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Advances in Understanding the Peptide Neurotransmitter NAAG and Appearance of a New Member of the NAAG Neuropeptide Family

Joseph H. Neale, **Rafal T. Olszewski**, **Daiying Zuo**[1], **Karolina J. Janczura**, **Caterina P. Profaci**, **Kaleen M. Lavin**, **John C. Madore**, and **Tomasz Bzdega**
Department of Biology, Georgetown University, Washington, D.C. 20075 USA

[1] Department of Pharmacology, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang, China, 110016

Abstract

A substantial body of data was reported between 1984 and 2000 demonstrating that the neuropeptide *N*-acetylaspartylglutamate (NAAG) not only functions as a neurotransmitter but also is the third most prevalent transmitter in the mammalian nervous system behind glutamate and GABA. By 2005, this conclusion was validated further through a series of studies *in vivo* and *in vitro*. The primary enzyme responsible for the inactivation of NAAG following its synaptic release had been cloned, characterized and knocked out. Potent inhibitors of this enzyme were developed and their efficacy has been extensively studied in a series of animal models of clinical conditions, including stroke, peripheral neuropathy, traumatic brain injury, inflammatory and neuropathic pain, cocaine addiction, and schizophrenia. Considerable progress also has been made in defining further the mechanism of action of these peptidase inhibitors in elevating synaptic levels of NAAG with the consequent inhibition of transmitter release via the activation of presynaptic mGluR3 by this peptide. Very recent discoveries include identification of two different nervous system enzymes that mediate the synthesis of NAAG from *N*-acetylaspartate and glutamate and the finding that one of these enzymes also mediates the synthesis of a second member of the NAAG family of neuropeptides, *N*-acetylaspartylglutamylglutamate ($NAAG_2$).

Keywords

N-acetylaspartylglutamate; *N*-acetylaspartylglutamylglutamate; NAAG; NAAG2; metabotropic glutamate receptor 3; NAAG synthetase; stroke; traumatic brain injury; inflammatory pain; schizophrenia

Introduction

In the mid 1960s, high concentrations of *N*-acetylaspartylglutamate (NAAG) were discovered in the mammalian brain by two laboratories on opposite sides of the planet (Curatolo et al., 1965; Miyamoto et al., 1966). As it later was noted with respect to this discovery, “in science as in life timing is everything” (Neale et al., 2000). The 1960s were the wrong time for NAAG to be discovered. GABA had recently been recognized as a

Corresponding Author: Joseph H. Neale, Department of Biology, Georgetown University, Washington, D.C. 20057 USA, 202 6875574; 202 6875662 (fax), nealej@georgetown.edu.

Note: This review focused exclusively on NAAG research in the mammalian nervous system. For NAAG-related research in crayfish, see the very interesting work of A. K. Urazaev, R. M. Grossfeld, and E. M. Lieberman.

transmitter and a role for glutamate in neurotransmission was on slightly stronger footing after more than a decade of dispute as to its physiological relevance. Peptides were not regarded as significant players in chemical neurotransmission and despite the data on glutamate and GABA, transmitters generally were not believed to be present in high concentrations in the nervous system. As a result, the discovery of this peptide remained effectively dormant for nearly 20 years. While the discovery of the opiate peptides set off a stampede to discover and characterize additional neuropeptides, NAAG was left unattended in this “gold rush”. The consequence was that this peptide was overdue for exploration by the mid 1980s and a small number of research groups took up the challenge. As it turns out, proof of NAAG’s function was more complex than first imagined because of the prevalence of extracellular NAAG peptidase activity in the nervous system that converts NAAG to NAA and importantly to glutamate. The complexity was exacerbated by the discovery that NAAG activated a metabotropic glutamate receptor (mGluR3), rather than directly activating an ionotropic receptor, and by some data leading to the incorrect conclusion that this peptide was a low potency agonist, or alternatively antagonist, at NMDA receptors. Sorting this out took more than a decade.

