The mechanism by which ligand binding at extracellular receptor domains gates a transmembrane ion-conducting pore is insufficiently understood. Examining a channel’s activation reaction in the presence of agonists with distinct efficacies may inform this issue and may help identify agonist-dependent transitions. We have recently applied this approach to NMDA receptors composed of GluN1 and GluN2A subunits. Based on our results with several subunit-specific partial agonists we concluded that agonist effects were distributed over several of the multiple transitions that make up NMDA receptor gating and that these changes were subunit independent. Here we examine an additional GluN2A partial agonist, 4-fluoro-D,L-glutamic acid, and we summarize the observed kinetic changes of all nine partial agonists investigated. These results support the premise that regardless of the subunit-type to which they bind, agonists influence multiple equilibria within the NMDA receptor reaction and may stabilize a slightly different family of conformers.

Introduction

NMDA receptors are ligand-gated ion-channels that require both glycine (Gly) and glutamate (Glu) binding to extracellular receptor domains before the transmembrane pore opens.1,2 This distinctive feature of NMDA receptors results from specific binding-sites for Gly and Glu on GluN1 and GluN2 subunits, respectively and the incorporation of two GluN1 and two GluN2 subunits in the functional tetrameric receptor.3,5 In addition, the gating reaction is initiated only after all agonist-binding sites are occupied. A number of low efficacy ligands have been identified for both subunits.6-9 The ability to trace the contributions of GluN1- or GluN2-specific ligands to the overall gating mechanism has been exploited to investigate the mechanism of partial agonism at NMDA receptors and to identify possible subunit-specific steps within the gating reaction.9,11 Recently we examined current traces generated by individual GluN1/GluN2A receptors activated with series of partial agonists in the presence of the full co-agonist and compared these with traces obtained with the endogenous agonists Gly and Glu. Based on these results we concluded that partial agonists produced sweeping changes in the single-channel kinetics of the receptors studied, and that these changes were not subunit-specific.12 However, aside from common mechanisms observed across all the ligands tested our results also revealed agonist-specific kinetic effects. To focus on this latter, insufficiently emphasized aspect of partial agonism at NMDA receptors, we characterized the kinetics of GluN1/GluN2A receptors with an additional Glu-site partial agonist, 4-fluoro-D,L-glutamic acid (FGA), and we summarize here the observed kinetic changes of all nine partial agonists investigated. We interpret these results to propose that regardless of the subunit-type to which they bind, agonists influence multiple equilibria within the NMDA receptor reaction and may stabilize a slightly different family of conformers.
To investigate the effects of the partial agonist 4-fluoro-D, L glutamate (FGA) (Fig. 1A) on the gating kinetics of NMDA receptors, we recorded single-channel current traces from on-cell patches containing only one GluN1/GluN2A receptor after including in the patch pipette saturating concentrations of FGA, 1 mM (EC_{s0} = 0.1 mM)\(^7\) and the endogenous co-agonist Gly (0.1 mM, EC_{s0} = 2.6 μM).\(^8\) In these experimental conditions, we obtained four one-channel records that were each 10–70 min long and contained together >70,800 events over 137 min of recording.

The overall appearance of single-channel currents elicited by FGA and Gly were similar with those we obtained previously with Glu and Gly (Fig. 1B).\(^12\) The calculated open probability for the observed GluN1/GluN2A channels in the presence of FGA was \(P_o = 0.33 \pm 0.04\) (\(n = 4\)). This represents 52% of the reported single-channel activity with Glu and Gly (\(P_o = 0.64 \pm 0.06\), \(n = 5\))\(^12\) and is in close agreement with the 55% macroscopic efficacy measured for this agonist relative to Glu.\(^7\) This congruence suggested that the ensemble of behaviors we captured in our single-channel recordings represented a fair sample of the activity previously measured in whole-cell recordings.

