DEVELOPMENTS IN MOLECULAR AND CELLULAR BIOCHEMISTRY 1

The Biological Effects of Glutamic Acid and its Derivatives

edited by V. A. Najjar

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Introduction to 'Developments in molecular and cellular biochemistry'

Molecular and Cellular Biochemistry is an international journal that covers a wide range of biophysical, biochemical and cellular research. This type of coverage is intended to acquaint the reader with the several parameters of biological research that are relevant to various fields of interest. Unlike highly specialized journals, it does not bring into focus a particular field of investigation on a monthly basis. Accordingly, it has been decided to supplement its present wide scope by periodic presentations of a restricted area of research in the form of book-length volumes. These volumes will also be published in hard covers as a book series entitled, *Developments in Molecular and Cellular Biochemistry*. Each volume will focus on an active topic of interest which will be covered in depth. It will encompass a series of contributions that deal exclusively with one single well-defined subject.

The present volume, *The Biological Effects of Glutamate and Its Derivatives,* is the first one in the book series. The second volume will deal extensively with *Immunologically Active Peptides.*

It is the editor's hope that *Molecular and Cellular Biochemistry* will fulfill its intended role of service to the international community of biological scientists.

The biological effects of glutamic acid and its derivatives

During the past decade, there has been much active research into the effects of glutamic acid and its derivatives on several target organs, particularly the nervous system. It is also involved in the γ-glutamyl cycle through the activity of the enzyme γ-glutamyl transpeptidase. Equally important are the poly-γ-glutamyl derivatives of folic acid. These have been scattered in many scientific journals, some in basic science journals and others in technical publications. In view of this and the rapid advances in this area during the past decade, it was deemed advisable to bring together many of these investigations into one single publication. This has been done under the auspices of *Molecular and Cellular Biochemistry,* an international journal, and has now been published as Volume 1 of 'Developments in Molecular and Cellular Biochemistry'.

Victor A. Najjar, Editor in chief

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Hippocampal glutamate receptors

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Summary

For years, the hippocampus has been the privileged domain of anatomists and electrophysiologists for investigating various neurobiological processes. The present review deals with recent work which shows that this structure is also well suited to study the role of glutamate as a neurotransmitter and more particularly the characteristics of glutamate receptors and their possible involvement in hippocampal function. After a brief description of the main anatomical features of the hippocampus, we attempt a critical evaluation of the electrophysiological studies of hippocampal glutamate receptors. We then describe the properties of Na-independent ^{3}H-glutamate binding sites in hippocampal membranes, and discuss the possibility that these binding sites are related to postsynaptic glutamate receptors. Finally we show that these binding sites are extremely labile and that hippocampal membranes possess various mechanisms which regulate their number. In particular we develop the idea that the calcium-stimulation of ^{3}H-glutamate binding in hippocampal membranes may be the mechanism by which electrical activity regulates the number of glutamate receptors at hippocampal synapses and thus induces long-lasting changes in synaptic transmission.

Introduction

Because of its relatively simple anatomical organization, the hippocampus has become increasingly popular as a model for the study of basic neurobiological processes. The cell bodies of hippocampal neurones form two densely packed and widely separated layers while the dendrites are arranged in parallel arrays stretching for considerable distances from the somata. The major extrinsic and intrinsic fiber projections travel in well defined layers oriented at right angles to the axis of the dendrites. Thus a given lamina of the hippocampus consists, for the most part, of dendritic segments from one cell type and synapses generated by one or two afferents, a situation which vastly simplifies the problem of analyzing well-defined inputs and their targets.

While most of the work exploiting these properties has involved anatomical or physiological questions, neurochemists have begun using the hippocampus for the study of transmitters and their receptors. The possibility that acidic amino acids are used as transmitter in excitatory synapses in the central nervous system (CNS) has come under particularly intense investigation (1–5). The hippocampus proves to have effective systems for the uptake and release of glutamate (6) and there is evidence which indicates that glutamate or a related compound is released during synaptic transmission (7). The hippocampus also possesses high affinity binding sites for glutamate (8) and one category of these possesses certain characteristics which render it a reasonable candidate for a post-synaptic receptor (9). It is this subject which forms the topic of the present paper. In the following sections we will review physiological and biochemical studies of glutamate receptors in hippocampus and attempt

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to relate these to each other and to the possible role of amino acids as transmitters. We shall also summarize recent evidence that the number of glutamate binding sites in hippocampal membranes is regulated by calcium-sensitive enzymes and consider the possibility that this form of receptor 'plasticity' is related to certain rather extraordinary physiological properties of hippocampal synapses. Before beginning the discussion of binding sites, it is appropriate that we first outline the various pathways within hippocampus which have been used in physiological and biochemical studies.

Anatomy of the hippocampus

Fig. 1 illustrates the major pathways and subdivisions of hippocampal formation. The dentate gyrus is composed of a 'C' shaped row of granule cell bodies, the dendrites of which generate a homogeneous, essentially cell-free molecular layer. This dendritic field is innervated by afferents from the entorhinal cortex ('perforant path') and ipsilateral ('associational' projections) and contralateral ('commissural' projections) hippocampus proper.

