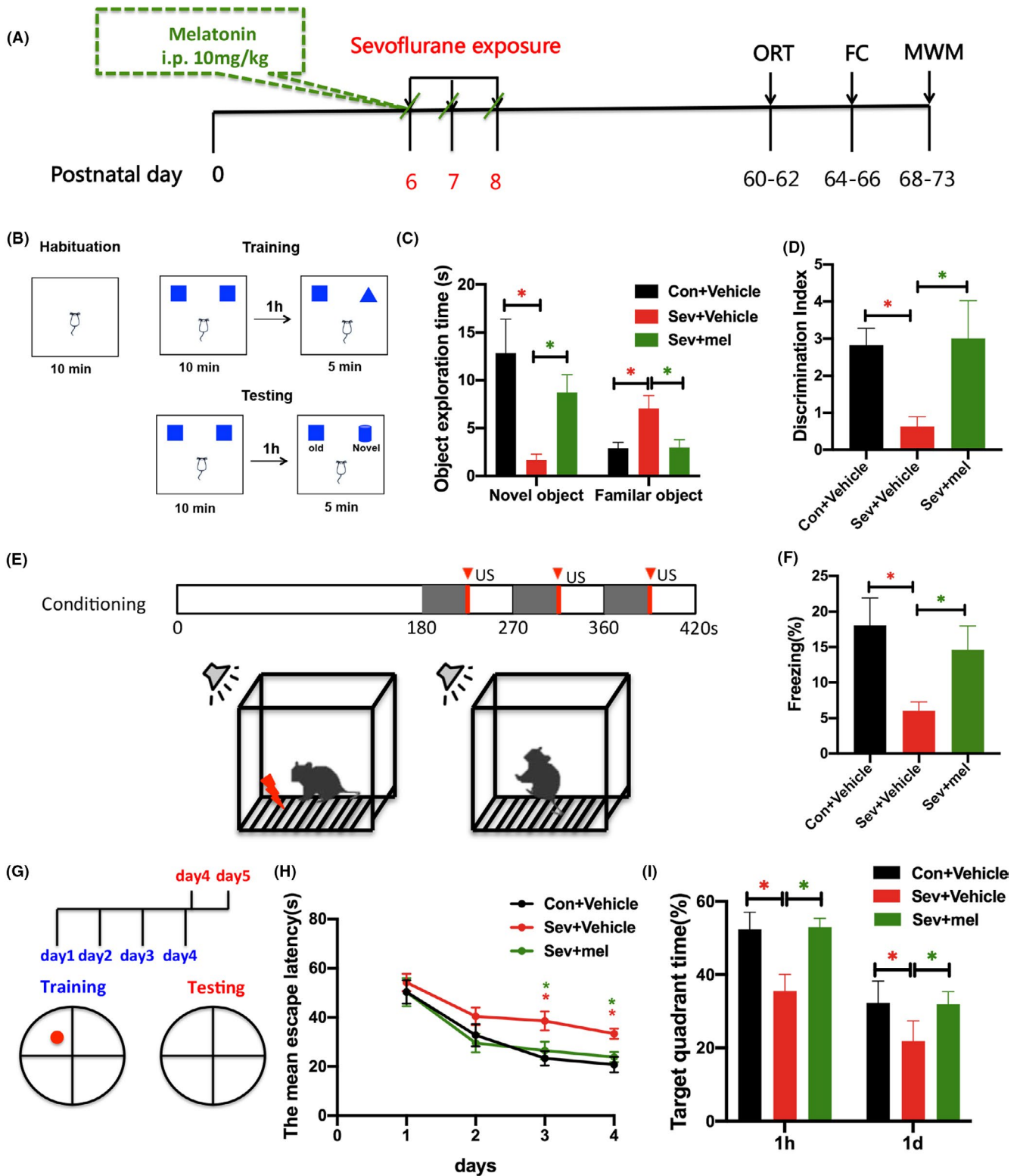
FIGURE 4 Beneficial effects of melatonin pretreatment on learning and memory in mice exposed to Sev at neonatal. (A) Experimental design. (B–D) Novel object recognition test. Melatonin pretreatment significantly increased the ability of novel object recognition, as compared with Sev-treated mice. (E, F) Fear conditioned memory. Notice the impairment of fear memory in Sev-treated mice and recovery of fear memory in melatonin-pretreated mice. (G–I) Morris water maze test of control mice, and Sev-treated, and Sev-melatonin-treated mice. Con, control. Sev, Sevoflurane. mel, melatonin. ORT, novel object recognition test. FC, fear conditioning test. MWM, Morris water maze test. $N = 8$ mice per group. *$p < .05$. One-way ANOVA

protective effects on the synaptic toxicity of repeated neonatal Sev treatment.

Because MT1 receptor was also up-regulated in NG2-positive oligodendrocyte precursors and chronic dysmyelination had been reported following Sev treatment,[37–39] we next examined whether melatonin could alleviate the toxic effects of Sev on oligodendrocytes. Sev treatment significantly inhibited the expression of PDGFRα, NG2, and Sox10 (markers of oligodendrocyte precursors), Olig2 (marker of cell fate determined oligodendrocyte), and MBP (marker of mature oligodendrocyte) (Figure 3A–F). Melatonin pretreatment significantly rescued the expression of PDGFRα, NG2 Sox10, Olig2, and MBP (Figure 3A–F, Figure S2B). Furthermore, electron microscopic study revealed that melatonin pretreatment significantly attenuated Sev-induced dysmyelination, as showed by g-ratio analysis (Figure 3G,H.). In addition, the levels of neurofilament were lower in Sev-treated mice and recovered in Sev-melatonin-treated mice, as compared with normal control (Figure S2C). These data are in line with the view that axon-myelin interaction provides mutual trophic support to each other.[40,41]

Next, we examined the effects of melatonin pretreatment on animal' learning ability (Figure 4A). In novel object recognition test which assessed animal's recognition memory, Sev-treated mice spent approximately 70% less time exploring novel object as compared with control mice. Melatonin pretreatment significantly prolonged the time exploring the novel object and the discrimination index (Figure 4B–D). In fear conditioning test which measured associative aversive learning and memory (Figure 4E), Sev-treated mice showed significantly less frequency of freezing upon conditioned stimulation, as compared with control. Mice pretreated with melatonin showed significant increase in freezing than the mice treated with Sev alone (Figure 4F). In Morris water maze test which evaluated spatial memory (Figure 4G), mice pretreated with melatonin used significantly less time for escaping after 3 d's training (Figure 4H). In addition, mice pretreated with melatonin spent significantly longer time in the targeted quadrant at 1 and 24 h after 4 d's training than the mice treated with Sev alone (Figure 4I). These data demonstrated that melatonin pretreatment effectively alleviated the long-term memory impairment induced by repeated neonatal Sev exposure.

3.3 | Melatonin exerts its protective effects through MT1 receptor

We next examined whether MT1 receptor mediated the protective effects of melatonin. Lentivirus expressing MT1-shRNA was injected into bilateral hippocampus of neonatal mice at P0 (Figure 5A). The efficiency of MT1-shRNA was analyzed at P11. Among three MT1-shRNAs, MT1-shRNA2 was most effective in silencing the expression of MT1 (Figure 5B,C). MT1-shRNA2(shMT1) was thus adopted in the following experiments. ShMT1 significantly blocked the rescuing effects of melatonin on the expression of GluR1, GluR2, Homer, PSD95, and vglut1 (Figure 5D,E). These data indicated that MT1 receptor might contribute to the protective effects of melatonin on synaptic development.

As to the changes of oligodendrocytes, shMT1 significantly compromised the up-regulation of PDGFRα, Sox10, NG2, and Olig2 in mice pretreated with melatonin (Figure 5F,G). These data indicated that MT1 might also mediate the protective effects of melatonin on myelination.

To further confirm the roles of MT1 receptor in this process, we evaluated the outcome of melatonin treatment in MT1 receptor knockout mice (MT1-KO) which had been exposed to Sev at their neonatal stage (Figure 6A). Western blotting confirmed the depletion of MT1 (Figure S3). Mutation of MT1 receptor significantly compromised the melatonin-induced expression of GluR1, GluR2, PSD-95, and vglut1 (Figure 6A–D). In regard of oligodendrocyte markers, significant lower levels of PDGFRα, Sox10, and Olig2 were detected in MT1 deficient mice treated with melatonin as compared with WT mice treated with melatonin (Figure 6E–G).

