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Deciphering the Biophysical Properties of Ion Channel Gating Pores by Coumarin–Benzodiazepine Hybrid Derivatives: Selective AMPA Receptor Antagonists

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Abstract

In the 1980s, the identification of specific pharmacological antagonists played a crucial role in enhancing our comprehension of the physiological mechanisms associated with α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (AMPARs). The primary objective of this investigation was to identify specific AMPA receptor antagonists, namely 2,3-benzodiazepines, that function as negative allosteric modulators (NAMs) at distinct locations apart from the glutamate recognition site. These compounds have exhibited a diverse array of anticonvulsant properties. In order to conduct a more comprehensive investigation, the study utilized whole-cell patch-clamp electrophysiology to analyze the inhibitory effect and selectivity of benzodiazepine derivatives that incorporate coumarin rings in relation to AMPA receptors. The study's main objective was to acquire knowledge about the relationship between the structure and activity of the compound and comprehend the potential effects of altering the side chains on negative allosteric modulation. The investigation provided crucial insights into the interaction between eight CD compounds and AMPA receptor subunits. Although all compounds demonstrated effective blockade, CD8 demonstrated the greatest potency and selectivity towards AMPA receptor subunits. The deactivation and desensitization rates were significantly influenced by CD8, CD6, and CD5, distinguishing them from the remaining five chemicals. The differences in binding and inhibition of AMPA receptor subunits can be attributed to structural discrepancies among the compounds. The carboxyl group of CD8, situated at the para position of the phenyl ring, substantially influenced the augmentation of AMPA receptor affinity. The findings of this study highlight the potential of pharmaceutical compounds that specifically target AMPA receptors to facilitate negative allosteric modulation.

Keywords AMPA receptor · Carboxyl group · Coumarin · Benzodiazepine · Inhibition · Antagonist

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Introduction

The ionotropic glutamate receptors (iGluRs) are classified into three primary types, namely N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate), based on the selective agonists that were initially identified to activate them. The native receptors of these families consist of tetrameric assemblies comprising multiple subunit types. The AMPA-sensitive ionotropic glutamate receptors are responsible for most rapid excitatory neurotransmission within the human brain's postsynaptic density of glutamatergic synapses [1, 2]. AMPA receptor channels are formed by combining four distinct subunits, specifically GluA1-4 [3, 4]. Each subunit contributes unique biophysical, pharmacological, and functional characteristics to the AMPAR, further diversifying the receptor's properties and functionality. The structural composition of each subunit comprises an N-terminal extracellular amino domain (NTD), a ligand binding domain (LBD), a channel membrane domain, and a C-terminal intracellular domain. The LBD also comprises two extracellular polypeptide segments, S1 and S2. The receptor channel domain comprises three helices that span the membrane (M1, M2, and M3) and a loop in the channel pore (M2) [2–4]. A proposal has been put forth suggesting that AMPARs exhibit a heterotetrameric arrangement consisting of dimer–dimers. The AMPARs exhibit rapid kinetics and play a crucial role in the manifestation of synaptic plasticity by initiating the opening of cation channels upon binding with glutamate, thereby leading to the depolarization of postsynaptic membranes [5, 6]. Within a short span of milliseconds, the pore undergoes closure due to either the release of ligand or the receptor's conformational alteration leading to a stable, closed, and desensitized state. The AMPA receptor channels exhibit rapid onset, offset, and desensitization properties, as reported in the literature [6].

The quantity and features of postsynaptic AMPARs are key determinants of synaptic transmission, and they are changed during synaptic plasticity to consistently increase or decrease transmission at particular synapses [7]. An augmentation in postsynaptic AMPAR expression typically elicits long-term potentiation (LTP), while a reduction promotes long-term depression (LTD). The GluA2 subunit is a prevalent constituent observed in the majority of AMPARs. Nevertheless, certain AMPARs do not possess GluA2, enabling the permeation of Ca^{2+} and Zn^{2+}. The presence of these particular AMPARs can be detected at specific synapses, and their initial temporary incorporation plays a crucial role in initiating both long-term potentiation (LTP) and long-term depression (LTD) [8, 9].

Moreover, the absence of GluA2 in AMPARs, which facilitates the influx of calcium ions, has been associated with excitotoxic neuronal demise. The hyperexcitability of excitable neurons is attributed to the entry of calcium ions (Ca^{2+}) through voltage-dependent calcium channels. Upon the entry of Ca^{2+} into the neuron, it initiates internal processes encompassing the modulation of membrane excitability, neurotransmitter release, and abnormal discharges and seizures [10–12]. Epileptic seizures are a multifaceted neurological disorder characterized by chronic and recurring episodes, as the World Health Organization states. These seizures are triggered by atypical and coordinated electrical activity in distinct clusters of overactive neurons dispersed throughout different brain regions. The excessive activation of these neurons results in heightened electrical discharges, manifesting as epileptic seizures. If these seizures occur repetitively over a prolonged period, they can elicit neurotoxic effects on the brain [10, 13, 14].

Understanding the physiological functions of AMPA receptors was achieved by the discovery of specific pharmacological antagonists in the 1980s. Quinoxalinediones were first made accessible in the late 1980s. Since then, a few more AMPAR antagonist drugs with varying chemical architectures have been synthesized. The 2,3-benzodiazepines, such as GYKI 52466, was identified as a new class of selective AMPA receptor antagonists in the early 1990s. Unlike previously identified AMPA receptor antagonists, 2,3-benzodiazepines function as negative allosteric modulators (NAMs) at a target other than the glutamate recognition site [13, 15, 16]. Such compounds have been shown to exhibit broad-spectrum anticonvulsant action [13]. In a clinical trial for refractory partial seizures, the GYKI 52466 homolog talampanel (GYKI 53773, LY300164) displayed effectiveness [17, 18].

Perampanel (PMP) is a pharmacological agent of the third generation of anticonvulsant drugs. It received approval from the Food and Drug Administration (FDA) in 2012 for its adjunctive therapy in treating refractory focal-onset seizures and generalized-onset tonic–clonic seizures [19–21]. It is postulated that the anticonvulsant properties of perampanel are attributable to its negative allosteric modulation of AMPA receptors. Numerous novel compounds have been examined for their potential as antagonists of AMPA receptors. Several affirmative results have been documented regarding the usage of systemically administered AMPA antagonists in diverse pharmacological models of epilepsy and neurological ailments that are believed to be associated with immoderate AMPA receptor stimulation [22–25]. According to existing literature, it has been observed that perampanel displays a binding affinity towards four distinct sites located at the interface of the extracellular and transmembrane domains of AMPA receptor channel subunits in their closed conformation [26]. The sites mentioned above

are situated at the S1-M1 and S2-M4 linkers, which significantly impact the regulation of channel gating [27]. PMP functions as a negative allosteric modulator by exerting inhibitory effects on the activity of AMPA receptors through non-competitive antagonism. A comprehensive comprehension of the inhibitory impact of PMP on AMPA receptors is yet to be fully elucidated.

