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RESEARCH ARTICLE

$GABA_B$ receptor agonist baclofen promotes central nervous system remyelination

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Abstract

Promoting remyelination is considered as a potential neurorepair strategy to prevent/limit the development of permanent neurological disability in patients with multiple sclerosis (MS). To this end, a number of clinical trials are investigating the potential of existing drugs to enhance oligodendrocyte progenitor cell (OPC) differentiation, a process that fails in chronic MS lesions. We previously reported that oligodendroglia express $GABA_B$ receptors ($GABA_B$Rs) both in vitro and in vivo, and that $GABA_B$R-mediated signaling enhances OPC differentiation and myelin protein expression in vitro. Our goal here was to evaluate the pro-remyelinating potential of $GABA_B$R agonist baclofen (Bac), a clinically approved drug to treat spasticity in patients with MS. We first demonstrated that Bac increases myelin protein production in lysolecithin (LPC)-treated cerebellar slices. Importantly, Bac administration to adult mice following induction of demyelination by LPC injection in the spinal cord resulted in enhanced OPC differentiation and remyelination. Thus, our results suggest that Bac repurposing should be considered as a potential therapeutic strategy to stimulate remyelination in patients with MS.

KEYWORDS
baclofen, $GABA_B$ receptor, multiple sclerosis, myelin, oligodendrocyte, remyelination

1 | INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterized by disseminated demyelination (Dendrou et al., 2015). As a consequence of inflammatory demyelination, action potential conduction is disrupted and axons are deprived from metabolic and trophic support, which leads to axonal loss (Lee et al., 2012; Saab et al., 2016), the main correlate of permanent disability in patients with MS (Trapp et al., 1999). The majority of currently available treatments for MS target CNS inflammation and associated relapses, but do not prevent long-term disability (Kremer, Akkermann, et al., 2019). Thus, the development of therapies to prevent axonal and neuronal loss remains an unmet therapeutic need for patients with MS (Lubetzki et al., 2020). Remyelination is the spontaneous regeneration of myelin that prevents axonal degeneration both in animal models (Irvine & Blakemore, 2008; Mei et al., 2016) and

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patients with MS (Kornek et al., 2000). However, in most patients, the efficiency of this process decreases significantly with age and disease progression (Franklin and ffrench-Constant, 2017). Therefore, the development of novel treatments that enhance remyelination is a major goal of current MS research, and includes the repurposing of existing drugs (Kremer, Göttle, et al., 2019).

A block in OPC differentiation (Kotter et al., 2006; Kuhlmann et al., 2008) and lack of myelin sheath formation by surviving mature oligodendrocytes (OLs) have been pointed out as important contributors to remyelination failure in MS (Duncan et al., 2018; Yeung et al., 2019; Heb et al., 2020; Franklin et al., 2021). Therefore, promoting OPC differentiation and improving OL myelination capacity are potential strategies for enhancing myelin repair and preventing neurodegeneration in this disease.

Neurotransmitters are important mediators of OPC-neuron communication with a clear influence on OPC behavior (Domercq et al., 2010; Fannon et al., 2015; Hamilton et al., 2017; Li et al., 2013; Serrano-Regal, Luengas-Escuza, et al., 2020; Zonouzi et al., 2015). As OPCs receive both excitatory and inhibitory synaptic inputs, mediated by glutamate and GABA (Bergles et al., 2000; Káradóttir et al., 2008; Kukley et al., 2008; Lin & Bergles, 2004), these molecules have been identified as key regulators of oligodendroglial maturation and myelination (Bai et al., 2021; Fannon et al., 2015; Gautier et al., 2015; Serrano-Regal, Bayón-Cordero, et al., 2020).

Regarding myelin repair, GABAergic signaling through $GABA_A$Rs has been associated with remyelination after focal demyelination in the rat *corpus callosum* (Kalakh & Mouihate, 2019), as well as in the caudal cerebellar peduncle (Cisneros-Mejorado et al., 2020). $GABA_B$Rs have also been suggested as important modulators of myelination given that $GABA_B$R antagonism increased OPC proliferation while decreasing their maturation and the production of myelin-related proteins in the developing rat *cingulum* (Pudasaini et al., 2022). However, the role of oligodendroglial $GABA_B$Rs in myelin regeneration remains to be investigated.

Baclofen (Bac), the best known $GABA_B$R agonist, is currently used as a therapeutic agent for spasticity in MS, and can be administered either intrathecally or orally because it crosses the blood–brain barrier (Ertzgaard et al., 2017). We previously reported that $GABA_B$R activation by Bac promotes differentiation and myelin protein expression in rat cortical OPC (Serrano-Regal, Luengas-Escuza, et al., 2020). Here, we investigated whether Bac modulates remyelination in lysolecithin (LPC)-demyelinated organotypic cerebellar slices as well as in LPC spinal cord lesions in adult mice. Our results demonstrate that Bac stimulates myelin protein production ex vivo and enhances remyelination in vivo, which suggests that this drug may also be a useful therapeutic agent to stimulate remyelination.

2 | MATERIALS AND METHODS

2.1 | Animals

All experiments were conducted with the approval of the ethical committee of the University of the Basque Country (UPV/EHU). Animals were handled in accordance with the European Directive 2010/63/EU and were housed under standard conditions with a 12 h light–dark cycle and ad libitum access to food and water. All possible efforts were made to minimize animal suffering and the number of animals used. Sprague Dawley rats, C57BL/6 mice, and transgenic mice expressing fluorescence reporter DsRed under the control of the glial-specific proteolipid protein promoter (PLP-DsRed; Hirrlinger et al., 2005), generously provided by Prof. Dr. F. Kirchhoff (University of Saarland, Homburg, Germany), were used in this study.

2.2 | Cerebellar organotypic slice culture

Slice cultures were made from cerebella of P5-P7 or P11-day-old Sprague Dawley rats and P11-day-old transgenic PLP-DsRed mice according to previously described procedures (Doussau et al., 2017; Dusart et al., 1997; Tan et al., 2018). Briefly, cerebella were cut with a tissue chopper (McIlwain) into 350 μm parasagittal slices. Meninges were removed and slices were plated onto 0.4 μm pore size Millicell CM culture inserts (Millipore), containing 2–3 slices each. Rat slices were maintained in six-well plates for 13–15 days and mice cerebellar slices for 11 days in culture medium consisting of 50% basal medium with Earle's salt (BME), 25% Hank's Balanced Salt Solution (HBSS), 25% inactivated horse serum (all from ThermoFisher Scientific), 5 mg/ml glucose (Panreac), 0.0025 mM L-glutamine (Sigma-Aldrich) and antibiotic-antimycotic solution (100 U/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B; ThermoFisher Scientific) at 37°C in a humidified atmosphere with 5% CO_2. Culture medium was replaced every 2–3 days. Slices were treated with GABAergic drugs starting on the day 2 in vitro (Table 1). Lysolecithin (LPC)-induced demyelination experiments were carried out in cerebellar slices from P11 animals at day 7 in vitro by incubation for 16 h with 0.5 mg/ml LPC (Sigma-Aldrich) (Birgbauer et al., 2004). Treatments were performed at the same time as the LPC-stimulus. Slices were fixed in culture inserts with 4% paraformaldehyde (PFA) solution in phosphate-buffered saline (PBS; pH 7.4) for immunochemistry or processed for western blot analysis at 4 and 6 days after treatment.