We next assessed the behavior of adult MT1 receptor mutant mice which were subjected to Sev-vehicle or Sev-melatonin treatment at neonatal (Figure 7A). In novel object exploration assay, no difference was found between these two groups in regard of the time exploring the novel object and the discrimination index (Figure 7B,C). In Morris water maze test, MT1-KO mice treated with Sev-melatonin showed similar escaping latency and spent similar time staying at the target quadrant as MT1-KO mice treated with Sev-vehicle did (Figure 7D–F).

3.4 | Neonatal Sev exposure leads to long-term suppression of Wnt signaling in hippocampal neurons and oligodendrocytes

To explore the underlying mechanism, we examined whether the change of above mentioned intracellular signaling lasted to adult. From 7 dps, there was no significant difference of PKA and CREB in the hippocampal of Sev-treated and control mice (Figure 8A,B), indicating a transient change of PKA signaling.
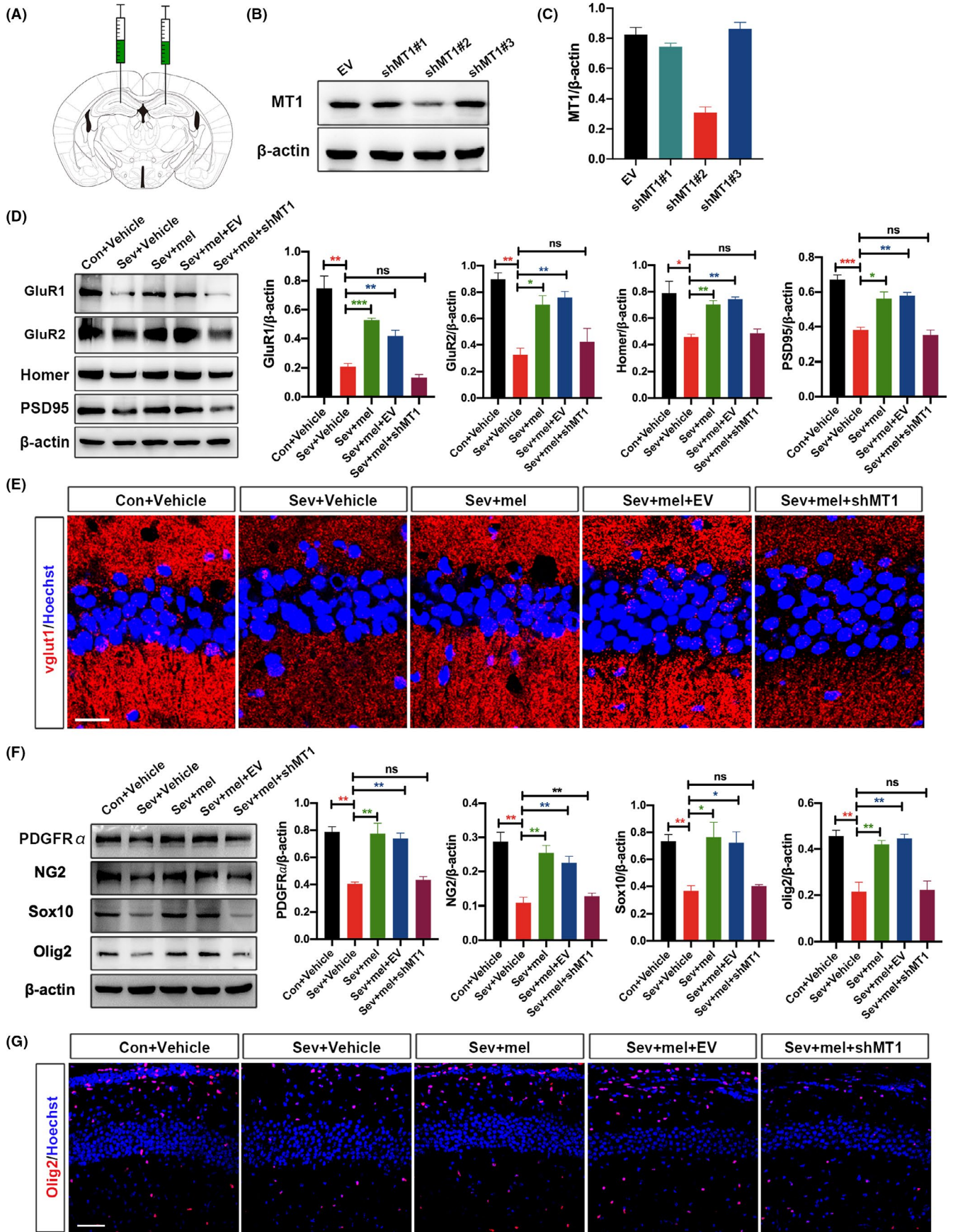
FIGURE 5 Blockage of the protective effects of melatonin on Sev-induced synaptic impairment and dysmyelination by MT1-shRNA (shMT1). (A) Illustration of injection of lentivirus expressing shMT1. (B, C) Western blotting and quantification of MT1 in hippocampus injected with different shMT1. ShMT1-#2 was most effective in silencing the expression of MT1 (D) Western blotting and quantification of GluR1, GluR2, Homer, and PSD95 in mice of the following groups: control + vehicle, Sev + vehicle, Sev + mel, Sev + mel + EV (EV, empty vector), Sev + mel + shMT1. Notice that shMT1 blocked the up-regulation of GluR1, GluR2, Homer, and PSD95 by melatonin. (E) Immunostaining of vglut1. Notice the weak immunoreactivity of vglut1 in mice treated with shMT1 as compared with that in mice treated with EV. (F) Western blotting and quantification of PDGFRα, NG2, Sox10, and Olig2 in mice of the following groups: control + vehicle, Sev + vehicle, Sev + mel, Sev + mel + EV, Sev + mel + shMT1. Notice that shMT1 blocked the up-regulation of PDGFRα, NG2, Sox10, and Olig2 induced by melatonin. (G) Immunostaining of Olig2. $N = 6$ mice per group. Con, control. Sev, Sevoflurane. mel, melatonin. $*p < .05$. $**p < .01$. $***p < .001$. One-way ANOVA

To dissect the long-lasting changes of intracellular signaling, we then performed RNA-seq analysis of the hippocampal tissue in adult mice which were either exposed to Sev or to Sev-melatonin at neonatal stage. We noticed that SFRP1 (soluble frizzled-related proteins 1), a Wnt signaling inhibiting peptide, was in the list of the top 10 up-regulated genes in Sev-treated mice as well as the top 10 recovered genes in Sev-melatonin-treated mice (Figure 8C). In addition, Wnt signaling pathway was also enriched by KEGG analysis, indicating that Wnt signaling was changed (Figure 8D). Western blotting confirmed the up-regulation of SFRP1 by Sev (Figure 8E). The levels of total and nuclear β-catenin, the core intracellular component of canonical Wnt signaling, were significantly reduced (Figure 8F,G) Consistently, the levels of p-β-catenin (Ser33/37/Thr41) and p-GSK-3β (T216), which were involved in the degradation of β-catenin, were significantly increased in mice exposed to Sev at neonatal (Figure 8F, Figure S4A). Both the protein and mRNA of Axin2, a faithful target gene of canonical Wnt signaling, were significantly down-regulated (Figure 8H,I). More importantly, Axin2 was mainly expressed by NeuN-positive neurons and CC-1 positive oligodendrocytes under normal condition (Figure 8J). Detailed time points analysis showed that levels of β-catenin, p-GSK-3β, and Axin2 changed from 7 dps (Figure S4B). Taken together, these data indicated the neonatal Sev treatment leads to a long-term suppression of Wnt/β-catenin signaling in hippocampal neurons and oligodendrocytes, which might be modulated by melatonin.

3.5 | Activating Wnt signaling rescues Sev-induced synaptic impairment and dysmyelination

We next investigated whether activating Wnt signaling could rescue Sev-induced synaptic impairment. We administered LiCl, an antagonist of GSK3β which could activate Wnt signaling, at a dose of 141.3mg/kg as described[42,43] immediately after each Sev exposure (Figure 9A). LiCl treatment restored the levels of β-catenin, p-GSK3β, and Axin2 (Figure S5A). Sev-LiCl treatment significantly increased the number of dendritic branching (Figure 9B,C), as compared with that in mice treated with Sev alone. The total dendrites length and spine density were increased in Sev-LiCl treated mice as well (Figure 9D,E). In regard of glutamate receptors and synaptic proteins, LiCl treatment significantly enhanced the expression of GluR1, GluR2, Homer, and PSD-95 (Figure 9F,G).