1984 to 1997 – NAAG meets each of the criteria of a neurotransmitter

The first step in defining the function of NAAG came from the discovery of NAAG-like immunoreactivity (IR) in neurons in the rat and cat nervous systems (Anderson et al., 1986; Cangro et al., 1987; Forloni et al, 1987; Tieman et al., 1987). Strikingly, this IR was not restricted to codistribution with a single small amine transmitter but was found in neurons in the brain, spinal cord, sensory ganglia and retina that coexpressed glutamate, GABA, acetylcholine, norepinephrine, dopamine, and serotonin (reviewed in Coyle et al., 1997). Several reports appeared in 1988 that demonstrated the calcium-dependent release of NAAG following depolarization of different neuronal preparations and this was quickly followed by an ultrastructural study in which NAAG-IR was identified within synaptic vesicles (Tsai et al., 1988; Williamson et al., 1988a, b; Zollinger et al., 1988). In parallel, some early studies took the approach of applying NAAG directly to neuronal preparations in order to elucidate a physiological function in spite of the understanding that peptide transmitters traditionally are inactivated by extracellular peptidases that in this case would release glutamate. Once Riveros and Orrego (1984) reported that NAAG was hydrolyzed by a brain peptidase activity, it was realized that some of the early physiologic data obtained by direct application of the peptide to neuronal preparations were severely compromised (Blakely et al., 1988).

Several hundred uM to 1 mM highly purified NAAG activated NMDA receptors in spinal cord neurons, olfactory bulb neurons and oligodendrocytes in culture and (Westbrook et al., 1986; Trombley and Westbrook, 1990; Kolodziejczyk et al., 2009). Due to the high concentration of peptide used in these studies, it is difficult to assess the physiological relevance of these data. Lower concentrations of NAAG have been reported to antagonize the NMDA receptor currents in hippocampal neurons. However, this effect was inexplicable eliminated in the presence of glycine, brining into question its significance (Bergeron et al., 2005; 2007). In contrast, several studies have directly demonstrated that NAAG is not a physiologically relevant NMDA receptor agonist or antagonist when applied to hippocampal and cerebellar granule cell neurons (Lea et al., 2000; Losi et al., 2004; Fricker et al., 2009).

In order to resolve the physiological role of NAAG and indeed to confirm its function as a neurotransmitter, the receptor that it activated needed to be rigorously identified. Here again, timing was important but in a positive way. That is, initial studies on the application of NAAG to cerebellar granule cells and later to astrocytes in cell cultures demonstrated that it reduced forskolin-stimulated levels of cAMP (Wroblewska et al., 1993; 1998). At about the same time, the metabotropic glutamate receptors (mGluRs) were cloned and several were found to be negatively coupled to adenylate cyclase. Following this lead and using cDNA

for mGluRs, NAAG was reported to selectively activate mGluR3 in stably transfected cell lines (Wroblewska et al., 1997; 1998; Lea et al., 2001). Later studies revealed that NAAG also negatively regulated cGMP levels via mGluR3 in cerebellar neurons and astrocytes as did group 2 agonists in mGluR3 transfected cells (Wroblewska et al., 2006; 2011)

The conclusion that NAAG selectively activates mGluR3 recently was challenged in two papers reporting data from *Xenopus* oocytes and HEK cell lines cotransfected with the receptor and a G-protein sensitive potassium channel (Chopra et al., 2009; Fricker, 2009). These studies confirmed an earlier report (Losi et al., 2004) that commercial NAAG often contained from 0.1–0.4% glutamate. Citing studies in which high levels of NAAG were reported to activate mGluR3, these reports concluded that those NAAG samples also might have contained sufficiently high levels of glutamate as to be responsible for the apparent activation of mGluR3 by NAAG. Unfortunately, these two papers failed to fully consider data in a series of reports that directly contradict this conclusion and demonstrate that NAAG, rather than glutamate contamination of NAAG, activates this receptor (Bischofberger and Schild, 1996; Wroblewska et al. 1997; 1998; Lea et al., 2001; Adedoyin et al., 2010; reviewed in Neale, 2011, submitted). Indeed, the laboratory directing all but one of these studies began routinely repurifying commercial NAAG in July of 1996 (Wroblewska and Neale, unpublished observation). However, these two reports that glutamate, but not NAAG, activate a G-protein regulated potassium channel in cells cotransfected with mGluR3 suggest that glutamate and NAAG interact somewhat differently with the ligand binding site of mGluR3 and thus the second messenger coupling. Indeed, different ligands for the same receptor have been well documented to activate different second messenger cascades in the same cells (reviewed in Ambrosio et al., 2011).

