ACS Chemical Neuroscience

Research Article

pubs.acs.org/chemneuro

Mechanism and Site of Inhibition of AMPA Receptors: Substitution of One and Two Methyl Groups at the 4-Aminophenyl Ring of 2,3-Benzodiazepine and Implications in the "E" Site

Congzhou Wang,[†] Andrew Wu,[†] Yu-Chuan Shen,[†] Roberta Ettari,[‡] Silvana Grasso,[§] and Li Niu*[,†]

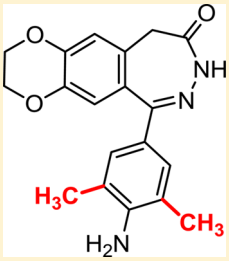
[†]Department of Chemistry and Center for Neuroscience Research, University at Albany, Albany, New York 12222, United States

[‡]Department of Drug Sciences and Products for Health, University of Messina, Viale Annunziata, 98168 Messina, Italy

[§]Department of Chemical Sciences, University of Messina, Via F. Stagno D'Alcontres 31, 98166 Messina, Italy

Supporting Information

ABSTRACT: 2,3-Benzodiazepines are a well-known group of compounds for their potential antagonism against AMPA receptors. It has been previously reported that the inhibitory effect of 2,3-benzodiazepine derivatives with a 7,8-ethylenedioxy moiety can be enhanced by simply adding a chlorine atom at position 3 of the 4-aminophenyl ring. Here we report that adding a methyl group at position 3 on the 4-aminophenyl ring, termed as BDZ-11-7, can similarly enhance the inhibitory activity, as compared with the unsubstituted one or BDZ-11-2. Our kinetic studies have shown that BDZ-11-7 is a noncompetitive antagonist of GluA2Q homomeric receptors and prefers to inhibit the closed-channel state. However, adding another methyl group at position 5 on the 4-aminophenyl ring, termed as BDZ-11-6, fails to yield extra inhibition on GluA2Q receptors. Instead, BDZ-11-6 exhibits a diminished inhibition of GluA2Q. Site interaction test indicates the two compounds, BDZ-11-6 and BDZ-11-7, bind to the same site on GluA2Q, which is also the binding site for their prototype, BDZ-11-2. Based on the results from this and our earlier studies, we propose that the binding site that accommodates the 4-aminophenyl ring must contain two interactive points, with one preferring polar groups like chlorine and the other preferring nonpolar groups such as a methyl group. Either adding a chlorine or a methyl group may enhance the inhibitory activity of 2,3-benzodiazepine derivatives with a 7,8-ethylenedioxy moiety. Adding any two of the same group on positions 3 and 5 of the 4-aminophenyl ring, however, significantly reduces the interaction between these 2,3-benzodiazepines and their binding site, because one group is always repelled by one interactive point. We predict therefore that adding a chlorine atom at position 3 and a methyl group at position 5 of the 4-aminophenyl ring of 2,3-benzodiazepine derivatives with a 7,8-ethylenedioxy moiety may produce a new compound that is more potent.



KEYWORDS: *Glutamate receptors, AMPA receptors, antagonist, 2,3-benzodiazepine, structure−activity relationship*

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are a subtype of ionotropic glutamate receptors.[1,2] AMPA receptors mediate fast synaptic neurotransmission in the central nervous system and are essential for brain activities, such as memory and learning.[1,2] Abnormal calcium influx through Ca^{2+}-permeable AMPA receptors has been implicated in neuronal injury and cell death, which is thought to contribute to a number of neurological disorders and diseases such as epilepsy, cerebral ischemia, and amyotrophic lateral sclerosis (ALS).[3−7] Potent and selective antagonists of AMPA receptors can be used to control excessive activity of these AMPA receptors in vivo. As such, developing such antagonists has attracted a sustained interest because of the therapeutic utility of these antagonists and a broad spectrum of neurological disorders and diseases that involve AMPA receptors.

2,3-Benzodiazepine (2,3-BDZ) derivatives, also known as GYKI compounds, are a class of compounds designed to inhibit AMPA receptors.[8] 2,3-BDZs are more selective toward AMPA receptors and exhibit neuroprotective properties in both cellular and animal models of various diseases.[8−10] The prototypic compound in this family, GYKI 52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine; see Figure 1 for its chemical structure), was discovered in the 1980s, and has been used as a template for the synthesis and the activity evaluation of a large number of derivatives carrying the 2,3-benzodiazepine backbone.[8,9,11−13] Using rapid kinetic techniques, we have studied the structure−activity relationship for a series of 2,3-BDZs. We have identified three noncompetitive binding sites for 2,3-BDZs, at least on the GluA2 AMPA receptor, one of the four homomeric AMPA receptors.[14−17] The presence of both the C-4 methyl group and the 7,8-methylenedioxy moiety in the 2,3-benzodiazepine structures defines the "M" site,[16] whereas replacing the C-4 methyl group with a C-4 carbonyl group results in 2,3-benzodiazepin-4-ones that bind to the "O" site.[14,15] Enlarging the 7,8-methylenedioxy ring into the 7,8-ethylenedioxy renders resulting compounds binding to the "E" site.[17] We have also shown that the structure−activity relationship for these 2,3-BDZs is site-specific. For instance, addition of a methyl-carbamoyl group to the N-3 position of the benzodiazepine ring strengthens the potency of a

Received: February 13, 2015
Revised: April 8, 2015
Published: May 12, 2015

DOI: 10.1021/acschemneuro.5b00064
ACS Chem. Neurosci. 2015, 6, 1371−1378

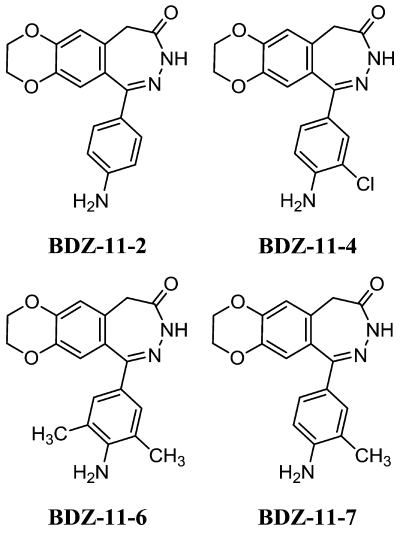


Figure 1. Chemical structures of BDZ-11-2 (1-(4-aminophenyl)-3,5-dihydro-7,8-ethylenedioxy-4*H*-2,3-benzodiazepin-4-one), BDZ-11-4 (1-(4-amino-3-chlorphenyl)-3,5-dihydro-7,8-ethylenedioxy-4*H*-2,3-benzodiazepin-4-one), BDZ-11-6 (1-(4-amino-3,5-dimethylphenyl)-3,5-dihydro-7,8-ethylenedioxy-4*H*-2,3-benzodiazepin-4-one), and BDZ-11-7 (1-(4-amino-3-methylphenyl)-3,5-dihydro-7,8-ethylenedioxy-4*H*-2,3-benzodiazepin-4-one).

resulting compound that binds to the "M" site,[16] but weakens a similar compound that binds to the "O" site.[14]

