Published in final edited form as:

Cell Tissue Res. 2012 August ; 349(2): 459–471. doi:10.1007/s00441-012-1455-z.

Ablation of TNAP function compromises myelination and synaptogenesis in the mouse brain

János Hanics,

Department of Anatomy, Histology and Embryology, Semmelweis University, Budapest, Hungary

János Barna,

Department of Anatomy, Histology and Embryology, Semmelweis University, Budapest, Hungary

Jinsong Xiao,

Centre de Recherche Cerveau et Cognition, CNRS and Université de Toulouse, UPS, Toulouse, France. Zhongnan Hospital of Wuhan University, Hubei, People's Republic of China

José Luis Millán,

Sanford Children's Health Research Center, Sanford-Burnham Medical Research Institute, La Jolla, CA, USA

Caroline Fonta, and

Centre de Recherche Cerveau et Cognition, CNRS and Université de Toulouse, UPS, Toulouse, France. CerCo, UMR5549, Pavillon Baudot, CHU Purpan, BP 25202, 31052 Toulouse Cedex, France

László Négyessy

Department of Theory, Institute for Particle and Nuclear Physics, Wigner Research Centre for Physics, Hungarian Academy of Sciences, P.O. Box 49, Budapest 1525, Hungary

Caroline Fonta: caroline.fonta@cerco.upstlse.fr; László Négyessy: negyessy.laszlo@wigner.mta.hu

Abstract

Mutations in the tissue-nonspecific alkaline phosphatase (TNAP) gene can result in skeletal and dental hypomineralization and severe neurological symptoms. TNAP is expressed in the synaptic cleft and the node of Ranvier in normal adults. Using TNAP knockout (KO) mice (*Akp2*^{-/-}), we studied synaptogenesis and myelination with light- and electron microscopy during the early postnatal days. Ablation of TNAP function resulted in a significant decrease of the white matter of the spinal cord accompanied by ultrastructural evidence of cellular degradation around the paranodal regions and a decreased ratio and diameter of the myelinated axons. In the cerebral cortex, myelinated axons, while present in wild-type, were absent in the *Akp2*^{-/-} mice and these animals also displayed a significantly increased proportion of immature cortical synapses. The results suggest that TNAP deficiency could contribute to neurological symptoms related to myelin abnormalities and synaptic dysfunction, among which epilepsy, consistently present in the *Akp2*^{-/-} mice and observed in severe cases of hypophosphatasia.

Keywords

Alkaline phosphatase; Electron microscopy; Node of Ranvier; Hypophosphatasia; Epilepsy

© Springer-Verlag 2012

Correspondence to: Caroline Fonta, caroline.fonta@cerco.upstlse.fr; László Négyessy, negyessy.laszlo@wigner.mta.hu.

János Hanics and János Barna equally contributed to data collection and analyses.

Introduction

The severe form of hypophosphatasia, an inborn-error-of-metabolism caused by mutations of the gene (*ALPL* in humans and *Akp2* in mice) expressing the tissue-nonspecific isozyme of alkaline phosphatase (TNAP), results in life-threatening diseases during early developmental periods (Whyte et al. 1995). TNAP has an important function in skeletal tissues where it is involved in mineralization (Hessle et al. 2002; Wennberg et al. 2000). However, the most severe forms of hypophosphatasia also manifest neurological symptoms, most notably epilepsy (Baumgartner-Sigl et al. 2007; Henthorn et al. 1992; Whyte 1995). Importantly, a recent investigation found the expression of the bone-type TNAP mRNA by neural cells in the brain of different species including humans (Brun-Heath et al. 2011). In addition, a putative functional role for TNAP during neuronal development is supported by the observations of TNAP activity in neural structures (in addition to the vascular localization) of rats (Sugimura and Mizutani 1979; Zisapel and Haklai 1980), monkeys (Fonta and Imbert 2002; Fonta et al. 2004, 2005; Friede 1966), and human (Negyessy et al. 2011). However, the specific biological role of TNAP in the brain is currently unknown.

Several substrates (vitamin B6, nucleotides, phosphoproteins) and interacting molecules (collagen, PrPc) of TNAP have been found, suggesting that this enzyme can have multiple possible functions in the brain. The glycoprotein TNAP is an ectozyme with phosphomonoesterase activity, which hydrolyzes pyridoxal 5'-phosphate (PLP), a B6 vitamer (Whyte et al. 1985). Consequently, TNAP activity has been implicated in the metabolism of different neurotransmitters, such as gamma-aminobutyric acid (GABA) or serotonin, whose synthesizing enzymes utilize PLP as the cofactor (Amadasi et al. 2007). Human neonates presenting hypophosphatasia exhibit functional deficiency in brain aromatic-L-amino acid decarboxylase (AADC) enzyme required for the synthesis of dopamine and serotonin (Balasubramaniam et al. 2010). TNAP is also potentially involved in purinergic transmission by producing nucleotide receptor substrates through the degradation of ATP to adenosine (Abbracchio et al. 2009). In addition, TNAP may be involved in extracellular matrix and cell membrane interactions through its collagen binding domain (Bossi et al. 1993) or its extracellular phosphatase activity (Diaz-Hernandez et al. 2010; Ermonval et al. 2009). Moreover, the finding of Ermonval and collaborators (Ermonval et al. 2009) demonstrates that TNAP is associated to the cellular prion protein in rafts of differentiated bio-aminergic neurons. In addition, the localization of TNAP in the synaptic cleft (Fonta et al. 2004) and nodes of Ranvier (Fonta et al. 2005) supports the role of TNAP in the regulation of neuronal activity in the cerebral cortex. All these findings suggest a functional role for TNAP in neuronal transmission and homeostasis (Ermonval et al. 2009).

There is also evidence suggesting a role for TNAP in neuronal plasticity and development. Visual deprivation studies suggest that expression of TNAP activity depends on neuronal activity in the primate visual cortex (Fonta et al. 2005) raising the possibility that TNAP is involved in activity-dependent neurodevelopmental processes. It was also shown that TNAP activity expresses a complementary pattern to that of myelin staining and gradually disappears as myelination proceeds during the development of the white matter in the primate brain (Fonta et al. 2005). A recent in vitro study (Diez-Zaera et al. 2011) showing that TNAP is involved in the control of axon extension and branching of hippocampal neurons adds further evidence for the possible role of TNAP in the maturation of neural connections and pathways. Interestingly, TNAP also regulates the differentiation of neural stem cells in vitro (Kermer et al. 2010).