Inspection of the mean durations of closed and open intervals (Fig. 1C and D) indicated that the lower efficacy of FGA...
as compared to Glu could be attributed mainly to 15 ± 3 ms (FGA) vs. 6.4 ± 1.7 ms (Glu) since the calculated reduction in mean open durations was not statistically significant: 7.4 ± 0.8 ms (FGA, n = 4) vs. 11.2 ± 1.1 ms (Glu, n = 5, p > 0.08).

Because each of the records analyzed reflected the activity of the same channel throughout, it was informative to compare entire closed interval distributions for the two agonists tested. This analysis showed that the increase in the mean closed time could be assigned specifically to an increase in the time constants for the second and third exponential components E 2 and E 3.

A qualitative summary of gating changes elicited by a series of partial agonists, including FGA is illustrated Table 1. This survey led us to conclude that when compared to the endogenous agonists Glu and Gly, each of the ligands studied was less efficacious in unique ways. For example, although ALA, ACBC, SYM and QA alike decreased all three forward rates (k12, k21, k42), their effect on the magnitude of the reverse rate constants was dissimilar: QA had Glu-like closing rate constants; ALA increased only k12; whereas ACBC and SYM, despite acting at separate subunits, increased the same two closing rate constants k12 and k21. Based on this analysis we conclude that at least for the GluN1/GluN2A receptors investigated here, each partial agonist is ‘partial’ for different reasons. Still, in all cases the effects were scattered across all rate constants. ACBC and HCA showed the most pervasive changes, with eight out of ten rate constants being significantly different (p < 0.05). In contrast, DCS and FGA showed the most localized effect, changing only three rate constants: k23, k41 and k13 for DCS; and k11, k21 and k31 for FGA.

The results summarized in Table 1 also suggest that almost all the rate constants postulated in the reaction mechanism are sensitive to the identity of the agonist activating the channel. Although none of the ligands tested changed all rate constants, each rate constant was sensitive to the identity of the agonist activating the channel. Although none of the ligands tested changed all rate constants, each rate constant was sensitive to the identity of the agonist activating the channel.

### Table 1. Agonists-specific gating of GluN1/GluN2A receptors

| Rate constant | DCS | ALA | ACPC | ACBC | HCA | SYM | HQA | QA | FGA | GluN1 agonists | GluN2A agonists |
|---------------|-----|-----|------|------|-----|-----|-----|-----|-----|----------------|----------------|
| Activation   |     |     |      |      |     |     |     |     |     |     |                |                |
| k_{33}       | ↓   |     |     |     |     |     |     |     |     |     | 7               |                |
| k_{21}       |     | ↓   |     |     |     |     |     |     |     |     | 7               |                |
| k_{10}       |     |     |     |     |     |     |     |     |     |     | 6               |                |
| Deactivation |     |     |      |      |     |     |     |     |     |     |                |                |
| k_{33}       |     |     |     |     |     |     |     |     |     |     | 2               |                |
| k_{21}       |     |     |     |     |     |     |     |     |     |     | 6               |                |
| k_{10}       |     |     |     |     |     |     |     |     |     |     | 5               |                |
| Entry into desensitized state |     |     |      |      |     |     |     |     |     |     |                |                |
| k_{33}       |     |     |     |     |     |     |     |     |     |     | 8               |                |
| k_{24}       |     |     |     |     |     |     |     |     |     |     | 3               |                |
| Recovery from desensitized state |     |     |      |      |     |     |     |     |     |     |                |                |
| k_{33}       |     |     |     |     |     |     |     |     |     |     | 0               |                |
| k_{42}       |     |     |     |     |     |     |     |     |     |     | 3               |                |

Summary of agonists-specific changes, relative to the natural agonist, in the rate constants optimized by fits with the model in Figure 1E. Arrows indicate significant (p < 0.05) increase (upward arrow, red) or decrease (downward arrow, blue) in the value for the respective rate constant; *, number of rate constants changed significantly. Except for FGA investigated in this study, all other data are compiled from Riusi and Popescu.12
that this rate constant and the recovery rate from desensitization, $k_{32}$, are the least agonist-sensitive transitions. Most ligands decreased the activation rate constants $k_{32}$ and $k_{21}$, and also decreased the desensitization rate constant $k_{53}$. This result may indicate that agonists that are less effective at opening the channel are also less effective at desensitizing the receptor.

