MINI REVIEW ARTICLE

Non-genetic therapeutic approaches to Canavan disease

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ABSTRACT:

Canavan disease (CD) is a rare leukodystrophy characterized by diffuse spongiform white matter degeneration, dysmyelination and intramyelinic oedema with consequent impairment of psychomotor development and early death. The molecular cause of CD has been identified as being mutations of the gene encoding the enzyme aspartoacylase (ASPA) leading to its functional deficiency. The physiological role of ASPA is to hydrolyse N-acetyl-L-aspartic acid (NAA), producing L-aspartic acid and acetate; as a result, its deficiency leads to abnormally high central nervous system NAA levels. The aim of this article is to review what is currently known regarding the aetiopathogenesis of CD with emphasis on the non-genetic therapeutic strategies, both at an experimental and a clinical level, by highlighting: (a) major related hypotheses, (b) the results of the available experimental simulatory approaches, as well as (c) the relevance of the so far examined markers of CD neuropathology. The potential and the limitations of the current non-genetic neuroprotective approaches to the treatment of CD are particularly discussed in the current article, in a context that could be used to direct future experimental and (eventually) clinical work in the field.

List of abbreviations: Ac: acetate; Acetyl-CoA: acetyl-coenzyme A; ACS: acetyl-coenzyme A synthetase; Asp: L-aspartic acid; ASPA: aspartoacylase; AspNAT: aspartate N-acetyltransferase; BBB: blood-brain barrier; CA: calcium acetate; CD: Canavan disease; CNS: central nervous system; CoA: coenzyme A; ECF: extracellular fluid; Gln: glutamine; Glu: glutamate; GMR3: metabotropic glutamate receptor 3 (mGluR3); GTA: glyceryl triacetate; HPLC: high-performance liquid chromatography;

Li: lithium; LiCl: lithium chloride; $Li_3C_6H_5O_7$: lithium citrate; MRI: magnetic resonance imaging; MRS: magnetic resonance spectroscopy; NAA: *N*-acetyl-L-aspartic acid *or* *N*-acetylaspartate; NAAG: *N*-acetylaspartylglutamic acid; NPCs: neural progenitor cells; TCA: tricarboxylic acid.

Introduction

Canavan disease (CD) is a rare leukodystrophy characterized by diffuse spongiform white matter degeneration, dysmyelination and intramyelinic oedema with consequent impairment of psychomotor development and early death (Hoshino and Kubota, 2014; Kumar *et al.*, 2006; Matalon and Michals-Matalon, 1999a). Globus and Strauss (1928) were the first to recognise the spongy deteriorative effects of the disease, although the first clinical description was provided by Myrtelle Canavan (Canavan, 1931). Almost two decades later, Van Bogaert and Bertrand (1949) reported its autosomal recessive nature and increased prevalence amongst Ashkenazi Jewish population; notably, CD is also known as the "Canavan-Van Bogaert-Bertrand" disease. The molecular cause of CD was identified many years later (Matalon *et al.*, 1988) as the presence of mutations within the gene encoding the enzyme aspartoacylase (ASPA; EC 3.5.1.15; *N*-acetyl-L-aspartate amidohydrolase) located on the short arm of chromosome 17 (17p13.3). However, since the purification and characterization of ASPA (Kaul *et al.*, 1991) and the cloning of the human *ASPA* cDNA (Kaul *et al.*, 1993), over 54 loss-of-function mutations have been identified (Kaul *et al.*, 1993; Stenson *et al.*, 2003). Only two point mutations are responsible for 98% of the Ashkenazi Jewish cases (Glu285Ala and Tyr231X), while other (non-Ashkenazi Jewish) cases may result from a range of mutations (Michals and Matalon, 2011; Zayed, 2015). The physiological role of ASPA is to hydrolyse *N*-acetyl-L-aspartic acid (NAA), producing (e.g. in oligodendrocytes) L-aspartic acid (Asp) and acetate (Ac) (see Figure 1 for more details); as expected, its deficiency leads to abnormally high central nervous system (CNS) NAA levels (>35%) (Janson *et al.*, 2006b), with vast amounts of NAA being excreted in urine (*N*-

acetylaspartic aciduria). It should be noted that urinary NAA levels are 10-100 fold higher in CD patients than age-matched healthy subjects, and this is the preferred method of CD diagnosis (Hagenfeldt, 1987).

Three forms of CD have been distinguished, differing in symptom onset and severity: (a) the "congenital form" (severest and symptoms appear within weeks of life), (b) the "infantile form" which is most common (also severe, with an onset of 2-6 months) and (c) the "juvenile form" (mildest and symptoms begin by the age of 5) (Adachi *et al.*, 1973; Baslow and Resnik, 1997). Early symptoms of the disease include irritability, lack of head control and hypotonia, while as the disease progresses macrocephaly develops and developmental delays (particularly in motor and verbal skills) are noted, becoming increasingly apparent with age (Matalon and Michals-Matalon, 2000). At later stages some infants may come to suffer from blindness due to optic atrophy, hypotonia can evolve into spasticity, voluntary movements (including swallowing) often become impossible and seizures may occur (Gascon *et al.*, 1990; Matalon *et al.*, 1995). Although patients with some milder forms live beyond the age of 20, the majority of CD patients do not reach adolescence (Hoshino and Kubota, 2014).

