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Oligodendrocyte Differentiation and Myelination Is Potentiated via $GABA_B$ Receptor Activation

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Abstract—Differentiation of oligodendrocyte precursor cells (OPCs) into mature oligodendrocytes (OLs) is a key event for axonal myelination in the central nervous system (CNS). Several growth factors and neurotransmitters like GABA are postulated as important regulators of that process, and different protein kinases may also participate in OL differentiation and myelination. However, the molecular mechanisms underlying the regulation of myelination by neurotransmitters are only partially known. In the present study, we provide evidence showing that GABA receptors (GABARs) play an important role in OL differentiation. First, we observed that OPCs and OLs synthesize GABA and expressed GABAR and transporters, both *in vitro* and *in vivo* and, in contrast to $GABA_A$Rs, the subunits $GABA_{B1}$R and $GABA_{B2}$R are expressed in OLs over time. Then, we found that exogenous GABA increases the number of myelin segments and MBP expression in DRG-OPC cocultures, indicating that GABA regulates myelination when OLs are in contact with axons. Notably, in purified rat OPC cultures, chronic treatment with GABA and baclofen, specific $GABA_B$R agonist, accelerates OPC differentiation by enhancing the processes branching and myelin protein expression, effects that are reverted in presence of $GABA_B$R specific antagonist CGP55845. Exposure of OPCs to baclofen promotes the Src-phosphorylation, and the baclofen-induced maturation is attenuated in presence of the Src-family kinases inhibitor PP2. None of these effects are mediated by the $GABA_A$R agonist muscimol. Together, these results highlight the relevance of the GABAergic system in OL differentiation, and indicate that this functional role is mediated through $GABA_B$R involving the participation of Src-family kinases.*This article is part of a Special Issue entitled: SI: Miledi's contributions.* © 2019 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: $GABA_B$ receptors, GABAergic system, OPCs, OLs, differentiation, myelination.

INTRODUCTION

Oligodendrocytes (OLs) are the myelinating glia in the vertebrate central nervous system (CNS), allowing the saltatory conduction of neural action potentials and providing protection and metabolic support to axons (Baumann and Pham-Dinh, 2001). Differentiation of oligodendrocyte precursor cells (OPCs) into mature OLs is the most relevant event for myelination during development and for adult remyelination in the context of demyelinating diseases such as multiple sclerosis (MS) (Franklin and Ffrench-Constant, 2017; Kremer et al., 2019). Failure of efficient remyelination in MS is largely due to the incapacity of OPCs to efficiently differentiate into myelinating OLs (Fancy et al., 2011), so that understanding the mechanisms that control the differentiation of OLs is essential for identifying therapeutic strategies to promote myelin repair and limit disability in MS.

OL myelination is a complex process mediated by neuron–glia interactions that requires the participation of several regulators like neurotransmitters and growth factors. The finding that even very immature OLs express a range of neurotransmitter receptors (Verkhratsky and Steinhaüser, 2000) suggests that they may be involved in the regulation of OL development and it has been described that ATP, adenosine, glutamate or GABA can modulate the proliferation, differentiation and migration of OPCs as well as OL survival and myelination (Domercq et al., 2010; Li et al., 2013; Fannon et al., 2015; Zonouzi et al., 2015; Hamilton et al., 2017).

GABA is a major neurotransmitter in the neonatal brain and the main inhibitory neurotransmitter in the CNS. It is known that it can be synthesized through two different pathways: the classical one, in which it is synthesized

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from glutamate through the action of $GAD_{65/67}$ and the second pathway, in which GABA is synthesized from putrescine through the action of MAO_B (Angulo et al., 2008; Yoon et al., 2014).

GABA acts on two main receptor types, ligand-gated ionotropic $GABA_A$ receptors ($GABA_ARs$), responsible for fast inhibitory neurotransmission, and G protein-coupled metabotropic $GABA_B$ receptors ($GABA_BRs$), which mediate slow and prolonged inhibitory neurotransmission. The $GABA_B$ receptor signaling pathways can involve different effector proteins: voltage-gated Ca^{2+} channels, G-protein activated inwardly-rectifying K^+ channels (GIRK) and adenylate cyclase system (Frangaj and Fan, 2018). However, the precise coupling of $GABA_B$ receptor to the molecular effector can vary depending on the cell type and region analyzed (Booker et al., 2018). Two major $GABA_BR$ isoforms, $GABA_{B1}$ and $GABA_{B2}$, have been described (Bettler et al., 2004; Bettler and Tiao, 2006). In CNS neurons, $GABA_{B1}$ and $GABA_{B2}$ are widely co-expressed and were found to generate functional receptors in a heterodimeric assembly (Kaupmann et al., 1998; Kuner et al., 1999; Marshall et al., 1999). While $GABA_{B1}$ binds orthosteric ligands, $GABA_{B2}$ couples with G protein and contains the allosteric ligand-binding site. GABA and the clinical drug *R*-baclofen are two of the best understood $GABA_B$ receptor agonists (Frangaj and Fan, 2018) and they are thought to bind via their carboxylic group to the hydroxyl groups of S246 and Y366 (Bettler et al., 2004). Ca^{2+} increases the affinity of GABA to the receptor and enhances G protein activation. However, this allosteric effect is not observed for baclofen (Galvez et al., 2000; Frangaj and Fan, 2018). A large range of antagonists with low and high affinities for the $GABA_BR$ has been described. The attachment of 3,4-dichlorobenzyl or 3-carboxybenzyl substituents to the existing molecules produced compounds with affinities about 10,000 times higher than previous antagonists, which is the case for CGP55845 (Bowery et al., 2002).

Three different mechanisms have been involved in the desensitization of $GABA_BRs$. In cerebellar granule cells, G protein receptor kinase (GRK) 4 and 5 associate with the receptors and induce its desensitization in a phosphorylation-independent manner. In cortical and hippocampal neurons, this process implicates the participation of NEM-sensitive fusion protein (NSF) and PKC. Some members of the potassium channel tetramerization domain-containing (KCTD) protein family as KCTD-12 or 12b also lead the receptor to desensitization (Benke et al., 2012). Concerning to $GABA_BR$ trafficking, these receptors display fast constitutive internalization, being endocytosed via the clathrin and dynamin-dependent pathway. $GABA_BRs$ are predominantly recycled back to the plasma membrane with only a minor fraction degraded in lysosomes (Benke et al., 2012).

Besides neurons, it is also known that OPCs express functional $GABA_ARs$ in culture (Hoppe and Kettenmann, 1989; Von Blankenfeld et al., 1991; Kirchhoff and Kettenmann, 1992; Borges et al., 1995; Williamson et al., 1998; Cahoy et al., 2008) and *in situ* in hippocampal slices (Lin and Bergles, 2004), and $GABA_BRs$ are also expressed in developing OLs (Luyt et al., 2007). However, sensitivity to GABA in mature OLs is greatly reduced (Berger et al., 1992). In addition, more recently, colocalization studies between GAT-1 GABA transporter and specific markers for OLs showed that GAT-1 was expressed in both immature and mature OLs in subcortical white matter, and *in vitro* functional assays showed that OLs exhibit GAT-1 dependent GABA uptake (Fattorini et al., 2017). All these data are suggesting that these cells could communicate with each other and with neurons through GABAergic pathways, and suggest a specific role for GABA signaling in the oligodendroglial lineage development and during the initial stages of myelination and/or axon recognition (Vélez-Fort et al., 2012; Zonouzi et al., 2015).

In this line, we have previously reported that the expression and function of $GABA_A$ receptors in cultured OLs are driven by axonal cues and that GABA signaling may play a relevant role during axon–glia recognition or in myelination (Arellano et al., 2016). However, this program of communication and differentiation requires multiple integrated extrinsic and intrinsic molecular signals that are poorly understood.

Cells in the oligodendrocyte lineage demand precise signaling mechanisms to transition through specific stages of their development. Several molecular pathways have been involved in this process. Erk1/2 MAPK and mTOR signaling sequentially regulates distinct stages of OL progenitor differentiation (Guardiola-Diaz et al., 2012) and GSK3β negatively regulates OL differentiation and myelination (Azim and Butt, 2011). The tyrosine kinase Src-family has been implicated in OL and Schwann cell differentiation and myelination (White and Krämer-Albers, 2014; Melfi et al., 2017), and it has also been related with the GABAergic system as Barati et al. (2015) have described that specific $GABA_BR$ activation mediates chemotaxis and cytoskeletal rearrangement via PI3-K/Akt/Src kinases signaling.

Here, we demonstrate in different *in vitro* and *in vivo* models that OLs express GABA and several GABAergic components. In addition, OLs maintain the expression of $GABA_B$ receptors, GAT-1 and GAT-3 GABA transporters and GABA-synthesizing enzymes over time, suggesting that the GABAergic system plays an important role in OL differentiation. Interestingly, we have found that GABA regulates myelination in a DRG-OPC coculture system. Moreover, in purified rat OPC cultures, GABA and the specific $GABA_BR$ agonist baclofen accelerates OPC differentiation, enhancing process branching and promoting local translation of MBP in peripheral areas. In the same way, chronic treatment with baclofen induces Src-phosphorylation and the baclofen-induced OL differentiation effect is attenuated in the presence of PP2 (an Src-family kinases inhibitor). Altogether, these results suggest that the effect of GABA on OPC differentiation and myelination is mainly mediated through $GABA_BRs$ and involves the participation of molecules from the Src-kinase family.

EXPERIMENTAL PROCEDURES

Ethics statement

All procedures with animals were carried out with the approval of the internal Animal Ethics Committee of the University of the Basque Country (UPV/EHU) and the European Communities Council Directive. In particular, all protocols were approved by the "Ethics Committee on Animal Experimentation" (CEEA) which is a collegiate authority into the operational structure of the Ethics Commission for Research and Teaching (CEID) of the University of the Basque Country. All possible efforts were made to reduce the number of animals used in this study and their suffering.

Rat forebrain oligodendrocyte culture

Purified OPCs were prepared from mixed glial cultures obtained from newborn (P0–P2) Sprague–Dawley rat forebrains according to the protocol of McCarthy and de Vellis (1980) with minor modifications (Chen et al., 2007; Bernal-Chico et al., 2015; Canedo-Antelo et al., 2018; Sánchez-Gómez et al., 2018). Briefly, forebrains were removed from the skulls, and the free of meninges cortices were isolated and digested by incubation (15 min, 37 °C) in Hank's balanced salt solution (without Ca^{2+} and Mg^{2+}) containing 0.25% trypsin and 0.004% DNAse (both from Sigma-Aldrich). Cells were dissociated by passage through needles (21G and 23G), centrifuged and resuspended in Iscove's modified Dulbecco's medium (IMDM; Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone; Gibco). Cells were seeded into 75-cm^2 flasks coated with poly-D-lysine (1 μg/ml; Sigma-Aldrich) and maintained in culture at 37 °C and 5% CO_2 with a medium change every 3–4 days. After 7 days in culture, flasks were shaken (400 rpm, 1 h, at 37 °C) to remove adherent microglia. The remaining OPCs present on top of the confluent monolayer of astrocytes were detached by shaking overnight at 400 rpm. The resulting cell suspension was filtered through a 10-μm pore size nylon mesh and preplated in 100-mm coated Petri dishes (ThermoFisher Scientific) for 30 min at 37 °C and 5% CO_2, allowing microglia to become firmly attached while OPCs were loosely attached and could be collected with a gentle twisting of the Petri dishes. The collected forebrain OPC cell suspension was filtered again through a 10-μm pore size nylon mesh, centrifuged and resuspended in a chemically defined medium (OL differentiation medium) composed of: Dulbecco's modified Eagle's medium (Gibco) supplemented with 5 μg/ml insulin, 100 μg/ml transferrin, 62.5 ng/ml progesterone, 40 ng/ml sodium selenite, 16 μg/ml putrescine, 1 mg/ml bovine serum albumin (BSA), 63 μg/ml N-acetyl-cysteine, 2 mM L-glutamine (all of from Sigma-Aldrich), 100 U/ml penicillin/streptomycin (Lonza), 30 ng/ml triiodothyronine, 40 ng/ml thyroxine (both from Sigma-Aldrich) plus 10 ng/ml CNTF and 1 ng/ml NT_3 (both from Peprotech). Cells were plated onto poly-D-lysine coated 14-mm-diameter coverslips in 24-well plates at densities between 4×10^3 and 8×10^4 cells/well. The purity of oligodendroglial cultures was confirmed by immunostaining with cell type-specific antibodies. After 1 day in culture, platelet-derived growth factor receptor α^+ (PDGFRα^+) OPCs represented 97% ± 5% of the total cells, and after 3 days in OL differentiation medium, at least 98% were MBP^+ cells.

