

Induction of Glutamate Binding Sites in Hippocampal Membranes by Transient Exposure to High Concentrations of Glutamate or Glutamate Analogs

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The number of Na⁺-independent, Cl⁻-dependent glutamate binding sites in rat hippocampal membranes is increased two- to fourfold after pre-exposing isolated membranes or hippocampal slices to high concentrations (0.1–10 mM) of L-glutamate or of glutamate analogs with high affinity for this binding site, such as quisqualate, homocysteate, or amino adipate. N-Methylaspartate and kainate are ineffective. A similar binding increase is induced by transient exposure to the dipeptide tyrosyl-glutamate. The newly induced binding sites appear to be identical with pre-existing Cl⁻-dependent binding sites by several criteria: They have a similar pharmacological profile, they are sensitive to low concentrations of Na⁺, and the number of sites can be further increased by transient exposure to micromolar calcium concentrations. Moreover, binding of [³H]APB, a ligand selective for the Cl⁻-dependent glutamate binding sites, is also increased after glutamate preincubation. The induction of binding sites by high glutamate concentrations, described herein, is calcium-independent, not inhibited by leupeptin and, therefore, different from the previously described activation of binding sites by a calcium-sensitive protease. The high concentration of ligand needed to induce increased binding suggests the presence in hippocampal membranes of a binding site with low, millimolar affinity that is functionally related to the known high-affinity binding sites. Several interpretations of the observed effects and their implications for the possible relationship between the binding site and the synaptic receptor are discussed.

An increasing body of evidence indicates that certain types of glutamate binding sites in brain synaptic membranes are affected by neurophysiological and behavioral manipulation. Thus long-term potentiation, a very long-lasting form of synaptic facilitation caused by brief periods of high-frequency stimulation, is accompanied by an increase in the number of these sites (Lynch et al., 1982). Similar effects have been found in the hippocampus after the induction of seizure proneness ("kindling") by repeated episodes of intense afferent stimulation (Savage et al., 1982), or following the learning of a conditioned response (Mamounas et al., 1984). In each of these cases, the experimental manipulation produces both a lasting modification of hippocampal physiology and a change in membrane glutamate binding sites. These changes seem to occur within one subgroup of glutamate binding sites, which is characterized by its dependence on Cl⁻ and its high affinity for amino-phosphonobutyrate and quisqualate (Savage

et al., 1982). Since there is evidence linking these binding sites to transmitter receptors (Baudry and Lynch, 1984; Fagg et al., 1982), it is not unreasonable to assume that the biochemical and physiological effects are causally related.

It is not known which aspects of physiological activity trigger the observed changes in putative receptors, but an accumulation of glutamate (or a related compound) in the synaptic zone is an attractive possibility. The initiating event for both long-term potentiation (LTP) and kindling is the rapid stimulation of a large contiguous group of fibers, a circumstance in which the capacity of the reuptake process might be exceeded by unusual amounts of released transmitter. This led us to test the effects of high concentrations of glutamate and several of its analogs on the number and affinity of glutamate binding sites. We have found that exposure of membrane preparations and *in vitro* hippocampal slices to these compounds results within minutes in the appearance of Cl⁻-dependent glutamate binding sites with characteristics comparable to those of basal sites. The implications of this finding for the understanding of the modulation of synaptic receptors and transmitter physiology will be discussed.

A preliminary report of some of the results has been presented (Baudry et al., 1981b).

Materials and Methods

Preparation of membranes from rat hippocampus

Two hippocampi were homogenized in a glass/Teflon homogenizer with a motor-driven pestle in 5 ml of 0.32 M sucrose and 1 mM EGTA/K⁺, pH 6.8 (buffer S). The pellet P₂ (1st spin: 1000 × g for 10 min; 2nd spin: 27,000 × g for 20 min) was suspended in 5 ml buffer L (5 mM Tris/HCl, 1 mM EGTA/K⁺, pH 8.1), sparing the brown core of the pellet, lysed for 40 min on ice, homogenized in a glass/Teflon homogenizer by three manual strokes, and spun for 20 min at 6000 × g. The upper whitish layer of the pellet ("buffy coat") was resuspended in the supernatant and spun at 40,000 × g for 20 min. The resulting pellet was resuspended in 8 ml of buffer T (50 mM Tris/HCl, 50 μM EGTA/K⁺, pH 7.4) and dispersed with a tip sonicator at low power for 3 sec. The last centrifugation, with subsequent sonication, was repeated two more times. The final pellet was suspended in buffer T to give a concentration of 150 μg protein/ml. Aliquots of the suspension (1.5 ml) were frozen at -70°C. All experiments were performed at least once with fresh membranes; frozen and fresh membranes gave qualitatively identical results, the only difference being a 10–40% reduction in the absolute amount of binding in frozen/thawed membranes.

Binding assays

In the standard binding assay, 40 μl of membrane suspension was mixed with 10 μl of a solution containing 0.5 μM [³H]L-glutamate (ICN, Radiochemicals, 50 Ci/mmol; final concentration, 100 nM) or 1 μM [³H]APB (2-amino-4-phosphonobutyric acid) (New England Nuclear, 26.1 Ci/mmol, final concentration, 200 nM). After 40 min incubation

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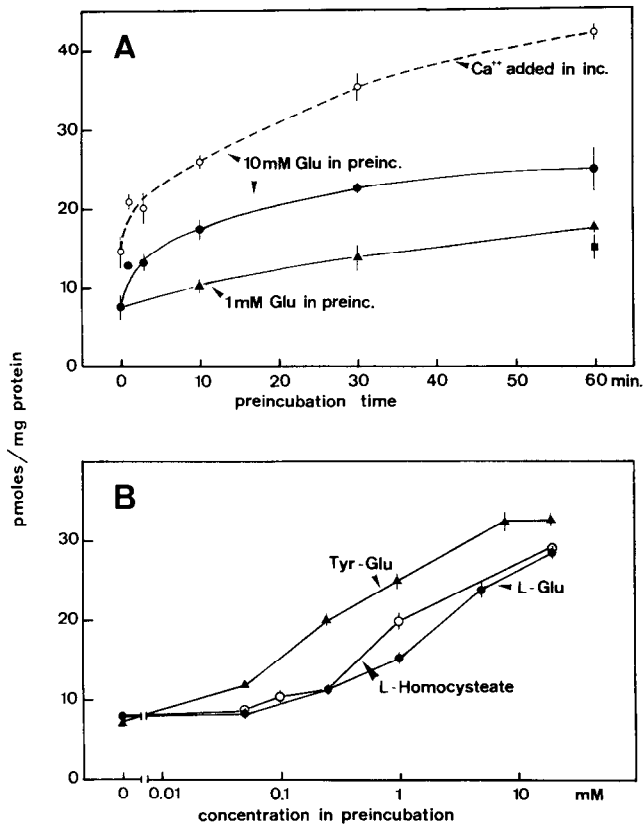


