Mechanism of inhibition of the GluA2 receptors by N-3 derivatives of 2,3-Benzodiazepines with C-4 methyl group

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Mechanism of Inhibition of the GluA2 Receptors by N-3 Derivatives of 2,3-Benzodiazepines with C-4 Methyl Group

by

Congzhou Wang

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ABSTRACT

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are one of the three subtypes of ionotropic glutamate receptors. AMPA receptors mediate fast synaptic neurotransmission in the central nervous system (CNS). Over-activation of calcium permeable AMPA receptors causes intracellular calcium overload, which leads to neurodegeneration and cell death. As such, AMPA receptors have been implicated in a number of neurological disorders and diseases, such as epilepsy, amyotrophic lateral sclerosis (ALS), and Parkinson’s disease. 2,3-Benzodiazepine derivatives (or GYKI compounds) are a group of structurally similar compounds synthesized as inhibitors of AMPA receptors, and they have been used as potential drug candidates for the treatment of various neurological disorders involving excessive activity of AMPA receptors. However, the detailed mechanism of action of these inhibitors has not been well understood, and a structure-activity relationship has not been defined at the molecular level.

To investigate the mechanism of action and establish a detailed structure-activity relationship at the molecular level, I used rapid kinetic techniques, primarily a laser-pulse photolysis technique that provided a microsecond time resolution, combined with whole-cell patch clamp recording. Specifically, I investigated the effect of an inhibitor on the channel-opening rate process that occurs within the microsecond-to-millisecond time span. I particularly focused on a group of compounds that all contain a C-4 methyl and additional functional groups attached to the N-3 position on the diazepine ring.
My results show several key findings. 

(i) These compounds are noncompetitive inhibitors of AMPA receptors with a preference of closed-channel over the open-channel conformation.

(ii) All of these compounds show a strong specificity for GluA2 and GluA1 AMPA receptor subunits but not GluA3 and GluA4.

(iii) The $R$ configuration, but not the $S$, of the 4-methyl group on the 2,3-benzodiazipine ring is crucial for the activity of resulting compounds as AMPA receptor inhibitors.

(iv) All of these 4-methyl group-containing compounds bind to the same noncompetitive site on the GluA2 receptors. This is the same site where GYKI 52446 binds as well.

(v) The N-3 on the 2,3-benzodiazipine ring is a desirable position in that attaching functional groups to this position strengthens potency, as compared with GYKI 52466, which is the prototypic compound of this family and is underivatized at N-3 position. Among those, compounds with heterocyclic functional groups attached to the N-3 position exhibit the strongest inhibitory potency.

These results are useful for design of new 2,3-benzodiazipine compounds with predictable molecular properties, including selectivity, potency and conformational specificity. My results also suggest new directions for developing more effective 2,3-benzodiazipine compounds as potential drug candidates for the treatment of neurological disorders and diseases involving over-activation of AMPA receptors.
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CHAPTER 1

INTRODUCTION

1.1 GLUTAMATE RECEPTORS

1.1.1 Ionotropic Glutamate Receptors

There are two different types of glutamate receptors that can be activated by L-glutamate in the central nervous system (CNS): metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors (iGluRs). Metabotropic glutamate receptors act through G proteins and therefore belong to G-protein coupled receptors (GPCRs). Ionotropic glutamate receptors are ligand-gated ion channels that mediate fast synaptic neurotransmission in the CNS. Glutamate molecules released from a pre-synaptic cell to a post-synaptic terminal bind to iGluRs expressed at the postsynaptic membrane. The binding of glutamate leads to a conformational change of a receptor, which causes the opening of the channel pore to allow small cations to flow through the channel. As a result, the membrane potential of the postsynaptic neuron becomes temporarily depolarized (1-3).

iGluRs are divided into three major subtypes by Watkins and coworkers (4): N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainate receptors (see Figure 1.1). They
are classified, in part, because these subtypes can be selectively activated by different glutamate analogs: NMDA, AMPA, and kainate, respectively. Yet, glutamate is the endogenous ligand.

Glutamate is the primary excitatory neurotransmitter in the vertebrate CNS (3). Fast glutamatergic neurotransmission at these synapses is predominantly mediated by two different types of iGluRs: AMPA receptors and NMDA receptors. Because NMDA receptors are blocked by magnesium ions at resting membrane potentials, AMPA receptors are responsible for most basal neurotransmission. Based on several decades of intense research, activity-dependent changes in excitatory synaptic efficacy such as long-term potentiation (LTP) and long-term depression (LTD) are believed to be cellular basis for learning and memory (5-7). NMDA receptors have classically been thought of as coincidence detectors in that coincidence of glutamate release and postsynaptic depolarization for the induction of long-term plasticity expressed as changes in AMPA receptors-mediated transmission (8, 9). In other words, changes in NMDA receptor function or expression can effectively modify the induction threshold for AMPA receptor-mediated plasticity, a phenomenon commonly known as metaplasticity (10). It is widely accepted that AMPA receptor regulation is a key component in the expression of postsynaptic forms of LTP and LTD, as well as homeostatic synaptic plasticity of excitatory synapses. There are two regulatory mechanisms by which AMPA receptors modulate synaptic plasticity: one is by regulation of its synaptic strength by trafficking and the other is by alterations in phosphorylation of its subunits (3). For instance, the insertion or removal of the receptor may increase or decrease the synapse strength (11-13). Unlike NMDA receptors and AMPA receptors, kainate receptors can be found at
both presynaptic and postsynaptic locations (14). Kainate receptors at hippocampal mossy fiber synapses control the synaptic integration and spike transmission (15). Presynaptically located kainate receptors may participate in the mechanism regulating the glutamate release in the hippocampus in the different synapses (14, 16).

1.1.2 Glutamate Receptor Genes and Receptor Assembly

*In vivo*, most of the iGluRs are thought to be heteromeric tetramers composed of receptor subunits from the same subtype (see Figure 1.1). iGluR subunits are encoded by at least six gene families (1, 3). For human, GRIA1-4 encode the four AMPA receptor subunits. Kainate receptor subunits are encoded by GRIK1-3 and GRIK4-5, and NMDA receptor subunits are encoded by GRIN1, GRIN2A-D, and GRIN3A-B, respectively. The primary sequences of rat AMPA receptors are shown schematically in Figure 1.2.

NMDA receptors usually are heterotetramers assembled from GluN1 and others, i.e., GluN2A-D and/or GluN3A/B (1, 3). AMPA receptor channels can be homomeric tetramers composed of four identical subunits: GluA1-4 (previously called GluR1-4 or GluRA-D). *In vivo*, however, most of the AMPA receptors are heterotetramers that usually contain two pairs of different subunits, as in the form of GluA1/2 or GluA2/3, the two prominent AMPA receptor channel types found in hippocampus (17). Crystallographic studies have indeed revealed a tetrameric structure of a dimer of dimers (see Figure 1.3) for an antagonist-bound rat GluA2 receptor (18). There are five kainate receptor subunits: GluK1-5 (1, 3). Among those, GluK1 and GluK2 can form homomeric
functional channels themselves, whereas GluK4 and GluK5 can only form functional
channels with GluK1 or GuK2 (19).
Figure 1.1 Ionotropic glutamate receptor subtypes and subunits.
Figure 1.2 The primary sequences of rat AMPA receptors. The alternative splicing and editing sites of AMPA receptor subunits are depicted schematically. The green boxes show the four membrane domains, including a re-entrant loop (i.e., M2). The orange region shows the Q/R editing site, which is unique to GluA2. The red spots show the position of R/G editing sites. The yellow regions show the flip/flop alternative splicing regions.
Figure 1.3 The crystal structure of the homotetrameric GluA2 receptor bound to a competitive antagonist. The left panel shows the view of the “broad” face of the receptor; the right panel, the view of the “narrow” face of the receptor. Each subunit is marked with a different color and consists of an extracellular amino-terminal domain (ATD), a ligand binding domain (LBD), and transmembrane domains (TMD). Figure 1.3 is from reference (18).
1.1.3 AMPA Receptors

Since my research focuses on the function and regulation of AMPA receptors, I provide a more detailed introduction on the structure and function of AMPA receptors.

AMPA receptors are expressed in various regions of the mammalian CNS, and are indispensable for brain functions like memory and learning (Reviewed by Dingledine et al, 1999 and Traynelis et al, 2010). Functional AMPA receptor channels can be either homomeric or heteromeric tetramers possibly composed of four subunits: GluA1-4 (1-3). Each subunit contains ~900 amino acids, and the molecular weight of each subunit is therefore around 105 kDa (20). The AMPA receptor subunits have similar molecular architectures (1). All the subunits have an extracellular N terminal domain and an extracellular agonist-binding domain formed by two segments, i.e., S1 and S2. S1 and S2 form two globular ligand binding domains called D1 and D2. The closure of the binding cleft between D1 and D2 induced by a full agonist molecule (e.g. L-glutamate) results in the channel opening. The topology of a single subunit, shown in Figure 1.4, containing three transmembrane domains, M1, M3, and M4 and a re-entrant membrane loop M2 (1, 3). Four subunits assemble into a functional tetrameric channel with the M2 domain lining the channel pore (21). It has been shown that the intracellular C terminus contains sites of protein-protein interaction that involves different cytoplasmic proteins in receptor trafficking and synaptic plasticity as well as phosphorylation (1, 22).

1.1.4 Post-transcriptional Modifications of AMPA Receptors

AMPA receptor subunits undergo post-transcriptional modifications (reviewed by Dingledine, 1999). Post-transcriptional modifications of AMPA receptor subunits are
realized by alternative splicing and RNA editing, which generates a higher functional diversity, as described below.

All the four AMPA receptor subunits are subject to alternative splicing that generates “flip” and “flop” splice variants or isoforms. The flip/flop splicing site is located at extracellular region between S2 and the M4 transmembrane domain (23) (see Figure 3A and B). The "flip" and "flop" variants differ in their kinetic and pharmacological properties. For instance, the "flop" splice variants desensitize several times faster than the "flip" variants for GluA2-4, but not for GluA1, and are less affected by an allosteric modulator cyclothiazide (1, 22, 24-27). In additional, an alternative splicing site locates at the C terminus of the GluA1, GluA2 and GluA4 subunits, yielding “long” isoforms (22, 28). The short isoform in GluA2 is more abundant, accounting for >90% of total GluA2 (29). For GluA4, the long isoform is predominant (28). However, the “short” isoform of GluA1 has not been reported, and GluA3 has a short C terminus that does not have a splicing site. The long/short splicing isoforms have been implicated in the interaction with different proteins in the C-terminal region. Only the short form contains the PDZ binding motif (1, 22, 30).

Post-transcriptional RNA editing causes single amino acid exchange in AMPA receptors (reviewed by Seeburg, 1996 (31)). One example is that a glutamine (Q) codon (CAG) in the M2 domain of GluA2 (shown in Figures 1.1B and 1.2) can be edited to an arginine (R) codon (CIG) in the primary transcript. This is due to the conversion from adenosine (A) to inosine (I) on the double-stranded RNA (dsRNA) by a hydrolytic deamination reaction catalyzed by the enzyme called adenosine deaminases acting on RNA (ADARs). This single amino acid exchange from Q to R (see Figure 1.2) results in
lowered channel conductance (32), lowered calcium permeability (33), and double rectification in both the homomeric and heteromeric receptors (34). This is thought to be due to the presence of the positive charge on the arginine's side chain at the critical region of the channel-forming domain of the receptor (Figure 1.4). The Q/R editing is developmentally regulated. GluA2 receptors found in healthy adult brain and spinal cord are nearly 100% edited (or in the R form) (35-37). However, the Q/R editing defect has been observed in epilepsy and amyotrophic lateral sclerosis (ALS) (38, 39). Another RNA editing site, which changes the desensitization and resensitization, occurs at the arginine/glycine (R/G) site of GluA2-4 (40), and this site is located in a region immediately before the flip/flop alternative splicing site in the extracellular region (shown in Figure 3B). The R/G editing site may be involved in epilepsy and ischemia (22).
Figure 1.4 The topology of an AMPA receptor subunit. Each subunit consists of an extracellular amino-terminal domain (ATD), two discontinuous extracellular domains S1 and S2 that form ligand binding domains D1 and D2, three transmembrane segments (M1, M3 and M4), a re-entrant loop (M2), and an intracellular C-terminal domain. The flip/flop alternative splicing site located at extracellular region between S2 and the M4 transmembrane domain results in variants with different channel's kinetic and pharmacological properties. The Q/R RNA editing site is exclusively associated with the GluA2 subunit (or precisely on the M2 domain of GluA2). This site determines channel conductance, calcium permeability, and double rectification in both the homomeric and heteromeric receptors from the Q to the R isoform. The R/G RNA editing site that changes the desensitization and resensitization is located at a region immediately before the flip/flop alternative splice region.
1.1.5 Excitotoxicity of AMPA Receptors and Neurological Disorders and Diseases

Elevated influx of calcium into the cytoplasm due to the glutamate-induced, over-activation of calcium permeable iGluRs may lead to neuron injury or death, which is termed excitotoxicity. AMPA receptor-mediated excitotoxicity underlies a number of neurological disorders and diseases such as epilepsy, ALS, and Parkinson’s disease, etc. (41-45). For example, ALS is a progressive motor neuron disease. The pathogenic hallmark of ALS is the selective loss of motor neurons, the nerve cells in CNS that control muscle movement via axons (46, 47). About 90% of all ALS cases are sporadic while less than 10% of ALS cases are familial or inherited. In the latter, known genetic mutations in various genes cause the disease (48). For instance, one of the common types of mutations in familial ALS is caused by mutation in the superoxide dismutase-1 gene (SOD1) on chromosome 21q22.1. To the contrary, the pathogenesis of sporadic ALS remains unclear; yet a number of hypotheses have been proposed. Among those, excitotoxicity seems to be a leading pathogenic mechanism (47) for both familial and sporadic ALS cases (49-52). Specifically, RNA editing efficiency at the Q/R editing site on the GluA2 subunit has been found incomplete in the motor neurons of ALS patients as compared to nearly 100% in the healthy human controls (53). As such, abnormal expression of calcium permeable, unedited GluA2 or GluA2-containing AMPA receptors makes motor neurons more vulnerable to glutamate insult.

AMPA receptors have also been shown to play a pivotal role in the delayed neurological degeneration after ischemic stroke. Ischemic stroke is a rapid loss of brain functions due to the insufficient blood supply to the brain (54). When the brain tissue is deprived of oxygen and glucose transported by the blood, the concentration of glutamate
outside neurons will increase due to the shutdown of the uptake carriers and the depolarization of the pre-synaptic membrane. Consequently a higher glutamate concentration leads to overactivation of AMPA receptors, and the AMPA receptor-mediated excitotoxicity causes cellular injury and even death (55).

Because excessive activity of AMPA receptors causes neurodegeneration, antagonists of AMPA receptors are shown to be neuroprotective (21, 56-58) and can be used as drugs for potential treatment of neurological disorders and diseases (54, 59, 60). For instance, AMPA antagonists are highly potent neuroprotectants in rodent and gerbil models of global forebrain ischemia (61-64). Although AMPA receptor antagonists have shown remarkable neuroprotective effects in animal models (1, 21, 65), none of these antagonists have yet succeeded in clinical trials. Instead, almost all existing AMPA receptor inhibitors show considerable side effects in clinical trials but insignificant, if any, therapeutic outcomes.

1.2 AMPA RECEPTOR ANTAGONISTS AND 2,3-BENZODIAZEPINE DERIVATIVES

1.2.1 Types of AMPA Receptor Antagonists

Structurally different classes of AMPA receptor antagonists have been made, and they show different properties. In 1980s, 2,3-dihydroxy-6-nitro-7-sulphamoylbenzo[F]quinoxaline (NBQX) was synthesized (66) and was first shown to have therapeutic values in animal models of neurological diseases. The quinoxaline template has since then become the prototypic template of synthesizing various competitive
AMPA receptor antagonists with better potency, selectivity and water solubility. These properties are thought to be desirable to translate inhibitors into clinical drugs with better pharmacokinetic properties and less serious adverse effects. The new compounds now include isatin oximes, decahydroisoquinolines and isoxazole derivatives (67-69). However, drugs that act as competitive inhibitors are generally not considered desirable, because they become less effective when agonist or glutamate concentration becomes higher; glutamate concentration does become higher under to excitotoxic conditions (65, 70). Furthermore, a competitive inhibitor generally does not discriminate against different subunits or isoforms of either iGluRs in general or AMPA receptors in particular. Consequently, these compounds bind and act “promiscuously”, causing often a greater adverse effect clinically.

The second class of AMPA receptor agonists is uncompetitive inhibitors or open-channel blockers, although the list of these compounds that block the open channels gated by AMPA/kainate receptors is very short. These compounds include mostly polyamine molecules, such as philanthotoxin-343 (PhTX-343) (71), argiotoxin-636 (ArgTX-636) (71, 72), and Joro spider toxin (JSTX) (73), and adamantane derivatives (74).

1.2.2 Noncompetitive AMPA Receptor Antagonists

Noncompetitive antagonists of AMPA receptors represent a growing list of compounds that have been recognized as a superior class, because their selectivity towards AMPA receptors is better (since they bind to sites distinct from the agonist binding site) (65, 75). One well-known family of AMPA receptor inhibitors are 2,3-
benzodiazepine derivatives, also referred as GYKI compounds. There are also several different types of noncompetitive AMPA receptor inhibitors that are structurally unrelated to 2,3-benzodiazepines, such as phthalazine derivatives, aryl-quinazoline-4-one derivatives, and phenyl-1,2,3-oxadiazolyl-phenoxo-ethylamine (65). All of them have shown strong inhibitory effect on AMPA receptors (21).

As the first member of 2,3-benzodiazepine derivatives, GYKI 52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine; see the chemical structure in Figure 1.5) was developed by Tarnawa et al. in 1989 (76). Donevan and Rogawski (77) later showed GYKI 52466 caused a concentration-dependent reduction in the maximal current mediated by AMPA receptors, but did not affect the EC50 value, suggesting that this compound noncompetitively inhibited AMPA receptors. To date, hundreds of compounds have been designed and synthesized from the original structure (65, 76).

1.2.3 Current Understanding of the Mechanisms of Action for 2,3-Benzodiazepines

The exact mechanism by which 2,3-benzodiazepines exert their inhibitory effects is not well understood. Initially, 2,3-benzodiazepines were thought to act to enhance AMPA receptor desensitization (78). This hypothesis was supported by the observation that benzothiadiazine cyclothiazide, which slows AMPA receptor desensitization but potentiates receptor currents (78-81) could reverse antagonistic effects of 2,3-benzodiazepines (78, 80, 82). However, electrophysiological studies with the use of rapid perfusion techniques showed that 2,3-benzodiazepines did not affect the rate of AMPA
receptor desensitization (77, 83) and that both cyclothiazide and 2,3-benzodiazepines could bind to their respective sites independently (84, 85). Therefore, a conclusion was drawn, suggesting that inhibition of AMPA receptors by 2,3-benzodiazepines is not the result of an enhancement of desensitization (84, 85).