Recent research suggests that discrete binding sites on AMPA receptors for both PMP and decanoic acid may exist. The PMP molecule has demonstrated a propensity for binding to the S1-M1 and S2-M4 linkers of the receptor. The scholarly works have identified where PMP binds to GluA2 AMPA receptor subunits. The PMP protein directly interacts with the pre-M1 linker and the M1, M3, and M4 helices in each subunit. Furthermore, there is a potential for encountering M3 from a neighboring subunit at the location of S615 [28]. Binding studies have revealed that NAMs from the 2,3-benzodiazepine, quinazolinone, and pyridone families have the same binding site, which differs from the well-known binding sites for positive allosteric modulators (PAMs) at LBD interfaces [29–32]. Recent research has revealed that NAMs bind to the interface between the ion channel (M3) and the transmembrane-ligand binding domain linkers (pre-M1, S2-M4) within a single subunit, creating a large and flexible binding pocket that allows for the binding of modulators with different structures. This binding site is critical in preventing the M3 helix from shifting during channel opening and is a promising target for developing new therapies for AMPA receptor–related neurological diseases [29–31, 33].

Previously, the coumarin derivatives were identified using spectroscopic techniques [34]. Multiple research teams began intensive chemical and pharmacological research in the 1990s to characterize the structure–activity relationship of benzodiazepines and to propose new derivatives with a more favorable pharmacological profile. A few hundred analogs have resulted in a thorough knowledge of replacing various side chains at various places on the fundamental chemical structure of 2,3-benzodiazepine. In a previous study, our laboratory researched two distinct groups of newly discovered 2,3-benzodiazepine derivatives. The primary objective was to better understand the relationship between the compound's structure and activity. Specifically, we focused on investigating the impact of modifications in the side chains on the compound's ability to potentially restrict excitotoxicity. As a result, adding an electron-withdrawing group to the phenyl ring was reported to strengthen the interaction of the compound with the AMPA receptor [22, 23]. The extent to which chemical structural modifications impact the selectivity of recently developed drugs for AMPA receptors remains uncertain. Based on the existing research on 2,3-benzodiazepine compounds, it is hypothesized that the eight CD compounds now under investigation (CD1-CD8) will have inhibitory characteristics on AMPA receptor subunits. These hybrids, which mix coumarin and benzodiazepine, are expected to have a unique affinity for AMPA receptors, modifying their gating processes and producing alterations in their functional characteristics (Fig. 1). This work intends to expand our knowledge of the link between structure and activity by evaluating CD drugs' inhibitory effect and selectivity on homomeric and heteromeric AMPA receptor subunits.

Furthermore, we want to investigate the effect of changes to the compounds' side chains on their capacity to prevent negative allosteric regulation. Furthermore, this research aims to determine the effect of chemical alterations on the specificity of AMPA receptor targeting shown by these newly discovered pharmaceutical compounds. This work seeks to contribute substantially to understanding CD drugs' medicinal potential by targeting AMPA receptors.

Our major goal is to use a comprehensive technique to examine the intricate link between the chemical makeup of CD compounds and their ability to block AMPA receptors. The findings of this research have the potential to aid in developing novel therapeutic approaches that increase AMPA receptor selectivity and inhibitory regulation. The advancements mentioned above can potentially have significant ramifications in neuroscience, paving the way for novel approaches to improve the efficacy and precision of treatments for various neurological disorders associated with AMPA receptor dysregulation.

Methods

Synthesis

The target molecules used in this study were synthesized according to the relevant literature [34]. In the reaction where β-toluenesulfonic acid was used as a catalyst, the β-dicarbonyl compound and o-phenylenediamine derivatives were dissolved in 30 ml xylene in equivalent amounts (1:1) and refluxed. After 24 h, the yellow product precipitated in the reaction medium. The crude product was filtered and washed with n-butanol and diethylether, respectively. The target product CD was then crystallized using *N*, *N*-dimethylformamide (DMF).

HEK293 Cell Culture and Transfection

The QIAGEN Plasmid Mini Kit was used for DNA preparation, producing high-copy plasmid DNA up to 20 µg [35]. The original cDNAs encoding the homomeric GluA1, GluA2, and heteromeric GluA1/2 and GluA2/3 AMPA receptors, GluK1 and GluK2 kainate receptors, and NMDA receptors GluN1a/2A and GluN1a/2B were kindly provided

