CHAPTER 5

Activation of Single AMPA- and NMDA-Type Glutamate-Receptor Channels

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A. Introduction

I. Rates of Physiological Events

In real life, the thing that matters about glutamate receptors is their response to the brief pulse of agonist (glutamate) that is released by a presynaptic nerve during synaptic transmission. The brief nature of this pulse makes it likely that postsynaptic channels will experience only a single activation (see below). The receptor is not at equilibrium, and it is the rate at which it works that controls its physiological properties. The study of rates (kinetics) is, therefore, crucial to the understanding of physiology. For some purposes, an empirical description of how things are observed to change is quite sufficient, and such empirical descriptions may be described appropriately as models; but, in order to understand how the receptor actually works, this is not good enough. For this purpose, we must aim to describe the receptor in terms of physical realities. The states in a kinetic mechanism must not be convenient abstractions but actual physical structures.

There is some hope that we may be near to this ideal for the (muscle-type) nicotinic receptor (Colquhoun and Sakmann 1985; Sine et al. 1995), but it has not yet been achieved for *N*-methyl-D-aspartate (NMDA) or α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors. To the extent that we can succeed in this aim, the description is better defined as a mechanism than as a model because the latter term (purposely) evades the crucial question of physical reality. The attempt to do so is important, not just for intellectual curiosity and not just for physiology. It is also essential for understanding how proteins work, the effects of mutations on receptors and the relationship (insofar as one is discernible) between the structure of drugs and their effects. Many attempts have, of course, been made to understand these questions in the absence of much knowledge about mechanisms. But, it is now clear that these attempts are, at best, inspired guesswork (which does not, of course, mean that they are always wrong). This question has recently been reviewed in detail (Colquhoun 1998).

It has been a long-standing goal of physiologists to explain the time course of synaptic currents. In most cases, and certainly in the case of synaptic currents mediated by NMDA receptors, the time course is determined by the rate

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at which channels close after the removal of free agonist (often referred to as their "deactivation" rate) (ANDERSON and STEVENS 1973). The next natural question concerns the nature of the single-channel events that underlie the synaptic current.

In the simplest case (latency followed by single opening) the macroscopic time course of the synaptic current would be the convolution of the distributions of first latency and of the length of the channel opening (COLQUHOUN and HAWKES 1995a), as used, for example, by ALDRICH et al. (1983) for sodium channels. This description works, however, only if there is a single sort of open state and the latency is followed by a single opening. This is not the case for the NMDA or AMPA receptors (or for most other agonist-activated channels).

The general relationship that is to be expected between single-channel activations and the macroscopic time course has only recently been given (COLQUHOUN et al. 1997), and experimental results on NMDA receptors have been found to be consistent with this theory (WYLLIE et al. 1998). The latter paper also serves to emphasise the high resolving power of single-channel measurements compared with those of macroscopic currents. For example, the distribution of the length of individual channel activations for the recombinant NR1a/NR2D receptor contains (at least) six exponential components, so it would be expected that the macroscopic response to a fast concentration jump would also contain six exponential components with very similar time constants (COLQUHOUN et al. 1997; WYLLIE et al. 1998). The observed responses to concentration jumps were consistent with this being correct, but a free fit to the macroscopic response could not resolve more than two or, at best, three of the components. This example makes it clear that it will generally be hard to discern physically realistic mechanisms from macroscopic responses alone.

II. The Definition of a Channel Activation

The idea of an individual receptor activation provides the crucial link between single-channel events and macroscopic (synaptic) currents. The term 'activation' defines the sequence of events that take place between the first opening that follows binding of agonist and the last opening before complete dissociation of agonist. In general, this definition implies that partially liganded shut states may form part of an activation. The property that defines activations is that they are separated by one or more sojourns in the resting state (no agonist bound), and so they must become progressively further and further apart as the agonist concentration is reduced. Experimentally, they are defined as bursts of openings (for NMDA receptors, these bursts are so long, and have so much sub-structure that they have been referred to as super-clusters).

The most direct way to see an individual receptor activation is to do a very brief concentration jump on a one-channel patch (WYLLIE et al. 1997); insofar as the agonist concentration is zero after the jump, no re-association can take

place and the whole of the observed signal is one activation (Fig. 5). It is also possible to define individual activations in steady-state recordings if they are made at a sufficiently low concentration such that activations are well separated. For NMDA receptors, the activations are so long that this means using very low concentrations, usually 5–100 nM glutamate (GIBB and COLQUHOUN 1991, 1992; WYLLIE et al. 1998). Insofar as the concentration is near zero in these experiments, the distributions of activation lengths should have time constants similar to those seen in the jump experiments, but the initial condition at the start of an activation may be very different for the two sorts of experiments so the areas attached to these components may be quite different, as discussed below.

III. The NMDA Receptors

The NMDA receptor has been the most intensely studied of all agonist-activated ion channels over the past decade and has been demonstrated to play a pivotal role in a variety of physiological and pathophysiological functions, not to mention an ever-increasing number of 'phenomena'. Furthermore, it is clear that NMDA receptors can be modulated by factors ranging from protein kinases to protons and from polyamines to simple membrane stretch. Two properties of the NMDA receptor are, however, likely to give rise to this macromolecule being so widely involved in biological responses. First, the NMDA receptor is highly permeable to Ca^{2+} and, second, glutamate remains bound to and activates NMDA receptors for several hundreds of milliseconds. Molecular biology and site-directed mutagenesis have allowed us to gain insights into what particular amino acids control the Ca^{2+} permeability of these channels (see Chaps. 1 and 7). Our knowledge of the factors that give rise to the very complex activation structure of these channels is far from being complete, but it is fundamental to a full understanding of the NMDA receptor.

IV. The AMPA Receptors

AMPA receptors mediate the fast component of glutamate-evoked synaptic currents in the central nervous system (CNS). Functionally, therefore, this receptor class can be considered to be similar to nicotinic acetylcholine receptors that are found at the endplate of skeletal muscle; they provide a fast depolarisation of the postsynaptic membrane. One should, however, be careful not to extend this analogy too far because, unlike nicotinic receptors at the endplate, AMPA receptors in the CNS are a heterogeneous class of ligand-gated ion channels. Such heterogeneity does not arise simply from the fact that there are four different AMPA-receptor subunits (GluR1, 2, 3 and 4, also termed GluR-A, B, C and D) each of which can form homomeric channels, but also from the fact that these four can form many heteromeric combinations. The existence of cellular mechanisms that generate different splice variants for each subunit, and of ribonucleic acid (RNA) editing, complicate still further

the number of different AMPA subunits that can be synthesised, and means that potentially thousands of possible receptor combinations could be present in the CNS (see Chaps. 1 and 9). Given this, it is perhaps not surprising that, as discussed below, there have been a wide variety of single-channel conductances and kinetic parameters reported for both native and recombinant AMPA receptors.

B. Comparison of Native and Recombinant Receptors

The question of the subunit composition of native receptors continues to be of paramount interest, and, although progress has been made, the job is very far from being completed. An important tool in this quest is to make comparisons between native receptors and recombinant receptors in some heterologous expression system.

It is the bane of all work on recombinant receptors that one can never be sure exactly what proteins one's expression system has produced, and to what extent their behaviour will mimic quantitatively the behaviour of native receptors. This has been a serious problem for neuronal nicotinic receptors, for which even single-channel conductances are unreliable (LEWIS et al. 1997; SIVILOTTI et al. 1997). To a lesser extent, it has also been a problem for muscle-type nicotinic receptors, for which conductances seem to be reliable, but kinetic behaviour may not be (GIBB et al. 1990; and unpublished observations, discussed in EDMONDS et al. 1995).

A recent review (SUCHER et al. 1996) emphasised the differences between recombinant and native NMDA receptors. However, many of the studies that were cited used shortcut methods that do not allow firm conclusions to be drawn on this question. It has been a basic principle of pharmacology for many years that, if one wishes to compare two receptors, it is necessary to measure some quantity (classically, the equilibrium constant for binding of a competitive antagonist) that should be characteristic of the receptor, but independent of differences between one tissue and another, and of irrelevant experimental variables, e.g. nature and concentration of the agonist. Differences in EC_{50} values often cannot be interpreted unambiguously because of differences in conditions such as calcium and glycine concentration and different application methods (with consequent differences in desensitisation). Even more worrying is the widespread use of the IC_{50} as a measure of the activity of antagonists like 2-amino-5-phosphonovaleric acid (APV).

It has been well known, at least since 1949, that it is impossible to estimate real equilibrium constants from IC_{50} curves, and, consequently, such measurements cannot be used for quantitative comparisons of receptors in different tissues or expression systems. LEFF and DOUGALL (1993) tried to resuscitate the IC_{50}, but their attempt to modify the Hill equation to allow for competitive antagonism is, like the Hill equation itself, entirely empirical; it cannot be derived from any physical mechanism. It merely describes parallel-

shifted Hill curves and, as such, is useful only as a means of implementing the Schild method. The "general Cheng-Prusoff equation" which they derive from it has no sound physical basis and so cannot be relied on to estimate real equilibrium constants, though it has been seized upon by some authors as a convenient shortcut. There is, however, one condition under which the IC_{50} should give a good estimate of the antagonist equilibrium constant, and that is when the application of agonist is so brief that there is insufficient time for the occupancy by antagonist to change perceptibly (COLQUHOUN et al. 1992). In general, though, the correct method will be the Schild analysis (ANSON et al. 1998); this takes a little longer to do, and there are few reliable measurements in the literature.

The use of IC_{50} measurements to compare magnesium sensitivities is equally unsound. It has been well known since the work of ADAMS (1976) that the action of a channel blocker is expected to depend on the fraction of channels that are open, e.g. on the agonist concentration. The fact that this varies from one set of experiments to the other makes it impossible to compare the IC_{50} values. If magnesium were a simple open-channel blocker, it would be possible to measure the association and dissociation rate constants for its combination with the open channel, values that would be characteristic of the channel. However, magnesium does not act like a simple open-channel blocker (NOWAK et al. 1994), and its action is not fully understood, so there is no known method of extracting from experimental results a quantity, such as an equilibrium constant or rate constant, that could reasonably be expected to be independent of things like the nature of the agonist and its concentration.

When single-channel properties of NMDA receptors are measured, a rather different picture emerges from that presented by SUCHER et al. (1996). The conductances and subconductances of NMDA receptors expressed in oocytes and in mammalian cell lines are quantitatively very similar (STERN et al. 1994), and there is good reason to think that they are similar to native receptors too, as discussed below.

C. Single-Channel Conductances of NMDA Receptors

Measurements of the single-channel conductance of NMDA-receptor channels have proved useful for identification of native receptors. For a comprehensive review of these single-channel conductance values, the reader is referred to a recent review by CULL-CANDY et al. (1995). NMDA-receptor channel conductances do not appear to depend on the glutamate concentration, though no systematic study has appeared, and judged by results obtained before the requirement for glycine (JOHNSON and ASCHER 1987; KLECKNER and DINGLEDINE 1988) was known, they appear not to depend on the glycine concentration either. However, the conductances do depend on the extracellular calcium concentration. The main single-channel conductance level for the most common sort of NMDA-receptor channel is about 50 pS in 1 mM calcium,

but 42 pS in 2.5 mM calcium and 66 pS in ethylenediamine tetraacetate (EDTA)-buffered solution (GIBB and COLQUHOUN 1992), and a similar dependence is also found in a "low-conductance" channel (recombinant NR1a/NR2D; WYLLIE et al. 1996). The values given below all refer to 1 mM calcium in bicarbonate buffer (about 0.85 mM free Ca^{2+}), or 0.85 mM Ca^{2+} in hydroxyethylpiperazine ethanesulfonic acid (HEPES) buffer.

In outside-out patches from *Xenopus* oocytes, the combinations NR1/NR2A and NR1/NR2B both give single-channels with a main conductance level of about 50 pS and a subconductance level of about 40 pS (Fig. 1) (STERN et al. 1992; also see TSUZUKI et al. 1994). These channels resemble closely the native receptors that have been described in cerebellar granule cells as well as the very similar channels in hippocampal neurones (HOWE et al. 1991; GIBB and COLQUHOUN 1991, 1992). This similarity was not restricted only to the two conductance levels, but it was also found that the frequency of transitions between different conductance levels was in close quantitative agreement with the measurements of HOWE et al. (1991) in cultured cerebellar granule cells. Furthermore, the quantitative similarity extended to the distributions of open times, shut times and burst lengths, etc. The conclusion of this work was that low-concentration equilibrium single-channel records are indistinguishable for (a) NR1/NR2A channels expressed in oocytes, (b) NR1/NR2B channels expressed in oocytes, and (c) the '50-pS' channels observed in various cultured, dissociated or brain-slice neurones. However, recombinant NR1/NR2A and NR1/NR2B channels are clearly distinguishable on the basis of their deactivation rates (MONYER et al. 1994; VICINI et al. 1998), their glycine sensitivity (which is ten times lower for the NR1/NR2A combination than for NR1/NR2B (KUTSUWADA et al. 1992; STERN et al. 1992), as well as by antagonists such as ifenprodil (WILLIAMS 1993).

