Received: 19 February 2021 | Revised: 26 July 2021 | Accepted: 20 September 2021

DOI: 10.1002/dneu.22853

RESEARCH ARTICLE

WILEY

Postnatal myelination of the immature rat cingulum is regulated by $GABA_B$ receptor activity

Samipa Pudasaini[1] | **Vivien Friedrich**[1,2] | **Christoph Bührer**[1] | **Stefanie Endesfelder**[1] | **Till Scheuer**[1] | **Thomas Schmitz**[1]

[1] Department of Neonatology, Charité University Hospital Berlin, Augustenburger Platz 1, Berlin 13353, Germany

[2] Berlin Institute of Health (BIH), Anna-Louisa-Karsch-Straße 2, Berlin 10178, Germany

Correspondence
Thomas Schmitz, Klinik für Neonatologie, Charité Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany.
Email: Thomas.Schmitz@charite.de

Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: Sche 2078/2-1; Förderverein für frühgeborene Kinder an der Charité e.V.; Lydia Rabinowitsch Foundation

Abstract

Myelination of axons in the neonatal brain is a highly complex process primarily achieved by oligodendroglial cells (OLs). OLs express receptors for γ-aminobutyric acid (GABA) which is released from cortical interneurons on a basal level, while glial cells can be a source of GABA, too. We investigated GABA-induced oligodendroglial maturation, proliferation, apoptosis, and myelin production after pharmacological inhibition of $GABA_A$ and $GABA_B$ in the neonatal rat brain. Daily injections of the reverse $GABA_A$ receptor agonist (DMCM) and the $GABA_B$ receptor antagonist (CGP35348) were performed from postnatal day 6 (P6) to P11. MBP expression was examined by Western blots and immunohistochemistry. Furthermore, we determined the number of $CC1^+OLIG2^+$ and CNP^+OLIG2^+ cells to assess maturation, the number of $PCNA^+OLIG2^+$ oligodendrocytes to assess proliferation, the number of oligodendrocyte precursor cells ($PDGFR\alpha^+OLIG2^+$), and apoptosis of OLs ($CASP3A^+OLIG2^+$) as well as apoptotic cells in total ($CASP3A^+DAPI^+$) at P11 and P15. In addition, we analyzed the expression *Pdgfrα* and CNP. MBP expression was significantly reduced after CGP treatment at P15. In the same animal group, CNP expression and CNP^+OLIG2^+ cells decreased temporarily at P11. At P15, the proliferation of $PCNA^+OLIG2^+$ cells and the number of $PDGFR\alpha^+OLIG2^+$ cells increased after $GABA_B$ receptor antagonization whereas no significant differences were visible in the *Pdgfrα* gene expression. No changes in apoptotic cell death were observed. CGP treatment induced a transient maturational delay at P11 and deficits in myelin expression at P15 with increased oligodendroglial proliferation. Our in vivo study indicates $GABA_B$ receptor activity as a potential modulator of oligodendroglial development.

KEYWORDS
γ-aminobutyric acid (GABA), neonatal brain, oligodendroglia, myelination, proliferation

1 | INTRODUCTION

γ-aminobutyric acid (GABA) is well-known as the main inhibitory neurotransmitter in the adult brain. Prenatally, however, it carries the potential to trigger excitatory currents (Ben-Ari, 2002; Ganguly et al., 2001; Kirchhoff & Kettenmann, 1992; Leinekugel et al., 1999). Its ionotropic and metabotropic receptors are characterized by a high heterogeneity as their function and the composition of their subunits can vary immensely depending on the brain region and the developmental stage (Balia et al., 2015; Serrano-Regal et al., 2020; Vogt, 2015).

In contrast to neuronal GABA receptors, the functional expression of GABA receptors in oligodendroglial lineage cells has not been investigated in great detail (Gilbert et al., 1984; Serrano-Regal et al., 2020; Steinhauser et al., 1994). Recent studies assessed the expression of $GABA_A$ receptors and $GABA_B$ receptors in oligodendrocytes and discovered their presence as being more pronounced in oligodendrocyte precursor cells (OPCs) compared to mature oligodendrocytes (Arellano et al., 2016; Káradóttir & Attwell, 2007; Serrano-Regal et al., 2020). Myelinating oligodendrocytes, which evolve from OPCs, are responsible for a proper myelination of neuronal axons in the central nervous system (CNS) to guarantee a high transmission speed of electric impulses (Baumann & Pham-Dinh, 2001). GABA can be assumed to influence myelin sheath development by activating oligodendroglial GABA receptors. However, results concerning this matter have so far shown inconsistency and heterogeneity (Balia et al., 2017; Luyt et al., 2007; Tong et al., 2009). Hamilton et al. described a decrease in oligodendroglial cell death as well as an enhanced oligodendroglial maturation, proliferation and myelination resulting from blocking $GABA_A$ receptor activity in cerebral cortex slices of mice (Hamilton et al., 2017). The research by Zonouzi et al., in contrast, indicated an increase in OPC proliferation and a delay of oligodendroglial maturation in the cerebellar white matter after injection of the $GABA_A$ receptor antagonist bicuculline in mice (Zonouzi et al., 2015). In a recent study, Serrano-Regal et al. showed that GABA stimulates oligodendroglial differentiation and myelin basic protein (MBP) expression in oligodendrocyte cultures via $GABA_B$ receptors in vitro, while there was no effect on the proliferation of OPCs (Serrano-Regal et al., 2020). To further define the effects that GABA receptor activation exerts on oligodendrocytes, additional experiments will be needed to elucidate interactions between GABAergic interneurons and immature oligodendroglia cells during brain development.

Developmental myelination has been described as a vulnerable process which is influenced by oligodendrocytes themselves (Baumann & Pham-Dinh, 2001), neuronal stimuli (Gibson et al., 2014), soluble growth factors and neurotransmitters (Barateiro et al., 2016), as well as the absence of axonal inhibitors such as Leucine rich repeat and Immunoglobin-like domain-containing protein 1 (LINGO-1) or L1CAM (L1) (Barbin et al., 2004; Mi et al., 2005). It is estimated that the lack of these axonal inhibitors in oligodendroglial cultures (Jakovcevski et al., 2009), may affect in vitro results as compared to experiments in in vivo models. Also, Arellano et al. demonstrated in their in vitro studies the relevance of neuronal crosstalk for a long-lasting $GABA_A$ receptor stability (Arellano et al., 2016). This and the general complexity of the interaction between GABA, oligodendroglia, neurons, and myelin sheaths further underline the need for new studies based on in vivo models. Therefore, we used in vivo rat experiments to analyze the effects of $GABA_A$ and $GABA_B$ receptor inactivation on oligodendroglial proliferation, maturation, apoptotic cell death, and myelination in the cingulum.

In our study, we demonstrate in an in vivo rat model that pharmacological inhibition of $GABA_B$ receptors with CGP causes a reduction of MBP levels as well as an increase in the proliferation rate of oligodendroglial cells in the cingulum. Both effects are not present after application of the $GABA_A$ reverse agonist DMCM. Further, we detected a temporary maturational delay among the CGP treated animals. No changes in apoptosis were present after DMCM and CGP application in oligodendrocytes and cells of the cingulum in total. Taken together, our results support the idea that $GABA_B$ receptors are highly relevant for developmental myelination.

2 | METHODS

2.1 | Animals and antagonization

All animal experiments were performed based on the institutional guidelines for good laboratory practice, as well as the ARRIVE guidelines, and were approved by the animal welfare committees of Berlin, Germany (LAGeSo, approval number G-0075/18). Six days old Wistar rats (P6) were divided into three experimental groups and received a daily intraperitoneal (i.p.) application of either *(1)* a vehicle (100 μl 0.9% NaCl/10 g bodyweight), *(2)* the $GABA_A$ inverse agonist methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate (DMCM) hydrochloride with a concentration of 50 μg/kg or *(3)* the $GABA_B$ antagonist 3-Aminopropyl-diethoxymethyl-phosphinic acid (CGP35348) with a concentration of 10 mg/kg (Figure 1). These concentrations were chosen because of their convulsion-avoiding effects (Kulick et al., 2014; Tyagi et al., 2015) and are based on preliminary experiments on dose optimization. Rats were housed under 12:12h light:dark cycle together with their lactating mothers until analyses at P11 and P15 (Figure 1). No

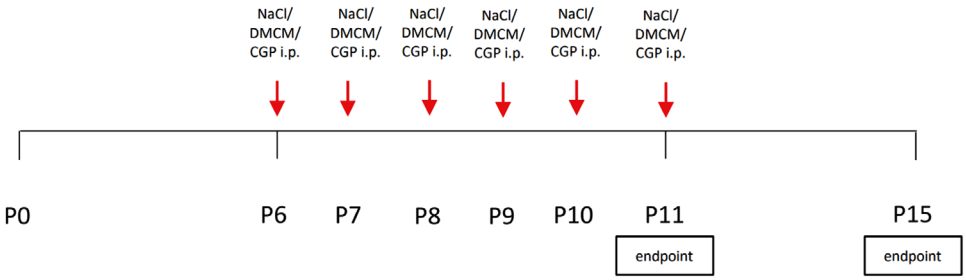

FIGURE 1 Schematic illustration of the experimental antagonization of $GABA_A$ and $GABA_B$ receptors via i.p. application of DMCM or CGP. Vehicle treatment or GABA antagonization was performed on a daily basis between P6 and P11. Two endpoints, P11, directly after the final injection, and P15, four days afterwards, were analyzed

major weight losses and no other morbidities were recognized in all pups during and after the treatment.

