Chapter

6

Tissue Transglutaminase activity is involved in the differentiation of oligodendrocyte precursor cells into myelin-forming oligodendrocytes during CNS (re)myelination

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Abstract

During normal brain development axons are myelinated by mature oligodendrocytes (OLGs). Under pathological, demyelinating conditions within the CNS, axonal remyelination is only partially successful as oligodendrocyte precursor cells (OPCs) largely remain in an undifferentiated state resulting in a failure to generate myelinating OLGs. Tissue Transglutaminase (TG2) is a multifunctional enzyme, which amongst other functions, is involved in cell differentiation. Therefore, we hypothesized that TG2 contributes to differentiation of OPCs into OLGs and thereby stimulates (re)myelination. Rat OPCs in vitro expressed TG2 protein and activity which reduces when the cells have matured into OLGs. Interestingly, TG2 immunoreactivity is also present in OPCs in developing human cerebellum. Furthermore, when TG2 activity is pharmacologically inhibited in vitro, the development of OPCs into myelin forming OLGs is dramatically reduced. Subsequent in vivo studies, using the cuprizone model for de- and remyelination in $TG2^{-/-}$ and wild-type mice, showed that during remyelination expression of proteolipid protein in the corpus callosum lags behind in the $TG2^{-/-}$ mice resulting in less myelin formation and, moreover, impaired recovery of motor behavior. We conclude that TG2 plays a prominent role in OPC differentiation into myelin forming OLGs. Therefore, manipulating TG2 activity may represent an interesting new target for remyelination in demyelinating diseases.

Introduction

Oligodendrocytes (OLGs) are the myelinating cells of the central nervous system (CNS). They exhibit processes that contact and wrap around axons to facilitate salutatory axonal conduction. Differentiation of oligodendrocyte precursor cells (OPCs), the oligodendrocyte-type 2 astrocyte (O2A) progenitors, into mature OLGs is a prerequisite for myelin production.[291] During embryonic brain development, but also in the adult brain, there is a large pool of OPCs present which implies an endogenous capacity for normal myelination, but also remyelination after insults or demyelinating diseases.[64] Remyelination of demyelinated axons in the central nervous system (CNS) occurs when OPCs proliferate, migrate to the site of damage, locally differentiate into mature OLGs and finally produce new myelin sheets that wrap the naked axon.[118] However, under demyelinating conditions within the CNS, such as Multiple Sclerosis (MS) and traumatic spinal cord- and/or brain injury, remyelination does occur but is often incomplete[119] and gives rise to so-called shadow plaques.[24] Moreover, recent evidence indicates that remyelination is most prominent in early MS lesions whereas the remyelination capacity deteriorates when the disease turns into a chronic phase.[133] This impaired remyelination is identified by the accumulation of OPCs that remain in an undifferentiated stage resulting in a failure to generate myelinating OLGs.[133] Extracellular matrix proteins, particularly fibronectin, can be considered as another important factor in the non-permissive nature for repair in demyelinated CNS lesions. It has been shown that fibronectin prevents proper formation of myelin sheets by mature oligodendrocytes.[342] Alternatively, impaired proliferation and differentiation of OPCs into myelin producing OLGs can be limiting for remyelination.[118] Indeed, growth factors such as PDGF-A, FGF-2, IGF-1 and TGFβ have been identified within the adult CNS as important factors in the regulation of OPC proliferation and differentiation.[120] Administration of these growth factors has however only been partly successful in preclinical studies of remyelination.[52, 200] Therefore, it remains of utmost importance to identify novel factors that are involved in the differentiation of OPCs into mature myelin-forming OLGs.

Tissue Transglutaminase (TG2) is one of such factors that we propose to contribute to OPC differentiation and subsequent (re)myelination. TG2 is a 78-kD multifunctional enzyme that belongs to a multigene family of Ca^{2+}-dependent protein cross-linking enzymes.[222] TG2 has various functions, amongst which is stimulation of cell differentiation. In this context, TG2 has been shown to stimulate differentiation of a number of cell types, including neurons, astrocytes and fibroblasts.[20, 56, 377] During differentiation of cells including OPCs, activated RhoA GTPase plays an important role in triggering intracellular pathways ultimately leading to essential morphological changes.[79] It is of interest to note that TG2 has been shown to enhance RhoA activity.[341] Based on these observations, we hypothesize that TG2 plays a prominent role in the differentiation of OPCs into myelin-forming OLGs. To this end, we first identified the presence of TG2 in rat OPCs *in vitro* as well as in developing human cerebellum. Subsequently, we investigated the role of TG2 in the differentiation of OPCs into myelin-forming OLGs *in vitro* and the link to RhoA activity. Finally, we studied the involvement of TG2 in the remyelination process of cuprizone-treated wild-type and $TG2^{-/-}$ mice, concomitant with the effect on motor behavior and mitochondrial activity in these mice.

Materials and Methods

Cell culture

Primary OLGs were cultured as described previously.[229] Briefly, the frontal cortex of newborn Wistar rats (day 1 or 2, Harlan, Horst, The Netherlands) were dissected and homogenized in minimal essential medium (MEM, Sigma, St. Louis, MO, USA) containing 30 U/ml papain (Worthington, Lakewood, NJ, USA), 0.24 mg/ml cysteine (Sigma), and 40 μg/ml DNAase I type IV (Sigma). Supernatant was then removed, 1 ml of ovomucoid trypsin inhibitor solution (OVO, containing 1 mg/ml trypsin inhibitor) (Boehringer-Mannheim, The Netherlands), 50 μg/ml BSA and 40 μg/ml DNase was added. The cells were homogenized by aspirating 20 times with a 1 ml pipette. Cells were centrifuged (5 min at 1,000 rpm) and the homogenate was cultured on poly L-lysine-coated (5 μg/ml; Sigma) 75 cm^2 cell culture flasks (Nalge Nunc, Naperville, IL) in DMEM (Gibco BRL, Paisley, UK) containing 10% FCS, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL). OPCs grown on top of a layer of astrocytes were collected by shake-off (pre-shake of 1 h at 180 rpm to remove microglia, then 20 h at 240 rpm to shake off O2A cells) and further purified by differential adhesion. OPCs were cultured on PLL-coated 75 cm^2 culture flasks in a defined SATO medium,[54] in the presence of PDGF-AA (10 ng/ml) and FGF-2 (10 ng/ml) for 2 days (PeproTech, Rocky Hill, NJ, USA) for synchronization of the precursor cells (oligodendrocyte type-2 astrocyte, O2A stage). The purity of the enriched OPCs cultures was at least 97%. OLG differentiation was started by removing the growth factors and culturing the cells in SATO medium containing 0.5% FCS. Cells will differentiate into galactosylceramide expressing cells (GC-stage; day 3), myelin producing cells (expressing myelin basic protein, MBP stage; day 7) and finally into MBP+ stage (forming myelin sheets; day 10).