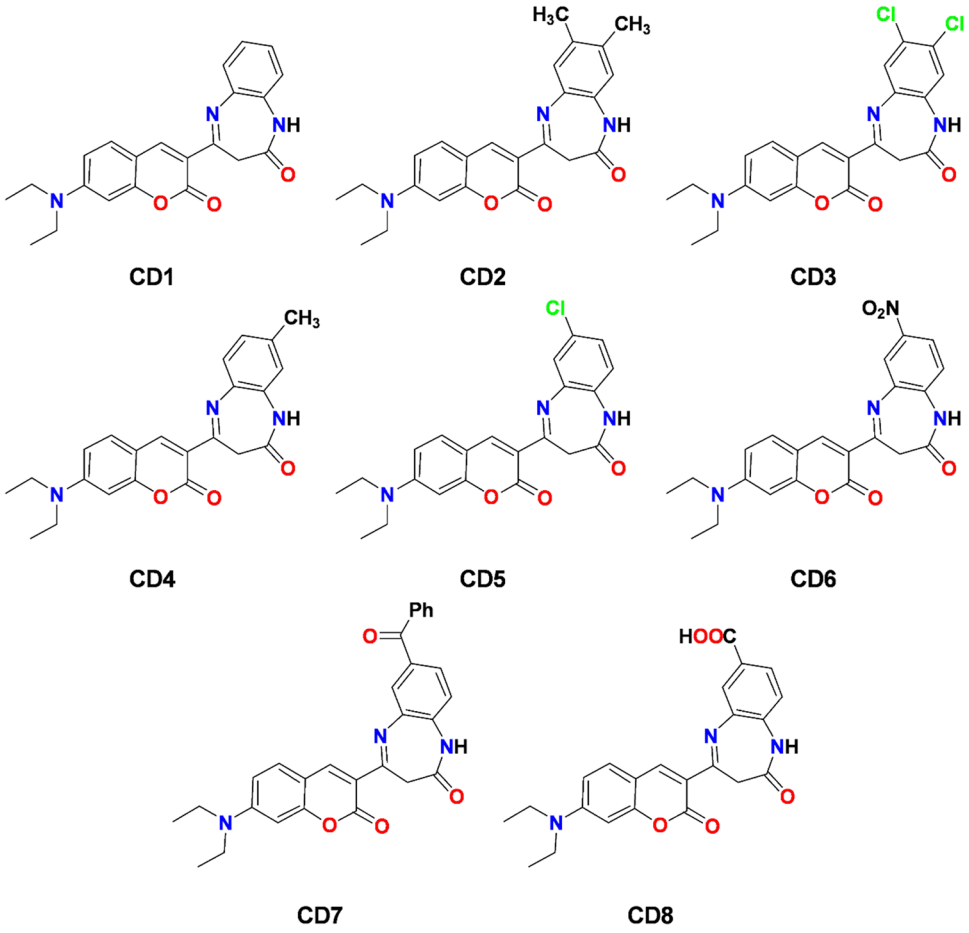


Fig. 1 Chemical structures of the eight coumarin–benzodiazepine hybrid compounds (CD1–CD8)

by S. F. Heinemann (Salk Institute, La Jolla, CA, USA). All the receptors were transiently expressed in human embryonic kidney (HEK293) cells. Previous research described DNA preparation, cDNA transient transfection in HEK293 cells, and HEK293 cell culture and transfection [33, 36]. In brief, HEK293 cells were grown in a humidified incubator at 37°C with 5% CO_2 and in Dulbecco's modified Eagle's medium (Sigma, Germany) supplemented with 10% fetal bovine serum, 0.1mg/ml streptomycin, and 1 mM sodium pyruvate (Sigma, Germany). The humidified incubator in which the cells developed was adjusted at 37°C and 5% CO_2. It should be mentioned that all of the compounds were tested with AMPA, kainate, and NMDA receptors in our investigation. In the transfection of AMPA and kainate receptors, 2–30 µg of a specific cDNA plasmid was utilized in a 10:1 ratio with the plasmid encoding a green fluorescent protein (GFP). A 1:1 ratio of NMDA receptor plasmids was employed to transfect NMDA receptors (GluN1a/GluN2A and GluN1a/GluN2B heteromeric subunits. To prevent excitotoxicity, 1 mM ketamine was added to the NMDA receptors solution. The medium was removed for recording and thoroughly washed with a ketamine-free recording solution several times. Repetitive glutamate administration before data collection guaranteed that the blocker was completely removed before the studies [37–39].

Whole-Cell Current Recordings

A patch was situated anterior to a swift perfusion mechanism consisting of a theta tube flow conduit affixed to a piezoelectric translator, which was employed to cleanse the cell from one barrel incessantly. At the same time, the other dispensed the desired compound to the cell. The recordings were performed at a temperature of 22°C, with a membrane potential of −60 mV, 36–48 h post-transfection. The currents were enhanced using an integrated patch amplifier (IPA) manufactured by Sutter Instruments in Novato, CA, USA. The amplified currents were then subjected to a 2-kHz filter and digitized at a rate of 10 kHz with SutterPatch Software v. 1.1.1 [23, 40]. The experiment employed pipettes with a resistance range of 2–4 MΩ. These pipettes were filled with a solution containing millimolar concentrations of the following substances: 110 CsF, 30 CsCl, 4 NaCl, 0.5 $CaCl_2$, 10 trypsin-EDTA solution B (0.25%), EDTA (0.05%), and 10 HEPES.

The pH of the solution was adjusted to 7.2 using CsOH. The composition of the extracellular medium contained (in mM) as follows: 150 NaCl, 2.8 KCl, 0.5 $MgCl_2$, 2 $CaCl_2$, and 10 HEPES, pH 7.4 adjusted with NaOH. The pH of the medium was adjusted to 7.4 using sodium hydroxide. The membrane potential at rest was commonly observed to be −60 mV. The solution exchange time (10–90% to peak) was estimated through open tip control, lasting 500 ms after the recording. The inhibition process was determined by comparing the current observed in a cell supplied with glutamate alone versus the current observed in the same cell after being supplied with glutamate and the CD compounds. The primary tube containing exclusively glutamate was reintroduced into the cell to ensure cellular safety and validate the antagonist's results. This measure was taken to ensure that the current induced by glutamate, observed before the administration of the antagonist, was nearly identical to the current observed after the administration. The sample size was determined based on the viable cells' requirement to compute the derivative's mean inhibition under investigation. The experiment was conducted with the dual objectives of ascertaining the ratio of whole-cell current amplitude in the absence and presence of compounds (A/A_I) and deriving a constant inhibitory value that is not dependent on rate measurement. As a supplementary technique, a rapid exchange solution flow methodology was utilized as an independent control method. This involved piezoelectric actuators, and a swift piezo-driven translation of parallel solution streams over an outside-out patch (provided by Automate Scientific, Berkeley, CA, USA). This method was chosen because it provides the fastest exchange solution, with open tip current rise times ranging from 100–300 µs [35, 41]. The purpose of this technique was to evaluate the amplitude of the whole-cell current.

The comprehensive analysis of the process mentioned above is presented in the supplementary materials (Tables S1-S8). The data obtained were analyzed using Igor Pro7 software (Wave Metrics, Inc.). The estimation of receptor desensitization (τ_{des}) and deactivation rates was done through a two-exponential fitting procedure. This fitting was performed on the current decay, which commenced from 95% of the peak and extended to the baseline current. The activation and deactivation of currents were achieved by applying 10 mM glutamate for 500 ms and 1 ms, respectively, to induce desensitization. AMPAR-current deactivation and desensitization were modeled using a dual exponential function. The resulting weighted tau (τ_w) was determined by the equation $\tau_w = (\tau_f \times a_f) + (\tau_s \times a_s)$, where τ_f and τ_s represent the time constants of the fast and slow exponential components, respectively, and a_f and as represent their relative amplitudes [42–44].

Statistical Analysis

The statistical information is presented as the mean value accompanied by the standard error of the mean (SEM). The term "sample size," denoting a value of n=10, pertains to the quantity of HEK293 cells subjected to examination. The statistical technique of one-way analysis of variance (ANOVA) was employed to examine the variations between the groups and the wild-type. The threshold for statistical significance was set at $p< 0.05$, with (*) indicating significance at this level, (**) indicating significance at $p< 0.01$, and (***) indicating significance at $p< 0.001$. The abbreviation "ns" denote a lack of statistical significance. The Hill equation fits concentration-response correlations as composite curves through GraphPad Prism version 6.01 (GraphPad Software). The findings were derived from a minimum of three distinct trials.

