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OVERVIEW

Are Astrocytes the Missing Link Between Lack of Brain Aspartoacylase Activity and the Spongiform Leukodystrophy in Canavan Disease?

Morris H. Baslow · David N. Guilfoyle

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Abstract Canavan disease (CD) is a genetic degenerative brain disorder associated with mutations of the gene encoding aspartoacylase (ASPA). In humans, the CD syndrome is marked by early onset, hydrocephalus, macroencephaly, psychomotor retardation, and spongiform myelin sheath vacuolization with progressive leukodystrophy. Metabolic hallmarks of the disease include elevated *N*-acetylaspartate (NAA) levels in brain, plasma and CSF, along with daily excretion of large amounts of NAA and its anabolic metabolite, *N*-acetylaspartylglutamate (NAAG). Of the observed neuropathies, the most important appears to be the extensive demyelination that interferes with normal neuronal signaling. However, finding the links between the lacks of ASPA activity in oligodendrocytes, the buildup of NAA in white matter (WM) and the mechanisms underlying the edematous spongiform leukodystrophy have remained elusive. In this analytical review we consider what those links might be and propose that in CD, the pathological buildup of NAA in limited WM extracellular fluid (ECF) is responsible for increased ECF osmotic–hydrostatic pressure and initiation of the demyelination process. We also hypothesize that NAA is not directly liberated by neurons in WM as it is in gray matter, and that its source in WM ECF is solely as a product of the catabolism of axon-released NAAG at nodes of Ranvier by astrocyte NAAG peptidase after it has docked with the astrocyte surface metabotropic glutamate receptor 3. This hypothesis ascribes for the first time a possible key role played by astrocytes in CD, linking the lack of ASPA activity in myelinating oligodendrocytes, the pathological buildup of NAA in WM ECF, and the spongiform demyelination process. It also offers new perspectives on the cause of the leukodystrophy in CD, and on possible treatment strategies for this inherited metabolic disease.

Keywords Canavan disease · *N*-Acetylaspartate · *N*-Acetylaspartylglutamate · Metabotropic glutamate receptor 3 · Aspartoacylase · NAAG peptidase

Abbreviations Used

Ac	Acetate
AQP4	Aquaporin 4
Asp	Aspartate
ASPA	Aspartoacylase
atm	Atmospheres
CD	Canavan disease
CNS	Central nervous system
CSF	Cerebrospinal fluid
ECF	Extracellular fluid
ECS	Extracellular space
fMRS	Functional magnetic resonance spectroscopy
Glc	Glucose
Glu	Glutamate
GM	Gray matter
GRM3	Metabotropic Glu receptor 3
IND's	Investigative new drugs

CD, a rare genetic disorder that compromises a physiologically important tri-cellular brain metabolic system.

M. H. Baslow (✉)
Center for Neurochemistry, Nathan S. Kline Institute for Psychiatric Research, 140 Old Orangeburg Road, Orangeburg, NY 10962, USA
e-mail: baslow@nki.rfmh.org

D. N. Guilfoyle
Center for Advanced Brain Imaging, Nathan S. Kline Institute for Psychiatric Research, Orangeburg, NY 10962, USA

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Me^+	Monovalent metal cation
MWP	Molecular water pump
NAA	*N*-Acetylaspartate
NAAG	*N*-Acetylaspartylglutamate
ODS	Osmotic demyelination syndrome
Pn	Paranodal
WM	White matter

Introduction

Background

Nature of Canavan Disease

Canavan disease (CD) is a rare recessive genetic neurodegenerative brain disorder of a universally important tricompartmental metabolic system that is associated with many different mutations in the gene encoding aspartoacylase (ASPA). Other genes may also be affected, and in the first genome-wide study performed on CD patients, it has been reported that 1,440 genes were significantly modulated, with 78% down-regulated and 22% up-regulated [1]. In humans, the CD syndrome is marked by early onset, hydrocephalus, macroencephaly, head–lag, ataxia, blindness, psychomotor retardation, and widespread spongiform myelin sheath vacuolization with progressive demyelination [2–4]. In addition to the hydrocephalus and spongiform vacuolization, the global osmotic nature of the disease is also apparent in the swelling of astrocytes, as well as in increased cerebrospinal fluid (CSF) space [5]. Metabolic hallmarks of the disease include a 20–30% elevation in the total of neuron-specific *N*-acetylaspartate (NAA) and its anabolic product *N*-acetylaspartylglutamate (NAAG) in brain gray matter (GM) and white matter (WM) [2]. There are also elevated levels of NAA and NAAG in CSF and increased diffusion of NAA and NAAG from brain extracellular fluid (ECF) down their gradients across the blood brain barrier (BBB) into the vascular system with daily excretion of large amounts of both substances [6]. Clinical symptoms generally appear within months following birth and death usually occurs during early childhood.

A Rationale for the Observed Clinical Course in CD Due to Lack of ASPA Activity

There are two distinct aspects of the disease that can explain its clinical course as a result of the lack of ASPA activity. One is related to consequences of an increase in osmotic pressure between brain and vascular compartments that has been suggested to be due to the buildup of unhydrolyzed NAA in brain ECF. The increase in osmotic pressure is estimated to be a daily addition of 1.94 lbs/in^2 to the normal brain osmotic–hydrostatic pressure level of 110.3 lbs/in^2 (about 7.5 atmospheres (atm) at 300 mosmols/L). This represents a potential daily 1.8% increase in osmotic pressure over the vascular compartment that, if sustained, is sufficient to reverse the normal negative osmotic pressure gradient of about −0.5% from brain ECF to blood, to a positive osmotic pressure gradient from blood to brain ECF [7]. The second aspect of the disease is the association of lack of ASPA activity in WM with a progressive spongiform leukodystrophy. In CD, a global osmotic pressure difference can explain the water retention, cell-swelling and resulting brain and head enlargement phenotypes, whereas the widespread spongiform leukodystrophy can explain the wide variety of other observable neuropathies.