Identification of mGluR3 as the NAAG receptor represented a breakthrough not simply because it advanced understanding of the neurobiology of this peptide, but because of the growing behavioral and neurochemical literature on the efficacy of heterotropic agonists at mGluR2/3 receptors *in vitro* and *in vivo*. This literature provided important leads as to potential roles of NAAG in inhibiting transmitter release, including glutamate release, via presynaptic receptors and ultimately in animal models of significant clinical disorders. Among the first reports of physiological actions of group II mGluR agonists was the finding that it functioned presynaptically to reduce transmitter release. Thus, it was not surprising that as little as 1 uM NAAG was shown to reduce voltage dependent calcium currents and transmitter release in olfactory bulb neurons via a group II mGluR (Bischofberger and Schild, 1996). This NAAG-induced inhibition of transmitter release was subsequently confirmed in cerebral cortical nerve cells and in amygdaloid neurons in vitro with both processes being blocked by an mGluR2/3 antagonist (Zhao et al., 2001; Adedoyin et al., 2010).

Critical developments in revealing the functions of endogenous NAAG in the nervous system were the discovery and purification of NAAG peptidase activity and cloning of the genes for NAAG peptidase enzymes, glutamate carboxypeptidase II and III (GCPII, GCPIII) (Riveros and Orrego, 1984; Slusher et al., 1990; Carter et al., 1996; Bzdega et al., 1997; 2004; Luthi-Carter et al., 1998; Bacich et al., 2001). GCPII and GCPIII are zinc metalopeptidases and members of the transferrin superfamily. They share 70% sequence homology with the former being expressed at a much higher level in the brain (Bzdega et al., 1997; 2004). While GCPII appears to be expressed exclusively or nearly so by glia, GCPIII is expressed at a higher level in cerebellar and cerebral cortical neurons than astrocytes (Bzdega et al., 2004), suggesting different sites of action. The crystal structures of both enzymes have been examined and their pharmacophore pockets compared (Barinka et al., 2007; Hlouchova et al., 2009). Several motifs associated with their active sites differ and these differences appear important in their interactions with peptidase inhibitors. For

example, the IC_{50} values for 2-PMPA at GCPII and GCPIII are 7nM and 1 nM respectively (Bzdega et al., 2004). The characterization of these peptidases can be seen as closing the loop on the traditional benchmarks for confirming the status of NAAG as a peptide neurotransmitter.

1998–2008: NAAG Peptidase Inhibitors and Preclinical Models

In order to better understand the role of NAAG as an mGluR3 agonist *in vivo*, a series of structurally divergent NAAG peptidase inhibitors have been synthesized and characterized with the aim of increasing synaptic levels of this peptide (reviewed in Neale et al., 2005; Zhou et al., 2005; Thomas et al., 2006; Tsukamoto et al., 2007). Important in interpretation of their effects, these peptidase inhibitors do not directly activate any mGluRs (Yamamoto et al., 2004; 2007) but rather increase synaptic levels of NAAG (Slusher et al., 1999; Zhong et al., 2006) that activates presynaptic mGluR3 to inhibit subsequent transmitter release (Figure 1A). Using one of these peptidase inhibitors, 2-PMPA, Slusher et al. (1999) published a breakthrough paper on the efficacy of endogenous NAAG *in vivo*. Systemic administration of 2-PMPA reduced the elevation of extracellular glutamate levels and consequent nerve cell death following cerebral ischemia in rat brain. Consistent with this result, GCPII knock out mice exhibit no overt differences in standard neurological testing but are less sensitive to ischemic brain injury than their wild type littermates (Bacich et al., 2002; 2005). While these mice clearly lack the full GCPII gene and fail to express GCPII message or protein, another lab inexplicably reported that knocking out GCPII is embryonic lethal (Tsai et al., 2003; Han et al., 2009)

GCPII was first cloned as prostate specific membrane antigen and used as a marker of prostate hypertrophy before its NAAG peptidase activity was independently discovered. This coincidence has led to a potentially important advance in diagnosis and treatment of prostate cancer as high affinity antagonists of this enzyme, such as ZJ43, are being used to both image the human prostate and to deliver drugs to prostate cells expressing high levels of this surface protein (reviewed in Zhou et al., 2005; Zaheer et al., 2009; Sanna et al., 2011).

