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NON-NMDA GLUTAMATE RECEPTOR ANTAGONIST ^{3}H-CNQX BINDS WITH EQUAL AFFINITY TO TWO AGONIST STATES OF QUISQUALATE RECEPTORS

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Abstract—Binding of ^{3}H-CNQX to rat cortical membranes is saturable and reversible. Apparently, ^{3}H-CNQX binds to a single site with $K_D = 39$ nM. However, studies using AMPA as inhibitor revealed a biphasic inhibition of ^{3}H-CNQX binding. The results suggest that CNQX binds with the same affinity to two different sites. The molecular target size of ^{3}H-CNQX binding (51.8 ± 3.4 kD) is equivalent to the size of the high affinity ^{3}H-AMPA binding sites, but different from the high affinity ^{3}H-kainate binding sites. A monoexponential decay curve for the high energy radiation inactivation analysis of ^{3}H-CNQX binding indicates that the two ^{3}H-CNQX binding sites have the same molecular weight and therefore might be two different conformations of the same receptor. The standard excitatory amino acids quisqualate, AMPA and kainate have a different rank order of potency as binding inhibitors at the two conformations of the quisqualate receptor.

Excitatory amino acids are considered to have a major role as neurotransmitters in the mammalian central nervous system (for review see Ref. 1). From electrophysiological [2]—and binding [3]—studies, there appear to be at least three subtypes of glutamate receptors, tentatively named *N*-methyl-D-aspartate (NMDA)-, quisqualate- and kainate receptors.

Glutamate receptor subtypes sensitive to quisqualate and kainate as a group are often referred to as non-NMDA receptors, due to similarities in the response after stimulation of either receptor and similarities in the sensitivities to antagonists [4]. On the other hand, receptor studies using binding techniques and the agonist radioligands ^{3}H-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (^{3}H-AMPA) (for quisqualate receptors) and ^{3}H-kainate (for kainate receptors) have shown striking differences with respect to selectivities (see for example, Ref. 5) and regional distribution (see, for example, Refs 6–8). Furthermore, high affinity ^{3}H-kainate binding sites have a molecular target size of 76.6 kD and are sensitive to Ca^{2+} ions [9], whereas quisqualate receptors determined by ^{3}H-AMPA binding have a molecular target size of 51.6 kD [10], are insensitive to Ca^{2+}, but coupled to a modulatory site and exist in two different conformations with the equilibrium being affected by SCN^- ions [11].

Recently, a potent and competitive non-NMDA antagonist, CNQX (6-cyano-7-nitro-quinoxaline-2,3-dione), was described [12]. In binding experiments CNQX showed a five-fold higher affinity for ^{3}H-AMPA binding sites as compared to ^{3}H-kainate binding sites indicating selectivity for quisqualate receptors. On the other hand, CNQX has comparable antagonistic potency after microelectrophoretic application near rat spinal neurones excited with either quisqualate or kainate [12]. This discrepancy between the binding experiments and the electrophysiological experiments, may have several possible explanations. One possibility being that kainate mediates its excitatory effects via quisqualate receptors.

In order to disclose which receptor sites CNQX interacts with we investigated the binding of ^{3}H-CNQX to rat brain membranes.

MATERIALS AND METHODS

8-^{3}H-CNQX (sp. act. 5.47 Ci/mmol) with chemical purity ≥97% and radiochemical purity ≥98% was synthesized by Chemsyn Science Laboratories (Lenexa, KA). CNQX was synthesized at Ferrosan Research Division (Soeborg, Denmark). [^{3}H]AMPA (sp. act. 25 Ci/mmol) was obtained from New England Nuclear (Boston, MA). All other chemicals were of the purest grade available from regular commercial sources.

Membrane preparations. Tissue preparation was done at 0° unless otherwise indicated. Frozen rat cortex was thawed and homogenized by an Ultra-Turrax homogenizer in 2 × 5 ml 30 mM Tris–HCl buffer, pH 7.1, containing 2.5 mM $CaCl_2$, and the homogenate was centrifuged at 30,000 *g* for 15 min. The pellet was washed three times by homogenization in the above buffer and centrifugation at 30,000 *g* for 10 min. The washed pellet was homogenized in 10 ml of buffer and incubated at 37° for 30 min, followed by centrifugation at 30,000 *g* at 25° for 10 min. The pellet was washed once by homogenization in buffer and centrifuged at 30,000 *g* for 10 min. After homogenization in buffer, the homogenate was frozen at −20°.

