GAMMA-AMINOBUTYRIC ACID AND GLUTAMIC ACID IN HUNTINGTON'S DISEASE:
INVESTIGATION OF NEUROTRANSMITTER RECEPTORS USING
RADIOLABELED AGONISTS

by

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I hereby recommend that this dissertation prepared under my direction by Kevin Beaumont entitled \textit{Gamma-Aminobutyric Acid and Glutamic Acid in Huntington's Disease: Investigation of Neurotransmitter Receptors Using Radiolabeled Agonists} be accepted as fulfilling the dissertation requirement for the degree of Doctor of Philosophy.

As members of the Final Examination Committee, we certify that we have read this dissertation and agree that it may be presented for final defense.

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SIGNED:  [Signature]
To Lorraine
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ABSTRACT

$^3$H-Muscimol, a structural analog of $\gamma$-aminobutyric acid (GABA), binds to rat brain synaptic membranes in a saturable manner, with a high affinity dissociation constant of 2.2 nM. Binding is increased in membranes that have been treated with Triton X-100 and is not dependent upon sodium. High affinity $^3$H-muscimol binding is regionally distributed, with highest binding density in the cerebellum > cerebral cortex > hippocampus, brainstem, midbrain, hypothalamus > pons-medulla, spinal cord. $^3$H-Muscimol binding is most highly concentrated in the synaptic membrane fraction. These properties are consistent with those reported for sodium-independent $^3$H-GABA binding to brain GABA receptors. $^3$H-Muscimol is displaced from synaptic membranes only by compounds which interact in neurophysiological studies with GABA receptors, indicating that the effects of muscimol result from activation of GABA receptors.

$^3$H-Kainic acid, a structural analog of L-glutamic acid, binds to rat brain membranes in a saturable manner with a dissociation constant of 5 nM. $^3$H-Kainic acid binding is regionally distributed with highest density in the striatum > hippocampus > cerebellum, cerebral cortex > midbrain, pons-medulla. $^3$H-Kainic acid is displaced potently by L-glutamate (IC$_{50}$ = 0.12 $\mu$M), although physiological antagonists of L-glutamate-induced excitations are ineffective at inhibiting $^3$H-kainic acid binding. Compounds related to L-aspartic acid are also very weak inhibitors of $^3$H-kainic acid binding, suggesting that
excitatory amino acid receptors exist that are distinct from the sites mediating kainic acid-induced depolarizations. Several cations effectively displace $^3$H-kainic acid binding, divalent cations being more potent in this respect than monovalent cations.

Injection of kainic acid into the rat striatum has been shown to produce alterations that are neurochemically and histologically similar to those occurring in Huntington's Disease (HD), as intrinsic striatal neurons are destroyed while afferent terminals and axons of passage are unaffected. Following kainic acid lesion, $^3$H-kainic acid binding undergoes a slow decrease over a period of 48 days. At 30 days after lesion, $^3$H-muscimol binding density in lesioned striatum is not significantly different than in control striatum. These results indicate that kainic acid binding sites are located on neurons originating in the striatum, while striatal GABA receptors appear to be localized on endings of neuronal afferents originating outside the striatum or on glia.

The density of $^3$H-kainic acid and $^3$H-muscimol binding was determined in regions of HD and control human brains. $^3$H-Kainic acid binding density is significantly reduced from control in HD caudate nucleus (-55%) and putamen (-53%), but not in HD cerebellum, frontal cortex, or globus pallidus. $^3$H-Muscimol binding density, determined using brain membranes that had been treated with Triton X-100 to remove endogenous inhibitors of GABA receptor binding, was significantly decreased compared to control in HD caudate nucleus (-36%) and putamen (-33%) but not in HD frontal cortex.
CHAPTER 1

INTRODUCTION

The amino acids gamma-aminobutyric acid (GABA) and glutamic acid appear to function as neurotransmitters in the vertebrate central nervous system (Curtis and Johnston, 1974). Muscimol and kainic acid are heterocyclic analogs of these amino acids which have recently been isolated from natural sources. The physiological and neurochemical changes following iontophoresis or injection of these analogs indicate that they mimic GABA and glutamic acid at the neuronal receptors for these amino acids. Recently, techniques have been developed for directly studying neurotransmitter receptors through the binding of radiolabeled compounds (Yamamura, Enna, and Kuhar, 1978). Using these techniques, we have investigated the binding of radiolabeled muscimol and kainic acid to brain tissue, both in order to verify the site of action of these compounds and to describe GABA and glutamic acid receptors.

In Huntington's Disease (HD), a hereditary neuropsychiatric disorder, a prominent neurochemical feature is the degeneration of GABA-synthesizing neurons of the basal ganglia (Shoulson and Chase, 1975). Injection of kainic acid into the rat striatum produces a similar destruction of GABAergic neurons, and additionally produces other neurochemical alterations characteristic of HD (Coyle et al., 1977). Therefore, kainic acid-induced striatal degeneration may be
useful as an animal model for HD. Because of the reduced striatal GABA levels, treatment with a GABA agonist, possibly muscimol, is often cited as a potential therapy. Because of the alterations in GABA-synthesizing neurons in HD and the similarities with kainic acid-induced degeneration, we have used $^3$H-muscimol and $^3$H-kainic acid binding to investigate amino acid receptors in HD and its animal model.

The approach taken here in studying the binding of radiolabeled GABA and glutamic acid analogs is based on the concept that these amino acids function as neurotransmitters, and therefore act at specific neuronal receptors. The criteria used to identify central neurotransmitters have been described by Werman (1966). Briefly, the properties which identify a compound as a neurotransmitter are its presence within neurons along with synthetic enzymes, existence of an inactivating enzyme or uptake system to prevent extracellular accumulation, release from neurons coincident with depolarization, an action upon neuronal membrane excitability and ion flux which is identical to the action of a neuronally-released transmitter, and pharmacological identity with the endogenous transmitter, that is, sensitivity to the same antagonists and potentiating agents. The extent to which GABA and glutamic acid fulfill these criteria has been reviewed in detail by Curtis and Johnston (1974), Krnjevic (1974), and Davidson (1976). The evidence that indicates these amino acids are neurotransmitters will be considered in the following sections, along with the experimental evidence implicating muscimol and kainic acid as GABA and glutamate agonists.
GABA as a Neurotransmitter

GABA functions as a neurotransmitter in both vertebrate and invertebrate nervous systems. Work implicating GABA as a neurotransmitter began in 1954, when Florey extracted a heat-stable substance from mammalian brain which had a depressant action upon excitable tissues (Florey, 1954). This substance was later shown to be GABA, which had been isolated from mammalian brain 4 years earlier (Roberts and Frankel, 1950). Subsequent work has shown that GABA fulfills all of the criteria that have been developed for identifying a substance as a central neurotransmitter.

Distribution

In vertebrates, GABA is located almost exclusively in the central nervous system. The free concentration of GABA varies from 1 to 10 μmole/gram tissue among brain regions of the rat. Highest levels occur in the substantia nigra (10 μmole/gm) followed by the globus pallidus and hypothalamus (8 μmole/gm). Intermediate levels occur in the cerebellum and cerebral cortex (3 μmole/gm) while lowest GABA concentrations are found in white matter (1 μmole/gm) (Fahn, 1976).

Synthesis

GABA is synthesized by decarboxylation of L-glutamic acid, catalyzed by glutamic acid decarboxylase (GAD). Pyridoxal-5'-phosphate is a required cofactor for GAD activity, and substances that lower pyridoxal phosphate levels, such as the hydrazides, interfere with GABA synthesis and cause convulsions (Davidson, 1976). Immunohistochemical studies indicate that GAD is associated exclusively with neuronal
endings (Saito, 1976). However, glioma and peripheral tissues de-carboxylate glutamic acid, though this activity proceeds at a lower rate than in neuronal tissue, and is affected differently by inhibitors than in neuronal GAD activity. Whether this GAD activity is catalyzed by a distinct "GAD II" or is the result of artifacts in the assay procedure is still unclear. Neuronal GAD has a regional distribution similar to that of GABA (Fahn, 1976), and is frequently used in lesion studies as a marker for GABAergic nerve endings.

Mechanism of Inactivation

A mechanism must exist for rapid inactivation of a neurotransmitter if discrete frequency-coded signals are to be generated. For GABA, uptake and enzymatic systems are present in nervous tissue to insure rapid removal and metabolism of extracellular GABA. Slices from various regions of rat brain have both high and low affinity uptake systems for GABA, with Kms of approximately 10-20 µM and 400 µM, respectively (Iversen and Neal, 1968; Bond, 1973). The low affinity system is distributed fairly evenly throughout the rat brain. The high affinity system shows a 5-fold regional variation in maximal velocity, with highest transport velocities present in the hypothalamus, cerebral cortex, and hippocampus, and lowest velocities in the medulla-pons and cerebellum (Bond, 1973; Iversen and Johnston, 1971). The high affinity GABA transport system requires sodium ions, which, along with high affinity, is a consistent feature of transmitter-related uptake systems (Bennett, Mulder, and Snyder, 1974). The high affinity sodium-dependent transport of GABA consists of both glial and neuronal systems,
with the neuronal system predominating in the central nervous system (Iversen and Kelly, 1975). The neuronal transport system, which appears to be located specifically on GABAergic cell bodies and terminals, is more sensitive to inhibition by L-2,4-diaminobutyric acid (DABA) than is the glial transport system. Glial uptake, on the other hand, is specifically inhibited by β-alanine. (-)Nipecotic acid is a particularly potent inhibitor of GABA transport into rat brain slices, while itself being a very weak depressant of neuronal firing (Johnston, 1976). GABA is metabolized by transamination with α-ketoglutarate, catalyzed by the enzyme GABA-transaminase (Davidson, 1976).

Release

The release of endogenous GABA in vivo into the fourth ventricle has been demonstrated following electrical stimulation of cerebellar Purkinje fibers that innervate the lateral vestibular (Deiter's) nucleus, which is situated adjacent to the fourth ventricle (Obata and Takeda, 1969). GABA is released by brain slices incubated in vitro following depolarization by K⁺ (Mulder and Snyder, 1974). The release from neuronal and synaptosomal compartments is Ca²⁺-dependent (Sellstrom and Hamberger, 1977). Exogenous and radiolabeled GABA is also released by glial cells of dorsal root ganglia following exposure to elevated K⁺ concentrations, but this glial release is not Ca²⁺-dependent (Munchin, 1974).

Identity of Action

The most prevalent type of inhibitory post-synaptic potential (IPSP) recorded within the mammalian central nervous system is generated
by an increase in membrane conductance to Cl\(^-\) (Eccles, 1969). This increased Cl\(^-\) conductance clamps the membrane potential near the equilibrium potential for Cl\(^-\), which is, in most cases, slightly negative to the resting potential (Krnjevic, 1976). Iontophoretic application of GABA at sites throughout the central nervous system depresses neuronal firing rates. GABA raises the membrane conductance in the cerebral cortex and other areas (Krnjevic and Schwartz, 1967a), and studies of the reversal potential indicate that the increased conductance is to Cl\(^-\) ions (Krnjevic, 1976). GABA receptors appear to be located on the outer surface of neuronal membranes, since intracellular injection of GABA does not produce inhibition (Krnjevic, 1976) and glial membranes are not hyperpolarized by GABA (Krnjevic and Schwartz, 1967b).

**Pharmacological Identity**

Several naturally-occurring amino acids other than GABA produce neuronal inhibition, including glycine, taurine, and \(\beta\)-alanine (Curtis and Watkins, 1960). In order to identify GABA as the endogenous inhibitory neurotransmitter at a particular synapse, antagonists which specifically block GABA-mediated inhibition are required. The inhibitory actions of GABA upon vertebrate neurons are blocked by several convulsant compounds, the most widely investigated being bicuculline and picrotoxin. Both of these compounds block evoked inhibitory postsynaptic potentials (IPSPs) as well as GABA-induced neuronal inhibition in several brain areas (Curtis and Johnston, 1974). Glycine-induced inhibitions, on the other hand, are blocked by the convulsant
strychnine, but not by bicuculline in concentrations which antagonize the actions of GABA (Johnston, 1976). Therefore, sensitivity to bicuculline and insensitivity to strychnine are characteristics used to identify synapses at which IPSPs are produced by GABA (Curtis et al., 1971). Such synapses are located throughout the central nervous system, while bicuculline-insensitive, strychnine-sensitive IPSPs, which are probably produced by glycine, are located primarily in the spinal cord. At several sites, GABA-induced inhibition is not antagonized by bicuculline, indicating the possible existence of bicuculline-insensitive GABA receptors (De Feudis, 1977). However, bicuculline is only sparingly soluble in aqueous media and is unstable under physiological conditions (Olsen et al., 1975), therefore technical difficulties may account for the variability in inhibitory potency reported in different studies. Bicuculline is also a weak acetylcholinesterase inhibitor and neuronal excitant (Johnston, 1976). However, these effects are produced equally by the (+) and (-) stereoisomers, while convulsant activity and antagonism of GABA are produced selectively by the (+) stereoisomer of bicuculline. $^3$H-GABA binding to rat brain membranes is displaced stereospecifically by (+)bicuculline (Enna, Collins, and Snyder, 1977). Picrotoxin, which does not displace $^3$H-GABA binding, appears to inhibit the chloride ionophore associated with the GABA receptor (Ticku et al., 1978).

Several structural analogs of GABA are potent bicuculline-sensitive inhibitors of neuronal firing. Muscimol is one of the most potent GABA-mimetics yet studied (Curtis et al., 1971). Imidazole-4-acetic acid, 3-aminopropanesulfonic acid, and trans-4-aminocrotonic acid
are all potent GABA agonists (Bowery, 1976; Johnston, 1976; Nicoll, 1977). Trans-3-aminocyclopentane-1-carboxylic acid is a strong GABA-mimetic, while its cis-isomer is less potent. β-guanidinopropionic acid is a somewhat weaker GABA-mimetic (Johnston, 1976). These compounds are useful in verifying that the receptor site measured in binding assays is pharmacologically similar to the receptor in vivo.

GABA Receptors

GABA receptors have been studied directly through binding studies using $^3$H-GABA and, recently, through the binding of $^3$H-bicuculline (Mohler and Okada, 1977). Early studies of GABA binding to brain in the presence of sodium represented binding to uptake sites (Varon et al., 1965), since the affinity of several compounds for the binding site paralleled their ability to block GABA uptake rather than their potency as neuronal depressants. Zukin, Young, and Snyder (1974) reported that $^3$H-GABA binds to rat brain synaptic membranes in the absence of sodium. Inhibition of this sodium-independent binding by various inhibitory amino acids paralleled their GABA-mimetic potency as determined in iontophoretic studies and did not correlate with their ability to inhibit GABA transport (Enna and Snyder, 1975). Sodium-independent GABA binding is inhibited by (+) but not (-)bicuculline and is enriched in synaptosomal membrane fractions of rat brain. It has a varied regional distribution, with highest levels in the cerebellum and lowest in the brain stem and spinal cord. Thus, in the absence of sodium, $^3$H-GABA appears to bind to a synaptic receptor which mediates GABA-induced neuronal inhibition.
The properties of sodium-independent $^3$H-GABA binding have been studied by several investigators in rat (Enna and Snyder, 1977; Wong and Horng, 1977; Krogsgaard-Larsen and Johnston, 1978; De Feudis, 1977), mouse (Olsen et al., 1978), monkey (Enna, Kuhar, and Snyder, 1975), and human brain (Lloyd, Shemen, and Hornykiewicz, 1977; Enna, Bennett et al., 1977). An important finding by several investigators (Enna and Snyder, 1977; Wong and Horng, 1977) is that treatment of brain membranes with the nonionic detergent Triton X-100 substantially increases the affinity of sodium-independent GABA binding while destroying sodium-dependent GABA binding to transport sites. Therefore, binding data can be more accurately determined using Triton treated membranes. This effect of Triton has been attributed to exposure of high affinity binding sites and to removal of endogenous inhibitors of GABA binding. It has been recently demonstrated that a washing procedure involving extensive freezing and thawing of the tissue has an effect similar, though less pronounced, to that of Triton in increasing the affinity of sodium-independent GABA binding (Greenlee, Van Ness, and Olsen, 1978). After these treatments, both high affinity (Kd = 20 nM) and low affinity (Kd = 150 nM) $^3$H-GABA binding sites are present. Both of these sites have pharmacological characteristics expected of neuronal GABA receptors (Enna and Snyder, 1977).