The conclusion that GYKI compounds are possibly noncompetitive AMPA receptor inhibitors also came from equilibrium binding studies using of [³H]AMPA. These studies show that 2,3-benzodiazepines do not share the same binding site on AMPA receptors with agonists or positive modulators like cyclothiazide (83). For example, thiocyanate (SCN⁻), a chaotropic anion, is thought to enhance [³H]AMPA binding to brain membranes (86, 87), by acting as an allosteric inhibitor of AMPA receptors (88). A study by Donevan and Rogawski (89) on 2,3-benzodiazepines, together with SCN⁻ and cyclothiazide using electrophysiological recording on native AMPA receptors, showed that 2,3-benzodiazepines and SCN⁻ block AMPA receptor currents in a noncompetitive fashion, most likely by binding to individual allosteric regulatory sites; yet these sites are not the agonist binding site.

Based on the study using site-specific mutagenesis and electrophysiological assay, Yael Stern-Bach and coworkers (90) proposed that a GYKI binding site is located at the linker region between the transmembrane domain and the S1 and S2 segments. They suggested that the interaction between a GYKI compound and the S1-M1/S2-M4 linker region stabilizes the conformation of these linkers, and interrupts the conformational change induced by the binding of agonists that initiates the channel-opening reaction. Despite the fact that a number of high resolution structural studies have been conducted such as those using NMR and X-ray crystallographic studies (91-97), including a study
that revealed the structure of a total, homomeric AMPA receptor channel (18), to date, there is no structural information for noncompetitive sites. An attempt to map the detailed site(s) for any noncompetitive inhibitors of AMPA receptors has not been reported.
Figure 1.5 Chemical Structures of GYKI Compounds. This list contains a total of 19 2,3-benzodiazepine derivatives used in our research. These 19 compounds are classified into two groups. Group 1 compounds were synthesized by Dr. Solyom’s group and are named from $a$ to $h$ with a prefix of BDZ. Group 2 were from Professor Grasso’s group and are called BDZ-0 to BDZ-12. The only exception is GYKI 52466, which keeps its most common name for the purpose of easy reference.
1.2.4 2,3-Benzodiazepine Derivatives and Structure-Activity Relationship

The discovery of 2,3-benzodiazepine derivative GYKI 52466 as a selective AMPA antagonist (Figure 1.5) in the late eighties and the recognition of advantages of using noncompetitive antagonists of AMPA receptors as drug candidates, as compared with competitive antagonists, has set off an extensive effort to developing more potent and more selective 2,3-benzodiazepine compounds (65). To date, hundreds of 2,3-benzodiazepine compounds have been synthesized. To develop them into better inhibitors and drugs, a structure-activity relationship study is required.

What is the structure-activity relationship? The structure–activity relationship is a relationship between the chemical structure of a molecule and its biological activity, but often this relationship is described in a group of structurally similar compounds. Compounds are often grouped together because they have structural characteristics in common including shape, size, stereochemistry and distribution of functional groups. Understanding a structure-activity relationship for a group of structurally similar compounds will enable the determination of a chemical group(s) responsible for evoking a biological effect at the target. In turn, chemical structures can be changed in a predictable way to improve the potency of a bioactive compound (typically a drug).

For hundreds of 2,3-benzodiazepine derivatives, previous studies of the structure-activity relationship have been based virtually all on classical, biological assays, such as the maximal electroshock seizures (MES) and the excitatory amino acids induced spreading depression in isolated chicken retina (SD). Through these studies, several structural features that are favorable to the inhibitory activities of those derivatives have
been revealed. For instance, the 4-amino group on the phenyl ring (see the first structure or structure for GYKI 52466 for the naming of atoms in Figure 1.5, labeled in red color) was proven to be indispensable for strong antagonistic effects. The transposition of this group is not favorable for the activity. Enlargement or breakage of the dioxolane ring tends to diminish the antagonism (Figure 1.5). In addition, an sp2 hybridization at carbon 4 of the ring system with an adjacent H-bond acceptor group is favorable for the biological activity (65).

The 7,8-methylenedioxy group seems to have a strong relationship with some unique properties GYKI 52466. Replacement of this moiety by two methoxy groups or two halogen atoms has been found to increase the potency in MES as compared to GYKI 52466 (65). However, the single substitution with a 7-methoxy or 7-halogen atom usually eliminates the antagonism from new compounds, whereas an additional substitution at C-8 can recover the activity (65).

The single substitution of the 4-methyl group with a carbonyl oxygen results in a higher potency and lower toxicity in the MES and other biological assays (65, 75). However 1-(4-aminophenyl)-3,5-dihydro-7,8-methylenedioxy-2,3-benzodiazepine-4-one (BDZ-2) (Figure 1.5) did not show much improvement as compared with GYKI 52466 in the whole-cell recording experiment of AMPA receptors, as reported from our lab (98, 99). The saturation of the 3,4-double bond and a new methylcarbamoyl group at N-3 position gives rise to 1-(4-aminophenyl)-3-N-methylcarbamoyl-7,8-methylenedioxy-5H-2,3-benzodiazepine-4-one (BDZ-3) (Figure 1.5). BDZ-3 has an improved potency, albeit to a lesser degree than BDZ-2 has, in biological assays (65, 75). One possible explanation is that the 3-N-methylcarbamoyl group is bulky and may therefore prevent the
conformational change from the loose, initial binding intermediate to the tighter, fully inhibited complex as compared to BDZ-2 (the chemical structures of BDZ-2 and BDZ-3 are shown in Figure 1.5) (99).

By replacing the ortho hydrogen on the 4-aminophenyl ring with various groups, studies showed a striking improvement in the biological activity of the resulting compounds as compared with GYKI 52466. The rank order of the substitution in the AMPA antagonist potency was determined to be $\text{CH}_3 \approx \text{Cl} > \text{OH}, \text{OCH}_3$ (21, 100).

1.2.5 Current Deficiencies in the Understanding of the Mechanism and the Structure-Activity Relationship for 2,3-Benzodiazepines

Two areas of deficiency have thus far seriously hampered the development of GYKI compounds into potentially powerful therapeutics targeting AMPA receptors.

First, in drug development, functional assays are required in evaluation of structure-activity relationship studies. However, among 6-7 most widely used biological tests (65), no method exists to characterize the mechanism of inhibitor/drug-receptor interaction and the structure-activity relationship within the microsecond ($\mu$s)-to-millisecond (ms) time domain. This time resolution is required because an AMPA receptor opens its channel in the $\mu$s time scale (101) and desensitizes within a few ms in the continued presence of glutamate (102). A noncompetitive inhibitor, for example, is presumed to inhibit both the ligand-bound, closed-channel state and the open-channel state. Thus, to measure the receptor channel opening and the mechanism of inhibition, a kinetic technique with a $\mu$s time resolution is required. Otherwise, any mechanistic information obtained with
insufficient time resolution would be most likely relevant to the desensitized receptor, which is an inactivated, channel-closed state with agonist remaining bound. In fact, the conclusion that GYKI compounds are noncompetitive AMPA receptor inhibitors and their corresponding $IC_{50}$ values largely came from equilibrium binding studies using of $[^3H]$AMPA, where only the desensitized receptor form(s) were present. How these inhibitors act when the channel is in the functional state, i.e., non-desensitized state, is therefore unclear. Single-channel recording can measure inhibitor kinetics and has worked elegantly with other types of ligand-gated channels, such as nicotinic acetylcholine receptors (103-106). To date, however, no report has yet appeared for the study of inhibitor/drug-receptor interaction with AMPA channels. This is most likely because an AMPA channel has a very short mean burst length or lifetime (96, 107), making it perhaps extremely difficult to resolve the “flickering” in open channel bursts in the presence of inhibitors (103, 104). On the other hand, rapid solution flow techniques are used routinely, but these techniques provide a time resolution of ~200 µs (1), which is insufficient to measure the channel-opening kinetics of AMPA receptors.

Second, there is a lack of systematic study of the structure-activity relationship of GYKI compounds for rational design of subunit-selective and conformation-selective compounds, nor a study revealing binding characteristics of structurally similar compounds, despite the fact that hundreds of GYKI compounds have been synthesized (65). A detailed characterization of the structure-activity relationship at the molecular level, rather than at a whole organism level (65), is required, because all of these compounds were supposed to specifically inhibit the AMPA receptors. As such, a comprehensive study or functional screening of these compounds against other glutamate
receptor subtypes and subunits other than AMPA receptors is also required. It would be desirable to identify and remove any structural motif that potentially induces cross activity with either NMDA or kainate receptors.

1.3 OVERALL RESEARCH OBJECTIVE AND SIGNIFICANCE

My thesis work focused on a rapid kinetic investigation of the mechanism of action and the structure-activity relationship for 2,3-benzodiazepine compounds. Specifically, I have singled out four compounds that represent perhaps the most potent 2,3-benzodiazepines, after a functional screening of the compounds shown in Figure 1.5. These compounds, i.e., BDZ-d (with its stereoisomer, BDZ-e), BDZ-f, BDZ-g, and BDZ-h, represent a group of inhibitors with the 4-methyl group but N-3 derivatization as compared with GYKI 52466. Among these compounds, BDZ-d and BDZ-e are a pair of stereoisomers; BDZ-f has an additional N-3 methylcarbamoyl group as compared with GYKI 52466; BDZ-g, and BDZ-h are structurally similar but significantly different from all other compounds in this family (Figure 1.5) in that there is a heterocyclic ring structure at the N-3 position in either one of these compounds.

With these compounds, my aim was to characterize (i) the mechanism of action, (ii) the inhibition constants for both the open- and the closed-channel conformations of the GluA2Qflip receptor channels, and (iii) the site of binding relative to GYK52466 (or competition binding probed by the kinetic measurements).

To make these studies possible, I have used a rapid kinetic technique, i.e., a laser-pulse photolysis technique and a photolabile precursor of glutamate or caged glutamate.
(e.g., 4-methoxy-7-nitroindolyl-caged-L-glutamate or MNI-caged L-glutamate). This technique provided a microsecond time resolution, sufficient to measure the rate of channel opening, and thus the effect of an inhibitor on the rate process.

My study is significant because of the following reasons.

(a) My results are generally useful. Excessive activities of AMPA receptors have been strongly implicated in various neurological diseases such as epilepsy, stroke, ALS and Parkinson’s disease. Therefore my data from the proposed in-depth investigation of the mechanism of action and the structure-activity relationship are broadly useful for development and use of 2,3-benzodiazepines as therapeutics for these neurological diseases.

(b) There is new mechanistic information from my study. My studies reveal the mechanism of action of these inhibitors with functional receptor states of GluA2 prior to the channel desensitization. Furthermore, the affinity of an inhibitor for the closed- and the open-channel states are separately obtained. As such, the most effective way of using such an inhibitor can be established (e.g., a closed-channel preferred inhibitor is more effective at a lower agonist concentration). Therefore, the mechanistic information obtained is useful for designing and synthesizing more potent and more subunit-selective GYKI noncompetitive inhibitors in the future in a more predictable, mechanism-driven fashion. It should be emphasized that my study was carried out at a physiologically relevant time scale, which has not been previously possible. Obtaining these results is an important step towards developing and achieving a more quantitative control of excessive receptor activity by using these inhibitors as drugs.
(c) Critical properties of the inhibitory sites on GluA2 are revealed. A double-inhibitor experiment reveals not only the number of inhibitory sites on the GluA2 receptor but, more importantly, whether these sites “cross talk” to each other. For example, if two inhibitory sites are negatively affected by occupancy of either site first, using two inhibitors as drugs for double occupancy is therapeutically counterproductive. In addition, using two inhibitors at the same time that compete for the same site will be also less productive than using two inhibitors that bind to separate sites, resulting in an “additive” inhibition. For these reasons, my results provide much needed insights into the properties of the putative inhibitory sites on GluA2. Without the information, the use of these inhibitors as therapeutics will not likely produce predictable therapeutic outcome.

1.4 MATERIALS AND METHODS

1.4.1 The Advantage of the Laser-Pulse Photolysis Technique

The traditional fast solution-exchange method (flow measurement) has a time resolution of ~0.2 ms (101). This time resolution is not good enough to measure the channel-opening reaction of AMPA receptors that occurs in the μs-to-ms time scale, separate from fast desensitization reaction on a millisecond time scale, and is unsuitable for investigation of a detailed mechanistic study of the mechanism of inhibition of AMPA receptors (101).

The laser-pulse photolysis technique our lab has used enables us to deliver free glutamate to the cell on a μs time scale through rapid photolysis of MNI-caged-L-glutamate so that one can measure the channel-opening kinetics with a μs time resolution.
The caged glutamate (Figure 1.6A) has no activity on glutamate receptors, i.e., it is neither an agonist nor an inhibitor, but can be rapidly photolyzed to release free glutamate (300–380 nm excitation). At room temperature the photolysis reaction of MNI-caged-L-glutamate has a time constant of ~200 ns (108), which is suitable for measuring the channel-opening rate of AMPA receptors. In a laser-pulse photolysis experiment, the caged glutamate was first allowed to equilibrate with the receptors. Then a single laser pulse was delivered to photolyze the caged glutamate, releasing free glutamate. The photolytically released glutamate would then bind to the receptor, evoking the whole-cell current. This technique provides a sufficient time resolution that helps us investigate the mechanism of the rapid channel-opening reaction, and therefore characterize how an antagonist acts on the receptor before the receptor becomes desensitized or transiently inactivated.

It should be emphasized that studying the kinetics of the inhibition of 2,3-benzodiazepines using this technique has allowed me to characterize the inhibition mechanism and structure-activity relationship of those compounds on a time scale relevant to its function, which has not been previously possible. Furthermore this study has yielded useful mechanistic information for design of new inhibitors and drug candidates that would have higher potency and selectivity.

1.4.2 Preparation of AMPA, Kainate, and NMDA Receptor cDNAs

My thesis work focused on AMPA receptors. However, to identify AMPA receptor-selective inhibitors and carry out mechanistic studies, I also tested these inhibitors with
kainate and NMDA receptors. This is because any activity from an inhibitor on either kainate or NMDA receptors will be viewed as unwanted, side effect and may diminish the potential of an inhibitor to become a drug candidate specifically targeting the AMPA receptor subunits or subtype.

The rat AMPA receptor subunits, i.e., GluA1-3, and GluK2 subunit cDNAs were generously provided by Dr. S. F. Heinemann (Salk Institute for Biological Studies). GluA4 and GluK1 were from Dr. P. Seeburg from (Max Planck Institute for Medical Research, Germany). The cDNA plasmids encoding the rat GluN1a, GluN2A, GluN2B subunits were generously provided by Dr. J. J. Woodward (Medical University of South Carolina). All of the AMPA and kainate receptor genes were subcloned into the pcDNA3.1 vector (Invitrogen, CA). The plasmids were propagated in the *Escherichia coli* host (DH5α) and purified using a kit from QIAGEN (Valencia, CA) (101).

### 1.4.3 Transient Transfection of Receptors in HEK-293S Cells and Cell Culture

In order to study the effect of a GYKI compound on a receptor described above, the receptor must be expressed in a heterologous expression system. In my study, I used human embryonic kidney 293S (HEK-293S) cells for transient expression of the receptors. The protocol of transient transfection is as follows.

HEK-293S cells were grown in 35×10 mm tissue culture dishes (Becton Dickinson and Company, Franklin Lakes, NJ) filled with ~2 mL Dulbecco’s modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA), containing 10% fetal bovine serum (FBS, Invitrogen), 100 units of penicillin/mL and 0.1 mg streptomycin/mL (Sigma-Aldrich, St.
Louis, MO). The cells grew in a humidified incubator set at 37 °C, 5% CO₂. The cells were transferred to a new dish 24 hr prior to transfection, and grew to ~50% confluency. The transfection for GluA1-4 and GluK1-2 cDNAs followed the standard calcium phosphate method (109). In each transfection, 2~30 µg of the desired cDNA plasmid was added into the transfection solution which also contained a plasmid encoding green fluorescent protein (GFP) and an additional plasmid encoding simian virus large T-antigen (TAg) at a ratio of 5:1:0.5. The transfection for GluN1a, GluN2A/B cDNAs in HEK0293S cells followed the Lipofectamine 2000 protocol (Invitrogen). In each transfection, 15 µg of the GluN1a and 15 µg of the GluN2A (or GluN2B) containing vectors were added into the transfection solution with 0.5 µg of enhanced green fluorescent protein (eGFP). The medium in which NMDA-receptor-transfected cells grew was supplemented with 0.3 mM ketamine. The cells were usually ready for recording 48 hr after transfection.

1.4.4 Whole-cell Recording and Flow Measurement

Whole-cell recording technique was used for measuring the glutamate-induced receptor response in the presence and absence of an inhibitor. Whole-cell current was recorded with an Axopatch 200B and pCLAMP 8.0 software (MDS) at -60 mV and 25 °C. Electrode resistance was ~3 MΩ after an electrode was filled with the internal buffer: 110 mM CsF, 30 mM CsCl, 4 mM NaCl, 0.5 mM CaCl₂, 5 mM EGTA, and 10 mM HEPES (pH 7.4 adjusted by CsOH). The extracellular buffer contained 150 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES (pH 7.4 adjusted by
NaOH). In the experiments for NMDA receptors, the electrode solution contained 140 mM CsCl, 1 mM MgCl₂, 0.1 mM EGTA, and 10 mM HEPES (pH 7.2 adjusted by CsOH). The extracellular buffer contained 135 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 10 mM glucose, and 5 mM HEPES (pH 7.2 adjusted by NaOH) (110). As the NMDA receptor co-agonist, glycine was added to all the extracellular buffer and inhibitor solutions with the final concentration of 100 µM. Transfected cells were re-plated into a 35×10 mm tissue culture dish 1-3 hr prior to recording. The culture medium was then replaced with the corresponding extracellular buffer.

