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3-aminoglutarate is a "silent" false transmitter for glutamate neurons

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ABSTRACT

Understanding the storage and release of the excitatory neurotransmitter, L-glutamate by synaptic vesicles has lagged behind receptor characterizations due to a lack of pharmacological agents. We report that the glutamate analog, 3-aminoglutarate (3-AG) is a "silent" false transmitter for glutamate neurons that may be a useful tool to study storage and release mechanisms. Like L-glutamate itself, 3-AG is a high-affinity substrate for both the plasma membrane (EAATs) and vesicular (vGLUT) glutamate transporters. As such, EAATs facilitate 3-AG entry into neuronal cytoplasm allowing 3-AG to compete with L-glutamate for transport into vesicles thus reducing glutamate content. In a synaptosomal preparation, 3-AG inhibited calcium-dependent endogenous L-glutamate release. Unlike L-glutamate, 3-AG had low affinity for both ionotropic (NMDA and AMPA) and G-protein coupled (mGlu1-8) receptors. Consequently, 3-AG behaves as a "silent" false transmitter that may be used in physiological experiments to probe synaptic vesicle storage and release mechanisms for L-glutamate. The companion paper by Wu et al. (submitted) describes initial experiments that explore the effects of 3-AG on glutamate synaptic transmission under physiological and pathophysiological conditions.

1. Introduction

As the major excitatory neurotransmitter in the mammalian CNS, the synaptic functions of L-glutamate are the subject of intense interest and investigation. Pharmacological tools have been instrumental in providing insights into L-glutamate neurotransmission (Traynelis et al., 2010; Niswender and Conn, 2010; Swanson et al., 2005). These have mainly been focused on the sub-types of receptors that mediate L-glutamate neurotransmitter function, such as the N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate ionotropic receptors, and the eight sub-types of G-protein coupled glutamate receptors (mGlu1-8). Agonists and antagonists for these receptors have proved enormously useful in understanding the complex physiology of L-glutamate-mediated neurotransmission. In addition, pharmacological tools for the cell membrane (EAAT1-5) and vesicular transporters (vGLUT1-3) that mediate cellular accumulation and synaptic vesicle loading of glutamate have been identified (Bridges and Esslinger, 2005; Shigeri et al., 2004; Dunlop, 2006). A majority of studies have used these pharmacological agents to address questions primarily focused on glutamate receptor function subsequent to transmitter release. Pharmacological tools that interact directly with the vesicular storage and release of glutamate have not been available. A useful addition to the glutamate pharmacopeia would be an effective false transmitter agent.

A false transmitter is a compound that enters synaptic vesicles and is released along with the endogenous neurotransmitter upon synaptic activation (Kopin, 1968). The false transmitter may have direct receptor activity similar to the endogenous neurotransmitter. However, a false transmitter that has reduced receptor activity is of particular interest. Following vesicular uptake of such a molecule, the false transmitter reduces the concentration of the endogenous transmitter in the vesicle because of competition for transport and occupation of vesicular capacity. When the vesicle undergoes release, both the endogenous and false transmitters enter the synapse, however a reduced receptor response occurs since the false transmitter cannot evoke a full receptor response and the endogenous transmitter is present in lower concentration. A reduced receptor response of the false transmitter may be due to lower receptor affinity, reduced efficacy or both. This kind of "silent" false transmitter would be an invaluable tool for studying the relationship between vesicle storage and release of

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neurotransmitters and how this influences physiological and pathophysiological responses. False transmitters have been identified in numerous neurotransmitter systems, such as those for monoamines (Kopin, 1968), γ-aminobutyric acid (GABA; Lerma et al., 1985) and acetylcholine (Newton et al., 1985). Recently, Gubernator et al. (2009) described fluorescent monoamine analogs that acted as false transmitters and were useful to study dopamine release mechanisms from presynaptic terminals. A "silent" false transmitter for the L-glutamate system would be beneficial as a research tool and could also have therapeutic potential in disorders where L-glutamate over-activity is implicated (eg epilepsy, neurodegeneration) and reduced L-glutamate release is the desired therapeutic approach.

