JOURNAL OF CHEMICAL INFORMATION AND MODELING

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J. Chem. Inf. Model., **Just Accepted Manuscript** • DOI: 10.1021/ci500559b • Publication Date (Web): 24 Nov 2014

Thermodynamic Characterization of New Positive Allosteric Modulators Binding to the Glutamate Receptor A2 Ligand-Binding Domain: Combining Experimental and Computational Methods Unravels Differences in Driving Forces

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Keywords: Ionotropic glutamate receptor A2, positive allosteric modulators, isothermal titration calorimetry, thermodynamic integration, one step perturbation, enthalpy-entropy compensation

Abbreviations

ADHD, attention deficit hyperactivity disorder; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; BTD, 3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide; CNS, central nervous system; GluA2, AMPA receptor subunit 2; ITC, isothermal titration calorimetry; LBD, ligand-binding domain; MD, molecular dynamics; OSP, one step perturbation; PEG, poly(ethylene glycol); RMS, root mean square; TI, thermodynamic integration; TMS, tetramethylsilane.

Abstract

Positive allosteric modulation of the ionotropic glutamate receptor GluA2 presents a potential treatment of cognitive disorders, *e.g.* Alzheimer's disease. In the present study, we describe the synthesis, pharmacology, and thermodynamic studies of a series of monofluoro-substituted 3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxides. Measurements of ligand binding by isothermal titration calorimetry (ITC) showed similar binding affinities for the modulator series at the GluA2 LBD, but differences in the thermodynamic driving forces. Binding of **5c** (7-F) and **6** (no-F) is enthalpy driven, **5a** (5-F) and **5b** (6-F) are entropy driven, and for **5d** (8-F) both quantities were equal in size. Thermodynamic integration (TI) and one step perturbation (OSP) were used to calculate the relative binding affinity of the modulators. The OSP calculations had a higher predictive power than those from TI, and combined with the shorter total simulation time, we found the OSP method to be more effective for this setup. Furthermore, from the molecular dynamics simulations we extracted the enthalpies and entropies and along with the ITC data, this suggested

that the differences in binding free energies are largely explained by the direct ligand-surrounding enthalpies. Furthermore, we used the OSP setup to predict binding affinities for a series of polysubstituted fluorine compounds and monosubstituted methyl compounds, and used these predictions to characterize the modulator binding pocket for this scaffold of positive allosteric modulators.

Introduction

The α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors mediate influx of metal ions across post-synaptic membranes in the mammalian central nervous system (CNS) in response to the neurotransmitter glutamate.[1] AMPA receptors form homo- and heterotetrameric ion channels; each subunit consists of an N-terminal domain (NTD), a ligand-binding domain (LBD), a membrane-spanning region, and an intracellular C-terminal domain. In the full-length homotetrameric glutamate receptor A2 (GluA2) the LBD subunits are arranged as a dimer of dimers.[2] The endogenous ligand (*S*)-glutamate binds in the agonist binding site, whereas positive allosteric modulators have been shown to bind at the dimer interface (Figure 1A-C). Upon (*S*)-glutamate binding, the LBD closes like a clamshell, which leads to a structural rearrangement and opening of the ion channel.[3] The receptor may undergo desensitization, triggered by a rearrangement of the LBD dimer where (*S*)-glutamate is still bound but the ion channel is closed.[1] Positive allosteric modulators potentiate the effect of (*S*)-glutamate by either stabilizing the (*S*)-glutamate bound conformation and thus slowing deactivation or by stabilizing the subunit interface, postponing the interface rearrangement and subsequent closure of the ion channel with (*S*)-glutamate bound and hence slowing desensitization.[4, 5] This fine-tuning of receptor signaling represents a promising therapeutic strategy in the treatment of several neurological diseases such as Alzheimer's disease, schizophrenia, and attention deficit hyperactivity disorder (ADHD).

In recent years, significant improvements in the thermodynamic characterization of small-molecule inhibitor complexes with receptors and enzymes have been made, both by experimental as well as by computational means. Currently, the main experimental approach is isothermal titration calorimetry (ITC),[6] while computationally, the necessary simulation time has become accessible to compute free-energy differences from statistical mechanical approaches.[7] Whereas successful calculations of binding free energies are now more common,[8] calculation of the individual enthalpic

and entropic contributions remain more challenging.[9] Furthermore, difficulties in drug design arise from enthalpy-entropy compensation,[10, 11] a thermodynamic concept where changes in the entropic contribution are counteracted by changes in the enthalpic contribution and *vice versa*. Moreover, it was shown that binding affinity is correlated to neither enthalpy nor entropy alone.[12] For using computational methods in drug development, it is important to understand how the individual interactions in the protein-ligand system contribute to the enthalpy-entropy compensation. It has been shown that the energetic contributions from the solvent reorganization exactly cancel[13-15] and therefore, the calculation of the direct ligand-surrounding contributions rather than the contributions of the interactions of the surroundings with itself can lead to additional insight. The contribution of the interactions of the surroundings with itself are also included in the experimentally determined data from ITC and thus, the combined use of experimental and computational methods allows for a more thorough decomposition of the energetic contributions, where the ligand-surrounding energy terms are analyzed. This provides a more detailed analysis of the energetic contributions that contribute directly to ligand binding, which would not be possible using experimental methods alone.

In the present study, we report the synthesis and pharmacological characterization of three new positive allosteric modulators, where the substitution pattern of fluorine on the benzene nucleus of 3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide (BTD) is varied. These modulators are derived from the previously described 4-cyclopropyl-7-fluoro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide (5c)[16] (Figure 2). We characterized the thermodynamics of binding to the dimeric GluA2 LBD of this modulator series using ITC. To complement the experimentally determined thermodynamic details of binding, we used both thermodynamic integration (TI) and one step perturbation (OSP) to set up a method to calculate the relative free energy of binding. TI is a highly accurate method to estimate free-energy differences between compounds, but it also has a high

computational expense.[17] The OSP method takes advantage of using a single reference molecule that is representative of several compounds of interest, and thus significantly improves the efficiency.[18] From these simulations we extracted the reduced enthalpies and entropies to perform a detailed analysis of the direct enthalpy-entropy contributions to ligand binding. Finally, we used the OSP data to make predictions of new compounds covered by the reference molecule, allowing for a detailed QSAR analysis of the modulator binding pocket. To our best knowledge, the present work represents the first free energy calculations of GluA2 LBD positive allosteric modulators.

Materials and Methods

Chemistry

All commercial chemicals (Sigma-Aldrich, Belgium; Appolo Scientific, United Kingdom and Fluorochem, United Kingdom) and solvents were reagent grade and used without further purification. Melting points were determined on a Stuart SMP3 apparatus in open capillary tubes and are uncorrected. NMR spectra were recorded on a Bruker Avance 500 spectrometer (^{1}H: 500 MHz; ^{13}C: 125 MHz) using DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal standard; chemical shifts are reported in δ values (ppm) relative to internal TMS. Elemental analyses (C, H, N, S) were carried out on a Thermo Flash EA 1112 series elemental analyser and were within ± 0.4% of the theoretical values. This analytical method certified a purity of ≥95% for each tested compound. All reactions were followed by TLC (silica gel 60F_{254} Merck) and visualization was accomplished with UV light (254 or 366 nm). The synthesis of **5c** starting from 2,5-difluorobenzenesulfonamide (**2c)** has been previously described.[16] Synthesis of the other compounds was performed according to the scheme in Figure 3.

General procedure for the synthesis of fluoro-substituted 2-fluorobenzenesulfonamides (**2**): Glacial acetic acid (30 mL) introduced in a round bottom flask (500 mL) was saturated for 30 min.

with gaseous sulfur dioxide. An aqueous solution of cupric chloride (1.5 g in 10 mL) was added to this solution giving a suspension of cuprous chloride (suspension A). In another flask, the appropriate fluoro-substituted 2-fluoroaniline (**1**; 5 g) was dissolved in a mixture of glacial acetic acid (30 mL) and 12N HCl (15 mL) and the resulting solution (solution B) was cooled on an ice/salt bath (-5 °C). A solution of sodium nitrite (2.5 g) in water (10 mL) was added drop wise to solution B and the resulting mixture was slowly added to suspension A and stirred on an ice bath for 15 min. The reaction mixture was then poured on water (200 mL) and diethyl ether (200 mL). The organic layer was separated, washed with water (100 mL) and concentrated to dryness under reduced pressure. The residue of the fluoro-substituted 2-fluorobenzenesulfonyl chloride was dissolved in dioxane (25 mL) and this solution was poured under stirring onto a cooled mixture of concentrated ammonia (25 mL) and water (10 mL). After 30 min, the reaction mixture was concentrated under reduced pressure and the resulting precipitate of the final compound was collected by filtration, washed with water, and purified by crystallization in methanol-water (yield: 60-70%).

2,6-Difluorobenzenesulfonamide (**2d**) was used from a commercial source (Fluorochem, ref 017310).

