Original article

Folia Neuropathologica

Phenotypic diversity resulting from a point mutation in a plp gene in *paralytic tremor* rabbit

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Folia Neuropathol 2006; 44 (4): 244–250

Abstract

Paralytic tremor (pt), a hereditary neurological disorder of rabbits, is a recessive, X-linked point mutation in exon 2 of the plp gene, responsible for substitution of 38 His by Glu in the PLP molecule. Pt genotype is expressed in a range of phenotypes, distinguished by the severity of neurological symptoms. Variable course of the disease, from totally asymptomatic to serious disorder, is observed even within the offspring of one breeding pair. The two most typical phenotypes have been chosen for the studies: one representing mild course of the disease and the other reflecting the most severe course. Since previous developmental studies proved that myelination is not only deficient but also delayed in pt rabbits, the age groups of animals have been selected with the aim of spanning the period of most active myelinogenesis. As revealed by experiments, the degree of CNS hypomyelination, which is the main future of pt mutation, is highest in the most affected animals. The amounts of mutated gene products, PLP and DM-20, examined both at mRNA and protein levels, exhibited a strong dependence on phenotype. Down-regulation of MBP and CNP was also observed. In contrast, MAG expression was normal or only slightly changed in mutants. The results lead to the conclusion that pt mutation in the plp gene affects a panel of events that governs myelinogenesis and is modulated in each individual that is manifested by gradation of neurological symptoms.

Key words: *point mutation, plp gene, phenotype, hypomyelination, gene expression*

Introduction

Paralytic tremor (*pt*) is an X-linked recessive mutation responsible for severe myelin deficiency in the central nervous system. The mutation was discovered within a Chinchilla rabbit lane in "Łomna" stock (Mińsk Maz., Poland) in 1964 [30,31]. The molecular base of the *pt* disease is a point mutation in the plp gene resulting in a changed 36th aa (His→Glu) [49], thus including the mutated rabbit in a specific group of plp mutants [55] serving as animal models of human Pelizaeus-Merzbacher disease (PMD). This severe neurological disease [14,16,38] results from various types of plp gene alterations, such as point mutations, deletions, duplications, or even triplication [55], and thereafter manifests as a spectrum of neurological disorders.

Also the *pt* mutation is characterised by gradation of neurological symptoms – from totally asymptomatic to severe disorders. Coarse body tremor and limb paresis develop on about the 10th postnatal day. Among

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affected animals (carefully screened for their *pt* trait) those with various phenotypes could be distinguished [33], even among homo(hemi)zygotic offspring of the same breeding pair. A four-grade scale has been conventionally established, based on the severity of neurological symptoms, in order to classify the variety of expressed phenotypes:

- pt_I: absolutely asymptomatic through the whole lifespan, which is normal or only slightly reduced;
- pt_{II}: asymptomatic at the beginning, mutants gradually develop a coarse body tremor and exaggeration; partial recovery often occurs thus resembling phenotype I; the typical, most frequent "mild" case of the disease;
- pt_{III}: symptoms as above, clearly visible from about the 10^{th} postnatal day, their severity gradually increases; longevity is considerably reduced;
- pt_{IV}: the most severe, quite frequent case of the disease; besides coarse body tremor and exaggeration, affected animals soon develop spastic limb paresis (first of the hindlimbs, then of the forelimbs); mutants try to move on the belly with their limbs shifted aside; they survive no longer than 3 months.

Previous studies on the developmental expression of several myelin proteins in *pt* rabbits with mild phenotype [43] revealed that the expression of almost all investigated proteins was significantly deficient and delayed when compared to controls. Quantification of their content in *pt* myelin showed an approximately 50% deficiency of both mutated proteins, PLP and DM-20. Amounts of other proteins (MBP,CNP,MAG) were also reduced, although not so drastically [44]. Significant and approximately constant hypomyeliation during the entire investigated period of development (i.e. first postnatal trimester) was observed. Considering the variety of phenotypes generated from the *pt* genotype, we aimed to determine whether there are any differences in the degree of hypomyelination and/or the expression of myelin-specific proteins in the myelin of mutants representing different phenotypes. Pt_{II} and pt_{IV} were selected for the purpose of the study.