2.3 | Optic nerve-derived organotypic slice culture

Cultures were obtained from optic nerves of P11-day-old transgenic PLP-DsRed mice. Optic nerves together with the retina were extracted in

TABLE 1 GABAergic agonists and antagonists used in this study

Product	Reference	Supplier	Concentration (in vitro)
GABA	A2129	Sigma-Aldrich	100 μM
Gabazine	SR-95531	Sigma-Aldrich	50 μM
Baclofen	0796	Tocris Bioscience	100 μM
Muscimol	0289	Tocris Bioscience	100 μM

order to maintain tissue organization and cellular connections. Meninges and residual tissue were removed in supplemented (2 μl/ml gentamicin, 1 mg/ml bovine serum albumin, BSA and 2 mM L-glutamine) HBSS under the microscope, and the optic nerve-retina units were maintained in 0.4 μm pore size Millicell CM culture inserts (Millipore), containing one unit each. Explants were placed in six-well plates for 3 days in the culture medium as described above for cerebellar organotypic cultures and in the same conditions. To favor appropriate feeding of the optic nerve-retina unit, 50 μl of culture medium were added directly over the tissue (Azim & Butt, 2011). $GABA_BR$ specific agonist baclofen (100 μM) was added to the medium immediately after plating and maintained for 3 days with daily renewal. Optic nerves without retina were fixed with 4% PFA in PBS and whole-mounted on slides with Prolong™ Gold antifade (Invitrogen).

2.4 | EdU labeling and detection

5-ethynyl-2′-deoxyuridine (EdU; Invitrogen) (10 μM) was added to the organotypic medium at day 5 in vitro and left for 48 h, to label proliferating cells. EdU was revealed using Click-iT Alexa Fluor 647 Imaging Kit according to the manufacturer's instructions (Invitrogen).

2.5 | Demyelinating lesion induction

Demyelinating lesions were induced in the spinal cord of 10-week-old female C57BL/6 mice by a stereotaxic injection of 0.5 μl of 1% LPC (Sigma-Aldrich) in sterile 0.9% NaCl solution, as previously described (Tepavcevic et al., 2014). Mice were anesthetized by intraperitoneal injection (i.p.) of a solution of ketamine (90 mg/kg; Fatro)/xylazine (20 mg/kg; Calier). Buprenorphine (0.1 mg/kg; Dechra) was subcutaneously administered as postoperative analgesic treatment. Daily i.p. injections of vehicle (saline solution) or baclofen (8 mg/kg) were performed from 5 to 16 days post lesion (dpl). Mice were sacrificed at 12 or 16 dpl, and the tissue was processed for immunohistochemical (IHC) or transmission electron microscopy (TEM) analysis, respectively.

2.6 | Perfusion and tissue processing

Mice were euthanized with ketamine/xylazine and transcardially perfused with 2% PFA solution in PBS for IHC analysis or 4% glutaraldehyde in 0.1 M PB for TEM studies. For IHC analysis, spinal cords were post-fixed with the same PFA solution, cryoprotected in 15% sucrose solution (Panreac) and frozen in 7% gelatin (Sigma-Aldrich)/15% sucrose solution in PBS. Samples were cut using a cryostat CM3050 S (Leica) to obtain 12 μm-thick coronal sections. For TEM studies, spinal cords were postfixed overnight, washed in 0.1 M PB, and cut into 2 mm-thick blocks. The tissue was postfixed in 1% osmium solution in 0.1 M PB, dehydrated and embedded in epoxy resin (Sigma Aldrich). Semithin (1 μm-thick) and ultrathin (55 nm-thick) sections were cut with an ultramicrotome RMC Boeckeler.

2.7 | Immunochemistry

Cerebellar slices were washed in PBS, permeabilized and blocked in 4% goat serum and 0.1% Triton X-100 in PBS (blocking buffer) for 1 h and incubated overnight at 4°C with primary antibodies (Table 2). Slices were washed in PBS with 0.1% Triton X-100 and incubated with Alexa fluorophore-conjugated secondary antibodies (1:400; Invitrogen) in blocking buffer for 1 h at RT. Slides with cryostat spinal cord sections were air-dried for 1 h, rehydrated in Tris buffer saline (TBS; 20 mM Tris and 1.4 M NaCl in dH_2O; pH 7.6) and pre-treated with absolute ethanol (Sharlab) for 15 min at −20°C. For APC and Olig2 immunostaining, antigen retrieval was performed by heating the sections in low-pH retrieval buffer (Vector Laboratories) for 45 s using a microwave. After washing, samples were incubated in blocking buffer solution (1% BSA, 5% goat serum, and 0.1% Triton X-100) for 30 min at RT, and then with the primary antibodies diluted in blocking buffer overnight at 4°C (Table 2). Sections were washed in TBS, and incubated with Alexa fluorophore-conjugated secondary antibodies (1:500; Invitrogen) in blocking solution for 1 h at RT. Cell nuclei were counterstained with DAPI (4 μg/ml, Sigma-Aldrich) and sections were mounted with Fluoromount-G (SouthernBiotech).

TABLE 2 Antibodies used in this study for immunohistochemistry

Antibody	Host	Dilution (rat tissue)	Dilution (mouse tissue)	Supplier	Reference
Anti-$GABAR_{B1}$	Rabbit	1:200	1:200	Alomone labs	AGB-001
Anti-$GABAR_{B1}$	Mouse	—	1:200	Abcam	#55051
Anti-$GABAR_{B2}$	Rabbit	1:200	1:200	Alomone labs	AGB-002
Anti-APC (clone CC1)	Mouse	1:200	1:200	Calbiochem	#OP80
Anti-Olig2	Mouse	1:200, 1:1000	1:500	Millipore	#MABN50
Anti-MBP	Chicken	—	1:200	Millipore	#AB9348
Anti-PDGFRα	Rat	—	1:300	BD Biosciences	#558774
Anti-Iba1	Guinea pig	—	1:200	Synaptic systems	234,004
Anti-Nkx2.2	Mouse	—	1:20	Developmental Studies Hybridoma Bank	#Q4818001B
Anti-GFAP	Rabbit		1:100	Millipore	#AB5804

2.8 | Image acquisition and analysis

Images from cerebellar organotypic slices and optic nerve explants were acquired using Zeiss LSM800 and/or Leica TCS SP8 laser scanning confocal microscopes. Cells in cerebellar slices were counted blindly along the z-stack using a 20× objective in Leica TCS SP8 confocal microscope. At least 3 different fields from 2 slices per experiment were analyzed by using LAS AF Lite software (Leica). The fluorescence signal corresponding to the PLP-DsRed OLs was quantified by ImageJ software and data were expressed as arbitrary units of fluorescence for each experimental situation. Images from spinal cord sections were collected using Zeiss LSM800 and/or Leica TCS SP8 confocal microscope and imported to *ImageJ* software. Area lacking myelin basic protein (MBP) staining within the *dorsal funiculus* of the spinal cord (area of demyelination) was delimited as region of interest (ROI) and measured. Cells positive for the markers of interest were counted from at least 3 different slices per animal. Results are presented as percentage of positive cells per lesion area measured or percentage area of lesion occupied by the corresponding markers. Same settings were kept for all samples (control and treated) belonging to a specific experiment. All images are shown as projections from z-stacks. For TEM studies, semi thin sections stained with Richardson's Blue were used to identify the lesion area. Ultrathin sections were cut and contrasted by incubation in 4% uranyl acetate and lead citrate solution for its visualization in Philips CM200 transmission electron microscope. Remyelinated axons were counted. Remyelination was determined as the percentage of OL and Schwann cell (SC)-remyelinated axons within the total numbers of axons initially demyelinated (those remyelinated + those demyelinated).

2.9 | Western blot

After treatments, cerebellar slices were directly resuspended in sodium dodecyl sulfate sample buffer on ice to enhance the lysis process and avoid protein degradation. Samples were boiled at 99°C for 8 min, size-separated by sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) in 4%–20% Criterion TGX Precast gels and transferred to Trans-Blot Turbo Midi PVDF Transfer Packs (Bio-Rad, Hercules). Membranes were blocked in 5% BSA (Sigma-Aldrich) in Tris-buffered saline/ 0.05% Tween-20 (TBS-T) and proteins were detected with specific primary antibodies (Table 3). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000; Sigma-Aldrich) and were developed by using an enhanced chemiluminescence detection kit according to the manufacturer's instructions (Supersignal West Dura or Femto; ThermoFisher Scientific). Protein bands were detected with a ChemiDoc XRS Imaging System (Bio-Rad) and quantified by volume using *ImageLab* software (version 3.0; Bio-Rad).