2.2 | Immunohistochemistry

2.2.1 | Immunofluorescence

The rats were anesthetized according to the guidelines of the animal welfare committee at the experimental endpoints P11 or P15 with an i.p. injection of ketamine (100 mg/kg), xylazine (20 mg/kg) and acepromazine (3 mg/kg). They were transcardially perfused with phosphate buffered saline (PBS), followed by 4 % paraformaldehyde (PFA). After dissection, the brains were fixed in 4 % PFA/PBS at 4˚C overnight. Brains were paraffin-embedded and processed for histological staining. Tissue sections were cut at 6 μm and stored at room temperature until usage.

Paraffinized sections were deparaffinized by a treatment with Roti-Histol (Carl Roth, Karlsruhe, Germany) and rehydrated in solutions of falling ethanol concentration. Heat mediated antigen retrieval was performed in the microwave at 600 Watt (citrate buffer, pH 6.0) for 10 minutes. Afterwards, sections were left for at least one hour in blocking solution [3% Bovine Serum Albumin, 0.2% Triton X-100 in PBS] at room temperature. Following primary antibodies were diluted in an antibody diluent (Zymed Laboratories, San Francisco, California, USA): polyclonal rabbit antibody to myelin basic protein (MBP, Abcam, #40390, 1:500), polyclonal mouse antibody to proliferating cell nuclear antigen (PCNA, Abcam, #ab29, 1:500), monoclonal mouse antibody to APC (CC1, Calbiochem, #OP80, 1:250), monoclonal rabbit antibody to cleaved caspase 3 (CellSignaling, #9664, 1:2000), monoclonal rabbit antibody to platelet derived growth factor receptor α (PDGFRα, Cell Signaling, #3174, 1:500), monoclonal mouse antibody to 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase, Sigma-Aldrich, MAB326, 1:400) and polyclonal goat antibody to oligodendrocyte 2 (OLIG2, R&D systems, AF2418, 1:2000). The incubation was performed at 4˚C overnight. Following secondary antibodies were afterwards diluted and applied to the brain sections: goat-anti-mouse Cyanine 5 (Jackson ImmunoResearch Laboratories, 1:100), goat-anti-rabbit Alexa Fluor 546 (Molecular Probes, #A11010, 1:200), goat-anti-mouse Alexa Fluor 546 (Molecular Probes, #A11003, 1:200) and 594 (Molecular Probes, #A11032, 1:200) and donkey-anti-goat Alexa Fluor 488 (Life Technologies, #A11055, 1:200). All sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma, #32670, 1:2000). The incubation lasted for one or two hours, depending on the antibody, at room temperature. Mounting was performed with mounting media (Shandon Immu-Mount, Thermo Fisher Scientific).

2.2.2 | Microscopy and cell density measurements

Immunohistochemically stained cerebral sections were viewed blindly using a Keyence compact fluorescent microscope BZ 9000 (Keyence, Osaka, Japan) with a 10x or 20x objective lens. The fluorescent properties of DAPI, MBP as well as CC1, CASP3A, PCNA, PDGFRα and CNP were visualized at the cingulum region.

For analysis of oligodendroglial maturation, proliferation, and apoptosis, CC1, CNP, PCNA, PDGFRα and CASP3A stainings were co-labelled with OLIG2. In average, two images out of two to four sections from one animal were obtained with help of the BZ-II Viewer software (Keyence). The merging process was performed by the BZ-II Analyzer software (Keyence). In order to analyze cell numbers in $CC1^+OLIG2^+$, CNP^+OLIG2^+, $PCNA^+OLIG2^+$, $PDGFR\alpha^+OLIG2^+$, $CASP3A^+OLIG2^+$ and $CASP3A^+DAPI^+$ co-stainings, brightness and contrast were adapted with minimal manipulation and cells were counted manually and blindly via Adobe Photoshop CS6 (Adobe Systems Incorporated). MBP and CNP stainings were analyzed using the ImageJ software (National Institute of Health) by measuring the pixel intensity as well as the area (per field).

2.3 | Molecular analysis

For molecular analysis, the brains of the rats were perfused with PBS, freezed in liquid nitrogen and stored at −80°C until protein and ribonucleic acid (RNA) extraction were performed.

2.3.1 | Protein extraction

Brain tissue was homogenized in a 4°C radioimmunoprecipitation assay (RIPA) buffer solution (Thermo Fisher Scientific) in order to extract proteins. For measurement of protein concentration, the Pierce BCA kit (Pierce/Thermo Fisher Scientific) was used. Spectrophotometry was performed with the iMark™ Microplate Absorbance Reader (BioRad, Life Science Research, Munich, Germany) at a wavelength of 570 nm.

2.3.2 | Western blotting

Aliquots of proteins were equally loaded (20 μg per lane) on 4–20 % Criterion™ TGX™ Precast Gels (BioRad) and separated according to their molecular weight by using PowerPac HC BioRad (180 V, 0.10 A, 300 W). The gels were transferred onto Trans-Blot Turbo nitrocellulose membrane (0.2 μm pore, BioRad) via Trans-Blot Turbo Transfer System and afterwards blocked in Roti Block (Carl Roth) for one hour. Following primary antibodies were diluted in Tris-buffered saline (TBS): monoclonal mouse antibody to MBP (Covance, #SMI-99P0, 1:1000), polyclonal rabbit antibody to 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase) (Thermo Fisher, #PA5-27972, 1:1000) and, for normalization, monoclonal mouse antibody to β-ACTIN (Sigma, #A5316, 1:5000). Membranes were incubated in primary antibodies at 4°C overnight. Afterwards, they were treated with horseradish-peroxidase-conjugated (HRP) secondary antibodies (polyclonal goat-anti-rabbit, Dako, P0448 and goat-anti-mouse, Dako, P0447) which were diluted to 1:2000 in TBS, for one hour. Chemiluminescent detection was performed using Clarity™ Western ECL Substrate (BioRad) or Supersignal West Pico (Pierce, Rockford, Illinois, USA) kits according to manufacturers' directions. The bands were measured using ChemiDoc™ XRS+ system (BioRad) and quantified with Image Lab™ (BioRad). All protein levels were normalized to β-ACTIN expression level. Due to similar molecular weights of CNP and β-ACTIN, membranes were stripped, in this particular case, between analyses with Restore™ Western blot stripping buffer (Thermo Fisher Scientific), according to the manufacturers' instructions.

2.3.3 | RNA extraction and quantitative real-time PCR

Total RNA was isolated from snap frozen brain tissue by acidic phenol/chloroform extraction (peqGOLD RNAPure™; PEQLAB Biotechnologie, Erlangen, Germany). 2 μg of RNA were treated with DNase and reverse transcribed. The RNA expression of Hypoxanthine phosphoribosyltransferase 1 (*Hprt*) and platelet derived growth factor receptor alpha (*Pdgfrα*) were quantified in triplicate with the sequences summarized in Table 1A. PCR and detection were performed with qPCR BIO Mix Hi-ROX (NIPPON Genetics Europe, Düren, Germany). *Hprt* was used as an internal reference. The expression of target genes was analyzed with the StepOnePlus real-time PCR system (Applied Biosystems/Life Technologies, Carlsbad, California, USA) according to the $2^{-\Delta\Delta CT}$-method (Livak & Schmittgen, 2001).

2.4 | Statistics

For statistical analysis, the mean value ± Standard Error of Mean (SEM) of two images out of two to four sections from one animal were obtained. Here, *n* reflects the number of rats per control as well as per experimental group included into the statistical analyses. The analysis as well as the creation of graphs were performed via Graph Pad Prism 8.0 software (GraphPad Software, La Jolla, California, USA). One-way analysis of variance (ANOVA) with Bonferroni post hoc tests were used when comparing the control animals with both experimental groups. An unpaired t-test (two-tailed) was applied in case of a one-to-one comparison between a control and a test group. If a significant difference appeared among means ($p < 0.05$), Brown-Forsythe and Welch ANOVA with Dunnett's T3 multiple comparisons test was applied. Additionally, a Shapiro-Wilk test was used to examine the distribution. If values were not normally distributed, a Kruksal-Wallis test was performed. Results were defined as significant in case of a p-value < 0.05.

