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A novel fluorescent labeling compound for GluN2A containing *N*-methyl-D-aspartate receptors identified by Autodisplay of GluN1/GluN2A ligand binding domains

Alexander Dombovski[1], Ruben Steigerwald[1], Nadine Ritter[2], Paul Disse[2], Gunnar Goerges[2], Jana Osthues[1], Isabel Aymanns[2], Carina Dilkaute[1], Julian Schreiber[1,2], Martina Düfer[1], Guiscard Seebohm[2], Bernhard Wünsch[1], Joachim Jose[1]

[1] University of Münster, Institute of Pharmaceutical and Medicinal Chemistry, PharmaCampus, Corrensstraße 48, D-48149 Münster, Germany
[2] University of Münster, Institute for Genetics of Heart Diseases (IfGH), Department of Cardiovascular Medicine, University Hospital Münster, D-48149, Münster, Germany

Abstract

Autodisplay was used for the co-display of GluN1 and GluN2A ligand binding domains (LBDs) of the *N*-methyl-D-aspartate (NMDA) receptor in *E. coli* [1][2]. LBDs were confirmed to be located at the cell surface and form dimers, similar to local LBD heterodimers present in full-length NMDA receptors. Flow cytometry was used to evaluate binding of fluorescently labeled TCN-201 derivatives to cells with co-displayed LBDs. TCN-201 is a negative allosteric modulator of GluN2A containing NMDA receptors that binds at the LBD heterodimer interface of GluN1 und GluN2A [3]. Among three TCN-201 derivatives, compound **8** was identified as a novel ligand that bound to cells co-displaying both LBDs but not to cells displaying exclusively one type of LBDs. This was to indicate that compound **8** addressed the same binding site as TCN-201. An apparent dissociation constant of 6.8 ± 1.6 µM for compound **8** was determined. Two-electrode voltage-clamp experiments showed that compound **8** did not inhibit GluN1/GluN2A NMDA receptor-mediated currents. However, compound **8** abolished the current inhibition by TCN-201, indicating competitive binding to the same binding site. Subunit selectivity of compound **8** was evaluated by fluorescence staining of recombinant NMDA receptors in mouse L(tk-) cells. Here, selective staining of GluN2A in contrast to GluN2B containing NMDA receptors with compound **8** was confirmed. Additionally, staining was prevented by preincubation with TCN-201, once more reaffirming the competitive binding mode. This work describes the identification of compound **8** which appears to be the first fluorescent small molecule labeling compound that selectively addresses GluN2A containing NMDA receptors.

Background

For evaluating the involvement of different *N*-methyl-D-aspartate (NMDA) receptor subtypes in the pathogenesis of known diseases, selective markers for *in situ* and *in vivo* analysis are required. NMDA receptors are heterotetrameric ion channels most commonly consisting of two GluN1 and two GluN2 subunits. The subunits multimerize *via* amino terminal domains (ATD), ligand binding domains (LBD) and transmembrane domains (TMD). Hereby, the LBDs assemble as a pair of local heterodimers. Previously, isolated LBDs were expressed as soluble recombinant proteins and used for ligand binding studies and x-ray crystallography [2]. Interestingly, isolated LBDs maintained their characteristic properties as observed for LBDs in full-length subunits, such as forming heterodimers and binding a plethora of ligands. In this work, the Autodisplay technique was used for recombinant co-expression of the LBDs of GluN1 and GluN2A subunits as membrane-anchored autotransporter fusion proteins on the surface of *E. coli* [1]. Subsequently, this approach was used to obtain cell-anchored GluN1/GluN2A LBD heterodimers, establish a ligand binding assay and evaluate GluN2A selective and fluorescently labeled ligands. These were synthesized based on the scaffold of TCN-201 which was the first reported GluN2A selective NMDA receptor modulator with a binding site located at the interface of dimerized GluN1/GluN2A LBDs [3].

