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Specific Binding of [^{3}H]Kainic Acid to Receptor Sites in Rat Brain

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SUMMARY

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The binding of [^{3}H]kainic acid to washed membranes from rat forebrain was saturable with dissociation constants at 2° of 4-16 nM and 27-66 nM for high and low affinity binding, respectively. The Hill coefficients for binding to high and low affinity sites were 0.99 and 0.85, respectively. At 2° [^{3}H]kainic acid receptor binding reached equilibrium by 60 minutes. Dissociation of the ligand-receptor complex at 2° was biphasic. The kinetically-determined dissociation constant for the high affinity binding site corresponded well with the value obtained in equilibrium studies. Of the substances tested, kainic acid and quisqualic acid were the most potent in competing for [^{3}H]kainic acid binding sites. L-glutamic acid was 40-fold less potent than kainic acid itself but 2500 times more effective than D-glutamic acid; dihydrokainic acid was 200-fold less potent than kainic acid at the low affinity site and 1000-fold less active in competing for binding to the high affinity site. Among the poor displacers of [^{3}H]kainic acid (IC_{50} > 0.1 mM at 50 nM [^{3}H]kainic acid) were D- and L-aspartic acids as well as several proposed glutamate antagonists. In brain, there was considerable variation in the regional distribution of high affinity binding sites: striatum > frontal cortex = hippocampus > cerebellum > medulla-pons; more than 90% of the total specific binding in the cerebellum and medulla-pons was to low affinity sites. Thus, on the basis of the kinetics of binding, differential affinity for competitors and differences in regional distribution in brain, the high and low affinity binding sites represent independent entities. Negligible specific binding was obtained (< 1 fmole/mg tissue) in liver, lung, kidney and intestine. Ablation of the intrinsic neurons of the caudate nucleus by injecting 10 nmole of kainic acid 28 days before sacrifice was associated with a 57-64% reduction in high and low affinity binding in this region.

INTRODUCTION

Kainic acid, a dicarboxylic acid containing pyrrolidine isolated from the seaweed Digenea simplex (1, 2), is a neuronal depolarizing agent (3-9). With the possible exception of domoic acid, kainic acid is the most potent known neuroexcitant in the mammalian central nervous system, being approximately 50-fold more effective than the putative neurotransmitter, L-glutamate (3, 4, 10). Because kainic acid contains the sequence of glutamic acid, it has been hypothesized that the potent excitatory effects of kainate reflect its restricted conformation which is optimal for interacting with excitatory glutamate receptors (4, 7). Modifications of the kainic acid molecule such as reduction of the isopropylene side chain, esterification of the carboxyl groups,

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acetylation of the ring nitrogen and alteration of its stereoisomerism abolish or markedly attenuate the neuroexcitatory properties of the compound (4, 11). Thus, the recognition site mediating kainate's action on neurons appears to be highly selective.

Recent studies have shown that *in situ* injection of kainic acid into brain produces a characteristic pattern of degeneration in which neurons with cell bodies near the area of infusion rapidly degenerate whereas axons from extrinsic neurons terminating in or passing through the region are relatively unaffected (12–15). Since excitatory amino acid receptors are localized on neuronal dendrites and possibly perikarya (16, 17), the microanatomic selectivity of kainic acid's neurotoxic action is consistent with its neuroexcitatory effects. Other neuroexcitants including glutamic acid and homocysteic acid have been shown to produce similar patterns of neuronal degeneration when administered systemically to immature rodents (18). The fact that structural modifications of the kainate molecule that alter its electrophysiologic activity also abolish its neurotoxicity (19) provides additional evidence that neurotoxicity may be related to its electrophysiologic effects.

The marked potency and structural specificity of kainate's electrophysiologic and neurotoxic effects suggest that kainate interacts with specific high affinity receptors. Simon *et al.* (20) have previously demonstrated saturable, specific binding of [^{3}H]-kainic acid to rat brain membranes. In this report, we have examined in detail the kinetics of [^{3}H]kainic acid binding to brain membranes in an attempt to correlate binding with the electrophysiologic and neurotoxic action of the agent.

MATERIALS AND METHODS

[^{3}H]Kainic acid. Two batches of kainic acid (Lot numbers: K-0250 and 116C-0404) obtained from Sigma Chemical Company (St. Louis, MO) were custom labeled by catalytic tritium exchange by Amersham Corporation (Arlington Heights, IL; Lot TRQ 1176, 4.1 Ci/mmole; Lot TRQ 1061, 4.0 Ci/mmole). The radioactive product was at least 97% pure, and migrated isographically with authentic kainic acid in thin-layer chromatography. The radioactive product was equipotent with unlabeled kainic acid in terms of selective neurotoxicity when stereotaxically injected into the corpus striatum as described by Coyle and Schwarcz (13).

Preparation of washed membranes. Tissue samples from male Sprague Dawley rats (175–250 g) were frozen and stored in liquid nitrogen until the time of assay; such storage did not affect receptor binding. The tissues were sonified in 100 volumes of ice-cold glass distilled water (GDW) with a Sonifier Cell Disrupter (Model W185; Heat Systems - Ultrasonics, Inc.; setting 4; 30–60 sec). Tissue suspensions were centrifuged at 48,000 × *g* for 10 min at 2° and the supernatant fluid was discarded. The membrane pellets were resuspended, pelleted and washed sequentially in 100 vol of GDW and 50 mM Tris-citrate buffer, pH 7.1. Finally, the membrane pellets were routinely resuspended in 50–100 vol (original homogenizing volume) of ice-cold 100 mM Tris-citrate buffer, pH 7.1 at 2°. Protein content of the membrane suspensions was determined by the method of Lowry *et al.* (21). For routine assays, membranes prepared from rat forebrain (frontal cortex plus striatum) were used. In other experiments, membrane suspensions were prepared from various regions of the rat brain dissected according to the method of Glowinski and Iversen (22) or samples of lung, liver, kidney and intestine.

Standard [^{3}H]kainic acid binding assay. To duplicate or triplicate 15 ml Sorvall teflon centrifuge tubes were added 1000 μl of washed membrane suspension (0.1–0.25 mg protein), 10 to 100 pmole of freshly diluted [^{3}H]kainic acid in a volume of 100 μl, various concentrations of potential radioligand displacers and GDW to bring the volume to 2 ml. During preparation and subsequent incubation, the tubes were kept in an ice-water bath maintained at 2°. Incubations were run for 60 min and terminated by centrifugation at 48,000 × *g* for 10 min in a Sorvall refrigerated centrifuge maintained at 2°. The resultant pellets were rinsed twice superficially with 5 ml of ice-cold water and then dissolved by incubating for 30 min at 55° with 1.5 ml of Protosol (New England Nuclear Corp.; Boston, MA). The dissolved pellets were counted in a

liquid scintillation spectrometer in 10 ml of LCS (Yorktown Research, New York) containing 40 ml of glacial acetic acid per gallon to eliminate chemoluminescence. Counting efficiency was 45%. Total specific binding of [^{3}H]kainic acid was defined as the difference between the total binding with radioligand alone and nonspecific binding measured in the presence of 0.1 mM unlabeled kainic acid; concentrations of kainic acid greater than 0.1 mM did not result in additional displacement of radioligand.