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Standard binding assay. The frozen homogenate was thawed and centrifuged at 48,000 *g* for 10 min, followed by washing twice by homogenization in 30 mM Tris–HCl buffer, pH 7.1, and centrifuged at 48,000 *g* for 10 min. The final pellet was homogenized in buffer (100 vol. per g of original tissue) and used for binding assays. In experiments where ^{3}H-AMPA was used as radioligand the final pellet was homogenized in 30 mM Tris–HCl buffer, pH 7.1 containing 2.5 mM $CaCl_2$ and 100 mM KSCN (50 vol. per g of original tissue). Standard assays were done by incubating aliquots (0.5 ml) in triplicate for 45 min at 0° with 2 nM ^{3}H-CNQX in the absence and presence of 0.7 mM L-glutamate for determination of non-specific binding. Free and bound radioactivity were separated by centrifugation at 17,500 *g* for 5 min, followed by rapid superficial rinsing of the pellet with 3 × 3 ml ice-cold buffer.

Data were analysed using the software "Kinetic, EBDA, Ligand Lowry" from Elsevier-Biosoft.

Radiation inactivation. Whole frozen rat cortices were exposed to ionizing radiation with high energy electrons using the 10 MeV linear accelerator at Risø, Denmark. The dose of radiation was determined using calibrated thermo dosimeters (water). The samples were cooled (*ca.* −10°C) during radiation which was delivered in runs of 0.5–2.0 Mrad. Between runs, samples were cooled to −15° for at least 2 min to ensure that they remained completely frozen during the whole irradiation process. At least 24 hr after the irradiation the cortices were thawed and subjected to the membrane preparation procedure described above.

RESULTS

Binding of ^{3}H-CNQX to membranes prepared from rat cortex was saturable and reversible (data not shown). Investigation of pH in the range 5.0–8.0 showed maximal binding at pH 7.5 (data not shown). Due to a very fast off-rate of the binding (on- and off-rate was too fast to measure), separation of bound and free radioligand by filtration gave loss of specific binding and a high standard deviation in repeated measurements. Specific binding obtained using filtration was 3% of the binding obtained using centrifugation (data not shown). Separation of bound and free radioactivity by centrifugation, using standard conditions (see experimental section) gave a total binding of 2350 ± 105 cpm/assay, with non-specific binding of 690 ± 35 cpm/assay (mean ± SE of ten independent experiments in hexdruplicate). Binding of ^{3}H-CNQX was linear with tissue concentrations up to 20 mg of original tissue/ml. The binding was not sensitive to Ca^{2+} up to a concentration of 5 mM, but 50 mM Ca^{2+} inhibited 60% of the specific binding.

Specific ^{3}H-CNQX binding was determined at different temperatures (Fig. 1). ^{3}H-CNQX binding was maximal at 0°. Accurate determinations of K_D at various temperatures were not attempted, but a van't Hoff plot of the specific ^{3}H-CNQX binding showed that binding is driven by enthalpy changes. The shift in the slope of the curve indicate enthalpy changes at approximately 23°.

Tissue suspended in 30 mM Tris–HCl and incubated 45 min at 0° was chosen as standard assay conditions.

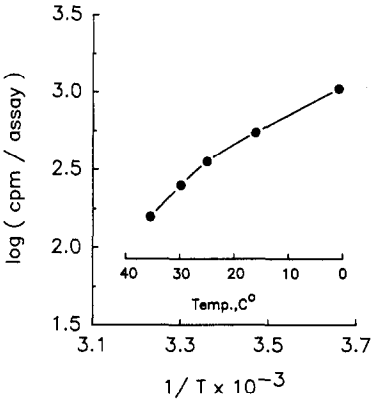

Fig. 1. Temperature dependence of specific ^{3}H-CNQX binding (final concentration 2 nM) to rat cortical membranes. Error bars (SE of three experiments) are within the size of the filled circles.