One of the techniques I used to apply ligand or glutamate with and without an inhibitor was a solution flow technique. This technique contained a Ψ-shaped device composed of a U-tube and a central tube (see a schematic drawing of this device in Figure 1.7). During whole-cell recording, each cell was tested for its response to glutamate at a certain concentration, which was delivered from the hole on the U-tube in the Ψ-shaped device (Figure 1.7). Pure glutamate solution was used as control to calculate the inhibitory effect, and was also used to determine if the cell/seal was stable under repeated exposures of glutamate to the same cell or after a laser flash. The central tubing in the Ψ device was filled with pure inhibitor solution for pre-incubation of inhibitor with a cell voltage-clamped at -60 mV. Based on my studies, I found that it took 8 sec for an inhibitor to fully equilibrate with receptors with a HEK-293 cell, consistent with all studies conducted in our lab (98, 99, 111). A reduction of the current response in the presence of an inhibitor was verified by the use of control before and after the exposure of inhibitor. The control was also used to ensure that the receptor/cell was fully recovered from the inhibition (after the exposure of first inhibitor solution and then
external buffer). The current response I collected included the amplitude and the desensitization rate profile from which the rate of desensitization was also measured (see 1.4 DATA ANALYSIS AND RESEARCH DESIGN).

1.4.5 Laser-Pulse Photolysis Measurement

The laser-pulse photolysis technique was used to measure the channel-opening and channel-closing constants ($k_{op}$ and $k_{cl}$) in the channel-opening reaction of AMPA receptors. The U-tube on the Ψ-shaped device delivered caged glutamate, such as MNI-caged-L-glutamate (Tocris Bioscience, MS), to the cell in absence and presence of the inhibitor. The cell was equilibrated with MNI-caged-L-glutamate for at least 250 ms before a laser pulse was applied to liberate free glutamate (see the photochemical reaction that releases free glutamate from MNI-caged glutamate in Figure 1.6A). As shown in Figure 1.6B, a single laser pulse at 355 nm generated by a pulsed Q-switched Nd:YAG laser (Continuum, CA), with a pulse length of 8 ns and energy varying in the range of 200-1000 µJ, was transmitted via optical fiber (300 µm core diameter) to irradiate the cell. The power of the laser and/or the concentration of the MNI-caged-L-glutamate were varied to obtain different concentrations of photolytically released glutamate. Two consecutive controls using free glutamate applied by the flow device described above were used to calibrate the amplitude of the laser-induced current by comparing each other with reference of the dose-response relationship. One more glutamate control with the same glutamate concentration was used to monitor any damage to the receptors and/or
the cell. The inhibitor was delivered to the cell by the same way as described in the flow measurement.
**Figure 1.6**  (A) The photolysis reaction of MNI-caged-L-glutamate releases free glutamate; (B) A single laser pulse at 355 nm generated by a pulsed Q-switched Continuum Nd:YAG laser is applied on the cell via optical fiber in order to liberate free glutamate. The cell is equilibrated with caged glutamate delivered by the U-tube before the application of laser.
Figure 1.7 The Ψ-shape flowing device used in the solution flow and the laser-pulse photolysis experiments. The left part is a magnified image of the device tip. A is the center tube that delivers the inhibitor solution; B, the U-tube that delivers the glutamate solution; C, a HEK 293S cell attached on a glass electrode pipette. The size of the hole is about 120 nm.
1.5 EXPERIMENTAL DESIGN AND DATA ANALYSIS

1.5.1 Channel-opening Kinetics of AMPA Receptors

In the measurement of channel-opening rate process of GluA2Qflip induced by a laser-pulse photolysis I have consistently observed a single-exponential rate expression for \( \sim 95\% \) of the rising phase \((101)\). As such, a first order constant, \( k_{\text{obs}} \), can be estimated for the rising phase of whole-cell current by using Equation 1,

\[
I_t = I_{\text{max}}(1 - e^{-k_{\text{obs}}t})
\]

(Eq. 1)

where \( I_t \) represents the current amplitude at time \( t \), \( I_{\text{max}} \) the maximum current amplitude.

If \( k_{\text{obs}} \) represent the rate of channel opening, the mechanism of the channel opening can be rationalized using Figure 1.8A, which shows a minimal mechanism \((101, 112)\). On the basis of this mechanism of channel opening, Equation 2 is derived for the observed rate constant of the whole current rise, \( k_{\text{obs}} \) \((101)\).

\[
k_{\text{obs}} = k_{\text{cl}} + k_{\text{op}}\left(\frac{L}{L + K_1}\right)^2
\]

(Eq. 2)

In deriving this equation using the mechanism shown in Figure 1.8A, it is assumed that the rate of glutamate (and/or an inhibitor) binding is fast relative to the rate of channel opening. This assumption is consistent with the observation of a single-exponential expression for \( \sim 95\% \) of the rising phase \((101)\). With a series of known \( k_{\text{obs}} \) as a function of glutamate concentration, both \( k_{\text{cl}} \) and \( k_{\text{op}} \) can be then estimated by a linear fit of the data to Equation 2. Note when glutamate concentration is low (i.e., \( L << K_1; K_1 \) is the...
intrinsic equilibrium dissociation constant), Equation 2 can be reduced to $k_{\text{obs}} \approx k_{\text{cl}}$. This shows that at a low glutamate concentration, the magnitude of $k_{\text{obs}}$ is reflective of the channel-closing rate constant (101).
Figure 1.8 (A) A minimal mechanism for the channel-opening reaction of the AMPA receptor. In the scheme, \( A \) represents the active, unliganded form of the receptor, \( L \) the ligand (glutamate), \( AL \) and \( AL_2 \) the ligand-bound closed channel forms, and \( AL_{\text{op}} \) the open channel form of the receptor, \( k_{\text{op}} \) the channel-opening rate constant, \( k_{\text{cl}} \) the channel-closing rate constant. For simplicity and without contrary evidence, it is assumed that glutamate binds to the two sites with equal affinity, represented by the same intrinsic equilibrium dissociation constant, \( K_1 \). (B) A minimal mechanism for the non-competitive inhibition of the AMPA receptor. \( K_I \) represents the inhibition constant associated with closed-channel state of the AMPA receptor, \( K_{I,1} \), the inhibition constant associated with open-channel state, \( I \), the inhibitor, \( k_{\text{op}}' \) the channel-opening rate constant of the inhibited AMPA receptor, \( k_{\text{cl}}' \) the channel-opening rate constant of the inhibited AMPA receptor. All the species with a bar refer to open-channel state.
1.5.2 Determination of Inhibition Constants Using Rate Measurement

The mechanism of inhibition of AMPA receptors can be rigorously investigated by measuring the effect of an inhibitor on $k_{\text{cl}}$ and $k_{\text{op}}$. Based on the previous studies of structurally similar GYKI compounds published from our group (98, 99, 111), a minimal mechanism of inhibition of the channel-opening reaction of the GluA2Qflip AMPA receptor can be described by the scheme shown in Figure 1.8B. In this mechanism, an inhibitor is assumed to act as a noncompetitive inhibitor. As such, an inhibitor will affect the channel-opening rate process or $k_{\text{obs}}$ as formulated in Equation 3

$$k_{\text{obs}} = k_{\text{cl}} \left( \frac{K_1'}{K_1' + I} \right) + k_{\text{op}} \left( \frac{L}{L + K_1} \right)^2 \frac{K_1'}{K_1' + I} \quad \text{(Eq. 3)}$$

where $K_1'$ represents the inhibition constant for closed-channel state, $\overline{K}_1'$ the inhibition constant for open-channel state, $I$ the inhibitor at a molar concentration. A further assumption is made when $L << K_1$, thus giving rise to Equation 4. Inspection of Equation 4 shows that the effect of the inhibitor on the $k_{\text{cl}}$ can be determined or $\overline{K}_1'$ can be estimated from the linear form of this equation.

$$\frac{1}{k_{\text{obs}}} = \frac{1}{k_{\text{cl}}} + \frac{1}{k_{\text{cl}} \overline{K}_1'} \quad \text{(Eq. 4)}$$

At a higher glutamate concentration, $K_1'$ is determined from the difference between $k_{\text{obs}}$ and $k_{\text{cl}}'$, as shown in Equation 5 (99, 113).
\[(k_{\text{obs}} - k_{\text{cl}})'^{-1} = \left[\frac{k_{\text{op}}L^2}{(L + K_1)^2}\right]^{-1} (1 + \frac{I}{K_1}) \]

(Eq. 5)

Thus from a linear plot, \(K_1'\) can be measured. If an inhibitor is a competitive inhibitor, the \(k_{\text{cl}}\) term in unaffected in Equation 3. In other words, \(k_{\text{cl}}\) is not inhibited. On the other hand, if an inhibitor is uncompetitive, the \(k_{\text{op}}\) term is unaffected in Equation 3. In this case, \(k_{\text{op}}\) is not inhibited. Therefore, by measuring the effect of an inhibitor on both the \(k_{\text{op}}\) and the \(k_{\text{cl}}\), one can determine the mechanism of inhibition.

1.5.3 Determination of \(K_1\) from Dose-Response Relationship Using Flow Measurement

On the basis of the channel-opening mechanism or Figure 1.8A, Equation 6 is derived to describe the dose-response relationship where the current amplitude is a function of glutamate concentrations. In this case, the measurement of the whole-cell current response can be readily obtained by using the U-tube flow device and a complete range of free glutamate concentrations, namely from a low to a saturation concentration of glutamate (e.g., from 0.1 to 10 mM glutamate for GluA2). From a dose-response curve, \(K_1\) can be estimated with the use of Equation 6 using a non-linear regression program such as Origin 7.0 software (OriginLab, Northampton, MA).

\[I_A = I_M R_M \frac{L^2}{L^2 + \Phi(L + K_1)^2} \]

(Eq. 6)
In Equation 6, $I_A$ represents the maximum current amplitude, $I_M$ the current per mole of receptor, $R_M$ the number of moles of receptors on the cell surface, $\Phi^{-1}$ the channel-opening equilibrium constant.

The current amplitude was used for constructing a dose-response curve. However, it should be mentioned that the current amplitude obtained from the flow measurement (not from the laser-pulse photolysis measurement) was corrected for the receptor desensitization during the rise time by using Equation 7 and Origin 7.0 (114). In the flow measurement, due to the uneven flow rates of the glutamate over the cell surface, each receptor on the membrane might not be evoked by glutamate at the same time. Some of the receptors have desensitized while some others are in the channel-opening procedure or even unliganded forms. The correction allows deduction of the desensitization from the rising phase of the observed current amplitude ($A_{obs}$) and produces the total current amplitude ($A$) assuming no desensitization occurred during the initial rising phase.

$$A = (e^{n\Delta t} - 1) \sum_{i=1}^{n} (A_{obs})\Delta t_i + (A_{obs})\Delta t_n$$

(Eq. 7)

In this method the time course of the current is first divided into a constant time interval ($\Delta t$), and then the observed current is corrected for the desensitization that occurs during each time interval. $A$ obtained from this correction method is independent of the solution velocities (101, 115). The correction program was generously provided by Prof. George P. Hess from Cornell University.
1.5.4 Determination of Inhibition Constants Using Flow Measurement

A solution flow device such as the $\Psi$ device described earlier was used to determine the apparent overall inhibition constant for both a closed-channel ($K_1$) and an open-channel state ($K_0$) for a compound that inhibited a receptor channel. In order to obtain the constants for the two states, two separate measurements are needed, which are defined by the use of two different glutamate concentrations. Specifically, glutamate concentrations of 100 µM and 3 mM were chosen for measuring the homomeric GluA2Qflip receptor, which corresponds to the fraction of the open-channel form at ~4% and at ~95%, respectively (98, 99, 111).

When current amplitude is collected in the presence ($A_I$) and absence ($A$) of an inhibitor, the overall inhibition ratio $A/A_I$ (the amplitudes are corrected) is plotted as a function of the inhibitor concentration, from which an apparent overall inhibition constant at a defined agonist concentration can be estimated by Equation 8a.

$$\frac{A}{A_I} = 1 + I \frac{(AL_2)_O}{K_1}$$

(Eq. 8a)

where $(AL_2)_O$ represents the fraction of the receptors in open-channel state, and is proportional to the current amplitude (99). This fraction is expressed as a function of the fraction of all receptor forms in Equation 8b.

$$\frac{(AL_2)_O}{AL_2} = \frac{AL_2}{A + AL + AL_2 + AL_2} = \frac{L^2}{L^2 (1 + \Phi) + 2K_1L\Phi + K_1^2\Phi}$$

(Eq. 8b)
From this equation, the apparent inhibition constant, $K_{I,\text{app}}$, can be estimated. It can be readily seen that $K_{I,\text{app}}$ depends on a varied agonist concentration.

### 1.5.5 Double-Inhibitor Experiment

A structure-activity relationship study is best served when the two compounds are known to bind or compete for the same site. To address this question, a double inhibition experiment is carried out by running flow measurement in order to determine whether the two inhibitors share the same binding site on the same receptor (note the rate measurement can be also used, but the experiment can be more readily done by measuring the effect of two inhibitors on the current amplitude).

The overall inhibition ratios ($A/A_1$) in the presence of two inhibitors were measured and compared with those in the presence of only one inhibitor. In the experiment involving two inhibitors, the concentration of one inhibitor was kept constant whereas the other one varied. According to Equation 9, if the two inhibitors bind to the same site, the slope obtained from the linear fit ($A/A_1$ against inhibitor concentration) will remain invariant (i.e., $K_I$ is constant) (alternatively, the two inhibitors bind to different sites, but the binding of the two sites would be mutually exclusive) (99). In contrast, if the two inhibitors bind to two sites, the slope will be steeper, because the $K_{\text{app}}$ is larger, as shown by Equation 10.

$$\frac{A}{A_{1,p}} = \left(1 + \frac{p}{K_P}\right) + \frac{l}{K_I}$$

(Eq. 9)
\[
\frac{A}{A_{1,P}} = \left(1 + \frac{P}{K_p}\right) + \left(1 + \frac{P}{K_P}\right) \frac{I}{K_I}
\]

(Eq. 10)

In both equations, \(I\) represents one inhibitor and \(P\) represents the other one, all at molar concentrations.

In my study, GYKI 52466/BDZ-\(f\) and GYKI 52466/BDZ-\(g/BDZ-h\) were selected for this double-inhibitor experiment on the basis of their chemical structures and inhibitory properties. Only GluA2Q_{flip} was used as the receptor for my experiment since GluA2Q plays a key in those neurodegenerative diseases.

1.5.6 **Investigation of Inhibition Selectivity**

All the 19 GYKI compounds involved in this study were tested for their selectivity on different homomeric or heteromeric glutamate receptor channels, including homomeric AMPA receptor GluA1-4, homomeric kainate receptor GluK1 and GluK2, heteromeric NMDA receptors composed of GluN1a/2A or GluN1a/2B, etc. More specifically, the overall inhibition ratios for different receptors at both closed and open-channel states were determined for each compound at a fixed concentration of 20 \(\mu\)M. My objective was to remove any compound with cross activity on either kainate or NMDA receptors, and then identify the most potent inhibitors of AMPA receptors for studying their mechanisms and the structure-activity relationship.
CHAPTER 2

SCREENING 2,3-BENZODIAZEPINES AGAINST GLUTAMTE RECEPTOR SUBUNITS

2.1 INTRODUCTION

A total of 19 2,3-benzodiazepine derivatives were involved in the initial phase of my study. These 19 compounds are classified into two groups, and their chemical structures are shown in Figure 1.5. In the first group, compounds are named from $a$ to $h$ with a prefix of BDZ. All compounds in group 1 share C-4 methyl group with the exception of BDZ-$c$. In the second group, compounds are named as BDZ-$0$ to BDZ-$12$. Almost all the compounds in this group carry a carbonyl group at the C-4 position of the diazepine ring. GYKI 52466 is used for the same name because it is the most widely used reference compound in this family (65). As seen (Figure 1.5), most of the chemical modifications within this list of compounds are on the N-3 position of the diazepine ring.

Because these compounds are synthesized as specific AMPA receptor inhibitors (65, 116), they are not expected to have any cross activity with either kainate or NMDA receptors. However as I explained before, this question is not currently known. Initially in my study, I wanted to identify whether these inhibitors were indeed AMPA receptor-selective. Thus, I assayed each of these inhibitors with not only AMPA receptor subunits
but kainate and NMDA receptor channels as well. The aim was to identify and remove any compounds that may have any activity on either kainate or NMDA receptors from additional, in-depth mechanistic studies. It is important to note that unwanted effects of an inhibitor with multiple sites of actions on functionally different targets often lead to clinical side effects. Any inhibitor that possesses such a promiscuous property should not be used for further development.

Experimentally, a fast solution flow technique was used to screen and characterize inhibitory activities of the 19 2,3-benzodiazepine derivatives on all the four homomeric AMPA receptors, and other glutamate receptor channels, including GluK1, GluK2, GluN1a/2A, and GluN1a/2B.

2.2 MATERIALS AND METHODS

The cell culture, whole-cell current recording, and data analysis have been described in the MATERIALS AND METHODS section of Chapter 1. All the compounds were tested at 20 µM with exception of BDZ-g and BDZ-h. Due to the large experimental error caused by potent inhibition from these two compounds, the inhibition ratio normalized to 100% (or A(I)/A, the current amplitude in the presence and absence of an inhibitor) for BDZ-g and BDZ-h against GluA2Qflip and GluA1flip were estimated using the data obtained at lower concentrations (e.g. 1 µM). The results are plotted vs. the corresponding receptors in terms of closed-channel state or open-channel state (Figure 2.1 to Figure 2.6). Two cells were tested for each compound in all the screening assays. In the individual assay for BDZ-g and BDZ-h, each data point shown in the plots (Figure
2.1, Figure 2.2) is an average of the ratio of inhibited and control current amplitude shown in percentage from at least three cells.

2.3 RESULTS

2.3.1 Inhibitory Effects of 2,3-Benzodiazepines in Group One

The screening assay was first conducted on the four homomeric AMPA receptors formed by GluA1fip, GluA2Qflip, GluA3fip and GluA4fip. The compounds were tested on both the closed-channel and open-channel states of each receptor by fixing the glutamate concentrations at 100 µM (for closed-channel of GluA2, 3, and 4) or 50 µM (for closed-channel of GluA1) and 3 mM (for open-channel of GluA2, 3, and 4) or 2 mM (for open-channel of GluA1), respectively. In our initial assays, we decided to exclude BDZ-a and BDZ-b considering that they are racemic compounds.