The specific etiology of these other neuropathies is related to uncoupling of associations between neuron axons and myelinating oligodendrocytes that were formed during the latter half of the gestation period [8]. This uncoupling is observed as a splitting of extant or re-forming compact myelin sheath layers at their intraperiod lines, producing a spongiform appearance in WM due to the formation of large fluid-containing vacuoles in these opened spaces [9]. As the disease progresses, there is continuous and extensive demyelination as newly forming and already formed internodal compact myelin sheaths increasingly lose their structural integrity. Consequently, there is a corresponding loss of axonal ensheathment, and of integrated CNS function that depends on the isolation of individual axons, even though both neurons and oligodendrocytes may be spared. However, finding a link between the lack of ASPA activity, the formation of these intraperiod vacuoles, and the resulting spongiform leukodystrophy has remained elusive [10].

Hypotheses Regarding the Leukodystrophy Component in CD

There are two hypotheses that have been proposed to account for the demyelination associated with CD; one is a metabolic hypothesis, the acetate (Ac) transport hypothesis in which it is reasoned that myelinating oligodendrocytes normally require NAA-derived Ac in order to carry out the energy dependent process of myelin synthesis, and where a lack of NAA-derived Ac is considered to inhibit this process [4]. The second is a metabolic–mechanical hypothesis, the osmotic–hydrostatic hypothesis which suggests that the cause of the demyelination in CD is not due to a lack of Ac obtained from NAA, but that it is the presence of unhydrolyzed NAA itself that is the cause of the problem [7].

The Acetate Transport Hypothesis

The Ac transport hypothesis is widely accepted and suggests that Ac derived from ASPA catabolism of neuronal NAA liberated to ECF in WM is required by myelinating oligodendrocytes as a substrate for fatty acid and myelin synthesis. Therefore, it has been proposed that in CD, the mechanism of demyelination is a function of the inability to hydrolyze NAA and generate Ac, and that this results in failure to build and maintain myelin sheaths. As attractive as this hypothesis is, it fails to adequately explain the observed cellular and extracellular edema that results in hydrocephalus, as well as the intraperiod myelin sheath splitting and spongiform vacuolization in WM that are characteristics of the CD syndrome. Furthermore, it does not explain why other inborn errors affecting the synthesis of myelin do not result in a spongiform condition [8]. This hypothesis also does not take into account that oligodendrocytes are connected to astrocytes via gap junctions forming an oligodendrocyte–astrocyte syncythium, and that glucose (Glc) and many other substances supplied by astrocytes from the vascular system, in addition to Ac, can also be used by oligodendrocytes to synthesize myelin [11]. Based on this hypothesis, it has been proposed that Ac supplementation might be an effective therapy for CD [4, 12].

While the lack of ASPA activity and buildup of NAA in WM appear to be linked with the demyelination process in CD, another problem with this hypothesis is that there is no compelling evidence that NAA is liberated directly from neuron axons to ECF in WM either at internodal regions or at nodes of Ranvier, although this is suggested to be the case [13]. Finally, one must also consider that ASPA activity in brain is global in nature which leads to the conclusion that the catabolism of NAA by non-myelinating oligodendrocytes in GM must have some other purpose since there is no myelin to build, and energy in the form of Glc is widely available from both ECF and astrocytes in GM, so that there is no need to use the energy-wasting process of the proposed WM NAA to Ac transfer mechanism. The presence of ASPA activity in GM, which constitutes 60% of brain [14], also suggests that the primary role of ASPA in brain is to rapidly remove NAA from ECF, and that the uptake and metabolism of Ac released by oligodendrocytes in both GM and WM is incidental to this role. NAA is an enigmatic amino acid present in neurons, in that it is only apparent role is to be continuously synthesized, liberated to ECF and immediately destroyed by ASPA.

The osmotic–Hydrostatic Hypothesis

The osmotic–hydrostatic hypothesis [7] proposes that it is not a lack of Ac from NAA hydrolysis in WM that is responsible for the demyelination process in CD, but instead, that it is the presence of large amounts of NAA in WM ECF that is the problem. If this is the case, then the root cause of the destructive vacuolization must be attributed to some factor or factors in which NAA buildup plays a role in the WM pathology of CD. However, as with the Ac transport hypothesis, the source of the buildup of NAA in WM ECF in the osmotic–hydrostatic hypothesis is also unclear. A second difficulty with this hypothesis is that a mechanism by which elevated NAA in WM ECF results in the myelin sheath splitting at intraperiod lines and initiation of the spongiform condition is not immediately apparent.

In this communication we consider what the links between the lack of ASPA activity, the buildup of NAA in WM, and the resulting spongiform leukodystrophy in CD might be, and develop a new hypothesis suggesting that astrocytes play a pivotal role in this process and that the nodes of Ranvier are the primary sites for initiation of demyelination.

Discussion

NAA and NAAG Function as a Metabolically Linked System in the Brain

NAA and NAAG function as a tri-compartmental metabolically linked system in the brain that requires the participation of three cell types for its complete sequence [6]. Neurons synthesize NAA in mitochondria, and then form NAAG, an adduct of NAA and glutamate (Glu), both of which are distributed throughout their cytosol. However, these substances generally cannot be catabolized by neurons. Thus, for its further metabolism NAA is released to ECF where it interacts with oligodendrocyte surface ASPA with formation of the products Ac and aspartate (Asp). The NAAG is also released to ECF where it interacts with astrocyte surface NAAG peptidase producing NAA and Glu. Absent any ASPA activity in astrocytes [15], the catabolically formed NAA then interacts sequentially with oligodendrocyte surface ASPA where it is further hydrolyzed to form Asp and Ac completing its catabolic cycle.

NAA and NAAG are Released to ECF by Stimulated Neurons

It has been observed that there is an increase in both NAA and NAAG release by neurons in response to stimulation, and attempts to associate their pulsed stimulation-induced release with physiological functions have been made. For NAA, it has been proposed that upon stimulation, each NAA molecule with 32 molecules of obligated water is released and may function as a molecular water pump

(MWP) [7] to transport neuronal metabolic water to ECF for its removal from the brain via aquaporin 4 (AQP4) channels present on the surfaces of astrocytes and vascular endothelial cells [16]. It is suggested that neurons, which appear not to express aquaporins [17], require such a MWP mechanism in order to transport metabolic and other cellular water to ECF from which it can then be removed from the brain.