**Muscimol**

Muscimol is a psychoactive compound isolated from the mushroom, *Amanita muscaria*, by Muller and Eugster (1965) and from *Amanita stabiliformis* by Takemoto, Nakajima, and Sakuma (1964). Its structure
is 3-hydroxy-5-aminomethylisoxazole (Brehm, Hjeds, and Krogsgaard-Larsen, 1972), and it is a conformationally restricted analog of GABA (Fig. 1). In man, oral administration of muscimol produces a decrease in ability to estimate elapsed time, dysphoria, depersonalization, muscle twitches, involuntary movements, and sleep alterations (Waser, 1967; Theobald et al., 1968).

Fig. 1. Structures of GABA and muscimol.
Microiontophoretic studies have shown muscimol to be a potent neuronal depressant at bicuculline-sensitive, strychnine-insensitive sites (Curtis et al., 1971; Johnston et al., 1968). Muscimol is also a potent inhibitor of Na\(^+\)-independent \(^3\)H-GABA binding to rat brain membranes (Enna and Snyder, 1977). Muscimol is relatively ineffective at blocking GABA uptake by rat brain slices (Johnston, 1971).

The neurochemical alterations following peripheral injection of muscimol indicate that it has central GABA-mimetic effects. Peripheral injection of muscimol in the rat prevents the seizures and the increase in cerebellar cyclic GMP which result when GABA synthesis is blocked, but is less effective in preventing strychnine-induced seizures (Naik, Guidotti, and Costa, 1976). However, muscimol injection directly into the rat substantia nigra produces circling contralateral to the injected nigra (Scheel-Kruger, Arnt, and Magelund, 1977; Oberlander, Dumont, and Boissier, 1977) which is opposite to the direction predicted from the action of GABA in inhibiting nigrostriatal dopaminergic neurons (Tarsy et al., 1975).

Muscimol has complex interactions with dopaminergic systems, depending on the location of injection. Injection of muscimol into the substantia nigra prevents the activation of striatal tyrosine hydroxylase by haloperidol (Gale and Guidotti, 1976). Bilateral injection of muscimol into the substantia nigra produces behavioral stimulation and stereotypy, and antagonizes catalepsy induced by peripheral neuroleptic injection (Scheel-Kruger, Arnt, and Magelund, 1977). However, bilateral injection of muscimol into the nucleus accumbens produces catalepsy and blocks the locomotor activity induced by a dopamine agonist, ergometrine.
In studies involving central injection, GABA itself is far less potent and has more short-lasting effects than muscimol (Scheel-Kruger, Arnt, and Magelund, 1977), probably due to rapid inactivation by transport. Therefore, muscimol is being increasingly used as a tool for investigating central GABA effects. Because of the inhibition of dopaminergic neurons produced by muscimol at some sites, it has been proposed as a potentially useful neuroleptic potentiating agent for the treatment of schizophrenia (Van Kammen, 1977). Therefore, the precise determination of muscimol's site of action is necessary.

**Glutamate as a Neurotransmitter**

The evidence that glutamate is a neurotransmitter in vertebrates, while less compelling than that supporting such a role for GABA, is nevertheless fairly strong. Hayashi (1954) was the first to demonstrate that L-glutamate has a powerful excitatory action when applied to the cerebral cortex. The development of microiontophoretic techniques allowed the identification of several dicarboxylic amino acids that have neuroexcitatory effects similar to glutamate (Curtis, Phillis, and Watkins, 1960). Neuronal depolarization in response to glutamate and other dicarboxylic amino acids has since been observed in practically every region of the CNS and in several vertebrate species (Curtis and Johnston, 1974). L-Glutamate and L-aspartate occur in the CNS at much higher concentrations than other excitatory dicarboxylic amino acids, and have therefore been most extensively examined as potential...
neurotransmitters. The evidence supporting such a role for L-glutamate will be briefly summarized.

Distribution

The concentration of L-glutamate acid in the mammalian brain is higher than that of any other amino acid. In rat brain, the concentration of L-glutamate varies from 8 to 13 μmoles/gram tissue (Liebschutz et al., 1977). The highest levels are present in the amygdala, cerebral cortex, and thalamus, while the lowest levels occur in the pons and medulla. At first, the high concentration and relatively even distribution of glutamate were invoked as evidence that glutamate was not a neurotransmitter, as transmitters were thought to exist in small quantities distributed unevenly throughout brain structures, depending upon innervation by nerve endings utilizing the transmitter. However, unlike the more classical neurotransmitters upon which these generalizations were based, glutamate functions as an intermediate in the metabolic reactions of brain and peripheral tissue. Glutamate is involved in numerous transamination reactions, ammonia metabolism, the formation of GABA, and the formation of peptides and proteins. Therefore, a large percentage of brain glutamate may not function in neurotransmission. At least two metabolic pools of glutamate exist in the brain. A large, heterogeneous pool is apparently associated with neuronal cell body metabolism and includes neurotransmitter glutamate, while a smaller, rapidly turning-over pool appears to be associated with glia (Van den Berg, 1974). Thus, although glutamate is present in nerve endings, analysis of its distribution is unlikely to provide
clear information concerning the location of neurotransmitter glutamate.

Synthesis

The major source of brain glutamate is synthesis from α-ketoglutarate and ammonia by glutamic dehydrogenase. Glutamate can also be formed through the transamination of α-ketoglutarate with various amino acids, including GABA. In addition, glutamate can be formed through the deamination of glutamine by glutaminase. Since the blood-brain barrier is fairly impermeable to glutamate, glutamate is synthesized almost entirely within the brain from α-ketoglutarate derived from glucose metabolism. Due to the presence of several pools of glutamate, none of the enzymes involved in the synthesis of glutamate is localized specifically within neurons. Therefore, the synthetic enzymes are not useful markers for the identification of glutamatergic neuronal tracts. In this regard, neither glutamate dehydrogenase nor glutaminase activity in the hippocampus is reduced after lesion of putative glutamatergic afferents to this area (Nadler, White, et al., 1978).

Mechanism of Inactivation

Transport systems represent the primary mechanism for inactivating glutamate released into the synaptic cleft. In support of this view, inhibitors of glutamate transport prolong the neuroexcitatory effects of iontophoretically-administered glutamate, while inhibitors of glutamate-metabolizing enzymes do not have this potentiating action (Curtis, Duggan, and Johnston, 1970). At least two transport systems
for glutamate exist in mammalian brain. A low affinity system (Km = 1 mM) and a high affinity system (Km = 20 μM) are present in synaptosomes prepared from rat cerebral cortex (Logan and Snyder, 1972). The high affinity system requires sodium, a property that is characteristic of the high affinity transport systems of other neurotransmitters. A low affinity and a sodium-dependent high affinity transport system are also present in glia, since isolated dorsal root ganglia take up radio-labeled glutamate by both processes (Roberts and Keen, 1974), and following uptake the radioactivity is localized exclusively within satellite glial cells (Schon and Kelley, 1974).

It has been proposed that glia is the major site of uptake and metabolism of glutamate, since exogenous glutamate is rapidly converted to glutamine by glutamine synthetase, an enzyme that has been shown by immunohistochemistry to exist predominantly within the glial cells (Martinez-Hernandez, Bell, and Norenberg, 1977). It has been suggested that transport into synaptosomal preparations in vitro represents uptake into vesicularized glial membranes (gliasomes), which contaminate such preparations. However, destruction of at least three putative glutamatergic neuronal tracts results in major reductions in high-affinity glutamate uptake in the areas innervated by these tracts. Destruction of afferents to the striatum originating in the cortex (Divac, Ponnun, and Storm-Mathisen, 1977), of afferents to the hippocampus originating in the entorhinal cortex (Nadler, White et al., 1978), and of cerebellar granule cells (Snyder et al., 1974) results in at least a 50% reduction of glutamate uptake in the areas innervated. It therefore appears that glutamate is inactivated by uptake into both nerve terminals and glia,
a mechanism similar to that which inactivates GABA. After uptake into glia, glutamate is rapidly converted to glutamine, which may diffuse or be transported into nerves for use in the synthesis of aspartate, GABA, and glutamate (Bradford and Ward, 1976).

Release

The release of glutamate within the CNS has been difficult to demonstrate, possibly due to the widespread presence of active glutamate transport systems. However, several investigators have demonstrated the release of glutamate in vitro from brain tissue. Glutamate is released in a Ca\textsuperscript{++}-dependent manner by electrical stimulation or K\textsuperscript+-induced depolarization of cortical synaptosomes (de Belleroche and Bradford, 1972) and of brain slices (Benjamin and Quastel, 1973). The Ca\textsuperscript{++}-dependent, but not Ca\textsuperscript{++}-independent, release of glutamate from brain slices is blocked by tetrodotoxin, which strongly suggests that this release is associated with the depolarization of neurons within the tissue slice (Potashner, 1978). Ca\textsuperscript{++}-dependent release of glutamate from hippocampal slices in vitro is reduced by 50\% after lesion of putative glutamatergic inputs from the entorhinal cortex, suggesting that at least a good portion of Ca\textsuperscript{++}-dependent release in vitro is from glutamatergic nerve-terminals (Nadler, White et al., 1978). The release of glutamate, along with other neurotransmitters, has been observed in vivo from the surface of the rat gracile and cuneate nuclei following stimulation of dorsal column or medial lemniscal afferents (Roberts, 1974a).
Identity of Action

Glutamate depolarizes most neurons in the central nervous system (Curtis and Johnston, 1974), which has led to the idea that it is a non-specific depolarizing agent. However, this idea is based on a generalization from the classical neurotransmitters, such as acetylcholine and norepinephrine, which are less widely distributed than the amino acid transmitters. Glutamate appears to produce depolarization through an action at synapses, since it does not depolarize glia (Krnjevic and Schwartz, 1967b) or neurons without synapses, such as are present in dorsal root ganglia (Nishi, Soeda, and Kokitsu, 1965). The properties of glutamate-induced depolarization have been studied and compared with the properties of synaptic depolarization caused by stimulation of excitatory neuronal inputs. Like the endogenous excitatory transmitter, glutamate produces depolarization with rapid onset and cessation, and is associated with an increased membrane conductance (Krnjevic, 1974). The reversal potential of 0 to -30 mV is more positive than the reversal potential of synaptically-evoked excitatory postsynaptic potentials (EPSPs). This discrepancy has been attributed to technical difficulties which prevent the measurement of reversal potentials within dendrites, where excitatory synapses are located (Curtis et al., 1972). Membrane depolarization evoked by both glutamate and the endogenous transmitter appear to be caused by increases in both Na\(^+\) and K\(^+\) permeability. Neither depolarization induced by glutamate nor synaptically-evoked EPSPs are blocked by tetrodotoxin, and are therefore not mediated by the same Na\(^+\) channels involved in propagation of action potentials (Curtis et al., 1972).
A major impediment in identifying glutamate with an endogenous excitatory transmitter is the nearly identical actions of L-aspartate and other dicarboxylic amino acids, which also may function as neurotransmitters in the vertebrate CNS. In several brain areas, DL-homocysteate and L-cysteate are more potent, while L- and D-aspartate are similar in potency to L-glutamate (Curtis and Watkins, 1963). While some investigators have found D-glutamate to be only slightly less potent than the L-isomer (Curtis and Watkins, 1963), others have found the D-isomer to be considerably less potent, at least in the cat cerebral cortex (Krnjevic and Phillis, 1963). However, it is difficult to directly compare the potencies of these amino acids from iontophoretic experiments alone because of their widely varying affinities for transport systems. For example, some potent excitants, such as D-homocysteate and D-glutamate, are poor substrates for the high-affinity L-glutamate uptake system (Balcar and Johnston, 1972), and therefore may diffuse over a wider area and depolarize the membrane at a greater number of sites than L-glutamate. For other amino acids which are potent inhibitors of L-glutamate uptake, such as cysteine-sulphinic acid, an increase in the concentration of glutamate at the synaptic cleft may contribute to their neuroexcitatory potency.

Certain synthetic amino acids, most notably N-methyl-D-aspartate, are very effective neuronal depolarizing agents. In addition, several cyclic alkaloids isolated from natural sources are considerably more potent than L-glutamate in causing depolarization. These include kainic, domoic, ibotenic, and quisqualic acids (Biscoe et al., 1976). It has been suggested that the high potency of these
natural compounds, which are structural analogs of glutamate, is due to their restriction in a favorable conformation for activation of glutamate receptors, as well as their low affinity for transport sites (Buu, Puil, and Van Gelder, 1976).

Pharmacological Identity

The identification of glutamate and other excitatory amino acids with endogenous neurotransmitters has long been hampered by the absence of specific antagonists. Of the many compounds that have been investigated, L-glutamic acid diethylester (L-GDEE) is the most consistent and specific antagonist of glutamate-induced excitations. In addition to blocking depolarization produced by iontophoretically-applied glutamate, GDEE blocks the depolarization of striatal cells resulting from stimulation of excitatory afferents originating in the cortex (Spencer, 1976). GDEE also blocks the depolarization of thalamic neurons caused by stimulation of afferents coursing through the brachium conjunctivum (Haldeman et al., 1972). While some investigators find that GDEE does not block depolarization in response to acetylcholine, Curtis et al. (1972) have reported that GDEE blocks acetylcholine-induced depolarization of some central neurons. In addition, GDEE blocks aspartate- and homocysteate-induced depolarization, although McLennan (1974) reports that its antagonism of these amino acids is relatively weak compared to its effectiveness against L-glutamate. GDEE is therefore probably not useful in differentiating excitatory amino acid receptor populations.
Other compounds that have been proposed as excitatory amino acid antagonists (McLennan, 1975) include L-methionine-DL-sulfoximine (Curtis et al., 1972), 1-hydroxy-3-aminopyrrolid-2-one (HA-966) (Davies and Watkins, 1973), D-lysergic acid diethylamine (LSD) (Boakes et al., 1970), and 2-methoxyaporphine (Curtis et al., 1972). Recently, Bisce et al. (1977) have demonstrated that α-aminoadipate, which slightly potentiates the action of L-glutamate, may be a selective antagonist of aspartate-induced depolarization.

To date, agonists have been more useful than antagonists in differentiating glutamate receptors from other excitatory amino acid receptors. Topographical variations of neuronal sensitivity to glutamate in comparison with aspartate or homocysteate occur within at least three areas of the CNS. In the lateral geniculate nucleus, a relay station in the visual system, cells receiving input from the center of the visual field are more sensitive to glutamate than to aspartate, while the reverse sensitivity holds for cells with peripheral receptive fields (Morgan, Vrbova, and Wolstencraft, 1972). In the thalamus, neurons in the ventrolateral region are more sensitive to L-glutamate relative to DL-homocysteate than are more dorsally located neurons (Haldeman et al., 1972). In the spinal cord, Renshaw cells are slightly more sensitive to aspartate than to glutamate, while the reverse is true for dorsal spinal interneurons (Duggan, 1974). Spinal Renshaw cells and dorsal interneurons have a much greater difference in sensitivity to excitation by N-methyl-D-aspartate and kainic acid (McCulloch et al., 1974). This experimental finding, as well as analysis of the conformation of these compounds (Buu et al., 1976),
suggests that N-methyl-D-aspartate preferentially activates aspartate receptors, while kainic acid is more potent at glutamate receptors. This proposal is supported by the greater antagonism of α-aminoadipate against N-methyl-D-aspartate than against kainic acid (Biscoe et al., 1977).