Rat optic nerve oligodendrocyte culture

Primary cultures of optic nerve OLs were prepared from 11-day-old Sprague–Dawley rats as previously described (Barres et al., 1992; Sánchez-Gómez et al., 2018). Cells were seeded into poly-D-lysine coated 14-mm-diameter coverslips in 24-well plates at a density of 5×10^3 cells per well. Cells were maintained at 37 °C and 5% CO_2 in the chemically defined medium described above (OL differentiation medium). After 3–5 days *in vitro* (DIV), cultures were constituted of at least 98% cells positive for O4 antigen and myelin basic protein ($O4^+$/MBP^+). Most the remaining cells were positive for the glial fibrillary acidic protein (GFAP) immunostaining. No $A2B5^+$ cells or microglial cells were identified in these cultures (Alberdi et al., 2002).

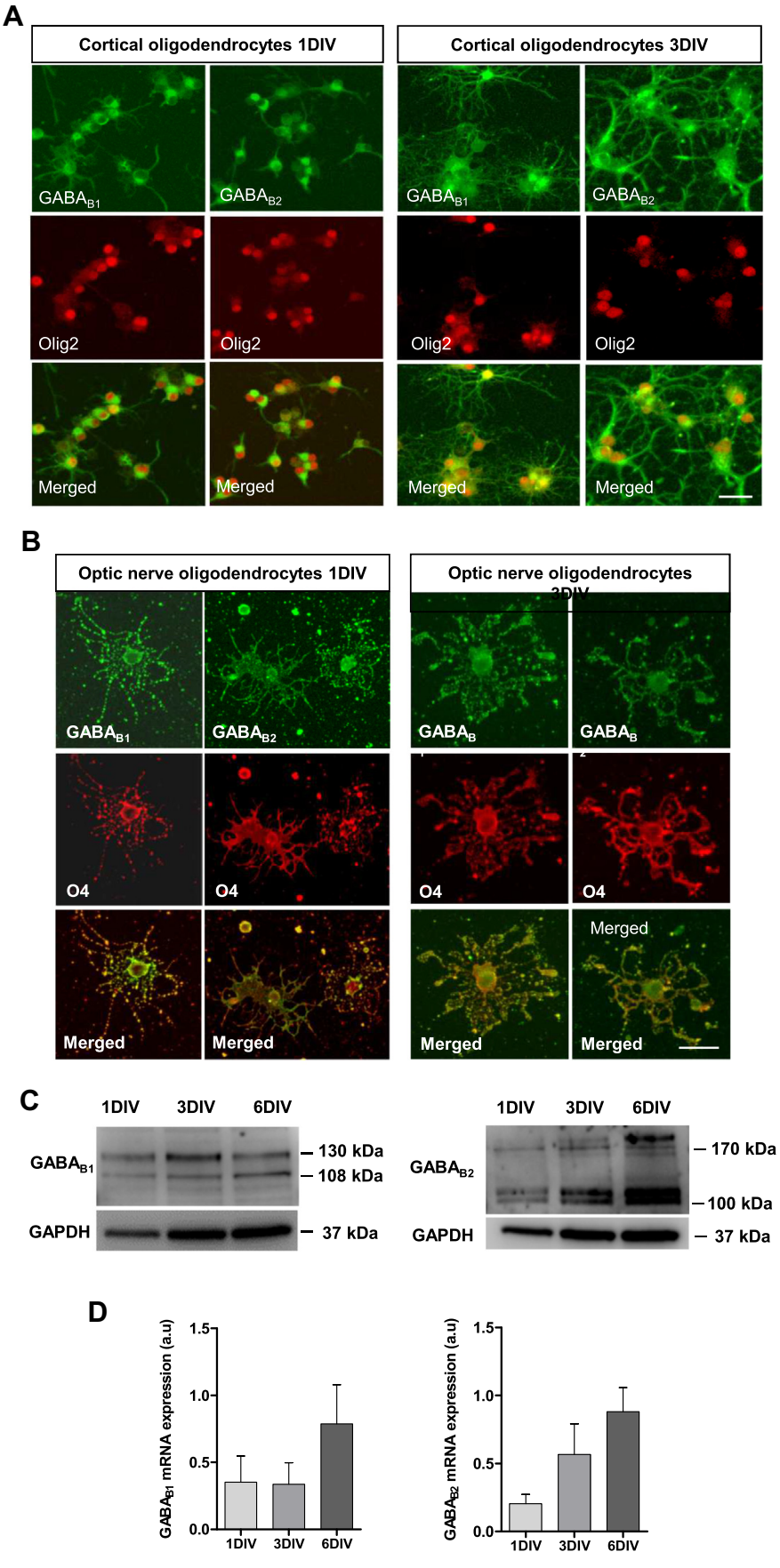
DRG neurons-OPCs coculture

DRG neurons were obtained from E15 rat embryos as previously described (Arellano et al., 2016). The dissociated cells were resuspended in DRG medium composed of Neurobasal medium (Gibco) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 50 ng/ml nerve growth factor (ThermoFisher Scientific) and 2% B27 supplement (Gibco). Cells were plated onto poly-D-lysine-laminin coated coverslips at a density of 5×10^4 cells per coverslip. DRG neurons were cultured for at least 2 weeks in DRG medium alone with fresh medium added every 3 days.

Isolated OLs (either from optic nerve or forebrain) prepared as above were seeded on a 2- to 3-week-old DRG neuron culture at a density of 2×10^4 cells/coverslip. The medium was changed to a 50:50 mixture of OL differentiation medium and DRG medium, without nerve growth factor, and cocultures were left for 14–21 days in control conditions or in presence of 10 μM or 50 μM GABA. Cultures were immunostained with mouse anti-MBP antibody (1:500, Ref. 808402; Biolegend) to visualize myelin expression and rabbit anti-neurofilament-L antibody (1:200, Ref. 2837; Cell Signaling) to visualize axons. Level of myelination was evaluated by different approaches. The total expression of MBP protein was quantified by western blot using the protocol indicated below. In addition, MBP expression

Table 1. . Primers used to analyze $GABA_{B1}$ and $GABA_{B2}$ gene expression.

Gene	Forward primer sequence	Reverse primer sequence
Gabbr1	AGATTGTGGACCCCTTGCAC	AGAAAATGCCAAGCCACGTA
Gabbr2	CACCGAGTGTGACAATGCAAA	CCAGATTCCAGCCTTGGAGG



was analyzed using *ImageJ* software (NIH, Bethesda, MD) in 20 random fields per coverslip in images captured with a 20× objective. Finally, the number of MBP$^+$/NF-L$^+$ segments was counted on a minimum of 30 random fields per coverslip at 40× magnification. All data were showed as percentage with respect to untreated control coculture.

Immunocytochemistry

Cells on the coverslips were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 20 min at room temperature (RT) and rinsed three times with PBS. Then, fixed cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) and blocked with 4% goat serum (Palex) in 0.1 M PBS for 1 h at RT. Cells were processed for immunofluorescence with mouse anti-Olig2 (1:1000, Ref. MABN50; Millipore), rabbit anti-Olig2 (1:800, Ref. AB15328; Millipore), rabbit anti-PDGFRα (1:200, Ref. sc-338; Santa Cruz), rabbit anti-NG2 (1:200, Ref. AB5320; Millipore), mouse anti-MBP (1:500, Ref. 808402; Biolegend), rabbit anti-PLP (1:100, Ref. ab28486; abcam), rabbit anti-GABAR$_{B1}$ and rabbit anti-GABAR$_{B2}$ (1:50, Refs. AGB-001, AGB-002; Alomone), guinea pig anti-GAT$_1$ (1:100, Ref. 274104; Synaptic Systems), rabbit anti-GAT$_3$ (1:100, Ref. AGT-003; Alomone), mouse anti-GAD$_{65/67}$ (1:250, Ref. sc-365180; Santa Cruz), rabbit anti-MAO$_B$ (1:100, Ref. HPA002328; Sigma-Aldrich) and rabbit anti-Ki67 (1:500, Ref. VP-RM04; Vector). After incubation, primary antibodies were detected with secondary antibodies: Alexa Fluor 488 or 594-conjugated anti-mouse IgG (H + L) (1:400, Refs. A11001 and A11032; Invitrogen), Alexa Fluor 488 or 594-conjugated anti-rabbit IgG (H + L) (1:400, Refs. A11008 and A11012; Invitrogen) and Alexa Fluor 647-conjugated anti-guinea pig IgG (H + L) (1:400, Ref. 706-605-148; Jackson Labs) secondary

antibodies, for 1 h at RT. Incubation for 5 min with DAPI (4 μg/ml; Sigma-Aldrich) was used to identify cell nuclei. Cells were washed three times in 0.1 M PBS and mounted on glass slides with fluorescent mounting medium (Prolong gold antifade reagent; ThermoFisher Scientific).

For GABA immunostaining, cells were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M PBS for 20 min at RT, permeabilized and blocked as described before and incubated with mouse anti-GABA (1:7000, Matute and Streit, 1986).

For immunostaining of A2B5 or the O4 antigen, live cells were incubated for 1 h or 30 min respectively at 37 °C with mouse anti-A2B5 IgM (1:10, kindly supplied by Dr. Paola Bovolenta, Center of Molecular Biology Severo Ochoa, Madrid) and mouse anti-O4 IgM (1:40, kindly supplied by Dr. Christine Thomson, University of Glasgow) in OL differentiation medium. Then, cells were rinsed with 0.1 M PBS, fixed with 4% paraformaldehyde in PBS as described before, and incubated for 1 h at RT with secondary Alexa Fluor 488-conjugated anti-mouse IgM (1:200, Ref. A21042; Invitrogen) and Texas Red-conjugated anti-mouse IgM (1:200, Ref. 401296; Calbiochem) secondary antibodies, diluted in PBS containing 4% goat serum. After being rinsed, coverslips were mounted on glass slides.

Immunohistochemistry

To improve detection of OPCs in $GABA_B$Rs localization experiments, we used 1-month-old transgenic mice expressing EYFP under the control of the NG2 promoter (NG2-EYFP) as markers of OPCs (Karram et al., 2008), generous gift from Dr. Jacqueline Trotter (Gutenberg University Mainz, Germany) and Dr. Susana Mato (University of the Basque Country, UPV/EHU, Spain), and 3-month-old transgenic mice expressing fluorescence reporter DsRed under the control of the glial-specific proteolipid protein promoter (PLP-DsRed; Hirrlinger et al., 2005), generous gift from Dr. Frank Kirchhoff (University of Saarland, Homburg, Germany).