NAAG peptidase Inhibition and Peripheral Neuropathy

The initial findings of the neuroprotective effects of a NAAG peptidase inhibitor in the stroke model prompted assessment of these inhibitors in peripheral neuropathies resulting from trauma, diabetes or chemotherapy. Chronic treatment of type 1 diabetic BB/Wor rats with the NAAG peptidase inhibitors GPPI-5232 and 2-MPPA reduced the development of hyperalgesia while improving sciatic nerve function and morphology (Zhang et al., 2002; 2006). Using an *in vitro* model of hyperglycemia, Berent-Spillson and colleagues found that NAAG acting via mGluR3 blocked glucose induction of caspase activity in sensory neurons, that the NAAG peptidase inhibitor 2-PMPA reversed glucose-induced programmed cell death in these neurons and that these effects were mediated by mGluR3 receptors on Schwann cells (Spillson and Russell, 2003; Berent-Spillson et al., 2004; Berent-Spillson and Russell, 2007). Similarly, NAAG peptidase inhibition attenuates the neurotoxicity induced by several different chemotherapeutic regimens (Carozzi et al., 2010).

In the sciatic nerve crush model of peripheral neuropathology, GCPII knockout mice suffered less injury and faster recovery than their wild type littermates (Bacich et al., 2005), consistent with the concept that NAAG peptidase inhibition is protective in peripheral neuropathy. Similarly, NAAG peptidase inhibition attenuates mechanical allodynia induced by partial sciatic nerve cell ligation (Yamamoto et al., 2004). The expression of NAAG in dorsal sensory ganglion neurons (Cangro et al., 1987), of mGluR3 receptors on these neurons and Schwann cells (Bruno et al., 1998) and of GCPII by Schwann cells (Chiechio et

al., 2006; Berger and Schwab, 1996) further support the view that this peptide system plays a role in dorsal sensory neuron function.

Traumatic Brain Injury

Fluid percussion injury to the rat cerebral cortex causes neuron and glial cell death in the hippocampus ipsilateral to the injury. As is known for stroke, percussive brain injury leads to cell death via elevated release of glutamate and a combination of apoptosis and necrosis over the 24-hour interval following injury. Systemic injection of the NAAG peptidase inhibitor ZJ43 just before and 8 and 16 hours after injury reduced neuronal and glial cell death by increasing extracellular NAAG levels and reducing the trauma-induced elevation in release of other transmitter levels, including glutamate, aspartate and GABA (Zhong et al., 2005; 2006). Each of these effects of ZJ43 was blocked by co-administration of the mGluR2/3 antagonist LY341495, a result supporting NAAG-mediated inhibition of transmitter release via a group II receptor. Consistent with NAAG activation of mGluR3 in these studies, neuroprotection induced by group II mGluR agonists appears to be mediated by this receptor rather than mGluR2 (Corti et al., 2007).

Inflammatory and Neuropathic Pain and Hyperalgesia

The analgesic efficacy of group II mGluR agonists (reviewed in Neugebauer, 2001) stimulated testing several NAAG peptidase inhibitors in animal models of inflammatory, neuropathic pain and metastatic cancer pain (Yamamoto et al., 2001; 2004; 2007; Carpenter et al., 2003; Saito et al., 2006). Analgesia induced by systemically administered NAAG peptidase inhibitors appears to be mediated both spinally and via brain pathways. NAAG is expressed at millimolar levels in the spinal cord (Fuhrman et al., 1994) and intrathecal administration of NAAG peptidase inhibitors induces an analgesic response to inflammatory pain in the hindlimb. Similarly, introduction of NAAG peptidase inhibitors directly into the ipsilateral lateral ventricle reduced responses to footpad inflammation (Yamamoto et al., 2008). NAAG peptidase inhibition also has been shown to reduce induction of contralateral hindlimb allodynia 24 hours after an inflammatory insult (Adedoyin et al., 2010). These data suggest that NAAG has a central role in moderating pain perception.