The NR1/NR2C channels, however, have lower conductance. They have a main conductance level of 36 pS with a sublevel about half the amplitude of the main level and with a briefer open time (STERN et al. 1992). These channels resemble closely the low conductance channels observed in brain slices by FARRANT et al. (1994). They described the development of NMDA receptors in rat cerebellum, and found that, in 14-day-old rat cerebellar slices, the channels all appeared to be of the '50-pS' type, described above. However, in older animals they found that mature post-migratory granule cells contain a subtype of receptor that shows conductance levels of 33 pS and 20 pS in 1 mM calcium; these values are similar to those found with the NR1/NR2C combination. This result is in beautiful agreement with in situ hybridisation studies (WATANABE et al. 1992; MONYER et al. 1994), which show that there is little messenger RNA for NR2C present in the cerebellum at 7 days after birth, but after 14 days the amount of NR2C message has increased to something near to the adult level.

The NR1/NR2D channels have very similar conductance levels (35 pS and 17 pS) to NR1/NR2C but differ from them in two ways: firstly, the mean duration of the subconductance level is longer than that of the main level, and,

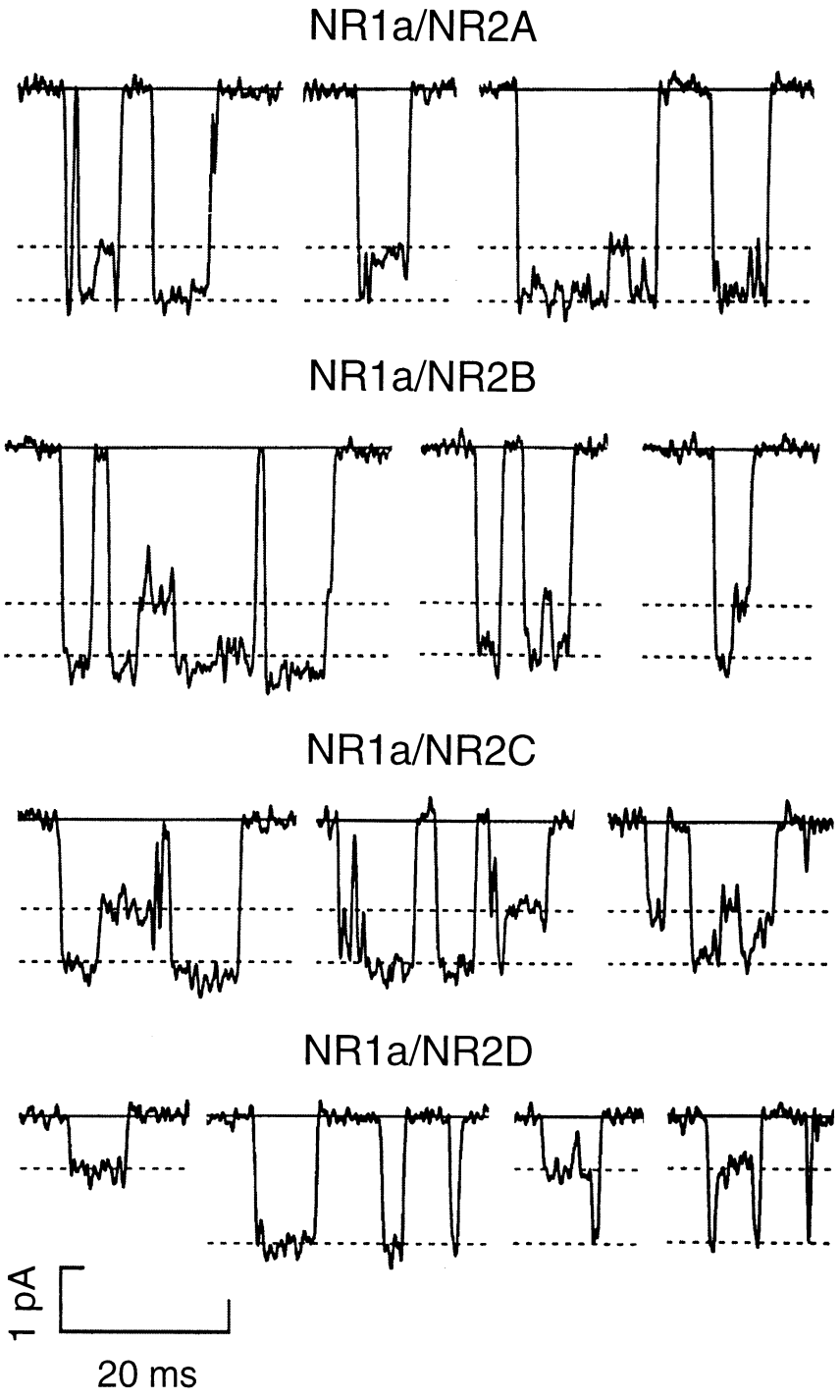


Fig. 1. Recombinant *N*-methyl-D-aspartate (NMDA) receptor channel currents. Examples of recombinant NMDA single-channel currents recorded in outside-out patches held at –60 mV. NR1a/NR2A and NR1a/NR2B channels give 'high conductance' channels with a main level at about 50 pS and a sublevel at 40 pS. In contrast NR1a/NR2C and NR1a/NR2D channels are lower in conductance, possessing a main level at around 35 pS and a sublevel at 18 pS

secondly, the records show temporal asymmetry. This asymmetry is manifested by the fact that direct transitions from the 35-pS level to the 17-pS level are more common than transitions in the opposite direction (Wyllie et al. 1996). Such asymmetry is not observed with any of the other subunit combinations or with native 'high conductance' channels. A similar asymmetry was, however, reported by Cull-Candy and Usowicz (1987). They reported single channels in cultured "large cerebellar neurones". Their records apparently contained two sorts of channels: one of which was the usual 50/40-pS type, but the other had conductance levels of 18 pS and 38 pS, which are similar values to those found in the NR1/NR2C and NR1/NR2D combinations, but the lower conductance type showed asymmetry which resembles only the NR1/NR2D combination. Momiyama et al. (1996) investigated Purkinje cells in cerebellar slices and found, up to P12, very similar low conductance channels (only); after this time, NMDA-receptor channels are absent from Purkinje cells. The suggestion that these channels are the NR1/NR2D type agrees very well with the results of in situ hybridisation studies, which show that only NR1 and NR2D mRNAs are detectable in Purkinje cells at this age (Akazawa et al. 1994). Very similar low-conductance channels, along with typical high conductance channels, were detected in deep cerebellar nuclei and in spinal cord, dorsal-horn neurones by Momiyama et al. (1996), and these too may be NR1/NR2D channels (Ishii et al. 1993; Tölle et al. 1993; Monyer et al. 1994).

An additional NMDA-receptor subunit has been cloned, which was termed initially χ-1 (Ciabarra et al. 1995) or NMDA-L (Sucher et al. 1995), but is now referred to as NR3A (Das et al. 1998). This subunit, which has a low homology with the others, when expressed together with NR1 and NR2A subunits, seems to give recombinant NMDA-receptor channels with conductances lower than those of NR1/NR2A channels alone.

D. Single-Channel Conductances of AMPA Receptors

It would appear that there are very few values of single-channel conductance, up to about 35 pS, that have *not* been reported to be the conductance of AMPA receptors. As with all electrophysiological measurements, differences in recording conditions undoubtedly play a major role in generating such a range of values. This is clearly not the only factor because, even under nominally constant conditions, AMPA receptors still exhibit a variety of unitary conductances. There has been no systematic study of the dependence of their conductance on calcium concentration. Furthermore, the determination of channel conductances is hindered by the fact that openings of AMPA receptors are very brief [as, of course, is to be expected because these channels mediate a rapidly decaying excitatory post-synaptic conductance (EPSC)], as well as having predominantly small amplitudes. Recently, however, a clearer picture has begun to emerge as the single-channel conductances of some recombinant AMPA receptors have been reported.

I. AMPA-Receptor Channels in Cerebellar Granule Cells

One of the best-characterised cell types is the cerebellar granule cell, so we shall concentrate on AMPA-receptor channels in these cells. Single-channel conductances have been measured (CULL-CANDY et al. 1988; HOWE et al. 1991; WYLLIE et al. 1993), and the AMPA subunit mRNA present in these cells has been analysed (MONYER et al. 1991; MOSBACHER et al. 1994). Recordings of single-channel currents in outside-out membrane patches from HEK 293 cells expressing recombinant AMPA receptors (SWANSON et al. 1997) has permitted the elucidation of some of the factors that contribute to determining the conductance of AMPA-receptor channels.

In recordings from membrane patches excised from granule cells, activation of non-NMDA-receptor channels produces three types of response. The 'high-conductance' response is composed of channel openings to three conductance levels – 12, 21 and 31 pS for glutamate, and is similar for AMPA and kainate (WYLLIE et al. 1993). The conductances are thought to represent multiple conductances of a single receptor type as direct transitions are seen occasionally between the three levels and the relative proportion of each type of opening is constant between different patches (WYLLIE et al. 1993). The 'low-conductance' response again gives discrete channel openings, but this time to levels of around 6 pS and 10 pS, again with transition between these levels being observed. Channels with extremely low conductances mediate the third type of response. No discrete channel openings are observed in this 'femtosiemens' response, which is characterised by a small noise increase in the glutamate-evoked membrane current. In situ hybridisation of mRNAs coding for AMPA subunits in granule cells indicate that both the 'flip' and 'flop' isoforms of GluR2 and GluR4 exist in these cells. Homomeric $GluR4(Q)_{flip}$ receptors ("Q" here refers to the fact that a glutamine residue is to be found at the Q/R site) give channels with multiple conductance levels of 8, 15 and 24 pS when activated by glutamate (similar conductances are observed if AMPA is used to activate the channel). Heteromeric channels comprised of $GluR2(Q)_{flop}/GluR4(Q)_{flip}$ also give channels with the same multiple conductance levels as is seen with homomeric $GluR4_{flip}$. However, as GluR2 subunits are most likely to be fully edited, that is contain an arginine (R) residue at the Q/R site, such heteromeric channels are unlikely to exist in vivo.

The question arises then, are the 'high-conductance' channels seen in granule cell patches composed of homomeric $GluR4(Q)_{flip}$ subunits? Similarities in conductance levels alone should not, of course, be the sole basis for such a proposition, but other pieces of evidence are also consistent with such a conclusion. The relative proportions of each of the three conductance levels are similar for both native and $GluR4(Q)_{flip}$ channels. $GluR4(Q)_{flip}$ channels and native high-conductance channels are sensitive to intracellular polyamines and, thus, are likely to be Ca^{2+} permeable (SWANSON et al. 1997). Although it is true that 'high-conductance' channels in granule cells have linear single-channel current-voltage relationships (see WYLLIE et al. 1993), which would

not be expected of homomeric GluR4(Q)$_{flip}$ channels, it is likely that this resulted from the 'wash-out' in outside-out patches of the intracellular factor(s) responsible for generating the inward rectification seen with such channels. One clear discrepancy does remain, however – native 'high-conductance' channels give 10, 20 and 30 pS openings when kainate, instead of glutamate or AMPA, is used to activate the channel. With homomeric GluR4(Q)$_{flip}$ channels, kainate does not give discrete channel openings, but rather spectral analysis of the kainate-evoked current suggests the channels underlying the response have a weighted mean conductance of around 2.5 pS (SWANSON et al. 1997). This is a clear difference between homomeric GluR4(Q)$_{flip}$ channels and 'high-conductance' channels in granule cells. It also appears to be a rare example of the channel conductance being determined by the nature of the agonist used to open the channel (though if kainate openings were extremely brief, spectral analysis could underestimate the single-channel conductance). To date, there have been no reports as to the single-channel conductance of GluR4(Q)$_{flop}$ channels other than for kainate, which, once again, produces a noise increase with no discernible channel openings (weighted conductance ~4 pS). Given that the flip and flop isoforms of GluR subunits differ in their amino acid sequences in the extracellular region between the third and fourth transmembrane segment, it would be surprising if alterations in this area of the protein had a major effect on the conductance of the channel.

What then of the 'low-conductance' channels? It appears that the co-expression of GluR4(Q)$_{flip}$ with either GluR2(R)$_{flip}$ or GluR2(R)$_{flop}$ results in a reduction of single-channel conductance. The 4-pS and 9-pS events seen when such heteromeric channels are activated by AMPA are very reminiscent of 'low-conductance' events seen in native channels.

