Published in final edited form as:

Glia. 2018 November ; 66(11): 2324–2339. doi:10.1002/glia.23468.

Enhanced oligodendrocyte maturation and myelination in a mouse model of Timothy syndrome

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Abstract

To study the role of L-type voltage-gated Ca^{++} channels in oligodendrocyte development, we used a mouse model of Timothy syndrome (TS) in which a gain-of-function mutation in the $\alpha 1$ subunit of the L-type Ca^{++} channel Cav1.2 gives rise to an autism spectrum disorder (ASD).

Oligodendrocyte progenitor cells (OPCs) isolated from the cortex of TS mice showed greater L-type Ca^{++} influx and displayed characteristics suggestive of advanced maturation compared to control OPCs, including a more complex morphology and higher levels of myelin protein expression. Consistent with this, expression of Cav1.2 channels bearing the TS mutation in wild-type OPCs triggered process formation and promoted oligodendrocyte-neuron interaction via the activation of Ca^{++} /calmodulin-dependent protein kinase II. To ascertain whether accelerated OPC maturation correlated with functional enhancements, we examined myelination in the TS brain at different postnatal time points. The expression of myelin proteins was significantly higher in the corpus callosum, cortex and striatum of TS animals, and immunohistochemical analysis for oligodendrocyte stage-specific markers revealed an increase in the density of myelinating oligodendrocytes in several areas of the TS brain. Along the same line, electron microscopy studies in the corpus callosum of TS animals showed significant increases both in the percentage of myelinated axons and in the thickness of myelin sheaths. In summary, these data indicate that OPC development and oligodendrocyte myelination is enhanced in the brain of TS mice, and

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Conflict of Interest: The authors declare that there is no conflict of interest regarding the publication of this article.

suggest that this mouse model of a syndromic ASD is a useful tool to explore the role of L-type Ca⁺⁺ channels in myelination.

Keywords

oligodendrocyte; voltage-gated calcium channels; Cav1.2; myelination; Timothy syndrome; autism

INTRODUCTION

L-type voltage-gated Ca⁺⁺ channels are well known for their role in promoting Ca⁺⁺ entry to drive synaptic plasticity, (Morgan and Teyler, 1999), as well as local translation (Lenz and Avruch, 2005) and global transcription (West et al., 2002). We have recently identified novel functions of L-type Ca⁺⁺ channels as modulators of intracellular Ca⁺⁺ levels in oligodendrocyte progenitor cells (OPCs) that are crucial for their normal development (Paez et al., 2007; 2009a; b; 2010). For instance, OPCs lacking the pore-forming subunit of the L-type Ca⁺⁺ channel Cav1.2 are less able to form contacts with neurons, and to differentiate into oligodendrocytes in vitro (Cheli et al., 2015a). Consistent with this, we found a significant hypomyelination in the brains of Cav1.2 conditional knockout mice in which Cav1.2 deletion was induced in OPCs during the first two postnatal weeks (Cheli et al., 2016). These results indicate that the L-type Ca⁺⁺ channel Cav1.2 modulates oligodendrocyte development, and suggest that Ca⁺⁺ influx mediated by L-type voltage-gated Ca⁺⁺ channels in OPCs is necessary for normal myelination.

Timothy syndrome (TS) is a rare autosomal dominant disorder characterized by physical malformations, as well as neurological and developmental defects, including long QT, arrhythmias and structural abnormalities in the heart, as well as autism spectrum disorders (ASD). There are two recognized types of TS, the milder TS1 and the more severe TS2. Both variants arise from missense mutations in alternatively spliced exons that cause the same glycine-to-arginine mutation in the gene encoding the pore-forming subunit of the L-type Ca⁺⁺ channel, Cav1.2 (Splawski et al., 2004). In a mouse model of TS2, this gain-of-function mutation alters voltage-dependent channel inactivation and results in behavioral phenotypes reminiscent of ASD symptoms (Bader et al., 2011). The TS2 mutation in these mice causes death at high levels of expression, but leaves basic functioning mostly unaffected at lower levels (Bader et al., 2011). No homozygous TS2 mice survived, but heterozygous TS2 mice (with the mutation and the neocassette in situ) had a normal appearance and survived to reproductive age. Gross brain anatomy was not markedly different in TS2 mice compared to control littermates, and no missing structures were noted (Bett et al., 2012). Thus, the TS2 mouse provide an important tool to understand the role of L-type Ca⁺⁺ channels activity in both TS and myelination.

In this study, we used the TS2 mouse to study the effects of the mutation that causes TS on oligodendrocyte development in vitro and in vivo. Our results indicate that OPC maturation is accelerated by the expression of the mutant Cav1.2 channel. In vitro, wild-type OPCs expressing the TS2 channel, as well as OPCs isolated from the cortex of TS2 mice, showed earlier morphological development with longer primary and secondary processes and higher

levels of myelin protein expression than control cells. TS2 channels stimulate OPC process extension whereas decrease process retraction, this effect was mediated by Ca^{++} influx and by the activation of Ca^{++} /calmodulin-dependent protein kinase II. Additionally, the postnatal myelination of TS2 mice was significantly augmented; numerous CNS structures were overmyelinated in TS2 animals and displayed greater numbers of mature oligodendrocytes. These results will contribute significantly to our understanding of the fundamental role of L-type Ca^{++} channels in brain development and begin to establish a link between TS and myelination.

MATERIALS AND METHODS

Transgenic mice

All animals used in the present study were housed in the UB Division of Laboratory Animal Medicine vivarium, and procedures were approved by UB's Animal Care and Use Committee, and conducted in accordance with the guidelines in "Guide for the Care and Use of Laboratory Animals" from the National Institutes of Health. The heterozygous TS2 line was obtained from Dr. Bett GC and Dr. Rasmusson RL (University at Buffalo) and was maintained as heterozygous. For all the experiments presented in this work mice of either sex were used.