Results

Electrophysiological Recordings of AMPAR Subunits' Responses in HEK293 Cells

Our patch-clamp experiments initiated the investigation by examining the effects of each of the eight CD compounds on the responses of HEK293 cells expressing GluA1, GluA2, GluA1/2, and GluA2/3 (**Tables S1-S8**). This study aimed to acquire significant knowledge regarding the impact of CD compounds on various subunit combinations of AMPA receptors, thereby providing a better understanding of their modulatory characteristics on these specific receptor subtypes. As illustrated in Fig. 2, the examined chemicals exhibited a negative potentiation effect on all the tested AMPAR subunits, reducing current amplitude. Applying CD1 and CD7 reduced the A/A_I ratio by approximately 2–3-fold. In this context, A denotes the current produced solely by glutamate, while A_I represents the current induced by the combination of glutamate and CD compounds (Fig. 2a, c, e, g). CD2, CD3, CD4, and CD5 demonstrated a 3–5-fold inhibition of AMPAR subunits, as depicted in Fig. 2b, d, f, h. In addition, it was observed that CD6 exhibited a significant reduction in the currents of all AMPAR subunits, resulting in a decrease of approximately 8–9 times. The IC_{50} values for this reduction ranged from 3.45 to 3.7 µM. The researchers observed that the CD8 compound exhibited the highest potency level on AMPAR subunits. The approximate IC_{50} values for the GluA1 subunit, GluA1/2, GluA2, and GluA2/3 were determined to be 3.02 µM, 3.10 µM, 3.19 µM, and 3.37 µM, respectively (Fig. 3, **Table S9**).

In addition, the investigated CD compounds exhibited a decline in the activity of AMPAR subunits that was dependent on the dosage. This reduction commenced at

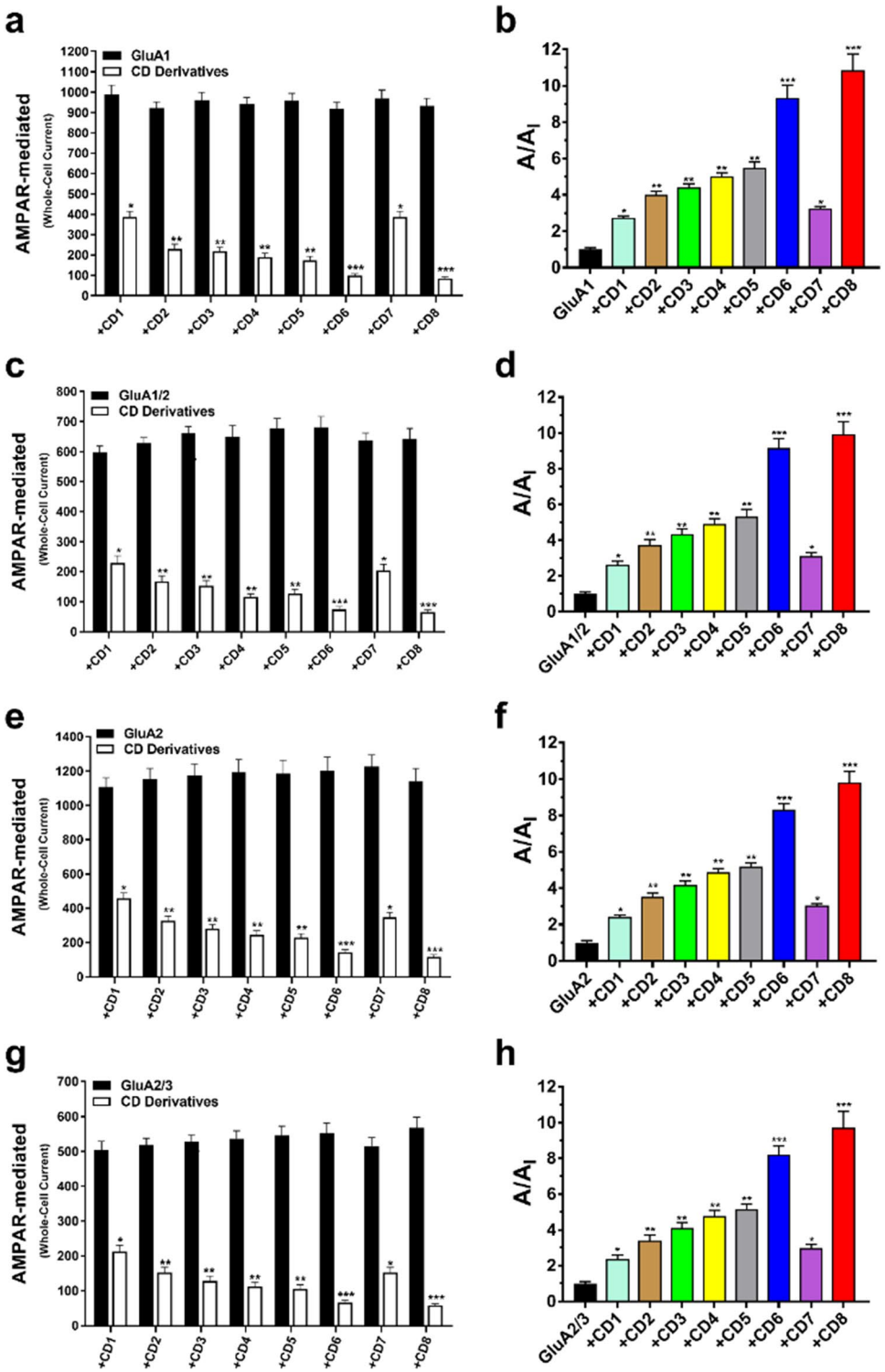


Fig. 2 Effect of the eight CD compounds on AMPAR synaptic currents. Representative whole-cell current responses of GluA1 (**a**), GluA1/2 (**c**), GluA2 (**e**), and Glua2/3 (**g**) expressed in HEK293 cells to 10 mM glutamate alone (black) and glutamate+ CD compounds (12 µM) (white). The whole-cell current (pA) was measured at −60 mV, pH 7.4, and 22°C. For comparison, one-way analysis of variance (ANOVA) was used: *$p< 0.05$; **$p< 0.01$; ***$p< 0.001$. **b**, **d**, **f**, and **h** indicate the A/A_I ratio, where A represents the current formed by glutamate alone, and A_I represents the current induced by glutamate + CD compounds obtained after each CD compound administration on each AMPAR subunit. Ten cells were evaluated in each experiment, and all data were provided as means ± SEM

a concentration of 2 µM and continued until reaching the maximum efficacy at the plateau concentration of 16 µM (Figure S1 a-d). Initially, a concentration of 10 mM of glutamate was employed. Subsequently, a combination of glutamate and CD chemicals was introduced at escalating concentrations (expressed in µM) during each recording interval, following the 500-ms protocol. Finally, glutamate alone was reintroduced to verify the viability of the cells.

