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N-Acetyl aspartyl-glutamate, NMDA Rceptor AND Psychosis

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Glutamate is recognized as one of the most abundant and important neurotransmitters in the brain. Glutamate and its receptors are implicated in both normal behavior and in various neuropsychiatric disorders, including schizophrenia where the ionotropic *N*-methyl-D-aspartate-sensitive glutamate receptor (NMDAR) subtype has received particular attention for possible hypo- or dysfunction. In part, this implication arises from observations that the NMDAR antagonist phencyclidine (PCP or "angel dust") can induce a syndrome that closely resembles schizophrenia, and exacerbates psychotic symptoms in patients with chronic schizophrenia.

Glutamate receptors

Glutamate acts at two distinct classes of receptors: ionotropic (iGluRs) and metabotropic (mGluR) receptors. On the basis of sequence homology, second messenger coupling and pharmacology, eight mGluR are grouped into three classes: group 1 (mGluR1 and mGluR5), group 2 (mGluR2 and mGluR3) and group 3 (mGluR4,6,7,8) [1]. Clarification of the physiological roles of *N*-acetylaspartylglutamate (NAAG) at glutamatergic receptors remains complicated and controversial. Because of its co-localization with glutamatergic systems, it was originally hypothesized to serve as an agonist at ionotropic glutamate receptors [2]. However, ionophoretic studies in hippocampal slices indicated that NAAG rarely and only weakly depolarized pyramidal cells [3, 4], arguing against a direct action at these glutamate ionophoric receptors. The fact that NAAG is co-localized to subpopulations of cortical GABAergic interneurons, motor neurons and locus coeruleus noradrenergic neurons further undermined the assumption that this acidic peptide was simply a glutamatergic agonist.[5]

NAAG and NMDA receptor

NMDARs are oligomeric ligand-gated ion channels formed by the assembly of different subunits, an essential NR1 subunit, which encodes the ion channel, and various NR2 subunits. NMDARs have specialized characteristics including voltage-dependent block by magnesium, calcium permeability, slow deactivation kinetics, and a complex pharmacology that includes multiple binding sites. A unique feature of NMDARs is the requirement that two different ligands bind to open the ion channel:, glutamate binds to a site on the NR2 subunit and glycine or D-serinea binds to a modulatory binding site on the NR1 subunit. Ligand binding studies have shown that NAAG specifically interacts with NMDAR but not with the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) or kainic acid (KA) receptor [6]. Westbrook et al showed that NAAG behaved as a very weak agonist at NMDARs in electrophysiological studies of the rat spinal cord [7]. The weak agonist effects of NAAG at NMDARs noted in some electrophysiological studies point to a

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second counterintuitive mechanism of action of NAAG as an NMDAR antagonist. Sekiguchi et al in patch clamping studies showed that NAAG inhibited NMDARs [8]. Consistent with this finding, Puttfarcken et al found that NAAG antagonized the release of [3H]norepinephrine from hippocampal slices evoked by glutamate and NMDA [9]. In hippocampal slices prepared young animals (3 weeks of age),, low concentrations of NAAG (20 M) reduced isolated intra-synaptic NMDAR currents. The antagonism of the NMDAR induced by NAAG was still observed in the presence of LY-341495, a potent and selective mGluR3 antagonist, suggesting that the antagonism induced by NAAG at NMDAR located on CA1 pyramidal neurons was not mediated by mGluR3.

Localization of NMDAR

Another aspect to consider when one is investigating the action of NAAG is the fact that NMDARs are present in both intra- and extra-synaptic areas, but they are found at higher density within the synapse [10, 11]. Shortly after birth, intra-synaptic NMDARs of cortical neurons display a relatively large and long-duration NMDAR-mediated EPSC [12, 13] and have a low open probability [14–16]. However, during postnatal development, the decay of intra-NMDAR kinetics becomes faster [17–19]. These changes are accompanied by a decline in the ability of the highly NR1/NR2B-selective antagonist ifenprodil to inhibit NMDAR current [20–22]. After the peak of synaptogenesis, the NR1/NR2A complex, characterized by rapid offset kinetics, dominates at the synapse, while the NR1/NR2B complex, characterized by slow kinetics, predominates in the extra-synaptic area [23–27]. The activation of extra-synaptic NMDARs by glutamate escaping from the synaptic cleft during episodes of high synaptic activity suggests that they may have a different role [28–33]. Moreover, extra-synaptic NMDARs are the target of astrocytic glutamate release [34]. While extra-synaptic NMDAR-mediated signaling may contribute to the overall dynamics of neuronal synchrony, its presence raises a series of intriguing questions on its possible role in pathological changes [35]. It has been proposed that extra-synaptic NMDARs are important mediators of excitotoxicity, whereas intra-synaptic NMDARs appear neuroprotective [33, 36, 37]. Another major difference between intra-synaptic and extra-synaptic NMDARs is that the former co-localize and interact with many signaling proteins within the post-synaptic compartment [38]. The action of NAAG on extra-synaptic NMDARs has not yet been investigated. It is reasonable to speculate that the antagonism of NAAG at NMDAR could be different depending it localization (synaptic vis-à-vis extra-synaptic).