**Glutamate Receptors**

Very few direct binding studies have been carried out on vertebrate glutamate receptors. De Robertis and Fiszer de Plazas (1976) have isolated a hydrophobic protein fraction by chromatography of chloroform-methanol extracts of synaptic membranes on a sephadex column equilibrated with chloroform. After incubation with labeled amino acids, the protein fractions are collected by rechromatography on sephadex columns and elution with chloroform. With this procedure, aspartate-binding to proteins is displaceable by N-methyl-D-aspartate, while glutamate-binding is displaced by kainic acid. However, the hydrophobic conditions required for collecting the proteins make extrapolation to aqueous physiological conditions difficult.

Roberts (1974b) determined the binding of C\textsuperscript{14}-glutamate to synaptic membranes by a filtration method, and found two kinetically distinct binding sites. In the presence of Na\textsuperscript{+} and N-methyl-D-aspartate to block amino acid receptor sites, glutamate bound to a putative uptake site, with a K\textsubscript{d} of 4 μM. In the absence of Na\textsuperscript{+}, glutamate bound to a putative receptor site with a K\textsubscript{d} of 8 μM. Both sites were present at a considerably greater density than is usual for central neurotransmitter receptors.
The most promising method to date for directly examining the glutamate receptor is through binding of $^3$H-kainic acid (Simon, Contrera, and Kuhar, 1976). These investigators reported that $^3$H-kainic acid binds to rat brain synaptic membranes with high affinity, and is displaceable by L-glutamate. These results will be discussed further in the next section.

**Kainic Acid**

Kainic acid was first isolated from the red marine alga, *Digenea simplex*, by Murakami et al. (1953). Its structure is 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (Fig. 2). In the naturally occurring compound, L-α-kainic acid, the 2-carboxy and 3-carboxymethyl groups are trans-oriented, and the 3-carboxymethyl and 4-isopropenyl groups have a cis orientation. The other available stereoisomers of kainic acid are relatively inactive, as is dihydrokainic acid, the analog in which the isopropenyl side-chain is reduced to an isopropyl moiety (Nadler, in press). Kainic acid has been in use for several years in Japan for the treatment of Ascariasis.

Shinozaki and Konishi (1970) first discovered the neuroexcitatory effects of kainic acid while investigating the actions of several anthelmintics and insecticides that contain the structure of glutamate. They reported that L-α-kainic acid was more powerful than L-glutamate when applied to rat cortical neurons, in terms of the frequency of spike discharges produced by electrophoretic currents of equal strengths. The cessation of discharge following kainic acid application was much more delayed than cessation following glutamate
Fig. 2. Structures of glutamic acid and kainic acid.
application, suggesting that kainic acid is less rapidly removed by transport systems, or that its dissociation from the active site is slower. Shinozaki and Konishi (1970) also reported that a subthreshold dose of kainic acid increased the slope of the dose response curve for glutamate-induced spike discharges, whereas subthreshold doses of L-glutamate, ibotenic acid, or L-α-allo-kainic acid shifted the dose response curve to the left. A leftward shift would be expected for substances acting in an additive manner, while the change in slope produced by kainic acid suggests that, unlike the other excitatory compounds, it somehow potentiates the action of L-glutamate. When large amounts of kainic acid were applied, spike amplitude decreased and eventually discharge ceased, probably due to blockade following persistent depolarization, similar to that observed in response to excess glutamate. Other investigators have confirmed the potency of kainic acid in depolarizing spinal neurons of cat (Johnston et al., 1974), frog (Constanti and Nistri, 1976), and rat (Biscoe et al., 1976) and thalamic neurons of rat (Hall, Hicks, and McLennan, 1978).

Several investigators have studied the effects of kainic acid upon invertebrate muscle, where glutamate is thought to be the natural excitatory transmitter. Kainic acid appears to be less potent than glutamate in exciting invertebrate muscle. However, it does potentiate the excitatory action of glutamate, although only when glutamate is iontophoresed outside of the synaptic junction (Shinozaki and Shibuya, 1974; Takeuchi and Onodera, 1975). It has been suggested that kainic acid does not alter the sensitivity of the junctional receptor, but rather sensitizes an extra-junctional receptor of invertebrate muscle to
L-glutamate (Takeuchi and Onodera, 1975). Although the mechanism of action of kainic acid at invertebrate muscle may be quite different than at vertebrate central neurons, its action in potentiating glutamate is similar in both preparations, and indicates the possible existence of extra-junctional receptors on vertebrate neurons.

Few studies have appeared concerning the ability of antagonists to inhibit kainic acid-induced depolarization. Pole and Haefely (1977) reported that 5,6-dimethoxyaporphine ((-)-nuciferine) blocks the stimulatory effects of kainic acid, but not of N-methyl-D-aspartate, upon spinal neurons. Since the compounds in this study were administered by intraperitoneal injection, the locations at which excitatory and depressant effects were initiated may not be the same. Recently, Hall et al. (1978) reported that the depolarization of rat thalamic neurons by kainic acid is less sensitive to blockade by L-GDEE than is depolarization induced by L-glutamate. Thus, specific antagonists of kainic acid have not yet been clearly identified.

By far the greatest use of kainic acid has been in producing selective neuronal lesions by direct injection into the central nervous system. Prior to the discovery of kainic acid's excitatory action, investigators had demonstrated that peripheral administration of glutamate to immature animals, in which the blood-brain barrier is not fully developed, causes destruction of neurons within the central nervous system (Olney, 1969). The arcuate nucleus of the hypothalamus and the retina are especially sensitive to peripheral glutamate administration. Direct application of glutamate to rat cortical neurons is also toxic (Van Harreveld and Fikova, 1971). In this study,
Iontophoretic application of less than 1 μg of glutamate over a one hour period resulted in necrosis of neurons at the site of injection, while local pre-synaptic endings and glia underwent a reversible swelling and survived the treatment. Olney, Ho, and Rhee (1972) found that the neurotoxic potency of a series of dicarboxylic and sulfur-containing amino acids, peripherally injected in immature mice, is related to their neuroexcitatory potency. He proposed the term "excitotoxic" to describe these amino acids, suggesting that their neurotoxicity may be the result of excess depolarization. As a test of the excitotoxic hypothesis, Olney, Rhee, and Ho (1974) injected kainic acid peripherally in infant mice, and found that it was very toxic to neurons of the arcuate nucleus, as predicted by its high neuroexcitatory potency. In addition, neurotoxicity in the hippocampus that is uncharacteristic of the excitotoxic amino acids was noted, although these alterations may have been the result of convulsive activity associated with kainic acid injection.

Noting the selectivity of the excitotoxic amino acids in damaging neuronal post-synaptic elements at the injection site, Coyle and Schwarcz (1976) injected kainic acid into the rat striatum. This procedure produced a specific chemical lesion of intrinsic striatal cholinergic and GABAergic neurons, while sparing terminals of afferent dopaminergic neurons originating in the substantia nigra. In this study, a dose of 5 μg kainic acid injected into the striatum produced an 80% decrease in the activities of glutamic acid decarboxylase (GAD) and choline acetyltransferase (CAT) after 48 hours, whereas the activity of tyrosine hydroxylase, a marker for neurons synthesizing dopamine, was increased by 80% at this time. For up to 24 hours following the
injection, rats demonstrated a pronounced rotational movement away from the injected side and clonic limb movements. These investigators suggested that the neuronal degeneration, neurochemical changes, and behavioral responses following intrastriatal kainic acid injection resemble those occurring in Huntington's Disease, and that the procedure could provide an animal model for this disorder. These results were soon verified (McGeer and McGeer, 1976) and several investigators have since studied the effects of intrastriatal kainic acid injection upon a number of neurochemical parameters (review—Coyle et al., 1977). Intra-striatal injection of much higher doses of L-glutamate are required to produce similar neurotoxic effects (Olney and de Gubareff, 1978b).

Kainic acid probably destroys neurons by the same mechanism as other excitotoxic amino acids, since similar tissue elements are affected. Analysis of the events following glutamate application to brain slices, as reviewed by Krnjevic (1970), suggests a possible mechanism for this toxicity. Immediately following the addition of glutamate, there is an increase in Na\(^+\) content and a decrease in K\(^+\) content of the intracellular space. These ion fluxes are probably associated with neuronal depolarization. Following this initial phase there is a period during which Na\(^+\) influx continues, and is accompanied by K\(^+\) and H\(_2\)O influx and depletion of high-energy phosphate compounds. It is likely that the increased intraneuronal Na\(^+\) content following depolarization activates Na-K ATPase, which exchanges internal Na\(^+\) with external K\(^+\) at the expense of high energy phosphate compounds. An influx of H\(_2\)O accompanies the influx of Na\(^+\) and K\(^+\) in order to maintain
internal osmolarity, resulting in swelling and eventual lysis of neuronal membranes.

The toxicity resulting from kainic acid application appears also to be associated with excess depolarization. N-Acetyl kainic acid and dihydrokainic acid, derivatives of kainic acid which are not excitatory, are also not neurotoxic (Schwarcz, Scholz, and Coyle, 1978). In the rat hippocampus, CA3 pyramidal cells are most sensitive, followed by CA1 pyramidal cells, and dentate granule cells are least sensitive to destruction following intraventricular kainic acid (Nadler, Perry, and Cotman, 1978). These neuronal types show the same order of sensitivity to depolarization by kainic acid (Ryan and Cotman, 1978).

The idea that kainic acid acts at glutamate receptors is based on its similar structure, depolarizing action, and neurotoxicity. This idea is further supported by histological studies, demonstrating that swelling and vacuolization following kainic acid injection first occurs in neuronal perikarya and dendrites (Hattori and McGeer, 1977), the sites of responsiveness to iontophoresed glutamate (Curtis et al., 1972). Kainic acid injection into the cerebellum causes the destruction of neurons that are post-synaptic to granule cells, but does not affect the granule cells themselves, except in the immediate vicinity of the injection site (Herndon and Coyle, 1977). Since cerebellar granule cells are thought to release glutamate as their neurotransmitter, the selective destruction of neurons innervated by these cells is consistent with a toxic action mediated by glutamate receptors.

However, some of the results obtained with kainic acid are more consistent with a potentiating action upon glutamate depolarization than
with a direct action at glutamate receptors. Following cortical ablation, which destroys cortico striatal glutamatergic fibers, injection of a toxic dose of kainic acid into the striatum no longer produces significant reductions in cholinergic and GABAergic neuronal markers (McGeer, McGeer, and Singh, 1978). This result indicates that glutamate innervation may be required for kainic acid to produce neurotoxicity. However, kainic acid does not release glutamate from striatal slices (Friedle, Kelly, and Moore, 1978), nor is it an effective inhibitor of glutamate uptake ($IC_{50} > 300 \mu M$) (McGeer et al., 1978; Friedle et al., 1978). Furthermore, kainic acid is toxic to neurons grown in culture, a system in which blockade of glutamate uptake or stimulation of glutamate release is not likely to be a factor, at a concentration (5 $\mu M$) at which kainic acid does not block glutamate uptake in any case (Honegger and Richelson, 1977). Biziere and Coyle (1978a), in comparing the effects of kainic acid with those of glutamate on striatal slices in vitro, found that 1 $\mu M$ kainic acid did cause increased $Na^+$ influx, while increased $Na^+$ influx did not occur with concentrations of less than 10 mM glutamate. However, kainic acid did not cause increased $K^+$ or $H_2O$ influx at up to 10 mM, the concentration at which glutamate produced these effects. Although kainic acid by itself did not alter ATP content of the slices, at high concentrations it potentiated the ATP-depleting effect of glutamate. Thus, the mechanism of kainic acid-induced neurotoxicity has not yet been clearly identified, although it appears to involve depolarization mediated by excitatory amino acid receptors, possibly through a potentiating rather than a direct action.
Simon et al. (1976) have reported that $^3$H-kainic acid binds to rat brain membranes. The specific binding was saturable, with an apparent maximal binding density in synaptic membranes of 1 pmole/mg protein. The dissociation constant (Kd) was reported to be 60 nM. The amount of specific binding to non-neural tissues (liver, kidney, lung) was very small compared to brain tissue. $^3$H-kainic acid binding was displaced most potently by kainic acid, with quisqualic acid slightly less effective. L-Glutamate was the next most potent compound tested in displacing $^3$H-kainic acid, being 4% as potent as kainic acid itself. L-Apartic acid and D-glutamic acid were considerably less effective displacers than L-glutamic acid, while the glutamate antagonist L-GDEE was approximately one-half as potent as L-glutamate. Specific $^3$H-kainic acid binding varied 5-fold in various regions of rat brain. Highest density of binding sites was measured in the striatum, followed by cerebral cortex and hippocampus. Lowest binding densities were found in the midbrain and medulla-pons, and binding was indetectable in white matter (corona radiata) of monkey brain. The localization of binding in grey matter of the CNS, enrichment in synaptosomal membranes, and varied regional distribution are consistent with binding to a neurotransmitter receptor. Furthermore, the quantity of binding sites is similar to that measured for other neurotransmitter receptors.

Kainic acid-induced alterations of the rat striatum have been of particular interest since they are similar to changes occurring in Huntington's Disease. Kainic acid-lesion of the striatum may serve as an animal model for this disorder, and indicates a possible defect involving glutamate metabolism in Huntington's Disease.
Huntington's Disease

Huntington's Disease (HD) is a hereditary neuropsychiatric disorder characterized by marked atrophy of the neostriatum and a lesser degeneration of the cerebral cortex and other brain regions (Shoulson and Chase, 1975). In the majority of cases, the primary symptoms of dementia and involuntary movements first appear in the fourth or fifth decade of life. The arrhythmic, choreic movements increase during voluntary effort or stress and disappear during sleep. Mental alterations, which are associated with 50-80% of HD patients, can include forgetfulness, irritability, inability to concentrate, personality changes, affective disorders, and psychosis (Garron, 1973; Shoulson and Chase, 1975). The disease undergoes an irreversible and progressive course, death occurring usually from respiratory causes at an average of 16 years after onset of symptoms (Myrianthopoulos, 1966; Edmonds, 1966). The genetic defect is autosomal and dominant, but, due to the late onset of symptoms, patients are usually past child-bearing age before the disease can be diagnosed. Six to ten per cent of the cases exhibit early onset with rapid progression (the rigid-hypokinetic or Westphal variant) and are marked by rigidity rather than the usual choreic movements (Dewhurst and Oliver, 1970).

Widespread neuronal degeneration accompanies adult-onset HD. The caudate nucleus and putamen are the most severely affected areas, and the third, fourth, and sixth layers of the cerebral cortex are also often involved (Enna, Stern et al., 1977a). In a study of postmortem brain tissue, Perry, Hansen, and Kloster (1973) have reported that GABA levels are significantly reduced in the substantia nigra, caudate
nucleus, putamen, and globus pallidus of HD brains. Furthermore, glutamic acid decarboxylase (GAD) activity is reduced in the substantia nigra, caudate nucleus, putamen, globus pallidus, and dentate nucleus of HD brains (McGeer, McGeer, and Fibiger, 1973; Bird and Iversen, 1974), suggesting that neurons of the basal ganglia which synthesize GABA undergo degeneration in HD. In addition, the concentration of GABA in cerebrospinal fluid from HD patients is significantly reduced (Enna, Stern et al., 1977b; Glaeser et al., 1975). However, choline acetyltransferase (CAT) activity is also reduced in corpus striatum of HD brains (McGeer et al., 1973; Wastek et al., 1976; Enna, Stern et al., 1977a) indicating that degeneration is not limited to GABAergic neurons, but also includes cholinergic neurons. On the other hand, neither tyrosine hydroxylase nor dopamine-beta-hydroxylase, presynaptic markers for dopamine and noradrenergic neurons, are altered in HD striatum, indicating that some specificity of degeneration exists (Bird and Iversen, 1977).