In order to characterize separately the binding to high affinity and to low affinity receptor binding sites, advantage was taken of the fact that the radioligand dissociates slowly ($t_{1/2}$ = 90 min) from high affinity sites but nearly instantaneously from low affinity sites (see RESULTS). After 60 min of incubation of the membranes with [^{3}H]-kainic acid, 200 nmole of unlabeled kainic acid were added to some experimental tubes to bring the final concentration of unlabeled ligand to 0.1 mM. After 5 min of additional incubation, the tubes were centrifuged and the binding of [^{3}H]kainic acid was measured. The addition of the 200 nmole of unlabeled ligand 5 min prior to centrifugation resulted in total displacement of radioligand from low affinity binding sites, but did not affect nonspecific binding; thus, the difference between binding in these preparations and those incubated in the presence of radioligand alone represents binding to low affinity receptor sites. The difference between binding occurring in the continuous presence of unlabeled ligand (nonspecific binding) and that occurring with the 5 min exposure to an excess of unlabeled ligand represents binding to high affinity receptor sites. Deviations from these methods for measurements of association, dissociation and saturation isotherms are described in the text.

Striatal kainate lesion. Male Sprague Dawley rats (160–180 g) were anesthetized with Equithesin (0.65 ml, i.p.: Jensen Salisbury Labs) and positioned in a David Kopf small animal stereotaxic apparatus. Kainic acid (Sigma Chemical Company St. Louis, MO; Lot number 105C-0064) was dissolved in artificial cerebrospinal fluid buffered to pH 7.4; 1 μl of the solution containing 10 nmole of kainic acid was infused into the left corpus striatum over a period of 40 sec as previously described (23) using stereotaxic coordinates: 0.8 mm anterior to bregma, 2.6 mm lateral to the midline and 4.8 mm ventral to the pial surface. Adequacy of the kainate lesion was determined by assay of choline acetyltransferase activity by the method of Bull and Oderfeld-Nowak (24); reduction in choline acetyltransferase activity reflects the degeneration of cholinergic neurons intrinsic to the striatum that are vulnerable to kainic acid (13, 25).

Compounds. Unlabeled kainic acid, L- and D-glutamic acids, L-glutamine, L- and D-aspartic acids, α-methyl-D, L-aspartic acid, L-glutamic acid diethylester, 2-amino-4-phosphonobutyric acid and γ-aminobutyric acid were purchased from Sigma Chemical Co. N-methyl-D,L-aspartic acid was purchased from K and K Laboratories (Plainview, NY). D,L-Homocysteic acid and ouabain were purchased from Calbiochem (San Diego, CA). Veratridine was purchased from Aldrich Chemical Co. (Milwaukee, WI). Quisqualic and ibotenic acids were gifts from Dr. A. Padjen of the University of Toronto. Muscimol was a gift from Dr. S. J. Enna of the University of Texas School of Medicine. Dihydrokainic acid was prepared by Drs. I. McDermott and C. H. Robinson of this Department of Pharmacology. Nuciferine was supplied by Dr. Haefly, Hoffman, La Roche, Basil. All other compounds utilized were reagent grade.

RESULTS

Conditions for specific binding. Under standard assay conditions, specific binding of [^{3}H]kainic acid to forebrain membranes was linear with tissue concentration in the range tested: 2.5–30 mg tissue wet weight or 0.03–0.35 mg membrane protein (Fig. 1). This precluded the presence of such artifacts as ligand degradation during incubation or contamination by unrecognized endogenous ligands. The pH optimum for specific binding was broad and flat between pH 6.0 and 7.1 at 2°; extremes of pH at 5 or 8 resulted in greater than 70% reduction in total specific binding (Fig. 2). Total specific

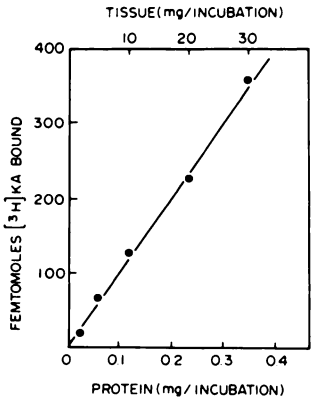

FIG. 1. *Total specific binding of [^{3}H]kainic acid to forebrain membranes as a function of tissue or protein content*

Forebrain membranes were prepared as under MATERIALS AND METHODS and incubated for 60 min at 2° with 50 nM [^{3}H]kainic acid. Specific binding was defined as the difference between binding obtained in the absence and presence of 0.1 mM unlabeled kainic acid. Incubations were terminated by centrifugation at 48,000 × *g*. The resultant pellets were rinsed twice with 5 ml of ice-cold Tris-citrate buffer. Points shown are the means of triplicate assays.

binding after 60 min of incubation did not vary significantly between 2° and 37°; however, at 50° specific binding was nearly abolished (Fig. 3). Incubation of the membranes with Triton X-100 at a concentration of 0.025% (vol/vol), a technique shown to enhance GABA receptor binding (26), completely eliminated the specific binding of [^{3}H]kainic acid.

Kinetics of [^{3}H]kainic acid binding. The specific binding of [^{3}H]kainic acid to forebrain membranes at 2° was saturable with a binding maximum of 1580 ± 42 fmole/mg protein. Non-specific binding was linearly related to the amount of [^{3}H]kainic acid added to the incubation mixture (Fig. 4). The ratios of specific to non-specific binding at 5 and 50 nanomolar [^{3}H]kainic acid were approximately 8:1 and 2:1, respectively. Scatchard analysis of the saturation isotherm revealed two apparent populations of receptor binding sites. Mean K_D values of 7.8 ± 1.8 nM and 37.6 ± 4.7 nM were obtained for the high and low affinity sites, respectively (Fig. 5A). These estimates of dissociation constants for total specific binding at equilibrium were similar using two separate batches of [^{3}H]kainic acid whether K_D values were determined by saturation isotherms with varying concentrations of [^{3}H]kainic acid (high affinity: 6–16 nM; low affinity: 32–47 nM) or by dilution of a fixed amount of radioligand with

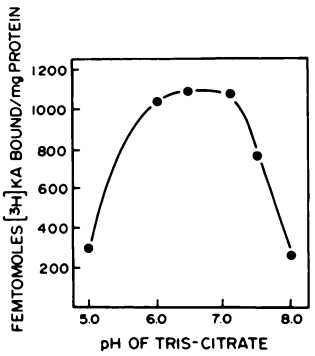

FIG. 2. *Total specific binding of [^{3}H]kainic acid to forebrain membranes as a function of pH*

Forebrain membranes were prepared as under MATERIALS AND METHODS except that they were finally resuspended in glass distilled water instead of Tris-citrate buffer. Membrane suspensions (20 mg tissue, original wet weight) were incubated with 50 nM [^{3}H]kainic acid and 1 ml of 100 mM Tris-citrate buffer at the pH's indicated for 60 min at 2°. Specific binding was defined as the difference between binding obtained in the absence and presence of 0.1 mM unlabeled kainic acid. Points shown are the means of triplicate assays.