Finally, it would appear that 'femtosiemens' channels are formed by homomeric GluR2(R)$_{flip}$ and GluR2(R)$_{flop}$ channels. Thus, in addition to affecting Ca^{2+} permeability and its influence on the current–voltage, the presence of a GluR2(R) subunit reduces the single-channel conductance of AMPA receptors (SWANSON et al. 1997) (in a manner that is analogous to the introduction of N → R mutated subunits in the NMDA receptor; BÉHÉ et al. 1995).

E. Structure and Stoichiometry of Glutamate Receptors

I. Location of the Agonist-Binding Sites

Since the AMPA receptors, GluR1 to GluR4, will all form homomeric channels, there is, presumably, one glutamate-binding site for each subunit (unless the subunits are arranged asymmetrically with the binding sites at interfaces). When the NR1 subunit of the NMDA receptor was first cloned (MORIYOSHI et al. 1991), the fact that it was found by expression cloning in oocytes led to the presumption that the glutamate-binding site must be on the NR1 subunit. This has, subsequently, turned out not to be the case (the response seen with

NR1 alone in oocytes may depend on an endogenous subunit that has been found in oocytes; SOLOVIEV and BARNARD 1997). In fact it is the glycine-binding site that appears to be on the NR1 (KURYATOV et al. 1994; WAFFORD et al. 1995; HIRAI et al. 1996; WILLIAMS et al. 1996), as judged by the fact that single amino acid mutations reduced glycine potency by up to four orders of magnitude, with only small effects on glutamate potency. These studies suggested that the glycine-binding site has a bi-lobar structure, similar to that found by crystallography for a family of bacterial periplasmic amino acid-binding proteins (OH et al. 1993, 1994), in which the ligand is bound in a "Venus-flytrap" mechanism. One lobe (the 'S1 domain') is part of the pre-M1 N-terminal region; the other is C terminal of M3 (the S2 domain). More recently, similar experiments have located the glutamate-binding site on the homologous regions of the NR2 subunit (LAUBE et al. 1997; ANSON et al. 1998; LUMMIS et al. 1998).

Single amino acid mutations in NR2A or NR2B produced increases in the EC_{50} for glutamate of up to 1000-fold, with little or no change in Hill slope and little effect on glycine potency. The most effective mutant found by ANSON et al. (1998), NR2A(T671A), was in the S2 domain, which had a 1000-fold increase in EC_{50} for glutamate, and they adduced several lines of evidence to support the view that such a large change could not result primarily from effects on gating (it would require something of the order of a million-fold reduction in the gating constant; COLQUHOUN 1998). They also found that the T671A mutant had a 250-fold increase in the equilibrium constant for binding of the competitive antagonist APV, which again suggests a change in the binding site itself. The T671A mutant produced single-channel openings that were similar to the wild-type NR1/NR2A, but had a much faster deactivation rate after 100 ms concentration jump, which is consistent with the same interpretation. LUMMIS et al. (1998) also found a 1000-fold increase in EC_{50} with the nearby NR2A(G669S) mutation in the S2 domain. LAUBE et al. (1997) found a 120-fold increase in EC_{50} with NR2B(S664G) in the S2 domain (homologous with S670G in NR2A), but, oddly, the IC_{50} for APV was decreased, not increased, with this mutation. Similar effects were found by mutations in the S1 domain, the most effective being NR2B (E387A), which is homologous with E394A in NR2A (240-fold increase in glutamate EC_{50}; LAUBE et al. 1997), and NR2A(H466A) (220-fold increase in glutamate EC_{50}; ANSON et al. 1998).

Although, in principle, the use of competitive antagonists is a good way of avoiding the binding-gating problem (COLQUHOUN 1998), the results found by LAUBE et al. (1997) and ANSON et al. (1998) suggest that the correlation between effects of mutations on agonists and antagonists may be imperfectly correlated and further investigation of this would be interesting.

II. Which Subunits Co-Assemble in NMDA Receptors?

Almost all of the native NMDA-receptor channels that have been described in sufficient detail so far (including the two cases discussed above) can be

closely matched by expression of NR1 with only one of the NR2 subunit types. The only obvious exception, so far, is a preliminary report by Paleček et al. (1998) that motor neurones contain an NMDA-receptor channel with a conductance that is larger than that seen in any recombinant receptor. Nevertheless, there are now quite good reasons to believe that more than two sorts of subunit can assemble to form "triplet" receptors. As with other aspects of stoichiometry, there is little unanimity about the details, perhaps because of the indirectness of the methods that have to be used. The majority of studies use immunoprecipitation. Several studies have found coprecipitation of NR1/NR2A/NR2B, with less agreement about NR1/NR2A/NR2C (Chazot et al. 1994; Sheng et al. 1994; Didier et al. 1995), but Chazot and Stephenson (1997) found that in mouse forebrain 46% of the NR1 immunoreactivity was associated with the NR2B subunit, and, of this, only 13% (6% of total) was NR1/NR2A/NR2B. Dunah et al. (1998) found in the thalamus that anti-NR2D precipitates 93% of NR2D and 48% of NR1, but only 25% of NR2A and 36% of NR2B, so binary NR1/NR2A was also present, but in the midbrain NR2A or NR2B were always co-precipitated. The immunoprecipitation method has the benefit of identifying proteins, but has the disadvantage that there is no way to be sure that the protein that is detected has been correctly inserted into the membrane and is functional. Blahos and Wenthold (1996) used isolated synaptic membranes (from rat forebrain), and they found that most receptors appeared to be either NR1/NR2A or NR1/NR2B, with little evidence for much triplet formation. They, and Chazot and Stephenson (1997), also found that more than one splice variant of NR1 can co-assemble in one receptor, with little preference for any particular NR2 subunit.

Electrophysiological studies have mostly been carried out on recombinant receptors. They have the advantage that they demonstrate that the receptors are inserted in the membrane and are functional, but the disadvantage that identification of which subunits are involved is indirect. No good single-channel studies have yet been published; inferences so far have been based on whole-cell responses. The first report by Wafford et al. (1993) suggested, on the basis of glycine sensitivity curves, that NR1, NR2A and NR2C could co-assemble, because co-expression produced an intermediate EC_{50} with little change in Hill slope for glycine. Köhr and Seeburg (1996) suggested co-assembly of NR1/NR2A/NR2B on the basis of intermediate deactivation rates when all three were transfected in HEK293 cells. However, Vicini et al. (1998), also using transfected HEK293 cells, found that most cells showed responses to short concentration jumps that were like those of either NR1/NR2A or of NR1/NR2B, with only a low proportion of putative triplets (NR1/NR2A/NR2B). Buller and Monaghan (1997) suggested co-assembly, in *Xenopus* oocytes, of NR1/NR2A/NR2D on the basis of IC_{50} values for antagonists, but they did not determine equilibrium constants for the antagonists (instead, a correction of IC_{50} values proposed by Durand et al. 1992, was used, but this correction, though soundly-based for binding experiments, is not valid for responses).

In summary, there is good evidence that some triplets can form, but considerable uncertainty about how many native receptors are triplets.

III. Stoichiometry of Recombinant NMDA Receptors

Despite the intense interest in glutamate receptors over the last decade or so, there is still serious doubt about whether they are tetramers or pentamers. The proposed membrane topology, which is now generally accepted, bears a superficial resemblance to that of a potassium channel, and that has made the idea of a tetrameric structure popular, but hard evidence is still lacking. There have been three electrophysiological approaches to the problem, each using somewhat different approaches, but with little agreement in the results.

Béhé et al. (1995) used an N → R mutation in the NR1a and NR2A subunits, at the "QRN site". This mutation causes a great reduction in single-channel conductance. When wild-type and mutant NR1 subunits were expressed with wild-type NR2A, only one intermediate conductance level was observed, so it was inferred that there are probably two NR1 subunits in the receptor. However, when the same mutation was made at the homologous position in the NR2A subunit, the co-expression of wild-type and mutant NR2A together with wild-type NR1 gave a baffling array of conductance levels, which had no easy interpretation.

Premkumar and Auerbach (1997) used a similar approach but with quite different results. They used the NR2B subunit and an N → Q mutation rather than N → R. Furthermore, the wild-type and mutant NR2 subunits were coexpressed, not with wild-type NR1 but with mutant NR1. This approach gave relatively simple results for the NR2 subunit; one intermediate was found, so it was suggested that there were two NR2 subunits in the receptor. However, coexpression of wild-type and mutant NR1 (with mutant NR2) produced a complex mixture of types, which the authors interpreted as suggesting the presence of three NR1 subunits in direct contradiction with the results of Béhé et al. (1995). They thus concluded that the receptor is a pentamer.

This sort of approach is clearly susceptible to errors in either direction. It is quite possible that all possible subunit combinations will not be expressed in sufficient amounts to be detected, or that some combinations are indistinguishable, so causing an underestimate of the number of subunits present. It is equally possible that the properties of the receptor will depend on the order in which the subunits are arranged round the channel, so causing an overestimate of the number of subunits. It was an advantage of the N → R mutation used by Béhé et al. (1995) that the large differences in conductance made it possible to show the entire results, and there was no need for subtle kinetic analysis. However, the complex results obtained by Premkumar and Auerbach (1997) for the NR1 subunit were separated into different classes by hidden Markov methods, the reliability of which (through no fault of the authors) is impossible for the reader to assess. The matter remains undecided.

A study by Laube et al. (1998) used a different method. They too used coexpression of wild-type and mutant subunits, but they used NR1(Q387K), which has a low sensitivity to glycine, and NR2B(E387A), which has a low sensitivity to glutamate. They then attempted to assess the number of components in macroscopic concentration–response curves, by fitting multiple Hill-equation components to them. The results were compatible with the view that the NR1/NR2B receptor was a tetramer that contained two subunits of each type. The resolving power of this method is much lower than that of single-channel analysis, and the Hill equation is empirical. Although the Hill equation often provides a tolerable fit to concentration–response curves, it is obviously not the correct equation, and it is not clear that it is good enough for distinguishing subtle differences in a multicomponent fit. Unfortunately, in the absence of a good kinetic mechanism, the equation that should be fitted is not known. The effect, if any, of these problems on the conclusions cannot realistically be assessed at the moment.

IV. Stoichiometry of Recombinant AMPA Receptors

There is also disagreement about stoichiometry in the case of AMPA receptors. Ferrer-Montiel and Montal (1996) and Mano and Teichberg (1998) both used the approach of MacKinnon (1991), which is now known to have given the correct tetrameric stoichiometry of a potassium channel (Doyle et al. 1998). This method uses coexpression of wild-type subunits with mutated subunits that have a reduced affinity for an inhibitor. In both studies, GluR1 receptors were used. Ferrer-Montiel et al. (1996) used a mutation that reduces the sensitivity of the GluR1 receptor to the inhibitors PCP and MK801, whereas Mano and Teichberg (1998) used, more dubiously on theoretical grounds, a mutation that reduced the sensitivity of the receptor to desensitisation by quisqualate. The former study concluded that the GluR1 receptor contains five subunits, and the latter concluded that it contained four.

The Mackinnon method involves plotting, against concentration of inhibitor, of the measured values of $\ln(R_{\text{mix}}/R_{\text{mut}})/\ln(p_{\text{mut}})$, where R_{mix} and R_{mut} are the responses to (an arbitrary concentration of) glutamate in the presence of the specified inhibitor concentration for mixed and all wild-type receptors respectively, and p_{mut} is the overall fraction of mutant subunits that were transfected into the cell. The asymptote of this plot, for high inhibitor concentration, is taken as an estimate of n, the number of subunits. It does not seem to have been widely noticed that this asymptote is, in general, less than n. The expression for the asymptote is actually

$$\left(\frac{1}{\ln(p_{\text{mut}})}\right)\ln\left[\sum_{i=0}^{n-1}\frac{K_i}{K_n}\binom{n}{i}p_{\text{mut}}^{i}(1-p_{\text{mut}})^{n-i}+p_{\text{mut}}^{n}\right],$$

where K_i is the equilibrium constant for binding of the antagonist to a receptor that contains i mutant subunits (and hence n–i wild-type subunits). This asymptote is always less than n. It will be close to n if the all mutant receptor

has a much lower affinity (large K_n) for antagonist than any other receptor (say 1000-fold higher to be on the safe side), so the first term in square brackets becomes negligible relative to the second. In general, though, the value for the asymptote is quite sensitive to errors in the estimation of p_{mut}, the fraction of subunits that contain the mutation in the assembled receptors. Because it has to be assumed that p_{mut} is the same as the ratio of the amounts of RNA that are injected, accuracy is not easy to guarantee.

