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Glutamic acid-insensitive [^{3}H]kainic acid binding in goldfish brain

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Kainic acid is supposed to be a specific agonist for a subclass of excitatory glutamate receptors in the vertebrate CNS. An investigation of (2 nM) [^{3}H]kainic acid binding sites in goldfish brain, using quantitative autoradiography, has revealed evidence for two types of kainic acid receptors which differ in sensitivity to glutamic acid. L-Glutamic acid (0.1–1 mM) displaced over 95% of specific [^{3}H]kainic acid binding elsewhere in the brain but only 10–50% in the cerebellum and cerebellar crest. These structures apparently contain [^{3}H]kainic acid binding sites that are extremely insensitive to glutamic acid. The glutamic acid-insensitive [^{3}H]kainic acid binding was not displaced by quisqualic acid, kynurenic acid, α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA), or *N*-methyl-D-aspartatic acid, but was completely displaced by the kainic acid analogue domoic acid. The data indicate that two types of high affinity binding sites for [^{3}H]kainic acid exist in the goldfish brain: glutamic acid-sensitive and glutamic acid-insensitive. High affinity [^{3}H]kainic acid binding may therefore not always represent binding to subsets of glutamic acid receptors.

INTRODUCTION

L-Glutamic acid is a non-selective agonist of excitatory amino acid (EAA) receptors in the vertebrate central nervous system and it may be the principal endogenous neurotransmitter in excitatory pathways[9,36]. At least 3 subtypes of ion channel-linked ('ionotropic') and one type of second-messenger-linked ('metabotropic') EAA receptor are recognized based on electrophysiological and biochemical experiments which have been carried out primarily in mammals. The subtypes are selectively activated by the agonists *N*-methyl-D-aspartate (NMDA), quisqualic acid or α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA), and kainic acid[25,30,36].

The regional distribution of [^{3}H]kainic acid binding sites in goldfish brain has been described using membrane preparations[14,21,22,38] and qualitative autoradiography[38]. We are investigating the distribution of kainic acid binding sites in goldfish brain using quantitative autoradiography. Here we report evidence for a surprising heterogeneity in specific [^{3}H]kainic acid binding sites in the brain. L-Glutamic acid (0.1 mM) displaced over 95% of specific [^{3}H]kainic acid binding elsewhere in the brain but only 10–35% in various regions of the cerebellum and in the cerebellar crest. We conclude that the fish cerebellum and cerebellar crest contain high affinity binding sites for [^{3}H]kainic acid that are strikingly insensitive to glutamic acid.

MATERIALS AND METHODS

Nine goldfish, 8–10 cm body length, were used to investigate in vitro binding of [^{3}H]kainate in brain sections[10]. The fish were anesthetized in 0.1% tricane (Sigma Chemical Co., St. Louis, MO), the brains removed and fast-frozen in O.C.T. medium. Three brains were embedded side-by-side in a single block. The blocks were cut in a cryostat to obtain 10-μm transverse sections of the 3 brains, each slice being thaw-mounted on a glass slide. To label [^{3}H]kainic acid binding sites the slides were prewashed at 4°C for 15 min in 50 mM Tris acetate, pH 7.4, or 50 mM Tris HCl, pH 7.2, containing 2.5 mM $CaCl_2$, and dried in a flow of warm air. The slides were next incubated at 4°C for 30 min with 2 nM [^{3}H]kainic acid, 58 Ci/mmol (New England Nuclear, Boston, MA). Glutamic acid, kainic acid, kynurenic acid (Sigma Chemical Co.), NMDA, quisqualic acid, domoic acid, or AMPA (Cambridge Research, Wilmington, DE) were added to the incubation medium as indicated. Non-specific binding measured in the presence of 100 mM kainic acid was less than 5% of the total [^{3}H]kainic acid binding.

Following incubation slides were quickly rinsed with ice-cold acetone containing 2.5% glutaraldehyde, dried in a flow of warm air and exposed to Hyperfilm (Amersham Corp., Arlington Heights, IL) for 4–5 days at room temperature. The concentration of binding sites was measured using a computer-assisted, video densitometer (Imaging Research, St. Catherines, Ont., Canada) method with calibrated radioactivity standards[28]. In each experiment the 3 brains were examined individually section by section. Density values for each structure were measured on both sides of the brain, where possible, and in every section, to obtain the average density for each structure in that experiment. Protein content was calculated using previously calibrated standards consisting of brain paste in which ^{3}H or ^{14}C had been admixed[28].