TABLE 3 Antibodies used in this study for western blot analysis

Antibody	Host	Dilution	Supplier	Reference
Anti-MAG	Mouse	1:500	Santa Cruz	SC-376145
Anti-CNPase	Mouse	1:1000	Sigma-Aldrich	#C5922
Anti-MBP	Mouse	1:1000	Biolegend	#SMI 99
Anti-GAPDH	Mouse	1:1000	Millipore	#MAB374
Anti-β-tubulin	Mouse	1:5000	abcam	AB7291

2.10 | Statistical analysis

All data are presented as mean ± SEM. Statistical analyses were performed using *GraphPad Prism* statistical software (version 8.0; GraphPad software). Comparisons between multiple experimental groups were made using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. For comparisons between two groups, we used the two-tailed Student's *t*-test assuming equal variance. In all instances, statistical differences were considered significant where $p < .05$. All the images shown represent the data obtained from at least three independent experiments.

3 | RESULTS

3.1 | Baclofen treatment increases myelin protein levels in organotypic slice cultures

We first validated the role of the GABAergic signaling in regulating oligodendroglial differentiation and myelination in organotypic cultures obtained from P5-P7 rats. We investigated $GABA_{B1}$ and $GABA_{B2}$ receptor-subunit expression during myelination ex vivo, and found that oligodendroglial cells–labeled using anti-Olig2 antibody–, and more specifically mature OLs–labeled using anti APC antibody–, express the two $GABA_BR$ subunits (Figure 1a,b), as we previously observed in OLs in vitro and in vivo (Serrano-Regal, Luengas-Escuza, et al., 2020).

Then, we were interested in evaluating whether GABA agonists could also modulate myelin-protein expression levels in cerebellar organotypic cultures. Exposure to 100 μM GABA or 100 μM muscimol (Mus; $GABA_AR$ specific agonist) did not change the levels of expression of myelin-associated glycoprotein (MAG), 2′,3′-cyclic nucleotide-3′- phosphodiesterase (CNPase) and MBP, compared with control slices (Supplementary Figures S2 and S3). However, treatment with 100 μM Bac (Figure 1c) induced a significant increase in the expression of MAG and MBP myelin proteins (2.0 ± 0.34 Bac vs. 1.0 ± 0.19 control for MAG, Figure 1d; and 1.29 ± 0.14 Bac vs. 1.0 ± 0.12 control for MBP, Figure 1f), together with a non-significant increase in the expression of CNPase (1.23 ± 0.11 Bac vs. 1.0 ± 0.14 control for CNPase, Figure 1e). This increase in MBP and MAG is similar to the effect of Bac in cultured OPCs, and becomes abrogated in the presence of $GABA_BR$ specific antagonist CGP55845 (Supplementary Figure S1). To investigate whether this effect of Bac was associated with changes in oligodendroglial proliferation, cerebellar organotypic cultures were exposed to EdU for 48 h, in the absence or presence of GABA or Bac (100 μM). Neither GABA nor Bac modified the percentage of mature OLs (APC^+Olig2^+)

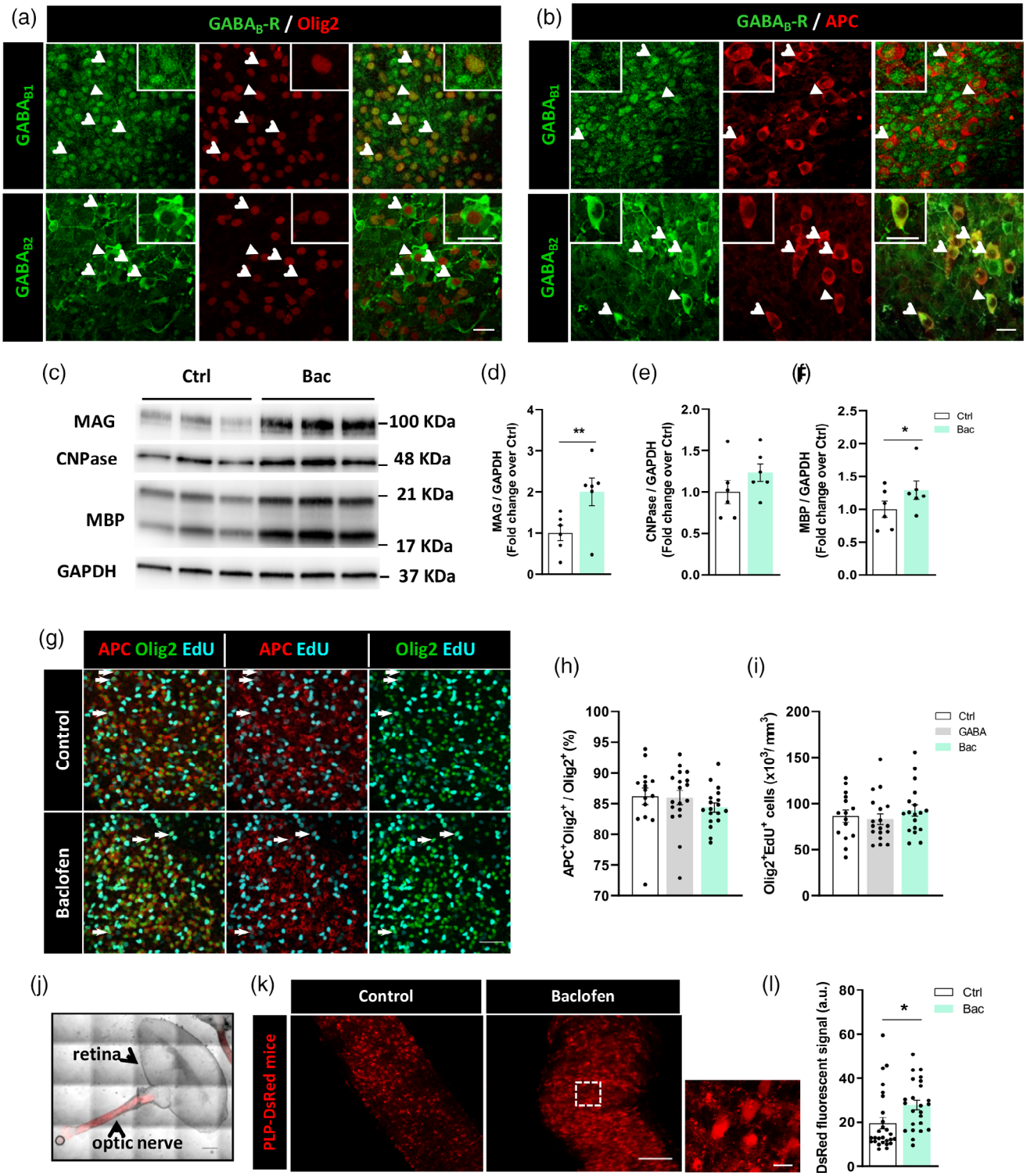

FIGURE 1 Legend on next page.

among total oligodendroglial cells, nor Olig2$^+$ cells that underwent proliferation in this time period (Olig2$^+$Edu$^+$) (Figure 1g–i), suggesting that GABA$_B$R activation promotes myelin generation by mature OLs without affecting OPC proliferative capacity. Additionally, we examined the effect of Bac in optic nerve explants of transgenic PLP-DsRed mice (Figure 1j). Quantification of PLP-DsRed-fluorescent signal (Figure 1k) revealed a significant increase in those optic nerves treated with Bac compared to controls (27.82 ± 2.27 for Bac vs. 19.62 ± 2.59 for control; Figure 1l). Together, these results show that Bac enhances myelin protein production in murine organotypic cultures and in optic nerve explants, confirming our previous observations in isolated OLs (Serrano-Regal, Luengas-Escuza, et al., 2020) in a complex environment more similar to physiological conditions.