Analysis of receptor density by binding of radiolabeled compounds indicates that the density of muscarinic cholinergic receptors, serotonin receptors, and binding sites for butyrophenone neuroleptic drugs are decreased in the neostriatum of HD brains (Enna, Bennett et al., 1976; Enna, Stern et al., 1977a; Wastek et al., 1976; Reisine et al., 1977). Conflicting data exist regarding the condition of GABA receptors, as detected by $^3$H-GABA binding, in the HD striatum. Enna, Bennett et al. (1976) have reported that the GABA receptor density of HD caudate nucleus is not significantly reduced, while Lloyd, Dreksler, and Bird (1977) report a 70-80% decrease in GABA receptor density of HD
caudate nucleus and putamen. Both investigators report that the GABA receptor density of HD frontal cortex is not significantly altered. Enna, Bennett et al. (1976) have also reported an increase in $^3$H-GABA binding in HD substantia nigra.

Current therapy of HD involves treatment with neuroleptic butyrophenones and phenothiazines, which afford some symptomatic relief of chorea and have antipsychotic activity (Shoulson and Chase, 1975). Chlordiazepoxide (Warick and Barrows, 1964) and diazepam (Farrell and Hofmann, 1968) also ameliorate the motor symptoms of HD, though less effectively than the neuroleptics. Because of the degeneration of GABA neurons in HD, several therapies aimed at potentiating GABAergic transmission have been tested clinically. Trials of the GABA agonists imidazole-4-acetic acid (Shoulson et al., 1975) and of GABA in combination with dipropylacetic acid, a GABA-transaminase inhibitor (Shoulson, Kartzinel, and Chase, 1976), were unsuccessful in improving the motor symptoms of HD, possibly because neither imidazoleacetic acid nor GABA penetrate the blood brain barrier to a significant extent. Recently, Shoulson et al. (1978) have reported that muscimol administration did not improve motor nor cognitive functions of 9 out of the 10 HD patients in the study, although the most severely hyperkinetic patient showed some improvement. The doses of muscimol employed (5-10 mg per day) were sufficient to produce adverse reactions in half of the patients, consisting of increased irritability, agitation, lethargy, lack of attention, loss of appetite, insomnia, and dystonia. These adverse central reactions indicate that muscimol or a metabolite is reaching the central nervous system. In view of the ineffectiveness of muscimol
and therapy with GABA agonists, it is most important to confirm muscimol's site of action as well as to determine the state of GABA receptors in HD.

**Rationale**

Both muscimol and kainic acid are of interest in ongoing research of HD. Muscimol, the GABA analog, is a theoretically useful therapeutic drug, although clinical trials with muscimol have failed to demonstrate a beneficial effect. Kainic acid, the glutamate analog, produces alterations in the striatum which are strikingly similar to the degeneration occurring in HD. Both compounds are being increasingly used in studies of GABA and glutamate neurotransmission.

The ineffectiveness of drugs designed to increase GABAergic transmission in relieving the symptoms of HD could be caused by a number of factors, such as failure to achieve adequate levels within the central nervous system, nonspecificity for GABA receptors, or production of nontherapeutic responses at GABA synapses distant from the proposed site of action in the striatum. Furthermore, these proposed therapies depend upon the viability of the neurons upon which GABA has its effects. We undertook studies of $^3$H-muscimol binding in order to determine first, whether muscimol acts specifically at GABA receptors; and second, whether these receptors are intact in HD brains.

The kainic acid-injected rat striatum is being increasingly used as an animal model for HD, and has led to proposals that glutamate neurotransmission may be altered in this disease. We have studied the binding of kainic acid to brain membranes in order to investigate its
site of action and to determine the state of kainic acid receptors in HD. Both muscimol and kainic acid binding were studied in kainic acid-lesioned rat striatum, both in order to verify the accuracy of this animal model for HD and to determine the location of the binding sites in the striatum.
MATERIALS AND METHODS

Radiolabeled Compounds

$^3$H-Muscimol was custom tritiated at New England Nuclear, Boston, Mass., by decarboxylation of ibotenic acid. Ott, Wheaton, and Chilton (1975) described the synthesis of unlabeled muscimol by a similar procedure. Briefly, ibotenic acid, isolated from Amanita pantherina, was decarboxylated in the presence of tritiated $H_2O$ by heating in 0.1 M formic acid (Fig. 3). After removal of labile tritium under vacuum pressure, $^3$H-muscimol was purified from the reaction product by electrophoresis and thin-layer chromatography. The purity of the $^3$H-muscimol, as determined by thin-layer chromatography on cellulose sheets (Kodak), was found to be 92% in an acidic solvent system (butanol:acetic acid: $H_2O$, $R_f = 0.52$), 84% in a neutral solvent system (methanol:$H_2O$, 9:4, $R_f = 0.65$), and 95% in a basic solvent system (isopropanol:$NH_4OH:H_2O$, 5:1:2, $R_f = 0.58$).

Fig. 3. Preparation of $^3$H-muscimol from ibotenic acid — Ibotenic acid was dissolved in a solution of tritiated water containing 0.1 M formic acid and the mixture was gently shaken for 10 minutes at 25°C prior to a 3 hour incubation at 100°C; *indicates isotope.
The specific activity of $^3$H-muscimol was determined by the fluorescamine assay for primary amines (Udenfriend et al., 1972), using Fluram Reagent (Roche) and unlabeled muscimol to generate a standard curve. The specific activity of $^3$H-muscimol was determined to be 0.3 Ci/m mole. After the initial studies, $^3$H-muscimol was made commercially available by New England Nuclear at a specific activity of 12.1 Ci/m mole and a purity of greater than 99%. This $^3$H muscimol was used for later studies involving human brains and brain lesions.

$^3$H-Kainic acid was purchased from New England Nuclear. It was labeled to a specific activity of 2.3 Ci/m mole by an acid-catalyzed tritium exchange procedure, using unlabeled kainic acid isolated from *Digenea simplex* as starting material. Its purity was greater than 98%, as determined by thin-layer chromatography.

**Preparation of Tissue**

Male Sprague-Dawley rats, 150-250 grams, were used for all studies requiring rat tissue. Crude synaptic membranes were prepared according to the procedure described by Zukin et al. (1974). Rats were decapitated and the brains removed and homogenized in 15 volumes of ice-cold 0.32 M sucrose, using ten strokes of a Potter-Elvejham glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 1,000 x g for 10 minutes to obtain the crude nuclear pellet ($P_1$) containing nuclei and cellular debris, which was discarded. The supernatant was centrifuged at 20,000 x g for 20 minutes to obtain the crude mitochondrial pellet ($P_2$), consisting of mitochondria, synaptosomes, and myelin. The supernatant was centrifuged for 20 minutes at 48,000
x g to obtain the crude microsomal membrane fraction (P3). To obtain the crude synaptic membrane fraction, the crude mitochondrial (P2) pellet was suspended in distilled H2O with a Brinkman Polytron, then centrifuged for 20 minutes at 8,000 x g. This centrifugation yields a firm pellet, the mitochondria-myelin fraction, surrounded by a loosely attached layer of membranes. The tube was shaken to resuspend the lighter layer in the supernatant, which was then decanted into a separate tube and centrifuged at 48,000 x g for 20 minutes. This final crude synaptic membrane pellet was used the same day when fresh tissue was required or frozen for at least 18 hours at -20°C when frozen tissue was needed. In either case, the pellets were washed thoroughly prior to assay by resuspending in 50 ml of fresh buffer and re-centrifuging. This washing procedure was repeated 3 times with fresh buffer for tissues used in muscimol binding assays, 4 times for tissues used in kainic acid binding assays. This procedure removes GABA, glutamate, and possibly other endogenous substances which interfere with the assay and cause lowered binding in less thoroughly washed tissue preparations. Dissection of rat brains into regions was done by the method of Glowinski and Iversen (1966).

Human Brain Tissue

Specimens of human brain tissue were obtained by Dr. E. D. Bird and Dr. E. Spokes of Addenbrookes Hospital, England, upon autopsy from patients with Huntington's Disease and from individuals without infectious or malignant disease of the central nervous system. Tissue samples were immediately frozen and shipped on dry ice to this...
laboratory, where they were stored at -70°C until assayed. Age, drug history, cause of death, and duration of HD symptoms were obtained from patient records. Mean patient age at time of death was 59 years (range = 42-74 years) for HD patients and 57 years (range = 17-88 years) for controls.

The whole particulate fraction was used for studies involving human brain tissue. On the day of the assay, brain tissues were homogenized with a Brinkman Polytron at setting 5 for 15 seconds, then centrifuged for 20 minutes at 48,000 x g. Pellets were thoroughly washed, as described for the synaptic membrane preparation, prior to assay.

**3H-Muscimol Binding Assay**

In the standard assay procedure, 500 µl portions of crude synaptic membrane suspensions (approximately 1 mg protein) were placed into 15 ml Sorvall tubes containing 5.5 mls of 0.05 N tris-citrate buffer at pH 7.1, and 3H-muscimol at a concentration varying from 0.5 to 300 nM. Samples were incubated in triplicate for 30 minutes, followed by a 10 minute centrifugation at 48,000 x g. The resulting pellet was surface-rinsed rapidly with 5 ml, then 10 ml, of ice cold distilled H₂O. Five ml of a Triton-toluene-omnifluor solution (1 l.-2 l.-16 gm) were added to the tubes, and the pellets were dispersed with wooden dowels. After an overnight incubation at room temperature, the dissolved pellets were transferred to counting vials and the tubes rinsed with another 5 ml of scintillation cocktail. Radioactivity was monitored by liquid scintillation spectrometry (Searle Mark III) at a counting
efficiency of 44-48%. The amount of radioactivity bound in the presence of a large excess of unlabeled GABA (200 μM) was termed nonspecific binding and was subtracted from the total amount bound to obtain specific $^3$H-muscimol binding. Thin-layer chromatography of the membrane-bound isotope indicated that greater than 95% was unchanged $^3$H-muscimol.

$^3$H-Kainic Acid Binding Assay

Initial $^3$H-kainic acid binding studies were performed by the method described by Simon et al. (1976), using concentrations of $^3$H-kainic acid varying from 0.5 to 300 nM. This method is similar to that described for $^3$H-muscimol binding, except that the final assay volume was 2 ml, and an excess of unlabeled L-glutamate (100 μM) was used to determine nonspecific binding. In later experiments, the assay was modified to allow the use of 4 ml disposable Biovials (Beckman). After the standard incubation, Biovials were centrifuged in a Sorvall GSA rotor, fitted with Beckman Biovial adapters, at 30,000 x g for 10 minutes. Pellets were then surface-rinsed twice with 4 ml distilled H$_2$O. Two hundred μl of NCS tissue solubilizer (Amersham-Searle) was added to each vial, followed by a one hour incubation, with constant shaking, at 45°C. After addition of 4 ml of toluene-omnifluor (1 liter=10.7 gm), and neutralization with glacial acetic acid, the Biovials were capped, vortexed, and placed in counting vials for monitoring by liquid scintillation spectrometry. With this procedure, tritium was determined at an efficiency of 40%. This procedure gives results similar to those obtained by the previously described method,
while it allows centrifugation of more tubes at once and more rapid pellet extraction, eliminates the need for transferring tube contents, lowers the requirement for scintillation cocktail, and prevents contamination due to reuse of incubation tubes.

**Choline-Acetyltransferase (CAT) Activity**

CAT activity was determined by the method of Yamamura, Gardner, and Goldberg (1972) as modified by Wastek et al. (1976). The enzyme transfers radiolabeled acetate from $^{14}$C-acetylCoA to choline, which is added in the incubation medium. The reaction is stopped by addition of tetraphenyl boron, and the newly synthesized radiolabeled acetylcholine is separated from unreacted acetylCoA by partitioning into an organic phase. The amount of radiolabeled acetate transferred to choline per unit time is a measure of CAT activity.

To measure CAT activity, 5 µl of unwashed tissue homogenate (3.3% in 50 mM NaKPO$_4$ buffer) were added to 25 µl of an assay mixture containing 0.4 ml Na$_2$HPO$_4$/NaH$_2$PO$_4$ buffer (0.2 M, pH 7.4), 0.17 ml eserine salicylate (0.001 M), 0.06 ml MgCl$_2$ (0.1 M), 0.12 ml NaCl (3 M), 0.12 ml choline chloride (0.02 M), and 0.1 ml $^{14}$C-acetyl CoA (0.02 mCi/ml). After 20 minutes incubation at 37°C, 0.1 ml of tetraphenyl boron in 3-heptanone (50 mg/ml) was added and the mixture was cooled in an ice bath for 5 minutes, then centrifuged for 2 minutes in a Beckman Microfuge B. The radioactivity in 50 µl of the organic layer was determined by liquid scintillation spectrometry.
Kainic Acid Lesions

Rats were anesthetized with pentobarbital and placed in a Kopf stereotaxic apparatus. Two μg of kainic acid, dissolved in a volume of 1 μl 0.9% NaCl, was injected into the right striatum at coordinates 7.8 anterior, 2.6 lateral, and 4.8 vertical, according to the atlas of König and Klippel (1967). The solution was injected over a period of 3 minutes, and the needle was left in place an additional 2 minutes before withdrawal. At various times after lesioning, animals were sacrificed by decapitation and left and right striata were dissected out and frozen. The striatum contralateral to the lesion served as control for each animal.

Protein Assay

Protein concentrations were determined by the method of Lowry et al. (1951).
CHAPTER 3

RESULTS

Muscimol Binding in Rat Brain

Characteristics of Specific $^3$H-Muscimol Binding

In a typical experiment using 2 nM $^3$H-muscimol, 800 dpm/mg protein were bound to membranes in the absence of displacer and only 140 dpm/mg protein were present on the membranes when 200 µM unlabeled GABA was present in the incubation. The difference between these two values, 660 dpm/mg protein, represents specifically bound isotope. Thus, over 80% of the bound ligand is specifically attached to the receptor site. This compares favorably with the 85% specific to non-specific ratio for $^3$H-GABA binding to rat brain membranes using a similar concentration of $^3$H-GABA.

Specific $^3$H-muscimol binding increases linearly as the concentration of synaptic membranes in the incubation is increased up to at least 1.8 mg protein/6 ml assay (Fig. 4). Specific binding appears to reach equilibrium within 15 minutes. As the pH of the buffer is varied from 6.5 to 7.7, specific $^3$H-muscimol binding remains fairly constant, only decreasing significantly at pH 7.7 (Fig. 5). Specific binding to membranes which have been previously frozen is approximately twice that observed using freshly prepared membranes, and remains constant in tissue stored at -20°C (Table 1). These results were used
Fig. 4. Tissue linearity of $^3$H-muscimol binding — Specifically bound
$^3$H-muscimol was determined at a concentration of 2 nM. Values
represent the means of triplicate determinations which varied
less than 10%.
Fig. 5. Effect of pH on $^3$H-muscimol binding — Specific $^3$H-muscimol binding was determined at a concentration of 2 nM in 0.05 M tris-citrate buffer of the designated pH. Values are the means of triplicate determinations, which varied less than 14%.
Table 1. Effect of freezing on specific $^3$H-muscimol binding --
Specific $^3$H-muscimol binding was determined at a concentration of 2 nM. Freshly prepared synaptic membranes were either used immediately or frozen at -20 C for the times indicated. Values represent the means of triplicate determinations, which varied less than 10%.