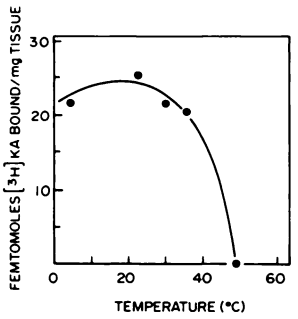

FIG. 3. *Total specific binding of [^{3}H]kainic acid to forebrain membranes as a function of temperature*

Forebrain membranes from 20 mg tissue (original wet weight) were prepared as under MATERIALS AND METHODS and incubated with 50 nM [^{3}H]kainic acid for 60 min at the temperatures indicated. Specific binding was defined as the difference between binding obtained in the absence and presence of 0.1 mM unlabeled kainic acid. Points shown are the means of triplicate assays.

various concentrations of unlabeled kainate (high affinity: 4–10 nM; low affinity: 54–66 nM). K_D values obtained by saturation studies at 25° were similar to those obtained at 2°.

The binding of [^{3}H]kainic acid to forebrain membranes was reversible, and the dissociation was biphasic at 2° (Fig. 6A). After incubating the membranes with radioligand for 60 min, the addition of 200 nmole of unlabeled kainic acid (final concentration 0.1 mM) reduced specifically-bound radioactivity by 50% within 2 min. [^{3}H]Kainic acid bound to higher affinity sites dissociated more slowly with a half-life of the radioligand-receptor complex approximating 90 min. The dissociation rate constant[1] obtained by logarithmic analysis for the high affinity component was 8.24×10^{-3} min^{-1} (Fig. 6B). Dissociation of bound [^{3}H]kainic acid was also measured by a dilution technique. After incubation for 60 min with radioligand, the membranes were pelleted by centrifugation; the supernatant fluid was discarded; and the membranes were gently resuspended in 100 mM Tris-citrate buffer, pH 7.1 at 2°. Membranes were isolated at various times after this resuspension, and the remaining bound radioactivity was measured. Approximately 50% of the total specifically bound [^{3}H]-kainate dissociated immediately upon resuspension; a second population of binding sites with a slower dissociation gave a rate-constant for dissociation of 11.8×10^{-3} min^{-1} in good agreement with the value obtained when rate of dissociation was measured by displacement with unlabeled kainic acid.

Since the rapidly dissociating low affinity binding sites could be occluded by brief exposure to unlabeled ligand, thus revealing the high affinity sites alone, this technique was used to examine the kinetics of association for the two populations of binding sites (Fig. 7). With 50 nM [^{3}H]kainic acid, a pseudo first-order rate constant (k_{OBS}) of 3.24×10^{-2} min^{-1} was determined for the association of [^{3}H]kainic acid with the high affinity receptors. The calculated second-order rate constant [$k_1 = (k_{OBS} - k_2)$/ [Kainic Acid] was 5.36×10^{-4} nM^{-1} min^{-1} using the k_2 obtained from the dissociation by the displacement experiment. From the two kinetic rate constants, a K_D of 15 nM was calculated ($K_D = k_2/k_1$) which approximated the K_D of the high affinity binding sites measured in saturation isotherms. To obtain more accurate estimates of high and low affinity specific binding at equilibrium, saturation isotherms were performed in

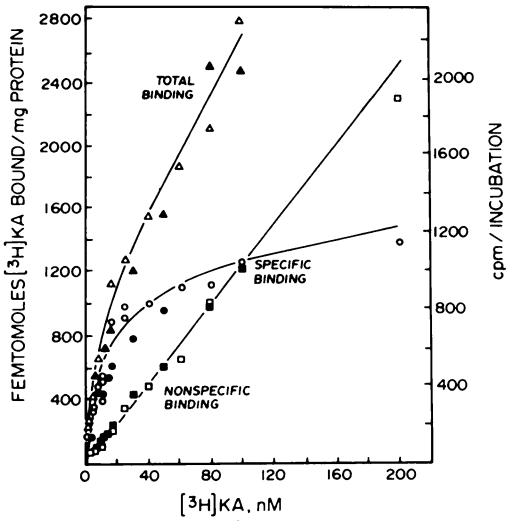

FIG. 4. *Binding of [^{3}H]kainic acid to forebrain membranes as a function of [^{3}H]kainic acid concentration*

Forebrain membranes from 10 mg tissue (original wet weight) were prepared as under MATERIALS AND METHODS and incubated for 60 min at 2° with [^{3}H]-kainic acid at the concentrations indicated. Nonspecific binding was defined as that occurring in the presence of 0.1 mM unlabeled kainic acid. Specific binding was defined as the difference in binding obtained in the absence and presence of 0.1 mM unlabeled kainic acid. Each value represents the mean of duplicate determinations. Closed and open symbols represent determinations from two separate representative experiments. This experiment was repeated six times.

[1] Kinetic rate constants were calculated from the reaction [kainic acid + $[R] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [KA{-}{-}R]$, where $[KA]$ is the free concentration of the radioligand, $[R]$ is the free concentration of receptor binding sites, and $[KA{-}{-}R]$ is the bound ligand concentration. k_1 is the true second order association rate constant and k_2 is the first order dissociation rate constant. Association kinetics were determined under conditions of ligand excess ($[KA]$ = 50 nM, [high affinity R] = 62.9 pM). The pseudo first order rate constant k_{OBS} was used to calculate the true association rate constant by the reaction $k_1 = ((k_{OBS} - k_2)/[KA])$ (27).

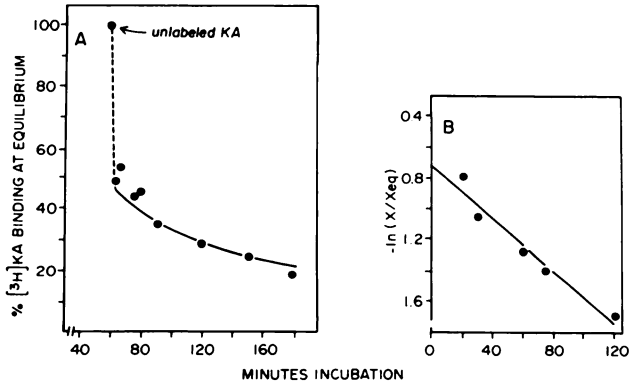

FIG. 5A. *Dissociation of [^{3}H]kainic acid ([^{3}H]KA) from forebrain membranes*

Forebrain membranes from 20 mg tissue (original wet weight) were incubated with 4 nM [^{3}H]kainic acid for 60 min at 4°; then 200 nmole of unlabeled kainic acid were added as indicated by the arrow, bringing the final concentration of unlabeled kainic acid to 0.1 mM. Each value represents the mean of duplicate determinations from two separate experiments.