Primary cultures of cortical OPCs

Primary cultures of cortical OPCs were prepared as described by Amur-Umarjee et al. (1993). First, cerebral hemispheres from 1 day old mice were mechanically dissociated and were plated on poly-D-lysine-coated flasks in DMEM/F12 (1:1 v/v) (Invitrogen), supplemented with 10% fetal bovine serum (FBS) (Life Technologies). After 4h the medium was changed and the cells were grown in DMEM/F12 supplemented with insulin (5 $\mu\text{g}/\text{ml}$), apo-transferrin (50 $\mu\text{g}/\text{ml}$), sodium selenite (30nM), d-Biotin (10mM) and 10% FBS. 2/3 of the culture media was changed every 3 days. After 14 days, OPCs were purified from the mixed glial culture by the differential shaking and adhesion procedure of Suzumura et al., (1984) and allowed to grow on poly-D-lysine-coated coverslips in DMEM/F12 supplemented with insulin (5 $\mu\text{g}/\text{ml}$), apo-transferrin (50 $\mu\text{g}/\text{ml}$), sodium selenite (30nM), 0.1% BSA, progesterone (0.06ng/ml), putrescine (16 $\mu\text{g}/\text{ml}$) (Sigma) and 1% FBS. OPCs were kept in mitogens, PDGF (20ng/ml) and bFGF (20ng/ml) (Peprotech), for 2 days and then induced to exit from the cell cycle and differentiate by mitogen withdrawal and T3 (15nM) addition.

OPC transfection

Construction of the wild-type- and TS2-Cav1.2 mutant plasmids has been previously described (Krey et al., 2013). One day after plating, OPCs were transfected using Lipofectamine 2000 (Life Technologies). Briefly, 1 μg of plasmid DNA was used to transfect 4×10^4 cells/coverslip. While the DNA was complexing, the cells were washed for 5min with serum free DMEM/F12. The complexed DNA mixture was then applied to the coverslips and incubated for 6h. The cells were washed with serum free DMEM/F12 and subsequently incubated in DMEM/F12 supplemented with insulin (5 $\mu\text{g}/\text{ml}$), apo-transferrin (50 $\mu\text{g}/\text{ml}$), sodium selenite (30nM), 0.1% BSA, progesterone (0.06ng/ml), putrescine (16 $\mu\text{g}/\text{ml}$)

(Sigma) and 1% FBS. OPCs were kept in mitogens, PDGF (20ng/ml) and bFGF (20ng/ml) (Peprotech) for 2 days and then induced to mature by mitogen withdrawal and T3 (15nM) addition for another 2 days.

Co-culture of cortical neurons and OPCs

Cortical neurons were prepared from the brains of 1- to 2-day-old mouse pups using established procedures (Echeverria et al., 2005) with minor modifications. Briefly, brains were removed aseptically and placed into DMEM/F12. After the brains were dissected, the blood vessels and meninges were removed under a dissecting microscope. Brain cortices were isolated and dissociated by digestion with a solution of 0.05% trypsin (Sigma) containing DNase I (0.06%) in Neurobasal medium for 10min at 37°C. The digestion reaction was stopped with Neurobasal containing 10% fetal bovine serum (Life Technologies) and triturated by repeated passages (20 times) through a 10ml pipette. The cell suspension was filtered through a sterile cell strainer (70µm; BD Biosciences) into a 50ml centrifuge tube. The cells were pelleted by centrifugation at 200g for 5min, and resuspended in Neurobasal plus 2% (v/v) B27 supplemented with 0.25mM GlutaMax I (Invitrogen) and 100µg/ml gentamicin (Life Technologies). High-density cultures (5×10^5 cells, ~ 2500 cells/mm²) were plated onto 20mm² tissue culture wells coated with poly-D-lysine (Sigma). After 7 days, cortical neuron cultures consisted of neurons essentially free from non-neural cells. Co-cultures were prepared by the addition of OPCs to the cultures of cortical neurons at a density of 3×10^5 cells/ml. These cultures were maintained in DMEM/F12, 1% FBS for 7 days.

Immunocytochemistry

Cells were stained with antibodies against several oligodendrocyte stage specific markers following the protocol outlined in Cheli et al. (2016). Fluorescent images were obtained using a spinning disc confocal microscope (Olympus, IX83-DSU). The percentage of positive cells was calculated counting the antigen-positive and DAPI-positive cells (total number of cells) in 20 randomly selected fields, which resulted in counts of more than 2000 cells for each experimental condition. Oligodendrocyte morphology was evaluated using the procedure described by Sperber and McMorris (2001). Briefly, individual MBP-positive OPCs were scored according to their morphological complexity in four different categories, based on the length and number of primary processes, whether the processes were radially distributed, the relative development of secondary and tertiary processes, and the overall size of the cell, including the process arbor. At least 500 MBP-positive cells were analyzed per experimental condition using the MetaMorph software (Molecular Devices). Data represent pooled results from at least 5 independent cultures. Quantification was performed blind to the experimental condition. The primary antibodies used for immunocytochemistry were against: caspase-3 (1:2000; Cell Signaling), CC1 (1:300; Calbiochem), Ki67 (1:250; Abcam), MAP2 (1:200; Millipore), MBP (1:1000; Millipore), MOG (1:200; Millipore), NG2 (1:400; Millipore), Olig1 (1:500; Millipore) and Olig2 (1:500; Millipore).

Calcium imaging

Methods were similar to those described previously (Paez et al., 2007). Briefly, primary cultures of cortical OPCs were incubated for 25min in serum and phenol red-free DMEM

containing a final concentration of 4 μ M fura-2 (AM) (Life Technologies) plus 0.08% Pluronic F127 (Life Technologies). Cells were then washed four times in DMEM and stored in DMEM for 10min before being imaged. Calcium influx and resting Ca⁺⁺ levels were measured in serum and phenol red-free HBSS containing 1.3mM Ca⁺⁺ and 1mM Mg⁺⁺. The fluorescence of fura-2 was excited alternatively at wavelengths of 340 and 380nm every 2s by means of a high-speed wavelength-switching device (Lambda DG4; Sutter Instruments). A spinning disc confocal inverted microscope (Olympus, IX83-DSU) equipped with a CCD camera (Hamamatsu ORCA-R2) measured the fluorescence. Calcium influx and resting Ca⁺⁺ levels were measured on individual cell bodies using the image analysis software MetaFluor (Molecular Devices). More than 600 cells for each experimental condition were analyzed and the results from 5 separate experiments were pooled.

Time-lapse imaging

Two days after transfection with wild-type- and TS2-Cav1.2 plasmids, OPCs were cultured in DMEM/F12 supplemented with insulin (5 μ g/ml), apo-transferrin (50 μ g/ml), sodium selenite (30nM), 0.1% BSA, progesterone (0.06ng/ml), putrescine (16 μ g/ml), T3 (15nM) and 1% FBS. Cultures were placed in a stage top chamber with 5% CO₂ at 37°C (Live-Cell Control Unit), which was located on the stage of a spinning disc confocal microscope (Olympus, IX83-DSU) equipped with a motorized z-stage. Time-lapse images of cells were taken every 6min over a period of 6h at 20x magnification using a CCD camera (Hamamatsu ORCA-ER). Imaging was performed on four wells, with at least 6 images taken per well. Individual YFP-positive oligodendrocytes were chosen in a blinded fashion such that the researcher was unaware of the treatment condition. The change in the number and length of primary processes and branch processes for each cell was measured and calculated every half an hour using MetaMorph software (Molecular Devices).

