

Charcot-Marie-Tooth type 1A drug therapies: role of adenylyl cyclase activity and G-protein coupled receptors in disease pathomechanism

Artur J. Kiepusa and Andrzej Kochański

Neuromuscular Unit, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland,

** Email: akiepusa@imdik.pan.pl*

Charcot-Marie-Tooth type 1A (CMT1A) is a dysmyelinating disease of the peripheral nervous system that results in a slow progressive weakening and wasting of the distal muscles of the upper and lower limbs. Despite extensive research and clinical trials there is still no treatment for CMT1A that results in complete neurological improvement. Recent studies investigating various pharmacological modulators of adenylyl cyclase activity, including ascorbic acid and ligands of G protein-coupled receptors (GPCRs), provide hope for future treatments of this type of hereditary motor and sensory neuropathy. A review of mechanisms of action of several compounds tested for CMT1A in pre-clinical and clinical studies ascorbic acid, onapristone, PXT3003 (baclofen, naltrexone, and sorbitol), and ADX71441, very clearly indicates an important role for adenylyl cyclase activity and GPCRs in the pathomechanism of the disease. Metabotropic γ -aminobutyric acid receptors (GABA_BR), subtype mu (μ) opioid receptors (MOR), and muscarinic acetylcholine receptors (mACh) appear to be particularly significant in both pathogenesis and treatment, and their activation may exert a similar and synergistic effect on the physiology of Schwann cells as well as neurons. These receptors participate in proliferation and differentiation of Schwann cells and influence excitatory transmission in neurons. We also hypothesize that onapristone might act through a non-classical mechanism via membrane progesterone receptor (mPR) and cAMP signaling. This review endeavors to outline a pathway leading inversely from therapy to an indispensable role for adenylyl cyclase activity and GPCRs in the modulation of dosage sensitive peripheral myelin protein (*PMP22*) gene expression.

Key words: CMT1A, gene dosage effect of *PMP22*, drug therapies, GPCRs, adenylyl cyclase activity, cAMP signaling pathway

INTRODUCTION

Major advancements in our understanding of the pathomechanism and genetic background of peroneal muscular atrophy have been achieved since Charcot and Marie, in Paris, and Tooth, in London, first described the heterogeneous syndrome in 1886. More than 80 years later Dyck and Lambert (1968), on the basis of neurologic, genetic, and electrophysiological findings, differentiated two main types of hereditary motor and sensory neuropathy (HMSN): type I (demyelinating) and type II (axonal), also known as Charcot-Marie-Tooth disease type 1 (CMT1) and type 2 (CMT2), respectively (Genigiani and Marbini 2001). With the advent of molecular

biology it has become clear that genetic heterogeneity between CMT types exists. Further development of new molecular biological techniques enabled the advancement of molecular genetic studies leading to the identification of nearly 100 genes responsible for many types of CMT disease. Charcot-Marie-Tooth disease type 1A (CMT1A) constitutes almost 60% of all known CMT cases (Liu et al. 2014). Disease onset occurs in the first decade of life and is usually identified because of frequent falls and clumsiness of gait. Initial symptoms are recognized as high-arched feet, which is a consequence of atrophy of the intrinsic feet muscles. Usually CMT disease begins with a slow progressive wasting and weakening of the distal muscles of the lower limbs. The atrophy spreads

to other muscle groups of the lower limbs, i.e., peroneal, anterior tibialis, and triceps, yielding a characteristic phenotype of inverted bottle legs. Muscle atrophy can also affect intrinsic muscle groups of the hands, spreading to 1/3 of the lower parts of the forearms. Tactile sensory disruption may be concomitant and observed in gloves and stockings areas (Kochański 2008). CMT1A disease is characterized by significant clinical heterogeneity in terms of the age of onset, degree of muscle weakness, comorbidity with other symptoms, and an anticipation (De Jonghe 1998).

The primary feature of CMT1A disease is a decrease of conduction velocity in both motor and sensory fibers to about 20 m/s. In sural nerve biopsy, numerous tomaculae (loops of unfolded myelin) surrounding demyelinating and remyelinating fibers are observed. Despite very heterogeneous clinical features, the genetic causes of CMT1A disease are relatively homogenous. In an overwhelming majority of cases, CMT1A disease is caused by a large duplication located on the short arm in region 17p11.2-p12, which is occupied by the *PMP22* gene encoding 22 kDa peripheral myelin protein (PMP22) – one of the major protein constituents of the myelin sheath of peripheral nervous system (PNS) neurons (Lupski et al. 1991, Patel et al. 1992, Raeymaekers et al. 1991). However, in 1993 a point mutation in the *PMP22* gene was identified for the first time (Roa et al. 1993). In this report an amino acid substitution (S79C) was shown to have the same phenotypic effect as duplication of the *PMP22* gene. Additionally, point mutations were absent in patient groups with duplication of *PMP22* (Warner et al. 1996).