From the cultured cells, TG2 mRNA, protein, and activity levels were determined at different stages of OLG development. To this end, cells were collected at different time points after the onset of differentiation, i.e. collected at OPC stage (day 0), GC stage (day 3) and MBP stage (day 7) and stored at -80^{0}C or directly used for immunocytochemical analysis. Subsequently, the role of TG2 during OLG development was determined. For that purpose, an irreversible inhibitor of TG2 activity (0.05 mM KCC009[422] in 0.1% DMSO) or a competitive inhibitor of TG activity (0.025 mM cystamine[86] in 0.1% PBS) were added at various stages of OLG development: at the OPC stage (O2A; day 0), at the differentiating OLG stage (GC; day 3) or at the myelination stage (MBP; day 7) and replenished with every medium change (every 3-4 days). When the cells reached the MBP+ stage (day 10), they were directly used for immunofluorescent analysis.

Quantitative RT- PCR

For quantitative RT-PCR, 1x10^6 cells were homogenized in Trizol reagent (Invitrogen, Carlsbad, USA) and total RNA was isolated as described by the manufacturer. RNA concentration and purity was determined by measuring the absorbance at 260 nm and 280 nm in a microtiter plate reader (Spectramax 250, Molecular Devices). One μl of RNA was reverse transcribed into cDNA using the Reverse Transcription System (Promega, Madison, WI, USA) with oligo-dT primers and AMV enzyme, according to the manufacturer's instructions. The RT reaction was carried out at 42^{0}C for 30 min, followed

by deactivation of the enzyme at 95^{0}C for 5 min and 4^{0}C for 5 min. For the PCR reaction, the SYBR Green PCR Core reagents kit (Applied Biosystems, Foster City, CA, USA) was used. Intron-spanning primers were designed using Primer Express Software (Applied Biosystems) and purchased from Eurogentec (Seraing, Belgium). Amplification of cDNA was performed in MicroAmp Optical 96-well Reaction Plates (Applied Biosystems) on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The reaction mixture (20 μl) was composed of 1x SYBR Green buffer, 3 mM $MgCl_2$, 875 μM dNTP mix, 0.3 U AmpliTaq gold, 0.12 U Amperase UNG, 15 pmol of each primer (GAPDH forward: 5' TCAAGGGCATCCTGGGCTAC 3', reverse: 5' CGTCAAGGTGGAGGAGTGG 3' and TG2 forward: 5' GGCCTGACCAAGGAACAGAAG 3', reverse: 5' CAATATCAGTCGGGAACAGGTC 3'), 12.5 ng cDNA and nuclease free H_2O. The reaction conditions were an initial 2 min at 50^{0}C, followed by 10 min at 95^{0}C and 40 cycles of 15 sec at 95^{0}C and finally extension for 1 min at 59^{0}C. The mRNA expression levels were quantified relatively to the level of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) using the following calculation: *2–(Threshold cycle of target mRNA – Threshold cycle of GAPDH) x 100*.

Western blotting

To determine TG2 protein levels, OLGs were homogenized in lysis buffer containing 10 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 μM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 10 μg/ml aprotinin (all from Sigma). Homogenates were sonificated and then cleared by centrifugation (14,000 rpm for 30 min at 4^{0}C) and protein concentrations of supernatants were determined by using the BCA method (Pierce Biotechnology, Perbio Science, Etten-Leur, The Netherlands). Of each sample, 10 μg of protein was loaded into the assay. SDS-polyaclylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide gel was performed and separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen). To visualize TG2 protein, the membrane was blocked with 5% BSA in TBS/1% Tween-20. Subsequently, the membrane was incubated o/n at 4^{0}C with a mouse monoclonal antibody to TG2 (Ab3, Labvision, 1:1,000). For subsequent antigen detection, blots were incubated for 2 h with goat anti-mouse Immunoglobulins/HRP (Dako 1:10,000). Bands were visualized using the enhanced chemiluminescence (ECL) detection system SuperSignal West Dura (Pierce Biotechnology) and a Chemidoc image capture system (Bio-Rad, Veenendaal, The Netherlands). Signal intensity of the bands was semi-quantified using Quantity One software (Bio-Rad).

TG activity assay

TG activity was measured at various stages during OLG development. Additionally, the effect of KCC009 or cystamine treatment on TG activity was measured. Therefore, primary OPCs (O2A stage) were left untreated or treated for 4 h with KCC009 or cystamine at 37^{0}C. Protein lysates were prepared as described for western blotting and activity was measured by using the TG Covtest TCMA (Transglutaminase Colorimetric Microassay; Covalab) following manufacturer's protocol.[323] In short, immobilized CBZ-Gln-Gly was coated onto the wells as the first TG substrate. Subsequently, 10 μg of protein from each sample was added/well, followed by addition of biotinylated cadaverine as a second substrate. After 30 min incubation at 37^{0}C, plates were washed with Tween-20 buffered saline (TTBS) and streptavidin-labeled peroxidase (HRP) diluted in TTBS was added to the wells for 15

min. After washing, peroxidase activity was revealed using 100 μl of 0.01% H_2O_2 as HRP substrate and (0.1 mg/ml) tetramethyl benzidine as electron acceptor (chromogen). The reaction was stopped by the addition of 50 μl of 2.5 N H_2SO_4. TG activity was detected by absorbance measurement of streptavidin-labeled peroxidase activity in each well on a microplate reader (SpectraMax 250, Molecular Devices) at 450 nm. Purified guinea pig TG2 (Sigma) was used to prepare a standard curve.

Immunofluorescence microscopy

OLGs in vitro

For immunofluorescence microscopy, OPCs were grown on Lab-Tek plastic chamber slides (Nalge Nunc) at a density of 15,000 cells per well. Cells were fixed with 2% paraformaldehyde (PFA) for 15 min at RT, followed by 4% PFA for another 15 min at RT. For staining of myelin basic protein (MBP), cells were permeabilized for 5-10 min with ice-cold methanol, subsequently blocked with 4% BSA in PBS for 30 min at RT, followed by a 1 h incubation with mouse anti-TG2 (Ab2, Labvision, Fremont, CA, USA, 1:1,000) or rat anti-MBP (Serotec, Oxford, UK, 1:10). After washing the cells with PBS, they were incubated for 30 min with a corresponding TRITC-conjugated goat anti-mouse (Jackson Immunoresearch Laboratories, West Grove, PA, 1:50) or a goat anti-rat antibody (Jackson Immunoresearch Laboratories, 1:50). Antibody staining of galactosphingolipids, i.e., cell surface components was performed on live cells at 4^{0}C. After blocking non-specific binding with 4% BSA in PBS for 10 min, primary OLGs were incubated with Ranscht mouse monoclonal antibody recognizing galactosylceramide and sulfatide[308] (IgG3, a kind gift of Dr. Guus Wolswijk, The Netherlands, 1:10) for 30 min at 4^{0}C, washed three times with ice-cold PBS and incubated for 25 min with TRITC-conjugated goat anti-mouse IgG3 (Jackson Immunoresearch Laboratories, 1:50). Subsequently, the cells were fixed with 4% PFA for 20 min at RT. Nuclei were stained with DAPI (1 μg/ml) and after washing with PBS, the cells were covered with 2.5% 1.4-diazobicyclo(2.2.2)ocatane (DABCO, Sigma) in 90% glycerol/10% PBS to prevent image fading. The cells were analyzed with a conventional fluorescence microscope (Olympus ProVis AX70) equipped with analySIS software. All MBP positive cells, cells with MBP positive sheets and cells with Ranscht positive sheets were counted. In all quantifications the values for the TG2 inhibitor-treated cells were related to the value of vehicle-treated cells at the MBP+ stage, which was set at 100%. Results are represented as mean ± s.e.m. of at least four independent experiments.