Results of functional magnetic resonance spectroscopy (fMRS) studies of the human visual cortex in response to visual stimulation [18, 19] also indicated that NAA metabolism is tightly coupled to neurostimulatory events in GM. The average reduction in NAA MRS Signal after 600 s of intense visual stimulation at 8 Hz (4,800 stimulations) was about 13%, with almost full recovery of the signal during a subsequent 600 s period without stimulation. Based on the rapidity and magnitude of the response in GM, it was suggested that the site of NAA release was extensive, perhaps the entire soma plasma membrane-ECF boundary, and that the mechanism of its release was probably vesicular [20], voltage-gated, and associated with the stimulation-induced depolarization process. Direct evidence that neuron depolarization results in the efflux of both NAA and NAAG has been obtained using superfused rat brain slices [21]. In that study it was observed that stimulation at 15 Hz for 3 min (2,700 stimulations) resulted in release of both NAA and NAAG to about 300% of their baselines, peaking at 8 min followed by a return to baseline in about 12 min. Application of tetrodotoxin at 10 μM, which blocks membrane depolarization, eliminated these stimulation-induced releases.

One of the functions of NAAG is that of a dynamic neurotransmitter, specifically targeted to the astrocytic surface metabotropic glutamate receptor 3 (GRM3), activation of which initiates intracellular calcium transients [22] and secondary astrocyte–astrocyte and astrocyte–vascular system signals that increase focal blood flow [23, 24]. A timely increase in focal blood perfusion is necessary in order to remove neuronal waste products including metabolic water, and to rapidly supply additional energy and oxygen as needed in response to neurostimulation. Thus, NAA and NAAG functions in the brain appear to be not only metabolically linked, but physiologically linked as well, and together they serve to provide a housekeeping function to maintain neuron performance by supplying energy and removing waste products in a timely fashion. Released NAAG first docks with the juxtaposed astrocyte surface GRM3, initiating a signaling sequence that results in focal hyperemia when hydrolyzed by NAAG peptidase, an enzyme also present on the astrocyte surface, perhaps in the form of a linear receptor-enzyme complex [25]. As a result, some catabolically formed NAA from NAAG is also produced in response to neurostimulation. NAAG appears to serve as a specific non-excitotoxic Glu delivery system to the GRM3 receptor-enzyme complex [26], since purified NAAG is reported to have no agonist activity at this receptor in the absence of NAAG peptidase [27]. The astrocyte GRM3 and NAAG peptidase are current therapeutic targets for treatment of schizophrenia [25].

Lack of Evidence that NAA is Directly Released in White Matter

As far as is known there is no comparable study to that of the visual stimulation paradigm in GM, or any other experimental evidence that has demonstrated the direct release of NAA in WM, nor is there any reason for its release upon neurostimulation in restricted WM extracellular space (ECS) since NAA has no known neurotransmitter functions at physiological levels normally found in ECF. Therefore, we hypothesize that NAA is only released from neurons directly to ECF in GM. This hypothesis is logical if one considers that the highly vascularized GM is the primary site of energy production, and that in GM there are large unrestricted neuronal surface areas (somata, axon hillocks, axon spikes, unmyelinated axonal surfaces and synapses) where influx of Glc and efflux of NAA-water can occur. This is not the case in WM where the available unrestricted neuronal surface area is only 0.2% of that found in GM as a result of the multilayer myelin-ensheathment of axons. In WM the axon ensheathment is almost complete, with the exception of sparsely distributed unmyelinated nodes approximately 2 μm wide, separated by myelinated diffusion-restricted internodal distances of about 1 mm (1,000 μm). Add to this that most of the axon surface in WM is covered by a transport-restricting axolemma, a double plasma membrane [28], except at nodal areas. Clearly, based only on the lack of available axonal surface area for transport of osmolytes, WM appears to be unsuitable for the rapid efflux and hydrolysis of large quantities of NAA in response to neurostimulation. Lastly, measurements of diffusion of axonal NAA using MR diffusion tensor spectroscopy show that in WM, axial diffusivity parallel to the main fiber axis, is significantly higher (0.26 μm^2/ms), than radial diffusivity perpendicular to the main fiber axis (0.13 μm^2/ms) [29], suggesting that radial movement of NAA in the entire intra-axonal space is highly restricted.

The Sources of NAA in Gray and in White Matter ECF Appear to be Different

Based on the results of these studies, we believe that the sources of NAA in GM ECF and WM ECF are very different, and that in GM anabolically formed NAA is directly liberated to ECF upon neurostimulation for a physiological

osmotic function [7], whereas in WM it is not liberated at all. In WM, we propose that only NAAG is liberated upon neurostimulation for its specific physiological GRM3 agonist function, and that NAA is then catabolically formed by the action of astrocytic NAAG peptidase after NAAG has docked with GRM3 receptors on astrocyte nodal endfoot surfaces as illustrated in Fig. 1.

Although, just as for NAA, there is no direct evidence that NAAG is released at nodes of Ranvier, several studies support this conclusion. First, based on NAAG-hydrolyzing activity per mg of protein, the highest level of NAAG peptidase activity is seen in the astrocyte-rich white matter of the brain [30]. Second, a total of eight metabotropic Glu receptors are known, arranged in 3 structurally related groups; group I consisting of GRM1 and 5; group II consisting of GRM2 and 3, and group III including GRM4, as well as 6, 7 and 8 [31]. Of these, the brain GRM's 1–5 have been tested by in situ hybridization with selective probes and only GRM3, the natural astrocytic target for NAAG, has been reported to be present in WM [32]. Thus, when we consider the circumstantial evidence that neuronal (axonal) NAAG levels are elevated relative to NAA in WM, and that the highest affinity of NAAG is for the GRM3 receptor, a receptor whose presence on juxtaposed astrocyte surfaces in WM is not only unique among GRM's, but is also associated with the highest levels of astrocyte NAAG-peptidase activity in brain, we reasonably conclude that this coincident NAAG-receptor-enzyme association in WM is of physiological importance. If so, it also follows that the catabolic production of NAA in WM from NAAG by astrocytic NAAG peptidase is an important metabolic sequence and that NAA from this source is continuously being liberated to WM nodal ECF.