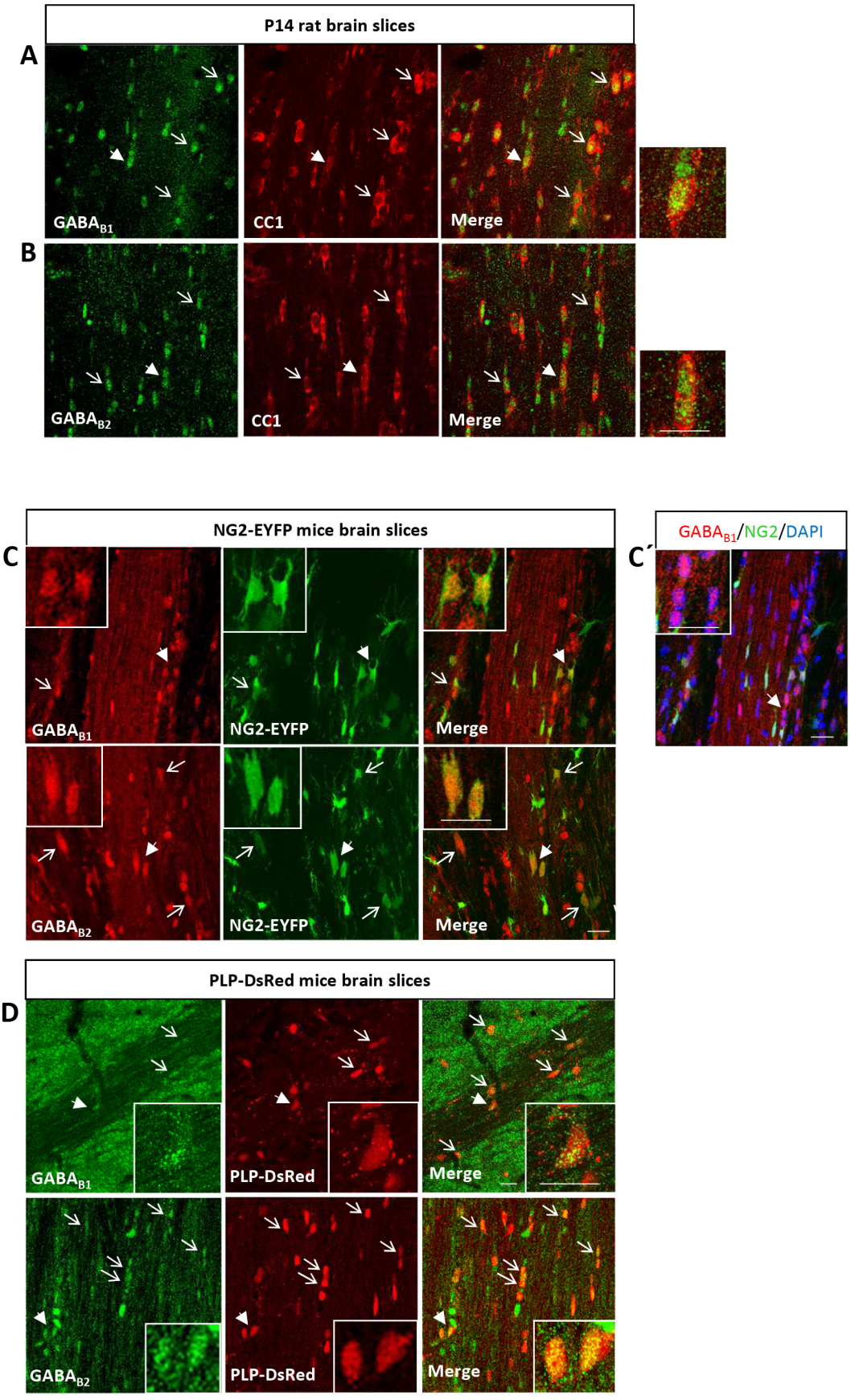
Sprague–Dawley P14 rats and transgenic mice were anesthetized with chloral hydrate and transcardially perfused with 4% paraformaldehyde in 0.1 M PB. Brains were extracted and postfixed with the same solution for 3 h at RT, placed in 0.1 M PBS with 0.02% sodium azide (Sigma-Aldrich) at 4 °C and maintained in cryoprotectant solution (30% ethylene glycol (Sigma-Aldrich), 30% glycerol (Merck) and 10% PB 0.4 M in dH_2O) at −20 °C. Tissue was cut using a Microm HM650V vibrating blade microtome to obtain coronal 40-μm-thick sections.

Free-floating vibratome sections were permeabilized with 0.1% Triton X-100 and blocked with 5% goat serum with shaking for 1 h at RT. Then, slices were incubated in blocking solution with 1% goat serum, overnight at 4 °C with gentle shaking, with primary antibodies: rabbit anti-$GABA_{B1}$R and rabbit anti-$GABA_{B2}$R (1:200, Refs. AGB-001, AGB-002; Alomone), guinea pig anti-GAT_1 (1:500, Ref. 274104; Synaptic Systems), rabbit anti-GAT_3 (1:100, Ref. AGT-003; Alomone) and mouse anti-APC (CC1, 1:200, Ref. OP80; Calbiochem). After three washes in 0.1 M PBS, slices were incubated in the same solution as primary antibodies, containing fluorochrome-conjugated secondary antibodies and DAPI (4 μg/ml) for 1 h at RT. The secondary antibodies used were as follows: Alexa Fluor 594-conjugated anti-mouse IgG (H + L) (1:400, Ref. A11032; Invitrogen), Alexa Fluor 488 or 594-conjugated anti-rabbit IgG (H + L) (1:400, Refs. A11008 and A11012; Invitrogen) and Alexa Fluor 488-conjugated anti-guinea pig IgG (H + L) (1:800, Ref A11073; Invitrogen). After that, slices were washed in 0.1 M PBS and mounted on glass slides with fluorescent mounting medium (Prolong gold antifade reagent; ThermoFisher Scientific).

Western blot analysis

Cells were washed twice in 0.1 M PBS and scraped in 60 μl of SDS sample buffer (2 wells and 8×10^4 cells per well). All the process was performed on ice to enhance the lysis and avoid protein degradation. Lysates were boiled at 99 °C for 8 min, size-separated by SDS-PAGE in 4–20% Criterion TGX Precast gels and transferred to Trans-Blot Turbo Midi PVDF Transfer Packs (Bio-Rad). Membranes were blocked with 5% BSA or with 5% Phosphoblocker blocking reagent (for phosphorylated protein detection; Cell Biolabs) in Tris-buffered saline/0.05% Tween-20 (TBS-T) for 90 min at RT. Then, they were incubated in the same solution overnight at 4 °C with gentle shaking with the following primary antibodies: mouse anti-$GABA_{B1}$R (1:500, Ref. ab55051; abcam), rabbit anti-$GABA_{B2}$R (1:200, Ref. AGB-002, Alomone), rabbit anti-GAT_3 (1:500, Ref. AGT-003; Alomone), mouse anti-$GAD_{65/67}$ (1:500, Ref. sc-365180; Santa Cruz), rabbit anti-MAO_B (1:500 Ref. HPA002328; Sigma), mouse anti-MAG (1:500, Ref. sc-376145; Santa Cruz), mouse anti-MBP (1:1000, Ref. 808402; Biolegend), rabbit anti-phospho-Src [Tyr418] (1:1000, Ref. 44-660G; ThermoFisher Scientific), rabbit anti-Src [36D10] (1:1000, Ref. 2109; Cell Signaling), mouse anti-GAPDH (1:1000, Ref. MAB374; Millipore) and rabbit anti-β-actin (1:2000, Ref. A2066; Sigma-Aldrich). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000, Refs: A6782, A6154; Sigma-Aldrich) for 90 min at RT and were developed using and enhanced chemiluminescence detection kit according to the manufacturer's

Fig. 1. Oligodendrocytes *in vitro* express $GABA_B$R and maintain their expression over time in culture. **(A)** Cultured cortical oligodendrocytes, identified as $Olig2^+$ cells (red), express both $GABA_{B1}$ and $GABA_{B2}$ subunits of $GABA_B$Rs (green) at 1DIV and 3DIV. Scale bar = 30 μm. **(B)** $GABA_{B1}$ and $GABA_{B2}$ subunits (green) are also observed in $O4^+$ optic nerve-derived oligodendrocytes (red), after 1DIV and 3DIV. Scale bar = 20 μm. **(C and D)** Western Blot analysis and RT-qPCR confirm expression of $GABA_{B1}$ and $GABA_{B2}$ subunits in cortical immature oligodendrocytes (1DIV) and mature oligodendrocytes after 3DIV or 6DIV in differentiation medium.


P14 rat brain slices
A
GABA$_{B1}$
CC1
Merge
B
GABA$_{B2}$
CC1
Merge
NG2-EYFP mice brain slices
C
GABA$_{B1}$
NG2-EYFP
Merge
GABA$_{B2}$
NG2-EYFP
Merge
C´
GABA$_{B1}$/NG2/DAPI
PLP-DsRed mice brain slices
D
GABA$_{B1}$
PLP-DsRed
Merge
GABA$_{B2}$
PLP-DsRed
Merge

instructions (Supersignal West Dura or Femto; ThermoFisher Scientific). Images were acquired with a Chemi-Doc XRS imaging system (Bio-Rad) and protein bands were quantified by adjusted volume using *ImageLab* software (version 3.0; Bio-Rad).

Antibody for phosphorylated Src was stripped using Restore Western Blot stripping buffer (ThermoFisher Scientific) for 15 min at RT with shaking. Membranes were then washed in TBS-T, blocked with 5% BSA and incubated with Src primary antibody.

Quantitative PCR

Total RNA was extracted from cultured forebrain OLs (at least two wells and 8×10^4 cells per well) with Trizol reagent (ThermoFisher Scientific) according to the manufacturer's recommendations (Invitrogen). First, strand cDNA synthesis was performed with reverse transcriptase SuperScript™ III (Invitrogen) using random primers as previously described (González-Fernández et al., 2014). Specific primers for $GABA_{B1}$ and $GABA_{B2}$ were obtained from ThermoFisher Scientific (see Table 1). Real-time quantitative PCR (qPCR) reactions were performed with SYBR-Green (Bio-Rad) using a Bio-Rad CFX96™ Real-time detection system. Data were normalized to GAPDH and CicA housekeeping genes using a normalization factor obtained in *geNorm* software.

Cell viability assay

Culture forebrain OLs (10^4 cells per well) were incubated with 1 μM calcein-AM (ThermoFisher Scientific) for 30 min at 37 °C in OL differentiation medium and then washed twice in pre-warmed 0.1 M PBS. Emitted fluorescence was measured using a Synergy-HT fluorimeter (Bio-Tek) as indicated by the supplier (485-nm excitation wavelength and 528-nm emission wavelength). All experiments were performed in triplicate. The values provided are the averages of at least three independent experiments.

Substances

γ-Aminobutiric acid (GABA; Ref. A2129) and gabazine (Ref. SR-95531) were from Sigma-Aldrich. Baclofen (Ref. 0796), CGP55845 (Ref. 1248) and muscimol (Ref. 0289) were all from Tocris Bioscience. PP2 (Src-family kinases inhibitor) was from Selleckchem.

Image acquisition and analysis

Images were acquired using a Zeiss Axioplan2 fluorescence microscope (Department of Neurosciences, UPV/EHU), an Olympus Fluoview FV500 (Analytical and High-Resolution Microscopy Service in Biomedicine, UPV/EHU) and a Leica TCS STED SP8 laser scanning confocal microscope (Achucarro Basque Center for Neuroscience). Same settings were kept for all samples within one experimental group. Data quantification on isolated OPCs/OLs was performed as follows: $NG2^+$, $A2B5^+$ and $Ki67^+$ cells were counted from at least 10 fields per coverslip using 20× objective.

MBP^+ cells and values of MBP^+ cells occupied area were derived from at least 15 fields per coverslip using 20× objective. All these quantifications were obtained by using *ImageJ* software. Morphology of MBP^+ cells was analyzed by using *Concentric circles* macro (created by Jorge Valero, Achucarro Basque Center for Neuroscience, Spain). Concentric circles at 10-μm intervals emerging from the center of the cell nucleus were created and MBP protein fluorescence signal was quantified after background subtraction. Data were represented as percentage of MBP in soma or periphery with respect to MBP total signal for each cell analyzed. All quantifications were performed on a minimum of 50 cells per coverslip, two coverslips per treatment of at least three independent experiments. The selection of the cells was performed on a minimum of 15 independent fields per coverslip, avoiding the central area where the cell density was higher and made more difficult the identification of each cell. To avoid the effect of the variations observed in basal differentiation between trials, all experimental conditions were paired with internal control performed in parallel and derived from common pools of cells.

Statistical analysis

All data are presented as mean ± S.E.M. Statistical analysis was performed using *GraphPad Prism* statistical software (version 5.0; *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 a single experimental group and a control group, we used two-tailed paired Student's t-test assuming equal variance. In all instances, statistical differences were considered significant where $p < 0.05$. All the images shown in this article represent the data obtained, at least, in three independent experiments with similar results.

RESULTS

Cultured oligodendrocytes express $GABA_BR$ *in vitro*

We first characterized the expression of $GABA_BRs$ over time in cultured oligodendrocytes by double immunocytochemistry, using $GABA_BR$ subunit-specific antibodies and oligodendroglial lineage-specific markers. We observed that

Fig. 2. $GABA_BRs$ are expressed in oligodendrocytes in the rat brain during postnatal development and in the adult. $CC1^+$ (red) oligodendrocytes express $GABA_{B1}$ **(A)** and $GABA_{B2}$ **(B)** subunits (green) of $GABA_BR$ in the rat corpus callosum at postnatal day 14. Scale bar = 20 μm. **(C)** $NG2^+$ cells (green) are positive for $GABA_{B1}$ (red, top) and $GABA_{B2}$ (red, bottom) subunits in the corpus callosum of 1-month-old NG2-EYGF transgenic mice. **(D)** PLP^+ oligodendrocytes (red) are positive for $GABA_{B1}$ (green, top) and $GABA_{B2}$ (green, bottom) subunits in the corpus callosum of 3-month-old PLP-DsRed transgenic mice. Scale bar = 20 μm. (**C´**) $NG2^-$ cells identified as oligodendrocytes are positive for $GABA_{B1}$ (red) in the corpus callosum NG2-EYGF transgenic mice. Scale bar = 20 μm. Arrowheads show the magnified cell in each photograph.

