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Astroglial D-serine is the endogenous co-agonist at the presynaptic NMDA receptor in rat entorhinal cortex

Alex M. Lench [a], Peter V. Massey [a], Loredano Pollegioni [b], Gavin L. Woodhall [c], Roland S.G. Jones [a,*]

[a] Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom
[b] Dipartimento di Biotecnologie e Scienze della Vita, Università degli studi dell'Insubria, Varese, Italy
[c] School of Life and Health Sciences, Aston University, Birmingham B4 7ET, United Kingdom

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ABSTRACT

Presynaptic NMDA receptors facilitate the release of glutamate at excitatory cortical synapses and are involved in regulation of synaptic dynamics and plasticity. At synapses in the entorhinal cortex these receptors are tonically activated and provide a positive feedback modulation of the level of background excitation. NMDA receptor activation requires obligatory occupation of a co-agonist binding site, and in the present investigation we have examined whether this site on the presynaptic receptor is activated by endogenous glycine or D-serine. We used whole-cell patch clamp recordings of spontaneous AMPA receptor-mediated synaptic currents from rat entorhinal cortex neurones *in vitro* as a monitor of presynaptic glutamate release. Addition of exogenous glycine or D-serine had minimal effects on spontaneous release, suggesting that the co-agonist site was endogenously activated and likely to be saturated in our slices. This was supported by the observation that a co-agonist site antagonist reduced the frequency of spontaneous currents. Depletion of endogenous glycine by enzymatic breakdown with a bacterial glycine oxidase had little effect on glutamate release, whereas D-serine depletion with a yeast D-amino acid oxidase significantly reduced glutamate release, suggesting that D-serine is the endogenous agonist. Finally, the effects of D-serine depletion were mimicked by compromising astroglial cell function, and this was rescued by exogenous D-serine, indicating that astroglial cells are the provider of the D-serine that tonically activates the presynaptic NMDA receptor. We discuss the significance of these observations for the aetiology of epilepsy and possible targeting of the presynaptic NMDA receptor in anticonvulsant therapy.

1. Introduction

Transmitter release at central synapses and, hence, the strength of synaptic transmission is controlled on a moment-to-moment basis by activation of auto or heteroreceptors on presynaptic nerve terminals. At glutamate synapses, presynaptic NMDA receptors (preNMDArs) exert a tonic facilitatory effect on glutamate release and, thus, mediate an instantaneous form of synaptic plasticity. We first demonstrated this functional facilitatory effect at synapses in the rat medial entorhinal cortex (EC; Berretta and Jones, 1996a) and subsequently showed that it was mediated via Ca^{2+}-entry via the receptor ionophore (probably di-heteromeric GluN1-GluN2B receptors) and could also contribute to activity-dependent facilitation (Woodhall et al., 2001; Chamberlain et al., 2008). It is now apparent that preNMDArs are expressed at many sites throughout the CNS, and a widespread role of the receptors in control of transmitter release is likely (see Duguid and Smart, 2009). In particular, attention has increasingly focussed on a role in long-term synaptic plasticity of excitatory transmission (Humeau et al., 2003; Sjostrom et al., 2003; Samson and Pare, 2005; Corlew et al., 2007; Yang et al., 2008; Rodríguez-Moreno et al., 2010; Larsen et al., 2011). Our observation that preNMDAr are

Abbreviations: 2-AP5, D-2-Amino-5-phosphonopentanoic acid; BsGO, *Bacillus subtilis* glycine oxidase; CGS15943, (9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-*c*] quinazolin-5-amine); CPPG, (*RS*)-α-Cyclopropyl-4-phosphonophenylglycine; DCKA, 5,7-Dichlorokynurenic acid; DIC, differential interference contrast; DREO, Departmental Research Ethics Officer; EC, entorhinal cortex; EPSC, excitatory postsynaptic current; MCPG, (*RS*)-α-Methyl-4-carboxyphenylglycine; mEPSC, miniature excitatory postsynaptic current; NFAc, sodium fluoroactetate; NMDAr, NMDA receptor; preNMDAr, presynaptic NMDA receptor; postNMDAr, postsynaptic NMDA receptor; RgDAAO, *Rhodotorula gracilis* D-amino acid oxidase; sEPSC, spontaneous excitatory postsynaptic current; tetrodotoxin, TTX.

* Corresponding author. Tel.: +44 1225 383935; fax: +44 1225 386114.
E-mail address: r.s.g.jones@bath.ac.uk (R.S.G. Jones).