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RESULTS

Total [^{3}H]kainate binding

The autoradiograms showed that most of the [^{3}H]kainate binding sites occurred in neuropil or molecular layers of specific structures as reported by Ziegra et al.[38]. High concentrations of label occurred in the vagal lobe and cerebellar crest of the medulla and parts of the cerebellum (Table I). The greatest density of binding was localized in the periventricular fiber layer of the vagal lobe[26], designated zone 1. The central region of the vagal lobe, designated zone 3, was moderately to heavily labeled. The remaining regions of the vagal lobe, zones 2 and 4, and the facial lobe were lightly labeled. The cerebellar crest, a molecular layer covering the octavolateralis area of the medulla, which fuses rostrally with the molecular layer of the cerebellum[1,20] was moderately labeled.

In the cerebellum, binding was distinctively localized in the molecular layers of the valvula (Fig. 1) and corpus[1] and vestibulolateral lobe. One granular layer of the vestibulolateral lobe of the pars medialis was heavily labeled, while the other, the eminetia granularis, was not (see ref. 38). The values for the vestibulolateral lobe in Table I are for the molecular layer and the pars medialis. The granular layer of the corpus and valvula were only lightly labeled, as can be seen in Fig. 1. Moderate concentrations of the binding sites occurred in the hindbrain reticular area adjacent to ventricle IV and the periventricular fiber layer of the optic tectum. The labeled reticular area was continuous rostrally with the labeled molecular layers of the lateral lobes of the valvula cerebellum and caudally with the labeled zones of the vagal lobes, facial lobe and cerebellar crest. In the optic tectum, the granular layer of the periventricular zone and the more superficial lamina[27] were unlabeled (Fig. 1).

The cerebrum was moderately labeled while the olfactory bulb was only lightly labeled. Small, lightly to moderately labeled profiles in the preoptic area, pretectum and dorsal thalamus were omitted owing to an insufficient number of sections. The hypothalamus was lightly and relatively uniformly labeled, including the nucleus anterior tuberis (not shown), the large nucleus diffusus and the entire nucleus glomerulosus complex[4] (Fig. 1). The results were similar to those illustrated by Ziegra et al.[38].

Competing ligand experiments

Glutamic acid or kynurenic acid (0.1 mM) displaced 92–100% and 94–100% of binding, respectively,in other brain structures but only 22–35% and 48–69%, respectively, in the cerebellum and the cerebellar crest (Table I). In the latter structures, glutamic acid competed less

TABLE I

Density of [^{3}H]kainic acid binding sites in brain regions

The values are means ± S.E.M. for 9 brains. Total binding is expressed as pmol/mg protein and displacement is expressed as the percentage of the total that was displaced.

Region	*Total binding [^{3}H]kainic acid*	*Displacement of binding (% of total)*			
	(2 nM)	*NMDA (0.1 mM)*	*Quisqualic acid (2.5 μM)*	*Kynurenic acid (0.1 mM)*	*Glutamic acid (0.1 mM)*
Cerebellum and cerebellar crest					
Valvula					
lateral lobe	1.64 ± 0.12	8 ± 5	10 ± 4	53 ± 6	35 ± 12
medial lobe	1.41 ± 0.05	8 ± 5	13 ± 3	69 ± 10	30 ± 7
Corpus	1.31 ± 0.08	12 ± 6	5 ± 4	56 ± 4	22 ± 12
Vestibulolateral lobe	1.48 ± 0.07	5 ± 1	10 ± 5	48 ± 1	29 ± 10
Cerebellar crest	0.98 ± 0.08	8 ± 5	8 ± 8	48 ± 6	28 ± 10
Other brain structures					
Olfactory bulb	0.45 ± 0.11	17 ± 5	12 ± 8	100 ± 0	100 ± 0
Cerebrum	1.12 ± 0.10	41 ± 9	11 ± 1	97 ± 2	96 ± 2
Optic tectum					
Perivent. lyr.	1.08 ± 0.15	32 ± 5	11 ± 2	96 ± 2	95 ± 3
N. diffusus	0.89 ± 0.04	27 ± 8	3 ± 2	96 ± 3	94 ± 3
N. glomerulosus	0.70 ± 0.09	33 ± 10	17 ± 6	99 ± 2	99 ± 0
Reticular area	1.04 ± 0.06	19 ± 3	7 ± 3	94 ± 1	96 ± 1
Facial lobe	0.40 ± 0.03	40 ± 13	17 ± 10	100 ± 0	99 ± 1
Vagal lobe					
zone 1	1.70 ± 0.07	15 ± 7	7 ± 3	97 ± 2	92 ± 1
zone 2	0.42 ± 0.04	24 ± 7	-2 ± 5	100 ± 0	98 ± 2
zone 3	1.23 ± 0.07	28 ± 4	9 ± 3	98 ± 1	92 ± 1
zone 4	0.54 ± 0.03	36 ± 13	10 ± 1	100 ± 0	99 ± 1