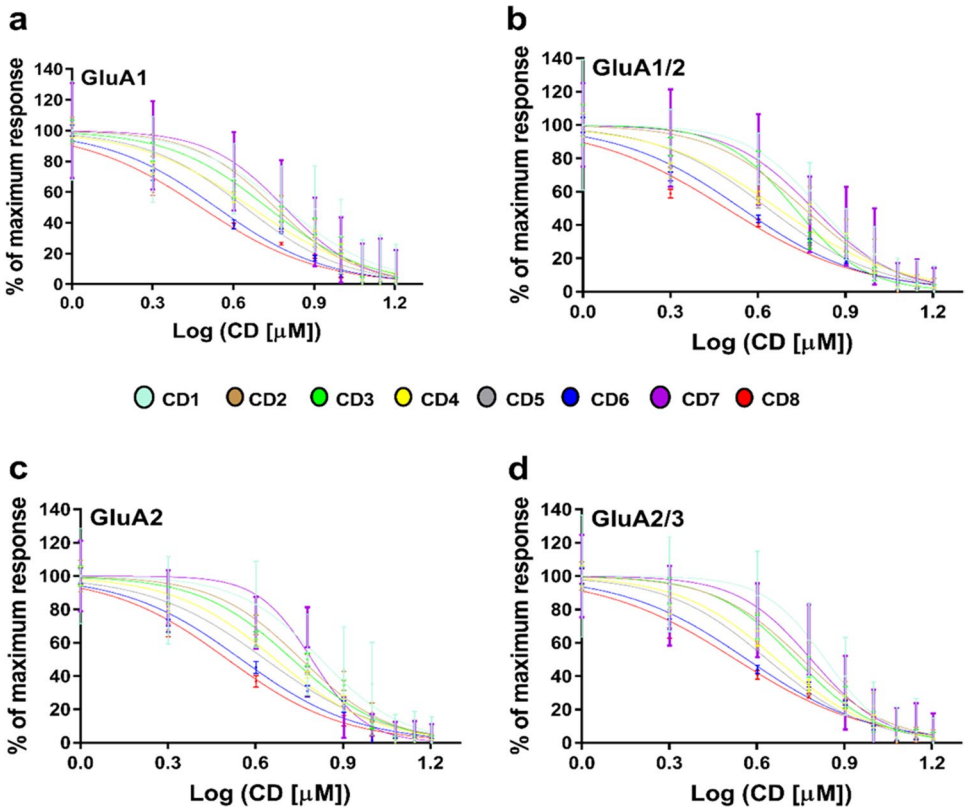


Fig. 3 Comparison of CD compounds' influence on AMPAR subunits' responses. Whole-cell responses to inhibitors were adjusted to currents (pA) and represented as a percentage of the control value. The IC_{50} values for CD compounds on the whole-cell peak amplitude in HEK293 cells are shown in (**a**, **b**, **c**, **d**). IC_{50} values were calculated using GraphPad Prism. The whole-cell current was measured at −60 mV, pH 7.4, and 22°C. Cells were subjected to CD compound concentrations (ranging from 2–16 μM). Each concentration was administered to the cell for 500 ms before being washed for 20 ms. Following many washout periods, glutamate (10 mM) was administered alone. The symbols/error bars reflect the mean ± SEM for 10 cells, and each data point represents the average of three to four different experiments

CD8 is a Potent and Selective AMPAR Subunits Inhibitor

The present study focused on investigating the effects of the CD8 compound on homomeric GluK1 and GluK2 kainate receptor channels, as well as GluN1a/2A and GluN1b/2B NMDA receptor channels. These receptor channels were expressed in HEK293 cells. This study aimed to comprehensively compare the CD compounds' activities on AMPA, kainate, and NMDA receptors. Furthermore, our objective was to ascertain whether the robust impact of CD8 was limited to AMPA receptors. In order to accomplish this objective, a series of comprehensive experiments were carried out, and the obtained results were thoroughly analyzed. This process, as illustrated in Fig. 4a, enabled us to make significant comparisons and gain valuable insights into the functional attributes of CD8 across the various receptor types. In contrast to the observed inhibitory effect of CD8 on AMPAR homomeric subunits (specifically, GluA1 and GluA2), as depicted in Fig. 4b, no significant inhibition of these channels by CD8 was observed when the A/A_I ratio was less than a 2-fold increase for the tested kainate and NMDA subunits. The concentration of CD8 used in all evaluated subunits was consistent at 12 μM, and the number of cells utilized in each experiment was standardized to 10.

The CD Compounds Modify the AMPAR Subunit's Kinetics

In order to examine the reactions of recombinant AMPAR subunits, a series of whole-cell recording experiments were performed on HEK293 cells that had been transiently transfected. The whole-cell recording technique facilitated the prompt and precise delivery of solutions, thereby enabling the accurate characterization of the swift deactivation and desensitization kinetics demonstrated by AMPAR subunits. The conducted experiments played a pivotal role in acquiring accurate data and delineating the functional properties of the AMPAR subunits that were being examined (**Tables S1-S8**). The 500-ms protocol was employed to investigate the phenomenon of AMPA receptor desensitization, while the 1-ms protocol was utilized to assess receptor deactivation. The results of our study indicate that the impact of the examined CD compounds on AMPAR gating characteristics varied. Specifically, CD1–4 and CD7 did not significantly affect deactivation or desensitization, as illustrated in Fig. 5a, c, e, g and Fig. 6a, c, e, g. In contrast, the administration of CD5 resulted in a notable augmentation of the weighted mean time constant of deactivation (τ_w deact) by a factor of 1–2, as demonstrated in Fig. 5a, c, e, and g. In addition, it was observed that CD6 resulted in a 2–3-fold increase in the deactivation rate (τ_w