3.2 | $GABA_BR$ activation elevates the levels of major myelin proteins during remyelination ex vivo

Since Bac promoted myelin protein synthesis in organotypic slices, we next studied the impact of $GABA_BR$ activation under experimental conditions mimicking damage to myelin. P11 rat-derived cerebellar slices were maintained for 7 days to allow myelination ex vivo and then exposed to LPC for 16 h. In the first set of experiments, slices were daily treated with GABA (100 μM) or Bac (100 μM) for 6 days after LPC exposure (Supplementary Figure S4A, B) and MAG and MBP proteins were analyzed by western blot. We found that LPC induced a significant decrease in both proteins (0.99 ± 0.11 LPC vs. 1.44 ± 0.11 fold control for MAG, and 1.00 ± 0.08 LPC vs. 1.50 ± 0.09 fold control for MBP). Bac treatment post-LPC significantly increased MAG levels (1.44 ± 0.11 Bac vs. 0.99 ± 0.11 LPC; Supplementary Figure S4C) while MBP levels were not affected (Supplementary Figure S4D). We then investigated whether previous application of these agonists during demyelinating phase may be more effective in recovering the levels of myelin protein expression after exposure to LPC. We exposed cerebellar slices to LPC concomitantly to GABA or Bac (100 μM) application. After LPC removal, GABA or Bac was maintained in the medium for 6 more days (Figure 2a). Under this experimental paradigm, we observed a significant increase in the expression levels of both MAG and MBP (2.75 ± 0.35 GABA and 2.81 ± 0.26 Bac vs. 1.0 ± 0.26 LPC for MAG, and 3.39 ± 0.52 GABA and 2.82 ± 0.29 Bac vs. 1.0 ± 0.37 LPC for MBP; Figure 2b–d).

We also used immunofluorescence to investigate the effect of $GABA_BR$-mediated signaling on OL differentiation and myelin protein production ex vivo by taking advantage of PLP-DsRed reporter mice, in which changes in PLP-associated endogenous fluorescence can be exploited to track changes in PLP-expression. We prepared organotypic cerebellar slices and maintained these in culture for 7 days, after which we exposed the slices to LPC in combination with drug treatments for 4 days. We applied GABA (100 μM), Bac (100 μM), and GABA plus gabazine (50 μM)–a $GABA_AR$ antagonist–, in order to study the effect of GABA directly over $GABA_BRs$, or gabazine alone (50 μM) (Figure 2e, f). As shown in Figure 2g, the PLP-DsRed fluorescent signal increased significantly in Bac- and GABA plus gabazine-treated slices compared to those exposed to LPC without treatment (10.42 ± 0.65 Bac and 20.74 ± 5.32 GABA plus gabazine vs. 4.5 ± 0.38 LPC; Figure 2g), indicating that $GABA_B$ receptor stimulation in these slices increases PLP production. Overall, these results indicate that $GABA_BR$ activation with Bac favors remyelination ex vivo in cerebellar organotypic slices.

3.3 | Baclofen administration promotes OPC differentiation in adult mouse CNS

We then aimed to assess the effect of Bac administration on CNS remyelination in vivo. We first validated the expression of $GABA_BR$ subunits in OPCs in normal spinal cord tissue of adult mice (Figure 3a), and observed expression of both B1 and B2. We then confirmed that this expression was maintained after induction of demyelination by LPC injection in the *dorsal funiculus*, both on reactive OPCs (Figure 3b) and by newly generated OLs (Figure 3c), which suggested these cells could be targeted by Bac.

To investigate the effect of Bac on remyelination, at 5 days post lesion (dpl), daily i.p. injections of vehicle or Bac (8 mg/kg/day) were initiated and administered during 7 days. Bac administration was initiated at 5 dpl to ensure that the treatment would not affect the extent of demyelination, as demyelination in LPC model is complited by 2 dpl. OPC differentiation was investigated at 12 dpl, given that the peak of OPC differentiation occurs during the second week post demyelination (Figure 4a).

Then, we explored the changes induced by Bac administration in OPC numbers and microglia/macrophage response at 12 dpl using anti-PDGFRα antibody as OPC marker and anti-Iba1 antibody as microglia/macrophage marker (Figure 4b). Quantification of $PDGFR\alpha^+$ cells per mm^2 (578.2 ± 117.3 Bac vs. 461.3 ± 49.93 vehicle; Figure 4c) or the percentage of lesion area occupied by Iba-1 (60.10 ± 5.52% Bac vs. 49.87 ± 11.84% vehicle; Figure 4d) revealed no variations between control vs Bac-treated mice. In addition, we used anti-Nkx2.2 antibody to label reactive OPCs and anti-GFAP antibody as

FIGURE 1 Baclofen increases myelin-related protein synthesis in organotypic cultures without altering the proliferation ratio of oligodendroglial lineage. (a) Oligodendroglial cells, distinguished as $Olig2^+$ cells (red), are positive for $GABA_{B1}$ and $GABA_{B2}$ subunits (green) of $GABA_BRs$ in cerebellar slices of P5-P7 rats. (b) Mature oligodendrocytes (OLs), identified as APC^+ cells (red), express $GABA_{B1}$, and $GABA_{B2}$ subunits (green) of $GABA_BRs$ in the same preparations. Arrows indicate double-stained cells and arrowheads show the cell magnified in the corresponding inset. Scale bars = 20 μm. (c) Representative western blot image-showing expression of myelin-associated glycoprotein (MAG), CNPase, and MBP proteins in control and baclofen-treated cerebellar slices. Quantification of MAG (d), CNPase (e), and myelin basic protein (MBP) (f) expression normalized to GAPDH values. *$p < .05$ and ** $p < .01$ versus control; paired Student's *t*-test. (g) Representative images showing immunofluorescence of mature OLs (APC^+, red) and total oligodendroglial cells ($Olig2^+$, green) co-labeled with EdU (cyan) to identify mature OLs (APC^+) and $Olig2^+$ cells in cerebellar slices in the indicated condition. Arrows indicate mature OLs (APC^+Olig2^+) or newly generated oligodendroglial cells ($Olig2^+EdU^+$). Scale bar = 50 μm. Quantification of (h) percentage of mature OL from total oligodendroglial cell pool ($APC^+Olig2^+/Olig2^+$) and (i) density of newly formed oligodendroglial cells ($Olig2^+EdU^+$), in the indicated conditions. One-way ANOVA followed by Tukey's post-test. (j) Optic nerve-retina unit from P11 PLP-DsRed transgenic mice. Scale bar = 500 μm. (k) Optic nerves explants cultured in control conditions (left) or in presence of baclofen (right) showing DsRed fluorescent signal. Scale bars = 100 μm; higher magnification scale bar = 315 μm. (l) Quantification of DsRed fluorescent signal in control and treated optic nerve explants. *$p < .05$ versus control; unpaired Student's *t*-test. (a–f): Control and bac 6 slices from different animals. (g–i): Control 15, GABA 18, and bac 18 images from cerebellar slices. (j–l): Control 27, bac 24 images from optic nerve explants