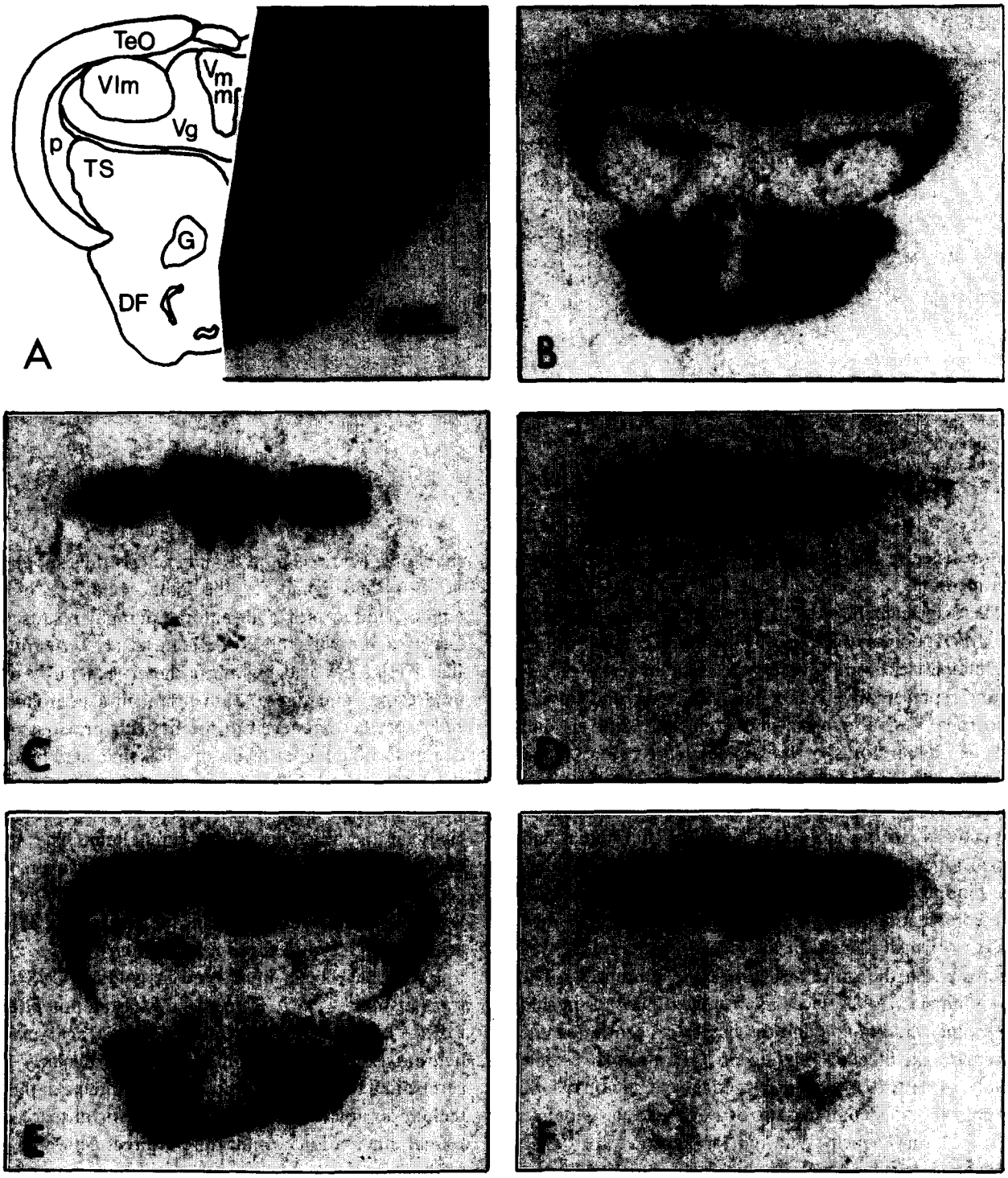

Fig. 1. A: transverse, Nissl-stained section showing the optic tectum (TeO) including the periventricular fiber layer (p), the valvula cerebellum granular layer (Vg) and molecular layer of the lateral (Vlm) and medial lobes (Vmm), the torus semicircularis (TS) in the ventral midbrain, and the n. glomerulosus (G) and n. diffusus (DF) in the caudal hypothalamus. B: autoradiogram of the same section showing total [^{3}H]kainic acid binding. C–F: autoradiograms of nearby sections showing [^{3}H]kainic acid binding in the presence of 0.1 and 1 mM glutamic acid (C and D, respectively), 0.1 mM AMPA (E) and 0.1 mM AMPA plus 0.1 mM glutamic acid (F).