Consistent with the expression of NAAG-immunoreactivity in large and some mid size spinal sensory neurons (Cangro et al., 1987), the expression of mGluR3 by these neurons (Carlton and Hargett, 2007), and the analgesic efficacy of group II mGluR agonists on peripheral neurites (Yang and Gereau, 2003), NAAG and NAAG peptidase inhibitors were shown to be analgesic when injected into the hindpaw prior to induction of an inflammatory insult, raising the possibility of topical analgesia via application of an inhibitor in a medium that facilitates penetration of the skin.

In each of these studies, the analgesia induced by peptidase inhibition was blocked by co-administration of the group II mGluR antagonist, LY341495, supporting the conclusion that the process is mediated by NAAG activation of mGluR3. The extent to which the analgesic effects of NAAG are due, if any, to interactions with other transmitters in the ascending and descending pain pathways is not known. Nonetheless, proof of the concept that NAAG peptidase inhibition is an efficacious analgesic strategy is particularly important because it represents a completely novel approach to pain perception.

NAAG Peptidase Inhibition as Drug Abuse Therapy

One element in the behavioral and addictive properties of cocaine is the stimulation of dopamine release in the nucleus accumbens. Based on the codistribution of NAAG with dopamine in some neurons (Forloni et al., 1987) and the efficacy of NAAG peptidase inhibitors in reducing transmitter release (Slusher, et al., 1999; Sanabria et al., 2004; Zhong

et al., 2006; Adedoyin et al., 2010), these inhibitors were tested in animal models of cocaine abuse where they inhibited cocaine-induced conditioned place preference, reinstatement of drug seeking behavior and cocaine self-administration under progressive ratio reinforcement conditions (Slusher et al., 2000; 2001;Peng et al., 2010; Xi et al., 2010a; 2010b). Additionally, microinjection of a peptidase inhibitor or NAAG into the nucleus accumbens inhibited cocaine self-administration and drug-induced reinstatement of drug seeking behavior while systemic injection of the inhibitor dose dependently reduced cocaine-induced release of dopamine and glutamate in this nucleus. Reinforcing the conclusion that NAAG mediated these effects via mGluR3, coinjection of a group II mGluR antagonist, systemically or directly into the nucleus accumbens reversed the effects of the peptide and peptidase inhibitor in these studies. The influence of NAAG on the opiate circuits is somewhat different with peptidase inhibition attenuating tolerance but not dependence in mice (Kozela et al., 2005)

Schizophrenia

A decade ago, a substantial body of data emerged on the efficacy of group II mGluR agonists in PCP, dizocilpine and *d*-amphetamine based animal models of schizophrenia (review in Niswender and Conn, 2010). These open channel NMDA receptor antagonists induce schizophrenia-like behaviors in humans and animals while stimulating the flux of dopamine and glutamate in the prefrontal cortex (Moghaddam and Adams, 1998). Given NAAG's role as a group II agonist and its efficacy in inhibition of transmitter release, the NAAG peptidase inhibitor ZJ43 was tested in a series of animal models of this disorder (Olszewski et al., 2004, 2008; Takatsu et al., 2011; Profaci et al., 2011). NAAG peptidase inhibition significantly reduced the motor activation and stereotypic movement effects of PCP and MK801 in both rat and mouse models, reduced PCP-induced social withdrawal in the resident-intruder assay and attenuated MK801 but not PCP induced reduction in prepulse inhibition of acoustic startle. In each study, the effects of the peptidase inhibitors were blocked by the co-administration of a group II mGluR antagonist.

Consistent with data on the efficacy of mGluR2 positive allosteric modulators, a heterotropic group II mGluR agonist reduced the effects of PCP in mice that were null mutant for mGluR3, but not in mGluR2 knockout mice, a result that called into question the selectivity of NAAG for mGluR3 in these schizophrenia assays. Using the same strains of mice, however, a NAAG peptidase inhibitor was recently found to be effective in reducing PCP-induced motor activation in the mGluR2 but not mGluR3 knockout mice (Olszewski et al., submitted). These data further strengthen the conclusion that NAAG is mGluR3 selective *in vivo* and suggest that both mGluR2 and mGluR3 activation have therapeutic relevance in schizophrenia.