False transmitters have previously been proposed for the glutamate system, but they have disadvantages for routine use in physiological experiments. D-glutamate is a substrate for vesicular glutamate transporters (Naito and Ueda, 1985; Moriyama and Yamamoto, 1995) and reduces post-synaptic glutamate receptor responses when loaded into synaptic vesicles (Pan and Jahr, 1993). However, D-glutamate is a poor substrate for cell membrane glutamate transporters (EAATs; Arriza et al., 1994), necessitating its delivery into cells through a patch-clamp electrode for these experiments. L- and D-aspartate and the analog 3-hydroxyaspartate are good substrates for EAATs (Arriza et al., 1994), and can be transported and released from neuronal preparations (Fagg and Foster, 1983; Fleck et al., 2001; Nadler, 2011). However, these amino acids are not substrates for vGLUTs (Naito and Ueda, 1985; Moriyama and Yamamoto, 1995), and there is no evidence that they reduce endogenous glutamate release (McMahon and Nicholls, 1990; Fleck et al., 2001). In addition they have significant ionotropic glutamate receptor activity (Grimwood et al., 1991). Ideally, a "silent" false transmitter for the glutamate system should have three properties: (1) high-affinity substrate activity (close to that for L-glutamate) for the cell membrane transporters (EAATs), to allow access into neurons; (2) high-affinity substrate activity (close to that for L-glutamate) for the vesicular transporters (vGLUTs) to allow loading into synaptic vesicles to reduce endogenous glutamate release upon depolarizing stimuli; (3) reduced activity at ionotropic and mGlu L-glutamate receptors (at least 10-fold less than L-glutamate) to minimize direct receptor activation. Theoretically, the reduced receptor activity of a "silent" false transmitter could be due to reduced affinity for the receptor or reduced efficacy. However, a compound that retained high affinity with low efficacy is expected to behave as an antagonist or partial agonist with the potential to inhibit the response of endogenous glutamate. In this sense it would not be a "silent" false transmitter since it would negatively impact the receptor response to endogenously released glutamate. Here, we present neurochemical evidence that 3-aminoglutarate (3-AG; beta-glutamate) fulfills these criteria and in the companion paper by Wu et al. (2015) show that 3-AG acts as a "silent" false transmitter for glutamate in physiological experiments and is able to reduce epileptiform activity.

2. Materials and methods

2.1. Animals

All experiments were approved by the Allergan IACUC and carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

2.2. Chemicals

3-aminoglutarate (β-glutamate; 3-AG) was purchased from Sigma–Aldrich. [^{3}H-G]3-AG was custom synthesized by Perkin Elmer with a specific activity of 16.7 Ci/mmol. [^{3}H]D-aspartate and [^{3}H]L-glutamate were from Perkin Elmer. All other common laboratory reagents used were supplied by Sigma–Aldrich.

2.3. Synaptosome and synaptic vesicle preparation

A crude synaptosome (P2) fraction was prepared from rat hippocampus as described by Fedele and Foster (1992). Synaptosomes were utilized immediately in transport assays. A crude synaptic vesicle preparation was prepared from rat forebrain (whole brain minus cerebellum, underlying brainstem and olfactory bulb) as described by Kish and Ueda (1989). Synaptic vesicles were stored in aliquots at −80 °C until the day of assay.

2.4. Synaptosome and synaptic vesicle transport assays

Freshly-prepared crude synaptosomes (approx. 5 μg protein) were incubated for 2 min at room temperature with either [^{3}H]D-aspartate or [^{3}H]3-AG at a final concentration of 1 μM (0.3 μCi) and test compounds in a final volume of 200 μl. The assay buffer had the following composition: NaCl 128 mM, KCl 5 mM, NaH_2PO_4 1.5 mM; $MgSO_4$ 1 mM, $CaCl_2$ 1 mM, D-glucose 10 mM, in 10 mM Tris-acetate buffer, pH 7.4. To examine sodium-dependence, equimolar choline chloride replaced sodium chloride and to examine temperature-dependence, tubes were incubated at 4 °C. The incubation was terminated by the addition of excess assay buffer at 4 °C and tissue collected on Whatman GF/C filters with additional washing to remove free radiolabel. Filters were dried, and radioactivity determined by liquid scintillation counting. Synaptic vesicles (approx. 80 μg protein) were pre-incubated for 5 min at 30 °C before addition of [^{3}H]L-glutamate or [^{3}H]3-AG (1 μCi; final concentration 50 μM) and ATP (final concentration 2 mM) in a final volume of 100 μl. The assay buffer had the following composition: sucrose 218 mM, $MgCl_2$ 4 mM in 5 mM HEPES:KOH buffer, pH 7.4. The incubation was terminated by the addition of excess 0.15 M KCl at 4 °C and tissue collected on cellulose filters (Millipore catalog # HAWP02500) with additional washing to remove free radiolabel. Filters were dried, and radioactivity determined by liquid scintillation counting.