Human cerebellum

Formalin-fixed paraffin embedded tissue sections (6 μm) of human cerebellum obtained at gestational week 28 till post-partum month 2 (n=8) were used to determine the presence of TG2 in OPCs. The sections were deparaffinized, rehydrated, and pretreated with citrate buffer (pH 6.0) for antigen retrieval. Subsequently, the sections were co-incubated o/n at 4^{0}C with a mouse monoclonal antibody directed against TG2 (Ab3, 1:500 Labvision) and a goat antibody directed to PDGFRα (R&D systems, Abingdon, UK, 1:100) or a rabbit anti-NG2 (Chemicon, 1:200) to identify oligodendrocyte precursor cells or a rabbit antibody against GFAP (DAKO, 1:400) to identify astrocytes. After rinsing the sections with TBS, the sections were co-incubated with appropriate fluorescently labeled secondary antibodies to detect PDGFRα, NG2, or GFAP (Alexa Fluor-488, Invitrogen, 1:400) and TG2 (Alexa

Fluor-594, Invitrogen, 1:400) immunoreactivity. Sections were embedded in Vectashield mounting medium (Vector Laboratories) and examined on a Leica confocal laser scanning microscope (Leica, Rijswijk, The Netherlands).

Analysis of cell viability

OPCs were seeded into a PLL-coated 24-wells plate in 500 μl of SATO medium at 1.0×10^6 cells/ml. The cells were treated with cystamine (0.025 mM) or KCC009 (0.05 mM), starting from day 0 (O2A), day 3 (GC) or day 7 (MBP). The TG2 inhibitors were continuously present for the subsequent duration of the experiment to determine their effect on cell survival. Vehicle-treated and staurosporine-treated cells served as negative and positive controls, respectively. After 3 days of treatment, the medium was collected and centrifuged for 7 min at 7,000 rpm. The level of lactate dehydrogenase (LDH) was measured using a commercial available LDH Cytotoxicity Detection Kit (Roche Diagnostics) following manufacturer's protocol. To compare multiple experiments (n=4), the values obtained for the TG2 inhibitor-treated cells were related to the value of vehicle-treated control cells, which was set at 100%.

RhoA GTPase assay

The presence of active RhoA GTPase was determined in OPCs (O2A stage) after treatment with KCC009 or 0.1% DMSO (vehicle control) for 1 h. OLGs ($4x10^6$ cells per treatment) were homogenized in lysis buffer containing 50 mM Tris/HCl pH 7.5, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 10 mM $MgCl_2$, 100 μM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 10 μg/ml aprotinin (all from Sigma). Protein lysates were cleared as described for western blotting. Cleared lysates were incubated with bacterially produced GST-RDB (Rhotekin)[310] and visualized using western blotting as described.[324] In short, lysates were incubated with 60 μg GST-RDB for 30 min and the GST-RDB beads were subsequently washed with wash buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Triton X-100, 10 mM $MgCl_2$ 100 μM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 10 μg/ml aprotinin (all from Sigma)). Beads and 10 μl of total cell lysates were loaded onto a 15% SDS-PAGE gel and separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen). Mouse anti-RhoA antibody (Santa Cruz Biotechnology, 1:1,000) was used to detect the active form of RhoA (RhoA-GTP) and total RhoA followed by goat anti-mouse IgG/HRP (Dako, 1:10,000). Bands were visualized using the enhanced chemiluminescence (ECL) detection system SuperSignal West Dura (Pierce Biotechnology) and a Chemidoc image capture system (Bio-Rad, Veenendaal, The Netherlands). Signal intensity of the bands was semi-quantified using Quantity One software (Bio-Rad).

Cuprizone demyelination model

For *in vivo* experiments, 8 weeks old C57 Bl/6J $TG2^{-/-}$ mice[85] and litter mate wild-type (wt) mice were used. Mice were originally obtained from G. Melino (Rome, Italy) and bred at our own facility. To induce demyelination, a diet supplemented with 0.2% cuprizone (bis-cyclohexanone oxaldihydrazone, Sigma-Aldrich) was used as described previously.[256] In short, 21 wt mice and 21 $TG2^{-/-}$ mice were fed ad libitum with 0.2% (wt/wt) cuprizone in the ground breeder chow for 35 days and thereafter fed with normal chow only. At 35, 40 and 50 days after the initial start of the experiment, groups of animals were

sacrificed and brains were removed, fixed overnight in 4% PFA in 0.2 M phosphate buffer and subsequently embedded in paraffin. Hearts were removed and directly frozen in liquid nitrogen and stored at -80^{0}C for mitochondrial activity measurements. All animal experiments were approved by the local animal ethical committee.

Immunohistochemistry

Paraffin-embedded brains of cuprizone treated mice were cut in 5 μm sections at the level of the corpus callosum (between approximately bregma levels -0.94 to 0 mm according to mouse atlas by Paxinos and Franklin[287]), the most frequently investigated white matter tract in this model.[194, 358] Sections were deparaffinized in xylene and transferred to distilled water via degrading 90%, 70% and 50% ethanol series. Before staining, endogenous peroxidase was blocked by incubation in methanol with 0.02% H_2O_2 for 30 min at RT. This was followed by antigen retrieval by heating the sections for 90 min in 0.05 M EDTA in 0.01 M Tris/HCl buffer (pH 8.5) in a household food steamer device (MultiGourmet FS 20; Braun, Kronberg/Taunus, Germany). Sections were incubated with 10% fetal calf serum (FCS) in 0.1 M phosphate-buffered saline (FCS/PBS). Next, primary antibodies were diluted in FCS/PBS and the sections were incubated o/n at 4^{0}C. The following primary antibodies were used: rat anti-MBP (Chemicon, Millipore, Amsterdam, The Netherlands, 1:100), rabbit anti-NG2 (Chemicon, 1:500) and mouse anti-Mac3 (BD Pharmingen, NJ, USA, 1:500). After washing with PBS, appropriate biotinylated secondary antibodies in FCS/PBS were applied for 1 h at RT. We used biotinylated donkey anti-rabbit, donkey anti-rat and sheep anti-mouse IgG's (Amersham Pharmacia Biotech, Uppsala, Sweden; 1:200). After washing, the sections were incubated for 1 h at RT with peroxidase-labeled streptavidin (Sigma, 1:100). Immunoreactivity was visualized with 3,3 diaminobenzidine-tetrahydrochloride (DAB, Sigma).