Figure 1. A. Freshly prepared hippocampal membranes were preincubated at 35°C with 10 mM (○, ●) or 1 mM (▲) L-glutamate/Tris for the time indicated on the x-axis. The membranes were washed by four centrifugations and glutamate binding was assayed as described in Methods. Membranes preincubated in 10 mM glutamate were incubated both in the absence (●) and presence (○) of 0.5 mM CaCl₂. One aliquot of membranes was preincubated in 10 mM glutamate at 0°C instead of 35°C (■). Bars indicate SEM of quadruplicate determinations. This figure shows a representative experiment. The average binding increase elicited by 10 mM L-glutamate was +187% ($n = 2$) after 5 min and +216 ± 18% ($n = 12$) after 10 min preincubation time. B. Dependence on concentration of inductants. Membranes were preincubated for 20 min at 35°C with L-glutamate (●), L-homocysteate (○), or L-tyrosyl-L-glutamate (TG, ▲) at the concentrations shown on the abscissa, and further processed as described in Methods.

at 35°C the sample was diluted with 3 ml ice-cold 50 mM Tris/Cl, pH 7.4, and filtered through 0.45 μ m pore size Millipore filters. The filters were washed twice with the same ice-cold dilution medium. Nonspecific binding was determined in the presence of 1 mM unlabeled L-glutamate and subtracted from all binding values.

Induction of new binding sites

Aliquots of fresh or frozen/thawed membranes were incubated at 33–35°C with 0.1–20 mM concentrations of glutamate or glutamate analogs, which were added from stock solutions adjusted to neutral pH with Tris (variation of the pH during the preincubation of between 5.0 and 9.5 in the absence of glutamate did not affect binding; therefore, minor changes in pH during the preincubation cannot account for the observed changes in glutamate binding). To wash the membranes free of glutamate, the preparations were centrifuged 4 \times in a cooled (4°C) Beckman airfuge at 130,000 \times g for 5 min; in order to promote the release of any trapped or bound glutamate, the pellets were resuspended in ice-cold buffer T by vigorous pipetting and left at 35°C for 5 min between spins. The final pellet was again resuspended in buffer T and immediately used for the binding assay.

Preparation of slices and preincubation with glutamate analogs

Transverse slices from rat hippocampi were cut at 400 μ m on a McIlwain tissue chopper. Each slice was transferred to a separate glass vial con-

taining 0.6 ml incubation medium (in mM: NaCl, 124; KCl, 3.3; KH₂PO₄, 1.25; MgSO₄, 2.4; CaCl₂, 3.1; NaHCO₃, 26; D-glucose, 10; urea, 2; Na-ascorbate, 3), and was continuously gassed with 95% O₂/5% CO₂. The slices were incubated for 1 hr at 35°C with gentle shaking. Slices treated in this way usually showed good electrophysiological responses when tested in a recording chamber. After the 60 min recovery period, L-glutamate or glutamate analogs were added from stock solutions adjusted to neutral pH with NaOH. At the end of the incubation, the slices were transferred to ice-cold buffer S (see above) for about 5 min and homogenized in a 1 ml glass/Teflon homogenizer in 200 μ l buffer S by 30 manual strokes. The homogenate was spun at 500 \times g for 5 min and the supernatant was centrifuged in a Beckman airfuge for 5 min (130,000 \times g). The pellet P₂ was suspended by vigorous pipetting in lysis buffer L, left on ice for 20 min, and centrifuged 3 \times for 5 min in the airfuge. After the first of these spins, the pellet was resuspended in buffer L, and after the subsequent spins, in buffer T. After the second spin, the resuspended membranes were warmed to 35°C for 5 min. Membranes prepared with up to four additional centrifugations and additional 5 min incubations between spins gave essentially identical values for specific glutamate binding.

Protein assay

Protein was determined with the assay according to Bradford (1976), using BSA as standard. Aliquots of the membrane suspensions (20 μ l) were first mixed with 90 μ l 0.1 N NaOH to make the membrane proteins more accessible to the dye, and then with 900 μ l of the commercially available Bio-Rad reagent.

Determination of residual glutamate and L-tyrosyl-L-glutamate by HPLC

Membrane aliquots prepared from hippocampus plus cortex were incubated for 40 min with 10 mM L-glutamate or L-tyrosyl-L-glutamate (TG) and washed as described above. One aliquot of the final suspension was used for a binding assay. The rest of the membranes were pelleted and resuspended in a small volume of 100 mM KCl. The protein was then precipitated by adding 2.5 volumes of ethanol, and spun down. Glutamate was determined in the supernatant by reverse-phase high-performance liquid chromatography (HPLC), using a precolumn derivatization method with *o*-phthalaldehyde (Lindroth and Mopper, 1979) and a 3 μ m ODS Spherisorb 10 cm, 4.6 mm ID column. The HPLC equipment consisted of a Spectra-Physics Model 8700 pump, a Kratos FS-950 fluorimeter, and a HP 3390A recording integrator. TG was determined by electrochemical detection of the tyrosyl phenolic group using a LC-4 amperometric controller (Bioanalytical Systems) set at 1.0 V and a mobile phase consisting of 6.8 gm sodium phosphate (monobasic), 6.9 gm sodium acetate, and 0.9 gm sodium lauryl sulfate (Sigma) in 1 liter of 75% distilled water and 25% acetonitrile, adjusted to pH 3 with phosphoric acid. The same HPLC pump, ODS column, and integrator were used.

Results

Transient exposure to glutamate increases [³H]L-glutamate binding

Specific binding of [³H]glutamate to hippocampal membranes was increased three- to fourfold when the membranes were preincubated for several minutes in millimolar concentrations of glutamate (Fig. 1A). The minimum glutamate concentration necessary in the preincubation to induce a noticeable binding increase was in the order of 100 μ M, while concentrations of about 10 mM were required to obtain maximum effect (Fig. 1B). At the latter concentration, half-maximal activation was obtained with preincubation times of 5 min or less. This binding increase was a highly reliable phenomenon; the average increase in all the experiments where membranes were exposed to 10 mM L-glutamate for 5–30 min was +370% ($n = 45$); the binding increase was in no case less than +60%, and only in three cases less than +130%.