General procedure for the synthesis of fluoro-substituted 2-cyclopropylaminobenzene-sulfonamides (**3**): A solution of the appropriate fluoro-substituted 2-fluorobenzenesulfonamide (**2**) (3 g) in dioxane (30 mL) and cyclopropylamine (3 mL) was heated in a closed vessel at 100-110 °C for 24 h. The solvent and the excess of amine were removed by distillation under reduced pressure and the residue was dissolved in methanol (20 mL). The methanolic solution was cooled and mixed with water (60 mL) under stirring. The resulting precipitate was collected by filtration, washed with water, dried and used in the next step without further purification (yield: 80-90%).

General procedure for the synthesis of fluoro-substituted 4-cyclopropyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxides (**4**): In an open vessel, a mixture of the appropriate fluoro-substituted 2-cyclopropylaminobenzenesulfonamide (**3**) (2 g) and triethyl orthoformate (20 mL) was heated at 130-150 °C for 24-48 h. The resulting suspension was cooled on an ice bath and the insoluble material was collected by filtration, washed with diethyl ether, dried and crystallized in methanol (yield: 70-80%).

General procedure for the synthesis of fluoro-substituted 4-cyclopropyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide (**5**): A mixture of the appropriate fluoro-substituted 4-cyclopropyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4**) (1.9 g) and isopropanol (50 mL) was supplemented with finely divided sodium borohydride (1 g) and then heated at 50-55 °C for 5-10 min. The solvent was removed by distillation under reduced pressure and the residue was taken up with water (50 mL). The resulting suspension was slightly acidified by the addition of 6N HCl. The title compound was extracted three times with dichloromethane (3 x 30 mL). The combined organic layers were dried over magnesium sulfate and the filtrate was concentrated under reduced pressure. The residue was crystallized in methanol-water (yield: 80-85%).

Effect on AMPA-evoked membrane depolarization (*in vitro* fluorescence assay). This assay was performed on rat primary brain cultures using fluorescent membrane potential dyes and an imaging based plate reader (FDSS, Hamamatsu, JP) following our previously published procedure.[16, 19]

Effect on AMPA-mediated release of noradrenaline on rat hippocampal slices. Potentiation of noradrenaline release on rat hippocampal slices was measured according to our previously reported

procedure.[20, 21]

Protein expression and purification

The dimeric GluA2-LBD double mutant L483Y-N754S was expressed and purified as previously described.[22] Briefly, *Escherichia coli* Origami B (DE3) cells were transformed with the GluA2-LBD-L483Y-N754S pET-22b(+) plasmid. Cells were grown in LB medium to an OD_{600} of 0.9 where the cells were subsequently cooled on ice to 20°C. Protein expression was induced by addition of 0.5 mM isopropyl β-D-thiogalactopyranoside. Cells were harvested and lysed after 18 hours, and the soluble protein was initially purified by Ni^{2+}-affinity chromatography. The *N*-terminal His-tag was cleaved by tryptic digest, and the cleaved protein was further purified by anion-exchange chromatography and size-exclusion chromatography.

Isothermal titration calorimetry

An ITC200 MicroCalorimeter (GE Healthcare) with a cell volume of 200 µL was used for ITC at 25ºC. Titrations with **5b**, **5d**, and **6** were direct titrations, whereas the binding affinity of **5a** was determined in a displacement assay with **5c**. The protein solution consisted of GluA2-LBD-L483Y-N754S in 100 mM HEPES, 100 mM NaCl, 2 mM KCl, 5 mM L-glutamate, pH 7.0. UV absorption was used to determine the protein concentration, which was: 47 µM (**5b** titration), 31 µM (**6** titration), 29 µM (**5d** titration), and 20 µM (**5a** titration). The protein solution buffer was also used to dissolve the compounds to the following concentrations used for ITC: 695 µM **5b**, 490 µM **6**, and 543 µM **5d**. For the displacement ITC assay of **5a** with **5c**, 285 µM **5c** was used to displace 100 µM **5a**. All experiments were set up with 20 injections with 3 min intervals; first injection was 0.4 µL, the remaining 2 µL. Data analysis was performed using the Origin 7.0 software (MicroCal)

(single binding site model). For each binding curve the first data point was discarded and the heat of dilution of injecting ligand into buffer was subtracted prior to fitting. All experiments were performed three times and the reported K_d, ΔH, and $-T\Delta S$ are mean values of three independent titrations.

Simulation setup

The GROMOS11 suite of simulation programs was used for all simulations.[23] The GROMOS++ suite of programs was used to set up and analyse the simulations.[24]

The coordinates for the protein were extracted from the crystal structure of GluA2-LBD-L483Y-N754S in complex with BPAM97 at 1.95 Å (PDB code 3TDJ; the X-ray structure of GluA2-LBD-L483Y-N754S in complex with BPAM344 was not available when the simulations were initiated).[22] The GROMOS 54a8 force field was applied for MD simulations.[25] The endogenous ligand *L*-glutamate was described according to the same force field in the zwitter-ionic form and bearing a net charge of -1 *e*. Building blocks for the allosteric modulators were derived from similar functional groups available in the force field. In particular, the sulphonamide group was previously described in simulations of sildenafil[26] and the charge distributions for the fluorine-substituents are taken from a model of tri-fluoroethanol.[27] The building blocks of all species are available in the Supplementary Material. For the protein, all Glu, Asp, Lys, and Arg residues were described in their charged states while the optimal protonation states of His (neutral) were chosen such that the potential number of hydrogen bonds is maximised.

The protein-ligand complex was placed in a rectangular box with a size of 7.8 x 8.7 x 9.9 nm^3 with a minimum solute-solvent distance of 0.23 nm, with 13 Cl^- and 5 Na^+ ions added and containing approximately 19000 simple point charge (SPC) water molecules. To obtain the free-energy

differences of the free compounds in water, which are required to calculate the free energy of binding, all real compounds and the reference compound (see below) were also simulated free in solution. For this, a box with 727 SPC water molecules was used.

To relax unfavorable contacts between the protein-ligand complex and the solvent or ligand and solvent, all systems were energy minimized before simulations, using a steepest descent energy minimization with an energy threshold of 0.1 kJ mol^{-1}. Initial velocities were subsequently assigned randomly from a Maxwell-Boltzmann distribution corresponding to 60 K. In the thermalisation and equilibration phase, the temperature was gradually increased from 60 K to 300 K using the weak coupling scheme[28] over six discrete simulation steps, while decreasing the force constant of an initial positional restraint from 25000 kJ mol^{-1} nm^{-2} to zero. In a seventh step, the Nosé-Hoover chains algorithm[29] was used involving three chains and a reference temperature of 300 K.

Preliminary simulations showed rotations of the modulator up to +/- 90º relative to the crystal structure and therefore, a distance restraint was applied between the centre of geometry of C4, C6, and C10 of one modulator to the F2 on the other modulator with an optimal distance of 0.36 nm and a force constant of 4000 kJ mol^{-1} nm^{-2} (for atom numbering, see the molecular building block in the Supplementary Material).

All production simulations were performed with a constant volume, a constant temperature (300 K), and a constant number of particles. Stability of the simulations was confirmed by calculating the atom-positional root-mean-square deviations with respect to the initial starting structure and by monitoring the occurrence of hydrogen bonds and secondary structure elements over the course of the simulations. The presence of hydrogen bonds was determined using a geometric criterion requiring a maximum hydrogen-acceptor distance of 0.25 nm and a minimum donor-hydrogen-

acceptor angle of 135°.[30] The occurrence of secondary structure elements was determined using the Kabsch-Sander rules,[31] as implemented in the GROMOS++ suite of analysis programs.

Free-energy calculations

The relative free energy of binding between two compounds A and B can be calculated using a thermodynamic cycle,[32] from which the following equations can be derived:

$$\Delta\Delta G_{bind} = \Delta G_{bind}(B) - \Delta G_{bind}(A) = \Delta G_{AB}(prot) - \Delta G_{AB}(wat) \quad (1)$$

where ΔG_{bind} (B) and ΔG_{bind} (A) represent the binding free energy of compound B and A, respectively, and ΔG_{AB} is the free energy of changing compound A into compound B as calculated in the protein (prot) or free in solution (wat).

Thermodynamic integration

In thermodynamic integration (TI) ΔG_{AB} is calculated by gradually modifying compound A into compound B using a coupling parameter, λ, which connects the Hamiltonians describing the two compounds. The Hamiltonian, $H(\lambda)$, is parameterized such that at $\lambda = 0$ it represents compound A ($H(0) = H^A$) and at $\lambda = 1$ it represents compound B ($H(1) = H^B$). Here, the GROMOS parameterization of the Hamiltonian is used[33] with a softcore potential[34] with softness parameters $\alpha_{LJ} = 0.5$ and $\alpha_{CRF} = 0.5$ nm^2.