Gene dosage effect of *PMP22* in CMT1A disease

A gene dosage effect has been hypothesized and is generally accepted as an underlying mechanism of CMT1A disease, proposed by Lupski et al. (1992), and is defined by an increase in expression of *PMP22* mRNA in patients with CMT1A phenotype (Schenone et al. 1997, Yoshikawa et al. 1994). Although a gene dosage effect represents the most likely molecular mechanism underlying CMT1A, it does not fully explain the pathogenesis of the disease. Indeed, duplication of a large submicroscopic region of DNA (1.5 Mb), observed at both the transcriptional and translational level, is certainly involved in the pathogenesis of CMT1A disease, but phenotype severity seems to correlate only loosely and reaches a plateau above a certain level. In light of the hypothesis, we could assume that patients with four copies of the *PMP22* gene are predicted to be affected more severely than those with three copies; however, they do not actually manifest a more severe phenotype. Though, in a subpop-

ulation of CMT1A patients, a triplication causing a more severe phenotype was found (Di Vincenzo et al. 2014). It is clear that broad heterogeneity in the clinical phenotype of CMT1A is also observable, with *PMP22* mRNA and protein levels being highly variable among patients and lacking correlation with disease severity and clinical outcome measures (Katona et al. 2009, Lewis et al. 2013, Nobbio et al. 2014, Scherer and Wrabetz 2008, Visigalli et al. 2016).

The gene dosage effect hypothesis has been fully confirmed in animal models where phenotype severity correlated with *PMP22* gene copy number (Schenone et al. 1997, Sereda et al. 2003). However, the animal models were developed by integration of extra copies of the *PMP22* gene into their genomes (Huxley et al. 1996, 1998, Magyar et al. 1996, Robaglia-Schlupp et al. 2002, Sereda et al. 1996, Verhamme et al. 2011). Additionally, some data indicate that duplication of a small region (186 kb) downstream of the dosage sensitive region of the *PMP22* gene is sufficient to cause the phenotype of the neuropathy, as recently found in six families. Such copy number variation (CNV) is not usually assessed during routine diagnostic screens for CMT1A because copy numbers of *PMP22* are normal (Weterman et al. 2010).

Thus, no biological basis for clinical variability of CMT1A is known. However, a recent study of a large cohort of CMT1A/HNPP-affected patients conducted by Sinkiewicz-Darol et al. (2015) determined that the LITAF 192V sequence variant predisposes patients to an earlier age of onset of CMT1A as well as hereditary neuropathy with liability to pressure palsies (HNPP) diseases. It is noteworthy that HNPP is characterized by a single copy of *PMP22* (Chance et al. 1993), but point mutations have also been reported (Nicholson et al. 1994). Moreover, Visigalli et al. (2016), focusing on alternative splicing of *PMP22*, found three new gene transcripts in human sural nerve biopsies and also demonstrated altered expression of *Qki* in rats, which is a critical splicing regulator during the myelination process. These data suggest a possible role for alternative splicing in the clinical variability of CMT1A neuropathy.

Drug therapies in CMT1A

In laboratory transgenic animals, *Pmp22* gene copy number has been shown to correspond with the clinical course of neuropathy (Schenone et al. 1997, Suter and Nave 1999). Moreover, an experimental approach involving reduction of *Pmp22* expression resulted in an improved clinical course of CMT in transgenic mice and rats (Passage et al. 2004, Sereda et al. 2003), which led to large clinical trials. However, the human trials did not confirm the findings from animal models, probably due

to a lack of correlation between gene dosage and clinical outcome of CMT1A (Sinkiewicz-Darol et al. 2015).

Currently available drug therapies for CMT1A aim to abolish the dosage effect/toxic gain of function of the overexpressed *PMP22* gene (Table I). Although pharmacological therapies aimed toward decreasing gene dosage of *PMP22* failed or showed some minor effect, they have been very informative in terms of unraveling CMT1A pathogenesis. Here we describe various pharmacological approaches in the treatment of CMT1A disease that modulate adenylyl cyclase activity. A complete list of currently available compounds tested in CMT1A therapy has previously been published by Ekins et al. (2015).

Ascorbic acid

Initial CMT1A therapy attempts involved ascorbic acid (AA, vitamin C), which is predominantly known for its antioxidant properties. However, the mechanism of action of AA is multifaceted due to its more than 20 metabolites, which differ in various tissues (Passage et al. 2004). Besides its role as an antioxidant, AA has an important neuromodulatory role in the brain, aiding in the release of some neurotransmitters while also inhibiting neurotransmitter binding to receptors (Rosa et al. 2005). Although the exact mechanism by which AA exerts its action remains unclear, a number of studies have recently demonstrated that AA can regulate the expression of a battery of genes encoding extracellular matrix proteins and myelin proteins (Belin et al. 2010). An experiment using neuron-Schwann cell co-culture supplemented with AA showed that AA plays a vital role in *in vitro* myelination (Clark and Bunge 1989). In-