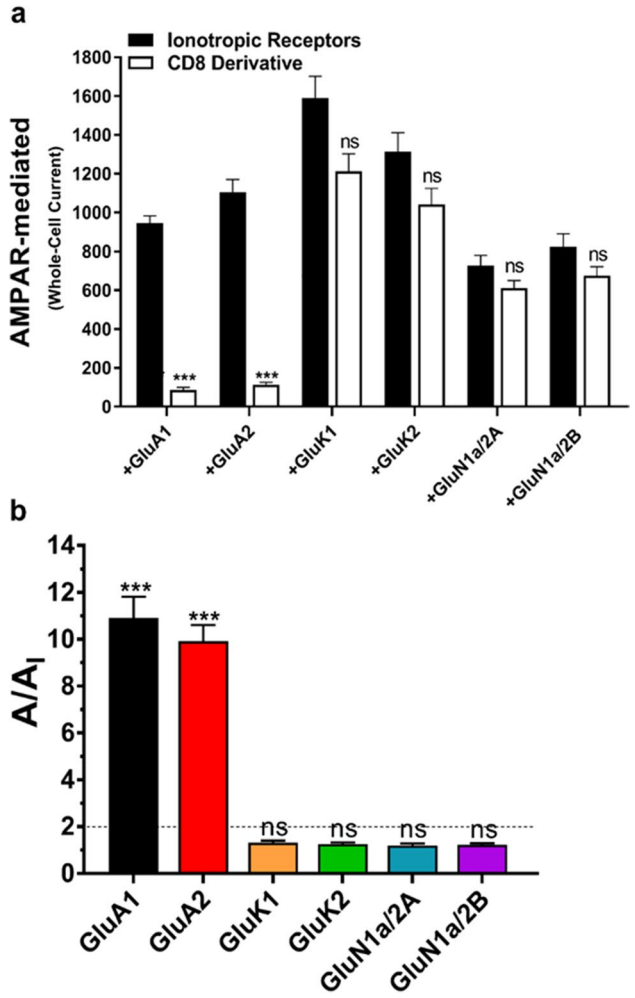


Fig. 4 CD8 selectivity to AMPAR subunits. The effects of the most effective CD compound, CD8, on homomeric AMPAR subunits (GluA1 and GluA2), kainate (GluK1 and GluK2), and NMDA receptor subunits (GluN1a/2A and GluaN1a/2B) are shown (**a**). The percentage of the current response in the absence and presence of CD8 (A/A_I) indicates an inhibitory impact of CD8 on any of these subunits. Each subunit received 12 μM of CD8, and each point averaged at least three measurements from 10 cells. The whole-cell current was measured at −60 mV, pH 7.4, and 22°C. One-way analysis of variance (ANOVA) was performed for comparison: ***$p< 0.001$, ns, not significant. The data are shown as mean ± SEM. CD8 selectively inhibited AMPA receptor homomeric subunits, as shown in (**b**), since the effect on kainate and NMDA subunits was less than 2-fold and regarded as negligible

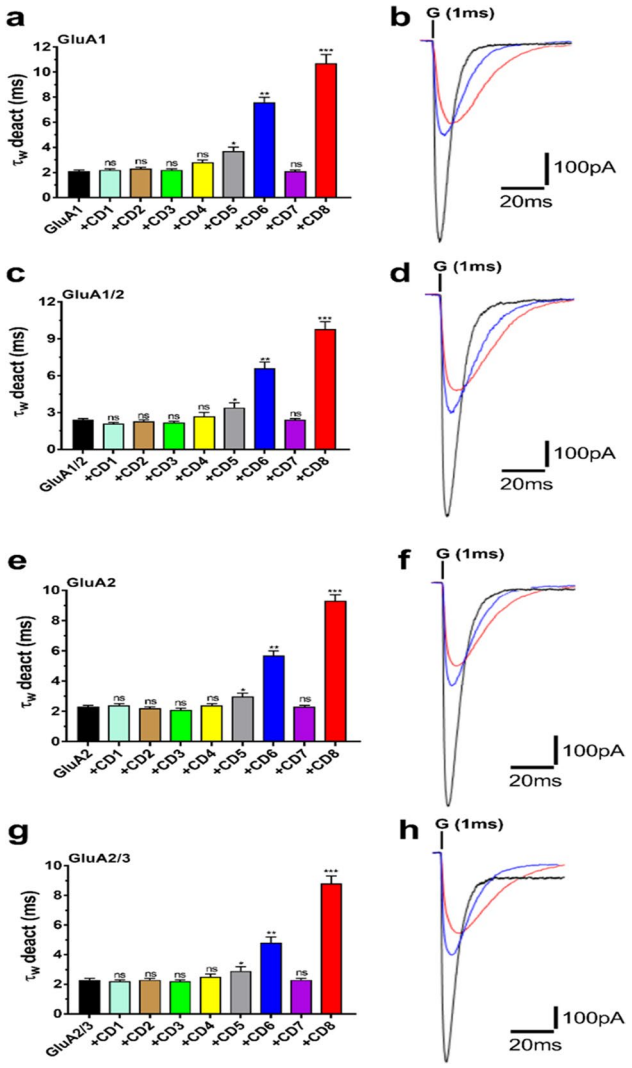


Fig. 5 Deactivation rate (τ_w deact) of AMPAR subunits after applying the CD compounds. The figures (**b**, **d**, **f**, and **h**) show the normalized whole-cell current traces (pA) of AMPAR subunits recorded upon 1 ms application of 10 mM glutamate (G, indicated above the current trace) to whole-cell recording from HEK293 cells expressing homomeric GluA1 (**a**), GluA2 (**c**), heteromeric GluA1/2 (**b**) and heteromeric GluA2/3 (**d**), glutamate alone (black) or in combination with the CD compounds as represented in colors. The whole-cell current recording was conducted at −60 mV, pH 7.4, and 22°C. Data are shown as mean ± SEM; $n = 10$ (number of patch cells in the whole-cell configuration). Significance (one-way ANOVA): where (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, ns, not significant

deact), while CD8 led to a 4–5-fold increase (Fig. 5b, d, f, h). The weighted mean time constant of desensitization (τ_w des) was reduced by CD6 and CD8 by factors of 1–2 and 2–3, respectively, as shown in Fig. 6b, d, f, and h. The application of CD8 resulted in a significant decrease in the desensitization rate (τ_w des) of GluA1/2, with a reduction of approximately 6-fold.

Discussion

The findings of our study highlight the notable pharmacological potential of the eight CD compounds, as they exhibit distinct abilities to decrease AMPAR currents and modulate the gating characteristics of these receptors. The results