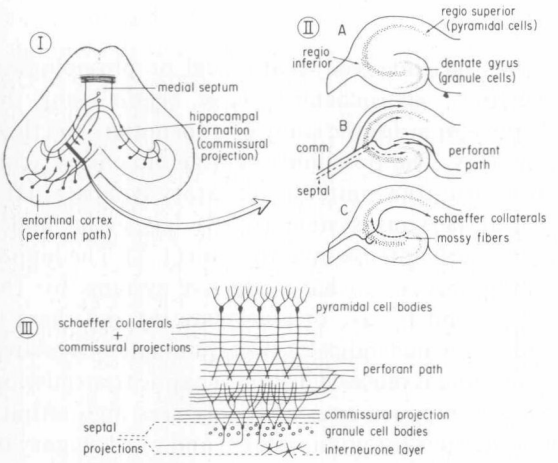

Fig. 1. Schematic illustration of major subdivisions and pathways of the hippocampus. The drawing at the top left is a horizontal view of the forebrain and illustrates the relative positions of hippocampus, entorhinal cortex and the septum (I). Three successive sections through the hippocampus are shown on the right; these indicate the subdivison (dentate gyrus, regio inferior, and regio superior) extrinsic and intrinsic pathways of the structure (II). At the bottom is shown a picture of the cell types of the regio superior and dentate gyrus including the distribution of key inputs (III).

These inputs are strictly segregated with the perforant path innervating the outer 3/4 of the molecular layer while the pyramidal cell projections share the inner portions of the dendritic trees (see ref. (10) for a review). The septum sends a cholinergic projection to the dentate gyrus but it is not yet clear if this terminates on the granule cells directly or instead is targeted for a population of interneurones (see ref. (11) for a review). The granule cell bodies are densely innervated by a group of subjacent basket cells; there is good evidence that these cells use GABA as their transmitter (12).

The granule cell axons, the mossy fibers, collect together on the inner face of the 'C' formed by the dentate gyrus and travel together as a bundle across the proximal dendrites of the pyramidal cells of the nearby regio inferior of the hippocampus proper. These fibers generate enormous boutons which are unusual in that they contain high concentrations of zinc and in all probability, enkephalin (13). The remaining portions of the pyramidal cell dendrites are innervated by the pyramidal cells of the ipsilateral and contralateral hippocampus as well as by the entorhinal cortex. The regio inferior pyramidal cells produce a dense bilateral projection to the apical dendrites of the regio superior pyramidal cells; this is the so-called Schaeffer-commissural projection and has been the subject of many neurophysiological studies.

Neurophysiological studies of amino acid receptors in hippocampus

Effects of various amino acids on cell physiology

A variety of neurophysiological techniques have been used to study amino acid receptors in hippocampus and in general the data accord well with those obtained for other structures. Attempts have also been made to determine if these receptors are used by several of the synaptic systems in hippocampus and, while certainly not conclusive, the results have been encouraging.

When applied by iontophoresis, glutamate and aspartate both cause a marked increase in the firing rates of hippocampal pyramidal and granule cells (14, 15). These effects are produced by application to the dendritic trees as well as to the cell bodies, occur with extremely short latencies, and can be

elicited by simply removing the 'holding' current on the glutamate electrode (15). The excitatory action of both amino acids is completely suppressed by iontophoretic application of glutamic acid diethyl ester (GDEE) in the regio superior; however, this drug has no effect on aspartate in the dentate gyrus (16). GDEE appears to have no effect on cell firing changes produced by acetylcholine in dentate gyrus (16, 17) and hippocampus proper (15). Segal (18) has measured the effects of pressure application of amino acids to the dendrites on the membrane potential and conductance of the CA1 pyramidal cell body and reports that glutamate produces a marked depolarization of the pyramidal cells followed by a long lasting hyperpolarization. He also found evidence for an inhibitory interneurone which appears to have an extremely rapid response to glutamate. Somewhat surprisingly, D-glutamate proved to have about the same potency as the L-isomer. Quisqualic and D,L-homocysteic acids were about 25 times more potent than glutamate while kainic acid was the most powerful amino acid tested, being some 100 times as effective as glutamate. However, kainic acid had a slower onset time than did glutamic acid and its effects persisted for a much longer period than did glutamate, suggesting that the two amino acids may be operating in a qualitatively different fashion. The effects of glutamate were blocked by D-glutamylglycine (DGG), 2-amino-5 phosphonovaleric acid (2APV) and GDEE, in that order of efficiency.

Effects of amino acid antagonists on synaptic physiology

There have been several recent attempts to block synaptic potentials in hippocampus with antagonists of the excitatory amino acids. Two of the better controlled studies have employed iontophoretic application of these drugs to the granule cells of the dentate gyrus. Wheal & Miller (17) report that GDEE, at concentrations which block iontophoretically applied glutamate, inhibits cell discharges elicited by stimulation of the perforant path but not by stimulation of the medial septum. Atropine, which blocks ACh but not glutamate-induced excitation of the granule cells, suppresses cell discharges elicited by stimulation of the medial septum but not those caused by perforant path activation. Hicks & McLennan (16) found that iontophoretically applied GDEE blocked cell discharge elicited by glutamate but not those recorded in response to aspartate or its analogue N-methyl-DL-aspartate (NMDA) (as noted above, however, GDEE has not been reported to block aspartate in the *regio superior*). D-a-aminoadipate (D-α-AA) produced a converse set of results: it blocked the aspartate response without influencing the response of the granule cells to glutamate. GDEE inhibited the discharge of the granule cells to perforant path but not commissural stimulation while D-α-AA produced the opposite pattern of results in 7 cells (Table 1).