NAAG and Astrocytes

The influence of NAAG on mammalian glia remains relatively unexplored. Astrocytes express high levels of mGluR3 message, respond to NAAG via a pertussis toxin sensitive G protein to negatively regulate cAMP and cGMP levels and are the primary, if not exclusive, source of GCPII in the nervous system (Wroblewska et al., 1997; Berger et al., 1999). Yet the primary observations on the role of NAAG and mGluR3 in astrocytes relates to their release of transforming growth factor beta (TGF-β) following activation of mGluR3 (Figure 1A) and the consequent neuroprotection that this provides in culture (Bruno et al., 1998; D'Onofrio et al, 2001; Thomas et al., 2001a; 2001b). Beyond this, Gehl et al. (2004) demonstrated that cortical astrocytes in cell culture had the capacity to synthesize low levels of NAAG from N-acetylaspartate and [3H]-glutamate.

2010–2011 – Two Synthetases and Two Neuropeptides: NAAG and NAAG2

Two breakthrough papers were published in 2010 in which independent research groups identified two nervous system enzymes, NAAG synthetase I and NAAG synthetase II, which mediate the synthesis of NAAG *in vitro* and in transfected cells (Becker et al., 2010; Collard et al., 2010). Previous reports demonstrated that NAAG is not synthesized via post translational processing as is the case for other mammalian peptides, except carnosine, but rather it is synthesized from *N*-acetylaspartate and glutamate (Cangro et al., 1987, Gehl et al., 2004; Arun et al., 2006). However, the enzymes mediating NAAG synthesis remained elusive for nearly 50 years. Both NAAG synthetase I and II are expressed in the rat brain and spinal cord with expression patterns that are similar to the distribution of NAAG. Both are members of the ATP grasp family of synthetases. NAAG synthetase I also mediates the synthesis of β-citrylglutamate and has two splice variants with somewhat different relative distributions among brain, spinal cord and testis (Becker et al., 2010).

More stunning was the very recent report that NAAG synthetase II also mediates the synthesis of the tripeptide *N*-acetylaspartylglutamylglutamate ($NAAG_2$) and that this peptide found in brain at 30–50-fold lower concentrations than NAAG (Lodder-Gadaczek et al, 2011). Due to the low concentration of $NAAG_2$ in brain tissue samples and its poor chromatographic separation from NAAG, its resolution required tandem MS fragmentation. This important discovery significantly advances this field and suggests that, like other neuropeptides, NAAG and $NAAG_2$ are members of a peptide family.

It is possible that previous studies in which antibodies were used to localize NAAG via immunohistochemistry or to assess its synaptic release may also have recognized $NAAG_2$. It seems unlikely, however, that such cross reactivity, if it did occur, would have significantly affected the results, given the 1–2 orders of magnitude difference in concentration of the two peptides in the nervous system and the fact cells transfected with NAAG synthetase II synthesized several fold more NAAG than $NAAG_2$. Rather, this discovery supports the conclusion that some of the cells in which NAAG has been localized also contain $NAAG_2$, albeit at a much lower concentration. These issues will need to be clarified via the development of $NAAG_2$ specific antibodies and assay of microdialysis samples using LS-MS.

NAAG and NAAG2 Model

A central question arising from the discovery of $NAAG_2$ is its role relative to that of NAAG in nervous system function. Extracellular GCPII hydrolyzes $NAAG_2$ to NAAG and glutamate and hydrolyzes NAAG to *N*-acetylaspartate, releasing a second glutamate (Loder et al., 2011). If $NAAG_2$ is released synaptically, its levels also are likely to be elevated by NAAG peptidase inhibitors with the resulting elevated levels of activation of a still hypothetical $NAAG_2$ receptor (Figure 1B). Despite the observation that NAAG synthetase II produces much more NAAG than $NAAG_2$, it is possible that neurons expressing this enzyme produce NAAG solely to serve as the precursor for the synthesis of $NAAG_2$. In contrast, neurons expressing NAAG synthetase I do not produce $NAAG_2$ (Figure 1A), suggesting the possibility that there are distinct NAAG- and $NAAG_2$-ergic neurons. Emerging from this model (Figure 1B) and from the traditions of neuropeptide peptide families is the hypothesis that $NAAG_2$ also functions in neurotransmission with its likely receptor candidates being the mGluRs. This theory waits testing in neurons, astrocytes and transfected cells.