Neuropathologically, CD is an *encephalitis periaxialis diffusa* (Baslow, 2000) where: (i) the failure of the white matter-seated oligodendrocytes to myelinate the local neuraxons leaves the latter intact but unsheathed (and, thus, underfunctioning), and (ii) the white matter bears a characteristic spongy degeneration due to the development of swelling (oedema) and the formation of multiple intramyelinic and extracellular fluid vacuoles (Leone *et al.*, 1999). The vacuoles are empty, can measure up to 200 μm and are primarily spotted subcortically; the deep white matter and the superficial cortex are

less affected. Macroscopically, the brain usually appears to be heavier than expected, and bears a soft, gelatinous white matter. Microscopically, CD patients' brains are characterized by: (i) splits in the myelin lamellae and (ii) astrocytic swelling (evident both in the perikaryon and the processes) accompanied by elongated mitochondria (Adornato *et al.*, 1972; Kumar *et al.*, 2006; Matalon and Michals-Matalon, 1999b; Surendran *et al.*, 2003)[1].

The aim of this mini-review article is to provide an overview of the currently-available knowledge on the aetiopathogenesis of CD with emphasis on the current non-genetic approaches to its treatment at both an experimental and a clinical level, by highlighting: (a) the major related hypotheses, (b) the results of the available experimental simulatory approaches, as well as (c) the relevance of the so far examined markers of CD neuropathology. In particular, the potential and the limitations of the current non-genetic neuroprotective approaches to the treatment of CD are particularly discussed in the current article, in a context that could be used as a basis for a redrafting of both future experimental and (eventually) clinical work in the field.

Hypotheses on the pathogenesis of CD

Various hypotheses have attempted to explain how the lack of ASPA activity results in the neurodegeneration and oedema seen in CD. One of them, the "Ac-lipid-myelin" hypothesis (Table 1) proposes that deficiency in NAA-derived Ac impedes myelin lipid synthesis (Baslow and Guilfoyle, 2013; Hagenfeldt *et al.*, 1987). Although the

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[1] for more details on the apparent oligodendrocyte number increase in the areas of dysmyelination, the phenotype of the astrocytic (Alzheimer Type 2) populations and the vacuole formation, readers are referred to the review article of Kumar *et al.* (2006).

incorporation of acetyl moieties from NAA into myelin lipids has been observed (D'Adamo and Yatsu, 1966), it is believed to be involved in - but not being essential to - myelination, due to documentation of rare cases of CD where ASPA does not function yet myelination proceeds as normal (Janson *et al.*, 2006a; Velinov *et al.*, 2008). However, it should be noted that experimental evidence has suggested that ASPA is predominantly expressed in oligodendrocytes (Madhavarao *et al.*, 2004) and that the developmental expression pattern of the ASPA gene in the postnatal rat brain closely correlates with myelination in the CNS (Kirmani *et al.*, 2003).

A second hypothesis is the "osmotic-hydrostatic" hypothesis (Table 1), which suggests that NAA cycling between brain cells acts as a molecular water pump, removing metabolic water from neurons (Baslow *et al.*, 1999). Once produced, NAA exists in a complex with 32 water molecules and a cation (Baslow, 2003); when depolarised, neurons in grey matter have been shown to release this NAA complex into the surrounding extracellular fluid (ECF), thus transporting water against its gradient (Sager *et al.*, 1997). In a healthy subject, this complex rapidly diffuses into oligodendrocytes where it undergoes hydrolysis and water molecules are released (Wang *et al.*, 2007); this free water then diffuses down its concentration gradient into the vasculature (Vajda *et al.*, 1999). However, in CD patients, the NAA-water complex is still released upon neuronal depolarisation, but is not cleared from the ECF and instead diffuses into the vasculature (which has restricted permeability to hydrophilic molecules) (Redzic, 2011). The NAA-water complex builds up in the ECF which could lead to changes in local osmotic pressure. If a similar NAA cycle disruption occurs in white matter, the resulting osmotic pressure changes could account for the CD-related osmotic pathologies (Baslow and Guilfoyle, 2013). Although the

depolarisation of white matter neurons has not been documented to cause NAA release into the ECF, it has been suggested that the *N*-acetylaspartylglutamic acid (NAAG) released from neurons (Figure 1) could provide a source of NAA in this case (Baslow and Guilfoyle, 2013). It is known that NAAG-peptidase within the astrocyte end feet at the nodes of Ranvier hydrolyzes NAAG to produce NAA; this is the proposed source of NAA for white matter oligodendrocytes. However, in white matter, the limited extracellular space at nodes means that a potential failure of oligodendrocyte removal of NAA and its consequent accumulation will likely have severe osmotic consequences that could explain vacuole formation and the observed splitting between myelin sheath layers (Baslow and Guilfoyle, 2013).