As shown in Figure 2.1, BDZ-e caused whole-cell current reduction by ~17%, but BDZ-d, BDZ-f, BDZ-g and BDZ-h significantly inhibited the channel response (e.g., by as much as >98% for BDZ-g and BDZ-h). As shown in Table 2.1a, on the closed-channel state of GluA2 receptors, BDZ-g and BDZ-h are ~9-fold and ~19-fold stronger than BDZ-d, respectively. However the potency of these inhibitors either as a whole or as an individual inhibitor dropped dramatically when tested on GluA3 or GluA4. Even BDZ-h could only reduce the currents mediated by the closed-channel state of GluA3 or GluA4 to ~40% and ~55%, respectively. The differential activities indicated that GluA1 and GluA2 may share a similar negative modulation mechanism and/or sites more favorable for these compounds, but not so with either GluA3 or GluA4.
Figure 2.1 The screening of inhibitory effects of the 2,3-benzodiazepine derivatives in group 1 or BDZ-a to BDZ-h was conducted against the closed-channel state (50 µM glutamate for GluA1flip and kainate receptors, and 100 µM for the others) of AMPA receptors and kainate receptors using rapid solution flow technique. The results are shown as the ratios of controls and inhibited current amplitudes, and plotted vs. the corresponding receptors. Each point is an average of the ratios of inhibited current amplitude and control shown in percentage. All the compounds were tested at 20 µM except BDZ-g and BDZ-h. Due to the large experimental error when measuring 20 µM BDZ-g and BDZ-h, the data for these two compounds against GluA2Qflip and GluA1flip were estimated using the data obtained at lower concentrations (e.g., 1 µM).
Figure 2.2 The screening of inhibitory effects of the 2,3-benzodiazepine derivatives in group 1 or BDZ-a to BDZ-h was conducted against the open-channel state (2 mM glutamate for GluA1flip, the kainate receptors, and 3 mM glutamate for others) of AMPA receptors and kainate receptors using rapid solution flow technique. The results are shown as the ratios of controls and inhibited current amplitudes, and plotted vs. the corresponding receptors. Each point is an average of the ratios of inhibited current amplitude and control shown in percentage. All the compounds were tested at 20 µM except BDZ-g and BDZ-h. Due to the large experimental error when measuring 20 µM BDZ-g and BDZ-h, the data for these two compounds against GluA2Qflip and GluA1flip were estimated using the data obtained at lower concentrations (e.g., 1 µM).
Figure 2.3 The screening of inhibitory effects of the 2,3-benzodiazepine derivatives in group 1 (BDZ-a to BDZ-h) was conducted against NMDA receptors using rapid solution flow technique. The results are shown as the ratios of controls and inhibited current amplitudes, and plotted vs. the corresponding receptors. Each point is an average of the ratios of inhibited current amplitude and control shown in percentage. 50 μM glutamate and 100 μM glycine were used to evoke the NMDA receptors. All the compounds were tested at 20 μM.
Table 2.1a The screening of inhibitory effects of the 2,3-benzodiazepine derivatives (BDZ-\textit{a} to BDZ-\textit{h}) has been conducted against the four homomeric glutamate receptors using rapid solution flow technique. The results are shown as inhibition ratio (a ratio of control and inhibited current, $A/A_I$, based on Equation 8). All the compounds were tested on each receptor for both the closed-channel (50 µM glutamate for GluA1\textit{flip}, and 100 µM for the others) and open-channel (3 mM glutamate) states. All the compounds were tested at 20 µM except BDZ-\textit{g} and BDZ-\textit{h}. Due to the large experimental error when measuring BDZ-\textit{g} and BDZ-\textit{h} at 20 µM, the inhibition ratios for these two compounds against GluA2Q\textit{flip} and GluA1\textit{flip} were estimated using the data obtained at lower concentrations (e.g. 1 µM).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition Ratio $A/A_I$</th>
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<td>GluA1\textit{flip}</td>
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<td>GluA4\textit{flip}</td>
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<td>GYKI 42466</td>
<td>1.34 ± 0.10</td>
<td>1.65 ± 0.17</td>
<td>2.31 ± 0.15</td>
<td>1.35 ± 0.06</td>
<td>1.18 ± 0.13</td>
<td>1.26 ± 0.36</td>
<td>1.06 ± 0.01</td>
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<td>BDZ-\textit{c}</td>
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<td>1.12 ± 0.11</td>
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<td>BDZ-\textit{d}</td>
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<td>1.67 ± 0.24</td>
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<td>BDZ-\textit{f}</td>
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<td>BDZ-\textit{g}</td>
<td>23.6 ± 2.7</td>
<td>11.6 ± 0.6</td>
<td>21.6 ± 0.9</td>
<td>28.0 ± 0.8</td>
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<td>1.61 ± 0.02</td>
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<td>BDZ-\textit{h}</td>
<td>35.2 ± 7.8</td>
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<td>46.2 ± 0.19</td>
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<td>1.92 ± 0.04</td>
<td>1.80 ± 0.04</td>
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\textsuperscript{a}The glutamate concentrations are 100 µM and 3 mM for the closed-channel and open-channel states of GluA2Q\textit{flip}, GluA3\textit{flip}, and GluA4\textit{flip}, respectively. The glutamate concentrations are 50 µM and 2 mM for the closed-channel and open-channel states of GluA1\textit{flip}, respectively.
Table 2.1b The screening of inhibitory effects of the 2,3-benzodiazepine derivatives (BDZ-\textit{a} to BDZ-\textit{h}) has been conducted against two kainate receptors and two NMDA receptors using rapid solution flow technique. The results are shown as inhibition ratios (a ratio of control and inhibited current, \( A/A_1 \), based on Equation 8). On the kainate receptors all the compounds were tested for both the closed-channel (50 \( \mu \)M glutamate) and open-channel (2 mM glutamate) states. Note that the two NMDA receptors were only tested with 50 \( \mu \)M glutamate and 100 \( \mu \)M glycine. All the compounds were tested at 20 \( \mu \)M.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition Ratio ( A/A_1 )</th>
<th>GluK1 mutant</th>
<th>GluK2Q</th>
<th>GluN1a/2A</th>
<th>GluN1a/2B</th>
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<tr>
<td>GYKI 42466</td>
<td>0.99 ± 0.07</td>
<td>1.06 ± 0.08</td>
<td>0.90 ± 0.01</td>
<td>1.02 ± 0.01</td>
<td>0.95 ± 0.06</td>
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<tr>
<td>BDZ-\textit{d}</td>
<td>0.97 ± 0.16</td>
<td>1.05 ± 0.05</td>
<td>0.99 ± 0.08</td>
<td>1.01 ± 0.01</td>
<td>1.07 ± 0.06</td>
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<tr>
<td>BDZ-\textit{f}</td>
<td>0.96 ± 0.02</td>
<td>1.02 ± 0.11</td>
<td>1.03 ± 0.09</td>
<td>1.04 ± 0.09</td>
<td>1.01 ± 0.09</td>
</tr>
<tr>
<td>BDZ-\textit{g}</td>
<td>1.05 ± 0.07</td>
<td>1.03 ± 0.17</td>
<td>0.97 ± 0.04</td>
<td>1.06 ± 0.01</td>
<td>1.03 ± 0.08</td>
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<tr>
<td>BDZ-\textit{h}</td>
<td>0.96 ± 0.11</td>
<td>0.91 ± 0.05</td>
<td>1.03 ± 0.01</td>
<td>1.22 ± 0.14</td>
<td>1.06 ± 0.01</td>
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</table>

\( ^b \) The glutamate concentrations are 50 \( \mu \)M and 2 mM for the closed-channel and open-channel states of GluK1 and GluK2Q, respectively. The NMDA receptors were tested with 50 \( \mu \)M glutamate and 100 \( \mu \)M glycine.
Previous studies have revealed a number of features in the structure-activity relationship for 2,3-benzodiazepine derivatives (Reviewed by Solyom et al., 2002). For instance, removing the 4-amino group on the phenyl ring will eliminate any antagonistic effect on whole animal assay. Introduction of a substituent at N-3 position on the diazepine ring that has sp² hybridization at the first atom with an adjacent H-bond acceptor group will dramatically increase the potency of resulting compounds. BDZ-d and BDZ-f are two examples in this scenario. In my study, I found that BDZ-f was at least 4-fold stronger than GYKI 52466 when tested against GluA2 (98, 117).

Note that in order to keep an inhibitory activity, the configuration of the methyl group on C-4 of the diazepine ring must be R configuration; the S configuration, on the other hand, will diminish the activity (58, 118). My results also supported this conclusion with GluA1 and GluA2 receptors, as shown by the inhibitory properties of BDZ-d and BDZ-e (see Figure 2.1). As seen, 20 μM BDZ-e can only reduce the current mediated by the closed-channel state of GluA2 by ~17%, whereas 20 μM BDZ-d reduced the current by ~59%. Since BDZ-d and BDZ-e are enantiomers and the only difference between the two compounds is the stereochemistry of the methyl group on C-4, one may hypothesize that the binding pocket prefers to accommodate the R configuration and the steric interaction between the binding pocket and the C-4 methyl group in the S configuration may be restrained. Based on these results, I excluded BDZ-e BDZ-c from additional assays because of their low potency on AMPA receptors.

Next I assayed these compounds with kainate and NMDA receptors, including homomeric GluK1 and GluK2 receptors, and heteromeric GluN1a/2A and GluN1a/2B receptors. Both the closed-channel and open-channel states of kainate receptors were
tested by fixing the glutamate concentrations at 50 µM for closed-channel and 2 mM for open-channel. The NMDA receptors were tested only with 50 µM glutamate and 100 µM glycine. All the data are plotted together with those for the closed-channel state of AMPA receptors and kainate receptors (Figure 2.2 and Figure 2.3). As shown in Figure 2.2 and Figure 2.3 and Table 2.1b, there was not any significant inhibition in all the tests, indicating those GYKI compounds we selected in this list did not affect either the kainate or the NMDA receptors.

2.3.2 Inhibitory Effects of 2,3-Benzodiazepines in Group Two

Similar to my assays of compounds in group one, I also carried out the screening assay of compounds in group two on both the closed-channel and open-channel states of the four homomeric AMPA receptor channels. BDZ-3 was excluded from my assay because of its chemical instability, which caused a larger error. As shown in Figures 2.4 and 2.5 as well as Table 2.2a, I found, by simply comparing $A_I/A$ values, that BDZ-0, BDZ-2, BDZ-4, BDZ-9 and BDZ-12 were among the strongest inhibitors in this group. However, those values or percentage of current reduction are not as large as those in group one. Rezessy et al (119) reported that BDZ-0 that bears a condensed heterocyclic ring on the 2,3-benzodiazepine skeleton has similar potency with BDZ-d in the inhibition of excitatory amino acid induced spreading depression in isolated chicken retina. This is constant with my result: an $A/A_1$ of 2.1 µM (closed) and an $A/A_1$ of 1.8µM (open) on GluA2 were obtained for BDZ-0, which are similar to those of BDZ-d.
BDZ-4 can be considered as a BDZ-2 derivative whose 1,2-double-bond is saturated. As shown in Table 2.2a, the saturation makes BDZ-4 slightly weaker on GluA1 and GluA2 as compared with BDZ-2, but slightly stronger on GluA4. BDZ-5 can be considered as the 3-methylcarbamoyl derivative of BDZ-4. The structural relationship between BDZ-4 and 5 are identical to that between BDZ-2 and BDZ-3. Similar to BDZ-2 and BDZ-3 (98, 99), the inhibitory effect of BDZ-5 is weaker than BDZ-4 on GluA2 (Table 2.2a). However BDZ-5 showed similar $A/A_1$ or potency on GluA1 as compared with BDZ-4, indicating possibly a different inhibition mechanism between GluA1 and GluA2 for the same compounds.

The low potency observed on BDZ-7 and BDZ-8 suggests that reducing the size of the diazepine ring is not favorable as inhibitors of AMPA receptors. On the other hand, breaking the diazepine ring may not lose the activity (e.g., BDZ-9). But it seems the 1,2-double-bond should be preserved. For instance, BDZ-10 has no 1,2-double-bond, and it showed a minimal inhibitory activity against all the four AMPA receptors (see Figure 2.4 and Table 2.2a).

Based on these data, I excluded BDZ-6, BDZ-7, and BDZ-10 for additional assays due to their low potency on AMPA receptors. For the rest of the compound in this group, I further tested them on kainate and NMDA receptors that included homomeric GluK1 and GluK2, and heteromeric GluN1a/2A and GluN1a/2B receptors. Both the closed-channel and open-channel states of kainate receptors were tested by fixing the glutamate concentration at 50 µM for closed-channel and 2 mM for open-channel states, respectively. The NMDA receptors were tested only with 50 µM glutamate and 100 µM glycine, and the data for the NMDA receptors are plotted together with those for the
closed-channel state of AMPA receptors and kainate receptors. As shown in Figure 2.6 and Table 2.2b, no appreciable inhibition was observed in any of these compounds, indicating the GYKI compounds in group 2 were selective to AMPA receptors.
Figure 2.4 The screening of inhibitory effects of the 2,3-benzodiazepine derivatives in group 2 or BDZ-0 to BDZ-12 was conducted against the open-channel state (2 mM for GluA1 flipped and the kainate receptors, and 3 mM glutamate for the others) of AMPA receptors and kainate receptors using rapid solution flow technique. The results are shown as the ratios of controls and inhibited current amplitudes, and plotted vs. the corresponding receptors. Each point is an average of the ratios of inhibited current amplitude and control shown in percentage. All the compounds were tested at 20 µM.
Figure 2.5 The screening of inhibitory effects of the 2,3-benzodiazepine derivatives (BDZ-0 to BDZ-12) was conducted against the open-channel state (2 mM for GluA1_{flip} and the kainate receptors, and 3 mM glutamate for the others) of AMPA receptors and kainate receptors using rapid solution flow technique. The results are shown as the ratios of controls and inhibited current amplitudes, and plotted vs. the corresponding receptors. Each point is an average of the ratios of inhibited current amplitude and control shown in percentage. All the compounds were tested at 20 µM.
Figure 2.6 The screening of inhibitory effects of the 2,3-benzodiazepine derivatives (BDZ-0 to BDZ-12) was conducted against NMDA receptors using rapid solution flow technique. The results are shown as the ratios of controls and inhibited current amplitudes, and plotted vs. the corresponding receptors. Each point is an average of the ratios of inhibited current amplitude and control shown in percentage. 50 µM glutamate and 100 µM glycine were used to evoke the NMDA receptors. All the compounds were tested at 20 µM.
Table 2.2a The screening of inhibitory effects of the 2,3-benzodiazepine derivatives (BDZ-0 to BDZ-10) has been conducted against the four homomeric glutamate receptors using rapid solution flow technique. The results are shown as inhibition ratios (a ratio of control and inhibited current, \( \frac{A}{A_I} \), based on Equation 8). All the compounds were tested on each receptor for both the closed-channel (50 µM glutamate for GluA1flip and 100 µM for the others) and open-channel (2 mM glutamate for GluA1flip and 3 mM glutamate for others) states. All the compounds were tested at 20 µM.

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<tr>
<th>Compound</th>
<th>( \text{Inhibition Ratio} \frac{A}{A_I} )</th>
<th>GluA1flip</th>
<th>GluA2Qflip</th>
<th>GluA3flip</th>
<th>GluA4flip</th>
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<tr>
<td>GYKI 42466</td>
<td>1.34 ± 0.10</td>
<td>1.65 ± 0.17</td>
<td>2.31 ± 0.15</td>
<td>1.35 ± 0.06</td>
<td>1.18 ± 0.13</td>
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<tr>
<td>BDZ-0</td>
<td>2.04 ± 0.30</td>
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<tr>
<td>BDZ-2</td>
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<td>BDZ-4</td>
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<td>BDZ-5</td>
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<td>BDZ-7</td>
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\(^a\) The glutamate concentrations are 100 µM and 3 mM for the closed-channel and open-channel states of GluA2Qflip, GluA3flip, and GluA4flip, respectively. The glutamate concentrations are 50 µM and 2 mM for the closed-channel and open-channel states of GluA1flip, respectively.
Table 2.2b The screening of inhibitory effects of the 2,3-benzodiazepine derivatives (BDZ-0 to BDZ-12) has been conducted against two kainate receptors and two NMDA receptors using rapid solution flow technique. The results are shown as inhibition ratios (a ratio of control and inhibited current, $A/A_I$, based on Equation 8). On the kainate receptors all the compounds were tested for both the closed-channel (50 µM glutamate) and open-channel (2 mM glutamate) states. Note that the two NMDA receptors were only tested with 50 µM glutamate and 100 µM glycine. All the compounds were tested at 20 µM.

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<td>GYKI 42466</td>
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<td>BDZ-5</td>
<td>0.92 ± 0.06</td>
<td>1.01 ± 0.18</td>
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<td>1.10 ± 0.12</td>
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<td>BDZ-8</td>
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<td>1.07 ± 0.06</td>
</tr>
<tr>
<td>BDZ-9</td>
<td>1.11 ± 0.04</td>
<td>1.02 ± 0.01</td>
<td>1.02 ± 0.01</td>
<td>1.03 ± 0.01</td>
<td>1.05 ± 0.07</td>
<td>1.08 ± 0.10</td>
</tr>
<tr>
<td>BDZ-12</td>
<td>0.93 ± 0.07</td>
<td>0.96 ± 0.01</td>
<td>0.93 ± 0.07</td>
<td>0.98 ± 0.01</td>
<td>1.06 ± 0.03</td>
<td>1.02 ± 0.02</td>
</tr>
</tbody>
</table>

The glutamate concentrations are 50 µM and 3 mM for the closed-channel and open-channel states of GluK1 and GluK2Q, respectively. The NMDA receptors were tested with 50 µM glutamate and 100 µM glycine.
2.3.3 Overall Inhibition Constants of BDZ-\textit{g} and BDZ-\textit{h} on GluA1 and GluA2 Homomeric Receptors

Previous studies on animal models have revealed that adding a specific substituent like methyl group on the \textit{ortho} position of the 4-aminophenyl ring can improve significantly the biological activity of 2,3-benzodiazepines (21, 100). The rank order of the substitution that increases the potency has been determined to be \( \text{CH}_3 \approx \text{Cl} > \text{OH}, \text{OCH}_3 \). BDZ-\textit{g} and BDZ-\textit{h} thereby have been designed and synthesized based on this “rule” or prediction; in other words, both compounds contain an extra methyl group on the 4-aminophenyl ring. However, the fact that BDZ-\textit{g} and BDZ-\textit{h} are the strongest inhibitors on this list (Figure 1.5) may also be attributed to the nature of the substituents attached to the N-3 positions.

