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L-[^{3}H]Glutamate labels the metabotropic excitatory amino acid receptor in rodent brain

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A quantitative autoradiographic assay for a novel L-[^{3}H]glutamate binding site in rodent brain has been developed. Binding to this site was distinguished by its high affinity for quisqualate (QA), ibotenate, glutamate and *trans*-1-amino-cyclopentyl-1,3-dicarboxylic acid (*trans*-ACPD), but low affinity for [RS]-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and *N*-methyl-D-aspartate (NMDA). 'AMPA-insensitive, QA-sensitive [^{3}H]glutamate binding' (AiQsGB) had a heterogeneous distribution in rat brain with high levels observed in molecular layer of cerebellum, striatum, and lateral septum. AiQsGB was reduced in molecular layer of cerebellum in mice lacking Purkinje cells. AiQsGB appears to represent binding to the 'metabotropic' neuronal excitatory amino acid receptor linked to phosphoinositide metabolism.

Excitatory amino acids (EAA) are believed to be the predominant excitatory neurotransmitters within the mammalian central nervous system [3,10]. EAA exert their effects via multiple receptors. At least two of these receptors, the NMDA receptor and the AMPA/quisqualate/kainate receptor are linked to ion channels. Selective antagonists for the AMPA/quisqualate/kainate receptor have been identified [4]. Recently, the existence of another EAA receptor has been proposed which, in contrast to the ion channel-linked, or 'ionotropic' EAA receptors, is a 'metabotropic' receptor linked to phosphoinositide metabolism [11, 18, 19]. This receptor appears to play an important role in development and response to injury. The lack of specific antagonists and binding assays for this receptor, however, has hindered our ability to define its role in normal brain function. This metabotropic receptor is stimulated by quisqualate, L-glutamate and ibotenate and is insensitive to AMPA, NMDA and kainate [11–13, 16–18]. We have characterized L-[^{3}H]glutamate binding that likely represents binding to the putative metabotropic receptor.

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TABLE I

THE PHARMACOLOGY OF [^{3}H]AMPA BINDING AND AMPA-INSENSITIVE, QUISQUALATE-SENSITIVE [^{3}H] GLUTAMATE BINDING IN CEREBELLAR MOLECULAR LAYER

Values represent the mean of at least 3 experiments each carried out in triplicate.

	IC_{50} (μM $\pm$ s.e.m.)	
Competitor	[^{3}H]AMPA binding*	[^{3}H]Glutamate binding
Quisqualate	0.081 $\pm$ 0.015	0.052 $\pm$ 0.025
Glutamate	1.78 $\pm$ 0.49	0.181 $\pm$ 0.058
AMPA	0.252 $\pm$ 0.024	> 100
Ibotenate	> 100	3.15 $\pm$ 0.77
trans-ACPD	> 100	10.7 $\pm$ 1.9
CNQX	0.358 $\pm$ 0.031	> 100
DNQX	0.339 $\pm$ 0.011	> 100
APB	> 100	> 100
GDEE	> 100	> 100
γDGG	> 100	> 100
Kynurenate	> 100	> 100
Cystine	n.d.**	> 100
SITS	n.d.	> 100

*[^{3}H]AMPA binding (37 nM, spec. act. 28 Ci/mmol) was carried out in 50 mM Tris-HCl buffer with 2.5 mM $CaCl_2$ and 100 mM potassium thiocyanate as previously described [15]. Non-specific binding was determined in the presence of 1 mM glutamate. AMPA-insensitive, quisqualate-sensitive [^{3}H]glutamate binding (82 nM) was carried out in 50 mM Tris-HCl buffer, pH 7.2, with 2.5 mM $CaCl_2$, 100 mM thiocyanate, 10 μM AMPA and 100 μM NMDA. Nonspecific binding was determined in the presence of 2.5 μM quisqualate.