We then examined the effects of LiCl treatment on the Sev-induced dysmyelination. The levels of MBP and neurofilament in LiCl-treated mice recovered to the similar level as that of normal control (Figure 9H,I, Figure S5B–C). Furthermore, the thickness and integrity of myelin increased significantly in Sev-LiCl treated mice, as compared with that in mice treated with Sev alone (Figure 9J,K).

To evaluate the effects of LiCl on Sev-induced memory impairment, we assessed spatial memory and novel object recognition at 2 month after Sev and Sev-LiCl treatment (Figure 10A). In Morris water maze test (Figure 10B), Sev-LiCl treated mice displayed significantly reduction in escape latency (Figure 10C) and spent significantly longer time in the targeted quadrant than the Sev-LiCl treated mice did (Figure 10D). In novel object recognition test, Sev-LiCl treated mice exhibited significant increase in novel object exploration and better discrimination index than the Sev-treated mice (Figure 10E,F). These data indicated that that activating canonical Wnt/β-catenin signaling could rescue the Sev-induced synaptic deficits and dysmyelination.

Previous studies have demonstrated that high dose of melatonin could, in some circumvents, activate 5-HT signaling,[44] or had anti-oxidant effects.[45] We next assessed the 5-HT signaling and oxidation/redox levels in hippocampus following Sev and Sev-melatonin treatment. The results showed that there was no change of 5-HT1A, 5-HT2C, and PLC at both 3 days and 60 dps (Figure S6A–E). Reactive oxygen species (ROS) and malondialdehyde (MDA) transiently increased in Sev-treated mice at 3 dps (Figure S6F,G). Total anti-oxidant activity (T-AOC)
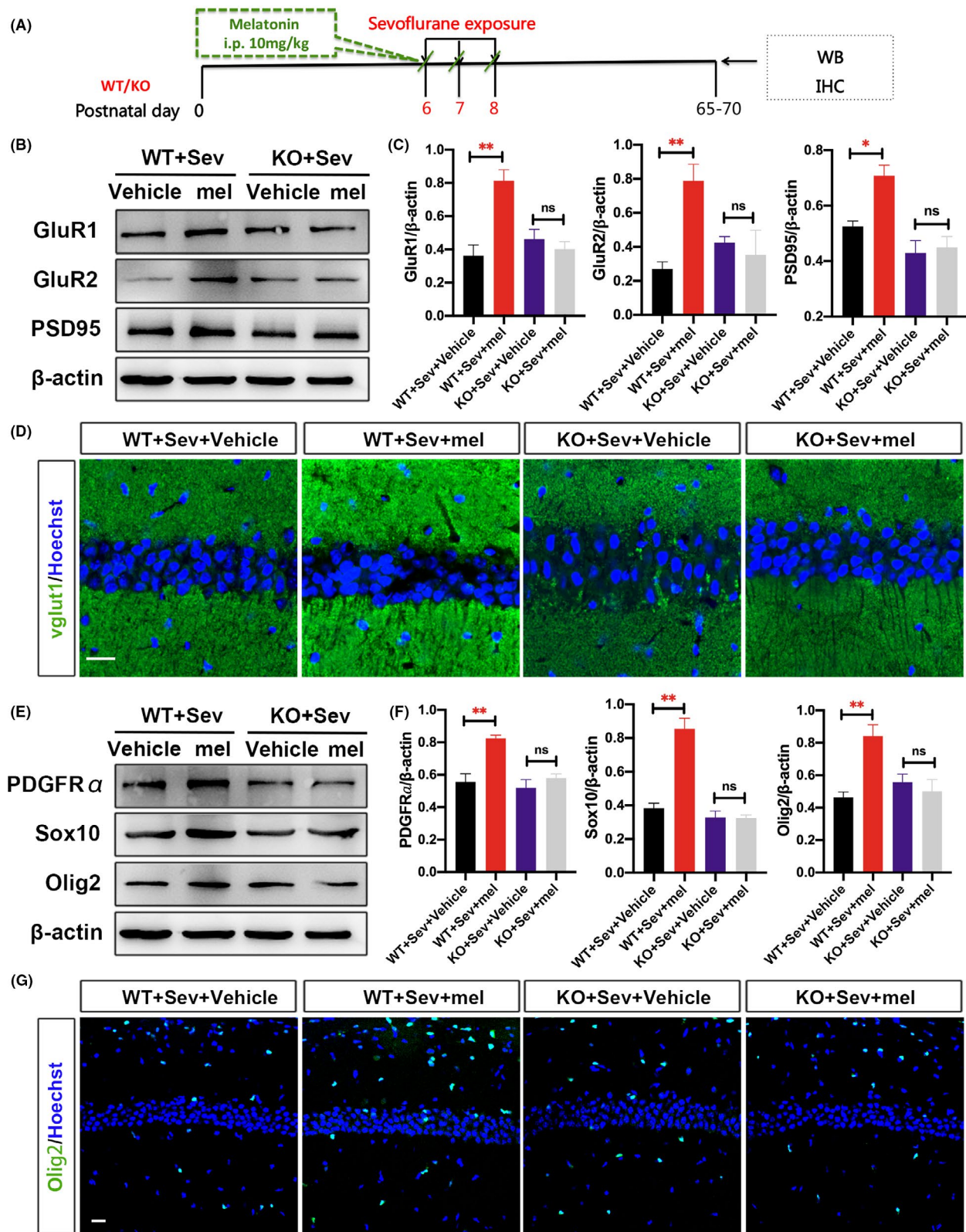
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FIGURE 6 Compromising the protective effects of melatonin on Sev-associated toxicity by MT1 depletion. (A) Experimental design. (B, C) Western blotting and quantification of GluR1, GluR2, and PSD95 in WT and MT1 knockout mice treated with Sev or Sev-melatonin. (D) Immunostaining of vglut1. Melatonin did not exert protective effects on the expression of GluR1, GluR2, PSD95, and vglut1 in MT1-KO mice. (E, F) Western blotting and quantification of PDGFRα, Sox10, and Olig2 in WT and melatonin knockout mice treated with Sev or Sev-melatonin. (G) Immunostaining of Olig2. Melatonin did not exert significant effects on the expression of PDGFRα, Sox10, and Olig2 in MT1 knockout mice. $N = 6$ mice per group. Con, control. Sev, Sevoflurane. mel, melatonin. WT, wild type. KO, MT1 knockout. $*p < .05$. $**p < .01$. One-way ANOVA. Bars = 20 μm
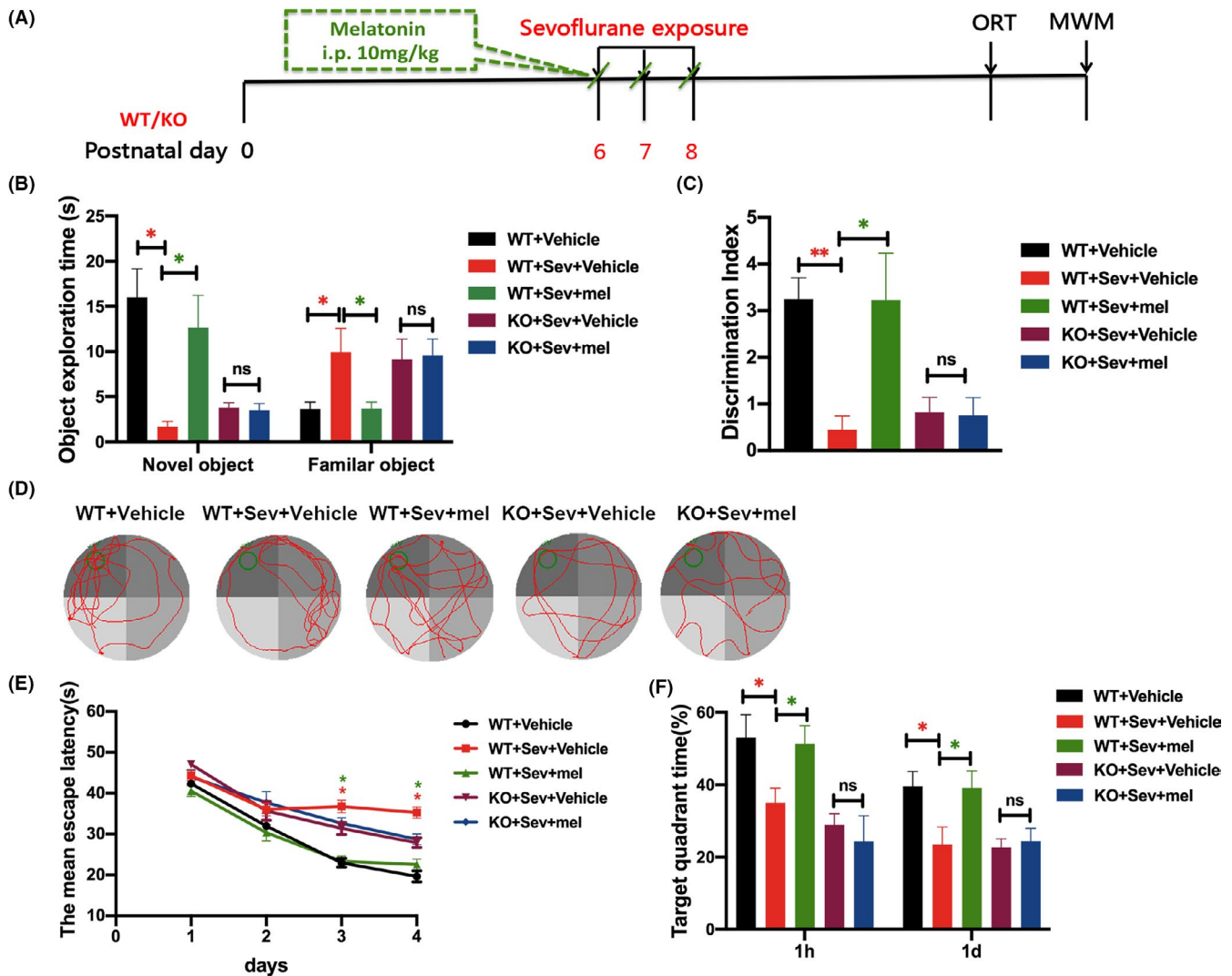
FIGURE 7 Roles of MT1 knockout in the memory promoting effects of melatonin on Sev-treated mice. (A) Experimental design. (B, C) Novel object recognition assay. Notice that MT1 knockout mice treated with either Sev or Sev-melatonin exhibited similar novel object recognition time and similar discrimination index. (D–F) Water Morris maze. MT1 knockout mice treated with either Sev or Sev-melatonin showed similar escaping time and spent similar time in the target quadrant. $N = 8$ mice per group. Con, control. Sev, Sevoflurane. mel, melatonin. WT, wild type. KO, MT1 knockout. $*p < .05$. $**p < .01$. One-way ANOVA