This analysis suggests that there is a dichotomy in how NAA and NAAG are normally metabolized in gray and white matter. In GM it is proposed that both NAA and NAAG are liberated to ECF in response to neurostimulation, and that their catabolism occurs simultaneously, with only a small amount of NAA being produced as a product of NAAG catabolism. However, in WM, we now propose that only NAAG is liberated to ECF upon neurostimulation and that NAA is exclusively produced at axonal nodes and synapses sequentially as a product of NAAG catabolism by astrocytes. In both GM and WM, the presence of oligodendrocyte ASPA is required to rapidly clear NAA from ECF in order to maintain these dynamic processes. The proposed simultaneous and sequential catabolic relationships between NAA and NAAG metabolism in normal gray and white matter are depicted in Fig. 2.

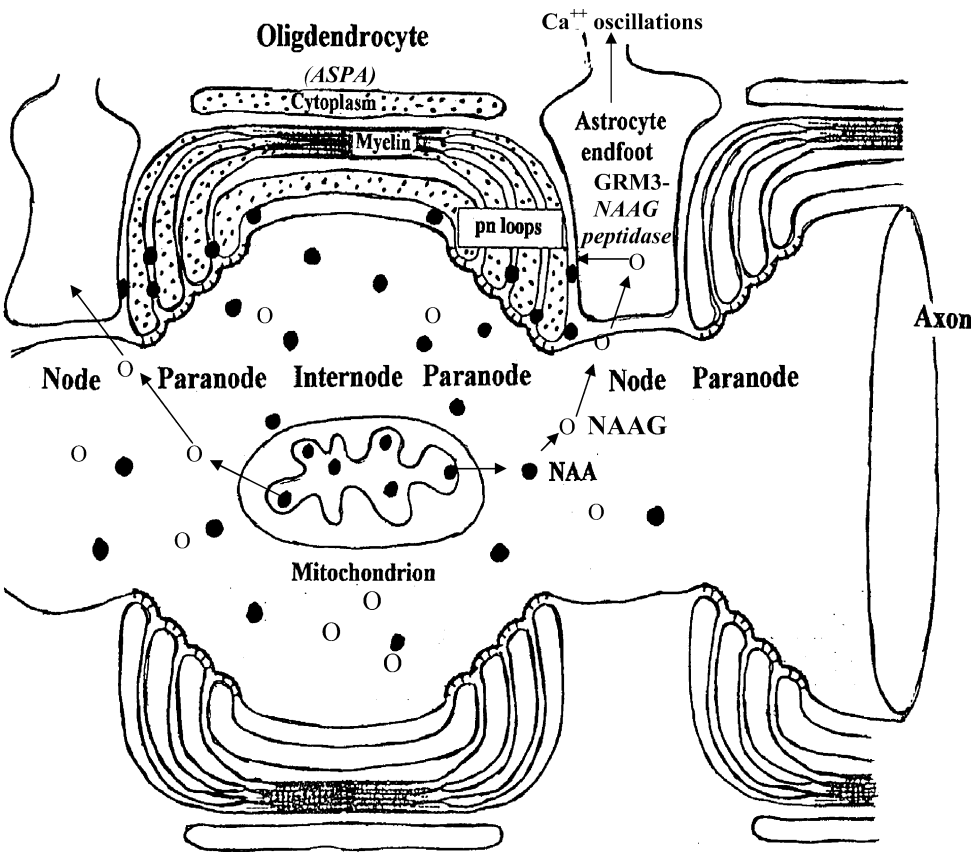

Fig. 1 Proposed site of liberation of NAAG at neuron axonal nodes in response to stimulation. Liberated NAAG first docks with astrocyte GRM3 and is then rapidly hydrolyzed by astrocyte end-foot surface NAAG peptidase initiating Ca^{++} oscillations and producing NAA and Glu. In this scheme (*arrows*), anabolically formed NAA serves as the substrate for formation of NAAG, but is itself not liberated at axonal nodes. However, catabolically formed NAA in ECF from NAAG hydrolysis is then further hydrolyzed by oligodendrocyte Pn loop ASPA, forming Asp and Ac for recycling, and liberating bound water for transport via astrocyte end-foot AQP4 water channels. Nodes are spaced approximately 1 mm apart, and are about 2–3 μm wide. In this illustration, internodes and compact myelin are highly compressed. NAAG, *open circles*; NAA, *filled circles*. Adapted from [53]