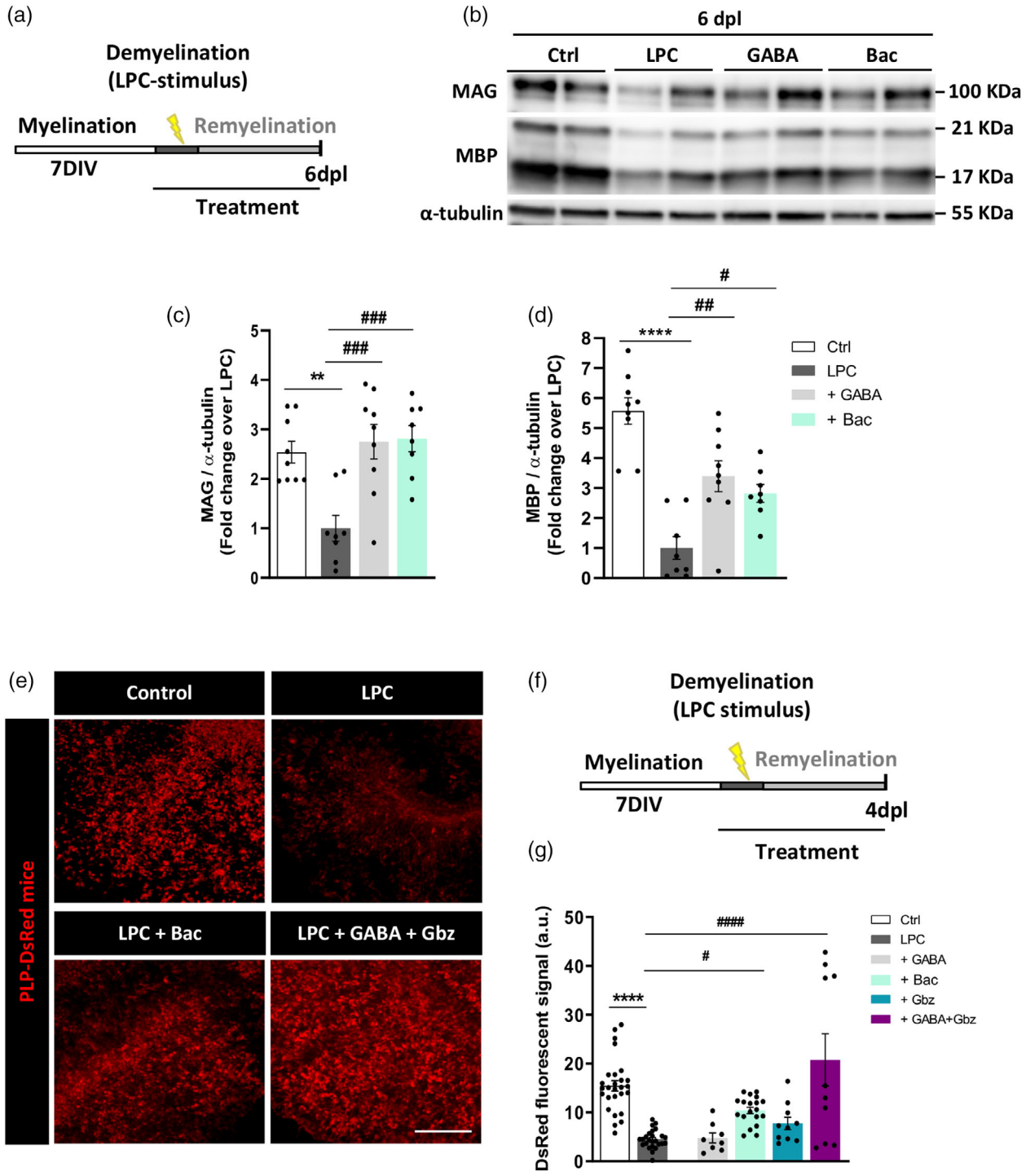

FIGURE 2 $GABA_BRs$ modulate remyelination in lysolecithin (LPC)-treated cerebellar organotypic slices. (a) Time course showing the experimental design in LPC-induced demyelination in cerebellar organotypic cultures obtained from P11 rats. (b) Representative western blot image showing in duplicates the effect of GABA and baclofen in modulating myelin-related protein restoration in LPC-treated organotypic cultures following the paradigm shown in a. (c, d) Quantification of myelin-associated glycoprotein (MAG) (c) and myelin basic protein (MBP) (d) levels in indicated conditions. $^{**}p < .01$ and $^{****}p < .0001$ versus control, $^{\#\#}p < .01$ and $^{\#\#\#}p < .001$ versus LPC; one-way ANOVA followed by Tukey's post-test. (e) Representative images of cerebellar slices from P11 PLP-DsRed transgenic mice showing DsRed fluorescence in indicated conditions. Scale bar = 100 μm. (f) Treatments were added to the slices in conjunction with LPC for 16 h and maintained thereafter for 4 days after. $GABA_BRs$ were selectively activated with baclofen or with GABA plus the $GABA_AR$ antagonist gabazine. (g) Quantification of DsRed fluorescent signal in indicated conditions. $^{****}p < .0001$ versus control, $^{\#}p < .05$ and $^{\#\#\#\#}p < .001$ versus LPC; one-way ANOVA followed by Tukey's post-test. (b–d): Control 9, LPC 8, GABA 9, bac 8 slices from different animals. (e–g): Control 27, LPC 25, GABA 8, bac 19, Gbz 10, GABA + Gbz 10 images from optic nerve explants

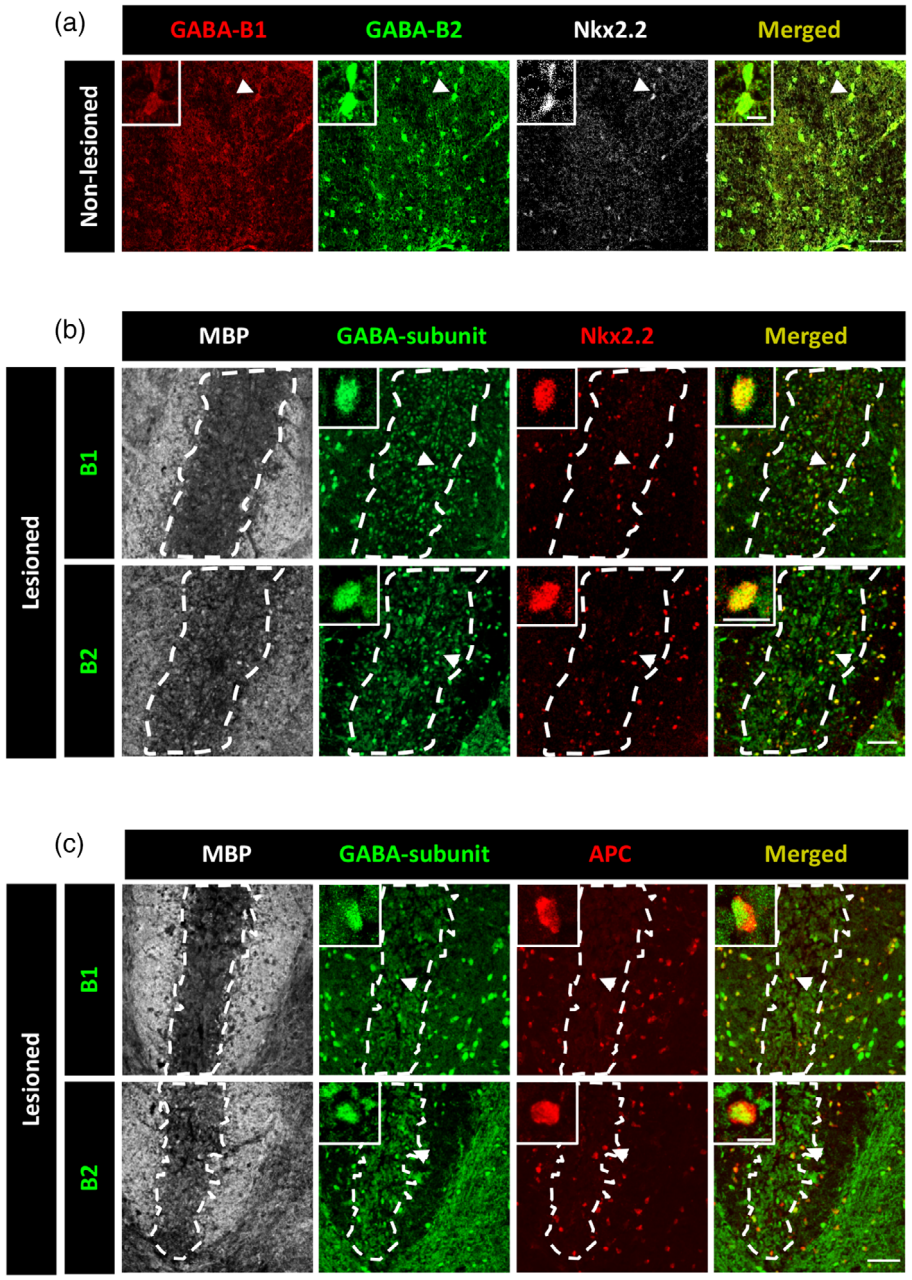

FIGURE 3 $GABA_B$ receptors are expressed by oligodendrocyte progenitor cell (OPCs) and mature oligodendrocytes from the mouse spinal cord. (a) Confocal images showing OPCs ($Nkx2.2^+$, gray) expressing $GABA_{B1}$ (red) and $GABA_{B2}$ (green) subunits of $GABA_B$Rs in the *dorsal funiculus* of the spinal cord of unlesioned mice. (B, C) Confocal images showing OPCs ($Nkx2.2^+$, red) (b) and mature OLs (APC^+, red) (c) expressing $GABA_{B1}$ (green, top) and $GABA_{B2}$ (green, bottom) subunits of $GABA_B$Rs in the *dorsal funiculus* of the spinal cord of control lysolecithin (LPC)-injected mice. White dash line indicates lesion border. Arrowheads point at cells shown at higher magnification in each photograph. Scale bars = 50 μm. Higher magnification = 10 μm