and total superoxide dismutase (T-SOD) exhibited no change following Sev exposure. Melatonin pretreatment exerted no significant effects on the levels of MDA, T-AOC, and T-SOD at all time points examined, except for a reduction in ROS levels at 3 dps (Figure S6G–I). These data precluded the involvement of 5-HT signaling and anti-oxidation in the protective effects of melatonin we observed.

3.6 | MT1 receptor interacts with β-catenin and regulates Wnt signaling

We next investigated whether there were any crosstalk or interaction between melatonin and Wnt/β-catenin signaling. As above, neonantal mice (P6-8) were exposed to Sev treatment. At 7 day following last Sev treatment, mice were sacrificed and double immunostaining was conducted. The results showed that MT1 receptor co-expressed well with β-catenin and Axin2 in the hippocampus under normal condition (Figure 11A,B, left panels). Upon Sev treatment, the number of MT1/β-catenin-positive cells and MT1/Axin2-positive cells decreased significantly (Figure 11A,B, right panels), consisting with above observation. Because β-catenin also localized at cell membrane, we tested whether there were interaction between MT1 receptor and β-catenin. Protein co-immunoprecipitation (CO-IP) assay demonstrated a strong interaction between MT1 and β-catenin (Figure 11C). Sev treatment dramatically reduced the

MT1/β-catenin interaction (Figure 11C). This MT1/β-catenin interaction was diminished in MT1-KO mice (Figure S7A). Since melatonin binds to the extracellular N terminal of MT1, we tested whether MT1 could bound β-catenin with its intracellular C-terminal (MT1-C). 293T cells were transfected with plasmids expressing MT1-C-Flag to test this possibility (Figure S7B). MT1-C-Flag co-localized well with β-catenin at cell membrane (Figure 11D). CO-IP assay revealed strong interaction between MT1-C with β-catenin (Figure 11E). Interestingly, in MT1-C overexpressing cells, the protein level of Axin2 was significantly increased while that of SFRP1 significantly decreased (Figure 11F). The effects of MT1-C on the expression of Axin2 and SFRP1 were effectively abolished by shMT1 (Figure 11G), indicating a potential role of MT1 receptor in facilitating Wnt signaling. To explore if MT1/β-catenin interaction could contribute to Wnt signaling activation, we stimulated MT1-overexpressing cells with melatonin. Immunocytochemistry showed that β-catenin shifted quickly from cytoplasm/membrane to nucleus (Figure 11H), suggesting that internalized MT1 receptor might help the nuclear translocation of β-catenin. Altogether, these data demonstrated a novel interaction between MT1 receptor and β-catenin and suggested this interaction might regulate Wnt/β-catenin signaling.

Importantly, in vivo experiments further demonstrated that melatonin pretreatment promoted the MT1/β-catenin interaction, as compared with that in Sev-treated mice (Figure 12A). Higher levels of β-catenin were detected in Sev-mel treated mice, as compared with that in mice treated with Sev alone (Figure 12B,C). Sev-mel treatment significantly reduced the levels of SFRP1 and p-GSK-3β (T216) (Figure 12B,C). Furthermore, both Western blotting and immunohistochemistry revealed restoration of Axin2 by melatonin pretreatment (Figure 12D,E). All these melatonin-induced changes of Axin2, β-catenin, and p-GSK-3β were diminished in MT1-KO mice (Figure 12F). These data demonstrated that melatonin pretreatment could restore Wnt signaling in Sev-treated mice.
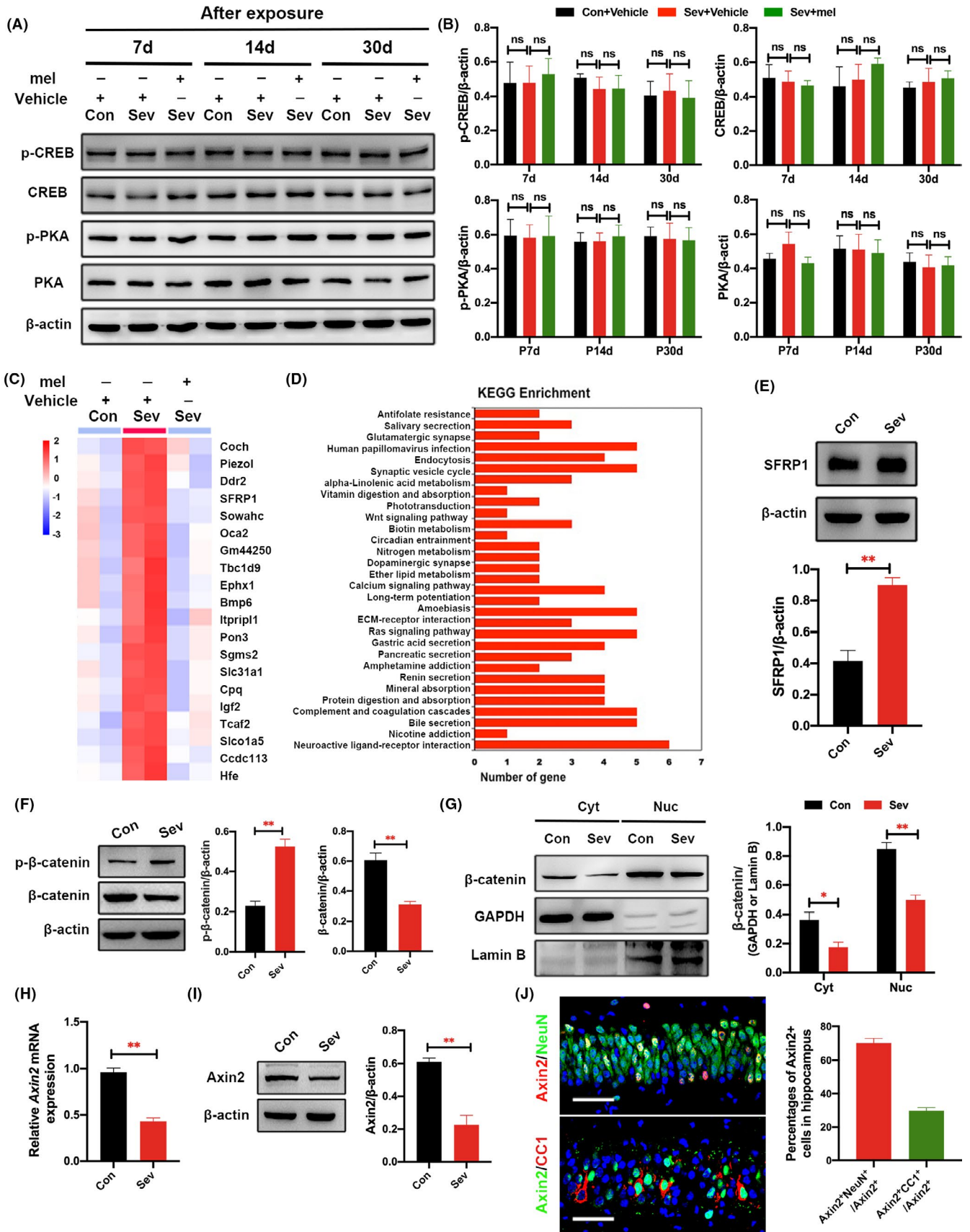
To further investigate the roles of canonical Wnt signaling in the protective effects of melatonin, we treated primary neurons with Sev, Sev-melatonin, Sev-melatonin-XAV939 (an Axin2 stabilizer which acts as an inhibitor of Wnt signaling), or Sev-XAV939 and assessed synaptic development. Two uM XAV939 significantly blocked the up-regulation of β-catenin in melatonin-treated cells (Figure S8A). The up-regulation of GluR1, GluR2, Homer, PSD95, and vglut1 by melatonin was almost completely abolished by XAV939 (Figure S8B). These data indicated that Wnt/β-catenin might act as the downstream signaling of the protective effects of melatonin in rescuing the toxicity of Sev.