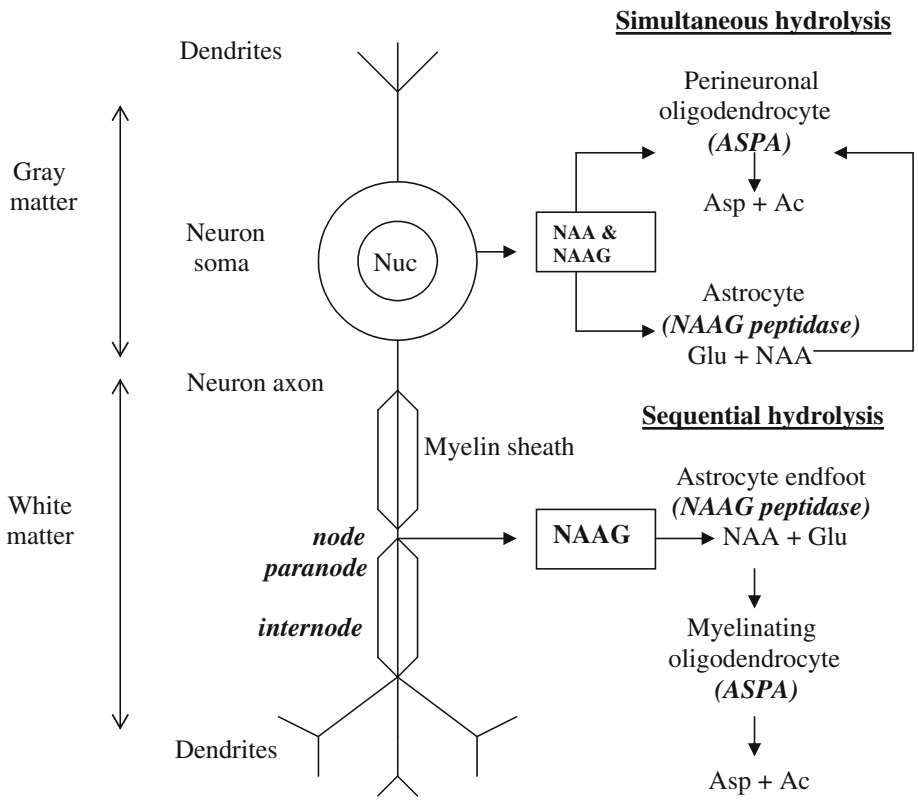

Fig. 2 Proposed differences in sites of efflux of NAA and NAAG in somata and axons of stimulated neurons. NAA is widely distributed in the neuron cytosol and is present in high concentration in both somata and axons. NAAG is also widely distributed, but is much more concentrated in axons. Upon stimulation, it is proposed that the major source of NAA in GM ECF is by its efflux from neuron somata, with a small amount from catabolism of liberated NAAG. In WM, it is hypothesized that NAA is not liberated, and that its source is from catabolism of liberated NAAG. In both cases, the final products of simultaneous or sequential hydrolytic sequences for NAA are Asp and Ac

An Astrocyte Mediated NAAG-NAA Link Between the Lack of ASPA Activity and the Demyelination Process in CD

While the source of NAA in WM ECF in CD still remains to be demonstrated, we propose that a major source is its continuous production due to catabolism of NAAG by astrocytes at axon nodes. Further, that the buildup of NAA in WM ECF from NAAG hydrolysis in CD is due to the lack of ASPA activity needed to metabolize it, and that the resulting elevation in NAA and its obligated water is in turn responsible for myelin sheath deconstruction by NAA-related osmotic–hydrostatic mechanisms. The astrocyte endfeet that surround axon nodes and paranodal regions are also rich in AQP4, the major water channel in brain that regulates bidirectional water flux between blood and brain [33]. NAAG has also been proposed to be involved in pathogenic myelination-related processes in Pelizaeus–Merzbacher-like hypomyelinating disease where mutations in the GjA12 gene that codes for oligodendrocyte-to-neuron axon connexins at the node of Ranvier may be involved [34]. To further develop the astrocyte NAAG to NAA hypothesis with regard to CD, we briefly consider the structure of the myelin sheath, the locations of ASPA and NAAG peptidase activity in normal WM, and then examine several possible osmotic–hydrostatic mechanisms that might be involved in the demyelination process.

Structure and Function of the Myelin Sheath

The structure of the myelin sheath can be visualized as a flat pad derived from a single oligodendrocyte process that is wrapped around the axon. An oligodendrocyte cytosolic layer is present only at the edges of the pad, so that upon winding around the axon, the oligodendrocyte cytosol, while contiguous with the cell body, is mostly restricted to the first axonal winding (inner cytoplasmic tongue), and an outer cytoplasmic winding (outer cytoplasmic tongue) leading to the cell body, as well as to enlarged cytoplasmic loops that attach to the axon via connexins at the edges of each node. These are designated as the paranodal (Pn) loops. The node itself is about 2–3 μm wide, and is separated from the next node by about 1,000 μm of compact myelin where both the cytosolic and intra-loop ECF layers have been compressed. Mean axonal diameter of myelinated axons in the corpus callosum, a representative WM area, is about 0.8 μm [35]. The function of the myelin sheath is not only to expedite transmission of signals, but also to insulate meaningful trains of impulses in one axon fiber from those in another. The release of the neurotransmitter NAAG at nodal gaps (10 nodes per cm of myelinated axon) is envisioned as a signaling device to the astrocyte syncytium allowing for gliotransmission [36] needed for tracking service requirements all along the designated axonal route, with the rate of nodal release a function of the frequency of impulses.

In the intranodal region of the myelin sheath several observable bands are formed: the myelinated plasma membranes of a single cellular loop merge by reducing the enclosed cytosol forming a major dense line (band 1), and the myelinated membrane layer of one loop merges with the myelinated membrane of the next loop reducing interstitial ECF and forming a minor dense line (band 2) [37]. This creates compact myelin, a chemically complex dehydrated hydrophobic structure [8] where there is little cytoplasm, and where the former ECF layer (band 2) between windings becomes the observable intraperiod bilayer that is of importance in CD pathology. The water content of the dehydrated WM in humans is about 70% compared to approximately 80% in GM [38], a 12% reduction that must be continuously maintained by active metabolic processes of myelinating oligodendrocytes. Failure to do so will result in an edematous condition where both the former cytosolic layer (band 1), and former ECF layer (band 2) may swell and disrupt the compact myelin structure. It has been suggested that if this occurs, it could produce a confluency between periaxonal and extracellular spaces, rendering the sheath useless [37].

Location of ASPA Activity

The location of ASPA activity reveals how neurons and oligodendrocytes may interact. In a rat brain immunoreactivty study, it was observed that ASPA activity was restricted to oligodendrocyte somata in both WM and GM [39]. In another immunohistochemical study [40], it was again observed that in the rat CNS, oligodendrocyte somata were also positive for ASPA, and that cross-sections of spinal tract axons showed that the myelin sheaths were unstained. From these studies, it would appear that compact myelin itself is without ASPA activity, but that the first axonal winding and the Pn loops which contain oligodendrocyte cytosol probably contain both cytosolic and membrane-bound ASPA and are the presumptive sites of NAA catabolism in WM. If correct, this would limit the possible interactions of NAA and myelin sheath oligodendrocyte ASPA to the elongated first axonal winding, and to the limited Pn regions surrounding each WM node. These would also be the limited sites for transfer of NAA-derived Ac to myelinating oligodendrocytes. The singular purpose of oligodendrocyte ASPA activity in WM and certainly in GM appears to be to rapidly remove NAA from ECF and thus to continuously maintain the brain tissue/ECF osmotic balance.

