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The structure and function of glutamate receptors: Mg²⁺ Block to X-ray diffraction

Mark L Mayer

NINDS, NIH, Bethesda MD 20892

Abstract

Experiments on the action of glutamate on mammalian and amphibian nervous systems started back in the 1950s but decades passed before it became widely accepted that glutamate was the major excitatory neurotransmitter in the CNS. The pace of research greatly accelerated in the 1980s when selective ligands that identified glutamate receptor subtypes became widely available, and voltage clamp techniques, coupled with rapid perfusion, began to resolve the unique functional properties of what cloning subsequently revealed to be a large family of receptors with numerous subtypes. More recently the power of X-ray crystallography and cryo-EM has been applied to the study of glutamate receptors, revealing their atomic structures, and the conformational changes that underlie their gating. In this review I summarize the history of this field, viewed through the lens of a career in which I spent 3 decades working on the structure and function of glutamate receptor ion channels.

Introduction

I was lucky to start working on glutamate receptor ion channels at a period when the work of Jeff Watkins and other medicinal chemists was coming to fruition. The identification of NMDA, AMPA and kainate subtypes, and the tools provided by Jeff and his colleagues, combined with the birth of patch clamp, allowed us to do biophysical experiments on mammalian CNS cell cultures that would have been impossible just a few years before. Later on, the timing was just right to enter into the field of structural biology. When I started doing crystallography in Eric Gouaux's lab in New York, third generation synchrotrons were in the pipeline, and huge advances in data processing software made it possible for even novice structural biologists to do high quality work. Most recently I was able to hitch a ride on the booming field of cryo-EM and study full length AMPA and kainate receptors trapped in different conformational states.

Bristol and the London School of Pharmacy

My interest in pharmacology started soon after I finished A-levels. Before entering university I worked as a technician in the Division of Molecular Pharmacology at the

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National Institute for Medical Research in London, purifying dihydrofolate reductase for pioneering protein NMR experiments in the lab of GCK Roberts. I was captivated by the idea that drugs worked on receptors, and that these were biochemically discrete objects that could be purified and studied in molecular detail. However, following the advice of a student who told me there were no jobs for Biochemistry graduates, I decided to look into other degree subjects. I was exposed to the field of glutamate receptor research very early in my career, when I was an undergraduate in the Department of Pharmacology at the University of Bristol. Here Jeff Watkins and Dick Evans were beginning to make progress in identifying iGluR subtypes, and by the time I completed my PhD in 1980 they had discovered NMDA, AMPA and kainate receptors (Watkins and Evans, 1981).

While this work was going on I was studying the effect of iontophoretic application of GABA and glycine on the firing rate of neurons in the hypothalamus, using extracellular recordings in anesthetized rats for my PhD thesis at the London School of Pharmacy. This gave only an indirect readout of the underlying chloride current that inhibited action potential discharge, and because many neurons were silent I had to drive their activity with an excitatory amino acid. I quickly discovered that D,L-homocysteic acid was much better than glutamate at mediating sustained firing, a finding Gary Westbrook and I would make sense of in a few years time. By contrast to my rather primitive work, the voltage clamp experiments of Paul Adams and David Brown on the M-current in bullfrog sympathetic neurons that were also going on at the same time marked the dawn of a new age for the study of neurotransmitter receptors (Brown and Adams, 1980). David gave a Departmental Seminar on this work before it was published and inspired by this I decided to learn biophysics as soon as I finished my PhD.

NIH and Biophysics

I was fortunate to be awarded a Harkness Fellowship which enabled me to work in the US, first at the University of Texas in Galveston, where I learned intracellular recording in Joel Gallagher's laboratory in the Department of Pharmacology, while also sneaking across the road to visit Paul Adams' laboratory in the Department of Physiology and Biophysics, where a flux of English visitors were doing voltage clamp experiments on neurons in brain slices and various ganglia. It was during these visits that I learned the rudiments of biophysics. I then moved to Phil Nelson's lab at the NIH to learn how to work with mammalian neurons in cell culture. When I arrived, Phil's setup was heavily used and the time available for experiments was limited. Peter Guthrie and Gary Westbrook, recently arrived postdocs, were in the process of building a second rig, which had a two-electrode voltage clamp amplifier that was designed and built by Bruce Smith, an electronics engineer in the research services branch. Gary and I pooled our time on this rig and decided to look for voltage-dependent currents in mouse DRG neurons that were large enough to survive impalement with two micro electrodes. It took us a while to get going but we eventually focused on a hyperpolarization activated inward current that was similar to that recently discovered in heart cells by Dario DiFrancesco at Oxford (Brown and DiFrancesco, 1980), and in hippocampal pyramidal cells in brain slices by James Halliwell and Paul Adams in Galveston (Halliwell and Adams, 1982). We named the current I_h (Mayer and Westbrook, 1983). When we had finished the experiments and written a paper for submission to the