4 | DISCUSSION

In the present study, we investigated the effects of melatonin pretreatment on the long-term neural toxic effects of neonatal repeated Sev exposure. By analyzing the expression of melatonin receptor, gene expression profile, and intracellular signaling pathways, we explored the possible mechanism underlying melatonin's protective effects. Our data illustrated a beneficial effect of melatonin pretreatment on the long-term synaptic toxicity, dysmyelination, and learning/memory impairment in mice which were exposed to Sev at neonatal and a novel interaction between MT1 and Wnt signaling in this process (Figure 13).

Because of the increasing concerns on the long-term safety of neonatal repeated Sev inhalation, many studies have attempted to protect animals from its neurotoxicity. Most of such researches focused on the acute toxicity, for example, the apoptosis induced by Sev.[46,47] In regard of chronic changes induced by Sev treatment, such as synaptic deficits and dysmyelination, there still lacks effective treatment. We have demonstrated that inhibiting HIPK2 could reduce the synaptic toxicity of Sev.[16] However, the protection on dysmyelination still remains an important issue to be addressed. In the present study, by using Golgi staining, EM, and electrophysiology technology,

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FIGURE 8 Long-term effects of neonatal Sev exposure on MT1 and Wnt signaling in adult hippocampus. (A, B) Western blotting and quantification of p-CREB, CREB, p-PKA, and PKA in hippocampus of control, Sev, and Sev-melatonin-treated mice at different time points. No changes of p-CREB, CREB, p-PKA, and PKA from 7 dps. (C) Heat map of RNA-seq results of top 20 differential genes in control, Sev-, and Sev-melatonin-treated mice. Notice the changes of SFRP1. (D, E) KEGG enrichment of signaling pathways in Sev-treated mice and Western blotting of SFRP1. (F) Western blotting and quantification of β-catenin, p-β-catenin (S33/S37/T41) in adult hippocampus of control and Sev-treated mice. (G) Western blotting and quantification of cytoplasmic and nuclear β-catenin in adult hippocampus of control and Sev-treated mice. Notice the decrease in nuclear β-catenin in Sev-treated mice. (H, I) qPCR and Western blotting and quantification of Axin2 in adult hippocampus of control and Sev-treated mice. (J) Double-immunohistochemistry and quantification of Axin2/NeuN- and Axin2/CC1-positive cells in normal hippocampus. $N = 6$ mice per group. Con, control. Sev, Sevoflurane. Cyt, cytoplasm. Nuc, nucleus. *$p < .05$. **$p < .01$. Student's t test. One-way ANOVA. Bars = 50 μm

(A) After exposure

(B) Con+Vehicle Sev+Vehicle Sev+mel

(C)

(D) KEGG Enrichment

(E)

(F)

(G)

(H)

(I)

(J)

(A)

(B)

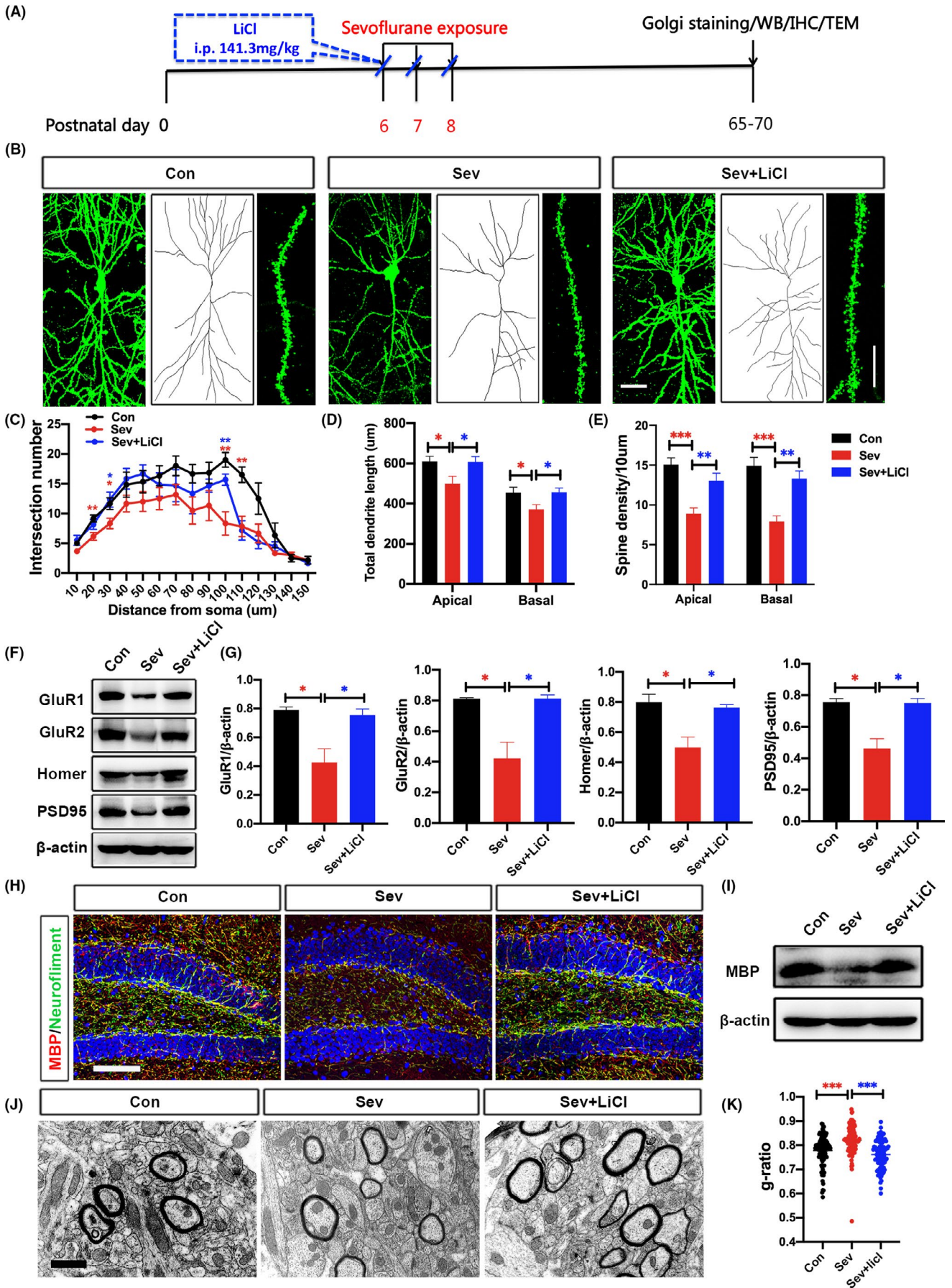(C) (D) (E)