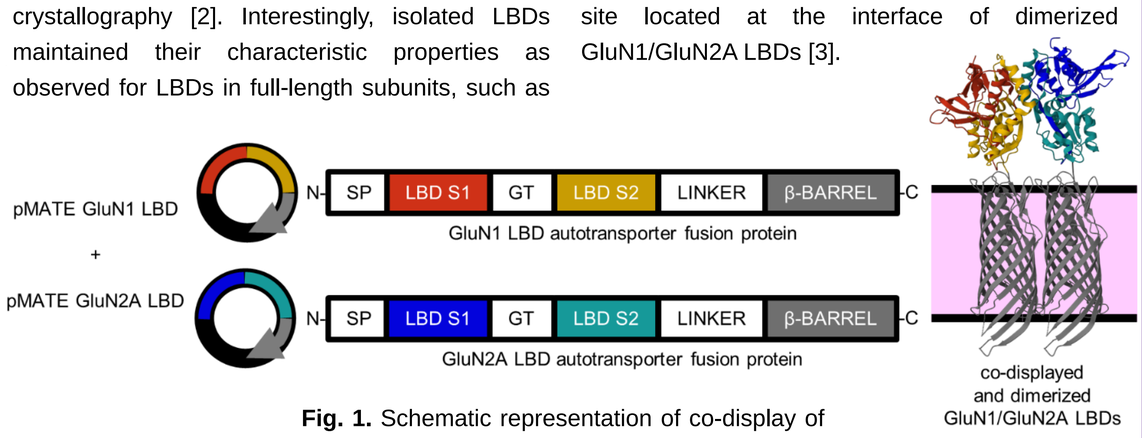

Fig. 1. Schematic representation of co-display of GluN1/GluN2A LBDs in *E. coli via* Autodisplay.

Results

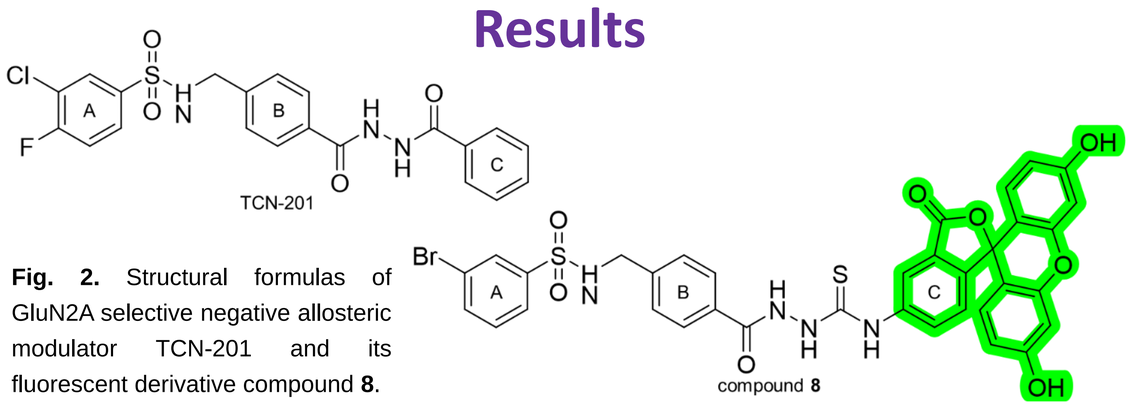

Fig. 2. Structural formulas of GluN2A selective negative allosteric modulator TCN-201 and its fluorescent derivative compound **8**.