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highly mobile in the presynaptic terminal membrane in the EC (Yang et al., 2008) could indicate an important role in metaplasticity as well. Enhanced expression or sensitivity of the preNMDAr may be involved in pathological plasticity associated with epilepsy (Steffens et al., 2005; Yang et al., 2006).

NMDAr are modulated by a number of intrinsic regulatory mechanisms. In addition to partial relief from voltage-dependent Mg^{2+}-blockade, NMDAr activation requires binding of two agonists; the glutamate binding site is located on the GluN2 subunit and a co-agonist binding site resides on GluN1 subunits. Both glycine and D-serine are available as physiological ligands for the co-agonist site. Although glycine has generally been considered to be the endogenous co-agonist, recent studies have indicated that D-serine is more likely to be the activator of the binding site at the postsynaptic NMDAr (postNMDAr), and that glycine is maintained at concentrations well below its affinity for the binding site by high-affinity transporters in the synaptic cleft (Fossat et al., 2012; Papouin et al., 2012). The source of the endogenous synaptic D-serine may well be adjacent astrocytes (Henneberger et al., 2010; Fossat et al., 2012), although a neuronal origin has not been ruled out (e.g. Wolosker et al., 1999; Kartvelishvily et al., 2006; Rosenberg et al., 2010).

The EC plays a crucial role in declarative and spatial memory storage (e.g. Eichenbaum, 2001; Squire et al., 2004; Witter and Moser, 2006) and in limbic seizure generation and propagation in temporal lobe epilepsy (see Jones and Woodhall, 2005). To help understand the role of the preNMDAr in synaptic function in the EC we have investigated whether they are subject to regulation by endogenous glycine or D-serine, and whether this regulation may be necessary for tonic facilitation of spontaneous glutamate release. Li and Han (2007) previously showed that an antagonist at the NMDAr co-agonist site was able to reduce the spontaneous release of glutamate in visual cortex, an effect that was reversed by addition of glycine suggesting that endogenous activation of the co-agonist-binding site regulated tonic activation of preNMDArs in this area. In the present study we show that preNMDArs in the EC undergo a similar endogenous modification, that the endogenous agonist responsible for this is D-serine not glycine, and that the source of the D-serine is likely to be astroglial cells.

2. Materials and methods

2.1. Ethics statement

All experiments were performed in accordance with the U.K. Animals (Scientific Procedures) Act 1986, European Communities Council Directive 1986 (86/609/EEC) and the University of Bath ethical review document, which requires that the number of animals used is kept to a minimum, and every precaution was taken to reduce suffering and stress. At this institution, all research work involving use of animal tissue requires submission of a consideration of ethical implications by the principal investigator. This is reviewed by a second investigator, external to the research group, and by the Head of Department, and requires signatory approval from both before being reviewed by a Departmental Research Ethics Officer (DREO). The DREO discusses any issues raised with the investigator and submits a report to the University Ethics Committee detailing the ethical implications of all research within the Department. This ensures that the ethical implications of the research have been considered and that there is a process in place for managing any ethical issues.

2.2. Slice preparation

Brain slices were prepared (Jones and Heinemann, 1988) from juvenile Wistar rats (50–100 g; P28–38). They were decapitated following cervical dislocation and the brain removed and submerged in oxygenated artificial cerebrospinal fluid (aCSF; see below for composition) at 4 °C. Combined EC-hippocampal brain slices (350 μm thick) were cut at 4 °C using a Campden Vibroslice and subsequently stored in oxygenated aCSF at room temperature. Slices were left to recover for 1 h before being used for electrophysiological recording. The aCSF contained (in mM) NaCl (126), KCl (4), $MgSO_4$ (1.25), NaH_2PO_4 (1.4), $NaHCO_3$ (24), $CaCl_2$ (2), D-glucose (10), ascorbic acid (0.57), sodium pyruvate (5) and creatinine monohydrate (5). To increase neuronal survival and viability, ketamine (4 μM), indomethacin (45 μM), aminoguanidine (25 μM), and Coomassie Brilliant Blue (250 nM) were included in the cutting solution, and the antioxidants, n-acetyl-L-cysteine (2 μM) and uric acid (100 μM), added to both cutting and storage solutions. We have established that the use of the additives facilitates production robust and viable slices, but does not affect the pharmacology of glutamate transmission.

2.3. Whole-cell patch clamp recordings

After a period of recovery at room temperature, slices were transferred to a chamber on an Olympus BX50WI microscope perfused (2 ml/min) with oxygenated aCSF at 31–32 °C, where they were allowed to equilibrate before recording commenced. Individual neurones were visualized using DIC optics and an infrared video camera.