A very recent third hypothesis, proposed by Francis *et al.* (2012), is that disruption to NAA catabolism during key myelination stages in early development causes oxidative stress in myelinating oligodendrocytes resulting in dysmyelination and other CD-related pathologies. In this "oxidative stress" hypothesis (Table 1), NAA is suggested to reduce oxidative stress by preventing the coupling of fatty acid synthesis to oxidative energy metabolism through the provision of Ac. The latter can be converted to acetyl-coenzyme A (Acetyl-CoA) by acetyl-coenzyme A synthetase (ACS; Figure 1.c). Oxidative stress was shown to precede dysmyelination in *nur7* mice (Francis *et al.*, 2012) and, even more recently, Francis *et al.* (2014) supported their hypothesis by showing that reduction of oxidative stress by triheptanoin administration (which increases Krebs cycle intermediates) was shown to alleviate CD-related pathologies.

Therapeutic approaches to CD

Due to uncertainty concerning causative mechanisms, CD is currently incurable, with available therapies being only palliative. Non-genetic treatment is focused principally on (a) the provision of adequate hydration and nutrition by gastronomy and nasogastric feeding, (b) the use of anticonvulsants to minimise seizures (Hoshino and Kubota, 2014) and (c) the reduction of intracranial pressure using acetazolamide (Matalon and Michals-Matalon, 2000). Research into possible therapies, including lithium (Li) supplementation, Ac-supplementation, stem cell replacement and gene therapy will be discussed in this article, with a focus on non-genetic approaches.

The aim of gene therapy in CD treatment is to restore ASPA activity through *ASPA* gene insertion. This approach has seen a degree of success in improving the major disease symptoms; however, progress is still to be made. The use of liposome encapsulated plasmids and adeno-associated viruses as vectors to insert the *ASPA* gene into CD mice, tremor mice and human CD subjects has resulted in enhanced ASPA production and activity and, in some cases, reduced CNS NAA levels and improved spongiform degeneration (Ahmed *et al.*, 2013; Klugmann *et al.*, 2005; Leone *et al.*, 2000; Leone *et al.*, 2012; McPhee *et al.*, 2005). However, it must be noted that in some cases the observed decrease in NAA and spongiform pathology is transient (Leone *et al.*, 2012), while in others the effects are limited within a confined area surrounding the region of intracerebral injection (Leone *et al.*, 2000; 2012). Improved motor function was noted in certain cases (Ahmed *et al.*, 2013; Leone *et al.*, 2012; McPhee *et al.*, 2005), but not in all (Klugmann *et al.*, 2005). Moreover, where motor defects were improved, they did not disappear altogether and were shown in a longitudinal study to relapse later in life (Ahmed *et al.*, 2013). However, one pathologic marker of the

disease, early lethality, was entirely rescued by Ahmed *et al.* (2013) when single intravenous injections of primate-derived adeno-associated viruses carrying the human *ASPA* gene were administered as late as postnatal day 20 (P20) in short-lived *ASPA*^{-/-} mice; a fact that is certainly a significant step forward towards a gene-replacement-mediated treatment of CD.

Non-genetic therapeutic approaches to CD

The majority of the non-genetic strategies towards CD treatment employed to date have focused on addressing either the accumulation of NAA throughout the CNS or the deficit in metabolites which results from impaired NAA hydrolysis (Figure 1). Both issues have been implicated in the pathological phenotype of CD, namely osmotic-mediated pathologies and dysmyelination. A synopsis of the major compounds tested as a non-genetic therapeutic approach to CD at an experimental and/or clinical level is provided in Table 2.

Reduction of CNS NAA levels

Those believing pathogenesis in CD to result principally from NAA accumulation in the CNS through mechanisms such as disrupted osmoregulation, have looked for substances that could reverse the observed CD pathology by lowering CNS NAA levels, determined through high-performance liquid chromatography (HPLC) analysis. Whilst experimental studies have identified ethanol (Baslow *et al.*, 2000; Hirakawa *et al.*, 1994), sodium valproate (O'Donnell *et al.*, 2000) as well as pyrazole and a number

of its derivatives[2] (Baslow *et al.*, 2000) to be potential candidates due to their ability to reduce NAA levels in non-diseased animal brains, when these compounds were tested in a CD animal model, no significant reductions were observed (Baslow *et al.*, 2002) (Table 2). In this case, the CD-simulating animal model used was the CD-like tremor rat, which possesses a CD-like syndrome as a result of a naturally-occurring deletion of the ASPA gene (Kitada *et al.*, 2000).