Location of NAAG Peptidase Activity

The location of NAAG peptidase activity reveals how neurons and astrocytes may interact. NAAG peptidase is present in astrocytes in both GM and WM, but the highest level of NAAG peptidase activity is in the astrocyte-rich white matter of the brain [30]. The enzyme also appears to be part of an astrocyte G-protein surface bound linear GRM3-NAAG peptidase complex that requires docking with the GRM3 by NAAG before it can hydrolyze the NAAG Glu component [25]. The singular purpose of NAAG peptidase on the astrocyte surface appears to be to hydrolyze receptor-docked NAAG and thus continuously reactivate the GRM3. A byproduct of this hydrolysis is the production of NAA in both GM and WM which is then rapidly recycled by the action of oligodendrocyte ASPA.

Two Possible Osmotic–Hydrostatic Demyelinating Mechanisms in CD

In CD there is a buildup of NAA in GM and WM, and we envision two possible osmotic–hydrostatic mechanisms, based on the buildup of NAA-water in WM ECF due to the lack of ASPA activity, that may be responsible for initiating the spongiform degeneration process and splitting the myelin sheath at its double membrane intraperiod lines.

An Osmotic Pressure Difference Across a Semipermeable Membrane

The first mechanism proposed considers that two osmotic ECF compartments exist in brain separated by a semipermeable membrane. The first osmotic compartment consists of the linked vascular and brain ventricular fluids, and the second is the mostly contiguous brain ECF enclosed between these spaces. The encompassing semipermeable membranes confining the ECF in brain consist of the ECF-plasma membrane interface of vascular endothelial cells, and the ECF-plasma membrane interface of CSF endothelial lining cells. These are the sites of the BBB, and the CSF-brain barrier respectively, with brain ECF entrapped between them [16]. If NAA-water builds in brain ECF and cannot readily pass through either of these barriers, NAA-water will be retained and hydrostatic pressure can increase throughout the entire brain ECF compartment. We have indicated that the increase due to daily synthesis and efflux of NAA, and its buildup in ECF in CD appears to be relatively small (1.94 lbs/in^2 or + 1.8%), but any level of resulting ECF edema would be expected to affect the spatial relationships between cells in GM and WM, and perhaps in the dehydrated intraperiod WM space as well. Since brain ECF is contiguous, although sub-compartmentalized and restricted at certain sites such as synapses and myelin sheath coverings, it is anticipated that an edematous condition will develop a hydrostatic force that will be exerted against plasma membranes of all cells, including between the WM protein-anchored Pn loops of

oligodendrocytes. In this edematous ECF condition, all brain cells would be moved apart as ECF expands, and this osmotic–hydrostatic process would be clinically apparent in young children with still open cranial bony sutures as the macroencephaly phenotype in CD.

Penetration of NAA-Water into the Myelin Sheath by Diffusion Down its Gradient As osmotic–hydrostatic pressures increase due to the brain ECF retention of NAA in CD, and as each hydrophilic NAA with its 32 molecules of obligated water accumulates in WM nodal ECF, it would diffuse to oligodendrocyte Pn surfaces, the likely site of normal ASPA activity in WM and its natural metabolic target [41–44]. As NAA-water builds up at these surfaces, some NAA-water will penetrate between myelin sheath windings at the oligodendrocyte Pn loops by mass movement (J) based on Fick's law [45]. NAA-water can then diffuse down its gradient between Pn loops further into dehydrated WM internodal space, approaching a zone of transition between the Pn loops and the internodal compact myelin layers. Here NAA-water encounters compact myelin with its very limited intraperiod laminar water space layer surrounded by hydrophobic myelinated lipid and protein-containing, laminar membranes. Clearly, as NAA builds up in WM ECF, and its concentration gradient increases, its mass movement into laminar space will also increase.

An Osmotic–Hydrostatic Pressure Mechanism Known to Cause Spongiform Demyelination

The effect of acute episodes of brain edema in WM has been observed in a condition known as the "osmotic demyelination syndrome" (ODS), previously called "central pontine myelinolysis" and also known as "water intoxication", that is related to end-stage kidney failure and several other pathologies [46, 47]. These patients present with a variety of neuropathological symptoms, the basic cause of which can be traced to the formation of extensive WM vacuolization and loss of oligodendrocytes in many brain areas. The spongiform demyelination resulting from acute induced brain edema appears to be initiated by hydrostatic separation of the inner oligodendrocyte cytoplasmic tongue from the axon with enlargement of the periaxonal space. This pathology has been observed histologically in a rat model of ODS which showed extensive WM vacuolization with microglial infiltration, loss of oligodendrocytes and degeneration of neuron cell bodies [48]. We have proposed that obligated water associated with NAA formed at nodes of Ranvier in CD cannot enter the small astrocytic AQP4 water channels at these nodes and therefore that NAA-water exerts osmotic pressure at these nodes causing injury to oligodendrocytes. Another demyelinating disease associated with an edematous condition at nodes of Ranvier is neuromyelitis optica, also known as optic spinal multiple sclerosis, a disease where AQP4 channels are selectively lost and water builds up at the nodes [33]. Thus, it appears that buildup of osmotic pressure at nodes of Ranvier, whether by binding water molecules to a large unhydrolyzable solute molecule as in CD, by rapid changes in blood osmolarity as in ODS, or by elimination of AQP4 water channels as in neuromyelitis optica may be a general phenomenon, all of which can result in injury to myelinating oligodendrocytes and initiate a demyelination process.