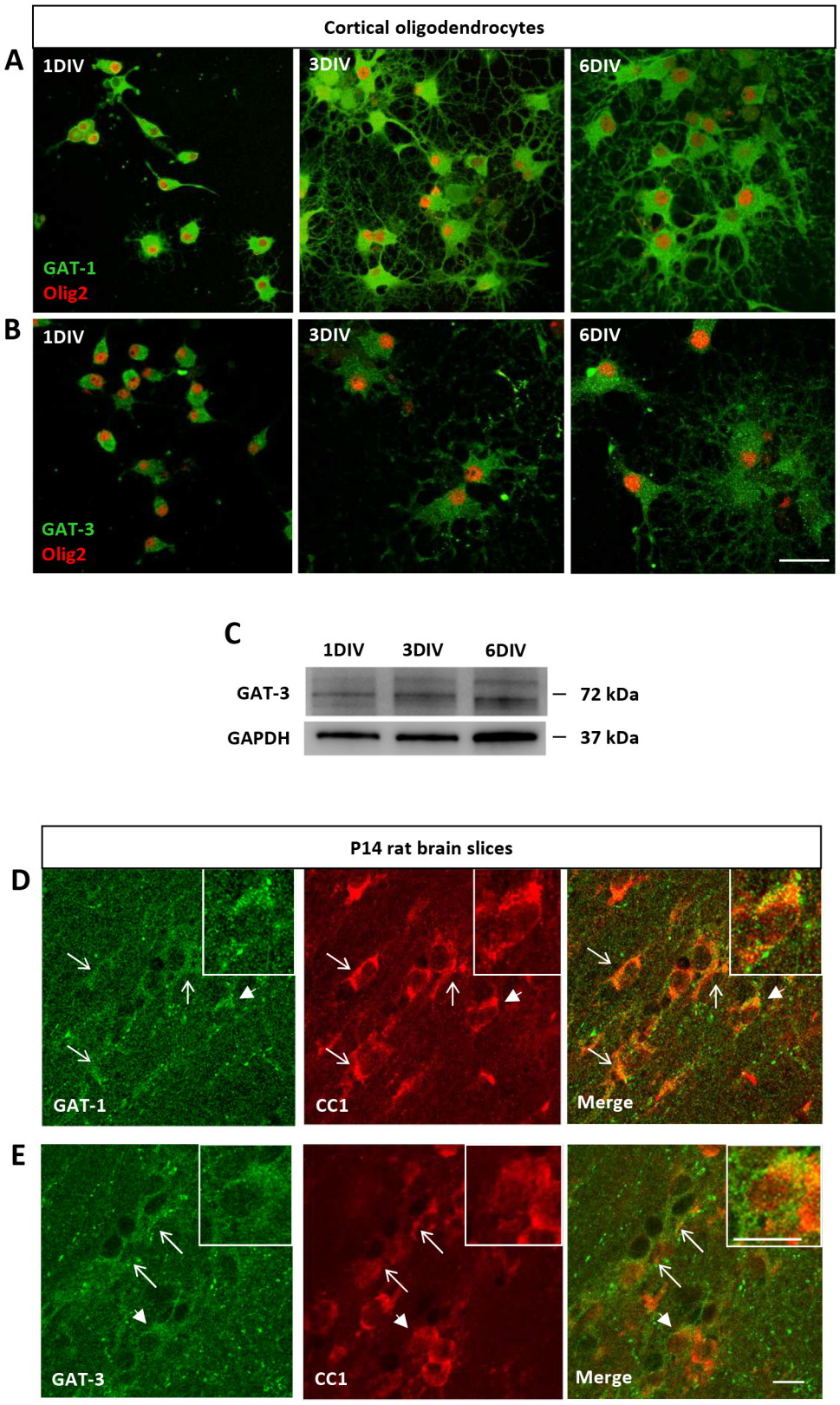


Fig. 3. Oligodendrocytes *in vitro* and *in vivo* express GABA transporters GAT-1 and GAT-3 and maintain their expression overtime. Cultured cortical oligodendrocytes, identified as Olig2$^+$ cells (red), express GAT-1 **(A)** and GAT-3 **(B)** transporters (green) at 1DIV, 3DIV and 6DIV. Scale bar = 20 μm. **(C)** Western Blot analysis shows protein expression of GAT-3 transporter in cortical oligodendrocytes at 1, 3 and 6DIV. CC1$^+$ (red) oligodendrocytes express GAT-1 **(D)** and GAT-3 **(E)** transporters (green) in the corpus callosum of P14 rats. Scale bar = 10 μm. Arrowheads show the magnified cell in each photograph.

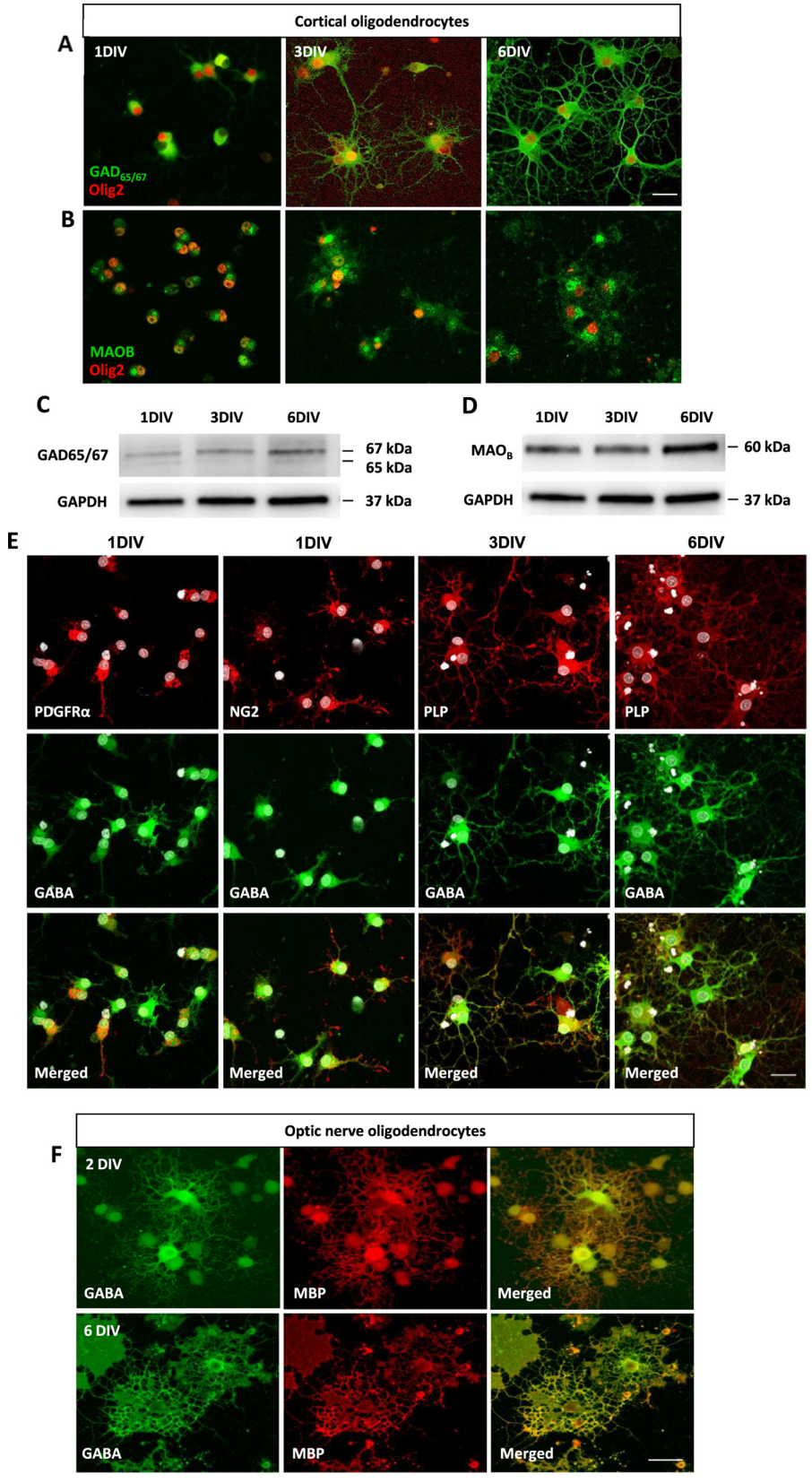


Fig. 4. Oligodendrocytes *in vitro* express the two GABA-synthesizing enzymes and synthesize GABA over time. Isolated cortical oligodendrocytes, distinguished as Olig2$^+$ cells (red), express $GAD_{65/67}$ **(A)** and MAO_B **(B)** enzymes (green) at 1, 3 and 6DIV. Scale bar = 30 μm. Western blot images confirm expression of $GAD_{65/67}$ **(C)** and MAO_B **(D)** enzymes at 1, 3 and 6DIV. **(E)** PDGFRα$^+$, NG2$^+$ immature oligodendrocytes and PLP$^+$ mature oligodendrocytes (red) express GABA (green) at 1DIV and 3 and 6DIV, respectively. Scale bar = 20 μm. **(F)** Optic nerve-derived MBP$^+$ mature oligodendrocytes (red) synthesize GABA (green) at 2 and 6DIV. Scale bar = 20 μm.

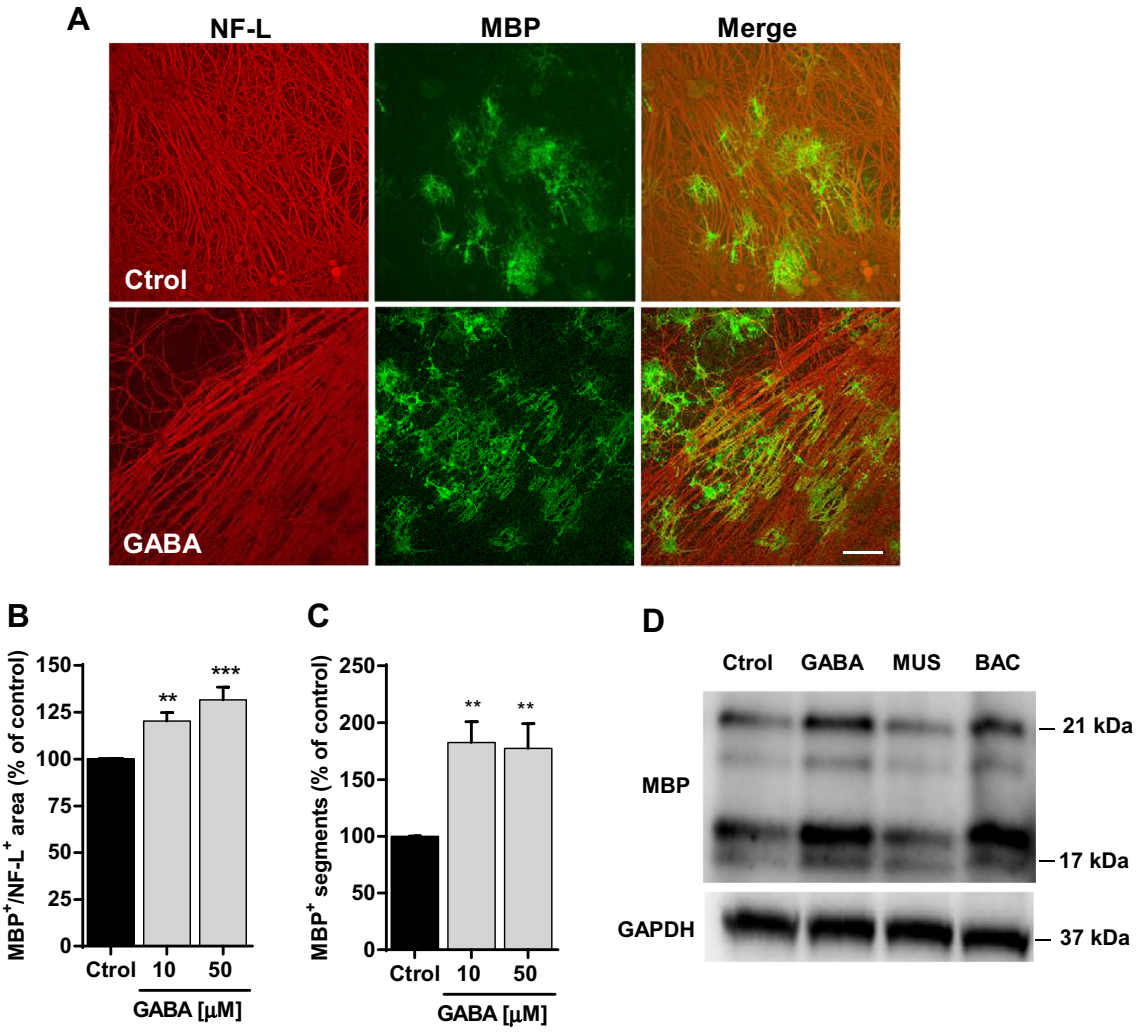


Fig. 5. Myelination of axons in a DRG-OPC coculture is potentiated in the presence of GABA. **(A)** Representative images showing MBP^+ oligodendrocytes (green) and $NF\text{-}L^+$ axons (red) in control conditions (top) or chronic treatment with 50 µM GABA (bottom) for 21-day coculture. Scale bar = 50 µm. **(B)** Quantification of occupied area by MBP relative to NF-L area in control and continuously treated cocultures with 10 µM or 50 µM GABA. $^{**}p < 0.01$, $^{***}p < 0.001$ vs control (n = 10 coverslips for each condition). **(C)** Quantification of MBP positive segments following exposure to GABA, expressed as percentage with respect to control $^{**}p < 0.01$ vs control (n = 10 coverslips for each condition). **(D)** Representative blot of MBP expression in control cocultures and after treatment with 50 µM GABA, 20 µM muscimol (MUS) or 50 µM baclofen (BAC).