**Discussion**

Fast intercellular communication in the nervous system is largely accomplished by chemical activation of ion-channels. The mechanism by which the energy resulting from the ligand-binding reaction is allocated within the agonist-receptor complex remains poorly understood. A seminal advance in attempting quantitative descriptions of fast synaptic transmission was achieved when the theory put forward for substrate-enzyme pairs in 1913 by Michaelis and Menten was extended in 1957 by del Castillo and Katz to ligand-channel pairs to postulate both quiet (AR) and active (AR*) conformations for agonist-receptor complexes. This formalism divided binding from gating and defined agonist efficacy as the ratio of opening and closing rate constants. It postulated that the AR ↔ AR* equilibrium is agonist-dependent and the AR and AR* complexes are energetically, structurally and functionally similar regardless of the identity of bound ligand. Modern views of gating mechanisms in neurotransmitter-gated channels attest to a more complex picture.

The vast majority of ligand-gated channels appear to assemble as heteromeric complexes of multiple subunits; have multiple, often non-equivalent ligand-binding sites, and in the case of NMDA-sensitive glutamate receptors, have dual-agonist requirement. The ideal concept of a two-state gating mechanism is most likely inadequate; a fact plainly demonstrated by the multiplicity of states observed directly in single-channel recordings for many receptors. Given this molecular complexity, it is not surprising that a mechanistic and quantitative description of agonist efficacy is still lacking. We owe the current view of agonism at glutamate-gated channels to accumulating structural information about various receptor elements.

The observation that glutamate receptor subunits have structurally distinct modules and that the isolated domains preserve their function upon excision from the core protein, initiated a rewarding line of inquiry in the structure and functionality of isolated modules. In particular, the investigations focused on extracellular agonist-binding domains have shaped the modern view of agonist-mediated gating. Essentially, these studies have demonstrated that the agonist-binding modules have an overall clamshell shape; ligands bind at the interface of two mobile domains; and the principal difference between the apo and agonist-bound forms rests with a more compact, closed clamshell conformation of the liganded form. Importantly, for soluble ligand-binding domains partial agonists induced conformations with intermediate degrees of cleft closure as compared to the full agonists on one end and antagonists at the other. In addition these seminal findings also revealed that regardless of the degree of closure induced, each ligand may stabilize subtly different conformations of the complex. It is not known whether the agonist-specific conformations observed in the structures of the isolated ligand-binding domains also translate to distinct families of conformers for full-length functional receptors. Insight into this important issue must come from investigations at the single-molecule level because this is the only known experimental technique that can classify receptor populations based on their active-inactive status and life-time distributions.

In the experiments summarized in this article, all ligands tested had similar although not identical effects on the gating of GluN1/GluN2A receptors and all gating transitions observed showed agonist-sensitivity. The differences observed in the mechanisms by which each ligand modified receptor activity did not correlate with the subunit-type specifically recognized by the particular agonist suggesting that the free-energy gained from agonist binding at either the GluN1 or the GluN2A subunit makes mechanistically similar contributions to channel activation. However, each agonist produced activations with distinct kinetic patterns perhaps reflecting that entities with unique chemistry and architecture produce specific rearrangements within the binding-pocket.

The observations that each agonist initiated different gating reactions and that all gating steps were sensitive to the nature of bound-agonist strongly suggest that the family of conformations elicited by each agonist are not identical. The implication is that agonist-sensitive transitions extend beyond the closing of the clamshell to yet-to-be identified downstream gating elements over which both subunit types have equivalent control.