Severe hypophosphatasia is phenocopied in *Akp2* knockout mice, null for TNAP activity (Narisawa et al. 1997). *Akp2*^{-/-} mice are viable only for about 1–2 weeks after birth and, beside the characteristic defects in skeletal mineralization, these animals develop epileptic seizures prior to death, which have been linked to a deficiency in PLP and GABA metabolism (Narisawa et al. 2001; Waymire et al. 1995). Here, we have studied the structural organization of the central nervous system of the *Akp2*^{-/-} mice at different postnatal ages both at the light and electron microscopic levels in search for further insights into the biological role of TNAP during brain myelination and synaptogenesis. Myelination was studied in the spinal cord where it proceeds prenatally and has a more advanced development compared to the cerebral hemispheres, and in the cerebral cortex where myelination initiates after birth in rodents (Coffey and McDermott 1997; Foran and Peterson 1992). Synaptogenesis in the cerebral cortex of different species, including the mouse (Li et al. 2010), also takes place mainly postnatally. Preliminary findings have been published in abstract form (a, Barna et al. 2009b; Xiao et al. 2006, 2007).

Materials and methods

Animals and histology

Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 1996), European Directive 86/609 and the guidelines of the local institutional animal care and use committee. The study was approved by the Regional (Midi Pyrénées) Ethics Committee (ref. no. MP/01/32/10/09). Inactivation of the mouse *Akp2* gene to generate TNAP null mice has been previously described (Narisawa et al. 1997). Animals were genotyped using dedicated primers (mTNAP-3': 5'-AGT CCG TGG GCA TTG TGA CTA-3', mTNAP-5': 5'-TGC TGC TCC ACT CAC GTC GAT-3').

Light microscopic analyses included 16 wild-type (WT) and 16 knockout (KO) mice aged from postnatal days 1 to 10 (P1 to P10; Table 1) perfused transcardially with a fixative containing 4 % paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Coronal sections 40 µm thick were cut from the cervical spinal cord with a freezing microtome. They were stained for myelin according to a protocol modified from Gallyas (1979) described in Nagyessy et al. (2011) to reveal the white matter. Additional staining was made by cresyl violet to reveal cytoarchitecture in the gray matter. TNAP activity was detected by histochemistry on the neighboring sections, using 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt (BCIP) as substrate and nitro-blue tetrazolium chloride (NBT) as chromogenic in a 0.1 M Tris solution pH 9.5 (Fonta and Imbert 2002; Fonta et al. 2004).

Electron microscopic analyses included 4 WT and 4 KO animals of different postnatal ages (P2, P7, and P8), which were transcardially perfused with a fixative containing 4 % paraformaldehyde and 2 % glutaraldehyde in PB. Coronal, vibratome sections were cut from the cervical spinal cord while the parietal cortex was cut in the parasagittal plane, both at 60 µm. The sections were washed, osmicated, dehydrated in ascending series of ethanol, and flat-embedded in Durcupan ACM (Fluka, Buchs, Switzerland). Studies were performed on semi- and ultrathin sections (1 µm and ~60 nm thick in that order) after staining with toluidin blue azure II and lead citrate, respectively (for more details of the techniques used, see Nagyessy et al. 1995; Fonta et al. 2004, 2005).

Quantitative analyses

Light microscopy analyses—Image analysis was performed by using Mercator software (Explora Nova®) on photos acquired with a Leica microscope DMR, equipped with a CCD camera interfaced with an IBM compatible computer. Percentage of the white matter (WM%) in the spinal cord was calculated at 7 different postnatal ages (P1, P2, P4, P6, P7,

P8, and P10) in the WT and KO mice from measurements made on several myelin-stained sections per animal (Table 1; Fig. 1a). The total cross-sectional area of the spinal cord and the gray matter were hand delimited and the surface area values were automatically obtained. The WM% was calculated relative to the total cross sectional area of the spinal cord sections. Growth rate was calculated by subtracting the 1st postnatal day (P1) cross-section area value from the one measured at a given postnatal day and divided by the P1 value.

Electron microscopy analyses—A Jeol 1010 transmission electron microscope equipped by a Morada digital camera (Olympus) was used. Qualitative and quantitative electron microscopic analyses of the KO and WT mice were made on P2, P7, and P8 on digital images. Measurements were made by ImageJ (Rasband 1997–2011) and analySIS (Olympus) softwares. Qualitative ultrastructural examinations led us to classify the stage of myelination into 3 types of fibers as follows: Class I fibers were encapsulated by processes of oligodendrocytes containing cytoplasmic substance, Class II fibers distinguished by the additional appearance of the compact myelin lamellae around the processes (i.e. forming two layers around the axolemma), and Class III fibers were encapsulated by matured, laminated myelin sheath only (Fig. 2a–e). The 3 classes of fibers were counted in a $64\text{-}\mu\text{m}^2$ area of an ultrathin section of each of the 6 different cases (3 ages P2, P7, P8 in the WT and KO, one animal per case) by capturing 50 electron microscopic images per animal at 20 K magnification (resulting in the $64\text{-}\mu\text{m}^2$ field of view on each image) in the ventral fascicle of the cervical segment of the spinal cord (25–25 shots were taken from the left and right fascicles, respectively). Altogether, we counted 100,877 axons, 41,641 in the KO and 59,236 in the WT (Table 2).

Myelinated axons of the spinal cord were further studied by measuring cross-sectional areas of the three classes of myelinated axons selected randomly in two KO and two WT mice at P7. We used 30 electron microscopic images of 20 K magnification from each animal. The images were overlaid with a $2\times 2\text{ }\mu\text{m}^2$ grid and axons (three axons/image from each of the three classes) were sampled in randomly chosen squares of the grid (altogether $30\times 4\times 3\times 3=1080$, axons). The following variables were measured: (1) total cross-sectional area of the myelinated axons (all classes), (2) cross sectional area of the bare axon (all classes), (3) cross-sectional area of the oligodendrocytic processes around the axons (Class I, II), and (4) cross-sectional area of the myelin sheath (Class II, III) (Fig. 2e). All the areas were hand delimited. Finally, the square root of the measured areas was computed for calculating the g-ratio (ratio of the size of the axon to the size of the whole fiber on the cross-sections) and also for statistical comparisons.