$GABA_{B1}$ and $GABA_{B2}$ subunits were expressed in both cortical oligodendrocytes (Fig. 1A) and primary cultures derived from optic nerves (Fig. 1B) at different time points *in vitro* (1DIV and 3DIV). Similar results were obtained by Western Blot analysis. In all samples, we detected two bands for $GABA_{B1}$ above 108 kDa and 130 kDa, corresponding to the $GABA_{B1a}$ and $GABA_{B1b}$ isoforms, respectively (Magnaghi et al., 2004) and other two bands for $GABA_{B2}$ (one above 100 kDa and the other above 170 kDa, Fig. 1C). We also detected $GABA_{B1}$ and $GABA_{B2}$ receptor mRNA expression from precursor state (at 1DIV) to mature OLs (at 6DIV) by RT-qPCR (Fig. 1D) in cortex-derived OLs.

Expression of $GABA_BR$ in OLs *in vitro* and *in vivo*

To corroborate the expression of $GABA_BRs$ in OLs *in vivo* we checked, by double immunohistochemistry, the presence of $GABA_BR$ subunits in brain slices of rat and mouse of different ages (Fig. 2). First, we analyzed P7 (data not shown) and P14 rat brain slices and we detected $GABA_{B1}$ and $GABA_{B2}$ subunits expression in $CC1^+$ mature OLs in corpus callosum of rats at both ages (Fig. 2A, B). Finally, we examined the expression *in vivo* of $GABA_BRs$ in transgenic 1-month old NG2-EYFP and 3-month-old PLP-DsRed mice brain slices and found that the two subunits were present in $NG2^+$ cells and PLP^+ differentiated OLs in the corpus callosum of these mice, respectively (Fig. 2C, D). In addition, $GABA_BRs$ were also detected in NG2 negative cells observed in corpus callosum of NG2-EYFP mice, identified as OLs based on their characteristic alignment in white matter tracts (Fig. 2C´ shows the signal for $GABA_{B1}$).

Together, these results indicate that OLs exhibit a sustained expression of $GABA_{B1}$ and $GABA_{B2}$ subunits over time, both *in vitro* and *in vivo*. This is in contrast to

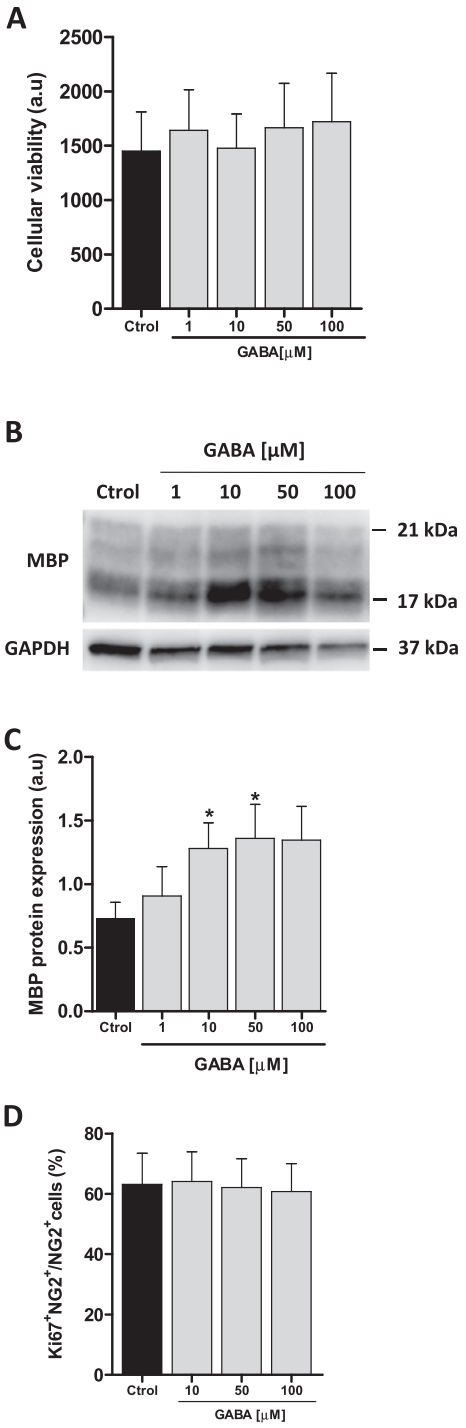


Fig. 6. GABA increases myelin protein expression in isolated OPC cultures without affecting cell viability or proliferation. **(A)** Quantification of cellular viability in cultured cortical OLs exposed to different concentrations of GABA for 24 h. **(B)** Representative western blot analysis of MBP protein in cortical oligodendrocytes cultured for 3 days in control and in the presence of increasing GABA concentrations. **(C)** Histogram showing the increase of MBP levels induced by GABA. Values for each condition were obtained from at least three independent experiments and normalized with respect to actin. *p < 0.05, **p < 0.01 vs control. **(D)** Quantification of the percentage of proliferating $Ki67^+NG2^+$ cells with respect to total $NG2^+$ cells showing no significant changes after exposure to GABA. All quantifications were performed in two or more coverslips per treatment in at least three independent experiments.

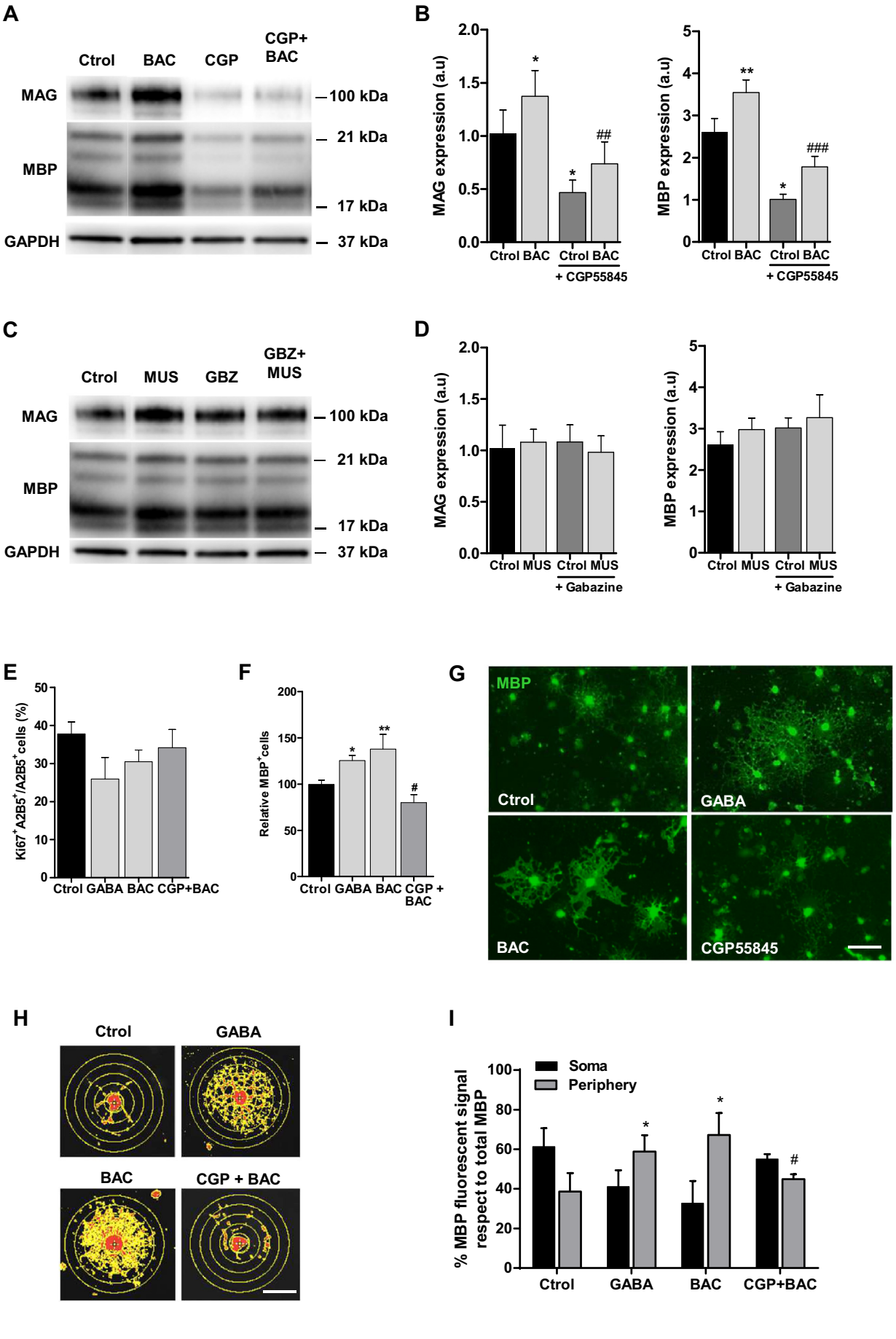
$GABA_A$Rs, whose expression in oligodendrocyte lineage changes during differentiation (Berger et al., 1992; Kirchhoff and Kettenmann, 1992; Arellano et al., 2016)

Expression of GABA transporters in oligodendroglial cells *in vitro* and *in vivo*

To deepen in the study of the GABAergic system in OLs, we investigated the expression of the GABA transporters GAT-1 and GAT-3 in isolated cultured OLs as well as in OLs from rat brain slices. We found that cortical OLs express GAT-1 at 1, 3 and 6 DIV (Fig. 3A), as previously observed in culture OLs obtained from rat optic nerves (Fattorini et al., 2017). In addition, we also detected the expression of GAT-3 transporter in OPC cultures (1DIV) as well as in OLs at different stages of maturation, after 3DIV and 6DIV, examined by immunostaining (Fig. 3B). These data were confirmed by Western blot analysis using total protein obtained from these *in vitro* samples, where we detected two bands close to the 72-kDa molecular weight (Fig. 3C), similar to described by other authors in astrocytic processes (Minelli et al., 1996). Consistent with these results, $CC1^+$ mature OLs in the corpus callosum of P14 rat brain sections express both GAT-1 and GAT-3 GABA transporters (Fig. 3D, E, respectively). F3