Western blot

Total proteins were collected from the mouse corpus callosum and cerebellum. The final protein pellet was homogenized in lysis buffer containing 50mM Tris-HCl, 0.25% (w/v) sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1% (w/v) Triton X-100, 0.1% (w/v) SDS, 1mM sodium vanadate, 1mM AEBSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin and 4 μ M sodium fluoride. Western blots were performed as previously described (Cheli et al., 2016). Twenty-five micrograms of total proteins were loaded onto a 4–12% Tris-glycine gel (Life Technologies). Protein bands were detected by chemiluminescence using the Amersham ECL kit (GE Healthcare) with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) and scanned with a C-Digit Bot Scanner (LI-COR). Relative intensities of the protein bands were quantified using the Image Studio™ Software (LI-COR). Data represent pooled results from at least four brains per experimental group. The primary antibodies used for Western blots were against: CNP (1:1000; Millipore), GAPDH (1: 10,000; Genetex), MBP (1:1000; Covance) and MOG (1:1000; Millipore).

Immunohistochemistry

All animals were anesthetized with isoflurane and then perfused with 4% of paraformaldehyde in PBS via the left ventricle. The brains were post-fixed overnight in the same fixative solution at 4°C. Coronal brain slices of 50 μ m thick were obtained using a

vibratome (Leica Biosystems, VT1000-S). Free-floating vibratome sections were incubated in a blocking solution (2% normal goat serum and 1% Triton X-100 in PBS) for 2h at room temperature and then incubated with the primary antibody overnight at 4°C. Sections were then rinsed in PBS and incubated with Cy3 or Cy5 conjugated secondary antibodies (1:400; Jackson Immuno Research) for 2h at room temperature followed by a counterstain with the nuclear dye DAPI (Life Technologies). After washing, the sections were mounted on to Superfrost Plus slides (Fisher) using coverslips and mounting medium (Aquamount; Thermo Scientific). The primary antibodies used in the present study were against: caspase-3 (1:1000; Cell Signaling), CC1 (1:300; Calbiochem), Ki67 (1:250; Abcam), MBP (1:1000; Millipore), MOG (1:200; Millipore), Olig1 (1:500; Millipore) and Olig2 (1:500; Millipore).

The staining intensity for myelin proteins as well as the number of positive cells was assessed in the central area of the corpus callosum, between the midline and below the apex of the cingulum (0.5mm²), in the motor and cingulate cortex including M1, M2, Cg1, Cg2 (0.5mm²) (Franklin and Paxinos, 2008; Figure 24) and in the dorsal/caudal striatum, immediately underneath the corpus callosum (0.5mm²). The integrated fluorescence intensity was calculated as the product of the area and mean pixel intensity using MetaMorph software (Molecular Devices). The density of myelin in control mice was given the arbitrary value of 100, and the density of myelin in TS2 mice was determined as a percentage of that in control mice. For all experiments involving quantification of positive cells and fluorescent intensity in tissue sections, data represent pooled results from at least 5 brains per experimental group. Seven slices per brain (50µm each) were used and quantification was performed blind to the genotype of the sample.

Electron microscopy

Mice were perfused with 3% paraformaldehyde and 1% glutaraldehyde for resin embedding. A block of approximately 1x1x2mm³ was removed from the body of the corpus callosum at the level of the anterior-dorsal hippocampus. Semi thin sections were stained with 1% toluidine blue (Sigma-Aldrich), and examined by light microscopy. Detailed evaluation of myelin integrity in the corpus callosum was performed on uranyl acetate and lead citrate stained thin sections using a FEI TecnaiTM F20 transmission electron microscope. Extent of myelination was quantitatively compared by determining *g*-ratios, which were calculated by dividing the diameter of the axon by the diameter of the entire myelinated fiber as previously described (Cheli et al., 2016). Furthermore, the percentage of myelinated axons from the total number of axons was calculated. At least five animals per experimental group and 300 fibers per animal were analyzed using the MetaMorph software (Molecular Devices).

Statistical Analysis

All data set were tested for normal distribution using Kolmogorov-Smirnov tests. Single between-group comparisons were made by the unpaired Student's t-test, using a confidence interval of 95%. Multiple comparisons were investigated by one-way ANOVA followed by Bonferroni's test to detect pair-wise between-group differences. All statistical tests were performed in Graphpad Prism (Graphpad Software). A fixed value of $p < 0.05$ for one tailed tests was the criterion for reliable differences between groups. Data are presented as mean $\pm$ SEM.

RESULTS

TS2 OPCs exhibit increased intracellular Ca^{++} elevations upon plasma membrane depolarization

To begin to explore a role for L-type channels in OPC development in vitro, we first examined the expression of these channels in cortical OPCs. OPCs were obtained from the cortex of P1 TS2 mice and were kept under proliferative conditions in the presence of PDGF and bFGF. After 2 days in vitro, the activity of L-type voltage-gated Ca^{++} channels was examined in control and TS2 OPCs using fura-2 ratiometric Ca^{++} imaging experiments. Elevated external potassium (K^+) was used as a depolarization agent (Paez et al., 2007; 2009a; b; 2010; Cheli et al., 2015a) (Figure 1A). In control cells, bath application of a solution containing high K^+ caused an average Ca^{++} increase of ~144% (Figure 1B). In contrast, in TS2 OPCs, the Ca^{++} transient induced by high K^+ was significantly higher (~221%) (Figure 1B). The rise in intracellular Ca^{++} concentration in control as well as in TS2 cells was blocked by the specific L-type channel inhibitors nifedipine and verapamil and was eliminated with the removal of extracellular Ca^{++} ($-\text{Ca}^{++}$) (Figure 1B). We did not find changes in the percentage of responding cells or in the basal intracellular Ca^{++} concentration (Figure 1C, D and E), but we did find a significant alteration in the shape of the Ca^{++} influx curve (Figure 1C and D) in TS2 OPCs compared to controls. In control cells, high K^+ induced a biphasic increase in intracellular Ca^{++} . The first phase consisted of a sharp peak characteristic of a transient, large increase in Ca^{++} ; this was followed by a second phase, in which intracellular Ca^{++} concentration sharply declined (Figure 1C and D). TS2 OPCs displayed a higher transient increase in cytoplasmic Ca^{++} , and the second phase, which represents channel inactivation, was significantly extended in these cells (Figure 1C and D). Together, this evidence demonstrates the presence of active L-type voltage-gated Ca^{++} channels in cortical OPCs and suggests the expression of mutant gain-of-function Cav1.2 Ca^{++} channels in TS2 OPCs.