PLP in situ hybridization, immunohistochemisty and quantification

Detection of PLP mRNA, as an index of remyelination, and PLP protein was performed on paraffin sections from wt and TG2$^{-/-}$ mouse brains as described previously.[26] In short, sections were incubated with digoxigenin-labeled anti-sense probes specific for proteolipid protein (PLP) followed by alkaline phosphatase-labeled anti-digoxigenin (Boehringer-Mannheim) and PLP mRNA was visualized using NBT/BCIP (Boehringer-Mannheim) as a substrate. To detect PLP protein, rabbit anti-PLP (Serotec, 1:1,000) followed by donkey anti-rabbit IgGs (Jackson, 1:1,000) was used. Sections were counterstained with haematoxylin. The number of PLP mRNA expressing cells in the corpus callosum (CC) was counted in 3 sections per animal (n=6/group) using a grid and expressed per mm^2.

Rotarod experiments

Motor performance was evaluated using a rotarod apparatus (MED Associates Inc., St. Albans, VT, USA) which consists of a motor-drive rotarod equipped with variable speeds. Mice were tested on the rotarod just before they were sacrificed, on day 0, 35, 40 and 50 after starting the cuprizone diet. Performance was defined as the mean duration that an animal remained on the rotarod as a function of increasing drum speed of 16, 24 and 32 rpm/trial with a maximum of 60 sec/trial.[117]

Measurement of mitochondrial complex enzyme activity

For detection of mitochondrial respiratory chain activity of complex II and complex IV enzymes in hearts of $TG2^{-/-}$ mice (n=4) and wt mice (n=4), animals were sacrificed at day 40 after they were tested on the rotarod. Fresh frozen 5 μm sections were air dried for 15 min at RT and enzyme activities were measured as described.[87, 258] In short, the activity of complex II was determined by incubating the sections in succinate dehydrogenase (SDH) medium (130 mM succinate, 200 μM phenazine methosulphate, 1 mM sodium azide, 1.5 mM tetranitroblue tetrazolium chloride in 0.2 M phosphate buffer, pH 7.0) at 37^0C for 7 min. To determine the activity of complex IV, sections were incubated in cytochrome c oxidase (COX) medium (100 μM cytochrome c, 4 mM diaminobenzidine tetrahydrochloride in 0.2 M phosphate buffer, pH 7.4) at 20^0C for 5 min. The absorbance of the formazan precipitate (SDH) and the DAB precipitate (COX) was determined on 3 sections per animal, using a Leica DMRB Microscope (Wetzlar, Germany) fitted with calibrated grey filters using an interference filter at 660 nm or 436 nm, respectively. Images were collected with a Sony XC-77CE camera (Towada, Japan) connected to a LG-3 frame grabber (Scion; Frederick, MD) in an Apple Power Macintosh computer, and analyzed using NIH Image software (http://rsb.info.nih.gov/nih-image/). Grey values were converted to absorbance values using the grey filters and calibrate options in NIH Image.

Statistics

Data were analyzed by one-way or two-way ANOVA, followed by a t-test for independent measurements (Fisher's LSD test). The statistical evaluation was carried out by using the NCSS 2007 statistical program (NCSS, East Kaysville, Utah, USA). Error bars represent standard error of the mean (s.e.m.).

Results

TG2 is expressed during early oligodendrocyte development in vitro

To examine the levels of TG2 protein and mRNA during OLG development, rat OPCs were allowed to differentiate into myelin producing cells. Cells were collected at various stages of OLG development; the O2A stage, GC stage and MBP stage, of which TG2 mRNA, protein and activity levels were determined. TG2 immunoreactivity was clearly present in the OPCs at the O2A stage (Fig. 1A). TG2 mRNA levels were significantly lower in the GC and MBP stage compared to the levels in progenitor cells (O2A stage) (Fig. 1A). Moreover, TG2 protein levels and TG activity were lower in the GC and MBP stage compared to the O2A stage (Fig. 1B).

TG2 immunoreactivity in developing human cerebellum

To determine whether TG2 could be involved in human OPC differentiation, the presence of TG2 immunoreactivity was studied during human cerebellar development when numerous differentiating OPCs are present. Indeed, TG2 immunoreactivity was identified in developing human cerebellum, at least between gestational week 28 and 2 months postnatally. Using immuno double labeling, we observed that within this developmental period, TG2 immunoreactivity in the cerebellum was present in NG2 (an integral membrane chondroitin sulfate proteoglycan expressed by OPCs) (Fig. 2A) and platelet-derived growth factor receptor alpha (PDGFRα) (Fig. 2B) positive OPCs. TG2 immunoreactivity was not detected in GFAP positive astrocytes (Fig. 2C).

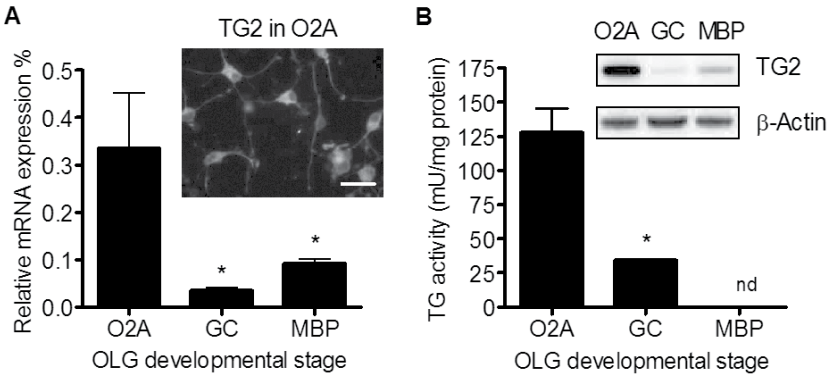


Figure 1: TG2 expression during OLG development. **A)** TG2 mRNA levels during OLG development and TG2 immunofluorescence in cultured OLGs in O2A stage (OPCs). Scale bar: 50 μm. **B)** TG2 protein levels visualized on Western blot and TG activity measured with the TG Covtest TCMA at various stages of OLG development. Data are expressed as mean + s.e.m., n=4/group, *P<0.05, nd= not detectable.

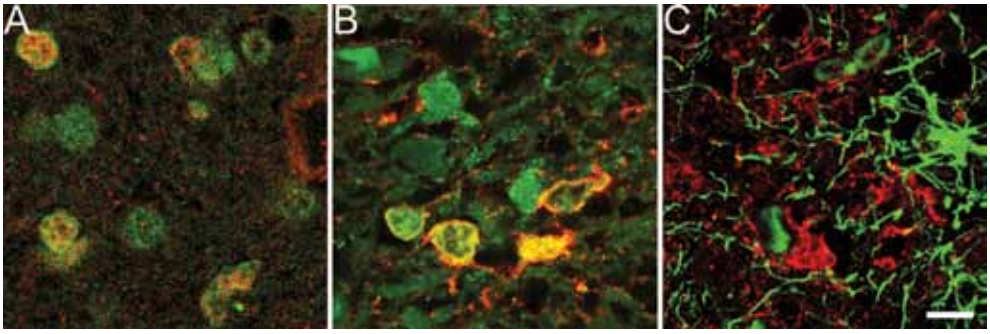


Figure 2: TG2 immunoreactivity in white matter of developing human cerebellum. Immunofluorescent double labeling of TG2 and **A)** NG2 or **B)** PDGFRα, at gestational week 28. Immunofluorescent double labeling of TG2 and **C)** GFAP, 2 months postnatally. TG2 is in red, and NG2, PDGFRα and GFAP are in green. Scale bar: 10 μm.