Table 1. Non-NMDA-receptors regional distribution

Region	Bound (cpm)* ^{3}H-AMPA	^{3}H-CNQX
Frontal cortex	2758 ± 230 (4)	1707 ± 41 (4)
Parietal cortex	2515 ± 64 (4)	1858 ± 41 (4)
Occipital cortex	3003 ± 47 (4)	1965 ± 18 (4)
Hippocampus	2924 ± 106 (4)	1865 ± 30 (4)
Cerebellum	632 ± 34 (4)	1506 ± 55 (4)
C.striatum	3258 ± 631 (4)	1274 ± 14 (3)
Pons-medulla	384 ± 53 (4)	398 ± 12 (4)
Hypothalamus	1180 ± 47 (4)	544 ± 38 (4)
Spinal cord	269 ± 27 (4)	430 ± 16 (4)

* Values are mean ± SE. Number of experiments is given in parenthesis.

Dissected tissue [22] was subjected to the standard membrane preparation procedure, and used for binding experiments.

The regional distribution of ^{3}H-CNQX binding (Table 1) was comparable to the regional distribution of ^{3}H-AMPA binding except in the cerebellum and spinal cord, where ^{3}H-CNQX binding is high compared to ^{3}H-AMPA binding.

The molecular weight or more correctly the molecular target size of ^{3}H-CNQX binding sites was determined using the high energy irradiation technique (for methodological details, see Ref. 13). This method is based on the fact that high energy particles by a single "hit" (energy transfer) can inactivate proteins following classical target theory. The functional mass (M) is determined *in situ* using the following relationship between functional inactivation (F_o–F_D) and radiation dose (D): $F_D = F_o \exp(-kD)$; M = calibration constant × k. The method has been validated for a number of enzymes and receptors [14–16]. The advantage of the technique is, that molecular weight (functional target size of a protein) can be determined without isolation of the protein from membranes and other environments.

Membranes obtained from radiated whole frozen

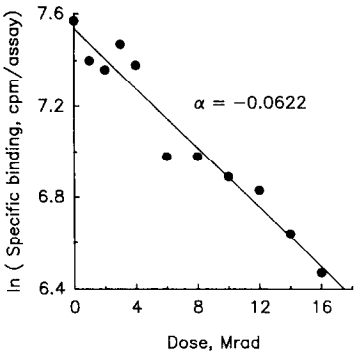

Fig. 2. Radiation inactivation of ^{3}H-CNQX (final concentration 2 nM) binding sites in rat cortical membranes. The depicted experiment is representative of three experiments which gave a molecular weight of 51.8 ± 3.4 kD (mean ± SE).

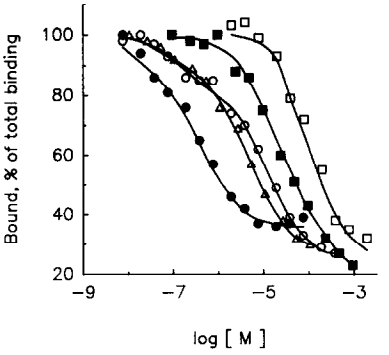

Fig. 4. Inhibition of ^{3}H-CNQX binding (final concentration 2 nM) to rat cortical membranes by quisqualate △, kainate ■ and AMPA in the absence (○) and presence (●) of KSCN (100 mM). A two-site fit is significantly better ($P < 0.001$; F-test) than a one-site fit for these compounds. The GAMS inhibition curve □ is a normal sigmoid curve, representing monophasic inhibition.

rat cortex (dose range: 0–20 Mrad) showed a monoexponential decay of specific ^{3}H-CNQX binding (Fig. 2). The radiation inactivation constant (k) was 0.0674 ± 0.0045 (mean ± SE determined from three separate experiments). Calibration was done by radiation inactivation of seven enzymes of known molecular weights (MW 67–148 kD), which gave a calibration constant of 768 kD. Molecular target size was 51.8 ± 3.4 kD (mean ± SEM determined from three separate experiments). The molecular target size of ^{3}H-CNQX binding site is similar to the target size of ^{3}H-AMPA binding (51.6 kD) [10], but different from high affinity ^{3}H-kainate binding (76.6 kD) [9], ^{3}H-3-(2-carboxypiperazine-4-yl)propyl-1-phosphonic acid (CPP) binding (209 kD) [17] and ^{3}H-glycine binding (115 kD) (Honoré *et al.*, unpublished).