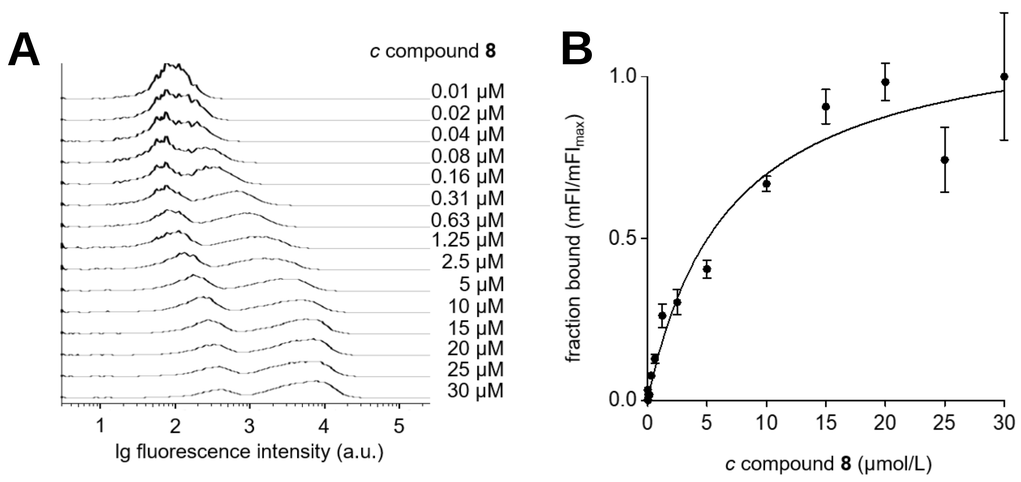

Fig. 3. Quantifying binding affinity of compound **8** to surface displayed GluN1/GluN2A LBD heterodimers by flow cytometry. **(A)** Offset overlay of histograms of compound **8** binding to GluN1/GluN2A LBD co-displaying cells. Non-specific binding was quantified in an analogous manner but with cells displaying exclusively the GluN1 LBD, and therefore lacking the TCN-201 binding site (histograms not depicted). Specific binding was calculated by subtracting non-specific binding from total binding for each concentration. **(B)** Saturation binding curve of specific binding of compound **8** to surface displayed GluN1/GluN2A LBD heterodimers. An apparent K_d value of 6.8 ± 1.6 µM was determined. Mean values ± SEM of n = 3 samples are presented.

Results

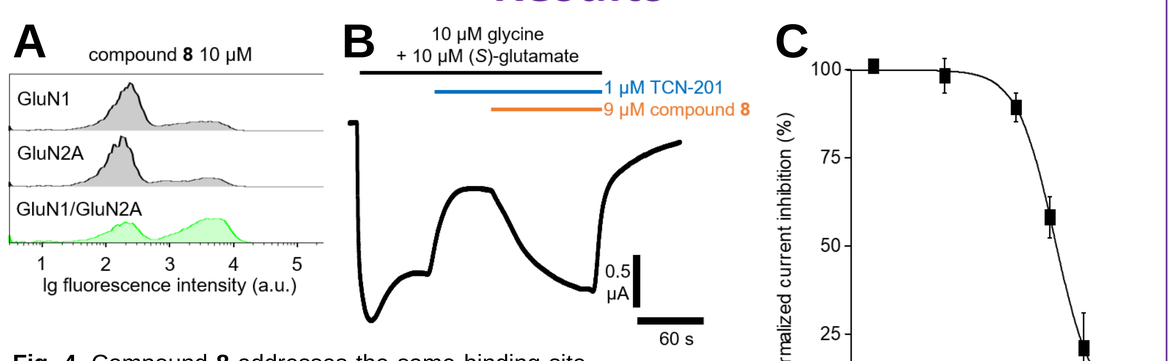

Fig. 4. Compound **8** addresses the same binding site as TCN-201, as shown by a flow cytometry and electrophysiology. **(A)** Flow cytometry-based binding assay to investigate compound **8** binding to GluN1/GluN2A co-displaying cells (green) and control cells displaying only one type of the LBDs (black). Notably, compound **8** does not bind to the cells in absence of the TCN-201 binding site. **(B)** Two-electrode voltage-clamp (TEVC). Current trace of a GluN1-1a/GluN2A expressing *X. laevis* oocyte activated by 10 µM glycine and 10 µM (*S*)-glutamate (black bar), inhibited by 1 µM TCN-201 (blue bar) and additional application of 9 µM compound **8** (orange bar). Compound **8** itself does not lead to a current inhibition (data not shown), however it is capable of displacing TCN-201. **(C)** Dose response curve of inhibition reduction by compound **8**. Current inhibition was evoked by 1 µM TCN-201 and reduction of inhibition by increasing concentrations of compound **8** (0.01−9 µM). An IC_{50} of 3.5 ± 1.6 µM was determined, which is defined as concentration of compound **8** required for half-maximal reduction of the current inhibition by 1 µM TCN-201.