The derivative of the Hamiltonian with respect to λ is calculated at each λ point and the ensemble average is numerically integrated providing the free energy difference between compounds A and B.[35] In the current work, performed at constant volume, we calculate the Helmholtz free-energy

difference, ΔA. For the small alchemical changes described here, the volume-pressure work will be negligible and the Helmholtz and Gibbs free energies will be directly comparable.

$$\Delta G \approx \Delta A = \int_{\lambda=0}^{\lambda=1} \langle \frac{\partial H(\lambda)}{\partial \lambda} \rangle \, d\lambda \quad (2)$$

In practice, the ensemble average, $<\partial H(\lambda)/\partial \lambda>$, is calculated from at least 11 independent simulations performed at 11 equidistant λ-values. At every λ-value, a short equilibration was performed for 100 ps, followed by production simulations of at least 1 ns. To obtain a smooth TI curve up to three additional simulations were performed at intermediate λ-values. The free-energy differences between the allosteric modulators were calculated as outlined in Figure 4A for the compounds free in solution and while bound to the protein. In the protein simulations, both modulators were modified simultaneously, with a distance restraint between them. The ensemble averages were unbiased prior to the integration as in umbrella sampling[36, 37] using

$$\langle \frac{\partial H}{\partial \lambda} \rangle_{ub} = \frac{\langle \frac{\partial H}{\partial \lambda} e^{U_{bias}/k_B T} \rangle_b}{\langle e^{U_{bias}/k_B T} \rangle_b} \quad (3)$$

where U_{bias} is the energy of the restraint, the subscript *b* indicates the ensemble averages obtained in the presence of the bias while the subscript *ub* indicates the ensemble average in the unbiased case, k_B is the Boltzmann constant, and T is the absolute temperature.

One step perturbation

To estimate the free energy difference using OSP,[38] a reference molecule, which can sample the relevant phase space for all real compounds, was designed (Figure 4B). The challenge lies in designing the reference compound to have the best possible resemblance to all real compounds.[18] The reference compound contains four soft fluorine atoms for which the charge was set to 0 and the

van der Waals interaction was softened[34] to remove the singularity at the origin with a softness parameter of $\alpha_{LJ} = 1.51$.[39]

To calculate the free energy difference between a real compound and the reference compound, the Zwanzig perturbation formula was applied to a single simulation of the reference state.[40] Thus, the free energy difference between a real compound, A, and the reference compound, R, is calculated via:

$$\Delta A_{RA} = A_A - A_R = -k_B T \ln\langle e^{-(H^A - H^R)/k_B T}\rangle_R \quad (4)$$

where angular brackets indicate the ensemble average obtained using molecular dynamics (MD) simulation of the reference compound, R. Again, in view of the small perturbations considered here, we use the approximation $\Delta G_{RA} \approx \Delta A_{RA}$. In this particular case H^R also contains the restraining energy between the two reference molecules, such that the free energy of releasing the restraint is included in ΔG_{RA}. The free energy difference between the real compounds is subsequently calculated according to $\Delta G_{AB} = \Delta G_{RB} - \Delta G_{RA}$. Production simulations of the reference state free in solution and when bound to the protein were performed for 10 ns, writing the coordinates every 0.5 ps for later analysis.

Enthalpy-entropy compensation

Theoretically, the enthalpic and entropic contributions to the relative binding free energies are also accessible from the simulations. The enthalpy difference can be obtained from the difference in the average total energy for two compounds, incremented by the volume-pressure work:

$$\Delta H^{AB} = \langle H^B\rangle_B - \langle H^A\rangle_A + V\Delta p \quad (5)$$

where $\langle H^A \rangle_A$ and $\langle H^B \rangle_B$ indicate the ensemble average of the Hamiltonian of compound A and B, respectively, as calculated over the ensemble for compounds A and B. The volume-pressure work as appropriate in simulations in the NVT ensemble and due to the alchemical modifications described in this work will be negligible. The entropy is subsequently calculated from the difference between the enthalpy and free energy.[41]

However, equation (5) is known to converge poorly, as it is based on the total energy of the entire system. Still, for a pairwise additive force field, the total enthalpic change can be divided into the contributions due to the ligand and its surroundings as well as the contributions due to the interactions within the surroundings (protein and solvent).

$$\Delta H_{ls}^{AB} = \langle H_{ls}^{B} \rangle_B - \langle H_{ls}^{A} \rangle_A \quad (6)$$

with H_{ls} describing the ligand-surrounding energy terms of the Hamiltonian. It can be proven that the surrounding-surrounding enthalpies are explicitly included in the corresponding entropy change, largely explaining the often-observed enthalpy-entropy compensation.[15] The reduced term ΔH_{ls} does not correspond to an experimental observable, but can be considered as the direct enthalpic driving force. Moreover, it can be readily computed from molecular simulations and may help to dissect the origins of the experimentally determined thermodynamic quantities. Equation 6 was applied directly to the end-state simulations of the TI calculations.

Results and Discussion

Chemistry

The synthesis of the monofluoro-substituted 4-cyclopropyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxides **5** is described in Figure 3. The appropriate *o*-fluoroaniline **1** bearing a second fluorine

atom in another position of the benzene nucleus was converted into the corresponding *o*-fluorobenzenesulfonamide **2** by means of the Meerwein variation of the Sandmeyer diazotization reaction.[42] Nucleophilic substitution of the fluorine atom of intermediates **2** by cyclopropylamine, providing the corresponding 2-cyclopropylaminobenzenesulfonamides **3**, was performed in a closed vessel by heating the mixture at 130-150°C for 24-48 h. The use of *o*-fluorobenzenesulfonamides instead of the corresponding *o*-chlorobenzenesulfonamides was preferred because of the greater reactivity of fluoro-substituted aromatic compounds to nucleophilic substitution. Moreover, this reaction was greatly facilitated by the fact that the fluorine atom was located at the *ortho* position of the electron-withdrawing sulfonamide group. In the case of intermediate **2b**, which bears a fluorine atom in the *para*-position of the sulfonamide group, thus in competition with the *ortho*-position for the reaction with the amine, the nucleophilic substitution was mainly observed in the *ortho*-position. Ring closure of intermediates **3** by heating them in triethyl orthoformate provided the fluoro-substituted 4-cyclopropyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxides **4**, which were converted with sodium borohydride into the corresponding "saturated" final compounds **5**. The characterization of all compounds is given in the Supplementary Material.

Biological evaluation

The new fluoro-substituted BTD compounds **5a** (5-F), **5b** (6-F), and **5d** (8-F) were evaluated as AMPA receptor potentiators in an *in vitro* fluorescence assay and their activity was compared to that of the previously reported BTD compound **5c** (7-F) as well as the unsubstituted analogue **6**.[16] The experiments were performed on primary cultures of neurons from rat embryonic cortex, measuring the effect of the modulators on AMPA-evoked membrane depolarization, as previously described.[16, 19] For each compound, the EC_{2x} value was determined (concentration of modulator giving a 2-fold increase of the fluorescence induced by 300 μM AMPA), as well as their maximum

effect (E_{max} value normalized to unity for AMPA-evoked response taken as x1) (Table 1). It was observed that the 8-fluoro-substituted compound **5d**, with an EC_{2x} value of 0.33 μM and a high value of maximal potentiation of x15, exhibited a potentiator effect on AMPA receptors comparable to that of the 7-fluoro-substituted reference compound **5c**,[16] while their 5- and 6-fluoro-substituted analogues, although very active (EC_{2x} ~ 2 μM), where clearly less efficient. This result indicated that the best positions for a fluorine atom on the BTD ring system are the 7- and the 8-positions. Such an observation was already made with their corresponding monochloro-substituted BTDs. However, the fluoro-substituted analogues were systematically found to be more active on AMPA receptors than the corresponding chloro-substituted compounds.[43]

It is known that positive allosteric modulators of the AMPA receptor are able to potentiate noradrenaline (NA) release in rat hippocampal slices, an effect linked to their interaction with presynaptic AMPA receptors.[21, 44] At 300 μM, most of the compounds induced a strong enhancement of the (*S*)-AMPA (10 μM) evoked [^{3}H]-NA release (100% representing the effects shown by (*S*)-AMPA alone). This result indicated that the new monofluoro-substituted compounds **5a**, **5b**, and **5d**, as previously observed with compounds **5c** and **6**,[16] were able to act on presynaptic AMPA receptors, almost with the same efficacy.

Thermodynamic details of modulator binding

To measure modulator binding independently of dimer formation, we used a double mutant of the GluA2 LBD, GluA2-LBD-L483Y-N754S. This mutant is a preformed dimer in solution, which mimics the endogenous binding environment found in the dimer of dimers structure of the full-length GluA2 receptor. We have previously used this mutant for ITC measurements, and the mutations do not interfere with modulator binding.[16, 22]

For compounds **5b**, **5d**, and **6** the thermodynamic details of binding were determined in a direct titration with GluA2. For **5a** we were not able to measure a saturation curve in a direct titration and therefore, the thermodynamic properties were determined in an ITC displacement assay with the previously reported **5c**.[16] The ITC experiments showed that for all four compounds, binding is an exothermic process (Figure 5). The binding affinities (K_d) and thermodynamic quantities are given in Table 2. The following rank order in binding affinity (K_d) was observed, with the most potent compound listed first: **5c** (7-F) > **5d** (8-F) > **6** (no-F) > **5b** (6-F) > **5a** (5-F). For compound **6** the complex formation is primarily enthalpy driven as also previously seen for **5c**. On the contrary, the complex formation for **5a** and **5b** is primarily entropy driven, whereas **5d** complex formation is equally enthalpy and entropy driven (Table 2).