**n.d. signifies 'not determined'.

L-[^{3}H]Glutamate binds to the NMDA and AMPA receptors and to a population of sites that are sensitive to quisqualate but not AMPA [2, 14]. In these experiments, 20 μm sections of rodent brain were rinsed for 30 min in 50 mM Tris-HCl buffer with 2.5 mM $CaCl_2$, pH 7.2, and then dried. Sections were incubated for 45 min in L-[^{3}H]glutamate (82–200 nM, spec. act. 28–46 Ci/mmol, Amersham) in the above buffer with 100 mM potassium thiocyanate in the presence or absence of various compounds. After incubation, sections were rinsed 3 times with cold buffer and once with cold 2.5% (v/v) glutaraldehyde in acetone and rapidly dried under a stream of warm air (total rinse time was less than 10 sec). Sections were placed in X-ray cassettes, apposed to tritium-sensitive film (Hyperfilm, Amersham Corporation), exposed for two weeks at 4°C and developed in D-19 (Kodak). Autoradiographic

images were analyzed by computer-based densitometry (Imaging Research, Inc., St. Catherine's, Ontario, Canada) [2].

In autoradiographic L-[^{3}H]glutamate binding studies, calcium and chloride specifically stimulate quisqualate-sensitive L-[^{3}H]glutamate binding. Unlike the chloride and calcium stimulated L-[^{3}H]glutamate 'binding' or sequestration observed in fresh neuronal and astrocytic membrane preparations, however, this quisqualate-sensitive L-[^{3}H]glutamate binding was insensitive to cystine, the anion channel blocker 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (SITS), and L-2-amino-4-phosphonobutyrate (L-APB) [2](Table I). Furthermore, two components of this quisqualate-sensitive L-[^{3}H]glutamate binding could be defined, AMPA-sensitive and AMPA-insensitive binding. The AMPA-sensitive, quisqualate-sensitive L-[^{3}H]glutamate binding had the properties of the ionotropic AMPA receptor, i.e., it had an almost identical regional distribution to that of [^{3}H]AMPA binding and inhibition by AMPA, quisqualate, and glutamate but not by ibotenate [2, 14]. AMPA's ability to displace L-[^{3}H]glutamate binding was enhanced by thiocyanate, which stimulates [^{3}H]AMPA binding [5, 14]. Nevertheless, a considerable component of quisqualate-sensitive L-[^{3}H]glutamate binding remained in 50 mM Tris-chloride plus 2.5 mM $CaCl_2$, 100 mM thiocyanate, 100 μM NMDA and 10 μM AMPA (nonspecific binding was defined as that binding remaining in the presence of 2.5 μM quisqualate). We studied the properties of this AMPA-insensitive, quisqualate-sensitive glutamate binding (AiQsGB).

The pharmacology of AiQsGB was different from that of AMPA-sensitive L-[^{3}H]glutamate binding or [^{3}H]AMPA binding to the presumed ionotropic receptor (Table I). AiQsGB was greatest at 4°C and was reduced at 23°C and 37°C. The anion channel blocker, SITS (100 μM), displaced only 30% of binding and cystine (100 μM), which potently inhibits chloride dependent glutamate sequestration, blocked less than 25% of binding [8, 10]. Quisqualate was the most potent displacer of AiQsGB, followed by glutamate, ibotenate and *trans*-ACPD. The latter two drugs had no effect on [^{3}H]AMPA binding. L-APB, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 6,7-dinitroquinoxaline-2,3-dione (DNQX), kainate, L-aspartate, glutamyl-diethyl ester (GDEE), γ-D-glutamylglycine (γ-DGG) and kynurenate were all ineffective displacers (IC_{50}s > 100 μM) of binding. The effective displacers of AiQsGB, quisqualate, glutamate, *trans*-ACPD and ibotenate, have all been shown to increase inositol phospholipid metabolism in tissue slices, neuronal cultures and/or synaptoneurosomes [6, 7,11–13, 16–19]. The present IC_{50} values for these compounds are lower than previously reported EC_{50} values for stimulation of inositol phospholipid metabolism. This difference may be due to a number of factors, including the temperature of the assay and the removal of inactivation systems for glutamate. In other neurotransmitter agonist binding studies, binding constants are frequently one to two orders of magnitude lower than the EC_{50} for stimulation of a physiological response, e.g. acetylcholine, GABA and dopamine. Thus, the pharmacology of this binding site was consistent with that of the metabotropic receptor and distinct from that of the AMPA-sensitive ionotropic receptor [2, 10, 14, 17, 18].