The results from the screening assay for BDZ-\textit{g} and BDZ-\textit{h} showed their remarkable potency against GluA1 and GluA2 (Figure 2.1, Figure 2.2 and Table 2.1a). I then further characterized their inhibition constants for GluA2 using Equation 8. A representative BDZ-\textit{g}-inhibited current mediated by GluA2 was compared to its control in Figure 2.7A. At the closed-channel state (Figure 2.7B), BDZ-\textit{g} inhibited GluA2 with an inhibition constant of 0.74 \( \mu \text{M} \left( K_i \right) \), and at the open-channel state, it inhibited with an inhibition constant of 0.47 \( \mu \text{M} \left( \bar{K}_i \right) \). The two constants for BDZ-\textit{h} are 0.60 \( \mu \text{M} \) and 0.47 \( \mu \text{M} \) (Figure 2.7C) for the close-channel and the open-channel state, respectively. It is apparent that no any other compounds in both groups of compounds we have or I have tested have shown an inhibition constant lower than 1 \( \mu \text{M} \) (98, 99, 117) other than these two compounds, again demonstrating that BDZ-\textit{g} and BDZ-\textit{h} are the strongest inhibitors we have ever studied or ever documented in literature (65).
The other distinctive property of BDZ-g and BDZ-h is that both compounds show roughly the same potency towards the closed-channel and the open-channel state of GluA1 or GluA2. This distinguishes BDZ-g and BDZ-h from all the other GYKI compounds we previously reported (98, 99, 117) and those I tested here. This excellent property should be attributed to a combination of the ortho methyl group on their aminophenyl rings and their unique additions of functional groups on the N-3 position in the diazepine ring.
Figure 2.7 (A) Representative whole-cell currents mediated by the open-channel state of GluA2Qflip receptors in the absence (left) and presence (right) of BDZ-g. The concentrations of glutamate and BDZ-g are 3 mM and 0.5 µM, respectively. The inhibition ratio ($A/A_i$) is ~1.7. The whole-cell recording was conducted at -60 mV, pH 7.4 and 22 ºC. (B) Effect of BDZ-g on the whole-cell current amplitude of GluA2Qflip receptors obtained from the rapid solution flow technique. Each datum point is the average of at least three separate measurements from different cells. A $K_i$ of 0.47 ± 0.02 µM was determined using Equation 8a for the closed-channel state (100 µM glutamate, ●); A $K_{i1}$ of 0.74 ± 0.02 µM was obtained for the open-channel state (3 mM glutamate, ○). (C) Effect of BDZ-h on the whole-cell current amplitude of GluA2Qflip receptors obtained from the rapid solution flow technique. Each datum point is the average of at least three separate measurements from different cells. A $K_i$ of 0.47 ± 0.02 µM was determined using Equation 8a for the closed-channel state (100 µM glutamate, ●); A $K_{i1}$ of 0.60 ± 0.04 µM was obtained for the open-channel state (3 mM glutamate, ○).
I also measured the inhibition constants against homomeric GluA1flip receptors for both compounds. At the open-channel state (Figure 2.8A), BDZ-g inhibited GluA1 with an inhibition constant of 1.2 µM ($K_i$), and at the closed-channel state, it inhibited GluA1 by an inhibition constant with 0.68 µM ($K_{i}$). The two constants for BDZ-h are 0.82 µM and 0.58 µM (Figure 2.8B) for the open-channel state and the closed-channel state, respectively. However, BDZ-h is slightly stronger on GluA1 than BDZ-g, while on GluA2, the two compounds are equally potent.
Figure 2.8 (A) Effects of BDZ-\textit{g} on the whole-cell current amplitude of GluA1\textsubscript{flip} receptors obtained from the rapid solution flow technique. Each datum point is the average of at least three separate measurements from different cells. A $K_I$ of 0.68 $\pm$ 0.08 µM was determined for the closed-channel state (50 µM glutamate, ●); A $\overline{K}_I$ of 1.16 $\pm$ 0.06 µM was obtained for the open-channel state (2 mM glutamate, ○). (B) Effects of BDZ-\textit{h} on the whole-cell current amplitude of GluA1\textsubscript{flip} receptors obtained from the rapid solution flow technique. Each datum point is the average of at least three separate measurements from different cells. A $K_I$ of 0.58 $\pm$ 0.06 µM was determined for the closed-channel state (50 µM glutamate, ●); A $\overline{K}_I$ of 0.82 $\pm$ 0.05 µM was obtained for the open-channel state (2 mM glutamate, ○).
2.3.4 GYKI 52466, BDZ-\(g\), and BDZ-\(h\) Bind to the Same Site on GluA2Qflip

Based on our earlier work, we have previously hypothesized that the 2,3-benzodiazepine compounds with the azomethine group on the diazepine ring, which contains the 4-methyl group (see Figure 1.5), bind to the same site, whereas 2,3-benzodiazepine compounds with an \(\varepsilon\)-lactam structure, which contains the 4-carbonyl group, bind to a different site (98). Therefore, BDZ-\(g\) (or BDZ-\(h\)) and GYKI 52466 were expected to compete for binding to the same site, given that BDZ-\(g\) (or BDZ-\(h\)) and GYKI 52466 share the same 4-methyl group (although BDZ-\(g\) or BDZ-\(h\) contains an extra N-3 methylcarbamoyl group) (Figure 1.5). To investigate this question, I performed a double-inhibitor experiment, designed to test whether two structurally similar inhibitors would actually bind to the same site on the same receptor. Specifically, the two inhibitors were applied onto the receptors individually first, and then, together. The concentration of one inhibitor was kept constant while the concentration of the other was varied. An apparent inhibition constant obtained from the two-inhibitor experiment (or the slope of the \(A/A_{1,IP}\) plot; see Equations 9 and 10) was compared to that obtained from one-inhibitor experiment (or the slope of the \(A/A_{1}\) plot; see Equation 8a). The comparison can readily tell whether the two inhibitors bind to the same site or two separate sites on the same receptor. Using this method, Ritz et al. (98) have previously identified two binding sites for GYKI compounds or 2,3-benzodiazepines, which are represented by GYKI 52466 and BDZ-2.

Using the method, I tested BDZ-\(g\) with GYKI 52466 on the closed-channel state of GluA2Qflip receptors (100 µM glutamate). The concentration of GYKI 52466 was fixed at 20 µM while that of BDZ-\(g\) varied from 0.5 µM to 3 µM. As shown in Figure 2.9A,
the double-inhibition constant, $K'_I$, was determined to be 0.45 µM, which was comparable with $K_I$ of 0.47 µM for BDZ-g alone. The result suggested that BDZ-g bound to the same site with GYKI 52466 on GluA2Qflip. If they bound to two different sites, the double-inhibitor regression would fit to the dashed line assuming that the two inhibitors bind to two different sites with an apparent double-inhibition constant of ~2.2 µM. The same conclusion was drawn from the double-inhibition experiment with BDZ-h and GYKI 52466 (Figure 2.9B). To confirm this result, I further tested BDZ-g and BDZ-h as a pair in the double-inhibitor experiment with GluA2Qflip. If they both bind to the same site with GYKI 52466 on GluA2Qflip, the two compounds should also share the same binding site with each other. As shown in Figure 2.9C, my result was affirmative.
Figure 2.9 (A) The result of a double-inhibitor experiment for GYKI 52466 and BDZ-g on the closed-channel state of GluA2Q flip (100 µM glutamate). The concentration of GYKI 52466 was fixed at 20 µM while that of BDZ-g varies from 0.5 µM to 3 µM. The double-inhibition constant, $K'_I$, was determined to be 0.45 ± 0.02 µM (filled circles, ●), compared with $K_I$ of 0.47 ± 0.02 µM for BDZ-g alone (open circles, ○). The dashed line simulates the $A/A_I$ plot by assuming that the two inhibitors bind to two different sites with a double-inhibition constant of ~2.2 µM (when GYKI 52466 was fixed at 20 µM). (B) The result of a double-inhibitor experiment for GYKI 52466 and BDZ-h on the closed-channel state of GluA2Q flip (100 µM glutamate). The concentration of GYKI 52466 was fixed at 20 µM while that of BDZ-h varies from 0.2 µM to 1.5 µM. The double-inhibition constant, $K'_I$, was determined to be 0.49 ± 0.05 µM (filled circles, ●), compared with $K_I$ of 0.47 ± 0.02 µM for BDZ-h alone (open circles, ○). The dashed line simulates the $A/A_I$ plot by assuming that the two inhibitors bind to two different sites with a double-inhibition constant of ~2.2 µM (when GYKI 52466 was fixed at 20 µM). (C) The result of a double-inhibitor experiment for BDZ-g and BDZ-h on the closed-channel state of GluA2Q flip (100 µM glutamate). The concentration of BDZ-h was fixed at 0.5 µM while that of BDZ-g varies from 0.1 µM to 1.5 µM. The double-inhibition constant, $K'_I$, was determined to be 0.49 ± 0.03 µM (filled circles, ●), compared with $K_I$ of 0.47 ± 0.02 µM for BDZ-h alone (open circles, ○). The dashed line simulates the $A/A_I$ plot by assuming that the two inhibitors bind to two different sites with a double-inhibition constant of ~0.23 µM (when BDZ-h was fixed at 0.5 µM).
2.4 SUMMARY

In this section, I conducted a screening assay for a series of GYKI compounds against a total of 8 different glutamate receptors, including homomeric GluA1\textsubscript{flip}, GluA2Q\textsubscript{flip}, GluA3\textsubscript{flip}, GluA4\textsubscript{flip}, GluK1, GluK2, and heteromeric GluN1a/2A and GluN1a/2B receptors. The results were shown in the ratio of whole-cell current amplitude in the presence and absence of an inhibitor or $A/A_1$, as in Tables 2.1 and Table 2.2, and in Figures 2.1 to 2.6. Comparison of those ratios allows me to select the most potent inhibitors of AMPA receptors yet without any cross activity on either kainate or NMDA receptors. Based on my results of the screening assay, BDZ-f, BDZ-g, and BDZ-h were selected for further investigation of their inhibitory effects and their inhibition mechanisms on GluA2. Furthermore, the inhibition constants of BDZ-g and BDZ-h were determined on both GluA1 and GluA2. My data revealed that GYKI 52466, BDZ-g, and BDZ-h all bind to the same site on GluA2.

However, due to the overlap of their maximum absorption wavelengths and the wavelength of the laser flash required to liberate free glutamate from the caged glutamate, BDZ-g and BDZ-h are not suitable for the laser-pulse photolysis study, which would have allowed me to investigate the detailed mechanism of action for these two most potent inhibitors in the GYKI compound family. It may be possible that development of another photolabile precursor and laser source in the future could solve this problem so that a rapid kinetic study of the inhibition mechanism for both BDZ-g and BDZ-h on AMPA receptors becomes a reality.
CHAPTER 3

MECHANISM OF INHIBITION OF GLUA2 BY BDZ-f

3.1 INTRODUCTION

AMPA receptors open their channels, in response to the binding of glutamate, the endogenous neurotransmitter, in the microsecond time scale but desensitize in the millisecond time scale (101). Therefore a kinetic investigation of the mode of action of a 2,3-benzodiazepine compound must be carried out using a technique that provides sufficient time resolution to measure the channel-opening rate of an AMPA receptor (99). However, commonly used techniques, such as solution flow and single-channel recording, do not have sufficient time resolution for characterizing the effect of a 2,3-benzodiazepine compound on the channel-opening rate process. To overcome that limitation, I used a laser-pulse photolysis technique in this study, together with a caged glutamate (112, 120) (the detailed photochemistry and the set-up for this technique are provided in “Materials and Methods” in Chapter 1). This technique provides a time resolution of ~60 μs (101, 112), which is sufficient for measuring the rate of channel opening and thus for investigating the mechanism of inhibition without the complication of channel desensitization in the millisecond time scale (99, 112).
In this chapter, I describe the work on investigating the mechanism by which the GluA2Qflip receptor channel-opening rate process is inhibited by BDZ-f using the laser-pulse photolysis technique. The questions I wanted to ask are: What is the mechanism of action of BDZ-f? Does the addition of an N-3 methylcarbamoyl group affect potency and specificity for the open-channel and the closed-channel conformations? Do GYKI 52466 and BDZ-f bind to the same site, or does the addition of an N-3 methylcarbamoyl group affect the binding site? Answers to these questions will allow us to determine whether addition of this group at the N-3 position in the diazepine ring will make BDZ-f a better inhibitor than the parent compound, i.e., GYKI 52466.

3.2 MATERIALS AND METHODS

The cell culture, whole-cell current recording, and data analysis are as described in in the section of Materials and Methods of Chapter 1.

3.3 RESULTS

3.3.1 BDZ-f Inhibited the Channel-Opening Process of GluA2Qflip

Using the laser-pulse photolysis technique, I characterized the effect of BDZ-f on the channel-opening rate process of GluA2Qflip. As shown in a pair of representative whole-cell recording traces initiated by laser-pulse photolysis of the caged glutamate (Figure 3.1A), the current rise was slowed and the current amplitude was reduced in the presence of BDZ-f, indicating that BDZ-f inhibited the opening of the GluA2Qflip receptor
channel. The observed rate constant in the absence ($k_{obs}$) and presence of an inhibitor ($k_{obs}'$) followed a first-order rate process for over 95% of the rising phase (i.e., the solid lines in Figure 3.1A). This kinetic feature was observed without exception in all inhibitor and glutamate concentrations used, not only in this study but also in our earlier studies of GluA2Qflip in the absence (101) and presence of other inhibitors (98, 99). These results were therefore consistent with the notion that the rate of the current rise in the laser-pulse photolysis measurement was pertinent to the channel-opening rate, and the reduction of the rate of the current rise was ascribed to the inhibition of the channel opening by an inhibitor (98, 99), such as BDZ-f.

To investigate the mechanism of inhibition, I characterized the effect of BDZ-f on both $k_{op}$ and $k_{cl}$ (98, 99) – the rationale for this mechanistic study is provided in the “Materials and Methods” in Chapter 1. Specifically, at the 100 µM glutamate concentration where $k_{cl}$ was measured, $K^*_1$, the inhibition constant for the open-channel state, was found to be 20 ± 3 µM for BDZ-f (Figure 3.1B). At the 300 µM glutamate concentration, where $k_{obs} > k_{cl}$ and thus $k_{op}$ was measurable, $K^*_1$, the inhibition constant for the closed-channel state, was 22 ± 1 µM (Figure 3.1C). The fact that BDZ-f inhibited both $k_{cl}$ and $k_{op}$ was consistent with it being a noncompetitive inhibitor. In contrast, an uncompetitive inhibitor would be expected to inhibit only $k_{cl}$ but not $k_{op}$, whereas a competitive inhibitor would be expected to inhibit $k_{op}$ but not $k_{cl}$ (98, 99).
Figure 3.1 (A) Representative whole-cell traces from the laser-pulse photolysis experiment showing that BDZ-f inhibited both the rate and the amplitude of the opening of the GluA2Qflip channels. The whole-cell current amplitude was measured in the absence and presence of BDZ-f. The top trace (○) is the control ($k_{\text{obs}} = 2439 \text{ s}^{-1}; A = 0.74 \text{nA}$), and the lower one (●) was recorded with 2 µM BDZ-f ($k_{\text{obs}} = 1988 \text{ s}^{-1}; A = 0.53 \text{nA}$). The concentration of the photolytically released glutamate was estimated to be ~100 µM in both cases. (B) Effect of BDZ-f on $k_{\text{cl}}$ obtained at 100 µM of photolytically released glutamate and as a function of BDZ-f concentration. A $K_I$ of 20 ± 3 µM was determined using Equation 4. (C) Effect of BDZ-f on $k_{\text{op}}$ obtained at 300 µM of photolytically released glutamate and as a function of BDZ-f concentration. A $K_I$ of 22 ± 1 µM was determined using Equation 5. (D) Effect of BDZ-f on the whole-cell current amplitude of GluA2Qflip receptors obtained from the laser-pulse photolysis measurement. Using Equation 8a, a $K_I$ of 4.0 ± 0.2 µM was determined at 100 µM of photolytically released glutamate (●); a $K_I$ of 4.7 ± 0.3 µM was obtained at 300 µM of photolytically released glutamate (○).
3.3.2 Effect of BDZ-f on the Amplitude of Whole-Cell Current Observed in the Laser-Pulse Photolysis Measurement

In the measurement of laser-pulse photolysis (Figure 3.1A), BDZ-f also inhibited the amplitude of the whole-cell current response. The magnitude of the reduction in current amplitude was also used to estimate an inhibition constant using Equation 8a. From the ratio of the current amplitude in the absence of BDZ-f to the amplitude in presence of BDZ-f (Figure 3.1D), I found a $K_1$ of 4.0 ± 0.2 µM for the closed-channel state (i.e., at 100 µM glutamate as in Figure 3.1D, solid circles). Similarly, a $K_1$ of 4.7 ± 0.3 µM was estimated at 300 µM glutamate (Figure 3.1D, open circles). All the inhibition constants obtained from the laser photolysis measurement (and solution flow measurement, described in details below) are summarized in Table 3.1.

I compared the magnitude of the inhibition constants obtained from the rate with those obtained from the amplitude, but found that the inhibition constants calculated from the amplitude were about five-fold smaller than those calculated from the rate, even from the same measurement, i.e., the laser-pulse photolysis experiment (Figure 3.1A). To make sure that this discrepancy between the corresponding inhibition constants determined from the amplitude and rate measurements was real, I further evaluated the inhibition constants using a solution flow technique (see Experimental Procedures). The flow measurement using free glutamate concentrations served as the control to determine an inhibition constant from the amplitude of whole-cell current in the absence and presence of BDZ-f.
Table 3.1 Inhibition Constants of BDZ-\(f\) Obtained from Rate and Amplitude Measurements for the Closed- and Open-Channel States of GluA2Q\(_{\text{flip}}\)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Rate Measurement(^a)</th>
<th>Amplitude Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_i^r (\mu M)^b) (K_i^o (\mu M)^b)</td>
<td>(K_i (\mu M)^{b,d}) (K_i (\mu M)^{b,e}) (K_i (\mu M)^{c,d}) (K_i (\mu M)^{c,f})</td>
</tr>
<tr>
<td>BDZ-(f)</td>
<td>22 ± 1 (20 ± 3)</td>
<td>4.0 ± 0.2 (4.7 ± 0.3) (3.8 ± 0.4) (5.4 ± 0.8)</td>
</tr>
<tr>
<td>GYKI 52466(^g)</td>
<td>61 ± 11 (128 ± 30)</td>
<td>15 ± 1 (16 ± 1) (14 ± 1) (30 ± 2)</td>
</tr>
<tr>
<td>BDZ-(3)^(h)</td>
<td>514 ± 60 (204 ± 18)</td>
<td>200 ± 18 (69 ± 4) (210 ± 20) (38 ± 10)</td>
</tr>
</tbody>
</table>

\(^a\) The constants obtained from rate measurements represent those in the first step of inhibition as in Figure 4, whereas those obtained from the amplitude measurements represent the overall inhibition constants. \(^b\) Laser-pulse photolysis measurement. \(^c\) Flow measurement. \(^d\) Measurements at 100 \(\mu M\) glutamate for the closed-channel state. \(^e\) Measurements at \(~300\ \mu M\) glutamate. \(^f\) Measurements at 3 mM glutamate. \(^g\) Ritz et al, 2011. \(^h\) Ritz et al, 2008.
3.3.3 Effect of BDZ-f on the Amplitude of Whole-Cell Current Observed in the Flow Measurement

As shown in glutamate-evoked whole-cell current traces in the absence (left trace of Figure 3.2A) and presence (right trace) of BDZ-f in the solution flow measurement, BDZ-f inhibited the current amplitude from the GluA2Q\textsubscript{flip} receptor channels. By setting glutamate concentrations to be 100 µM and 3 mM, I determined that the $K_1$ was 3.8 ± 0.4 µM for the closed-channel and $K_1$ was 5.4 ± 0.8 µM for the open-channel state (these values are also summarized in Table 3.1).