As an example, consider the case where $n = 4$ and $p_{mut} = 0.9$, as in MacKinnon (1991); with his values for K_i this gives an asymptote of 3.91, close enough to 4 to enable MacKinnon to get the correct answer. If, however, n had actually been 5, but p_{mut} had been overestimated and was actually 0.45 rather than 0.9, then the asymptote of the plot would have been exactly 4 despite the fact that $n = 5$, and a quite incorrect conclusion might have been drawn. Apart from this hazard, there are several other potential problems. For example, the whole argument depends on random assembly of subunits, i.e. assembly and insertion must be unaffected by the mutation. It is also assumed that the response that is measured is directly proportional to the number of unblocked channels. This is reasonable with a high-affinity blocker in the potassium channel case, but not so for an agonist-activated channel; indeed, it will not be true unless the response can be tested with a pulse of agonist so short that no blocker dissociates (not always easy in a whole oocyte).

A third study, by Rosenmund et al. (1998) used a different approach. They used mainly a chimaeric AMPA/kainate channel (GluR3/GluR6). They stepped from a high antagonist concentration into a high agonist concentration in order to slow the activation of the channel. They found, unusually, that the single-channel conductance depended on the number of agonist molecules that are bound (a phenomenon that has been shown even more clearly for the ion channels that are activated by intracellular cyclic guanosine monophosphate (cGMP); Ruiz and Karpen 1997). The results were interpreted in terms of a simple mechanism, which suggested that the largest conductances were produced when four agonist molecules were bound. If the mechanism they used is sufficiently close to the truth, this implies that four agonist molecules are needed to open the channel optimally (see below). They interpreted this result as meaning that the channels were tetrameric, though it could also be that a fifth molecule produced no further increase in conductance. As the authors are careful to point out, the inference of a tetrameric structure depends on an assumption of the simplest binding mechanism. Whether or not this has foiled this ingenious approach to the stoichiometry problem remains to be seen.

Electrophysiological methods, such as those just described, face many problems of interpretation (though they have given correct answers in other cases). In the face of the many uncertainties, it could well be that only biochemical, and ultimately structural, studies will provide unambiguous answers. One recent biochemical study suggested a tetrameric structure, but several others have been ambiguous (Wu et al. 1996, and references therein).

V. How Many Agonist Molecules Are Needed to Activate Glutamate Receptors?

One of the first questions that one would like to answer concerns the number of agonist molecules that must be bound in order to open the ion channel (which is not necessarily the same as the number of agonist-binding subunits). There may, of course, be no unique answer to this question. In principle, the channel might be expected to open more efficiently as more ligands become bound (Ruiz and Karpen 1997; Colquhoun 1998).

AMPA receptors must have an agonist-binding site on each subunit because all the subunits (GluR1–4) can form homomeric receptors, but it is important to know how many of these sites must be occupied in order to open the channel efficiently. In the case of NMDA receptors, the number of glutamate-binding sites is presumably the same as the number of NR2 subunits, i.e. two or three (see above). The Hill slope of equilibrium concentration–response curves provides a clue about the minimum number of agonist molecules that are needed. Many such curves have been published for AMPA and NMDA receptors and there is general agreement that Hill slopes range from 1 to 2, which implies that more than one agonist must be bound before the channel can open. Further interpretation is complicated because of desensitisation and the fact that Hill slopes are dependent not only on binding of agonist but also on their ability to open the channel once bound (Colquhoun 1998; Lewis et al. 1998). Alternatives to the concentration–response curve approach have, however, provided additional insight.

By studying the kinetics of AMPA receptor responses (in cultured hippocampal neurones) to rapid applications of low concentrations of quisqualate, Clements et al. (1998) have demonstrated clearly that the current elicited by this agonist has a sigmoid rising phase, thus suggesting at least two agonists need to be bound for the channel to open. Fit of simple sequential mechanisms suggests a (modest) improvement of the fit when it is assumed that two rather than three bindings are needed to open the channel. Even if the simple models used are adequate, this does not tell us much about what happens when three, four or possibly five agonist molecules are bound (as is presumably possible in a homomeric receptor). However, the time course of recovery after removal of agonist, shows little sigmoidicity. Some sigmoidicity would be expected if it was necessary to wait for two or three agonist molecules to dissociate before the channel could shut. The authors suggest that this may mean that such dissociations are too fast to be seen, and that this means that agonist binding shows negative co-operativity, such that the affinity for bindings beyond the second is low.

The recent report by Rosenmund et al. (1998) suggested that up to four agonist molecules must be bound to open an AMPA-receptor channel (actually a GluR3/GluR6 chimaera) optimally (see above). This appears to disagree with the results of Clements et al. (1998), though the two studies were on different sorts of channel. However, the results could be compatible if the last

two bindings in the study by CLEMENTS et al. (1998) showed sufficient negative co-operativity. The two studies were interpreted on the basis of quite different postulates about the channel mechanisms. CLEMENTS et al. (1998) supposed that all subunits underwent a concerted conformation change so that there was only one sort of open conformation, whereas the results of ROSENMUND et al. (1998) were clearly inconsistent with a concerted conformation change. Once again, the interpretation of observations is hindered by lack of knowledge about the physical reaction mechanism.

BENVENISTE and MAYER (1991) measured the rate of antagonist binding to NMDA receptors and compared the results with reaction schemes that involved the simple sequential binding of either one or two molecules; they concluded that two binding sites fitted better than one. CLEMENTS and WESTBROOK (1991) looked at NMDA receptors in outside-out patches from cultured hippocampal neurones. They measured the time course of the averaged response that followed a step into various glutamate concentrations (with fixed glycine concentration). To interpret the results, they postulated a simple linear reaction scheme in which only fully-liganded channels were able to open. On this assumption, the results were fitted better by the assumption that there were two rather than three binding sites for glutamate (though the distinction depended on quite small differences in shape between the predicted curves). A similar conclusion was reached for glycine sites by stepping into low glycine concentrations (with a fixed glutamate concentration). The problem is, once again, that it is really possible to distinguish subtle changes of shape (in this case, of the sigmoidicity of the onset of the response) if one has an adequate physical description of the channel. It is obvious from single-channel results that a simple linear binding scheme is not an adequate description of the NMDA receptor, but it is not clear at all whether this is a sufficiently serious problem to invalidate the conclusions. It is, for example, very unlikely that it would be possible to distinguish in this way between the case where there are two binding sites that must both be occupied to open the channel, and the case were there are three binding sites, occupation of two (or more) of which is sufficient to open the channel with reasonable efficiency.

The mechanisms that are used do, of course, provide an adequate fit of macroscopic currents, despite the fact that single-channel results show they cannot really be 'right'. This is, perhaps, an illustration of the point mentioned in the introduction. An empirical description, one that is not based on physical reality, may well provide curves that go through the data, but one cannot expect such descriptions to have much predictive ability.

Although it is not easy to tell whether binding of a third agonist molecule occurs or not (ROSENMUND et al. 1998), it does seem very likely that two glutamate molecules are sufficient to open both NMDA- and AMPA-receptor channels quite effectively. There is, however, no suggestion thus far that singly-liganded channels can open (GIBB and COLQUHOUN 1992), though this is quite well documented now for muscle-type nicotinic receptors (COLQUHOUN and SAKMANN 1981; JACKSON 1986).

F. Activation of NMDA Receptors

I. Steady-State Recordings: Can We Reach a Steady State?

The theory on which the interpretation of experiments with constant agonist concentration is based (COLQUHOUN and HAWKES 1982, 1995a) assumes that the channel has reached a steady state. It does not assume equilibrium, so it is still applicable to channels such as NR1/NR2D, which show non-equilibrium behaviour in the steady state (see below). In fact, judging by stability plots (COLQUHOUN and SIGWORTH 1995), it does seem to be possible to achieve something close to a steady state for quite long periods in the best patches (though certainly not in all). Despite the numerous reports describing rundown of NMDA receptor-channel activity in dialysed cells (MACDONALD et al. 1989; MEDINA et al. 1995, 1996) and the evidence for regulation of channel activity by phosphorylation (LIEBERMAN and MODY 1994; WANG and SALTER 1994; WANG et al. 1994), the single-channel activity assessed on excised patches appears remarkably stable from a few minutes to hours after excision, even when the cytoplasmic side of the channel is exposed to nothing else than salts and ethyleneglycol tetraacetic acid (EGTA). Perhaps this favourable circumstance is because of the constancy of the milieu, the fact that patch excision isolates the channel from cytoskeletal influences (ROSENMUND and WESTBROOK 1993), and because the use of very low agonist concentration which ensures that the internal calcium level will remain well buffered. Cell-attached single-channel recordings resolve receptor activations that are similar to those in outside-out patches (GIBB and COLQUHOUN 1992; KLECKNER and PALLOTA 1995), so we may hope that results on the latter are relevant to real cells.

Several authors have reported the rare occurrence of 'high P_{open}' periods in both native and recombinant NR1/NR2A receptors (JAHR and STEVENS 1987; HOWE et al. 1991; GIBB and COLQUHOUN 1992; STERN et al. 1992). During these episodes, in which shut times suddenly become shorter, the channel is open for most of the time for periods that can be hundreds of milliseconds. Their cause is still not known, but they occur in both cell-attached and excised patches, in the absence of adenosine triphosphate (ATP) etc., and with or without added glycine. In our steady-state recordings, such periods are excised (by visual inspection of stability plots) to avoid distortion of shut time distributions. However, their occurrence during synaptic currents and in concentration-jump experiments would not be obvious and could provide an additional complicating factor.

II. Activations in Low-Concentration Steady-State Records

Steady-state recordings of single-channel activity from central neurone patches exposed to a low concentration of NMDA in the presence of glycine revealed the complex bursting behaviour of this glutamate-receptor subtype

(Ascher et al. 1988; Howe et al. 1988; Cull-Candy and Usowicz 1989; Howe et al. 1991). These studies were all carried out before the requirement for glycine was known. As a consequence, the effective agonist concentration was uncontrolled, and, although bursting behaviour was obvious on a short time scale, it was not possible to identify the channel activations, which are long.

Lester et al. (1990) used brief concentration jumps to show elegantly that the time course of the synaptic current was determined by the rate at which the channel shuts in the absence of agonist. This was followed by more detailed studies using both steady-state single-channel recording (Gibb and Colquhoun 1992; Kleckner and Pallota 1995) and concentration jumps (Edmonds and Colquhoun 1992; Lester and Jahr 1992; Dzubay and Jahr 1996; Wyllie et al. 1998). We shall review the contribution made by each method.

One complicating factor in investigation of NMDA receptors is the fact that glycine is also required for the channel to open (Johnson and Ascher 1987; Kleckner and Dingledine 1988). The EC_{50} for glycine (at an arbitrary but high glutamate concentration) has been estimated as $2\,\mu M$ for NR1/NR2A receptors, and about tenfold less for the other three combinations (Ikeda et al. 1992; Kutsuwada et al. 1992). Therefore, it is now usual to include 10–$20\,\mu M$ of glycine in the recording solution. At the synapse, the resting glycine concentration is not really known. Attwell et al. (1993) showed that, in theory, glycine transporters might keep the glycine concentration as low as $0.2\,\mu M$. The glycine concentration is hard to measure experimentally, but microdialysis has suggested a value around $5\,\mu M$ (rising during ischaemia) (Baker et al. 1991), so this is a reasonable upper limit for the concentration in the synaptic cleft. Notice that even this concentration is barely enough to saturate NR1/NR2A receptors. It is, therefore, quite possible that changes in cleft glycine concentration could affect synaptic transmission. Such changes could occur as a result of reverse transport consequent on a rise in intracellular sodium concentration (Attwell et al. 1993), but their actual importance is not known, and experimental evidence (Thomson et al. 1989) is difficult to obtain and inconsistent.