Materials and methods

The animals were supplied by the Department of Comparative Neurology, Polish Academy of Sciences (Mińsk Maz. Poland). Mutants (homozygous or hemizygous) were strictly controlled for both their *pt* trait and the phenotype; those presenting either phenotype II or IV were selected for the study. Age--matched control animals derived from the same Chinchilla rabbit lane. Four groups of animals were collected, aged respectively 28, 42, 68 and 80 days. Sacrification was made by decapitation. Each brain, without cerebellum, was immediately frozen in liquid nitrogen and stored in –70°C until use. One hemisphere was used for myelin isolation and protein determination; the other one served for mRNA analysis.

Myelin preparation. The hemispheres were homogenised in 0.32 M sucrose, 20% of homogenate sample was spun for 15 min, 14 krpm, and the pellet was suspended in water with protease inhibitors (0.1 mM PMSF; 10 µg/ml antipain; 5 µg/ml leupeptin; 5 µg/ml pepstatin; 2 mM EDTA; 2 mM EGTA). The remaining homogenate was used for myelin isolation according to Norton and Poduslo [28].

Protein concentration was determined by the method of Lowry [19].

ELISA for MBP antigen. For determination of MBP antigen content by ELISA test, homogenates were dissolved with SDS to the final concentration of 1% in water. After heating the mixture at 100°C for 5 min. the insoluble residue was removed by centrifugation and the clear supernatant was dialysed overnight against the water. Anti-MBP antibody was used in 1:500 dilution and the second anti-mouse-IgG antibody (1:5000) was conjugated directly with peroxidase (BioRad). TMB Peroxidase EIA Substrate Kit (# 172-1066) was supplied by BioRad. All washing steps were done with PBS containing either 1% BSA or 5% delipidated milk. We screened for homogenate samples of 0.1 to 10 µg protein/well.

Immunoblotting. Samples containing 80 µg of proteins, prepared as described by Amiquet et al. [2], were separated on 12% polyacrylamide gel [18] and subsequently either stained with Commassi blue or transferred to nitrocellulose membranes (Amersham) using the electroblotting technique [51]. The major myelin-specific proteins were detected with the following antibodies: Polyclonal anti-PLP/DM-20 and anti-MBP antibodies, prepared in Prof. J.M. Matthieu's laboratory, were used after 1:400 dilution. Monoclonal anti-MAG, diluted for the experiments to 1:500, was a kind gift from Dr Ch. Quarles [35]. Monoclonal anti--CNPase was purchased from SIGMA Immunochemicals and diluted to 1:200 before application. The immunoblots were incubated with the horseradish peroxidase-conjugated secondary antibody: goat anti-

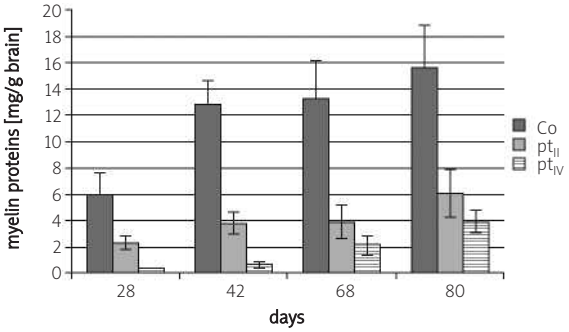


Fig. 1. Amounts of myelin isolated from rabbits of different phenotypes (n=4 in each group): control, pt_{II} (moderate disability) and pt_{IV} (severe course of the disease), as deduced from comparison of myelin protein content. Dependency of hypomyelination degree on phenotype is striking

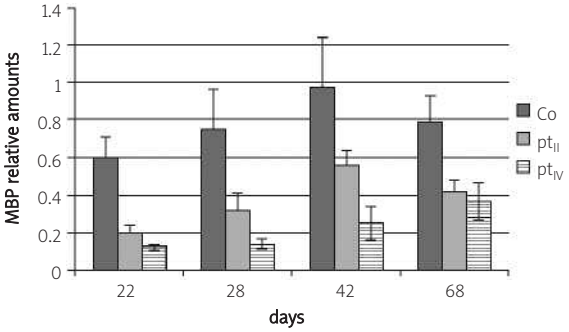


Fig. 2. ELISA test for the content of MBP antigen in the brain homogenete samples. Detected amounts of MBP as a specific myelin marker indicate severe hypomyelination of pt mutant brains, depending on phenotype

-rabbit IgG (SIGMA) for anti-PLP/DM-20 and anti-MBP or sheep anti-mouse IgG (Amersham) for anti-MAG, anti-MOG and anti-CNPase and detected by ECL technique (Amersham). The times of immunoblot exposure to Hyperfilm™-ECL (Amersham) varied from 0.5 up to 30 min.