Taken together these results make a reasonable case for the following conclusions: 1) a 'glutamate' receptor is used in the perforant path synapses but not in the connections arising from the commissural projections; 2) an 'aspartate' (or NMDA preferring) receptor is involved in transmission across the commissural but not perforant path synapses. It bears emphasizing that the physiological measures used in these studies, namely spike discharges, are both physically and temporally removed from the transmission process: the experiments measure the effects of the drugs on the responses of the granule cells to EPSP's but not the effects of the drugs on the EPSP's themselves. The selectivity of drug action (e.g., GDEE blocks one

Table 1. The effect of three drugs applied by iontophoresis on the granule cell discharges elicited by putative transmitters or electrical stimulation of dentate gyrus afferents (see text for abbreviations).

	Glutamate	Aspartate	Acetylcholine	Perforant path	Comm. project	Medial septum
GDEE[a,b]	block	no effect	no effect	block	no effect	no effect
D-α-AA[a]	no effect	block	not tested	no effect	block	not tested
atropine[b]	no effect	not tested	block	no effect	not tested	block

[a] Based on Hicks & McLennan (16).
[b] Based on Wheal & Miller (17).

input but not another) tends to rule out generalized effects on the electronic properties of the granule cells; however, the afferents used are located at different levels of the dendritic trees and the possibility that they are differentially influenced by manipulations of the physiological properties of the primary dendrites and somata of the granule cells cannot be excluded. Hicks & McLennan (16) also report that D-α-AA causes an increase in the background firing rate of the granule cells. Since the increased firing is blocked by the GABA antagonist bicucilline, they assume that the effect is due to the actions of α-AA on a group of aspartate-driven inhibiting interneurones. While this is a reasonable interpretation, it must be noted that bicuculline has been shown to have multiple effects on hippocampal neurones (19) and therefore its actions in this pivotal control experiment cannot be assumed with certainty to be due to an antagonism of GABA. Furthermore, removal of tonic inhibition by α-AA would have been expected to produce an enhanced response by the granule cells to both glutamate and perforant path stimulation and this apparently did not occur.

Local application of antagonists via pressure injection has also been used to study the effects of antagonists on synaptic transmission in hippocampus. Segal (18) found that D-glutamylglycine (DGG). 2-amino-5-phosphonovaleric acid (2APV), and GDEE infusion into the apical dendrites of the regio superior pyramidal cells blocked EPSP's (recorded intracellularly) produced by stimulation of the Schaeffer-commissural inputs to these regions. However, he does not describe controls for potential non-specific effects of the drugs and accordingly these data must be considered as preliminary in nature.

Electrophysiological studies using the in vitro hippocampal slice

The *in vitro* slice method affords the opportunity to perfuse the hippocampus with drugs at known concentrations, as well as to identify their locus of action. While these features should be of great value in assessing the possible role of amino acid receptors in synaptic transmission, the pharmacological results thus far obtained with the slice have not been conclusive. Aminophosphonobutyric acid (APB) has been reported to block the synaptic field potentials generated by stimulation of the perforant path in the dentate gyrus (20) and by the Schaeffer-commissural fibers in the regio superior (20, 21). Interestingly, GDEE was found to be ineffective with bath applications although, as noted above, it does block responses when applied by pressure to the dendritic fields of regio superior or by iontophoresis to the cell bodies of the granule neurones (see Table 1).

While suggestive, these experiments do not satisfactorily establish that the observed actions of the drugs are due to post-synaptic receptor blockade. The concentrations of APB used in the above studies cause an increased discharge of the pyramidal cells (20) and are close to levels which produce pronounced physiological disturbances in slices. White *et al.* (20) argue that the increase in the frequency of cell firing produced by APB is due to the reduction of activity of inhibitory interneurones (via a suppression of amino acid innervation of these cells) but the effect could as well be due to a partial depolarization of the neurones. It has been reported that APB does not reduce antidromic potentials or fiber volleys in the slice (20); however, it is not clear if these extracellular measures are sufficiently sensitive to detect the small degree of depolarization needed to reduce release and post-synaptic excitatory potentials. It should be emphasized that relatively small drops in the membrane potential of nerve terminals have been shown to cause substantial decrements in transmitter release. In light of this, it is imperative that the sensitivity of controls for depolarization and non-specific effects etc. be carefully calibrated in pharmacological experiments using slices and to date this has not been done.

In summary, several lines of evidence point to the conclusion that hippocampus contains a full complement of acidic amino acid receptors. Furthermore, while individual experiments are not entirely satisfactory, the body of data suggests that these receptors are involved in synaptic transmission. There are points of confusion which require attention; chief among these is that GDEE blocks synaptic responses when applied by iontophoresis but appears relatively ineffective when perfused through slices.