As part of the ongoing characterization of the structure–activity relationship for 2,3-BDZs, the present study concerns the mechanism and the site of inhibition of GluA2 AMPA receptors by two structurally similar compounds, BDZ-11-7 (i.e., 1-(4-amino-3-methylphenyl)-3,5-dihydro-7,8-ethylenedioxy-4*H*-2,3-benzodiazepin-4-one) and its derivative BDZ-11-6 (i.e., 1-(4-amino-3,5-dimethylphenyl)-3,5-dihydro-7,8-ethylenedioxy-4*H*-2,3-benzodiazepin-4-one(see their chemical structures in Figure 1). The two compounds are supposed to bind to the "E" site, because both contain a 7,8-methylenedioxy ring.[17] However, BDZ-11-6 contains an extra *ortho*-methyl group on the 4-aminophenyl ring, as compared with BDZ-11-7. The 4-aminophenyl ring is the core structural element of the hydrophobic center essential for biological activity of 2,3-BDZs. The hydrophobic center is present in the two pharmacophore models predicted by Rezessy and Solyom[12] using a detailed Structure–Activity Relationship (SAR) examination and DIStance COmparison (DISCO) computational approach. Previously we have shown that substituting an *ortho*-Cl atom on the 4-aminophenyl ring increases the inhibitory potency of the resulting compound without affecting of the site of binding.[17] The questions we specifically ask for in the present study are as follows. How does the substitution with a 3-methyl group at the 4-aminophenyl ring affect the potency of the resulting compound? We note that a methyl group is an electron-donating group, unlike a Cl atom; and a substitution with even one methyl group is expected to perturb the hydrophobic center of a 2,3-BDZ. What happens to the potency if positions 3 and 5 at the 4-aminophenyl ring are both occupied by a methyl group? Do these chemical substitutions cause the change of binding site for the resulting compounds? These questions are important in designing more potent "E" site inhibitors. Because substituting an *ortho*-methyl group on the 4-aminophenyl ring is known to slow *in vivo* metabolic rate of the resulting compounds,[13,18] substituting two methyl groups at positions 3 and 5 may also affect the rate of its metabolism of the resulting compound.

To address these questions, we have investigated the mechanism of inhibition of the GluA2Q$_{flip}$ AMPA receptors by BDZ-11-7 and BDZ-11-6. GluA2 is a key AMPA receptor subunit, and the unedited GluA2 or GluA2Q (at the Q/R site or the glutamine/arginine site) is a calcium-permeable isoform of GluA2. Editing defect at this site possibly leads to expression of GluA2Q isoform in some neurological disorders such as transient forebrain ischemia[19] and ALS.[4] In this study, we expressed GluA2 receptors in human embryonic kidney (HEK)-293 cells, and measured both the glutamate-induced whole-cell current amplitude and the rate of current rise in the presence and absence of an inhibitor. For measuring the rate of current rise, we used a laser-pulse photolysis technique that provided a ~60 μs time resolution. We have also measured the inhibition of the GluA2 current amplitude in the presence of two inhibitors, including BDZ-11-2 (see Figure 1), to characterize the site of inhibition for both BDZ-11-7 and BDZ-11-6. The implication of our results on both the structure–activity relationship for the BDZ-11 series (see Figure 1) and on the receptor binding site that accommodates the 4-aminophenyl ring is presented.

RESULTS AND DISCUSSION

BDZ-11-6 and BDZ-11-7 Inhibited the Whole-Cell Current of GluA2Q$_{flip}$ AMPA Receptors. We first measured the effect of an inhibitor on the amplitude of glutamate-induced whole-cell current through GluA2Q$_{flip}$ channels expressed in HEK-293 cells. We found that the amplitude was reduced in the presence of either BDZ-11-6 or BDZ-11-7 (Figure 2a), indicating that both compounds inhibited the GluA2Q$_{flip}$ receptor. To quantitatively characterize an inhibition constant, we measured the ratio of the whole-cell current amplitude in the absence and presence of an inhibitor (i.e., A/A_I) as a function of inhibitor concentration. Because the receptor could exist in either the open-channel or the closed-channel state, we used two glutamate concentrations (i.e., 100 μM and 3 mM) at which an inhibition constant could be estimated with respect to each of the receptor states of GluA2Q$_{flip}$. Specifically, glutamate concentrations of 100 μM and 3 mM correlated to the fraction of the open-channel state in the entire receptor population being ~4% and ~96%, respectively, for GluA2Q$_{flip}$.[20] Using eq 1, we determined that the inhibition constant of BDZ-11-7 was 3.7 ± 0.2 μM for the closed-channel state (K_I) and 9.0 ± 0.3 μM for the open-channel state ($\overline{K_I}$) (Figure 2b; eq 1 and all other equations are listed and described in the Supporting Information). Likewise, K_I of 94 ± 15 μM and $\overline{K_I}$ of 164 ± 8 μM were determined for BDZ-11-6 (Figure 2c). All of these inhibition constants are also summarized in Table 1.

The following can be concluded from the results described above: (a) both compounds inhibited GluA2Q$_{flip}$ receptors; (b) both compounds preferred to inhibit the closed-channel state of GluA2Q$_{flip}$; (c) BDZ-11-7 was 25-fold and 18-fold more potent than BDZ-11-6 for the closed-channel and the open-channel state of GluA2Q$_{flip}$, respectively.

BDZ-11-7, BDZ-11-6, and BDZ-11-2 Bound to the Same Site or the "E" Site on GluA2Q$_{flip}$. It is interesting to find that substitution with one *ortho*-methyl group at the 4-aminophenyl ring strengthened the potency; yet substitution with two *ortho*-methyl groups weakened the potency, as compared with BDZ-11-2, the unsubstituted one (Figure 1). To understand this apparent opposite effect, we asked whether the two compounds

DOI: 10.1021/acschemneuro.5b00064
ACS Chem. Neurosci. 2015, 6, 1371–1378

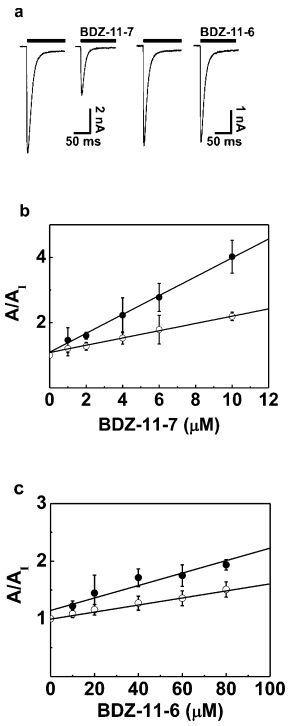


Figure 2. (a) Representative whole-cell current traces from HEK-293 cells expressing GluA2Q$_{flip}$ receptors, obtained from solution flow experiments. The bar above each current trace represents the time course of 3 mM glutamate used to evoke receptor response recorded at −60 mV, pH 7.4, and 22 °C. The pair on the left was from the same cell showing that BDZ-11-7 at 10 μM inhibited the current response as compared with the control; the pair on the right was from another cell showing that BDZ-11-6 at the same concentration, i.e., 10 μM, only weakly inhibited the receptor response. (b) Effect of BDZ-11-7 on the whole-cell current amplitude of GluA2Q$_{flip}$ receptors. A and A_I represent the current amplitude in the absence and presence of an inhibitor, respectively, and I represents inhibitor. A K_I of 3.7 ± 0.2 μM was determined, by using eq 1 (in the Supporting Information), for the closed-channel state (100 μM glutamate, ●); a $\overline{K_I}$ of 9.0 ± 0.3 μM was obtained for the open-channel state (3 mM glutamate, ○). (c) Effect of BDZ-11-6 on the whole-cell current amplitude of GluA2Q$_{flip}$ receptors obtained from the flow measurement. Similarly, a K_I of 94 ± 15 μM was determined for the closed-channel state (100 μM glutamate, ●); a $\overline{K_I}$ of 164 ± 8 μM was obtained for the open-channel state (3 mM glutamate, ○).