In order to resolve individual channel activations, it proved necessary to use very low concentrations of glutamate (Gibb and Colquhoun 1991, 1992). This is because individual activations may contain quite long shut times (mean length 20–250 ms, depending on subunit combination). Therefore, to distinguish these from the shut times that separate one activation from the next, the activations must be several seconds apart (on average). It is difficult to show a typical recording because a single-channel activation can consist of as few as one-channel opening, or as many as several tens of openings. Figure 2 shows some activations of the recombinant NR1a/NR2A receptor channel. In this experiment, in which the glutamate concentration was 100 nM (+$20\,\mu M$ glycine), the longest and second longest components of the shut-time distribution had "time constants" of 3211 ms and 23.9 ms, so bursts ("super-clusters") were defined by a critical shut time of 88 ms, and these were taken as

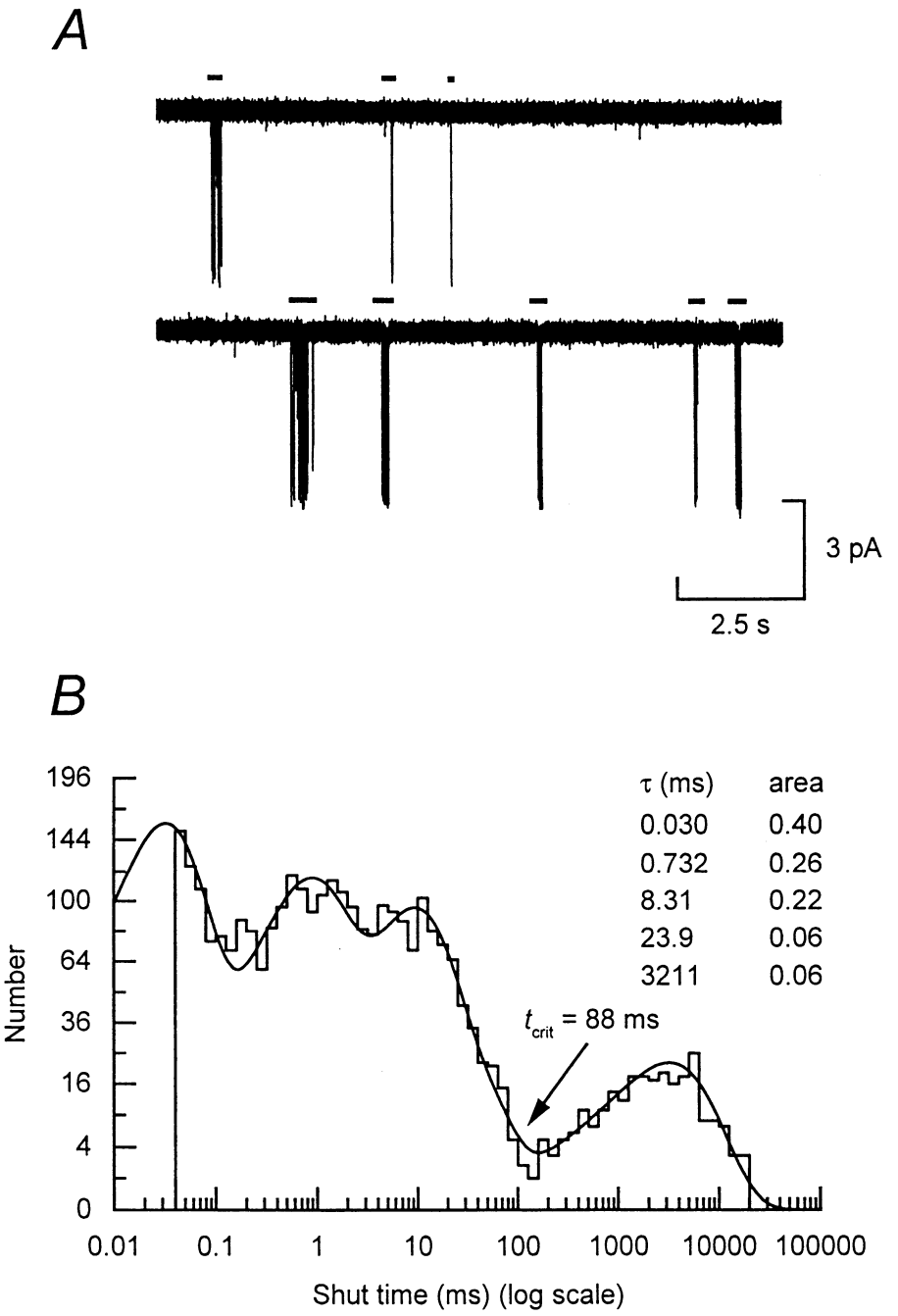


Fig. 2A,B. Steady-state NR1a/NR2A receptor channel activity. **A** 25 s of NR1a/NR2A receptor channel activity (evoked by 100 nM glutamate plus 20 μM glycine) taken from a recording lasting 20 min in total. The lines above each series of openings indicate separate channel activations. **B** Shut-time distribution for the whole recording fitted with a mixture of five exponential components with means and areas as indicated. A critical gap length (t_{crit}) of 88 ms was calculated that achieved optimal separation of gaps contained in the fourth and fifth components of the distribution. This t_{crit} was then used to identify the channel activations in **A**. Adapted from WYLLIE et al. 1998

estimates of the individual activations of the channel, each being marked with a line above the trace.

Figure 3 shows examples of distribution of the durations of channel activations. They are shown for recombinant NR1a/NR2A and NR1a/NR2D. These two receptor subtypes have vastly different kinetic parameters (WYLLIE et al. 1998). The NR1a/NR2A activations are relatively short (overall mean length 36 ms), with an average of seven (6.78 ± 1.01) openings per activation and are open for a substantial fraction of the time ($P_{open} = 0.36$). In contrast, NR1a/NR2D receptor activations are extremely long (overall mean 1602 ms) with an average of 40 (40.19 ± 8.61) openings per activation and have a very low $P_{open} = 0.04$. These means are, however, not very informative because the range is enormous – for NR1a/NR2D the number of openings per activation may be anything from one to several hundred.

One question that has yet to be addressed is regarding the extent to which the long shut times that often occur with a single activation can be considered as short-lived desensitised states, in the way that has been suggested for gamma aminobutyric acid (GABA) receptors (JONES and WESTBROOK 1995). Desensitisation of NMDA receptors has proved to be a complex and controversial matter. It is not even settled whether desensitisation occurs mainly from open or shut states (LIN and STEVENS 1994; COLQUHOUN and HAWKES 1995b). From a single channel perspective, it would be interesting to look at the activation structure in which macroscopic glycine-independent desensitisation has been altered by mutation of amino acids in the *N*-terminal region of the NR2A subunit (KRUPP et al. 1998).

III. Are the Rate Constants Constant?

The conventional way of analysing single-channel records, just like the conventional way of analysing any chemical kinetic problem, assumes that the system exists in a smallish number of discrete states, and that the rate constants that describe the frequency of transitions between states really are constant (do not vary with time). There are two sorts of ways in which this may not be true. The first, and trivial, way is that rate constants that depend on membrane potential and rate constants for association reactions, which depend on ligand concentration, will not be constants if potential or concentration, respectively, vary with time. That is why we always try to keep them constant by using voltage clamps and by doing fast concentration jumps. There is, however, a much more fundamental way in which the rate constants could vary. It could, for example, be the case that the rate constant for dissociation of a bound molecule was not constant, but depended on the length of time for which the molecule had been bound. In this case, the system would be described as non-Markovian. The normal assumption is that the tendency of the molecule to dissociate in the next microsecond (say) is the same, regardless of how long the molecule has been bound already – this is the Markov property. It implies that ligand–receptor complex "has no memory", so its

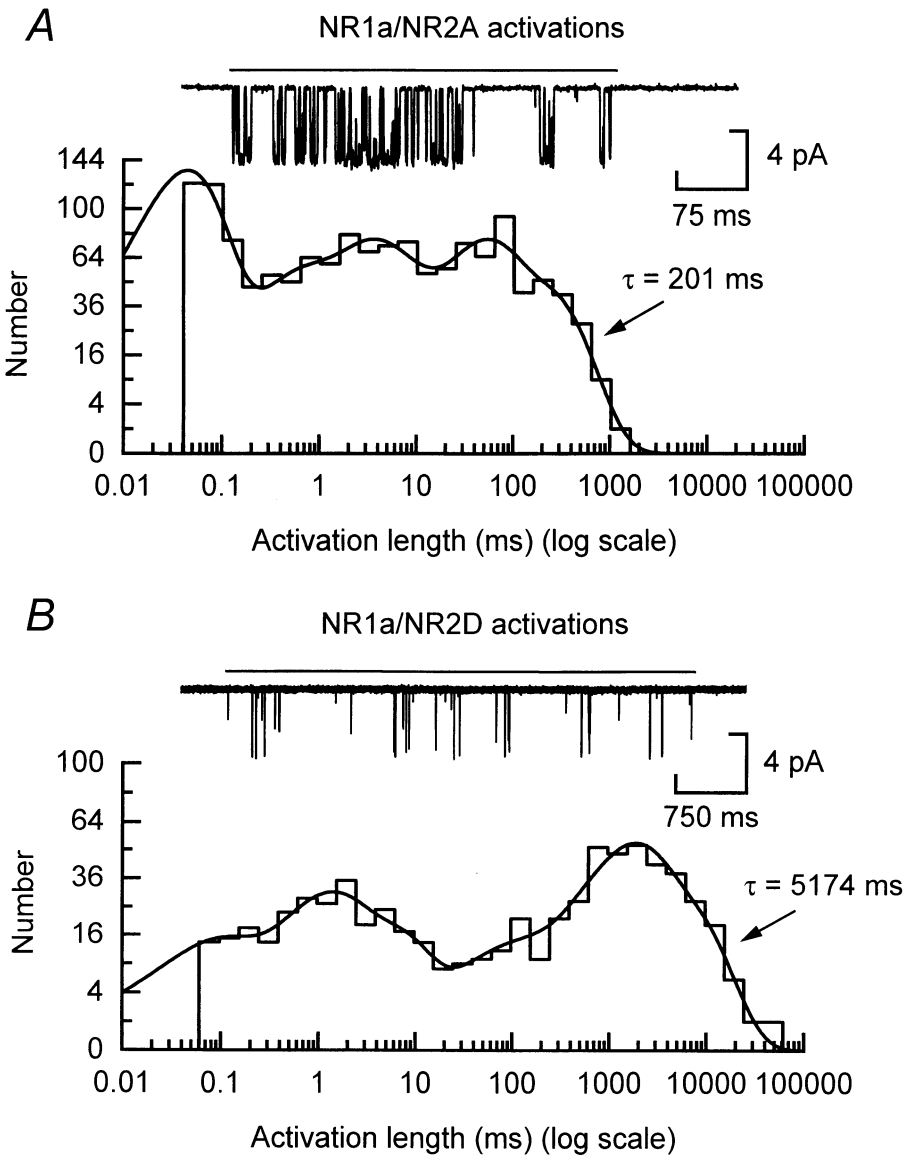


Fig. 3A,B. Activation distributions for NR1a/NR2A and NR1a/NR2D receptor channels. **A** Distribution of NR1a/NR2A receptor channel activations fitted with a mixture of six exponentials with means and areas of 42 μs (39%), 0.380 ms (8%), 1.88 ms (8%), 4.08 ms (14%), 40.6 ms (17%) and 201 ms (14%). The overall mean of the distribution is 35.8 ms. **B** Distribution of NR1a/NR2D receptor channel activations fitted with a mixture of six exponentials with means and areas of 71 μs (8%), 1.03 ms (18%), 4.71 ms (14%), 65.6 ms (6%), 1405 ms (31%) and 5174 ms (23%). In each case the slowest component of each distribution carries the majority of the charge. Examples of channel activations found in the slowest of each distribution are shown. Adapted from WYLLIE et al. 1998

future behaviour depends only on its present state, not on its past history (Colquhoun and Hawkes 1995a). There is little concrete reason to believe that there is anything seriously wrong with the Markov assumptions.

Obviously, a protein molecule can, in principle, adopt an essentially infinite number of conformations, but all the evidence suggests that a small number of conformations are predominant. The single-channel record is probably one of the most direct and striking demonstrations of this fact, together with crystal structures that have been determined for many proteins. Nevertheless, it has been suggested (Liebovitch 1989) that fractal analysis might be more appropriate (despite the fact that the results it provides are quite uninformative about physical mechanisms). There is much evidence against this view (McManus et al. 1988; McManus and Magleby 1989; Gibb and Colquhoun 1992; Wyllie et al. 1998).

IV. In How Many States Can the Receptor Exist?

The number of shut states of a channel is at least the number of components in its shut-time distribution, and the number of open states is at least the number of components in the open-time distribution or the distribution of the total open time per burst (Colquhoun and Hawkes 1982). The NMDA receptor is a great deal more complicated than the nicotinic acetylcholine receptor. The latter appears to exist, predominantly, in exactly the number of states that would be expected on physical grounds: three shut states (with zero, one and two agonist molecules bound) and two open states (with one and two agonist molecules bound). Its activation consists of two or three openings in quick succession, separated by openings that are mostly very short.

In contrast, the NMDA receptor shows five (NR1a/NR2A) or six (NR1a/NR2D) components in the shut-time distribution so there are probably at least six shut states. The components are not all easy to separate and show no very obvious concentration dependence, but the distribution of log(shut time) is certainly not unimodal as expected for a fractal process. Both native and recombinant NMDA receptors seem to have four detectable open states, two at each conductance level. These all seem to be interconnected, and at least two of them are directly accessible from the shut states. Thus, we are probably dealing with at least ten states altogether. All of the shut-time components, except the slowest, appear to be 'within an activation'. Thus there are four or five shut-time components 'within an activation', and these, together with the four open states suggest that there should be eight or nine components in the distribution of the length of the activation (Colquhoun and Hawkes 1982; Wyllie et al. 1998). In fact, only six components could be resolved in the distribution of activation lengths (Wyllie et al. 1998). It is, of course, only too easy to miss components that have either small areas, or time constants that are not well separated from others. In any case, it is clear that at least ten states must be postulated to account for the single-channel properties of the NMDA receptors. At present it is not known what physical structures these

states correspond with, and it is a future challenge to attempt to clarify this question. Clearly it will be a much harder job than for the muscle-nicotinic receptor.