Biochemical studies of hippocampal glutamate receptors

In addition to the physiological measures described above, receptors can also be analyzed using biochemical events associated with the interaction between them and their transmitters. In many cases this event can be the stimulation of an enzymatic activity linked to the receptor (e.g. β-adrenergic receptor and adenylate cyclase) (22). While excitatory amino acids induce an increased level of cyclic GMP in cerebellar slices (23), with a pharmacology relatively similar to that of the electrophysiological receptors (24), there have been no attempts to use this approach in hippocampal slices. Another biochemical approach to the study of receptors consists in measuring changes in ionic fluxes elicited by the transmitter, as exemplified in studies of the acetylcholine receptors (25). A recent study (26) showed that glutamate modifies sodium fluxes in striatal slices, but this procedure has not yet been applied to the identification of hippocampal glutamate receptors. The most popular technique for studying receptors in the past decade has been to measure the binding of an appropriate ligand to purified membranes. While simple and direct, these procedures suffer from the drawback that they do not provide any guarantee that the binding sites are part of a physiologically active receptor. Therefore, binding studies typically incorporate efforts to establish if the sites under study possess various properties which *a priori* would be expected of a 'true' receptor (27). Several of these anticipated properties are: 1) an affinity in the concentration range over which the ligand is known to produce physiological effects, 2) saturability, 3) reversibility, 4) a regional and subcellular localization consistent with those of its action, 5) a relative binding affinity of analogues which correlates with their physiological effects. Several groups have used some or all of these criteria in attempts to identify glutamate receptors in a number of preparations (27–34). Although kainic acid was initially thought to label glutamate receptors (35), more recent studies have clearly shown that it binds to a site separate from the glutamate receptors (36, 37). In the absence of specific agonists or antagonists, the ligand which has been generally used is glutamate itself, which raises the problem of distinguishing presynaptic uptake and postsynaptic receptor sites. In the case of GABA, the binding to uptake sites requires sodium and this feature has been used to distinguish between these 2 categories of sites (38). Although Roberts (28) reported the existence in cortical membranes of what appeared to be Na-independent and Na-dependent sites for glutamate, a later study on cerebellar membranes (32) did not substantiate this point. On the other hand, Vincent & McGeer (39), using rat striatal membranes, showed that binding of glutamate in the presence of sodium was labeling sites likely associated with high-affinity uptake. Given that the data on this crucial point were not in complete agreement, it was necessary to begin the analysis of glutamate binding sites in hippocampus with an analysis of the effects of monovalent cations.

Effects of monovalent cations on hippocampal ^{3}H-glutamate binding

When the effects of a wide range of concentrations were tested, it was found that sodium exerts a remarkable biphasic effect on ^{3}H-glutamate binding to hippocampal membranes (Fig. 2) (9). Very low concentrations of sodium (0.1 to 5 mM) induce a dose-dependent inhibition of the binding with an IC_{50} of about 0.75 mM and a maximum inhibition (at 2.5 mM) of 80%. Concentrations of sodium higher than 10 mM cause a dose-dependent stimulation of the binding with an

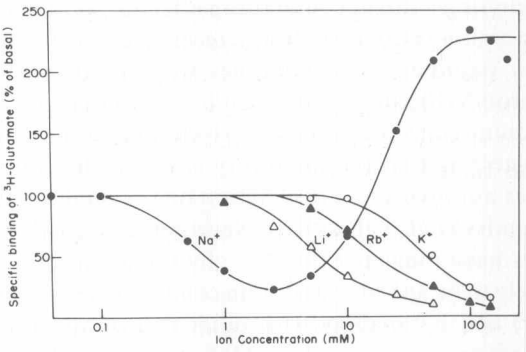

Fig. 2. Effects of monovalent cations on ^{3}H-glutamate binding to rat hippocampal membranes. Rat hippocampal membranes were prepared and immediately assayed for ^{3}H-glutamate binding as described in Baudry & Lynch (9,59), in the presence of the various cations. Results represent specific binding of ^{3}H-glutamate determined by the amount of binding displaced by 0.1 mM cold glutamate and are expressed as percent of the binding measured in the absence of sodium. Mean of three different experiments. Data from Baudry & Lynch (9,40).

EC_{50} of about 25 mM and a maximum stimulation of about 250%. The inhibitory effect of the sodium ion is shared by several monovalent cations (Fig. 2), with lithium, caesium, and potassium exhibiting an IC_{50} of 6.5 mM, 20 mM and 55 mM, respectively (40). This order of potency suggests that the inhibitory effect is relatively selective for sodium since there is a 10-fold difference in IC_{50} between sodium and lithium and almost a 100 fold difference between sodium and potassium. This pattern of results is most easily explained by the existence of two glutamate binding sites in hippocampus, one of which ('Na-independent') is inhibited by sodium and one of which ('Na-dependent') requires the cation. As will be developed below, pharmacological experiments provide strong support for this hypothesis.

A similar biphasic effect of sodium is also found in cerebellar membranes although, possibly due to a different balance of the Na-independent and the Na-dependent binding sites in this structure compared to hippocampus, high concentrations of sodium still produce a net inhibitory effect; this probably explains the apparently contradictory results of Roberts (28) and Foster & Roberts (32).

When the binding of various concentrations of ^{3}H-glutamate was measured in the presence of various monovalent cation concentrations, it was found that the maximum number of binding sites was decreased without any obvious changes in the apparent affinity of glutamate for the receptors excluding a direct competition effect of sodium on the binding (40). In addition, recent studies showed that treatment of membranes with the detergent Triton X-100 suppresses the inhibitory effect of low sodium concentrations on ^{3}H-glutamate binding, suggesting that the inhibitory effect of the cation does not involve a direct action on the binding site (Baudry *et al.*, submitted). Neurophysiological studies have indicated that the glutamate receptor is likely to be linked to a sodium conductance channel (41) and by analogy with other neurotransmitter receptors (for example GABA with chloride (42) and glycine with chloride (43)) it is possible that the sodium-induced decrease in binding reflects some interaction between the sodium channel and the glutamate receptors. Conceivably the inhibitory effect is due to a feed-back regulatory mechanism in which opening and subsequent interactions of the channel with sodium ions result in an inhibition of the association of glutamate to its recognition site.