Cells in the oligodendroglial lineage express several neuroligand receptors that are linked to Ca^{++} homeostasis, including ionotropic N-methyl-D-aspartate (NMDA) and AMPA receptors, metabotropic glutamate receptors (mGluR) (Bergles et al., 2000; Cahoy et al., 2008; Kukley et al., 2010) and ionotropic (P2X) and metabotropic (P2Y) ATP receptors (Agresti et al., 2005; Hamilton et al., 2010). In order to analyze the activity of these ligand-gated Ca^{++} channels and receptors, control and TS2 OPCs were stimulated with ATP and glutamate (Figure 1F). While we observe large increases in intracellular Ca^{++} upon both ATP and glutamate stimulation, these channels and receptors are unaffected in TS2 OPCs, indicating that TS2 OPCs have a selective defect in Ca^{++} entry through Cav1.2 (Figure 1F).

OPCs isolated from the TS2 brain mature earlier than control cells

In order to study the maturation of TS2 OPCs in vitro, immunocytochemical analysis for oligodendrocyte stage specific markers was performed in control and TS2 primary cultures. After two days in vitro, OPCs were stimulated to mature by mitogen withdrawal and T3 addition. In this pro-differentiation culture medium, there is a general decline in early immunocytochemical markers, such as Olig1 and NG2, and an increase in mature (e.g. CC1, MOG) markers consistent with differentiation of the OPCs. The number of oligodendrocytes

expressing CC1 and MOG was significantly higher in TS2 cultures than in control preparations, and the proportion of OPCs expressing the immature markers Olig1 and NG2 was concomitantly reduced, suggesting an accelerated maturation in TS2 OPCs. (Figure 2A and B). Additionally, we used the mitotic marker Ki67 and the apoptotic indicator caspase-3 in combination with the oligodendrocyte marker Olig2 to study oligodendrocyte proliferation and cell death (Figure 2C). We found that TS2 OPCs did not proliferate or undergo apoptosis at levels above that of control OPCs in vitro, as the percentage of Olig2/Ki67 and Olig2/caspase-3 did not significantly differ from controls (Figure 2C). Furthermore, TS2 cells showed a more complex morphology with longer primary and secondary processes and larger MBP positive sheets than control cells (Figure 2D, E and F). In summary, these data indicate that TS2 OPCs mature faster than control cells in vitro, and that the expression of the TS2 L-type channel is not affecting the survival and/or proliferation of TS2 OPCs.

Enhanced development of OPCs expressing Cav1.2 channels with the TS2 mutation

To further investigate the effects of the mutation that causes Timothy syndrome on OPC development, we introduced plasmids encoding wild-type Cav1.2 channels (**WT-Cav1.2**) or Cav1.2 channels with the mutation (G406R) that causes TS2 (**TS2-Cav1.2**) together with YFP into mouse primary OPCs. OPCs transfected with an empty plasmid expressing the YFP were used as a control. By means of ratiometric Ca^{++} imaging, we found that both L-type Ca^{++} channels were active in OPCs three days after transfection (Figure 3A, B and C). As expected, OPCs expressing the TS2-Cav1.2 channels had a substantially higher intracellular Ca^{++} rise than control cells or cells expressing the WT-Cav1.2 channels (Figure 3A, B and C), indicating that increased Ca^{++} influx through TS2-Cav1.2 led to an increase in intracellular Ca^{++} in OPCs expressing these channels. Relative to controls, we also detected a slight but significant increase in Ca^{++} influx in cells expressing the WT-Cav1.2 channel, but this change was minimal compared to the increased Ca^{++} elevations observed with introduction of the TS2 mutant channel (Figure 3C). Since both L-type Ca^{++} channels contain a T1036Y mutation, which renders the channels resistant to dihydropyridines, Ca^{++} imaging experiments were also performed in the presence of nifedipine to block endogenous Cav1.2 channels. In Figure 3C the response to depolarization in the presence or in the absence of nifedipine is shown for all the experimental conditions. While the response of control cells to depolarization was almost entirely blocked by nifedipine, Ca^{++} influx mediated by WT or TS-Cav1.2 exogenously-expressed channels was not affected (Figure 3C).

To determine whether the expression of TS2-Cav1.2 channels affects OPC maturation, we performed immunocytochemical analysis for oligodendrocyte stage-specific markers. As shown in Figure 4A and B, OPC cultures expressing TS2-Cav1.2 channels contained lower numbers of NG2 positive cells and higher quantities of positive cells for mature markers such as CC1 and MBP as compared to control or WT-Cav1.2 cultures. Importantly, WT and TS2-Cav1.2 expressing OPCs showed normal levels of proliferation and apoptotic cell death (Figure 3C). We also found significant changes in the morphology of TS2-Cav1.2 transfected cells after two days in a pro-maturation culture media (Figure 4D). TS2-Cav1.2 expressing oligodendrocytes exhibited an increase in the total number of processes, in the

volume of myelin like sheets (Figure 4A and D) and in the length of primary and secondary processes (Figure 4E). Furthermore, time-lapse experiments reveal that processes from OPCs expressing TS2-Cav1.2 underwent more extension and less retraction events than WT-Cav1.2 transfected cells, leading to a faster growth of the process arbor (Figure 5A, B and C). To provide evidence that process extension caused by TS2-Cav1.2 was a consequence of elevated intracellular Ca^{++} , we reduced extracellular Ca^{++} concentrations in OPCs expressing TS2-Cav1.2 and measured process growth by time-lapse. Reducing extracellular Ca^{++} to 0.2mM in TS2-Cav1.2 expressing cells prevent process extension (Figure 5C and D), strongly suggesting that Ca^{++} influx is required for this effect. Furthermore, to identify intracellular pathways we investigated Ca^{++} /calmodulin-dependent protein kinases that could be linked to signaling cascades known to alter the cytoskeleton in oligodendrocytes (Waggener et al., 2013). Blocking calmodulin (**CaM**) activation using the CaM antagonist W-7 completely inhibit process formation in TS2-expressing oligodendrocytes (Figure 5D), suggesting that CaM activation is the first step in the signaling cascade. Next, OPCs were treated with KN-93, a membrane-permeable inhibitor of the Ca^{++} /calmodulin-dependent protein kinase II (**CaMKII**). Blocking CaMKII drastically prevent process extension in OPCs expressing both, WT- and TS2-Cav1.2 channels (Figure 5D). Likewise, the myristoylated form of the autocamtide-2-related inhibitory peptide (Myr-AIP), a highly potent and specific substrate competitive inhibitor of CaMKII, negatively influence process development in WT- as well as in TS2-positive OPCs (Figure 5D). Thus, these results demonstrate that TS2 channels stimulate OPC process extension by Ca^{++} influx and that the activation of Ca^{++} /calmodulin-dependent protein kinase II is necessary for this effect.