An Osmotic–Hydrostatic Reverse Micelle Mechanism There is a second and potentially more powerful mechanism where osmotic–hydrostatic pressures exerted by the buildup of NAA-water in WM ECF may not only be able to induce vacuole formation, but may also be able to split the myelin sheath at its intraperiod lines, a characteristic of the CD syndrome. This is based on hydrostatic forces generated by the formation of reverse hydrophilic micelles in the hydrophobic environment of compact myelin. Unlike the edematous osmotic–hydrostatic process described above, reverse micelles are capable of exerting enormous hydrostatic pressures at the molecular level. However, it should also be recognized that these two osmotic–hydrostatic mechanisms are not mutually exclusive, and in fact may operate in tandem in WM to first breech the Pn loop-neuron protein connections, and then to pry apart myelin sheath laminar structures allowing the further penetration of ECF and formation of vacuoles.

Reverse Micelles and Their Interactions with NAA Reverse micelles are comprised of a small amount of polar hydrophilic solvent (water) sequestered by a relatively large amount of a hydrophobic organic phase (lipids/oils) creating spherical micelles with diameters of about 0.3–20 nm [49]. Since 1 nm = 10 Å, these micelles exist in the range of molecular sizes. At 0.3 nm, the diameter (d) is about 3–4 Å, approximately the size of a water molecule. The structure of NAA-water and several other amino acid–water containing reverse micelles has been proposed [49], and each NAA molecule is pictured as a dimer with 28 molecules of obligated water per NAA molecule. In theory, this value would be 31, and experimentally, it has been measured at 32 [50]. The spherical shape of these micelles is a function of surface tension (T) which is the driving force to diminish its surface area to the minimum area for a given volume. The forces involved are intermolecular attraction balanced by the resistance of the liquid to compression, in which configuration there is no net inward force exerted. These reverse micelles also have the ability to host various hydrophilic molecules including NAA

which in turn alter the behavior and properties of the reverse micelles [49]. Studies with NAA at 5–30 mM in vitro, the range of probable brain ECF levels in CD, indicated that NAA resides in the core of the reverse micelles and its increase in concentration results in a progressive increase in droplet size and diameter. With the increase in water content of reverse micelles in the micro-emulsion, formation of swollen micelles with greater surface areas and diameters are formed by the combination of contents of fusing NAA-containing droplets.

Reverse Micelles Hosting NAA-Water-Me^+ May Initiate the Delamination Process

As the most stable structure for such reverse micelles is that of a sphere, upon fusion of droplets, surface area and diameter are increased but a spherical shape is maintained as the micelle tries to assume its most stable thermodynamic condition. To increase the surface area and (d) a quantity of work is required, and it is speculated that this work expended would result in increased hydrostatic pressure on the hydrophobic walls surrounding the intraperiod space, forcing the walls apart. Continuous liberation of NAA could be the source of such energy. The internal water pressure for a droplet increases with decreasing radius, and for reverse micelles at standard temperature and pressure in the range of $d = <20$ nm the increase in atmospheric pressure becomes enormous. For a water droplet at $d = 20$ nm, the increase in pressure is 143 atm, about 19 times that of normal opposing osmotically induced hydrostatic tissue pressure of 7.52 atm at 300 mosmol/l. In addition to reverse micelles producing hydrostatic forces that may initiate separation of laminar windings, as the amount of intraperiod water increases and proteins, including myelin basic protein, that have been folded to their highest compressibility values in restricted reverse micelle water space begin to hydrate and unfold, they add to hydrostatic forces that can further separate the laminar windings [51].

Astrocytes and Their Possible Role in CD Leukodystrophy

A continuous liberation of NAA from NAAG hydrolysis by astrocytes in limited WM nodal ECS in CD where NAA cannot be hydrolyzed fits well with the hypothesis that the buildup of NAA at WM nodes may result in increased hydrostatic pressures sufficient to initiate myelin sheath deconstruction. In time, it is reasonable to assume that this chronic process will result in formation of the larger spongiform vacuolar lesions observed at later stages of the disease. Based on this hypothesis, we now offer a new perspective for understanding the cause of the demyelination process in CD, and also for a possible treatment.

A Potential Drug Treatment for CD

If NAAG catabolism by astrocytes is a key step that results in the increase in NAA in WM ECF and the initiation of the demyelination process in CD, it then suggests a possible treatment for this CD phenotype based on the actions of several investigative new drugs (IND's) that compete

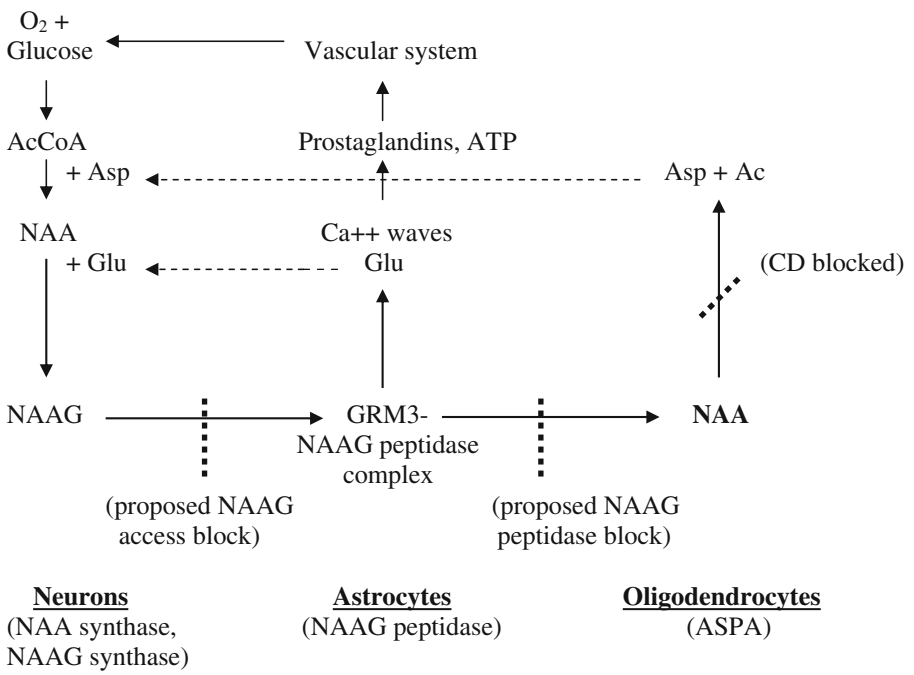

Fig. 3 Metabolic sequences for the NAAG cycle between neurons, oligodendrocytes and astrocytes in the brain, and possible sites of pharmacological interventions for CD. In CD, ASPA is inactive and liberated NAA cannot be metabolized causing it to rise to pathological levels in ECF. In WM, it is proposed that the sole source of NAA is from hydrolysis of GRM3-docked NAAG at nodes of Ranvier so that in CD, by blocking access of NAAG to the GRM3, or to NAAG peptidase, the rate of buildup of NAA at nodes may be slowed, and it is osmotically induced internodal spongiform demyelinating pathology reduced