Kinetic and pharmacological properties of ^{3}H-glutamate binding

When the association of ^{3}H-glutamate to hippocampal membranes was studied in the absence of sodium, the binding occurred rapidly and equilibrium was reached within about 15 min. (9). The second-order association constant (K_1) was estimated to be 0.57 μM^{-1} min^{-1}. Equilibrium was achieved more rapidly for the Na-dependent binding (less than 5 minutes) and the second-order association constant was found to be 1.18 μM^{-1} min^{-1}. The dissociation of ^{3}H-glutamate bound to hippocampal membranes induced by adding an excess of gold glutamate was extremely rapid at 30 °C; the time for half-dissociation of the Na-independent binding was about 1 minute corresponding to a dissociation rate constant (K_{-1}) of 0.65 min^{-1}, whereas the corresponding values for the Na-dependent binding were 0.5 min and 1.3 min^{-1}. The ratio of K_{-1} to K_1 gives an estimate of the equilibrium dissociation constant Kd which according to the above data would be about 1 μM for both the Na-independent and the Na-dependent sites.

^{3}H-Glutamate binding to hippocampal membranes in the absence or presence of sodium is saturable and Scatchard analysis revealed a homogeneous population of binding sites in the range of glutamate concentrations studied (50 nM to 10 μM). The Na-independent binding exhibited a Kd of about 500 nM and a maximum number of sites of 6.5 pmol/mg protein, whereas the Na-dependent binding exhibited a Kd of 2500 μM and a maximum number of sites of 75 pmol/mg protein (9). These numbers provide an explanation for the two to three fold increase in binding elicited by high sodium concentration observed at a ^{3}H-glutamate concentration of 100 nM. In both cases the Hill coefficient was not significantly different from unity indicating the absence of apparent cooperativity of the binding of glutamate to the two sites.

Thus the equilibrium dissociation constants estimated from saturation kinetics or association and dissociation kinetics, are in reasonably good agreement, although the very rapid dissociation rate of the binding may be responsible for imprecise values of the dissociation rate constants. It should be noted that the above-described studies used a filtration method, and there are theoretical considerations (44) which indicate that this technique

should not measure such relatively low affinity binding sites. However, because the association rates are relatively slow as compared to an association reaction governed solely on free diffusion, the filter method appears to give a reasonably accurate estimate of the binding properties. In the case of the Na-dependent binding the slow association rate probably reflects glutamate binding to a sodium receptor complex as has been suggested from uptake studies (45). The slow association rate for the Na-independent binding might reflect some conformational changes taking place at the receptor level.

As stressed previously, the pharmacological properties of the physiological receptors in hippocampus are still poorly characterized in part due to uncertainty about efficacy and specificity of the various agonists and antagonists. Nevertheless we attempted to measure the ability of several putative agonists and antagonists to compete with ^{3}H-glutamate binding in the absence or presence of sodium. In the absence of sodium, ^{3}H-glutamate binding exhibited some degree of stereoselectivity: the L-isomers of glutamate or aspartate were 10–20 times more potent in inhibiting the binding than

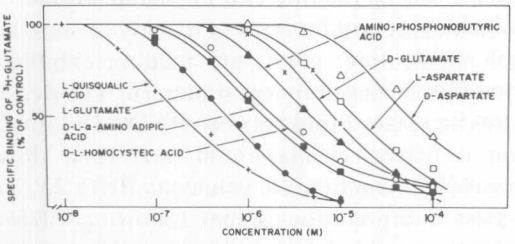

Fig. 3 Inhibition of Na-independent ^{3}H-glutamate binding by various analogs. Concentration-response curves for various analogs were obtained for ^{3}H-glutamate binding to rat hippocampal membranes at a ^{3}H-glutamate concentration of 100 nM. Results are expressed as percent binding measured in the absence of any analogs and are means of three experiments.

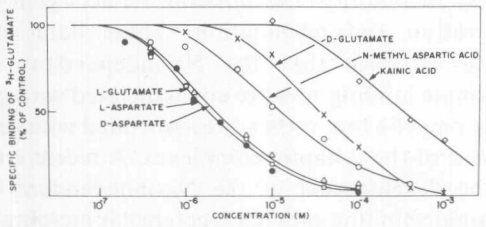

Fig. 4. Inhibition of Na-dependent ^{3}H-glutamate binding by various analogs Same legend as in Fig. 3 except that the binding was conducted in the presence of 150 mM sodium chloride.

were the D-isomers, while glutamate was 10 times more effective than was aspartate (Fig. 3). Several excitatory amino acids, among which L-quisqualic, L-ibotenic and DL-homocysteic acids, were very potent inhibitors of glutamate binding, with K_I values of 0.12, 1.2 and 2 μM respectively. Although all these compounds have powerful excitatory effects on neurones, only the physiological actions of quisqualic acid have been linked to a glutamate receptor (41). Kainic acid and N-methyl-D,L-aspartic acid (NMDA) were totally devoid of inhibitory effect on Na-independent binding whereas they produce a concentration-dependent inhibition of the Na-dependent binding (Fig. 4); this correlates well with their inhibitory effect towards the high-affinity uptake (8). Among the putative antagonists, D-α-aminoadipate (αAA) and α-amino-phosphonobutyric acid (APB) proved to be strong inhibitors of Na-independent ^{3}H-glutamate binding, but had no significant effect on the Na-dependent ^{3}H-glutamate binding. On the other hand, glutamate diethylester (GDEE) was a very poor inhibitor of the Na-independent ^{3}H-glutamate binding, requiring very high concentrations to induce partial blockade. A very similar pharmacological profile has been reported for the Na-independent ^{3}H-glutamate binding in cerebellar membranes by Foster & Roberts (32). This pattern of results does not accord well with the findings of studies using iontophoretic application of drugs. As described above GDEE is a rather specific antagonist of glutamate-induced excitation of neurones while D-α-AA has little effect in this regard – this is the reverse of their effects on Na-independent glutamate binding. Perhaps the simplest explanation is that multiple sites exist and the Na-independent binding site is distinct from that involved in mediating the effects of iontophoretically applied glutamate. If this were to prove to be the case, the question of which site, if either, is associated with synaptic receptors would assume paramount importance. It bears repeating in this context that GDEE proved to be a very poor blocker of synaptic potentials in hippocampus when applied by perfusion, although it has been reported to be effective when administered by iontophoresis. It is also possible that the artificial environment in which binding assays are conducted distorts their properties such that aberrant pharmacological profiles emerge – the potent effects of various cations on