We next used a co-culture system of OPCs with cortical neurons to analyze the role of TS2 L-type channels on the early phases of myelination. Twenty-four hours after transfection with plasmids encoding WT-Cav1.2 or TS2-Cav1.2 channels, OPCs were added to cortical neuron cultures prepared from the brains of mouse pups (Echeverria et al., 2005). After 7 days in vitro, we analyzed the interaction of OPCs with neurites and the morphological maturation of these cells, as described previously (Cheli et al., 2015a). Although in WT-Cav1.2 transfected cells, there was a change in cell morphology when co-cultured with cortical neurons, process formation and elaboration of membranous sheets were much less extensive than in TS2-Cav1.2 expressing cells (Figure 6A and C). Analysis of the morphological complexity showed that the expression of TS2-Cav1.2 channels in OPCs markedly decreased the percentage of cells with medium morphology and increased the proportion of cells with high complexity in co-culture (Figure 6B). Furthermore, the average number of contact points with neurites (Figure 6A and C, white arrowheads) as well as the length of primary and secondary processes, was significantly increased when the co-culture included TS2-Cav1.2 transfected OPCs (Figure 6D).

Abnormal postnatal myelination in the TS2 brain

To examine the effect of the Cav1.2 gain-of-function TS mutation in oligodendrocyte development and function in vivo, we analyzed myelin production as well as OPC maturation in the TS2 mice postnatally. We first performed immunohistochemical experiments in the corpus callosum, cortex and striatum of TS2 mice to evaluate the expression of myelin proteins and the density of myelinated oligodendrocytes at different

developmental time points. We found that the expression of myelin proteins (MBP and MOG) was significantly higher in the TS2 brains (Figure 7 and 8). At P15 as well as at P30, TS2 animals showed an increased number of MBP and MOG positive fibers in the cingulate cortex, higher fluorescence intensity for these myelin proteins in the central area of the corpus callosum, and more myelinated tracts in the striatum (Figure 7A, B and C). Similar results were observed in the corpus callosum, cortex and striatum of P60 TS2 animals (Figure 8A and B). We also collected total protein from the corpus callosum and cerebellum of TS2 mice to evaluate the expression of myelin proteins by western blot (Figure 8C). Consistent with the results described above, the expression levels of MBP, MOG and CNP were higher in TS2 brains than in control samples at P60 (Figure 8C).

We next used electron microscopy to evaluate the ultrastructure of axon and myelin integrity in the TS2 brains. The degree of myelination was analyzed in P30 mice by calculating the axon diameter, myelin thickness and mean *g*-ratio of myelinated axons, as described previously (Cheli et al., 2016) (Figure 9). In line with our previous results, a significant increase in the percentage of myelinated axons was found in the corpus callosum of TS2 animals (Figure 9A and D). *G*-ratios, a measure of myelin sheath thickness calculated as the ratio of axon diameter to myelinated fiber diameter, were also determined for TS2 mice and control littermates (Figure 9B, C and F). The mean *g*-ratio of myelinated axons from TS2 mice was significantly lower compared with controls, indicating that the myelin sheaths were thicker in TS2 mice (Figure 9C). Plotting *g*-ratios against axonal diameters demonstrated that the myelin thickness was higher in axons of all sizes (Figure 9B) and the distribution of *g*-ratio values clearly shows a shift to the left (lower values) (Figure 9F). Importantly, no axonal loss and/or degeneration, and no changes in the mean diameter of myelinated axons were found in the corpus callosum of TS2 mice (Figure 9E). These data indicate that myelination is increased in the brain of young TS2 mice, and suggest that our mouse model is a useful tool to explore the role of L-type Ca^{++} channels in oligodendrocyte maturation.

Increased number of mature oligodendrocytes in the TS2 mice

To further examine the effect of the TS2 mutation in OPC development, specific oligodendrocyte markers such as Olig2 and CC1 were used to measure oligodendrocyte numbers in vivo. No changes in the number of total oligodendrocytes (Olig2 positive cells) was found in the TS2 corpus callosum, cortex or striatum at P30 (Figure 10A and B), but we detected a substantial increase in the density of myelinating oligodendrocytes (CC1 positive cells) in these brain regions (Figure 10A and C). Likewise, at later developmental stages (P60), TS2 animals displayed a significant increase in the density of CC1 positive cells in the corpus callosum and cortex with no changes in the density of Olig2 positive cells (Figure 10E and F). To evaluate the proportion of mature oligodendrocytes as a fraction of the total population, we measured the percentage of Olig2 positive cells that stained positive for CC1. TS2 mice displayed an increased ratio of Olig2/CC1 double positive cells in the corpus callosum, cortex and striatum at P30 (Figure 10D), and in the corpus callosum and cortex at P60 (Figure 10G), suggesting an accelerated maturation rate of oligodendrocytes. We next evaluated OPC proliferation using the cell cycle marker Ki67 in combination with the oligodendrocyte marker Olig2 as well as the proportion of immature oligodendrocytes by

co-immunostaining cells with Olig2 and the transcription factor Olig1 (Figure 11). The corpus callosum, cortex and striatum of TS2 mice exhibited normal levels of Olig2/Ki67 and Olig2/Olig1 double positive cells at P30 as well as P60 (Figure 11A, B, E and F), indicating no alterations in the rate of OPC proliferation. Finally, in order to assess cell death, the proportion of Olig2/Caspase-3 double positive cells was quantified in the brain of P30 and P60 animals. In all experimental groups, the percentage of apoptotic oligodendrocytes was lower than 8% with no statistical differences between genotypes (Figure 11D and G). In summary, our results indicate that OPC proliferation and survival are not affected by the TS2 mutation, but TS2 OPCs yield more mature myelinating oligodendrocytes than control cells in the postnatal brain.