3 | RESULTS

3.1 | $GABA_B$ receptor antagonization reduces MBP levels in vivo

To analyze the function of GABA on oligodendroglial cells, we antagonized $GABA_A$ and $GABA_B$ receptors in the brains of neonatal rats by daily injection of $GABA_A$ inverse agonist DMCM and $GABA_B$ antagonist CGP, respectively, from P6 to P11. We investigated cellular effects with regards to oligodendroglial proliferation, maturation, and apoptosis rate via immunohistological labelling of specific markers. Also, since oligodendrocytes carry the major role in myelinating neuronal axons (Back, 2006; Baumann & Pham-Dinh, 2001), possible impairments on myelin production resulting from DMCM and CGP treatment were examined by Western blot and immunohistochemistry via MBP analysis at ages P11,

TABLE 1 **(A)** Sequences of oligonucleotides and gene locus labelled with FAM/TAMRA. ***Hprt***: Hypoxanthine phosphoribosyltransferase 1. ***Pdgfrα***: Platelet derived growth factor receptor alpha

A Gene	Oligonucleotide sequence 5'-3'	Accession number (NCBI)
Hprt :		
Forward Primer	GGAAAGAACGTCTTGATTGTTGAA	NM_012583.2
Reverse Primer	CCAACACTTCGAGAGGTCCTTTT	
Probe	CTTTCCTTGGTCAAGCAGTACAGCCCC	
Pdgfrα :		
Forward Primer	CCTGCGCCGTCTTTAACAAT	NM_012802.1
Reverse Primer	TCTCCTCCAGCATGGTGATG	
Probe	TTACCCTGGAGAGGTGAGAA	

immediately after antagonization, and P15, after further 4 days without treatment. As shown in Figure 2a–c, antagonization of $GABA_A$ and $GABA_B$ receptors did not significantly alter MBP immunostaining intensity of the white matter at P11 but caused a significant reduction of MBP protein levels, normalized to β-ACTIN, in CGP treated animals at the same time point (CGP: 0.8; ± 0.03; p = 0.001; n = 7) (Figure 2d and e). At P15, in contrast, there was a significant reduction of MBP pixel intensity for both treatment groups compared to controls (DMCM: 35 ± 10; p = 0.01; CGP: 38 ± 9; p = 0.01; n = 6) (Figure 2b). The same applies to the measurement of MBP area (per field) (DMCM: 33.3 ± 10; p = 0.01; CGP: 27.31 ± 5; p = 0.007, n = 6) (Figure 2c). The findings for MBP reduction in CGP treated animals at P15 were confirmed by our Western blot results as shown in Figure 2e (CGP: 0.7 ± 0.06; p = 0.03; n = 6), however not for the DMCM group. In total, CGP treatment caused a consistent decrease, predominantly at P15, in MBP production.

3.2 | $GABA_B$ receptor antagonization increases proliferation of oligodendroglia in vivo

The oligodendroglial lineage is characterized by its progression from oligodendroglial precursor cells, pre-oligodendrocytes, immature/premyelinating to mature/myelinating oligodendrocytes (Barateiro et al., 2016; Jakovcevski et al., 2009; Miron et al., 2011). A high proliferation rate is seen in progenitor and immature stages, while proliferation is downregulated at more mature stages in favor of initiating myelin production (Barateiro et al., 2016). Immunohistochemistry was performed to assess the effects of DMCM and CGP treatment on proliferation of oligodendroglial lineage cells at P11 and P15. Rates of $PCNA^+$ (i.e. proliferating) cells were examined for all oligodendroglial cells by co-staining for OLIG2. The results indicate no significant elevation of $(OLIG2^+ + PCNA^+)/OLIG2^+$ rates at P11 in both animal groups receiving $GABA_A$ or $GABA_B$ receptor antagonist injections (Figure 3a and b. A significant increase was detected at P15 after CGP treatment in comparison to age matched controls (CGP: 0.8 ± 0.01; p = 0.002; n = 6) (Figure 3b). After antagonization with DMCM, no significant rise was observed at P15 (DMCM: 0.7 ± 0.06; p = 0.06; n = 6) (Figure 3b). However, as shown in Figure 3c, a change of total $OLIG2^+$ cell number was not detected at any time point.

3.3 | $GABA_B$ receptor antagonization increases OPC population of oligodendroglia in vivo

For further analysis of effects on cell proliferation, we performed real-time PCR for *Pdgfrα* gene expression quantification as a marker for OPCs. The results did not show expression changes of the investigated OPC marker (Figure 4c). To investigate possible effects of $GABA_B$ receptor antagonization on the OPC population, specifically, we performed immunohistochemical stainings of $PDGFR\alpha^+$ $OLIG2^+$ cells at P11 and at P15 (Figure 4a and b). As a result, there was a significant increase in the $(OLIG2^+ + PDGFR\alpha^+)/OLIG2^+$ ratio in CGP treated animals at age P15 (CGP: 0.3 ± 0.02; p = 0.007; n = 6) (Figure 4b). No significant changes in the proportion of OPCs over total $OLIG2^+$ cells were found at P11 (CGP: 0.5 ± 0.03; p = 0.18; n = 6) (Figure 4b).

3.4 | $GABA_B$ receptor antagonization shows a delay in early maturation of oligodendrocytes in vivo

Proper oligodendroglial lineage progression is essential for developmental myelination (Barateiro et al., 2016; Jakovcevski et al., 2009). To examine effects of $GABA_A$ and $GABA_B$ antagonization on oligodendroglial maturation, we performed immunostainings with CC1 as a maturation marker in these cells. Analysis of the ratio of $(OLIG2^+ +$