Synaptogenesis was quantified in the parietal cortex of 6 animals (2 KO and 2 WT at P7 and one mouse per group at P8) by classifying synaptic contacts into 2 groups. Synapses exhibiting well-developed pre- and postsynaptic membrane specializations, clear synaptic cleft, and numerous synaptic vesicles were classified as matured (Fig. 6). Immature synapses were characterized by noticeably thinner synaptic membrane specializations and by either the lack or a low number and scattered distribution of synaptic vesicles (Fig. 6) (Li et al. 2010). Synapses were counted on 50 electron microscopic images per animal at 20 K magnification. The number of synapses is presented in Table 3. To confirm that our synaptic classification was not the result of a tangential cut, 11 and 7 synaptic contacts were also reconstructed in 3D from serial sections in the KO and WT, respectively, at P7 by using the Reconstruct software (Fiala 2005). The series included the total extent of the postsynaptic densities.

Statistics

Spinal cord growth and white matter maturation of the WT and KO mice were compared by linear regression and analyses of correlation. Non-parametric Mann–Whitney test was applied to compare growth rates. All other comparisons (total cross-sectional areas, cross-sectional areas of the bare axons, oligodendritic processes, myelin sheath, ratio of bare axon/total section area and synapse numbers) were made by ANOVA and Student's *t* test.

Results

Spinal cord

The central nervous system of the *Akp2*^{−/−} mice did not exhibit major macro-anatomical alterations (for the spinal cord, see Fig. 1a). Both in the KO and WT mice, the size of the spinal cord measured by the cross-sectional area, linearly increased with age ($p < 0.01$, linear regression, WT: $r = 0.86$; KO: $r = 0.83$) (Fig. 1b). Due to the constant growth shown on Fig. 1b, the average cross-sectional area was 1.6 and 1.7 times higher at P10 than at P1 in the KO and WT, respectively. However, spinal growth exhibited a slower growing rate in the KO than in WT mice as indicated by the shallower slope of the KO trend line (dashed line) in Fig. 1b. This slowing of the spinal growth of the KO group was especially obvious after the fourth postnatal day (Fig. 1b). Accordingly, the spinal cross-sectional area was significantly smaller in the KO than in the WT mice when ages P6–10 were grouped together ($p = 0.014$, Mann–Whitney *U* test). In contrast, grouping younger animals of ages P1–4 did not exhibit significant difference between the spinal cross-sectional areas of the KO and WT mice ($p = 0.85$, Mann–Whitney *U* test).

The developmental growth of the spinal cross-sectional area resulted in an increase in the size of both the gray matter and the white matter (Fig. 1c). On average, in the WT and KO mice, the cross-sectional area of the gray matter increased similarly by 52 and 50 %, respectively, as shown by the growth rate at P10 (Fig. 1c). However, the white matter of the WT exhibited a much higher growth rate than that of the KO animals at P10 (117 vs. 83 %, respectively) (Fig. 1c). At the same time, the age-dependent increase of WM amount strongly correlated with the increasing size of the spinal cross-sections during the postnatal days studied (WT: $r = 0.98$; KO: $r = 0.86$). We have therefore calculated the relative amount of the white matter (WM%) and plotted it as a function of age (Fig. 1d). Figure 1d indicates that the WM% linearly increased in the case of both the KO and WT animals ($p < 0.01$, linear regression, WT: $r = 0.8$; KO: $r = 0.7$). However, the difference of the slopes indicates a considerably decreased growth of WM% in the KO (slope of 0.7) when compared to the WT mice (slope of 1.4) (Fig. 1d). The smaller increase of the WM% of the KO relative to the WT mice was especially evident after P4/P6 (Fig. 1d).

The fine structure of the spinal white matter appeared normal at a superficial examination except that the shape of axonal cross-sections appeared more irregular in the KO than in the WT mice (Fig. 2a–d). However, quantitative electron microscopic examination showed that the total density of myelinated fibers (i.e. including Classes II and III) in the KO mice was significantly lower at P2 and gradually reached the level found in the WT mice by P8 (Fig. 2f; Table 2). Considering myelination, the number and proportion of the different types of axonal processes (Fig. 2e) changed differently in the WT and KO mice (Fig. 2f). At P2, the ratio of myelinated fibers calculated by pooling the number of Class II and III fibers (as opposed to non-myelinated Class I fibers), was twice as much in the WT (20 %) than in KO mice (10 %). Of the myelinated fibers (Class II and III together), more than 92 % were found belonging to class II in the WT mice. No mature myelinated fibers were found in KO mice (Fig. 2f). At P8, a similar ratio of myelinated fibers was observed both in the WT and KO mice (80 and 77 %, respectively). However, the ratio of Class III fibers in the WT (27

%) was double that of the KO mice (14 %), suggesting a delay in myelination as a result of *Akp2* gene inactivation (Fig. 2f).

Myelination was characterized by the g-ratio calculated as the ratio of the square root of the size of the cross-section area of the axonal processes itself (marked by A in Fig. 2e) to the square root of the total cross-section area including oligodendrocytic processes (marked by O in Fig. 2e) in the case of Class I and II axons, and/or the matured myelin sheath engulfing the axons (marked by M in Fig. 2e) in the case of Class II and III axons. At P7, significant changes of this ratio were found in all three classes of fibers. In the case of Class I fibers, the ratio increased from 0.67 in the WT to 0.7 in the KO mice ($p=0.002$, one-way ANOVA). Similarly, g-ratio was significantly smaller in the WT (0.71) than in the KO mice (0.73) ($p=0.02$, one-way ANOVA). In contrast, g-ratio decreased in the case of Class III axons from 0.78 in the WT to 0.76 in the KO ($p<0.01$, one-way ANOVA). In Class I fibers, the difference of the ratio was associated with the decreased thickness of the oligodendrocytic processes in the KO as compared to the WT mice ($0.53 \pm 0.37 \mu\text{m}^2$ vs. $0.84 \pm 0.9 \mu\text{m}^2$) respectively ($p<0.001$, student t-test). In Class III fibers the thickness of the neuronal process, i.e. the axon, was decreased in the KO ($1.68 \pm 0.85 \mu\text{m}^2$) compared to that of WT mice ($1.96 \pm 0.96 \mu\text{m}^2$) ($p=0.048$, Student's *t* test). In contrast, the thickness (i.e. area) of the myelin sheath of Class III axons was not statistically different between KO and WT mice. In the case of Class II fibers, axonal thickness tended to be increased in the KO when compared to the WT mice; however, the difference was not statistically different.

In addition structural abnormalities of myelination reminiscent of cellular degradation were frequently observed around the para- and juxtanoal regions of the nodes of Ranvier in the KO mice (Fig. 3c–f). Similar ultrastructural alterations were not found in the WT mice (Fig. 3a, b). Some of the affected axonal regions were serially cut to see whether degeneration affected only the nodal including para- and juxtanoal regions. Cellular degradation was apparently localized in the cytoplasm of the oligodendrocytes (Fig. 4) and was characterized by the presence of lysosomes, endosomes and multivesicular bodies in the para and/or juxtanoal regions (Fig. 3, 4).