FIG. 5B. *Logarithmic analysis of the dissociation of high affinity binding*

Time 0 is the time unlabeled KA was added. Xeq represents high affinity [^{3}H]kainic acid binding prior to the addition of the unlabeled ligand. X represents high affinity binding after addition of unlabeled kainic acid. The slope of this semilogarithmic plot was calculated by regression analysis (r = 0.98) and represents the first order rate constant k_2 = 0.00824 min^{-1} (at 4 nM [^{3}H]kainic acid).

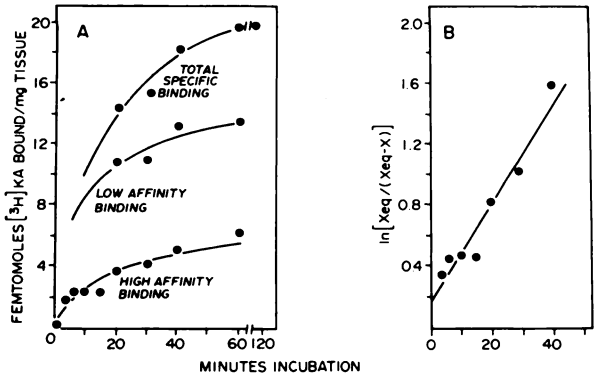

FIG. 6A. *Specific binding of [^{3}H]kainic acid ([^{3}H]KA) to forebrain membranes as a function of time*

[^{3}H]Kainic acid (50 nM) was incubated with forebrain membranes from 20 mg tissue (original wet weight) for the indicated times at 2°. To some incubations were added 200 nmole of unlabeled kainic acid, bringing the final concentration of unlabeled kainic acid to 0.1 mM. The bound radioactivity remaining after 5 min of incubation at 2°, less nonspecific binding (see MATERIALS AND METHODS), was considered as specific high affinity binding. Low affinity binding was defined as the difference between total specific binding and specific high affinity binding. Each value represents the mean of duplicate determinations.

FIG. 6B. *Logarithmic analysis of [^{3}H]kainic acid binding to the high affinity site*

X represents the amount of [^{3}H]kainic acid bound at time t, Xeq represents the amount of [^{3}H]kainic acid bound at equilibrium. The slope of this semilogarithmic plot was calculated by regression analysis (r = 0.96) and represents the pseudo-first order association rate constant (k_{OBS} = 0.032 min^{-1}) at 50 nM [^{3}H]kainic acid (26).

which an excess of cold kainate was added to some tubes at the end of incubation (Fig. 5B, C). Under these conditions, the K_D for binding to high affinity sites was 5.3 nM with a maximum of 382 fmole bound per mg protein; the apparent low affinity K_D was 27 nM with a maximum of 1,080 fmole bound per mg protein. Hill coefficients for high and low affinity specific binding were 0.94 (r = 0.99) and 0.85 (r = 0.94), respec-

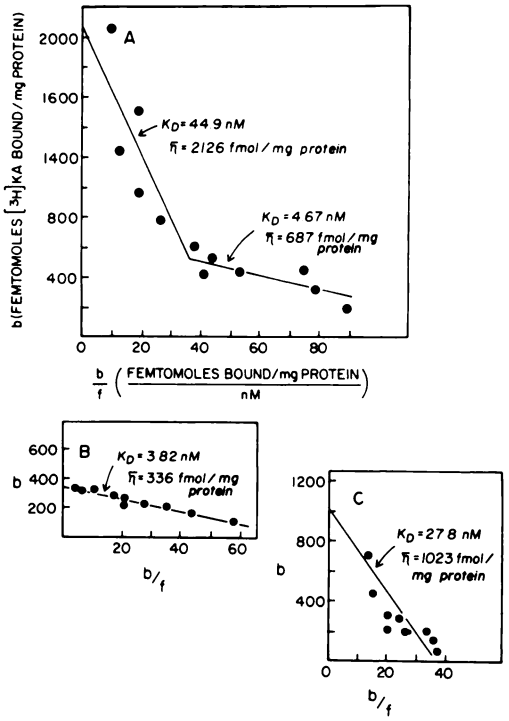

FIG. 7. *Eadie plots of [^{3}H]kainic acid binding to forebrain membranes*

Forebrain membranes (10 mg tissue, original wet weight) were incubated for 60 min as 2°, as described under MATERIALS AND METHODS, with various concentrations of [^{3}H]kainic acid. High and low affinity binding were defined as in Fig. 6. Points shown are the means of duplicate assays. A, Eadie plot of total specific binding, showing K_D values of 4.7 nM and 45 nM with receptor densities of 687 fmole/mg protein and 2126 fmole/mg protein for high and low affinity components, respectively. This plot is from a representative experiment which was repeated six times. B, Eadie plot of high affinity binding, showing a K_D value of 3.8 nM and receptor density of 336 fmole/mg protein. This plot is from a representative experiment which was repeated twice. C, Eadie plot of low affinity binding showing a K_D value of 28 nM and receptor density of 1023 fmole/mg protein. This experiment was repeated twice.

tively.

Inhibitors of specific binding of [^{3}H]-kainic acid. The abilities of unlabeled kainic acid, L-glutamic acid and dihydrokainic acid to compete with [^{3}H]kainic acid for specific binding are shown in Fig. 8. Unlabeled kainic acid displaced 5 nM [^{3}H]kainic acid with a K_i value of 1.5 nM for the high affinity binding site and 19 nM for the low affinity site, which is within the broad range of the K_D values obtained with saturation isotherms. Maximal displacement observed with 0.1 mM kainic acid was identical to that occurring with 0.1 mM L-glutamic acid; incubation with both compounds at 0.1 mM concentration did not produce additional inhibition of binding. The calculated K_i's for several compounds structurally related to glutamic acid at both the high and low affinity binding sites are presented in Table 1. Quisqualic acid, the most potent competitor, had a K_i approximately 15 times that of kainic acid at both the high and low affinity binding sites. L-Glutamic acid was the next most potent displacer with a K_i approximately 40 times that of kainic acid at both the high and low affinity sites. Dihydrokainic acid, which differs from kainic acid only with respect to the reduction of the isopropylene side chain, exhibited a 4,000-fold lower affinity for the high affinity binding site and a 200-fold lower affinity for the low affinity binding site than did kainic acid. The marked stereoselectivity of binding was demonstrated by the 2500-fold lower potency of D-glutamic acid as compared with L-glutamic acid at the high affinity binding site. Generally, the K_i ratios for the relative affinity of the competitors for the low versus high affinity binding sites were about 11; notable exceptions were dihydrokainic acid, L-glutamine and D-glutamic acid which exhibited higher affinities for the low affinity than the high affinity binding sites. A variety of agents with neuroexcitatory properties exhibited IC_{50} values greater than 0.1 mM in inhibiting total specific binding of [^{3}H]kainic acid at 50 nM (Table 1). Notably, the neuroexcitants L- and D-aspartic acid and N-methyl aspartic acid had negligible activity. In addition, reputed antagonists of glutamate neuroexcitation, glutamic acid diethylester (28, 29), morphine (30) and 2-amino-4-phosphonobutyric acid (31) were also ineffective as competitors. Several anticonvulsants including phenobarbital, diphenylhydantoin, diazepam and trimethadione had negligible activity at the receptor.

