Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 488 (2004) 101–109
www.elsevier.com/locate/ejphar

Analogues of homoibotenic acid show potent and selective activity following sensitisation by quisqualic acid

Henrik T. Vestergaard[a,*,1], Stine B. Vogensen[b,1], Ulf Madsen[b], Bjarke Ebert[c]

[a] Department of Pharmacology, The Danish University of Pharmaceutical Sciences, 2 Universitetsparken, DK-2100 Copenhagen Ø, Denmark
[b] Department of Medicinal Chemistry, The Danish University of Pharmaceutical Sciences, 2 Universitetsparken, DK-2100 Copenhagen Ø, Denmark
[c] Department of Molecular Pharmacology, H. Lundbeck A/S, 9 Otilliavej, DK-2500 Valby, Denmark

Received 16 October 2003; received in revised form 4 February 2004; accepted 10 February 2004

Abstract

Quisqualic acid induces sensitisation of neurones to depolarisation by analogues of 2-amino-4-phosphonobutyric acid (AP4), phenylglycine, and homoibotenic acid (HIBO). Thus, after administration of quisqualate these analogues become active at concentrations at which they are otherwise inactive. The mechanisms behind quisqualate-induced sensitisation are poorly understood and have not previously been quantified properly. In this study, we have tested the activity of a number of 4-alkyl- and 4-aryl-substituted analogues of HIBO as regards quisqualate-sensitisation, and present a method for quantifying the sensitisation induced by quisqualate at cortical neurones. These analogues are generally more potent and selective than (*S*)-AP4 or its homologue (*S*)-AP5 following quisqualate-sensitisation. Furthermore, we found a statistically significant correlation between the ligands' ability to inhibit $CaCl_2$-dependent (*S*)-[^{3}H]glutamate uptake into rat cortical synaptosomes, and their potency following quisqualate-induced depolarisation. This demonstrates the involvement of a transport system in the mechanism underlying the quisqualate-effect.

Keywords: Quisqualic acid; Quisqualate-effect; Quisqualate-priming; $CaCl_2$-dependent [^{3}H]glutamate uptake; Cortical wedge preparation; 2-Amino-4-phosphonobutyric acid

1. Introduction

(*S*)-Glutamic acid (Fig. 1) is the major excitatory neurotransmitter in the central nervous system. Glutamate operates via activation of two distinct receptor classes: ionotropic glutamate iGlu receptors, which mediate fast neuronal transmission, and metabotropic glutamate mGlu receptors, which are G-protein coupled to second-messenger systems (Bräuner-Osborne et al., 2000). The ionotropic glutamate receptors are subdivided into *N*-methyl-D-aspartic acid (NMDA) receptors and non-NMDA receptors. The glutamate non-NMDA receptors are further divided, according to selective agonists, into 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) and kainic acid receptors. It is generally agreed that the glutamatergic system is vital to brain function and the glutamatergic system is a potential target for therapeutic intervention in a number of neurological disorders (Bleakman and Lodge, 1998; Bräuner-Osborne et al., 2000; Parsons et al., 1998).

Quisqualic acid is a naturally occurring compound activating glutamate non-NMDA receptors and glutamate mGlu receptors (Bräuner-Osborne et al., 2000). Furthermore, quisqualate has an unresolved effect on synaptic transmission. Hence, quisqualate is able to induce an enhanced sensitivity of neurones to depolarisation by analogues of 2-amino-4-phosphonobutyric acid (AP4) as first described by Robinson et al. (1986). The effect has subsequently been variously termed quisqualate-sensitisation, quisqualate-effect and quisqualate-priming. Several hypotheses regarding the mechanism of the quisqualate-effect have been proposed in the literature (Charpak et al., 1992; Harris et al., 1987; Price et al., 1994; Schulte et al., 1993). A central question is whether a receptor or an uptake site is the primary site of action. If the site of action is a receptor, quisqualate either directly or indirectly sensitises a receptor to subsequent activation by AP4.

* Corresponding author. Tel.: +45-35-30-65-19; fax: +45-35-30-60-20.
E-mail address: htv@dfuni.dk (H.T. Vestergaard).
[1] These authors have contributed equally to this work.

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.02.024

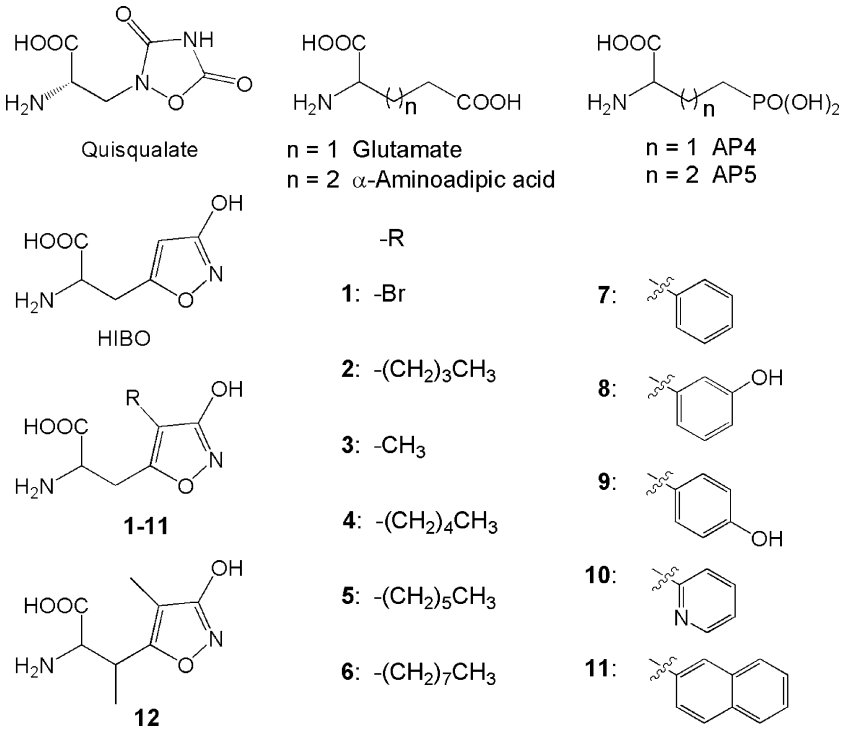

Fig. 1. Structures of quisqualate, α-aminoadipic acid, glutamate, AP4, AP5 and the HIBO analogues (**1**–**12**).