deed, Passage et al. (2004) demonstrated in a transgenic mouse model that high doses of AA decreased *Pmp22* expression and ameliorated CMT1A phenotype in terms of functional and histopathological findings. This was further confirmed by Kaya et al. in 2007, who additionally determined the mechanism by which AA reduced *Pmp22* expression (Kaya et al. 2008). AA was proposed as a competitive inhibitor of adenylyl cyclase activity that exerts an effect on intracellular cyclic adenosine monophosphate (cAMP). Additionally, the inhibitory effect of AA was abolished by administration of vitamin A or E (Kaya et al. 2008). Promising findings in experimental models resulted in various clinical trials of AA administration in CMT1A patients. However, the first and largest study, conducted over two years, reported no significant differences between AA and placebo groups (Pareyson et al. 2011). Although clinical studies revealed that AA treatment had no impact on CMT1A, it is thought to carry out an important function in peripheral nerve myelination and possibly in remyelination (Gess et al. 2011). An informative and well-designed experiment was conducted by Gess et al. (2011) who studied sodium-dependent vitamin C transporter 2-heterozygous (SVCT2^{-/-}) mice. In this study, they showed reduced expression of SVCT2 mRNA and protein levels as well as decreased AA concentrations in sciatic nerves of KO mice. Histopathology of sciatic nerve also revealed reduced myelin thickness, which was reflected in sensorimotor performance and electrophysiological tests of the studied animals. Furthermore, they provided evidence for defects in collagen synthesis both in sciatic nerves and in Schwann cell cultures under SVCT2-inhibiting conditions. Interestingly, SVCT2 mutation is associated with myelin protein zero (MPZ) reduction and simultaneously with an

Table I. Compounds tested in preclinical and clinical settings for CMT1A that modulate adenylyl cyclase activity and cAMP levels.

Compound	Targeted GPCR	Research stage	Outcome	Reference
Ascorbic Acid	-	Pre-clinical	Improvement (myelination, myelin thickness, PMP22 level)	Passage et al. 2004
		Clinical	No improvement	Pareyson et al. 2011
Onapristone*	mPR	Preclinical	Improvement (myelination, PMP22 level)	Sereda et al. 2003
PXT3003 Baclofen Naltrexone Sorbitol	GABA _B opioid receptor muscarinic acetylcholine receptor	Pre-clinical	Improvement (myelination, PMP22 level)	Chumakov et al. 2014
		Phase 2 clinical trial	Minor improvement	Attarian et al. 2014
ADX71441	GABA _B	Pre-clinical	Improvement (myelination, PMP22 level, electrophysiology)	Addex Therapeutics 2013

* Onapristone is hypothesized by author A.J.K. of this manuscript as an mPR modulator which has not yet been confirmed in any study.

increase (2.8-fold), though not statistically significant, in (mRNA) *Pmp22* expression levels (Gess et al. 2011). The statistically insignificant rise in *Pmp22* expression might be sufficient to yield a disease phenotype. This fact is interesting when considered together with AA's function in modulating extracellular matrix (ECM). AA is necessary for collagen synthesis in the PNS and collagen in turn is vital for correct myelination (Petersen et al. 2015). Moreover, it has been suggested that an interaction between ECM and the expression of myelin genes is possible (Chernousov et al. 2008). AA would be necessary for the myelination process by an, as of yet, unclear mechanism. However, myelination might be controlled by non-canonical cAMP signaling, including exchange protein activated by cAMP (EPAC) exerting its activity via direct binding to cAMP, soluble adenylyl cyclase (sAC), which is activated independently of GPCRs, and the highly conserved orphan GPCR GPR126 that controls myelination via cAMP (Bacallao and Monje 2015, Monje 2015).

GPR126 is known to interact with the extracellular matrix proteins collagen and laminin-211 (Monk et al. 2009, 2011) and may constitute a functional link between two elements (ECM and expression of myelin genes).

Onapristone-progesterone receptor antagonist

Progesterone is considered to be a neuroactive steroid because it is synthesized in the nervous system by glial cells independently of endocrine production. The literature suggests a mutual interplay between the GABAergic system (via GABA_A and GABA_B receptors) and neuroactive steroids, such as progesterone and its derivatives. Neuroactive steroids act as amplifiers of GABA neurotransmission (Magnaghi et al. 2006). Progesterone elicits its action via two mechanisms: genomic – through the specific binding of progesterone receptor (PR) to progesterone-responsive elements (PRE) and further stimulation of activity of the promoters of myelin protein genes and non-genomic – through activation of signaling pathways that may be mediated by membrane-associated PRs (Singh et al. 2013). The *MPZ* promoter, *PMP22* promoter 1, and *PMP22* exon1 do not contain PREs. Thus, progesterone may indirectly activate these promoters by inducing a Schwann cell-specific transcription factor (Desernaud et al. 1998) and increasing mRNA concentrations of myelin genes (Melcangi et al. 1998). Moreover, derivatives of progesterone, dihydroprogesterone (DHP) and tetrahydroprogesterone (THP or allopregnenolone), increased myelin gene expression by activation of GABA_A steroid receptors on Schwann cells in both *in vitro* and *in vivo* experiments (Melcangi et al. 1999). THP is a steroid that is unable to