Inhibition of TG2 activity reduces OPC differentiation and myelin formation in vitro

To analyze the functional role of TG2 activity during OLG development, rat OPCs were treated with two different TG2 inhibitors, KCC009 and cystamine, starting treatment from various stages of OLG development until the vehicle-treated cells produced myelin-like sheets (MBP+ stage).

First, the effect of KCC009 and cystamine treatment on the number of MBP positive cells and of MBP positive sheets formed was studied. In vehicle-treated cells, a dense network of MBP expressing OLG processes and membrane sheets was observed, which was clearly reduced in the KCC009-treated (Fig. 3A) or in the cystamine-treated cells (Fig. 3B). KCC009 and cystamine significantly attenuated the number of MBP positive cells when treatment started from O2A and GC stage onwards. This effect was most evident when treatment started at the O2A stage (Fig. 3C). Also the number of MBP positive sheets was significantly reduced when cells were treated with each TG2 inhibitor from the O2A or GC stage onwards (Fig. 3D). Again most clearly when treatment was started at the O2A stage (Fig. 3D). When KCC009 or cystamine treatment started at the MBP stage, no significant effect on the number of MPB positive cells or sheets was observed (Fig. 3C,D).

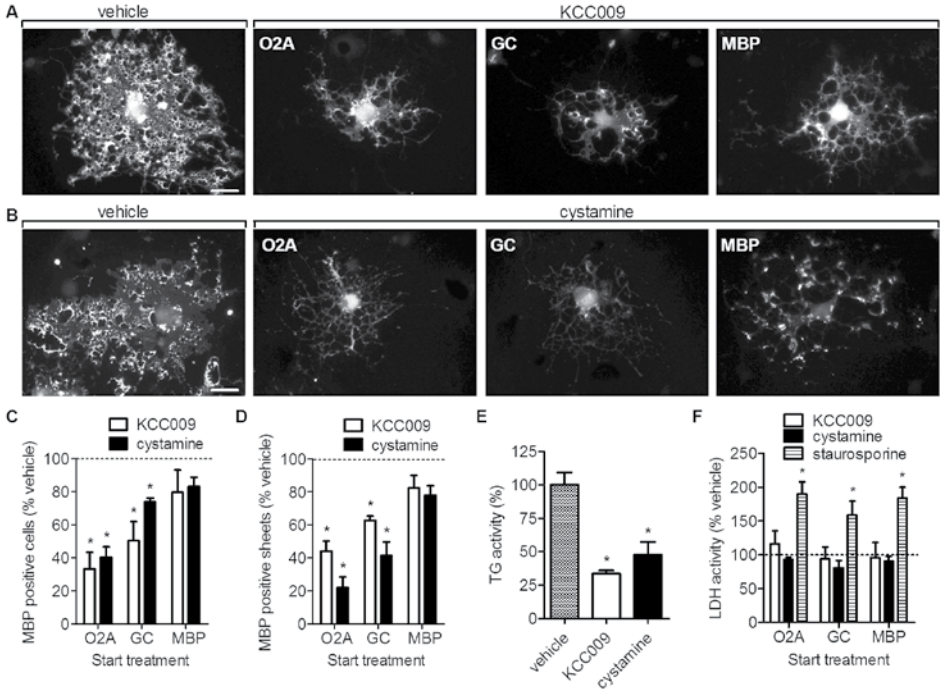
Of importance is that treatment with KCC009 or cystamine reduced TG activity in OPCs by approximately 65% or 52%, respectively (Fig 3E). Both inhibitors did not influence cell viability (Fig. 3F) whereas staurosporine treatment increased LDH activity by approximately 80% (Fig. 3F).

As effects of KCC009 and cystamine on OLGs were similar, we continued our studies by using KCC009 only, being a specific TG2 inhibitor. Subsequently, OLG membrane formation was studied. Irreversible inhibition of TG2 activity by KCC009 starting from O2A stage onwards reduced the number of galactosphingolipid-positive myelin-like membrane sheets formed by 35% compared to vehicle-treated cells as detected by the Ranscht antibody (Fig. 4A,B). When KCC009 treatment started in the GC or MBP stage, no significant effect on the number of galactosphingolipid-positive myelin-like membrane sheets formed was found.

Based on our observations that TG2 inhibition reduced OLG membrane sheet formation, we studied whether this also affects RhoA GTPase activity. Rho GTPases are essential in cytoskeleton rearrangements, and consequently the outgrowth of OLG processes.[79] Indeed, treatment of OPCs with KCC009 reduced the amount of active RhoA with approximately 75%, leaving total RhoA protein levels unaffected (Fig. 4C).

Impaired remyelination in cuprizone-treated TG2$^{-/-}$ mice

As we showed that TG2 was involved in OLG development *in vitro*, we studied whether TG2 is involved in remyelination *in vivo*. To address this question, TG2$^{-/-}$ mice and litter mate wild-type (wt) mice were fed a cuprizone diet for 35 days which resulted in demyelination of the CC. Subsequently, remyelination was studied by combined PLP in situ hybridization and PLP immunohistochemistry when the cuprizone diet had finished (day 35) and at 2 time-points during remyelination (days 40 and 50). As illustrated in Fig. 5A, PLP mRNA and protein was present in the CC of untreated TG2$^{-/-}$ and wt mice (day 0). After the cuprizone diet (day 35), and also during remyelination (days 40 and 50) the amount of PLP mRNA and protein expressing cells was altered (Fig. 5A). After the cuprizone diet had finished and when demyelination was evident (day 35), the number of PLP mRNA expressing cells was reduced similarly in TG2$^{-/-}$ mice and wt mice compared to their corresponding untreated controls (day 0) (Fig. 5B). During remyelination, i.e. at day 40 and day 50, the number of PLP mRNA expressing cells in the CC of wt animals was significantly increased compared to the number of cells at day 35 (demyelination). In contrast, the number of PLP mRNA expressing



cells in the CC of TG2$^{-/-}$ mice at day 40 and 50 remained similar to the number of cells at day 35. Consequently, the number of PLP mRNA expressing cells during remyelination (days 40 and 50) was significantly lower in TG2$^{-/-}$ mice than in wt mice (Fig. 5B). Subsequently, MBP and NG2 immunoreactivity was studied in the CC of TG2$^{-/-}$ and wt mice. Both proteins are expressed by oligodendrocytes, either by mature myelin-producing OLGs (MBP) or by OPCs (NG2). MBP immunoreactivity followed a similar temporal expression pattern as PLP mRNA and protein in wt and TG2$^{-/-}$ mice (Fig. 6A). The observation that there were less PLP and MBP producing cells present in the CC of TG2$^{-/-}$ mice during remyelination was