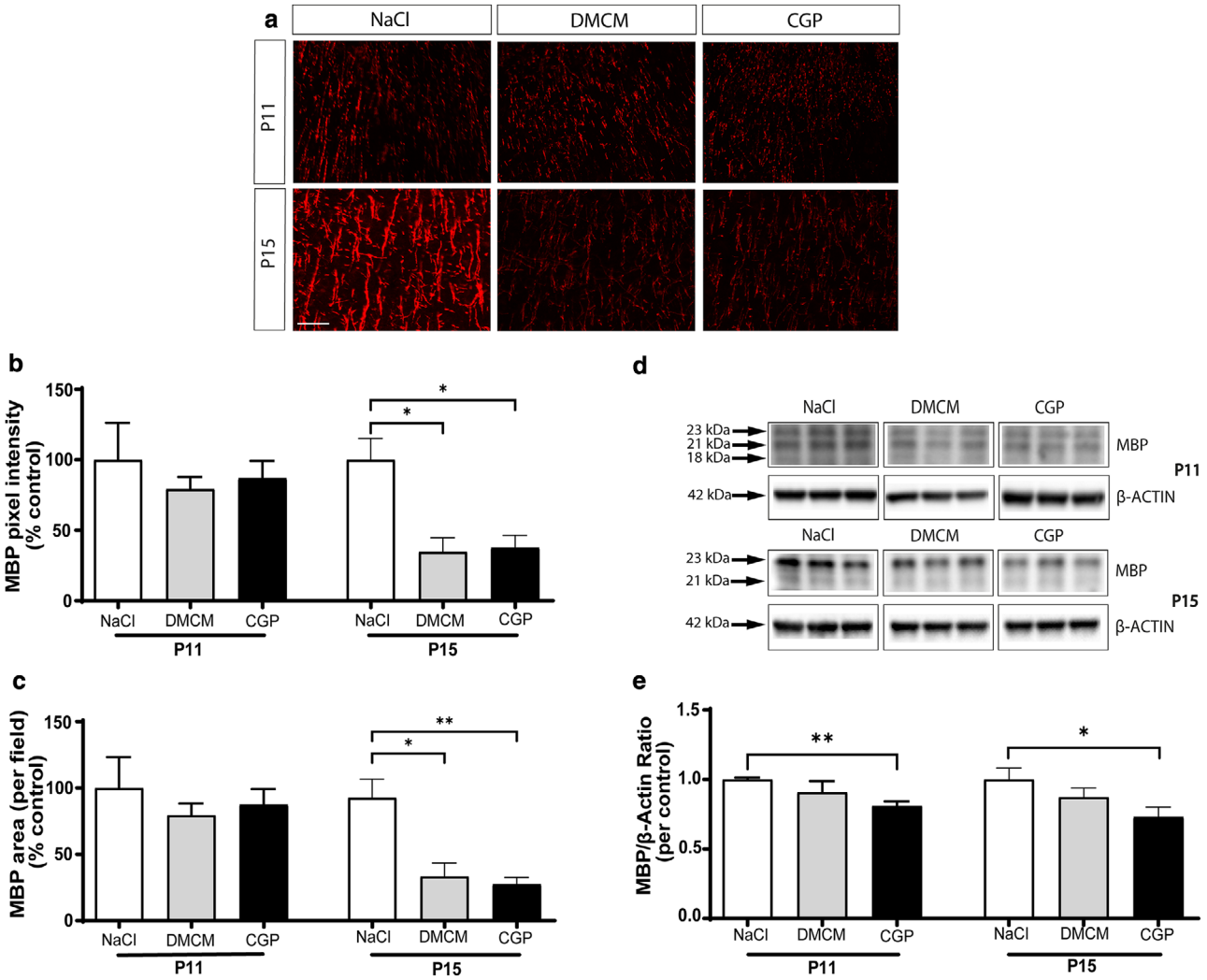

FIGURE 2 $GABA_B$ receptor antagonization reduces MBP levels in vivo. (a–c) Immunohistochemical analysis of the cingulum region shows no significant change in MBP fluorescence intensity and in area (per field), caused by $GABA_A$ and $GABA_B$ receptor antagonization at P11. At P15, however, a significant reduction of MBP intensity and area (per field) was measured in both experimental groups. (d, e) The MBP expression was quantified by Western blot analysis. A ratio of MBP and β-ACTIN was determined. After DMCM treatment, MBP expression was not significantly reduced at P11 and P15. MBP expression after CGP treatment showed a significant decrease at P11 and P15. A x10 objective lens was used for P11 and a x20 objective for P15. Scale bar = 50 μm (n = 4–7 (control), 6–7 (DMCM), 6–7 (CGP); post-hoc ANOVA test *P < 0.05, **P < 0.01 versus control group with vehicle application)

$CC1^+$)/$OLIG2^+$ cells at the ages P11 and P15 did not reveal effects of $GABA_A$ or $GABA_B$ receptor antagonization with DMCM or CGP, respectively (Figure 5a and b). For further analysis of maturation towards premyelinating and myelinating oligodendroglial stages, CNP production was quantified from brain tissue samples via Western blot analysis at both time points. As demonstrated in Figure 6a and b, the ratio of CNP and β-ACTIN protein levels showed a significant reduction (0.7 ± 0.04; $p < 0.05$; n = 7) at P11 after treatment with CGP for $GABA_B$ receptor antagonization. Treatment with DMCM did not show similar effects. At P15, no significant difference was found in any treatment group (Figure 6b). Our immunohistochemical stainings for analysis of CNP^+OLIG2^+cells (15.2 ± 2.4; p = 0.002; n = 6) (Figure 6c and d), and for measurement of CNP pixel intensity (61.0 ± 7; p = 0.015; n = 6) (Figure 6c and e) and area (per field) (59.5 ± 6; p = 0.015; n = 6) (Figure 6c and f) confirmed the findings of CNP reduction after CGP application at age P11. Taken together, there was a decrease of CNP protein production in oligodendrocytes at age P11 after $GABA_B$ receptor antagonization without changes in the ratio of $CC1^+$ oligodendrocytes.

3.5 | $GABA_A$ and $GABA_B$ antagonization does not cause apoptosis in oligodendrocytes and total cells in vivo

Stainings with cleaved caspase 3 (CASP3A) antibodies were performed at both endpoints to determine possible

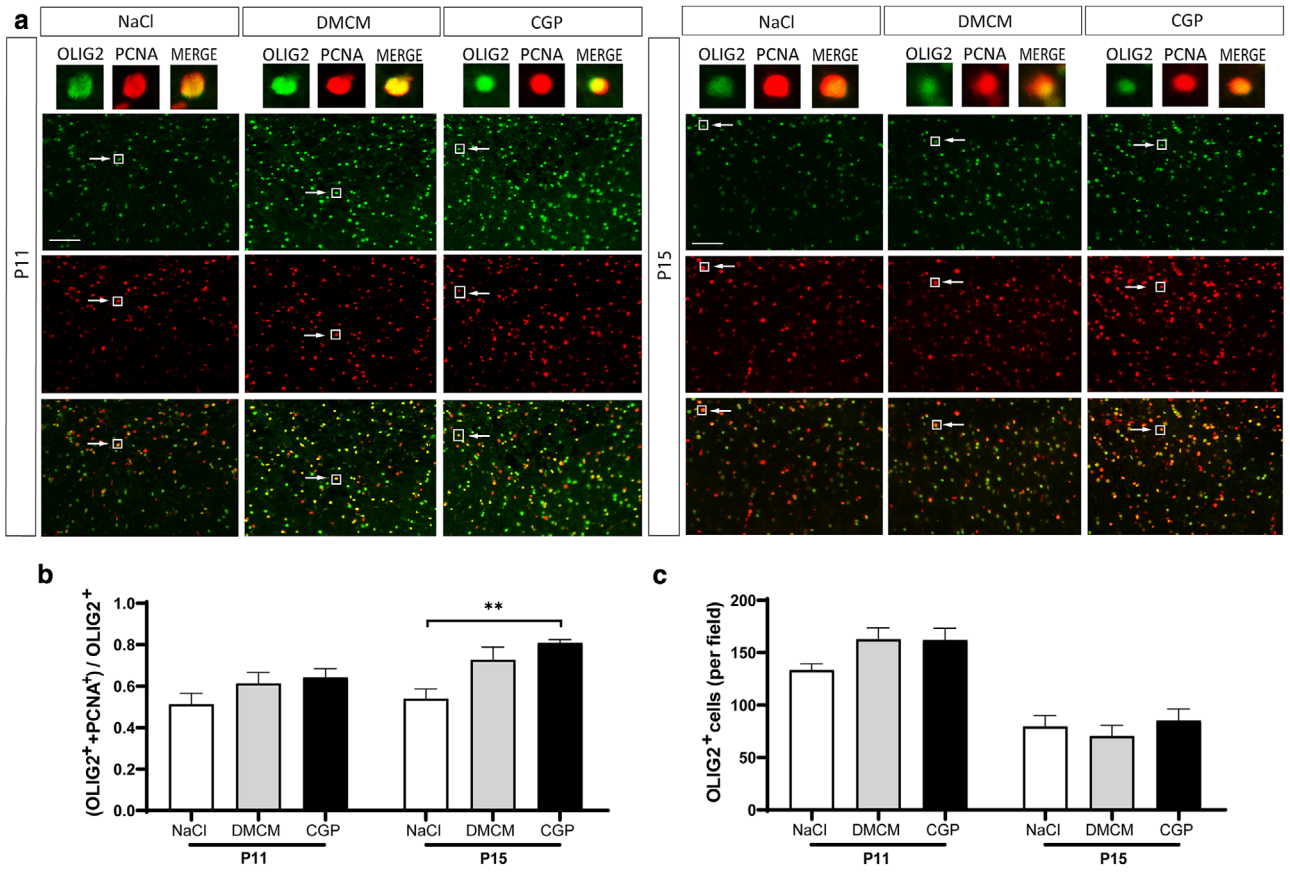

FIGURE 3 $GABA_B$ receptor antagonization increases proliferation of oligodendroglia in vivo. (a, b) Immunohistochemical analysis of the 6 μm sections of the cingulum region to label proliferating $PCNA^+$ oligodendrocytes at P11 and P15. The ratio of $PCNA^+$ oligodendrocytes showed no changes in the cingulum of rats after $GABA_A$ and $GABA_B$ antagonization at P11. A significant increase of proliferating oligodendrocytes was measured at P15, after CGP treatment, while in DMCM treated animals, no significant rise was detectable. (c) $OLIG2^+$ cells (per field) showed no significant differences compared to the control group at stage P11 and P15. A x20 objective lens was used. Scale bar = 50 μm (n = 6 (control), 6 (DMCM), 6 (CGP); post-hoc ANOVA test $^{**}P < 0.01$ versus control group with vehicle application)

alterations through $GABA_A$ and $GABA_B$ antagonization on apoptotic cell death of oligodendroglial cells as well as of all $DAPI^+$ cells in total. The results in Figure 7a and b present that there are no differences when comparing $(OLIG2^+ + CASP3A^+)/OLIG2^+$ cells of animals of both experimental groups to control rats. Similar results can be seen when looking at $(DAPI^+CASP3A^+)/DAPI^+$ cells at P11 and P15, indicating no altered apoptosis activity amongst all cells after a treatment with DMCM and CGP (data not shown).

4 | DISCUSSION

In this study, we demonstrate that $GABA_B$ receptor antagonization in newborn rats by daily systemic injections (P6-P11) of DMCM and CGP, followed by analysis at the endpoints P11 and P15, respectively, caused a significant reduction of MBP production during brain development. In addition, our experiments revealed a significant increase of proliferating oligodendroglial cells in CGP treated rats. Remarkably, both events were most pronounced during further development until P15. Our treatment with DMCM and CGP did not influence the numbers of mature $CC1^+$ oligodendroglia. However, solely after CGP application, a maturational delay in early stages of oligodendrogenesis was observed by a decrease in CNP^+ oligodendrocytes (P11) and at P15, an increased number of $PDGFR\alpha^+$ oligodendrocyte progenitor cells. Antagonization of GABA receptors did not lead to changes in oligodendroglial apoptosis or total cell numbers of oligodendroglial lineage cells of the cingulum.