To date, the only substance that has persistently decreased CNS NAA levels in both animals and humans, diseased as well as non-diseased, is Li in both the form of Li chloride (LiCl) and Li citrate ($Li_3C_6H_5O_7$)[3]. O'Donnell *et al.* (2000) have shown LiCl to reduce brain NAA levels by 9% when given to rats at a daily dosage of 170 mg/kg of body weight for 2 weeks, but it was Baslow *et al.* (2002) that highlighted its potential therapeutic value in CD and tested it on CD-like tremor rats (Table 2). A 13% reduction in brain NAA levels was measured through HPLC analysis of CD-like tremor rat CNS samples following administration of LiCl at a dose of 300 mg/kg of body weight, daily for 4 days (Baslow *et al.*, 2002). Interestingly, $Li_3C_6H_5O_7$ was later tested on a human CD patient (Janson *et al.*, 2005) and achieved statistically-significant reduction of NAA levels in only one of the four CNS regions studied; nonetheless, NAA levels declined in all studied CNS regions in both the white and the grey matter, as measured through proton magnetic resonance spectroscopy (MRS) studies. The 18-month-old female CD patient ingested $Li_3C_6H_5O_7$ to up to 45 mg/kg of

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[2] more specifically, the following (pyrazole-deriving) compounds which are known inhibitors of alcohol dehydrogenase: 4-pyrazolecarboxylic acid, 1-*H*-pyrazole-1-carboxamidine-HCl, 4-methypyrazole and, pyrazole-3,5-dicarboxylic acid monohydrate.
[3] readers are reminded that Li compounds are used as mood stabilizing agents; their action is not yet characterized by a specific biochemical mechanism, but recent evidence suggests that Li might exert neuroprotection through a concentration of mitochondrial dysfunction (Malhi *et al.*, 2013); a potential reason for its herein discussed effectiveness in CD.

body weight per day for 4 months and her magnetic resonance imaging (MRI) scans showed that T1 changes in white matter showed greater similarity to age-matched values for healthy human subjects (compared with theoretical values for CD patients), suggesting that myelination was more normal as a result of the Li-treatment (Janson *et al.*, 2005). Her clinical symptoms also appeared mildly improved, as the patient showed increased awareness of her surroundings, increased alertness and minor improvements in language and gross motor function (Janson *et al.*, 2005).

A second human study on a cohort of six patients and over a time period of 60 days (Assadi *et al.*, 2010), showed $Li_3C_6H_5O_7$ to be non-toxic at the same dosage (45 mg/kg per day), demonstrated a statistically-significant drop in NAA levels in the basal ganglia (through proton MRS) and indicated a mildly improved myelination in the frontal white matter (from MRI T1 relaxation times). Improved alertness and social interactions were also noted, while gross motor function testing on the other hand failed to show any statistically-significant improvement (Assadi *et al.*, 2010).

In a more recent case report (Solsona *et al.*, 2012), $Li_3C_6H_5O_7$ was again reported to lower CNS NAA levels when given to a 3-month-old female CD patient for a year at a dosage of 45 mg/kg per day: a 20% reduction of CNS NAA levels was achieved and the substance was again well-tolerated. Moreover, alertness and visual tracking were reported to have improved, but hypotonia and spasticity remained (Solsona *et al.*, 2012).

In all cases (Table 2), any symptomatic improvements from Li-administration were mild, but results were consistent and no adverse effects were reported following intake for up to a year. It is possible that higher dosages might be required to see greater therapeutic benefit; therefore studies on the safety of higher Li dosages in

animals might be a beneficial prerequisite prior to any clinical attempt. The mechanism by which Li lowers CNS NAA levels is unknown[4]. It has been suggested to prevent NAA release from neurons or increase expulsion from the CNS by affecting blood-brain barrier (BBB) permeability (Janson *et al.*, 2005). An alternative mechanism that could be targeted is inhibition of the NAA synthetic pathways (Baslow and Guilfoyle, 2009). The latter could either be accomplished by competitive or irreversible inhibition of aspartate *N*-acetyltransferase (AspNAT; the enzyme that synthesises NAA from Asp and Acetyl-CoA) (Figure 1.c) or by inhibiting NAAG-peptidase in order to prevent NAA synthesis from NAAG hydrolysis; the latter is further discussed below.

Water removal

Baslow and Guilfoyle (2013) have recently proposed that maintenance of a sustained increase in plasma NAA levels in CD patients, by oral NAA-administration, might be therapeutic as a means of reducing oedema and other osmotic brain pathologies seen in the disease. Baslow and Guilfoyle (2013) claim that NAA accumulation in plasma would increase plasma oncotic pressure, and since NAA is thought to be unable to pass through the BBB, an outward water gradient from the CNS ECF to the vasculature should be established and water should exit the brain. However, studies have yet to be carried out to test this hypothesis.

Accumulation of NAA and oxidative stress

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[4] it is worth noting that recent experimental studies have shown that Li can enhance remyelination of peripheral nerves (Makoukji *et al.*, 2012) and influence white matter microstructure (Benedetti *et al.*, 2013) by (probably) acting as a glucogen synthase kinase 3-beta (GSK3-β) inhibitor.

Due to evidence that CNS NAA accumulation may be damaging through its initiation of oxidative stress (Pederzolli *et al.*, 2007; 2009; Surendran and Bhatnagar, 2011), antioxidants have been considered as a potential treatment for CD. Lipoic acid, being known to cross the BBB and to have a high potency, was tested in its ability to reduce signs of NAA-induced oxidative stress in 14-day-old Wistar rats (Pederzolli *et al.*, 2010): when given before acute NAA-administration (40 mg/kg of body weight, intraperitoneally, 2-days prior to NAA), all signs of oxidative stress induced by NAA were prevented. Whereas rats that received NAA alone showed increased lipid peroxidation, protein oxidation and DNA damage and decreased enzymatic and non-enzymatic defences, none of these effects were seen in rats pre-treated with lipoic acid (Pederzolli *et al.*, 2010). These results suggest that dietary supplementation with lipoic acid could be a worthwhile approach to the treatment of CD, but further studies must first produce evidence of symptomatic improvements in more appropriate CD animal models.