OLs synthesize GABA *in vitro*

GABA transporters are commonly present in cells synthesizing GABA itself. To assess that possibility, we analyze whether OLs indeed synthesize GABA. To that end, we first examined the presence of the GABA-synthesizing enzymes in cultured oligodendroglial cells. GABA can be synthesized by $GAD_{65/67}$ from glutamate or alternatively from putrescine, which requires MAO_B (Yoon et al., 2014). Therefore, we did again double immunocytochemistry using anti-Olig2 antibody as cellular lineage marker and specific anti- $GAD_{65/67}$ and anti-MAO_B antibodies. We found that cortical OLs express both $GAD_{65/67}$ and MAO_B enzyme at all times analyzed, namely 1, 3 and 6DIV after platting (Fig. 4A, B). Western Blot assays, analyzing the total protein collected from OL cultures, confirmed those results. For $GAD_{65/67}$, we detected a band of 65/67 kDa and for MAO_B, a band of approximately 60 kDa, according to the predicted molecular weights (Fig. 4C, D, respectively). We next examined the presence of GABA in cultured OLs derived from rat cerebral cortex and optic nerves using immunocytochemistry with an antibody against GABA. Double-staining with differentiation stage-specific markers showed that GABA was present throughout the cortical oligodendroglial lineage, from $PDGFR\alpha^+$ and $NG2^+$ OPCs to PLP^+ mature OLs (Fig. 4E). We also observed that optic nerve-derived MBP^+ OLs express GABA both at 2 and 6DIV (Fig. 4F). These results indicate that cultured rat OPCs and OLs synthesize GABA both from glutamate through $GAD_{65/67}$ enzyme, and from putrescine through MAO_B enzyme.F. F4


A
Ctrol
BAC
CGP
CGP+ BAC
MAG
MBP
GAPDH
100 kDa
21 kDa
17 kDa
37 kDa
B
MAG expression (a.u)
MBP expression (a.u)
Ctrol BAC
Ctrol BAC + CGP55845
C
Ctrol
MUS
GBZ
GBZ+ MUS
D
Ctrol MUS
Ctrol MUS + Gabazine
E
Ki67+A2B5+/A2B5+cells (%)
Ctrol GABA BAC CGP+BAC
F
Relative MBP+cells
Ctrol GABA BAC CGP + BAC
G
MBP
Ctrol
GABA
BAC
CGP55845
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Ctrol
GABA
BAC
CGP + BAC
I
Soma
Periphery
% MBP fluorescent signal respect to total MBP
Ctrol
GABA
BAC
CGP+BAC

GABA regulates myelination in DRG-OPC coculture system

We next studied the possibility that activation of GABAR may modulate OPC differentiation and myelination. For that, we developed a DRG-OPC coculture system to analyze myelination by quantifying the level of myelin basic protein and the number of myelinated segments present in the cocultures. Control medium or GABA at 10 μM or 50 μM was added immediately after plating OPCs on well-established DRG culture and cocultures were maintained for 21 days. In these conditions, OPCs differentiated and myelinated DRG axons, as assessed by immunostaining for MBP and NF-L (Fig. 5A). Notably, exposure to GABA for 21 days induced a higher MBP expression, as assessed by the increase in the area occupied by MBP with respect to NF-L area, reaching values of 120.3 ± 2.6 for 10 μM GABA and 131.7 ± 3.8 for 50 μM GABA, as compared to control values (Fig. 5B). In addition, OLs in GABA-treated cocultures displayed longer myelin sheaths parallel to axons, as evidenced by immunostaining for MBP (Fig. 5A). In turn, quantification of the number of myelin segments produced in these cocultures revealed that GABA induced a robust increase in axonal myelination with 182.7 ± 10.5 segments of MBP^+ axons for GABA 10 μM and 177.3 ± 12.6 for GABA 50 μM, with respect to culture controls (Fig. 5C). Finally, analysis of MBP expression by Western Blot confirmed that effect (Fig. 5D). In these blots, the four isoforms of MBP were detected (molecular weights: 17, 18.5, 20 and 21.5 kDa) and the quantification of all of them revealed significant MBP increments for GABA (163.22 ± 4.1% with respect to control). In addition, we observed that treatment with baclofen 50 μM triggered a similar MBP enhancement (171.3 ± 4.9% with respect to control) while muscimol was ineffective (95.43 ± 2.1% with respect to control). These results demonstrate that activation of GABAR potentiates the myelination capacity of OLs co-cultured with DRG neurons and suggest a potent involvement of $GABA_BR$ in this process.

GABA upregulates MBP expression in OL cultures

To determine whether the increase in myelination observed in DRG-OL cocultures was due to a direct effect of GABA stimulation on the oligodendrocyte lineage, we carried out assays of differentiation *in vitro* on isolated OPCs. Purified OPCs were plated on coverslips in differentiation medium, without or with GABA at various concentrations (0, 1, 10, 50 and 100 μM) for different times. We initially tested if GABA was deleterious as it is excitatory acting via $GABA_AR$s in OLs, and like glutamate analogs may cause excitotoxicity in these cells (Canedo-Antelo et al., 2018). Using calcein viability assays, we observed that GABA exposure did not affect OPC viability at none of the concentrations analyzed (Fig. 6A). To study the possible effect of GABARs on OPC differentiation, cells were incubated with GABA for 72 h and total protein was harvested to evaluate MBP levels. Quantitative analysis of MBP by Western blot showed that GABA stimulation yielded a remarkable increase in MBP expression (1.3-fold to 2.5-fold; Fig. 6B, C). These results indicate that GABA promotes direct OL maturation. However, to rule out the possibility that increasing of myelin proteins was due to an initial effect of GABA on OPC proliferation, we analyzed the number of $Ki67^+$ and $NG2^+$ cells in OPC cultures treated with vehicle or GABA, as above (Fig. 6D). We found that GABA did not induce proliferation on isolated OPCs. Together, these results indicate that GABA favors oligodendroglial differentiation.

$GABA_BR$ stimulation enhances myelin protein levels and OL process branching

To determine which type of GABAR mediates GABA-induced maturation, purified cortical OPCs were plated on coverslips in differentiation medium with or without $GABA_AR$ and $GABA_BR$ specific agonists and antagonists. As described above for GABA, we first performed calcein viability assays for all drugs and we warranted that GABAergic agonists and antagonists were not toxic for cultured OPCs at the concentrations used (data not shown). After 4 days, whole protein extracts from control and treated OLs were analyzed by Western blot. The results revealed that treatment with the $GABA_BR$ specific agonist baclofen (BAC; 50 μM) significantly increased the levels of MAG and MBP myelin proteins more than 1.5-fold, as compared to non-treated controls (Fig. 7A, B). This effect was attenuated in the presence of the $GABA_BR$ specific antagonist CGP55845 (CGP; 20 μM), which restored the levels of myelin protein to control levels. However, treatment with the $GABA_AR$ specific agonist muscimol (MUS), alone or in conjunction with the antagonist gabazine (GBZ), both at 20 μM, did not accelerate OPC differentiation or changed MAG and MBP protein levels (Fig. 7C and D). MAG protein was detected at the predicted molecular weight (100 kDa) and four isoforms of MBP were identified (MW: 17, 18.5, 20

Fig. 7. $GABA_BR$ but not $GABA_AR$ stimulation induces oligodendrocyte differentiation. **(A, C)** Representative western blots of cultured cortical oligodendrocytes in indicated condition for 4 days. **(B, D)** Quantification of MAG and MBP expression with respect to GAPDH values. Representative blots were show from at least three independent experiments. *$p < 0.05$, **$p < 0.01$ vs control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs baclofen. **(E)** Quantification of the percentage of $Ki67^+A2B5^+$ cells with respect to total $A2B5^+$ cells in indicated situation. **(F)** Quantification of MBP^+ cells in cultures treated for 3 days with the indicated drugs. **(G)** Representative images of cortical oligodendrocytes cultured in indicated condition for 3 days and immunostained with anti-MBP antibody. Scale bar = 20 μm. **(H)** Illustrative scheme of quantification by using concentric circle macro. Six concentric circles at 10-μm intervals emerging from the center of the cell nucleus were obtained. **(I)** Quantification of MBP fluorescent signal, expressed as percentage, in the soma and periphery of cortical oligodendrocytes for each condition. Quantifications were performed on a minimum of two coverslips per treatment of at least three independent experiments.*$p < 0.05$ vs control. #$p < 0.05$ vs baclofen.

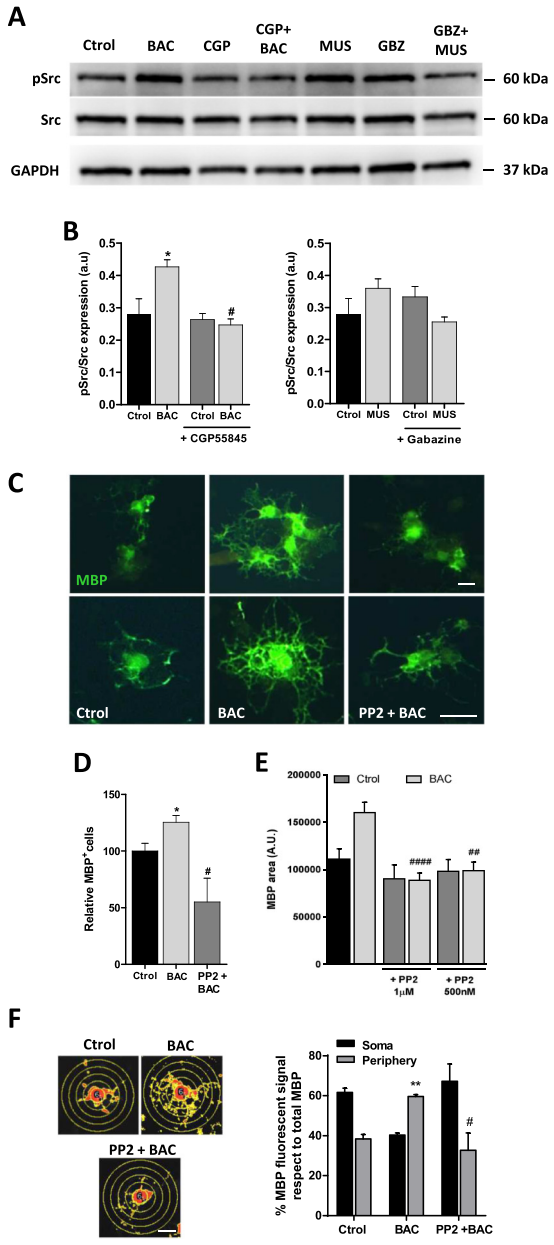

Fig. 8. $GABA_BR$-induced OL differentiation involves the participation of Src-family kinases. **(A)** Representative blot of phosphorylation of Src (pSrc) and Src in cultured cortical oligodendrocytes for 4 days following the indicated condition. **(B)** Quantification of pSrc expression in A. The expression of pSrc was normalized with total Src. GAPDH was analyzed as a load control. **(C)** Representative images of MBP^+ oligodendrocytes in control condition, baclofen (BAC) and PP2 + BAC treatment. Scale bar = 20 μm. **(D)** Quantification of MBP^+ cells per mm^2 was performed from images captured at 20× magnification, as showed in C, top. **(E)** Quantification of occupied area by the MBP staining per cell was carried out from images captured with 40× objective, and representative cells are showed in Fig. 8C, bottom. All quantifications were performed on a minimum of two coverslips per treatment of at least three independent experiments. *$p < 0.05$, **$p < 0.01$ vs control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs baclofen. **(F)** Analysis of MBP fluorescence intensity at soma and distal processes (periphery) of cortical oligodendrocytes in indicated situation, expressed as percentage of total MBP signal. Quantifications were performed in a minimum of two coverslips per treatment of at least three independent experiments. **$p < 0.01$ vs control. #$p < 0.05$ vs baclofen. Scale bar = 20 μm.

and 21.5 kDa) and were all quantified. Again, and to test if the pro-differentiation effect of baclofen was due to an increase in OPC population, we performed double immunocytochemistry to identify the dividing cells in cultures. After 48 h of culture, the percentages of $Ki67^+$ cells of the total of $A2B5^+$ early progenitor cells were quantified and found to be similar in all instances (Fig. 7E**)**. Thus, our data indicate that baclofen specifically regulates OPC differentiation without affecting their proliferation.

OPC differentiation is also characterized by the transition from a simple bipolar morphology to a more complex branched shape. To check the possible morphological changes induced by addition of GABA and baclofen during OPC differentiation *in vitro*, we developed morphological analysis based on MBP expression and its cellular distribution. First, we observed that the percentage of MBP^+ cells was enhanced in cultures treated with GABA and baclofen, both at 50 μM, for 72 h (Fig. 7F, G). The number of MBP^+ cells was 125.5 ± 4.01% for GABA and 137.9 ± 7.8% for baclofen, with respect to control (100%), while this effect was abolished in the presence of the $GABA_BR$ antagonist CGP55845 (79.95 ± 6.04% with respect to control). In addition, the morphological features revealed by MBP fluorescence indicated that cultures treated with GABA or baclofen displayed a bigger branching in comparison with non-treated cultures (Fig. 7G).