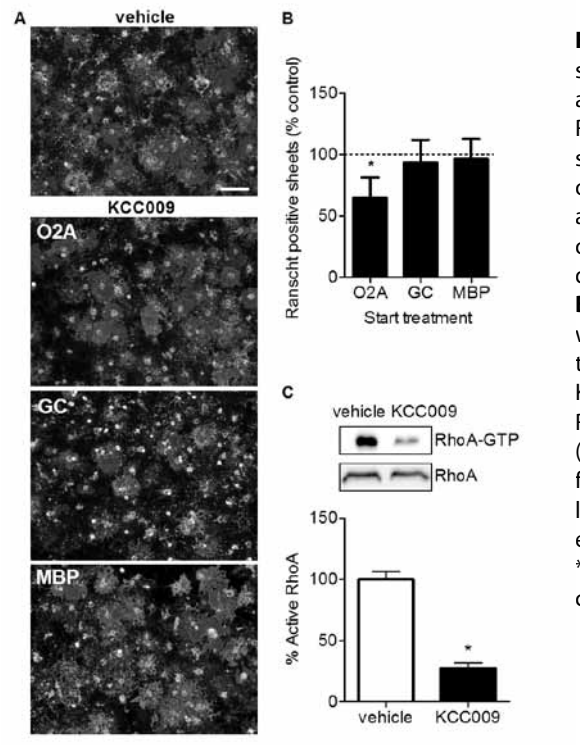


Figure 4: Galactosphingolipid positive sheets after treatment with KCC009 and effect of KCC009 treatment on RhoA activity. **A)** Immunofluorescent staining using the Ranscht antibody on OLGs collected in the MBP+ stage after treatment with 0.05 mM KCC009 or vehicle from different stages of OLG development onwards. Scale bar: 100 μm. **B)** The number of Ranscht positive sheets were counted and corrected for vehicle treated cells (set at 100%). **C)** Effect of KCC009 treatment on the activity of RhoA visualized with Western blot. OPCs (day 0, O2A) were treated with KCC009 for 1 h and RhoA-GTP and total protein levels of RhoA were measured. Data are expressed as mean + s.e.m., n=4/group, *P<0.05 compared to vehicle-treated control (set at 100%).

◀— **Figure 3**: Effect of KCC009 and cystamine treatment on myelin formation. **A)** Immunofluorescent staining of MBP on primary rat OLGs. Cells were treated with 0.05 mM KCC009 or vehicle from different stages of OLG development onwards and fixed in MBP+ stage. Scale bar: 20 μm. **B)** Immunofluorescent staining of MBP on primary rat OLGs. Cells were treated with 0.025 mM cystamine or vehicle from different stages of OLG development onwards and fixed in MBP+ stage. Scale bar: 20 μm. **C)** The number of MBP positive sheets were counted and compared to the number of MBP positive sheets formed by vehicle-treated cells (set at 100%). **D)** The number of MBP-positive cells were counted and compared to the number of MBP positive vehicle-treated cells (set at 100%). **E)** TG activity measured in protein lysates of OPCs (day 0, O2A) that were treated with KCC009, cystamine or vehicle for 4 hours. **F)** LDH activity in KCC009-, cystamine- and vehicle-treated cells. Data are expressed as mean + s.e.m., n=4/group, *P<0.05.

not explained by an absence of local NG2 positive OPCs in these mice as these cells were equally present in both mouse types (Fig. 6B). In fact, at day 40, NG2 immunoreactivity in the TG2$^{-/-}$ mice appeared elevated compared to that in wt mice. Furthermore, no clear difference was found in Mac3 immunoreactivity between TG2$^{-/-}$ mice and wt mice (Fig. 6C), showing an increase in Mac3 expression during demyelination (day 35) which was reduced during remyelination (days 40 and 50).

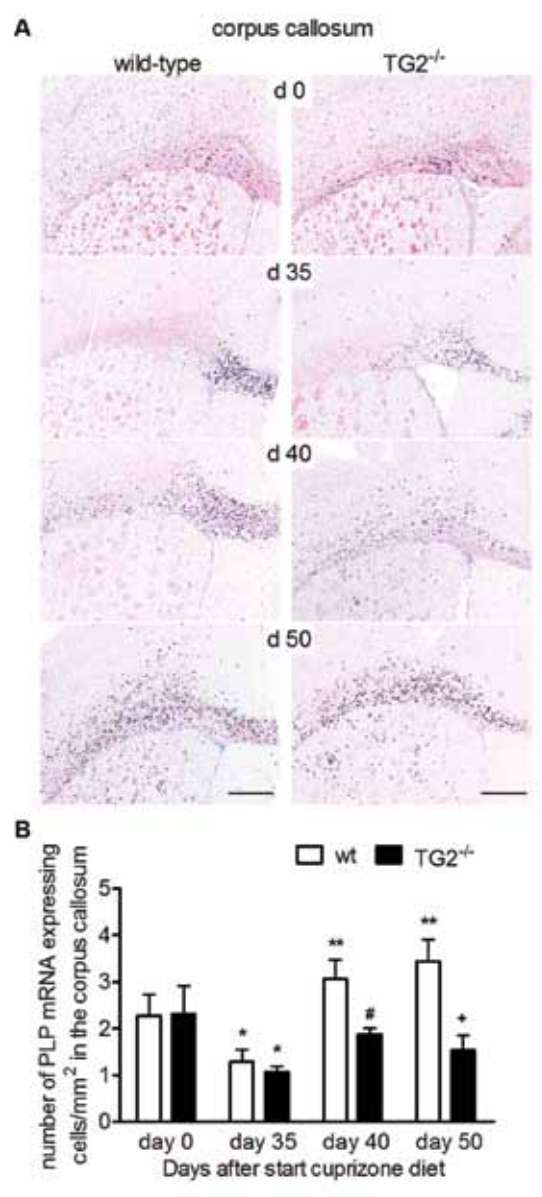


Figure 5: PLP in situ quantification in the corpus callosum of cuprizone treated wt and TG2$^{-/-}$ mice. **A)** Representative images of PLP in situ hybridization in paraffin sections, scale bar: 250 μm. **B)** quantification of PLP mRNA positive cells in the corpus callosum of wt and TG2$^{-/-}$ mice treated with cuprizone for 35 days and subsequently fed with a normal diet for 5 (day 40) or 15 (day 50) days. Data are expressed as mean + s.e.m., n=7/group, *P<0.05 compared to wt day 0, **P<0.05 compared to wt day 35, $^{\#}$P<0.05 compared to wt day 40 and +P< 0.05 compared to wt day 50.