RNA isolation and analysis. One hemisphere of each brain was homogenised in 20 mM $MgCl_2$, 200 mM Na Cl and 20 mM Tris (pH= 8), diluted with one volume of 3% SDS and 40 mM EDTA, then spun briefly at 5000 x g. RNA was purified from the resulting supernatant by phenol/chloroform extraction and precipitated in ethanol. Purified RNA samples were then separated on formaldehyde/MOPS agarose gel [36] and transferred to Zeta-Probe membranes (Bio--Rad). Blots were hybridized with ^{32}P labelled specific cDNA probes and exposed to Hyperfilm™-MP (Amersham). Autoradiograms were analysed on a GelScan XL densitometer (Pharmacia). Specific cDNA probes – p27 for PLP, pMBP1 and WG7 for CNP mRNA – were a kind gift of Dr J. M. Matthieu. Actin probe used as a housekeeping control was kindly provided by Dr J. Siedlecki.

Results

Quantitative examination of myelin protein amounts isolated from different phenotypes indicated severe hypomyelination of *pt* central nervous system (CNS) and showed that degree of myelin deficit correlates well with expressed phenotype (Fig. 1). This observation was confirmed by determination of MBP antigen content in the brain homogenates by ELISA test (Fig. 2). MBP served as a specific protein marker of myelin and its diversified content indicated that myelin deficiency corresponds to the severity of neurological symptoms and is highest in the animals most affected by the disease. Whereas the amounts of MBP as well as of both proteins modified by the mutation, i.e. PLP and DM-20, in the brain homogenates were much lower in phenotype IV than in II, the contents of MAG and CNP were approximately equal in both phenotypes and showed reduction to 50~60%.

Examination of the protein composition of the myelin showed significant decrease and dependence on the phenotype in the content of PLP and DM-20, the protein products of the mutated gene. Although developmental expression of PLP is different to DM-20, the amounts of both proteins in *pt* myelin were much lower than in controls and they were reduced to approximately 50% of control values in phenotype II, whereas their deficiency in phenotype IV achieved more than 90% (Fig. 3). A less drastically dampened expression of CNP and 18.5 kD MBP isoform was also marked. The highest reduction typically was observed in phenotype IV, although the differences in 18.5 kD MBP and CNP contents between the two phenotypes are not statistically significant. In contrast, MAG and 21,5 MBP isoform levels seem to be almost intact in *pt* mutants (Fig. 4).

Altered expression of some of the myelin-specific protein genes at the translational level prompted examination of their transcription by hybridization with specific cDNA probes. It revealed reduction of PLP mRNA amounts that increased with age and was most pronounced in the groups of 42- and 80-day-old mutants, especially in those of phenotype IV (Fig. 5). Substantially lowered contents of MBP mRNA and CNP mRNA were also observed during densitometric analysis of autoradiograms. This indicates that transcription of the mutated gene was not severely impaired and secondary mechanism(s) responsible for further suppression of plp as well as the other myelin connected genes was induced.

Discussion

Alternative splicing of the plp gene generates two proteins, PLP and DM-20, which lack amino acids 116–150 of the PLP sequence [26]. Both proteolipids comprise about 50% of the total myelin protein fraction and are the major transmembrane proteins of the CNS myelin. They are supposed to regulate oligodendrocyte maturation, to stabilize myelin periodicity and to act as an ionophore. Mutations, duplications or additional copies of intact PLP lead to neurological disorders. One of them is *paralytic tremor* disease, caused by a point mutation. It results in CNS hypomyelination, as has been proven in many morphological [46,47,48,56] and biochemical

A

PROTEIN	PHENOTYP IV	PHENOTYP II
CNP	67.0±15.6	86.7±8.1
MAG	89.3±14.3	90.1±8.3
MBP 21.5 kD	94.4±11.6	95.7±13.6
MBP 18.5 kD	66.8±12.1	71.5±17.0

B

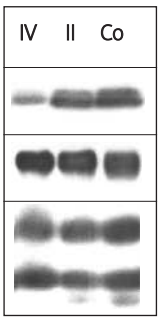


Fig. 4. Relative contents of CNP, MAG and two MBP isoforms in the myelin fractions, expressed as percent of control. Values are means ±SD of at least four experiments (n=4 in each phenotypic group) based on immunoblotting, densitometry and statistical analysis