To decipher the mode of action of the modulators, we compared the relative difference in EC_{2x} for a compound **x** (EC_{2x}(**x**)/EC_{2x}(**5c**)) with the relative difference in the K_d: (K_d(**x**)/K_d(**5c**)). A direct comparison between EC_{2x} and K_d is not possible, since the EC_{2x} is the concentration of modulator that leads to two-fold increase in receptor response to a given agonist compared to the agonist alone and only indirectly contains information on binding affinity. The comparison of the relative differences in EC_{2x} to the ITC data enables a decomposition of the EC_{2x} values into the binding effect and the modulatory strength. Thus, it suggests if the relative difference between the effects of two compounds is purely due to binding affinity or if it is due to a more efficient modulation: If the EC_{2x}(**x**)/EC_{2x}(**5c**) equals K_d(**x**)/K_d(**5c**), it suggests that these compounds are different in their binding affinity only and hence, the two compounds should have similar modulatory effects. If EC_{2x}(**x**)/EC_{2x}(**5c**) does not equal K_d(**x**)/K_d(**5c**), then binding affinity alone does not explain the differences and therefore, the two compounds likely have different modulatory strengths. From data in Table 1 and Table 2, it is seen that for all compounds the EC_{2x}(**x**)/EC_{2x}(**5c**) is lower than K_d(**x**)/K_d(**5c**). Thus, differences in the binding affinity between the modulators are most likely not

the sole cause of the difference between these compounds. The largest difference is seen for **5a** relative to **5c** where K_d(**5a**)/K_d(**5c**) amounts to 35, while EC_{2x}(**5a**)/EC_{2x}(**5c**) is only 8. The other three compounds show smaller differences between the fractions. This suggests that **5a** may have a stronger modulatory effect than **5c**, which is partially compensated by a weaker binding of **5a** relative to **5c**. Therefore, it may be of interest to improve the binding affinity by introducing modifications elsewhere in **5a**.

Stability of the simulations

To set up a system for calculation of binding affinities, we used the GluA2-LBD-L483Y-N754S protein in complex with either two copies of **5c** for TI (Figure 4A) or two copies of the reference molecule for OSP (Figure 4B).

To ensure that the MD simulations represent stable protein structures, we evaluated the root mean square (RMS) deviation of the protein backbone from the initial starting structure. The RMS deviation remained below 0.25 nm, which indicates stability of the protein. Furthermore, the average occurrence of hydrogen bonds as well as the occurrence of secondary structural elements remained stable throughout the simulations (data not shown). However, a closer inspection of modulator binding in the protein showed that the modulators were turning ±90º relative to the crystal structure of GluA2-LBD-L483Y-N754S with **5c** (PDB code 4N07) in all MD simulations. Therefore, we used two distance restraints between the two modulator molecules located at the binding site, from the fluorine atom of one modulator to the centre of geometry on the benzene ring of the other modulator and *vice versa* (see methods).

Free energy calculations

The results of the free energy calculations using both TI and OSP are shown in Table 3 along with the relative free energy differences calculated from ITC. As a consistency check, the free energy differences from **5d** to **5a**, from **5a** to **6**, from **6** to **5b**, and from **5b** to **5d** were calculated in water as well, and the maximum free energy difference along the closed cycles was -0.19 kJ/mol. Comparing the relative free energy calculated by TI with the free energy determined by ITC, we get an RMS error of 5.1 kJ/mol, which is slightly higher than what is typically considered 'chemical accuracy' (~4 kJ/mol),[45] but just below 5.6 kJ/mol corresponding to a factor 10 in K_d at room temperature. This high RMS error of the TI calculations is primarily due to the poor prediction of **5a** relative to **5c** (-0.4 kJ/mol *vs*. 8.8 kJ/mol determined by ITC), but also the prediction for **6** is relatively poor (2.5 kJ/mol *vs*. 6.0 kJ/mol), although still within chemical accuracy.

Next, we calculated the free energy of binding using the OSP method, which is a computationally more efficient method than TI. When we compare the OSP free energy calculations with the relative free energy differences from ITC we obtain an RMS error of 2.1 kJ/mol, which is even below thermal fluctuations (k_BT = 2.5 kJ/mol). Thus, for this series of compounds we find that the OSP setup gives better predictions. Similar to the TI calculations the relative free energy of binding of **5a** was the most difficult to calculate (11.9 kJ/mol *vs*. 8.8 kJ/mol). However, the OSP method performs slightly better than the TI method for the difference between **5c** and **6**, and conversely, slightly worse for **5b**.

Thus, the calculations of the relative binding free energy of **5a** showed the poorest correlation for both TI and OSP in comparison to the experimentally determined relative free energies from ITC. For this setup we found the OSP method to have a somewhat higher predictive power compared to the TI method. This is promising because for the four TI calculations in Figure 4A, we have performed at least 4 x 11 x 1 ns = 44 ns of simulation, while the OSP calculations are based on a single simulation of 10 ns of the reference state.

Ligand orientations, conformations and interactions

The modulators bind in the dimeric interface of the GluA2 ligand binding domain. The only direct hydrogen bond observed is between the N2 hydrogen and the carbonyl oxygen of Pro494 (Figure 1B & 1C.[16, 22] For **5c** and its analogue BPAM97, a weak hydrogen-fluorine bond has been observed to Ser497, otherwise the binding of the modulators is stabilized primarily through non-bonded interactions and shape complementarities. To check that the experimentally observed binding pose was also seen in the simulations, we examined the final structures from the end-state simulations of the TI and compared them to the experimentally observed structure of **5c** (Figure 6). The structures of **5b** and **6** match best with the experimental structures for both copies of the modulators (Figure 6A and 6C). An alternative puckering of the ring system relative to the experimental structure was observed for **5d** in one of the modulators (Figure 6B). The puckering seen in the crystal structure enables the only hydrogen bond from the N2 hydrogen of the modulator to the carbonyl oxygen of Pro494.

The advantage of the OSP method lies in the usage of a reference structure, which may sample all relevant conformations of multiple molecules of interest. For the OSP calculations, the number of structures in the trajectories that contribute significantly to the free energy calculation was determined for each real compound by counting the number of structures for which the energy difference $H^A - H^R$ in equation (4) was less than the final free energy difference increased by k_BT ($H^A - H^R < \Delta G_{AR} + k_BT$). The number of contributing structures was found to be in the range of 0.3-12% of the entire set of 20,000 structures, with the lowest amount of structures contributing to the free energy estimates of compound **5a**.

We extracted the single structure, which contributed the most to the free energy calculation for each of the real compounds from the OSP simulation. Figures 7A and 7C show the front view of the single structures with the highest probability for each of the compounds in line representation together with the X-ray crystal structure of **5c** in complex with GluA2-LBD-L483Y-N754S. The RMS deviations of the most contributing ligand structures were found to be: 2.4 Å (**5a**), 1.3 Å (**5b**), 0.95 Å (**5c**), 2.6 Å (**5d**), and 1.1 Å (**6**), with respect to the X-ray structure of **5c**. For both copies of the compounds some translation of the simulated structures relative to the crystal structure (in green) is seen, the best overlap with the X-ray structure is seen for **5c** and **6** (Figure 7A and 7C).

The side view of the same structures reveals a different puckering of the ring system for **5a** and **5d** in both copies of the modulator (Figures 7B and 7D) relative to the puckering observed in the X-ray structure of **5c**. The observation of both conformations indicates that the reference structure simulates a broad ensemble of conformations and the relevant structures for the individual compounds may be selected. Furthermore, the cyclopropyl group is rotated 180º for **5a** and **5d** (Figure 7B and 7D) and 90º for **5b** (Figure 7B) relative to the conformation seen in the X-ray crystal structure of **5c** in GluA2-LBD-L483Y-N754S.

We also calculated the average puckering for the real compounds by reweighting the reference state simulation (data not shown) and confirmed our observations of the single highest contributing structures. Comparing the results from the TI and OSP, it is seen that **5a** consistently goes to the alternative puckering in the OSP, while it remains in the experimentally observed puckering in the TI calculations. This may explain the differences observed between the free energy values using TI and OSP (Table 3).

To see if the two different ring conformations represent an intrinsic effect of the compounds or if they are induced by the protein, we also extracted the highest contributing structure for each of the

real compounds from the simulation in water (Figures 7E and 7F). Here, the two different ring conformations were also observed where **5c** and **5d** showed preference for the conformation seen in the crystal structure, and **5a, 5b**, and **6** were found with the other ring conformation. The observation of both conformations in the water simulation suggests that there is not a large energy difference associated with the transition in water and thus, the protein may easily influence the preference for either of the conformations. **5c** and **5a** are the only compounds, which preserve the intrinsic conformation seen in the water simulation when bound to the protein. For **5b**, **5d,** and **6** the binding to the protein may result in a slightly shifted orientation, which induces another puckering than seen in the water simulation (considering only the single most contributing structure). For **5a** the fluorine substituent may influence the puckering, by some steric hindrance caused by the cyclopropyl group, thus favoring the alternative conformation to the X-ray structure both intrinsically and in the protein, severely hampering the formation of the N2 hydrogen bond to the carbonyl oxygen of Pro494, and possibly explaining the low number of contributing structures to its free energy estimate.

These observations suggest that **5c** shows the overall combined best modulatory effect and binding affinity, since it is the only compound with preserved intrinsically favored conformation, which is also the optimal conformation for hydrogen bonding when bound to the protein. Furthermore, **5d** also shows the alternative puckering of the ring in the protein and this ligand is shifted most to the right from the X-ray structure. This observation may give a molecular explanation for the previously suggested difference in modulation effects as compared to compounds **5a**, **5b** and **5c**.