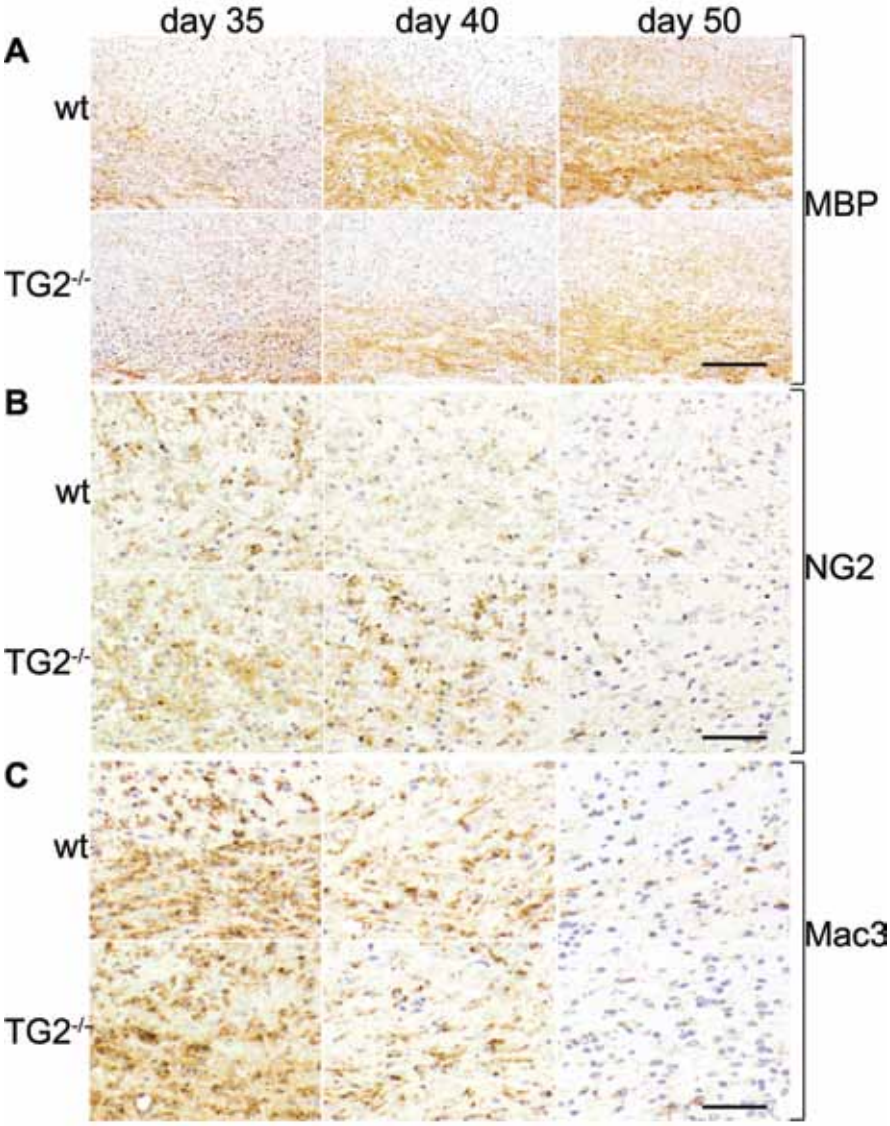


Figure 6: Immunoreactivity of various markers present in the corpus callosum of TG2$^{-/-}$ and wt mice after treatment with cuprizone. Immunohistochemical analysis of wt and TG2$^{-/-}$ mice treated with cuprizone for 35 days and allowed to recover for 5 (day 40) and 15 (day 50) days. **A**) MBP, scale bar: 100 μm. **B)** NG2, scale bar: 50 μm. **C)** Mac3, scale bar: 50μm.

Rotarod performance in TG2$^{-/-}$ and wt mice during de- and remyelination.
Motor coordination was evaluated using a rotarod apparatus. Untreated TG2$^{-/-}$ and wt mice (day 0) performed equally well and stayed approximately 50 seconds on the rotarod apparatus without falling off. After finishing the cuprizone diet (day 35), the motor performance of wt and TG2$^{-/-}$ mice was impaired compared to day 0 (Fig. 7A). In the wt and TG2$^{-/-}$ mice, motor performance was subsequently improved during remyelination (day 40 and 50) (Fig. 7A). However, this improvement in motor performance was significantly less and lagged behind in time in TG2$^{-/-}$ mice (Fig. 7A).

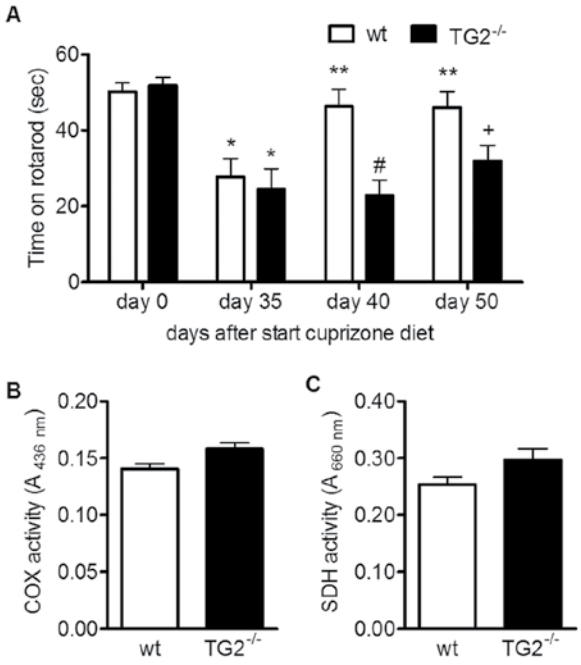


Figure 7: Rotarod performance and mitochondrial activity in TG2$^{-/-}$ and wt mice treated with cuprizone. **A)** Motor performance of wt and TG2$^{-/-}$ mice before and after feeding the 0.2% cuprizone diet determined with the rotarod test. Diet starts at day 0, day 35 is the end of the cuprizone diet (demyelination), and animals had a normal diet (remyelination) until day 40 or 50. The graph represents the time on the rotarod spend until the first fall per trial and averaged per 3 subsequent trials (16, 24 and 32 rpm, max 60 sec/trial). Data are expressed as mean + s.e.m., n=7/group, *P<0.05 compared to wt day 0, **P<0.05 compared to wt day 35, $^{\#}$P<0.05 compared to wt day 40 and $^{+}$P< 0.05 compared to wt day 50.
Mitochondrial activity in hearts of TG2$^{-/-}$ mice compared to wt mice during remyelination (day 40). **B)** cytochrome c oxidase (COX) and **C)** succinate dehydrogenase (SDH) were measured. Data are expressed as mean + s.e.m., 3 sections/animal, n=4 animals/group.

To determine whether the impaired motor performance in the TG2$^{-/-}$ mice was an effect of reduced mitochondrial respiratory chain activity, we collected the hearts of TG2$^{-/-}$ and wt mice directly after rotarod performance on day 40 and measured activity levels of mitochondrial respiratory chain complex IV, i.e. cytochrome c oxidase (COX) and complex II, i.e. succinate dehydrogenase (SDH). No significant difference was found in the activity of both mitochondrial complex enzymes between TG2$^{-/-}$ and wt mice (Fig. 7B,C).

