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Endogenous Wnt/β-catenin signaling in Müller cells protects retinal ganglion cells from excitotoxic damage

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Purpose: To analyze whether activation of endogenous wingless (Wnt)/β-catenin signaling in Müller cells is involved in protection of retinal ganglion cells (RGCs) following excitotoxic damage.

Methods: Transgenic mice with a tamoxifen-dependent β-catenin deficiency in Müller cells were injected with N-methyl-D-aspartate (NMDA) into the vitreous cavity of one eye to induce excitotoxic damage of the RGCs, while the contralateral eye received PBS only. Retinal damage was quantified by counting the total number of RGC axons in cross sections of optic nerves and measuring the thickness of the retinal layers on meridional sections. Then, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed to identify apoptotic cells in retinas of both genotypes. Western blot analyses to assess the level of retinal β-catenin and real-time RT-PCR to quantify the retinal expression of neuroprotective factors were performed.

Results: Following NMDA injection of wild-type mice, a statistically significant increase in retinal β-catenin protein levels was observed compared to PBS-injected controls, an effect that was blocked in mice with a Müller cell-specific β-catenin deficiency. Furthermore, in mice with a β-catenin deficiency in Müller cells, NMDA injection led to a statistically significant decrease in RGC axons as well as a substantial increase in TUNEL-positive cells in the RGC layer compared to the NMDA-treated controls. Moreover, in the retinas of the control mice a NMDA-mediated statistically significant induction of leukemia inhibitory factor (Lif) mRNA was detected, an effect that was substantially reduced in mice with a β-catenin deficiency in Müller cells.

Conclusions: Endogenous Wnt/β-catenin signaling in Müller cells protects RGCs against excitotoxic damage, an effect that is most likely mediated via the induction of neuroprotective factors, such as Lif.

Wingless (Wnt) signaling is involved in various processes, such as embryonic development, maintenance of homeostasis in adults, or tumor growth in cancer biology. Members of the Wnt family are secreted glycolipoproteins, which bind with a high affinity to frizzled receptors. The central signaling molecule for the canonical Wnt signaling pathway is β-catenin, which is constitutively expressed in most cell types. In the absence of Wnt proteins, β-catenin is constantly removed by its degradation complex. After Wnt proteins bind to their frizzled receptors, a complex with its coreceptor, low-density lipoprotein receptor-related protein (LRP) 5 or 6, is formed to inactivate the β-catenin degradation complex by recruiting it to the plasma membrane. After accumulation in the cytoplasm, β-catenin translocates into the nucleus to induce the expression of Wnt-specific target genes [1].

In the retina, Wnt/β-catenin signaling is involved in the development and maintenance of vasculature, as well as in homeostasis of neurons. A predominant mediator of retinal Wnt/β-catenin signaling is the secreted protein Norrin, which is specifically expressed in Müller cells [2]. Via binding to frizzled 4 or leucine-rich repeat-containing G-protein-coupled receptor (LGR) 4, Norrin activates canonical Wnt/β-catenin signaling in the presence of its coreceptor LRP 5 or 6 [3-5]. During the development of the retinal vasculature, Norrin-mediated Wnt/β-catenin signaling is crucial for vessel outgrowth on the inner retinal surface toward the ora serrata, the formation of the intraretinal plexus as well as the differentiation and maintenance of the inner blood–retinal barrier [6-8]. In addition to an angiogenic function in development, Norrin promotes vessel regrowth into ischemic retinal areas in a mouse model of retinopathy of prematurity [9]. Several reports demonstrated an additional neuroprotective role of Norrin for retinal neurons. A continuous loss of retinal ganglion cells (RGCs) was observed in Norrin-deficient mice in addition to the vascular changes, while transgenic overexpression of Norrin during development led to enhanced proliferation of retinal progenitor cells [6,7]. In addition, following acute damage of RGCs with N-methyl-D-aspartate (NMDA) and of photoreceptors by light exposure, Norrin protects both cell types against apoptosis [10,11]. In both

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animal models, Wnt/β-catenin dependent induction of neuroprotective factors, such as leukemia inhibitory factor (Lif), endothelin (Edn)-2, brain-derived neurotrophic factor, ciliary neurotrophic factor, or fibroblast growth factor (Fgf)-2, was observed. In a recent study, we showed that following NMDA injection into the vitreous cavity Norrin mediates its protective effects on RGCs via the induction of Lif [12]. Moreover, in DBA/2J mice that have increased intraocular pressure and chronic degeneration of RGCs, Norrin protects the cells via Wnt/β-catenin-mediated induction of insulin-like growth factor-1 [13].

As Müller cells have the distinct potential to transmit protective effects on damaged retinal neurons via enhanced expression of neuroprotective factors [14], we hypothesized that activation of endogenous Wnt/β-catenin signaling in Müller cells could mediate protective effects on RGCs following acute excitotoxic damage. To this end, a mouse model with a tamoxifen-dependent, conditional β-catenin deficiency in Müller cells was generated, and excitotoxic damage of RGCs was investigated after induction of β-catenin deficiency and intravitreal injection of NMDA. The results provide evidence that the endogenous Wnt/β-catenin pathway in Müller cells mediates protective properties that are critical for RGC survival after injury, an effect that involves increased expression of neuroprotective factors in Müller cells.

METHODS

Animals: All animal procedures in this study conformed with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were authorized by the local authorities (Regierung der Oberpfalz, Regierung, Germany). Mice were housed in the animal facility of the University of Regensburg according to standard conditions. All experiments were performed in mice of both sexes.

Mice with two loxP restriction sites including exon 2 to 6 of *Ctnnb1* (*Ctnnb1*$^{fl/fl}$; a gift from Rolf Kemler) [15] were crossed with mice that express tamoxifen-dependent Cre-recombinase under the control of the Slc1a3 promoter (*Slc1a3-Cre*^{ERT}; Jackson Laboratories, Bar Harbor, ME) to generate *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice. Both mouse strains were bred in the C57BL/6J genetic background. To analyze the expression and activity of Cre-recombinase in the retinas of the *Slc1a3-Cre*^{ERT} transgenic animals, the mice were crossed with Rosa26-LacZ reporter animals [16]. Transgenic mice were identified with PCR genotyping, using the primer pairs 5′-ACA ATC TGG CCT GCT ACC AAA GC-3′ and 5′-CCA GTG AAA CAG CAT TGC TGT C-3′ for the *Slc1a3-Cre*^{ERT} mice (fragment size about 600 bp), 5′-ATC CTC TGC ATG GTC AGG TC-3′ and 5′-CGT GGC CTG ATT CAT TCC-3′ for the Rosa26-LacZ animals (fragment size about 300 bp), and 5′-AAG GTA GAG TGA TGA AAG TTG TT-3′ and 5′-CAC CAT GTC CTC TGT CT ATTC-3′ for the *Ctnnb1*$^{fl/fl}$ mice (the fragment size for the wild-type and loxP allele was 221 bp and 324 bp, respectively). The thermal cycle profile of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s for 35 cycles was used to identify Rosa26-LacZ, *Slc1a3-Cre*^{ERT}, and *Ctnnb1*$^{fl/fl}$ mice. All primers were purchased from Thermo Fisher (Waltham, MA). To induce Cre-mediated recombination, 50 µl tamoxifen (20 mg/ml) were injected intraperitoneally in isoflurane-anesthetized 6-week-old *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$, *Slc1a3-Cre*^{ERT}/Rosa26-LacZ, Rosa26-LacZ, and *Ctnnb1*$^{fl/fl}$ mice twice a day for 5 days.

NMDA-induced retinal damage: The NMDA injection was performed as previously described [10]. Briefly, to induce retinal excitotoxic damage, 8-week-old *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice and *Ctnnb1*$^{fl/fl}$ controls were anesthetized with isoflurane followed by an injection of 3 µl NMDA (10 mM dissolved with 1X PBS; 144 mg/l KH_2PO_4, 9 g/l NaCl, 795 mg/l Na_2HPO_4-$7H_2O$, pH 7.4) into the vitreous cavity of one eye while the contralateral eye was injected with 3 µl PBS. The eyes, optic nerves, and retinas were prepared at the indicated time.