glutamate binding may prove pertinent to this point. These issues will be considered further in a later section.

Localization and ontogeny of ^{3}H-glutamate binding sites

The number of Na-dependent binding sites exhibits a marked regional difference which correlates with the regional distribution of high-affinity uptake sites (Fig. 5), while the Na-independent binding sites show little variation in numbers across brain areas (9). This absence of regional variation is not totally surprising since glutamate is able to excite most neurones in the CNS, suggesting a widespread distribution of glutamate receptors, which is also in agreement with the proposed role of glutamate as an excitatory neurotransmitter of a large number of anatomical pathways (46).

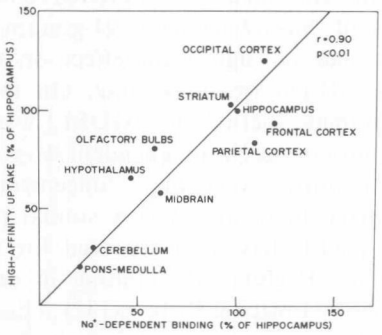

Fig. 5. Correlation between the regional distribution of Na-dependent ^{3}H-glutamate binding and high-affinity uptake. Data are from Baudry & Lynch (9) and are expressed as percent of the values found in the hippocampus. Mean of 4 different experiments.

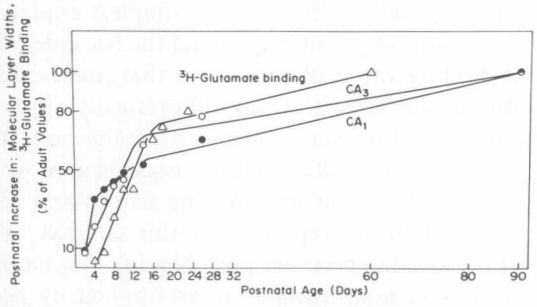

Fig. 6. Postnatal increase in Na-independent ^{3}H-glutamate binding in rat hippocampal membranes and widths of CA_1 and CA_3 molecular layers. The data for the ^{3}H-glutamate binding are from Baudry *et al.* (47) and those for the changes in the widths of CA_1 and CA_3 molecular layers have been generously provided by Dr R. Loy. They are expressed as percentage of the adult values.

More interesting is the developmental pattern of the Na-independent binding sites in hippocampus. The amount of binding sites, expressed as pmol/hippocampus, increases 40 times between the posnatal day (PND) 4 and adult. The binding sites are added at a very fast rate between PND 6 and 18 and at a slower pace thereafter (47). This time-course is very similar to that of synapse addition in the hippocampal formation (48). Hippocampal pyramidal cells undergo their final division before birth and during the first two postnatal weeks they extend dendrites and establish synaptic contacts with their afferent connections (49). This is reflected by an increase in the size of the dendritic trees of the pyramidal cells (50) and thus in the width of the molecular layers of regio superior and regio inferior (Fig. 6); in fact the addition of ^{3}H-glutamate binding sites seems to parallel very closely the increase in molecular layer widths. The analysis of the changes in the dentate gyrus is complicated by the fact that granule cells continue to be added after birth (51); however as is the case with the pyramidal cell fields, the dentate gyrus is invaded by its major afferents during the first two postnatal weeks.

When expressed in terms of density of sites, i.e., pmol/mg protein, glutamate binding exhibits a very different developmental pattern. The density of sites increases 3 fold between PND 4 and PND 9 when it reaches a maximum, and then slowly decreases to reach adult values at PND 23. The simplest interpretation is that following PND 9, proteins are added faster than are ^{3}H-glutamate binding sites. In this regard it is noteworthy that astroglial cells invade the hippocampus at the beginning of the second postnatal week. Myelination does not start before the second or third postnatal week and therefore the reduction in density of binding sites possibly reflects glial cell maturation and myelination. These data offer strong evidence that the Na-independent ^{3}H-glutamate binding sites are not associated with glial cells or myelin but, rather, are associated with some element of the synaptic complexes. A recent study on the development of the Na-independent ^{3}H-glutamate binding sites in rat cerebellar membranes reached a similar conclusion (33).

Subcellular localization of the Na-independent ^{3}H-glutamate binding sites also indicated that these

sites are associated with synapses, since they were highly enriched in synaptic plasma membranes and in synaptic junctions (52), whereas only small amounts of binding were associated with myelin or mitochondria (32).

Are the Na-independent glutamate binding sites glutamate receptors?