DISCUSSION

We recently found that Cav1.2 loss of function alters myelination in the postnatal brain, and that Ca^{++} influx facilitated by Cav1.2 is necessary for the development of oligodendroglial cells through the modulation of multiple cellular events, such as process extension and cell migration (Cheli et al., 2015a; 2016; Paez et al, 2007; 2009a; b; 2010). The goal of this work was to further determine the involvement of Cav1.2 channels in oligodendrocyte development and function in vivo, and to test whether Cav1.2 gain of function was sufficient to induce changes in oligodendrocyte maturation. To achieve this, we used a mouse model of Timothy syndrome (TS) in which the normal function of the Cav1.2 channel is altered by the TS2 point mutation (Bader et al., 2011). Timothy syndrome is a multisystem disorder characterized by cardiac arrhythmias, syndactyly, hypoglycemia and ASD. It is caused by a dominant mutation in an alternatively spliced exon of the *CACNA1C* gene, which encodes the $\alpha 1$ subunit Cav1.2 (Splawski et al., 2004; Krey et al., 2013). The mutation that causes TS results in a glycine-for-arginine substitution at position 406 in the first of three intracellular loops in Cav1.2, altering the ability of Cav1.2 to undergo voltage-dependent and Ca^{++} -dependent inactivation. This mutation thus presents an excellent opportunity to examine the importance of these biophysical properties in oligodendrocyte development and function.

TS2 OPCs displayed enhance maturation and Ca^{++} homeostasis alterations

In order to study the effect of this Cav1.2 gain-of-function mutation on oligodendrocyte development, we used primary cultures of OPCs isolated from the cortex of TS2 mice. The expression of Cav1.2 channels with the TS2 mutation was detected in OPCs by Ca^{++} imaging experiments, in which the cells were depolarized by high extracellular K^{+} and were treated with specific inhibitors for L-type Ca^{++} channels. We found that the TS mutation decreased the inactivation of the channel and increased the Ca^{++} rise triggered by depolarization of OPCs. Functionally, we demonstrated that TS2 OPCs matured earlier and extended longer primary and secondary processes than control cells. TS2 oligodendrocytes were positive for mature oligodendrocyte markers and produced high levels of myelin proteins in vitro. Importantly, the proliferation and survival of TS2 OPCs were not affected, suggesting that the expression of the mutant TS2 L-type Ca^{++} channel selectively affects maturation in these cells.

These results were confirmed in wild-type OPCs transfected with plasmids encoding the TS2-Cav1.2 channel. Relative to control cells, TS2-Cav1.2-transfected OPCs showed a ~128% rise in L-type Ca^{++} influx and, similar to OPCs isolated from the cortex of TS2 animals, presented a mature phenotype with longer processes and increased expression of differentiation markers compared to control oligodendrocytes. Furthermore, TS2-Cav1.2 expressing oligodendrocytes were more capable of interacting with cortical neurons in vitro than control or WT-Cav1.2 expressing OPCs. After seven days in the presence of cortical neurons, TS2-Cav1.2-expressing OPCs matured substantially and developed the classic morphology of myelinating oligodendrocytes with multiple parallel process along neighboring axons. OPCs expressing the WT-Cav1.2 channel displayed a ~24% increase over endogenous L-type Ca^{++} channel activity, but unlike TS2-Cav1.2-expressing cells, these OPCs showed no significant morphological changes and no important modifications in the expression of developmental markers compared to control cells. Collectively, these results demonstrate that increased Ca^{++} influx mediated by the TS2 Ca^{++} channel promotes OPC development in vitro and that L-type channels are key regulators of oligodendrocyte maturation.

In neurons, a major function of Cav1.2 is to regulate dendritic extension and retraction in response to electrical activity (Redmond et al., 2002; Wong and Ghosh, 2002; Krey et al., 2013). Bolsover (2005) have shown that Ca^{++} -sensitive sites that evoke processes elongation in neurons, are located close to L-type channels. Furthermore, the C-terminus region of Cav1.2 channels can translocate to the nucleus and act as a transcription factor (Gomez-Ospina et al., 2006). This factor modulates the transcription of several genes and promotes neurite outgrowth (Gomez-Ospina et al., 2013). We have established that the expression of the mutated TS2 Ca^{++} channel induces the extension of primary and secondary processes in OPCs. In previous publications, we have shown that the activation of L-type channels induces similar morphological changes in OPCs, as well as in immature neurons (Cheli et al. 2015a; b). For example, activation of L-type Ca^{++} channels triggers OPCs morphological maturation and stimulates myelin protein expression in vitro. Conversely, blocking the expression of Cav1.2 channels with specific siRNAs prevents the morphological differentiation of primary OPCs (Cheli et al., 2015a).

In this work, we have found that TS2 channels stimulate OPC process extension through the activation of Ca^{++} /calmodulin-dependent protein kinase II, an event initiated by voltage-gated Ca^{++} influx. A role of CaMKII in regulating oligodendrocyte morphology via stabilization of the actin cytoskeleton was recently proposed (Waggener et al., 2013). Furthermore, in vivo analysis of developmental myelination has demonstrated that CaMKII is involved in the regulation of myelin thickness (Waggener et al., 2013). It has been proposed that activation of CaMKII serves to trigger a period of dramatic cytoskeleton reorganization following Ca^{++} influx (McVicker et al., 2015). For example, profilin (Ryan et al., 2005) as well as cofilin (Xie et al., 2007) can be phosphorylated by CaMKII through independent pathways, being the net effect of both an enhancement of actin polymerization. Additionally, CaMKII has recently been shown to bind to microtubules near active synapses, indicating that CaMKII may play a role in the reorganization of both the actin and microtubule cytoskeleton during processes growth (Lemieux et al., 2012). Since process elongation in OPCs is crucial for myelin protein synthesis and for the initial stages of

myelination (Bercury and Macklin, 2015), it is possible that the earlier morphological maturation of TS2 OPCs induced by an overactive L-type Ca^{++} channel and the concomitant activation of CaMKII stimulate the synthesis of myelin proteins and the full differentiation of these cells.

Increased myelination during the postnatal development of the TS2 brain

Brain imaging studies of autism developmental abnormalities often report an increased total brain volume (Hazlett et al., 2005) and early acceleration in brain growth (Nicolson and Szatmari, 2003). Studies examining the differential contributions of gray and white matter to this abnormal growth in autistic patients have detected a significant increase in myelin production (Nicolson and Szatmari, 2003). White matter excesses in autism have been interpreted in functional neuroanatomical terms as suggesting that brain connectivity may be impaired in regions showing volume excesses. The abnormally rapid growth of the brain overall, observed during early infancy, may be the result of an abnormal myelination process during childhood (Carmody and Lewis, 2010, Gozzi et al., 2012). As such, it is plausible that excesses in white matter may be attributable to an overproduction of myelin in infancy, an abnormality in myelin packing, or anomalies in the production or anatomical distribution of oligodendrocytes that produce myelin throughout the white matter.