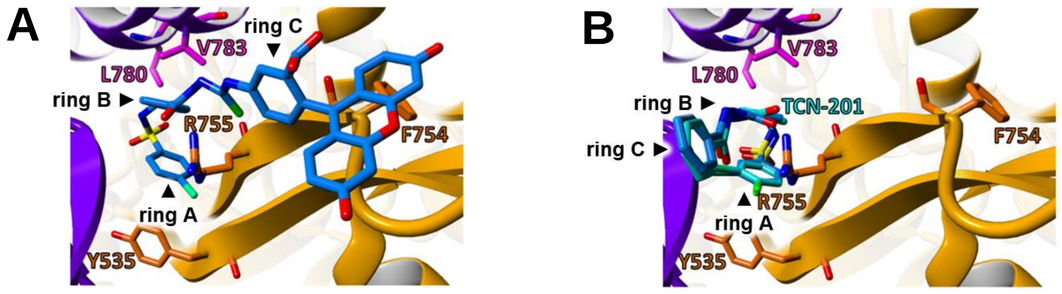

Fig. 5. The binding pose of **(A)** compound **8** was determined *in silico* and showed a similar π-π stacking interaction of rings A and B, as observed for **(B)** TCN-201 (crystallized pose in cyan; docked pose in blue). However, in comparison to TCN-201, the rest of the molecule adjacent to ring B is oriented into an opposite direction. Considering that a cation-π interaction of GluN1 R755 and ring C is crucial for inhibition, the reorientation of compound **8** at the binding site might be the reason for its lack of inhibitory activity.

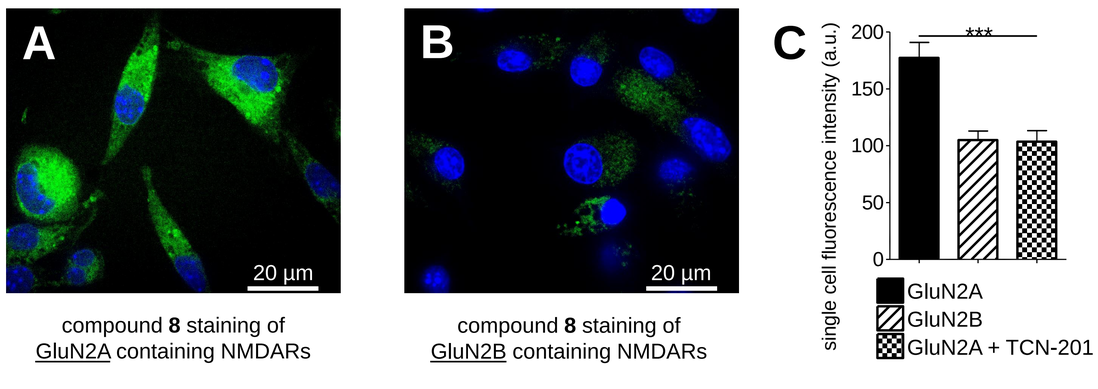

Fig. 6. Fluorescence microscopy of mouse L(tk−) cells expressing recombinant **(A)** GluN1-1a/GluN2A or **(B)** GluN1-1a/GluN2B subunits that were stained with compound **8** (10 µM, 90 min). **(C)** Quantitative analysis of single cell fluorescence intensities after staining with compound **8**. When indicated, GluN1-1a/GluN2A expressing mouse L(tk−) cells were preincubated with TCN-201 (10 µM, 90 min) before they were stained with compound **8**. Mean values ± SEM of n = 7−16 single cells are presented.

Conclusions

Here, it is described for the first time how to use Autodisplay for the surface display of NMDA receptor LBDs in *E. coli*. This approach shows that autodisplayed domains of an eukaryotic NMDA receptor remain functional even though a β-barrel domain serves as a membrane anchor. Autodisplay facilitated identification and characterization of compound **8**, which is a novel fluorescent and GluN2A selective ligand. Compound **8** was confirmed to bind to the same binding site as TCN-201, as shown by flow cytometry, TEVC, fluorescence microscopy and docking studies. Compound **8** appears to be a valuable small molecule labeling probe, in particular when application of an antibody is not possible or not desired.

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E-mail: alexander.dombovski@uni-muenster.de

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