The involvement of glutamate mGlu receptors has been proposed (Whittemore and Cotman, 1991). However, substantial evidence supports a hypothesis based on a heteroexchange mechanism (Chase et al., 2001; Harris et al., 1987; Harris, 1989; Madsen et al., 1993; Roon and Koerner, 1996; Sheardown and Thomsen, 1996). Quisqualate is transported into a subset of interneurones during sensitising exposure of brain slices to this ligand (Chase et al., 2001; Price et al., 1994; Schulte et al., 1993). A subsequent exposure to extracellular AP4 causes a release of quisqualate into the synaptic cleft in exchange for AP4. The released quisqualate activates postsynaptic glutamate non-NMDA receptors, producing what appears to be an enhanced response to AP4. Depending on the specific interaction with this transport system, any compound with affinity for this heteroexchange mechanism could either induce sensitisation (like quisqualate), inhibit/prevent the quisqualate-effect, or produce release by heteroexchange (like AP4). Only a few compounds are known to induce a "quisqualate-like" sensitisation; quisqualate to date being the most potent of them (Madsen et al., 1993; Saitoh et al., 1998; Venkatraman et al., 1994). A group of compounds named "pre-blockers" or "reversers" are known to prevent the induction of the quisqualate-effect or reverse it (Charpak et al., 1992; Schulte et al., 1993; Whittemore and Koerner, 1991). The most studied compound in this group is α-aminoadipic acid. This amino acid is not only able to pre-block and reverse the quisqualate-effect (Harris, 1989; Roon and Koerner, 1996; Schulte et al., 1993; Whittemore and Koerner, 1989, 1991), but its depolarising effect is also potentiated upon pre-exposure of brain slices to quisqualate (Saitoh et al., 1998; Turner, 1993). The group of compounds for which quisqualate induces enhanced sensitivity comprise analogues of AP4 and phenylglycine (Johansen et al., 1998; Madsen et al., 1993; Roon and Koerner, 1996; Saitoh et al., 1998; Schulte et al., 1992, 1994; Sheardown and Thomsen, 1996; Subasinghe et al., 1992; Turner, 1993). Investigation of the quisqualate-effect has partly been hindered by the non-selective activity of these analogues, as they show additional glutamatergic effects at primarily glutamate mGlu receptors. The syntheses of several analogues of homoibotenic acid (HIBO, Fig. 1) have previously been published by our group (Bischoff et al., 1995; Christensen et al., 1992; Hansen et al., 1989; Johansen et al., 1998; Kromann et al., 2002; Madsen et al., 2001). In this context, we have reported that quisqualate is able to sensitise neurones to Br-HIBO (**1**, Fig. 1) (Madsen et al., 1993) and butyl-HIBO (**2**, Fig. 1) (Johansen et al., 1998). We now extend these two studies to include a number of 4-alkyl- and 4-aryl-substituted analogues of HIBO (**3**–**12**, Fig. 1). Furthermore, we report a method for quantifying the quisqualate-effect.

2. Materials and methods

2.1. Compounds

Analogues of HIBO were synthesised in our laboratory according to previously published methods (Bischoff et al.,

1995; Christensen et al., 1992; Hansen et al., 1989; Johansen et al., 1998; Kromann et al., 2002; Madsen et al., 2001). α-Aminoadipic acid, AP4, 2-amino-5-phosphonopentanoic acid (AP5), 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid, kainic acid, NMDA and quisqualic acid were purchased from Tocris Cookson (Bristol, UK). (*S*)-[^{3}H]Glutamate was purchased from Amersham Pharmacia Biotech UK (Buckinghamshire, UK). Stock solutions were either prepared in Tris-buffer (uptake assay) or Krebs buffer (cortical wedge preparation) depending on the respective assay and usually in a concentration of 1 mM. Compounds with low solubilities were sonicated until dissolution, but for no longer than 1 hour (h). Due to low solubility, compound **6** was initially dissolved in 100% dimethyl sulfoxide (DMSO) before dilution in buffer to a final concentration of DMSO $<0.5\%$.

2.2. $CaCl_2$-dependent (S)-[^{3}H]glutamate uptake assay

A modified version of the $CaCl_2$-dependent (*S*)-[^{3}H]glutamate uptake assay described by Honoré et al. (1986) was used to determine the transport of glutamate into cell membrane vesicles. Frozen whole rat cerebral cortex from male Sprague–Dawley rats (150–300 g) was thawed and homogenised by an Ultra-Turrax homogeniser for 5–10 s in 40 ml Tris-buffer (in mM): Tris–HCl 30, $CaCl_2$ 2.5; pH = 7.4 (adjusted with NaOH). The homogeniser was rinsed with 10 ml of Tris-buffer and the combined suspension was centrifuged for 10 min at 13,000 rpm. The pellet was washed twice with 2×10 ml Tris-buffer followed by centrifugations at 13,000 rpm for 10 min. After homogenisation of the resulting pellet in 10 ml Tris-buffer, the suspension was incubated at 37 °C for 30 min followed by centrifugation at 25 °C for 10 min at 13,000 rpm. The pellet was washed once in 10 ml Tris-buffer, and centrifuged at 13,000 rpm for 10 min. The final pellet was resuspended in 10 ml cold Tris-buffer (5 °C) and tissue concentration was adjusted to 0.75 mg original tissue per ml in Tris-buffer (5 °C). The cold membrane preparation was heated (37 °C) for 6 min prior to use.

A 100 μl (*S*)-[^{3}H]glutamate was mixed with 100 μl test compound, 100 μl Tris-buffer (total uptake) or 100 μl 1.2 mM (*S*)-glutamate (non-specific uptake). Test compounds were determined in triplicate, generally of 13 different concentrations distributed equally among 4–6 decades on a logarithm axis. The samples were thoroughly mixed and incubated at 37 °C for 20 min. Samples were immediately filtered through polyethylamine-coated glass microfibre filters GF/B (Whatman® Paper, Gaithersburg, MD, USA) using a Cell Harvester M-48 (Brandel, MD, USA) and washed three times with 3 ml Tris-buffer (5 °C). Membrane bound tritium was determined by conventional liquid scintillation spectrometry using a Liquid Scintillation Analyser 2000CA (Packard Instrument, Groningen, The Netherlands).