competed to the binding of the same site or the "E" site. The "E" site is where BDZ-11-2 binds, as previously shown.[17]

To address the site question, we carried out a double inhibitor experiment in which the concentration of one inhibitor was held constant while the concentration of the second inhibitor was varied (see the Supporting Information for details). Using this approach, we first investigated whether substitution with one methyl group at the 4-aminophenyl ring changed the site of binding as compared with the unsubstituted 4-aminophenyl ring structure or BDZ-11-2. Experimentally, BDZ-11-2 and BDZ-11-7 were applied on GluA2Q$_{flip}$ simultaneously while the concentration of BDZ-11-2 was kept at 20 μM and the concentration of BDZ-11-7 varied from 1 to 10 μM. The apparent, double-inhibition constant was found to be 4.0 ± 0.5 μM (upper solid line in Figure 3a) by the use of eq 2. This value

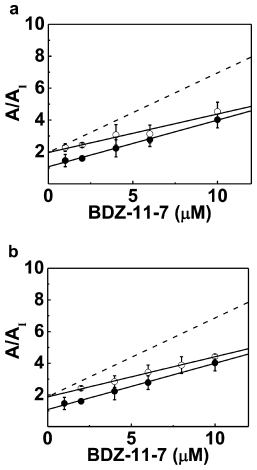


Figure 3. (a) Double-inhibition experiment for BDZ-11-7 and BDZ-11-2 on the closed-channel state of GluA2Q$_{flip}$. The concentration of BDZ-11-2 was fixed at 20 μM, while that of BDZ-11-7 varied from 1 to 10 μM. The apparent, double-inhibition constant was determined to be 4.0 ± 0.5 μM (○) using eq 2, as compared with a K_I of 3.7 ± 0.2 μM for BDZ-11-7 alone (●). The dashed line is the simulation of the A/A_I ratio by assuming that the two inhibitors bound to two different sites with an apparent, double-inhibition constant of ∼2.0 μM when BDZ-11-2 was fixed at 20 μM, using eq 3. (b) Double-inhibition experiment for BDZ-11-7 and BDZ-11-6 on the closed-channel state of GluA2Q$_{flip}$. The concentration of BDZ-11-6 was fixed at 80 μM, while that of BDZ-11-7 varied from 2 to 10 μM. The apparent, double-inhibition constant was determined to be 4.0 ± 0.3 μM (○), as compared with a K_I of 3.7 ± 0.2 μM for BDZ-11-7 alone (●). The dashed line is the simulation of the A/A_I ratio by assuming that the two inhibitors bound to two different sites with an apparent, double-inhibition constant of ∼2.0 μM, when BDZ-11-6 was fixed at 80 μM, using eq 3.

Table 1. Inhibition Constants of 2,3-Benzodiazepines Obtained from Rate and Amplitude Measurements for the Closed-channel and Open-channel States of GluA2Q$_{flip}$

	rate measurement[a]		amplitude measurement			
inhibitor	K_I^* (μM)[b] (closed channel)	$\overline{K_I}^*$ (μM)[b] (open channel)	K_I (μM)[bd]	K_I (μM)[be]	K_I (μM)[cd] (closed channel)	$\overline{K_I}$ (μM)[cf] (open channel)
BDZ-11-2[g]	39 ± 9	43 ± 11	19 ± 1	24 ± 4	21 ± 0.1	33 ± 1
BDZ-11-4[g]	9.0 ± 3.0	24 ± 5.0	2.3 ± 0.2	2.4 ± 1.0	2.0 ± 0.1	14 ± 0.4
BDZ-11-6					94 ± 15	164 ± 8
BDZ-11-7	29 ± 5	15 ± 1	4.4 ± 0.3	5.4 ± 0.4	3.7 ± 0.2	9.0 ± 0.3
GYKI 52466[h]	22 ± 1	20 ± 3	4 ± 1	5 ± 1	4 ± 1	5 ± 1

[a]The constants obtained from rate measurements represent those in the first step of inhibition as in Figure 5, whereas those obtained from the amplitude measurements represent the overall inhibition constants. [b]Laser-pulse photolysis measurement. [c]Flow measurement. [d]Measurements at 100 μM glutamate for the closed-channel state. [e]Measurements at ∼300 μM glutamate. [f]Measurements at 3 mM glutamate. [g]Reference 17. [h]Reference 15.

DOI: 10.1021/acschemneuro.5b00064
ACS Chem. Neurosci. 2015, 6, 1371−1378

was identical, within experimental error, to K_I of 3.7 ± 0.2 μM for BDZ-11-7 alone (lower solid line in Figure 3a). This result suggested that BDZ-11-7 and BDZ-11-2 competed for binding to the same site on the GluA2Q$_{flip}$ receptor. If the two inhibitors bound to two different sites, an apparent double-inhibition constant of ∼2 μM or a much stronger inhibition would be expected (i.e., the dashed line in Figure 3a; the dashed line was simulated using eq 3). Therefore, this result suggested that substitution with one *ortho*-methyl group at the 4-aminophenyl ring, which resulted in BDZ-11-7, did not change the binding site. The site to which BDZ-11-7 bound was the "E" site, previously defined with BDZ-11-2.[17]

Using the same method as described above, we carried out another double inhibitor experiment where BDZ-11-6 and BDZ-11-7 were applied together to inhibit the GluA2Q$_{flip}$ receptor. This experiment was to show whether the substitution with one more *ortho*-methyl group at BDZ-11-7, resulting BDZ-11-6, could cause the change of binding site. We found that the slope of the A/A_I plot in the presence of two inhibitors, that is, BDZ-11-6 and BDZ-11-7, as a function of BDZ-11-7 concentration was identical to that of BDZ-11-7 alone (Figure 3b). Specifically, the apparent inhibition constant of the two inhibitors was calculated to be 4.0 ± 0.3 μM (upper solid line in Figure 3b). As a comparison, the inhibition constant of BDZ-11-7 alone was 3.7 ± 0.2 μM (lower solid line in Figure 3b). This result was consistent with the conclusion that (i) both BDZ-11-6 and BDZ-11-7 competed to the same site, and (ii) this site was the "E" site (Figure 3a) where the two inhibitors, together with BDZ-11-2 (Figure 3a), competed binding. Therefore, these results suggested that acylating the two *ortho*-positions or positions 3 and 5 apparently weakened the potency of the resulting compound, that is, BDZ-11-6, but did not change the site of the resulting compound. That the two compounds competed for the same site may not be surprising given the two compounds both contain a 7,8-ethylenedioxy ring.[17] If the two compounds each bound to its own site, an apparent "additive" inhibition would have been observed, yielding in a stronger inhibition (or steeper slope as in the dashed line in Figure 3b).

