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REVIEW

An answer to "The Nagging Question of the Function of N-Acetylaspartylglutamate"

Morris H. Baslow

Nathan S. Kline Institute for Psychiatric Research, 140 Old Orangeburg Road, Orangeburg, NY 10962, USA

Correspondence: Morris H. Baslow
E-mail: baslow@ nki.rfmh.org
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In 1997, a paper was published entitled "The Nagging Question of the Function of N-Acetylaspartylglutamate". N-acetylaspartylglutamate (NAAG) is an unusual acetylated dipeptide that is synthesized by neurons, but whose complete metabolism requires four different enzymes and a specific receptor distributed among three different cell types in the brain. In 2007, it was hypothesized that the primary function of NAAG was that of a homeostatic neuronal-astrocyte-vascular system control mechanism. The purpose of this mechanism being to initiate focal hyperemic responses increasing the availability of oxygen and glucose to stimulated neurons in order to replenish their energy stores and thus maintain their ability to transmit a full range of meaningful frequency-encoded messages. In this communication, evidence is presented supporting the NAAG feedback control hypothesis. This evidence shows that NAAG is not required by individual neurons for survival, for myelination, or for their basic "spiking" activity by which signals are transmitted at synapses. However, in the absence of NAAG, or if its complex intercellular metabolism is compromised, higher brain cognitive functions and coordinated motor activities that are dependent on the integrity of frequency-encoded signals are negatively affected.

Keywords: Astrocytes; Brain; Canavan disease; Hypoacetylaspartia; NAA; NAAG; Neurons; Oligodendrocytes

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Introduction

In 1997, a paper was published entitled "The Nagging Question of the Function of N-Acetylaspartylglutamate" [1]. A decade later it was proposed that the primary function of N-acetylaspartylglutamate (NAAG) was that of a "neuronal astrocyte-vascular feedback signal that regulates activation induced focal hyperemic responses" [2]. Now, based on results ofrecent studies, a reasoned response to the original questionis provided. In this report, it is shown that neither NAAG nor its precursor N-acetylaspartate (NAA) are required by neurons for survival, myelination, or for their basic "spiking" function by which a signal is transmitted to a second neuron. This is demonstrated in a single human case of hypoacetylaspartia (HA), an inborn error (IE) where NAA synthase is inactive and neither NAA or its glutamate (Glu) adduct NAAG are synthesized [3,4] and in an animal model of HA where NAA synthase is knocked out (KO) [5,6]. While the physiological function of neuronal NAA is still unclear, NAAG has been proposed to be a component of a neurovascular complex that communicates a neuron's real-time requirement for energy needed for maintenance of its full range of frequency-encoded language transmitted at synapses [2]. As such, the role of NAAG appears to be a subtle one in that its absence is not fatal, but its presence is associated with support of higher cognitive brain functions including social behaviors [5] and coordinated motor activities [7]. In this article we evaluate the role of NAAG in brain as evidenced by IE's in human and animal metabolism,