These findings expand our view on the role of GABA receptors on oligodendroglial proliferation, and maturation, as well as on myelination of the immature brain. In our rat experiments, the impact of $GABA_B$ receptor antagonization on myelination are in accordance with the findings presented by Serrano-Regal et al. (Serrano-Regal et al., 2020). The same applies to results of $GABA_A$ receptor antagonization, which showed no significant changes in myelin sheath development (Serrano-Regal et al., 2020). These results,

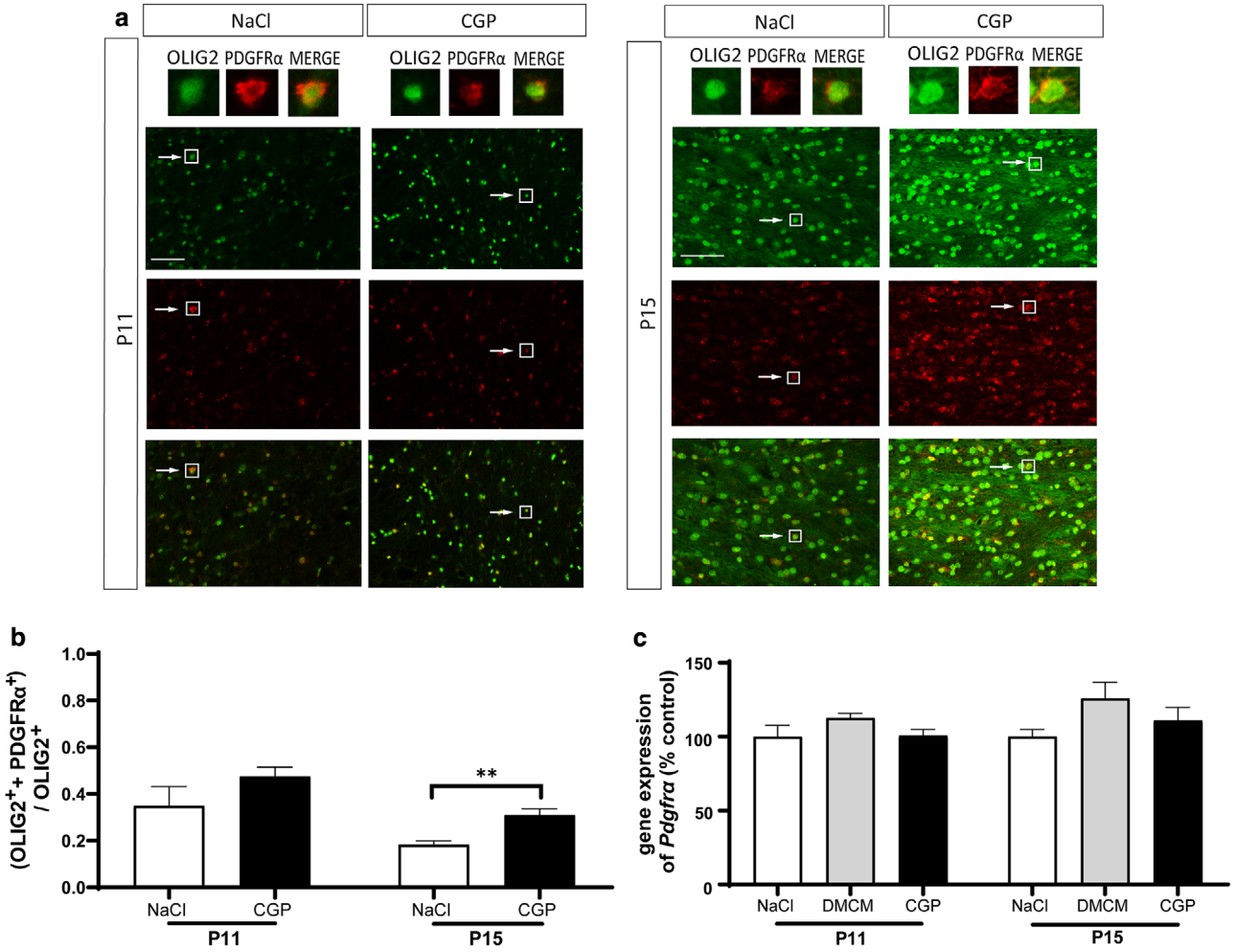

FIGURE 4 $GABA_B$ receptor antagonization increases OPC population of oligodendroglia in vivo. (a, b) Immunohistochemical analysis of the 6 μm sections of the cingulum region to label PDGFRα^+ oligodendrocytes of CGP treated animals at P11 and P15. The ratio of PDGFRα^+ oligodendroglial cells showed no changes after $GABA_B$ antagonization with CGP treatment at age P11, while at age P15, after four days recovery, a significant increase was measured. (c) Real-time PCR to measure the gene expression of *Pdgfrα* at both time points. No significant differences were found in the experimental groups at any time. A x20 objective lens was used. Scale bar = 50 μm (n = 6 (control), 6 (DMCM), 6 (CGP); unpaired t-test for (b), post-hoc ANOVA test for (c), **P < 0.01 versus control group with vehicle application)

after inhibition of $GABA_A$ receptors, are incoherent with observations made by Hamilton et al., who presented an increase of myelination of rodent brain slice cultures after treatment with the antagonist GABAzine (Hamilton et al., 2017). A possible explanation for this inconsistency is delivered by Jakovcevski et al. (Jakovcevski et al., 2009), who in their study described an increase in the number of MBP^+ oligodendrocytes in human fetal slice cultures of the forebrain, in comparison to frozen sections, and explained it, on the one hand, with the possible absence of myelin inhibitors, such as polysialylated neuronal cell adhesion molecule (PSA-NCAM), and, on the other hand, with the stimulation of myelination caused by the culture medium itself (Jakovcevski & Zecevic, 2005; Jakovcevski et al., 2009; Jakovcevski et al., 2007).

Our data show that the proliferation rate of overall $OLIG2^+$ oligodendroglial cells is increased after $GABA_B$ antagonization at P15, i.e., four days of recovery time after final pharmacological treatment at P11. However, numbers of overall $OLIG2^+$ cells did not differ after $GABA_B$ receptor antagonization. Apparently, the effect of higher proliferation accounts for a subset of oligodendroglial lineage cells but does not enhance the number of the whole $OLIG2^+$ cell population in a statistically relevant manner. To specify cell numbers for progenitor stages, we further determined PDGFRα^+ OPC numbers in animals after $GABA_B$ receptor antagonization which demonstrated a significant increase in the defined OPC population at age P15 in comparison to controls without antagonist treatment. In contrast to these results after $GABA_B$ receptor antagonization, no changes in the proliferation of