Scatchard analysis of specific ^{3}H-CNQX binding (final concentration 1–1000 nM) to rat cortical membranes revealed a linear plot (Fig. 3a) suggesting a single binding site, with $K_D = 39 \pm 2$ nM and $B_{max} = 0.56 \pm 0.02$ pmol/mg of original tissue (mean ± SE of three separate experiments). The Hill coefficient was 0.92 ± 0.01.

Inhibition of ^{3}H-CNQX binding by AMPA gave a biphasic inhibition curve (Fig. 4), with $K_i = 0.3 \pm 0.2$ and 22 ± 9 μM (mean ± SE of four independent experiments). In each experiment a two-site fit was significantly better than a one-site fit ($P < 0.001$, F-test [18]). The high affinity AMPA site (tentatively named conformation A) corresponded to 28 ± 6% of the binding (mean ± SEM of four independent experiments), the low affinity site (72 ± 6%) is named conformation B. AMPA is a specific agonist at quisqualate receptors, which apparently binds with different affinity to two states of the quisqualate receptors [11]. The monophasic Scatchard plot of ^{3}H-CNQX binding to rat cortical membranes together with AMPA giving biphasic inhibition curves, suggest that ^{3}H-CNQX binds with the same affinity to the two conformational states of quisqualate receptors. Furthermore, in saturation binding studies under standard conditions for ^{3}H-CNQX, the presence of 200 nM AMPA gave biphasic Scatchard plots (Fig. 3b) indicating selective inhibition of one component of the ^{3}H-CNQX binding by AMPA.

It has recently been suggested that chaotropic ions, such as SCN^--ions, affect the equilibrium between the two states of quisqualate receptors, shifting the

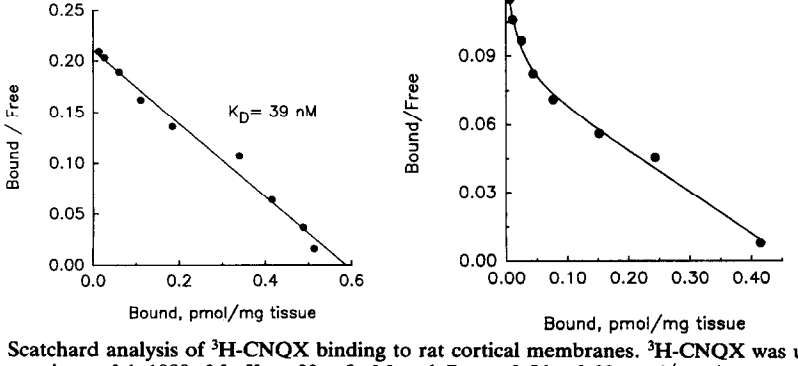

Fig. 3. (a) Scatchard analysis of ^{3}H-CNQX binding to rat cortical membranes. ^{3}H-CNQX was used in final concentrations of 1–1000 nM. $K_D = 39 \pm 2$ nM and $B_{max} = 0.56 \pm 0.02$ pmol/mg (mean ± SE of three experiments). (b) Scatchard analysis of ^{3}H-CNQX binding to rat cortical membranes in the presence of AMPA (200 nM final concentration). Curvilinearity was significant ($P < 0.001$, F-test).

conformation towards the one with high affinity for AMPA [11]. Scatchard analysis of a saturation binding study using ^{3}H-CNQX as radioligand in the presence of 100 mM KSCN gave a linear plot with K_D = 160 ± 15 nM and B_{max} = 0.73 ± 0.05 pmol/mg of original tissue (mean ± SE of three separate experiments). Apparently, KSCN marginally reduces the affinity and increased the density of sites.