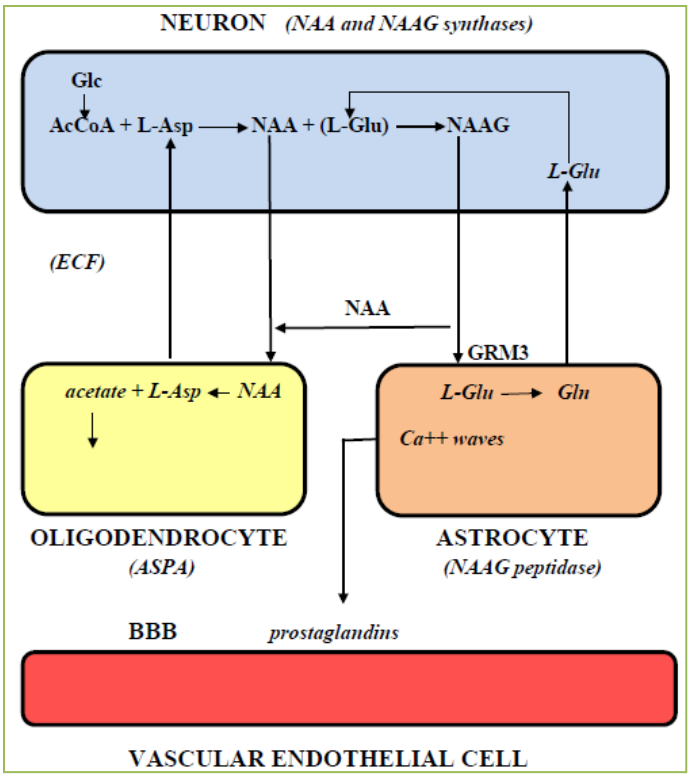

Figure 1.Tri-cellular metabolism of NAA and NAAG. This cartoon is based on known metabolism of NAA and NAAG. The system serves neurons by increasing sink capacity for metabolic wastes and for obtaining energy supplies in response to the momentary state of neuronal activation. Adapted from [11].

results of drug administration, and of genetic engineering, all of which provide novel insights into the role of NAAG. In these cases, basic neuron signaling ability is retained, but higher cognitive and motor functions are affected.

Discussion

NAA and NAAG metabolism

NAA is synthesized by neurons from L-aspartate (Asp) and acetyl Co-enzyme A (AcCoA), by NAA synthase [4]. NAAG is then synthesized from NAA and glutamate (Glu) by NAAG synthase [8] and most neurons in homeotherm brain synthesize and store mM quantities of both substances. However, neurons cannot catabolize either these substances. For their metabolism they are exported to extracellular fluid (ECF) upon neuron depolarization [2]. NAA is targeted to oligodendrocytes where it is hydrolyzed by aspartoacylase (ASPA) liberating Ac and Asp[9], and NAAG is targeted to the metabotropic Glu receptor 3 (mGluR3, a.k.a. GRM3) on the astrocyte surface where the Glu is released by NAAG peptidase [10]. This activates astrocytes to initiate Ca^{++} waves and the release second messengers to signal the vascular system and at the same time liberating NAA to ECF which is then hydrolyzed by oligodendrocyte ASPA. The unusual and indeed unique tri-cellular metabolism of NAAG with two synthetic and two hydrolytic enzymes distributed between three cell types and the mGluR3-NAAG peptidase-Glu trigger mechanism on the astrocyte surface that signals the vascular system has been called the "operating system" of the brain since failure of some parts of the system in humans have been observed to lead to grossly abnormal brain function [11]. This is evidenced by the single human case of HA presenting with profound loss of brain function and motor coordination, where NAA synthase is inactive and NAA and NAAG are not present[4]. It is also the outcome of many different IE's observed in human Canavan disease (CD) where ASPA is inactive and NAA cannot be hydrolyzed[12]. In CD this leads to a buildup of both NAA and NAAG in brain ECF that is associated with extensive spongiform demyelination. In this report, the NAAG homeostatic neuronal-astrocyte-vascular system control mechanism hypothesis is seen to be supported by studies in which each component of the tri-cellular NAAG cycle is selectively blocked. The normal tri-cellular metabolism of NAAG is shown in figure 1.

How neurons communicate with one another and why energy supply is a critical factor

The human brain constitutes about 2% of body weight and uses approximately 20% of the body's daily energy intake. In order to better understand the possible role of NAAG and its mGluR3-astrocyte-vascular system trigger mechanism in brain function, it is informative to review how neurons interact. Neurons communicate with one another using frequency-encoded signaling in the range of about 0.1-1000 Hz [13,14]. These communications are meaningful and have in many cases been able to be translated into specific neuronal spike/pause words and phrases [15]. Neuron signaling is an energy expensive process that involves a rapid depolarization followed by a rapid re-polarization of the plasma membrane usually in less than 1 ms, followed by varying periods of quiescence during which time the signals may be modulated into sequences of frequency pulsed information. The ability to rapidly re-polarize the membrane is a function of the membrane bound enzyme Na+/K+ adenosine tri-phosphatase (Na/K ATPase) using energy in the form of high energy adenosine tri-phosphate (ATP). A product of this re-polarization process is the formation of lower energy adenosine di-phosphate (ADP) which must be rapidly regenerated into ATP or the maximum possible rate of re-polarization will be reduced and the meaning of some frequency-encoded messages may be lost. A neuron has minimal glucose (Glc) reserves and has ATP stores for only a few minutes so that energy in the form of Glc to restore ATP from ADP must be delivered via the vascular system in a timely fashion and in sufficient amounts to specific brain