A major step forward in this task would be to discover how the states are connected. Single-channel analysis offers a tool for doing this that is not available in any other approach, the investigation of correlations between successive events. This will be discussed next.

V. Correlations in Single-Channel Records – How Are the States Connected?

The fact that a Markov process is (in the sense mentioned above) without memory does not mean that there cannot be correlations between, for example, the length of one opening and the next (FREDKIN et al. 1985; BLATZ and MAGLEBY 1989; COLQUHOUN and HAWKES 1987; COLQUHOUN and HAWKES 1995a; COLQUHOUN et al. 1996). Steady-state, low-concentration recordings (cell-attached or outside-out) from NMDA receptors show a negative correlation between the length of an opening and the length of the adjacent shut time, and a positive correlation between the length of one opening and the next (GIBB and COLQUHOUN 1992). The distributions of the open-time durations for openings that are adjacent to short shuttings had the same time constants as for those that were adjacent to long shuttings, as predicted for a Markov process. The fact that the mean of the former was greater than that of the latter was entirely a result of the different areas that were associated with each time constant.

These sorts of correlation are qualitatively similar to those seen with the muscle-nicotinic receptor (COLQUHOUN and SAKMANN 1985; LABARCA et al. 1985), and with large-conductance, calcium-dependent, potassium channels (MCMANUS and MAGLEBY 1989). Qualitatively, they can occur when a long-lived open state is connected with a short-lived shut state, which in turn connects to a longer shut state from which brief openings can occur. We have also found that the first opening in an activation tends to be shorter than average, and this too places a constraint on how states are connected. It is likely that further investigation of the correlation structure will provide further information about connections between states, along the lines described by BLATZ and MAGLEBY (1989), MAGLEBY and WEISS (1990), MAGLEBY and SONG (1992) and COLQUHOUN et al. (1996). At the moment, we are still a long way from having a complete kinetic mechanism for any NMDA receptor. Perhaps the best attempt so far is that of KLECKNER and PALLOTTA (1995), but this was designed to describe only the short bursts that occur within an activation, not the entire channel activation, and it also fails to account for the observed correlations.

At present, the prospects for being able to identify structural counterparts of the states that are inferred from kinetic analysis, do not seem as good as for the nicotinic receptor, but that, nevertheless must be the aim.

VI. Temporal Asymmetry

Any reaction scheme that is capable of reaching true equilibrium must obey the principle of microscopic reversibility (see, for example COLQUHOUN and HAWKES 1982). In single molecule terms, this implies that the behaviour is symmetrical in time. If a single-channel record were recorded on tape, this means that it would be impossible to tell whether the tape was being played forwards or backwards. Several cases have been reported in which channels do not behave in this way (HAMILL and SAKMANN 1981; TRAUTMANN 1982; CULL-CANDY and USOWICZ 1987). In all of these cases, the phenomenon has been manifested as unequal transition frequencies for transitions from a main conductance level to a subconductance level. It is also possible (but thus far unreported) that this sort of asymmetry could be manifested as, for example, a difference between the mean duration of the first and last opening in a burst that contained three openings (COLQUHOUN and HAWKES 1982).

In the case of the NMDA receptor, no sign of asymmetry is seen with recombinant NR1a/NR2A, NR1a/NR2B or NR1a/NR2C receptors. However, recombinant NR1a/NR2D receptors do show the phenomenon (WYLLIE et al. 1996), as do some native receptors that, by other criteria, are very likely to be NR1/NR2D receptor channels (MOMIYAMA et al. 1996). At the more fundamental level, it implies that the channel does not obey the principle of microscopic reversibility and cannot come to a true equilibrium. It is perhaps surprising that this phenomenon seems, so far, to be relatively rare. As pointed out by LÄUGER (1983, 1985), the ion flux through a channel is not at equilibrium, so if there is any interaction between the flow of ions and the opening and shutting of the channel, the process as a whole cannot be at equilibrium. This appears to be what is happening with the NR1a/NR2D channel for which the extent of asymmetry depends strongly on the ionic composition of the recording solutions on each side of the membrane (unpublished data of PB). An elegant analysis of asymmetry in a mutant NMDA receptor has been provided by SCHNEGGENBURGER and ASCHER (1997) based on the principles enunciated by LÄUGER.

VII. Concentration Jumps: Differences Between Subunit Combinations

Studies by MONYER et al. (1994) and KÖHR and SEEBURG (1996) established that the deactivation of NR1/NR2A receptors is faster than that of NR1/NR2B receptors, whereas that of NR1/NR2D receptors is very slow. More recently, brief (1 ms) pulses of agonist have been used in order to mimic more closely synaptic conditions (VICINI et al. 1998 in HEK cells, and WYLLIE et al. 1998 in oocytes). These studies confirmed that NR1/NR2A receptors have the fastest deactivation rate. VICINI et al. found the rate to be much the same for four different splice variants of the NR1 subunit, and that NR1/NR2C showed a deactivation rate comparable with that of NR1/NR2B, whereas NR1/NR2D was

much slower. The decay (deactivation) at zero agonist concentration, after the end of the 1 ms pulse of agonist, usually shows more than one exponential component. VICINI et al. (1998) state their results as weighted mean constants, the individual time constants being weighted with the relative amplitudes of the components. This makes the results a bit hard to interpret since it is not clear at which time point the relative amplitudes were measured (the results will be strongly dependent on this).

In both studies, a substantial amount of variability in decay rates was found from one patch to another. WYLLIE et al. (1998) observed, with NR1a/NR2A, a tendency for patches that contained more channels to show a slower deactivation rate. The reason for this is not known.

The study by WYLLIE et al. (1998) concentrated on a comparison of the fastest and slowest types. They found that the current through NR1a/NR2A channels following a concentration jump from zero to 1 mM glutamate for 1 ms was well fitted by three exponential components with time constants of 13 ms (rising phase), 70 ms and 350 ms (decaying phase). Similar concentration jumps on NR1a/NR2D channels were well fitted by two exponentials with means of 45 ms (rising phase) and 4408 ms (decaying phase) components. During prolonged exposure to glutamate, NR1a/NR2A channels desensitised with a time-constant of 649 ms while NR1a/NR2D channels exhibited no decline during the prolonged exposure (though this is not sufficient to conclude that there is no desensitisation; see FELTZ and TRAUTMANN 1982).

VIII. Latency to the First Opening

The distribution of the length of time that elapses (the first latency) between applying a pulse of agonist and the first opening of the channel is potentially very informative about the reaction mechanism. JAHR (1992) and DZUBAY and JAHR (1996) reported an indirect estimate of the mean first latency for NMDA receptors in primary cultures of hippocampal neurones. Their method was to use the channel-blocking antagonist, MK-801; if this blocks only open channels, and is essentially irreversible, then it is possible to estimate the first latency and the probability of a channel being open. To do this, it is also necessary to assume that every channel that opens will become blocked during its first opening. Under these conditions, DZUBAY and JAHR (1996) assume that the time course of the current will be approximately proportional to the distribution of first latencies. They suppose that any channel that opens for more than about 2 ms will become irreversibly blocked, and so they estimate that the mean first latency is of the order of 10 ms. This value is in fair agreement with the directly-observed value for recombinant NR1a/NR2A channels (see below). The time course of the current cannot, however, be expected to give a very precise estimate of the shape of the first latency distribution, even if its mean is roughly correct. This is illustrated in Fig. 4, which shows two examples of the calculated time course of the probability of being open (solid lines)

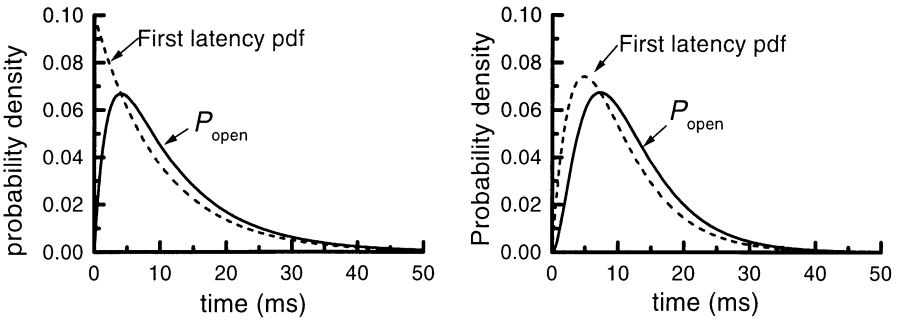


Fig. 4. Estimation of first latency by use of a channel blocker. The *dashed lines* show two assumptions about the shape of the first latency distribution, the mean first latency being 10 ms in both cases. The *solid lines* show the corresponding probability of being open, which is calculated on the assumption that the latency is followed by a single opening of mean duration 2 ms. The mean first latency inferred from the *solid curve* would be 12 ms in both cases (not very different from the correct value of 10 ms), but the *solid curves* give little idea of the shape of the first latency distribution

when the mean first latency is 10 ms and the mean open time is 2 ms. These are calculated by convolving the first latency distribution with the open time distribution on the assumption that the channel is blocked the first time it opens so there is only ever one opening (as in COLQUHOUN and HAWKES 1995a). The left-hand graph assumes a single exponential first latency (mean 10 ms), and the right-hand graph, more realistically, assumes a double exponential first latency (found by convolving shut times with means of 4 ms and 6 ms, so the mean latency is still 10 ms). Clearly the P_{open} curves (solid lines) in Fig. 4 do not describe the shape of the latency distribution well, but they do give a reasonable idea of the average latency (the mean inferred from the solid lines is actually the mean latency plus the mean open time, i.e. 12 ms in both cases). Their results are also consistent with those of BENVENISTE and MAYER (1995) who used a related method.

Direct observation of the first latency distribution requires a patch that contains only one channel, and which lasts for a long time (there is only one latency per concentration jump, and data acquisition following jumps has to be long for NMDA receptors). This has been achieved only rarely, but WYLLIE et al. (1997) found, in this way, a mean first latency of 20–30 ms for NR1a/NR2A receptors expressed in oocytes. Figure 5 shows three channel activations elicited by a 1 ms pulse of glutamate to an outside–out patch containing a single NR1a/NR2A NMDA-receptor channel. It is clear that the channel does not open immediately following glutamate application but rather does so after a delay (first latency) of up to a few tens of milliseconds. The average of 248 such activations gives a slowly decaying current that has a time-course resembling that of a NMDA synaptic current.

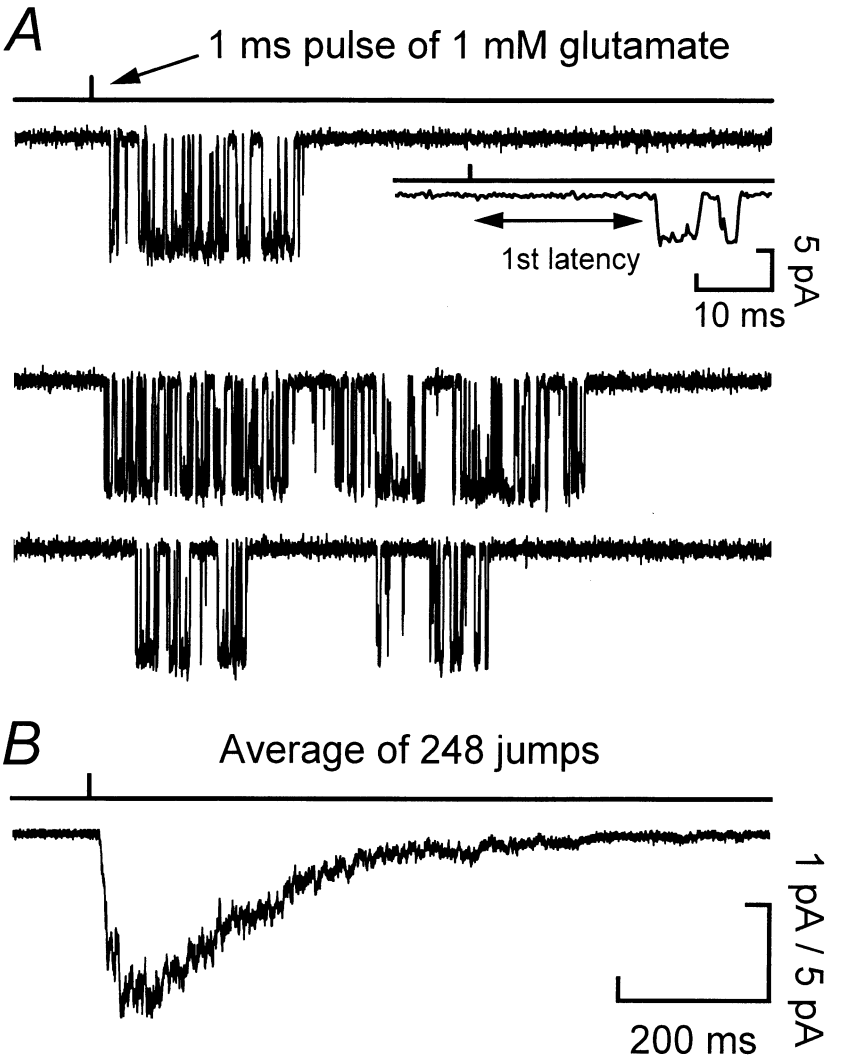


Fig. 5A,B. NR1a/NR2A receptor channel activations evoked by 1 ms concentration jumps. **A** Examples of three channel activations recorded in an outside–out patch containing only one channel. Channel openings were evoked by exposing the patch to 1 mM glutamate for 1 ms. The *inset* in the first trace shows the activation on an expanded time base to illustrate more clearly the first latency. **B** Average of 248 jumps gives a mean current with a peak amplitude of 1.7 pA and a slowly decaying time course. Single-channel currents in this patch had a mean amplitude of around 5.5 pA giving an estimate of the P_{open} at the peak of 0.3. Adapted from WYLLIE et al. 1997

IX. The Relationship Between Steady-State Activity and Concentration Jumps

It is only recently that a fairly complete theoretical background has been given for the behaviour of non-stationary single-channel records, such as the channels that are elicited by a jump in agonist concentration (COLQUHOUN et al. 1997). It is shown there (see also WYLLIE et al. 1998) that the following measurements should all have the same number of exponential components and the time constants for these components should be essentially the same (for the precise assumptions see Appendix to WYLLIE et al. 1998).