BDZ-11-7 Inhibited the Channel-Opening Rate of GluA2Q$_{flip}$. Next, we investigated the mechanism of action of BDZ-11-7 on the channel-opening rate of GluA2Q$_{flip}$. For this experiment, we used a laser-pulse photolysis technique, together with a photolabile precursor of glutamate or caged glutamate (see details in Methods).[21] Specifically, a single laser pulse (with a pulse length of 8 ns) was used to photolyze the "caged" glutamate, releasing free glutamate with a $t_{1/2}$ of ∼30 μs.[21] The time resolution was therefore high enough to enable us to measure the channel-opening kinetics of GluA2[20] and the effect of an inhibitor on the channel-opening rate for a mechanism study[14−17] without the complication of channel desensitization that occurs in the millisecond (ms) time region. In this study, we chose BDZ-11-7 instead of BDZ-11-6 because the two compounds bound to the same site but the inhibition by BDZ-11-7 was much stronger.

Figure 4a displays two whole-cell current traces, obtained from the laser-pulse photolysis experiment, from a HEK-293 cell that expressed GluA2Q$_{flip}$ channels. The upper trace was a response to glutamate photolytically released at time zero with amplitude of 1.31 nA; the observed rate constant of current rise (k_{obs}) was determined to be 2040 s^{-1} (see the Supporting Information, eq c). In the presence of BDZ-11-7, the current amplitude was reduced to 0.68 nA and the rate of current rise was slowed to 1692 s^{-1} as in the lower trace (see the legend of Figure 4a for the

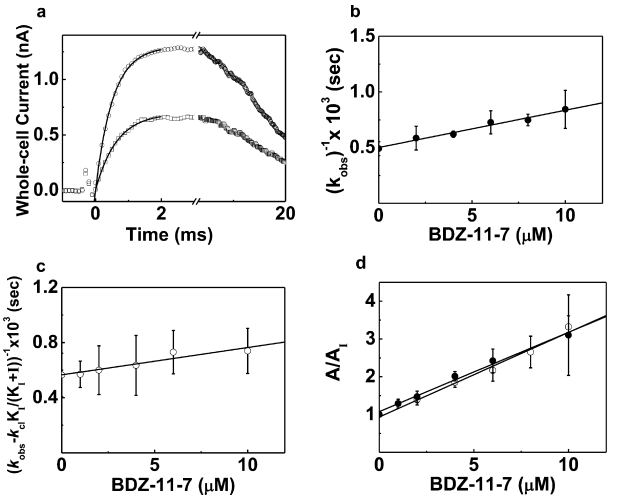


Figure 4. (a) BDZ-11-7 inhibited both the rate and the amplitude of the opening of the GluA2Q$_{flip}$ channels, shown as the representative whole-cell current traces from an HEK-293 cell obtained from the laser-pulse photolysis experiment. The upper trace (○) was the control (k_{obs} = 2040 s^{-1}; A = 1.31 nA), and the lower trace (□) contained 4 μM BDZ-11-7 (k_{obs} = 1692 s^{-1}; A = 0.68 nA). The concentration of the photolytically released glutamate was estimated to be ∼100 μM in both traces. (b) Effect of BDZ-11-7 on k_{cl} obtained at 100 μM of photolytically released glutamate and as a function of BDZ-11-7 concentration. A $\overline{K_I}^*$ of 15 ± 1 μM was determined using eq 4. (c) Effect of BDZ-11-7 on k_{op} obtained at 300 μM of photolytically released glutamate and as a function of BDZ-11-7 concentration. A K_I^* of 29 ± 5 μM was determined using eq 5. (d) Effect of BDZ-11-7 on the whole-cell current amplitude obtained from the laser-pulse photolysis measurement. A K_I of 4.4 ± 0.3 μM was determined at 100 μM of photolytically released glutamate (●); a K_I of 5.1 ± 0.4 μM was obtained at 300 μM of photolytically released glutamate (○).

experimental detail). This result indicated that BDZ-11-7 inhibited both the observed rate and the amplitude of the current rising phase for the opening of the GluA2Q$_{flip}$ channels. The fact that the time course of the current rise remained single exponential at this and all concentrations of photolytically released glutamate in our experiment (i.e., between 100 ± 20 and 300 ± 50 μM) as well as in the presence of BDZ-11-7 (for up to 10 μM) was consistent with the assumption that the binding of the inhibitor/glutamate was fast relative to the opening of receptor channel.[20,22] Therefore, in the range of glutamate concentrations we used in this study, the rate constant we measured was pertinent to the rate of receptor channel opening, rather than ligand binding. Consequently, the reduction of the rate and the amplitude in Figure 4a was attributed to the inhibition of the receptor channel opening by BDZ-11-7.

To investigate the mechanism of action of BDZ-11-7 on the GluA2Q$_{flip}$ channel opening, we characterized the effect of BDZ-11-7 on both the channel-opening rate constant (k_{op}) and the channel-closing rate constant (k_{cl}) (Figure 4b and c). In the first experiment, we kept the concentration of photolytically released glutamate at ∼100 μM, and varied the concentration of BDZ-11-7. Under this condition, we measured the effect of BDZ-11-7 on k_{cl} (Figure 4b), and further determined the inhibition constant for the open-channel state, $\overline{K_I}^*$, to be 15 ± 1 μM using eq 4. At ∼300 μM of glutamate released from the laser photolysis, we determined the effect of BDZ-11-7 on k_{op}. Using eq 5, we calculated the inhibition constant for the closed-channel state, K_I^*, to be 29 ± 5 μM (Figure 4c). That BDZ-11-7 affected both

DOI: 10.1021/acschemneuro.5b00064
ACS Chem. Neurosci. 2015, 6, 1371−1378

k_{op} and k_{cl} was consistent with a noncompetitive mechanism.[17] In fact, BDZ-11-7 acted similarly with BDZ-11-2 and BDZ-11-4, which we reported previously.[17] It should be noted as well that all of these compounds share the same structural features and bind to the "E" site on GluA2Q$_{flip}$.

BDZ-11-7 Inhibited the Channel Opening of GluA2Q$_{flip}$ by a Two-Step Process. As seen in the laser-pulse photolysis experiment (Figure 4a), BDZ-11-7 inhibited not only the rate of current rise, which we have described above, but also the whole-cell current amplitude. Using the ratio of the current amplitude in the absence and presence of BDZ-11-7 from the laser-pulse photolysis measurement, we also calculated the inhibition constant of 4.4 ± 0.3 and 5.1 ± 0.4 μM for 100 and 300 μM glutamate concentration, respectively (Figure 4d).