Light microscopy: Following tamoxifen treatment and 2 weeks after the NMDA injection, the eyes and the attached optic nerves were enucleated and fixed in cacodylate buffer with 2.5% paraformaldehyde (PFA) and 2.5% glutaraldehyde overnight. After washing with cacodylate buffer and postfixing with OsO_4, the eyes and the optic nerves were dehydrated and embedded in Epon (Carl Roth, Karlsruhe, Germany) according to standard protocols. Semithin meridional sections (1 µm) of the eyes and the optic nerves were stained with fuchsin and methylene blue, or paraphenylenediamine, respectively, according to standard protocols and analyzed with light microscopy using an Axiovision microscope (Carl Zeiss, Oberkochen, Germany). Quantification of the axons in the optic nerve was performed following a protocol published previously [17]. Briefly, one central and four peripheral squares (40 µm × 40 µm each), which cover more than 10% of the total optic nerve area, were projected onto the optic nerves, quantified, and extrapolated to the total area of the optic nerve.

To evaluate the thickness of the retinal layers, meridional retinal semithin sections were analyzed as described previously [18]. Briefly, the distance between the ora serrata and the optic nerve head was divided into tenths, and the thickness of the total retina, the inner nuclear layer, the outer nuclear layer, the inner plexiform layer, and the outer plexiform layer

was measured between each tenth using Axiovision software 4.8 (Carl Zeiss).

X-Gal staining: Two weeks after tamoxifen treatment, the eyes of the *Slc1a3-Cre*^{ERT}/Rosa26-LacZ mice and Rosa26-LacZ littermates were prepared and fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) at 4 °C for 1 h. After three washings for 30 min each (0.01% sodium deoxycholate, 0.02% NP-40, 2 mM $MgCl_2$, and 0.1 M phosphate buffer, pH 7.3), the eyes were incubated in LacZ staining buffer (0.1 M phosphate buffer, pH 7.3, 3 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg/ml X-Gal) overnight. After three additional washings with PBS, the eyes were embedded in paraffin according to standard protocols. Meridional sections were analyzed on an Axiovision fluorescent microscope (Carl Zeiss).

Immunohistochemistry and TUNEL labeling: For immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) labeling, the eyes were fixed with 4% PFA for 4 h and embedded in paraffin according to standard procedures. For Sox6 staining, the sections were treated with 1x Target Retrieval Solution (Dako North America, Carpinteria, CA) at 100 °C for 20 min, washed with 1x PBS at room temperature for 5 min, and incubated in 0.25% Triton X-100 in 1x PBS at room temperature for an additional 10 min. After blocking with 3% bovine serum albumin (BSA) and 0.1% Triton X-100 in 1x PBS for 60 min, the sections were incubated with rabbit anti-Sox9 antibodies (1:50, Merck, Darmstadt, Germany) in 0.2% BSA and 0.01% Triton X-100 in 1x PBS overnight at 4 °C. Following three washes (10 min each) with 1x PBS, the samples were incubated with Cyc3 coupled anti-rabbit antibodies (1:1,000; Thermo Fisher) for 60 min. After the final incubation, all specimens were washed again three times, mounted in fluorescent mounting medium containing 1:50 of 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA), and analyzed on an Axio Observer 7 fluorescent microscope (Carl Zeiss).

Apoptotic retinal cells were identified with the Deadend Fluorometric TUNEL system (Promega, Fitchburg, WI). TUNEL labeling of the paraffin sections was performed according to the manufacturer’s instructions. Slides were analyzed on an Axiovision fluorescent microscope (Carl Zeiss). For quantification, the total number of TUNEL-positive cells was quantified in the RGC layer and in the inner nuclear layer (INL) of the meridional sections and calculated as the number of TUNEL-positive cells per 1,000 µm retinal length.

RNA isolation, cDNA synthesis, and real-time RT–PCR analyses: For the expression analyses, the retinas were dissected 24 h after injection of 3 µl NMDA (10 mM) into the vitreous body of one eye and 3 µl PBS into the contralateral eye. The retinas were homogenized in peqGOLD TriFast (Peqlab, Erlangen, Germany), and total RNA was isolated according to the manufacturer’s instructions. The RNA concentration and the OD260/OD280 ratio were measured with the NanoDrop spectrophotometer ND-2000c (Peqlab). Only total RNA with a 260/280 ratio between 1.8 and 2.0 was used for first-strand cDNA synthesis with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) in accordance with the manufacturer’s recommendations. Quantitative real-time RT-PCR analyses were performed on a Bio-Rad iQ5 Real-Time PCR Detection System (Bio-Rad). HotStart Taq DNA polymerase (Qiagen, Hilde, Germany) was used for PCR according to the manufacturer’s protocol. PCR was performed in a volume of 15 µl, consisting of 1.5 µl of 10x PCR buffer (Qiagen), 0.6 µl of 25 mM $MgCl_2$ (Qiagen), 0.12 µl of dNTPs (25 mM each, Qiagen), 0.06 µl of HotStart Taq (5 U/µl), 0.16 µl of primer mix (1 µM each, Thermo Fisher) and 0.39 µl of 1x SYBR Green I solution (Qiagen). The following temperature profile was used: 40 cycles with 20 s denaturation at 95 °C, 10 s annealing at 60 °C, and 20 s extension at 70 °C. RNA that had not been reverse-transcribed served as negative control for real-time RT-PCR. All PCR primers were designed to span exon–intron boundaries and were purchased from Thermo Fisher (Table 1). All PCR products were analyzed with DNA sequencing. For relative quantification, the reference gene *Gnb2l1* was used. Results were analyzed using the iQ5 optical system software (Version 2.1).

Protein preparation and western blot analyses: To analyze retinal β-catenin expression, dissected retinas of 8-week-old *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice and *Ctnnb1*$^{fl/fl}$ controls with or without intravitreal injections of 3 µl NMDA into the vitreous body of one eye and 3 µl PBS into the contralateral eye were homogenized in peqGOLD TriFast (Peqlab), and retinal proteins were isolated according to the manufacturer’s instructions. Protein concentration was measured with the bicinchoninic acid (BCA) assay according to the manufacturer’s instructions (Interchim, Wörgl, Austria). Up to 25 µg of total protein were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Roche, Rotkreuz, Switzerland) with semidry blotting. After blocking with 5% BSA in Tris-buffered saline and Tween 20 (TBST), the membranes were incubated overnight with rabbit anti-β-catenin antibodies (1:1,000; Cell Signaling, Cambridge, UK) in TBST with 0.5% BSA. Horseradish peroxide (HRP)-conjugated anti-rabbit antibodies were used as secondary antibodies (Santa Cruz, Santa Cruz, CA). Antibody labeling was visualized using the Immobilon HRP substrate (Millipore,

Darmstadt, Germany) and documented on an LAS 3000 Imager work station (Fujifilm, Tokyo, Japan). As loading control, HRP-conjugated anti-GAPDH antibodies were used (Cell Signaling). Densitometry of western blot analyses was performed with the Aida Advanced Image Data Analyzer v.4.06 (Raytest, Straubenhardt, Germany).

Statistics: All values are expressed as mean ± standard error of the mean (SEM). For statistical analyses, one-way analysis of variance (ANOVA) was performed to compare the mean variables, followed by a least significant difference (LSD) post hoc test for data that met the criteria of the assumption of homogeneity of variances and a Games-Howell post hoc test for data that did not meet the criteria of homogeneity of variances. P values of less than 0.05 were considered statistically significant.