In summary, in the spinal cord, TNAP gene deletion resulted in an alteration specific of the white matter. It was the most pronounced from the fourth day of postnatal life. The volumetric decrease of the white matter amount at the light macroscopic level was accompanied by a decreased ratio of the myelinated fibers, the thinning of the myelin sheath, the decrease in axon size and degradation of myelin in the nodal regions at the ultrastructural level, which all suggest a delay or inhibition of spinal cord myelination.

Cerebral cortex

The laminar organization of the parietal cortex of the KO mice was similar to that of WT mice in each age group (P2, P7, P8) examining toluidin blue azure II-stained semithin sections (P2 is shown in Fig. 5a, b). However, at the ultra-structural level, myelination and synaptic organization appeared disrupted in the KO animals.

At P2 of the WT cases, myelination of some axonal fibers had just initiated (Fig. 5c). In contrast, signs of engulfing the axonal fibers by myelin sheath were not observed in the KO mice. Myelinated axonal process-like structures were rarely observed in the KO animals. In contrast, we observed disorganized structures such as that illustrated in Fig. 5d with myelin-like formation surrounding a deteriorated membranous structure. At later ages (P7–8), myelinated fibers could be easily found in the WT mice (Fig. 5e, g). However, in the KO mice of the same age, only a few myelinated axons were found (Fig. 5f), which exhibited abnormal structural properties as illustrated in Fig. 5h.

Considering synaptogenesis, contacts of recognizable synaptic membrane specializations were not found at P2 in the cortex of the WT and KO mice. At P7 and P8, numerous synaptic contacts could be observed in both groups (Fig. 6a, b). However, compared to the WT mice, the characteristic features of the synapses such that the pre- and postsynaptic membrane specializations, as well as the presence, density, and arrangement of the synaptic vesicles, were in many cases underdeveloped in the KO mice at P7 and P8 (Fig. 6a, b). Synapses were classified accordingly as mature and immature (Fig. 6a, b) and their ratios compared in the two groups. The comparison indicated a decreased proportion of mature synapses in the KO mice (Fig. 7; Table 3). Accordingly, repeated measure two-way ANOVA indicated significant effect of *Akp2* gene ablation on the number of mature and immature synapses after grouping the data by ignoring age differences ($p=0.03$). Post hoc comparisons indicated a significant increase of the number of immature synapses compared to the mature ones of the KO group ($p=0.03$, *t* test) (Table 3). Interestingly, in the WT mice, there was no significant difference between the numbers of the two types of synapses. However, in the WT group, case P8 was an outlier with an exceptionally low number of immature synapses and resulting in a large variance of the data (Table 3). Selected number of synapses was cut serially via the full extent of their synaptic densities. Serial reconstructions of mature-like synapses (11 in KO and 7 in WT mice) were possible on considerably shorter series in the KO (average 2.5 sections) than in the WT mice (5.7 sections in average) at P7, which suggest that mature-like synaptic contacts have a smaller size in the KO animals than in the WT (Fig. 6c–f).

In summary, delay in myelination and in synaptogenesis was observed in the cortex of *Akp2*^{-/-} mice. Generally, cortical synapses also appeared under-developed in the TNAP null animals.

Discussion

This is the first time that the consequences of ablating TNAP function on the developing brain were investigated. Based on previous work providing relationships between TNAP activity in the synaptic cleft and nodes of Ranvier (Fonta et al. 2004, 2005), we presently targeted our investigations on the effect of TNAP gene deletion on

myelination and synaptogenesis. The present study explored several structural and ultrastructural alterations indicating that TNAP plays an important role in the development of these above-mentioned structures, which are responsible for signal propagation and transmission in the central nervous system. These findings are in accordance with the expression of TNAP by neuronal cells shown recently by Brun-heath et al. (2011). Consequently, our results suggest possible neurobiological causes contributing to the idiopathic epilepsy and poor coordination and orientation abilities recorded in case of TNAP deficiency (Narisawa et al. 1997, 2001). Additionally, considering that impaired myelin formation and synaptic transmission are implicated in many neuropsychiatric diseases, the findings of the present study urge further investigation on the role of TNAP in such conditions.

Our findings in the spinal cord of KO mice indicating decreased WM relative amount at the light microscopic level can most likely be attributed to impaired development of the white matter, rather than a disproportional development of the gray matter. Accordingly, the developmental increase of the proportion of spinal cord gray matter did not differ between the WT and KO mice. The ultrastructural examination suggests that the reduced number of axonal fibers destined to be myelinated fibers (presently including three classes), and especially the reduction of the number of the fully developed, Class III myelinated axons, could explain the decrease of the WM% in the KO mice. In addition, it was also found that

the diameter of Class III axons was decreased in the TNAP null mice. All these factors could reduce the intensity, quality, and speed of activity propagation in the spinal cord of the KO mice. Regarding the development of the myelin sheath, the present comparisons showed that processes of the oligodendrocytes initially encapsulating the axons (Class I types) are thinner in the KO than in the WT mice. Together with the ultrastructural observations of degradatory cellular processes in the myelin structure around the nodal axonal regions of the spinal cord, these findings strongly suggest that TNAP activity is necessary for the normal development of myelin in the central nervous system. Our data may explain Narisawa and collaborators (1997) findings that describe thinner nerve roots in the lumbar spinal cord of 10-day-old KO mice. These abnormalities, which were not induced by cell death, were attributed to defect in the development of the neural tube during embryonic life (Narisawa et al. 1997). Our hypothesis on the role of TNAP in oligodendrocyte maturation is also supported by the almost complete lack of myelinated processes in the cerebral cortex at P8 in the KO animals when myelinated fibers are regularly found in the age-matched WT animals. It should be noted, that the present data, obtained in postnatal animals, may also be partly the result of prenatal developmental processes since strong TNAP activity is present at the axon level at the embryonic stage in the mice (Brun-Heath et al. 2011; Langer et al. 2007; Narisawa et al. 1994). Meanwhile, TNAP null embryos do not morphologically distinct from WT embryos and embryonic mortality is not associated with TNAP deficiency (Narisawa et al. 1997). Nevertheless, the role of TNAP in the development of the central nervous system during embryonic life has not yet been studied.

Cortical synaptogenesis was also impaired as suggested by the decreased number of matured synapses in the KO mice at P7 and P8, i.e. a very few days before they usually die. Furthermore, even mature-like cortical synapses appeared smaller and contained fewer if any synaptic vesicles in the KO than in the WT mice. Therefore, the absence of TNAP activity could hamper neurotransmission via the synaptic contacts in the cortex of KO mice.