Discussion

In the present study, we demonstrate that TG2 plays a prominent role in the differentiation of OPCs into OLGs and is involved in myelin protein formation. This is of interest for myelin formation during normal brain development, but also for demyelinating conditions within the CNS, e.g. MS and traumatic brain injury, when remyelination is only partially successful. At first we showed that during OLG development *in vitro*, TG2 mRNA, protein and activity levels are primarily present during the early OPC differentiation phase (O2A), and reduced when the OPCs start to produce myelin. Together with previous observations that TG2 is involved in differentiation of astrocytes and neurons[56, 377] and a link between TG2 and myelination of Schwann cells was identified,[298] our data suggest that TG2 can play an important role in the differentiation of OPCs. Additionally, we identified the presence of TG2 immunoreactivity in NG2 and PDGFRα positive cells in human cerebellum at gestational week 28. These cells are likely OPCs, based on the cell markers they express.[274] Moreover, in the third trimester of pregnancy, differentiation of early OPCs into NG2 and PDGFRα expressing cells is quite evident and progresses thereafter.[189] Surprisingly, TG2 immunoreactivity was not detectable in astrocytes in the human developing cerebellum, although astrocytes have been shown to produce TG2 *in vitro*.[255] This may be explained by the developmental human material we studied TG2 expression in, which is rather different from astrocyte cultures expressing TG2 as previously published. Our data suggest thus far that TG2 can also play a role in OPC differentiation in human developing cerebellum.
To elucidate the functional role of TG2 in OLG development, rat OLGs were treated with TG2 activity inhibitors from various developmental stages onwards. As expected, KCC009 and cystamine significantly reduced the activity of TG2. Importantly, the concentrations of TG2 inhibitors used were not toxic to the cells. Treatment of OPCs with KCC009 reduced the production of galactosphingolipid positive myelin-like membranes. When cells were treated from the GC or MBP stage onwards, it did not affect the formation of galactosphingolipid expressing myelin-like membranes which is probably due to the presence of these membranes already at those OLG developmental stages,[306] and which are likely not influenced in their formation anymore by TG2 activity. Thus, after inhibition of TG2 activity, differentiation of OPCs into the GC-stage was reduced. Interestingly, further differentiation into myelinating OLGs (MBP-stage) was also perturbed, i.e., inhibition of TG2 activity clearly reduced the expression of MBP, a major myelin protein.[277] Particularly, cells treated from the O2A and GC stage onwards show dramatically less myelin producing cells and myelin sheets formed. This can be explained by the fact that OLGs in the MBP stage have already started to produce myelin[291, 424] which cannot be inhibited anymore. Furthermore, the finding that OLG differentiation and myelin formation is

reduced most prominently when TG2 was inhibited from O2A stage onwards, together with the observation that TG2 expression and activity reduced when OPCs had started to differentiate, suggest that TG2 is involved in the timing of differentiation.

Investigation of the underlying mechanism points to a role for RhoA GTPase activity in TG2-mediated OPC differentiation. It is known that Rho-GTPases are expressed by OLGs[99] and their activity is essential for promoting process extension during OPC differentiation.[214] As we observed that TG2 is clearly present in OPCs, and TG2 can transamidate RhoA GTPase thereby increasing its activity,[341] we expected that inhibition of TG2 activity would attenuate RhoA activity. Indeed, RhoA activity was reduced in OPCs upon KCC009 treatment, while leaving total RhoA levels unaffected. Activated RhoA GTPase can subsequently enhance the activity of the mitogen-activated protein kinase (MAPK) pathway, which in turn is involved in expression of new genes[341] that regulate OLG elongation and branching.[420] Thus, TG2 in OPCs probably acts by upregulating RhoA activity which subsequently activates intracellular pathways, e.g. the MAPK pathway, thereby promoting differentiation of OPCs into myelin forming OLGs.

As we now demonstrated that TG2 is involved in the development of OPCs into myelin-forming OLGs, it is of importance to determine whether TG2 can play a role in remyelination *in vivo* which is an essential process of recovery in demyelinating diseases. To that end, we used the well characterized cuprizone model for de- and remyelination in wt and $TG2^{-/-}$ mice. We observed that during the remyelination phase, the number of PLP mRNA expressing cells in the CC was lower in $TG2^{-/-}$ mice than in wt mice. Moreover, less PLP and MBP immunopositive cells were present in $TG2^{-/-}$ mice. These observations are not due to a compromised presence of OPCs in the CC of $TG2^{-/-}$ mice as shown by comparable NG2 immunoreactivity in wt and $TG2^{-/-}$ mice. In fact, more NG2 positive cells were detected in the CC of $TG2^{-/-}$ mice during the remyelination phase (day 40) which may indicate that OPC differentiation lagged behind in $TG2^{-/-}$ mice compared to wt mice. Therefore, TG2 can be an early trigger in the timing of differentiation from NG2 positive OPCs towards differentiated and, finally, MBP forming OLGs. Furthermore, cuprizone-induced demyelination is accompanied by robust microglia activation.[153] Activated microglial cells produce inflammatory mediators including cytokines, such as tumor necrosis factor-α (TNFα) and interferon gamma (IFNγ), and nitric oxide (NO), which can reduce remyelination.[152] Importantly, we did not observe a difference in microglia activation status between $TG2^{-/-}$ and wt mice as shown by comparable Mac3 immunoreactivity. Moreover, we observed a similar time-dependent reduction in Mac3 immunoreactivity during the remyelination phase of wt and $TG2^{-/-}$ mice. It is therefore unlikely that activated microglia contribute to the impaired remyelination in $TG2^{-/-}$ mice. All together, our data convincingly show that TG2 is involved in remyelination *in vivo*.

We finally studied whether the impaired remyelination in $TG2^{-/-}$ mice has functional consequences. The rotarod system is well known to monitor motor performance in rodents. We observed a reduced motor function in $TG2^{-/-}$ and wt mice after demyelination, which quickly recovered during remyelination in wt mice. In contrast, the motor performance in $TG2^{-/-}$ mice slightly improved but the functional recovery clearly lagged behind that of wt mice. Interestingly, the impaired motor performance in the $TG2^{-/-}$ mice coincides with reduced PLP mRNA expression in the CC of those animals, suggesting that myelination of the CC is essential for motor performance. Indeed, demyelination of the CC

has been shown to correlate with modified motor function,[150] perhaps as a consequence of reduced transfer of visual information between the cerebral hemispheres.[81] To exclude that the impaired motor performance of the $TG2^{-/-}$ mice as observed is due to reduced mitochondrial complex enzyme activity in the heart resulting in less energy (i.e. ATP) production as suggested by others,[37, 238] we studied in the hearts of both mice types the activity of mitochondrial complex II and IV enzymes i.e. succinate dehydrogenase and cytochrome c oxidase, respectively. We observed no difference in mitochondrial activity between wt and $TG2^{-/-}$ mice, indicating that under the conditions studied, ablation of the TG2 gene does not compromise mitochondrial enzyme activity. Thus TG2 is involved in myelination *in vivo* which has functional consequences for e.g. motor performance.
Overall, we conclude that TG2 is an interesting new player in CNS (re)myelination. Inhibition of TG2 activity reduces OPC differentiation into myelin-forming OLGs and subsequent remyelination *in vivo*. We put forward that manipulating TG2 activity may create a possibility to regulate the differentiation status of OLG, and hence represents an innovative therapeutic target for remyelination.

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