RESULTS

Tamoxifen-induced β-catenin deficiency in Müller cells of Slc1a3-Cre^{ERT}/Ctnnb1$^{fl/fl}$ mice: To confirm the expression of Cre-recombinase in Müller cells of *Slc1a3-Cre*^{ERT} mice, which express tamoxifen-dependent Cre-recombinase under the control of the *Slc1a3* promoter, *Slc1a3-Cre*^{ERT}/Rosa26-LacZ mice with LacZ expression after Cre-mediated recombination were generated. Two weeks after tamoxifen treatment, X-Gal staining of the eyes of 8-week-old *Slc1a3-Cre*^{ERT}/Rosa26-LacZ mice was performed while Rosa26-LacZ animals without additional Cre expression served as negative controls. In the *Slc1a3-Cre*^{ERT}/Rosa26-LacZ mice, blue staining in the middle of the inner nuclear layer was observed, strongly indicating cytoplasmic labeling of Müller cells. From these labeled cells, radial extensions to the inner and outer limiting membrane with enhanced staining in the RGC layer and the inner limiting membrane were detected that likely corresponded to the end feet of the Müller cells (Figure 1B). In Rosa26-LacZ mice without Cre expression, no positive signal for β-galactosidase was detected (Figure 1A).

To verify that tamoxifen treatment leads to β-catenin deficiency in retinas of *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice, retinal proteins were investigated by western blot analyses. In retinal proteins from tamoxifen-treated *Ctnnb1*$^{fl/fl}$ mice, an intense signal for β-catenin was detected, whereas in the tamoxifen-injected *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice only a faint signal for retinal β-catenin was observed (Figure 1C). Quantification with densitometry of several experiments demonstrated a more than 35% decrease in the β-catenin protein levels in *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice compared to the *Ctnnb1*$^{fl/fl}$ controls ($p<0.05$; Figure 1D). The difference was statistically significant ($p<0.05$), strongly suggesting that tamoxifen treatment promotes substantially reduced β-catenin expression in the retinas of *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice.

Then, to rule out that Wnt/β-catenin deficiency in Müller cells by tamoxifen treatment leads to a loss of Müller cells, immunohistochemical staining against Sox9, a glial marker for Müller cells and astrocytes [19], as well as real-time RT–PCR for Sox9 mRNA were performed. In the retinas from the tamoxifen-treated 8-week-old *Ctnnb1*$^{fl/fl}$ mice and the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice, staining for Sox9 was predominantly observed in the nuclei of the INL of both genotypes and to a lesser extent in the RGC layer (Figure 1F,G). No obvious difference in the number or distribution of Sox9-positive nuclei in the INL was seen between the *Ctnnb1*$^{fl/fl}$ and *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice. Furthermore, 2 weeks after

Table 1. Primers used for real-time RT-PCR amplification.

Gene	Accession No.	Sequence	Position	Product size
Lif	NM_008501	5’-CGCCAATGCTCTCTTCATTT-3‘	400 - 419	80 bp
		5‘-TAGGCGCACATAGCTTTTCC-3‘	479 - 460	
End2	NM_007902	5‘-ACCTCCTCCGAAAGCTGAG-3‘	720 - 738	76 bp
		5‘-TTTCTTGTCACCTCTGGCTGTA-3‘	795 - 774	
Fgf2	NM_008006	5’- CGGCTCTACTGCAAGAACG-3’	285 - 303	108 bp
		5’- TGCTTGGAGTTGTAGTTTGACG-3’	392 - 371	
Gfap	NM_001131020	5’-TCGAGATCGCCACCTACAG-3’	1156 - 1174	67 bp
		5’-GTCTGTACAGGAATGGTGATGC-3’	1222 - 1201	
Sox9	NM_011448	5’-GTACCCGCATCTGCACAAC-3’	753 - 771	94 bp
		5’-CTCCTCCACGAAGGGTCTCT-3’	846 - 827	
GNB2L1	NM_008143	5’- TCTGCAAGTACACGGTCCAG-3’	514 - 533	89 bp
		5’- ACGATGATAGGGTTGCTGCT-3’	601 - 582	