areas and especially to individual cells as a function of focal rates of activation. Thus, the depolarization-induced NAAG release to ECF and its astrocyte-vascular trigger mechanism can be viewed as a way for each individual spiking neuron to communicate its specific needs for energy in real time over distances of < 1mm to the regulatory endothelial cells that form the blood-brain-barrier (BBB) of the focal capillary system.

Why neurons use NAAG instead of Glu to communicate with astrocytes

The mGluR3 receptor on the surface of astrocytes can also be activated by its physiological Glu "key". However, this would require Glu to be released by neurons to ECF where it could pose a risk of injurious excitotoxic effects. Therefore, it has been proposed that neurons synthesize the relatively inert neurotransmitter NAAG for this purpose and release it upon depolarization in order to safely signal astrocytes. This has been described as a "key-lock" mechanism used by neurons to avoid autointoxication [16]. After NAAG transit across extracellular space and docking with the mGluR3, the Glu key is then safely released by the action of mGluR3-coupled NAAG peptidase and the astrocyte is activated to release second messengers to the vascular system. This NAAG "Glu" inactivated biofeedback mechanism thus insures that the limited energy resources of the brain are channeled to activated neurons and neuron networks without any untoward effects on other neurons. The Glu released by NAAG peptidase is converted to less toxic glutamine (Gln) by astrocytes and recycled to neurons. The NAA released to ECF is also non-excitotoxic even at high concentrations and is subsequently metabolized by oligodendrocyte ASPA.

NAAG and the "BOLD" response

The NAAG signaling cascade initiated by activated neurons communicates their specific individual needs for increased amounts of energy to restore levels of ATP expended for membrane re-polarization. As focal blood flow increases in response to neuronal activation and astrocyte-vascular signaling, the ratio of oxyhemoglobin to deoxyhemoglobin changes at these sites as oxygen-enriched blood is channeled to specific areas of activation. These changes occur about 1-3 s after spiking and can be measured using magnetic resonance imaging (MRI) as a blood oxygenation level-dependent (BOLD) effect and show areas of brain "activation" in response to specific inputs [2]. These kinds of measurements form the basis of most of our present knowledge of regional areas of brain activation and especially of the interconnectivity of widely separated brain areas in response to a variety of stimuli. While neurons differ in morphology, synaptic contacts, neurotransmitters released, the nature of their frequency-encoded messages as well as their interconnectivity, all neurons require large amounts of energy delivered on a timely basis to maintain a full range of neuronal messaging. Thus, the homeostatic NAAG biofeedback mechanism while operating at the level of a single neuron, enables ensembles of neurons to transform Glc energy via ATP into a collective "mind" the aggregate of all higher cognitive brain processes including perception, thought, insight, foresight, imagination and social behaviors [15].

Evidence of the role of the NAA/NAAG metabolic cycle in brain

An animal NAA synthase KO where NAA and NAAG are not synthesized

An NAA synthase KO mouse has been created in which NAA and NAAG are not present in brain [5]. The importance of this model is that it demonstrated for the first time that neither NAA nor NAAG are required by neurons for their survival, ability to signal or for their myelination. However, in this mouse model there was an observed increase in time spent in rearing and grooming, reduced interaction with a novel mouse in an unfamiliar environment and an increased exploration time of a novel object, all indicators of a change in higher levels of social behaviors. These authors concluded that the absence of the gene associated with synthesis of NAA or its adduct NAAG resulted in a mouse with a reduction in social interactions. In another study using these mice, they were observed to be 20 % heavier than their wild-type littermates and to have no evidence of NAA in their brain, but were otherwise undistinguishable [6]. Of great importance, these authors produced a NAA synthase KO mouse that also had no ASPA. The lack of ASPA normally results in CD, a profound demyelinating syndrome in both humans and mice. However, in these CD mice the lack of NAA synthase resulted in no brain NAA or NAAG being produced and remarkably, in a complete rescue of CD as evidenced by lack of vacuolization and a complete recovery of their rotorod motor performance. One long-standing hypothesis was that CD was caused by a lack of NAA with its component Ac, considered to be required by oligodendrocytes for successful myelination. This hypothesis is now resolved.