2.3. Rat cortical wedge preparation

The rat cortical wedge preparation was used according to previously published methods (Harrison and Simmonds, 1985) with slight modifications (Ebert et al., 2002). Briefly: An adult male Sprague–Dawley rat (150–300 g) was decapitated and the brain rapidly removed. Wedges (500 μm thick) of rat brain containing cerebral cortex and corpus callosum were placed with the corpus callosum part between two layers of nappy liner and constantly superfused with Mg^{2+}- and Ca^{2+}-free oxygenated Krebs buffer (in mM): NaCl 118, KCl 2.1, KH_2PO_4 1.2, D-glucose 11, $NaHCO_3$ 25. The cortex part was likewise placed between layers of absorbent fiber and superfused with Mg^{2+}-free oxygenated Krebs buffer (in mM): NaCl 118, KCl 2.1, KH_2PO_4 1.2, D-glucose 11, $NaHCO_3$ 25, $CaCl_2$ 2.5. The two parts were electrically insulated with a grease gap. Ag/AgCl electrodes (Dri-Ref™, World Precision Instruments, Sarasota, FL, USA) were placed in contact with the nappy liner on each side of the grease gap and the potential difference between electrodes recorded on a Yokogawa LR 4220E chart recorder (Yokogawa Electric, Tokyo, Japan). Test compounds were dissolved in Mg^{2+}-free oxygenated Krebs buffer and applied to the cortex part of the wedges. All compounds were applied for 90 s with a minimum interval of 15 min between each application. Before each experiment, 10 μM NMDA ($\sim EC_{50}$, (Ebert et al., 1996)) was applied until 3 subsequent applications gave identical amplitudes. Compounds were tested (6–7 points) over a concentration range of 1–1000 μM.

2.4. Data analyses

Inhibition of the $CaCl_2$-dependent (*S*)-[^{3}H]glutamate uptake was analysed using the non-linear curve fitting program GraFit 4.0 (Erithacus Software) and the logistic equation:

$$\%\text{Binding} = \frac{Total - NS}{1 + \left(\frac{[\text{Inhibitor}]}{IC_{50}}\right)^{n_H}} + NS$$

Total and *NS* are the total and non-specific uptake, respectively, and n_H is the Hill coefficient.

When experiments in the cortical wedge preparation exceed the duration of 4 h, responses generally diminish over time, and it was therefore necessary to correct for rundown. Glutamate NMDA receptors are not involved in the quisqualate-effect (Harris et al., 1987; Roon and Koerner, 1996; Sheardown and Thomsen, 1996) and NMDA was therefore used for correction of rundown. NMDA (10 μM) was applied every 0.5–1.5 h during the application protocol and used to measure loss of response. This loss could usually be described by a 3rd order polynomial function, but in a very few cases a 5th or 7th order polynomial function was used. 2-Amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (3.5 μM $\sim EC_{50}$, (Johansen et al., 1998)) was used instead

of NMDA for the compounds (*R*)- and (*S*)-AP5, since both these compounds are competitive glutamate NMDA receptor antagonists (Davies and Watkins, 1982). The data obtained were analysed using the non-linear curve fitting program GraFit 4.0 (Erithacus Software) and the logistic equation:

$$E = \frac{E_{max} \cdot [\text{Agonist}]^{n_H}}{EC_{50}^{n_H} + [\text{Agonist}]^{n_H}}$$

where E is the observed drug response at a specific agonist concentration, E_{max} is the maximal drug response, EC_{50} is the concentration, which produces the half maximum response, and n_H is the Hill coefficient. Non-linear curve fits were determined on individual slices. It was not always possible to obtain full concentration–response curves (concentrations>1000 μM) due to low solubility of some of the compounds. In these cases, the concentration–response curve was determined by visual approximation of a sigmoidal curve to the data points using GraFit. The visual approximation gave a Hill coefficient within the interval 1.2 to 1.8, and the maximal response (E_{max}) was estimated from the response amplitude of 80 μM NMDA (>EC_{95}). Since high concentrations of NMDA can cause loss of response due to neurotoxicity, no slice was used after application of 80 μM NMDA.

2.5. Statistical analyses

Data were analysed using SigmaStat (version 2.03, SPSS). EC_{50}- and IC_{50}-values were assumed to follow a log Gaussian distribution and are presented as mean [pMean ± S.E.M.]. One-way analysis of variance (ANOVA) was used for comparisons. Significance was assumed at $P \leq 0.05$. Correlation was evaluated by Pearson product moment correlation analysis.

3. Results

3.1. Inhibition of $CaCl_2$-dependent (S)-[^{3}H]glutamate uptake

The $CaCl_2$-dependent uptake assay is believed to cover a heterogeneous population of uptake systems and is associated with a high non-specific uptake; reported as high as 70% (Butcher et al., 1983; Fagg and Lanthorn, 1985; Monaghan et al., 1983; Sheardown and Thomsen, 1996). The levels of non-specific uptake were in our case 5–45% of the total bound (*S*)-[^{3}H]glutamate. The absence of $CaCl_2$ in the Tris-buffer during the washing procedure did not significantly affect the levels of non-specific ($P_{NS} = 0.178$, $n = 24$) or total uptake ($P_{Total} = 0.401$, $n = 24$) indicating that the presence of $CaCl_2$ during the washing procedure did not reverse the transport of (*S*)-[^{3}H]glutamate.

Neither NMDA (100 μM), 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (100 μM), kainic acid (100 μM) nor a mixture of these (100 μM of each) showed any affinity for the $CaCl_2$-dependent uptake site ($n = 3$, data not shown). Neither the addition of, NMDA (100 μM, $n = 12$), nor 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (100 μM, $n = 13$) nor kainic acid (100 μM, $n = 13$) affected the inhibition of (*S*)-[^{3}H]glutamate uptake by (*RS*)-**2** within the tested concentration range (0.001–100 μM; data not shown).