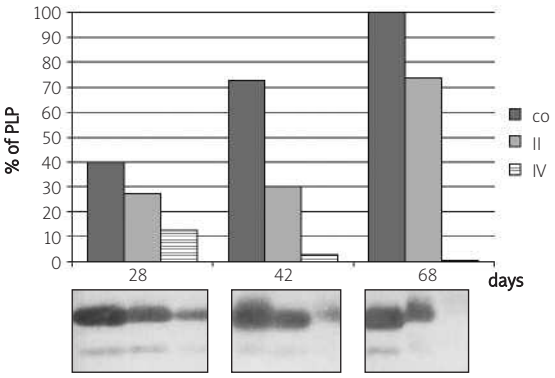
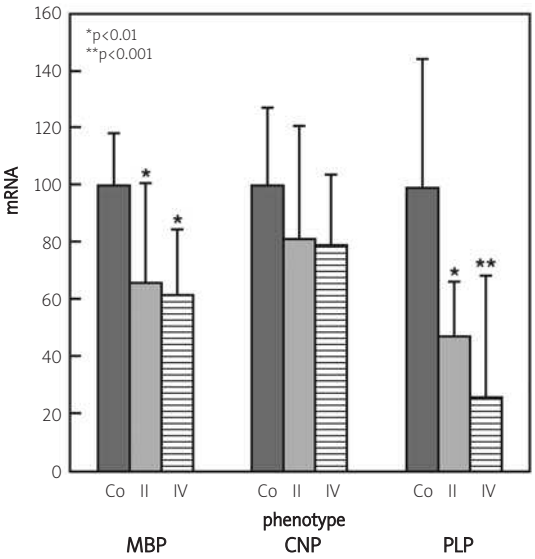


Fig. 3. Relative amounts of PLP in controls pt_{II} and pt_{IV}, respectively, as revealed by immunoblotting. The graph shows a densitometric analysis of the most representative immunoblot



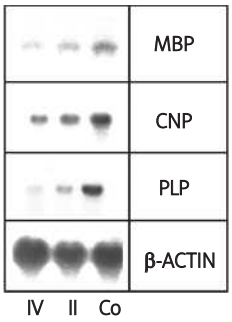


Fig. 5. Relative amounts of mRNA for PLP, MBP and CNP, as estimated by hybridization with ^{32}P-labelled specific cDNA probes, followed by autography, densitometry and statistical analysis. Lowered gene expression is observed in mutants, in the case of PLP mRNA strictly depending on phenotype. Statistical significance: *p<0.01, **p<0.001

studies [8,44]. This work shows correlation between degree of hypomyelination and severity of neurological symptoms exhibited by affected animals in the symptomatic period of disease, which was anticipated considering the role myelin sheaths play in the nervous system. Microscopic examination of the brain cross sections (cerebral cortex, subcortical white matter, pons, brain stem, corpus collosum) previously suggested that after the period of active myelination, when myelin deficiency is comparable in all mutants, it tends to increase to a greater extent in severely affected animals during the symptomatic period of the disease [56].

It is generally known that CNS hypomyelination is caused by several mutations in the plp gene, resulting in a human Pelizaeus-Merzbacher disease [14] and many inherited neurological disorders in animals [15,17,25]. In the majority of mutations a huge myelin deficiency and loss of oligodendrocytes (predominantly by apoptosis), accompanied by down-regulation of most myelin-specific proteins, is observed [12,37,40,41]. In *pt* rabbits hypomyelination is moderate (in asymptomatic and mild cases), number of oligodendrocytes is normal or even slightly elevated, and in spite of their abnormal morphology, no typical signs of apoptosis are observed [45]. Similar properties are shown by murine *rumpshaker* mutation. However, among other plp mutants, only *pt* rabbit is distinguished by a variety of phenotypes, which could be observed even within the offspring of a single breeding pair. This feature of *pt* mutation reflects human Pelizaeus-Merzbacher disease, where a varied course of the disease could be observed within one family [14,38]. Both the pathology and the variation in the disease course indicate *pt* mutant as a possibly suitable animal model for PMD.

In the presented studies, alterations in plp gene expression on both its transcriptional and translational level have been observed in rabbits that differ in their phenotype (from normal to severely affected by the disease). Examination of phenotype pt_{II} indicates that in spite of the mutation, both proteins, PLP and its alternatively spliced variant DM-20 [26], are synthesized, although deficiently (~50% of controls), and they could be incorporated into forming myelin. However, in phenotype pt_{IV} an about 70% reduction of PLP mRNA amount and over 90% reduction of translated proteins content has been observed.