After preincubation, the membranes were extensively washed by four centrifugations. Careful washing was mandatory in these experiments, since the concentration of free glutamate had to be lowered from 10 mM to less than 100 μ M in order to avoid

Table 1. Sensitivity of glutamate-induced binding sites to Cl^- , Na^+ , and Saponin

Composition of the incubation medium	^3H glutamate bound (mean \pm SD) (pmol/mg protein)	
	"Standard" binding	Binding after glutamate induction
0 mM Cl^- , 0.3 mM APB	0.96 \pm 0.07	2.7 \pm 0.1
30 mM Cl^-	10.2 \pm 0.1	40.1 \pm 2.2
30 mM Cl^- , + 2 mM Na^+	2.7 \pm 0.1	9.0 \pm 0.3
30 mM Cl^- , + 10 mM Na^+	1.66 \pm 0.1	2.3 \pm 0.3
After transient exposure to Saponin:		
0 mM Cl^- , 0.3 mM APB	1.19 \pm 0.07	1.15 \pm 0.05
30 mM Cl^-	1.62 \pm 0.05	2.13 \pm 0.22

Membranes were preincubated for 30 min without (for "standard" binding) or with 10 mM L-glutamate and washed 4 \times in 50 mM MES/Tris, 50 μM EGTA, pH 7.4. KCl (30 mM) and Na-acetate (2 or 10 mM) were added to the incubation medium, as indicated in the table. APB was added to the Cl^- -free media to suppress residual Cl^- -dependent binding (Fagg et al., 1983). Aliquots of the membranes were incubated with 0.02% Saponin for 20 min at 35°C. The membranes were then washed free of the detergent by two centrifugations, resuspended in MES/Tris buffer and used for the binding assay. This detergent treatment solubilized less than 10% of the membrane protein. Cl^- -dependent glutamate binding was similarly sensitive to low concentrations of other detergents, such as Triton X-100 (0.01%). The table shows a representative experiment. All parts of the experiment were performed at least 4 \times with essentially identical results.

interference with the binding assay. We have routinely used four centrifugations, although three centrifugations proved to be sufficient, and additional centrifugations up to a total of eight had no effect on the magnitude of specific binding. After each centrifugation, the membranes were left for 5 min at 35°C to promote release of bound or trapped glutamate.

The induced sites are Cl^- -dependent, APB-sensitive glutamate binding sites

The observed stimulation of glutamate binding resulted entirely from an increase in the number of sites and not from a change in the affinity for glutamate (Fig. 2). The K_d value of 0.95 μM and the Hill coefficient of 0.98 are in good agreement with values reported for glutamate binding by various authors (Foster and Fagg, 1984). Glutamate binding measured after induction displayed all the characteristics of one subclass of glutamate binding sites, typified by strict dependence on Cl^- (a detailed description of these sites is given in Fagg et al., 1982, 1983; Mena et al., 1982; Monaghan et al., 1983): (1) Binding was reduced by more than 90% when Cl^- was omitted from the incubation medium (Table 1). (2) Binding was increased up to twofold after exposure to micromolar concentrations of Ca^{2+} (Fig. 1A). (3) Binding was inhibited by millimolar concentrations of Na^+ (Table 1). (4) Quisqualate, homocysteate, and D- and L-aminoadipate inhibited glutamate binding with a potency similar to that of L-glutamate itself (Table 2). D-Glutamate and L- and D-aspartate had 5–50 \times lower affinities. N-Methyl-DL-aspartate (NMA) and kainate were ineffective in displacing glutamate binding. APB was clearly preferred over the analog 2-amino-5-phosphonovaleric acid (APV), which is believed to interact preferentially with the NMDA receptor. This pharmacological profile is congruent with that reported for the Cl^- -dependent glutamate binding site (Fagg et al., 1983; Foster and Fagg, 1984). (5) Binding of ^3H APB was increased in membranes preincubated in 10 mM glutamate (Fig. 3B). Using tritium-labeled APB, Monaghan et al. (1983) demonstrated that APB interacts selectively with the Cl^- -dependent glutamate binding site. (6) Binding was very sensitive to even low concentrations of detergents, which are in themselves insufficient to solubilize membranes;

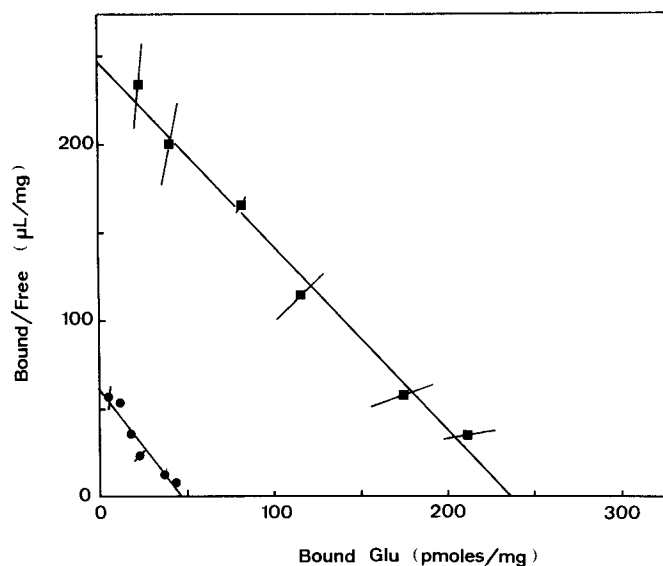


Figure 2. Scatchard plot of basal binding and after induction of glutamate binding sites. Aliquots of the membrane preparation were preincubated with 0 (●) or 10 mM (■) L-glutamate for 20 min. The Scatchard plot was determined from glutamate binding at 0.1, 0.2, 0.5, 1, 3, and 6 μM glutamate. The bars represent the SD of duplicate or triplicate measurements. Nonspecific binding was determined in the presence of 1 mM glutamate and subtracted from all binding values. The Hill coefficients were 1.03 for untreated membranes and 0.98 for glutamate preincubated membranes. ■—■, $K_d = 0.95 \mu\text{M}$; $B_{\text{max}} = 236 \text{ pmol/mg}$. ●—●, $K_d = 0.73 \mu\text{M}$; $B_{\text{max}} = 46 \text{ pmol/mg}$.

transient exposure to 0.02% Saponin irreversibly inactivated binding (Table 1); the Cl^- -dependent glutamate binding site in untreated and in glutamate-preincubated membranes was found to be similarly sensitive to low concentrations of Triton X-100, Digitonin, and other detergents (data not shown).

Binding-site induction by glutamate analogs

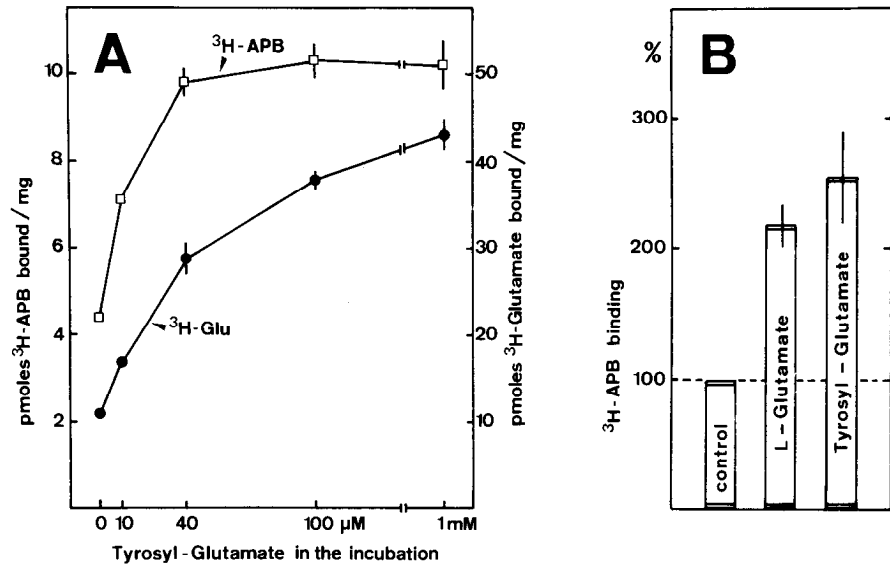
The ability to generate new binding sites was not confined to L-glutamate. All glutamate analogs that can displace ^3H glutamate from the Cl^- -dependent high-affinity binding sites were also capable of inducing new binding sites, and vice versa, with the single exception of TG (see below), and the order of potency of all the analogs tested appeared to be about the same under both conditions, quisqualate, homocysteate, and amino-adipate being the most potent inducers aside from L-glutamate itself (Fig. 4; compare with Table 2). However, the concentrations required to induce new sites were consistently in the millimolar range and thus about a factor of 1000 higher than the apparent affinities for the high-affinity binding site. NMA and kainate, two analogs that interact selectively with Cl^- -independent binding sites, did not induce a binding increase; instead they regularly caused a slight depression of glutamate binding.