Journal of Physiology we asked Phil if he would like to be an author; although Gary and I had worked independently, our work was done in Phil's lab, and he had earlier described the time dependent sag in hyperpolarizing electronic potentials that I_h now explained; he generously declined.

Magnesium Block and my Return to NIH

When my Harkness fellowship expired I had to return to the UK. John Kelly, the Chair of the Department of Pharmacology at St. Georges Hospital Medical School, recently vacated by Humphrey Rang, offered me space in his laboratory, and the Beit Memorial foundation paid my salary. Leaving NIH was hard for many reasons, but especially because at that time labs in the US were better funded compared to resources available in the UK in the days before the Wellcome Trust started supporting biomedical research. After less than a year at St. George's I applied for a Travel Award from the Royal Society which paid my airfare for a trip to NIH where I spent four months in the summer of 1983 working on glutamate receptors. By that time the rig that Gary and I used became ours by squatters rights and we used it exclusively for our experiments over the next three years. The preparation we used, mouse spinal cord motor neurons in dissociated cell culture were, like DRG neurons, large enough for two-electrode voltage clamp. We used this approach for our experiments that led to discovery of the Mg^{2+} block of NMDA receptors, before switching to whole cell patch clamp a year later, learning to our chagrin both how much damage impalement with microelectrodes did to a neuron's input resistance, and how much easier it was to do whole cell recording.

The background for our experiments was set by prior studies from multiple groups that reported variously a decrease, no change, or an increase in membrane resistance during depolarizing responses to glutamate (Engberg et al., 1979). If the response of glutamate receptors was mediated by a voltage-dependent conductance, like that recently discovered by Adams and Brown for the action of acetylcholine on the M-current, it was possible that this, combined with the activation of endogenous voltage gated sodium and potassium currents, could account for the range of responses produced by glutamate. The solution was obvious: We would record responses to glutamate under voltage clamp instead of measuring depolarization combined with hyperpolarizing current pulses to determine changes in membrane resistance. It was surprising that nobody had conducted a series of experiments like this before, and we quickly established that all the agonists we tested activated a conductance with a reversal potential close to zero mV; that responses to NMDA had a region of negative slope conductance; and that responses to glutamate, which produced no change in slope conductance, could be mimicked by applying a mix of NMDA and kainate or quisqualate. We also found that the response to glutamate could be converted into a non-NMDA receptor, kainate or quisqualate like response, when co-applied with APV, an NMDA receptor antagonist recently developed by Watkins. Looking back to my days as a graduate student, we found that the D and L isomers of homocysteic acid produced different extents of activation of NMDA and non-NMDA receptors, and that the racemic mixture had just the right voltage dependence to keep neurons in a membrane potential range that supported action potential discharge. We published this work in the Journal of Physiology (Mayer and Westbrook, 1984b). It is still one of my favorites.

But why were responses to NMDA voltage-dependent? Our experiments eliminated the possibility, as had been suggested previously, that NMDA receptors gated a voltage-dependent potassium current, like the M-current of Adams and Brown. Several groups had subsequently postulated that NMDA receptors instead gated a voltage-dependent sodium or calcium current (Dingledine, 1983; MacDonald et al., 1982), and we next proceeded to test this idea. We were hoping to use classical voltage clamp techniques to look at the kinetics of current relaxations following step changes in membrane potential, but in our experiments responses to NMDA were always much smaller than those to kainate and quisqualate. As a result, analysis of their kinetics was difficult. Unfortunately, when we started this series of experiments the cultures died, and I had to return to the UK for two weeks to interview for a lectureship at the University of Bristol, which I did not get. This break gave me the chance to think things over, and a chat with Jeff Watkins while I was interviewing at Bristol reminded me that they had recently found that Mg^{2+} inhibited responses to NMDA (Ault et al., 1980).