Based on these results, I conclude the following: (i) that BDZ-f inhibited the whole-cell current response from both the closed-channel and the open-channel states of GluA2Q\textsubscript{flip} was consistent with its effect on the rate of channel opening, again suggesting that BDZ-f inhibited the GluA2Q\textsubscript{flip} channel in a noncompetitive fashion. (ii) Quantitatively, the $K_1$ values for BDZ-f calculated from the amplitude data for the closed-channel state were similar (4 µM vs. 3.8 µM from the flow and laser experiments, respectively; Table 3.1). (iii) The $K_1$ value of BDZ-f for the open-channel state was slightly higher than the value for the closed-channel state (5.4 µM vs. 3.8 µM, respectively; Table 3.1), suggesting that BDZ-f was slightly more selective for the closed-channel state. (iv) The inhibition constant obtained from the amplitude data (4.7 µM) collected from the laser experiment at 300 µM of glutamate was within this range (i.e., $3.8 < 4.7 < 5.4$ µM; see Table 3.1). This was expected, because the fraction of the open-channel form that corresponded to 300 µM was 10%, which was within the range of 4% – 95% (or 100 µM – 3 mM glutamate).
To calculate the inhibition constants from the current amplitude obtained from the solution flow experiments, I used the total amplitude of the current response in the absence and presence of BDZ-f (Figure 3.2B). However, I also examined an entire whole-cell current trace that included both the desensitizing and non-desensitizing or steady-state phase (Figures 3.2A and 3.2D). First, I found that BDZ-f did not affect the desensitization rate constant ($k_{\text{des}}$, in Figure 3.2C). As shown here (see the upper and lower hollow symbols in Figure 3C) and previously (101), $k_{\text{des}}$ was dependent only on the ligand concentration, yet was independent of inhibitor concentration (up to 10 µM, the highest concentration used in this study). This result indicated that BDZ-f reduced the current amplitude by inhibiting the channel opening through which the current was generated but did not affect the rate by which the channel desensitized. Second, the desensitization of the GluA2Qflip channels proceeded in two phases, i.e., a rapidly desensitizing phase (the major phase, whose rate constants are shown in Figure 3.2C), and a non-desensitizing phase (the minor phase, an example is shown in Figure 3.2D). The fraction of the non-desensitizing phase was glutamate-concentration dependent in that at a glutamate concentration of 100 µM, the percentage of the non-desensitizing phase was ~18%, and at a glutamate concentration of 3 mM it was only ~2% (based on the measurement of 55 and 49 cells, respectively). In addition, the absolute current amplitude of the non-desensitizing phase, collected from the same cell and at these two glutamate concentrations, seemed to be only slightly changed; however, the absolute amplitude of the non-desensitizing phase observed at either glutamate concentration was small (Table 3.2). A similar percentage of the non-desensitizing phase for other AMPA receptor subunits but at saturating glutamate concentration, ranging from 0.6% to 2.4%,
has been documented (see a review in [1]). It should be noted that the percentage of the non-desensitizing phase in GluA2, as I described here, is for the flip variant of GluA2Q, one of the two alternatively spliced isoforms of GluA2 (23). The flop variant desensitizes almost completely (23, 25, 121). The non-desensitizing phase is thought to link to the firing of action potential from AMPA-containing neurons and the propagation of action potential in post-synapses (122). Furthermore, it is thought that a sustained existence of the non-desensitizing phase, due to prolonged low-level glutamate exposure, causes cell death (123).

Based on the analysis described above, I separately examined the effect of BDZ-f on the current amplitude of the desensitizing and the non-desensitizing phases. For the closed-channel form, I obtained $K_i$ of $3.2 \pm 0.3$ µM from the desensitizing phase (Figure 3.2E, left panel) and $K_i$ of $6.9 \pm 2.3$ µM from the non-desensitizing phase (Figure 3E, right panel). Conversely, for the open-channel form or at 3 mM glutamate concentration, I calculated $K_i$ to be $5.4 \pm 0.3$ µM from the desensitizing phase (Figure 3.2F, left panel). However, I could not obtain a statistically significant inhibition constant from the non-desensitizing phase (Figure 3.2F, right panel), because of a large experimental error range. I then compared the inhibition constants from the fractional amplitude with those from the total amplitude. I found that for the open-channel state, $K_i$ of $5.4$ µM calculated from the fractional amplitude (i.e., the desensitizing phase only) was identical to $K_i$ of $5.4$ µM from the total amplitude. This was not surprising because at a high glutamate concentration, where the effect of BDZ-f on the open-channel form was measured, the desensitizing phase dominated the total current amplitude. At a low glutamate concentration, where the desensitizing phase became relatively less dominant and the
non-desensitizing phase became relatively more significant, $K_1$ of $3.2 \pm 0.3$ µM from the desensitizing phase was identical to the $K_1$ value ($3.8 \pm 0.4$ µM) calculated from the total amplitude. Therefore, these comparisons suggest that the use of the total current amplitude is reliable enough for estimating the inhibition constants for both the open-channel and closed-channel states.
Figure 3.2 (A) Representative whole-cell currents mediated by GluA2Qflip receptors expressed in HEK-293S cells in the absence (left) and presence (right) of BDZ-f, obtained by flow measurement. The concentrations of glutamate and BDZ-f are 3 mM and 2 µM, respectively. The inhibition ratio ($A/A_i$) for the pair is ~1.61. The whole-cell current was recorded at -60 mV, pH 7.4, and 22 °C. (B) Effect of BDZ-f on the whole-cell current amplitude of GluA2Qflip receptors obtained from the flow measurement. $K_i$ of 3.8 ± 0.4 µM was determined by using Equation 8a for the closed-channel state (100 µM glutamate, ●); $K_i$ of 5.4 ± 0.8 µM was obtained for the open-channel state (3 mM glutamate, ○). (C) The effect of BDZ-f on the channel desensitization rates for the closed-channel (lower, determined at 100 µM glutamate) and open-channel (upper, determined at 3 mM glutamate) states of GluA2Qflip. (D) A representative whole-cell current mediated by GluA2Qflip receptors at 100 µM of glutamate shows that the total current amplitude ($A_{tot}$) equals 425 pA with the amplitude of non-desensitizing phase ($A_{non-des}$) remaining at 61 pA (until glutamate was removed), and the desensitizing phase being 364 pA. The fraction of the non-desensitizing phase is 14%.
Figure 3.2 (E) Effect of BDZ-f on the two components of the whole-cell current of the closed-channel state of GluA2Q_{flip} receptors. In the left panel, the amplitude of the desensitized phase is plotted against inhibitor concentration, and a $K_i$ of $3.2 \pm 0.3 \, \mu\text{M}$ was determined. In the right panel, a $K_i$ of $6.9 \pm 2.3 \, \mu\text{M}$ was determined for the non-desensitizing phase. (F) Effect of BDZ-f on the two components of the whole-cell current of the open-channel conformation of GluA2Q_{flip}. In the left panel, the amplitude of the desensitized phase is plotted against inhibitor concentrations, and a $K_i$ of $5.4 \pm 0.3 \, \mu\text{M}$ was determined. An inhibition constant for the data on the right panel was not estimated due to large experimental error.
Table 3.2 Homomeric GluA2Q_{flip} receptors desensitize in two phases (Figure 3D), i.e., a fast desensitizing phase, and a non-desensitizing phase. The amplitudes of the desensitizing and non-desensitizing phases were measured at both closed-channel (determined at 100 µM of glutamate) and open-channel (determined at 3 mM of glutamate) states. The following list shows the ratio of the amplitude of non-desensitizing phase at 3 mM vs. 100 µM, collected from the same cell. The data are from a total of 8 cells.

<table>
<thead>
<tr>
<th>Glu (mM)</th>
<th>$A_{des}$ (nA)</th>
<th>$A_{non-des}$ (nA)</th>
<th>$A_{non-des}/A_{des}$</th>
<th>$A_{non-des}$ (3mM)/$A_{non-des}$ (100 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell 1</td>
<td>0.1</td>
<td>-0.29</td>
<td>-0.045</td>
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81
3.3.4 BDZ-\(f\) Inhibits the Channel Opening by a Two-Step Process

That BDZ-\(f\) inhibited \(k_{cl}\) and \(k_{op}\) as well as the whole-cell current amplitude at both high and low glutamate concentrations, which reflected the open-channel and closed-channel states, respectively, is consistent with a noncompetitive mechanism of inhibition. Quantitatively, the inhibition constants determined from the amplitude ratio from both the laser and flow measurements were in good agreement (Table 3.1). Those constants, however, were \(~5\)-fold smaller than the inhibition constants determined from the measurement of the channel-opening rate. The discrepancy between these inhibition constants and all other pieces of mechanistic evidence I obtained from this study led us to identify a minimal mechanism of inhibition for BDZ-\(f\) (Figure 3.3), i.e., the same mechanism we have proposed for other 2,3-benzodiazepine inhibitors of AMPA receptors (98, 99). By this mechanism, the initial binding of BDZ-\(f\) to the receptor is assumed to form a loosely bound intermediate (e.g., \(IAL_2^{--}\)) in both the closed-channel and open-channel states of the receptor. A receptor:inhibitor intermediate is partially conducting, which yields partial inhibition of channel activity. The second step, the receptor:inhibitor intermediate rapidly isomerizes into a more tightly bound complex (\(IAL_2\)), and such a complex is no longer capable of conducting ions.

The proposed mechanism of inhibition (Figure 3.3) is plausible because it can account for the results I obtained for BDZ-\(f\). First, both the rate and the amplitude measurements in the laser-pulse photolysis experiment with BDZ-\(f\) (as in Figure 3.1A) were associated with the channel-opening process. Amplitude, however, was an equilibrium measure (the channel-opening equilibrium is transient because the channel becomes desensitized in the millisecond time scale). Thus, a stronger inhibition or a
smaller $K_i$ value calculated from the amplitude suggested that a larger inhibition constant or a less than full inhibition obtained from the rate in the same experiment, i.e. the photolysis-triggered whole-cell traces (Figure 3.1A), reflected only a fraction of the overall inhibition. In other words, the effect of BDZ-f on the channel-opening rate was only partial; full inhibition would have to be produced from an additional step, i.e., the isomerization reaction, which turned the initial, partially conducting complex into a totally inhibiting complex. As such, only one step or the slow step in the mechanism was observable in the rate measurement. It should be noted that Equations 4 and 5 were derived based on a one-step process, and those equations allowed us to estimate the inhibition constants (Table 3.1) associated with the slow step, which limited the observed rate of the reaction in the presence of the inhibitor.
Figure 3.3 A minimal mechanism of inhibition for BDZ-f. 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 form or the state of the receptor (all the species with a bar sign refer to open-channel state), $k_{op}$ the channel-opening rate constant, and $k_{cl}$ the channel-closing rate constant. For simplicity and without contrary evidence, it is assumed that glutamate binds to the two steps with equal affinity, represented by the same intrinsic equilibrium dissociation constant, $K_1$. The initial binding of BDZ-f to the receptor is assumed to form a loosely bound, partially conducting intermediate (e.g., $\overline{I}AL_2^*$) in both the closed-channel and open-channel states of the receptor (middle row). In the second step (from the middle to the lower row), the receptor:inhibitor intermediate rapidly isomerizes into a more tightly bound complex ($\overline{IAL}_2^*$), and such a complex is no longer capable of conducting ions. The inhibition constants pertinent to various steps in this mechanistic scheme are shown in Table 3.1. $K_1$ represents the overall inhibition constant associated with the closed-channel state of the receptor (i.e., the values from Column 5 in Table 3.1), $\overline{K}_1$, the overall inhibition constant associated with open-channel state (i.e., the values from Column 6 in Table 3.1), $I$, the inhibitor, $k_{op}'$ the channel-opening rate constant of the inhibited AMPA receptor, $k_{cl}'$ the channel-closing rate constant of the inhibited AMPA receptor. In addition, the values for $K_1^*$ and $\overline{K}_1^*$ for step 1 can be found from Columns 1 and 2 in Table 3.1.
Evidence from this study suggested that the first step in the minimal mechanism of inhibition was the slow step (Figure 3.3) or the step in which the loose receptor:inhibitor intermediates are formed in the closed- and open-channel states. The assumption that the first step is slow compared with the second step was based on the observation that throughout the concentration range for both glutamate and inhibitor, only a single exponential rise for the opening of the channel was observed. If the rate of the second step was slow or comparable to that of the first step, one would expect complete or nearly complete inhibition because the inhibition constants calculated from the rate data would agree fully or nearly fully with those from the amplitude. Furthermore, the fact that \((1/k_{\text{obs}})\) increased linearly with increasing inhibitor concentration as predicted by Equations 4 and 5 (Figures 3.1B and C) in both the closed-channel and open-channel states of the receptor further supports the notion that the rate of the isomerization reaction from both the closed-channel and open-channel pathways (Figure 3.3) would have to be faster than the initial step. Consequently, the \(K_i^*\) value (Table 3.1) obtained from the rate measurement at a high glutamate concentration was thought to be pertinent to the inhibition of the open channel by BDZ-f by the initial inhibitor:receptor intermediate, whereas the \(K_i^*\) value (Table 3.1) was assigned to the inhibition of the closed-channel state by the initial inhibitor:receptor intermediate (Table 3.1).

Although the effect of BDZ-f on both \(k_{\text{op}}\) and \(k_{\text{cl}}\) was partial, which I determined from the rate of channel opening in the absence and presence of the inhibitor (Table 3.1), that BDZ-f inhibited both \(k_{\text{op}}\) and \(k_{\text{cl}}\) was consistent with its acting in a noncompetitive mechanism and inconsistent with either a competitive or an uncompetitive mechanism of inhibition. By a competitive mechanism, BDZ-f would compete with glutamate for the
same binding site. Consequently, only the effect on \( k_{\text{op}} \), but not on \( k_{\text{cl}} \), would be expected. In other words, there would be no \( [K_1^* / (K_1^* + I)] \) term associated with \( k_{\text{cl}} \) in Equation 3, and thus \( 1/k_{\text{obs}} \), as in Equation 4, would be independent of inhibitor concentration. By an uncompetitive mechanism, commonly known as an open-channel blockade, BDZ-f would inhibit the open-channel state only; i.e., only the effect on \( k_{\text{cl}} \), but not on \( k_{\text{op}} \), would be expected. In this scenario, the \( [K_1^* / (K_1^* + I)] \) term associated with \( k_{\text{op}} \) in Equation 3 would not exist. Consequently, the \((k_{\text{obs}} - k_{\text{cl}}')\) term, as in Equation 5, would not be dependent on inhibitor concentration.

3.3.5 BDZ-f Inhibited Almost Equally Strongly the Closed-Channel and Open-Channel States of Both the Flip and Flop Variants of GluA2Q