Supplementation of Ac

Other studies have focused on the "Ac-myelin-lipid" hypothesis and attempted to restore myelination through Ac-supplementation (Arun *et al.*, 2010; Madhavarao *et al.*, 2009; Mathew *et al.*, 2005; Namboodiri *et al.*, 2006; Segel *et al.*, 2011) (Table 2). To this end, calcium Ac (CA) and glyceryl triacetate (GTA) have both been tested in their ability to deliver Ac to the CNS (Mathew *et al.*, 2005; Namboodiri *et al.*, 2006). Intragastric administration of equivalent amounts of CA or GTA to 21-day-old

C57BL/6 mice showed GTA to raise CNS Ac levels much more effectively[5] and to have fewer adverse effects than CA, although both were found to be reasonably safe (Mathew *et al.*, 2005; Namboodiri *et al.*, 2006).

A few years later, GTA was shown to be safe when orally administered in low doses to CD infant patients and in high doses to tremor rat pups, although not efficiently alleviating the CD-associated symptomatology in the human trial (Madhavarao *et al.*, 2009). A later study (Arun *et al.*, 2010) on the tremor rats reported increased CNS galactocerebroside[6] levels, decreased spongy vacuolation and improved motor performance were noted due to high-dose administration of GTA. Moreover, once again, the treatment was well-tolerated as no toxic effects were reported (Arun *et al.*, 2010).

Given the successful tolerance of GTA at high dosage in rats, high-dose tests (4.5 g/kg of body weight per day) were carried out on 8-month- and 1-year-old CD infants over a period of 4.5 and 6 months respectively, throughout which time neither toxicity nor motor improvements were observed (Segel *et al.*, 2011). The latter was attributed to the late onset of treatment (Segel *et al.*, 2011). Moreover, it should be noted that at its highest dosage so far trialled, GTA has shown limited benefit in humans (Table 2), while treatment at an earlier age could lead to improved symptoms (as was seen in tremor rats) and should be considered when designing future studies.

Energetic substrates

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[5] it has been reported that when equimolar concentrations of GTA and CA were administered (intragastrically) to mice, CNS Ac levels were found to be higher in the GTA-fed mice; a finding suggested to reflect the ability of the hydrophobic GTA to easier penetrate cell membranes (Mathew *et al*., 2005; Namboodiri *et al.*, 2006).

[6] galactocerebroside (or galactosylceramide) is a critical CNS myelin-associated lipid that is recognised as an oligodendrocytic marker and that is found to be specifically-reduced in the CD-like tremor rat (Wang *et al.*, 2009).

Another dietary supplement proposed for use as a non-genetic therapeutic approach to CD is the anaplerotic triglyceride "triheptanoin". Building on the earlier observation that deficient NAA hydrolysis causes acute oxidative stress by increasing the coupling of fatty acid synthesis to oxidative energy metabolism (Francis *et al.*, 2012), Francis *et al.* (2014) attempted to restore oxidative integrity through provision of alternative substrates for energy production. In that study, *nur7* mouse mutants in their last week of prenatal life and 2-week-old *nur7* pups were fed a chow containing 35% (as caloric composition) triheptanoin until 12-weeks of age, and analysis of the animals' brains at 12-weeks showed oxidative stress, oligodendrocyte loss and dysmyelination to have been markedly reduced, while performance on accelerating rotarod testing at this age suggested improved motor function (Francis *et al.*, 2014) (Table 2). Notably, earlier-treated mice showed the greatest improvements in all aforementioned parameters (Francis *et al.*, 2014). However, it is not yet known how triheptanoin supplies substrates to oligodendrocytes, but triheptanoin was chosen based on its ability to produce TCA cycle intermediates whilst also providing substrates for lipogenesis.

Cell therapy

Since oligodendrocytes are an important source of CNS ASPA (Bhakoo *et al.*, 2001), if not the most important (Baslow *et al.*, 1999; Kirmani *et al.*, 2002; 2003; Klugmann *et al.*, 2003; Mhadavarao *et al.*, 2002; 2004), repopulation of the brain of a CD patient with functional oligodendrocytes could potentially rectify much of the disease phenotype through the restoration of normal NAA metabolism. Neural stem cells have been successfully used to generate oligodendrocytes in the CD mouse model

(Surendran *et al.*, 2004) and these oligodendrocytes expressed a myelin-specific enzyme indicative of myelin-producing ability, but clinical outcomes of such a treatment have yet to be investigated. Although the survival rate of neural progenitor cells (NPCs) was generally high in the juvenile treated mice (Surendran *et al.*, 2004), the migration of these cells was limited, suggesting that much progress has to be made before any results (if seen) will be widespread enough to impact upon the brain pathology of this condition. In addition to the migratory potential, the stability (Amariglio *et al.*, 2009) and safety of transplanted and differentiated NPCs in the CD mice CNS remains to be proven, while acquiring sufficient quantities of NPCs for human transplantation is likely to be difficult (Miranda *et al.*, 2013).