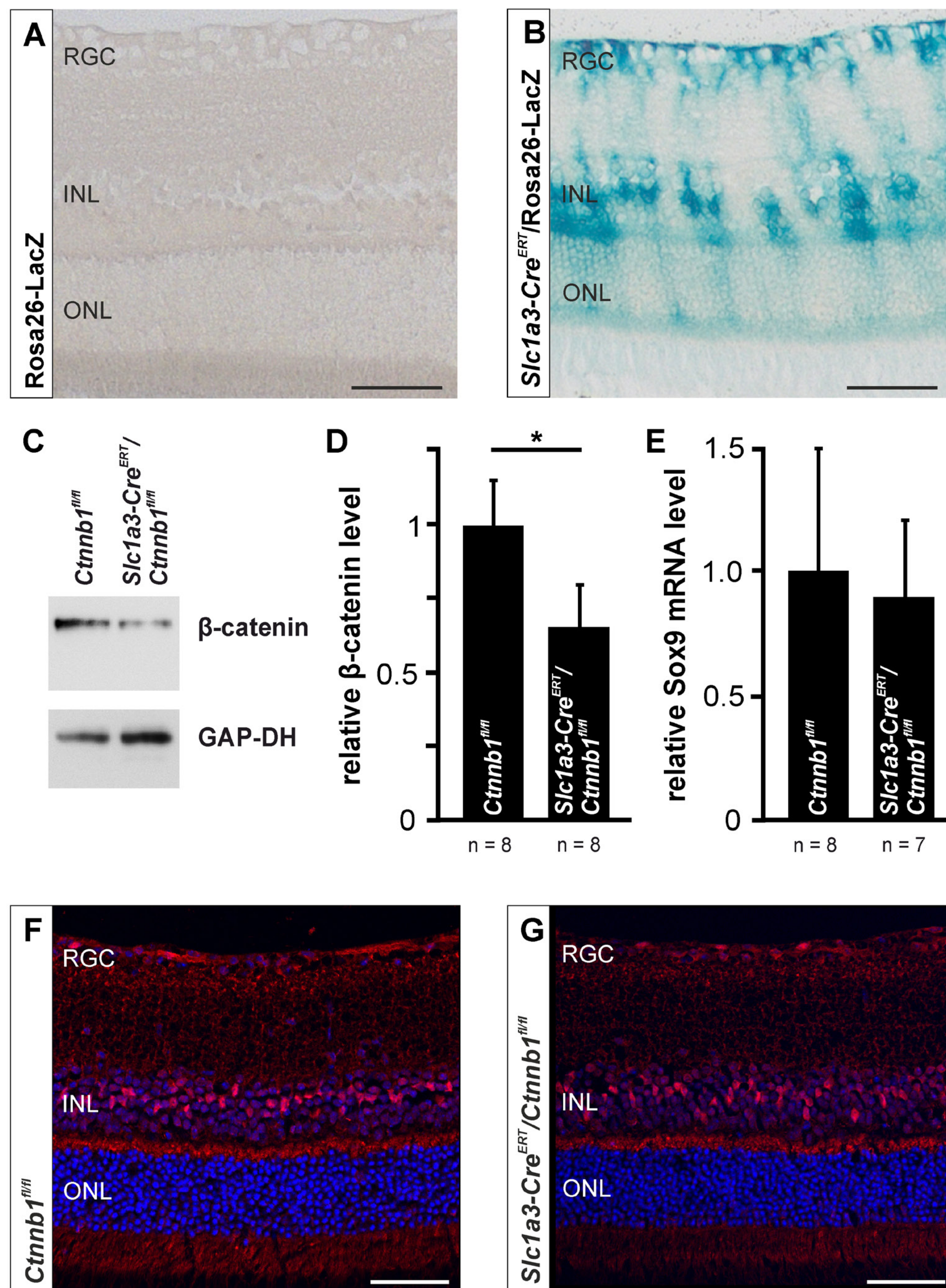


Figure 1. Tamoxifen-induced β-catenin deficiency in Müller cells of *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice. **A, B**: Representative β-galactosidase staining of 8-week-old Rosa26-LacZ reporter mice with or without additional Cre expression in Müller cells (*Slc1a3-Cre*^{ERT}) after treatment with tamoxifen (50 µl tamoxifen [20 mg/ml] i.p. 2x/day for 5 days). In the *Slc1a3-Cre*^{ERT}/Rosa26-LacZ mice, radial staining for β-galactosidase that spread from the inner to the outer limiting membrane was detected. In addition, the nuclei of the labeled cells were localized in the inner nuclear layer (**B**), whereas no β-galactosidase expression was detected in mice without Cre expression (**A**). Magnification bars = 50 µm. RGC, retinal ganglion cell, INL, inner nuclear layer; ONL, outer nuclear layer. **C, D**: Western blot analysis (**C**) and densitometry (**D**) for β-catenin from retinal proteins of 8-week-old *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice 2 weeks after treatment with tamoxifen (mean ± standard error of the mean, SEM; n = 8; *p<0.05). **E**–**G**: Real-time RT–PCR (**E**) and immunohistochemical staining (**F**, **G**) for Sox9 of 8-week-old mice 2 weeks after treatment with tamoxifen (50 µl tamoxifen [20 mg/ml] i.p. 2x/day for 5 days). No difference in the number or distribution of Sox9 positive nuclei (red) in the INL was seen between the *Ctnnb1*$^{fl/fl}$ (**F**) and *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ (**G**) mice. Magnification bars, 50 µm. Blue, 4′,6-diamidino-2-phenylindole (DAPI) staining; RGC, retinal ganglion cell, INL, inner nuclear layer; ONL, outer nuclear layer.

the tamoxifen treatment similar expression of Sox9 mRNA was detected with real-time RT-PCR in the retinal RNA of both genotypes (Figure 1E), strongly suggesting that the tamoxifen-induced loss of Wnt/β-catenin signaling in Müller cells does not lead to prompt degeneration of these cells.

Wnt/β-catenin deficiency in Müller cells enhances NMDA-mediated excitotoxic damage of retinal ganglion cells: To investigate whether endogenous Wnt/β-catenin signaling in Müller cells protects against NMDA-mediated excitotoxic RGC damage, the optic nerves from 10-week-old *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice and *Ctnnb1*$^{fl/fl}$ littermates were investigated 4 weeks after treatment with tamoxifen and 2 weeks after intravitreal injection of NMDA or PBS. After injection of PBS into the vitreous cavity, only a few darkly stained, degenerating myelin sheathes were detectable (Figure 2A–D). In contrast, an obvious loss of axons with darkly stained myelin whorls and extensive glial scars was seen after NMDA injection (Figure 2E–H). Furthermore, axonal damage was even more pronounced in the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice compared to the *Ctnnb1*$^{fl/fl}$ controls (Figure 2E–H). The quantitative analysis of the total number of axons in the optic nerves of both genotypes showed a substantial decline in the RGC axons following the NMDA injection to 17,029 ± 870 in the *Ctnnb1*$^{fl/fl}$ mice as well as 13,677 ± 775 in the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice compared to the PBS-injected controls (32,856 ± 961 in the *Ctnnb1*$^{fl/fl}$ mice; 32,451 ± 1,646 in the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice), a difference that was highly statistically significant for both groups ($p<0.001$; Figure 2I). Comparing the total number of optic nerve axons from the NMDA-treated eyes between the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice and *Ctnnb1*$^{fl/fl}$ littermates, we found almost 25% more axons in the *Ctnnb1*$^{fl/fl}$ mice, a difference that was statistically significant ($p<0.05$). In contrast, no statistically significant difference between the total number of optic nerve axons of the PBS-injected eyes from the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice and *Ctnnb1*$^{fl/fl}$ controls was observed. In summary, the results strongly suggest that deficiency of endogenous Wnt/β-catenin signaling in Müller cells enhances NMDA-mediated excitotoxic damage of RGCs.

To further analyze the effects of NMDA-mediated excitotoxic damage on retinal neurons in mice with a β-catenin deficiency in Müller cells, we analyzed the retinal morphology of *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice and *Ctnnb1*$^{fl/fl}$ controls on meridional semithin sections 2 weeks after intravitreal injection of NMDA or PBS and 4 weeks after treatment with tamoxifen. For quantification, the distance

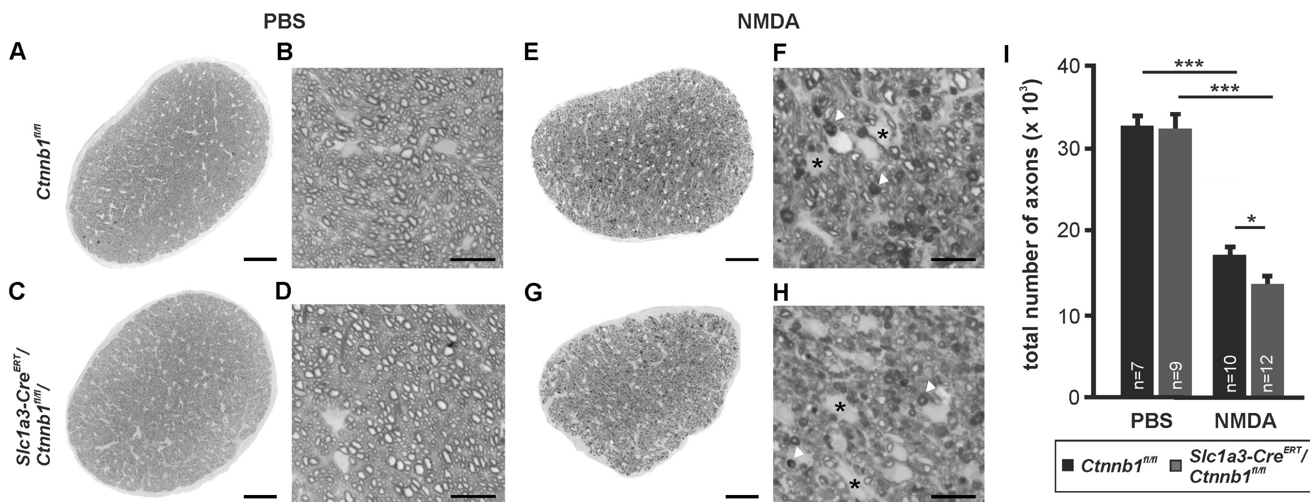


Figure 2. Wnt/β-catenin deficiency in Müller cells enhances axon loss in the optic nerve following acute excitotoxic damage of RGCs. **A**–**H**: Representative sagittal semithin sections through optic nerves from 10-week-old *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice and *Ctnnb1*$^{fl/fl}$ controls 2 weeks after intravitreal injection of 3 µl N-methyl-D-aspartate (NMDA) [10 mM] or PBS and treatment with tamoxifen at the age of 6 weeks (50 µl tamoxifen [20 mg/ml] i.p. 2x/day for 5 days). In the optic nerves of the NMDA-injected eyes, obvious loss of axons, broad glial scars (asterisk), and darkly stained myelin whirls (arrow heads) were observed **(E**–**H)**, whereas in the PBS-treated eyes only a few degenerating myelin sheathes were detectable **(A**–**D)**. In the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice, the axonal damage was even more pronounced compared to that in the *Ctnnb1*$^{fl/fl}$ controls **(E**–**H)**. Magnification bars: A, C, E, G, 50 µm; B, D, F, H, 10 µm. **I**: For quantification, the number of axons in the optic nerves from the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice and the *Ctnnb1*$^{fl/fl}$ controls was quantified and plotted as the total number per optic nerve (mean ± standard error of the mean, SEM; *$p<0.05$; ***$p<0.001$).

between the ora serrata and the optic nerve head was divided into tenths, and the thickness of the total retina, inner plexiform layer (IPL), INL, outer plexiform layer (OPL), and outer nuclear layer (ONL) was measured between each tenth.