On the basis of the overall inhibition constants (Table 3.1), it can be concluded that BDZ-f inhibited both the closed-channel and open-channel states of GluA2Q_{flip} receptors roughly equally, although there was a slight preference for the closed-channel state over the open-channel state, i.e., \( K_1 \) of 3.8 ± 0.4 \( \mu \)M vs. \( \bar{K}_1 \) of 5.4 ± 0.8 \( \mu \)M, respectively. Thus far the studies that led to these conclusions were carried out with the flip variant of GluA2Q (or precisely GluA2Q_{flip} receptors). However, these data raise two interesting questions. First, does BDZ-f show a similar potency and selectivity for the flop variant of GluA2Q? The flip and the flop variants are generated by alternative splicing, and GluA2Q_{flip} and GluA2Q_{flop} have only 9 different amino acids (23). However, the homomeric channels assembled from the flip and flop variants of GluA2Q have different kinetic properties, such that GluA2Q_{flop} has the same \( k_{\text{op}} \) but a larger \( k_{\text{cl}} \) than the flip variant (124). Using the flow technique, I measured the overall inhibition constants for
the closed- and open-channel states of the receptor. I found that BDZ-f had a similar
inhibitory property on the flop variant as it did on the flip variant. Specifically, BDZ-f
inhibited the GluA2Q_{flop} receptors expressed in HEK-293 cells with a $K_i$ of $3.8 \pm 0.3 \ \mu M$
for the closed-channel state and a $\bar{K}_i$ of $5.6 \pm 0.6 \ \mu M$ for the open-channel state (Figure
3.4). These inhibition constants were similar to those for GluA2Q_{flip} (Table 3.1).
Therefore, it can be concluded that BDZ-f is equally effective on the flop variant of the
same receptor but had a slight preference for the closed-channel state of the flop receptor
channels. Because BDZ-f did not show any preference in potency between the two
alternatively spliced variants and yet the flip/flop sequence segments differ by 9 amino
acids, it is unlikely that the flip/flop sequence segment of GluA2 is involved critically in
making up the noncompetitive site on GluA2.
Figure 3.4 Effect of BDZ-\(f\) on the whole-cell current amplitude of the GluA2Q\(_{\text{lop}}\) receptors obtained from the flow measurement. The inhibition constants were determined from this plot by using Equation 8a. \(K_1\) of 3.6 ± 0.2 µM was determined for the closed-channel state (100 µM glutamate, ●); a \(K_1\) of 5.7 ± 0.3 µM was obtained for the open-channel state (3 mM glutamate, ○).
Next I investigated whether BDZ-f binds to the same site as GYKI 52466 does. Whether a slight modification of a structure is enough to change the binding site for the new compound is one of the essential questions in defining the structure-activity relationship. We previously hypothesized that the 2,3-benzodiazepine compounds with the azomethine group on the diazepine ring, which contains the 4-methyl group (see Figure 1.3), bind to the same site, whereas 2,3-benzodiazepine compounds with an \( \varepsilon \)-lactam structure, which contains the 4-carbonyl group, bind to a different site (98). This was the same question that prompted us to investigate whether BDZ-g or BDZ-h competes with the same site to which GYKI 52466 binds. BDZ-f and GYKI 52466 are expected to compete for binding to the same site, given that BDZ-f and GYKI 52466 also share the same 4-methyl group, although BDZ-f contains an extra N-3 methylcarbamoyl group (Figure 1.3). To investigate this question, I performed a double-inhibitor experiment (see details in the section of 1.3 Materials and Methods in Chapter 1). Specifically, GYKI 52466 and BDZ-f were applied concurrently to the closed-channel state of the GluA2Q\textsubscript{flip} receptor in that the concentration of GYKI 52466 was fixed while the concentration of BDZ-f was varied (Figure 3.5A). I found that the double-inhibition constant, \( K_I' = 3.6 \pm 0.6 \ \mu\text{M} \), was identical to \( K_I = 3.8 \pm 0.4 \ \mu\text{M} \) for BDZ-f alone (Figure 3.2B and Table 3.1), indicating that the two inhibitors competed for the same site on the GluA2Q\textsubscript{flip} receptor. If GYKI 52466 and BDZ-f bound to two different noncompetitive sites on the same receptor, a much stronger inhibition would have been expected (Figure 3.5A, dashed line simulated by Equation 10). This was because the concentration of two inhibitors, each binding to its own site independently, would have been higher, thus
producing stronger inhibition. Furthermore, I performed an additional double-inhibitor experiment with BDZ-f and BDZ-2. BDZ-2 has a 4-carbonyl group on the diazepine ring or, precisely, a ε-lactam structure. BDZ-2 was shown before that it binds to a noncompetitive site different from the site to which GYKI 52466 binds. Thus, as a control, I expected that BDZ-2 and BDZ-f would bind to two separate sites. In fact, that was exactly what I found (Figure 3.5B). As expected, a larger slope was observed when both inhibitors were present. This was because the collective inhibition was attributed to the binding and inhibition from two sites. The dashed line in Figure 3.5B shows the simulation of a one-site binding/inhibition by the use of Equation 9.
Figure 3.5 (A) The result of a double-inhibition experiment for GYKI 52466 and BDZ-f on GluA2Q_{flip} determined at 100 µM-glutamate concentration. The concentration of GYKI 52466 was fixed at 15 µM. The double-inhibition constant, $K'_I$, was determined to be $3.6 \pm 0.6$ µM (filled circles), compared with $K_I$ of $3.8 \pm 0.4$ µM for BDZ-f alone (open circles). The dashed line simulates the $A/A_I$ ratio by using from Equation 10, assuming that the two inhibitors bind to two different sites with a double-inhibition constant of $\sim1.9$ µM (when GYKI 52466 was fixed at 15 µM). (B) The result of the double-inhibition experiment for BDZ-2 and BDZ-f on GluA2Q_{flip} determined at 3 mM-glutamate concentration. The double-inhibition constant, $K'_I$, was determined to be $3.1 \pm 0.2$ µM (filled circles) compared with $K_I$ of $5.4 \pm 0.8$ µM for BDZ-f alone (open circles). The dashed line represents the simulated $A/A_I$ values by using Equation 9, assuming that the two inhibitors bind to the same site with an inhibition constant of $\sim5.4$ µM (when BDZ-2 was fixed at 8 µM).
3.4 THE MECHANISM OF ACTION AND THE KINETIC PROPERTIES OF BDZ-\(f\) WITH A COMPARISON WITH GYKI 52466

In the present study, I characterized the mechanism of action of BDZ-\(f\) by measuring its inhibitory effect on the channel-opening and channel-closing rate constants as well as the whole-cell current amplitude of the GluA2Q_{\text{flip}} receptors, using a laser-pulse photolysis and a rapid solution flow technique. I further investigated whether BDZ-\(f\) interacts with two other known binding sites on GluA2Q_{\text{flip}}, which we reported earlier (98, 99); one site is where GYKI 52466 binds and the other is where BDZ-2 binds. Our findings established that (i) BDZ-\(f\) is a noncompetitive inhibitor with a slight preference for the closed-channel over the open-channel state; (ii) BDZ-\(f\) is a non-selective inhibitor for the flip and flop variants of GluA2Q because it inhibits both equally well; (iii) like other 2,3-benzodiazepine compounds we have characterized (98, 99), BDZ-\(f\) inhibits GluA2Q_{\text{flip}} by forming an initial, loose intermediate that is still partially conducting, yet this intermediate rapidly isomerizes into a tighter, fully inhibitory receptor-inhibitor complex; and (iv) BDZ-\(f\) binds to the same noncompetitive site as GYKI 52466 does on the GluA2Q_{\text{flip}} receptor. This site, however, is not the same site where BDZ-2 binds.

BDZ-\(f\) is synthesized as a structural derivative of, but supposedly better than, the parent 2,3-benzodiazepine ring structure or GYKI 52466, the prototypic 2,3-benzodiazepine compound (Figure 1.3) (58, 118). The results from the present investigation establish that BDZ-\(f\) is indeed a better inhibitor than GYKI 52466. I found that defined by the overall inhibition constant or \(\bar{K}_I\) associated with the open-channel state of GluA2Q_{\text{flip}} (Table 3.1), BDZ-\(f\) is six-fold stronger than GYKI 52466 (\(\bar{K}_I\) is 5.4 \(\mu\)M for BDZ-\(f\) and 30 \(\mu\)M for GYKI 52466; see Table 3.1). Furthermore, for the closed-
channel state, BDZ-f is four-fold better than GYKI 52466 ($K_i$ is 3.8 µM for BDZ-f and 14 µM for GYKI 52466).

The higher potency of BDZ-f, compared with GYKI 52466, can be best accounted for on the basis of the structure-activity relationship. (i) The finding that BDZ-f and GYKI 52466 bind to the same noncompetitive site on the GluA2Qflip receptor (Figure 3.5A) suggests that the addition of an N-3 methylcarbamoyl group on the diazepine ring of GYKI 52466 improves potency without changing the site of binding of the new compound, i.e., BDZ-f. (ii) The higher potency of BDZ-f seems to be realized even at the first step involving the formation of the initial, partially inhibitory receptor:inhibitor intermediate (Figure 3.3). Specifically, the GluA2Qflip:BDZ-f intermediate formed in the closed-channel state is about three-fold more inhibitory than the GYKI 52466:receptor counterpart ($K^*_i = 22$ µM for BDZ-f and 61 µM for GYKI 52466; see Table 3.1). The GluA2Qflip:BDZ-f intermediate formed in the open-channel state is six-fold more inhibitory than the GYKI 52466:receptor intermediate ($K^*_i$ is 20 µM for BDZ-f but 128 µM for GYKI 52466, respectively). This comparison suggests that the addition of an N-3 methylcarbamoyl group to the 2,3-benzodiazepine ring of GYKI 52466 makes BDZ-f more adaptable for binding to and interacting with the same noncompetitive site. (iii) GYKI 52466 shows a two-fold selectivity for the closed-channel over the open-channel state (i.e., the inhibition constants for the closed-channel and the open-channel states are 61 µM and 128 µM, respectively) (98). Addition of an N-3 methylcarbamoyl group to form BDZ-f reduces that selectivity (i.e., the inhibition constants for the closed- and open-channel states were ~3.8 µM and ~5.4 µM, respectively) but strengthened the overall inhibition constants for both the open- and closed-channel states.
Addition of an N-3 methylcarbamoyl group to the diazepine ring of GYKI 52466, resulting in BDZ-f, clearly yields a more potent inhibitor. This result stands in stark contrast with addition of the same N-3 methylcarbamoyl group to the diazepine ring of BDZ-2, thus resulting in a new compound termed BDZ-3 (99). BDZ-3, like BDZ-2, has an ε-lactam structure in which a 4-carbonyl group replaces the 4-methyl group as in the azomethine ring structure of GYKI 52466. However, BDZ-3, which contains an N-3 methylcarbamoyl group, is a weaker inhibitor than BDZ-2 although the mechanism of action of BDZ-3 and the binding site are the same as those of BDZ-2. These results suggest that the functional impact of the addition of an N-3 methylcarbamoyl group depends on the nature of the C-4 group. The addition of the N-3 methylcarbamoyl group to the azomethine structure of the diazepine ring gives rise to a stronger inhibitor (such as BDZ-f vs. GYKI 52466). In contrast, the addition of the same group to the ε-lactam structure of the diazepine ring yields a weaker inhibitor (BDZ-3 vs. BDZ-2) for GluA2Qflip (99) and is therefore undesirable. In either case, the addition of an N-3 methylcarbamoyl group does not change the destination of the resulting compound on the receptor; namely, the new compound continues to bind to the same site. On the contrary, the substitution of the azomethine group on the diazepine ring with an ε-lactam structure changes the binding site for these noncompetitive inhibitors (98). Consistent with this feature of the structure-activity relationship, BDZ-f competes at the same noncompetitive site with GYKI 52466, but this site is not the same one to which BDZ-2 binds (Figure 3.5B). Thus BDZ-2 and BDZ-f can independently bind to their respective sites on GluA2Qflip, thereby producing greater inhibition when the two are used together.
3.5 SUMMARY

In this section, I showed that the addition of an N-3 methylcarbamoyl group to the diazepine ring with the azomethine feature (i.e., GYKI 52466) does not alter the binding site of the resulting compound (i.e., BDZ-f) nor the mechanism of action but does make BDZ-f a better inhibitor of GluA2 than the original GYKI 52466. Further studies are needed to determine whether this structural feature and/or addition of other types of functional groups at the N-3 position imparts the same functionality, namely, a more potent effect but without changing the site of binding. Other factors, which include shape and stereochemical arrangement of the same size, may be also involved in defining the functionality of the resulting compounds and should also be explored. In addition, the results from this study suggest the possibility of making new 2,3-benzodiazepine derivatives with different properties, such as water solubility, as well as the possibility of converting new compounds into potentially useful photolabels for site mapping without losing the biological activity and site specificity. These new properties can be certainly generated by attaching appropriate chemical groups and/or substituents to the N-3 position of the benzodiazepine ring. The fact that BDZ-g and BDZ-h have better or much better potency yet they still bind to the same site as GYKI 52466 does further demonstrates that the N-3 position is a desirable and fertile site for chemical modifications. But again, this prediction is only applicable for 2,3-benzodiazepine compounds with the C-4 methyl group on the benzodiazepine ring.
CHAPTER 4

MECHANISM OF INHIBITION OF GLUA2 BY TALAM PANEL AND ITS STEREOISOMER

4.1 INTRODUCTION

2,3-Benzodiazepine derivatives are not just known as a group of better inhibitors of AMPA receptors, as compared with other structural templates, but they are also known for their strong neuroprotective properties against AMPA receptor-mediated neurotoxicity. For instance, BDZ-d, also known as Talampanel (or GYKI 53773, (-)-1-(4-aminophenyl)-3-acetyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine, Figure 1.3) is perhaps the best known among GYKI compounds (118, 125, 126). This is due to the fact that a number of animal and electrophysiological studies have demonstrated a strong potential for BDZ-d as a neuroprotective agent (58, 127-130). All of those promising findings, including initial clinical studies and trials in the past (131, 132), have culminated in a much publicized, high-profiled phase II clinical trial of this compound against ALS (sponsored by Teva, Israel; http://ir.tevapharm.com/phoenix.zhtml?c=73925&p=irol-newsArticle&ID=1555496). However, last May, the phase II clinical trial study reported that there is no significant
clinical benefit of using this compound for treatment of ALS. Yet, this compound shows serious side effects such as ataxia and sedation when tested in the phase II clinical trial.

What it is that we know about Talampanel or BDZ-\(d\)? It turns out that very few mechanistic details for this compound exist in literature. As a GYKI compound, how it inhibits AMPA receptor channels is lacking. To date, there has not been a systematic study of this compound with AMPA and other glutamate receptor subunits. At the molecular level, Talampanel or BDZ-\(d\) is not a very strong inhibitor as I showed in Chapter 2 from the screening assay of the inhibitory potency with respect of other 2,3-benzodiazepine compounds. For a detailed mechanistic study of this compound, I used a fast solution flow measurement together with the laser-pulse photolysis technique. These are the same techniques I used for investigation of the mechanism of action and the kinetic properties of BDZ-\(f\).

In this chapter, I describe the study of characterizing BDZ-\(d\). I also compare the result with that of BDZ-\(f\), because the structural similarity of BDZ-\(d\) and BDZ-\(f\) allows me to further define the structure-activity relationship for these 2,3-benzodiazepines. In this sense, I also used BDZ-\(e\), enantiomer of BDZ-\(d\). Moreover, the difference in the inhibition potency of 2,3-benzodiazepine enantiomers generated by a single configurational change at C-4 position on the diazepine ring had been revealed previously (e.g. BDZ-\(d\) and BDZ-\(e\)) \((118, 133)\). But to date no specific investigation has been done to compare the inhibitory effects of such a pair of enantiomers on the GluA2 and other homomeric AMPA receptors.
There are two important questions to address. First, the study of BDZ-e and its enantiomer of BDZ-d, which differs from the stereochemistry at the C-4 position, allows me to define how the stereochemistry affects the potency. This study is significant because the C-4 group is a key molecular determinant for a site of binding and generally the preference of receptor conformation. For instance, virtually all of the C-4 methyl compounds are selective towards the closed-channel conformation. Second, because both BDZ-e and BDZ-d have an identical N-3 derivatization, my study shall then enable me to validate new properties generated from derivatizations at the N-3 group, as we predicted before (see Chapter 3 conclusions). For one property, a double-inhibitor experiment is expected to show both BDZ-d and GYKI 52466 compete for the same site on GluA2.

4.2 MATERIALS AND METHODS

The cell culture, whole-cell current recording, and data analysis are as described in in the section of Materials and Methods of Chapter 1.

4.3 RESULTS

4.3.1 BDZ-d and BDZ-e Inhibit the Whole-cell Current of AMPA Receptors

The first aim in this work, as described in the first section, was to determine $K_I$ values of BDZ-d and BDZ-e against the homomeric GluA2Qflip receptor expressed in HEK-293 cells. By setting the glutamate concentrations at 100 µM and 3 mM, as is describe earlier (98, 99, 111, 117), I determined the inhibition constants for the closed-
channel and open-channel state of GluA2Q\textsubscript{flip}, respectively. Figure 4.1A shows the representative whole-cell current traces mediated by the closed-channel state (100 µM glutamate) of GluA2Q\textsubscript{flip} receptors in the absence (left) and presence (right) of 20 µM BDZ-d (this refers to a pair of traces on the left of Figure 4.1A), obtained by flow measurement. The inhibition ratio (control amplitude \textit{vs.} inhibited amplitude, \(A/A_i\)) was determined to be \(\approx 2.4\). Under the same condition, BDZ-e was barely inhibitory (see the pair of traces on the right panel of Figure 4.1A). Furthermore, the inhibition ratios were determined for BDZ-d and BDZ-e with various concentrations of respective inhibitor on both the closed-channel and open-channel states of GluA2Q\textsubscript{flip}. The results were plotted against inhibitor concentration in Figure 4.1B and Figure 4.1C, respectively. By linear regression using Equation 8a, the best fit produced the inhibition constant for BDZ-d to be 14 ± 1 µM for the closed-channel state \((K_I)\) and 30 ± 4 µM for the open-channel state \((\bar{K}_I)\). Similarly, a \(K_I\) of 201 ± 27 µM and a \(\bar{K}_I\) of 304 ± 32 µM were determined for BDZ-e. The comparison of the two sets of inhibition constants clearly shows that BDZ-d is at least 10-fold more potent than its enantiomer to inhibit GluA2Q\textsubscript{flip} at both the open-channel and the closed-channel states.
Figure 4.1 (A) Representative whole-cell currents mediated by GluA2Q\textsubscript{flip} receptors expressed in HEK-293S cells inhibited by BDZ-d or BDZ-e, respectively, obtained using flow measurement. The first pair was tested in the absence (left) and presence (right) of 20 µM BDZ-d, and the second pair was tested in the absence (left) and presence (right) of 20 µM BDZ-e. The bar above each current trace represents a pulse of 100 µM glutamate used to evoke each current. The inhibition ratio (current amplitudes in absence and presence of inhibitors, $A/A_I$) for the BDZ-d pair is ~2.4, and for the BDZ-e pair is ~1.0. All the whole-cell currents were recorded at -60 mV, pH 7.4, and 22 °C. (B) Effect of BDZ-d on the whole-cell current amplitude of GluA2Q\textsubscript{flip} receptors obtained from the flow measurement. $K_I$ of 14.5 ± 0.4 µM was determined by using Equation 8a for the closed-channel state (100 µM glutamate, ●); $K_{Ih}$ of 29.7 ± 3.6 µM was obtained for the open-channel state (3 mM glutamate, ○). (C) Effect of BDZ-e on the whole-cell current amplitude of GluA2Q\textsubscript{flip} receptors obtained from the flow measurement. $K_I$ of 201 ± 27 µM was determined by using Equation 8a for the closed-channel state (100 µM glutamate, ●); $K_{Ih}$ of 304 ± 32 µM was obtained for the open-channel state (3 mM glutamate, ○). (D) Effect of BDZ-d on the whole-cell current amplitude of GluA2Q\textsubscript{flop} receptors obtained from the flow measurement. $K_I$ of 14.7 ± 0.8 µM was determined by using Equation 8a for the closed-channel state (100 µM glutamate, ●); $K_{Ih}$ of 21.7 ± 1.4 µM was obtained for the open-channel state (3 mM glutamate, ○).
4.3.2 The Configuration of 4-methyl Group Distinguishes BDZ-\(d\) from BDZ-\(e\)

Similar to the assay with GluA2Q\(\text{flip}\), BDZ-\(d\) at different concentrations were tested on the homomeric flop channels of GluA2 or the GluA2Q\(\text{flop}\) receptor expressed in HEK293S cells. The inhibition ratios were plotted as a function of inhibitor concentrations in Figure 4.1D. A \(K_1\) of 15 ± 1 µM was determined for the closed-channel state and a \(K_1\) of 22 ± 1 µM was obtained for the open-channel state using Equation 8a. Therefore, the two sets of inhibition constants of BDZ-\(d\) against the flip and flop isoforms of GluA2 are identical (Figure 4.1B and 4.1D), within experimental errors. This result is consistent with my earlier observation with BDZ-\(f\). In both cases, I found that neither compound was sensitive to the flip/flop sequences, suggesting that the flip/flop amino acid sequence cassette located in the extracellular domain may not be involved in the formation of the binding site for these compounds.