In agreement with previously published results (Butcher et al., 1983; Fagg et al., 1983a; Honoré et al., 1986; Monaghan et al., 1983; Robinson et al., 1985) α-aminoadipic acid, (*S*)-glutamate and quisqualate were potent inhibitors. The majority of the HIBO analogues had similar affinities (Table 1); the exceptions were compounds (*RS*)-**6**, (*RS*)-**10**, and (*RS*)-**12**. The affinity of (*RS*)-**10** and (*S*)-AP4 were of the same magnitude, whereas compounds (*RS*)-**6** and (*RS*)-**12** were poor inhibitors of (*S*)-[^{3}H]glutamate in the presence of $CaCl_2$. In agreement with previously published results (Butcher et al., 1983; Honoré et al., 1986; Monaghan et al., 1983; Robinson et al., 1985) the *S*-enantiomers of α-aminoadipic acid, AP4, AP5 and glutamate were more potent than the respective *R*-enantiomers. Surprisingly, the analogues of HIBO (compounds **2** and **5**) did not show this stereochemical preference, since the *S*- and *R*-enantiomers inhibited (*S*)-[^{3}H]glutamate to the same extent.

3.2. Quantification of the quisqualate-effect in the cortical wedge preparation

Agonist-induced depolarisation of native cortical neurones was measured in the rat cortical wedge preparation. A method quantifying the sensitisation induced by quisqualate was developed in order to compare the induced potencies of the investigated analogues of HIBO. Compounds (*RS*)-**5** and (*RS*)-**11** were used to evaluate the application protocol, since they did not by themselves produce responses in the cortical wedge preparation (< 1000 μM). The quisqualate-effect is known to persist for several hours after a single application of quisqualate (Robinson et al., 1986). Repeated application of 30 or 100 μM (*RS*)-**5** gave diminishing responses after pre-exposure of the slices to a single application of quisqualate (100 μM). Over 6 applications the quisqualate-induced response to 30 and 100 μM (*RS*)-**5** faded to 48% [30;66% (95% confidence interval)] ($n = 6$) and 54% [19;88%] ($n = 6$), respectively, relative to the initial response, although the quisqualate-effect persisted over a time period of >4 h. If the slices were pre-exposed to quisqualate (100 μM) once again, the quisqualate-effect was partially restored for both concentrations of (*RS*)-**5** (84% [77;100%], $n = 5$ and 75% [55;95%], $n = 6$, respectively). The maximal effect of (*S*)-AP4 can be obtained by pre-exposure of slices to a concentration of 30 μM quisqualate (Sheardown and Thomsen, 1996). It was possible to reproduce the response during repeated applications of 500 μM (*RS*)-**11**, if quis-

Table 1
Inhibition of (*S*)-[^{3}H]glutamate transport into cell membrane vesicles (IC_{50}), the concentration giving half maximal response and 20% of maximal response after pre-exposure of brain slices to quisqualate (QC_{50} and QC_{20}, respectively), and the concentration giving half maximal response (EC_{50}) without prior exposure to quisqualate

Compound	IC_{50} (μM)[a]	[pIC_{50} ± S.E.M.]	QC_{50} (μM)[b]	[pQC_{50} ± S.E.M.]	QC_{20} (μM)[b,c]	[pQC_{20} ± S.E.M.]	EC_{50} (μM)
(*S*)-Glutamate	0.23	[6.66 ± 0.08]	>1000				>1000
(*R*)-Glutamate	6.2	[5.22 ± 0.07]	>1000				
(*RS*)-Glutamate	0.35	[6.47 ± 0.09]					
Quisqualate	0.27	[6.62 ± 0.12]					7.3[d]
(*S*)-AP4	1.3	[5.89 ± 0.06]	340	[3.48 ± 0.05]	168	[3.78 ± 0.03]	>1000
(*R*)-AP4	24	[4.72 ± 0.20]	>1000				>1000
(*S*)-AP5	8.8	[5.07 ± 0.08]	>1000		800	[3.11 ± 0.08]	>1000
(*R*)-AP5	20	[4.83 ± 0.22]	>1000				>1000
(*RS*)-AP5	5.3	[5.29 ± 0.07]					
(*S*)-α-AA	0.29	[6.54 ± 0.02]					
(*R*)-α-AA	0.87	[6.08 ± 0.08]					
(*RS*)-α-AA	0.35	[6.50 ± 0.14]					
(*R*)-**1**	0.40	[6.44 ± 0.14]	63	[4.21 ± 0.04]	26	[4.63 ± 0.04]	>1000[e]
(*RS*)-**1**	0.24	[6.17 ± 0.05]	95	[4.04 ± 0.07]			374[e]
(*S*)-**2**	0.37	[6.43 ± 0.12]	35	[4.47 ± 0.05]			21[d]
(*R*)-**2**	0.22	[6.71 ± 0.13]	70	[4.16 ± 0.05]	18	[4.81 ± 0.04]	>1000[d]
(*RS*)-**2**	0.36	[6.48 ± 0.12]	46	[4.36 ± 0.09]			23[d]
(*R*)-**3**	0.37	[6.43 ± 0.12]	61	[4.23 ± 0.07]	22	[4.59 ± 0.05]	>1000[d]
(*RS*)-**3**	0.30	[6.54 ± 0.05]	50	[4.31 ± 0.06]			37[d]
(*RS*)-**4**	0.17	[6.77 ± 0.06]	68	[4.17 ± 0.04]	22	[4.66 ± 0.02]	630[f]
(*S*)-**5**	0.18	[6.77 ± 0.12]	53	[4.31 ± 0.12]	20	[4.69 ± 0.07]	>1000[f]
(*R*)-**5**	0.13	[6.90 ± 0.06]	29	[4.54 ± 0.06]	14	[4.87 ± 0.06]	>1000[f]
(*RS*)-**5**	0.20	[6.78 ± 0.21]	38	[4.46 ± 0.14]	18	[4.81 ± 0.11]	>1000[f]
(*RS*)-**6**	24	[4.63 ± 0.04]	>1000				>500[f,g]
(*RS*)-**7**	0.34	[6.52 ± 0.10]	69	[4.20 ± 0.12]	23	[4.55 ± 0.02]	>1000[f]
(*RS*)-**8**	0.39	[6.41 ± 0.03]	64	[4.22 ± 0.10]	26	[4.54 ± 0.01]	990[h]
(*RS*)-**9**	0.41	[6.43 ± 0.08]	84	[4.09 ± 0.06]	33	[4.46 ± 0.06]	~ 1000 (IC_{50})[h,i]
(*RS*)-**10**	3.4	[5.50 ± 0.12]	63	[4.22 ± 0.08]			23[g,h]
(*RS*)-**11**	0.30	[6.57 ± 0.13]	60	[4.22 ± 0.01]	20	[4.70 ± 0.01]	715 (IC_{50})[h,i]
(*RS*)-**12**	13	[4.90 ± 0.07]	>1000		550	[3.26 ± 0.03]	