Whole cell patch clamp recordings were made with an Axopatch 200A amplifier using pipettes pulled from borosilicate glass on a Flaming-Brown microelectrode puller. AMPA receptor mediated spontaneous excitatory post synaptic currents (sEPSCs) were recorded using a Cs-gluconate based solution intracellular solution containing (in mM) D-gluconate (100), HEPES (40), QX-314 (1), EGTA (0.6), NaCl (2), Mg-gluconate (5), TEA-Cl (5), phosphocreatine (10), ATP-Na (4), GTP-Na (0.3) and MK801 (5 mM). The solution was adjusted to 275–290 mOsm by dilution and pH 7.3 with CsOH. MK-801 was included in the patch pipettes to allow us to record AMPA-receptor mediated responses in isolation and to monitor activity at preNMDArs uncontaminated by postsynaptic receptor effects. We developed and validated this approach (Berretta and Jones, 1996b; Woodhall et al., 2001; Yang et al., 2006, 2008), and others have used it successfully to block postNMDArs in recorded neurones (Sjostrom et al., 2003; Samson and Pare, 2005; Bender et al., 2006; Jourdain et al., 2007; Li and Han, 2007; Brasier and Feldman, 2008).

EPSCs were recorded at a holding potential of −60 mV. Signals were filtered at 2 kHz, digitised at 50 kHz, and stored using AxoScope software. Series resistance compensation was not employed, but access resistance was monitored at 5-min intervals throughout recording and cells where it varied by greater than 15% were excluded from analysis. Input resistances for the neurones recorded in these studies were of the order 500–570 MΩ. Data recording commenced 10–15 min after gaining whole cell access and then continued for at least 15 min during control and each drug treatment condition. Miniature EPSCs (mEPSCs) were isolated using tetrodotoxin (TTX; 1 μM). EPSCs were analysed offline over a stable 5-min period of recording where spontaneous events were detected using a threshold-crossing algorithm with Minianalysis software (Synaptosoft, Decatur).

The average frequency, calculated across a 5-min epoch, was compared before and after drug application. To compare amplitudes of spontaneous currents we determined median values for each neurone in the same 5-min period as these better reflect the population distributions (normal distribution with a slight skew towards larger amplitude events) than arithmetical means. Median values were then averaged in the neuronal populations for comparative illustrative purposes. We have used this approach in a previous study to characterize IPSCs in the same neuronal population (Woodhall et al., 2005). Event kinetics were compared via arithmetical means. Statistical comparison of drug effects on mean frequency and kinetics and mean median amplitudes within cells employed a two-tailed paired *t*-test. In experiments involving matched slices with and without prior enzyme treatment, comparison between neurones was assessed using a two-way ANOVA. In some experiments involving pooled data, we additionally compared cumulative probability distributions of interevent intervals using a Kolmogorov–Smirnov test.

2.4. Enzymatic depletion of endogenous D-serine and glycine

To determine whether glycine or D-serine is the endogenous activator of the co-agonist site we depleted one or other of the amino acids from the slices by pre-incubation with selective enzyme scavengers. An yeast D-amino acid oxidase (DAAO), was used to degrade D-serine, whereas a bacterial glycine oxidase (GO) was used to target glycine. Recombinant *Rhodotorula gracilis* DAAO (RgDAAO) and *Bacillus subtilis* glycine oxidase GO (BsGO) were overexpressed in *Escherichia coli* cells and then purified as described previously (Fantinato et al., 2001; Job et al., 2002). In experiments where they were employed, slices were incubated with enzymes at room temperature in the recording chamber for at least 45 min prior to recording, and then continuously during the recording period at 32 °C. Matched slices in normal buffer were treated in the same way and alternated with the enzyme treated slices. For each experiment a single neurone was recorded from an enzyme treated slice, and a second recording was made in an untreated slice from the same animal on that day, so that neurones were matched in that animal. The order of recording (treated or untreated) was varied from animal to animal.

2.5. Source of D-serine in EC

Previous studies have suggested that a principal source of D-serine in slices is via release from astroglial cells (Kartvelishvily et al., 2006; Miya et al., 2008; Rosenberg et al., 2010). The metabolic status of astroglial cells is compromised by sodium fluoroacetate (NFAc). Accordingly, we have determined the effect of NFAc on spontaneous release of glutamate in EC. In experiments where this was tested, slices were incubated with NFAc in the recording chamber for at least 35 min prior to recording, and also continuously during the recording period. Matched slices in normal buffer were treated in the same way and alternated with the NFAc treated slices.