The inhibition constant of 4.4 μM obtained from the amplitude at 100 μM glutamate, as presented above, is close to K_I of ~3.7 μM, also obtained at 100 μM glutamate, using the solution flow technique (Figure 2). A slightly larger K_I (i.e., 5.1 ± 0.4 μM) at 300 μM glutamate is not unreasonable, because 300 μM is closer to 100 μM at which the closed-channel state is measured than it is to 3 mM glutamate that corresponds to the measurement of the open-channel state (at 3 mM glutamate, $\overline{K_I}$ of 9.0 ± 0.3 μM has been determined using the solution flow technique; Figure 2, Table 1). The comparison of these results demonstrates that the inhibition constants we determined at 100 μM glutamate concentration are in good agreement, despite that these constants have been obtained from two different techniques, that is, the laser-pulse photolysis technique and the solution flow approach. It should be noted that because it was not possible to achieve 3 mM glutamate release by a single laser-pulse photolysis with an HEK-293 cell, we were not able to obtain an inhibition constant from the amplitude at 3 mM glutamate from the laser-pulse photolysis measurement.

Surprisingly, however, an inhibition constant for either the closed-channel or the open-channel state obtained from the amplitude measurement was at least 3-fold smaller than the corresponding value from the rate measurement (Table 1). This is especially noteworthy because even by the use of the same technique, that is, the laser-pulse photolysis approach (Table 1), the inhibition constant obtained at 100 μM glutamate from the rate data is 29 μM, as compared to ~4 μM calculated from the amplitude data (see Table 1 for BDZ-11-7 data). In fact, other compounds that bind to this site on GluA2Q$_{flip}$, such as BDZ-11-2 and BDZ-11-4, also show a similar trend (Table 1). In other words, from the rate data, the degree of inhibition (as measured by inhibition constant) seems to be less than full as compared with the degree of inhibition estimated using the amplitude data.

The discrepancy between the inhibition constants obtained from the amplitude measurement and from the rate measurement can be accounted for by a mechanism (Figure 5) we have previously proposed for 2,3-benzodiazepine compounds.[14−17] By this model, the inhibition of GluA2Q$_{flip}$ channel opening by BDZ-11-7 involves two steps, and each step contributes to the overall antagonism (Figure 5). The first step involves the formation of a loose, inhibitor-receptor intermediate, only producing partial inhibition. In the second step, the inhibitor-receptor intermediate rapidly isomerizes into a tighter, nonconducting complex, producing additional inhibition. As compared to the rate of the second step, the first step is slow. As such, from the rate measurement, we observed only the partial inhibition or larger inhibition constant from the first step or the slow step. In contrast, amplitude data was an equilibrium measure. From the amplitude data, which included additional

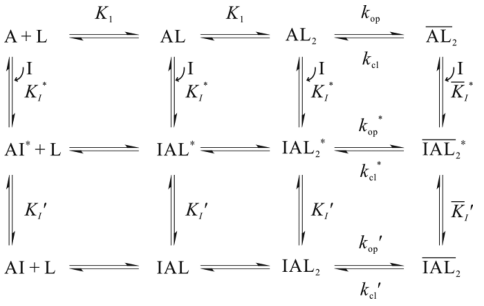


Figure 5. Minimal kinetic mechanism of inhibition for BDZ-11-7. The upper row shows the channel-opening reaction of the AMPA receptor. *A* represents the active, unliganded form of the receptor, *L* the ligand (glutamate), *AL* and AL_2 the ligand-bound closed-channel forms, $\overline{AL_2}$ the open-channel state of the receptor (all the species with a bar sign refer to open-channel state). Furthermore, k_{op} and k_{cl} are the channel-opening and channel-closing rate constants. For simplicity and without contrary evidence, it is assumed that glutamate binds to the receptor in the two steps with equal affinity, represented by the same intrinsic equilibrium dissociation constant, K_1. The initial binding of BDZ-11-7 to the receptor is assumed to form a loosely bound, partially conducting intermediate (e.g., $\overline{IAL_2}^*$) (middle row). In the second step (the lower row), the receptor:inhibitor intermediate rapidly isomerizes into a more tightly bound, inhibitory complex ($\overline{IAL_2}$). The inhibition constants pertinent to various steps in this mechanistic scheme are shown in Table 1. Specifically, K_I represents the overall inhibition constant associated with the closed-channel state of the receptor (i.e., column 5 in Table 1); $\overline{K_I}$, the overall inhibition constant associated with open-channel state (i.e., column 6 in Table 1). The values for K_I^* and $\overline{K_I}^*$ for step 1 can be found from columns 1 and 2 in Table 1.

inhibition from the second step, we observed a stronger or overall inhibition, which yielded smaller inhibition constants.

In the two-step model described above, the inhibitor and the receptor first form a loose complex, followed by a rapid conformational change that tightens the complex to give the final nonconducting complex. In this context, it is interesting to note that, for BDZ-11-7, the $\overline{K_I}^*$ of 15 μM (Figure 4b) for the open-channel state at step 1 is only 1.6-fold larger than $\overline{K_I}$ of 9 μM (Table 1). In contrast, for the closed-channel state, the K_I^* of 29 μM at step 1 is 7-fold larger than K_I of ~4 μM (Table 1). This comparison suggests that the closed-channel state must undergo a more significant conformational change to reach a tighter, nonconducting complex. In addition, BDZ-11-7 inhibits the closed-channel state twice as more strongly as it does with the open-channel state (Table 1). Based on these data, we speculate that the closed-channel state of the GluA2Q$_{flip}$ receptor is more flexible or less structured as compared with the open-channel state, at least from the viewpoint of the "E" site. For making stronger inhibitors targeting the "E" site, the closed-channel state is therefore a better target. In fact, BDZ-11-7 acts similarly to both BDZ-11-2 (Table 1) and BDZ-11-4.[17] We note that a larger conformational change could also be contributed by a binder not just the receptor. However, given that binders to this site are all small molecule inhibitors such as BDZ-11-7, it is unlikely that, upon binding, these compounds change their conformations as significantly as does the GluA2Q$_{flip}$ receptor.