bind PR, but actively interacts with some components of the GABA_A receptor. THP and the progestogens P and DHP in turn can influence the expression of GABA_B subunits in Schwann cells (Magnaghi et al. 2006). Importantly, Zhu et al. (2003a, 2003b) discovered and classified three subtypes of membrane progesterone receptors (mPR), which are GPCRs belonging to the progestin and adipoQ receptor family (PAQR) and mediate rapid action of progestogens in reproductive tissues and in the nervous system (Thomas and Pang 2012). Sereda et al. (2003) conducted an experiment using transgenic rats that suggested the PR of myelin-forming Schwann cells is a promising pharmacological target for therapy of CMT1A. In the study, administration of progesterone increased the expression of myelin genes in the sciatic nerve and resulted in enhanced Schwann cell pathology. In contrast, administration of onapristone (progesterone receptor antagonist) reduced overexpression of *Pmp22*, ameliorating the CMT1A phenotype (Sereda et al. 2003). These findings were confirmed by Meyer zu Horste et al. (2007). However, the latter reported that onapristone did not improve myelin sheath thickness. Clinical trials have not been started due to severe side effects of anti-progesterone treatment (Robertson et al. 1999). In light of these data it should be considered that treatment with anti-progesterone derivatives like THP may be more efficient than anti-progesterone itself, as THP can exert its action not only via classical PR but also via GABA_A receptors (Melcangi et al. 1999), which is certainly a more complex approach. Additionally, Schwann cells are able to synthesize progesterone locally and thus abolish the effect of onapristone by increasing the concentration of progesterone within the cell or possibly signaling via the GABA-ergic system may overwhelm the PR pathway. We also cannot exclude the possibility that onapristone exerts its action via mPR in Schwann cells and neurons. The importance of alternative pathways of progesterone signaling is supported by the observation of Jung-Testas et al. (1999) who showed that the structure of peripheral nerves was normal in PR knockout (PRKO) mice.

PXT3003

PXT3003 is a novel combination of three drugs – baflofen, naltrexone, and sorbitol – that was shown to lower *Pmp22* transgenic rats (Chumakov et al. 2014). Additionally, GPCRs targeted by the compound are expressed not only in Schwann cells but also in peripheral neurons, which indicates a wider mechanism of action (Stein and Lang 2009).

PXT3003 has already passed phase 2 clinical trials, which confirmed the safety and tolerability of low dos-

es of PXT3003 in the treatment of CMT1A affected individuals and showed improvement beyond stabilization (Attarian et al. 2014). Further investigation is currently being conducted in phase 3 clinical trials. Baclofen is a derivative of GABA and exerts its action by GABA_B receptors. It is used as a spasmolytic drug in multiple sclerosis (Hudgson et al. 1972) as well as in alcohol addiction therapy (Addolorato et al. 2002). Naltrexone is a reverse agonist of opioid receptors used in treatment of opioid (Resnick et al. 1978) and alcohol dependence (Morris et al. 2018). Its mechanism of action is exerted primarily via μ -opioid receptors (MOR). Sorbitol may act as a chaperone and/or bind muscarinic acetylcholine GPCRs (Liu et al. 2005).

ADX71441

Recently, a positive allosteric modulator (PAM) of GABA_B receptor N-(5-(4-(4-chloro-3-fluorobenzyl)-6-methoxy-3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3H)-yl)-2-fluorophenyl)acetamide, termed ADX71441, was studied in the transgenic CMT rat model, modeling symptoms in CMT1A patients and characterized by overexpression of *Pmp22* at transcriptional level, decreased nerve conduction velocity, and lower grip strength. Peripheral nerves of ADX71441-treated rats exhibited downregulation of *Pmp22* mRNA, reduction of hypomyelinated axons, and increased compound muscle action potentials (CMAP) in comparison with the