Therefore, to assess if $GABA_BR$ activation could modulate MBP distribution along the cell, we analyzed this protein level at soma and distal processes, an index that is associated with the arborization complexity and the degree of differentiation (Fig. 7H). We tested the effect of 50 μM GABA, 50 μM baclofen, and baclofen plus 20 μM CGP55845 for 72 h in cultured OPCs, and found that GABA and baclofen increased MBP fluorescence intensity at distal processes (periphery), while no significant differences were found at soma area **(**Fig. 7I**)**. These effects were not observed in the presence of the antagonist CGP55845. Overall, these results indicate that $GABA_BR$ stimulation upregulates MPB expression in cultured OLs, increases branching complexity and induces MBP localization at distal process.

Src-family kinases mediates baclofen-induced MBP upregulation in isolated OPCs

In order to investigate the molecular signals underlying baclofen-induced MBP upregulation, we focused on Src kinases, which are involved in myelin synthesis. Src kinases are upregulated during OL and Schwann cell differentiation and have been related to myelination (White and Krämer-Albers, 2014; Melfi et al., 2017). To analyze the putative role of Src family during $GABA_BR$-induced MBP increase, we first examined Src phosphorylation after exposure of OPCs to different GABAergic drugs (Fig. 8A). Treatment of OPCs with baclofen 50 μM for 72 h increased Src phosphorylation (pSrc) to 0.43 ± 0.02 with respect to 0.28 ± 0.05 of control (a 152.39% increase) while the incubation of baclofen plus antagonist CGP55845, 20 μM, reduced the levels of pSrc to 0.24 ± 0.18 (85.71% of level control) (Fig. 8B, left histogram**)**. The treatment of OPCs only with $GABA_BR$

F8

antagonist did not produce significant changes in pSrc level (0.26 ± 0.02; 94.04% of control).

On the other hand, and according to the absence of $GABA_AR$-induced MBP upregulation, the exposure of OPCs for 72 h to $GABA_AR$ agonist muscimol (20 μM), antagonist gabazine (20 μM), or both, did not promote significant phosphorylation of Src (Fig. 8B, right histogram).

To corroborate the involvement of Src kinase family in the baclofen-induced MBP overexpression, we tested if the observed effects on OL maturation were blocked by PP2, an Src kinase family inhibitor, at 1 μM and 500 nM. After 72 h of treatment with baclofen alone or together with PP2, cells were fixed and stained with MBP antibody (Fig. 8C) and the number of MBP^+ cells and the occupied area by the MBP staining per cell were quantified (Fig. 8D, E). In both cases, the presence of the PP2 inhibitor significantly blocked the baclofen-caused effects, reducing the number of MBP^+ cells (55.98 ± 14.9% with respect to 125.5 ± 4.01% induced by baclofen; Fig. 8D) and the occupied area by MBP (81.26 ± 10.7% for 1 μM PP2 and 89.04 ± 7.3% for 500 nM, with respect to 144.9 ± 3.9% produced by baclofen; Fig. 8E).

In addition, we observed that PP2 inhibitor, at 500 nM, significantly reverted the upregulation of MBP expression in OL distal processes promoted by 50 μM baclofen (Fig. 8F). So, baclofen increased the MBP expression in cellular periphery up to 60.71 ± 0.9% versus 38.39 ± 2.2% detected in control situation while presence of PP2 inhibitor reduced this level to 32.75 ± 8.6%.

Taken together, these results suggest that Src kinase family is involved in the upregulation of MBP expression induced by $GABA_BR$ stimulation in isolated OPC cultures.

DISCUSSION

In this study, we provide evidence that oligodendrocytes synthesize GABA and express the major components of the GABAergic system including $GABA_ARs$, $GABA_BRs$, GAT-1 and GAT-3 GABA-transporters as well as the two GABA-synthesizing enzymes, $GAD_{65/67}$ and MAO_B. Notably, we demonstrate a functional role of $GABA_BRs$ in OPC differentiation *in vitro* suggesting that GABA plays an important role in OL development and maturation.

Previous studies have reported that oligodendroglial cells express both $GABA_A$ (Hoppe and Kettenmann, 1989; Von Blankenfeld et al., 1991; Berger et al., 1992; Cahoy et al., 2008) and $GABA_B$ receptors (Luyt et al., 2007). However, while $GABA_AR$ expression decreases when OPCs differentiate into OLs (Von Blankenfeld et al., 1991; Vélez-Fort et al., 2012) and it is lost when OLs are cultured in isolation needing the presence of axons to be maintained (Arellano et al., 2016), our results show that the expression of $GABA_BRs$ in OLs remains largely stable over time even in absence of axons.

While $GABA_BR$-mediated mechanisms in neurons have been well characterized, functional identification of $GABA_BRs$ in OLs is less well understood (Xu et al., 2014; Booker et al., 2018). In order to resolve this question, we developed electrophysiological recordings and calcium imaging assays on OLs expecting to detect coupling between $GABA_BRs$ and Kir3 and L-type calcium channels, described for other cells types. However, we could not get results that confirmed this coupling (data not shown). In this line, a recent paper describes that $GABA_BRs$ are present on dendritic membranes of CA1 somatostatin interneurons, but do not activate the canonical Kir3 signaling cascade, while pyramidal neurons do it (Booker et al., 2018). Our data suggest that $GABA_BRs$ in OLs may signal through alternative effectors, whose nature will have to be studied in detail.

On the other hand, we also found that *in vivo* $GABA_BR$ expression occurs in OPCs ($NG2^+$ cells) and in mature oligodendrocytes ($CC1^+$ and PLP^+) in the juvenile rodent brain and persists to adulthood. These observations are in contrast with the idea of a downregulation of $GABA_BRs$ both in mature OLs (Charles et al., 2003; Luyt et al., 2007; Vélez-Fort et al., 2012) as well as in pre- and non-myelinating Schwann cells from sciatic nerve (Corell et al., 2015). However, and in line with the findings reported in the current study, GABA, via $GABA_BRs$, is a positive regulator of myelination without affecting Schwann cell proliferation.

We have previously described that oligodendrocytes express functional GAT-1 GABA transporter (Fattorini et al., 2017), and here we show that they also express GAT-3 GABA transporter. Again, the expression of both transporters is maintained over time as we have seen it at all stages in cultured oligodendrocytes, and *in vivo* in the rodent brain. The presence of these GAT-1 and GAT-3 in oligodendrocytes is not unexpected given the detailed relationship between GABA and oligodendrocytes. Thus, GAT-1 may contribute to the effects of GABA on oligodendrocytes by regulating GABA levels, either by taking up GABA or by releasing it, if operating in the reverse mode (Wu et al., 2007; Scimemi, 2014). GAT-3 might cooperate with GAT-1 in the regulation of GABA homeostasis in oligodendrocytes; however, its precise contribution will require further investigation.

GABA is synthesized from glutamate decarboxylation by $GAD_{65/67}$ enzyme, or alternatively, from putrescine through the action of MAO_B (Angulo et al., 2008; Yoon et al., 2014). Consistent with an existing database (Zhang et al., 2014), we have observed that OLs express both $GAD_{65/67}$ and MAO_B. Moreover, we have detected GABA itself in OPCs, $NG2^+$ cells and OLs at different stages of maturation, which confirms that oligodendrocytes synthesize GABA possibly for a proper differentiation and maturation. In line with that, Bergmann glia and Schwann cells have the ability to generate GABA and respond to this neurotransmitter, suggesting a putative autocrine/paracrine signaling with additional roles in axon–glia communication (Yoon et al., 2014; Corell et al., 2015).

GABA has been postulated as one of the modulators of oligodendroglial cell proliferation, differentiation and myelination (Zonouzi et al., 2015; Hamilton et al., 2017). Thus, endogenously released GABA, via $GABA_ARs$, reduces the number of oligodendroglial cells and, as a consequence, negatively modulates myelination *in situ* in cerebral cortical slices while activating or blocking $GABA_BRs$ had no effect on the number of OLs present in slices (Hamilton et al.,

2017), the latter in agreement with our data. In turn, we have observed in DRG-OPC cocultures that exogenous GABA and baclofen regulate myelination, specifically by increasing MBP levels and the myelin sheath segments around axons. These effects are likely direct as GABA stimulation of isolated OPC cells in culture accelerates differentiation into mature OLs as it induces an increase in MBP levels in the periphery of the cells, suggesting that these molecules are promoting local translation of MBP in peripheral areas. In addition, chronic treatment with baclofen in isolated oligodendrocytes produced an increase in the production of the myelin proteins MAG and MBP which was prevented by the $GABA_BR$ specific antagonist CGP55845. However, selective activation of $GABA_ARs$ did not significantly change the levels of these myelin proteins. These results indicate that potentiation by GABA of MBP expression and OL differentiation is mainly mediated by $GABA_BRs$, as observed in Schwann cells (Procacci et al., 2013; Corell et al., 2015), as well as by NMDA receptors in differentiation of oligodendrocyte precursor cells (Li et al., 2013; Lundgaard et al., 2013).

We then explored the downstream molecular mechanisms underlying $GABA_BR$-induced OL differentiation. In particular, we tested whether $GABA_BR$ activation initiated Fyn kinase signaling, a known integrator of neuronal signals that regulates the morphological differentiation of oligodendrocytes, the recruitment of cytoskeleton components and the MBP local translation (White and Krämer-Albers, 2014). Indeed, $GABA_BR$ stimulation mediates chemotaxis and cytoskeletal rearrangement which is dependent on signaling via PI3-K/Akt/Src kinases, and the modulation of this pathway may potentially be used to regulate cellular responses to injury and disease (Barati et al., 2015). Our data showed that chronic treatment with baclofen in rat OPC cultures induces Src-phosphorylation and $GABA_B$-R-induced OL differentiation is blocked by the Src-family kinases inhibitor PP2. Hence, our results suggest that Src-family acts downstream of $GABA_BR$ to favor OL differentiation and local translation of MBP in OL distal processes. Similarly, Src and phospho-FAK kinases activation promotes Schwann cells development and maturation, though these events lie under the control of $GABA_AR$-dependent mechanisms (Melfi et al., 2017).

In summary, the present study introduces an important role for the GABAergic system via $GABA_BRs$ in oligodendroglial cell differentiation from isolated OPCs *in vitro*. However, it is not known yet whether $GABA_BRs$ are essential for myelination during development and remyelination in pathological conditions. Nonetheless, the current study may lay the bases to address those questions. In particular, it will be relevant to study the role of GABA in demyelinating diseases, such as multiple sclerosis, in which reduced GABA levels are associated with cognitive impairment and physical disability (Cawley et al., 2015; Cao et al., 2018).

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REFERENCES

Alberdi E, Sánchez-Gómez MV, Marino A, Matute C. (2002) Ca(2+) influx through AMPA or kainate receptors alone is sufficient to initiate excitotoxicity in cultured oligodendrocytes. Neurobiol Dis 9 (2):234-243.

Angulo MC, Le Meur K, Kozlov AS, Charpak S, Audinat E. (2008) GABA, a forgotten gliotransmitter. Prog Neurobiol 86(3):297-303.

Arellano RO, Sánchez-Gómez MV, Alberdi E, Canedo-Antelo M, Chara JC, Palomino A. (2016) Pérez-Sanmartín, and Matute C. Axon-to-glia interaction regulates $GABA_A$ receptor expression in oligodendrocytes Mol Pharmacol 89(1):63-74.

Azim K, Butt AM. (2011) GSK3β negatively regulates oligodendrocyte differentiation and myelination *in vivo*. Glia 59(4):540-553.

Barati MT, Lukenbill J, Wu R, Rane MJ, Klein JB. (2015) Cytoskeletal rearrangement and Src and PI-3K-dependent Akt activation control $GABA_BR$-mediated chemotaxis. Cell Signal 27(6):1178-1185.

Barres BA, Hart IK, Coles HS, Burne JF, Voyvodic JT, Richardson WD, Raff MC. (1992) Cell death and control of cell survival in the oligodendrocyte lineage. Cell 70(1):31-46.

Baumann N, Pham-Dinh D. (2001) Biology of oligodendrocyte and myelin in the mammalian central nervous system. Physiol Rev 81(2):871-927.

Benke D, Zemoura K, Maier PJ. (2012) Modulation of cell surface GABA (B) receptors by desensitization, trafficking and regulated degradation. World J Biol Chem 3(4):61-72.

Berger T, Walz W, Schnitzer J, Kettenmann H. (1992) GABA-and glutamate-activated currents in glial cells of the mouse corpus callosum slice. J Neurosci Res 31(1):21-27.