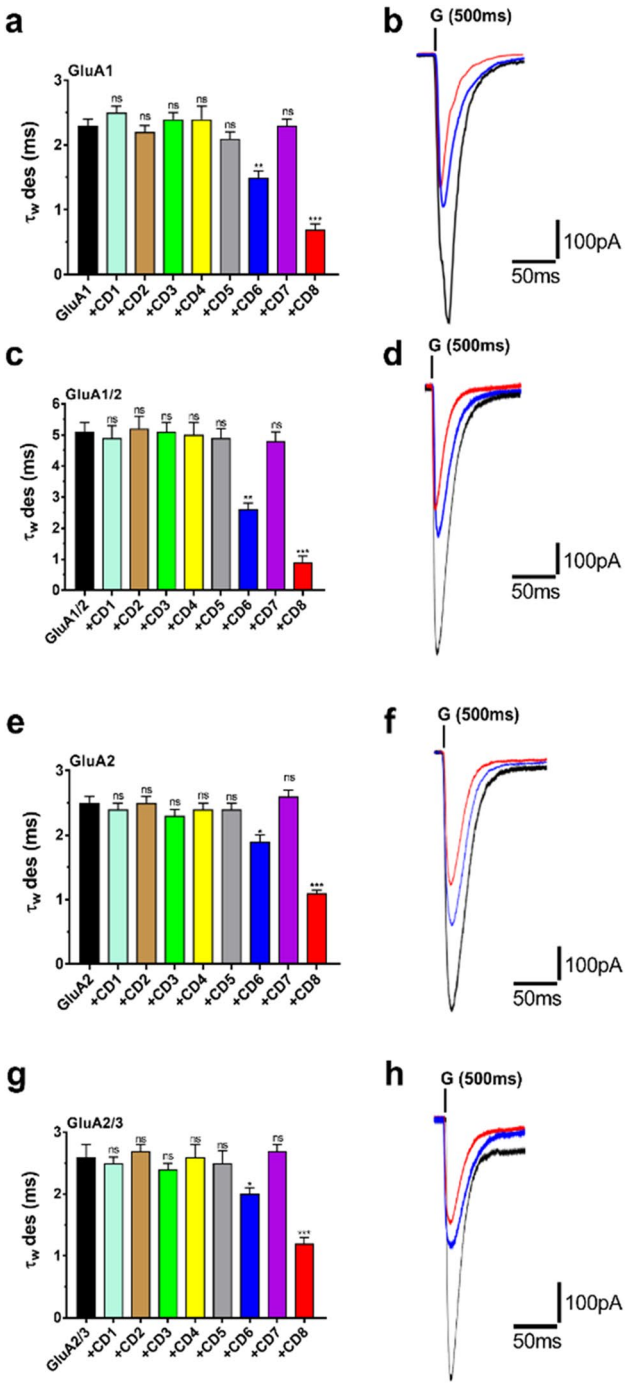


Fig. 6 Desensitization rate (τ_w des) of AMPAR subunits after applying the CD compounds. The figures (**b**, **d**, **f**, and **h**) show the normalized whole-cell current traces (pA) of AMPAR subunits recorded upon 500 ms application of 10 mM glutamate (G, indicated above the current trace) to whole-cell recording from HEK293 cells expressing homomeric GluA1 (**a**), GluA2 (**c**), heteromeric GluA1/2 (**b**) and heteromeric GluA2/3 (**d**), glutamate alone (black) or in combination with the CD compounds as represented in colors. The whole-cell current recording was conducted at −60 mV, pH 7.4, and 22°C. Data are shown as mean ± SEM; $n = 10$ (number of patch cells in the whole-cell configuration). Significance (one-way ANOVA): where (**) $p < 0.01$, (***) $p < 0.001$, ns, not significant

of our study definitively establish that the CD derivatives are powerful antagonists of the AMPA receptor, effectively reducing the activity of AMPAR. The electrophysiological recording data is of particular significance as it identifies CD8 as the most potent negative allosteric modulator (NAM) of AMPA receptors among the compounds examined. The aforementioned notable results emphasize the potential therapeutic possibilities of CD compounds in treating neurological disorders associated with AMPAR. The compounds examined in this study demonstrate a high level of efficacy in inhibiting all AMPAR subunits, underscoring their importance in furthering our comprehension and treatment of these conditions. By comprehensively examining the complex mechanisms involved in the modulation of AMPAR, we can lay the foundation for innovative therapeutic approaches to mitigate the effects of AMPAR dysfunction.

Additionally, this research presents new avenues for manipulating synaptic strength and facilitating memory formation processes. Our research study provides significant contributions that could have wide-ranging implications in developing precise and efficient treatments for neurological disorders linked to the dysregulation of AMPAR. Compounds with heterocycles that can participate in various interactions with the amino acids in the binding site of the AMPA receptor subunits have been extensively studied in recent years due to their potential therapeutic applications. These interactions can inhibit the activity of AMPA receptors, which can lead to anticonvulsant properties. The carbonyl groups, heteroatoms, and aromatic or heterocyclic rings present in our previous study of 2-oxo-3*H*-benzoxazole derivatives, and these compounds are important pharmacophore groups that can participate in various types of interactions with the amino acids in the binding site of the AMPA receptor subunits, leading to the inhibition of the receptor activity and the potential anticonvulsant properties of these derivatives [42].

Previous studies have successfully identified several crucial residues located in the vicinity of the binding pocket. These residues encompass the pre-M1, M3, and M4 helices and the S2-M4 linker. These residues are anticipated to significantly impact the interaction with compounds [43, 44]. The crystal structures of the GluA2 ligand binding core have provided insights into the molecular interactions between specific residues and established antagonists, including DNQX and ZK200775. The findings of our analysis suggest that these residues play a crucial role in the interaction with our CD compounds, thereby exhibiting antagonistic effects on AMPA receptors comparable to DNQX and ZK200775. This implies that the CD compounds exhibit AMPA receptor antagonistic properties, potentially utilizing a binding mechanism similar to the antagonists mentioned above [45, 46].

Additionally, our research unveiled a significant association between incorporating hydrophilic substituents (carboxyl, nitro, and chlorine) on the phenyl ring of the cyclodextrin compounds and their efficacy. Among the compounds studied, CD8, which possesses a carboxylic acid (COOH) functional group, exhibited the highest binding affinities. The observed interaction bears similarity to the behavior demonstrated by DNQX and ZK200775, thereby presenting compelling evidence that supports the antagonist properties of the CD compounds. On the other hand, CD1 and CD2, which either lack a substituent or possess a methyl group on the phenyl ring, demonstrated reduced efficacy. The primary distinction among these CD compounds is the substitution occurring at positions 7 and 8 within the benzodiazepine ring. The thorough comprehension of the ligand binding core of GluA2 and the mechanisms by which established antagonists such as DNQX and ZK200775 exert their effects have played a significant role in recognizing the potential of these CD compounds for the development of novel AMPA receptor antagonists. The aforementioned potential exhibits significant potential in managing neurological disorders linked to excessive stimulation of AMPA receptors, thereby underscoring their viability as therapeutic agents for addressing these conditions.

The current study outcomes greatly expand knowledge regarding CD compounds, specifically focusing on CD8 and CD6 and their strong inhibitory impacts on AMPAR subunits. The observed effects demonstrate a significantly greater magnitude than the other CD compounds, emphasizing the crucial significance of structural variations within these eight compounds. It is worth mentioning that CD8 is particularly noteworthy for its capacity to inhibit a significant portion of AMPA receptor subunits efficiently. This ability is attributed to a carboxyl group at the para position of the phenyl ring.