Important to understanding the role of NAAG and perhaps $NAAG_2$ is the traditional model of peptide release under conditions of high frequency stimulation into the *perisynaptic* space with subsequent activation of receptors in this space. The presence of mGluR3 receptors as

well as GCPII outside the immediate synaptic space supports this model of NAAG's action as does the peptide's seemingly redundant coexpression in some glutamatergic neurons. This model is based on the very active transport of glutamate from the synapse leaving NAAG to regulate perisynaptic neuronal and glial mGluR3. The codistribution of NAAG with other small amine transmitters supports a global role in biasing transmitter release to expand the dynamic range of the release process, particularly at high levels of synaptic activity. This is supported by the low basal levels of extracellular NAAG in the brain, the elevated levels induced by peptidase inhibitors in activated brain regions and the efficacy of these inhibitors in reducing extracellular levels of GABA as well as glutamate (Slusher et al., 1999: Zhao, 2001; Zhong et al., 2006) (Figure 1A).

Conclusion

Now nearly 50 years after its initial discovery, the peptide neurotransmitter NAAG remains much less widely recognized than other neuropeptides within the neuroscience community or the texts that are used to educate the newest generation of students. Nonetheless, understanding the functions of NAAG and $NAAG_2$ via the peptidase inhibitors offers substantial promise as this knowledge is translated in preclinical models.

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Abbreviations

Dizocilpine	an open channel NMDA receptor antagonist
LY341495	a group II mGluR antagonist
mGluR	metabotropic glutamate receptor
mGluR2	type 2 metabotropic glutamate receptor
mGluR3	type 3 metabotropic glutamate receptor
NAA	*N*-acetylaspartate
NAAG	*N*-acetylaspartylglutamate
$NAAG_2$	*N*-acetylaspartylglutamylglutamate
NAAGS I	NAAG synthetase I
NAAGS II	NAAG synthetase II
PCP	phencyclidine – an open channel NMDA receptor antagonist
TGF-β	Transforming Growth Factor Beta
ZJ43, 2-PMPA, GPPI-5232 and 2-MPPA	NAAG peptidase inhibitors

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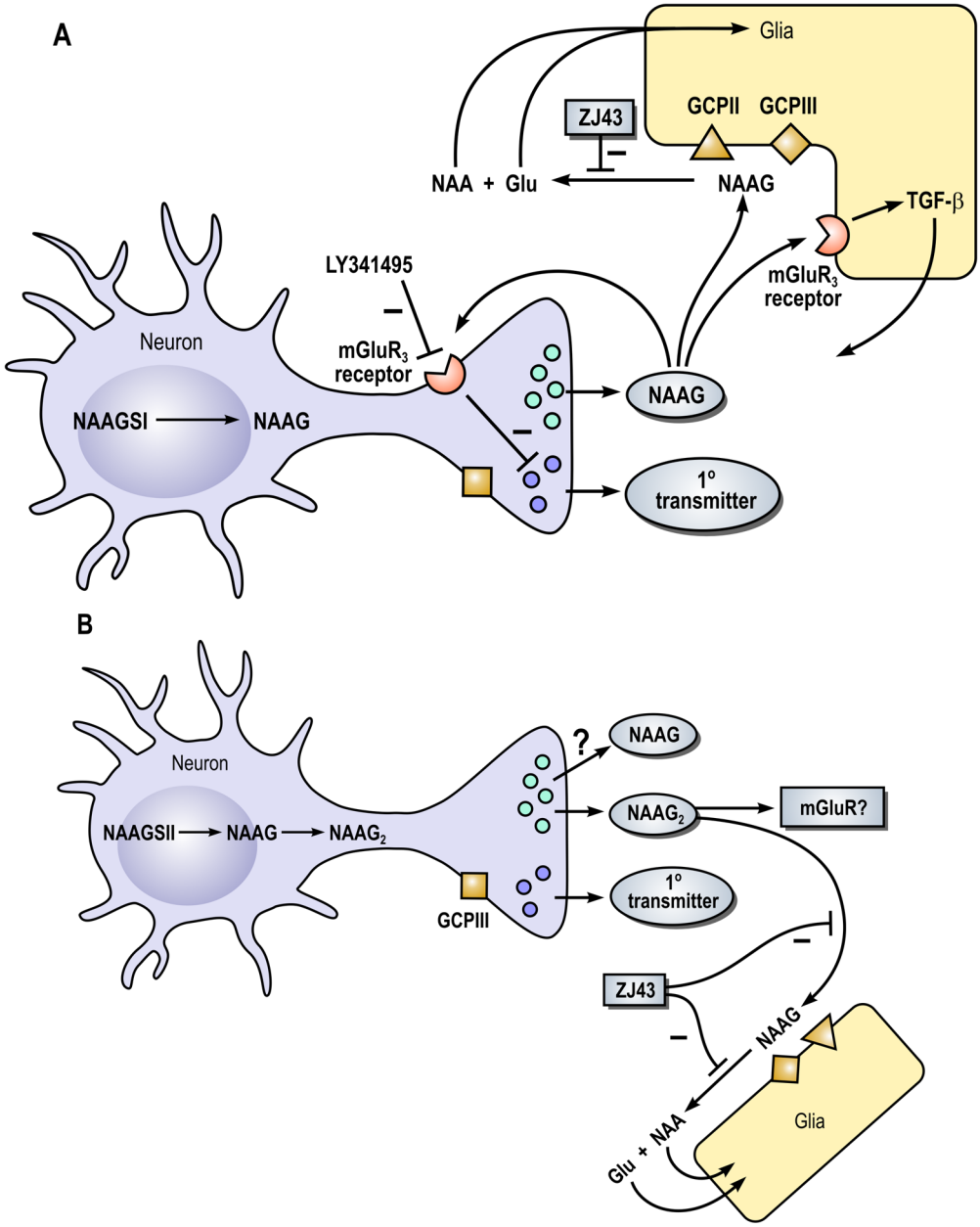

