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Neurosteroid binding to the amino terminal and glutamate binding domains of ionotropic glutamate receptors

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ABSTRACT

The endogenous neurosteroids, pregnenolone sulfate (PS) and 3α-hydroxy-5β-pregnan-20-one sulfate (PREGAS), have been shown to differentially regulate the ionotropic glutamate receptor (iGluR) family of ligand-gated ion channels. Upon binding to these receptors, PREGAS decreases current flow through the channels. Upon binding to non-NMDA or NMDA receptors containing an GluN2C or GluN2D subunit, PS also decreases current flow through the channels, however, upon binding to NMDA receptors containing an GluN2A or GluN2B subunit, flow through the channels increases. To begin to understand this differential regulation, we have cloned the S1S2 and amino terminal domains (ATD) of the NMDA GluN2B and GluN2D and AMPA GluA2 subunits. Here we present results that show that PS and PREGAS bind to different sites in the ATD of the GluA2 subunit, which when combined with previous results from our lab, now identifies two binding domains for each neurosteroid. We also show both neurosteroids bind only to the ATD of the GluN2D subunit, suggesting that this binding is distinct from that of the AMPA GluA2 subunit, with both leading to iGluR inhibition. Finally, we provide evidence that both PS and PREGAS bind to the S1S2 domain of the NMDA GluN2B subunit. Neurosteroid binding to the S1S2 domain of NMDA subunits responsible for potentiation of iGluRs and to the ATD of NMDA subunits responsible for inhibition of iGluRs, provides an interesting option for therapeutic design.

1. Introduction

Ionotropic glutamate receptors (iGluRs) are a family of ligand-gated ion channels located in the post-synaptic neural membrane, which play an important role in memory, learning, development, and neural plasticity [1,2]. There are four members of the iGluR family: AMPA,[1] kainate, NMDA, and δ receptors, all of which are functional tetramers. Each family has multiple subunits (AMPA: GluA1–4; NMDA: GluN1, GluN2A–D, GluN3A–B; kainate: GluK1–5; δ: GluD1–D2) that can be assembled in specific combinations. Although varying in primary amino acid sequence, each iGluR subunit has a similar membrane-spanning topology. There is an ~400 amino acid extracellular ATD which is important in subunit assembly, receptor trafficking, channel gating, and allosteric modulation [3] that is structurally similar to the clam-shell shaped bacterial leucine/isoleucine/valine binding protein, Fig. 1. The ATD is followed by an extracellular S1S2 domain which forms a second clam-shell shaped motif with structural similarity to the glutamine binding protein, three transmembrane spanning regions, a re-entrant loop, and an intracellular C-terminus.

In order to better study the iGluR extracellular neurotransmitter binding domain, S1S2 constructs have been designed that join S1 and S2 domains by a glycine-threonine linker, which replaces the intervening membrane spanning regions [4,5]. ATD constructs from AMPA GluA1 and GluA2 [6–8]; kainate GluK2, GluK3, and GluK5 [9–11]; NMDA GluN1, GluN2A, and GluN2B subunits [12–14]; as well as mGluR1 [15] have also been crystallized (Fig. 1). The structure and documented pharmacology of both of these domains supports their study in isolation from the intact protein. In addition, there is one full-length structure of an iGluR, a homo-tetramer of AMPA GluA2 subunits [16].

Since glutamate is the major fast excitatory neurotransmitter in the central nervous system, the activity of iGluRs is highly regulated. A number of endogenous compounds are known to be involved in this regulation, including the sulfated neurosteroids

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[1] *Abbreviations:* AMPA, amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ATD, iGluR amino terminal domain; iGluR, ionotropic glutamate receptor; λ_{max}, maximum wavelength; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate; PREGAS, pregnanolone sulfate (3α-hydroxy-5β-pregnan-20-one sulfate); PS, pregnenolone sulfate; S1S2, iGluR agonist binding domain.

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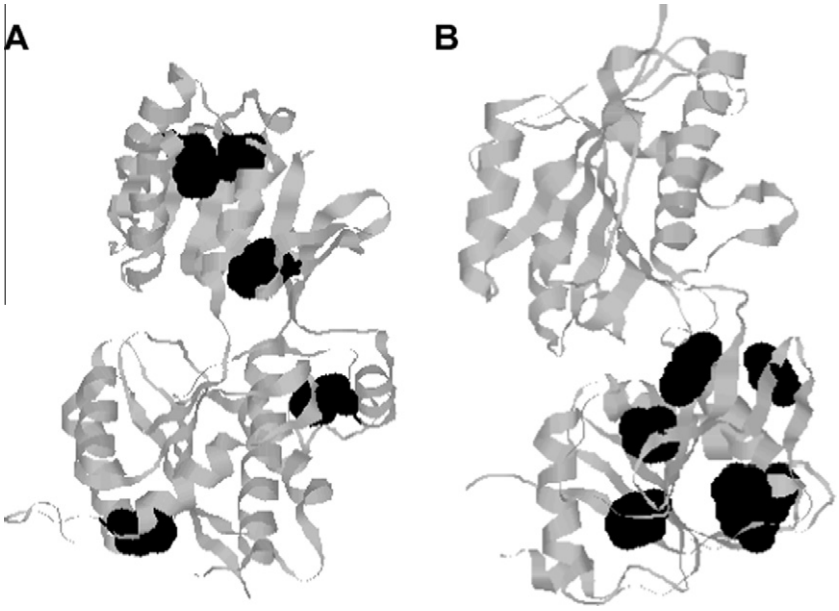