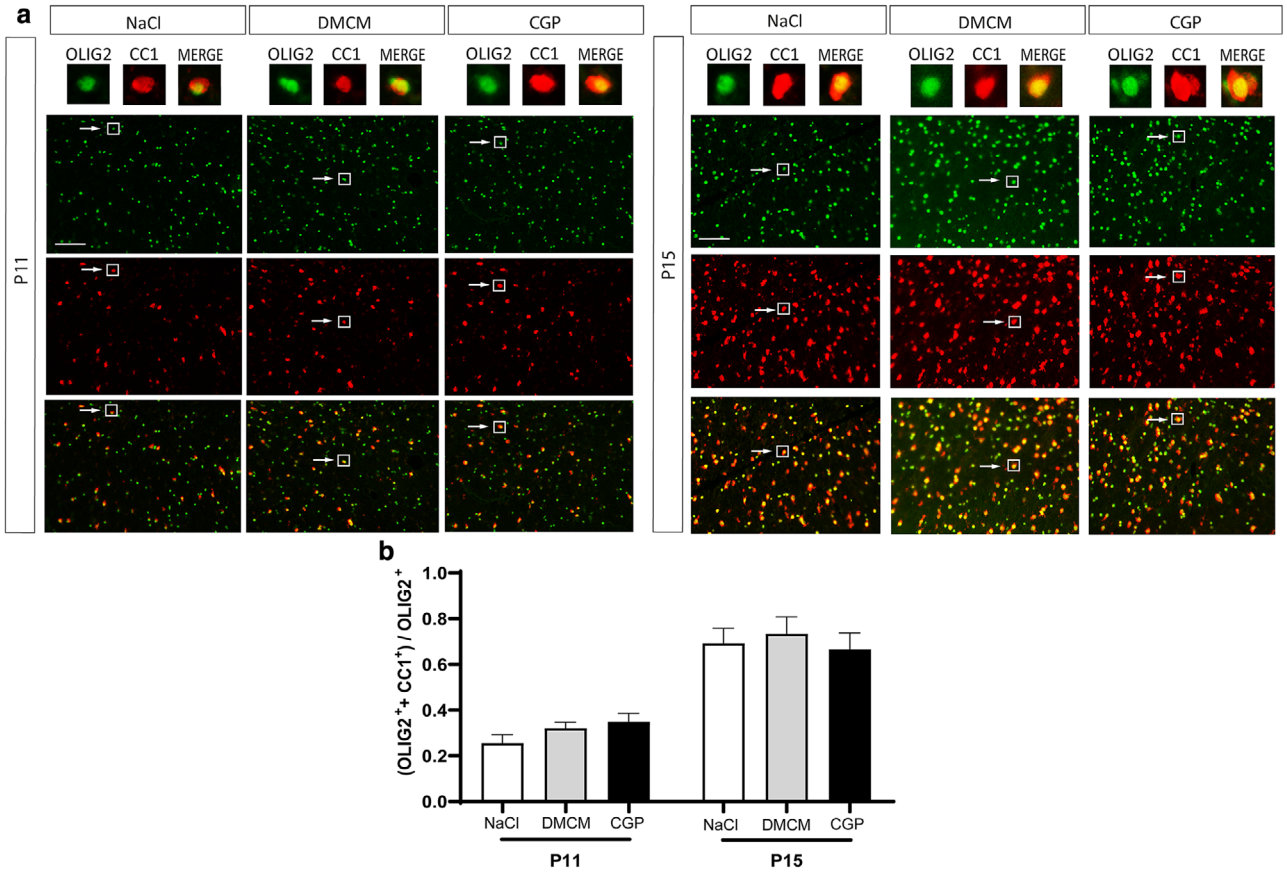

FIGURE 5 $GABA_B$ receptor antagonization shows no changes in maturation of $CC1^+$ oligodendrocytes in vivo. (a,b) Immunohistochemical analysis of the 6μm sections of the cingulum region to label mature $CC1^+$ oligodendrocytes at P11 and P15. No significant differences in the number of mature $CC1^+$ oligodendrocytes were observed in the cingulum of rats after $GABA_A$ and $GABA_B$ antagonization at P11 and P15 in comparison to the control pups. A x20 objective lens was used. Scale bar = 50 μm (n = 6 (control), 6 (DMCM), 6 (CGP); post-hoc ANOVA test *P < 0.05 versus control group with vehicle application)

oligodendroglial lineage cells and no alterations of *Pdgfrα* expression were found in our analysis after $GABA_A$ receptor antagonization. Hence, our findings after $GABA_A$ receptor antagonization differ to those from Zonouzi et al. who found an increased proliferation in OPCs in the mouse cerebellum after application of the $GABA_A$ receptor antagonist bicuculline (Zonouzi et al., 2015). Interestingly, the different subregions of the brain, i.e., cerebellum in the Zonouzi study versus cerebrum in our study, seem to coincide with different cellular responses to the blocking of $GABA_A$ receptor. It cannot be excluded, however, that the variation may be caused by the different species of rats and mice. Hamilton et al. likewise discovered an increase in proliferation after $GABA_A$ receptor blockade in cerebral mouse cortex slices (Hamilton et al., 2017). In theory, the absence of proliferative changes at P11, together with the clear enhancement of proliferation activity in OPCs at P15, suggests that GABA receptor antagonization may not have a direct effect on the proliferation of oligodendroglial lineage cells. Instead, the increased proliferation may occur as a late compensative response to the earlier myelin deficits at P11 after administration of the final injection. Since GABA receptor inhibition also applies to interneuronal GABA receptors, it has to be discussed that GABA receptor antagonization may exert effects on interneurons that impair interneuronal-oligodendroglial interactions relevant for oligodendroglial maturation and myelin production.

The altered maturation of oligodendroglial cells after CGP application at P11 documented by the temporary reduction of CNP levels in Western blots as well as by the decrease of CNP^+OLIG2^+ cells in the immunohistochemical stainings, seems to recover thereafter as indicated by CNP protein expression returning to control levels at P15. In contrast, $GABA_A$ receptor antagonization caused no effects on oligodendroglial maturation. The numbers of mature $CC1^+$ oligodendrocytes were not altered by $GABA_A$ and $GABA_B$ antagonization at any given time point. Since the expression of CNP is initiated in earlier stages of oligodendrogenesis than in the case of CC1 expression (Cai et al., 2010), our data could indicate that maturation is affected mainly in early oligodendroglial stages, i.e. during transition from OPCs to immature oligodendroglial stages. It has to be considered that CNP

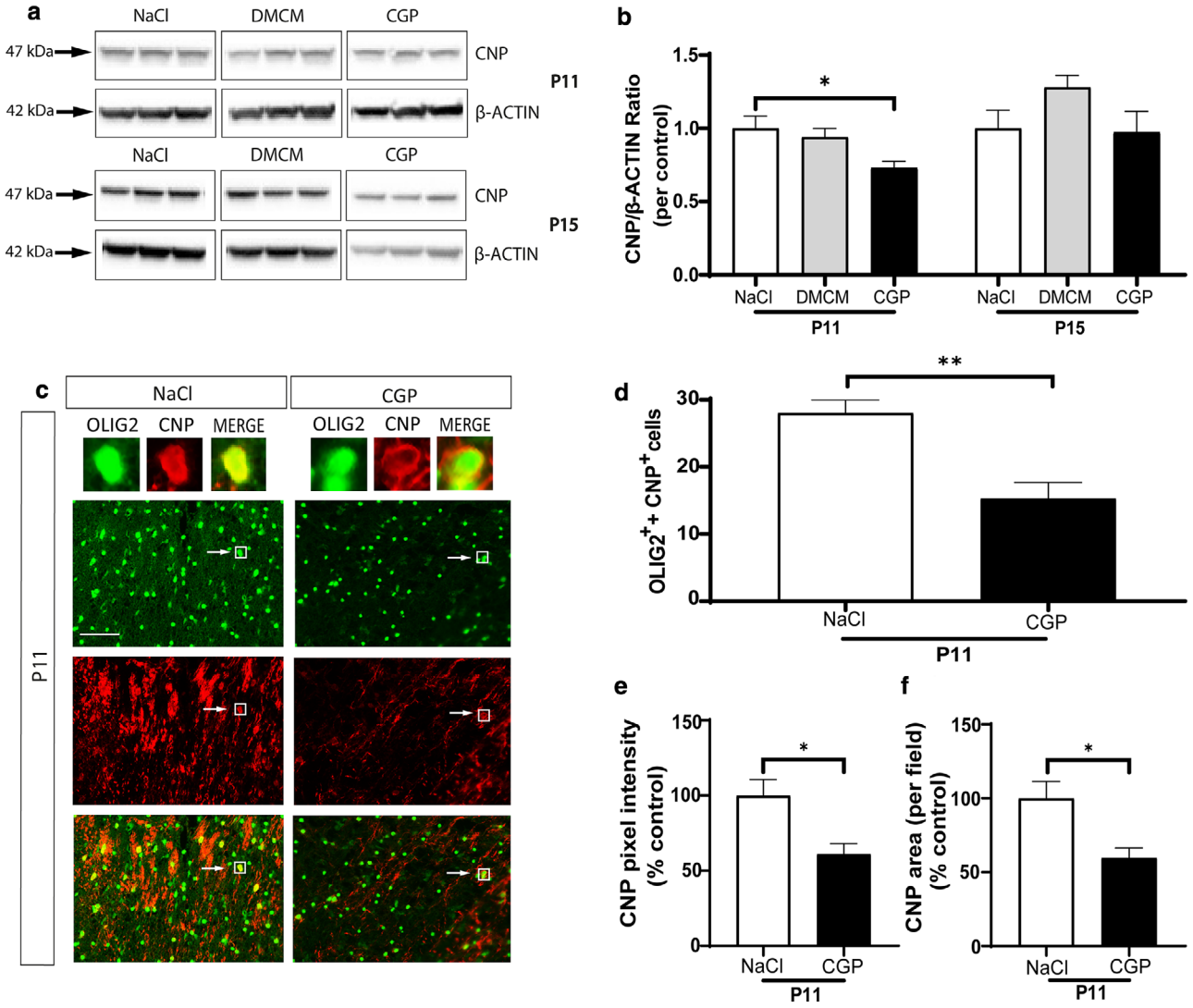

FIGURE 6 $GABA_B$ receptor antagonization shows a delay in early maturation of CNP^+ oligodendrocytes in vivo. (a, b) The CNP expression was quantified by Western blot analysis. A ratio of CNP and β-ACTIN was determined. After DMCM treatment, no significant difference could be measured at both time points. After CGP treatment, however, CNP showed a decrease at P11 and normalized again at P15. (c–f) Immunohistochemical analysis of the 6μm sections of the cingulum region to label mature CNP^+ oligodendrocytes of CGP treated animals at P11. The number of CNP^+ oligodendroglial cells shows a significant decrease after $GABA_B$ receptor antagonization at P11. Likewise, a significant reduction of CNP fluorescence intensity (in % control) and CNP area (per field) was measured. A x20 objective lens was used. Scale bar = 50 μm (n = 6–7 (control), 6–7 (DMCM), 6–7 (CGP); post-hoc ANOVA test (b), unpaired t-test for (d,e,f) *P < 0.05, **P < 0.01 versus control group with vehicle application)

belongs to the group of myelin associated proteins and plays a major function in processes that lead up to the formation of myelin sheaths (Gravel et al., 1996). Its altered protein expression after CGP treatment could therefore implicate a damage in the initiation of myelination. In contrast to our findings, Zonouzi et al. reported a reduction of $CC1^+$ cells after bicuculline application (Zonouzi et al., 2015) while Hamilton et al. described GABA as a neurotransmitter that causes a decrease in mature oligodendrocytes via its $GABA_A$ receptors, based on experiments with GABAzine (Hamilton et al., 2017). This discrepancy remains to be explained but may result from the use of different $GABA_A$ receptor antagonists as well as the varying experimental focus on either cerebrum or cerebellum.