Regional distribution of the specific binding of [^{3}H]kainic acid. The regional

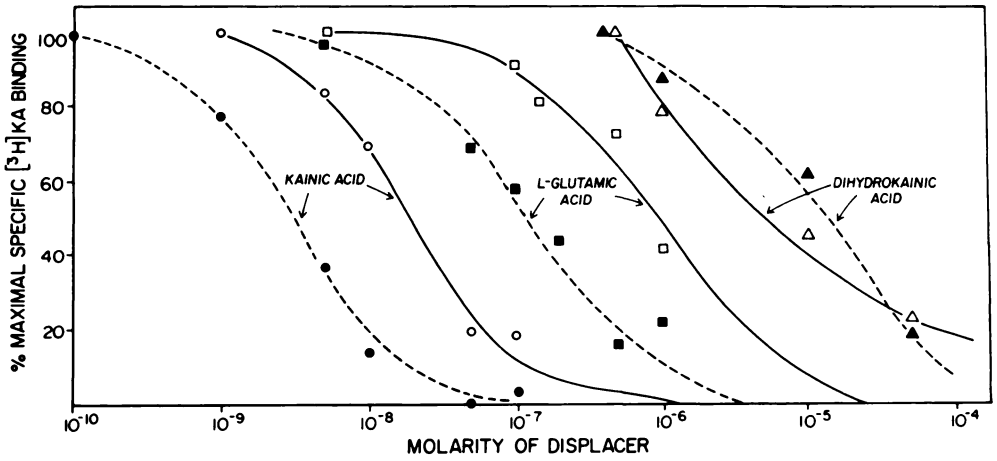

FIG. 8. *Inhibition of high and low affinity binding of [^{3}H]kainic acid*

Forebrain membranes (20 mg tissue, original wet weight) were incubated with various concentrations of kainic acid, L-glutamic acid and dihydrokainic acid for 60 min at 2° to which [^{3}H]kainic acid (5 nM) was added. High affinity binding (dotted lines) and low affinity binding (solid lines) were defined as in Fig. 6. Each point represents the mean of duplicate assays and each experiment was repeated.

TABLE 1

Affinities of various substances for [^{3}H]kainic acid binding sites

Forebrain membranes were incubated in triplicate with 4 nM [^{3}H]kainic acid for 60 min at 2° in the presence of several concentrations of the listed radioligand displacers.[a] To some incubations were added 200 nmol unlabeled kainic acid, bringing the final concentration of unlabeled kainic acid to 0.1 mM. The bound radioactivity remaining after 5 min of incubation at 2°, less nonspecific binding (see MATERIALS AND METHODS) was considered as specific high affinity binding. Low affinity binding was defined as the difference between total specific binding and specific high affinity binding. IC_{50} values were determined by log-probit analysis, and apparent K_i values calculated from the equation $K_i = IC_{50}/(1 + [^3H]\text{kainic acid}]/K_D)$. The K_D values used for high and low affinity binding, respectively, were 5 nM and 25 nM. Each experiment was performed once or twice.

Radioligand displacer	K_i at high affinity receptor	K_i at low affinity receptor	ratio low/high
	(nM)	(nM)	
Kainic acid	1.5	19	13
Quisqualic acid	23	255	11
L-Glutamic acid	63	722	11
Ibotenic acid	3360	35,700	11
Dihydrokainic acid	6110	3,150	0.5
D,L-Homocysteic acid	7120	87,200	12
L-Glutamine	19,400	6,430	0.3
D-Glutamic acid	155,000	42,000	0.3

[a] Forebrain membranes were also incubated in triplicate with 50 nM [^{3}H]kainic acid; no compound inhibited specific binding by more than 50% at 0.1 mM: L-aspartic acid, D-aspartic acid, L-proline, N-methyl-D,L-aspartic acid, γ-aminobutyric acid, baclofen, muscimol, veratridine, ouabain, L-glutamic acid diethylester, 2-amino-4-phosphonobutyric acid, nuciferine, morphine, phenobarbital, barbituric acid, secobarbital, pentobarbital, thalidomide, aminoglutethamide, diazepam, diphenylhydantoin, trimethadione, phensuximide, methsuximide, phenacimide, pemoline.

distribution of specific binding of [^{3}H]kainic acid to high and low affinity receptors in brain is shown in Table 2. Of all regions examined, the striatum exhibited the greatest total specific binding as well as the most binding at both high and low affinity sites. The frontal cortex and hippocampus exhibited somewhat less binding which was

TABLE 2

Regional distribution of specific [^{3}H]kainic acid binding

Washed membranes from various regions of rat brain were prepared as described under MATERIALS AND METHODS, and were incubated with 25 nM [^{3}H]kainic acid for 60 min at 2°. High and low affinity specific binding were determined as described in Table 2. Estimates of binding maximum ($\bar{\eta}$) were made using the formula: fraction of $\bar{\eta}$ at any ligand concentration = ([ligand]/K_D)/([ligand]/K_D + 1), and K_D values of 5 nM and 25 nM for high and low affinity binding, respectively. Values given are means ± standard errors for the numbers of experiments indicated in parentheses, each conducted in duplicate.

Region	Femtomoles [^{3}H]KA bound/mg protein			Estimated $\bar{\eta}$		
	Total specific	High affinity	Low affinity	High	Low	High/Low + High
Striatum (9)	1510 ± 180	506 ± 70	997 ± 185	612 ± 84	1990 ± 368	0.25
Frontal Cortex (9)	955 ± 78	393 ± 57	558 ± 80	476 ± 69	1110 ± 159	0.30
Hippocampus (10)	927 ± 58	280 ± 37	643 ± 52	339 ± 45	1280 ± 103	0.21
Cerebellum (10)	458 ± 53	31 ± 14	431 ± 64	38 ± 17	858 ± 128	0.04
Medulla-Pons (9)	66 ± 12	8 ± 4	66 ± 14	10 ± 5	132 ± 28	0.07

roughly comparable for both regions. The medulla pons had the lowest total specific binding. The ratio of high affinity to low affinity specific binding varied from region to region with the frontal cortex having the greatest proportion (30%) of high affinity binding; high affinity binding was virtually undetectable in both the cerebellum and medulla-pons. In this regard, Scatchard analysis of the [^{3}H]kainic acid saturation isotherm in the cerebellum yielded only one component with a K_D of 25 nM (Fig. 9), similar to the dissociation constant for the low affinity binding site observed in the forebrain. In other brain regions, total specific binding of [^{3}H]kainic acid as expressed in fmole/mg protein (N ≥ 4) were as follows: hypothalamus, 598 ± 56; locus coeruleus, 204 ± 32; septum, 322 ± 121; and the inferior and superior colliculi, 204 ± 32. Specific binding of [^{3}H]kainic acid to membrane preparations from lung, intestine, liver and kidney was virtually undetectable (<1 fmole/mg tissue).