When I returned to NIH we decided to continue our analysis in Mg^{2+} free solutions with the goal of recording larger currents. At first we thought something had gone terribly wrong, because the response to NMDA was no longer voltage-dependent, but within a couple of hours we realized instead that NMDA receptor ion channels are blocked by Mg^{2+} ions. We made this leap very quickly because in our prior work on I_h we had studied the voltage dependence of ion channel block by Cs^+ and were very familiar with this phenomenon. It was then easy to design experiments to tie these observations together, and when I returned to the UK in September, the story was complete. We presented this work at the December meeting of the Physiological Society in 1983 (Mayer and Westbrook, 1984a). We should not have waited! Unknown to us Philippe Ascher's group in Paris had independently discovered that Mg^{2+} blocked glutamate activated ion channels and already had a paper in press (Nowak et al., 1984). At first, Nature did not send our manuscript out for review, but with support from John Kelly I called the Editorial office and convinced them to review our paper which was published in May (Mayer et al., 1984). At the time I was an unknown postdoc, while Philippe Ascher was an eminent scientist with an outstanding track record, so at first our cause seemed hopeless, but the independence of our work, and its significance saved the day. Neither Philippe nor Gary and I set out to discover the mechanism of action of Mg^{2+} on NMDA receptors, but we all recognized the phenomenon of ion channel block as soon as we saw it. As mentioned above we were prepared by our work on the block of I_h by Cs^+ while Phillip's lab had previously worked on the voltage dependent block of ACh receptors in *Aplysia* (Ascher et al., 1978). In addition they quickly recognized the kinetic signature of ion channel block in single channel recordings, which had been previously reported for the action of local anaesthetics on neuromuscular junction ACh receptors (Neher and Steinbach, 1978).

Ca²⁺ Permeability and the action of other ions on NMDA receptors

In September 1984 I returned to the NIH to continue working as a postdoctoral fellow in Phil Nelson's lab, collaborating with Gary Westbrook for another two years until each of us set up our own groups (Fig. 1). We had one more encounter with Philippe during this period. After writing up our papers from the summer of 1983, which took far longer than it should

have, we set out to look at the ion selectivity of iGluR responses, this time using whole cell patch clamp recording, and later on hippocampal neurons which were much easier to prepare than spinal cord cultures. Using classical electrophysiological techniques we found a large depolarizing shift in the reversal potential for NMDA but not non-NMDA receptor responses when the extracellular Ca^{2+} concentration was raised, and calculated a relative permeability coefficient of $P_{\text{Ca}}/P_{\text{Na}}$ of 10.6 compared to 0.15 for non-NMDA receptors (Mayer and Westbrook, 1987). Around the same time Philippe's group concluded that the effect of calcium was due to a surface charge screening effect, raising doubts about our interpretation. Although we were confident about our data, we had a lucky break when Steve Smith who was en route to Yale stopped by the NIH and built a micro spectrophotometer in Jeff Barker's lab that was designed to measure absorbance changes by the Ca^{2+} indicator dye arsenazo III. Due to the arrival in less than a year of the fluorescent Ca^{2+} sensor Fura-2 synthesized by Roger Tsien, which was much more sensitive, this rig had a very short life! When Steve's work was done we learned about the rig and explained that we had a question that it was the perfect tool to address.

I spent a couple of months working with Amy MacDermott to show that in voltage clamped spinal cord neurons NMDA but not kainate produced large rises in intracellular Ca^{2+} concentration, and that this was due to ion flux through NMDA receptors (Fig. 2). These measurements, combined with the permeability measurements done with Gary, were enough to convince the skeptics, and our study was published in Nature (MacDermott et al., 1986). During the course of our work we examined a number of different ions for activity on NMDA receptors, leading to the joke that presumably we lacked the imagination to do anything new and were working our way through the Periodic table. One of these ventures, proposed by Gary, involved a study of the action of zinc, which is highly abundant in the hippocampus. We discovered that zinc inhibited both NMDA and GABA receptors, and that for NMDA receptors the mechanism was not ion channel block (Westbrook and Mayer, 1987). We had discovered an NMDA receptor allosteric regulatory site that years later Pierre Paoletti explored to gain the first insights into the structure and role of the amino terminal domain (Paoletti et al., 2000).