Bernal-Chico A, Canedo M, Manterola A, Victoria Sánchez-Gómez M, Pérez-Sanmartín A, Rodríguez-Puertas R, Matute C, Mato S. (2015) Blockade of monoacylglycerol lipase inhibits oligodendrocyte excitotoxicity and prevents demyelination in vivo. Glia 63(1):163-176.

Bettler B, Tiao JY. (2006) Molecular diversity, trafficking and subcellular localization of GABAB receptors. Pharmacol Ther 110(3):533-543.

Bettler B, Kaupmann K, Mosbacher J, Gassmann M. (2004) Molecular structure and physiological functions of GABAB receptors. Physiol Rev 84(3):835-867.

Booker SA, Loreth D, Gee AL, Watanabe M, Kind PC, Wyllie DJA, Kulik A, Vida I. (2018) Postsynaptic $GABA_BRs$ inhibit L-type calcium channels and abolish long-term potentiation in hippocampal somatostatin interneurosn. Cell Rep 22:36-43.

Borges K, Wolswijk G, Ohlemeyer C, Kettenmann H. (1995) Adult rat optic nerve oligodendrocyte progenitor cells express a distinct repertoire of voltage-and ligand-gated ion channels. J Neurosci Res 40(5):591-605.

Bowery NG, Bettler B, Froestl W, Gallagher JP, Marshall F, Raiteri M, Bonner TI, Enna SJ. (2002) International Union of Pharmacology. XXXIII. Mammalian gamma-aminobutyric acid(B) receptors: structure and function. Pharmacol Rev 54(2):247-264.

Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, Xing Y, Lubischer JL, Krieg PA, Krupenko SA, Thompson WJ, Barres BA. (2008) A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. J Neurosci 28(1):264-278.

Canedo-Antelo M, Serrano MP, Manterola A, Ruiz A, Llavero F, Mato S, Zugaza JL, Pérez-Cerdá F, Matute C, Sánchez-Gómez MV. (2018) Inhibition of casein kinase 2 protects oligodendrocytes from excitotoxicity by attenuating JNK/p53 signaling cascade. Front Mol Neurosci 11:333.

Cao G, Edden RAE, Gao F, LiH Gong T, Chen W, Liu X, Wang G, Zhao B. (2018) Reduced GABA levels correlate with cognitive impairment in patients with relapsing-remitting multiple sclerosis. Eur Radiol 28 (3):1140-1148.

Cawley N, Solanky BS, Muhlert N, Tur C, Edden RA, Wheeler-Kingshott CA, Miller DH, Thompson AJ, Ciccarelli O. (2015) Reduced gamma-aminobutyric acid concentration is associated with physical disability in progressive multiple sclerosis. Brain 138:2584-2595.

Charles KJ, Deuchars J, Davies CH, Pangalos MN. (2003) GABA B receptor subunit expression in glia. Mol Cell Neurosci 24(1):214-223.

Chen Y, Balasubramaniyan V, Peng J, Hurlock EC, Tallquist M, Li J, Lu QR. (2007) Isolation and culture of rat and mouse oligodendrocyte precursor cells. Nat Protoc 2(5):1044-1051.

Corell M, Wicher G, Radomska KJ, Daglikoca ED, Godskesen RE, Fredriksson R, Benedikz E, Magnaghi V, Fex Svenningsen A. (2015) GABA and its B-receptor are present at the node of Ranvier in a small population of sensory fibers, implicating a role in myelination. J Neurosci Res 93(2):285-295.

Domercq M, Pérez-Sanmartín A, Aparicio D, Alberdi E, Pampliega O, Matute C. (2010) P2X7 receptors mediate ischemic damage to oligodendrocytes. Glia 58(6):730-740.

Fancy SP, Chan JR, Baranzini SE, Franklin RJ, Rowitch DH. (2011) Myelin regeneration: a recapitulation of development? Annu Rev Neurosci 34:21-43.

Fannon J, Tarmier W, Fulton D. (2015) Neuronal activity and AMPA-type glutamate receptor activation regulates the morphological development of oligodendrocyte precursor cells. Glia 63(6):1021-1035.

Fattorini G, Melone M, Sánchez-Gómez MV, Arellano RO, Bassi S, Matute C, Conti F. (2017) GAT-1 mediated GABA uptake in rat oligodendrocytes. Glia 65(3):514-522.

Frangaj A, Fan QR. (2018) Structural biology of $GABA_B$ receptor. Neuropharmacology 136:68-79.

Franklin RJM, Ffrench-Constant C. (2017) Regenerating CNS myelin — from mechanisms to experimental medicines. Nat Rev Neurosci 18 (12):753-769.

Galvez T, Urwyler S, Prézeau L, Mosbacher J, Joly C, Malitschek B, Heid J, Brabet I, Froestl W, Bettler B, Kaupmann K, Pin JP. (2000) Ca(2+) requirement for high-affinity gamma-aminobutyric acid (GABA) binding at GABA(B) receptors: involvement of serine 269 of the GABA(B)R1 subunit. Mol Pharmacol 57(3):419-426.

González-Fernández E, Sánchez-Gómez MV, Pérez-Sanmartín A, Arellano RO, Matute C. (2014) A3 adenosine receptors mediate oligodendrocyte death and ischemic damage to optic nerve. Glia 62(2):199-216.

Guardiola-Diaz HM, Ishii A, Bansal R. (2012) Erk1/2 MAPK and mTor signaling sequentially regulates progression through distinct stages of oligodendrocyte differentiation. Glia 60(3):476-486.

Hamilton NB, Clarke LE, Arancibia-Carcamo IL, Kougioumtzidou E, Matthey M, Káradóttir R, Whiteley L, Bergersen LH, Richardson WD, Attwell D. (2017) Endogenous GABA controls oligodendrocyte lineage cell number, myelination, and CNS internode length. Glia 65(2):309-321.

Hirrlinger PG, Scheller A, Braun C, Quintela-Schneider M, Fuss B, Hirrlinger J, Kirchhoff F. (2005) Expression of reef coral fluorescent proteins in the central nervous system of transgenic mice. Mol Cell Neurosci 30 (3):291-303.

Hoppe D, Kettenmann H. (1989) GABA triggers a Cl^- efflux from cultured mouse oligodendrocytes. Neurosci Lett 97(3):334-339.

Karram K, Goebbels S, Schwab M, Jennissen K, Seifert G, Steinhäuser C, Nave KA, Trotter J. (2008) NG2-expressing cells in the nervous system revealed by the NG2-EYFP-knockin mouse. Genesis 46(12):743-757.

Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A, Bettler B. (1998) GABAB-receptor subtypes assemble into functional heteromeric complexes. Nature 396(6712):683-687.

Kirchhoff F, Kettenmann H. (1992) GABA triggers a [Ca2+]i increase in murine precursor cells of the oligodendrocyte lineage. Eur J Neurosci 4(11):1049-1058.

Kremer D, Akkermann R, Küry P, Dutta R. (2019) Current advancements in promoting remyelination in multiple sclerosis. Mult Scler 25(1):7-14.

Kuner R, Köhr G, Grünewald S, Eisenhardt G, Bach A, Kornau HC. (1999) Role of the heteromer formation in GABAB receptor function. Science 283(5398):74-77.

Li C, Xiao L, Liu X, Yang W, Shen W, Hu C, Yang G, He C. (2013) A functional role of NMDA receptor in regulating the differentiation of oligodendrocyte precursor cells and remyelination. Glia 61(5):732-749.

Lin SC, Bergles DE. (2004) Synaptic signaling between GABAergic interneurons and oligodendrocyte precursor cells in the hippocampus. Nat Neurosci 7(1):24-32.

Lundgaard I, Luzhynskaya A, Stockley JH, Wang Z, Evans KA, Swire M, Volbracht K, Gautier HO, Franklin RJ, Ffrench-Constant Charles, Attwell D, Káradóttir RT. (2013) Neuregulin and BDNF induce a switch to NMDA receptor-dependent myelination by oligodendrocytes. PLoS Biol 11(12)e1001743.

Luyt K, Slade TP, Dorward JJ, Durant CF, Wu Y, Shigemoto R, Mundell SJ, Váradi A, Molnár E. (2007) Developing oligodendrocytes express functional $GABA_{(B)}$ receptors that stimulate cell proliferation and migration. J Neurochem 100(3):822-840.

Magnaghi V, Ballabio M, Cavarretta ITR, Wolfgang W, Lambert JJ. (2004) Zucchi and Melcangi RC. GABAB receptors in Schwann cells influence proliferation and myelin protein expression. Eur J Neurosci 19:2641-2649.

Marshall FH, Jones KA, Kaupmann, K and Bettler B (1999) GABAB receptors — the first 7TM heterodimers Trends Pharmacol Sci 20 (10): 396–399.

Matute C, Streit P. (1986) Monoclonal antibodies demonstrating GABA-like immunoreactivity. Histochem 86:147-157.

McCarthy KD, de Vellis J. (1980) Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J Cell Biol 85 (3):890-902.

Melfi S, Montt Guevara MM, Bonalume V, Ruscica M, Colciago A, Simoncini T, Magnaghi V. (2017) Src and phosphor-FAK kinases are activated by allopregnanolone promoting Schwann cell motility, morphology and myelination. J Neurochem 141(2):165-178.

Minelli A, DeBiasi S, Brecha NC, Vitellaro Zuccarello L, Conti F. (1996) GAT-3, a high-affinity GABA plasma membrane transporter, is localized to astrocytic processes, and it is not confined to the vicinity of GABAergic synapses in the cerebral cortex. J Neurosci 16(19):6255-6264.

Procacci P, Ballabio M, Castelnovo LF, Mantovani C, Magnaghi V. (2013) GABA-B receptors in the PNS have a role in Schwann cells differentiation? Front Cell Neurosci 6:68.

Sánchez-Gómez MV, Serrano MP, Alberdi E, Pérez-Cerdá F, Matute C. (2018) Isolation, expansión and maturation of oligodendrocyte lineage cells obtained from rat neonatal brain and optic nerve. Methods Mol Biol 1791:95-113.

Scimemi A. (2014) Structure, function, and plasticity of GABA transporters. Front Cell Neurosci 8:161.

Vélez-Fort M, Audinat E, Angulo MC. (2012) Central role of GABA in neuron–glia interactions. Neuroscientist 18(3):237-250.

Verkhratsky A, Steinhaüser C. (2000) Ion channels in glial cells. Brain Res Rev 32(2–3):380-412.

Von Blankenfeld G, Trotter J, Kettenmann H. (1991) Expression and developmental regulation of a GABAA receptor in cultured murine cells of the oligodendrocyte lineage. Eur J Neurosci 3(4):310-316.

White R, Krämer-Albers EM. (2014) Axon–glia interaction and membrane traffic in myelin formation. Front Cell Neurosci 7:284.

Williamson AV, Mellor JR, Grant AL, Randall AD. (1998) Properties of GABA(a) receptors in cultured rat oligodendrocyte progenitor cells. Neuropharmacology 37(7):859-873.

Wu Y, Wang W, Díez-Sampedro A, Richerson GB. (2007) Nonvesicular inhibitory neurotransmission via reversal of the GABA transporter GAT-1. Neuron 56(5):851-865.

Xu C, Zhang W, Rondard P, Pin J-P, Liu J. (2014) Complex GABAB receptor complexes: how to generate multiple functionally distinct units from a single receptor. Front Pharmacol 5:1-8 Article 12.

Yoon B-E, Woo J, Chun Y-E, Chun H, Jo S, Bae JY, An H, Min JO, Oh S-J, Han K-S, Kim HY, Kim T, Kim YS, Bae YC, Lee CJ. (2014) Glial GABA, synthesized by monoamine oxidase B, mediates tonic inhibition. J Physiol 592:4951-4968.

Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O' Keeffe S, Phatnani HP, Guarnieri P, Caneda C, Ruderisch N, Deng S, Liddelow SA, Zhang C, Daneman R, Maniatis T, Barres BA, Wu JQ. (2014) An RNA-sequencing transcriptome and splicing database of glia, neurons and vascular cells of the cerebral cortex. J Neurosci 34(36):11929-11947.

Zonouzi M, Scafidi J, Li P, McEllin B, Edwards J, Dupree JL, Harvey L, Sun D, Hübner CA, Cull-Candy SG, Farrant M, Gallo V. (2015) GABAergic regulation of cerebellar NG2 cell development is altered in perinatal white matter injury. Nat Neurosci 18(5):674-682.

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