It is hypothesized that including the carboxyl group increases the binding strength between CD8 and AMPAR, potentially accounting for the observed discrepancies in inhibitory efficacy across these compounds. Moreover, it is conceivable that the carboxyl group demonstrates a more pronounced interaction with the amino acids that constitute the structure of the AMPA receptor compared to other chemical groups found in the CD compounds. The observations above provide valuable information regarding the fundamental mechanisms that give rise to the unique inhibitory characteristics of CD8. This knowledge contributes to the advancement of more precise and efficient pharmaceutical interventions for neurological conditions linked to the dysregulation of AMPAR.

In contrast to its counterparts, CD6 incorporates a nitro moiety at the para position of the phenyl ring, a structural characteristic that has been observed to influence ligand-gated ion channels. Similarly, CD5 is characterized by incorporating an electron-withdrawing moiety (chlorine) at the corresponding position on the ring. Chlorine has the potential to facilitate intermolecular interactions in ligand–protein complexes due to its significant electronegativity. Notably, CD3, which contains an additional electron-withdrawing group (two chlorine atoms on the phenyl ring), demonstrated significantly lower binding and inhibitory effectiveness towards AMPAR subunits than CD5. The reduced effectiveness observed could be attributed to the constraining impact of the chlorine atom on the 8 positions, thereby diminishing possible binding interactions.

The 2016 study by Yelshanskaya [47] provides a foundational perspective on the functioning of the AMPA receptor and offers key insights into the inhibitory mechanisms of non-competitive antagonists like benzodiazepine derivatives. Their use of crystallography to pinpoint the binding site for benzodiazepines and other NAMs, along with their proposed mechanism of NAM action, enrich our understanding of this complex process. The present investigation expands upon the existing knowledge to elucidate the interaction mechanism between our CD compounds, which possess both coumarin and benzodiazepine moieties, and the AMPA receptor. Our findings are largely consistent with Yelshanskaya's research. However, we have identified intriguing distinctions in how our compounds, specifically CD5, CD6, and CD8, interact with the receptor subunits. Essentially, our research confirms the significance of structural variations within NAMs. The study reveals that the presence of a carboxyl group, such as in CD8, enhances the affinity of AMPAR. On the other hand, the existence of a nitro group, as observed in CD6, or an electron-withdrawing group like chlorine, as in CD5, can regulate the gating properties of the receptor. The observations above underscore the necessity of considering the structural characteristics of NAMs in evaluating their therapeutic efficacy in conditions associated with AMPA receptors.

Moreover, the selective profile of CD compounds was compared to the ionotropic glutamate receptor family in limited research. However, it is generally accepted that the chemicals exhibit high selectivity for AMPA receptors instead of kainate and NMDA receptors. Tests showed that CD8 was highly selective for AMPA receptor subunits but had no impact on NMDA or kainate receptor subunits. Selectivity for a particular subtype of AMPA receptors is a relatively new finding. For instance, GYKI-52466, GYKI-53405, and GYKI-53655 seem to inhibit homomeric AMPA receptors expressed in HEK293 cells quite well, despite their much lower efficacy targeting kainate receptors [15, 48]. It is possible to learn more about the function of AMPA receptors in neurotransmission and disease by identifying selective antagonists that can tell apart responses induced by AMPA receptors from those evoked by other receptors.

Understanding synaptic transmission and maintenance under normal and pathological conditions and overcoming difficulties in interpreting electrophysiological data and

other factors such as voltage dependence, ionic flux, and the experimental environment will allow us to move forward with AMPA receptor research. Previous research has cast doubt on the feasibility of specifically targeting AMPA receptors; however, our findings contribute to a better understanding of how AMPA receptors work and how each subunit responds to antagonists, as well as new insights into how these coumarin and benzodiazepine derivatives exert inhibitory effects and bind AMPAR subunits, allowing us to gain a molecular understanding of this class of AMPAR inhibitors.

The present study was designed to clarify the specific-binding sites of negative allosteric modulators (NAMs) within AMPAR subunits. Identifying the binding mechanism and site of antagonists to AMPARs remains challenging, making this contribution significant to the scientific community. The inquiry also included the examination of homomeric and heteromeric subunits of AMPAR, specifically GluA1, GluA1/2, GluA2, and GluA2/3. Furthermore, the study revealed that the influence of CD6 and CD8 on the rates of desensitization exhibited atypical characteristics for an inhibitor of AMPAR. Additional research is necessary to determine the impact of AMPAR-binding compounds on the rates of deactivation and desensitization.

Conclusion

The current research investigation investigated the effects of eight CD compounds on the subunits of AMPA receptors in HEK293T cells. The findings of our study yielded statistically significant outcomes via rigorous patch-clamp experiments. The compounds exhibited notable inhibition of AMPAR subunits, with CD8 particularly remarkable for its pronounced effect on AMPA receptors. We found the structural disparities among the eight chemicals to be particularly intriguing. Introducing a carboxyl group at the para position of the phenyl ring in CD8 led to notable variations in AMPAR subunit binding, inhibition, and overall efficacy. It is worth mentioning that CD8 demonstrated considerable selectivity as an antagonist of AMPA receptors while having minimal effects on kainate and NMDA receptors. The findings of this study highlight a noteworthy prospect, emphasizing the potential utilization of coumarin and benzodiazepine derivatives as innovative therapeutic approaches for neurological disorders associated with the intricate AMPA receptor system. This study aims to provide a comprehensive understanding of the complex mechanisms these compounds exert their effects on AMPARs, thereby establishing a basis for developing novel therapeutic interventions. Neurological medicine is currently being influenced by the potential for significant advancements, which can be facilitated by a more comprehensive understanding of the landscape of AMPA receptors.

Abbreviations CD: coumarin-benzodiazepine hybrid compounds; iGluRs: ionotropic glutamate receptors; AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; NMDA: *N*-methyl-D-aspartate; HEK293: human embryonic kidney 293 cells; AMPARs: AMPA receptors; CNS: central nervous system; NTD: N-terminal extracellular amino domain; LBD: ligand binding domain; TMD: transmembrane domain; LTP: long-term potentiation; LTD: long-term depression; NAM: negative allosteric modulator; PAM: positive allosteric modulator; GFP: green fluorescent protein; DMF: *N*, *N*-dimethylformamide; ANOVA: a one-way analysis of variance

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Data Availability Data collected or analyzed in this investigation are included in this manuscript and its supplemental material file.

Declarations

Ethics Approval and Consent to Participate Not applicable.

Consent for Publication All authors have given their consent for publication.

Competing Interests The authors declare no competing interests.

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