A reasonable case can be made for the conclusion that the Na-dependent ^{3}H-glutamate binding sites are related to the high-affinity uptake of glutamate; their regional distribution, pharmacological profiles, and even their dependency on sodium, all resemble the properties of the high-affinity glutamate uptake process. Certain characteristics of the Na-independent sites suggest that these may be post-synaptic receptors but the evidence is not yet compelling. Lesions of hippocampal afferents do not cause a reduction in sodium-independent binding (unpublished observation), suggesting that these sites are either on target dendrites or glial cells and the developmental studies cited above tend to rule out the latter location. Furthermore, subcellular fractionation studies suggest that the sites are enriched in synaptic junctions. With regard to the criteria outlined earlier, the binding is saturable, reversible and has a regional and subcellular distribution which is consonant with what would be expected of a physiological receptor. Further evaluation of the relationship between the Na-independent sites and synaptic receptors will require additional evidence on two issues. First, it would be most helpful if an estimate could be made of the concentration range over which glutamate acts physiologically; this information could indicate whether or not the Na-independent site possess an appropriate Kd for glutamate. Experiments using slices or dissociated cells could conceivably provide data on this issue although the possibility of extrajunctional excitatory receptors with higher affinities than synaptic receptors as well as the very effective uptake systems found in neurones might well generate misleading conclusions. Second, and of more immediate interest, further work is needed on the pharmacology of both physiologically effective receptors and glutamate binding sites and the relationship of these sites to synaptic transmission. As discussed, there are enough discrepancies between the pharmacologies of the Na-independent site and iontophoretically applied glutamate to raise the possibility that two distinct sites are studied by these techniques. However, only the dentate gyrus has received detailed investigation using the iontophoretic approach and there are suggestions in the literature that drugs may operate somewhat differently elsewhere in hippocampus (for example, GDEE does not block aspartate in the dentate gyrus but is does in the regio superior). Furthermore, the effects of iontophoretic application of drugs on synaptic potentials does not agree well with the results of experiments using perfusion. These areas of confusion indicate that additional pharmacologial studies, perhaps combining techniques as well as using a broader range of analogues, will be needed before it will be possible to determine if the Na-independent site possesses the appropriate pharmacological profile for a physiologically effective glutamate receptor, to say nothing of a true post-synaptic receptor.

Possible involvement of glutamate receptors in hippocampal physiological plasticity

The hippocampus exhibits a remarkable degree of anatomical and physiological 'plasticity'. Very brief trains of high frequency stimulation produce an increase in synaptic efficiency which persists for weeks or even longer (long-term potentiation: 'LTP') (53,54). Neurophysiological studies have provided evidence that the LTP effect is due to a change in the synaptic connections ((55) for a review), thereby raising the possibility that post-synaptic receptors undergo some form of modification following high frequency activity. These findings, coupled with the development of a convenient and sensitive assay for a possible glutamate receptor, prompted us to investigate first, the possibility that hippocampal ^{3}H-glutamate binding sites are modifiable and second, the role, if any, receptor plasticity might play in the long-term potentiation effect. For two reasons, we began our studies with an analysis of the effects of calcium on ^{3}H-glutamate binding: a) experiments using a variety of systems have shown that calcium exerts profound effects on membrane organization, including surface receptors (see (56) for a review) and b) ion manipulation experiments using *in vitro* hippocampal slices suggest that the LTP effect is

quite sensitive to calcium levels (57) as well as to drugs which block the binding of calcium to calmodulin (58).

Effect of calcium on ^{3}H-glutamate binding and a possible mechanism for the regulation of binding sites

Whereas monovalent cations induce a marked inhibition of the Na-independent ^{3}H-glutamate binding to hippocampal membranes, several divalent cations produce a strong stimulation of this binding. Calcium, manganese and strontium increase the binding two to three fold with apparent EC_{50}'s of 30, 300 and 400 M respectively (Fig. 7). Magnesium also increases the binding by about 20% at 5 mM, whereas cobalt and barium are ineffective up to 10 mM and inhibit ^{3}H-glutamate binding at higher concentrations (40). Scatchard analysis of ^{3}H-glutamate binding in the presence of calcium indicates that calcium increases the maximum number of binding sites without changing their apparent affinity for glutamate. Moreover this stimulatory effect of calcium on the number of glutamate receptors is partially irreversible; when hippocampal membranes are preincubated with calcium and then washed to eliminate the cations, these membranes still exhibit a higher number of binding sites than control, untreated membranes.

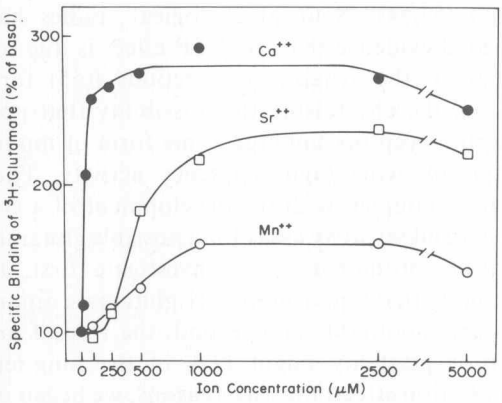

Fig. 7. Effect of various divalent cations on Na-independent ^{3}H-glutamate binding. Hippocampal membranes were prepared as described in Baudry & Lynch (9) except that 2.5 mM EGTA was included in all the solutions during the preparation. After washing the EGTA, ^{3}H-glutamate binding was assayed in the presence of various divalent cations (chloride salts). Results are expressed as percentage of the binding measured in the absence of added cations and are means of 3–6 experiments.