(F) (G)

(H) (I)

(J) (K)

FIGURE 9 Effects of LiCl on the long-term synaptic toxicity of neonatal Sev exposure. (A) Experimental design. (B) Representative images of Golgi staining in adult control, Sev-treated, and Sev + LiCl treated mice. (C–E) Quantification of dendritic length, dendritic branching, and spine density. Notice the recovery of dendritic length, dendritic branches, and spine density by LiCl treatment. (F, G) Western blotting and quantification of GluR1, GluR2, Homer, and PSD-95. Notice the restoration of GluR1, GluR2, Homer, and PSD-95 in Sev + LiCl treated mice. (H, I) Immunostaining and Western blotting of MBP in the hippocampus of adult control, Sev-treated, and Sev + LiCl treated mice. LiCl treatment restored the expression of MBP. (J, K) Representative EM images of myelin and the g-ratio in the hippocampus of adult control, Sev-treated, and Sev + LiCl treated mice. Notice the recovery of myelin thickness in LiCl-treated mice. $N = 6$ mice per group. $*p < .05$. $**p < .01$. $***p < .001$. One-way ANOVA. Bar = 20 μm (B, left panel), 10 μm (B, right panel), 50 μm (H), and 1 μm (J)
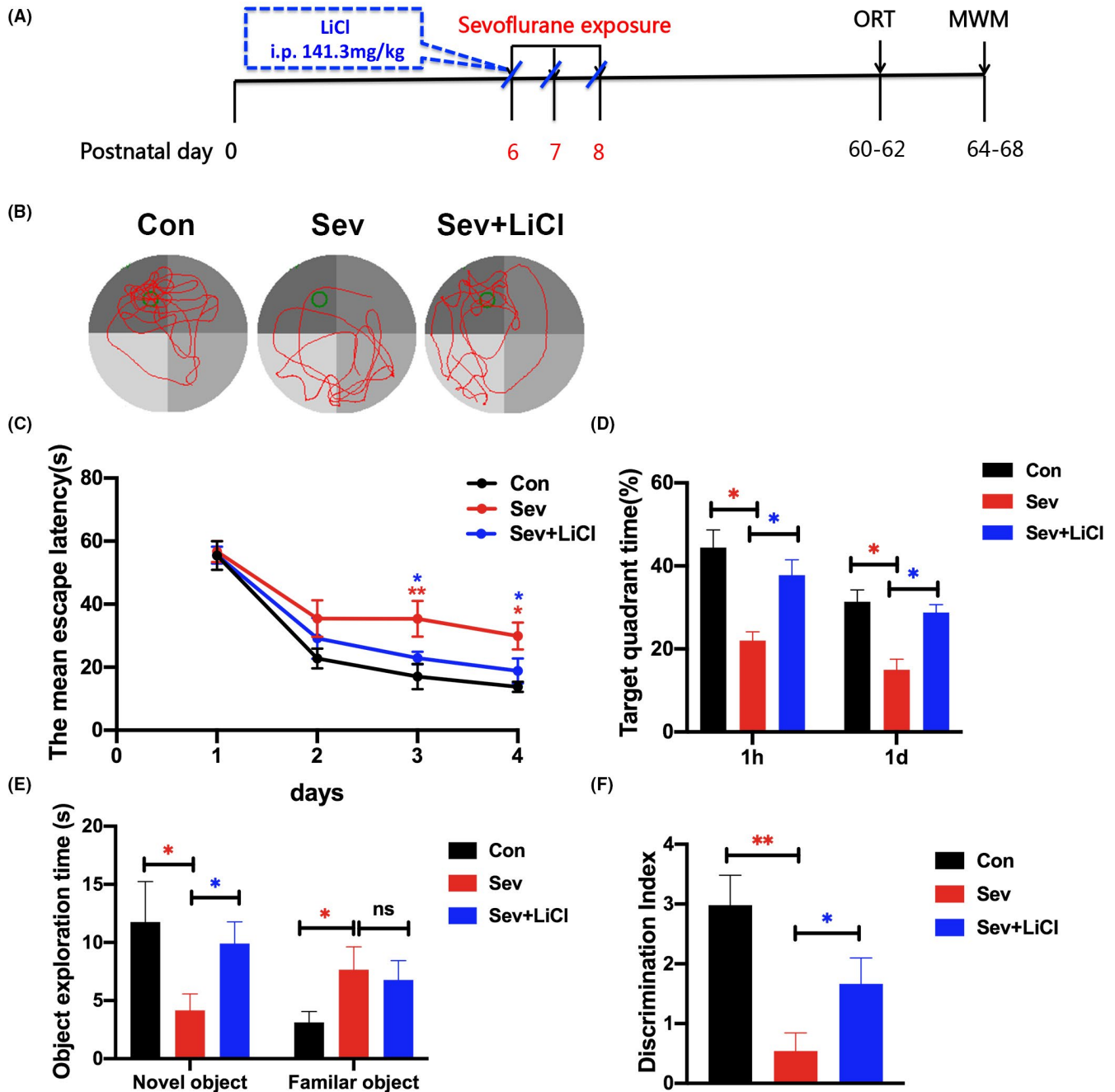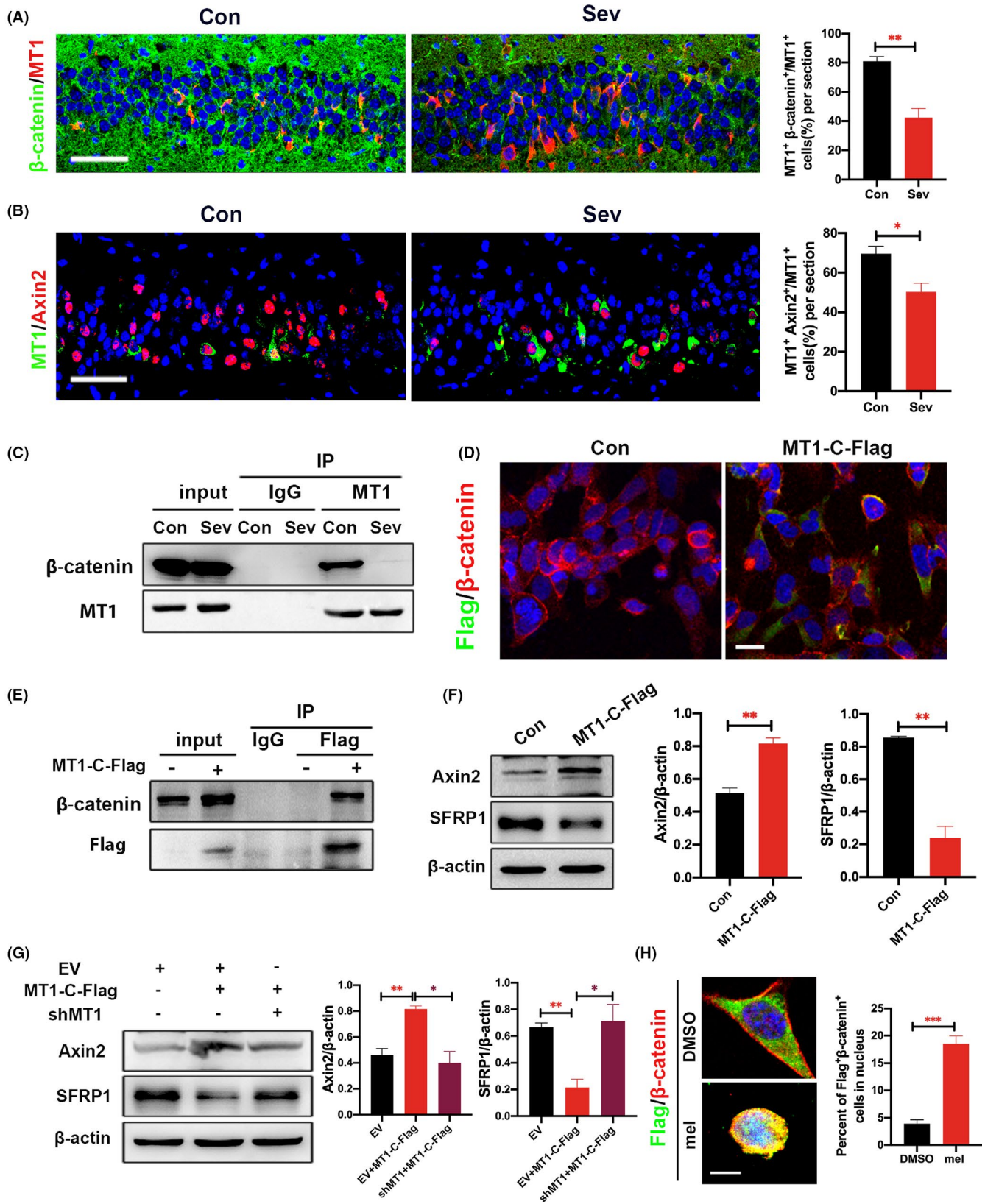