α-AA: α-aminoadipic acid.
[a] Data from 3–6 membrane preparations.
[b] Data from 3–5 slices.
[c] QC_{20}-values (the concentrations which produce 20% of maximal response after exposure to quisqualate) were calculated in the cases where this concentration of compound by itself (i.e. without prior exposure to quisqualate) evoked a response <10% of the response evoked after pre-exposure to quisqualate.
[d] Johansen et al. (1998).
[e] Bischoff et al. (1995).
[f] Madsen et al. (2001).
[g] Estimated value due to low solubility of the compound.
[h] Kromann et al. (2002).
[i] NMDA receptor antagonists.

qualate (30 μM) was applied before each application of (*RS*)-**11** ($P=0.964$, $n=4$; data not shown). Hence, to assure reproducible responses, the concentration–response relationships of the test compounds were determined by pre-exposure of the slices to a single application of quisqualate (30 μM) before each application of test compound (Fig. 2). The concentration of the half maximal response of the test compounds after pre-exposure of the slices to quisqualate is termed QC_{50} in order to distinguish the value from the traditional EC_{50}-value of the test compounds. The QC_{50}-values obtained are listed in Table 1. The application protocol is illustrated by a representative electrophysiological recording from the rat cortical wedge preparation (Fig. 2), and the concentration–response relationships of a few compounds are shown in Fig. 3. According to the concentration–response curves in Fig. 3 and the QC_{50}-values in Table 1, HIBO analogues are sensitised by quisqualate to a greater extent than (*S*)-AP4 and (*S*)-AP5; the only exception being compounds (*RS*)-**6** and (*RS*)-**12** (Table 1).

3.3. Correlation between (S)-[^{3}H]glutamate uptake inhibition and the quisqualate-effect

A number of the compounds tested produce responses in the cortical wedge preparation without prior exposure to quisqualate, due to their inherent glutamate AMPA receptor agonist activity. It is of course necessary to differentiate

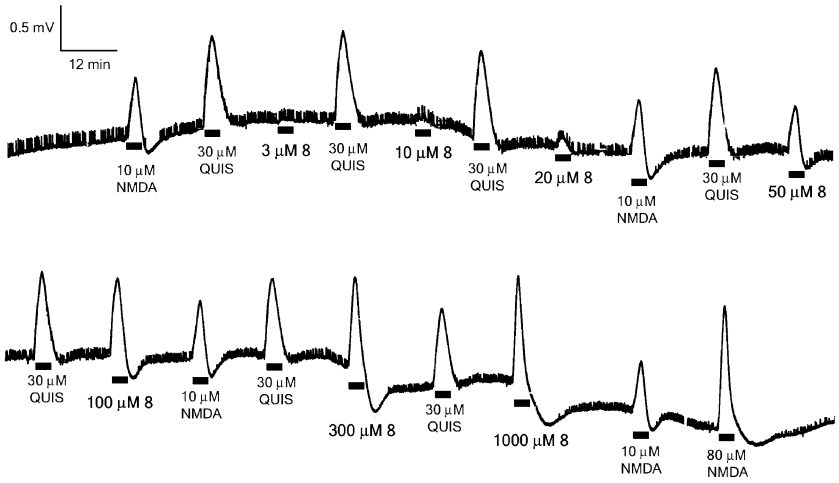

Fig. 2. An example of an electrophysiological recording (approximately 5.5 h) from the rat cortical wedge preparation, displaying the application protocol for (*RS*)-**8**. Note that only the concentration of (*RS*)-**8** is varied during the application protocol. Quisqualate (30 μM) is pre-applied before application of (*RS*)-**8** at each concentration. NMDA (10 μM) is used as a measurement of loss of response due to the prolonged application protocol. The baseline noise is due to spontaneous neuronal excitatory activity.

between the response evoked by the test compounds themselves and the sensitised responses induced by quisqualate, in order to make a quantitative comparison of the potentiations induced by quisqualate. It was not possible to use the QC_{50} value, since most test compounds evoked a response at this concentration without prior exposure to quisqualate. Therefore, we decided to use the concentration that produced 20% of maximal response after exposure to quisqualate (QC_{20}) but only in those cases where this concentration of compound by itself (i.e., without prior exposure to quisqualate) evoked a response of $<10\%$ of the response evoked after pre-exposure to quisqualate (Table 1). This resulted in exclusion of compounds (*RS*)-**1**, (*S*)-**2**, (*RS*)-**2**, (*RS*)-**3**, and (*RS*)-**10** from the correlation. For the remaining compounds, we found a correlation ($r=0.980$, $P<0.001$, $n=14$) between the IC_{50}-values determined in the $CaCl_2$-dependent (*S*)-[^{3}H]glutamate uptake assay, and the QC_{20}-values determined in the cortical wedge preparation (Fig. 4). All data points are within the 95% confidence interval for the population.

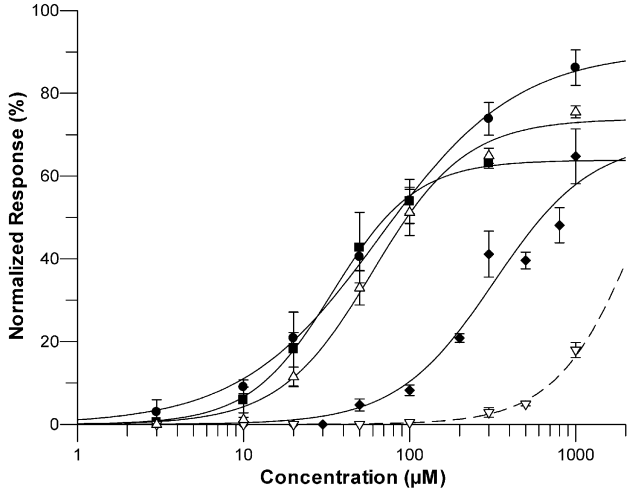

Fig. 3. The normalised average concentration–response relationships of the quisqualate-induced response to (*R*)-**2** (●), (*RS*)-**5** (■), (*RS*)-**8** (△), (*S*)-AP4 (◆) and (*S*)-AP5 (▽). The responses were normalised to the maximal response of NMDA (80 μM). Continuous lines are non-linear curve fits to the average data points. The dashed line is a sigmoidal curve approximated visually to the average data points. In both cases, the equation described in Materials and Methods was used. Note that non-linear curve fits were determined on individual slices (Table 1) instead of on the average data shown in this figure. Data from 3–5 slices.