Considering the high degree of correlation between the pharmacological profiles, it is of particular interest that binding-site induction differed from high-affinity glutamate binding in two aspects: (1) Binding-site induction did not require the presence of Cl^- and (2) it was not inhibited by Na^+ even at concentrations that completely abolished Cl^- -dependent glutamate binding (Table 3).

Binding-site induction by L-tyrosyl-L-glutamate

TG induced new binding sites with a higher potency than did glutamate or any of the above-mentioned glutamate analogs (Fig. 1B), although it did not seem to interact with the high-affinity glutamate binding site itself, even when present at a concentration of 10 mM during the incubation with

Figure 3. Binding of [^3H]amino-phosphonobutyrate (APB). *A*, Increased [^3H]APB binding in the presence of TG. Membranes were incubated for 60 min with $0.1\ \mu\text{M}$ [^3H]glutamate (\bullet) or $0.2\ \mu\text{M}$ [^3H]APB (\square) in the presence of the TG concentrations given on the abscissa. The figure shows means and SEM of triplicate determinations. *B*, Induction of [^3H]APB binding sites by glutamate and TG. Membranes were preincubated for 30 min at 35°C with no further addition (*left column* = 100%), with $10\ \text{mM}$ L-glutamate (*middle column*), or with $5\ \text{mM}$ TG (*right column*), washed by four centrifugations and incubated with $0.2\ \mu\text{M}$ [^3H]APB for 60 min. Means and SEM are shown for six experiments with three different membrane preparations.



[^3H]glutamate. Ferkany et al. (1983, 1984) have reported that several glutamyl dipeptides increase [^3H]glutamate binding when present in the binding incubation; they suggested that these peptides may control the number of available glutamate binding sites through a second, modulatory binding site. The use of a Cl^- -free buffer for their incubations suggested that TG regulated one of the Cl^- -independent glutamate binding sites, but we have found that binding increase in the presence of Cl^- is much more pronounced than in its absence, and also that the pharmacological profile of [^3H]glutamate binding in the presence of TG and Cl^- strongly resembles that for Cl^- -dependent binding (not shown). Moreover, TG, when added to the incubation medium,

also caused an increased binding of [^3H]APB (Fig. 3A), which appears to bind selectively to the Cl^- -dependent site. Whether only one or several glutamate binding sites are regulated through TG, or whether the Cl^- -dependent site is identical with one of the sites measured in the absence of Cl^- , possibly with a different pharmacological profile, remains to be elucidated.

Induction of glutamate binding by TG, as shown in Figure 1B, showed a high degree of similarity with induction by glutamate itself. The maximum inducible increase was the same in both cases and was not additive when membranes were exposed to $10\ \text{mM}$ glutamate and TG simultaneously or sequentially; however, calcium, when added to the incubation medium, doubled glutamate binding both in glutamate and in TG-preincubated membranes (not shown). In addition, membranes preincubated with TG maintained an increased number of binding sites for hours after washing, as is the case for glutamate-treated membranes. Experimentation with TG is subject to a possible error not encountered with glutamate induction: Whereas incomplete removal of glutamate during the wash procedure would lead to an apparent reduction of binding in the binding assay, residual TG might stimulate glutamate binding directly through its presence in the incubation. However, the amount of $1.1 \pm 0.7\ \text{nmol/mg}$ protein ($n = 6$) of residual TG determined in washed membranes via separation on HPLC could give a concentration of about $0.22\ \mu\text{M}$ free TG in the binding assay incubation, which is too far below the EC_{50} of $11\ \mu\text{M}$ (Ferkany et al., 1984) to cause a significant binding increase. Moreover, the percentage increase in binding in TG-preincubated membranes was the same at protein concentrations ranging from 6.5 to $260\ \mu\text{g/ml}$; at the lowest protein concentration, free TG, as determined by HPLC, could maximally be $8\ \text{nM}$.

Table 2. Pharmacological profile of glutamate binding after induction of new glutamate binding sites

Glutamate analog tested	K_d (μM) (mean and SEM)	(n)
Quisqualate	0.86 ± 0.11	8
L-Glutamate	1.05 ± 0.13	10
D-Glutamate	14.5 ± 4.0	5
L-Homocysteate	1.45 ± 0.30	5
D-Homocysteate	2.34 ± 0.37	4
L-Aminoadipate	1.12 ± 0.14	5
D-Aminoadipate	2.33 ± 0.32	5
L-Aspartate	20.0 ± 4.1	7
D-Aspartate	69.1 ± 11.7	6
APB (2-amino-4-phosphonobutyric acid)	9.8 ± 1.4	5
APV (2-amino-5-phosphonovaleric acid)	54 ± 17	4
GDEE (glutamate-diethylester)	580 ± 100	6
NMA (N-methyl-DL-aspartate)	284 ± 53	6
Kainate	>1000	6

Membranes were preincubated for 30 min with $10\ \text{mM}$ glutamate and washed as usual. Binding was determined without ligand added and at two or three concentrations of the listed glutamate analogs. The glutamate analogs were added to the membranes 15 min before addition of $0.1\ \mu\text{M}$ [^3H]glutamate. The K_d value was determined from the intersection of the regression line of $1/\text{bound}$ glutamate vs ligand concentration and the horizontal line at $1/B_{\text{max}}$. n is the number of independent K_d determinations, most of them done on separate membrane preparations. In some experiments the membranes were suspended in $50\ \text{mM}$ MES/Tris, pH 7.4, $20\ \text{mM}$ KCl, $50\ \mu\text{M}$ EGTA instead of buffer T; the K_d values obtained were not dependent on the choice of buffer.