<table>
<thead>
<tr>
<th>Days frozen</th>
<th>Specifically bound $^3$H-muscimol (pmoles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.187</td>
</tr>
<tr>
<td>1</td>
<td>0.303</td>
</tr>
<tr>
<td>19</td>
<td>0.315</td>
</tr>
<tr>
<td>45</td>
<td>0.309</td>
</tr>
</tbody>
</table>
to determine the assay conditions for \(^3\)H-muscimol binding. For routine experiments, \(^3\)H-muscimol binding was studied by incubating 1 mg of previously frozen membrane protein for 30 minutes at 4°C in pH 7.1 buffer.

**Kinetics of \(^3\)H-Muscimol Binding**

As the concentration of \(^3\)H-muscimol is increased, specific binding saturates, indicating the presence of a limited number of binding sites (Fig. 6). Non-specific binding is not saturable over this concentration range. Scatchard analysis (Scatchard, 1949) of the saturation curve reveals the presence of two populations of binding sites (Fig. 7). The higher affinity site has an apparent dissociation constant of 2.2 nM and binds maximally 0.7 pmole \(^3\)H-muscimol/mg protein. The lower affinity site has an apparent dissociation constant of 60 nM and binds maximally 2.8 pmoles \(^3\)H-muscimol/mg protein. Binding constants were derived from linear regression analysis of the Scatchard plot, using data from \(^3\)H-muscimol concentrations above 17 nM for low affinity calculations and below 7 nM for high affinity calculations. Correlation coefficients (r) of the linear regressions are 0.83 for the low affinity site and 0.94 for the high affinity site.

**Effect of Triton X-100 and Sodium on \(^3\)H-Muscimol Binding**

Prior treatment of synaptic membranes with the nonionic detergent Triton X-100 increases \(^3\)H-muscimol binding in a concentration-dependent fashion, with 0.05% Triton treatment doubling the amount of isotope bound (Fig. 8). Treatment with greater than 0.05% Triton
Fig. 6. Saturation of specific $^3$H-muscimol binding with increasing concentrations of $^3$H-muscimol -- Whole rat brain synaptic membranes (1 mg protein/tube) were incubated in tris-citrate (pH 7.1) containing various concentrations of $^3$H-muscimol in the presence and absence of unlabeled GABA (200 μM). Specific binding was determined by subtracting the amount of isotope bound in the presence of unlabeled GABA from that bound in the absence of the displacer. Values are the means of triplicate determinations, which varied less than 10%, from 6 separate experiments.
Fig. 7. Scatchard plot of specific $^3$H-muscimol binding using increasing concentrations of $^3$H-muscimol -- Data are transformed from those of Fig. 6. Binding constants were determined as described in the text.
Fig. 8. Effect of Triton X-100 on specific binding of \(^{3}\text{H}\)-muscimol. Previously frozen synaptic membranes were resuspended in 20 volumes of tris-citrate buffer and incubated with the indicated volumes of Triton X-100 for 30 minutes at 37 °C. Suspensions were centrifuged for 10 minutes at 48,000 x g then washed three times in fresh buffer. Specific binding of \(^{3}\text{H}\)-muscimol was determined at a concentration of 3 nM. Values represent the means of two experiments, each done in triplicate.
results in greater variability of results, apparently because the pellets are unstable and fragment easily during washing. Scatchard analysis of the binding data derived from experiments using membranes treated with 0.05% Triton X-100 yields one binding site with a Kd of 1.8 nM and a density of 2.6 pmoles/mg protein (Fig. 9). However, due to the slightly curvilinear nature of the Scatchard plot, the presence of two populations of binding sites in Triton treated membranes cannot be ruled out.

As the sodium concentration in the incubation medium is increased, the amount of $^3$H-muscimol bound decreases, though not markedly. This result is obtained with both freshly prepared and previously frozen membranes (Fig. 10).

Subcellular Distribution of $^3$H-Muscimol Binding

Specific $^3$H-muscimol binding is most enriched in the crude synaptic membrane fraction of rat brain (Table 2). This fraction also contains the greatest absolute number of $^3$H-muscimol binding sites, approximately 75% of the total observed in all of the subcellular fractions.

Pharmacological Specificity of $^3$H-Muscimol Binding

The potency of various neurotransmitters, amino acids, and drugs in displacing specifically bound $^3$H-muscimol from Triton treated membranes was analyzed. Substances which are known to interact neurophysiologically with the GABA receptor displayed a much greater affinity for the muscimol binding site than agents which are known to interact
Fig. 9. Scatchard plot of $^3$H-muscimol binding to whole rat brain synaptic membranes treated with 0.05% Triton X-100 — Values represent the mean of 2 experiments, each performed in triplicate. Values from the 2 experiments varied from 3-15%. Correlation coefficient ($r$) for the linear regression is 0.97.
Fig. 10. Effect of sodium on specific $^3$H-muscimol binding — NaCl was added to the buffer to give the final concentrations indicated. Specific $^3$H-muscimol binding was determined at a concentration of 2 nM to either fresh or previously frozen tissue. Values represent the means of 2 experiments.
Table 2. Subcellular distribution of $^3$H-muscimol binding in rat brain

Whole homogenate particulate is the pellet resulting from a 48,000 x g centrifugation of a 5% whole brain homogenate in 0.32 M sucrose. Other fractions were prepared as described in Materials and Methods. All fractions were washed three times in fresh buffer to remove endogenous GABA. Specific $^3$H-muscimol binding was determined at a concentration of 2 nM. Values are the means of two experiments, each performed in triplicate.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Density (fmole/mg protein)</th>
<th>Total binding/fraction (fmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole homogenate particulate</td>
<td>81</td>
<td>8000</td>
</tr>
<tr>
<td>Crude nuclear pellet ($P_1$)</td>
<td>123</td>
<td>2600</td>
</tr>
<tr>
<td>Crude microsomal pellet ($P_3$)</td>
<td>96</td>
<td>610</td>
</tr>
<tr>
<td>Osmotically shocked $P_2$ subfractions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondria-myelin pellet</td>
<td>103</td>
<td>550</td>
</tr>
<tr>
<td>Crude synaptic membrane pellet</td>
<td>225</td>
<td>6000</td>
</tr>
</tbody>
</table>
with other receptor sites. Thus, 3-aminopropanesulfonic acid, trans-4-aminocrotonic acid, GABA, and imidazole-acetic acid, all potent GABA agonists (Johnston, 1976), are about equipotent with muscimol in displacing $^3$H-muscimol (Table 3). $\beta$-guanidinopropionic acid, a moderately active GABA analog, is moderately active in displacing the isotope. Furthermore, $^3$H-muscimol binding displays structural specificity similar to that reported for the GABA receptor in that (+)-trans-3-amino cyclopentane-1-carboxylic acid, a neurophysiologically active GABA receptor agonist, is 100 times more potent on $^3$H-muscimol binding than the cis-isomer, which is much less active neurophysiologically (Johnston, 1976). Similarly, (+)bicuculline, a GABA receptor antagonist, is more potent neurophysiologically and as a convulsant than (-)bicuculline, and (+)bicuculline has over 5 times more affinity for the $^3$H-muscimol binding site than does (-)bicuculline. Whereas imidazole-acetic acid is a potent GABA agonist, 1-methylimidazole-acetic acid has little or no potency in this regard and also displays little or no potency in displacing specifically bound muscimol.

Furthermore, 2,4-diaminobutyric acid and nipecotic acid, inhibitors of neuronal GABA transport having little or no activity as GABA agonists, have essentially no affinity for the muscimol binding site. Picrotoxin, a GABA antagonist, does not interfere with $^3$H-muscimol binding, suggesting that muscimol binds to the GABA recognition site rather than to the associated ionophore.
Table 3. Substrate specificity of $^3$H-muscimol binding to rat brain membranes — Inhibition of specific $^3$H-muscimol binding by various concentrations of the different compounds was determined using the standard assay procedure with Triton treated membrane preparations. Membranes had been previously frozen. IC$_{50}$ values, the concentration which inhibits specific $^3$H-muscimol binding 50%, were calculated by log-probit analysis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscimol</td>
<td>5</td>
</tr>
<tr>
<td>3-Aminopropanesulfonic acid</td>
<td>5</td>
</tr>
<tr>
<td><strong>trans</strong>-4-Aminocrotonic acid</td>
<td>7</td>
</tr>
<tr>
<td>GABA</td>
<td>8</td>
</tr>
<tr>
<td>Imidazoleactic acid</td>
<td>8</td>
</tr>
<tr>
<td>$\beta$-Guanidinopropionic acid</td>
<td>40</td>
</tr>
<tr>
<td>(+)-<strong>trans</strong>-3-Aminocyclopentane-1-carboxylic acid</td>
<td>80</td>
</tr>
<tr>
<td>(+)-<strong>cis</strong>-3-Aminocyclopentane-1-carboxylic acid</td>
<td>8,000</td>
</tr>
<tr>
<td>(+)-Bicuculline</td>
<td>2,000</td>
</tr>
<tr>
<td>(-)-Bicuculline</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>1-Methylimidazoleactic acid</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Picrotoxin</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>2,4-Diaminobutyric acid</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>($\pm$)-Nipecotic acid</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

Note: Other compounds having an IC$_{50}$ > 10,000: L-glutamic acid, taurine, D-tubocurine, $\epsilon$-amino-n-caproic acid, norepinephrine, serotonin, histamine, dopamine, amphetamine, morphine, phenobarbital, pentobarbital, chlorpromazine, glycine, metharbitral, promazine, atropine, trifluperazine, halazepam, diazepam, amino oxyacetic acid, $\gamma$-hydroxybutyric acid, $\beta$-alanine, and L-glutamine.
Regional Distribution of $^3$H-Muscimol Binding

The amount of $^3$H-muscimol binding varies more than 15-fold among the different regions of the rat central nervous system (Table 4). At a $^3$H-muscimol concentration of 2 nM, the greatest density of binding sites is found in the cerebellum, which has about twice the binding capacity of the next highest regions, the cerebral cortex and olfactory bulb. The hippocampus, midbrain, corpus striatum, and hypothalamus possess a similar number of muscimol binding sites, about one-fifth that observed in the cerebellum. The medulla-pons and spinal cord display the least amount of binding, about 10% of the cerebellar level. The distribution of $^3$H-muscimol binding at a concentration of 200 nM is generally similar to the distribution at 2 nM, a notable exception being that the binding site density in the cerebellum is slightly less than in the cerebral cortex at this higher concentration.

No measurable specific $^3$H-muscimol binding was observed in tissues outside of the central nervous system, including heart, stomach, small intestine, diaphragm, liver, and kidney.

Kainic Acid Binding in Rat Brain

Characteristics of Specific $^3$H-Kainic Acid Binding

In a typical experiment using 5 nM $^3$H-kainic acid, a total of 200 counts/min/mg protein were bound to synaptic membranes, while 55 counts/min/mg protein were bound in the presence of 0.1 mM L-glutamate. A large excess of unlabeled kainic acid (0.01 mM) displaced the same percentage of bound ligand as did excess L-glutamate, and combination
Table 4. Regional distribution of $^3$H-muscimol binding in rat central nervous system — Binding experiments were performed on previously frozen, washed whole homogenate particulate fractions. The final concentration of $^3$H-muscimol in the medium was 2 nM or 200 nM. Each value is the mean ± S.E.M. of 3-4 experiments.

<table>
<thead>
<tr>
<th>Region</th>
<th>Specifically bound $^3$H-muscimol (fmole/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 nM $^3$H-muscimol</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>280 ± 14</td>
</tr>
<tr>
<td>Cerebral Cortex</td>
<td>147 ± 9</td>
</tr>
<tr>
<td>Olfactory Bulb</td>
<td>137 ± 33</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>60 ± 11</td>
</tr>
<tr>
<td>Corpus Striatum</td>
<td>60 ± 3</td>
</tr>
<tr>
<td>Midbrain</td>
<td>56 ± 8</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>50 ± 8</td>
</tr>
<tr>
<td>Medulla Oblongata-Pons</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>Spinal Cord</td>
<td>17 ± 2</td>
</tr>
</tbody>
</table>
of the two displacers in the same tube produced no further displacement. Thus, 70-75% of the bound $^3$H-kainic acid was specifically attached to a receptor site. When the whole particulate fraction of rat brain was used rather than synaptic membranes, only 50% of the $^3$H-kainic acid bound at a concentration of 5 nM was specific. Therefore, synaptic membranes were used for most studies. Specific $^3$H-kainic acid binding is linear with tissue concentration up to at least 1 mg protein/3 ml assay (Fig. 11) and reaches equilibrium within 30 minutes at 5°C.

Kinetics of $^3$H-Kainic Acid Binding

Specific $^3$H-kainic acid binding saturates as the concentration of $^3$H-kainic acid is increased (Fig. 12). Nonspecific binding does not saturate, increasing linearly throughout the concentration range that was examined, 0.5-300 nM. Binding constants were obtained by Scatchard analysis of saturation isotherms (Fig. 13). A dissociation constant (Kd) of 5.3 ± 1.8 nM and a receptor density (Bmax) of 182 ± 47 fmoles/mg protein were obtained for $^3$H-kainic acid binding to washed whole rat brain membranes (n = 5).

Pharmacological Specificity of $^3$H-Kainic Acid Binding

Several neuroexcitatory amino acids, proposed glutamate antagonists, and neurophysiologically active compounds were tested for their ability to displace $^3$H-kainic acid from whole rat brain synaptic membranes. The IC$_{50}$, the concentration of inhibitor required to displace 50% of the $^3$H-kainic acid specifically bound to synaptic membranes, was determined at a $^3$H-kainic acid concentration of 5 nM from
Fig. 11. Tissue linearity of $^3$H-kainic acid binding to whole rat brain membranes -- Specific $^3$H-kainic acid binding was obtained by subtracting nonspecific binding obtained in the presence of 0.1 mM L-glutamate from total binding obtained at a concentration of 5 nM $^3$H-kainic acid. Values represent the means of triplicate determinations.
Fig. 12. Saturation isotherm for $^3$H-kainic acid binding to whole rat brain membranes -- Total and nonspecific $^3$H-kainic acid binding was determined at various concentrations of $^3$H-kainic acid, using 0.1 mM L-glutamate as displacer. Data represent the means of triplicate determinations from a typical experiment.
Fig. 13. Scatchard plot of $^3$H-kainic acid binding to whole rat brain membranes -- Data are transformed from those of Fig. 12. Correlation coefficient for linear regression ($r$) = 0.90.
log-logit plots of displacement data. Half-maximal displacement of specifically bound $^3$H-kainic acid occurs with 0.006 $\mu$M unlabeled kainic acid, 0.12 $\mu$M L-glutamate, and 12 $\mu$M D-glutamate (Fig. 14). Thus, L-glutamate has approximately 5% of the affinity of kainic acid for the binding site, while D-glutamate is only about 1% as potent as the D-isomer. Determination of the slopes of Hill plots of displacement data yielded Hill coefficients of 0.96 for kainic acid and 0.99 for L-glutamate (Fig. 15).

Quisqualic acid, a heterocyclic alkaloid which is a potent neuroexcitant, has a slightly lower affinity for the binding site ($IC_{50} = 0.03 \mu$M) than does kainic acid (Table 5). Dihydrokainic acid, a relatively inactive analogue of kainic acid, is 1000-fold less potent than kainic acid in displacing the radiolabeled ligand from the receptor. DL-homocysteic acid and L-cysteic acid, compounds with neuroexcitatory potencies similar to or greater than L-glutamate, are only 1% as potent at L-glutamate in displacing $^3$H-kainic acid, as is L-glutamine, a compound without reported neuroexcitatory properties. N-methyl-D-aspartate, a potent neuroexcitant that may act selectively at aspartate receptors, is very ineffective at inhibiting $^3$H-kainic acid binding, having an $IC_{50}$ of 0.85 mM. The D- and L-isomers of aspartate have the same low affinity for the binding site, both with an $IC_{50}$ of 0.4 mM. DL-aminoadipic acid, a weakly excitatory compound that specifically antagonizes aspartate-induced excitations, is a weak inhibitor of $^3$H-kainic acid binding, with an $IC_{50}$ of 0.12 mM.