Enthalpy-entropy compensation

To investigate the thermodynamic details of ligand binding to the protein, we extracted the ligand-surrounding enthalpies, ΔH_{ls}, from the end-states of the TI simulations, according to equation (6) (Table 4). TI simulations were preferred over OSP simulation data, since the endstate TI are simulations of the real molecules. By comparing the computed ligand-surrounding enthalpy to the values that were obtained experimentally, we computed the changes in enthalpy due to the reorganization in the surroundings (protein and solvent; ΔH_{ss}). For ease of comparison, we have also explicitly calculated the thermodynamic quantities relative to **5c** ($\Delta\Delta H$, $\Delta\Delta H_{ls}$, and $\Delta\Delta H_{ss}$).

Comparing **6** with **5c**, we see that the $\Delta\Delta G$(ITC) of 6.0 kJ/mol is almost entirely explained by the ligand-surrounding enthalpy change ($\Delta\Delta H_{ls}$(TI) = 6.7 kJ/mol). Since also $-T\Delta\Delta S$(ITC) is almost zero, the surrounding-surrounding enthalpy is close to zero as well. Hence, for this compound there is no significant reorganization of the surroundings and the difference in affinity is explained from the direct interaction energy between the modulator and the protein. This is in line with the very similar position of **6** at the binding site compared to **5c** (Figure 7).

For **5d**, the $\Delta\Delta G$(ITC) is also very similar to the value of $\Delta\Delta H_{ls}$(TI) in Table 4 (5.1 vs. 5.0 kJ/mol). However, here the experiment indicates that the difference in binding of **5d** relative to **5c** is the result of an unfavorable enthalpy ($\Delta\Delta H$(ITC) = 13.5 kJ/mol), and favorable entropy ($-T\Delta\Delta S$(ITC) = -8.4 kJ/mol). This effect entirely compensates the enthalpy-change due to reorganization of the surroundings ($\Delta\Delta H_{ss}$ = 8.5 kJ/mol). So even if the experiment suggests entropic differences between the compounds, a closer analysis reveals that the enthalpic differences in the direct interaction ($\Delta\Delta H_{ls}$(TI)) may explain the differences in affinity.

Finally, for **5a** and **5b** the entropically driven binding is compensated by an even larger value of $\Delta\Delta H_{ss}$. Also for these compounds, the largest portion of $\Delta\Delta G$ may be explained from $\Delta\Delta H_{ls}$(TI), and the favorable entropic contributions are entirely compensated by enthalpically unfavorable

reorganization of the surroundings. Thus, the combination of experimental data with the calculated ligand-surrounding enthalpies allows us to dissect the origin of the measured enthalpies and entropies, and could give a better insight into the design of new compounds. It is arguably easier to design compounds that have more favorable ΔH_{ls}, than to design compounds to have an effect on the reorganization of the surroundings or on a largely compensated entropy.

Thus, our calculations of the energetic contributions to ligand binding indicate that enthalpic and entropic differences for this modulator series have large (compensating) contributions from either solvent reorganization or interactions between the protein and the solvent. This also shows that the differences in the binding affinities are mostly explained from direct interactions between the ligands and their surroundings.

Prediction of binding affinities

The strength of the OSP method lies in the need for only a single simulation of the reference compound to cover multiple compounds of interest (Figure 4B). Therefore, to characterize the modulator binding pocket with respect to the 4-cyclopropyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide modulator scaffold, we designed a series of additional fluorine-derivatives and calculated the free energy of these compounds relative to **6** for ease of comparison (Table 5).

Apart from **5c**, the 6,7-di-F compound was predicted to have the most favorable free energy ($\Delta\Delta G_{\text{bind}}$ = -2.2 kJ/mol) and the fluorine substituted compound which was predicted to have the least favorable energy was the 5,6,7,8-tetra-F compound with $\Delta\Delta G_{\text{bind}}$ = 24.6 kJ/mol. Comparing the predicted relative free energy for all the compounds with a fluorine at the 5-position the ΔΔG is 9–24 kJ/mol, whereas for the four remaining fluorine substituted compounds the ΔΔG is (-2)–3

kJ/mol (*i.e*. without fluorine in the 5-position). Thus, an electronegative substituent or hydrogen-bonding acceptor in the 5-position decreases the predicted binding affinity regardless of the number and position of other electronegative substituents, in line with the unfavorable binding of **5a** (calculated and experimental).

Table 5, combined with the OSP data described in Table 3, allows us to investigate if the contributions of the F substitutions are additive or not. The free energy of some compounds may be approximated, such as *e.g.* for the 5,8-di-F compound (9.4 kJ/mol relative to **6**), which is very close to the sum of the relative free energies for **5a** (7.0 kJ/mol) and **5d** (1.4 kJ/mol). Further examples of compounds for which additivity holds reasonably well are the 6,8-di-F and the 6,7-di-F compounds, while additivity does not hold for the 5,7-di-F, 5,7,8-tri-F, 5,6,7-tri-F, or 5,6,7,8-tetra-F compounds. This indicates that the predictions generated from the OSP simulation are more thorough than a simple QSAR model, which can be derived from looking at only the monosubstituted compounds, and take into account the mutual influences of the substitutions.

To investigate the impact of a small non-polar substituent on the binding affinity, we calculated the binding free energies relative to **6** for a series of mono-substituted methyl compounds (Table 5). From these four derivatives, it is seen that the 5-substituted compound binds with the least favorable energy ($\Delta\Delta G_{\text{bind}}$ = 63.3 kJ/mol). The predicted methyl derivative with the most favorable energy is the 6-methyl compound with $\Delta\Delta G_{\text{bind}}$ = 1.8 kJ/mol. Thus, for both mono-methyl and mono-fluorine compounds the 5-substituted position is predicted to result in the weakest binding compounds; however, the predictions indicate that it is more favorable to have an electronegative atom compared to a bulkier methyl substituent in this position. Also, for **5a** (the 5-F-compound) the lowest number of structures contributing to the OSP calculations was observed, and this molecule favors an alternative puckering. Therefore, an explanation may also be found in a steric clash of any

type of substituent in this position, which may favor an alternative puckering of the ring system, which in turn prevents the hydrogen bond between the modulator and the protein.

Comparing **5b** (6-F, $\Delta\Delta G$ = 0.1 kJ/mol) with the compound with a methyl in the 6-position ($\Delta\Delta G$ = 1.8 kJ/mol) shows that the two compounds with a substituent in the 6-position bind with similar relative free energies, thus, at this position there is no apparent preference for either a hydrophobic or electronegative substituent. For both the 7- and 8-position it is more favorable to have an electronegative substituent than a hydrophobic substituent, and it may also be concluded that it is more favorable for a substituent to be located in the 7- rather than the 8-position.

In summary, for the 5-, 7-, and 8-position it is less favorable to fill up space with a hydrophobic substituent than to introduce an electronegative substituent, with the largest effect seen for the 5-position. For the 5-position, this may be due to interactions with the cyclopropyl group, leading to an alternative puckering. For the 7- and 8-position, steric clashes with the protein environment cause unfavorable interactions. In contrast, for the 6-position the difference between a methyl- and an electronegative substituent is small. The 6-position is the position where a substituent would be the closest to its similar substituent on the other modulator molecule when bound to the protein and thus, a substituent in this position might strengthen interactions between the modulators.

Conclusion

This work is the first example of combining experimental and calculated free energies of positive allosteric modulator binding to the GluA2 LBD, and furthermore, it gives a detailed decomposition of the thermodynamic driving forces of modulator binding.

The experimentally measured thermodynamic details of binding of this fluorine substituted series of GluA2 positive allosteric modulators showed that despite similarities in the binding affinities, complex formation was primarily enthalpy driven for **6** as also previously seen for **5c**. The **5a** and **5b** complex formation was primarily entropy driven, and **5d** complex formation was equally enthalpy and entropy driven. Dissection of the thermodynamic contributions, however, indicated that the main driving forces of binding stem from the ligand-surrounding enthalpies for all compounds and that the experimentally determined enthalpic and entropic differences are largely explained by reorganizations in the protein and the solvent. Our comparison of the ITC, pharmacological, and simulation data suggested that since **5c** is the only compound, which preserves the intrinsically favored and optimal conformation for hydrogen bonding when bound to the protein, it shows the overall best combined modulatory effect and binding affinity. Our predictions generated using the OSP simulation provided an indication of favorable substitution positions in this modulator scaffold. While a larger substituent in the 5-, 7- or 8-position would be sterically unfavorable, a larger substituent in the 6-position might strengthen interactions between the modulators.

Supporting Information

Experimental characterization of intermediates and synthesis products, free energy profiles for TI and molecular building block for all simulated compounds. This information is available free of charge via the internet at http://pubs.acs.org.

Acknowledgements

The Danish Council for Independent Research (Mobility PhD grant) and GluTarget (A.B.N., L.O., J.S.K.) as well as the Vienna Science and Technology Fund (WWTF, grant number LS08-QM03, C.O.) and the European Research Council (ERC grant number 260408, C.O.) are acknowledged for financial support.