Figure 1. A model of the role of *N*-acetylaspartylglutamate (NAAG) peptidase inhibition and its influence on (A) NAAG and (B) N-acetylaspartylglutamylglutamate ($NAAG_2$) in the nervous system

In part A, the neuron expresses NAAG synthetase I (NAAGS I), an enzyme that mediates the synthesis of NAAG but not $NAAG_2$. In this cell, NAAG is co-released with a primary amine transmitter, such as glutamate, under conditions of elevated neuronal activity. While the primary transmitter is released into the immediate synaptic space, the peptide is released perisynaptically where it activates presynaptic and glial type 3 metabotropic glutamate receptors ($mGluR_3$). NAAG is inactivated by glutamate carboxypeptidases II (GCPII) and III (GCPIII), forming *N*-acetylaspartate (NAA) and glutamate (Glu), which are transported into glial cells. While GCPIII is expressed by neurons and glia in cell culture (Bzdega et al., 2004), its localization on presynaptic ending is purely speculative. High levels of glutamate-mediated neurotransmission are associated with several clinical disorders including

traumatic brain injury, stroke, peripheral neuropathy, inflammatory pain and schizophrenia. NAAG inhibits glutamate release by activation of presynaptic $mGluR_3$ receptors. Inhibition of the peptidases GCPII and GCPIII by a NAAG peptidase inhibitor, such as ZJ43, reduces inactivation of NAAG. In animal models of these disorders, the NAAG peptidase inhibitor-mediated elevation of peptide levels increases the activation of $mGlu_3$ receptors on axon endings, inhibiting further glutamate release and reducing the pathology. In a second neuroprotective pathway, NAAG activation of $mGlu_3$ receptors on glial cells stimulates the release of a trophic factor, transforming growth factor β (TGF-β).

In Part B, the neuron expresses NAAG synthetase II (NAAGSII), an enzyme that mediates the synthesis of NAAG and $NAAG_2$. In this model, we propose that $NAAG_2$ and perhaps NAAG are co-released with a primary amine transmitter, again as it the case for other neuropeptides, under conditions of elevated neuronal activity. The receptor that $NAAG_2$ might activate has not been identified but is likely to be defined in the near future. Since GCPII hydrolyzes both NAAG and $NAAG_2$, peptidase inhibitors such as ZJ43 can be predicted also to elevate levels of $NAAG_2$ and increase its activity.