2.5. Acridine orange assay

Synaptic vesicles (15 μg/ml protein final concentration) were incubated at 30 °C in the presence of 0.5 mM ATP, 0.2 μM acridine orange in a final volume of 2500 μl in a cuvette in a Perkin Elmer LS 50B spectrophotometer. The assay buffer had the following composition: sucrose: 218 mM, $MgCl_2$: 4 mM in 5 mM HEPES:KOH buffer, pH 7.4. Changes in fluorescence (excitation: 492 nm; emission: 540 nM) were measured before and after addition of test compounds and monitored for 8 min.

2.6. Glutamate release assay

Experiments were conducted as described by Nicholls et al. (1987) using a crude synaptosomal (P2) preparation from rat forebrain as described above for hippocampus. To evaluate the effects of 3-AG, DHK and D-aspartate, compounds were preincubated with synaptosomes for 10 min at 37 °C, placed on ice and then centrifuged at 10,000 rpm for 2 min at 4 °C and resuspended in 1.1 ml assay buffer without compound. This washing process was repeated twice before synaptosomes were used to measure endogenous glutamate release.

2.7. EAAT expression in Xenopus oocytes

The cDNA for the coding sequences of each of the human excitatory amino acid transporters (EAATs 1–5) were sub-cloned into the *Xenopus* oocyte expression vector pBSTA between the single *Bgl*II insertion site and an upstream XhoI site at position 676. The pBSTA vector contains a T7 promoter followed by 5′ noncoding β-globin sequences, 3′ noncoding β-globin sequences, a poly(A) tail, and a polylinker containing a NotI restriction site that allows the plasmid to be linearized before *in vitro* transcription (Shih et al., 1998). DNA was prepared on a small scale using the Promega Wizard Plus SV Miniprep Kit. Restriction enzyme digestions with Xmn I (EAAT 1, 4, 5), Hind III (EAAT 2), Sac II (EAAT 3) confirmed that there were no major rearrangements during growth of the bacteria. Large scale preparations of transporter DNA were then prepared and the fidelity of the coding regions was confirmed by restriction enzyme digestions and sequencing. RNA was transcribed using the Ambion mMessage T7 kit and expression of each transporter was confirmed by injection of the RNA into oocytes and subsequent voltage-clamping. The effect of L-glutamate and 3-AG in on each of the transporters was compared using a multi-step electrophysiological protocol.

2.8. Two-electrode whole-cell voltage clamp analysis of transporter currents in Xenopus oocytes

Oocytes injected with EAAT RNA were incubated for 1–4 days in ND96, and maintained in a solution of ND96 adjusted to a pH of 7.40 and an osmolarity of 200 ± 5 mOsm during experimental recording (Dascal, 2001). Transporter currents were recorded using an OpusXpress parallel oocyte voltage clamp (Molecular Devices, Sunnyvale, CA). The currents were low-pass-filtered at 1 kHz and digitized at 5 kHz. Microelectrodes were filled with 3 M KCl and had tip resistances of <1 MΩ. The bath was connected to ground by a 3 M KCl-agar bridge from the recording chamber to a 3 M KCl reservoir containing a Ag/AgCl electrode. The oocytes were held at −60 mV with an initial 18 s perfusion of ND-96 to generate a stable baseline. This was followed by a 90 s perfusion of 1 mM L-glutamate as a control to observe the current during maximum activation of the transporter, followed by a subsequent

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