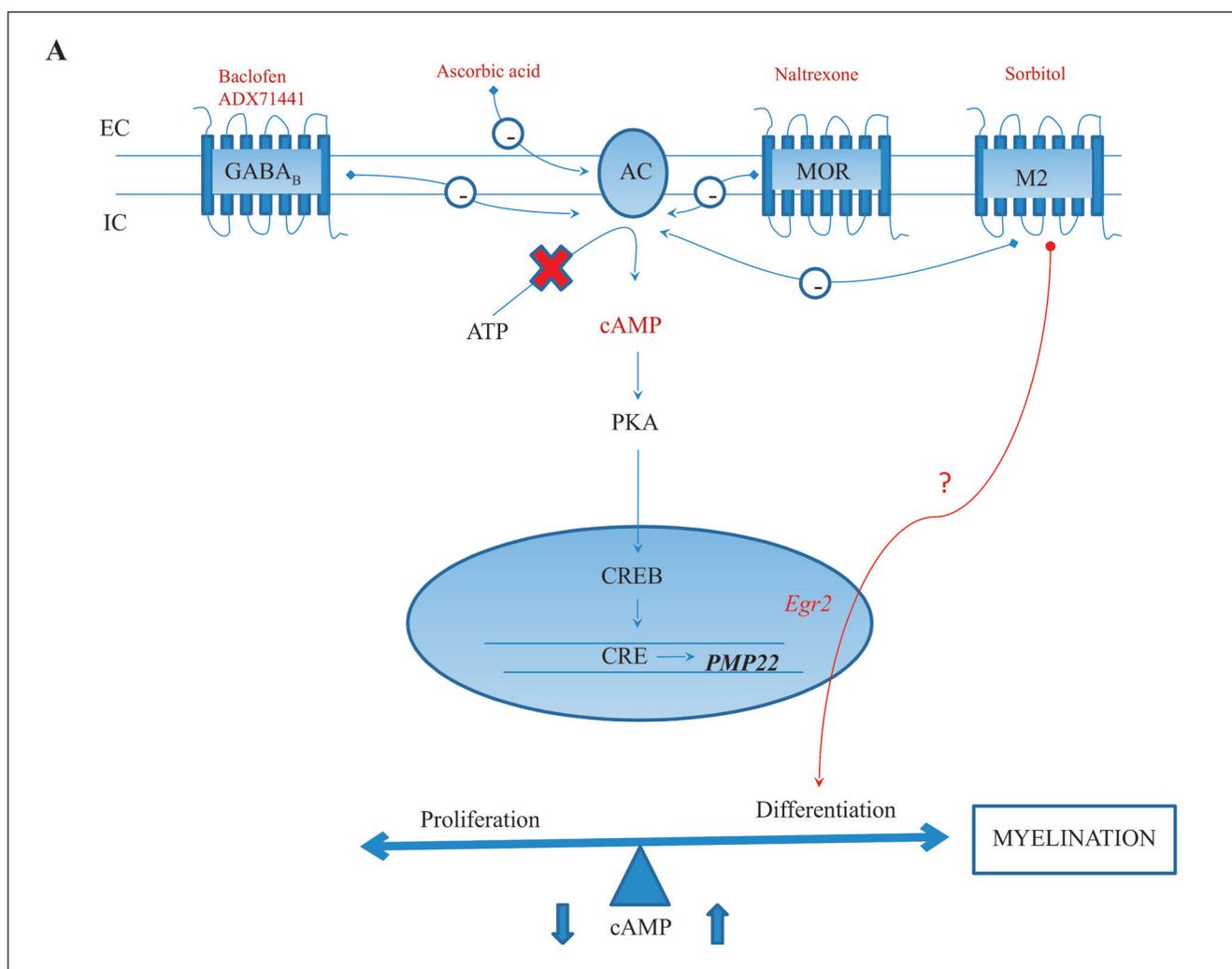


Fig. 1. (A) Determination of Schwann cell fate via ascorbic acid and GPCR modulators. Administration of ascorbic acid and pharmacological activation of GPCRs (GABA_B, opioid receptor or muscarinic receptor) in Schwann cell membrane leads to transmembrane adenylyl cyclase inhibition and concomitant decrease in cAMP level, inhibition of PKA phosphorylating activity, and finally effective suppression of myelin genes' transcription, which in turn shifts Schwann cells into proliferative state and inhibits the differentiation and myelination process. Thus, low levels of cAMP promote proliferation, while high levels promote differentiation of Schwann cells. (EC) extracellular; (IC) intracellular; (AC) adenylyl cyclase; (ATP) adenosine triphosphate; (cAMP) cyclic adenosine monophosphate; (PKA) phosphokinase A; (CREB) cAMP responsive element binding protein; (CRE) cAMP response element; (*PMP22*) peripheral myelin protein gene; (*Egr2*) (also termed *Krox20*) early growth response 2.

control group (Dyer 2013). It is noteworthy that neither onapristone nor PXT3003 increased CMAP, which is an important indicator of neuromuscular transmission. ADX71441 acts as an allosteric modulator amplifying the orthosteric agonist effect on its metabotropic receptor either by enhancing the affinity of binding or the functional efficacy (May et al. 2007). It has previously been approved for phase 1 clinical trials in overactive bladder (Kalinichev et al. 2014) as well as for anxiety, pain, and spasticity treatment (Kalinichev et al. 2017).

GPCRs influence Schwann cell fate and excitatory transmission in neurons

The GPCR superfamily constitutes the largest class of functionally selective drug therapy targets for PNS diseases (for review see Mogha et al. 2016). Data obtained from both pre-clinical and clinical trials allows for analysis of the mechanism of action of several receptors and downstream signaling pathways, and thus explain their role in the physiology and pathology of neural cells. A clear and essential role of GPCRs in the pathomechanism of CMT1A disease has been demonstrated. Particularly significant to both pathogenesis and treatment, are the GABA_BRs, the MORs, and possibly type 2 muscarinic receptors (M2), the activation of which may exert a similar and synergistic effect on the physiology of neurons (Christie and North 1988) as well as Schwann cells. We also hypothesize that mPR and

cAMP signaling could be modulated by onapristone, as described earlier in this text.

The common denominator in signal transduction by GPCRs is cAMP. Activation of the above-mentioned receptors inhibits adenylyl cyclase, which does not convert ATP into cAMP. Low levels of cAMP inhibits expression of transcription factors and myelin genes, switching the Schwann cell into a proliferative state (Fig. 1A). Moreover, cAMP maintains a balance between other signaling pathways like PI3K-AKT and MEK-ERK and integrates stimuli derived from other receptors, i.e., tyrosine kinases activated by neuregulin 1 (NRG1), which provides a proliferation or myelination signal in accordance with the concentration of cAMP. Activation of GPCRs can also directly influence the kinetics of potassium and calcium ion channels important to excitatory neurotransmission as well as indirectly influence by lowering cAMP levels (Fig. 1B).