NAAG and mGluR receptors

In a recombinant system in which NAAG was screened against individual cloned mGluRs, NAAG was shown to activate mGluR3, but not mGluR2 [39]. These data lead to the hypothesis that one role for NAAG in the nervous system is that of an endogenous agonist at mGluR3 receptors, localized pre-synaptically on neurons and on glia. These pre-synaptic mGluR3 receptors have been shown to inhibit release of neurotransmitters, including glutamate [40–46]. In an elegant study exploiting CHO cells transfected with rat cDNAs for mGluR1–6, Wroblewska et al demonstrated that NAAG is a full agonist at mGluR3 receptors [47]. Using patch-clamp techniques in the cultured olfactory bulb, Bischofberger and Schild reported that NAAG activates mGluR3s leading to an inhibition of voltage-dependent calcium currents in the frog olfactory bulb interneurons [48]. Neale et al have provided convincing evidence that that potent agonist effects of NAAG at mGluR3s is not an artifact of glutamate contaminating commercial NAAG and suggests that NAAG and glutamate in fact activate different transduction pathways at the mGluR3.[49] This pathway for NAAG's action appears to account for its neuro-protective effects as Slusher et al demonstrated that inhibition of NAAG's degradation markedly attenuated the excitotoxic neurodegeneration

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caused by middle cerebral artery ligation or hypoxia in brian cultures. [50] This protective effect was abrogated by mGluR3 antagonists.[51]

GCP-II inactivates NAAG

As most neurotransmitters are inactivated by specific processes such as reuptake or enzymatic cleavage, the uptake of NAAG was examined in synaptosomal preparations [52]. These studies indicated that [3H]NAAG was not taken up intact but rather that the [3H]glutamate derived from NAAG was accumulated within the synaptosomal fraction in a fashion that was potently inhibited by quisqualic acid (QA). Since QA does not inhibit glutamate uptake, this result suggested that a QA-sensitive peptidase was cleaving NAAG to N-acetylaspartate (NAA) and [3H]glutamate[53]. This QA-sensitive peptidase was characterized in brain membrane preparations and shown to have a remarkably high affinity for NAAG [54]. Based upon substrate specificity, it was designated N-acetylated α-linked acidic dipeptidase or NAALADase [54], although more recent evidence also indicates substantial activity at γ-glutamyl peptide bonds. Accordingly, the enzyme has more recently been designated glutamate carboxypeptidase type II (GCP-II) [55–57]. GCP-II (EC3.4.17.21) is also known as prostate specific membrane antigen (PSMA) or folate hydrolase 1 (FOLH1) because it is identical to the enzyme in the small intestine responsible for removing the poly-glutamate tail from dietary folate for its absorption. [58] It is a type II transmembrane glycoprotein, which has a molecular weight of approximately 100 kDa. A glycosylation of the proteins is essential for enzymatic activity. Zinc is its essential cofactor. [59]

The regional distribution of the activity of GCP-II corresponds reasonably well with the regional concentrations of NAAG in the rodent brain [60], with the possible exception of the cerebellum, where the activity of GCP-II is very high. Immunocytochemical studies in the rat brain demonstrate that GCP-II is expressed exclusively on astrocytes and Bergmenn glial cells.[61] Sacha at al have expanded these studies to the human brain where the enzyme is also expressed exclusively in astrocytes with activity in white matter several fold greater than that in gray matter. [62] The disproportionally high expression of GCP-II in rat cerebellum does not appear in the human brain[62].