A human IE in which NAA synthase is inactive and NAA and NAAG are not synthesized

In the singular human case of HA, NAA synthase is inactive and no NAA or NAAG are produced [4,7]. This individual is profoundly affected showing retardation and poor motor skills. There are two possible reasons for the

Table 1. Results of selective ablation of components of the NAAG tri-cellular metabolic cycle in brain

NAAG component	Mechanism*	Result	References
Synthesis via NAA	IE (HA)	No NAAG	[3, 4]
Synthesis via NAA	KO	No NAAG	[5]
mGluR3 receptor	KO	No astrocyte NAAG receptor	[20, 21]
mGluR3 receptor	DG	Astrocyte NAAG receptor blocked	[20]
NAAG peptidase	KO	No astrocyte NAAG peptidase	[22]
NAA acylase	IE (CD)	NAAG and NAA elevated in ECF	[12]

* IE, inborn error; HA, hypoacetylaspartia; KO, knockout; DG, drug; CD, Canavan disease

severity in this case. First, in many cases, a human having a more sophisticated brain will show much greater effects of a metabolic defect than in lower forms. This is the case in CD where mice and rats are much less affected than their human counterparts with the same metabolic lesion. Second, in the human HA case, there is no way to know at present if the lesion is limited to NAA synthase or if other genetic and metabolic factors may also be involved. However, as in the case of the NAA synthase KO mice, in the absence of NAA and NAAG, this individual's neurons survive, are myelinated and can still spike although higher cognitive and motor skills are lost.

Pharmacological evidence in which the mGluR3 is blocked and NAAG cannot dock with the receptor

There are a number of substances that mimic the structure of NAAG and can bind with the mGluR3 receptor, thus blocking NAAG access to this receptor and its hydrolysis by NAAG peptidase [17]. Most are based on the presence of a Glu-like moiety that can target the receptor. These substances may be receptor agonists or antagonists and/or NAAG peptidase inhibitors. In each case, the normal neuron-astrocyte-vascular system signaling role of NAAG is affected. One of these substances is the NAAG peptidase inhibitor 2-(phosphonomethyl)-pentanedioic acid (2-PMPA). Using this substance in an MRI BOLD study in mouse brain *in vivo*, it was observed that after an initial rise in the BOLD response lasting several minutes, the BOLD response was then depressed for an extended 30-min period [18]. The BOLD effect in MRI is based on the paramagnetic properties of deoxyhemoglobin, which dampens the MR water signal as opposed to oxyhemoglobin which does not. Thus, the ratio of oxyhemoglobin to deoxyhemoglobin provides a dynamic measure of the level of blood oxygenation and therefore of changes in focal blood perfusion, a widely used measure of brain "activation". This study supports the notion that the normal physiological role of the release of neuronal NAAG to ECF is to induce a focal hyperemic response via an astrocyte-vascular system link. In other studies using 2-PMPA in mice, it was observed that treated mice were negatively affected in learning and memory tasks showing altered behaviors [19], and that 2-PMPA enhanced the memory of mice in a novel object recognition test [20].Many of these substances that affect NAAG metabolism have been considered for their potential in treatment of a variety of human cognitive impairments [17].

Genetic intervention in which the mGluR3 is KO and neuronal NAAG released to ECF cannot dock on the astrocyte surface

A number of different mGluR3 KO mouse models have been generated for use in studies of the effects of selected drugs with an affinity for this receptor on locomotor activity [21]. In this study it was observed that mGluR3 KO mice are not significantly different in locomotor activity from wild-type mice under the same conditions further supporting the notion that this receptor is not required for neuronal survival or for basic cell to cell signaling.