There could be several molecular biological processes responsible for the structural alterations described above. In the central nervous system TNAP has several natural substrates including PLP, but also different nucleotides. Thus, some neuronal abnormalities observed in TNAP null mice could be correlated with Vitamin B6 deficiency. It was found that daily supplementation with pyridoxal during the postnatal period results in the lengthening of the survival time of *Akp2*^{-/-} mice by a few days, an improvement of spinal nerve root deficiencies and a delay in epileptic seizures onset (Narisawa et al. 2001). Meanwhile, many enzymes have PLP as a co-factor, including the GABA-synthesizing enzyme GAD. GABA is known to have neurotrophic role during development and also plays important role in the development of neuronal excitability (Represa and Ben-Ari 2005), acting both at pre-and postnatal stages. Consequently, disturbance of GABA synthesis could substantially impair neuronal development. Regarding myelination, it is known that myelin synthesis is also vitamin B6-dependent (Stephens and Dakshinamurti 1975). The brains of rodents deprived of vitamin B6 from the first day of gestation show a decrease in myelination (Kirksey et al. 1990). In humans with B6-dependent seizures, delay in myelination and decrease in white matter have also been reported (Jardim et al. 1994; Lott et al. 1978). Interestingly, delayed white matter maturation was reported in children with Mabry disease who have an elevated TNAP serum level without bone disease but with an important neurological compromise (seizures and psychomotor retardation), presumably due to low membrane-bound TNAP activity during brain development (Thompson et al. 2010). Thus, our data and these literature reports converge to suggest that TNAP level has a biological role on white matter development which could be explained through its enzymatic activity.

However, pyridoxal administration cannot rescue TNAP-deficient animals, who despite a slight prolongation of life span and a delay in the onset of epileptic seizures, die near weaning time (Narisawa et al. 2001), suggesting that mechanisms other than vitamin B6 metabolism may be at play. Indeed, TNAP is suspected to be involved in various functions through multiple substrates. TNAP is also an ectonucleotidase providing substrates for purinergic receptors, and it is known that the purinergic system plays an important role in neural development, beyond the control of neurogenesis (Mishra et al. 2006; Riquelme et al. 2008; Zimmermann, 2011). Specifically, ATP and its dephosphorylated nucleotide derivatives stimulate migration and differentiation of oligodendrocytes and promote myelination in the central nervous system (Fields and Burnstock 2006; Neary and Zimmermann 2009). It has also been shown that TNAP regulates axonal growth by controlling the extracellular level of ATP, which acts on P2X7 receptors of the axonal growth cone (Diez-Zaera et al. 2011). An alternative hypothesis is that, in the cortex, lack of myelination obstructs saltatory conduction of the action potentials, which not only hampers fast conduction but at the same time results in great energy deficiency as the point-to-point spread of membrane potential changes is energetically expensive. Energy deficit in turn could result in cellular malfunction hence developmental failures and/or a life-threatening state.

While the exact molecular mechanisms responsible for the changes observed in our study remain to be explored, it is very important to note that the findings of Kermer et al. (2010), showing in cell culture experiments that TNAP is essential for the differentiation of neurons and/or oligodendrocytes, are in great agreement with the results of the present study within the brain.

Whether the roles of TNAP in myelination and synaptic maturation are independent needs to be investigated since normal myelination is closely linked to axonal activity especially through the purinergic system (Butt 2006; Fields and Burnstock 2006). Therefore, analyses of the functions of TNAP in brain development appear to be an interesting opportunity to explore mechanisms of myelin defects and altered synaptic signaling, both being critical in learning and memory abilities (Fields 2010) and in several neurological diseases (such as multiple sclerosis, epilepsy, traumatic injury, schizophrenia, depression, and Alzheimer disease) (Stys 2011; Vardy et al. 2011).

Acknowledgments

The authors acknowledge Dr Myriam Ermonval for fruitful discussion, and Tünde Magyar, Magali Philippe, and Luc Renaud for technical assistance. This study was supported by PHC Egide (Balaton 17341UE), CNRS (PICS 4331), Hypophosphatasie Europe, University of Toulouse (ASUPS and ATUPS) (C.F), the French Embassy in Beijing (J.X), the joint funding by the National Office for Research and Technology (NKTH) and the Hungarian Science and Technology Foundation (TETALAP) (FR-16/2007) (L.N), and also supported in part by grant DE12889 from the National Institutes of Health, USA (J.L.M.).

References

- Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H. Purinergic signalling in the nervous system: an overview. *Trends Neurosci.* 2009; 32:19–29. [PubMed: 19008000]
- Amadasi A, Bertoldi M, Contestabile R, Bettati S, Cellini B, di Salvo ML, Borri-Voltattorni C, Bossa F, Mozzarelli A. Pyridoxal 5'-phosphate enzymes as targets for therapeutic agents. *Curr Med Chem.* 2007; 14:1291–1324. [PubMed: 17504214]
- Balasubramaniam S, Bowling F, Carpenter K, Earl J, Chaitow J, Pitt J, Mornet E, Sillence D, Ellaway C. Perinatal hypophosphatasia presenting as neonatal epileptic encephalopathy with abnormal neurotransmitter metabolism secondary to reduced co-factor pyridoxal-5'-phosphate availability. *J Inherit Metab.* 2010