The thickness of the total retina, the INL, and the OPL of the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice was reduced by approximately 6%, 10%, and 15%, respectively, in the central retina following PBS injection compared to the *Ctnnb1*$^{fl/fl}$ controls, suggesting that a lack of endogenous Wnt/β-catenin signaling leads to slight neuronal degeneration of the central retina (Figure 3A,B,D,E). In addition, the central ONL and the OPL were statistically significantly thicker in the eyes of mice from both genotypes following NMDA injection compared to the PBS-treated contralateral eyes (Figure 3E,F) suggesting that NMDA-mediated damage of the inner retina might lead to protective effects of the outer retina.

As expected, following treatment with NMDA both genotypes showed a pronounced and statistically significant reduction in the thicknesses of the total retina (up to 15%), the IPL (up to 50%), and the INL (up to 30%) compared to the PBS-injected controls (Figure 3A–D). In contrast, following NMDA injection, the thicknesses of the IPL and of the total retina of the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice were only slightly decreased compared to those of the *Ctnnb1*$^{fl/fl}$ controls, a result that was, at least in part, statistically significant (Figure 3A–C). The difference between the two genotypes after NMDA injection was most evident for the INL, which in the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice showed a uniform, statistically significant reduction in the INL thickness of up to 12% compared to the *Ctnnb1*$^{fl/fl}$ mice (Figure 3A,D). Overall, the data strongly suggest that Wnt/β-catenin signaling in Müller cells mediates effects that protect retinal neurons from excitotoxic retinal damage.

Endogenous Wnt/β-catenin signaling inhibits NMDA-induced apoptosis of RGCs: After NMDA treatment, RGC death is a result of apoptosis [20]. To evaluate whether a deficiency in Wnt/β-catenin signaling in Müller cells leads to an increase in NMDA-mediated apoptosis, TUNEL labeling on meridional sections of retinas was performed 24 h after injection of NMDA or PBS into the vitreous cavity of 8-week-old *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice and the *Ctnnb1*$^{fl/fl}$ controls and tamoxifen treatment.

In eyes of both genotypes, only a few TUNEL-positive cells were detected in the ONL following PBS injection (Figure 4A). In contrast, in the retinas of the NMDA-injected eyes from the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$, as well as to a lesser extent in the retinas of the NMDA-treated eyes from the *Ctnnb1*$^{fl/fl}$ mice, numerous apoptotic cells were found in the RGC layer and the inner parts of the INL (Figure 4A). Quantitative analysis confirmed that the number of TUNEL-positive cells in the RGC layer of the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice (56.0 ± 4.60 per 1,000 µm, mean ± SEM) was approximately 40% higher than in the RGC layer of the *Ctnnb1*$^{fl/fl}$ controls (39.8 ± 4.70 per 1,000 µm, mean ± SEM) following NMDA injection, a difference that was statistically significant (p = 0.023; Figure 4B,C). Intriguingly, no difference in the number of apoptotic cells of NMDA-injected eyes was detected in the INL between the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ and *Ctnnb1*$^{fl/fl}$ animals (Figure 4B,C).

Acute damage of RGCs enhances Wnt/β-catenin signaling in Müller cells: Next, we investigated whether the excitotoxic damage of RGCs leads to activation of Wnt/β-catenin signaling in Müller cells. Therefore, western blot analyses were performed on retinal proteins from NMDA- or PBS-injected *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice and *Ctnnb1*$^{fl/fl}$ littermates that served as controls for β-catenin expression. In the *Ctnnb1*$^{fl/fl}$ and *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice, a moderate signal for β-catenin was observed in retinal proteins after PBS injection (Figure 5A). In contrast, treatment of the *Ctnnb1*$^{fl/fl}$ controls with NMDA led to a marked increase in β-catenin levels compared to the PBS-injected contralateral eyes (Figure 5A). However, no difference in the retinal β-catenin levels was seen between the NMDA- or PBS-injected eyes of the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice (Figure 5A).

Quantification with densitometry demonstrated a statistically significant increase of more than 85% in the β-catenin protein level in the retinas of the *Ctnnb1*$^{fl/fl}$ animals following NMDA treatment compared to that of the PBS-injected contralateral eyes (1.87 ± 0.31; $p<0.05$; Figure 5B), whereas in the retinas of the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice, no difference between the PBS- (1.24 ± 0.21) and NMDA- (1.08 ± 0.19; Figure 5B) treated eyes could be observed. Overall, the results strongly suggest that excitotoxic damage of the RGCs leads to enhanced expression of β-catenin in Müller cells.

Wnt/β-catenin signaling in Müller cells after acute damage of RGCs but also its loss in undamaged retina enhances gliosis reaction: To analyze whether Müller cell gliosis is mediated via enhanced Wnt/β-catenin signaling in Müller cells, quantitative real-time RT–PCR for Gfap on retinal RNA from eyes of 8-week-old mice that had been injected with NMDA or PBS after tamoxifen treatment was performed. In the *Ctnnb1*$^{fl/fl}$ mice, a moderate but statistically significant induction in the mRNA level for Gfap (1.7 ± 0.3; $p<0.05$) was detected following NMDA treatment compared to the PBS-injected contralateral eyes (Figure 5C). Intriguingly, following PBS injection of *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice, a statistically significant increase in *Gfap* (2.5 ± 0.6; $p<0.05$) was observed compared to the PBS-injected *Ctnnb1*$^{fl/fl}$