GCP-II inhibition and NMDAR

One way of reducing NMDAR function is by increasing the synaptic concentration of the endogenous NAAG. To define better the interaction of NAAG with the NMDAR, we used the acute hippocampal slices to determine how alterations in synaptic NAAG affected NMDAR currents in CA1 pyramidal neurons (a sub-region known to be affected in schizophrenia). As we previously demonstrated, perfusion with exogenous NAAG attenuated NMDAR currents generated by stimulating the pre-synaptic Shaffer collaterals known to contain NAAG. Moreover, perfusion with the potent GCP-II inhibitor, 2-PMPA[63–69], also caused significant attenuation in the amplitude of NMDAR currents, consistent with the inference that endogenous synaptic NAAG was inhibiting the NMDAR and that GCP-II controls its synaptic concentration. Perfusion of the slice with soluble, recombinant GCP-II lacking the intracellular and transmembrane domain so as to be soluble [70]. resulted in increased amplitude of NMDAR currents but had no effect AMPAR currents. As a second strategy to reduce synaptic NAAG, the slice was perfused with purified antibodies against NAAG. Similar to soluble GCP-II, NAAG antibodies caused a significant increase in the amplitude of the NMDAR currents. These latter two experiments provide convincing evidence that endogenous NAAG is released from the Shaffer collaterals into the synaptic cleft [70]. All together, these results suggest that endogenous NAAG significantly modulates NMDAR.

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NMDA Receptor Hypofunction and Schizophrenia

Almost four decades ago (1976), phencyclidine (PCP, also called "angel dust") was introduced into clinical medicine as an anaesthetic agent, but it was soon withdrawn because it caused a high incidence of psychotic "emergence" reactions [71, 72]. Researchers in that era were struck by the "schizophrenomimetic" quality of the syndrome that was induced by PCP in normal subjects [73]. They were also impressed with the ability of PCP to trigger a prolonged recrudescence of the acute psychotic state in stabilized chronic schizophrenic patients [74]. Most schizophrenia investigators agree that the positive, negative and cognitive symptoms triggered by PCP and a related dissociative anaesthetic, ketamine, are a more faithful model for studying schizophrenia than amphetamine or LSD [75–77]. Not until 1988 was PCP found to be a potent, selective and non-competitive antagonist for the NMDAR. [78]

The hypothesis of hypofunction of NMDAR was articulated in 1991 when Zukin and Javitt [73] noted that the plasma concentration of PCP associated with psychotic symptoms corresponded to the levels that block NMDAR. Thus, at subanesthetic doses, PCP binds to a site within the ion channel blocking the influx of cations, thereby acting as a noncompetitive antagonist. Ketamine, another anesthetic that has approximately a 10-to-50 fold lower affinity for the NMDAR than PCP, continues to be used as an anesthetic in children. It is interesting to note that psychotomimetic effects associated with exposure to ketamine are reported to occur less frequently in children than in adults, suggesting a similar age dependence in vulnerability to psychoses associated with NMDAR hypofunction and onset of schizophrenia. Subsequent studies have shown that low dose ketamine reproduces the subtle cognitive impairments and several physiologic abnormalities such a elevated subcortical dopamine release after an amphetamine challenge, altered default mode fMRI and abnormal cortical event related potential.[79, 80]

The PCP model for schizophrenia was further supported by a report showing that mice expressing only 5 percent of wild-type levels of NMDAR exhibit the same behavioural abnormalities as PCP injection to rodents [81]. Furthermore, the behavioral equivalents of negative symptoms in the NMDAR hypomorph mice are reversed by the atypical antipsychotic, clozapine. Notably, various NMDAR antagonists, both competitive and noncompetitive, that cause psychotic reactions in humans also induce neurodegenerative changes in cortico-limbic regions of the rat or mouse brain similar to the pattern of structural changes that have been reported in post-mortem studies from the brains of schizophrenic patients [82].

NAAG and Schizophrenia

Given the duel role of NAAG as an NMDAR antagonist and a presynaptic inhibitor of glutamate release via mGluR3, it would seem that NAAG would be an obvious candidate for involvement in schizophrenia in the context of the hypothesized hypofunction of NMDA receptors. Tsai et al were the first to examine this issue in postmortem brain studies. [83] They found a significant reduction in the activity of GCP2 in the frontal cortex, hippocampus and para-hippocampus. NAAG levels were elevated in the hippocampus. Several magnetic resonance spectroscopy studies have shown reduced levels of N-acetylaspartate, a catabolic product of GCP2, in cortical limbic brain regions in schizophrenia. Reductions in GCP2 expression in cortico-limbic brain regions in schizophrenia have been independently confirmed. Tkachev et al used the micro arrays and Q-PCR to demonstrate a significant reduction in the mRNA for GCP2 in the prefrontal cortex in schizophrenia. [84] Ghose et al used *in situ* hybridization to quantify sub regional expression of GCP2 in the hippocampus and found a significant reduction in the anterior hippocampus, CA1 in particular, in