- Dis Barna, J.; Hanics, J.; Xiao, JS.; Fonta, C.; Négyessy, L. Ultrastructural studies of the CNS of TNAP-knock out mice. 12th Meeting of the Hungarian Neuroscience Society; Budapest (Hungary). 2009a. p. 215
- Barna, J.; Hanics, J.; Xiao, JS.; Millan, JL.; Fonta, C.; Négyessy, L. Ultrastructural studies of the CNS of TNAP-knock out mice. Annual meeting Society for Neuroscience; Chicago, III. 2009b.
- Baumgartner-Sigl S, Haberlandt E, Mumm S, Scholl-Burgi S, Sergi C, Ryan L, Ericson KL, Whyte MP, Hogler W. Pyridoxine-responsive seizures as the first symptom of infantile hypophosphatasia caused by two novel missense mutations (c.677T > C, p.M226T; c.1112C > T, p.T371I) of the tissue-nonspecific alkaline phosphatase gene. *Bone*. 2007; 40:1655–1661. [PubMed: 17395561]
- Bossi M, Hoylaerts MF, Millan JL. Modifications in a flexible surface loop modulate the isozyme-specific properties of mammalian alkaline phosphatases. *J Biol Chem*. 1993; 268:25409–25416. [PubMed: 8244974]
- Brun-Heath I, Ermonval M, Chabrol E, Xiao J, Palkovits M, Lyck R, Miller F, Couraud PO, Mornet E, Fonta C. Differential expression of the bone and the liver tissue non-specific alkaline phosphatase isoforms in brain tissues. *Cell Tissue Res*. 2011; 343:521–536. [PubMed: 21191615]
- Butt AM. Neurotransmitter-mediated calcium signalling in oligodendrocyte physiology and pathology. *Glia*. 2006; 54:666–675. [PubMed: 17006895]
- Coffey JC, McDermott KW. The regional distribution of myelin oligodendrocyte glycoprotein (MOG) in the developing rat CNS: an in vivo immunohistochemical study. *J Neurocytol*. 1997; 26:149–161. [PubMed: 9192283]
- Diaz-Hernandez M, Gomez-Ramos A, Rubio A, Gomez-Villafuertes R, Naranjo JR, Miras-Portugal MT, Avila J. Tissue-nonspecific alkaline phosphatase promotes the neurotoxicity effect of extracellular tau. *J Biol Chem*. 2010; 285:32539–32548. [PubMed: 20634292]
- Diez-Zaera M, Diaz-Hernandez JI, Hernandez-Alvarez E, Zimmermann H, Diaz-Hernandez M, Miras-Portugal MT. Tissue-nonspecific alkaline phosphatase promotes axonal growth of hippocampal neurons. *Mol Biol Cell*. 2011; 22:1014–1024. [PubMed: 21289095]
- Ermonval M, Baudry A, Baychelier F, Pradines E, Pietri M, Oda K, Schneider B, Mouillet-Richard S, Launay JM, Kellermann O. The cellular prion protein interacts with the tissue non-specific alkaline phosphatase in membrane microdomains of bioaminergic neuronal cells. *PLoS One*. 2009; 4:e6497. [PubMed: 19652718]
- Fiala JC. Reconstruct: a free editor for serial section microscopy. *J Microsc*. 2005; 218:52–61. [PubMed: 15817063]
- Fields RD. Neuroscience. Change in the brain's white matter. *Science*. 2010; 330:768–769. [PubMed: 21051624]
- Fields RD, Burnstock G. Purinergic signalling in neuronglia interactions. *Nat Rev Neurosci*. 2006; 7:423–436. [PubMed: 16715052]
- Fonta C, Imbert M. Vascularization in the primate visual cortex during development. *Cereb Cortex*. 2002; 12:199–211. [PubMed: 11739267]
- Fonta C, Negyessy L, Renaud L, Barone P. Areal and subcellular localization of the ubiquitous alkaline phosphatase in the primate cerebral cortex: evidence for a role in neurotransmission. *Cereb Cortex*. 2004; 14:595–609. [PubMed: 15054075]
- Fonta C, Negyessy L, Renaud L, Barone P. Postnatal development of alkaline phosphatase activity correlates with the maturation of neurotransmission in the cerebral cortex. *J Comp Neurol*. 2005; 486:179–196. [PubMed: 15844208]
- Foran DR, Peterson AC. Myelin acquisition in the central nervous system of the mouse revealed by an MBP-Lac Z transgene. *J Neurosci*. 1992; 12:4890–4897. [PubMed: 1281497]
- Friede RL. A quantitative mapping of alkaline phosphatase in the brain of the rhesus monkey. *J Neurochem*. 1966; 13:197–203. [PubMed: 4957290]
- Gallyas F. Silver staining of myelin by means of physical development. *Neurol Res*. 1979; 1:203–209. [PubMed: 95356]
- Henthorn PS, Raducha M, Fedde KN, Lafferty MA, Whyte MP. Different missense mutations at the tissue-nonspecific alkaline phosphatase gene locus in autosomal recessively inherited forms of mild and severe hypophosphatasia. *Proc Natl Acad Sci USA*. 1992; 89:9924–9928. [PubMed: 1409720]