Several antagonists of L-glutamate excitations are relatively ineffective at displacing $^3$H-kainic acid. Thus, $IC_{50}$s are greater than
Fig. 14. Displacement of $^3$H-kainic acid binding — The amount of $^3$H-kainic acid bound to whole rat brain synaptic membranes at a concentration of 5 nM was determined in the presence of the indicated concentration of unlabeled kainic acid, L-glutamate, or D-glutamate. Results are expressed as per cent of total binding in the absence of displacers. Data are from a single experiment, performed in triplicate.
Fig. 15. Hill plot of displacement of $^3$H-kainic acid — $^3$H-Kainic acid bound to whole rat brain synaptic membranes at a concentration of 5 nM was displaced by increasing concentrations of unlabeled kainic acid (●) or L-glutamic acid (○). Hill plots were constructed from displacement data. Hill constants, determined from the slopes of the Hill plots, are 0.96 for kainic acid and 0.99 for L-glutamic acid. Correlation coefficients (r) for the linear regressions are 0.96 for kainic acid and 0.97 for L-glutamic acid. Data are from a single experiment, performed in triplicate.
Table 5. Displacement of $^3$H-kainic acid from rat brain synaptic membranes — Values represent the means of 2-5 determinations.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (micromolar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kainic Acid</td>
<td>0.006</td>
</tr>
<tr>
<td>Quisqualic Acid</td>
<td>0.032</td>
</tr>
<tr>
<td>Dihydrokainic Acid</td>
<td>5</td>
</tr>
<tr>
<td>L-Glutamic Acid</td>
<td>0.12</td>
</tr>
<tr>
<td>D-Glutamic Acid</td>
<td>12</td>
</tr>
<tr>
<td>DL-Homocysteic Acid</td>
<td>13</td>
</tr>
<tr>
<td>L-Cysteic Acid</td>
<td>16</td>
</tr>
<tr>
<td>L-Homocysteic Acid</td>
<td>18</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>13</td>
</tr>
<tr>
<td>DL-$\alpha$-Aminoadipic Acid</td>
<td>118</td>
</tr>
<tr>
<td>D-Aspartic Acid</td>
<td>400</td>
</tr>
<tr>
<td>L-Aspartic Acid</td>
<td>400</td>
</tr>
<tr>
<td>N-Methyl-D-Aspartic Acid</td>
<td>850</td>
</tr>
</tbody>
</table>
0.1 mM for alpha-methyl-DL-glutamate, L-glutamic acid diethylester (L-GDEE), and L-methionine sulfoximine. L-glutamic acid dimethylester, reportedly a weak agonist and glutamate uptake inhibitor (Haldeman and McLennan, 1973), has an IC$_{50}$ greater than 0.1 mM. Putative glutamate antagonists (-)nuciferine, 2-amino-3-phosphonopropionic acid, and 2-amino-4-phosphonobutyric acid have IC$_{50}$s greater than 1 mM.

Several compounds tested produced little or no inhibition at a concentration of 0.1 mM. These included GABA, muscimol, bicuculline, strychnine, glycine, taurine, histamine, adenosine, dopamine, atropine sulfate, serotonin, naltrexone, L-carnosine, L-cysteine, L-histidine, hemicholinium, phenobarbital, diazepam, metrazol, diphenylhydantoin, clozapine, amitryptiline, theophylline, ouabain, 2,4-dinitrophenol, pyridoxal-5'-PO4, 2-mercaptoethanol, dithiothreitol, caffeine, ATP, ADP, AMP, GMP, GTP, uridine monophosphate, inosine-5'-PO4, inosine, cytosine, guanosine, L-ascorbic acid, 6-OHdopamine, 6-OHDOPA, o-phospho-L-serine, DL-alpha-aminopimelic acid, and gamma-hydroxybutyrate.

Regional Distribution of $^3$H-Kainic Acid Binding in Rat Brain

The $^3$H-kainic acid binding site varies 5-fold in density among several regions of the rat brain (Table 6). When measured at a concentration of 10 nM, the highest density of specific $^3$H-kainic acid binding is present in the striatum, with 258 fmole bound/mg protein. The hippocampus has 80% of the binding capacity of the striatum, while cerebellum and cerebral cortex have approximately 50% of the binding density of the striatum. Midbrain and medulla-pons have the lowest
Table 6. Regional distribution of $^3$H-kainic acid binding in rat brain -- Values are the means ± S.E.M., n = 4.

<table>
<thead>
<tr>
<th>Region</th>
<th>$^3$H-Kainic acid bound (fmoles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Striatum</td>
<td>258 ± 16</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>199 ± 30</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>146 ± 18</td>
</tr>
<tr>
<td>Cerebral Cortex</td>
<td>140 ± 14</td>
</tr>
<tr>
<td>Midbrain</td>
<td>55 ± 7</td>
</tr>
<tr>
<td>Medulla-pons</td>
<td>45 ± 7</td>
</tr>
</tbody>
</table>

density of binding sites of the regions measured, approximately 20% that of the striatum.

Effects of Ions and pH Upon $^3$H-Kainic Acid Binding to Rat Brain

Specific ions alter the relative affinities of agonists and antagonists for certain neurotransmitter receptors, such as the opiate (Pert and Snyder, 1974), and GABA (Enna and Snyder, 1977) receptors, in some cases dramatically increasing the affinity of antagonists. Therefore, the effects of several ions upon agonist and antagonist binding to the kainic acid receptor were studied. Monovalent alkali cations were found to inhibit $^3$H-kainic acid binding in a dose-dependent manner (Fig. 16). Inhibitory potency of the ionic species, as measured by the $IC_{50}$ for displacement of 5 nM $^3$H-kainic acid, decreases with increasing molecular weight of the cationic species. Thus, $Li^+$ is the most potent
Fig. 16. Displacement of $^3$H-kainic acid by monovalent cations -- Each point represents the mean of 2-4 experiments, performed in triplicate.
inhibitor, with an IC$_{50}$ of 32 mM, while Cs$^+$ is the least potent inhibitor of the alkali cations, with an IC$_{50}$ of 220 mM. Hill plots of the displacement data gave Hill constants varying from 0.85 to 1.55 for the monovalent cations (Table 7).

Table 7. Inhibition of $^3$H-kainic acid binding by ions -- Values were calculated from the data presented in Fig. 16. Ranges for the Hill constants represent 95% confidence intervals.

<table>
<thead>
<tr>
<th></th>
<th>IC$_{50}$ (mM)</th>
<th>Hill constant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monovalent Cations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Li$^+$ (Cl)</td>
<td>32</td>
<td>1.27 ± 0.16</td>
</tr>
<tr>
<td>Na$^+$ (Cl)</td>
<td>61</td>
<td>1.15 ± 0.14</td>
</tr>
<tr>
<td>K$^+$ (Cl)</td>
<td>149</td>
<td>1.55 ± 0.32</td>
</tr>
<tr>
<td>NH$_4^+$ (Cl)</td>
<td>171</td>
<td>0.95 ± 0.05</td>
</tr>
<tr>
<td>Rb$^+$ (Cl)</td>
<td>192</td>
<td>0.85 ± 0.17</td>
</tr>
<tr>
<td>Cs$^+$ (Cl)</td>
<td>197</td>
<td>1.79 ± 0.10</td>
</tr>
<tr>
<td><strong>Divalent Cations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn$^{++}$ (Cl$_2$)</td>
<td>2.8</td>
<td>0.55 ± 0.26</td>
</tr>
<tr>
<td>Ca$^{++}$ (Cl$_2$)</td>
<td>2.3</td>
<td>0.62 ± 0.34</td>
</tr>
<tr>
<td>Mg$^{++}$ (Cl$_2$)</td>
<td>24</td>
<td>0.49 ± 0.08</td>
</tr>
<tr>
<td><strong>Monovalent Anions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F$^-$ (Na)</td>
<td>184</td>
<td>1.27 ± 0.27</td>
</tr>
<tr>
<td>Cl$^-$ (Na)</td>
<td>61</td>
<td>1.15 ± 0.14</td>
</tr>
<tr>
<td>Br$^-$ (Na)</td>
<td>50</td>
<td>1.20 ± 0.18</td>
</tr>
<tr>
<td>I$^-$ (Na)</td>
<td>66</td>
<td>1.43 ± 0.57</td>
</tr>
</tbody>
</table>

The effects of the sodium salts of the halide anions were determined. NaCl, NaBr, and NaI inhibit $^3$H-kainic acid binding to a similar extent, having IC$_{50}$s of 50-70 mM (Fig. 17). However, NaF produces less displacement than other sodium halides, having an IC$_{50}$ approximately four times that of the other halides (Table 7). Thus,
Fig. 17. Displacement of $^3$H-kainic acid by anions — $^3$H-Kainic acid binding to synaptic membranes was determined in the presence of varying concentrations of the sodium salts of the halide anions. Values represent the means of 2-4 experiments, performed in triplicate.
fluoride may have some potentiating effect upon kainic acid binding. Chloride itself does not appear to inhibit $^3$H-kainic acid binding, since increasing the concentration of tris-Cl buffer from 10-200 mM does not inhibit binding (Fig. 18). Therefore, the inhibition produced by NaCl is probably due solely to Na$^+$. In fact, increasing the molarity of the buffer appeared to slightly increase $^3$H-kainic acid binding, both with tris-Cl buffer and with the normal incubation buffer, tris-citrate. Slightly greater binding was obtained with tris-citrate buffer (Fig. 18).

Divalent cations were also found to inhibit $^3$H-kainic acid binding (Fig. 19). Mn$^{++}$, Ca$^{++}$, and Mg$^{++}$ have $IC_{50}$s of 2, 3, and 24 mM, respectively, and thus are considerably more potent inhibitors than the monovalent cations tested. Hill plots of displacement data for divalent cations gave Hill coefficients varying from 0.49 to 0.62 (Table 7). Experiments involving divalent cations were performed in tris-Cl buffer rather than the usual tris-citrate, since citrate chelates divalent cations. Inhibition by divalent cations was greatly reduced when measured in tris-citrate rather than tris-Cl buffer. Substitution of tris-Cl for tris-citrate had no effect on results for monovalent cations, which are not chelated by citrate.

The nature of the inhibition produced by monovalent and divalent cations was investigated by performing saturation studies of $^3$H-kainic acid binding to whole rat brain synaptic membranes in the presence of either Na$^+$ or Mg$^{++}$. The saturation isotherms generated in the presence of 70 mM NaCl, a concentration which inhibits approximately 60% of specific $^3$H-kainic acid binding at 5 nM, exhibited a lower
Fig. 18. Effect of buffer molarity upon $^3$H-kainic acid binding. $^3$H-Kainic acid binding to synaptic membranes was determined in the presence of increasing concentrations of either tris-Cl or tris-citrate buffer. Values represent the means of 2 experiments, performed in triplicate.
Fig. 19. Effect of divalent cations upon $^3$H-kainic acid binding. $^3$H-Kainic acid binding to synaptic membranes was determined in tris-Cl buffer in the presence of increasing concentrations of MnCl$_2$, CaCl$_2$, or MgCl$_2$. Values represent the means of 2 experiments, performed in triplicate.
affinity for $^3$H-kainic acid than did control isotherms (14 nM with NaCl vs. 5 nM control). Maximal $^3$H-kainic acid binding was similar in the presence of NaCl to that obtained in Na$^+$-free buffer (Fig. 20). It should be noted that $^3$H-kainic acid binding to synaptic membranes has the same affinity but is more than twice as enriched per mg protein as binding to whole particulate fractions (Fig. 13). MnCl$_2$ had a similar effect in lowering the affinity of $^3$H-kainic acid binding (20 nM with Mn$^{++}$ vs. 9.6 nM without Mn$^{++}$) without altering the Bmax. In the study involving Mn$^{++}$, tris-Cl buffer was substituted for tris-citrate buffer because of the previously noted chelating effect of tris-citrate. The affinity of $^3$H-kainic acid binding appeared to be somewhat lower in tris-Cl than in tris-citrate buffer. These results are consistent with competitive rather than noncompetitive inhibition of $^3$H-kainic acid binding by Na$^+$ and Mg$^{++}$.

The effects of cations upon the affinities of glutamate agonists and antagonists for the kainic acid receptor were determined (Fig. 21). Seventy mM NaCl, a concentration which reduces $^3$H-kainic acid binding at 5 nM by approximately 60%, lowers the IC$_{50}$'s of L-glutamate, L-GDEE, and kainic acid for the kainic acid receptor. Concentrations of these compounds which produce 50% inhibition of binding in the absence of NaCl have very little effect on binding in the presence of 70 mM NaCl. Thus, Na$^+$ does not differentiate glutamate agonists from antagonists, as it lowers the affinities of both.

The effect of pH on $^3$H-kainic acid binding to rat brain synaptic membranes was determined by varying the pH of the buffer from 5.5 to 11. Specific $^3$H-kainic acid binding is greatest between the pHs of 6.5 and
Fig. 20. Scatchard plots of $^3$H-kainic acid binding $\pm \text{Na}^+$ -- Scatchard plots were constructed of saturation data generated for $^3$H-kainic acid binding to whole rat brain synaptic membranes in the presence of 70 mM NaCl and in control media.
Fig. 21. Effect of 70 mM NaCl upon displacement of $^3$H-kainic acid by agonists and antagonists — Displacement data were determined using whole rat brain synaptic membranes in control media or in the presence of 70 mM NaCl. Values represent the means of triplicate determinations from one experiment, which varied less than 15%.
8.5, and is decreased at pHs above and below this range (Fig. 22). Nonspecific $^3$H-kainic acid binding increases with increasing pH.

Fig. 22. Effect of pH on $^3$H-kainic acid binding -- Values represent means of triplicate determinations, which varied less than 10%, from a representative experiment.
Endogenous Inhibitor of $^3$H-Kainic Acid Binding

In preliminary studies, we found that brain tissue must be thoroughly washed to achieve maximal binding of $^3$H-kainic acid. The amount of an inhibitor of binding present in the supernatant is lower after each tissue wash (Table 8) and does not appreciably alter binding at the concentration present after four resuspensions of the tissue in fresh buffer.

Table 8. Inhibition of $^3$H-kainic acid binding by rat brain supernatant -- Frozen whole rat brain was washed by suspending in 30 volumes of fresh buffer and centrifuging four consecutive times. The amount of specific $^3$H-kainic acid binding to 0.5 ml of the tissue homogenate was determined after each centrifugation and resuspension. Two ml aliquots of the supernatant from each centrifugation were tested for ability to displace 5 nM $^3$H-kainic acid from thoroughly washed whole rat brain membranes, in a final assay volume of 4 ml. Values are expressed as per cent displacement of specific $^3$H-kainic acid binding.