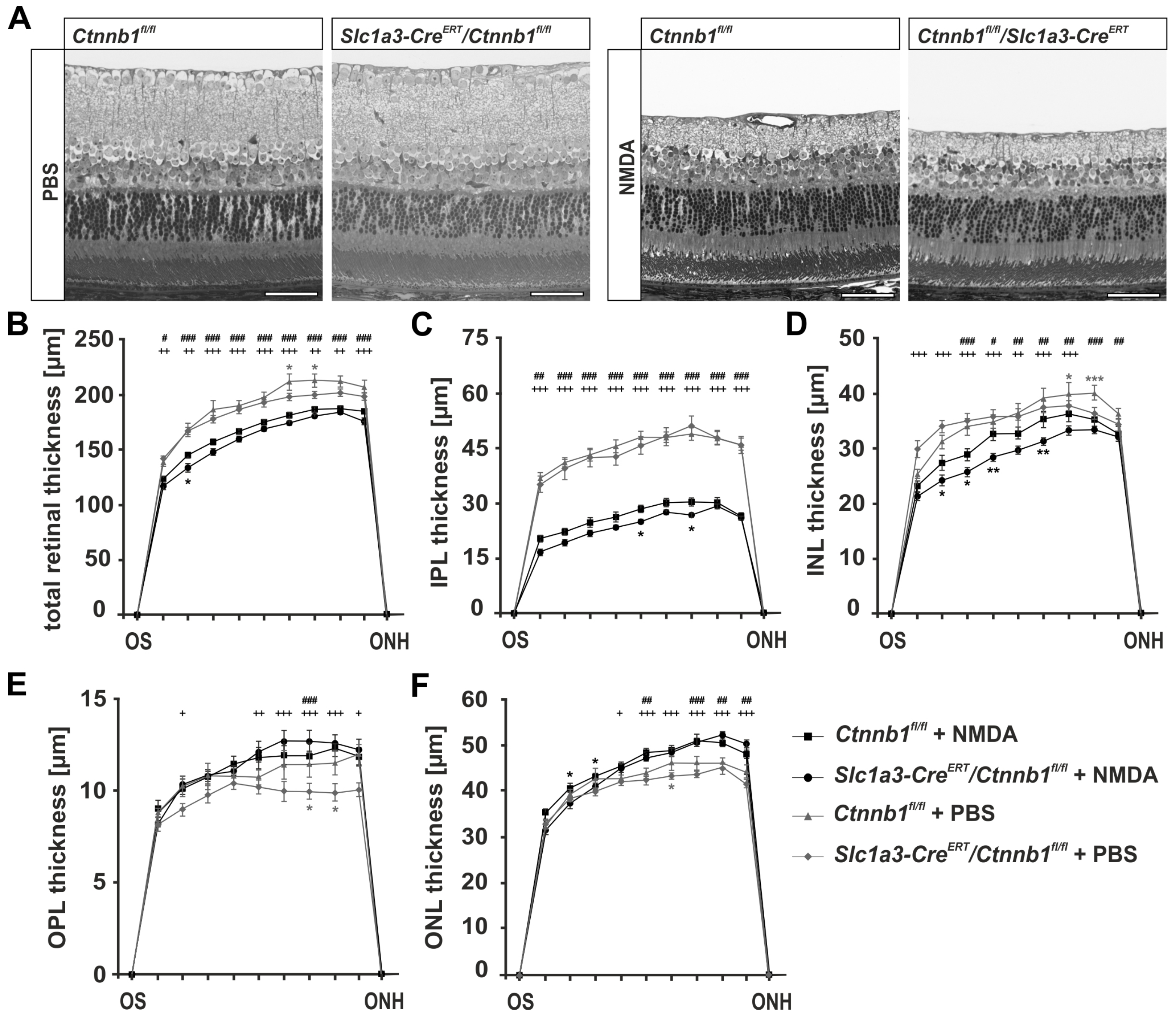


Figure 3. Deficiency of Wnt/β-catenin signaling in Müller cells enhances degeneration of the inner retina following acute RGC damage. **A**: Representative sagittal sections of retinas from 10-week-old *Ctnnb1*$^{fl/fl}$ and *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice 2 weeks after injection of N-methyl-D-aspartate (NMDA) or PBS into the vitreous cavity and treatment with tamoxifen at the age of 6 weeks. Scale bars, 50 µm. **B**–**F**: For quantification, the thickness of the (**B**) total retina, (**C**) the inner plexiform layer (IPL), (**D**) the inner nuclear layer (INL), (**E**) the outer plexiform layer (OPL), and (**F**) the outer nuclear layer (ONL) of 10-week-old *Ctnnb1*$^{fl/f}$ and *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice 2 weeks after injection of PBS or NMDA into the vitreous cavity and treatment with tamoxifen 4 weeks before was measured between every retinal tenth and plotted as a spider diagram. OS, ora serrata; ONH, optic nerve head; mean ± standard error of the mean, SEM; n≥8; *p<0.05, **p<0.01, ***p<0.001; * indicates statistical significance between the two genotypes with the same treatment; # (*Ctnnb1*$^{fl/f}$) and + (*Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$) indicate statistical significance between NMDA and PBS-injected eyes.

littermates. However, treatment of the *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice with NMDA did not lead to a further increase in the retinal Gfap mRNA level, suggesting that Wnt/β-catenin signaling in Müller cells might be required for gliosis reaction after acute excitotoxic damage of retinal neurons.

Wnt/β-catenin signaling in Müller cells induces expression of protective factors following acute excitotoxic damage of RGCs: In previous studies, we showed that intravitreal injection of NMDA leads to increased expression of neuroprotective factors, such as Lif in the retina [10,12]. We wondered whether the induction of protective factors is mediated by

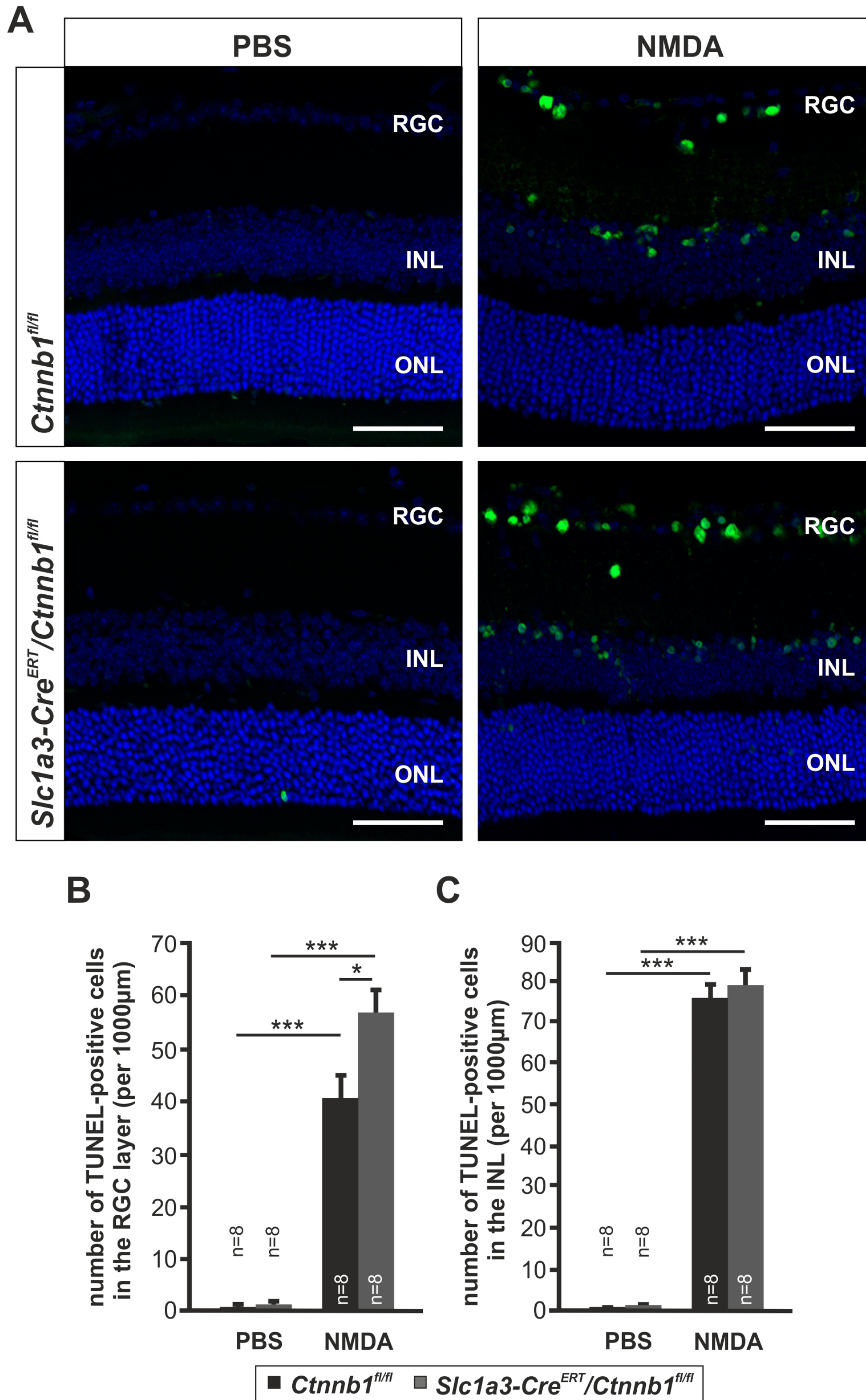
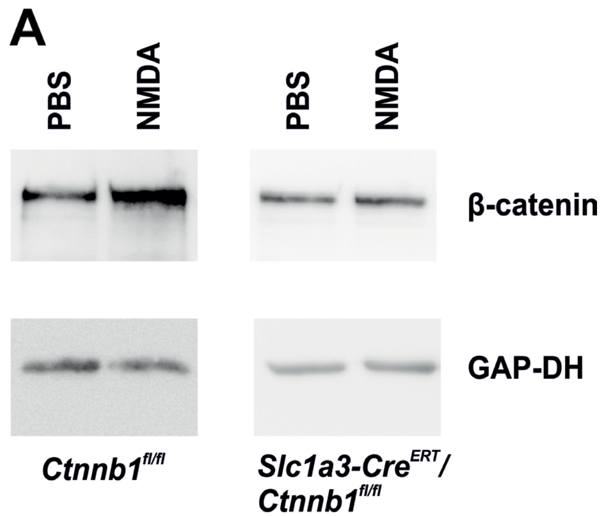
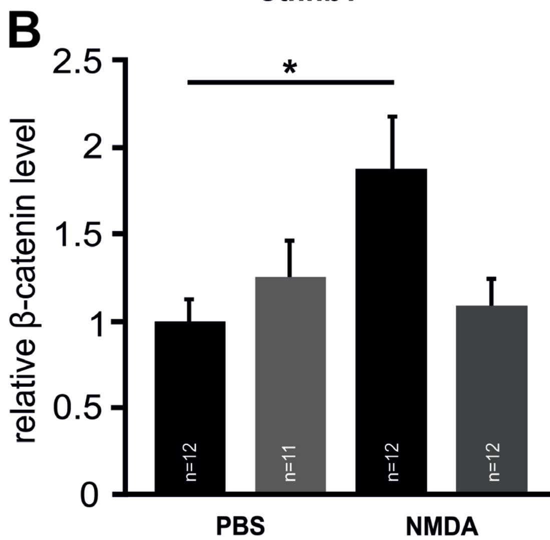


