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

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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 [12]. 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 fluorocyanically labeled TCN-201 derivatives to cells with co-displayed LBDs. TCN-201 is a non-competitive, allosteric modulator of GluN2A containing NMDA receptors that binds at the LBD heterodimer interface of GluN1 and 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 addresses the same binding site as TCN-201. An apparent dissociation constant of 6.8 ± 1.6 mM 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. Submit selectivity of compound 8 was evaluated by fluorescence staining of recombinant NMDA receptors in mouse Ltk- 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 platform of ligands. In this work, the Autodisplay technique was used for recombinant co-expression of the LBDs of GluN1 and GluN2A subunits as cell-anchored autotransporter fusion proteins on the surface of E.coli [1]. Subsequently, this approach was used to obtain cell-anchored GluN2A/GluN2B 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].

Results

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 (blue). 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 GluN1-1α/1β 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 by compound 8. Current inhibition was evoked by 1 µM TCN-201 and inhibition of inhibition by increasing concentrations of compound 8 (0.01-9 µM). An IC50 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. 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 (blue). 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 GluN1-1α/1β 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 by compound 8. Current inhibition was evoked by 1 µM TCN-201 and inhibition of inhibition by increasing concentrations of compound 8 (0.01-9 µM). An IC50 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.

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 facilitates 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 fluorescent molecule labeling probe, in particular when application of an antibody is not possible or not desired.