GABA_B receptors

GABARs are primarily known for their inhibitory actions on excitatory transmission in neurons, but they also have an important role in Schwann cell proliferation and myelination. A GABAR agonist, GABA, increases or decreases the synthesis of PMP22 protein, depending on the receptor involved (GABA_A or GABA_B). The activation of ionotropic GABA_A receptors exerts a stimulatory effect on *PMP22* gene expression (Magnaghi et al. 2001). Activation of metabotropic GABA_B receptors leads

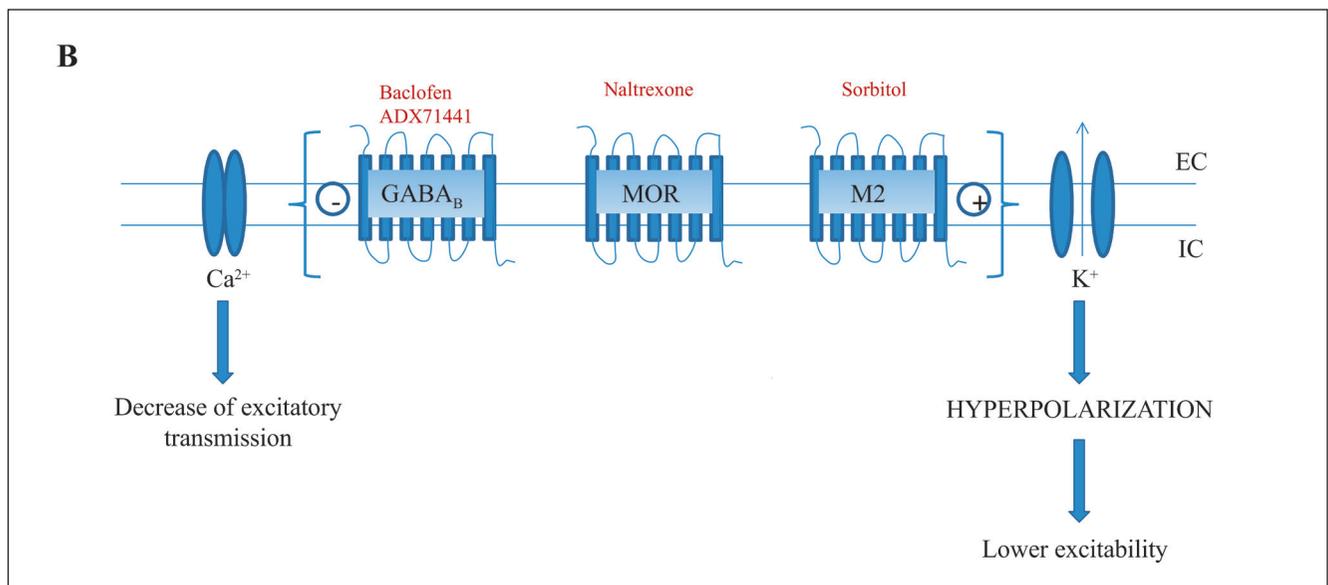


Fig. 1. (B) Activation of GPCRs (directly) and low level cAMP (indirectly – not shown in the Figure) in neurons influence potassium and calcium ion channel kinetics causing closing and opening, respectively, which evokes hyperpolarization (K⁺) of the cell and inhibits axonal transmission of neuronal stimuli (Ca²⁺).

to decreased levels of PMP22 and MPZ in Schwann cells (Magnaghi et al. 2004, Melcangi et al. 2005). This is the case, at least, for Schwann cells in culture absent of neurons. However, Corell et al. (2015) suggested that GABA_B receptor is not involved in developmental Schwann cell proliferation because neither baclofen (GABA_B receptor agonist) nor CGP55485 (GABA_B receptor antagonist) had an effect on proliferation of Schwann cells, which were co-cultured with DRG neurons. In this model myelin associated glycoprotein (MAG) and myelin basic protein (MBP) mRNAs were increased, suggesting that GABA_B receptor activation controls myelin protein expression and myelination itself. Importantly, upregulation of GABA_B receptor occurred after nerve crush injury. This is interesting because GABA_B activation decreased cAMP levels in Schwann cells as well as in neurons and additionally inhibited neuronal excitability and, thus, does not promote myelination. Since activation of the inhibitory GABAergic pathway promotes amelioration of the CMT1A phenotype, one question is whether over-activation of the glutamatergic pathway may be involved in the pathomechanism of CMT1A. If so, we should also consider the glutamatergic pathway as a potential novel target for drug therapies in CMT1A and other demyelinating diseases of the nervous system.

Opioid receptors

By targeting opioid receptors it is possible to modulate calcium and potassium ion channels. Opioids exert inhibitory effects on neuronal excitability by regulating Kir3 potassium channels and inhibiting calcium conductance (Al-Hasani 2011). Despite their function in neuronal excitability, opioid receptors are expressed on CNS oligodendrocytes, and the role of the opioid system in myelination is poorly understood. Vestal-Laborde et al. (2014) reported that perinatal exposure of rat pups to the opioid methadone elevated levels of the myelin proteins MBP, PLP, and MOG in the brain and increased the number of myelinated axons. Thus, *per analogiam*, myelin protein expression could be changed in the PNS through opioid receptor modulation.