Figure 4. Wnt/β-catenin deficiency in Müller cells amplifies apoptosis of retinal neurons. **A**: Representative terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining of 8-week-old, tamoxifen-treated *Slc1a3-Cre*^ERT^/*Ctnnb1*^fl/fl^ mice and *Ctnnb1*^fl/fl^ controls 24 h after injection of 3 µl of N-methyl-D-aspartate (NMDA; 10 mM) in one eye and PBS in the contralateral eye. In the retinas of the *Slc1a3-Cre*^ERT^/*Ctnnb1*^fl/fl^ mice, statistically significantly more TUNEL-positive cells in the retinal ganglion cell (RGC) layer were observed compared to those in the *Ctnnb1*^fl/fl^ controls. Intriguingly, no difference in the number of apoptotic cells was detected in the inner nuclear layer (INL) between the *Slc1a3-Cre*^ERT^/*Ctnnb1*^fl/fl^ and *Ctnnb1*^fl/fl^ animals. Magnification bars, 50 µm; ONL, outer nuclear layer. **B, C**: The number of TUNEL-positive cells in the RGC layer (**B**) and the INL (**C**) was quantified and correlated to the retinal length (mean ± standard error of the mean, SEM; *p<0.05; ***p<0.001).





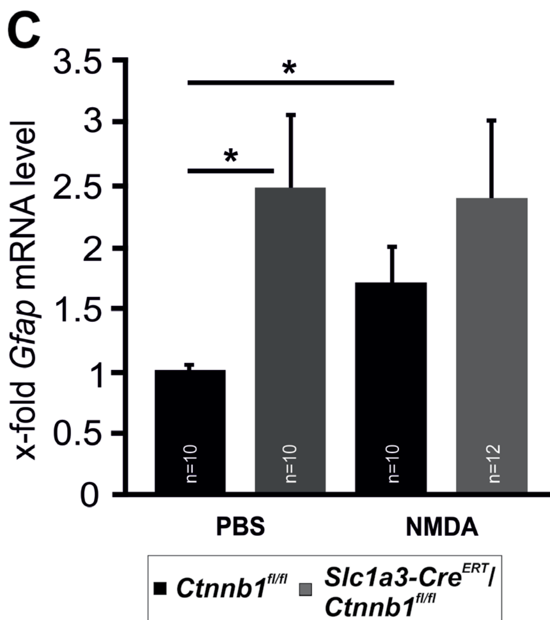


Figure 5. Acute damage of retinal ganglion cells enhances β-catenin expression in Müller cells and gliosis reaction via Wnt/β-catenin signaling in Müller cells. **A, B**: Western blot analyses (**A**) and densitometry (**B**) for β-catenin from retinal proteins of 8-week-old *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice and *Ctnnb1*$^{fl/fl}$ controls 24 h after injection of N-methyl-D-aspartate (NMDA) or PBS into the vitreous cavity and 2 weeks after treatment with tamoxifen (mean ± standard error of the mean, SEM; *p<0.05). **C**: Quantitative real-time RT–PCR for Gfap in RNA from retinas of 8-week-old *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice and *Ctnnb1*$^{fl/fl}$ controls 7 h after injection of 3 µl of NMDA (10 mM) or PBS into the vitreous cavity and tamoxifen treatment 2 weeks before (mean ± SEM; *p<0.05).

activation of Wnt/β-catenin signaling in Müller cells. To this end, quantitative real-time RT–PCR on retinal RNA from eyes of 8-week-old mice that had been injected with NMDA or PBS after tamoxifen treatment was performed.

Following injection of NMDA into the vitreous body of the *Ctnnb1*$^{fl/fl}$ mice, the mRNA expression levels for *Lif* (37.45 ± 6.430; p<0.001), *Fgf2* (2.22 ± 0.290; p<0.05), and *End2* (1.59 ± 0.18; p<0.05) were statistically significantly enhanced compared to those of the PBS-treated eyes, although the strength of the mRNA induction was different (Figure 6A-C). However, in NMDA-treated retinas from mice with a deletion of Wnt/β-catenin signaling in Müller cells, the mRNA level for *Lif* (16.94 ± 3.720) was statistically significantly lower (p<0.05; Figure 6A) compared to that of the *Ctnnb1*$^{fl/fl}$ controls. In addition, there was a trend toward lower levels of *Fgf2* (1.61 ± 0.16) and *End2* (1.39 ± 0.18) although it was not statistically significant (Figure 6B,C). Overall, the observation strongly suggested that excitotoxic damage of RGCs enhances Wnt/β-catenin signaling in Müller cells, which, in turn, induces the expression of neuroprotective factors, such as Lif.

DISCUSSION

We concluded that endogenous Wnt/β-catenin signaling in Müller cells has the distinct potential to protect RGCs from excitotoxic damage, an effect that is most likely mediated via enhanced expression of neuroprotective factors. Our conclusions are based on (1) the observation that acute excitotoxic damage leads to activation of the Wnt/β-catenin pathway, (2) the decrease in optic nerve axons and the increase in TUNEL-positive cells in the RGC layer following excitotoxic damage in mice with a β-catenin deficiency in Müller cells, and (3) the finding that the expression of neuroprotective factors after excitotoxic damage is substantially decreased in mice with a β-catenin deficiency in Müller cells.

The effects of endogenous Wnt/β-catenin signaling were analyzed in a mouse model that expresses tamoxifen-dependent Cre-recombinase under the control of the *Slc1a3* promoter fragment. The *Slc1a3* gene encodes for the high-affinity glutamate transporter GLAST, which was located with immunohistochemistry in retinal Müller cells and astrocytes [21-23]. In line with these reports, we found radially oriented staining in the reporter mice, which span from the inner to the outer limiting membranes of the retina and had triangular protrusions in the nerve fiber layer corresponding to the end feet of Müller cells. However, no obvious β-galactosidase staining for astrocytes was detected in the RGC layer, in which retinal astrocytes are located. Although we cannot rule out tamoxifen-induced activation