The NPCs used in the aforementioned study (Surendran *et al.*, 2004) were also tested on their ability to act as vectors for *ASPA* gene therapy. After 4 weeks of transplantation, enzyme activity assays carried out on juvenile CD mice showed ASPA activity to be 16% of that measured in wild-type mice (Surendran *et al.*, 2004). However, this activity was shown to decline in the adult mice 5 weeks after the transplantation, perhaps due to the short-term expression of the retroviral vector pLXIN *in vivo* (Surendran *et al.*, 2004); one should note that the use of adeno-associated virus vectors has been suggested to increase long-term efficacy of treatment. Although further studies should be carried out in order to clarify whether ASPA activity can be sufficiently increased through this method for there to be any phenotypic improvements in CD mouse models, yet again safety, stability and migration of NPCs will still need to be considered.

Novel pharmaceutical approaches

The anti-convulsant "topiramate" was found to decrease the velocity of head growth in two CD patients following its administration for 7 and 15 months, beginning at 6-months of age in both children (Topçu *et al.*, 2004). The mechanisms responsible for this effect are not certain, but Topçu *et al.* (2004) suggested that CNS water accumulation was reduced, perhaps involving carbonic anhydrase inhibition. Although the benefit of topiramate is very minor to CD patients, if the underlying mechanisms by which it acts are elucidated, more light might be shed upon the pathological basis of the disease and might be supportive of the aforementioned "osmotic-hydrostatic" hypothesis.

Blocking of NAAG catabolism

Considering the hypothesis that an important amount of NAA accumulating within the white matter of CD patients could derive from NAAG catabolism, Baslow and Guilfoyle (2009) have suggested that the use of NAAG-peptidase inhibitors or of metabotropic glutamate receptor 3 (GRM3 or mGluR3; the natural astrocytic surface target for NAAG)[7] agonists and antagonists (Baslow, 2008) could slow down the dysmyelination process.

Conclusion

There are still many aspects of the pathophysiology of CD that need to be clarified. Although the intracerebral NAA accumulation and dysmyelination appear to be the major regulators of the disease's progress and phenotype, experimental evidence

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[7] GRM3 and NAAG-peptidase are actually a complex reported to appear particularly in the white matter astrocytic cells (Baslow, 2008; Baslow and Guilfoyle, 2009; Fotuhi *et al.*, 1994).

suggests that CD mutations might have broader deregulating impacts on the developing CNS (Kumar *et al.*, 2006).

It is without doubt that an efficient gene therapy for CD would be an ideal treatment approach if applied at a very early age. As such an approach still needs to be addressed through the manufacturing of long-time expression effective vectors (Surendran *et al.*, 2006), some degree of near-term management of CD must be considered through NAA synthesis-blockage or NAA removal from the patients' CNS. To-date, the only non-genetic approach to the treatment of CD that has generated evidence towards a clinical improvement in CD patients is Li-supplementation. Interestingly, while drafting this article, Guo *et al.* (2015) have provided a very important study in support of the "osmotic-hydrostatic" theory (for which Li-supplementation appears as a therapeutic option). On the other hand, the supplementation of Ac through GTA has shown promise when in animal tests, but as of yet fails to achieve clinical improvements in human studies, perhaps due to late onset of the attempted treatment (Table 2).

Notably, early intervention has consistently shown to produce better results in all forms of treatment, supporting the theories that implicate disruption to early developmental myelination in the pathogenesis of CD. Targeting of oxidative stress seems likely to have a therapeutic benefit but clinical studies in these areas are still lacking. Future attempts incorporating larger cohorts, longer periods of analysis and wider dose schemes (especially in the case of studies focused on Li- and Ac-supplementation) could provide more conclusive evidence of the therapeutic benefit of these approaches. Due to the rarity of the disease, the launching of a multicentre coordination initiative might be necessary in order to achieve larger cohort studies in a

timely manner. However, even if such attempts do reach successful outcomes and produce a considerable clinical benefit, the course of the disease might not be fully obstructed until the problem is addressed at the axon-myelinating oligodendrocyte interface.