<table>
<thead>
<tr>
<th>Number of washes</th>
<th>Specific $^3$H-kainic acid binding (DPM)</th>
<th>% Inhibition of binding by supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>129</td>
<td>56%</td>
</tr>
<tr>
<td>2</td>
<td>295</td>
<td>44%</td>
</tr>
<tr>
<td>3</td>
<td>422</td>
<td>33%</td>
</tr>
<tr>
<td>4</td>
<td>436</td>
<td>18%</td>
</tr>
</tbody>
</table>
The supernatant of the first tissue centrifugation, containing high quantities of the inhibitor(s) of $^3$H-kainic acid binding, was treated by methods that might reveal the presence of an inhibitory protein (Table 9). Boiling the supernatant of the first tissue centrifugation did not alter its inhibitory potency, nor did incubation of the inhibitor with trypsin. Dialysis of the supernatant removed all inhibitory activity. Therefore, the endogenous inhibitor of $^3$H-kainic acid binding is heat-stable, trypsin-insensitive, and dialyzable. These properties are consistent with its being L-glutamate, which is present at mM concentrations in brain tissue and is a strong inhibitor of $^3$H-kainic acid binding.

Table 9. Treatment of endogenous inhibitor — Twenty μl of a 1:10 dilution of the supernatant from a centrifugation of a 3% rat cortex homogenate was added to a 2 ml assay, and specific $^3$H-kainic acid binding determined at 5 nM. Some samples of supernatant were boiled for 2 minutes, or treated with 10 μg/ml trypsin for 30 minutes at 37°C then boiled, prior to addition. Supernatant was also dialyzed at 4°C for 16 hours, then 200 μl added to the assay. Values are expressed as percent inhibition of specific $^3$H-kainic acid binding.

<table>
<thead>
<tr>
<th>Addition</th>
<th>% Inhibition of specific $^3$H-kainic acid binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>68%</td>
</tr>
<tr>
<td>Supernatant—Boiled</td>
<td>75%</td>
</tr>
<tr>
<td>Supernatant—Trypsin-treated, Boiled</td>
<td>63%</td>
</tr>
<tr>
<td>Dialyzed Supernatant</td>
<td>0%</td>
</tr>
<tr>
<td>0.1 mM L-glutamic acid</td>
<td>100%</td>
</tr>
</tbody>
</table>
Muscimol and Kainic Acid Binding Following Kainic Acid Lesion

Rats were lesioned by injection of kainic acid into the striatum, as described in Materials and Methods. Upon recovering from the anesthetic, all animals rotated vigorously in a direction contralateral to the lesioned side, as described previously (Coyle and Schwarcz, 1976). This rotation lasted not longer than 24 hours and was accompanied by clonic movements of the limbs.

Some animals were sacrificed at various times after lesioning for histological examination. Twelve hours following kainic acid injection, neurons in the injected striatum were shrunken and had pyknotic nuclei. Edema was apparent around neurons, blood vessels, and in the neuropil, while myelinated fibers appeared unaffected. Two days following injection, the grey matter showed striking edema and cell shrinkage. Myelinated fibers were, for the most part, unaffected. After 21 days, most of the caudate exhibited marked neuronal degeneration with replacement gliosis, while myelinated fiber bundles were relatively well preserved. The extent of lesion within the striatum varied somewhat among the animals. In all animals, the inferior medial portion of the caudate was spared. The extent of the uninvolved area varied among the animals, but in no case extended over more than 10-15% of the total volume of the caudate. The cerebral cortex immediately surrounding the needle track showed degenerative changes similar to those that occurred in the striatum. The average weights of control and lesioned striatum at 48 days after lesion were $46 \pm 2$ mg and $29 \pm 2$ mg, respectively ($n = 10$).
The activity of choline acetyltransferase (CAT), an enzyme located in cholinergic nerve terminals, was measured at 5, 8, and 48 days following lesion. CAT activity was reduced maximally to 33% of control within 5 days after the lesion, indicating destruction of cholinergic neurons within the striatum (Table 10).

Table 10. Alterations in CAT activity, \(^3\)H-muscimol binding, and \(^3\)H-kainic acid binding in kainic acid-lesioned striatum — Values are expressed as mean ± S.E.M. Significance was determined by student's paired "t" test. N.S. = not significant.

<table>
<thead>
<tr>
<th>Days after lesion: (n)</th>
<th>Control</th>
<th>Lesion</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAT activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(nmol/mg protein/hr)</td>
<td>(nmol/mg protein/hr)</td>
<td></td>
</tr>
<tr>
<td>5 (9)</td>
<td>185 ± 10</td>
<td>60 ± 6</td>
<td>33%, p &lt; 0.001</td>
</tr>
<tr>
<td>8 (9)</td>
<td>208 ± 5</td>
<td>80 ± 11</td>
<td>39%, p &lt; 0.001</td>
</tr>
<tr>
<td>48 (10)</td>
<td>163 ± 8</td>
<td>78 ± 8</td>
<td>47%, p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>(^3)H-muscimol binding</td>
<td>(^3)H-muscimol binding</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(fmol/mg protein)</td>
<td>(fmol/mg protein)</td>
<td></td>
</tr>
<tr>
<td>30 (5)</td>
<td>184 ± 20</td>
<td>249 ± 31</td>
<td>135%, N.S.</td>
</tr>
<tr>
<td></td>
<td>(^3)H-kainic acid binding</td>
<td>(^3)H-kainic acid binding</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(fmol/mg protein)</td>
<td>(fmol/mg protein)</td>
<td></td>
</tr>
<tr>
<td>5 (9)</td>
<td>149 ± 4</td>
<td>132 ± 7</td>
<td>89%, N.S.</td>
</tr>
<tr>
<td>8 (9)</td>
<td>177 ± 18</td>
<td>173 ± 18</td>
<td>98%, N.S.</td>
</tr>
<tr>
<td>14 (7)</td>
<td>136 ± 10</td>
<td>106 ± 5</td>
<td>78%, p &lt; 0.001</td>
</tr>
<tr>
<td>21 (4)</td>
<td>138 ± 16</td>
<td>81 ± 16</td>
<td>59%, N.S.</td>
</tr>
<tr>
<td>48 (10)</td>
<td>206 ± 13</td>
<td>52 ± 10</td>
<td>25%, p &lt; 0.001</td>
</tr>
</tbody>
</table>
The binding of $^3$H-muscimol to lesioned and unlesioned striata was measured at a concentration of 4 nM $^3$H-muscimol. The density of $^3$H-muscimol binding in lesioned striatum was not significantly different than the density measured in the contralateral control striatum (Table 10). Binding did not differ significantly from that measured in left (203 ± 8 fmol/mg protein) and right (247 ± 26 fmol/mg protein) striata of unlesioned rats.

$^3$H-Kainic acid binding to lesioned and control striatum was measured at a concentration of 10 nM at various times following lesion. The density of $^3$H-kainic acid binding was not significantly different from control at 5 or 8 days after lesioning (Table 10). However, binding was reduced to 78% of control after 14 days, and decreased to 25% of control at 48 days after lesion (Fig. 23).

$^3$H-Muscimol and $^3$H-Kainic Acid Binding in Huntington's Disease

The density of $^3$H-muscimol binding was determined in four regions of HD and control human brains, the frontal cortex, cerebellum, putamen, and caudate nucleus. Binding was determined at a concentration of 20 nM $^3$H-muscimol. Tissues were incubated with 0.05% Triton-X100 for 30 minutes at 37°C, then washed three times as described in Materials and Methods prior to assay.

$^3$H-Muscimol binding in 5 HD caudate nuclei was reduced significantly by 36% from control values (Table 11). Similarly, $^3$H-muscimol binding density of HD putamen was reduced significantly by 33%. $^3$H-Muscimol binding in HD cerebellum and frontal cortex was not significantly different than control values (Table 11).
Fig. 23. Changes in CAT activity and $^3$H-kainic acid binding after kainic acid lesion of the striatum -- Values represent means ± S.E.M. for 4-10 animals.
Table 11. $^3$H-Muscimol binding in HD — $^3$H-Muscimol binding was determined using Triton-treated, thoroughly washed HD and control brains at a concentration of 20 nM. Values are expressed as mean (fmoles/mg protein) ± S.E.M. Significance was determined by student's unpaired "t" test.

<table>
<thead>
<tr>
<th>Area</th>
<th>Control (n)</th>
<th>HD</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caudate</td>
<td>854 ± 65 (5)</td>
<td>544 ± 50 (5)</td>
<td>64%, p &lt; 0.01</td>
</tr>
<tr>
<td>Putamen</td>
<td>700 ± 61 (5)</td>
<td>470 ± 71 (5)</td>
<td>67%, p &lt; 0.05</td>
</tr>
<tr>
<td>Frontal Cortex</td>
<td>3250 ± 370 (5)</td>
<td>3488 ± 724 (5)</td>
<td>107%, n.s.</td>
</tr>
</tbody>
</table>
Preliminary studies of $^3$H-kainic acid binding in human brain indicated that the receptor has a similar affinity and binding density to the receptor of rat brain. Saturation isotherms were determined using cerebellum and temporal cortex of control human brain. Dissociation constants for $^3$H-kainic acid binding of 11 nM and 7 nM were obtained for these areas, with binding densities of 145 fmoles/mg protein and 118 fmoles/mg protein, respectively (Figs. 24 and 25). In both human cortex and cerebellum, $^3$H-kainic acid was displaced by L-glutamate, with IC$_{50}$s of 0.2 μM and 0.6 μM, respectively.

$^3$H-Kainic acid binding density was determined in 5 regions of HD and control human brains (Table 12). $^3$H-Kainic acid binding density was significantly decreased in the caudate nucleus and putamen of 12 HD brains as compared to 12 control brains. Binding was reduced to 45% of control in the caudate nucleus and to 47% of control in the putamen (p < 0.001 by student's "t" test) (Fig. 26). In the frontal cortex, cerebellum, and globus pallidus, the density of $^3$H-kainic acid binding in HD brains was not significantly different from control (Fig. 27).

$^3$H-Kainic acid binding density in the caudate nucleus and putamen was not significantly correlated with duration of HD symptoms, nor with age at time of death for either HD patients or controls.
Fig. 24. $^3$H-Kainic acid binding to HD cerebellum — Scatchard plot of saturation data from one experiment, performed in triplicate. Correlation coefficient of linear regression ($r$) = 0.80.
Fig. 25. $^3$H-Kainic acid binding to HD temporal cortex — Scatchard plot of saturation data from one experiment, performed in triplicate. Correlation coefficient of linear regression $(r) = 0.88$. 
Table 12. $^3$H-Kainic acid binding in HD — Values expressed as fmoles/mg protein ± S.E.M. (N). Significance was determined by student's unpaired "t" test.

<table>
<thead>
<tr>
<th>Region</th>
<th>Control (N)</th>
<th>HD (N)</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caudate</td>
<td>115.5 ± 7.7 (12)</td>
<td>51.5 ± 8.2 (11)</td>
<td>45%, p &lt; .001</td>
</tr>
<tr>
<td>Putamen</td>
<td>128.5 ± 8.7 (12)</td>
<td>70.0 ± 5.8 (11)</td>
<td>47%, p &lt; .001</td>
</tr>
<tr>
<td>Globus Pallidus</td>
<td>14.4 ± 2.5 (5)</td>
<td>15.3 ± 3.6 (5)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Frontal Cortex</td>
<td>139.1 ± 14.4 (7)</td>
<td>148.6 ± 13.9 (10)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>130.7 ± 14.9 (4)</td>
<td>151.4 ± 7.2 (3)</td>
<td>N.S.</td>
</tr>
</tbody>
</table>
Fig. 26. $^3$H-Kainic acid binding to HD and control caudate nucleus and putamen -- Each point represents a different brain.
Fig. 27. $^3$H-Kainic acid binding to HD and control frontal cortex and cerebellum -- Each point represents a different brain.
**Muscimol**

\(^3\)H-Muscimol appears to bind specifically to the synaptic GABA receptor of rat brain membranes. By several criteria, \(^3\)H-muscimol binding is similar to sodium-independent \(^3\)H-GABA binding and is distinct from sodium-dependent \(^3\)H-GABA binding, which is thought to represent attachment of the isotope to a transport site for GABA (Enna and Snyder, 1975). The two-fold increase in binding to previously frozen tissue, enhancement of binding by Triton treatment of membranes, and broad pH optimum centered around pH 7 are all characteristics of both \(^3\)H-muscimol binding and of sodium-independent \(^3\)H-GABA binding. In contrast, sodium-dependent \(^3\)H-GABA binding is reduced by 80% in previously frozen tissue, is absent after Triton treatment of membranes, and exhibits a sharp pH optimum at pH 7.7 (Enna and Snyder, 1975). The absence of any enhancement of \(^3\)H-muscimol binding to fresh tissue in the presence of Na\(^+\) is particularly significant, since 100 mM Na\(^+\) causes a ten-fold increase in \(^3\)H-GABA binding to fresh tissue, representing enhanced binding to a transport site. The subcellular distribution of \(^3\)H-muscimol binding, with the synaptosomal membranes containing the greatest density and absolute number of binding sites (75% of the total in the rat brain), is consistent with that expected of a synaptic receptor.
The substrate specificity of the $^3$H-muscimol binding site provides the strongest evidence that this alkaloid is highly specific for the GABA receptor. Thus, the $^3$H-muscimol receptor displays appreciable affinity and stereospecificity only for those drugs and amino acids which are known to interact neurophysiologically with the GABA receptor. $^3$H-Muscimol is strongly displaced from binding sites by several potent GABA agonists, most effectively by muscimol itself and 3-amino-propanesulfonic acid, and also by trans-4-aminocrotonic acid, GABA, and imidazole-acetic acid. As noted in the text, several other GABA agonists have relative potencies in displacing $^3$H-muscimol binding similar to their relative potencies as GABA agonists in neurophysiological tests. $^3$H-Muscimol is displaced from its binding site by the neurophysiologically active GABA antagonist and convulsant (+) stereoisomer of bicuculline, but not by a five-fold greater concentration of the relatively inactive (-) isomer of bicuculline.

The $K_i$ for unlabeled muscimol is 2.4 nM, as calculated from the $IC_{50}$ for muscimol displacement of $^3$H-muscimol and the concentration of $^3$H-muscimol in the displacement assay. This value is in good agreement with the $K_D$ of 2.2 nM derived from Scatchard analysis of saturation isotherms for $^3$H-muscimol binding, and indicates that tritiation of muscimol does not alter its binding characteristics. Furthermore, this $K_D$ is similar to the $IC_{50}$ (3 nM) for muscimol in displacing specifically bound $^3$H-GABA from rat brain GABA receptors (Enna and Snyder, 1977).

It is also notable that agents such as norepinephrine, dopamine, serotonin, and histamine do not inhibit muscimol binding, suggesting that the behavioral effects observed after muscimol administration are
not mediated by activation or inhibition of these neurotransmitter receptor sites. However, it is still possible that the actions of muscimol are partially mediated through other neurotransmitter systems by an effect on other transmitter processes such as synthesis, metabolism, or uptake. It is unlikely, though, that muscimol would have an affinity for any of these processes greater than its observed affinity for the GABA receptor.

Further support for the idea that \(^3\)H-muscimol binds specifically to the GABA receptor is provided by the nearly identical regional distribution of \(^3\)H-muscimol binding, when measured at a concentration of 2 nM (the \(K_D\) of the higher affinity binding site) to the distinctive regional distribution of sodium-independent \(^3\)H-GABA binding (Enna, Beaumont, and Yamamura, 1978). In addition, the \(B_{\text{max}}\) of 0.7 pmol/mg protein for high affinity \(^3\)H-muscimol binding to synaptic membranes is quite similar to the \(B_{\text{max}}\) for high affinity sodium-independent GABA binding of 0.69 pmol/mg protein, measured in frozen and washed rat brain synaptic membranes (Greenlee et al., 1978), and 0.7 pmol/mg protein, measured in Triton treated synaptic membranes (Enna and Snyder, 1977).