- Hessle L, Johnson KA, Anderson HC, Narisawa S, Sali A, Goding JW, Terkeltaub R, Millan JL. Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. *Proc Natl Acad Sci USA*. 2002; 99:9445–9449. [PubMed: 12082181]
- Jardim LB, Pires RF, Martins CE, Vargas CR, Vizioli J, Kliemann FA, Giugliani R. Pyridoxine-dependent seizures associated with white matter abnormalities. *Neuropediatrics*. 1994; 25:259–261. [PubMed: 7885536]
- Kermer V, Ritter M, Albuquerque B, Leib C, Stanke M, Zimmermann H. Knockdown of tissue nonspecific alkaline phosphatase impairs neural stem cell proliferation and differentiation. *Neurosci Lett*. 2010; 485:208–211. [PubMed: 20849921]
- Kirksey A, Morre DM, Wasynczuk AZ. Neuronal development in vitamin B6 deficiency. *Ann NY Acad Sci*. 1990; 585:202–218. [PubMed: 2356985]
- Langer D, Ikehara Y, Takebayashi H, Hawkes R, Zimmermann H. The ectonucleotidases alkaline phosphatase and nucleoside triphosphate diphosphohydrolase 2 are associated with subsets of progenitor cell populations in the mouse embryonic, postnatal and adult neurogenic zones. *Neuroscience*. 2007; 150:863–879. [PubMed: 18031938]
- Li M, Cui Z, Niu Y, Liu B, Fan W, Yu D, Deng J. Synaptogenesis in the developing mouse visual cortex. *Brain Res Bull*. 2010; 81:107–113. [PubMed: 19751806]
- Lott IT, Coulombe T, Di Paolo RV, Richardson EP Jr, Levy HL. Vitamin B6-dependent seizures: pathology and chemical findings in brain. *Neurology*. 1978; 28:47–54. [PubMed: 563538]
- Mishra SK, Braun N, Shukla V, Fullgrabe M, Schomerus C, Korf HW, Gachet C, Ikehara Y, Sevigny J, Robson SC, Zimmermann H. Extracellular nucleotide signaling in adult neural stem cells: synergism with growth factor-mediated cellular proliferation. *Development*. 2006; 133:675–684. [PubMed: 16436623]
- Narisawa S, Hasegawa H, Watanabe K, Millan JL. Stage-specific expression of alkaline phosphatase during neural development in the mouse. *Dev Dyn*. 1994; 201:227–235. [PubMed: 7533563]
- Narisawa S, Frohlander N, Millan JL. Inactivation of two mouse alkaline phosphatase genes and establishment of a model of infantile hypophosphatasia. *Dev Dyn*. 1997; 208:432–446. [PubMed: 9056646]
- Narisawa S, Wennberg C, Millan JL. Abnormal vitamin B6 metabolism in alkaline phosphatase knock-out mice causes multiple abnormalities, but not the impaired bone mineralization. *J Pathol*. 2001; 193:125–133. [PubMed: 11169525]
- Neary JT, Zimmermann H. Trophic functions of nucleotides in the central nervous system. *Trends Neurosci*. 2009; 32:189–198. [PubMed: 19282037]
- Négyessy L, Takács J, Mogensen J, Divac I, Hámori J. Synaptic reorganisation of the mediodorsal thalamic nucleus in adult rat following chronic prefrontal cortical lesions. *J Hirnforsch*. 1995; 36:433–441. [PubMed: 7560915]
- Négyessy L, Xiao J, Kantor O, Kovacs GG, Palkovits M, Doczi TP, Renaud L, Baksa G, Glasz T, Ashaber M, Barone P, Fonta C. Layer-specific activity of tissue non-specific alkaline phosphatase in the human neocortex. *Neuroscience*. 2011; 172:406–418. [PubMed: 20977932]
- Rasband, WS. ImageJ. U. S. National Institutes of Health; Bethesda, Maryland, USA: 1997–2011. <http://imagej.nih.gov/ij/>
- Represa A, Ben-Ari Y. Trophic actions of GABA on neuronal development. *Trends Neurosci*. 2005; 28:278–283. [PubMed: 15927682]
- Riquelme PA, Drapeau E, Doetsch F. Brain microecologies: neural stem cell niches in the adult mammalian brain. *Philos Trans R Soc Lond B Biol Sci*. 2008; 363:123–137. [PubMed: 17322003]
- Stephens MC, Dakshinamurti K. Brain lipids in pyridoxine-deficient young rats. *Neurobiology*. 1975; 5:262–269. [PubMed: 1202390]
- Stys PK. The axomyelinic synapse. *Trends Neurosci*. 2011; 34:393–400. [PubMed: 21741098]
- Sugimura K, Mizutani A. Histochemical and cytochemical studies of alkaline phosphatase activity in the synapses of rat brain. *Histochemistry*. 1979; 61:123–129. [PubMed: 457450]
- Thompson MD, Nezarati MM, Gillessen-Kaesbach G, Meinecke P, Mendoza-Londono R, Mornet E, Brun-Heath I, Squarcioni CP, Legeai-Mallet L, Munnich A, Cole DE. Hyperphosphatasia with

- seizures, neurologic deficit, and characteristic facial features: five new patients with Mabry syndrome. *Am J Med Genet A*. 2010; 152A:1661–1669. [PubMed: 20578257]
- Vardy ER, Kellett KA, Cocklin SL, Hooper NM. Alkaline Phosphatase Is Increased in both Brain and Plasma in Alzheimer's Disease. *Neurodegener Dis*. 2011
- Waymire KG, Mahuren JD, Jaje JM, Guilarte TR, Coburn SP, Mac-Gregor GR. Mice lacking tissue non-specific alkaline phosphatase die from seizures due to defective metabolism of vitamin B-6. *Nat Genet*. 1995; 11:45–51. [PubMed: 7550313]
- Wennberg C, Hessle L, Lundberg P, Mauro S, Narisawa S, Lerner UH, Millan JL. Functional characterization of osteoblasts and osteoclasts from alkaline phosphatase knockout mice. *J Bone Miner Res*. 2000; 15:1879–1888. [PubMed: 11028439]
- Whyte, M. Hypophosphatasia. In: Scriver, CR.; Beudet, AL.; Sly, WS.; Valle, D., editors. *The metabolic and molecular bases of inherited disease*. McGrawHill; NY: 1995. p. 4095-4112.
- Whyte MP, Mahuren JD, Vrabel LA, Coburn SP. Markedly increased circulating pyridoxal-5'-phosphate levels in hypophosphatasia. Alkaline phosphatase acts in vitamin B6 metabolism. *J Clin Invest*. 1985; 76:752–756. [PubMed: 4031070]
- Whyte MP, Landt M, Ryan LM, Mulivor RA, Henthorn PS, Fedde KN, Mahuren JD, Coburn SP. Alkaline phosphatase: placental and tissue-nonspecific isoenzymes hydrolyze phosphoethanolamine, inorganic pyrophosphate, and pyridoxal 5'-phosphate. Substrate accumulation in carriers of hypophosphatasia corrects during pregnancy. *J Clin Invest*. 1995; 95:1440–1445. [PubMed: 7706447]
- Xiao, JS.; Negyessy, L.; Barna, J.; Renaud, L.; Millan, JL.; Fonta, C. A role for the bone alkaline phosphatase in cortex myelination. 36th annual meeting Society for Neuroscience; Atlanta, GA. 2006.
- Xiao, JS.; Barna, J.; Negyessy, L.; Renaud, L.; Millan, JL.; Fonta, C. Myelin abnormalities in the Akp2 knock-out mice. 5th Int Alkaline Phosphatase Symposium; Huningue, France. 2007.
- Zimmermann H. Purinergic signaling in neural development. *Semin Cell Dev Biol*. 2011; 22:194–204. [PubMed: 21320621]
- Zisapel N, Haklai R. Localization of an alkaline phosphatase and other synaptic vesicle proteins. *Neuroscience*. 1980; 5:2297–2303. [PubMed: 7465056]

Fig. 1.

a Cross-sections of the spinal cord cervical segment in wild-type (*WT*) and TNAP knockout (*KO*) animals at postnatal days 8 and 10 (*P8*, *P10*). *Upper two sections* are stained for myelin and *lower left and right sections* are stained by cresyl violet and TNAP histochemistry, respectively. Note the high activity of TNAP in the gray matter and along fibers in the white matter. Ventral is down. Scale bar 200 μm . **b** Size of total cross-sectional area measured on the myelin-stained sections of the spinal cord is illustrated as the function of age. Individual cases are shown as *open triangles* in case of the *WT* as well as *open circles* in case of the *KO* animals. Linear fit is represented by the *straight line* in the case of *WT* and *dashed line* in the case of *KO* animals. *Filled symbols* represent averages of the *KO* (*circle*) and *WT* (*triangle*) data. **c** Growth rates of grey and white matter between P1 and P10 in the *WT* and *KO* animals. **d** Relative white matter content (WM%) in the two groups as the function of age. Conventions are the same as in (**b**)