Concentration Jump Experiments

The experiments that Gary and I did when working on Mg^{2+} block and ion permeability had all used focal application of ligand solutions from pressurized micropipettes positioned close to the cells we were recording from. This produced reproducible brief responses that were great for our analysis of ion permeation but were entirely unsatisfactory for other purposes because we did not know the concentration of ligand at the cell surface. There was a long standing rumor in the lab, and probably many other labs using nerve cell culture, that you could not perfuse the preparation because the neurons would die, presumably due to removal of survival factors in the complex, and expensive mix of serum, growth factors and media in which the cultures were grown. Today this seems nonsense but it held us back at the time. Jon Johnson, a postdoc in Philippe Ascher's lab, clearly showed that nerve cell cultures could be perfused, and more importantly developed a linear array of tubes mounted on a stepper motor that could be used to rapidly change solutions bathing a neuron. With this approach they discovered that NMDA receptors used glycine as a coagonist (Johnson and

Ascher, 1987), and started to do concentration jump experiments that used the same experimental principles used previously by biochemists for kinetic experiments on enzymes (Henderson et al., 1990). This was clearly a major step forwards, and with help from the same instrumentation group that built our first voltage clamp amplifier, we designed a similar system at NIH. This allowed us to do many sets of experiments, including with Doris Patneau a systematic analysis of concentration response relationships for different ligands and glutamate receptor subtypes. Ladia Vyklicky and later Morris Benveniste studied the allosteric interactions between glutamate and glycine at NMDA receptors, and the modulatory actions of polyamines on NMDA receptors. Linda Wong and Doris Patneau analyzed the action of 5-substituted willardiines on AMPA and kainate receptors.

When Derek Bowie joined the lab we had already begun to work with recombinant AMPA receptors using clones that were generously provided by Peter Seeburg's lab; these came with a huge list of conditions, including the specific requirement that they were to be named GluRA, GluRB etc. so as to distinguish them from the Heinemann lab GluR1 and GluR2 clones! Years later, neither of these nomenclatures is in use, and today the receptors are named GluA1 and GluA2 (Collingridge et al., 2009). When my lab was reviewed in 1990 I received the following critique regarding my proposed experiments using recombinant iGluRs: "*A second ambition is to start mutagenesis experiments to define structural feature important for channel function, along the lines pioneered by Sakmann, Imoto and Lester ... concerns at this stage must be directed to the general questionability of entering a field already trampled by the heavy-weight laboratories*". Fortunately I was not dissuaded and before taking up his analysis of polyamine block with Robert Bähring, Derek helped Kathy Partin finish up experiments that defined a single residue in the alternatively spliced flip/flop cassette identified by Peter Seeburg, that Kathy, later helped by Mark Fleck, showed controlled sensitivity to cyclothiazide (Partin et al., 1995; Partin et al., 1996).

Structural Studies on Glutamate Receptors

Although work in the lab was going well it became increasingly clear to me that our so called structure function studies, performed using site directed mutagenesis to manipulate the iGluR clones given to us by Peter Seeburg, had a major drawback. Without structures to place our data in a molecular framework, we were working in the dark. Viewed in a harsh light we were clueless about the origin of the effects that our mutants produced. The work that Kathy Partin did side stepped this problem for a while because nature had guided us to interesting parts of AMPA receptors, where single amino acid changes introduced by alternative splicing produced dramatic effects on the action of cyclothiazide, which abolished desensitization for flip but not flop splice variants. But I knew the end was approaching. My dilemma was solved when I reviewed an NIH grant written by Eric Gouaux, a structural biologist at Columbia University in New York, who convincingly proposed to study iGluRs using crystallography and X-ray diffraction. I asked Eric if he would be willing to let me work in his lab as a visiting scientist, and convinced the authorities at NIH to grant me a one year sabbatical.