effectively than kynurenic acid ($t = 6.29$, $P < 0.01$). [^{3}H]Kainic acid binding was comparatively insensitive to 0.1 mM NMDA or 2.5 μM quisqualic acid. NMDA displaced an average of 8.2% of binding in the combined parts of the cerebellum and cerebellar crest and 27.5% in the other structures. Quisqualate displaced an average of only 9.2 and 10.2% of binding in the cerebellum and cerebellar crest and the other structures, respectively. Overall, [^{3}H]kainic acid binding and the competing effects of the various ligands did not vary significantly with

TABLE II

Displacement of [^{3}H]kainic acid binding by AMPA and/or glutamic acid

The values for total [^{3}H]kainic acid binding are the average pmol/mg protein for 3 brains.

Region	*Total binding [^{3}H]kainic acid*	*Displacement of binding (% of total)*				
		Glutamic acid			*AMPA*	*Glu + AMPA*
	(2 nM)	*(0.1 mM)*	*(0.3 mM)*	*(1 mM)*	*(0.1 mM)*	*(0.1 mM each)*
Cerebellum						
Valvula						
lateral lobe	1.55	12	30	54	11	14
medial lobe	1.51	22	35	50	18	22
Corpus	1.42	9	26	48	8	11
Other brain structures						
Optic tectum						
Perivent. lyr	0.93	88	100	100	21	89
N. diffusus	0.78	86	100	100	20	86
N. glomerulosus	0.46	100	100	100	24	100
Reticular area	0.69	96	100	100	11	96

the presence or absence of Ca^{2+} and Cl^- in the incubation medium.

The displacement of [^{3}H]kainic acid binding produced by AMPA, domoic acid, and by increased concentrations of glutamic acid were investigated in brain sections containing representative cerebellum structures, the valvula or corpus, and also parts of the nucleus diffusus and nucleus glomerulosus or the reticular area (Fig. 1 and Table II). The presence of 0.1, 0.3 or 1 mM glutamic acid displaced an average of 14, 30 and 51% of the binding, respectively, in the molecular layers of the medial and lateral lobes of the valvula and the corpus cerebellum. The binding was similarly insensitive to 0.1 mM AMPA or a combination of AMPA and glutamic acid (0.1 mM each), which displaced an average of 12 and 14%, respectively. In the other structures, the presence of 0.1 mM glutamic acid displaced an average of 92% of specific [^{3}H]kainic acid binding and the higher concentrations of glutamic acid blocked binding of [^{3}H]kainic acid completely. AMPA (0.1 mM) displaced an average of 19% of binding and the combination of AMPA plus glutamic acid (0.1 mM each) blocked an average of 93%. [^{3}H]Kainic acid binding was completely blocked by the presence of 0.1 mM unlabeled kainic acid or domoic acid.

DISCUSSION

Glutamic acid or another related molecule is considered to be the predominant excitatory neurotransmitter within the vertebrate central nervous system. Based on experiments performed mainly in mammalian systems, the neuroexcitatory effects of glutamate are proposed to be mediated by several types of EAA receptors including the kainic acid receptor. Kainic acid is thus supposed to be a specific ligand for the kainic acid-type of EAA amino acid receptor. Our autoradiography results, which largely agree with those of Ziegra et al.[38], clearly show that [^{3}H]kainic acid binding is heterogeneously distributed throughout the goldfish brain, in a neuroanatomically specific manner. Consistent with previous studies demonstrating higher levels of [^{3}H]kainic acid binding in the brains of fish[8,14,19,21] compared to mammals[11,18,19], we found high levels of [^{3}H]kainic acid binding, especially in the vagal lobe and cerebellum. The striking concentration of labeling in the molecular layers of the cerebellum, excepting the granular pars medialis of the vestibulolateral lobe, resembles the pattern of [^{3}H]kainic acid binding seen in pigeon cerebellum[13].

EAAs have been implicated in goldfish CNS neurotransmission[16,17,22,34]. Kainic acid is also a potent neurotoxin[5]. Intracerebral injection of kainic acid produces neurotoxic lesions in goldfish brain, particularly in the cerebellum[35]. Kainic acid's neurotoxic effects in the optic tectum do not vary with the presence or absence of the retinal innervation[29], indicating a direct action of kainic acid.