Considering the structural similarity between BDZ-\(d\) and BDZ-\(e\), I confirmed the previous finding that the \(S\) configuration of the 4-methyl group on the diazepine ring of 2,3-benzodiazepines diminishes its activity against AMPA receptors. However, my results showed more when the data were compared with all other AMPA receptor subunits (see Figure 2.1). Specifically, BDZ-\(d\) is a relatively stronger inhibitor than BDZ-\(e\) on GluA1-4 individually. However, BDZ-\(e\) inhibits GluA1 and GluA2 with relatively the similar potency, albeit weakly. However, neither BDZ-\(d\) nor BDZ-\(e\) inhibits either GluA3 or GluA4 significantly. Overall, BDZ-\(e\) is not a strong inhibitor at all. On the other hand, BDZ-\(d\) exhibits a similar trend as BDZ-\(e\) does. However, when compared to other 2,3-benzodiazepine inhibitors, BDZ-\(d\) can be best described as a weak inhibitor. For a more detailed study, I characterized and compared the activities of both
enantiomers at 20 µM, in the form of inhibition ratios, on all of the four homomeric AMPA receptors, i.e., GluA1\textsubscript{flip}, GluA2Q\textsubscript{flip}, GluA3\textsubscript{flip}, and GluA4\textsubscript{flip}. Again, both compounds showed low potency against GluA3 and GluA4, as the inhibition ratios against GluA3 and GluA4 were measured at 100 µM and calibrated to the ratios of 20 µM (on the basis of the linear relationship between inhibition ratios and inhibitor concentrations). These results are summarized in Figure 4.2.

A few conclusions could be drawn by simply comparing the data sets in Figure 4.2. First, BDZ-\textit{d} showed higher potency than BDZ-\textit{e} on each of the four receptors. Second, both BDZ-\textit{d} and BDZ-\textit{e} inhibited GluA1 and GluA2, relatively speaking, more strongly than they do with either GluA3 or GluA4. Third, on GluA1 and GluA2, the inhibitory potency of BDZ-\textit{d} is significantly higher than that of BDZ-\textit{e} (Figure 4.2). Finally, BDZ-\textit{d} preferentially inhibited the closed-channel state on each of the four receptors (see Figure 4.2), but BDZ-\textit{e} does not have such a conformation selectivity. It should be mentioned that the potency, selectivity and receptor conformational preference defined at the subunit level of AMPA receptors have not been reported previously for either compound.
Figure 4.2 The inhibition ratios ($A/A_i$) of 20 µM BDZ-d and BDZ-e were determined for both the closed-channel and open-channel states of the four homomeric AMPA receptors: GluA1flip, GluA2Qflip, GluA3flip, and GluA4flip. The inhibition ratios against GluA3 and GluA4 were measured at 100 µM and calibrated to the ratios of 20 µM on the basis of the linear relationship between inhibition ratios and inhibitor concentrations.
4.3.3 BDZ-\textit{d} Inhibits the Channel Opening by a Two-Step Process

On the kinetic properties of these compounds, neither BDZ-\textit{d} nor BDZ-\textit{e} inhibited or affected the rate of the receptor desensitization at any glutamate or inhibitor concentrations. This observation was constant with the properties of other GYKI compounds previously reported by us and others (117, 134). Therefore in the following study I concentrated on the effects of these compounds on the channel opening rate process (as well as the current amplitude, since in the laser experiment, the current amplitude was simultaneously obtained).

The laser-pulse photolysis technique was used to investigate how BDZ-\textit{d} affected the channel opening rate process of GluA2Q\textsubscript{flip}. As a representative trace obtained from laser-pulse photolysis measurement shown in Figure 4.3A, the rise of the laser-induced whole-cell current was reflective of the opening of GluA2 channels. As compared to the control (upper trace), the current rise was slowed and the amplitude was reduced in the presence of BDZ-\textit{d} (lower trace), indicating that BDZ-\textit{d} inhibited the channel opening process of the GluA2Q\textsubscript{flip} receptor. The results further suggested that the binding of the inhibitor/glutamate was fast relative to channel opening of the GluA2Q\textsubscript{flip} receptor, because the observed rate constants in the absence or presence of the inhibitor exhibited a single-exponential rate process (observed at both 100 ± 20 µM glutamate and 300 ± 50 µM glutamate). As such, BDZ-\textit{d} inhibited the channel opening rate process rather than the rate process of ligand binding to the receptor (98, 99, 117).

The effects of BDZ-\textit{d} on the channel-opening and channel-closing rate constants were determined (Figure 4.3C, 4.3D) (as described in the section of Materials and
Methods in Chapter 1). The results suggest that BDZ-\textit{d} affected both \( k_{cl} \) and \( k_{op} \).

Specifically, a \( K_{i}^{*} \) of 57 ± 5 \( \mu \text{M} \) and a \( K_{i}^{*} \) of 66 ± 20 \( \mu \text{M} \) were determined from the rate measurement or the effect of the inhibitor on the time course of current rise. A \( K_{i} \) of 17.5 ± 1.6 \( \mu \text{M} \) was determined at 100 \( \mu \text{M} \) glutamate, and a \( K_{i} \) of 15.7 ± 0.5 \( \mu \text{M} \) was obtained at 300 \( \mu \text{M} \) glutamate from the effects of the inhibitor on the amplitudes of the current rise as observed from the laser experiment (see an example in Figure 4.3B). All the inhibition constants obtained from both laser measurement and flow measurement are summarized in Table 4.1 for comparison. The fact that BDZ-\textit{d} inhibited both \( k_{cl} \) and \( k_{op} \) was consistent with a noncompetitive mechanism of inhibition.

Interestingly, the inhibition constants for both the closed-channel and open-channel states obtained from the amplitude measurement are all smaller than those from the rate measurement. The discrepancy substantiates our previous hypothesis (98, 99, 117) that the inhibition of GluA2.Qflip channels by 2,3-benzodiazepines involves two steps, and each step contributes to the overall inhibition of the receptor (Figure 3.3). In the case of BDZ-\textit{d}, the inhibition constants for the closed-channel and open-channel states obtained from the amplitude measurement are 14.5 \( \mu \text{M} \) /29.7 \( \mu \text{M} \), comparing to the corresponding values 66 \( \mu \text{M} \) /57 \( \mu \text{M} \) from the rate measurement. By the model that involves a rapid isomerization step or the second step which we previously proposed (98, 99, 117), BDZ-\textit{d} first forms a loose receptor-inhibitor intermediate, producing partial inhibition before this intermediate turns into a tighter complex, yielding additional inhibition. The partial inhibition represented by a larger inhibition constant as observed from the rate measurement or from the single exponential rising phase in the presence of BDZ-\textit{d}, further suggested that the second step was much faster than the first step, and was not
observable in our experiment. But in the amplitude measurement, we did observe the entire effect of inhibition by this compound, because the amplitude was an equilibrium measure.

Now I wish to compare my data for both BDZ-\(d\) and BDZ-\(f\) with GYKI 52466. BDZ-\(d\) and BDZ-\(f\) are structurally different from their prototype GYKI 52466 first by an additional group on the N-3 position of the diazepine ring. The kinetic distinction between the inhibitory activities of BDZ-\(d\)/BDZ-\(f\) and GYKI 52466 can be found in the inhibition constant (\(K_i^+\)) for the open-channel state of GluA2Q\textsubscript{flip} determined in rate measurement. The measurable inhibition of GYKI 52466 shown on GluA2Q\textsubscript{flip} in the first step consists of two different inhibition constants: \(K_i^+\) of 61 \(\mu M\) and \(K_i^+\) of 128 \(\mu M\) (Table 4.1) (98). But for both BDZ-\(d\) and BDZ-\(f\), the two constants are roughly identical: 66 \(\mu M\)/57 \(\mu M\) and 22 \(\mu M\)/20 \(\mu M\), respectively (Table 4.1) (117). Thus, most likely the difference in this trend may be contributed by the N-3 substitution. In each case, an N-3 addition increased the inhibitory effect for both BDZ-\(d\) and BDZ-\(f\). It is likely that the hydrogen-bond acceptors on the N-3 substitution of a BDZ-\(d\)-like 2,3-benzodiazepine may play a key role in increasing the activity.
Figure 4.3 (A) Representative whole-cell traces from the laser-pulse photolysis experiment showing that BDZ-d inhibited both the rate and the amplitude of the opening of the GluA2Qflip channels. The top trace (○) is the control ($k_{obs} = 2079$ s$^{-1}$; $A = 0.74$ nA), and the lower one (□) was recorded in presence of 20 µM BDZ-d ($k_{obs} = 1591$ s$^{-1}$; $A = 0.38$ nA). The concentration of the photolytically released glutamate was estimated to be ~100 µM in both cases. (B) Effect of BDZ-d on the whole-cell current amplitude of GluA2Qflip receptors obtained from the laser-pulse photolysis measurement. Using Equation 8a, a $K_I$ of 17.5 ± 1.6 µM was determined at 100 µM of photolytically released glutamate (●); a $K_I$ of 15.7 ± 0.5 µM was obtained at 300 µM of photolytically released glutamate (○). (C) Effect of BDZ-d on $k_{cl}$ obtained at 100 µM of photolytically released glutamate and as a function of BDZ-d concentration. A $K_I$ of 57 ± 5 µM was determined using Equation 4. (D) Effect of BDZ-d on $k_{op}$ obtained at 300 µM of photolytically released glutamate and as a function of BDZ-d concentration. A $K_I$ of 66 ± 20 µM was determined using Equation 5.
Table 4.1: Inhibition Constants of 2,3-Benzodiazepines Obtained from Rate and Amplitude Measurements for the Closed-channel and Open-channel States of GluA2Q$_{nip}$

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Rate Measurement$^a$</th>
<th>Amplitude Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_i^*$ (µM)$^b$</td>
<td>$K_i$ (µM)$^{b,d}$</td>
</tr>
<tr>
<td></td>
<td>(closed channel)</td>
<td>(closed channel)</td>
</tr>
<tr>
<td>BDZ-d</td>
<td>66 ± 20</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>BDZ-e</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GYKI 52466$^g$</td>
<td>61 ± 11</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>BDZ-f$^h$</td>
<td>22 ± 1</td>
<td>4.0 ± 0.2</td>
</tr>
</tbody>
</table>

|             | $K_i^*$ (µM)$^b$     | $K_i$ (µM)$^{b,c}$    |
|             | (open channel)       | (open channel)        |
| BDZ-d       | 57 ± 5               | 16 ± 1                |
| BDZ-e       | -                    | -                     |
| GYKI 52466$^g$ | 128 ± 30            | 16 ± 1                |
| BDZ-f$^h$   | 20 ± 3               | 4.7 ± 0.3             |

$^a$ 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. $^g$ Ritz et al, 2011. $^h$ Wang et al, 2011.
4.3.4 BDZ-\(d\) and GYKI 52466 Bind to the Same Site on GluA2Q\(_{\text{flip}}\)

In the amplitude measurement, BDZ-\(d\) showed similar inhibition constants (see Table 4.1) for GluA2Q\(_{\text{flip}}\) receptors as compared with GYKI 52466. However, the inhibition constants of \(K_1^*\) determined from the rate measurement for the two compounds differ by about 2-fold (i.e. 57 \(\mu M\) for BDZ-\(d\) and 128 \(\mu M\) for GYKI 52466). This raised a question about whether the two compounds bind to the same site on the GluA2Q\(_{\text{flip}}\) in order to explain the effect of altered structural feature in BDZ-\(d\) on its improved biological activity as compared with GYKI 52466. A double-inhibitor experiment was therefore performed to address this question.

In this assay, BDZ-\(d\) and GYKI 52466 were applied on the receptor simultaneously. However, the concentration of GYKI 52466 was fixed at 20 \(\mu M\), while the concentration of BDZ-\(d\) varied from 5 \(\mu M\) to 40 \(\mu M\). As shown in Figure 4.4, the double-inhibition constant was determined to be 14.0 \(\pm\) 0.4 \(\mu M\), comparable with \(K_1\) of 14.5 \(\pm\) 0.4 \(\mu M\) for BDZ-\(d\) alone. This result suggested that BDZ-\(d\) would have to bind to the same site where GYKI 52466 bound on the GluA2Q\(_{\text{flip}}\) receptor. If the two inhibitors bound to two different sites, a double-inhibition constant of \(\sim 7 \mu M\) would be expected (using Equation 10). Because both compounds bind to the same site, the change of the inhibitory potency can be rationalized by the fact that the addition of an N-3 acetyl group does not change the binding site of the resulting compound on GluA2Q\(_{\text{flip}}\), but does improve the potency. This conclusion was consistent with my result with BDZ-\(f\), where a methylcarbamoyl group at the N-3 position of the diazepine ring did not change the binding site on GluA2Q\(_{\text{flip}}\) as compared with GYKI 52466, but like BDZ-\(d\), such as an addition led to a compound with an improved potency.
Figure 4.4 The result of a double-inhibitor experiment for GYKI 52466 and BDZ-d on the closed-channel state of GluA2Q_{flip} (100 µM glutamate). The concentration of GYKI 52466 was fixed at 20 µM while that of BDZ-d varied from 5 µM to 40 µM. The double-inhibition constant, $K'_i$, was determined to be $14.0 \pm 0.4$ µM (○), compared with $K_i$ of $14.5 \pm 0.4$ µM for BDZ-d alone (●). The dashed line simulates the $A/A_i$ plot by assuming that the two inhibitors bind to two different sites with a double-inhibition constant of ~7 µM (when GYKI 52466 was fixed at 20 µM).
4.4 THE MECHANISM OF ACTION AND THE KINETIC PROPERTIES OF BDZ-d AND BDZ-e WITH A COMPARISON WITH GYKI 52466

The comparison of the inhibitory effects of BDZ-d and BDZ-e (Figure 4.2) demonstrated the importance for the 4-methyl group of 2,3-benzodiazepines: \(4R\)-methyl group is a crucial structure-activity feature required perhaps for any 2,3-benzodiazepine compounds with a useful inhibitory property. Because all of the potent compounds in group one or in Figure 1.3 contain the C-4 methyl group, such as BDZ-d, BDZ-f, BDZ-g and BDZ-h as well as GYKI 52466, and they seem to bind to the same site, the difference in inhibitory properties for these compounds on GluA2\textsubscript{Qflip} can be accounted for by the difference in their structural variations. Therefore, there are several important conclusions that can be drawn from my studies.

First, as compared to the potency among these compounds, the results from my study of BDZ-d show that the 3-acteyl group is not a good functional group in the view point of improving potency for a resulting compound. Other groups, such as a methylcarbomoyl group is better as in BDZ-f. Second, the fact that BDZ-d and BDZ-e show a slightly better inhibition on GluA1 and GluA2 but barely any inhibition on GluA3 and GluA4 is surprising. This indicates that configuration of the 4-methyl group has less impact on the inhibitory activity of a compound against GluA3 or GluA4 than on GluA1 or GluA2. Third, when all of the compounds I studied are compared, i.e., BDZ-d, BDZ-f, BDZ-g and BDZ-h as well as GYKI 52466, there seems to be a trend that these compounds have all better inhibitory effect on GluA1 and GluA2 than GluA3 and GluA4. These observations are not understood at this point. However, these results could suggest
that the binding site for the same compound such as GYKI 52466 is more similar on GluA1 and GluA2 than on GluA3 and GluA4.

It should be noted that it is perhaps not desirable to leave any subunit of AMPA receptors uninhibited. For example some evidence exists that the expression of GluA3 mRNA (but not the other AMPA receptor subunits) is elevated in motor neurons (but not in either dorsal horn neurons or in white matter), after kainic acid infusion but before any death of motor neuron (135) (kainic acid can activate AMPA receptors without desensitizing them, and its infusion into animals is a model that mimics sporadic ALS). An increase in the GluA3 expression is also observed in the motor neurons of the human superoxide dismutase 1 G93A (DOD1G93A) transgenic mice (136), and these motor neurons display an increased vulnerability to glutamate (136). Yet, the animal survival can be prolonged by administering either GluA3 antisense peptide nucleic acid (137) or a noncompetitive AMPA receptor inhibitor (138). These results suggest that an elevated GluA3 expression is uniquely linked to the excitotoxicity underlying the selective neurodegeneration of motor neurons in both familial and sporadic ALS animal models. Thus, inhibiting the GluA3 subunit is expected to be therapeutically beneficial for ALS treatment.

Therefore, the data from my study on BDZ-d would strongly suggest that for a clinical trial for ALS, other compounds such as BDZ-f, BDZ-g and BDZ-h would have to be a better choice than a weak inhibitor BDZ-d. Coupled with its even weaker potency on GluA3 and GluA4, the choice would be even clearer.
Nonetheless, it is interesting to notice that BDZ-\textit{d} differs from BDZ-\textit{e} in the preference on inhibiting the closed-channel state of all the four AMPA receptors (Figure 4.2). This suggests a direction for developing inhibitors that differentially affect the closed-channel state over the open-channel state of AMPA receptors. Furthermore, the data from our lab also support the notion that the closed-channel state of GluA2 at the very least is more flexible than the open-channel state. Therefore, developing inhibitors targeting the closed-channel state may be a better choice than targeting the open-channel state. This conclusion can be qualitatively rationalized also from the inhibitory trend from the compounds in group 1 vs. group 2, as described in Chapter 2.

4.5 SUMMARY

In the work described in this chapter, I characterized the effect of stereochemistry on the structure-activity relationship of 2,3-benzodiazepine compounds. Specifically, I focused on the change of 4\textit{R}-methyl vs. 4\textit{S}-methyl group on the benzodiazepine ring. I further carried out my study by choosing a compound (i.e., BDZ-\textit{d} or Talampanel) that has failed an ALS clinical trial. The molecular mechanism of inhibition and subunit selectivity of this compound is currently unknown. My result demonstrates that 4\textit{R}-methyl group is crucial for all ensuing activity of resulting compounds, and the C-4 methyl defines a site to which all other compounds that carry the C-4 methyl group bind. I further demonstrate that the N-3 position is a site to which various chemical substituents can be attached. Among those, certain heterocyclic ring structures such as those on BDZ-\textit{h} and BDZ-\textit{g} produce the strongest potency ever known.
The results from my study therefore allow me to conclude that BDZ-d would not have been a choice for clinical trial due to its poor potency and poor subunit selectivity. It is apparent that further studies will be needed to refine the drug/inhibitor design strategy because my data show there is not a synthetic approach that can produce 2,3-benzodiazepine compounds that show higher specificity for GluA3 and/or GluA4. More importantly, my results imply that GluA3 and GluA4 have different structures than GluA1 and GluA2. Perhaps different synthetic strategy would have to apply to discover better compounds for GluA3 and GluA4. Alternatively, different chemical molecules with different structures may have to be found to become potent inhibitors.
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