Given the role of Cav1.2 in oligodendrocyte differentiation and myelination, the overproduction of myelin in the brain of autistic patients and the high penetrance of the Cav1.2 mutation in causing autism, we analyzed myelin production as well as oligodendrocyte maturation in the postnatal TS2 brain. We found that the expression of myelin proteins was significantly higher in TS2 brains at several postnatal time points. TS2 animals showed an increased number of MBP and MOG positive fibers in the cingulate cortex and in the central area of the corpus callosum, as well as more myelinated tracts in the striatum. These data were confirmed by western blot experiments for several myelin proteins performed with total protein lysates isolated from different brain regions.

Immunohistochemical analysis for oligodendrocyte stage-specific markers exhibited a similar outcome with the TS2 line showing an increased number of myelinating oligodendrocytes in the corpus callosum, as well as in the cortex. Importantly, no changes in the density of immature oligodendrocytes and in the rate of OPC proliferation was found, suggesting that the overproduction of myelin in TS2 brains is not a consequence of an enlarged OPC population. In line with these results, the corpus callosum of TS2 animals presented a significant increase in the percentage of myelinated axons and a substantial decrease in the mean *g*-ratio of myelinated axons, suggesting that more myelin per internode is being made by TS2 oligodendrocytes.

L-type Ca^{++} channels are also important mediators of neuronal function. Neuronal Cav1.2 channels trigger neurotransmitter release, generate dendritic Ca^{++} spikes and induce activity-dependent gene regulation (Simms and Zamponi, 2014). It was recently reported that Cav1.2 channels with the TS alteration cause activity-dependent dendrite retraction in rat and mouse neurons and in induced pluripotent stem cell-derived neurons from individuals with Timothy syndrome (Krey et al., 2013). We thus cannot completely exclude the possible contribution of abnormal neuronal development in the observed myelination changes in TS2 brains. Our

in vitro experiments, however, show that perinatal OPCs isolated from the cortex of TS2 mice have increased L-type Ca^{++} channel activity and mature earlier than control cells. Moreover, oligodendrocytes expressing TS2-Cav1.2 channels were better able to interact with wild-type cortical neurons in vitro as compared to control cells. As these experiments were performed in the absence of TS2 neurons, our data suggests that TS2 channels are affecting OPC development in a cell autonomous manner. We propose the general hypothesis that enhanced myelination, as well as increased oligodendrocyte differentiation, is a key event in the pathogenesis of TS. Consequently, our findings will significantly contribute to our understanding of the neuropathology of TS in particular and the role of L-type Ca^{++} channels in brain development in general.

Acknowledgments

NIH/NINDS grant 5R01NS078041 and National Multiple Sclerosis Society Grant RG4554-A-2. We thank Dr. Laura Feltri and Edward Hurley (Hunter James Kelly Research Institute at UB) and Ted Szczesny (Director of the electron microscopy lab, Department of Pathology and Anatomical Sciences, Jacobs School of Medicine and Biomedical Sciences at UB) for assistance with the electron microscopy experiments.

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Main Points

- Elevated activity of the L-type Ca^{++} channel Cav1.2 promotes oligodendrocyte maturation.
- Oligodendrocyte development and myelination is enhanced in a mouse model of Timothy syndrome.

FIGURE 1. Ca⁺⁺ imaging in TS2 oligodendrocytes

(A) L-type Ca⁺⁺ channels activity was examined in OPCs from control and TS2 mice using high K⁺ (50mM). Fura-2 images were obtained at 2s intervals for a total of 4min. An increased fura-2 fluorescence ratio is indicated by warmer colors. Scale bar = 80μm. (B) Ca⁺⁺ influx was stimulated in control and TS2 cells using high K⁺ (50mM) in the presence of nifedipine (5μM), verapamil (5μM) and zero Ca⁺⁺ medium (-Ca⁺⁺). The bar graph shows the average amplitude of the Ca⁺⁺ response, calculated from the responding cells expressed as a percentage of change of the emission intensities. (C) Average Ca⁺⁺ responses in control and TS2 OPCs after depolarization with 50mM K⁺ (n = 50 cells per condition). (D) Representative Ca⁺⁺ traces from individual control and TS2 OPCs are shown. (E) Percentage of responding cells in control and TS2 cultures. (F) Ca⁺⁺ influx was stimulated in control and TS2 cells using glutamate (25μM) and ATP (25μM). The bar graph shows the average amplitude of the Ca⁺⁺ response, calculated from the responding cells expressed as a percentage of change of the emission intensities. Values are expressed as mean ± SEM of at least four independent experiments. **p<0.01 vs. control.

FIGURE 2. Maturation assessment of control and TS2 OPCs in vitro

(A and B) Two days after mitogen withdrawal, OPCs were stained with antibodies against Olig2, Olig1, NG2, CC1 and MOG and the percentage of positive cells in each experimental condition was examined by confocal microscopy. Representative pictures of control and TS2 cultures immunostained for NG2, CC1 and MOG are shown. Scale bar = 80μm. (C) OPCs were stained with antibodies against Olig2, Ki67 and caspase-3 and the percentage of Olig2/Ki67 and Olig2/caspase-3 double positive cells was examined by confocal microscopy. (D) Representative pictures of control and TS2 cultures immunostained for MBP at 2, 3 and 4 days in vitro (DIV). Scale bar = 40μm. (E) Morphological complexity of MBP-positive cells was scored in four categories. (F) Average primary and secondary processes length in control or TS2 MBP-positive cells (n = 50 cells per condition). Values are expressed as mean ± SEM of at least four independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs. control.

FIGURE 3. The effect of TS2-Cav1.2 channels on WT OPCs Ca⁺⁺ influx

(A) Average Ca⁺⁺ responses in OPCs expressing WT-Cav1.2 or TS2-Cav1.2 after depolarization with high K⁺ (50mM) (n = 50 cells per condition). (B) Representative Ca⁺⁺ traces from individual OPCs expressing either WT-Cav1.2 or TS2-Cav1.2 are shown. (C) Ca⁺⁺ influx was stimulated in control OPCs and in cells expressing WT-Cav1.2 or TS2-Cav1.2 channels using high K⁺ (50mM) with and without 5μM of nifedipine. The bar graph shows the average amplitude of the Ca⁺⁺ response, calculated from the responding cells expressed as a percentage of change of the emission intensities. Values are expressed as mean ± SEM of at least four independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs. control; ##p<0.01 vs. WT-Cav1.2.