A lower affinity sodium-independent \(^3\)H-GABA binding site is present in thoroughly washed synaptic membranes with \(K_D = 146\) nM and \(B_{\text{max}} = 3.14\) pmol/mg protein (Greenlee et al., 1978), and also in Triton treated synaptic membranes, with \(K_D = 130\) nM and \(B_{\text{max}} = 5\) pmol/mg protein (Enna and Snyder, 1977). This lower affinity sodium-independent GABA receptor has pharmacological characteristics qualitatively similar to the high affinity sodium-independent GABA receptor. \(^3\)H-Muscimol also binds to a lower affinity site, although only in frozen but not Triton
treated membranes, with a Bmax (2.8 pmol/mg protein) similar to that of the low affinity GABA receptor. The regional distribution of the low affinity $^3$H-muscimol binding site is basically similar to that of the high affinity site, although binding is greatest for the low affinity site in the cerebral cortex rather than the cerebellum. Interestingly, binding density is also greater in the cerebral cortex than the cerebellum for $^3$H-(+)-bicuculine-methiodide (Mohler and Okada, 1977). The IC$_{50}$ of muscimol for displacing $^3$H-(+)-bicuculline is 58 nM, quite close to the low affinity $K_D$ for $^3$H-muscimol binding of 60 nM. These studies raise the possibility that the low affinity $^3$H-muscimol binding site may be equivalent to an antagonist binding site, whose existence was proposed by Mohler and Okada (1977). Alternatively, the two sites might represent functionally distinct thought pharmacologically similar GABA receptor subpopulations.

These studies indicate that the behavioral and neurochemical results observed after administration of muscimol are primarily the result of GABA receptor activation. Following the characterization of $^3$H-muscimol binding (Beaumont et al., 1977, 1978) and its detailed comparison with sodium-independent GABA binding (Enna et al., 1978), $^3$H-muscimol has been used in the autoradiographic localization of central nervous system GABA receptors (Chan-Palay and Palay, 1978). Thus, muscimol is a potent and specific compound for in vitro and in vivo studies of the GABA receptor.
Kainic Acid

The identity of the $^3$H-kainic acid binding site is less clear than that of the muscimol binding site. A previous report (Simon et al., 1976) demonstrated that kainic acid binding is enriched in synaptic membranes, is not detectable in peripheral tissues or in white matter of the central nervous system, and has a varied regional distribution, characteristics which are consistent with binding to a synaptic receptor. In our hands, the affinity of $^3$H-kainic acid binding to rat brain membranes is approximately ten-fold higher than the affinity of 60 nM reported by Simon et al. (1976). This higher affinity of 5 nM is confirmed by displacement studies using unlabeled kainic acid, which has an IC$_{50}$ of 6 nM in displacing $^3$H-kainic acid. Since the regional distribution of the high affinity binding site by our determination is quite similar to the distribution of $^3$H-kainic acid binding reported by Simon et al., the difference in affinities most likely represents methodological differences rather than distinct receptor sites. The only notable differences in methods between the two studies are the higher specific activity of the $^3$H-kainic acid and the more extensive tissue washing procedure utilized in our studies. The latter is a likely cause of the measured difference in affinities, since brain tissue contains high quantities of a competitive inhibitor of $^3$H-kainic acid binding which are removed only by extensive washing. Also, since the kainic acid, which is labeled by tritium exchange, is isolated from natural sources, the amount of less active stereoisomers present in different batches may be variable.
The ability of several compounds to displace $^3$H-kainic acid binding parallels their neuroexcitatory potency. Quisqualic acid, a heterocyclic alkaloid with a neuroexcitatory potency slightly greater than kainic acid's when tested on rat spinal neurons (Biscoe et al., 1976), has an affinity for the kainic acid binding site nearly equal to that of kainic acid. Dihydrokainic acid, which is more potent than kainic acid as an inhibitor of glutamate uptake (Biziere and Coyle, 1978b) but is over 100-fold less potent than kainic acid in depolarizing rat spinal neurons (Johnston et al., 1974), is also considerably less potent than kainic acid in displacing $^3$H-kainic acid binding. Kainic acid itself has a potency range reportedly 18-54 (Biscoe et al., 1976) or 8-80 (Johnston et al., 1974) times that of L-glutamic acid in depolarizing rat spinal neurons, and 3-25 times that of L-glutamic acid in depolarizing rat thalamic neurons (McLennan and Wheal, 1978). This neurophysiological potency range is in good agreement with the difference in affinities of these two compounds for the $^3$H-kainic acid binding site, kainic acid having an affinity 20-fold greater than L-glutamic acid. D-Glutamic acid, which is generally reported to be less potent than L-glutamic acid in microiontophoretic studies (Krnjevic and Phillis, 1963), has a lower affinity for the $^3$H-kainic acid binding site than does the L-isomer.

However, significant differences exist between the affinities of certain excitatory amino acids for the $^3$H-kainic acid binding site and their neuroexcitatory potencies. L-Cysteate and DL-homocysteate are equal to or greater than L-glutamate in neuroexcitatory potency, yet are considerably less effective than L-glutamate in displacing $^3$H-kainic
acid binding. Aspartic acid, which has a neuroexcitatory potency similar to that of L-glutamic acid, is a very weak inhibitor of $^3$H-kainic acid binding, with an IC$_{50}$ of 0.4 mM. These results suggest that aspartate, homocysteate, and cysteate act at a separate receptor for excitatory amino acids, possibly the "aspartate-preferring" receptor proposed by some investigators (Buu et al., 1976). This possibility is further supported by the very low affinity (IC$_{50} =$ 0.85 mM) for the kainic acid receptor of N-methyl-D-aspartate, an extremely potent neuronal excitant which reportedly acts preferentially at aspartate receptors (Johnston et al., 1974). Furthermore, $\alpha$-amino adipate, a compound which antagonizes L-aspartate but not L-glutamate induced excitation, is only a weak inhibitor of kainic acid binding. The inhibition that does occur with $\alpha$-amino adipate may represent its action as a partial agonist. Thus, the pharmacological specificity of the kainic acid binding site is consistent with that of a glutamate-preferring excitatory receptor, and clearly distinguishes this site from an excitatory receptor at which aspartate might act.

Several antagonists of L-glutamate-induced excitation were very weak or ineffective inhibitors of $^3$H-kainic acid binding. This may reflect the non-specificity of their action in depressing neuronal firing rates. Also, some antagonists may block the ionophores associated with glutamate receptors, as is the case with picrotoxin inhibiting GABAergic actions by blockade of the chloride ionophore associated with GABA receptors. Numerous neurotransmitters and centrally acting compounds were inactive in displacing $^3$H-kainic acid binding, including convulsants (metrazol, strychnine, bicuculine) and
anticonvulsants and neuronal depressants (diphenylhydantoin, phenobarbital, diazepam, and α-hydroxybutyrate).

For several receptor systems, specific ions alter the affinity of either agonist or antagonist binding, possibly causing a shift between two states of the receptor. For example, in the presence of iodide or thiocyanate, the IC$_{50}$ of the antagonist bicuculline in displacing $^3$H-GABA binding is lowered ten-fold, while the IC$_{50}$ of GABA remains unaltered (Enna and Snyder, 1977). We investigated the effects of several ions upon $^3$H-kainic acid binding to determine whether a similar increase in antagonist affinity might be effected. We found that monovalent and divalent cations strongly inhibit $^3$H-kainic acid binding. Hill constants for inhibition by monovalent cations are generally near to or greater than 1, while Hill constants for inhibition by divalent cations are grouped nearer to a value of 0.5, suggesting that divalent cations do not displace $^3$H-kainic acid from its binding site by a simple competitive interaction. Cations may inhibit binding by forming salt bridges between acidic groups within the receptor structure, thereby stabilizing the active site in a conformation less favorable for binding kainic acid. Such a mechanism holds for several enzymes which are allosterically inhibited by ions (Perutz, 1978). If such a mechanism is responsible for the inhibition of kainic acid binding by cations, then one would predict from the Hill constants that monovalent cations act at one location per receptor to alter the affinity of the binding site, while divalent cations act at two locations per receptor. Alternatively, monovalent cations may bind to anionic groups within the recognition site for $^3$H-kainic acid,
competitively displacing the isotope. Also, cations may chelate kainic acid and lower its effective concentration. Such a possibility holds especially for divalent cations, which often chelate compounds with multiple acidic groups. Except for fluoride, which appears to enhance the binding of \(^3\text{H}\)-kainic acid, the monovalent anions tested had little effect upon binding.

Neither monovalent (Na\(^+\)) nor divalent (Mn\(^{++}\)) cations were useful in differentiating antagonists from agonist binding or in converting conformations of the receptor, since the affinities of both were lowered. This result may, once again, reflect the lack of specific antagonists for glutamate receptors. The effects of pH and the distinctive effects of cations upon \(^3\text{H}\)-kainic acid binding may be useful in the future for comparison with binding studies of other ligands to excitatory amino acid receptors.

High quantities of endogenous substances which displace \(^3\text{H}\)-kainic binding were found in brain tissue, requiring thorough washing for removal. The endogenous inhibitors of binding are heat-stable, dialyzable, and trypsin-insensitive. Thus, an inhibitory protein, such as that recently found in association with GABA receptors (Toffano, Guidotti, and Costa, 1978), does not appear to be present. L-Glutamate would appear to be the major inhibitor of \(^3\text{H}\)-kainic acid binding present in brain tissue. However, we cannot rule out the possibility that small molecules other than glutamate may be present in brain tissue which interact with the kainic acid receptor. Furthermore, the marked inhibition of \(^3\text{H}\)-kainic acid binding by ions indicates that a portion of
the inhibition produced by brain supernatants is due to cations present in the tissue homogenates.

**Kainic Acid Lesions**

The behavioral events, histological alterations, and rapid decrease in CAT activity that we observed following kainic acid lesion of the striatum are consistent with findings reported in the literature (Coyle et al., 1977). $^3$H-Muscimol binding is not significantly different from control at 30 days following lesion. This finding indicates that GABA receptors are not located on neurons intrinsic to or arising from the striatum, which are destroyed by the lesion. Thus, GABA receptors may reside on terminals of afferents to the striatum, which originate in the cortex, substantia nigra, and thalamus, as well as other regions. However, preliminary evidence from this laboratory indicates that $^3$H-muscimol binding in the striatum is not altered 30 days after 6-hydroxy-dopamine injection into the substantia nigra, which destroys nigrostriatal dopaminergic neurons. Therefore, GABA receptors may reside in corticostriatal or thalamostriatal terminals, or on the endings of one of the minor tracts innervating the striatum.

These findings contrast with reports that $^3$H-GABA binding is increased several fold at 10 days after striatal lesion with kainic acid (Schwarcz and Coyle, 1977) and is decreased by 80% when measured 9 months after lesion (Zazcek, Schwarcz, and Coyle, 1978). Since striatal GAD activity and presumably therefore intrinsic GABAergic neurons are severely reduced within 3 days of lesioning, GABA receptors, if located on unaffected axonal terminals, may undergo denervation.
supersensitivity following lesion, resulting in the increased GABA binding measured at early time periods. The marked decrease in striatal 
$^3$H-GABA binding measured after 9 months may represent slow trans-
synaptic degeneration or functional alteration of striatal afferents.

The slow decrease in $^3$H-kainic acid binding following kainic acid lesion is unexpected, since dendrites and cell bodies, which would be expected to bear the glutamate receptors, are destroyed within 2-3 days of lesioning (Hattori and McGeer, 1977). However, Olney and de Gubareff (1978a) have recently reported that postsynaptic densities, visualized by electron microscopy, remain adhering to the presynaptic terminals at 21 days after kainic acid lesion. The postsynaptic densities are thought to contain receptors for neurotransmitters, and their continued presence after dendrites and cell bodies have degenerated may account for the relatively slow decline in kainic acid receptor density after lesion. The eventual 75% decrease in kainic acid binding 48 days after lesion suggests that striatal kainic acid receptors are located on neurons with cell bodies intrinsic to the striatum, possibly at sites postsynaptic to corticostriatal glutamatergic afferents.

**Huntington's Disease**

Previous reports that $^3$H-GABA binding in HD caudate is unaltered (Enna, Bird et al., 1976) or decreased by 70-80% (Lloyd, Dreksler, and Bird, 1977) utilized frozen tissue that had been washed twice. It has recently been reported that significant amounts of an endogenous inhibitor of GABA-binding, and possibly endogenous GABA as well, are
present unless the tissue is more thoroughly washed or treated with Triton (Greenlee et al., 1978; Toffano et al., 1978). Therefore, varying levels of endogenous inhibitor and GABA remaining in the tissue samples may have obscured measurement of the GABA receptor in these studies, especially considering the use in both studies of sub-saturating concentrations of $^3$H-GABA, which would be more easily displaced by inhibitors. Accordingly, we treated tissues with Triton prior to assay, and used a saturating concentration of $^3$H-muscimol. Under these conditions, we found significant decreases in $^3$H-muscimol binding of 36% in HD caudate nucleus and of 33% in HD putamen, but no significant alterations in HD frontal cortex. This finding contrasts with the lack of alteration in striatal $^3$H-muscimol binding 30 days after kainic acid lesion. This difference may reflect the longer process of degeneration in HD, possibly indicating late alterations in striatal afferents. On the other hand, the contrasting results may indicate that decreased GABA receptor density precedes degeneration in HD, rather than being a very late result of such degeneration. Decreased GABA receptor density and hence decreased inhibition of afferents to HD striatum, especially if localized to corticostriatal glutamate-releasing terminals, might precede and contribute to the degenerative process in HD.

$^3$H-Kainic acid binding was also found to be reduced in HD specifically in the caudate nucleus and putamen, by 47% and 52%, respectively. Kainic acid binding was not reduced in globus pallidus, frontal cortex, or cerebellum of HD brains. These results are a preliminary indication that destruction of elements postsynaptic to glutamate-releasing neurons is not generalized in HD, but rather is
restricted to the striatum. It should also be noted that a 50% reduc-
tion in $^3$H-kainic acid binding in the striatum following kainic acid
lesion is reached after approximately 30 days, proceeding to a 75%
reduction after 48 days. The extent of alterations at 30 days after
kainic acid lesion may therefore represent the average extent to which
striatal degeneration has proceeded in HD at the time of death.

Conclusion

The studies reported here demonstrate that muscimol acts
specifically at GABA receptors, and is a useful ligand both for in vitro
measurement of GABA receptors and for in vivo studies. Kainic acid
appears to act at glutamate-preferring receptors and does not interact
with aspartate-preferring receptors, although the development of
specific glutamate and aspartate antagonists and iontophoretic studies
of isolated vertebrate preparations are necessary to verify this site
of action.

The decrease in kainic acid binding in HD striatum parallels
the decrease measured after KA lesion, supporting the validity of the
KA lesion model for HD. This finding is consistent with the hypothesis
that excess glutamate-mediated neuroexcitation may be a factor in pro-
ducing striatal degeneration in HD. The decrease in muscimol binding in
HD striatum, though less pronounced than the decrease in kainic acid
binding, is significant and differs from the results obtained 30 days
following kainic acid lesion of the striatum, at which time no decrease
in muscimol binding was observed. This may reflect late degeneration
in HD of striatal afferent terminals, upon which GABA receptors appear
to be located, or may reflect an alteration in HD striatum that precedes the degenerative changes.

Thus, the ineffectiveness of muscimol in the treatment of HD does not appear to be due to a lack of specificity for GABA receptors nor to a dramatic decrease in GABA receptor density in HD brains. Muscimol may activate GABA receptors outside the striatum, such as in cortex and cerebellum, to produce adverse effects before therapeutic levels in the striatum are attained.

Future studies must focus on determining which populations of striatal afferents bear GABA receptors, upon isolation and characterization of GABA receptors and associated inhibitory factors, upon identification of the multiple receptors for excitatory amino acids, and upon determining the factors that regulate glutamate release in the striatum and their viability in HD.
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