Inhibition of ^{3}H-CNQX binding by AMPA in the presence of SCN^--ions gave K_i = 0.02 ± 0.01 and 0.5 ± 0.2 μM (mean ± SE of three separate experiments, Fig. 4). In each experiment a two-site fit was significantly better than a one-site fit ($P < 0.05$, F-test [19]).

The low-affinity AMPA binding site showed a 60-fold increase in affinity in the presence of SCN^--ions, whereas the high-affinity site only showed a five-fold increase in affinity.

A series of standard excitatory compounds were tested as inhibitors of ^{3}H-CNQX binding (Table 2). The experimental values were fitted to a one-site and a two-site model. When the two-site fit was not significantly better than the one-site fit, this was interpreted as the compound having the same affinity for the two sites.

Both AMPA and quisqualate have the highest affinity for the so-called conformation A representing 20% of the ^{3}H-CNQX binding sites. AMPA was the most potent. Both compounds are weak inhibitors of ^{3}H-CNQX binding to conformation B, where quisqualate shows higher affinity than AMPA.

Kainate has opposite to AMPA and quisqualate the highest affinity for conformation B. Similarly, DL-homocysteate has the highest affinity for conformation B. NMDA and D-APV have equal and low affinity for both conformations. The rank order of potency for the conformation A is CNQX > quisaqualate = AMPA > L-glutamate > GAMS > NMDA > kainate D-APV > DL-homocysteate. The rank order of potency for the conformation B is CNQX > quisqualate > kainate > AMPA = L-glutamate > GAMS > DL-homocysteate > NMDA > D-APV.

In order to investigate the specificity of ^{3}H-CNQX binding a series of standard compounds active in other transmitter systems were tested (Table 2). None of the compounds were active as inhibitors in concentrations below 100 μM, suggesting that ^{3}H-CNQX does not bind to the benzodiazepine/GABA-chloride ionophore complex (diazepam, flunitrazepam, clonazepam, β-CCE, FG 7142, DMCM, GABA, muscimol, bicuculline-methiodide, pentazol, pentobarbital, IPTBO); opiate receptors (morphine, naloxone); the NMDA gated ionophore (phencyclidine, ketamine, MK-801); dopamine receptors (D-butaclamol, haloperidol, chlorpromazine); 5-HT uptake carrier (paraoxetine); α-adrenergic receptors (phentolamine); muscarine receptors (atropine, scopolamine); adenosine receptors (caffeine); glycine receptors (glycine, D-serine, strychnine).

DISCUSSION

The recently described quinoxalinediones, CNQX

Table 2. Inhibition of ^{3}H-CNQX binding to rat cortical membranes

	K_i (μM)*	
	Conformation A	Conformation B
CNQX	0.039† ± 0.002 (3)	0.039† ± 0.002 (3)
AMPA	0.3 ± 0.2 (4)	22 ± 9 (4)
Quisqualate	0.21 ± 0.02 (3)	5.9 ± 0.2 (3)
L-glutamate	30 (2)	30 (2)
GAMS	70 (2)	70 (2)
NMDA	130 (2)	130 (2)
Kainate	240 ± 70 (3)	12 ± 4 (3)
D-APV	300 (2)	300 (2)
DL-homocysteate	1600 (2)	85 (2)

* Values are mean ± SE. Number of experiments is given in parenthesis.
† K_D from Scatchard analyses.

At least twelve different concentrations of the inhibitors were used under standard assay conditions as described in Materials and Methods. K_is were calculated using the program Ligand [18]. A biphasic inhibition curve is assumed when a two-site fit is significantly better ($P < 0.01$, F-test) than a one-site fit in all the N experiments. Binding to conformation A is assumed, when the percent binding = 28 ± 6 (= the amount of high affinity AMPA sites) and to conformation B, when percent binding = 72 ± 6.