Genetic intervention where astrocytic NAAG peptidase (a.k.a. glutamate carboxypeptidase II) is KO and docked NAAG cannot be hydrolyzed

A NAAG peptidase KO mouse model has been generated [22]. These mice developed normally into adulthood, exhibiting normal neurologic responses and behaviors including mating, open field activity and rotorod performance. These authors found that in NAAG peptidase KO mice there was little detectable effect on their neurochemistry or behavior under normal conditions, but suggested that such effects might become apparent under longer-term intense synaptic transmission. In another study it was observed that in a specific memory test in these KO mice memory was enhanced [20]. Once again, these studies demonstrate that neurons can survive, signal and perform some important tasks in mice even in the absence of an intact NAAG metabolic system.

Conclusions

The brain is a complex organ made up of neurons and several supporting chaperone cells, whose role is processing information for use in elicitation of behaviors. While neurons have given up their ability to reproduce in return for longevity, a varied and complex morphology and the ability to rapidly transmit meaningful frequency-encoded signals at synapses, they also had to develop a novel mechanism for communicating their needs for waste removal and for supply

of energy in real time. Thus, the physiological role of the neurotransmitter NAAG appears to be such a mechanism that allows individual neurons in a complex cellular environment to meet these needs. Based on recent studies where each part of the NAAG metabolic system has been ablated in some way, it is clear that NAAG synthesis, docking with the mGluR3 astrocyte receptor, or its hydrolysis are not required for the basic functioning of neurons (Table 1).

Without NAAG, neurons can still survive, signal and interact with oligodendrocytes to form myelin sheaths surrounding their axons. NAAG is normally released by neurons upon depolarization and targeted to astrocytes that in turn signal the vascular system for an increase in focal blood flow. However, in the absence of the mGluR3 receptor on astrocytes or astrocytic NAAG peptidase, neurons can still survive and function. The complete metabolism of NAAG is complex and depends on four enzymes distributed in three different cell types in order to produce a focal hyperemic response. As a metaphor, just as an isolated mechanical engine can be studied and understood, the function of the engine only becomes apparent when it is coupled with a vehicle. In the case of NAAG, its metabolic engine is well known (figure 1) but its role in the brain cannot be ascertained from a study of its engine alone. Therefore in this report and based on accruing evidence, it is proposed that the function of NAAG only becomes apparent when coupled with its specific vehicle, the frequency-encoded languages of neurons.

In answer to the original question asked in 1997, it is now reasoned that the physiological function of NAAG is that of a homeostatic feedback mechanism, operating at the level of individual neurons in a ms timeframe and over distances of microns in order for each of them to maintain the integrity of their specific frequency-encoded languages. Without formation of NAAG, and/or interference with its complex intercellular cycling, neurons are still able to survive, become myelinated and signal. However, many higher functions of the brain requiring precise encoded signaling between neurons that result in more subtle outcomes such as degree of sociability and the ability to perform complex cognitive tasks, are dependent on the timely availability of adequate energy supplies which, in the absence of an intact tri-cellular NAAG metabolic system, may be impacted. Of course, the hypothesis presented herein is "an answer" to the original question, but not necessarily the only answer. In this regard it has also been proposed that hypofunction of the N-methyl-D-aspartate-sensitive Glu receptor in response to NAAG may contribute to elements of human psychoses [23]. Again, suggesting involvement of the NAAG metabolic cycle in higher cognitive functions, but in this case without consideration of the primary hyperemic role of NAAG that is associated with its astrocyte mGluR3-targeted receptor.

Conflict of interest

No conflict of interest

List of Abbreviations

Ac: acetate; AcCoA: acetyl-coenzyme A; ADP: adenosine di-phosphate; Asp: L-aspartate, ASPA: aspartoacylase; ATP: adenosine tri-phosphate; ATPase: adenosine tri-phosphatase; BBB: blood brain barrier; BOLD: blood oxygenation level-dependent; CD: Canavan disease; DG: drug; ECF: extracellular fluid; Glc: glucose; Gln: glutamine; Glu: L-glutamate; HA: Hypoacetylaspartia; IE: inborn error; KO: knock out; mGluR3: metabotropic glutamate receptor 3; MRI: magnetic resonance imaging; NAA: N-acetylaspartate; NAAG: N-acetylaspartylglutamate; 2-PMPA: 2-(phosphonomethyl)-pentanedioic acid

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