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Research Report

Presynaptic D1 heteroreceptors and mGlu autoreceptors act at individual cortical release sites to modify glutamate release

Takuya Hikima[1], *Marianela Garcia-Munoz*, *Gordon William Arbuthnott*[*]

Brain Mechanism for Behaviour Unit, Okinawa Institute of Science and Technology Graduate University, Okinawa, Japan

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ABSTRACT

The aim of this work was to study release of glutamic acid (GLU) from one-axon terminal or bouton at-a-time using cortical neurons grown *in vitro* to study the effect of presynaptic auto- and heteroreceptor stimulation. Neurons were infected with release reporters SypHx2 or iGluSnFR at 7 or 3 *days-in-vitro* (DIV) respectively. At 13–15 DIV single synaptic boutons were identified from images obtained from a confocal scanning microscope before and after field electrical stimulation. We further stimulated release by raising intracellular levels of cAMP with forskolin (10 μM). Forskolin-mediated effects were dependent on protein kinase A (PKA) and did not result from an increase in endocytosis, but rather from an increase in the size of the vesicle readily releasable pool. Once iGluSnFR was confirmed as more sensitive than SypHx2, it was used to study the participation of presynaptic auto- and heteroreceptors on GLU release. Although most receptor agonizts (carbamylcholine, nicotine, dopamine D2, BDNF) did not affect electrically stimulated GLU release, a significant increase was observed in the presence of metabotropic D1/D5 heteroreceptor agonist (SKF38393 10 μM) that was reversed by PKA inhibitors. Interestingly, stimulation of group II metabotropic mGLU2/3 autoreceptors (LY379268 50 nM) induced a decrease in GLU release that was reversed by the specific mGLU2/3 receptor antagonist (LY341495 1 μM) and also by PKA inhibitors (KT5720 200 nM and PKI14-22 400 nM). These changes in release probability at individual release sites suggest another level of control of the distribution of transmitter substances in cortical tissue.

1. Introduction

Activation of presynaptic auto- and heteroreceptors contribute to neurotransmitter release by sensing the local presence of neurotransmitter to either inhibit or promote further release. Cortical output neurons are presynaptically modulated by GLU autoreceptors and heteroreceptors to a plethora of neurotransmitters. Although the presence of presynaptic receptors is required, a direct presynaptic input is not. Volume transmission and spillover allow neurotransmitters to reach receptors away from their immediate release sites. GLU is the agonist of two classes of receptors, ion channel linked (ionotropic)

[*]Correspondence to: Brain Mechanism for Behaviour Unit, Okinawa Institute of Science and Technology Graduate University, 1919-1 Tancha, Onna-son, Okinawa 904-0495, Japan.
E-mail addresses: takuya.h.shg@gmail.com (T. Hikima), marianela@oist.jp (M. Garcia-Munoz), gordon@oist.jp (G.W. Arbuthnott).
[1]Present address: Department of Neurosurgery, NYU-Langone Medical Center, 455 First Ave, New York, USA.

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receptors, which include NMDA, AMPA and kainate receptors, and metabotropic receptors (mGluRs) which couple via G-proteins to intracellular second messenger signaling pathways. Presynaptic modulation of GLU release by activation of ionotropic or metabotropic (mGLUR) receptors has been well documented (Garcia-Munoz et al., 1991a, 1991b; Lovinger et al., 1993; Maura et al., 1988; Nicholls, 1998; O'Donnell and Grace, 1994; West and Grace, 2002) to quote just a few. Depending on the nucleotide sequence of genes mGLURs are classified into 3 groups: mGLURs 1 and 5 (group I), mGLURs 2 and 3 (group II) and mGLURs 4, 6, 7 and 8 (group III). Groups II and III are usually found in presynaptic terminals as autoreceptors (Williams and Dexter, 2014). Since mGLURs have approximately 10-fold higher affinity for GLU than ionotropic receptors (Gerber, 2003) their location as sensors of presynaptic release has been considered ideal (Miller, 1998).

Evidence of modulation of GLU release by activation of heteroreceptors in cortical terminals has also been given for GABA (Logie et al., 2013; Waldmeier et al., 2008), acetylcholine (Giocomo and Hasselmo, 2007; Pancani et al., 2014), dopamine (Bamford et al., 2004; Cepeda et al., 2001), noradrenaline (Luo et al., 2014a, 2014b), adenosine (Bannon et al., 2014; Quiroz et al., 2009); somatostatin (Grilli et al., 2004); cholecystokinin (Deng et al., 2006); endocannabinoids (Ferreira et al., 2012; Lemtiri-Chlieh and Levine, 2010; Perez-Rosello et al., 2013) and serotonin (Aghajanian and Marek, 1999; Calcagno et al., 2006, 2009; Wang et al., 2006).