1. The macroscopic response that follows a concentration jump to zero concentration (NMDA receptor channels hardly ever open within a 1-ms pulse of agonist, so the entire response to a 1-ms pulse is measured at zero agonist concentration).
2. The distribution of the length of activations measured in steady-state records at very low agonist concentrations (estimated as bursts or super-clusters).
3. The macroscopic current synthesised by adding such low-concentration activations after aligning them on their first openings.

WYLLIE et al. (1998) showed that observations on NR1a/NR2A and NR1a/NR2D receptors are consistent with these predictions (and this constitutes additional evidence that the Markov framework is valid). When the macroscopic currents are fitted with the number of time constants (five or six) needed to fit the distribution of activation length, with the time constants fixed at the values found from that distribution, a good fit can be obtained in most cases. A free fit to the macroscopic currents can, of course, produce good estimates of only two or three of the time constants, but this is merely a reflection of the poor resolving power of macroscopic measurements. Figure 6 compares the time-course of aligned activations and macroscopic currents evoked by 1 ms pulses of agonist for NR1a/NR2A and NR1a/NR2D channels.

The fact that the time constants agree means that it is indeed possible to predict from steady-state measurements that a jump response will have a slow component (as in GIBB and COLQUHOUN 1991, 1992). What we cannot predict is how big that slow component will be. The relative amplitudes or areas that are attached to each time constant differ vastly between jumps and steady-state results. This is because the initial vector (the fraction of receptors in each state) at the start of an activation in the steady state is quite different from that for a concentration jump. The amplitudes of the components in a macroscopic jump cannot be calculated from the steady-state results without detailed knowledge of the kinetic mechanism. It would not be correct simply to convolve the observed first latency distribution (WYLLIE et al. 1997) with the distribution of activation lengths. If there is only one open state, it should be convolved with the macroscopic probability, $P_{11}(t)$, that the channel is open at time t, given that it is open at $t = 0$, but if, as for the NMDA receptor, there

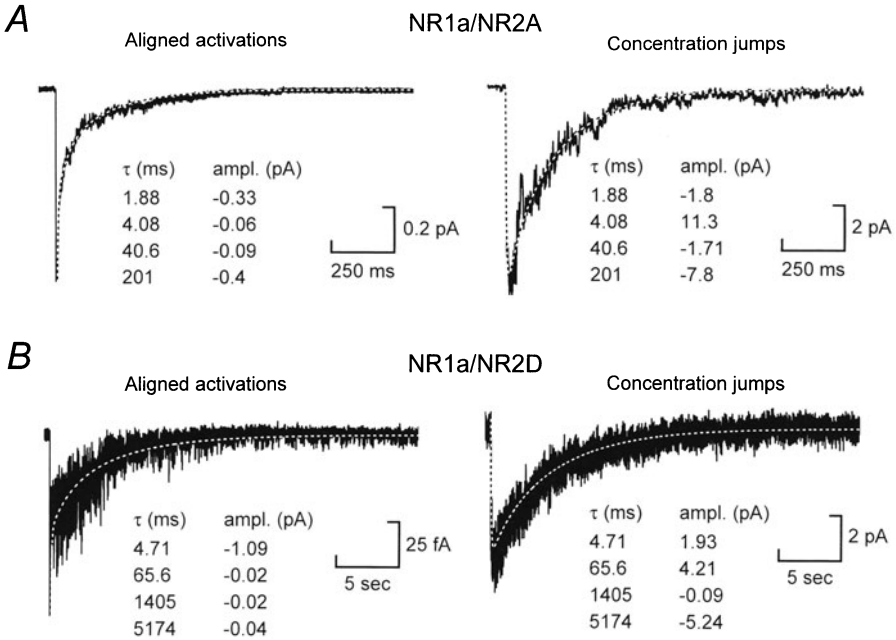


Fig. 6A,B. Comparison of aligned activations and concentration jumps on NR1a/NR2A and NR1a/NR2D receptor channels. *Left panels*, currents obtained from the alignment and averaging of NR1a/NR2A receptor channel activations (**A**) or NR1a/NR2D receptor channel activations (**B**). *Right panels*, examples of macroscopic currents mediated by NR1a/NR2A (**A**) or NR1a/NR2D (**B**) receptor channels and recorded following a 1 ms pulse of 1 mM glutamate. In each case the *white dashed line* shows the fit of these currents with a sum of exponentials with time constants fixed at the values obtained from the distributions of activation length shown in Fig. 3. Adapted from Wyllie et al. 1998

is more than one open state, the first latency distribution, although it contains valuable kinetic information, cannot be used directly in this way at all (see Colquhoun and Hawkes 1995a).

It is, presumably, this difference in the initial vector that accounts for the fact that one-opening activations are more frequent in steady-state activations than in activations elicited by a concentration jump (unpublished data). Despite this fact, the probability of being open is similar (around 0.3) at the peak of the jump response and for steady-state activations.

In an earlier study, Edmonds and Colquhoun (1992) found longer first latencies in dentate granule cells. The subunit composition of these receptors is not known, so it may be that some receptors have longer first latencies. It must also be noted that it is an ever-present hazard of latency measurements that a small background contamination by agonist (or spontaneous openings) could give rise to the appearance of long latencies. They also found that the decay of aligned super-clusters and super-cluster length distributions did not appear to share the same set of time-constants. Their suggestion that this dis-

crepancy could be explained in part by the existence of a long first latency was incorrect (see WYLLIE et al. 1998). It now seems likely that small amplitude of the slowest component in the aligned super-cluster decay prevented its detection.

G. Activation of AMPA Receptors

In contrast to NMDA single-channel activations, AMPA receptor activations would appear to be remarkably simple, consisting of bursts of one or two individual openings (for example see WYLLIE et al. 1993). As AMPA receptors mediate the fast component of glutamatergic EPSCs that last at most a few milliseconds, such brief activations are perhaps to be expected. However, despite the fact that the channel activation are brief, many factors contribute to determine the precise nature and duration of these events.

I. Macroscopic Currents from Recombinant AMPA Receptor Channels

Our understanding of the factors that control AMPA receptor channel kinetics has been advanced greatly from studies of recombinant receptor whole-cell currents and macroscopic currents in outside-out membrane patches. As mentioned above, mRNA coding for AMPA subunits exist in two splice variants which have been termed "flip" and "flop" (SOMMER et al. 1990). In this paper, it was suggested that some aspects of the gating kinetics of AMPA receptors were dependent on the particular splice variants of the AMPA subunits forming the receptor-channel complex. However, in this study, whole-cell recordings from HEK 293 cells expressing recombinant AMPA receptors would be unlikely to resolve fast components of the activation kinetics of these channels due to the fact that perfusion of these cells by agonist was relatively slow. This problem was overcome in a later study (MOSBACHER et al. 1994), which made outside-out patch recordings from *Xenopus* oocytes expressing AMPA receptors and used a piezo-electric device to achieve rapid solution exchanges. In response to brief (1 ms) pulses of glutamate homomeric GluR-D_{flip} and GluR-D_{flop} channels, each exhibit rapid decay kinetics ($\tau_{decay} = 0.6$ ms). Similarly, homomeric GluR-A_{flip} and GluR-A_{flop} channels each show similar deactivation kinetics albeit with slower decay time constants ($\tau_{decay} = 1.1$ ms). However, during sustained agonist exposure, differences in the response of flip and flop channels become apparent. Homomeric GluR-D_{flop} channels desensitise almost as rapidly as they deactivate following a brief concentration jump ($\tau_{desens} = 0.9$ ms) whereas GluR-D_{flip} channels desensitise more slowly ($\tau_{desens} = 3.6$ ms). Similarly, homomeric GluR-C_{flop} channels desensitise more rapidly than their homomeric flip counterparts. This difference between flip and flop desensitisation rates is not seen with homomeric GluR-A channels. As many native AMPA receptors are likely to be heteromers containing GluR-B sub-

units, it is interesting to note that GluR-D_{flop} channels still desensitise faster than GluR-D_{flip} channels when expressed with GluR-B subunits. The developmental regulation of expression levels of various splice variants of AMPA subunits (MONYER et al. 1991) provides a mechanism by which the kinetics of AMPA receptors may be controlled. The fact that certain AMPA receptors desensitise in less than a millisecond has important implications for synaptic transmission. The decay of the AMPA component of glutamatergic EPSCs has been suggested to reflect the closing rates of the AMPA receptor channels following exposure to glutamate (COLQUHOUN et al. 1992). However, under certain circumstances the desensitisation rate of AMPA receptor channels may contribute significantly to the time course of the synaptic current (TRUSSELL et al. 1993). Clearly in the case of GluR-D_{flop} channels, a change in the concentration profile of glutamate is unlikely to have a substantial effect on the decay of the synaptic current.

In addition to the well-documented RNA editing of the Q/R site of AMPA receptor subunits (SOMMER et al. 1991), a second site of RNA editing also exists (LOMELI et al. 1994). The last codon of exon 13 of the genes encoding the GluR-B, C and D subunits can undergo editing to switch the codon at this position from one that encodes an arginine residue (AGA) to one that gives a glycine residue (GGA). This residue precedes immediately the alternatively spliced flip and flop sequences of amino acids. GluR-A subunits appear to exist only in the non-edited arginine version. The presence of arginine or glycine residues does not appear to affect the rise time or decay time of AMPA currents following brief agonist exposure. The main kinetic difference between the two forms of subunit appears to be in the rate of recovery from desensitisation. In experiments where pairs of brief (1 ms) pulses of glutamate were applied to outside-out patches, AMPA receptor channels containing edited (glycine) residues showed less depression of the second response and faster rates of recovery than AMPA receptor channels containing non-edited residues. This is true for both homomeric and heteromeric channel combinations. Such findings have led to the proposal that in the CNS postsynaptic AMPA receptor channels that are edited at this site may be better able to convey high frequency presynaptic activity than non-edited AMPA receptor channels (LOMELI et al. 1994).

II. Identifying Native AMPA Receptors on the Basis of Macroscopic Deactivation and Desensitisation Kinetics

The characterisation of the kinetic properties of recombinant AMPA receptors together with in situ mRNA hybridisation studies has provided valuable information concerning the subunit combination of native AMPA receptors. Studies that combine electrophysiological recordings with reverse-transcription polymerase chain reaction of mRNAs present in individual cells may give further insights into how edited and differentially spliced subunits

affect the kinetics of native AMPA receptor channels (LAMBOLEZ et al. 1992, 1996; BOCHET et al. 1994; JONAS et al. 1994; GEIGER et al. 1995; ANGULO et al. 1997; GÖTZ et al. 1997).