Reversibility of induced glutamate binding

The induced binding increase lasted for hours after removal of glutamate (Table 4A). Binding decreased for the average of four experiments by only 26%, if glutamate-treated and -washed membranes were kept for 2 hr at 35°C prior to the binding assay. However, exposure to Na^+ after washing out glutamate caused a reversion to very low values for glutamate binding (Table 4B). A subsequent second exposure of Na^+ -treated membranes to $10\ \text{mM}$ glutamate restored glutamate binding to high levels similar to those following the first glutamate exposure, which suggests the possibility that Na^+ and high concentrations of glutamate exert a reversible, antagonistic effect on the state of the glutamate binding sites in these membranes.

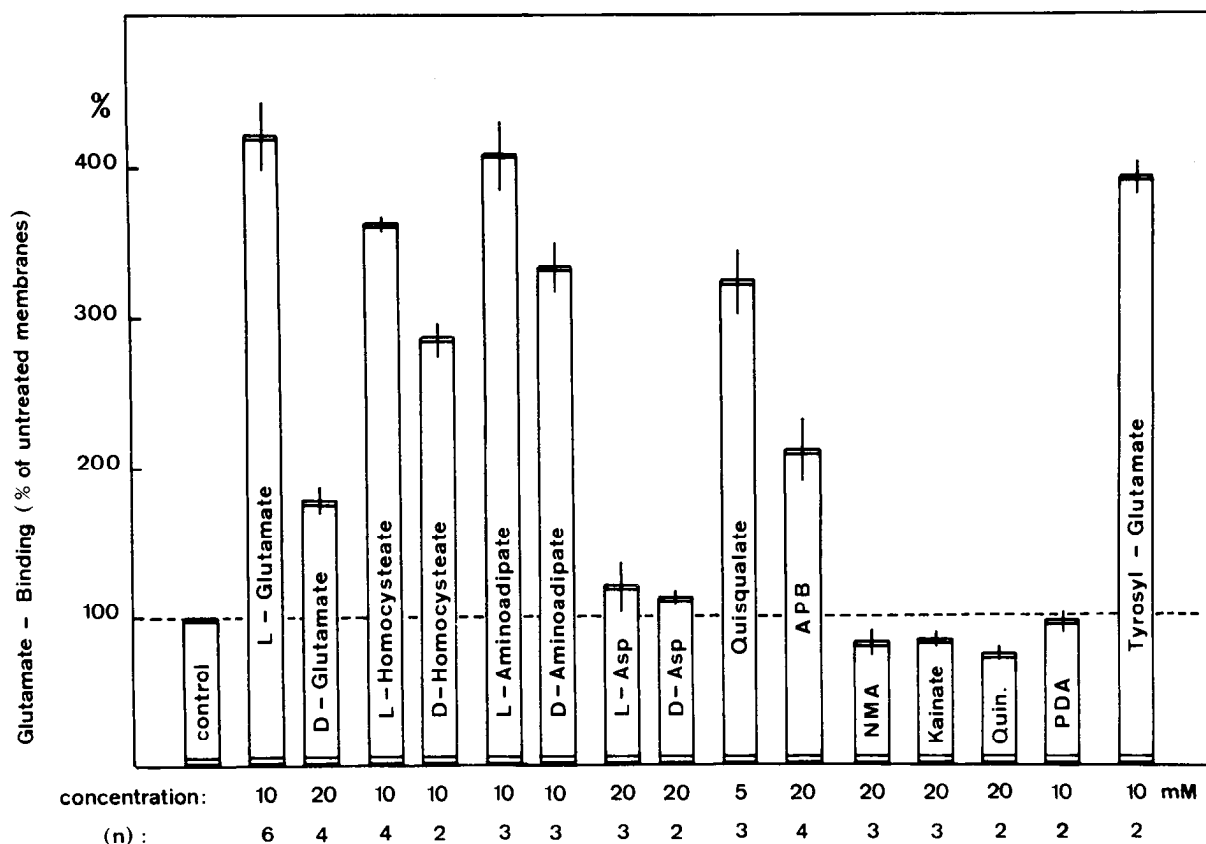


Figure 4. Potency of glutamate analogs to induce new glutamate binding sites. Freshly prepared membranes were suspended after the final centrifugation in 50 mM triethanolamine/HCl, pH 7.4, and 50 μ M EGTA. Glutamate and glutamate analogs were added to the membranes from stock solutions preadjusted to neutral pH with Tris, with the final concentration shown underneath the figure. The membranes were incubated with the various glutamate analogs for 20 min at 35°C, washed by four centrifugations, and incubated with [3 H]glutamate, as described in Methods. Glutamate binding in membranes not exposed to any ligand during the preincubation (*control*) was 7.9 pmol/mg protein (mean of nine determinations). Binding in the other membrane samples (mean and SEM) is expressed as percentage relative to binding in control membranes. The experiments were done on three different membrane preparations, the number of experiments (*n*) for each inductant being shown underneath the figure. The triethanolamine buffer was chosen for this experiment in order to provide an increased buffer capacity in the preincubation medium. It was further demonstrated (data not shown) that both glutamate binding in control membranes and induction by glutamate are little affected by varying the pH during the preincubation period between 6.8 and 7.8, thus making it unlikely that the differences in the potencies to induce glutamate binding arise from some small variations of the pH. APB, 2-Amino-4-phosphonobutyric acid; NMA, N-methyl-DL-aspartate; Quin., quinolinate; PDA, *cis*-2,3-piperidine dicarboxylate.

Some tests for possible mechanisms of binding-site induction

The protease inhibitor leupeptin did not influence the binding-site induction by glutamate or TG (Table 3). Moreover, in membranes in which glutamate binding had been maximally stimulated by a 60 min preincubation with 10 mM glutamate, calcium could still activate further glutamate binding sites (Fig. 1A). Inclusion of calcium in the preincubation with glutamate similarly increased glutamate binding (Table 3). These data suggest that binding-site induction by glutamate is based on a mechanism different from that for the calcium-induced binding increase.

No substance has yet been found which inhibits binding-site induction without permanently decreasing glutamate binding itself (as do thiol reagents). The protein phosphatase inhibitors Zn^{2+} and F^- have only minimally affected binding-site induction (Table 3). GTP and its non-hydrolyzable analog, Gpp(NH)p, did not cause any change in Cl^- -dependent glutamate binding, nor did they inhibit the induction of binding sites by glutamate or TG (not shown).

Induction of binding sites in hippocampal slices

A marked increase in glutamate binding could also be elicited in intact hippocampal slices if they were incubated in a medium containing millimolar concentrations of L-glutamate, homocysteate, or other analogs, including TG (Table 5). Time course,

concentration dependence, and pharmacological profile of binding-site induction in slices were similar to what has been described above for isolated hippocampal membranes. It is noteworthy that exposure of slices to 50 mM K^+ , which would presumably lead to transmitter release and massive ion fluxes, did not elicit an increase in binding. An important question, which necessarily complicates the interpretation of biochemical studies of slices, concerns the locus of the observed effects. Slices contain apparently intact, as well as damaged, neurons (Lee et al., 1981), and either or both could contribute to the results described above.