Several studies have shown that all subtypes of opioid receptors are negatively coupled to adenylyl cyclase and suppress cAMP formation via activation of GTP-binding proteins ($G_{i/o}$). However, Russell et al. (2001) demonstrated that activation of κ -opioid receptors in neurons of rabbit iris-ciliary body can evoke both stimulatory and inhibitory effects on the cAMP pathway depending on the concentration being used. Production of cAMP was inhibited at high concentrations and stimulated at low concentrations of bremazocine (κ -opioid receptor agonist) administration.

A similar response of opioid receptor activation on cAMP level was observed by Dziedzicka-Wasylewska and Przewłocki (1995) who showed that administration of U50,488H (κ -opioid receptor agonist) as well as morphine (μ -opioid receptor agonist) increased the cAMP level in rat hippocampal slices. These observations lend to the possibility that naltrexone (reverse agonist of opioid receptor) could also have a biphasic effect on cAMP level, suppressing its production at low doses. In fact, opiate withdrawal leads to a significant increase in cAMP-dependent protein kinase (PKA) and cAMP response element binding protein (CREB) in noradrenergic nuclei (Guitart et al. 1992), the expression of which was shown to be decreased in rats that received low doses of naltrexone in drinking water (Mannelli et al. 2004).

Muscarinic acetylcholine receptors

Schwann cells, by expressing muscarinic acetylcholine receptors M1, M2, M3, and M4 (for comprehensive review see Fields et al. 2017) participate in cholinergic signaling within the PNS (Loreti et al. 2006). The most abundant GPCR found in Schwann cell membranes is the M2 receptor. Morphometric and ultrastructural analysis of M2/M4 knock-out mouse sciatic nerve revealed altered myelin organization and degenerated axons (Uggenti et al. 2014). Additionally, cAMP production was inhibited by activation of M2 and M4 receptors, which in turn abolished Ca²⁺ conductance (Eglen 2006). This effect is similar to the action of opioid receptors, as mentioned previously. Uggenti et al. (2014) also showed that *in vitro* activation of M2 receptor, by its agonist arecaidine, upregulated transcription factors Sox10 and Krox20, involved in the promyelinating phase, and simultaneously downregulated proteins c-Jun, Notch-1 and Jagged-1, which in turn maintain Schwann cells in an undifferentiated state. It is important to note that upregulation of the transcription factor c-Jun in Schwann cells protected sensory neurons in a mouse model of CMT1A (Hantke et al. 2014). Thus, the cell cycle is arrested by M2 receptor activation, which inhibits cell proliferation and at the same time promotes cell differentiation and expression of myelin proteins (Uggenti et al. 2014). This is of interest because low levels of cAMP, induced by M2, act as a proliferation signal for Schwann cells and suggests that M2 receptor stimulation may exert a dual/multiple effect: lowering the level of proliferation-promoting cAMP and upregulating transcription factor Egr2 (Krox-20), which in turn promotes expression of myelin genes. However, another more convincing explanation is possible, that an upregulation of Krox-20 and Sox10 is likely independent of cAMP signaling or may use other

pathways or downstream effectors such as the previously mentioned EPAC, sAC, and GPR126 (Fig. 1). The second explanation is that d-sorbitol acts via different subtypes of muscarinic receptor, i.e., M1, M3, or M5, whose signal stimulates via Gq protein's phospholipase C activity and calcium signaling, thus increasing intracellular calcium concentration and leading to the expression of myelin genes (Fields et al. 2017). It is noteworthy that neuregulin 1 isoform III (NRG1), which was shown to alleviate CMT1A phenotype in rats, influenced the activity of the PLC pathway but through a different type of receptor – tyrosine kinase (Fledrich et al. 2014). Additionally, cAMP signaling is vital in switching β -neuregulin 1 (NRG1- β) from a proliferative signal to a myelin differentiation signal (Arthur-Farraj et al. 2011). cAMP could also switch d-sorbitol action in the same manner.

It seems that proliferation rather than differentiation relies on the activation of transmembrane adenylyl cyclase (tmAC). In turn, myelination may be controlled by non-canonical cAMP signaling including EPAC, sAC, which is activated independently of GPCRs, and GPR126, a highly conserved orphan GPCR that controls myelination via cAMP (Bacallao and Monje 2015, Monje 2015).

This can partially explain why therapy aiming at modulation of cAMP levels via GPCRs may not be as effective as predicted. Drugs inhibit tmAC without inhibiting its soluble form. Overactivity of sAC in CMT1A patients might lead to downstream signaling, inducing transcription factors responsible for overexpression of myelin genes including *PMP22*. The total effect of pharmacological therapy with the use of GPCR ligands could depend on the canonical (via tmAC) and non-canonical (via EPAC, sAC, and GPR126) pathway signaling ratio.