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schizophrenia. In normal individuals, they found a highly significant positive correlation between the expression of GCP2 and mGluR3 that was absent in schizophrenia.[85]

Guilarte et al used a highly specific radio-ligand, N-[N-(S)-1,3-dicarboxypropyl] carbamoyl]-S-3-[125I]iodo-L-tyrosine ([125I]DCIT), to label GCP2 for quantitative autoradiography. They found significantly reduced levels of GCP2 in the prefrontal cortex, entorhinal cortex and most subregions of the hippocampus as compared to controls. Bipolar subjects also exhibited some reductions in GCP2 expression, but they were more limited and less severe than in schizophrenia.

Coyle[86, 87] first proposed that reduced GCP2 expression could lead to the potentiation of NAAG's effects in the brain's of individuals with schizophrenia. Thus, like PCP/ ketamine, NAAG would block NMDA receptors and attenuate glutamate release. Although *FOLH1* has not appeared as a risk gene for schizophrenia in association or genome wide association studies (GWAS), Semple et al noted that the *FOLH1* gene lies in close proximity (11 q14.3) to the translocation breakpoint [t(1:11)(q42.1,q14.3)] for the DISC1 mutation that is associated with increased risk for schizophrenia.[88]

Preclinical studies may also shed light on the potential role of NAAG and GCP2 in the pathophysiology of schizophrenia. Bacich et al. (2002, 2005) previously described a null mutation of GCP II in which stop codons were inserted in exons 1 and 2. Mice homozygous for the null mutation did not express GCP II. [89, 90] However, a residual 7–18% of wild type NAAG peptidase activity was detected in their brain. Notably, no differences in the endogenous levels of NAAG, N-acetylaspartate or glutamate, and very modest and subtle differences in behavior were noted in the homozygous as compared to wild type. In contrast, Tsai et al. (2003) knocked out the zinc ligand domain essential for GCP2 enzyme activity by deleting exons 9 and10. Mouse fetuses homozygous for the null mutation did not survive past 8 days gestation, indicating that GCP 2 is essential for embryogenesis before brain development.[91] In an attempt to resolve the strikingly different outcomes for the two null mutations of GCP 2, Han et al exploited the Cre/LoxP system to remove exons 1 and 2, the exons targeted by Bacich et al. (2002) to determine the impact of reduced or absent GCP 2. Again, homozygotes were lethal prior to birth. Heterozygous mice (GCP2+/−) expressed half the protein and GCP enzyme activity of wild-types. Heterozygotes exhibited increased motor activity, reduced social interactions and a subtle deficit in working memory consistent with a schizophrenia endophenotype.[92]

NMDA receptor antagonists are associated with increased glutamate release, presumably due to the differential sensitivity of NMDA receptors on the rapid-firing, recurrent, parvalbumen-positive GABAergic interneurons, resulting in pyramidal neuron disinhibition.[93] Lilly developed a group of drugs that are agonists at the Group II mGluRs to act at the mGluR3 to inhibit glutamate release. The prototypical agent, LY 354740 selectively reduces the stereotypies and hyperactivity caused by phecyclidine[94] and in a clinical trial significantly reduced the symptoms of schizophrenia as compared to placebo but comparable to the established antipsychotic, olanzapine[95]. Following a similar line of reasoning, Olszewski et al hypothesized that a GCP2 inhibitor could potentiate the action of endogenous NAAG at mGluR3 and thereby reduce the symptoms of schizophrenia. [46] Using a novel NAAG analog that inhibits GCP2, Olszewski et al showed that it reduced hyperactivity, stereotypies and a behavioral homolog of negative symptoms produced by treatment with the potent NMDA receptor antagonist, dizocilpine[96]. An orally active GCP2 inhibitor, 2-MPPA, attenuates dizocilipine-induced prepulse inhibition deficits in mice. [97]

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Concluding remarks

NAAG is an endogenous agonist at the mGluR3 and antagonist at NMDAR, both of which receptors are implicated by genetics in the pathophysiology of schizophrenia. The decreased activity of GCP2 in some brain areas such as the dorsolateral prefrontal cortex and the hippocampus of patients with schizophrenia supports the hypothesis that NAAG-mediated signaling is disrupted in schizophrenia. Given that schizophrenia appears to the consequence of disrupted brain circuit function, the suggestion that it can be explained by hypofunction of a single receptor, the NMDAR, is simplistic. In this context, GCP2 may both contribute to symptoms and yet be a target for pharmacologic intervention in schizophrenia.

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