This was the start of a long, productive and mutually beneficial collaboration. While I was starting from scratch in Eric's lab, he benefited from having daily access to a walking

glutamate receptor encyclopedia, followed later by frequent email and telephone exchanges. I proposed to Eric that it would be interesting to crystallize the amino terminal domains of NMDA receptors, which contained binding sites for Zn^{2+} and ifenprodil. Fortunately for me, because it took Hiro Furukawa years of work to achieve this, my idea never took off and Eric suggested instead that I solve the structure of the ligand binding domain of GluR0, a bacterial ion channel that he had recently discovered, and for which for Changhai Cui and I did a functional analysis in a mad rush before I left for New York (Chen et al., 1999). Having a paper in Nature at the start of my sabbatical was a good start, but I soon realized how much biochemistry and crystallography I had to learn if I was going to succeed. Indeed, my unspoken vision at the start of the year was that if nothing worked I would at least learn to use the fancy molecular graphics programs that structural biologists used to make pretty pictures from X-ray coordinates in the protein data bank.

In fact Eric's lab already had GluR0 LBD crystals before I arrived. I was able to quickly reproduce these, but found that they diffracted X-rays to low resolution. The project took off when by mistake I forgot to add a protease inhibitor to one of my early GluR0 preps and found that the purified a protein was cleaved into two parts. Using N-terminal sequencing of the cleaved products I was able to establish where the protein was cut, and on the basis of this designed a new construct. I made this over the Christmas break, back in my lab at NIH, causing minor matrimonial disharmony when I had to go to the lab on Christmas day to set up cultures to sequence the new construct. At the start of the New Year I took the clone with me to New York, and in March collected my first MAD data set from selenomethionine substituted GluR0 crystals, which showed greatly improved diffraction. With help from Neali Armstrong and Rich Olsen, two graduate students in Eric's lab, I processed the data set and calculated my 1st electron density map using an SGI O2 stereo graphics work station that I had purchased for \$13,451 using funds from my lab at NIH (current desktop computers are 1/10 the cost and at least 10 times more powerful). What followed was a frustrating few weeks while I learned the rudiments of model building into an experimentally phased electron density map using the program O, a task which I could now complete in a couple of days. In addition to having to learn a huge number of what seemed to me to be very unfriendly computer programs, I was forced to manage a Unix work station with no background in computer science; the final straw towards the end, when I could take no more, was having to learn to use Photoshop to assemble figures for publication (Mayer et al., 2001). Today, programs like COOT, PHENIX, and the CCP4 suite have graphical interfaces, tutorials, and user guides that greatly facilitate this process.

Back at the NIH, while continuing to collaborate with Eric on the mechanism of AMPA receptor desensitization (Sun et al., 2002), I set about building up the facilities I would need to do biochemistry and by 2005 had solved a series of structures for kainate receptor ligand binding domains that nicely explained their selective ligand binding properties (Mayer, 2005). I discussed some of this work with Jeff Watkins who told me that he had never imagined that it would be possible to see the structure of a glutamate binding site in complexes with different ligands. It is interesting to compare the three site model he proposed (Curtis and Watkins, 1960) with the atomic structures solved 40 years later (Armstrong and Gouaux, 2000; Mayer, 2005). I believe Jeff took great pleasure in seeing his work put to such good use (Fig. 3). My efforts in structural biology continued with studies

on GluN3 ligand binding domains, the amino terminal domains of kainate receptors, and most recently novel Ctenophore glycine binding subunits.

Working with Andrew Plested, and later with Phil Biggin, we identified the binding sites for Na^+ and Cl^- ions that Derek Bowie had discovered modulate kainate receptor activity (Bowie, 2002; Wong et al., 2006). We established that the Na^+ binding site was non-selective, as revealed by electron density for K^+ , Rb^+ and even Cs^+ when crystals were soaked in solutions containing these ions. The conflict with Derek's earlier work, which revealed that Cs^+ did not support kainate receptor gating, was resolved when we measured the affinity and efficacy of ions for kainate receptor modulation. Our crystal structures were solved in the presence of 600 mM Cs^+ and even this concentration was not sufficient to produce full occupancy of the cation binding sites, while at the 150 mM concentration used for functional studies Cs^+ was bound very weakly; increasing the concentration of Cs^+ in our functional experiments restored kainate receptor gating, resolving the conundrum. Peter Schuck and Charu Chaudhry then used analytical ultracentrifugation to establish that both Na^+ and Cl^- ions regulate the strength of kainate receptor ligand binding domain dimer assembly (Chaudhry et al., 2009). Our results fit in nicely with prior work on AMPA and kainate receptor desensitization that established that when the ligand binding dimers dissociate, the receptors desensitize (Chaudhry et al., 2009; Sun et al., 2002; Weston et al., 2006). Thus we believe that for kainate receptors, all the ions do is maintain the ligand binding domain dimer assembly in a conformation that supports ion channel gating when glutamate binds, and I still do not believe the idea that ions are coactivators of kainate receptors!