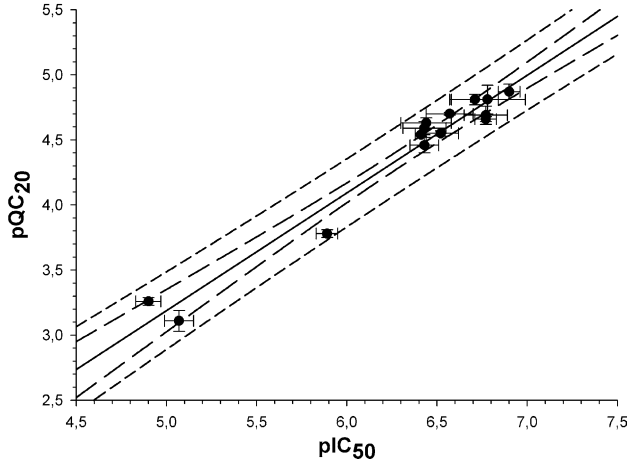

Fig. 4. The correlation between affinity for $CaCl_2$-dependent (*S*)-[^{3}H]glutamate uptake sites and functional potency determined in the cortical wedge preparation. Affinities are displayed as pIC_{50} with horizontal error bars displaying ± S.E.M. Functional potencies are displayed as pQC_{20} with vertical error bars displaying ± S.E.M. The solid line displays the correlation. The long and short dashed lines show the respective 95% confidence intervals for the regression and the population.

4. Discussion

There has been some debate in the literature as to whether the $CaCl_2$-dependent glutamate uptake site is involved in the quisqualate-effect (Harris et al., 1987; Schulte et al., 1992, 1993; Sheardown and Thomsen, 1996; Whittemore and Koerner, 1989, 1991). Substantial evidence seems to indicate that the $CaCl_2$-dependent uptake site does not correspond to an excitatory amino acid receptor but rather represents transport into synaptic vesicles (Bridges et al., 1986; Fagg and Lanthorn, 1985; Pin et al., 1984; Recasens et al., 1987; Zaczek et al., 1987). Our data seem to support this theory. NMDA, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid and kainic acid did not cause displacement of (*S*)-[^{3}H]glutamate, indicating that binding to the ionotropic glutamate receptors was not measured in this assay.

Evidence supports the involvement of a transport system in the quisqualate-effect (Chase et al., 2001; Harris et al., 1987; Harris, 1989; Madsen et al., 1993; Roon and Koerner, 1996; Sheardown and Thomsen, 1996). Recently, Chase et al. (2001) have proposed that a transporter with the properties of the cystine/glutamate exchange carrier, System x_c^-, is responsible for inducing of the quisqualate-effect. The involvement of an (*S*)-AP4-sensitive glutamate transporter was ruled out, because (*S*)-AP4 is a poor inhibitor of the quisqualate-effect. We report here a number of compounds with higher affinity for this transporter compared to (*S*)-AP4. Thus, these analogues of HIBO have made it possible to induce more specific inhibition of the transporter involved.

A heterogeneous population of uptake sites appear to be involved in $CaCl_2$-dependent glutamate uptake (Bridges et al., 1986). Previously as many as 3 distinct populations of sites have been proposed (Fagg et al., 1983b; Nadler et al., 1985; Werling and Nadler, 1982). We propose that the $CaCl_2$-dependent (*S*)-[^{3}H]glutamate uptake assay is an unspecific measurement of glutamate uptake comprising several different glutamatergic transport systems, possibly including System x_c^-, rather than a specific measurement of glutamate uptake by a single transporter, i.e., an AP4-sensitive transporter. All compounds with affinity for $CaCl_2$-dependent uptake sites seem to be involved in the quisqualate-effect, thus indicating a connection between the two. The only exception is glutamate, which has a very low excitatory effect in the cortical wedge preparation due to the high capacity of multiple glutamate transport systems (Harrison and Simmonds, 1985). Moreover, Sheardown and Thomsen (1996) found a correlation between the potencies of a series of phenylglycine analogues following pre-exposure to quisqualate, and their affinity for the $CaCl_2$-dependent (*RS*)-[^{3}H]AP4 transporter. We report here a correlation between affinity for $CaCl_2$-dependent (*S*)-[^{3}H]glutamate uptake sites, and the quisqualate-effect, for the series of HIBO analogues tested.

The involvement of glutamate mGlu receptors in the quisqualate-effect seems unlikely. Even though several of the compounds tested have shown affinity for glutamate mGlu receptors, there is no consistency between a ligand's subtype selectivity and its propensity for the quisqualate-sensitisation (Bräuner-Osborne et al., 1998; Bräuner-Osborne and Krogsgaard-Larsen, 1998; Thomsen et al., 1994). Furthermore, several selective glutamate mGlu receptor agonists and antagonists are not potentiated by quisqualate (Chase et al., 2001; Johansen and Robinson, 1995; Littman et al., 1995).

The method described here for quantifying the quisqualate-effect may prove useful in future studies. Furthermore, the potency and selectivity of some HIBO analogues holds promise for the future elucidation of the mechanisms underlying the quisqualate-effect and may help to further clarify the physiological and pharmacological significance of this effect. Our results support the hypothesis that a transport system is integral to the quisqualate-effect.

Acknowledgements

We gratefully acknowledged the technical assistance of Christian Thorkildsen and Gunilla Steven and Jeremy R. Greenwood for assistance with manuscript preparation.

References

Bischoff, F., Johansen, T.N., Ebert, B., Krogsgaard-Larsen, P., Madsen, U., 1995. Excitatory amino acid receptor ligands: asymmetric synthesis, absolute stereochemistry and pharmacology of (*R*)- and (*S*)-homoibotenic acid. Bioorg. Med. Chem. 3, 553–558.

Bleakman, D., Lodge, D., 1998. Neuropharmacology of AMPA and kainate receptors. Neuropharmacology 37, 1187–1204.

Bräuner-Osborne, H., Krogsgaard-Larsen, P., 1998. Pharmacology of (*S*)-homoquisqualic acid and (*S*)-2-amino-5-phosphonopentanoic acid [(*S*)-AP5] at cloned metabotropic glutamate receptors. Br. J. Pharmacol. 123, 269–274.