Fig. 2. Myelinated fibers in the spinal cord of normal (*WT*) and *Akp2* gene-ablated (*KO*) animals. Light (**a**, **b**) and electron microscopic (**c**, **d**) images taken from the ventral funiculus of WT and KO mice at postnatal day 8 (P8). Images of semithin (**a**, **b**) and ultrathin (**c**, **d**) sections taken from the same resin-embedded blocks of WTP8 (**a**, **c**) and KOP8 (**b**, **d**) are shown in *columns*. **a**, **b** Note strongly labeled circular structures corresponding to myelinated axons in the toluidin blue azure II-stained semithin sections. **c**, **d** Note irregular shape of the cross-section of myelinated fibers in the KO. I, II, and III mark the three classes of fibers (see definition in the text). **e** Higher resolution of (**c**). *M* myelin, *O* processes of oligodendrocytes, *A* axon. **f** Effect of TNAP gene deletion on the proportional distribution of the myelinated (Class II and Class III) fibers 2 days (P2) or 8 days (P8) after birth. Scale bars 10 μ m on the light micrographs (**a**, **b**) and 500 nm on the electron micrographs (**c**–**e**)

Fig. 3. Structure of the myelin sheath of individual fibers of the spinal cord is shown around the nodal regions (*stars*) both in the WT (**a, b**) and KO (**c–f**) animals at postnatal day 8 (P8). (**c, d**) *White arrows* show enlarged regions containing intracellular organelles responsible for cellular degradation as presented in (**e, f**). *M* myelin, *A* axon, *N* node of Ranvier or preterminal region, *lys* lysosome, *e* endosome, *v* endocytic vesicle, *mt* mitochondria, *encl* enclosure. *Scale bars* (**a–d**) 2 μ m, (**e, f**) 1 μ m

Fig. 4. Ultrastructural evidence of cellular degradation in the myelinated axons of the spinal cord in the TNAP KO (a–c). *M* myelin, *A* axon, *e* endosome, *MVB* multivesicular body, *lys* lysosome, ** myelin engulfing a structureless empty-like region probably resulted by cellular degradation. *Scale bars* 1 μm

Fig. 5. Myelination in the cerebral cortex of the WT and TNAP KO3 mice of postnatal days P2 and P8. **a, b** Toluidine blue azure II-stained semithin sections show similar laminar organization of the WT and KO at P2. **c, d** Ultrastructural differences between WT and TNAP KO mice at P2. **c** Developing myelin sheath (*arrow*) around an axonal fiber (*f*) in WT mice at P2. **d** *Arrow* indicates an apparently empty, myelinated fiber-like circular structure in KO mice at P2. **e, f** Absence of myelinated axons in the cerebral cortex of the TNAP KO mice is shown on the semithin sections at P8. **e** Myelinated fibers are shown by the *arrows* on a toluidine blue azure II-stained semithin section in WT P8 mice. **f** *Arrowhead* points to cross-section of

an axon-like structure partially enwrapped by oligodendrocytic-like processes (dark staining). Note darkly stained oligodendrocytes. **g, h** Electron micrographs showing the lack of axonal myelination in the TNAP KO mice. **g** Normal cortical myelination is shown at P8 in WT animals. **h** Electron microscopic image showing myelin-like sheath with an empty core (*arrow*) in KO mice at P8. *Scale bars* (**b**, and **a**) 250 μm , (**c**, **d**, **f**, **h**) 200 nm, (**e**, **g**) 25 μm

Fig. 6.

Synaptic organization of the cerebral cortex in the WT (**a, c, e**) and TNAP KO (**b, d, f**) mice at P7. **a, b** Arrows indicate synaptic contacts exhibiting all the ultrastructural characteristics of matured synapses. *Inset* shows non-matured synapses lacking synaptic vesicles with recognizable synaptic membrane specializations (arrows) and synaptic cleft in the KO mice. **c, d** Three-dimensional reconstructions showing the ultrastructural differences between developed and non-developed synaptic contacts. Axon terminal is shown *darker* and postsynaptic dendrite is in *lighter shading*. Dots represent synaptic vesicles in the axon terminals. Synaptic membrane specialization is shown in *black*. Note small synaptic membrane specialization and the paucity of synaptic vesicles in the KO. **e, f** Electron micrographs from the series used for the reconstructions shown in (**c, d**), where the synapses exhibited the most elaborated structural specialization. Arrows connect corresponding structures of the reconstructions and the electron micrographs. Arrowhead synaptic membrane specialization. *a* axon terminal, *d* postsynaptic dendrite. Scale bars (**a, b**) 1 μ m, (*inset* and **e, f**) 500 nm

Fig. 7.

The ratio of mature (*mat*) and immature (*immat*) synaptic contacts are shown in two animals at postnatal day 7 (*P7a*, *P7b*) (*diamonds* and *squares*) and in one animal at postnatal day 8 (*P8*) (*triangles*) in both WT and TNAP KO mice

Table 1

Number of animals used for the light microscopic measurements at the different postnatal ages (*P1–10*); the number of sections is indicated in parentheses

Age	WT	KO
P1	1 (4)	1 (4)
P2	2 (11)	2 (15)
P4	2 (20)	3 (30)
P6	2 (19)	2 (16)
P7	3 (28)	3 (29)
P8	4 (46)	3 (27)
P10	2 (19)	2 (18)
Total	16 (147)	16 (139)

WT wild-type, *KO* knockout

Number of axons in each of the three classes of fibers determined on electron microscopy images of cross-sectional area of the spinal cord (1 animal per case)

Table 2

Axon type	WT			KO				
	Class I	Class II	Class III	Subtotal	Class I	Class II	Class III	Subtotal
P2	1,341	305	26	1,672	493	54	0	547
P7	40,912	5,209	1,057	47,178	25,353	3,737	1,656	30,746
P8	2,085	6,069	2,232	10,386	2,355	6,854	1,139	10,348
Total	4,483	11,583	43,170	59,236	4,504	10,645	26,492	41,641

WT wild-type, KO knockout

Table 3

Number of matured and non-matured synapses in the cortex of 1-week-old WT and TNAP-KO animals

Cases	Matured	Non-matured	Subtotal	Cases	Mature	Non-mature	Subtotal
P7WT339	191	190	381	P7KO337	125	169	294
P7WT341	168	132	300	P7KO340	103	131	234
P8WT	176	89	265	P8KO	110	166	276
Mean ± sd	178.3 ± 11.7	137.0 ± 50.7	315.3 ± 59.5		112.6 ± 11.2	155.3 ± 21.1	268.0 ± 30.8