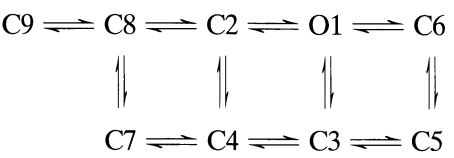
Several studies have shown that AMPA receptor channels in principal (excitatory) neurones in the hippocampus and cortex display slower deactivation and desensitisation rates than those found in inhibitory interneurones (for example, see COLQUHOUN et al. 1992; JONAS and SAKMANN 1992; HESTRIN 1993; JONAS et al. 1994; GEIGER et al. 1995; LAMBOLEZ et al. 1996; GÖTZ et al. 1997; see MONYER et al. Chap. 9, and GEIGER et al. Chap. 11; this volume). How do these kinetic differences correlate with levels of expression of different AMPA subunits? In the hippocampus and neocortex, principal neurones contain high levels of mRNA for the GluR-B subunit mainly in its flip form (GEIGER et al. 1995; LAMBOLEZ et al. 1996). In addition to conferring linearity to AMPA receptor channel current-voltage relationships in these cells, as well as their low Ca^{2+} permeability, it was suggested that the presence of this subunit gave rise to channels with relatively slow gating kinetics. The faster gating kinetics of AMPA receptors found in interneurones has been suggested to result from (a) the presence of higher levels of GluR-D (mainly in the flop version) in these cells (GEIGER et al. 1995), or (b) a more general increase in the levels of flop variants of each of the four AMPA receptor subunits (LAMBOLEZ et al. 1996). In cultures of hippocampal neurones, two types of neurone have been classified on the basis of current-voltage relationship and Ca^{2+} permeability of their AMPA receptors (OZAWA et al. 1991). AMPA receptor channels in type II neurones (putative GABAergic interneurones) have inwardly rectifying current-voltage relationships, high Ca^{2+} permeability, and contain only GluR1(A) and GluR4(D) subunits each in the flop variant (BOCHET et al. 1994). This result, therefore, supports both the proposal that GluR-D_{flop} specifically is an important regulator of fast-gating kinetics in interneurones as well as the proposal that it is the increased levels of flop variants of any AMPA subunits that determines rapid kinetics. More recently, however, a study of fast spiking and regular spiking non-pyramidal cells of the neocortex (ANGULO et al. 1997) has provided evidence that no single molecular parameter (e.g. ratio of flip to flop, presence/absence of particular subunit, R/G editing of subunits) is the sole determinant of the AMPA receptor channel kinetics. Furthermore, this study showed it was possible to obtain inwardly rectifying current-voltage relationships from cells with slow desensitisation kinetics and conversely fast desensitisation from patches with weak rectification. This latter observation has also been documented for GABAergic neurones in the substantia nigra (see also GÖTZ et al. 1997). These findings suggest, therefore, that the absence of GluR-B subunits in a receptor complex does not necessarily mean that receptors will have fast desensitisation kinetics. Thus, it is unlikely that one will be able to correlate the presence of specific subunits with particular kinetic properties in the same way that it has been possible to relate the presence of GluR-B subunits with Ca^{2+}-impermeable receptors with linear current-voltage relationships.

III. Single-Channel Kinetics

The fact that the non-NMDA receptor mediated component of glutamatergic EPSCs lasts at most a few milliseconds tells us that activations of AMPA receptors are brief. Unlike NMDA receptors, single-channel analysis of AMPA receptors has proved remarkably unpopular. This is no doubt due to the fact that the channel openings are so brief and open to poorly defined conductance levels (see above). Even among the reports that have appeared, few have used glutamate as an agonist on these channels. In cerebellar granule cells 'high conductance' AMPA receptor channels activated by glutamate have a mean apparent open time of 660 μs and a mean burst length of just 920 μs (WYLLIE et al. 1993). Given the similarity of the mean apparent open time and burst length, it is unsurprising that such bursts contain few resolvable brief gaps (on average 0.17 per burst). Homomeric $GluR4_{flip}$ channels also give briefing openings with glutamate (170 μs) and short bursts (overall mean 1.0 ms) (see SWANSON et al. 1997). As mentioned above, fast concentration jumps on $GluR4_{flip}$ channels (MOSBACHER et al. 1994) give rapidly deactivating macroscopic currents whose time constant is very similar to the mean burst length of 'high conductance' channels. However, it should be noted that, although the burst length of 'high conductance' channels is very similar to decay-time constant of the AMPA component of the EPSC at the mossy fibre-granule cell synapse (SILVER et al. 1992), it is unclear whether these 'high conductance' channels mediate the synaptic current (see above). Other reports of glutamate activation of AMPA receptor single-channel currents also indicate that openings are brief (for example see GREENGARD et al. 1991; TANG et al. 1991).

IV. Models of AMPA Receptor Activation

Several kinetic schemes have been proposed for AMPA-receptor activation (for example see RAMAN and TRUSSELL 1992; JONAS et al. 1993; HÄUSSER and ROTH 1997). Each of the schemes proposed has been based on observations made from the study of macroscopic currents and, to a large extent, are able to predict accurately the properties of currents evoked by short and long pulses of agonist application, recovery of receptors from desensitisation and the EC_{50} and Hill slopes of dose-response curves. How well these schemes can account for single-channel data is less clear as none of the models put forward has been based on data obtained from single-channel experiments. For the purposes of this review, therefore, we have simulated some single-channel data based on one of the published models for AMPA receptor channel activation. The model chosen is that from a study of AMPA receptors in cerebellar Purkinje cells (HÄUSSER and ROTH 1997). This model contains one open state and eight shut states, and is shown below as Scheme 1. Calculations were done with the rate constants specified by HÄUSSER and ROTH (1997). With a low glutamate concentration (1 μM), it is predicted that openings (mean duration 0.26 ms) occur in well-separated bursts (30.7 s apart for one channel), each



Scheme 1

burst containing on average 4.1 openings. The shut times within a burst (spent in states 2 to 8) are mostly short (43 μs, 96.5% of area), with some longer gaps (9.1 ms, 3.2% of area; the other five components have negligible area). The short gaps result almost entirely from oscillations between states 1 and 2 (fully-liganded open and shut, just as for the nicotinic receptor). The longer gaps within a burst result mostly from single sojourns in state 6, with some visits to states 3 and 5 too. The burst length distribution has a predominant component with a mean of 1.1 ms (88% of area) and a component of 10.2 ms (11.5% of area), the other six components being negligible; the overall mean is 2.3 ms. The macroscopic response to a very short (0.1 ms) pulse of 1 mM glutamate is predicted to have seven exponential components but the decay is dominated by a single exponential (1.1 ms). There is, as expected from the burst length distribution, a component with a time constant of 10.2 ms, but although this accounts for about 7% of the area, it has an amplitude only 1.4% of the 1 ms component and would be undetectable in practice.

The best resolution that can be achieved for small-amplitude events such as AMPA receptor channels, even with time-course fitting, is about 100 μs. When this resolution is imposed on the simulated record (for both openings and closings) most of the short gaps within bursts are undetected, resulting in a mean of only 1.4 openings per burst instead of the predicted 4.1 (see above).

The distribution of shut times and apparent burst lengths after such a resolution has been imposed is shown in Fig. 7A. A free maximum likelihood fit to this shut time distribution resolves only three of the eight components – the fitted time constants of 66 μs (53.1%), 9.3 ms (4.2%) and 34.4 s (42.7%) may be compared with three largest components of the true shut-time distribution, namely 44 μs (73.2%), 9.1 ms (2.45%) and 30.1 s (24.2%). This sort of distortion caused by limited time resolution can be dealt with by, for example, the methods described by Hawkes et al. (1992) and exemplified in Colquhoun et al. (1996).

The distribution of the apparent lengths of activations with 100 μs resolution is shown in Fig. 7B. Two components could be fitted, as might be expected from the very small areas (less than 1%) that are predicted for the other six components. The fitted components, 1.09 ms (89.8%) and 10.2 ms (10.2%), may be compared with the two main components of the true distribution, 1.09 ms (87.8%) and 10.2 ms (11.5%). This agreement is a good example of the fact that burst length distributions are far less susceptible to errors resulting from missed events than are open-time or shut-time distributions.

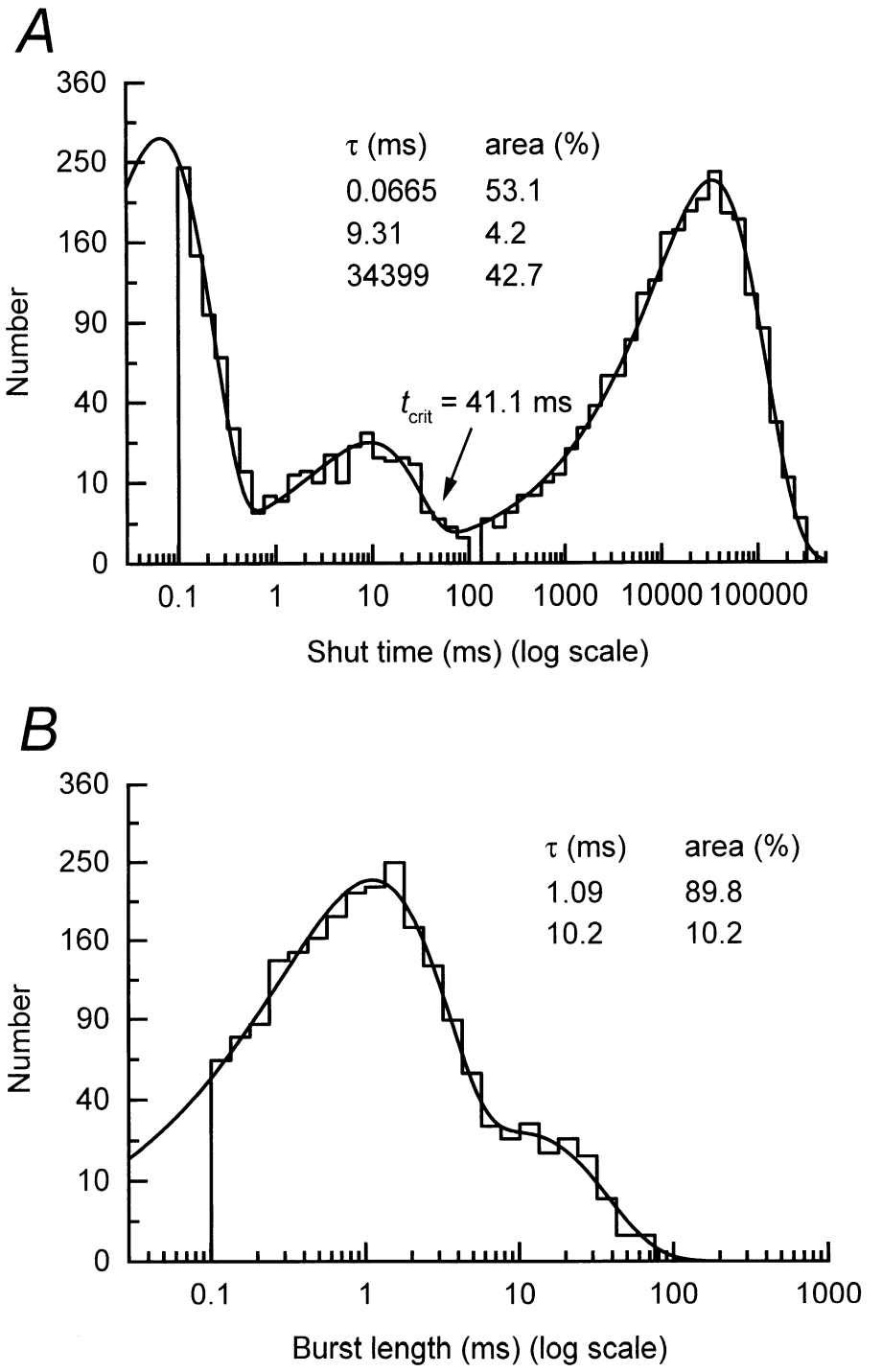

A
360
250
160
90
40
10
0
Number
τ (ms) area (%)
0.0665 53.1
9.31 4.2
34399 42.7
t_{crit} = 41.1 ms
0.1 1 10 100 1000 10000 100000
Shut time (ms) (log scale)
B
360
250
160
90
40
10
0
Number
τ (ms) area (%)
1.09 89.8
10.2 10.2
0.1 1 10 100 1000
Burst length (ms) (log scale)

Fig. 7. Shut time and burst length distributions for AMPA receptor channel activity simulated using the model shown in Scheme 1. **A**, Shut time distribution obtained from the simulation of AMPA receptor channel activity by 1 μM glutamate and based on the kinetic scheme proposed by HÄUSSER and ROTH (1997). Despite the fact that there are eight shut states in the scheme, only three components are visible in the steady-state shut-time distribution. This distribution gave a t_{crit} of 41.1 ms for the separation of the second and third components, and this value was used to calculate the burst length distribution which is shown in **B**

H. Conclusions

As stated in the introduction, one the goals for those of us working in this field is to generate kinetic mechanisms of channel activations which reproduce faithfully the nature of single-channel activity seen for both NMDA and AMPA receptor-channels. Understanding the single-channel activation structure of both these types of ligand-gated ion channel and how these activations can be controlled and/or modified will provide additional insights into synaptic physiology that can not be obtained from the study of macroscopic currents alone.

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