The following compounds inhibit ^{3}H-CNQX binding to rat cortical membranes with $IC_{50} > 100$ μM: diazepam, flunitrazepam, clonazepam, DMCM (ethyl 5,6-dimethoxy-4-ethyl-β-carboline-3-carboxylate), β-CCE (ethyl β-carboline-3-carboxylate), FG 7142 (*N'*-methyl β-carboline-3-carboxamide), GABA, muscimol, bicuculline-methiodide, pentazol, pentobarbital, morphine, naloxone, phencyclidine, ketamine, MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[*a*,*d*]cyclohepten-5,10-imine), D-butaclamol, haloperidol, chlorpromazine, paroxetine, phentolamine, atropine, scopolamine, caffeine, strychnine, glycine, D-serine.

and 6,7-dinitroquinoxaline-2,3-dione (DNQX), show selectivity for the ^{3}H-AMPA binding sites, and at the same time antagonize quisqualate and kainate induced effects equally well. No interaction with NMDA binding sites nor antagonism of NMDA-induced effects in electrophysiological experiments were seen. Recently, a weak non-competitive inhibition of NMDA-responses by CNQX and DNQX was published [20, 21]. The *in vivo* selectivity of these compounds may depend on the concentration of endogenous glycine. The selectivity for ^{3}H-AMPA binding sites is apparently not expressed in functional models involving non-NMDA receptors. Either the selective inhibition of ^{3}H-AMPA binding by these compounds is an artifact or the high-affinity binding of ^{3}H-kainate (in the absence of calcium ions) does not represent the receptors where kainate induces its physiological effect.

Opposite to ^{3}H-kainate binding where the high affinity component was totally inhibited by the presence of 2.5 mM Ca^{2+} [9], ^{3}H-CNQX binding was not affected by Ca^{2+} concentrations up to 5 mM. However, very high Ca^{2+} concentrations, i.e. 50 mM, inhibited 50% of the binding. Furthermore, the molecular target size of the ^{3}H-CNQX binding site (51.8 kD) is different from the high affinity ^{3}H-kainate site (76.6 kD), but equal to the low affinity ^{3}H-kainate site (52.4 kD [9]). The molecular target size of the low affinity kainate site is equal to the ^{3}H-AMPA binding site (51.6 kD). These results suggest that the ^{3}H-CNQX site is different from the high-affinity calcium sensitive kainate binding site, but may be identical to the low-affinity ^{3}H-kainate site (= quisqualate receptors). Consequently, CNQX sensitive kainate effects may not be mediated via the high affinity kainate site, but rather via the low affinity site.

The detailed study of ^{3}H-CNQX binding showed that this antagonist, although binding to the same receptor as the agonist ^{3}H-AMPA, had a different type of interaction. ^{3}H-CNQX binding revealed linear Scatchard-plots, but biphasic inhibition curves, when AMPA was used as inhibitor of the binding, i.e. ^{3}H-CNQX binds with the same affinity to the two sites defined by AMPA.

In molecular target size analysis ^{3}H-CNQX gave the same size as the high-affinity ^{3}H-AMPA binding site. This indicates that the high and low affinity AMPA-site has the same molecular size, and thereby suggest that the two sites are the same molecular entity. Furthermore, SCN^--ions which have almost no influence on ^{3}H-CNQX binding increased the apparent affinity of ^{3}H-AMPA dramatically. This is in accordance with a recent suggestion that the two sites which bind AMPA with different affinity are not two separate sites, but two interconvertible conformations of the same receptor. The equilibrium between the sites is affected by chaotropic ions, such as SCN^--ions [11].

The radiation inactivation curve of ^{3}H-CNQX binding was monoexponential opposite to the curvilinear inactivation curve of ^{3}H-AMPA binding [10]. This finding suggests that the high molecular weight modulatory unit, which is coupled to the quisqualate receptor and downregulate the affinity of the binding site for agonists, does not influence the affinity for antagonists.

Under the present experimental conditions ^{3}H-CNQX does not bind to glycine receptors. Firstly, neither glycine nor D-serine inhibit ^{3}H-CNQX binding. Secondly, the molecular target size of ^{3}H-glycine binding to rat cortical membranes (115 kD, Honoré *et al.*, unpublished) is markedly different from the ^{3}H-CNQX as well as the ^{3}H-AMPA site.

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