astrocyte marker (Figure 4e). We observed no significant differences in $Nkx2.2^+$ cells per mm^2 (437.4 ± 46.8 Bac vs. 496.5 ± 65.5 control; Figure 4f) nor in the percentage of lesion area occupied by GFAP (21.2 ± 4.0 Bac vs. 26.9 ± 2.0 control; Figure 4g) between vehicle versus Bac-treated animals. Finally, we investigated whether Bac administration accelerates OPC differentiation in the lesions by analyzing the numbers of mature OLs (APC^+Olig2^+) relative to the total number of oligodendroglial cells ($Olig2^+$) (Figure 4h). The percentage of APC^+ among total $Olig2^+$ cells was significantly increased in Bac-treated LPC-injected mice (41.91 ± 2.29% Bac vs. 27.95 ± 1.46% vehicle; Figure 4i), without altering the total numbers of $Olig2^+$ cells (928.0 ± 100.7 Bac vs. 814.8 ± 50.13 vehicle; Figure 4j). These results demonstrate that Bac treatment promotes differentiation of OPCs in LPC-induced demyelinating lesions.

3.4 | Baclofen administration accelerates remyelination

We next analyzed whether Bac administration accelerates remyelination. Vehicle and Bac injections were initiated at 5 dpl, and the proportion of remyelinated axons was analyzed at 16 dpl (Figure 5a), given that the onset of remyelination in the LPC model takes place at 14 dpl and is near completion at 21 dpl. At this time point, OL remyelination–identified as thin myelin sheaths–was found predominantly around lesion borders, while SC remyelination, a well-recognized feature of the LPC lesion in the spinal cord (Jeffery & Blakemore, 1995), was observed in the lesion center. TEM analysis revealed that the percentage of remyelinated axons (Figure 5b, c) was higher following Bac treatment (36.95 ± 2.18% Bac vs. 22.29 ± 1.85%

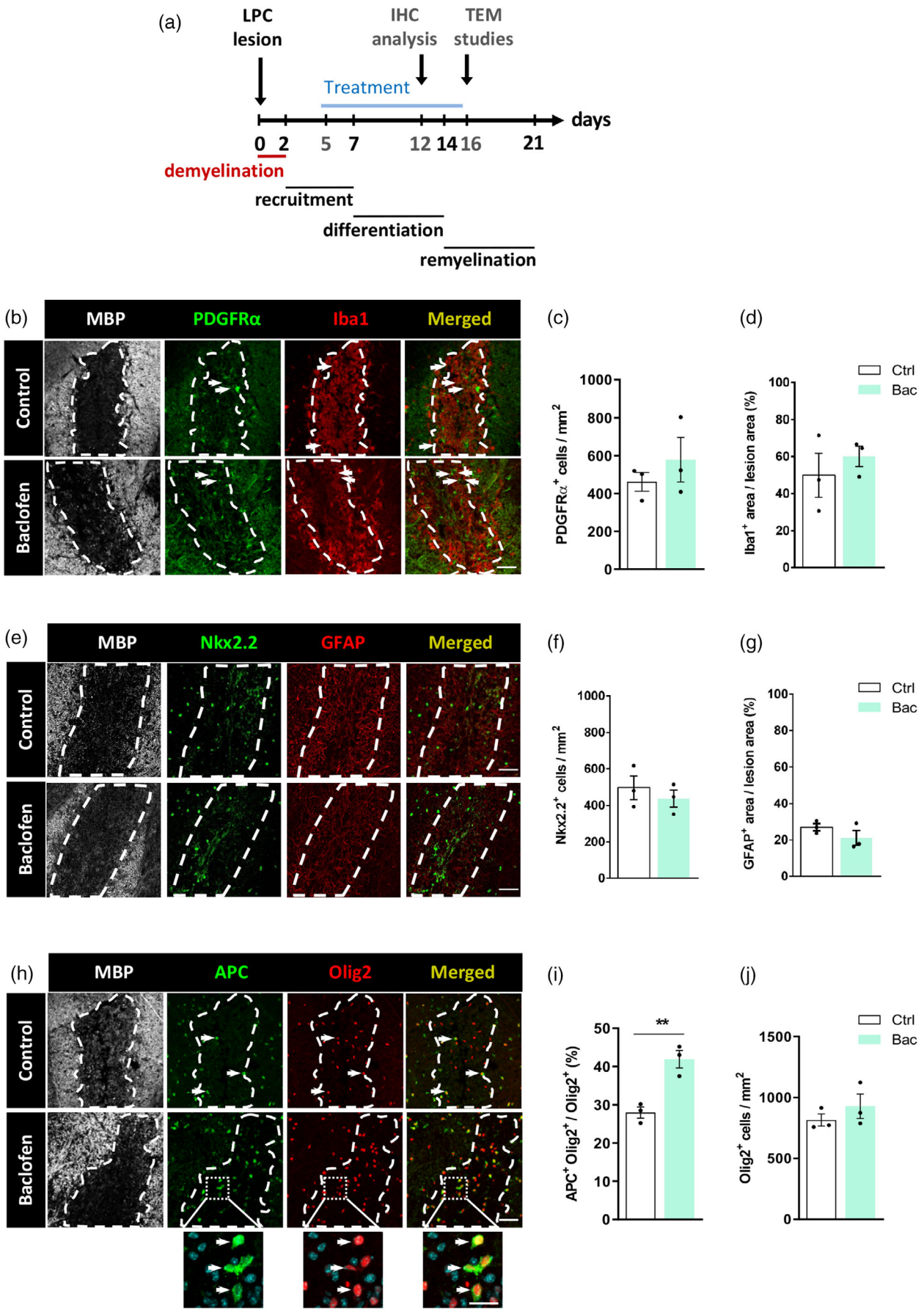

FIGURE 4 Legend on next page.

vehicle). In particular, the percentage of axons remyelinated by OLs was increased after Bac administration (33.45 ± 3.95% Bac vs. 19.75 ± 2.63% vehicle; Figure 5d), whereas SC-driven remyelination was not significantly altered (3.70 ± 2.02% Bac vs. 2.54 ± 1.82% vehicle; Figure 5e). Thus, Bac administration following LPC-induced demyelination accelerates the regeneration of myelin sheaths.

4 | DISCUSSION

Remyelination of demyelinated lesions varies considerably between MS patients, and it is associated with improved axonal preservation (Kornek et al., 2000) and less disability (Bodini et al., 2016). OPC differentiation arrest is an important contributor to failed myelin repair in MS (Kotter et al., 2006; Kuhlmann et al., 2008), and newly generated OLs are more pro-myelinating than those that survive injury (Neely et al., 2022). Therefore, promoting OPC differentiation may enhance remyelination and thus prevent axonal degeneration in MS (Irvine & Blakemore, 2008; Mei et al., 2016). In this study, we have identified that baclofen is a pro-remyelinating agent that can heal MS lesions.

We previously showed that $GABA_BR$-selective agonist Bac, a drug used for the treatment of spasticity in MS patients (Chisari et al., 2020) stimulates OPC differentiation in vitro via $GABA_BR$ activation (Serrano-Regal, Luengas-Escuza, et al., 2020). Interestingly, intrathecal Bac administration in MS patients improves cognitive performance (Farrell et al., 2021). Notably, higher cognitive performance in patients with MS correlates with quantitative improvements in myelin water imaging (Abel et al., 2020). Here, we show that Bac administration stimulates myelin protein synthesis and remyelination ex vivo and in vivo.