The Cavity for the 4-Aminophenyl Ring on the "E" Site is Half Polar and Half Nonpolar. As we have shown from this study, both BDZ-11-6 and BDZ-11-7 bind to the same site as BDZ-11-2, suggesting that methylating *ortho*-positions of the 4-aminophenyl ring does not change the site of binding for

DOI: 10.1021/acschemneuro.5b00064
ACS Chem. Neurosci. 2015, 6, 1371−1378

resulting compounds. It is surprising, however, that BDZ-11-7 with one *ortho*-methyl group on the 4-aminophenyl ring is more potent than its prototype BDZ-11-2, whereas BDZ-11-6 with two *ortho*-methyl groups or at positions 3 and 5 is less potent than BDZ-11-7; in fact, BDZ-11-6 is even less potent than BDZ-11-2, which has a nonalkylated 4-aminophenyl ring (Table 1). The most obvious hypothesis is that the two sides of the cavity that accommodate the two methyl groups at positions 3 and 5 of the 4-aminophenyl ring are nonidentical. In other words, the cavity itself is half polar and half nonpolar. This hypothesis is also supported by our earlier finding that BDZ-11-4, which has one chlorine atom at position 3 of the 4-aminophenyl ring, is more potent than BDZ-11-2 with the nonalkylated 4-aminophenyl ring.[17] Based on the present and the earlier study of the "E" site compounds,[17] we conclude that methylating only one *ortho*-position strengthens the potency of a resulting "E" site compound, most likely by strengthening the nonpolar interaction between the alkylated 4-aminophenyl ring and the amino acid residues that make up part of the cavity. A substituted 4-aminophenyl ring is able to adopt an optimal orientation within its cavity because the 4-aminophenyl ring and the 2,3-benzodiazepine ring are covalently linked by a σ bond (Figure 1). On the contrary, methylating both positions 3 and 5 is not desirable, because one of the methyl groups will always face the polar environment and thus be repelled by an unfavorable interaction. Potential steric hindrance caused by a disubstitution on the 4-aminophenyl ring could also contribute to the reduction of the potency. It should be pointed out, however, that we do not know where the "E" site is located in the GluA2Q_{flip} receptor. There is no structural information currently available for any other noncompetitive sites on this and any other AMPA receptor subunits. As a result, we do not know the amino acid composition for the cavity that accommodates the 4-aminophenyl ring of a 2,3-BDZ.

Implications in Design of New "E" Site 2,3-BDZs. The results from our current study of one and two methyl substituted 4-aminophenyl ring at the *ortho*-positions, along with our earlier report of a chloro-substituted compound,[17] suggest that the 4-aminophenyl ring is a useful scaffold for chemical substitutions at the *ortho*-positions for design of new compounds. Specifically, substituting either a chlorine atom or a methyl group seems to be beneficial in producing more potent inhibitors than the unsubstituted 4-aminophenyl compound, that is, BDZ-11-2 (Figure 1). In contrast, substituting two identical methyl groups (with the same size and same polarity) at both *ortho*-positions is significantly counterproductive in that the disubstituted compound, that is, BDZ-11-6, is less potent than even the unsubstituted ring structure. Based on these results, we propose that the binding pocket that accommodates the 4-aminophenyl ring contains two interactive points, with one preferring polar groups like chlorine and the other preferring nonpolar group such as a methyl group. Adding either a chlorine atom or a methyl group increases the inhibitory activity of 2,3-BDZs, most likely by strengthening the interaction between the receptor pocket and the 4-aminophenyl ring. Adding any two of the same group on positions 3 and 5 of the 4-aminophenyl ring will, however, weaken the interaction between these 2,3-benzodiazepines and their binding pocket, thereby reducing the inhibitory potency.

Our results help improve the understanding of the two pharmacophore models reported previously by Rezessy and Solyom.[12] These two models define two types of spatial arrangements of a set of key features present in 2,3-BDZs that interact favorably with the receptor binding site, leading to their biological function. In establishing the original models, the biological function was assessed using the efficacy of 2,3-BDZs in blocking both AMPA and kainate-induced cortical spreading depression in chicken retina.[12,23] Our study, however, exclusively examines the structure−activity relationship of 2,3-BDZs with homomeric AMPA receptors expressed in HEK-293 cells. Furthermore, the original models assumed all the 2,3-BDZs would bind to the same receptor site. We now know, however, that there are three, independent noncompetitive sites for 2,3-BDZs, such as the "E" site, the subject of this study. Nonetheless, the common pharmacophoric features in both models are a donor atom ($-NH_2$ group) and a hydrophobic center (the center of the aromatic ring) on the aminophenyl ring, depicted in green color (Figure 6). For example, talampanel [(*R*)-(−)-1-(4-aminophenyl)-3,4-dihydro-3-*N*-acetyl-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine] can fit to both models with different abilities. Thiadiazolyl 2,3-BDZs fit to the type 2 model, which has been defined by one of the lone pairs of C-4 carbonyl oxygen or that of the heteroatom of the attached heterocycle (for detailed description of the two models and interpoint distances, please refer to original paper by Rezessy and Solyom[12]). Type 2 pharmacophore was assumed to be better because compounds that fit to this model tend to have higher efficacy.[12] Indeed, the two thiadiazolyl 2,3-BDZs we reported recently have perhaps the highest potency among all 2,3-BDZs.[24] It is further important to note that no other structural features associated with the 4-aminophenyl ring were used in the construction of the two pharmacophores.[12] In the present study, we have investigated the effect of perturbing the polarity of the hydrophobic center, represented by the 4-aminophenyl ring. Our results clearly show that perturbation of the hydrophobic center by introducing different polarity significantly affects the potency of the resulting 2,3-BDZs that bind to the "E" site.

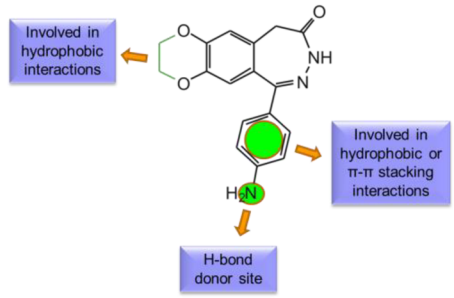


Figure 6. Donor atom ($-NH_2$ group) and the hydrophobic center (the center of the aromatic ring or the 4-aminophenyl ring) are highlighted in green colored circles. These elements are the common features of the two pharmacophore models as proposed by Rezessy and Solyom[12] (the detailed pharmacophore models and interpoint distance maps for the two models can be found in the same paper). Our results show that substituting the 4-aminophenyl ring at positions 3 and 5 with chlorine atom and/or a methyl group affects the potency of a resulting compound. This effect is most likely attributed to the perturbation of the interaction of the hydrophobic center with the amino acid surrounding of the "E" site on an AMPA receptor (the "E" site is defined with 2,3-BDZs that contain a 7,8-ethylenedioxy ring, shown in part in green color). The possible functional roles of the three green-color coded regions in an "E" site 2,3-BDZ are also presented.

For making more potent 2,3-BDZs that supposedly bind to the "E" site, we suggest additional ways to effect the polarity of the resulting compounds. For example, adding both a 3-chloro group and a 5-methyl group on the 4-aminophenyl ring of 2,3-

DOI: 10.1021/acschemneuro.5b00064
ACS Chem. Neurosci. 2015, 6, 1371−1378

benzodiazepine derivatives with a 7,8-ethylenedioxy moiety may produce a new compound that is more potent than a single *ortho*-substitution. Obviously, because the 4-aminophenyl ring is linked to the 2,3-benzodiazepine structure by a σ bond, the 3-chloro-5-methyl-substituted 4-aminophenyl ring can orientate to properly position itself in such a cavity so that the interaction of the compound with the cavity is strengthened. Furthermore, substituting two methyl groups may still lead to a better inhibitor but the second substitution should be at position 2. In this case, the two methyl groups at positions 2 and 3 respectively may reside at the same nonpolar environment. If this is the case, dielectrophilic substitutions with two chlorine atoms in the similar fashion could be also favorable. Finally, if we extend the prediction beyond a methyl group and a chlorine atom, other alkyl substitutions and other halogenation or even other types of electrophilic substitutions may be beneficial. Because we do not know the size of this cavity, at least extending the size of a substituent slightly with a similar polarity is a worthy attempt. It should be noted, however, that our predictions, described above, should apply only to the "E" site compounds. Whether our predictions apply additionally to the "M" site as well as the "O" site is not clear. It would be interesting, however, that compounds can be synthesized to test these predictions for the two other sites. Furthermore, our prediction is based on polarity. Steric hindrance, however, could invalidate our predictions, especially when the 4-aminophenyl ring is subject to more than one substitution.