This suggests that the effect of calcium is indirect and requires the participation of an unknown number of intermediate steps. This is also indicated by several other lines of evidence: a) neonatal hippocampal membranes do not exhibit the stimulatory effect of calcium on ^{3}H-glutamate binding until PND 10 (47). Thus glutamate binding sites appear before the onset of the calcium effect; b) Calcium ions do not stimulate ^{3}H-glutamate binding at temperatures below 15–20 °C (Baudry *et al.*, submitted); c) treatment of membranes with Triton X-100 at concentrations which only slightly reduce ^{3}H-glutamate binding, totally suppresses the effect of calcium on the binding (Baudry *et al.*, submitted); d) calcium ions at concentrations as high as 250 μM do not stimulate ^{3}H-glutamate binding in cerebellar membranes, whereas the effect is maximal in hippocampal, striatal or cortical membranes at these concentrations (59).

Pharmacological studies have provided important clues as to the possible cellular and molecular mechanisms which might be involved in the stimulatory effect of calcium on ^{3}H-glutamate binding (59). Thus it appears that reducing or alkylating agents as well as variety of proteinase inhibitors decrease both basal and calcium stimulated ^{3}H-glutamate binding (59); moreover some of these agents namely, N-ethyl maleimide (NEM) and leupeptin, a tripeptide generally considered as a specific inhibitor of thiol proteases (60), do not affect basal ^{3}H-glutamate binding but totally suppress the increase in binding sites produced by calcium (61, 62). This suggests that calcium exerts its actions by stimulating a membrane-bound calcium-sensitive proteinase. This hypothesis is strengthened by the fact that two exogenous proteinases, trypsin and chymotrypsin, induce an increased number of ^{3}H-glutamate binding sites in hippocampal membranes (59). Previous studies have shown that nervous tissue contains calcium and temperature-sensitive thiol proteinases (63–65) but these require concentrations of calcium a hundred times higher than that required to stimulate ^{3}H-glutamate binding. However, recent studies provided evidence that hippocampal and cortical synaptic membranes contain calcium-dependent, leupeptin-sensitive, proteolytic activity, with an apparent affinity for calcium of about 30 μM (61). The substrate of this calcium-dependent proteinase appears to be a high-molecular weight doublet

protein (Mr above 200 000) which has been found in purified postsynaptic densities preparations (66), and which may belong in the category of actin-binding proteins. This would agree well with what is known of the substrates of soluble calcium activated proteinases in brain and other tissues (65).

Taken together these data indicate that the calcium stimulation of ^{3}H-glutamate binding could be due to the calcium-induced activation of a membrane-bound proteinase; the subsequent proteolysis of its substrate protein might reveal binding sites which were inaccessible, possibly through a local change in membrane organization.

Long-term potentiation and changes in ^{3}H-glutamate binding sites

The regulatory mechanism described above has several characteristics which make it an obvious candidate for the substrate of the long-term potentiation effect (Table 2). First, the effect of calcium on glutamate binding is partially irreversible, which is consonant with the fact that LTP induction is dependent on calcium and is virtually irreversible. Second, during postnatal development, LTP cannot be elicited before postnatal day 10 in the regio superior (47,68) and this is close to the age at which the calcium stimulation effect appears. Third, cerebellar membranes do not exhibit this effect of calcium and it does not appear that LTP is found in cerebellum.

These arguments led us to test for the effects of brief bursts of high frequency stimulation on ^{3}H-glutamate binding in hippocampus. In a first set of experiments we found that hippocampal slices accumulated more ^{3}H-glutamate following stimulation than control, unstimulated slices; this increased accumulation was restricted to the stimulated terminal field and was still present 30 min after the stimulating train (69). No changes in GABA or tyrosine accumulation could be detected under these conditions. Moreover, membranes prepared from stimulated slices exhibited an increased number of glutamate binding sites, without changes in affinity for glutamate, as compared to membranes prepared from control, unstimulated slices (69).

In a second set of experiments we used a 'minislice' preparation which uses only the regio superior subfield; in this preparation the great majority of the synapses originate from the regio inferior pyramidal cells and through the use of multiple stimulating electrodes it is possible to activate very large numbers of these. Despite its very small size, the minislice exhibits physiological responses which are not greatly different from those recorded from hippocampus *in vivo*. Our preliminary data with this preparation confirms the earlier finding that high frequency stimulation causes a marked increase in ^{3}H-glutamate binding.

A molecular model for long-term potentation

Taken together, the above data can be integrated in the following multiple-stage molecular model (70, 71):

1) High-frequency stimulation results in an influx of calcium in the postsynaptic dendrites (72, 73).

2) This influx of calcium results, directly or indirectly through the eventual participation of mitochondria (71), in a transitory increase in cytoplasmic free calcium. Although this point is still controversial, it is not unlikely that calcium concentration, which in the resting state is in the order of 0.1 μM, can increase two or more orders of magnitude to reach concentrations as high as 10 or 100 μM, at least locally.

3) This, in turn, activates the postulated membrane-bound calcium-dependent proteinase, causing the proteolysis of some membrane-associated components such as neurofilaments or actin-bin-

Table 2. Correlation between the characteristics of LTP and the effect of calcium on ^{3}H-glutamate binding.

Characteristics of LTP	Calcium effect on ^{3}H-glutamate binding
Increased synaptic transmission	Increased number of ^{3}H-glutamate binding sites
Depends on external Ca^{++} (57)	Effect of Ca^{++} partly irreversible (40)
Ca^{++} can be substituted by Sr^{++} (67)	Mimicked by Sr^{++} and Mn^{++} (Fig. 7)
Absent before PND 10 (47,68)	Absent before PND 10 (47)
Never been reported in cerebellum	Absent in cerebellar membranes (49)