Despite these recent insights, the issue of partial agonism at ligand-gated channels is far from settled, with the first wave of single-channel investigations leading to somewhat dissimilar conclusions. For example, in the case of the pentameric channels of the nicotinic receptor family, agonist effects appeared localized to only the initial pre-gating step, called priming or flipping. For tetrameric, AMPA-type glutamate receptors partial agonists shifted the receptor occupancies toward lower conductance states. Further, within the NMDA receptor family, experiments done in excised patches identified two pre-opening transitions to be agonist- and subunit-specific, whereas our on-cell experiments summarized here, show distributed ligand-specific but subunit-independent effects of agonists on NMDA receptor gating transitions.

Whether these discrepancies reflect true mechanistic differences or limitations inherent to the preparations or methodologies used remains to be clarified by additional experimentation. To reveal the still hidden mechanism by which ligand binding produces gating it will be necessary to bring to congruence structural data on a variety of functional conformations and kinetic information regarding the route and time course of their interconversion.

**Materials and Methods**

**Cells and transfections.** HEK cells (ATCC CRL-1573, gift from Dr. Auerbach, Buffalo, NY) were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, Grand Island, NY) at 37°C in a 5% CO₂ atmosphere and were passed when reaching 80–90%
confluence. Passages 22–35 were used for transfections.

Plasmids encoding rat GluN1-1a (U08261), rat GluN2A (M91561), and GFP were gifts from Drs. Wenthgold (Washington, DC), Auerbach (Buffalo, NY) and Kosman (Buffalo, NY). Open reading frames were sub-cloned into pcDNA3.1(+) under the control of the CMV promoter. Complete sequence of each insert was confirmed periodically by sequencing.

Calcium phosphate-mediated transient transfections were done by incubating the cells for 2 hours with mixtures consisting of (in mM): 140 NaCl, 5 KCl, 0.75 Na2HPO4, 6 sucrose, 125 CaCl2, 25 HEPES/NaOH, pH 7.05 and ~1 μg cDNA (GluN1-GluN2A:GFP = 1:1:1) per 35-mm dish containing cells at ~50% density. After removing the precipitate, the cells were grown 24–48 hrs in DMEM supplemented with 2 mM Mg2+.

Before each experiment, cells were washed, covered with PBS, and placed on the stage of an inverted microscope. Individual cells were selected visually, based on fluorescence intensity and patch clamp recordings were obtained while the cells were still attached to the dish.

Electrophysiology. Steady-state single-channel currents were recorded with the cell-attached patch-clamp technique.35 The electrodes were filled with solutions containing (in mM): 150 NaCl, 2.5 KCl, 1 EDTA, 10 HEPBS, 0.1 glycin and 1 FGA (Sigma), adjusted to pH 8 (NaOH). Inward sodium currents were elicited by applying +100 mV through the recording pipette. Currents were amplified and low pass filtered at 10 kHz (Axopatch200B; 4-pole Bessel), sampled at 20–40 kHz (PCI-6229, M Series card, National Instruments, Austin, TX) and written onto computer hard drives with QUB acquisition software (www.qub.buffalo.edu, Buffalo, NY).

Processing and analyses of single-channel current records. Each digital file was displayed on computer monitors with QUB software and inspected visually for simultaneous openings, signal-to-noise ratios, high-frequency artifacts and baseline drift. In the conditions tested, due to low receptor desensitization rate constants (<10 s–1) and high open probability within bursts (Po, 0.3–0.6), records consisted of long, almost solid clusters of activity separated by zero-current periods lasting seconds (see Fig. 1B). As a result, records originating from patches containing two or more active channels revealed many superimposed bursts, and were easily detected and excluded from further analyses. Baseline drifts were corrected by resetting the baseline to zero-current levels, as necessary.

Preprocessed data were idealized in QUB with the SKM algorithm after digitally low-pass filtering at 12 kHz. Idealized records were subjected to kinetic analyses and modeling with the MIL algorithm in QUB as described previously.12,39

Significance of differences observed was evaluated with Student’s t-tests assuming equal variance; differences were considered significant for p < 0.05.

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