FIGURE 10 Effects of LiCl on the learning/memory impairment induced by neonatal Sev exposure. (A) Experimental design. (B–D) Morris water maze test of control, Sev-treated, and Sev + LiCl treated mice. (E, F) Novel object recognition test in control, Sev-treated, and Sev + LiCl treated mice. LiCl treatment restored the spatial memory and the ability of novel object recognition, as compared with Sev-treated mice. Con, control. Sev, Sevoflurane. mel, melatonin. ORT, novel object recognition test. MWM, Morris water maze test. $N = 8$ mice per group. $*p < .05$. $**p < .01$. One-way ANOVA

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we showed that melatonin pretreatment could effectively prevent both the chronic synaptic impairment and dysmyelination induced by Sev. In clinic, thanks to its hypnotic, antinociceptive, and anticonvulsant properties, melatonin is used as a preoperative sedative in combination with anesthetic.[48,49] Our data, for the first time, demonstrated a

FIGURE 11 Interaction between MT1 receptor and β-catenin. (A, B) Immunostaining of MT1/β-catenin and MT1/Axin2 in the control and Sev-treated mice and corresponding quantification at 7 dps. Sev treatment reduced the number of MT1/β-catenin- and MT1/Axin2-positive cells. (C) Protein CO-IP assay of MT1/β-catenin. Sev inhibited the interaction between MT1 and β-catenin. (D) Double immunostaining of MT1-C-Flag and β-catenin in control and MT1-C-Flag transfected cells. (E) Protein CO-IP assay of MT1--C/β-catenin. Notice the co-localization and interaction of MT1-C/β-catenin. (F) Western blotting of Axin2 and SFRP1 in control and MT1-C overexpressing cells. (G) Western blotting of Axin2 and SFRP1 in control cells, cells treated with MT1-C-Flag, and cells treated with combination of MT1-C-Flag and shMT1. MT1-C increased the expression of Axin2 and decreased the expression of SFRP1. (H) Immunostaining of Flag/β-catenin in MT1-Flag overexpressing cells under the stimulation of melatonin. Notice the nuclear localization of Flag and β-catenin upon the stimulation of melatonin. $N = 6$ mice per group in A–B. $N = 3$ batches of cells in C–H. *$p < .05$. **$p < .01$, ***$p < .001$. Student's t test or one-way ANOVA. Bars = 50 μm (A, B), 20 μm (D), and 10 μm (H)

beneficial effect of melatonin in reducing the long-term toxicity of general anesthesia. Given the very few or no side effects of melatonin, our study strongly indicated that melatonin could be clinically applied to enhance the safety of Sev anesthesia.

To investigate the potential mechanisms underlying melatonin's effects on synapse function and myelination, we focused on signaling pathways which (1) showed long-lasting changes till adulthood, (2) were induced both in neurons and oligodendrocytes, and (3) could be rescued by melatonin. Surprisingly, our data showed that the levels of PKA/cAMP/CREB, the intracellular downstream molecules of MT1 receptor, were up-regulated shortly after Sev treatment but remain unchanged in adult mice, suggesting that the response of MT1 signaling might be a compensatory response and that the long-term protective effects of melatonin might be achieved through alternative pathways.

Previous studies have reported that high dose melatonin inhibits oxidation, activates 5-HT signaling, and promotes interaction between 5-HT2C receptor with MT2 receptor.[50,51] We adopted a dose of 10 mg/kg which was higher than physiological level but had been used in rats without affecting diurnal rhythms and also been adopted in mice studies.[31–33] Sev exposure induced a transient increase in ROS and MDA, while not affecting T-AOC and T-SOD, which was consisted with previous reports that Sev exposure could lead to acute up-regulation of ROS.[52,53] The transient inhibitory effects of melatonin on ROS suggested that anti-oxidant mechanism may be involved in the acute effects of melatonin. The no changes of MDA, T-AOC, and T-SOD, and the no changes of 5-HT2A, 5-HT2C, and PLC precluded intracellular oxidation/redox level and 5-HT signaling as the key contributor of the melatonin's long-term protective effects.

Interestingly, gene profiling revealed a long-term up-regulation of SFRP1 in Sev-treated mice and the recovery of SFRP1 in Sev-melatonin-treated mice, indicating that melatonin might exert its protective effects through strengthening canonical Wnt signaling. It has been well documented that canonical Wnt

signaling plays important roles in synaptic maturation and myelination during normal development.[54–58] The co-expression of Axin2 with MT1 indicated the activation of canonical Wnt signaling in melatonin responsive cells. The long-term changes of β-catenin, p-GSK-3β, and Axin2 in Sev-treated mice and the rescue effects of LiCl suggested that Wnt signaling might act as a key intracellular pathway that mediated Sev's long-term toxicity. The restoration of β-catenin, p-GSK-3β, and Axin2 by melatonin pretreatment supported this possibility.

Previous studies have reported activation of Wnt signaling by melatonin.[59,60] However, how melatonin activated Wnt signaling remained unclear. For the first time, our data demonstrated interaction of MT1 with β-catenin. Manipulation of MT1 (overexpression and knockdown/ knockout) dramatically modulated the levels of intracellular Wnt signaling. Melatonin treatment stimulated nuclear translocation of β-catenin, indicating that melatonin regulates Wnt signaling via MT1 receptor. The detailed mechanism how the binding of melatonin to MT1 receptor activates the intracellular β-catenin signaling remains to be further investigated. What's more, inhibiting Wnt signaling by XAV939 significantly blocked the protective effects of melatonin, suggesting that Wnt signaling might act as the key intracellular signaling of the protective effects of melatonin. In all, our data revealed an important protective effects of melatonin on the long-term toxicity of Sev and a novel interaction between melatonin and canonical Wnt signaling, indicating a potential application of melatonin pretreatment for the safe use of Sev in pediatric anesthetics.