Effects of striatal kainate lesion on the specific binding of [^{3}H]kainic acid. Kainic acid was infused into the left striatum and the specific binding of [^{3}H]kainic acid to membranes was determined 7 and 28 days after placement of the lesion. Seven days after the kainate injection, the activity of choline acetyltransferase, a marker for cholinergic interneurons in the striatum, was reduced by 71% as compared with the contralateral striatum; however, total specific and high and low affinity [^{3}H]kainic acid receptor binding were not significantly different from contralateral striatal membrane preparations. At 28 days after the injection, the lesioned striata exhibited a 71% reduction in choline acetyltransferase activity, a 59% reduction in total specific binding of kainic acid with 64% and 57% reductions in the high and low affinity bind-

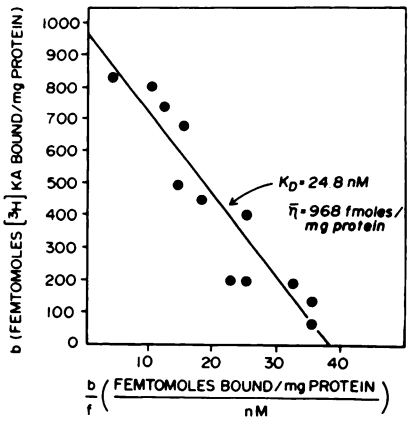

FIG. 9. *Eadie plot of total specific [^{3}H]kainic acid binding to cerebellar membranes showing a K_D value of 25 nM and receptor density of 968 fmole/mg membrane protein*

Cerebellar membranes (10 mg of tissue, original wet weight) were incubated for 60 min at 2°, as described under MATERIALS AND METHODS, with various concentrations of [^{3}H]kainic acid. Nonspecific binding was determined in the presence of 0.1 mM unlabeled kainic acid. Points shown are those obtained in a single experiment performed in duplicate.

ing, respectively (Table 3). In these studies there were no significant differences between striata contralateral to those injected with kainic acid and those taken from control, unlesioned rats in terms of kainic acid receptor binding and choline acetyltransferase activity. Thus, degeneration of neurons intrinsic to the striatum following kainate lesion was associated with delayed but marked reduction in the specific binding of [^{3}H]kainic acid.

TABLE 3

Effect of striatal kainate lesion on specific binding of [^{3}H]kainic acid and choline acetyltransferase activity

Rats were injected with 10 nmol kainic acid (KA) into the left corpus striatum as described under MATERIALS AND METHODS, and sacrificed 7 or 28 days later. Total specific, high and low affinity [^{3}H]kainic acid binding were assayed in washed striatal membrane preparations with [^{3}H]kainic acid as described in Table 2. Choline acetyltransferase activity was assayed in whole striatal homogenates. Values given are means ± standard errors for 5–6 preparations. Uninjected contralateral striata served as controls. Values of p were determined by Student's t-test.

	% Control	
	7 days	28 days
Total specific binding (fmole [^{3}H]KA bound/mg protein)	102 ± 5	41 ± 11*
High affinity binding (fmole [^{3}H]KA bound/mg protein)	89 ± 17	36 ± 13*
Low affinity binding (fmole [^{3}H]KA bound/mg protein)	117 ± 10	43 ± 16*
Choline acetyltransferase activity (nmole acetylcholine formed/mg striatum/hr)	29 ± 4	29 ± 6*

* Significantly different from control ($p < 0.01$).

DISCUSSION

These studies demonstrate specific, high affinity, saturable and reversible binding of [^{3}H]kainic acid to brain membranes. Kainic acid is a biological material that has three asymmetric carbons; previous electrophysiologic studies suggest that maintenance of stereoisomerism is essential for neurophysiologic activity (11). In this study, we have confirmed that the radiolabeled kainic acid is biologically active in terms of neurotoxic potency when compared to the unlabeled parent compound. Furthermore, with two separately prepared batches of [^{3}H]kainic acid of high specific radioactivity (4.0 and 4.1 Ci/mmole), similar saturation isotherms are obtained using various concentrations of the radioactive ligand or by diluting the radioactive ligand with unlabeled kainic acid. Thus, the radiolabeled kainic acid utilized in these studies appears to be comparable to the parent compound in terms of biological activity and kinetic characteristics.

Typical of the specific binding of better characterized agonists to putative neurotransmitter receptors, the specific binding of [^{3}H]kainic acid exhibits a relatively broad pH optimum between 6.0 and 7.1, and total specific binding at equilibrium is relatively independent of temperature between 2° and 37°. Analysis of the saturation isotherms at equilibrium at 2° and 25° reveals biphasic binding with apparent K_D's of approximately 5 and 30 nM. The biphasic dissociation of kainic acid demonstrated both by dilution of ligand as well as by competition with high concentrations of unlabeled ligand is compatible with the presence of two populations of binding sites with different affinities rather than negative cooperativity. In fact, the Hill coefficients for binding to the low and high affinity sites are near unity. Compelling evidence of the autonomy of the two binding sites is their differential distribution in the brain with 30% of total binding in the high affinity form in the frontal cortex and essentially no detectable high affinity binding in the cerebellum. The specific receptor binding of several other putative neurotransmitter agonists including acetylcholine (32), GABA (33) and serotonin (34) also exhibit separate high and low affinity components.

Both high and low affinity sites exhibit marked selectivity. Reduction of the isopropylene side chain of kainic acid results in a 200-fold loss in potency at the low affinity site and a 4000-fold loss of affinity for the high affinity site. Both binding sites are stereoselective with L-glutamic acid being considerably more avid than D-glutamic

acid. The distance between the carboxyl groups on the compound also plays a major role since the dicarboxylic acids, D- and L-aspartic acids and N-methyl aspartic acid, are virtually inactive at the receptor site. The importance of the dicarboxylic nature of the molecule is further emphasized by the rather poor results of substitution with the electrophilic sulphur or phosphate moiety for the γ-carboxyl group. The high affinity receptor, however, exhibits greater stereoselectivity and greater specificity for the isopropylene side chain of kainate than the low affinity site.

The cellular localization of the specific binding sites for [^{3}H]kainic acid must be considered. Specific binding is virtually undetectable in several peripheral tissues; accordingly the receptor sites are probably restricted to neuronal tissue. A previous study by Simon *et al.* (20) demonstrated an enrichment of the binding sites in synaptosomal fractions prepared from rat brain. Since local injection of kainic acid destroys neurons intrinsic to a brain region but spares glia and axons of extrinsic neurons (12–14), this technique was used to identify the association of receptors with neurons. Although six days after injection of kainate into the striatum [^{3}H]kainic acid receptor binding was virtually unaffected in spite of the complete degeneration of neurons intrinsic to the striatum, there was a 60% reduction in total specific binding of [^{3}H]-kainic acid and a comparable reduction in both the high and low affinity binding sites 28 days after the striatal kainate lesion. The delayed disappearance of the receptor binding sites does not preclude their localization on striatal intrinsic neurons and, in fact, is compatible with the ultrastructural evolution of the kainate lesion. In early stages after injection, in spite of the complete degeneration of intrinsic neurons, postsynaptic specializations, presumably sites of high receptor density, remain adherent to surviving terminal boutons of extrinsic neurons (14, 35). The postsynaptic densities as well as neuronal membranes phagocytized by Gitter cells are slowly cleared from the lesioned area (14). GABA receptors, like the [^{3}H]kainate binding sites, exhibit a delayed disappearance after striatal kainate lesions (36). Thus, on the basis of localization in brain, subcellular association with synaptosomes and vulnerability to selective neuronal degeneration, it is reasonable to conclude that the specific binding sites for [^{3}H]kainic acid are associated primarily with neurons.