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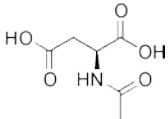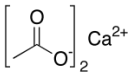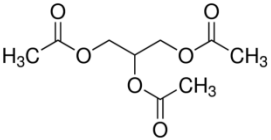
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Table 1. Overview of the current three hypotheses proposed for the pathogenesis of Canavan disease (CD), their mechanism(s) and the neuroprotective approaches that could prove effective in each case.
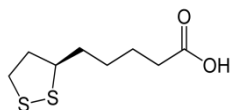
Hypothesis	Outline of the mechanism(s) and potential neuroprotectant(s)
Ac-lipid-myelin	– in CD patients, deficiency in NAA-derived Ac results in impeded myelin lipid synthesis in oligodendrocytes – potential therapeutic approach: CA, GTA
osmotic-hydrostatic	– NAA cycling between brain cells acts as a molecular water pump – in CD patients, the NAA-water complex builds up in the ECF, leading to changes in local osmotic pressure – in the white matter of CD patients, the limited extracellular space at nodes means that a potential failure of oligodendrocyte removal of NAA (and its consequent build up) is likely having severe osmotic consequences that could explain vacuole formation and the observed splitting between myelin sheath layers – potential therapeutic approach: ethanol, LiCl, $Li_3C_6H_5O_7$, NAA, pyrazole and its derivatives, sodium valproate, topiramate
oxidative stress	– in CD patients, disruption to NAA catabolism during key myelination stages in early development causes oxidative stress in myelinating oligodendrocytes, resulting in dysmyelination and other CD-related pathologies – potential therapeutic approach: lipoic acid, triheptanoin

Note: the use of topiramate as a potential therapeutic approach to CD within the "osmotic-hydrostatic" hypothesis is still to be clarified / confirmed. Abbreviations used: Ac: acetate; CA: calcium acetate; ECF: extracellular fluid; GTA: glyceryl triacetate; Li: lithium; LiCl: lithium chloride; $Li_3C_6H_5O_7$: lithium citrate; NAA: *N*-acetyl-L-aspartic acid *or* *N*-acetylaspartate; TCA: tricarboxylic acid.

Table 2. Synoptic presentation of the major experimental and/or clinical studies (if any) on the efficacy of potential therapeutic compounds for Canavan disease (CD).

| Compound name	Chemical structure	Major experimental and/or clinical studies and their findings
N-Acetyl-L-aspartate (NAA)		– hypothesis of use: "osmotic-hydrostatic" – specific aim: increase of plasma NAA levels as a means of increasing plasma oncotic pressure and generating an outward water gradient from the CNS ECF to the vasculature; suggestion made by Baslow and Guilfoyle (2013) – experimental study: --- – human trial: ---
Calcium acetate (CA)		– hypothesis of use: "Ac-lipid-myelin" – specific aim: Ac-supplementation towards restoration of myelination – experimental study: Mathew *et al.* (2005); Namboodiri *et al.* (2006) – major finding(s): increased CNS Ac levels in C57BL/6 mice; less efficient than GTA
Ethanol		– hypothesis of use: "osmotic-hydrostatic" – specific aim: reduction of CNS NAA levels – experimental study: Baslow *et al.* (2000) – major finding(s): reduction of CNS NAA levels in healthy Swiss-Webster mice – experimental study: Baslow *et al.* (2002) – major finding(s): unable to reduce CNS NAA levels in CD-like tremor rats
Glyceryl triacetate (GTA)		– hypothesis of use: "Ac-lipid-myelin" – specific aim: Ac-supplementation towards restoration of myelination – experimental study: Mathew *et al.* (2005); Namboodiri *et al.* (2006) – major finding(s): increased CNS Ac levels in C57BL/6 mice; more efficient than CA

- experimental study: Madhavarao *et al.* (2009)
- major finding(s): no detectable toxicity due to high-dose GTA administration in tremor rats
- experimental study: Arun *et al.* (2010)
- major finding(s): increased CNS galactocerebroside levels, decreased spongy vacuolation and improved motor performance in tremor rats due to high-dose administration of GTA
- human trial: Madhavarao *et al.* (2009)
- subject(s): two CD patients (8- and 13-month-old)
- major finding(s): no change in CNS NAA levels, no observable pathological changes over the course of the treatment, stabilization of clinical condition without improvement
- human trial: Segel et al. (2011)
- subject(s): two CD patients (8-month- and 1-year-old)
- major finding(s): no clinical improvement, good tolerance of the high dose

Lipoic acid

- hypothesis of use: "oxidative stress"
- specific aim: reduction of NAA-induced oxidative stress; crossing of the BBB
- experimental study: Pederzolli *et al.* (2010)
- major finding(s): reduction of NAA-induced oxidative stress-related changes in the Wistar rat CNS through pretreatment with lipoic acid

Lithium chloride
(LiCl)

LiCl

- hypothesis of use: "osmotic-hydrostatic"
- specific aim: reduction of CNS NAA levels / unknown mechanism
- experimental study: O'Donnell *et al.* (2000)
- major finding(s): reduction of CNS NAA levels in healthy Sprague-Dawley rats
- experimental study: Baslow *et al.* (2002)
- major finding(s): reduction (-13%) of CNS NAA levels in CD-like tremor rats