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TABLES

Table 1. Effects of monofluoro-substituted 4-cyclopropyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxides on the depolarization induced by 300 µM AMPA in primary neuronal cultures from rat embryonic cortex and on AMPA-mediated presynaptic noradrenaline (NA) release on rat hippocampal slices.

	AMPA-mediated depolarization			AMPA-mediated NA rel.[d] (%)
Cpd	**EC_{2X}[a] (µM)**	**E_{max}[b]**	**EC_{50}[c] (µM)**	
5a	2.14 [1.55; 2.96] (5)	>x7.20 ± 1.02 (5)	ND	320.63 ± 24.51 (4)
5b	2.11 [1.01; 4.39] (3)	x5.67 ± 0.33 (3)	6.69 [2.74; 16.34] (3)	363.44 ± 12.02 (2)
5c[e]	0.26 [0.15; 0.47] (3)[e]	x15.00 ± 2.65 (3)[e]	0.89 [0.53; 1.48] (3)[e]	330.33 (1)
5d	0.33 [0.22; 0.50] (3)	x11.67 ± 1.76 (3)	1.44 [0.61; 3.43] (3)	373.46 ± 21.97 (2)
6[e]	0.70 [0.30; 1.67] (3)[e]	x8.50 ± 0.76 (3)[e]	4.07 [2.08; 7.96] (3)[e]	394.37 (1)

[a-c] AMPA mediated depolarization; [a] EC_{2X} : concentration of modulator giving a 2-fold increase of the depolarization induced by 300 µM AMPA. [b] E_{max}: maximal effect obtained by compounds in the presence of 300 µM AMPA, normalized to unity for response evoked in the presence of AMPA alone taken as x1. [c] EC_{50}: concentration of modulator responsible for 50% of the maximal effect. [d] NA rel. : effect of compounds on AMPA-mediated noradrenaline release at 300 µM, normalized to the effect obtained in the presence of AMPA alone, taken as 100%. For EC_{2X} and EC_{50} results are expressed as geometric mean and upper and lower confidence intervals. For E_{max} and NA release, results are expressed as mean and standard error to the mean. Numbers in brackets indicate the number of independent experiments. ND: not determined. [e] Published compounds and results (see ref. 16, in this previous publication, all results were expressed as the arithmetic mean, showing very small differences from the geometric means reported here).

Table 2. Isothermal titration calorimetry on modulator binding to GluA2-LBD-L483Y-N754S.

	5a [b]	**5b**	**5c** [a]	**5d**	**6**
K_d (μM)	12.3±1.9[c]	7.0±0.6	0.35±0.02	2.8±0.6	3.9±0.4
ΔG_{bind} (kJ/mol)[d]	-28.0±0.4	-29.4±0.2	-36.8±0.1	-31.7±0.5	-30.8±0.3
ΔH (kJ/mol)	-10.0±2.1	-7.9±1.3	-31.4±0.8	-17.9±0.4	-25.1±1.3
$-T\Delta S$ (kJ/mol)	-18.0±2.1	-21.8±1.7	-5.4±0.8	-15.1±0.8	-5.9±1.3
n_H[e]	nd[f]	1.7±0.1	2.7±0.1	2.2±0.2	1.4±0.1

a) Values from ref 16, b) thermodynamic properties of **5a** was determined in an ITC displacement assay with **5c**, c) standard deviation over three independent experiments, d) calculated from $\Delta G = RT\ln(K_d)$, e) stoichiometry, f) not determined.

Table 3. Relative free energies of modulator binding to GluA2-LBD-L483Y-N754S.

Compound	$\Delta\Delta G_{bind}$ (ITC)	$\Delta\Delta G_{bind}$ (TI)	$\Delta\Delta G_{bind}$ (OSP)
5a	8.8	-0.4	11.9
5b	7.4	5.9	5.0
5c [a]	0.0	0.0	0.0
5d	5.1	7.2	6.3
6	6.0	2.5	4.9
RMS error	-	5.1	2.1

a) All values (in kJ/mol) are denoted as the free energy relative to **5c** as this was the compound for which the X-ray structure was solved and that is central in the TI calculations (Figure 4A).

Table 4. Reduced enthalpy and entropy terms from the TI simulation in kJ/mol.

	ΔH(ITC)	ΔH_{ls}(TI)[a]	ΔH_{ss}[b]	$-T\Delta S$(ITC)
5a	-10.0	-41.7	31.7	-18.0
5b	-7.9	-39.7	31.8	-21.5
5c	-31.4	-44.6	13.2	-5.4
5d	-17.9	-39.6	21.7	-13.8
6	-25.1	-37.9	12.8	-5.7
	$\Delta\Delta H$(ITC)[c]	$\Delta\Delta H_{ls}$(TI)[c]	$\Delta\Delta H_{ss}$[c]	$-T\Delta\Delta S$(ITC)[e]
5a	21.4	3.0	18.4	-12.6
5b	23.5	5.0	18.5	-16.1
5c	0.0	0.0	0.0	0.0
5d	13.5	5.0	8.5	-8.4
6	6.3	6.7	-0.4	-0.3

a) calculated from equation (6), b) calculated as $\Delta H_{ss} = \Delta H(\text{ITC}) - \Delta H_{ls}(\text{TI})$, c) relative to **5c**.

Table 5. Prediction of relative binding free energies using OSP relative to 6.

Substitution pattern [a]				$\Delta\Delta G_{bind}$ (kJ/mol)
R_5	R_6	R_7	R_8	
H	H	H	H	0.0
F	H	H	H	7.0
H	F	H	H	0.1
H	H	F	H	-4.9
H	H	H	F	1.4
F	F	H	H	12.8
F	H	F	H	9.6
F	H	H	F	9.4
H	F	F	H	-2.2
H	F	H	F	2.8
H	H	F	F	0.3
F	F	F	H	18.1
F	F	H	F	13.2
F	H	F	F	10.7
H	F	F	F	2.5
F	F	F	F	24.6
CH_3	H	H	H	63.3
H	CH_3	H	H	1.8
H	H	CH_3	H	7.1
H	H	H	CH_3	16

a) The MD simulation of the reference compound for the OSP calculations was used to predict the free energy of binding relative to **6** for selected compounds with different substitution patterns (Figure 2).

Figure 1. Cartoon representation of the previously reported structure of GluA2-LBD-L483Y-N754S in complex with glutamate and 5c.

A) Each LBD monomer (blue and magenta, respectively) adopts a clamshell-like form around the endogenous ligand glutamate (shown in cyan stick representation). The positive allosteric modulator binding pocket lies in the LBD dimer interface where two copies of the positive allosteric modulator **5c** (shown in green stick representation) bind. B & C) Zoom on the positive allosteric modulator binding pocket, C) is rotated 180º relative to B). Dashed lines indicate potential hydrogen bonds (**5c** to Pro494) or hydrogen-fluorine bonds (**5c** to Ser497). The figure was prepared using PyMOL (The PyMOL Molecular Graphics System. Version 1.5.0.5, Schrödinger, LLC) and structure coordinates from the Protein Data Bank (PDB code 4N07).