METHODS

Cell Culture and Receptor Expression. HEK-293S cells were cultured in a 37 °C, 5% CO_2, humidified incubator, and in the Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 100 units of penicillin/mL and 0.1 mg streptomycin/mL (Sigma-Aldrich, St. Louis, MO). The DNA plasmids were prepared as previously described.[25] The HEK-293S cells were transiently transfected to express the AMPA receptor by following a standard calcium phosphate method.[26] The cells were also cotransfected with a plasmid encoding green fluorescent protein (GFP) as a transfection marker and a separate plasmid encoding large T-antigen to enhance the receptor expression at the single cell level.[27] The weight ratio of the plasmid for GFP and the large T-antigen to that for AMPA receptor was 1:1:10, respectively. The plasmid used for transient transfection for AMPA receptors ranged from 5 to 15 μg per 35 mm dish. The cells were used for recording 24–48 h after transfection.

Whole-Cell Current Recording. Glutamate-induced whole-cell current from transfected HEK-293S cells was recorded on an Axopatch 200B (Molecular Devices, Sunnyvale, CA) at a cutoff frequency of 2–20 kHz by a built-in, 4-pole low-pass Bessel filter. The whole-cell current traces were digitized at a 5–50 kHz sampling frequency using a Digidata 1322A (Molecular Devices). All recordings were at −60 mV and room temperature. The pClamp 8 software (Molecular Devices) was used for data acquisition. A recording electrode was made from glass capillary (World Precision Instruments, Sarasota, FL) and had a resistance of ~3 MΩ when filled with the electrode solution. The electrode solution was composed of (in mM) 110 CsF, 30 CsCl, 4 NaCl, 0.5 $CaCl_2$, 5 EGTA, and 10 HEPES (pH 7.4 adjusted by CsOH). The external solution contained (in mM) 150 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (pH 7.4 adjusted by NaOH). All chemicals were from commercial sources.

Laser-Pulse Photolysis Measurement. The use of the laser-pulse photolysis technique to measure the channel-opening kinetics has been described previously.[16] In this experiment, a caged glutamate (e.g., 4-methoxy-7-nitroindolinyl-caged-L-glutamate from Tocris Bioscience) was dissolved in the external buffer and applied to a cell using a flow device.[16] A single laser pulse at 355 nm generated from a pulsed Q-switched Nd:YAG laser (Continuum, Santa Clara, CA), with a pulse length of 8 ns and energy output in the range of 200–1000 μJ, was applied to an HEK-293S cell via optical fiber. To determine the concentration of glutamate generated photolytically by laser photolysis, we calibrated the receptor response in the same cell by applying two solutions of free glutamate with known concentrations before and after laser flash, with reference to the dose–response relation.[16] These measurements also allowed us to monitor any damage to the receptors and/or the cell for successive laser experiments with the same cell. A flow device[28] was used to deliver free glutamate and/or caged glutamate solutions in the absence and presence of inhibitor. The time resolution of this flow device, determined by the rise time of the whole-cell current response (10–90%) to saturating glutamate concentrations, was 1.0 ± 0.2 ms, an average of the measurement from >100 cells expressing the same receptor.[29] Furthermore, we used an 8-s time protocol in preincubating all of the 2,3-benzodiazepine compounds in both flow and laser photolysis experiments in order to observe and record full inhibition by these inhibitors, a phenomenon we previously observed with other 2,3-benzodiazepine compounds.[16]

Experimental Design and Data Analysis. The design of the experiments for determining the inhibition constant for the closed-channel and the open-channel state from the amplitude by varying glutamate concentration has been described in the text. The design of the experiment for measuring the effect of an inhibitor on the rate constant of k_{op} and k_{cl} as a function of inhibitor concentration and at two glutamate concentrations is described in detail in the Supporting Information. All equations used for quantitative data analysis are also in the Supporting Information. Also described in the Supporting Information are the description and the equations for the use of double-inhibitors to assess whether the two inhibitors bind to the same site or two different sites on the same receptor. It should be noted that when free glutamate with or without an inhibitor was used to evoke the receptor response, by the use of the solution flow device, the amplitude of the whole-cell current was corrected for receptor desensitization during the rise time.[28] The corrected current amplitude was then used for data analysis. Origin 7 (Origin Lab, Northampton, MA) was used for both linear and nonlinear regressions (Levenberg–Marquardt and simplex algorithms). Unless otherwise noted, each data point shown in a plot was an average of at least three measurements collected from three different cells. The error reported refers to the standard error of the fits.

ASSOCIATED CONTENT

Supporting Information

Figures showing and an appendix listing all equations for data analysis. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00064.

AUTHOR INFORMATION

Corresponding Author

*Phone: 518-591-8819. Fax: 518-442-3462. E-mail: lniu@albany.edu.

Author Contributions

C.W. conducted the experiments, produced the plots and table, and wrote the draft; A.W. and Y.S. conducted part of the experiments; L.N. directed the research and wrote the paper based on the draft written by C.W. R.E. and S.G. synthesized and purified the compounds.

Funding

This work was supported by grants from NIH/NINDS (R01 NS060812) and the Muscular Dystrophy Association.

Notes

The authors declare no competing financial interest.

ABBREVIATION

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BDZ, 2,3-benzodiazepine derivatives; ALS, amyotrophic lateral

DOI: 10.1021/acschemneuro.5b00064
ACS Chem. Neurosci. 2015, 6, 1371–1378

sclerosis; GYKI 52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine; BDZ-11-2, 1-(4-aminophenyl)-3,5-dihydro-7,8-ethylenedioxy-4*H*-2,3-benzodiazepin-4-one; BDZ-11-6, 1-(4-amino-3,5-dimethylphenyl)-3,5-dihydro-7,8-ethylenedioxy-4*H*-2,3-benzodiazepin-4-one; BDZ-11-7, 1-(4-amino-3-methylphenyl)-3,5-dihydro-7,8-ethylenedioxy-4*H*-2,3-benzodiazepin-4-one; HEK-293 cells, human embryonic kidney 293 cells

REFERENCES

(1) Dingledine, R., Borges, K., Bowie, D., and Traynelis, S. F. (1999) The glutamate receptor ion channels. *Pharmacol Rev. 51*, 7−61.

(2) Traynelis, S. F., Wollmuth, L. P., McBain, C. J., Menniti, F. S., Vance, K. M., Ogden, K. K., Hansen, K. B., Yuan, H., Myers, S. J., and Dingledine, R. (2010) Glutamate receptor ion channels: Structure, regulation, and function. *Pharmacol Rev. 62*, 405−496.