Discussion

Characterization of induced binding sites

Transient exposure of hippocampal membranes to millimolar concentrations of glutamate caused an increase in the number of glutamate binding sites, typically from about 50 to more than 200 pmol/mg protein. Scatchard-plot analysis in glutamate-preincubated membranes revealed only one binding site, with a dissociation constant K_d of about 1 μ M, a value not significantly different from the K_d of basal glutamate binding (Fig. 2), and a Hill coefficient close to 1. However, it has become increasingly clear during the past years that several different Na^+ -independent binding sites with similar dissociation constants for glu-

Table 3. Effect of leupeptin and various ions on the generation of glutamate binding sites

Composition of medium during preincubation	% Increase in glutamate binding (mean \pm SEM)
A. 10 mM glutamate plus	
Buffer T	+215 \pm 14%
Buffer T + 1 mM leupeptin	+215 \pm 5%
Buffer T + 20 mM NaCl	+245 \pm 11%
Buffer T + 50 mM NaF/1 mM EDTA	+185 \pm 9%
Buffer T + 100 μ M Zn acetate	+124 \pm 15%
Buffer T + 0.5 mM CaCl ₂	+329 \pm 11%
Buffer T + 10 mM MgCl ₂	+183 \pm 19%
B. 10 mM glutamate plus	
PIPES buffer (Cl ⁻ -free)	+133 \pm 10%
PIPES buffer + 20 mM KCl	+137 \pm 13%
PIPES buffer + 50 mM KCl	+110 \pm 10%

Membranes were incubated for 10 min at 34°C in the solutions indicated above. Buffer T: 50 mM Tris/HCl, 50 μ M EGTA, pH 7.4; 1,4-piperazine-diethane sulfonic acid (PIPES) buffer: 50 mM PIPES/Tris, 50 μ M EGTA, pH 7.4. Binding in control membranes was 7.2 pmol/mg protein. Induction in solutions containing Good buffers such as PIPES, HEPES, MOPS, or MES was consistently somewhat reduced as compared to that in Tris/HCl or triethanolamine/HCl buffers, which is possibly due to some weak interaction of these zwitterionic buffers with the glutamate binding sites. The numbers shown are from a representative experiment done on one membrane preparation. All tests were done 3–6 \times on different membrane preparations, giving essentially identical results.

tamate are present in hippocampal membranes. Werling et al., (1983) proposed the existence of two binding sites that can be discriminated by a large difference in their affinities to quisqualate. Mena et al. (1982) similarly suggested that glutamate binding is composed of at least two components, one of which, tentatively identified by these authors to be the synaptic receptor, is strictly dependent on Cl⁻, inhibited by Na⁺, activated by Ca²⁺, and binds selectively the glutamate analog APB. Using these criteria, 50–80% of glutamate binding in untreated membranes and more than 90% of such binding in glutamate-preincubated membranes is to these Cl⁻-dependent binding sites, which suggests that most of the newly induced sites are of the Cl⁻-dependent, APB-sensitive type. Binding to Cl⁻-independent sites was somewhat elevated in glutamate-preincubated membranes (Table 1), but it has not yet been determined if this was due to some residual binding to Cl⁻-dependent sites or whether binding to one of the Cl⁻-independent sites was also modified by the glutamate preincubation.

The number of Cl⁻-dependent glutamate binding sites can be increased by at least two different mechanisms

Transient exposure of synaptic membranes to calcium has been shown to lead to an increase in the number of Cl⁻-dependent glutamate binding sites, which persists after calcium is removed from the medium by EGTA (Baudry and Lynch, 1980; Baudry et al., 1981a, 1983). This activation does not seem to be caused by a direct interaction between calcium and the binding protein, since it can be completely suppressed by leupeptin, a tripeptide that inhibits a calcium-activated protease present in these membranes and that has by itself no effect on glutamate binding.

One important conclusion from these earlier studies was that synaptic membranes contain a reservoir of binding sites that normally are not expressed without appropriate pretreatment. This report provides further evidence that binding measurements in freshly prepared membranes underestimate the overall number of Cl⁻-dependent glutamate binding sites potentially

Table 4. Reversibility of binding-site induction

Preincubation scheme	pmol/mg Protein bound (mean \pm SEM)
A. Reversibility at 35°C after removal of L-glutamate	
1. No glutamate exposure, membranes on ice during preincubation period	6.1 \pm 0.1
2. No glutamate exposure, membranes at 35°C during the 140 min preincubation	6.0 \pm 0.6
3. Membranes at 35°C for 120 min, then exposed to 10 mM glutamate for 20 min	22.2 \pm 1.4
4. Membranes exposed to 10 mM glutamate for 20 min, washed and incubated at 35°C for 120 min in glutamate-free medium	19.1 \pm 0.3
B. Reversal of binding-site induction by exposure to Na⁺	
1. No glutamate exposure	7.3 \pm 0.4
2. After exposure to 10 mM glutamate	36.5 \pm 0.7
3. After glutamate exposure and subsequent 20 min exposure to 50 mM Na ⁺ (followed by seven washes)	6.1 \pm 0.2
4. After glutamate exposure, subsequent Na ⁺ exposure and a second exposure to 10 mM glutamate	22.6 \pm 1.3

A. Membranes were incubated without (2) or with (3, 4) 10 mM glutamate at 35°C for 20 min, then washed by two centrifugations, resuspended and further incubated in glutamate-free buffer T for 100 min at 35°C. Glutamate (10 mM) was then added to sample 3 and the incubations continued for 20 min. All four samples were then washed by four centrifugations and immediately used for the binding assay. In the experiment shown, each condition was run in duplicate. The experiment was repeated three more times. The average loss of induced binding sites in all four experiments caused by the 120 min postincubation (condition 4 compared to condition 3) was 26%. B. All samples were incubated for 20 min without (1) or with (2–4) 10 mM glutamate. Samples 1 and 2 were then washed by five centrifugations. Samples 3 and 4 were washed twice, then incubated for 10 min with 50 mM NaCl (at 35°C) and washed three more times. Both samples were then incubated again in the absence (3) or presence (4) of 10 mM glutamate for 20 min, washed by four additional centrifugations as usual, and immediately used for the binding assay. The experiment was repeated 3 \times with identical results.

present in hippocampal membranes. In membranes transiently exposed to millimolar glutamate concentrations, the number of sites typically increased to more than 200 pmol/mg protein, and if these membranes were further exposed to calcium (together with or subsequent to glutamate exposure), the number of binding sites reached values of 300–500 pmol/mg protein. Thus, only about 10–15% of all the potentially available binding sites are expressed in untreated membranes.

Calcium- and glutamate-treated membranes are particularly well suited for the study of the kinetic and pharmacological properties of Cl⁻-dependent binding sites, since all the Cl⁻-independent binding sites together (NMDA site, quisqualate site, and kainate site) account for only 5% or less of total binding. Moreover, membranes thus enriched in Cl⁻-dependent binding sites might be the material of choice for starting the isolation of the glutamate binding protein.