5 | CONCLUSIONS

Melatonin pretreatment effectively alleviates the long-term synaptic deficits, dysmyelination, and learning/ memory impairment induced by neonatal repeated Sev exposure. Mechanistically, MT1 receptor interacts with β-catenin and the protective effects of melatonin are mainly mediated by canonical Wnt signaling. Our findings
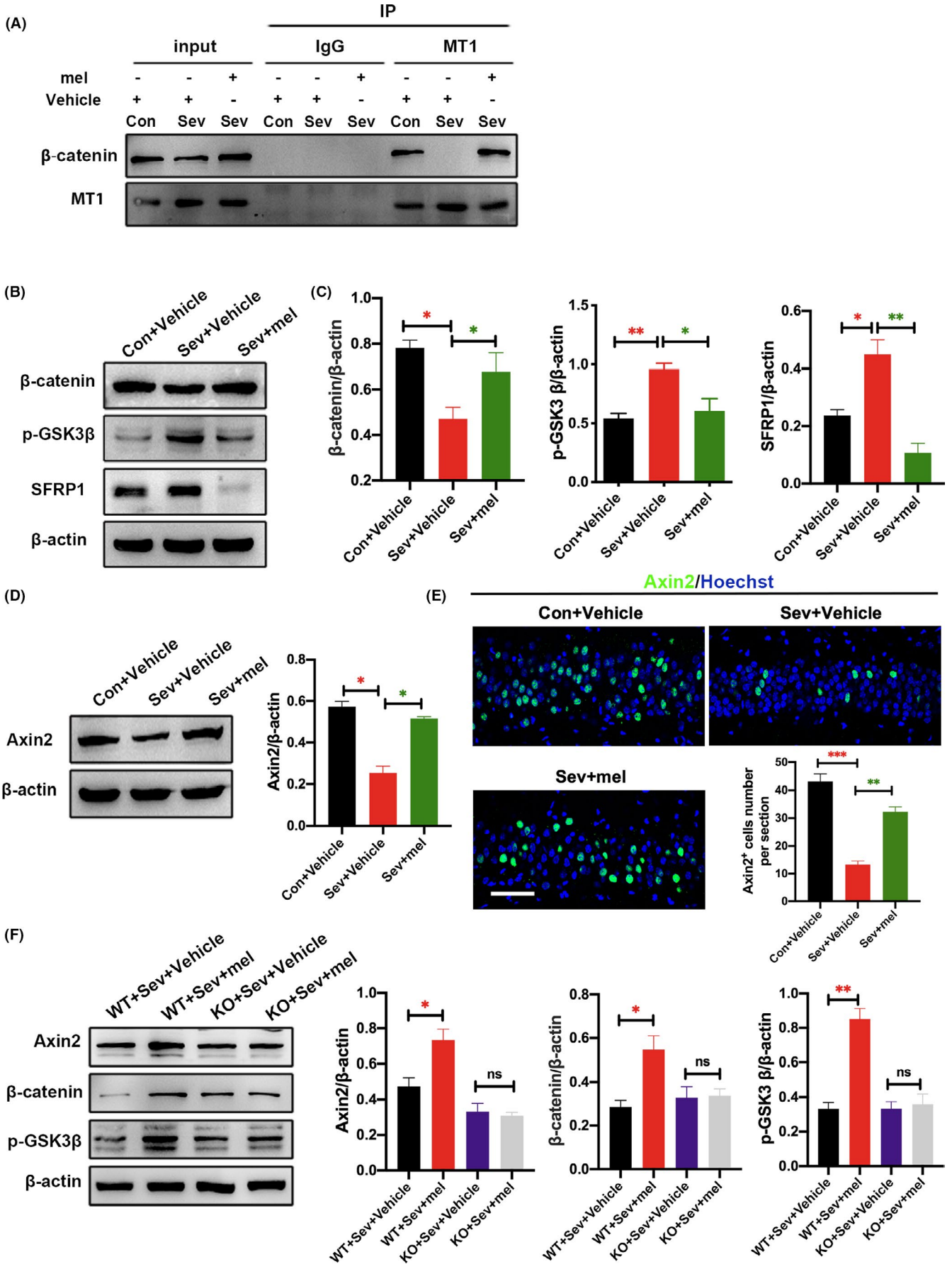
FIGURE 12 Long-term effects of neonatal melatonin pretreatment on the canonical Wnt signaling in adult hippocampus. (A) Protein CO-IP assay of MT1/β-catenin in adult hippocampus of mice treated by Sev or Sev-melatonin at neonatal. Notice that melatonin pretreatment restored the protein interaction between MT1 and β-catenin. (B, C) Western blotting and quantification of β-catenin, p-GSK-3β(T216), and SFRP1 in adult control, Sev-treated, and Sev-melatonin-treated mice. Melatonin pretreatment reversed the expression of β-catenin, p-GSK-3β(T216), and SFRP1. (D–E) Western blotting and immunostaining of Axin2. In comparison with Sev treatment, Sev-melatonin increased the expression of Axin2. (F) Western blotting and quantification of Axin2, β-catenin, and p-GSK-3β(T216) in WT mice and MT1 knockout mice (KO) treated with Sev, or Sev-melatonin. Sev, Sevoflurane. mel, melatonin. WT, wild type. KO, MT1 knockout. $N = 6$ mice per group. *$p < .05$. **$p < .01$. ***$p < .001$. One-way ANOVA. Bar = 50 μm
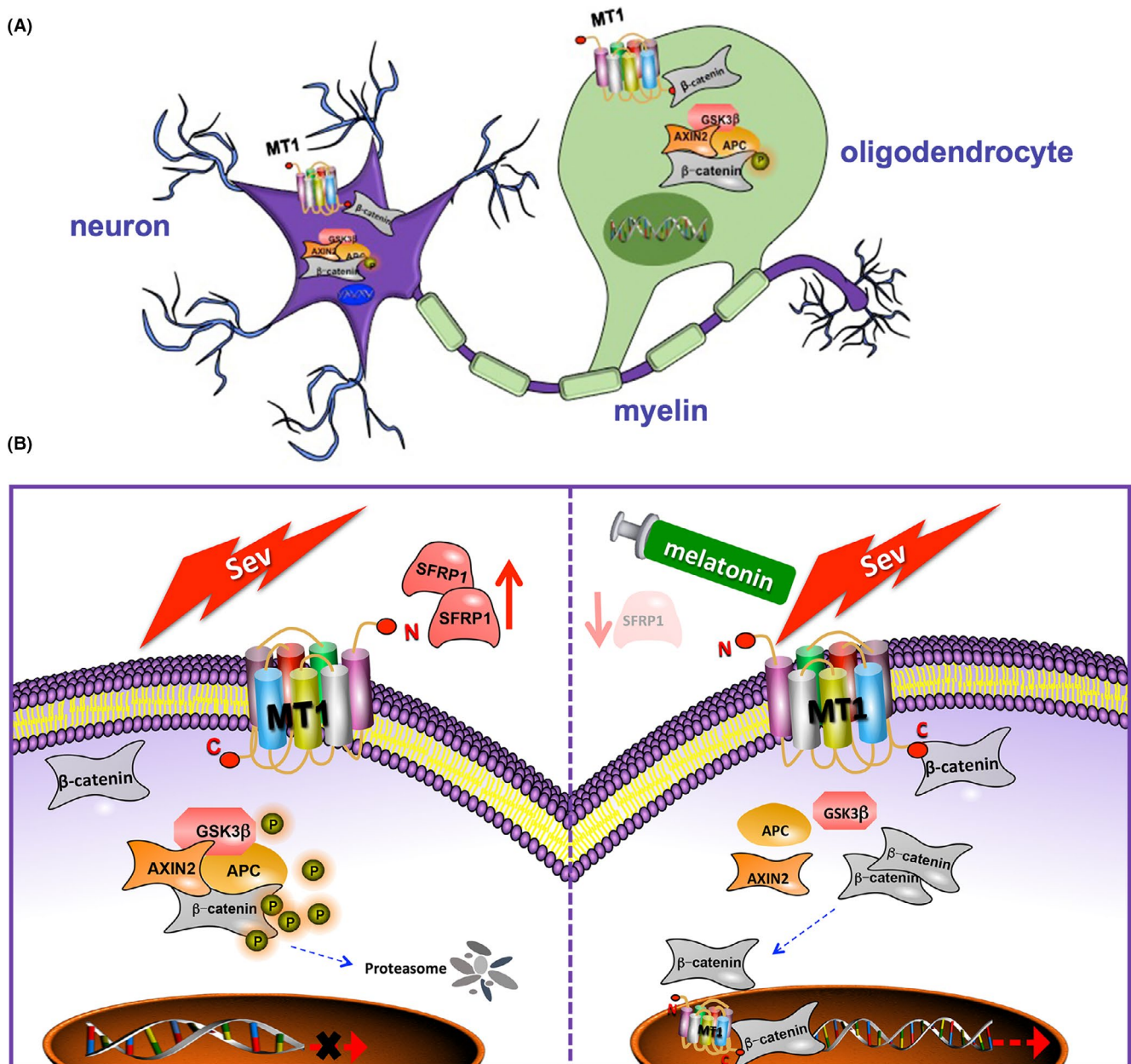

FIGURE 13 Schematic drawing of the major finding. (A) Under normal condition, hippocampal neurons and oligodendrocytes express MT1 receptor. MT1 co-localizes with β-catenin at membrane and binds to β-catenin with its C-terminal. (B) Left panel: upon Sev stimulation, β-catenin dissociates from MT1, leading to up-regulation of SFRP1 and suppression Wnt signaling. Right panel: at the presence of melatonin, MT1/β-catenin interaction is strengthened; thus, Wnt signaling is enhanced and SFRP1 lowered

suggested that melatonin may be used in clinic to improve the safety of general anesthesia.

CONFLICT OF INTEREST
The authors declare no conflicts of interests.

AUTHOR CONTRIBUTIONS
L.L., L.R., and Z.T. involved in behavior analysis. L.L. designed Golgi staining. L.L., Z.Y., and Z.L. involved in immunohistochemistry. L.L. and T.W. involved in electron microscopic study. L.L., M.Z, and Y.X. performed Western blotting. X.R., and B.L involved in patch-clamp recording. L.L., L.R., and Z.Y. analyzed the data. L.L., Z.H., and W.Y. prepared the manuscript. L.L., Z.H., W.Y., and W.S. designed the experiment. Z.H. and W.S. involved in financial support.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE
All experimental procedures were carried out according to the protocols approved by the Animal Care and Use Committee of FMMU.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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