It has been suggested that kainic acid is a specific agonist for excitatory glutamate receptors on neurons and that its marked potency results from the restricted cyclical structure that holds relevant portions of the molecule in a conformation favorable for fitting into glutamate recognition sites (4, 7). This hypothesis, however, is not entirely supported by other neurophysiologic data. In invertebrates, in which the physiology of agonist-receptor interactions can be examined under more controlled conditions, kainic acid has been found to be a rather weak agonist at excitatory glutamate receptor sites (8, 37). At the crustacean neuromuscular junction (38) and on certain neurons of Helix Aspersa (39), kainic acid appears to act at separate receptors that potentiate the neuroexcitatory action of glutamic acid. Studies by Shinozaki and Konishi (3) suggest this may also be the case in the mammalian cerebral cortex. In addition, the ionic requirements for kainate-induced neuronal depolarization differ from those of glutamate in the mammalian brain (40). Recently, Hall *et al.* (41) have shown that glutamate-induced excitation of rat cortical neurons can be antagonized specifically by drugs that are ineffective at blocking kainate-induced depolarization and conclude that they act at different receptors.

With the possible distinction between the neurophysiologic effects of kainic acid and glutamic acid kept in mind, it is important to consider whether the specific binding of [^{3}H]kainic acid to brain membranes may label a putative glutamate receptor. If the conformational rigidity of the kainic acid molecule, in particular the two carboxyl groups, confer its potent action at glutamate receptors, it is curious that reduction of the isopropylene side chain, which has no homology in the glutamate molecule, so dramatically attenuates its affinity for the receptor sites. It is possible, however, that

reduction of the double bond may have secondary effects on the conformation of the molecule. Although the heterocyclic compound, quisqualic acid, which is also a potent neuroexcitant (10), exhibits a relatively high affinity for the kainate receptor binding sites, another glutamate receptor agonist, ibotenic acid, is nearly inactive as a competitor. An important feature of glutamate's electrophysiologic action is the near equipotency between D- and L-glutamic acids (7). Although a certain degree of stereoselectivity of the receptor may be compensated by D-glutamate's lower susceptibility for various metabolic inactivation processes, it is difficult to rectify the 60- and 3,000-fold stereoselectivity of the low and high affinity kainate receptor binding sites for L-glutamate. Nevertheless, the receptors do exhibit significant specificity for the two carboxylic acid groups separated by a three carbon chain as evidenced by the negligible affinity for aspartate. Taken together, these characteristics of the high and low affinity binding sites for [^{3}H]kainic acid do not suggest that they represent a major receptor for L-glutamic acid, in agreement with the conclusion of Hall *et al.* (41); possibly these sites are a subset of glutamate receptors or are receptors for an unidentified endogenous substance containing L-glutamic acid.

Of particular interest is whether the receptor binding sites for [^{3}H]kainic acid mediate the neurotoxic action of this agent in brain. Kainate's neurotoxicity is a complex process and, at least in the striatum, requires the integrity of the afferent input (42, 43). Furthermore, *in vitro* (44) studies suggest that glutamate acts in a cooperative fashion with injected kainate to cause neuronal degeneration (43, 44). Thus, kainic acid does not appear to be a potent neurotoxin in and of itself, but involves the permissive effects of afferents, possibly glutamatergic. Destruction of striatal neurons by local injection of kainic acid results in a marked reduction in the binding sites for [^{3}H]kainic acid; this is consistent with the association of kainate receptors with neurons vulnerable to kainate. This interpretation is strengthened by the results of recent studies in the chick retina. Intra-ocular injection of kainate causes severe degeneration of neurons in the inner nuclear layer while sparing photoreceptor and ganglion cells; this lesion is associated with a 75% loss in the concentration of kainate receptors in retina, indicating a high degree of localization on vulnerable cells and a low concentration on insensitive cells.[2] Furthermore, neuronal sensitivity to the neurotoxic action of kainic acid in the striatum of the developing rat increases in association with increasing receptor binding of [^{3}H]kainic acid (45). Whether both high and low affinity receptor sites participate in the neurotoxic effects of kainic acid remains unclear; however, kainic acid is a potent and specific neurotoxin in cerebellum (15), a region which possesses only low affinity binding sites.

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REFERENCES

1. Ueno, Y., Nawa, H, Ueyanagi, J., Morimoto, H., Nakamori, R. & Matsuoka, T. (1955) Studies on the active components of Digenia simplex: Ag and related compounds. *J. Pharm. Soc. Japan* **75,** 807–811.
2. Murayama, K., Morimura, S., Nakamura, Y. & Sunagawa, G. (1965) Synthesis of pyrrolidine derivatives. (I) Synthesis of 2-decarboxykainic acid. *J. Pharm. Soc. Japan* **85,** 130–42.
3. Shinozaki, H. & Konishi, S. (1970) Actions of several anthelmintics and insecticides on rat cortical neurons. *Brain Res.* **24,** 368–371.
4. Johnston, G. A. R., Curtis, D. R., Davies, J. & McCulloch, R. M. (1974) Spinal interneurone excitation by conformationally restricted analogues of L-glutamic acid. *Nature, Lond.* **248,** 804–805.
5. McCulloch, R. M., Johnston, G. A. R., Game, C. J. A. & Curtis, D. R. (1974) The differential sensitivity of spinal interneurones and Renshaw cells to kainate and N-methyl-D-aspartate. *Exp. Brain Res.* **21,** 515–518.
6. Padjen, J. T. (1974) Pharmacology of acidic amino acids studied on isolated frog spinal cord. *Proc. 4th Annu. Meeting Soc. Neurosci.*

[2]K. Biziere and J. T. Coyle, submitted.