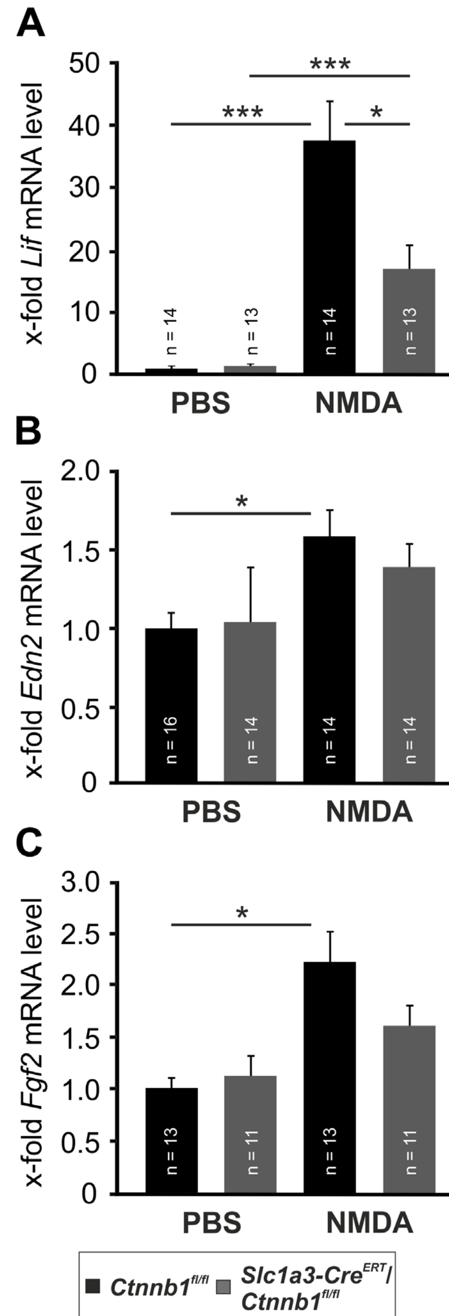


Figure 6. Acute damage of RGCs induces the expression of Lif via Wnt/β-catenin signaling in Müller cells. Quantitative real-time RT–PCR for (**A**) Lif, (**B**) Edn2, and (**C**) Fgf2 in RNA from retinas of 8-week-old *Slc1a3-Cre*^{ERT}/*Ctnnb1*$^{fl/fl}$ mice and *Ctnnb1*$^{fl/fl}$ controls 7 h after injection of 3 µl of N-methyl-D-aspartate (NMDA; 10 mM) or PBS into the vitreous cavity and tamoxifen treatment 2 weeks before (mean ± standard error of the mean, SEM; *p<0.05; ***p<0.001).

of the recombinase in a few astrocytes, several studies clearly demonstrated expression of Cre-recombinase in Müller cells of *Slc1a3-CRE*^{ERT} mice [24-26]. The observed discrepancy between the endogenous and transgenic *Slc1a3* promoter activity could be caused by the use of a *Slc1a3* promoter fragment from a BAC clone in the *Slc1a3-CRE*^{ERT} mice [27]. The protective effects of endogenous Wnt/β-catenin signaling in Müller cells were investigated following induction of NMDA-mediated excitotoxic damage of retinal neurons. After binding to NMDA-type glutamate receptors, NMDA induces apoptosis via an excessive Ca^{2+} influx [28]. In the retina, RGCs and amacrine cells are known to express these receptors and thus, are susceptible to excitotoxic damage [28], which is in accordance with our observations of TUNEL-positive cells in the RGCs and the INL. Although we found no obvious retinal phenotype or a loss of Müller cells in mice with a targeted disruption of Wnt/β-catenin signaling in these cells following tamoxifen treatment and PBS injection 4 and 2 weeks before analysis, respectively, the thickness of the total retina, the INL, and the ONL showed a moderate but statistically significant reduction in the central retina compared to those of the control mice. Several studies that examined light-mediated retinal degeneration reported enhanced damage of the central retina, which occurs because the lens focuses light on this region of the eye [29-31]. As we found the reduced retinal thickness of Wnt/β-catenin deficient mice only in the central retinal areas, but NMDA-mediated damage either occurred in the peripheral retina or was uniformly distributed, it remains to be determined whether endogenous Wnt/β-catenin signaling in Müller cells mediates protective effects on retinal neurons against damage induced by ambient light of the animal facility. Further on, in the central retinas of the NMDA-treated mice from both genotypes the ONL was thicker compared to that in the PBS-injected contralateral eyes. As following NMDA treatment the expression of protective factors is enhanced in the retina, it is tempting to speculate that these factors also protect photoreceptors against light-induced degeneration.

In several neurodegenerative diseases, such as Alzheimer or Parkinson disease, reduced Wnt/β-catenin signaling is associated with pathological progression [32,33]. In ocular disorders, enhanced expression of inhibitors of Wnt/β-catenin signaling has been observed in eyes of patients with glaucoma, retinitis pigmentosa, age-related macular degeneration, and diabetic macular edema, suggesting that impaired Wnt/β-catenin signaling might be involved in retinal degeneration [34-38]. In line with previous findings that enhanced Wnt/β-catenin signaling mediates protective effects on retinal neurons [10-13], we observed a reduced number of RGC axons and an increased number of apoptotic cells in the RGC layer in mice with a β-catenin deficiency in Müller cells. With light microscopy, approximately 32,500 RGC axons were detected in optic nerves from both genotypes following PBS injection, which were obviously lower than that observed by Williams and colleagues in a previous report of various mouse strains with electron microscopy [39]. This discrepancy might be explained by the use of mice in the C57BL/6J genetic background, which are known to have a smaller number of RGCs, and by the use of light microscopy for readout, which is less sensitive than electron microscopy. However, no effect of Wnt/β-catenin signaling on NMDA-damaged neurons in the INL was detected, in which amacrine cells are predominantly located. As amacrine cells were previously described as much more susceptible to excitotoxic damage than RGCs [40,41], we assume that the administered dose of NMDA may be excessive and covers potential neuroprotective effects of Wnt/β-catenin signaling on amacrine cells. In contrast, with light microscopy 2 weeks after NMDA injection a moderate reduction in the IPL and the INL was observed in the mice with a Wnt/β-catenin deficiency in Müller cells compared to the control animals. Two weeks after NMDA-induced damage, not only a marked loss of RGCs and amacrine cells were reported but also a moderate reduction in bipolar cells [42] suggesting that the acute degeneration of RGCs and amacrine cells could lead to secondary degeneration of bipolar cells. As in retinas with normal Wnt/β-catenin signaling in Müller cells the number of surviving RGCs is increased, and the expression of neuroprotective factors is enhanced, it is tempting to speculate that this scenario could protect retinal neurons in the INL.

A major cell type mediating potential neuroprotective effects are Müller cells. The important role of Müller cells for the maintenance of retinal homeostasis was pointed out in a transgenic mouse model with inducible ablation of Müller cells. Following selective ablation of Müller cells, degeneration of photoreceptors and enhanced retinal Wnt/β-catenin signaling were observed [19,43]. In line with these reports, the loss of Wnt/β-catenin signaling in Müller cells leads to enhanced expression of GFAP, strongly suggesting that Wnt/β-catenin signaling in these cells is required for normal retinal function. In addition, in retinas of mice with targeted disruption of Wnt/β-catenin signaling in Müller cells, no further expression of Gfap mRNA was observed following NMDA treatment compared to control littermates. However, the role of Wnt/β-catenin signaling in Müller cells for damaged retinal neurons has not been investigated thus far. RGC damage leads to reactive gliosis of Müller cells, which enables Müller cells, depending on the kind of RGC damage and its duration, to mediate protective or detrimental effects. For instance, following NMDA-induced damage of

RGCs, enhanced NFκB-mediated expression of TNF-α was observed, which can further enhance the susceptibility of RGCs to excitotoxic damage via increased insertion of Ca^{2+}-permeable AMPA receptors [44,45]. However, for reactive Müller cells, increased expression of protective factors, such as Lif, Cntf, or Edn2, following damage of RGCs has been described as well [10,46,47], which is in line with the observation of reduced *Lif* expression in mice with the deletion of Wnt/β-catenin signaling in Müller cells. Further on, following laser damage, retinal Wnt/β-catenin signaling was enhanced, and the increased retinal activity of Wnt/β-catenin signaling was shown to be associated with the more pronounced gliosis reaction of Müller cells [10,48], which is in line with the blocked induction of Gfap mRNA after NMDA treatment of mice with the targeted deletion of Wnt/β-catenin signaling in Müller cells. In the present study, we demonstrated that the damage-induced activation of Wnt/β-catenin signaling in the retina occurs predominantly in Müller cells, which, in turn, leads to the increased expression of neuroprotective factors. Overall, we identified the Wnt/β-catenin pathway in Müller cells as part of a neuroprotective network in the retina to promote RGC survival after acute damage, an effect that involves enhanced expression of protective factors.

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