As stated by Kessaris et al. and Sun et al., apoptotic cell death is an important regulator of the oligodendroglia cell pool and can be regarded as a tool of homeostasis between the different cell stages (Habermacher et al., 2019; Kessaris et al., 2006; Sun et al., 2018). Hamilton et al., for example, described $GABA_A$ receptor signaling as a stimulator of oligodendroglial lineage cell death (Hamilton et al., 2017). In our experiments, however, apoptosis was not regulated in response to altered GABA receptor activity. Generally, it needs to be pointed out that the results of our in vivo GABA receptor antagonization

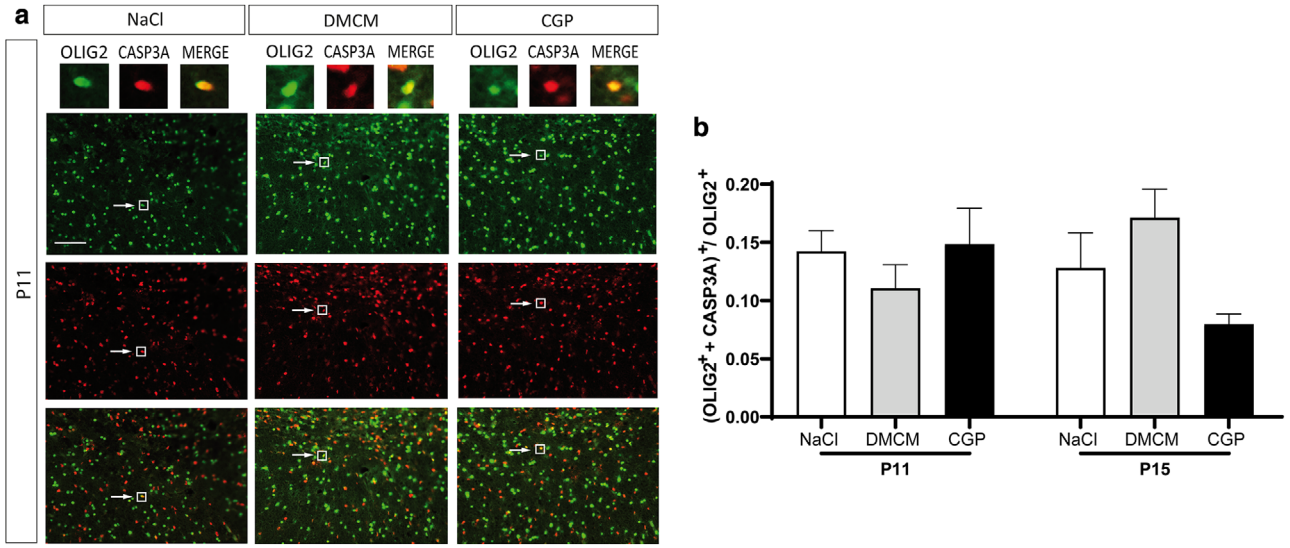

FIGURE 7 $GABA_A$ and $GABA_B$ receptor antagonization does not cause apoptosis of oligodendrocytes and total cells in vivo. (a, b) Immunohistochemical analysis of the 6 μm sections of the cingulum region to label $CASP3A^+$ oligodendrocytes at P11 and P15. Cell numbers of apoptotic oligodendrocytes showed no significant changes in the cingulum of rats after $GABA_A$ and $GABA_B$ antagonization at P11 and P15. A x20 objective lens was used. Scale bar = 50 μm (n = 6 (control), 6 (DMCM), 6 (CGP); post-hoc ANOVA test versus control group with vehicle application)

may be attributable to neuronal and/or glial receptors. Also when considering that the excitatory to inhibitory shift of GABA in the cortex of rats takes place in the first two postnatal weeks (Ben-Ari et al., 2007), the downregulated myelination, after our antagonization, may be a result of impaired neuronal stimuli and axon-glia-connection (Barateiro et al., 2016; Gibson et al., 2014). Therefore, changes in neuronal-oligodendroglial interplay are relevant for the cortical myelination process and are worth to be studied in the future.

In conclusion, our data in an in vivo rat model demonstrate that GABA and its type B receptors are of high relevance for oligodendroglial maturation and myelin production in the cingulum of the developing brain. In addition to the knowledge we have about GABA agonists, this study can lay the ground for a treatment with GABA in the field of neonatology to foster an adequate oligodendrogenesis and proper early myelination.

ACKNOWLEDGEMENTS

This research was funded by the Deutsche Forschungsgemeinschaft (grant number: Sche 2078/2-1), the *Förderverein für frühgeborene Kinder an der Charité e.V.* and the *Lydia Rabinowitsch Foundation*.

Also, we thank Mrs. Evelyn Strauss and Mrs. Ruth Herrmann for their support with paraffin sections and Western blots.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

ORCID

Samipa Pudasaini https://orcid.org/0000-0001-9123-5080
Till Scheuer https://orcid.org/0000-0002-9465-7200

REFERENCES

Arellano, R. O., Sanchez-Gomez, M. V., Alberdi, E., Canedo-Antelo, M., Chara, J. C., Palomino, A., Pérez-Samartín, A., & Matute, C. (2016). Axon-to-glia interaction regulates GABAA receptor expression in oligodendrocytes. *Molecular Pharmacology*, *89*(1), 63–74. https://doi.org/10.1124/mol.115.100594

Back, S. A. (2006). Perinatal white matter injury: The changing spectrum of pathology and emerging insights into pathogenetic mechanisms. *Mental Retardation and Developmental Disabilities Research Reviews*, *12*(2), 129–140. https://doi.org/10.1002/mrdd.20107

Balia, M., Benamer, N., & Angulo, M. C. (2017). A specific GABAergic synapse onto oligodendrocyte precursors does not regulate cortical oligodendrogenesis. *Glia*, *65*(11), 1821–1832. https://doi.org/10.1002/glia.23197

Balia, M., Velez-Fort, M., Passlick, S., Schafer, C., Audinat, E., Steinhauser, C., Seifert, G., & Angulo, M. C. (2015). Postnatal down-regulation of the GABAA receptor gamma2 subunit in neocortical NG2 cells accompanies synaptic-to-extrasynaptic switch in the GABAergic transmission mode. *Cerebral Cortex*, *25*(4), 1114–1123. https://doi.org/10.1093/cercor/bht309

Barateiro, A., Brites, D., & Fernandes, A. (2016). Oligodendrocyte development and myelination in neurodevelopment: Molecular

mechanisms in health and disease. *Current Pharmaceutical Design*, *22*(6), 656–679. https://doi.org/10.2174/1381612822666151204000636

Barbin, G., Aigrot, M. S., Charles, P., Foucher, A., Grumet, M., Schachner, M., Zalc, B., & Lubetzki, C. (2004). Axonal cell-adhesion molecule L1 in CNS myelination. *Neuron Glia Biology*, *1*(1), 65–72. https://doi.org/10.1017/S1740925%D7;04000092

Baumann, N., & Pham-Dinh, D. (2001). Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiological Reviews*, *81*(2), 871–927. https://doi.org/10.1152/physrev.2001.81.2.871

Ben-Ari, Y. (2002). Excitatory actions of gaba during development: The nature of the nurture. *Nature Reviews Neuroscience*, *3*(9), 728–739. https://doi.org/10.1038/nrn920

Ben-Ari, Y., Gaiarsa, J. L., Tyzio, R., & Khazipov, R. (2007). GABA: A pioneer transmitter that excites immature neurons and generates primitive oscillations. *Physiological Reviews*, *87*(4), 1215–1284. https://doi.org/10.1152/physrev.00017.2006