To date, only a few GPCRs that could be used for future dysmyelinating disease therapy have also been described in Schwann cells: GPR126/ADGRG6 (adhesion family member interacting with laminin-211, collagen IV, and Prp^c) (Küffer et al. 2016, Paavola et al. 2014, Petersen et al. 2015), GPR44 and LPA1 (rhodopsin family members) interacting with prostaglandin D2 (Trimarco et al. 2014) and lysophosphatidic acid (Anliker et al. 2013), respectively. For a comprehensive review see Mogha et al. (2016).

cAMP signaling in the pathomechanism of CMT1A

It seems that the cAMP signaling pathway, although very prominent in maintaining proper balance between proliferation and differentiation of Schwann cells, does not work in a unitary manner, but rather orchestrates other pathways, i.e., PI3K-AKT and MEK-ERK kinase cascades (Fledrich et al. 2014) and neurosteroid-mediated

signaling (Gellersen and Brosens 2003, Magnaghi et al. 2006) all of which are built around a common second messenger (Monje 2015). The targeting of multiple disease-relevant pathways by modulation of cAMP levels and inhibition of downstream effectors exerts a pleiotropic mechanism of action in both Schwann cells and neurons. Firstly, it leads to an imbalance between activity of PI3K-AKT and MEK-ERK cascades, favoring the latter. This in turn causes upregulation of dedifferentiation markers c-Jun and Sox-2 and the downregulation of myelin gene expression, including *PMP22*, which maintains an immature proliferative Schwann cell phenotype (Fledrich et al. 2014). Moreover, MEK-ERK activity induces upregulation of monocyte chemoattractant protein (MCP-1 or CCL2), which attracts phagocytic macrophages, removing myelin debris and degenerated axons from the site of injury (Martini 2014). Martini et al. (2013) also observed that altogether those events are harmful for healthy nerves but might be advantageous in the context of disease-injured axons. However, immaturity of Schwann cells that persists for a long time, along with defective differentiation, favors pathogenesis of CMT (Fledrich et al. 2014). In summary, dedifferentiation in pathological conditions is necessary but in intact axons is harmful. Secondly, activation of GPCRs and inhibition of cAMP signaling influences the excitability of neuronal cells and activates cytoprotective signaling pathways. As mentioned previously, the lack of proper neuronal stimulation of Schwann cells does not promote myelination (Wake et al. 2015); however, it may be advantageous for axon regeneration as excessive excitability could lead to secondary nerve injuries in CMT patients. Saitoh et al. (2016) showed that activation of metabotropic glutamate receptor class 2 (mGluR2) promotes Schwann cell proliferation and de-differentiation, while their inhibition promotes myelination by eliciting intracellular signaling downstream of a GPCR. Interestingly, mGluR2 activation enhances NRG1-induced ERK phosphorylation that promotes proliferation and dedifferentiation of Schwann cells, but not Akt phosphorylation that results in Schwann cell migration and/or sorting. mGluR signaling showed a significant effect on Schwann cell phenotype modulation *in vivo*. Thus, control of glutamate levels in peripheral nerve is crucial for Schwann cell proliferation, as well as differentiation. For this reason glutamate receptors could also contribute to the pathomechanism of CMT1A. Because differentiation of Schwann cells may counterbalance axon growth, therapy aiming to increase myelination should be applied independently of axon regenerating therapy or in later stages of regeneration (Monje 2015). As long as axons continue to grow, Schwann cells should be maintained in an immature state. Thus, highly important to any

pharmacological approach is the consideration of physiological chronology of individual processes. It appears that currently available therapies mainly aim for axon regeneration, and prolonged maintenance of Schwann cells in an undifferentiated state might be unfavorable for CMT phenotype alleviation.

CONCLUSIONS

Many of the currently available experimental therapeutics tested for effect in CMT1A in both pre-clinical and clinical studies impact adenylyl cyclase activity, changing the physiology of Schwann cells and neurons. PXT3003, ADX71441, and, hypothetically, onapristone modulate adenylyl cyclase via GPCRs. In turn, AA acts as a competitive inhibitor of adenylyl cyclase activity. All compounds tested for CMT1A were equally promising in pre-clinical studies in terms of remyelination and decreasing *PMP22* mRNA expression. However, they generally did not influence myelin thickness, which was increased only by AA administration and even in this case was only a minor improvement. Clinical trials that have been conducted with the use of AA did not show any significant effect on neuropathy compared with placebo after two years. However, the first and largest clinical trial established a systematic approach in clinical assessment of CMT1A – the CMT neuropathic scale was introduced and large multicentre patient groups appeared. Onapristone was not tested for CMT1A in clinical trials due to severe side effects of previously conducted antiprogestosterone treatment in women suffering from breast cancer. PXT3003 is currently under investigation in phase 3 clinical trials. It was tested for safety and tolerability but showed only minor improvement in phase 2 trials. The most promising compound in the treatment of CMT1A appears to be ADX71441, which exhibits the properties of previously tested drugs but additionally improves CMAP. Moreover, it is a positive allosteric modulator of GABA_B receptor and, thus, more selective and potent than orthosteric modulators. Applying new methods in structure based drug design to derive allosteric modulator drugs for the largest superfamily of cell surface receptors – GPCRs – should be a consideration in the future search for an effective CMT1A cure.

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