In order to gain insight into potential benefits of Bac application on remyelination, we first investigated the pattern of $GABA_BR$ expression of OPCs under normal and demyelinating conditions ex vivo and in vivo. While our previous in vitro and in vivo observations demonstrated the presence of both $GABA_BR$ subunits on oligodendrocyte membranes (Serrano-Regal, Luengas-Escuza, et al., 2020), the analyses of oligodendroglia in cerebellar organotypic cultures show an apparent nuclear pattern of $GABA_{B1}$ expression (Figure 1a,b), which suggests that $GABA_{B2}$ homodimers may be operating as functional $GABA_BRs$ in OLs in the organotypic slices. However, we detected a membrane pattern expression of $GABA_{B1}$ in OPCs and mature OLs in vivo in the spinal cord (Figure 3), which suggests that $GABA_{B1}$–$GABA_{B2}$ heterodimers may be operating in these cells. Together, these localization studies support the idea that expression of $GABA_BRs$ may vary across CNS regions.

Bac induces an increase in myelin proteins in cerebellar slices and optic nerve explants (Figure 1c–f; Figure 1k,l). Interestingly, the percentage of mature OLs (APC^+Olig2^+) was not affected by Bac in cerebellar slices (Figure 1g–i), which suggest that Bac is promoting OL maturation in this model. In our previous study however, Bac increased both OPC differentiation and maturation when cultured in isolation (Serrano-Regal, Luengas-Escuza, et al., 2020). The lack of effect of Bac on OPC differentiation observed in organotypic slices could be attributed to alternative actions of Bac in neurons and glial cells in this preparation. Alternatively, regional differences may also account as organotypic slices originate from cerebellum, while dissociated cultures of OPCs are obtained from cerebral cortex. Importantly, slices are prepared at a later stage in development (postnatal day 5–7), as compared to the OPC cultures (post-natal day 0–3), which means that isolated OPC cultures are much less differentiated to start with, and their differentiation is potentially more malleable, while organotypic slices may contain many more immature OLs rather than OPCs.

Another striking observation in the current study is that GABA does not induce changes in myelin proteins in cerebellar organotypic slices (Supplementary Figure S2), as opposed to what occurs in cortical cultured OPCs (Supplementary Figure S1). These differences may be due to the fact that OPCs cultured in the absence of axons lose $GABA_ARs$ and their electrophysiological responses to GABA (Arellano et al., 2016). Therefore, the positive effects of GABA in modulating OPC differentiation and OL maturation observed in cultured OPCs (Supplementary Figure S1) are likely mediated through $GABA_BR$ (Serrano-Regal, Luengas-Escuza, et al., 2020). This is consistent with our current data that $GABA_BR$ agonists (Figure 1c–f) but not $GABA_AR$ increase levels of myelin proteins. In the present study, we have used cerebellar organotypic slices in which, as suggested by electrophysiological recordings by Hamilton et al. (2017), OPCs maintain the expression of $GABA_ARs$, unlike cortical OPCs cultured in vitro. Our interpretation is that, in organotypic slices, GABA could be activating both types of oligodendroglial GABARs, and the positive effect of GABA via $GABA_BR$ may be abrogated by the activation of $GABA_ARs$. Consistently with that, activating specifically $GABA_ARs$ in these slices with the $GABA_AR$ agonist muscimol, did not affect OL

FIGURE 4 $GABA_B$ receptor activation by baclofen promotes oligodendrocyte differentiation but does not impact on oligodendrocyte progenitor cell (OPC), microglia/macrophague population or astrocytes in lysolecithin (LPC)-induced demyelinated spinal cords. (a) Diagram representing the time course of the studies in the LPC-induced demyelination model. (b) Spinal cord sections of LPC-injected control (top) and baclofen-treated (bottom) mice immunostained with anti-MBP (gray), anti-PDGFRα (green) and anti-Iba1 (red) antibodies. Dapi was used to identify cell nuclei. White dash line indicates lesion border. Quantification of number of $PDGFR\alpha^+$ cells per mm^2 (c) and percentage of $Iba1^+$ occupied area (d) in LPC-injected mice. (e) Spinal cord sections of LPC-injected control (top) and baclofen-treated (bottom) mice immunostained with anti-MBP (gray), anti-Nkx2.2 (green) and anti-GFAP (red) antibodies. Dapi was used to identify cell nuclei. White dash line indicates lesion border. Quantification of number of $Nkx2.2^+$ cells per mm^2 (f) and percentage of GFAP stained area (g) in LPC-injected mice. (h) Spinal cord sections of LPC-injected control (top) and baclofen-treated mice immunostained with anti-MBP (gray), anti-APC/CC1 (green) and anti-Olig2 (red) antibodies. Dapi was used to identify cell nuclei. White dash line indicates lesion border. Histograms showing percentage of APC^+Olig2^+ cells from total $Olig2^+$ cells (i), and number of $Olig2^+$ cells per mm^2 (j) in LPC-injected mice. At least 3 lesion areas from 3 different animals were analyzed. $^{**}p < .01$ versus control; unpaired Student's *t*-test, control 3 animals, bac 3 animals. Scale bars: *B*, *E*, *H* = 50 μm. Higher magnification = 20 μm. Arrows show positive staining