The induction of new glutamate binding sites—the subject of this report—and the previously described activation by calcium must be based on different mechanisms. Glutamate induction proceeds in calcium-free media and is not inhibited by the protease inhibitor, leupeptin. The two mechanisms are additive, i.e., the stimulation that can be achieved by a combination of these two treatments is larger than the maximal stimulation that can be induced by either calcium or glutamate preincubation

Table 5. Generation of glutamate binding sites in hippocampal slices

Agent-tested	Concentration (mM)	Exposure time	% Increase (mean with SEM or range)	Number of slices tested
DL-Homocysteate	1	5 min	38 ± 19%	4
DL-Homocysteate	5	5 min	81 ± 11%	17
DL-Homocysteate	5	10 min	130 ± 22%	5
L-Glutamate	10	5 min	68%/124%	2
D-Aminoadipate	6	30 min	142%/177%	2
NMA	1–5	10–20 min	–3% (–18 to +14%)	8
Kainate	0.1–0.2	5–10 min	–20% (–30 to –14%)	4
Kainate	1–5	10–20 min	–12% (–17 to 0%)	3
L-Tyrosyl-L-glutamate (TG)	5	30 min	65 ± 16%	7
K ⁺	50	10–20 min	–9% (–20 to +16%)	5
Reduction of homocysteate-elicited binding increase by 5 mM leupeptin			–9%	9

Hippocampal slices were gently shaken in glass vials in 0.6 ml of continuously gassed medium at 33°C for 1 hr. The agents indicated in the table were then added from 20-fold concentrated stock solutions, previously adjusted to neutral pH with NaOH. At the end of the incubation period, the slices were transferred into ice-cold buffer S and homogenized. Membranes were prepared in a scaled-down isolation procedure and immediately used for the glutamate binding assay (for further details, see Methods). Leupeptin, when added, was present in the slice medium throughout the 1 hr preincubation period and the incubation with homocysteate.

alone (Fig. 1A); in fact, since calcium approximately doubled glutamate binding, both in untreated and in glutamate-preincubated membranes, the binding increase in absolute numbers was much larger after glutamate induction. Whether glutamate binding increase induced by TG is based on still another regulatory mechanism remains to be elucidated.

An induction of Cl⁻-dependent glutamate-binding sites similar to the one described here has recently been reported by Malouf et al. (1984). Neuroblastoma cells exposed to millimolar concentrations of glutamate in the culture medium had 2.5 × more Cl⁻-dependent glutamate binding sites in their membrane fractions. The pharmacological profile of induction was essentially the same as that reported here. Calcium activation and induction by glutamate were also additive in their system. However, it is somewhat surprising that exposure times of at least 3 d were necessary to induce a maximal induction, whereas induction in hippocampal slices reaches maximal values within 20–60 min (Table 5). Thus, it remains to be determined whether induction in these two systems is caused by the same mechanism.

Possible mechanisms for glutamate binding induction; evidence for a low-affinity glutamate binding site

The experiments reported here lead to several interrelated questions: (1) What is the mechanism underlying binding site induction? (2) What is the nature of the binding site, which has to be occupied to induce the new, high-affinity glutamate binding sites? And (3) in what form did the induced binding sites exist prior to the induction?

Although the process underlying binding-site induction is not yet understood, the available data lead to certain hypotheses about probable mechanisms. However, we would like, first, to discuss some mechanisms that are most likely *not* involved in binding-site induction. First, nonspecific effects of glutamate on membranes or membrane proteins can be safely ruled out for several reasons: (1) Many glutamate analogs, such as L-aspartate, D-aspartate, quinolinate, etc., did not induce a binding increase; and D-glutamate, whose physicochemical properties are identical to those of L-glutamate, was much less potent than the latter. (2) Preincubating membranes at pH values between 6.5

and 8.5 did not elicit a binding increase (data not shown). (3) Large changes in the ionic composition of the incubation media, e.g., addition of 200 mM morpholinoethane sulfonic acid (MES)/Tris, pH 7.4, to the standard incubation medium did not lead to an increase in glutamate binding (data not shown).

Incomplete removal of glutamate is again most unlikely to cause a binding increase. Residual glutamate from the preincubation would displace [³H]glutamate from the binding sites and thus cause an apparent reduction in binding. Residual glutamate could only elicit a binding increase if binding would be highly cooperative. However, Hill coefficients for Cl⁻-dependent glutamate binding are approximately 1 (Fig. 2; Monaghan et al., 1983). We have further tested membrane samples from three membrane preparations, preincubated for 40 min with 10 mM glutamate, for residual glutamate, using separation by HPLC. These membrane samples showed a three- to fourfold binding increase over control membranes over a 40-fold protein concentration range (0.3–12 μg protein/incubation). The amount of 3.3 ± 0.8 (SEM, n = 3) pmol glutamate/μg protein found in these membranes could account for concentrations of 20–700 nM free glutamate in the incubation with [³H]glutamate, depending on the protein concentration chosen for the binding assay. However, the free glutamate concentration probably is much lower, since there is evidence that part of this residual glutamate is trapped in membrane vesicles and thus cannot interfere with [³H]glutamate binding. We also preincubated membranes for 20 min at 35°C with 0.5 μM unlabeled glutamate prior to adding [³H]glutamate to simulate possible effects of residual glutamate, and did not find any stimulation of binding by this pretreatment.

We thus believe that the induction of glutamate binding is based on a specific process that selectively controls the number or the state of the high-affinity Cl⁻-dependent glutamate binding sites. Obvious candidates for such regulatory functions are the numerous intracellular messenger systems and the phosphorylation/dephosphorylation processes they control. However, various observations suggest that none of those processes is involved in glutamate binding-induction. First, our experiments were done on lysed and sonicated membrane preparations, which could at best contain traces of ATP and messenger compounds. Addition of Mg²⁺ plus ATP, GTP, or Gpp(NH)p to the mem-

branes indeed had no effect on either basal binding or binding-site induction. Receptor dephosphorylation is unlikely, since the phosphatase inhibitor, F^{-} , does not prevent induction. Moreover, the reversible activation and deactivation of binding sites described in Table 4B would require the presence of ATP at least at some point in the cycle, if phosphorylation were involved.

The glutamate concentrations necessary, during the preincubation period, to induce a binding increase are in the millimolar range. This strongly suggests that these membranes possess a low-affinity site for glutamate, distinct from the high-affinity sites detected in the binding assay. Since there are no techniques that would detect binding sites with such low affinities, we must infer the properties of this hypothetical low-affinity site from the induction experiments. One remarkable characteristic is the close resemblance of this site's pharmacological profile with that of the high-affinity Cl^{-} -dependent binding site itself, in spite of the thousand-fold difference in affinities. This suggests that the low- and high-affinity sites might in fact be two interconvertible forms of the same binding site. Prolonged occupation of the low-affinity site by a ligand might trigger a conversion into the high-affinity form that persists for at least hours after removal of the ligand. Short exposure to Na^{+} might convert the high-affinity site back into the low-affinity form. This latter observation might raise doubts about the possible physiological significance of the induction process, since receptors are continuously exposed to Na^{+} in both the intra- and the extracellular compartments. However, it might well be the case that additional factors control the conversion between low- and high-affinity forms of the binding sites, since we find a stable population of high-affinity binding sites in freshly prepared membranes. Thus, the rapid conversion of high-affinity sites back into the low-affinity form might be confined to the *in vitro* situation.