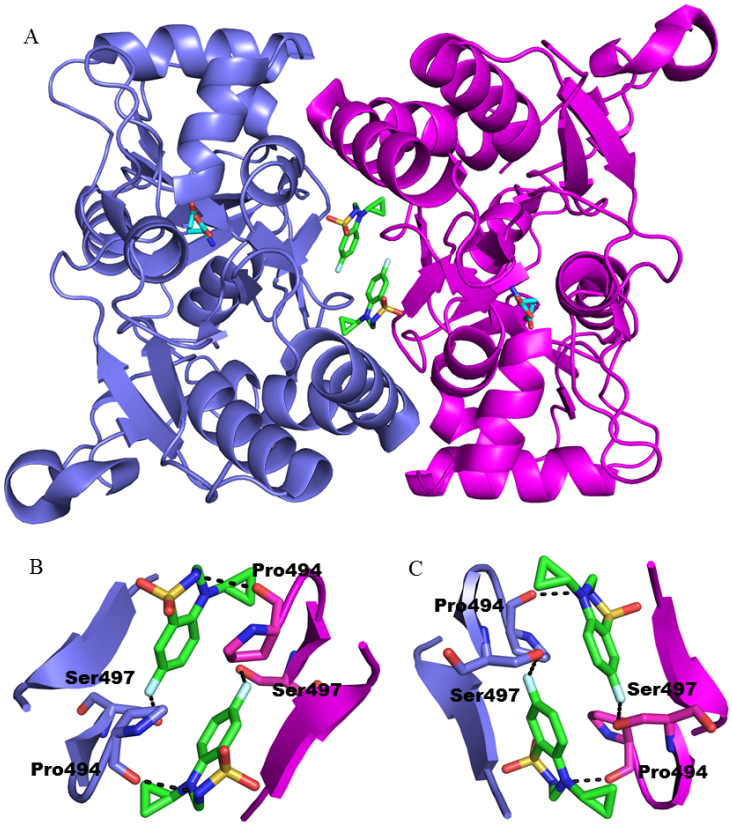
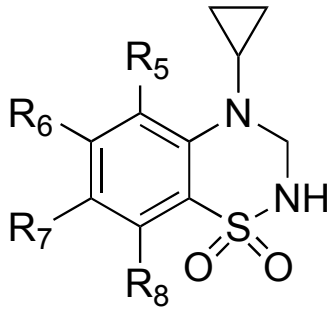
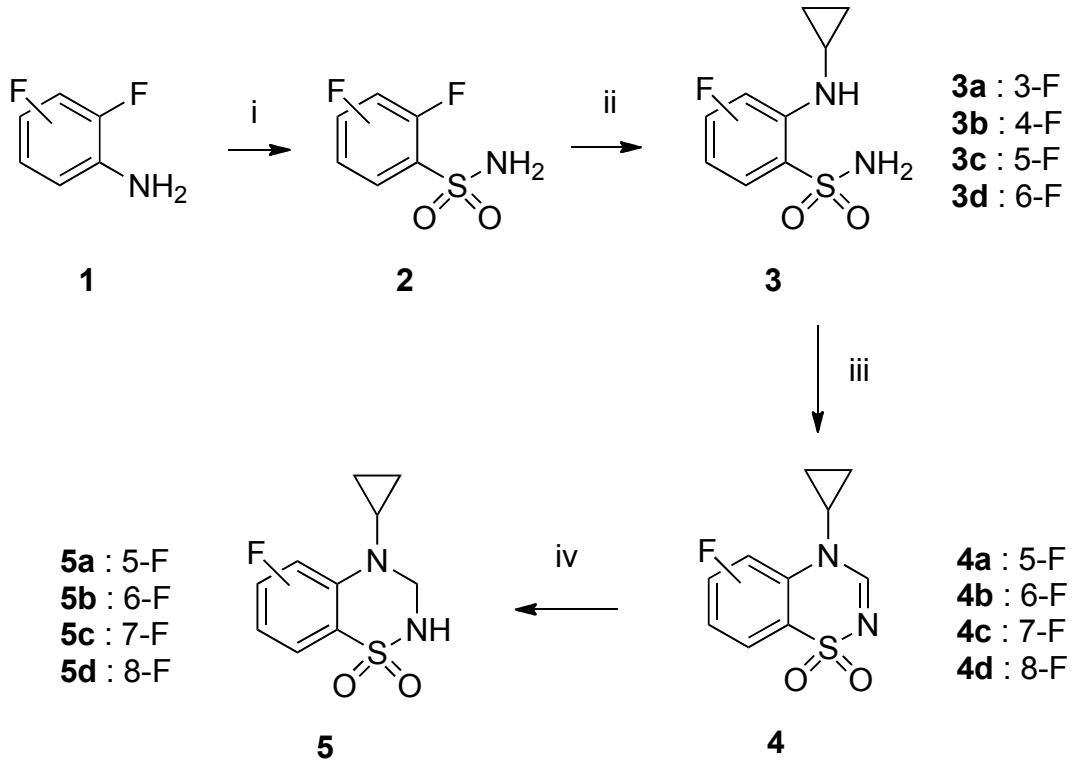


Figure 2. Chemical structure of the 4-cyclopropyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxides (BTDs) studied. All compounds differ only by the substitution pattern at positions 5, 6, 7, or 8.



Cpd	R_5	R_6	R_7	R_8
5a	F	H	H	H
5b	H	F	H	H
5c	H	H	F	H
5d	H	H	H	F
6	H	H	H	H

Figure 3. Schematic representation of the synthesis of compounds 5a, 5b, 5c, and 5d.



Reagents: i: 1. HNO_2, -5 °C; 2. SO_2, Cu_2Cl_2, 15 min; 3. NH_3 (60-70%); ii: cyclopropylamine, dioxane, 100-110 °C, 24 h (80-90%); iii: $HC(OEt)_3$, 130-150 °C, 24-48 h (70-80%); iv: $NaBH_4$, isopropanol, 50-55 °C, 5-10 min. (80-85%).

Figure 4. Schematic overview of the thermodynamic integration (TI) and one step perturbation (OSP) simulations.

A) TI simulations. Each TI simulation starts with **5c** (λ = 0) and simulates the perturbation to **5a**, **5b**, **5d**, or **6** (λ = 1), respectively. B) OSP simulation of a reference compound with four soft fluorine atoms (marked in red). From a single simulation of the reference compound, the difference in free energy relative to each of the real compounds (**5a**, **5b**, **5c**, **5d**, and **6**) can be calculated as well as predictions for other compounds represented by the references state, here marked by X, Y, and Z.

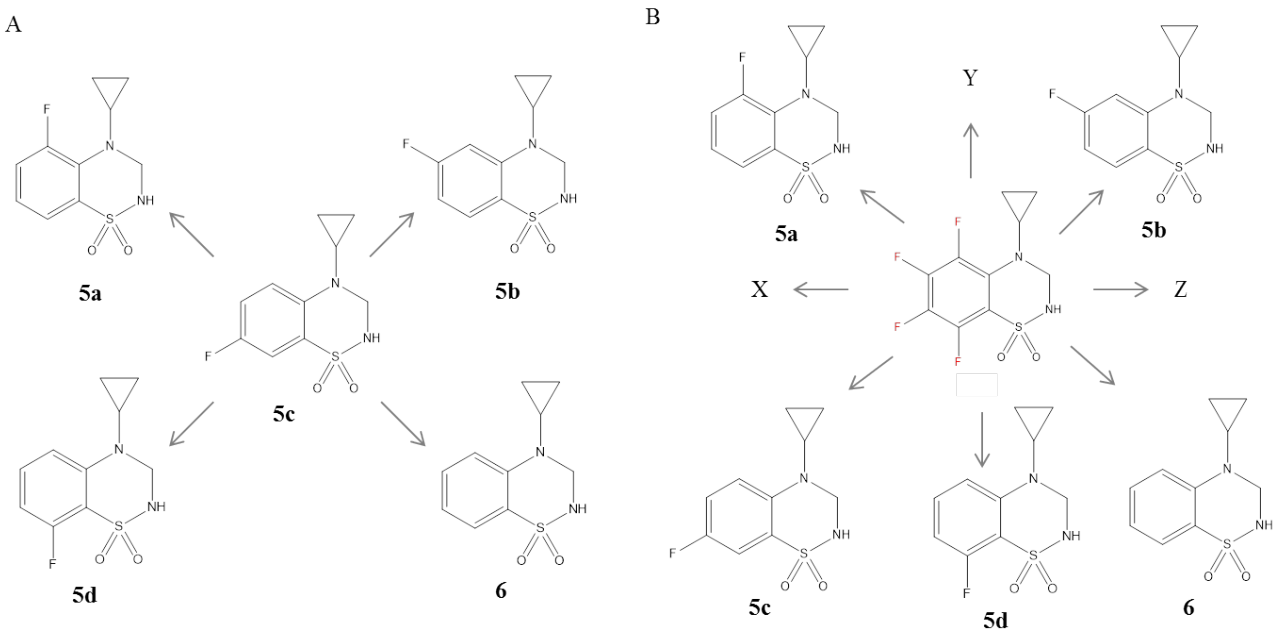


Figure 5. Isothermal titration calorimetry study of binding of 5a (A), 5b (B), 5d (C), and 6 (D) to GluA2-LBD-L483Y-N754S.

The top panel shows raw data and the bottom panel shows the derived isotherm. Heat is developed after each injection (exothermic reaction) and the signal is reduced during saturation of the protein with A) **5c** (for displacement of **5a**), B) **5b**, C) **5d**, and D) **6**, respectively. The shown experiments are a representative of three independent experiments.