Cai, J., Zhu, Q., Zheng, K., Li, H., Qi, Y., Cao, Q., & Qiu, M. (2010). Co-localization of Nkx6.2 and Nkx2.2 homeodomain proteins in differentiated myelinating oligodendrocytes. *Glia*, *58*(4), 458–468. https://doi.org/10.1002/glia.20937

Ganguly, K., Schinder, A. F., Wong, S. T., & Poo, M. (2001). GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. *Cell*, *105*(4), 521–532. https://doi.org/10.1016/s0092-8674(01)00341-5

Gibson, E. M., Purger, D., Mount, C. W., Goldstein, A. K., Lin, G. L., Wood, L. S., Inema, I., Miller, S. E., Bieri, G., Zuchero, J. B., Barres, B. A., Woo, P. J., Vogel, H., & Monje, M. (2014). Neuronal activity promotes oligodendrogenesis and adaptive myelination in the mammalian brain. *Science*, *344*(6183), 1252304. https://doi.org/10.1126/science.1252304

Gilbert, P., Kettenmann, H., & Schachner, M. (1984). gamma-Aminobutyric acid directly depolarizes cultured oligodendrocytes. *Journal of Neuroscience*, *4*(2), 561–569. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/6366154

Gravel, M., Peterson, J., Yong, V. W., Kottis, V., Trapp, B., & Braun, P. E. (1996). Overexpression of 2',3'-cyclic nucleotide 3'-phosphodiesterase in transgenic mice alters oligodendrocyte development and produces aberrant myelination. *Molecular and Cellular Neuroscience*, *7*(6), 453–466. https://doi.org/10.1006/mcne.1996.0033

Habermacher, C., Angulo, M. C., & Benamer, N. (2019). Glutamate versus GABA in neuron-oligodendroglia communication. *Glia*, *67*(11), 2092–2106. https://doi.org/10.1002/glia.23618

Hamilton, N. B., Clarke, L. E., Arancibia-Carcamo, I. L., Kougioumtzidou, E., Matthey, M., Káradóttir, R., & … Attwell, D. (2017). Endogenous GABA controls oligodendrocyte lineage cell number, myelination, and CNS internode length. *Glia*, *65*(2), 309–321. https://doi.org/10.1002/glia.23093

Jakovcevski, I., Filipovic, R., Mo, Z., Rakic, S., & Zecevic, N. (2009). Oligodendrocyte development and the onset of myelination in the human fetal brain. *Frontiers in Neuroanatomy*, *3*, 5. https://doi.org/10.3389/neuro.05.005.2009

Jakovcevski, I., Mo, Z., & Zecevic, N. (2007). Down-regulation of the axonal polysialic acid-neural cell adhesion molecule expression coincides with the onset of myelination in the human fetal forebrain. *Neuroscience*, *149*(2), 328–337. https://doi.org/10.1016/j.neuroscience.2007.07.044

Jakovcevski, I., & Zecevic, N. (2005). Sequence of oligodendrocyte development in the human fetal telencephalon. *Glia*, *49*(4), 480–491. https://doi.org/10.1002/glia.20134

Káradóttir, R., & Attwell, D. (2007). Neurotransmitter receptors in the life and death of oligodendrocytes. *Neuroscience*, *145*(4-5), 1426–1438. https://doi.org/10.1016/j.neuroscience.2006.08.070

Kessaris, N., Fogarty, M., Iannarelli, P., Grist, M., Wegner, M., & Richardson, W. D. (2006). Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. *Nature Neuroscience*, *9*(2), 173–179. https://doi.org/10.1038/nn1620

Kirchhoff, F., & Kettenmann, H. (1992). GABA triggers a [Ca2+]i increase in murine precursor cells of the oligodendrocyte lineage. *European Journal of Neuroscience*, *4*(11), 1049–1058. https://doi.org/10.1111/j.1460-9568.1992.tb00131.x

Kulick, C., Gutherz, S., Kondratyev, A., & Forcelli, P. A. (2014). Ontogenic profile of seizures evoked by the beta-carboline DMCM (methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate) in rats. *European Journal of Pharmacology*, *740*, 662–668. https://doi.org/10.1016/j.ejphar.2014.06.012

Leinekugel, X., Khalilov, I., McLean, H., Caillard, O., Gaiarsa, J. L., Ben-Ari, Y., & Khazipov, R. (1999). GABA is the principal fast-acting excitatory transmitter in the neonatal brain. *Advances in Neurology*, *79*, 189–201. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10514814

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.)*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Luyt, K., Slade, T. P., Dorward, J. J., Durant, C. F., Wu, Y., Shigemoto, R., Mundell, S. J., Váradi, A., & Molnar, E. (2007). Developing oligodendrocytes express functional GABA(B) receptors that stimulate cell proliferation and migration. *Journal of Neurochemistry*, *100*(3), 822–840. https://doi.org/10.1111/j.1471-4159.2006.04255.x

Mi, S., Miller, R. H., Lee, X., Scott, M. L., Shulag-Morskaya, S., Shao, Z., Chang, J., Thill, G., Levesque, M., Zhang, M., Hession, C., Sah, D., Trapp, B., He, Z., Jung, V., McCoy, J. M., & Pepinsky, R. B. (2005). LINGO-1 negatively regulates myelination by oligodendrocytes. *Nature Neuroscience*, *8*(6), 745–751. https://doi.org/10.1038/nn1460

Miron, V. E., Kuhlmann, T., & Antel, J. P. (2011). Cells of the oligodendroglial lineage, myelination, and remyelination. *Biochimica Et Biophysica Acta*, *1812*(2), 184–193. https://doi.org/10.1016/j.bbadis.2010.09.010

Serrano-Regal, M. P., Bayon-Cordero, L., Ordaz, R. P., Garay, E., Limon, A., Arellano, R. O., Matute, C., & Sanchez-Gomez, M. V. (2020). Expression and function of GABA receptors in myelinating cells. *Frontiers in Cellular Neuroscience*, *14*, 256. https://doi.org/10.3389/fncel.2020.00256

Serrano-Regal, M. P., Luengas-Escuza, I., Bayon-Cordero, L., Ibarra-Aizpurua, N., Alberdi, E., Perez-Samartin, A., Matute, C., & Sanchez-Gomez, M. V. (2020). Oligodendrocyte differentiation and myelination is potentiated via GABAB receptor activation. *Neuroscience*, *439*, 163–180. https://doi.org/10.1016/j.neuroscience.2019.07.014

Steinhauser, C., Jabs, R., & Kettenmann, H. (1994). Properties of GABA and glutamate responses in identified glial cells of the mouse hippocampal slice. *Hippocampus*, *4*(1), 19–35. https://doi.org/10.1002/hipo.450040105

Sun, L. O., Mulinyawe, S. B., Collins, H. Y., Ibrahim, A., Li, Q., Simon, D. J., Tessier-Lavigne, M., & Barres, B. A. (2018). Spatiotemporal control of CNS myelination by oligodendrocyte programmed cell death through the TFEB-PUMA axis. *Cell*, *175*(7), 1811–1826.e21. https://doi.org/10.1016/j.cell.2018.10.044

Tong, X. P., Li, X. Y., Zhou, B., Shen, W., Zhang, Z. J., Xu, T. L., & Duan, S. (2009). Ca(2+) signaling evoked by activation of Na(+) channels and Na(+)/Ca(2+) exchangers is required for GABA-induced NG2 cell migration. *Journal of Cell Biology*, *186*(1), 113–128. https://doi.org/10.1083/jcb.200811071

Tyagi, R. K., Bisht, R., Pant, J., Kumar, P., Majeed, A. B., & Prakash, A. (2015). Possible role of GABA-B receptor modulation in MPTP induced Parkinson's disease in rats. *Experimental and Toxicologic Pathology*, *67*(2), 211–217. https://doi.org/10.1016/j.etp.2014.12.001

Vogt, K. (2015). Diversity in GABAergic signaling. *Advances in Pharmacology*, *73*, 203–222. https://doi.org/10.1016/bs.apha.2014.11.009

Zonouzi, M., Scafidi, J., Li, P., McEllin, B., Edwards, J., Dupree, J. L., Harvey, L., Sun, D., Hübner, C. A., Cull-Candy, S. G., Farrant, M., Gallo, V., & Gallo, V. (2015). GABAergic regulation of cerebellar NG2-cell development is altered in perinatal white matter injury. *Nature Neuroscience*, *18*(5), 674–682. https://doi.org/10.1038/nn.3990

How to cite this article: Pudasaini, S., Friedrich, V., Bührer, C., Endesfelder, S., Scheuer, T., & Schmitz, T. (2022). Postnatal myelination of the immature rat cingulum is regulated by $GABA_B$ receptor activity. *Developmental Neurobiology*, *82,* 16–28. https://doi.org/10.1002/dneu.22853