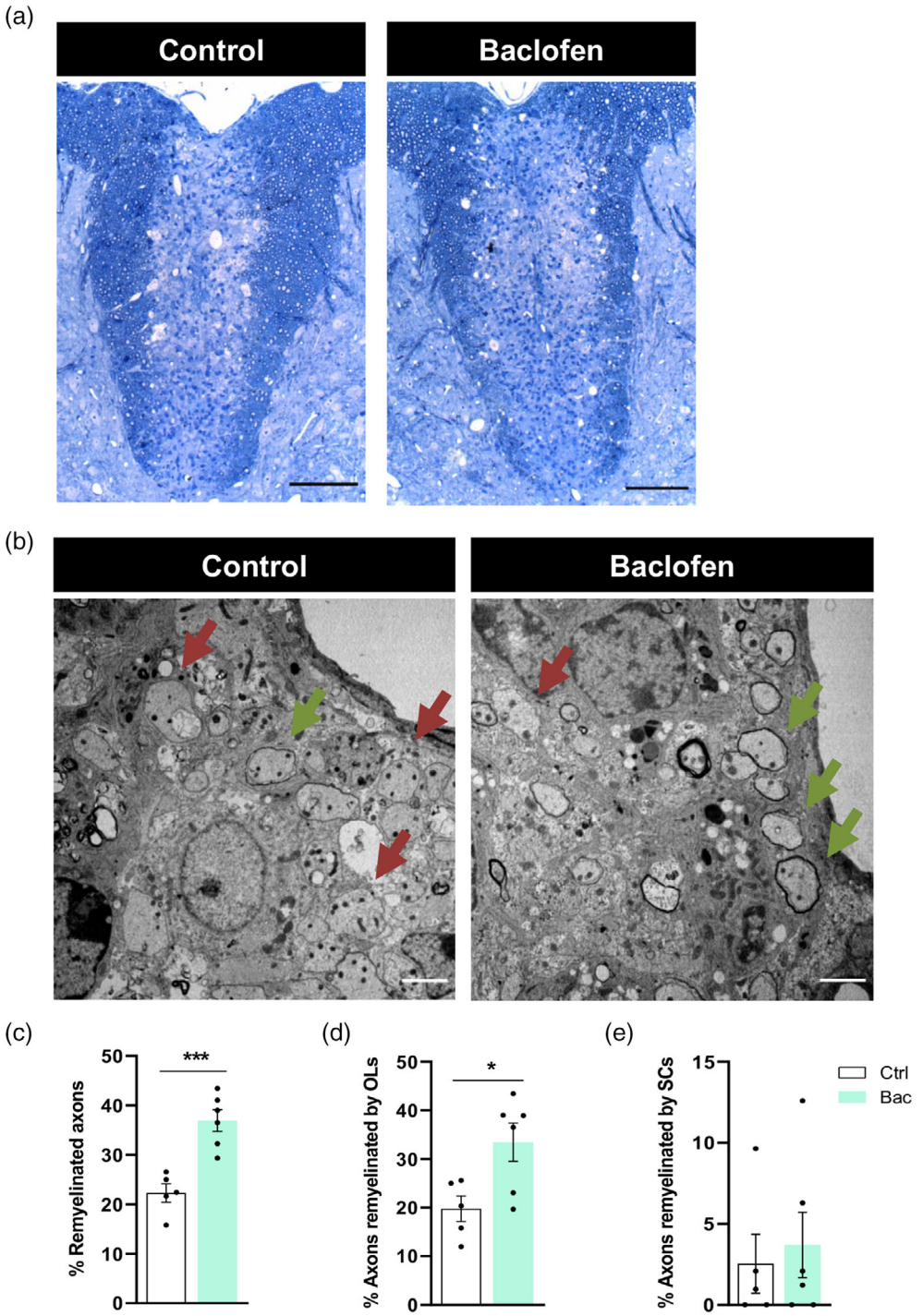

FIGURE 5 Baclofen treatment promotes remyelination following spinal cord lysolecithin (LPC)-induced demyelination. (a) Representative images of semithin sections of spinal cord control (left) and baclofen-treated (right) mice showing LPC-induced lesions, stained with Richardson's blue. (b) Electron micrographs of ultrathin spinal cord sections showing remyelinated (green arrows) and unmyelinated (red arrows) axons. Histograms showing percentage of total remyelinated axons within the lessions (c), axons remyelinated by oligodendrocytes (OLs) (d) and axons remyelinated by Schwann cells (SCs) (e). $^{*}p < .05$ and $^{***}p < .001$ versus control; unpaired Student's *t*-test, control 5 animals, bac 6 animals. Scale bars: *A* = 100 μm; *B* = 2 μm

maturation as it did not induce changes in the myelin protein levels (Supplementary Figure S3). Moreover, although GABA alone did not modulate PLP production after demyelination (Figure 2g), it did it when added together with gabazine, a $GABA_AR$ antagonist. Hence, $GABA_BR$-mediated effect is lost when GABA is activating both GABAR types.

Both GABA and Bac applied during demyelination with LPC in cerebellar organotypic slices resulted in increased levels of myelin proteins compared to LPC alone (Figure 2b–d) which suggests that Bac exerts anti-demyelination effects. Indeed, Bac has anti-inflammatory and neuroprotective properties preventing the release of pro-inflammatory cytokines by astrocytes and microglia and reducing oxidative stress (De Beaurepaire, 2018). Thus, it may be that the reduction of oxidative stress by Bac during LPC treatment protects OPCs, resulting in enhanced differentiation and myelin protein expression upon LPC withdrawal. Such a protective effect may be exploited for the repair of MS demyelinating lesions.

As mentioned above, GABA increased PLP levels after LPC stimulus in organotypic slices prepared from PLP-DsRed mice only when applied in conjunction with the $GABA_AR$ antagonist gabazine (Figure 2g), but it increased MBP and MAG levels when applied alone in cerebellar rat slices (Figure 2C-D). Thus, these different myelin

proteins and evaluation may respond differently to GABA stimulation, in terms of dynamics. Also, species and regional differences among these two preparations may account for the variable response of GABA on myelin proteins (Aguayo et al., 1994; Mendu et al., 2012; Pöltl et al., 2003). Nonetheless, our results demonstrate that Bac pro-myelinating effects prevail across regions and species.

In the current study, we used an LPC model of demyelination to evaluate the effect of Bac on remyelination. This model is, together with cuprizone, an inflammation-independent model of demyelination that allows for precise evaluation of potential pro-remyelinating agents. In this experimental setting, demyelination occurs within the first 2 days after LPC injection, after which remyelinating events initiate, and end by day 23 post-injection (Piaton et al., 2011; Tepavcevic et al., 2014; Wegener et al., 2015; Psenicka et al., 2021), which means that the process of remyelination can be manipulated following demyelination. In the cuprizone model, demyelination and remyelination occur concomitantly as the lesion develops, making it difficult to dissociate the effect on OPC responses from the protective effects on oligodendrocytes, or the effects on macrophages cleaning the myelin debris (Ransohoff, 2012). An alternative model for evaluating the therapeutic benefits of drugs in MS is experimental autoimmune encephalomyelitis (EAE), whereby myelin and oligodendrocytes are initially targeted by developing an immune response to MAG. Undoubtedly, it will be worth to examine the potential of Bac in attenuating EAE symptoms and structural damage. However, there are confounding factors that limit that study, as GABARs are also present on immune cells (Bhat et al., 2010) and Bac may impact the immune response thus making it difficult to analyze its remyelinating properties. Because of that we chose the LPC model which, in spinal cord lesions in vivo, is characterized by a defined sequence of events: demyelination within 2 dpl, followed by OPC recruitment and proliferation, OPC differentiation and remyelination (Figure 4a; Piaton et al., 2011; Tepavcevic et al., 2014, Wegener et al., 2015). Thus, the timing of Bac treatment (5–16 dpl) ensures no effect on demyelination/macrophage recruitment/debris removal. As we aimed to investigate whether Bac treatment accelerates remyelination, we quantified the proportion of remyelinated axons at 16 dpl, shortly after the onset of remyelination. Remarkably, Bac-treated mice showed an increase in the percentage of remyelinated axons, indicative of accelerated remyelination (Figure 5c,d).

Bac administration may increase OPC differentiation and consequently remyelination by multiple mechanisms. The first one is via activation of $GABA_BRs$, that, as we showed here, are expressed by oligodendroglial cells in the lesions. This mechanism would be consistent with our observations in vitro that Bac treatment of OPC cultures increases OPC differentiation, and that this effect is abrogated by application of $GABA_B$ antagonist. Whether the same holds during remyelination warrants future experiments using models with conditional ablation of $GABA_BRs$ on OPCs and/or OLs. Another potential mechanism underlying increased remyelination in Bac-treated mice could be the direct effects of Bac on microglia/macrophages, as these cells are important modulators of OPC differentiation (Miron et al., 2013). In addition, microglia-specific ablation of $GABA_{B1}Rs$ in mice impairs synaptic refinement by microglia, which leads to behavioral abnormalities (Favuzzi et al., 2021), thus suggesting that $GABA_BR$ modulation can modify microglial function. Our results show that Bac treatment did not alter the lesion area occupied by microglia/macrophage at 12 dpl (Figure 4c). However, it remains unclear whether Bac induces microglial/macrophage modifications that favor remyelination without altering their recruitment or proliferation. While further research should address the exact effect of Bac on microglia/macrophages in the context of remyelination/demyelination, it appears clear that, at least in the LPC model, Bac administration improves remyelination, either directly by stimulating $GABA_BRs$ in oligodendroglia, and/or by enhancing microglia/macrophage support of remyelination.

In conclusion, our results provide compelling evidence showing that Bac, a drug approved for spasticity treatment in MS patients, improves remyelination. Advanced imaging techniques for non-invasive measurement of myelin content/remyelination (Kolb et al., 2021) should evaluate whether MS patients treated with Bac indeed show evidence of enhanced remyelination. This may be an important first step in evaluating the suitability of this drug as a pro-remyelinating/neurorepair agent in MS.

AUTHOR CONTRIBUTIONS

Mari Paz Serrano-Regal: investigation, data curation, formal analysis, writing. Laura Bayón-Cordero: investigation, data curation, formal analysis, writing. Juan Carlos Chara Ventura: investigation. Blanca I. Ochoa-Bueno: investigation, data curation, formal analysis. Vanja Tepavcevic: conceptualization, investigation, formal analysis, writing. Carlos Matute: conceptualization, writing, funding acquisition. María Victoria Sánchez-Gómez: conceptualization, writing, funding acquisition.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available from the corresponding authors upon reasonable request.

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