Apart from the effects of neurotensin receptor activation that appear to be indirectly mediated by the induced release of another neurotransmitter (Ferraro et al., 2008; Matsuyama et al., 2003; Yin et al., 2008), direct modulation of cortical GLU release resulting from auto- and heteroreceptor stimulation typically occurs by stimulation of secondary intraterminal cascades the simplest of which is the facilitation of calcium entrance, by ionotropic GLU receptor induced depolarization, that favors vesicle release (Perkinton and Sihra, 1999). Stimulation of G-protein coupled metabotropic auto- and heteroreceptors leads to activation of intracellular signal transduction pathways targeting a variety of intracellular proteins including protein kinases. A second messenger such as calcium or cAMP typically regulates the activity of protein kinases. Protein kinase A (PKA) and protein kinase C (PKC) are associated with modulation of neurotransmitter release.

Studies of presynaptic events, such as those reported above, have used a variety of methods to detect endogenous release; stimulated release of preloaded neurons or synaptosomes, microdialysis, postsynaptic intracellular recordings of spontaneous and miniature depolarizing potentials or postsynaptic currents following pair-pulse stimulation. Those methods provide valuable data but in general their disadvantage is the large population of synapses involved. Although our basic knowledge about transmitter release is largely based on the much simpler and more robust neuromuscular junction, central synapses seem to have different properties the most striking of which is the intermittent nature of release from individual boutons. Methods recently available that use optical reporters of exocytosis allow the precise study of release at single boutons. In view of the variety of modulatory influences on cortical GLU release we decided to use two optical release reporters, synaptophysin-pHlourinx2 (SypHx2) and intensity-based GLU-sensing fluorescent reporter (iGluSnFR). SypHx2 is the fusion of synaptic vesicle protein synaptophysin with two molecules of the super ecliptic pHluorin, pH-sensitive green fluorescent protein (GFP) (Burrone et al., 2006; Zhu et al., 2009), iGluSnFR is a sensor for synaptically released GLU constructed using a bacterial periplasmic binding protein and circularly permutated GFP (Marvin et al., 2013). We first studied release and compared the sensitivity of the two reporters using cortical neurons grown *in vitro* and stimulated the production of cAMP with forskolin. Since cortical neuron synapses are smaller than those typically used in release studies (i.e., hippocampal or cerebellar) the sensitive iGluSnFR reporter proved superior to SypHx2 for the study of the effect of presynaptic receptors stimulation on facilitated release. iGluSnFR had a higher signal-to-noise ratio and detected release after only a single pulse of electrical field stimulation. The effect of specific presynaptic receptor stimulation before and after drug administration allowed us to detect changes in release at the level of single boutons. Here we report a major enhancing effect on GLU release at individual boutons induced by dopamine D1/D5 receptor stimulation and an inhibitory effect induced by a group II mGLU2/3 agonist. Both specific hetero- and autoreceptor-mediated effects were reversed by PKA antagonists. In summary, our results confirm presynaptic auto- and heteroreceptor modulation of single pulse-induced GLU release in individual synaptic *boutons en passage* of cortical neurons grown *in vitro* dependent on the AC/PKA pathway.

2. Results

The study of neurotransmitter release was significantly advanced by the use of synaptopHluorin (SypHx2), a fluorescent protein designed to detect vesicle fusion at the synapse. To detect the pH change that occurs along neurotransmitter release a particularly pH-sensitive variant of green fluorescent protein, ecliptic pHluorin, was fused to the vesicular protein synaptobrevin. Once transfected and expressed, pHluorin is quenched in the acidic lumen of vesicles (pH~5.5) and increases fluorescence about 20-fold after fusion with the plasma membrane. This reporter has been invaluable for the study of changes in neurotransmitter release (Balaji and Ryan, 2007; Gandhi and Stevens, 2003; Zhu et al., 2009). As is typical for this reporter, SypHx2 signal is optimal above 5 action potentials at 20 Hz (Gandhi and Stevens, 2003; Zhu et al., 2009). The iGluSnFR sensor for synaptically released GLU is produced under the synapsin promoter and uses the periplasmic binding-protein-dependent transport system for GLU and aspartate to anchor the protein to the extracellular plasma membrane. The binding of GLU to this periplasmic protein induces a conformational change in an inserted circularly permuted green fluorescent protein. The advantage of this reporter is that fluorescence can be detected in response of a single stimulus (Marvin et al., 2013). In our cultures, two weeks after transduction cortical neurons expressed either the SypHx2 or iGluSnFR sensor. To report release neurons expressing SypHx2 required five action potentials at 20 Hz (20 mA/1ms,