7. Buu, N. T., Puil, E. & Van Gelder, N. M. (1976) Receptors for amino acids in excitable tissues. *Gen. Pharmacol.* **7,** 5–14.
8. Constanti, A. & Nistri, A. (1976) A comparative study of the effects of glutamate and kainate on the lobster muscle fiber and the frog spinal cord. *Br. J. Pharmacol.* **57,** 359–368.
9. Polc, P. & Haefely, W. (1977) Effects of intravenous kainic acid, N-methyl D-aspartate, and (-) nuciferine on the cat spinal cord. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **300,** 199–203.
10. Biscoe, T. J., Evans, R. H., Headley, P. M., Martin, M. R. & Watkins, J. C. (1976) Structure-activity relations of excitatory amino acids on frog and rat spinal neurons. *Br. J. Pharmacol.* **58,** 373–382.
11. Shinozaki, H. & Shibuya, I. (1976) Effects of kainic acid analogues on crayfish opener muscle. *Neuropharmacol.* **15,** 145–147.
12. Olney, J. W., Sharpe, L. G. & de Gubareff, T. (1975) Excitotoxic amino acids. *Neurosci. Abstr.* **5,** 371.
13. Coyle, J. T. & Schwarcz, R. (1976) Model for Huntington's chorea: lesion of striatal neurons with kainic acid. *Nature, Lond.* **263,** 244–246.
14. Coyle, J. T., Molliver, M. E. & Kuhar, M. J. (1978) In situ injection of kainic acid: a new method for selectively lesioning neuronal cell bodies while sparing axons of passage. *J. Comp. Neurol.* **180,** 301–323.
15. Herndon, R. M. & Coyle, J. T. (1977) Selective destruction of neurons by a transmitter agonist. *Science* **198,** 71–72.
16. Curtis, D. R., Duggan, A. W., Felix, D., Johnston, G. A. R., Trebecis, A. K. & Watkins, J. C. (1972) Excitation of mammalian central neurons by acid amino acids. *Brain Res.* **41,** 283–301.
17. Zieglgänsberger, W. & Puil, E. A. (1975) Actions of glutamic acid on spinal neurons. *Exp. Brain Res.* **17,** 35–49.
18. Olney, J. W., Ho, O. L. & Rhee, V. (1972) Cytotoxic effects of acidic and sulfur containing amino acids on the infant mouse central nervous system. *Exp. Brain Res.* **14,** 61–76.
19. Schwarcz, R., Scholz, D. & Coyle, J. T. (1978) Structure activity relations for the neurotoxicity of kainic acid derivatives and glutamate analogues. *Neuropharmacol.* **17,** 145–151.
20. Simon, J. R., Contrera, J. F. & Kuhar, M. J. (1976) Binding of [^{3}H]kainic acid, an analogue of L-glutamate, to brain membranes. *J. Neurochem.* **26,** 141–147.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.
22. Glowinski, J. & Iversen, L. L. (1966) Regional studies of catecholamines in rat brain. I. The disposition of [^{3}H]-norepinephrine, [^{3}H]-dopamine and [^{3}H]-DOPA in various regions of the brain. *J. Neurochem* **13,** 655–669.
23. London, E. D. & Coyle, J. T. (1978) Pharmacologic augmentation of acetylcholine levels in kainate-lesioned rat striatum. *Biochem. Pharmacol.* **27,** 2962–2965.
24. Bull, G. & Oderfeld-Nowak, B. (1971) Standarization of a radiochemical assay of choline acetyltransferase and a study of the activation of the enzyme in rabbit brain. *J. Neurochem.* **19,** 935–947.
25. McGeer, E. G. & McGeer, P. L. (1976) Lesion of striatal neurones provides a model for Huntington's chorea. *Nature, Lond.* **263,** 517–519.
26. Enna, S. J. & Snyder, S. H. (1976) Influences of ions, enzymes and detergents on γ-aminobutyric acid binding in synaptic membranes of rat brain. *Mol. Pharmacol.* **13,** 443–453.
27. Jencks, W. P. (1978) *Catalysts in Chemistry and Enzymology.* McGraw Hill Book Co., New York, 568–588.
28. Haldeman, S. & McLennan, H. (1972) The antagonistic action of glutamic acid diethylester towards amino acid-induced and synaptic excitations of central neurons. *Brain Res.* **45,** 393–400.
29. Haldeman, S., Huffman, R. D., Marshall, K. C. & McLennan, H. (1972) The antagonism of the glutamate-induced and synaptic excitations of thalamic neurones. *Brain Res.* **39,** 419–425.
30. Satoh, M., Zieglgänsberger, W. & Herz, A. (1976) Supersensitivity of cortical neurones of the rat to acetylcholine and L-glutamate following chronic morphine treatment. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **293,** 101–103.
31. Cull-Candy, S. G., Donnelian, J. F., James, R. W. & Lunt, J. J. (1976) 2-Amino-4-phosphonobutyric acid as a glutamate antagonist on locust muscle. *Nature, Lond.* **262,** 408–409.
32. Birdsall, N. J. M. & Hume, E. C. (1976) Biochemical studies on muscarinic acetylcholine receptors. *J. Neurochem.* **27,** 7–16.
33. Campochiaro, P., Schwarcz, R. & Coyle, J. T. (1977) Characteristics and localization of the sodium-independent [^{3}H]GABA receptor site in rat striatum. *Brain Res.* **136,** 501–511.
34. Fillion, G. M., Rouselle, J. C., Fillion, M. P., Beaudoin, D. M., Goiny, M. R., Deniau, J. M. & Jacob, J. J. (1978) High-affinity binding of [^{3}H]5-hydroxytryptamine to brain synaptosomal membranes: comparison with [^{3}H]lysergic acid diethylamide binding. *Mol. Pharmacol.* **14,** 50–59.
35. Olney, J. W. & de Gubareff, T. (1978) The fate of synaptic receptors in the kainate-lesioned striatum. *Brain Res.* **140,** 340–343.
36. Zaczek, R., Schwarcz, R. & Coyle, J. T. (1978) Long-term sequelae of striatal kainate lesion.

Brain Res. **152,** 626–632.

37. Clements, A. N. & May, T. E. (1974) Pharmacological studies on a locust neuromuscular preparation. *J. Exp. Biol.* **61,** 421–442.
38. Shinozaki, H. & Shibuya, I. (1974) Potentiation of glutamate-induced depolarization by kainic acid in the crayfish opener muscle. *Neuropharmacol.* **13,** 1057–1065.
39. Walker, R. J. (1976) The action of kainic acid and quisqualic acid on the glutamate receptors of three identifiable neurones from the brain of the snail Helix aspersa. *Comp. Biochem. Physiol.* **55C,** 61–67.
40. Evans, R. H., Francis, A. H. & Watkins, J. C. (1977) Effects of monovalent cations on the responses of motorneurons to different groups of amino acid excitants in frog and rat spinal cord. *Experientia* **33,** 246–248.
41. Hall, J. G., Hicks, T. P. & McLennan, H. (1978) Kainic acid and the glutamate receptor. *Neurosci. Letts.* **8,** 171–175.
42. McGeer, E. G., McGeer, P. L. & Singh, K. (1978) Kainate-induced degeneration of neostriatal neurons: dependency upon cortico-striatal tract. *Brain Res.* **139,** 381–383.
43. Biziere, K. & Coyle, J. T. (1978) Influence of cortico-striatal afferents on striatal kainic acid neurotoxicity. *Neurosci. Letts.* **8,** 303–310.
44. Biziere, K. & Coyle, J. T. (1978) Effects of kainic acid on ion distribution and ATP levels of striatal slices incubated *in vitro*. *J. Neurochem.* **31,** 513–520.
45. Campochiaro, P. & Coyle, J. T. (1978) Ontogenetic development of kainate neurotoxicity: correlates with glutamatergic innervation. *Proc. Nat. Acad. Sci.* **75,** 2025–2029.