The present results indicate that the goldfish contains high affinity [^{3}H]kainic acid binding sites that are extremely insensitive to glutamic acid. The glutamate-insensitive sites were distinctively localized in the cerebellum, including the corpus, valvula, vestibulolateral lobe, and in the cerebellar crest of the medulla. Other brain structures, some containing similar or higher concentrations of [^{3}H]kainic acid binding sites, specifically the vagal lobe, hindbrain reticular area and the cerebrum, exhibited no evidence of glutamate-insensitive sites. The presence of 0.1 mM glutamic acid displaced

virtually all specific [^{3}H]kainic acid binding in these structures but only 10–35% in the cerebellum and cerebellar crest, and a 10-fold increase in the concentration increased the displacement to only 50%. Thus, the brain of the goldfish, and presumably of other teleosts, may contain at least two types of kainic acid receptor differing in sensitivity to L-glutamic acid. Both types appear to be specific kainate receptors, since [^{3}H]kainic acid binding was completely blocked by domoic acid and insensitive to AMPA. Kynurenic acid (0.1 mM), a glutamatergic receptor antagonist which blocks retinotectal neurotransmission in goldfish[14,15], displaced more [^{3}H]kainic acid binding than did (0.1 mM) glutamic acid in all regions tested.

The data imply that not all [^{3}H]kainic acid binding sites are recognized by glutamic acid. The glutamic acid-insensitive sites might, therefore, be distinct from the kainic acid EAA receptor defined in electrophysiological experiments in mammals. In contrast to [^{3}H]kainic acid binding sites which have been described in amphibians, birds, and mammals[8,13,15,18,24] the glutamate-insensitive [^{3}H]kainic acid site in goldfish brain were less than 50% displaced by 0.1 mM glutamic acid. Additionally, in contrast to [^{3}H]kainic acid binding in mammal brain[3], the [^{3}H]kainic acid binding in goldfish was not inhibited by calcium ions.

The [^{3}H]kainic acid binding sites in the goldfish brain were comparatively insensitive to the non-NMDA receptor agonist AMPA, particularly in the cerebellum, but highly sensitive to domoic acid (Table II). Domoic acid, an analogue of kainic acid, is a potent neurotoxin found in certain mussels[6,7,33]. Domoic acid, immobilized on affinity columns, has been used to isolate kainic acid receptors from frog brain[12,37]. Thus, the glutamic acid- and AMPA-insensitive [^{3}H]kainic acid binding sites in the goldfish brain specifically bound domoic acid, another 'kainate-specific' ligand.

The identity of these glutamic acid-insensitive [^{3}H]kainic acid binding sites is unknown. Recent investigations in fish and birds indicate that certain [^{3}H]kainic acid binding sites may be localized to glia cells, in particular Bergmann glia cells[32]. Our method did not allow us to distinguish whether the [^{3}H]kainic acid binding occurred in neurons or glia. [^{3}H]Kainic acid binding sites in goldfish cerebellum may be intracellular[22]. Recent experiments showed that a low molecular weight, heat stable substance extracted from goldfish CNS tissue inhibited [^{3}H]kainic acid binding in CNS membranes[23]. The substance did not inhibit glutamate binding in fish membranes nor did it displace [^{3}H]kainic acid binding in rat brain membranes. Sewell and Mroz[31] have recently purified from the goldfish inner ear a similar substance which has neuroexcitatory properties. The relationship between these substances is unclear but their existence implies that non-glutamatergic excitatory neurochemical systems are present in goldfish. The glutamic acid-insensitive [^{3}H]kainic acid binding sites in the goldfish cerebellum and cerebellar crest may represent a component of such systems. For example, it is conceivable that the binding may reflect non-glutamatergic excitatory neurotransmitter receptors.

In summary, there appear to be two types of [^{3}H]kainic acid binding sites in the goldfish CNS, one which is sensitive to glutamic acid and another, novel site which is highly insensitive to glutamic acid. The two types are distinctively localized in the brain. The glutamic acid-insensitive binding sites are highly concentrated in the cerebellum and the cerebellar crest. The high concentrations of the glutamic acid-insensitive sites in the cerebellum suggests that similar sites may occur in other vertebrates which exhibit high concentrations of [^{3}H]kainic acid binding sites in the cerebellum. Finally, our data suggest that classifications of EAA receptors based on experiments in mammals should be cautiously applied to non-mammalian species.

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