Lithium citrate
(Li$_3$C$_6$H$_5$O$_7$)
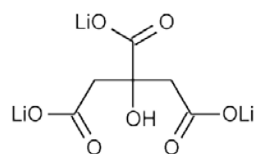
- hypothesis of use: "osmotic-hydrostatic"
- specific aim: reduction of CNS NAA levels / unknown mechanism
- human trial: Janson *et al.* (2005)
- subject(s): 18-month-old female CD patient
- major finding(s): reduction of CNS NAA levels, improved myelination, mild clinical improvement
- human trial: Assadi *et al.* (2010)
- subject(s): cohort of six CD patients (average age: 9.5 months)
- major findings: reduction of basal ganglia NAA levels, mildly improved myelination in frontal white matter, partial clinical improvement
- human trial: Solsona *et al.* (2012)
- subject(s): 3-month-old female CD patient
- major finding(s): reduction (-20%) of CNS NAA levels, partial clinical improvement

Pyrazole and derivatives

- hypothesis of use: "osmotic-hydrostatic"
- specific aim: reduction of CNS NAA levels
- experimental study: Baslow *et al.* (2000)
- major finding(s): reduction of CNS NAA levels in healthy Swiss-Webster mice
- experimental study: Baslow *et al.* (2002)
- major finding(s): unable to reduce CNS NAA levels in CD-like tremor rats

Sodium valproate
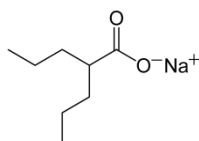
- hypothesis of use: "osmotic-hydrostatic"
- specific aim: reduction of CNS NAA levels
- experimental study: O'Donnell *et al.* (2000)
- major finding(s): reduction of CNS NAA levels in healthy Sprague-Dawley rats
- experimental study: Baslow *et al.* (2002)
- major finding(s): unable to reduce CNS NAA levels in CD-like tremor rats

Topiramate
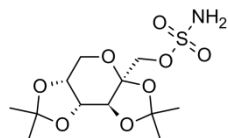
- hypothesis of use: "osmotic-hydrostatic" / to be clarified
- specific aim: presumably involving reduced CNS water accumulation due to carbonic anhydrase inhibition
- human trial: Topçu *et al.* (2004)
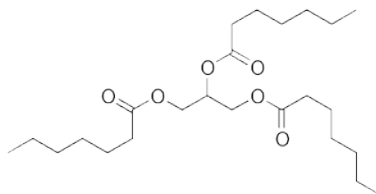
	–	subject(s): two CD patients (13- and 21-month-old)
	–	major finding(s): decline in head growth velocity (as a measure of megalencephaly progression)
Triheptanoin	–	hypothesis of use: "oxidative stress"
	–	specific aim: support of oxidative integrity, providing of ketone bodies capable of crossing of the BBB
	–	experimental study: Francis *et al*. (2014)
	–	major finding(s): reduction of CNS oxidative stress, increased thalamic myelin levels and cortical oligodendrocyte survival, reduced spongiform degeneration as well as improved motor function in *nur7* mice

Abbreviations used: Ac: acetate; BBB: blood-brain barrier; CNS: central nervous system; ECF: extracellular fluid.

Legend to the Figure:

Figure 1. Overview of the metabolic pathway of *N*-acetyl-L-aspartate (NAA) within the human central nervous system. (**a**, **b**): The synthesis of NAA occurs in neuronal mitochondria (1) and, from there, NAA is transported out of the mitochondria *via* a putative NAA-transporter and released from neurons or transported to oligodendrocytes at the junction of the neuraxon with the inner plasma membrane of the myelin sheaths (2). The NAA reaching the oligodendrocytes is used for fatty acid / myelin lipid synthesis and energy production. On the other hand, neuronal NAA could also combine with glutamate (Glu) to produce *N*-acetylaspartylglutamate (NAAG) (1). The latter is co-released from synaptic vesicles along with several neurotransmitters and can act on the astrocytic metabotropic glutamate receptor 3 (GRM3 or mGluR3) or be hydrolyzed at the surface of astrocytes to NAA and Glu (3). In the astrocytes, Glu can combine with ammonia to produce glutamine (Gln) (4). Neurons can uptake Gln (5) and use it as a source of Glu, while both astrocytic NAA and Gln can be released to the general circulation (6). (**c**): Schematic overview of NAA synthesis and metabolism in neurons and oligodendrocytes. In neuronal mitochondria, pyruvate (Pyr) undergoes decarboxylation by the pyruvate dehydrogenase (PDH) complex and produces acetyl-coenzyme A (Acetyl-CoA). The latter can either be introduced to the tricarboxylic acid cycle (TCA cycle or Krebs cycle) or be combined with L-aspartic acid (Asp) to synthesize NAA *via* aspartate *N*-acetyltransferase (AspNAT). One NAA is transferred out of the neuronal mitochondrion, it can either be used to synthesize NAAG, to be released from the neurons or to be transferred to the oligodendrocytes. In the oligodendrocytes, NAA is hydrolyzed by aspartoacylase (ASPA; the enzyme whose

deficiency is considered as the causing factor for Canavan disease) to produce Asp and acetate (Ac). The latter can be converted to Acetyl-CoA by acetyl-coenzyme A synthetase (ACS) which in turn can provide a substrate for the synthesis of fatty acid synthesis and myelination. Figure based on data from: Baslow and Guilfoyle (2009), Benarroch (2008), Hoshino and Kubota (2014) and Moffett *et al.* (2007).

[Figure 1]