Since glutamate binding in isolated membranes can be cyclically activated by high glutamate concentrations, deactivated by exposure to Na^{+} , and then reactivated by glutamate (Table 4B), the conversion between low- and high-affinity forms may be caused simply by reversible conformational changes within the binding protein, and thus might not require intracellular regulatory processes.

One further possibility is that the high-affinity and the postulated low-affinity sites are different, but functionally interconnected, binding sites. Transient occupation of the low-affinity site might trigger a long-lasting conversion of the high-affinity binding site from a nonfunctional to a functional state. This hypothesis is supported by the experiments involving TG. Since TG does not interact with the high-affinity site itself, one may assume that a modulatory site is present, in close association with the high-affinity site. It is then conceivable that the site for TG is identical with the low-affinity site for glutamate and glutamate analogs. Such a binding site complex, involving several nonequivalent sites, is, of course, at the moment purely hypothetical; but it is not implausible, given the multiplicity of the binding sites found in the GABA/benzodiazepine/barbiturate receptor complex (Olsen, 1981).

To conclude, the results presented here indicate that Cl^{-} -dependent glutamate binding cannot convincingly be interpreted in terms of a simple binding process to a stable population of immutable receptor sites. They suggest, instead, that a low-affinity site exists in hippocampal membranes which itself can be converted into a high-affinity site upon prolonged occupation, or which is a regulatory site in a multiple binding site complex.

Possible physiological significance of binding-site induction

The relationship between the Cl^{-} -dependent glutamate binding sites assayed in the present experiments and the synaptic receptors for various hippocampal pathways has been discussed

elsewhere (Baudry and Lynch, 1984). Suffice it to say that circumstantial evidence links the two but compelling experiments have yet to be reported. If Cl^{-} -dependent glutamate binding is a valid measure of functional synaptic receptors, then the induction described here might represent a physiologically relevant process, which leads to an increased number of synaptic receptors and to increased synaptic transmission, provided that synaptic strength is limited by receptor number. It thus could have important implications for the understanding of the variety of physiological changes that occur in the hippocampus after intense synaptic stimulation. Here, brief bursts of stimulation can cause an increase in glutamate binding sites as well as potentiation of synaptic potentials (Lynch et al., 1982). During these bursts, enough transmitter might accumulate in the synaptic cleft to temporarily convert some low-affinity protoreceptors into functional receptors. Additional receptors might then be irreversibly activated by intracellular calcium, and structural changes might be induced by the previously described mechanism involving a calcium-sensitive protease (Baudry and Lynch, 1984). The different temporal components in the decay of synaptic potentiation, with half-lives ranging from hours to days (Racine et al., 1983), might thus correlate with the different activation mechanisms for Cl^{-} -dependent glutamate binding sites.

Repeated subthreshold stimulation can also produce seizures in the hippocampus and, with repetition, the threshold for producing these is substantially reduced (kindling) (Racine and Burnham, 1984). Each seizure is followed by a prolonged depression of physiological activity, a period in which the cells are depolarized. While experimental studies of extracellular amino acid concentrations have not been reported, it is certainly possible that the processes that control synaptic glutamate concentrations are disturbed for considerable periods by the induction of kindling. If so, then the inductive effects reported above might explain the lowered threshold of hippocampal circuitry for seizures, as well as the increased number of binding sites found in the kindled hippocampus.

A transfer of a receptor from a low- to a high-affinity state does not necessarily indicate a greater physiological effect. Upon prolonged exposure to agonists, the nicotinic ACh receptor converts from a low-affinity state, which is the physiologically active form of the receptor, to a high-affinity state, in which the bound agonist no longer elicits opening of the ion-channel (Prinz and Maelicke, 1983; Rang and Ritter, 1970). This state of the receptor has been termed "desensitized." By analogy, the induction of binding sites after exposure to high glutamate concentrations might be interpreted as a "desensitization" process.

According to this alternative interpretation, the postulated low-affinity sites would be the actual, functional synaptic receptors, whereas the high-affinity sites revealed in the binding assay would represent a pool of desensitized receptors that do not respond to the binding of glutamate with channel-opening. The binding sites normally found in freshly prepared membranes might represent a small pool of desensitized receptors already present in the tissue, where they might be in dynamic equilibrium with the functional receptors. Receptor sites with millimolar affinities may at first seem unrealistic in view of the fact that most receptor systems studied have dissociation constants in the nanomolar concentration range. However, it should be borne in mind that the fast-switching synaptic receptors must have association and dissociation times for their natural ligands in the millisecond time scale, and that low-affinity sites are more likely to meet these demands. The slow association and dissociation rates of the Cl^{-} -dependent high-affinity glutamate binding site are in fact incompatible with the kinetics of a fast-switching synaptic receptor, and suggest that the receptor would have to undergo some kind of conversion into the high-affinity form identified in the binding assay. (However, mechanisms

other than the induction described here might cause this conversion into the slowly dissociating form, e.g., removal of Na⁺ during the membrane preparation.)

Desensitization is of unclear functional significance and conditions leading to desensitization may be different for each receptor type. Even if glutamate binding induction should represent receptor desensitization, any analogy with the ACh receptor should be made with caution. The conversion between the low- and high-affinity states would indeed be orders of magnitude slower in the case of the glutamate receptor, thus "freezing" receptors for hours in the desensitized state after removal of the ligand. One other important difference would be that glutamate receptor desensitization could be induced by both agonists and antagonists, whereas desensitization is restricted to agonists in the case of the ACh receptor.

The question of whether induction of new binding sites is accompanied by a change in synaptic strength and, if so, whether this reflects a reduction (as predicted from the "desensitization" interpretation) or a potentiation of synaptic responses must await future physiological experiments. The major difficulty faced by studies of this type is that the opening of channels throughout the entire neuron for extended periods can be expected to be deleterious to the cell, and indeed, amino acids do produce excitotoxic effects (Zaczek and Coyle, 1982). This does not rule out a physiological role for high-transmitter concentrations in regulating receptors since, under physiological circumstances, only a small fraction of the total synaptic population would be activated by the activity in a given afferent. Under these conditions, local potentiation could occur without disrupting the ionic and metabolic homeostasis throughout the target neuron. One possible way to alter the number of receptors by prolonged exposure to amino acids without producing excitotoxic effects would be to use antagonists of the synaptic receptor. Compounds such as APB and D-aminoadipate have only weak agonist properties but strong affinities for the Cl⁻-dependent site and, as described above, are powerful inducers of high-affinity sites. Accordingly, it should be possible to apply these compounds in high concentrations for extended periods, wash the slices clean, and test for changes in excitatory synaptic potentials.

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