As was shown [50], a point mutation (T→A transversion) in the plp gene leads to substitution of ^{36}His by Glu in *pt* rabbit. As observed in the present study, the *pt* mutation has an effect on other myelin-specific protein genes. Sequence analysis indicates possible regulatory fragments that are common for some of them, which suggests coordinated transcriptional control [6]. MBP (its 18.5 kD isoform) is, beside PLP, the most affected protein in *pt* mutation. Since that phenomenon could not be explained by altered protein transport, which is different for proteolipids [9,29,49] and basic proteins [3,7,52], a role of transcription regulating factor(s) is probable. Unlike MBP, which is synthesized in the region of its incorporation into myelin, PLP/DM-20 is translated in RER, modified in Golgi apparatus and transported to the cell processes. Defined proportions of both proteolipids seem to be crucial for at least PLP transport to the myelin membranes [39]. Studies on transgenic mice showed that even increased dosage of the plp gene disturbs myelination [22]. An alteration in PLP/DM20 ratio in known PLP mutants has also been reported. In *rumpshaker mouse* [37], *myelin deficient rat* [24] and *shaking pups* [54] PLP/DM20 proteins or transcripts ratio is decreased. Molecular studies [50] showed that the mRNA ratio of both proteolipid proteins is also changed in *pt* mutants. However, DM20 concentration seems to be altered to at least the same extent, as revealed by immunoblotting, where PLP/DM20 were detected by the same common polyclonal antibody. It was impossible however to calculate even approximate values due to difficulties in mutated protein detection: PLP amounts are reduced by 50 and 90% in phenotypes II and IV, respectively, and DM20 is a minor proteolipid in the investigated stage of development [20]. The altered proportion of both proteolipids may explain to some extent the more reduced protein level in myelin than their transcripts in the cytoplasm. However, a question about the reason for which the plp gene is less efficiently transcribed in the phenotype IV still remains unanswered. Moreover, the highest deficiency in plp gene expression in phenotype IV correlates with more significant alteration in MBP than that observed in phenotype II.

The pathomechanism(s) of the disorders caused by PLP alterations is still under discussion. It seems most probable that storage of malformed PLP deposits

evokes in oligodendrocytes so-called "endoplasmatic reticulum stress" [5,10,11]. ER "quality control" apparatus, involving most probably ER lectins calnexin and calreticulin [43], is activated and engaged in misfolded or unassembled protein degradation, thus allowing the cell to survive. If the accumulated proteins are not disposed of efficiently, they block ER and such a toxic event leads to oligodendrocyte apoptosis, as is observed in many PLP mutations. Besides malformed PLP molecules, also a role for PLP mRNA is discussed. It is known that PLP mRNA is extremely stable. It is postulated that the 3' untranslated region might regulate observed stability [21].

The variation in *pt* phenotypes might be explained by different degree of hypomyelination, which could influence the severity of neurological symptoms. As shown in that study however, the differences between the phenotypes have been observed already at the PLP mRNA level, which contributes to the intensive discussion about PLP expression patterns. The conclusion that *pt* mutation, except plp, also influences certain myelin protein genes (MBP, CNP) while the others (MAG) are not altered, leads to the hypothesis about the involvement of transcription regulating factors [4] or the signal transduction mechanism in the process. Due to its proposed structure [34] and properties [13], the PLP molecule probably itself plays a role in the signal transduction. The studies on the unique model represented by *pt* rabbits brought new insights to the discussion about PLP.

Hypomyelinated animals nowadays are especially useful for testing possible therapies based on transplantation of either stem cells or oligodendrocyte--like cell lines [1,23]. Since they are well characterised, the myelin mutants can serve as suitable models. The *shi* mice served for instance for successful transplantation of fetal and adult stem cells: cells migrated towards hypomyelinated areas and differentiated into mature oligodendrocytes [53]. The same study allowed a comparative analysis to be performed of fetal versus adult stem cells as a source for future transplantations. Numerous successful experiments that enforced oligoneogenesis in hypomyelinated mutants [27,33] offer a real approach for clinical use. The *pt* rabbit, as a mutant interesting in itself in the context of phenotypic diversity in the presented study, and well characterised at the molecular, biochemical, morphological and phenotypic levels, seems to be an excellent animal model for innovative, up-to-date studies.

Acknowledgements

Antiserum to PLP/DM-20 and p27, pMBP1, WG7 clones were kindly provided by Dr J.M. Matthieu. Anti-MAG antibody was a kind gift from Dr Ch. Quarles, and β-actin probe was generously provided by Dr J. Siedlecki.

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