Bräuner-Osborne, H., Nielsen, B., Krogsgaard-Larsen, P., 1998. Molecular pharmacology of homologues of ibotenic acid at cloned metabotropic glutamic acid receptors. Eur. J. Pharmacol. 350, 311–316.

Bräuner-Osborne, H., Egebjerg, J., Nielsen, E.Ø., Madsen, U., Krogsgaard-Larsen, P., 2000. Ligands for glutamate receptors: design and therapeutic prospects. J. Med. Chem. 43, 2609–2645.

Bridges, R.J., Hearn, T.J., Monaghan, D.T., Cotman, C.W., 1986. A comparison of 2-amino-4-phosphonobutyric acid (AP4) receptors and [^{3}H]AP4 binding sites in the rat brain. Brain Res. 375, 204–209.

Butcher, S.P., Collins, J.F., Roberts, P.J., 1983. Characterization of the binding of DL-[^{3}H]-2-amino-4-phosphonobutyrate to L-glutamate-sensitive sites on rat brain synaptic membranes. Br. J. Pharmacol. 80, 355–364.

Charpak, S., Thomsen, S.M., Gähwiler, B.H., Gerber, U., 1992. Characterization of L-2-amino-4-phosphonobutanoate action following sensitization by quisqualate in rat hippocampal slice cultures. Eur. J. Neurosci. 4, 491–499.

Chase, L.A., Roon, R.J., Wellman, L., Beitz, A.J., Koerner, J.F., 2001. L-quisqualic acid transport into hippocampal neurons by a cystine-sensitive carrier is required for the induction of quisqualate sensitization. Neuroscience 106, 287–301.

Christensen, I.T., Ebert, B., Madsen, U., Nielsen, B., Brehm, L., Krogsgaard-Larsen, P., 1992. Excitatory amino-acid receptor ligands. Synthesis and biological activity of 3-isoxazolol amino-acids structurally related to homoibotenic acid. J. Med. Chem. 35, 3512–3519.

Davies, J., Watkins, J.C., 1982. Actions of D-forms and L-forms of 2-amino-5-phosphonovalerate and 2-amino-4-phosphonobutyrate in the cat spinal-cord. Brain Res. 235, 378–386.

Ebert, B., Madsen, U., Søby, K.K., Krogsgaard-Larsen, P., 1996. Functional partial agonism at ionotropic excitatory amino acid receptors. Neurochem. Int. 29, 309–316.

Ebert, B., Storustovu, I., Mortensen, M., Frølund, B., 2002. Characterization of GABAA receptor ligands in the rat cortical wedge preparation: evidence for action at extrasynaptic receptors? Br. J. Pharmacol. 137, 1–8.

Fagg, G.E., Lanthorn, T.H., 1985. Cl^-/Ca^{2+}-dependent L-glutamate binding sites do not correspond to 2-amino-4-phosphonobutanoate-sensitive excitatory amino acid receptors. Br. J. Pharmacol. 86, 743–751.

Fagg, G.E., Foster, A.C., Mena, E.E., Cotman, C.W., 1983a. Chloride and calcium ions separate L-glutamate receptor populations in synaptic membranes. Eur. J. Pharmacol. 88, 105–110.

Fagg, G.E., Mena, E.E., Monaghan, D.T., Cotman, C.W., 1983b. Freezing eliminates a specific population of L-glutamate receptors in synaptic membranes. Neurosci. Lett. 38, 157–162.

Hansen, J.J., Nielsen, B., Krogsgaard-Larsen, P., Brehm, L., Nielsen, E.Ø., Curtis, D.R., 1989. Excitatory amino acid agonists. Enzymic resolution, X-ray structure, and enantioselective activities of (*R*)- and (*S*)-bromohomoibotenic acid. J. Med. Chem. 32, 2254–2260.

Harris, E.W., 1989. L-α-Aminodipate antagonizes the priming effect of quisqualate in hippocampal slices. Eur. J. Pharmacol. 161, 107–109.

Harris, E.W., Stevens, D.R., Cotman, C.W., 1987. Hippocampal cells primed with quisqualate are depolarized by AP4 and AP6, ligands for a putative glutamate uptake site. Brain Res. 418, 361–365.

Harrison, N.L., Simmonds, M.A., 1985. Quantitative studies on some antagonists of *N*-methyl-D-aspartate in slices of rat cerebral cortex. Br. J. Pharmacol. 84, 381–391.

Honoré, T., Drejer, J., Nielsen, M., Braestrup, C., 1986. Differentiation of Cl^-/Ca^{2+}-dependent and sodium dependent ^{3}H-glutamate binding to cortical membranes from rat brain by high energy radiation inactivation analysis. J. Neural. Transm. 65, 93–101.

Johansen, P.A., Robinson, M.B., 1995. Identification of 2-amino-2-methyl-4-phosphonobutanoic acid as an antagonist at the mGlu4a receptor. Eur. J. Pharmacol. 290, R1–R3.

Johansen, T.N., Ebert, B., Bräuner-Osborne, H., Didriksen, M., Christensen, I.T., Søby, K.K., Madsen, U., Krogsgaard-Larsen, P., Brehm, L., 1998. Excitatory amino acid receptor ligands: resolution, absolute stereochemistry, and enantiopharmacology of 2-amino-3-(4-butyl-3-hydroxyisoxazol-5-yl)propionic acid. J. Med. Chem. 41, 930–939.

Kromann, H., Sløk, F.A., Stensbøl, T.B., Bräuner-Osborne, H., Madsen, U., Krogsgaard-Larsen, P., 2002. Selective antagonists at group I metabotropic glutamate receptors: synthesis and molecular pharmacology of 4-aryl-3-isoxazolol amino acids. J. Med. Chem. 45, 988–991.

Littman, L., Chase, L.A., Renzi, M., Garlin, A.B., Koerner, J.F., Johnson, R.L., Robinson, M.B., 1995. Effects of quisqualic acid analogs on metabotropic glutamate receptors coupled to phosphoinositide hydrolysis in rat hippocampus. Neuropharmacology 34, 829–841.

Madsen, U., Ebert, B., Hansen, J.J., Krogsgaard-Larsen, P., 1993. The non-depolarizing D-form of bromohomoibotenic acid enhances depolarizations evoked by the L-form or quisqualate. Eur. J. Pharmacol. 230, 383–386.