FIGURE 4. The role of TS2-Cav1.2 channels on WT OPC development

(A and B) Two days after mitogen withdrawal, the percentage of YFP/Olig2, YFP/Olig1, YFP/NG2, YFP/CC1 and YFP/MBP double positive cells was determined in control, WT- and TS2-Cav1.2 transfected cultures. Representative pictures of WT- and TS2-Cav1.2 transfected cultures immunostained for NG2, CC1 and YFP are shown. Scale bar = 60μm. (C) OPCs were stained with antibodies against Ki67, caspase-3 and YFP and the percentage of YFP/Ki67 and YFP/caspase-3 double positive cells was examined by confocal microscopy. (D) Morphological complexity of YFP-positive cells was scored in four categories. (E) Average primary and secondary processes length in control, WT- and TS2-Cav1.2 transfected OPCs (n = 100 cells per condition). Scale bar = 60μm. Values are expressed as mean ± SEM of at least four independent experiments. **p<0.01, ***p<0.001 vs. control; #p<0.05, ##p<0.01 vs. WT-Cav1.2.

FIGURE 5. TS2-Cav1.2-mediated process extension is dependent of Ca^{++} /calmodulin protein kinase II activation

(A) Representative time course images of WT- and TS2-Cav1.2 transfected oligodendrocytes. White arrows mark processes that extend and yellow arrows mark processes that retract relative to the previous time point. Scale bar = 40 μm . (B) Change in total process outgrowth in oligodendrocytes expressing WT-Cav1.2 or TS2-Cav1.2 over 6h. (C) Percentage of processes with an increase (extended) or decrease (retracted) in length > 5 μm after 6h in **Basal** (2mM Ca^{++}) and in 0.2mM Ca^{++} solution (**-Ca $^{++}$**). (D) Percentage change in total process length in WT- and TS2-Cav1.2 expressing oligodendrocytes after 6h in **Basal** (2mM Ca^{++}), 0.2mM Ca^{++} solution (**-Ca $^{++}$**) and in the presence of inhibitors for **CaM** (W-7, 2.5 μM) and **CaMKII** (KN-93, 10 μM ; Myr-AIP, 10 μM). Results are the means $\pm$ SEM of at least 24 cells per experimental condition. * $p < 0.05$, ** $p < 0.01$ vs. WT-Cav1.2.

FIGURE 6. Co-culture of cortical neurons and TS2-Cav1.2 expressing OPCs

(**A and C**) 24h after transfection, control, WT-Cav1.2 and TS2-Cav1.2 expressing OPCs were co-cultured with cortical neurons for 7 days. Representative pictures of WT- and TS2-Cav1.2 transfected oligodendrocytes immunostained for YFP are shown. Arrows indicate oligodendrocyte-neurite contact points. Scale bar = 40μm. (**B**) Morphological complexity of YFP-positive cells was scored in four categories. (**D**) The average primary and secondary processes length and the number of contact points with neurites was analyzed in control, WT-Cav1.2 and TS2-Cav1.2 transfected cells. Results are the means ± SEM for three independent experiments. >200 cells were analyzed per experimental condition. **p<0.01 vs. control, #p<0.05, ##p<0.01 vs. WT-Cav1.2.

FIGURE 7. Evaluation of myelin proteins expression in the postnatal TS2 mouse
 (A) MBP and MOG immunostaining in the brains of control and TS2 mice at P30. Representative brain coronal sections are shown. Scale bar = 180 μ m. (B and C) Myelin was quantified by analyzing fluorescence intensity of MBP and MOG in the central area of the corpus callosum (CC), in the cingulate cortex (CX) and in the striatum (ST) at P15 and P30. Five brains per experimental condition were analyzed and values are expressed as mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$ vs. respective controls.

FIGURE 8. Evaluation of myelin proteins expression in the postnatal TS2 mouse

(A) MBP and MOG immunostaining in the brains of control and TS2 mice at P60.

Representative brain coronal sections are shown. Scale bar = 180µm. (B) Myelin was quantified by analyzing fluorescence intensity of MBP and MOG in the central area of the corpus callosum (CC), in the cingulate cortex (CX) and in the striatum (ST) at P60. (C)

Total proteins were collected from the corpus callosum and cerebellum (CB) at P60 to assess the expression of myelin proteins by western blot. Representative western blots are shown. GAPDH was used as the internal standard and data from five independent experiments are summarized based on the relative spot intensities and plotted as percent of controls. Five brains per experimental condition were analyzed and values are expressed as mean ± SEM.

*p<0.05, **p<0.01 vs. respective controls.

FIGURE 9. Electron microscopy of the TS2 corpus callosum

(A) Electron micrographs of axons in the corpus callosum of control and TS2 mice at P30. Scale bar = 8μm upper panel; 2μm lower panel. (B and C) Scatter plot and mean *g*-ratio values of control and TS2 mice. (D) Percentage of myelinated axons of control and TS2 mice. (E) Mean diameter of myelinated axons in control and TS2 mice. (F) Distribution of *g*-ratio values. Values are expressed as mean ± SEM. Five animals per experimental group and 300 fibers per animal were analyzed. **p*<0.05, ****p*<0.001 vs. control.

FIGURE 10. Analysis of oligodendrocyte maturation in the TS2 brain

(A) Representative brain coronal sections of control and TS2 mice at P30 immunostained with anti-Olig2 and anti-CC1 antibodies. Scale bar = 180µm. (B, C, E and F) The number of Olig2 and CC1 positive cells was stereologically quantified in the central area of the corpus callosum (CC), in the cingulate cortex (CX) and in the striatum (ST) of control and TS2 mice at P30 and P60. (D and G) The percentage of Olig2/CC1 double positive cells was measured in the same brain areas. Five brain per experimental condition were analyzed and values are expressed as mean ± SEM. *p<0.05, **p<0.01 vs. respective controls.

FIGURE 11. Examination of OPC proliferation and survival in the TS2 brain

(A) Representative brain coronal sections of control and TS2 mice at P30 immunostained with anti-Olig2, anti-Ki67 and anti-Olig1 antibodies. Scale bar = 180µm. (B–G) The number of Olig2/Ki67, Olig2/Olig1 and Olig2/caspase-3 double positive cells was stereologically quantified in the central area of the corpus callosum (CC), in the cingulate cortex (CX) and in the striatum (ST) of control and TS2 mice at P30 and P60. Five brain per experimental condition were analyzed and values are expressed as mean $\pm$ SEM.