(3) Meldrum, B. S. (1993) Excitotoxicity and selective neuronal loss in epilepsy. *Brain Pathol. 3*, 405−412.

(4) Kawahara, Y., Ito, K., Sun, H., Aizawa, H., Kanazawa, I., and Kwak, S. (2004) Glutamate receptors: RNA editing and death of motor neurons. *Nature 427*, 801.

(5) Sheardown, M. J., Nielsen, E. O., Hansen, A. J., Jacobsen, P., and Honore, T. (1990) 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)-quinoxaline: a neuroprotectant for cerebral ischemia. *Science 247*, 571−574.

(6) Liu, S. J., and Zukin, R. S. (2007) Ca^{2+}-permeable AMPA receptors in synaptic plasticity and neuronal death. *Trends Neurosci. 30*, 126−134.

(7) Rogawski, M. A. (2011) Revisiting AMPA receptors as an antiepileptic drug target. *Epilepsy Curr. 11*, 56−63.

(8) Tarnawa, I., Farkas, S., Berzsenyi, P., Pataki, A., and Andrasi, F. (1989) Electrophysiological studies with a 2,3-benzodiazepine muscle relaxant: GYKI 52466. *Eur. J. Pharmacol. 167*, 193−199.

(9) Solyom, S., and Tarnawa, I. (2002) Non-competitive AMPA antagonists of 2,3-benzodiazepine type. *Curr. Pharm. Des. 8*, 913−939.

(10) De Sarro, A., De Sarro, G., Gitto, R., Grasso, S., Micale, N., Quartarone, S., and Zappala, M. (1998) 7,8-Methylenedioxy-4H-2,3-benzodiazepin-4-ones as novel AMPA receptor antagonists. *Bioorg. Med. Chem. Lett. 8*, 971−976.

(11) Grasso, S., Micale, N., Zappala, M., Galli, A., Costagli, C., Menniti, F. S., and De Micheli, C. (2003) Characterization of the mechanism of anticonvulsant activity for a selected set of putative AMPA receptor antagonists. *Bioorg. Med. Chem. Lett. 13*, 443−446.

(12) Rezessy, B., and Solyom, S. (2004) Advanced pharmacophore model of non-competitive AMPA antagonist 2,3-benzodiazepines. *Lett. Drug Des. Discovery 1*, 217−223.

(13) Zappala, M., Pellicano, A., Micale, N., Menniti, F. S., Ferreri, G., De Sarro, G., Grasso, S., and De Micheli, C. (2006) New 7,8-ethylenedioxy-2,3-benzodiazepines as noncompetitive AMPA receptor antagonists. *Bioorg. Med. Chem. Lett. 16*, 167−170.

(14) Ritz, M., Micale, N., Grasso, S., and Niu, L. (2008) Mechanism of inhibition of the GluR2 AMPA receptor channel opening by 2,3-benzodiazepine derivatives. *Biochemistry 47*, 1061−1069.

(15) Ritz, M., Wang, C., Micale, N., Ettari, R., and Niu, L. (2011) Mechanism of Inhibition of the GluA2 AMPA Receptor Channel Opening: the Role of 4-Methyl versus 4-Carbonyl Group on the Diazepine Ring of 2,3-Benzodiazepine Derivatives. *ACS Chem. Neurosci. 2*, 506−513.

(16) Wang, C., Sheng, Z., and Niu, L. (2011) Mechanism of inhibition of the GluA2 AMPA receptor channel opening: consequences of adding an N-3 methylcarbamoyl group to the diazepine ring of 2,3-benzodiazepine derivatives. *Biochemistry 50*, 7284−7293.

(17) Qneibi, M. S., Micale, N., Grasso, S., and Niu, L. (2012) Mechanism of inhibition of GluA2 AMPA receptor channel opening by 2,3-benzodiazepine derivatives: functional consequences of replacing a 7,8-methylenedioxy with a 7,8-ethylenedioxy moiety. *Biochemistry 51*, 1787−1795.

(18) Szenasi, G., Vegh, M., Szabo, G., Kertesz, S., Kapus, G., Albert, M., Greff, Z., Ling, I., Barkoczy, J., Simig, G., Spedding, M., and Harsing, L. G., Jr. (2008) 2,3-benzodiazepine-type AMPA receptor antagonists and their neuroprotective effects. *Neurochem. Int. 52*, 166−183.

(19) Peng, P. L., Zhong, X., Tu, W., Soundarapandian, M. M., Molner, P., Zhu, D., Lau, L., Liu, S., Liu, F., and Lu, Y. (2006) ADAR2-dependent RNA editing of AMPA receptor subunit GluR2 determines vulnerability of neurons in forebrain ischemia. *Neuron 49*, 719−733.

(20) Li, G., Pei, W., and Niu, L. (2003) Channel-opening kinetics of GluR2Q(flip) AMPA receptor: a laser-pulse photolysis study. *Biochemistry 42*, 12358−12366.

(21) Wieboldt, R., Gee, K. R., Niu, L., Ramesh, D., Carpenter, B. K., and Hess, G. P. (1994) Photolabile precursors of glutamate: synthesis, photochemical properties, and activation of glutamate receptors on a microsecond time scale. *Proc. Natl. Acad. Sci. U. S. A. 91*, 8752−8756.

(22) Li, G., and Niu, L. (2004) How fast does the GluR1Qflip channel open? *J. Biol. Chem. 279*, 3990−3997.

(23) Sheardown, M. J. (1993) The triggering of spreading depression in the chicken retina: a pharmacological study. *Brain Res. 607*, 189−194.

(24) Wang, C., Han, Y., Wu, A., Solyom, S., and Niu, L. (2014) Mechanism and site of inhibition of AMPA receptors: pairing a thiadiazole with a 2,3-benzodiazepine scaffold. *ACS Chem. Neurosci. 5*, 138−147.

(25) Pei, W., Huang, Z., Wang, C., Han, Y., Park, J. S., and Niu, L. (2009) Flip and flop: a molecular determinant for AMPA receptor channel opening. *Biochemistry 48*, 3767−3777.

(26) Chen, C., and Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol. 7*, 2745−2752.

(27) Huang, Z., Li, G., Pei, W., Sosa, L. A., and Niu, L. (2005) Enhancing protein expression in single HEK 293 cells. *J. Neurosci. Methods 142*, 159−166.

(28) Udgaonkar, J. B., and Hess, G. P. (1987) Chemical kinetic measurements of a mammalian acetylcholine receptor by a fast-reaction technique. *Proc. Natl. Acad. Sci. U. S. A. 84*, 8758−8762.

(29) Han, Y., Wang, C., Park, J. S., and Niu, L. (2012) Channel-Opening Kinetic Mechanism of Wild-Type GluK1 Kainate Receptors and a C-Terminal Mutant. *Biochemistry 51*, 761−768.

DOI: 10.1021/acschemneuro.5b00064
ACS Chem. Neurosci. 2015, 6, 1371−1378