Madsen, U., Bräuner-Osborne, H., Frydenvang, K., Hvene, L., Johansen, T.N., Nielsen, B., Sanchez, C., Stensbøl, T.B., Bischoff, F., Krogsgaard-Larsen, P., 2001. Synthesis and pharmacology of 3-isoxazolol amino acids as selective antagonists at group I metabotropic glutamic acid receptors. J. Med. Chem. 44, 1051–1059.

Monaghan, D.T., McMills, M.C., Chamberlin, A.R., Cotman, C.W., 1983. Synthesis of [^{3}H]2-amino-4-phosphonobutyric acid and characterization of its binding to rat brain membranes: a selective ligand for the chloride/calcium-dependent class of L-glutamate binding sites. Brain Res. 278, 137–144.

Nadler, J.V., Wang, A., Werling, L.L., 1985. Binding sites for L-[^{3}H]glutamate on hippocampal synaptic membranes: three populations differentially affected by chloride and calcium ions. J. Neurochem. 44, 1791–1798.

Parsons, C.G., Danysz, W., Quack, G., 1998. Glutamate in CNS disorders as a target for drug development: an update. Drug News Perspect. 11, 523–569.

Pin, J.P., Bockaert, J., Recasens, M., 1984. The Ca^{2+}/Cl^- dependent L-[^{3}H]glutamate binding: a new receptor or a particular transport process? FEBS Lett. 175, 31–36.

Price Jr., R.H., Schulte, M.K., Renno, W.M., Koerner, J.F., Beitz, A.J., 1994. Immunocytochemical evidence that quisqualate is selectively internalized into a subset of hippocampal neurons. Brain Res. 663, 317–325.

Recasens, M., Pin, J.P., Bockaert, J., 1987. Chloride transport blockers inhibit the chloride-dependent glutamate binding to rat brain membranes. Neurosci. Lett. 74, 211–216.

Robinson, M.B., Crooks, S.L., Johnson, R.L., Koerner, J.F., 1985. Displacement of DL-[^{3}H]-2-amino-4-phosphonobutanoic acid ([^{3}H]APB) binding with methyl-substituted APB analogues and glutamate agonists. Biochemistry 24, 2401–2405.

Robinson, M.B., Whittemore, E.R., Marks, R.L., Koerner, J.F., 1986. Exposure of hippocampal slices to quisqualate sensitizes synaptic responses to phosphonate-containing analogues of glutamate. Brain Res. 381, 187–190.

Roon, R.J., Koerner, J.F., 1996. Persistent depression of synaptic responses occurs in quisqualate sensitized hippocampal slices after exposure to L-aspartate-beta-hydroxamate. Brain Res. 734, 223–228.

Saitoh, T., Ishida, M., Shinozaki, H., 1998. Potentiation by DL-α-aminopimelate of the inhibitory action of a novel mGluR agonist (L-F(2)CCG-I) on monosynaptic excitation in the rat spinal cord. Br. J. Pharmacol. 123, 771–779.

Schulte, M.K., Whittemore, E.R., Koerner, J.F., Johnson, R.L., 1992. Structure–function relationships for analogues of L-2-amino-4-phosphonobutanoic acid on the quisqualic acid-sensitive AP4 receptor of the rat hippocampus. Brain Res. 582, 291–298.

Schulte, M.K., Roon, R.J., Koerner, J.F., 1993. Quisqualic acid-induced sensitization and the active uptake of L-quisqualic acid by hippocampal slices. Brain Res. 605, 85–92.

Schulte, M.K., Roon, R.J., Chalmers, D.J., Sunter, D.C., Koerner, J.F., 1994. Utilization of the resolved L-isomer of 2-amino-6-phosphonohexanoic acid (L-AP6) as a selective agonist for a quisqualate-sensitized site in hippocampal CA1 pyramidal neurons. Brain Res. 649, 203–207.

Sheardown, M.J., Thomsen, C., 1996. Phenylglycines can evoke quisqualate-primed depolarizations in rat cingulate cortex: an effect associated with [^{3}H]DL-AP4 uptake. Eur. J. Neurosci. 8, 2599–2604.

Subasinghe, N., Schulte, M., Roon, R.J., Koerner, J.F., Johnson, R.L., 1992. Quisqualic acid analogs-synthesis of β-heterocyclic 2-aminopropanoic acid-derivatives and their activity at a novel quisqualate-sensitized site. J. Med. Chem. 35, 4602–4607.

Thomsen, C., Boel, E., Suzdak, P.D., 1994. Actions of phenylglycine analogs at subtypes of the metabotropic glutamate receptor family. Eur. J. Pharmacol. 267, 77–84.

Turner, J.P., 1993. Anion transport blockers inhibit DL-2-amino-4-phosphonobutyrate responses induced by quisqualate in the rat cerebral cortex. Br. J. Pharmacol. 109, 449–458.

Venkatraman, S., Roon, R.J., Schulte, M.K., Koerner, J.F., Johnson, R.L., 1994. Synthesis of oxadiazolidinedione derivatives as quisqualic acid analogs and their evaluation at a quisqualate-sensitized aite in the rat hippocampus. J. Med. Chem. 37, 3939–3946.

Werling, L.L., Nadler, J.V., 1982. Complex binding of L-[^{3}H]glutamate to hippocampal synaptic membranes in the absence of sodium. J. Neurochem. 38, 1050–1062.

Whittemore, E.R., Cotman, C.W., 1991. Agonists selective for phosphoi-

nositide-coupled receptors sensitize neurons to depolarization by L-2-amino-4-phosphonobutanoic acid (L-AP4). Brain Res. 555, 215–219.
Whittemore, E.R., Koerner, J.F., 1989. Novel recognition site for L-quisqualate sensitizes neurons to depolarization by L-2-amino-4-phosphonobutanoate (L-AP4). Brain Res. 489, 146–156.
Whittemore, E.R., Koerner, J.F., 1991. Pre-exposure to L-homocysteinesulfinic acid blocks quisqualate-induced sensitization to L-2-amino-4-phosphonobutanoic acid. Eur. J. Pharmacol. 192, 435–438.
Zaczek, R., Balm, M., Arlis, S., Drucker, H., Coyle, J.T., 1987. Quisqualate-sensitive, chloride-dependent transport of glutamate into rat brain synaptosomes. J. Neurosci. Res. 18, 425–431.