Cryo_EM and full length Glutamate Receptors

In December 2009 Eric Gouaux's group published the first crystal structure of a full length glutamate receptor: GluA2 trapped in the closed state with a competitive antagonist (Sobolevsky et al., 2009). This was a huge advance that revealed many unexpected details about how the receptor was assembled. Specifically, the structure resembled the letter Y, with the amino terminal and ligand binding domains both arranged as a dimer of dimers, with subunit cross over in the ligand binding domain layer. It also raised the question of whether other glutamate receptor subtypes had a similar structure, and more importantly how did the active and desensitized states differ from the resting state. After failing to crystallize the kainate receptor GluK2 we started a collaboration with Sriram Subramaniam whose lab had expertise in cryo electron microscopy. In our first experiments we were unable to obtain imaging conditions suitable for single particle analysis and so turned to tomographic reconstructions in which the grid is tilted in the microscope so that the same particles are imaged over a $\pm 60^\circ$ rotation angle.

Our results revealed that GluK2, engineered to bind a high affinity competitive antagonist, adopted a resting state structure that closely resembled that for GluA2, but the desensitized state looked nothing like that predicted by models based on a decades worth of work on isolated ligand binding domains. To our great surprise we found that in the ligand binding domain layer the receptor adopted a 4-fold symmetric arrangement (Schauder et al., 2013). Although the tomograms were convincing, the low resolution of these structures, around 21

Å, did not provide further insight into how this change occurred. When Joel Meyerson joined the project he solved the problem that had prevented single particle analysis, and we set out to look at the structural basis of the gating cycle. We used ligands to trap receptors in resting, activated, and desensitized states; because the necessary drugs for the full cycle are only available for AMPA receptors we started with GluA2. Again, a surprise emerged when we found that the desensitized state adopted multiple conformations, preventing a high resolution analysis; fortunately GluK2 was much better behaved yielding a desensitized state structure at 7.6 Å resolution (Meyerson et al., 2014). Shortly after our results were published, Eric Gouaux's group published a complimentary series of crystal structures that were in good agreement with the cryo-EM data (Durr et al., 2014).

Much remains to be done. Even for model homomeric receptor systems the resolution of the currently available structures leaves many issues open. Currently we still do not have the structure of an iGluR with an open channel; the structure of complexes with auxiliary proteins and the mechanisms by which they modulate iGluR function remain to be determined. The molecular mechanism of Mg^{2+} block is unknown. There is more than a career's worth of work for the next generation!

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Fig. 1.
Mark Mayer and Gary Westbrook photographed by Phil Nelson in the fall of 1986 prior to the laboratory's 4-year review.

Fig. 2.

Voltage dependent Ca^{2+} influx through NMDA receptor channels measured under voltage clamp using arsenazo III. (A) Titration of arsenazo III with increasing concentrations of Ca^{2+} measured in a benchtop spectrophotometer. (B) Responses to NMDA with a low extracellular concentration of Mg^{2+} recorded from a spinal cord neuron under voltage clamp over the membrane potential range -100 to $+40$ mV. (C) The simultaneously recorded increase in $[\text{Ca}^{2+}]_i$ measured as a change in differential transmittance for the wavelength pair $570 - 660$ nm.

Fig. 3. The ligand binding site of glutamate receptor ion channels. (A) The three point receptor hypothesis of Curtis and Watkins in which in 1960 they proposed that the α -carboxyl, α -amino and γ -carboxyl groups of glutamate bind to charge complimentary sites in the receptor. (B) The 1.65 Å resolution crystal structure of the GluK2 ligand site (PDB 1S50) revealed the molecular identity of these binding sites, but with one difference. There is no counter charge for the glutamate γ -carboxyl group, which instead binds via hydrogen bonds to a main chain amide, to the hydroxyl group of Thr659, and to a hydrogen bonded network

of molecules which are trapped in the ligand binding cavity together with glutamate. Displacement of these water molecules allows the binding of large heterocyclic amino acids such as quisqualic acid and AMPA.

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