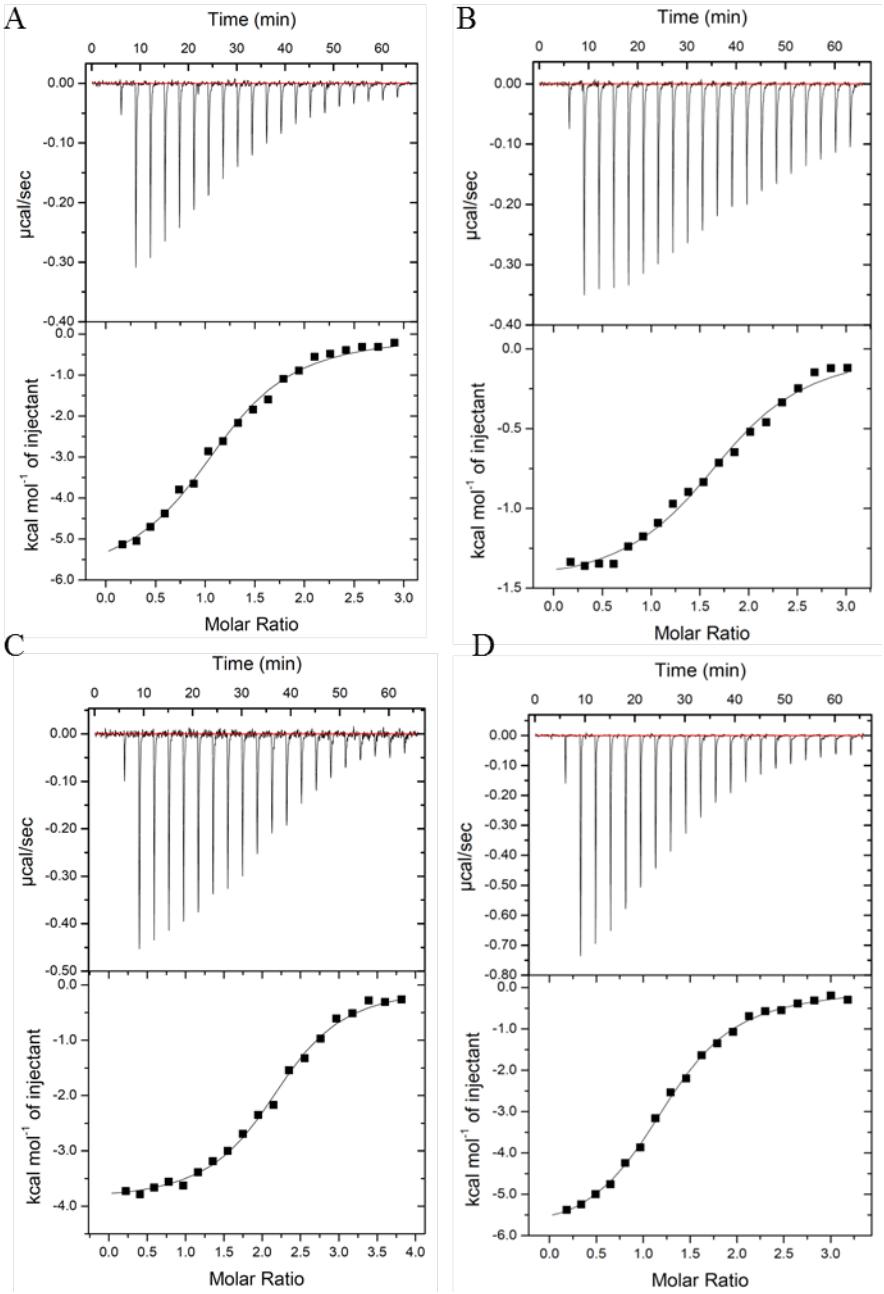


Figure 6. Binding mode of modulators in TI end-state simulations.

Structures of **5a**, **5b**, **5d**, and **6** from TI end-state simulations are shown in line representation with the X-ray crystal structure of **5c** in complex with GluA2-LBD-L483Y-N754S (PDB code 4N07, green lines). Color code: **5a**: salmon, **5b**: magenta, **5d**: grey, **6**: yellow. A) Front view of BTD molecule 1, B) Side view of BTD molecule 1, C) Front view of BTD molecule 2, D) Side view of BTD molecule 2.

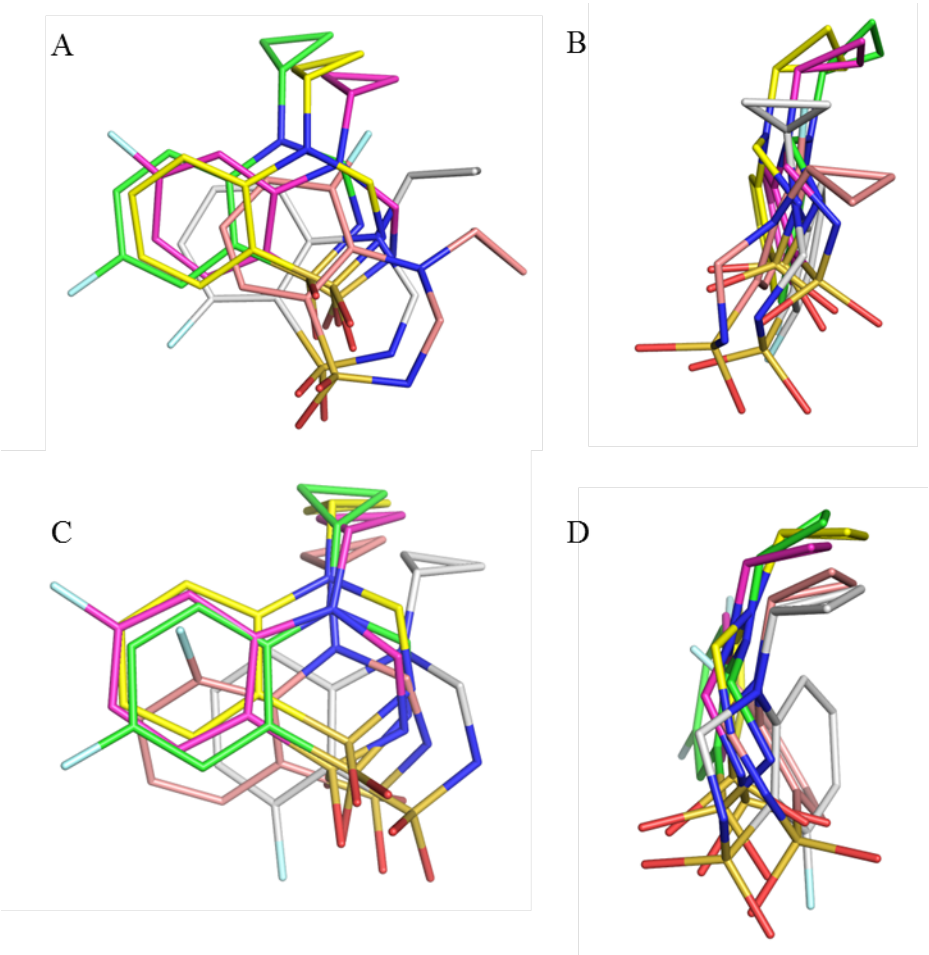


Figure 7. Overlay of the most contributing structures of the real compounds from the OSP simulation.

The most contributing structure of each real compound is shown in line representation along with the X-ray crystal structure of **5c** in complex with GluA2-LBD-L483Y-N754S (PDB code 4N07, green lines). Color code: **5a**: salmon, **5b**: magenta, **5c**: cyan, **5d**: grey, **6**: yellow. A) Front view of BTD molecule 1, B) Side view of BTD molecule 1, C) Front view of BTD molecule 2, D) Side view of BTD molecule 2, E) Front view of BTD compounds from OSP simulation in water, F) Side view of BTD compounds from OSP simulation in water. The figure was prepared using PyMOL (The PyMOL Molecular Graphics System. Version 1.5.0.5, Schrödinger, LLC).

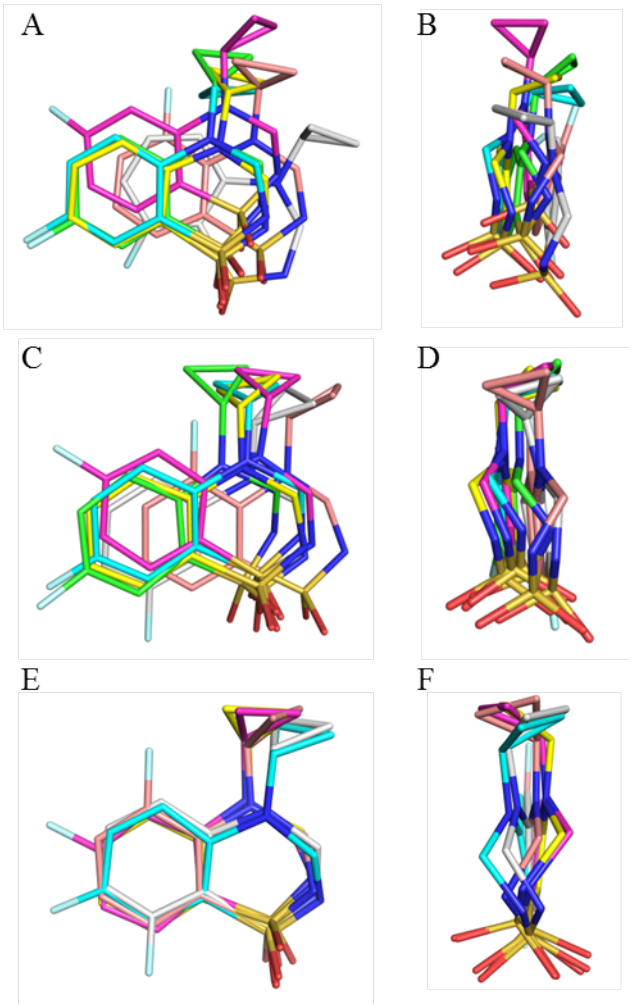
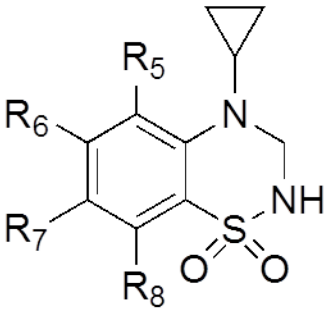


Table of Content Graphic



Cpd	R_5	R_6	R_7	R_8
5a	F	H	H	H
5b	H	F	H	H
5c	H	H	F	H
5d	H	H	H	F
6	H	H	H	H

$$\langle \frac{\partial H}{\partial \lambda} \rangle_{ub} = \frac{\langle \frac{\partial H}{\partial \lambda} e^{U_{bias}/k_B T} \rangle_b}{\langle e^{U_{bias}/k_B T} \rangle_b} \qquad \Delta A_{RA} = A_A - A_R = - k_B T \ln \langle e^{-(H^A - H^R)/k_B T} \rangle_R$$