with NAAG for the astrocyte surface GRM3-NAAG peptidase complex [25]. These include GRM3 agonists and antagonists, and NAAG-peptidase inhibitors, drugs that may all be able to block docking of NAAG with GRM3 and its subsequent hydrolysis that results in formation of NAA in white matter ECF. Metabolic sites of possible interventions are shown in Fig. 3.

Conclusions

NAA and NAAG Function as Linked Metabolic and Physiological Systems in Brain

In this communication, a hypothesis is presented that upon neurostimulation, NAA is released only from neuron somata where it may function as a MWP to transport metabolic water to ECF. Simultaneously, in response to stimulation, NAAG is released from neuron somata and from both unmyelinated and myelinated axons, as well as from synaptic membranes, and whose function is to dock with juxtaposed astrocytic endfeet GRM3 to initiate the process of astrocyte–vascular system signaling to increase focal blood flow. This is required in order to remove waste products, and to rapidly supply additional energy and oxygen when needed in response to stimulation. Thus, NAA and NAAG functions in the brain are seen to be linked, and they appear to serve to maintain neuron performance by supplying energy and removing waste products in a timely fashion.

NAA Has Different Functions in Gray and White Matter

Anabolically Formed NAA is Released Directly Only in GM for its MWP Function

Based on the hypothesis that NAA is not released from axons, it is now proposed that NAA has different functions in gray and white matter. The primary source of NAA in GM ECF is from efflux of anabolically formed NAA from stimulated neurons for its MWP function. However, in axons, NAA functions primarily as a cytosolic osmolyte, and as a substrate for the synthesis of NAAG, but is not released upon stimulation.

Catabolically Formed NAA is Produced from NAAG in Both GM and WM

It is further proposed that unlike NAA, NAAG is not only released from cell bodies, but also from axonal sites in both gray and white matter upon stimulation, where its function is the same, to dock with astrocyte GRM3. In GM, some NAA is formed as a catabolic product of NAAG hydrolysis and there is complete catabolism of both products by simultaneous hydrolysis of released NAA and NAAG. However, in WM, it is now posited that NAA is not released and that it is only formed as a catabolic product of NAAG hydrolysis. Thus, in WM complete hydrolysis of NAA to form Ac and Asp is sequential, relying on its first being produced by the catabolism of NAAG by astrocytes. The primary role of oligodendrocyte ASPA in both gray and white matter regions also being the same; to maintain osmotic equilibrium by rapidly removing NAA from ECF. Based on this hypothesis, observed differences in neuron morphology, neuronal NAA and NAAG distribution and different NAA-NAAG functions can be reconciled.

CD is an Osmotic Disease in Which Astrocytes May Play a Pivotal Metabolic Roll

Astrocytes are the Primary Source of NAA in WM ECF

In this analytical review, we hypothesize that CD is essentially a global osmotic brain disease caused by the buildup of unhydrolyzable NAA in GM and WM ECF, and that its sources are both by direct efflux from neurons, and indirectly by hydrolysis of NAAG. While the buildup of NAA in GM may be responsible for observable GM vacuoles in CD [9], that it is specifically the buildup of NAA from NAAG hydrolysis by astrocytes in limited WM ECF that is responsible for splitting the myelin sheath and the leukodystrophy component in CD.

Possible Osmotic–Hydrostatic Mechanisms Responsible for the Demyelination Process

We propose two non-exclusive osmotic–hydrostatic mechanisms that may be responsible for the leukodystrophy phenotype in CD. First, a low osmotic pressure mechanism wherein the buildup of unhydrolyzable NAA in brain ECF produces a global chronic edematous condition that by itself may act at the axon-myelin sheath interface and result in formation of spongiform WM vacuoles and loss of oligodendrocytes as has been observed in ODS. A second osmotic–hydrostatic mechanism proposed is that NAA-water in WM ECF penetrates between Pn loops, diffusing along myelin sheath double layer intraperiod lines down its gradient, forming molecular sized reverse micelles containing NAA-Water-Me^{+} at their core. As continuously forming micelles fuse, they can produce very high hydrostatic pressures sufficient to split the myelin sheath at its intraperiod bi-layer lines, leading to the distinctive spongiform leukodystrophy condition observed in CD.

Future Directions, a Possible Treatment for CD

If an important source of NAA in WM in CD is from NAAG catabolism, and if unhydrolyzable NAA in WM is responsible for the demyelination process in this disease, then by blocking NAAG access to GRM3 or to NAAG peptidase, it may be possible to slow this process. Both the GRM3 and NAAG peptidase are current targets for treatment of schizophrenia, and one selective NAAG peptidase inhibitor has been tested in humans in phase I clinical drug trials [52], as have two GRM3 agonists that compete with NAAG for the receptor complex [25]. Currently, there are several animal models with some similarities to human CD that are available, an ASPA knock-out mouse; the tremor rat, a CD model that has a natural deletion in the gene for ASPA; and, a recently described mouse model with a nonsense mutation in the ASPA gene [9]. We propose, based on the hypotheses presented in this report, that these animal models be considered for testing the effects of several GRM3-NAAG peptidase related IND's on expression of the spongiform leukodystrophy component of the CD syndrome, in the hope of developing a possible pharmacological intervention for treatment of CD.

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