The regional distribution of AiQsGB is different from that of other EAA receptors

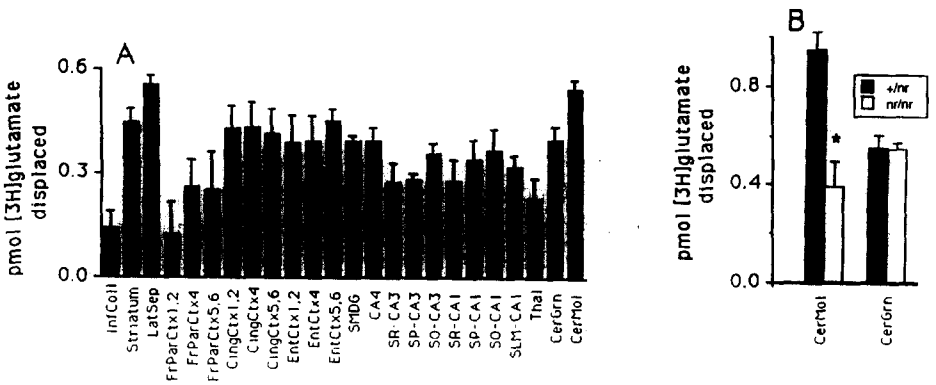

Fig. 1. Regional distribution of AMPA-insensitive, quisqualate-sensitive [^{3}H]glutamate binding in rat brain (A) and cerebellar levels of AiQsGB in nervous mutant mice (B). Sections were incubated in [^{3}H]glutamate (82 nM) in Tris-HCl plus 2.5 mM CaC_2, 100 mM thiocyanate, 10 μM AMPA and 100 μM NMDA. Adjacent sections were incubated as above with the addition of 2.5 μM quisqualate. Bars represent pmol of [^{3}H]glutamate displaced by 2.5 μM quisqualate. Binding in the cerebellar molecular layer of nervous mutant homozygotes (*nr/nr*) was significantly decreased compared to their heterozygous littermates (+/*nr*) ($^*P < 0.0006$), whereas AiQsGB levels were not significantly different in the granule cell layer. Abbreviations: InfColl, inferior colliculus; LatSep, lateral septum; FrParCtx, fronto-parietal cortex; CingCtx, cingulate cortex; EntCtx, entorhinal cortex; SMDG, stratum moleculare of dentate gyrus; SR-CA3, stratum radiatum of CA3; SP-CA3, stratum pyramidale of CA3; SO-CA3, stratum oriens of CA3; Thal, thalamus; CerGrn, cerebellar granule cell layer; CerMol, cerebellar molecular layer. Numbers after cortex labels refer to cortical layers.

[2, 10, 14] (Fig. 1A). Binding was highest in lateral septum and cerebellar molecular layer. Binding was also dense in striatum, cingulate and entorhinal cortices and lower in hippocampus. Thalamus, brainstem and frontal-parietal cortex had low binding densities.

In cerebellar Purkinje neurons, quisqualate produces longer lasting responses than other EAA's and these responses persist after diffusion of the quisqualate, suggesting the activation of a second messenger system [6]. In rat cerebellar slices, quisqualate and glutamate but not AMPA stimulate inositol phosphate turnover, whereas in cerebellar slices from mutant mice lacking Purkinje cells, glutamate stimulation of phosphoinositide turnover is abolished [1]. We examined the neuronal localization of AiQsGB in mouse cerebellum (Fig. 1B). In nervous mutant mice (which lack Purkinje cells), AiQsGB was markedly reduced in the cerebellar molecular layer, the area in which Purkinje cell dendrites are found, compared to binding in heterozygous littermate controls (Fig. 1B). [^{3}H]AMPA binding and total quisqualate-sensitive [^{3}H]glutamate binding also were very reduced in nervous mutants [9, 15]. Thus, both ionotropic and metabotropic quisqualate receptors appear to be concentrated on Purkinje cells. The ionotropic receptor is the likely mediator of fast synaptic transmission at the parallel fiber Purkinje cell synapses whereas the metabotropic receptor probably plays a role in the generation of the longer lasting quisqualate responses and long-term depression observed in cerebellum [6, 7]. The evidence from mutant mouse cerebella

strongly suggests that AiQsGB is localized on postsynaptic neuronal elements rather than on presynaptic or glial sites.

The present study suggests that AiQsGB represents L-[^{3}H]glutamate binding to the metabotropic EAA receptor. AiQsGB is distinct from [^{3}H]AMPA binding, is differentially distributed throughout the rat brain, and is likely localized to postsynaptic elements. The development of a binding assay for this receptor site should allow its evaluation in human hypoxic-ischemic and neurodegenerative brain pathology and facilitate the identification of more selective and potent agonists and antagonists.

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