Fig. 1. Rasmol rendering of the (A) AMPA GluA2 ATD (PDB: 2WJW [8]) and (B) NMDA GluN2B ATD (PDB: 3JPW [11]). Tryptophan residues are shown in black.

pregnenolone sulfate (PS) and 3α-hydroxy-5β-pregnan-20-one sulfate (PREGAS (Fig. 2), [17–21]). PS and PREGAS, neurosteroids that differ in the presence or absence of one double bond at the steroid A/B ring junction and the orientation of the sulfate group are our focus here, as PS has been shown to have the largest potentiating effect of NMDA receptors (150%) and PREGAS has been shown to have the largest inhibitory effect (66%), in this class of compounds [37]. More specifically, studies have shown that PS positively regulates the activity of NMDA receptors containing GluN2A or B subunits by increasing open channel probability. PS negatively regulates receptors containing GluN2C or D subunits and all non-NMDA iGluRs. PREGAS negatively regulates all iGluRs [19–21]. When binding to NMDA receptors, PREGAS has been shown to reduce open channel probability and time, and to increase receptor desensitization [22]. It has been hypothesized that PS may be a "mechanistically novel agent for cognitive and negative symptoms of schizophrenia" [23]. For use of PS or PREGAS as either a drug or lead compound, it is important to understand the differences in PS binding which lead to iGluR activation vs. inhibition. Our lab has shown that both PS and PREGAS bind to the S1S2 domain of the GluA2 subunit of the AMPA receptor, but that they do not bind to any extracellular region of the NMDA GluN1 subunit [24,25]. Due to the regulation of NMDA receptors by both of these neurosteroids, which has been shown to occur on the extracellular face, it seems likely that binding occurs either within GluN2 subunits or at a GluN1/GluN2 interface [17–21]. Here we present results probing the binding of both neurosteroids to the GluN2D, GluN2B, and GluA2 extracellular ATD and S1S2 domains.

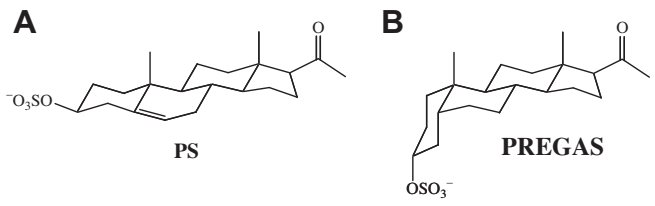

Fig. 2. Neurosteroid structures. (A) pregnenolone sulfate (PS) (B) 3α-hydroxy-5β-pregnan-20-one sulfate (PREGAS).

2. Experimental

2.1. Cloning

The S1 and S2 regions of the NMDA GluN2B (S1: D404-R540, S2: Q672-E814) and GluN2D (S1: T426-R564, S2: T687-D828) subunits were amplified using PCR (rat cDNA obtained from Stephen Traynelis). The restriction sites *Eco* RI (GluN2B 3′S1 and 5'S2), *Hind* III (GluN2D 3′S1 and 5'S2), *Xho* I (GluN2B 3′S2 and GluN2D 3′S2), and *Nco* I (GluN2B 5′S1 and GluN2D 5′S1) were included in the primers, as was coding for a glycine-threonine linker between the S1S2 domains. After restriction digestion of the 3′S1 and 5′S2 ends, the S1-GT-S2 domain was ligated and PCR amplified. The insert was then ligated into plasmid pET-GQ (from Eric Gouaux), and transformed into Origami 2(DE3) cells (Novagen). Colonies were screened by PCR and then sequenced.

The ATDs for the NMDA GluN2B, GluN2D, and AMPA GluA2 subunits (amino acids 21–404) were amplified using PCR (rat GluA2 cDNA from Mark Mayer). After restriction digestion (NcoI/XhoI for GluA2 and GluN2D and NcoI/BamHI for GluN2B), these inserts were ligated into plasmid pET-GQ, and treated as above.

2.2. Protein over-expression and purification

An overnight of GluN2B or GluN2D S1S2 in Origami 2(DE3) was used to seed 1.5 L LB-kan (37 °C), until an OD_{600} of 0.8 was reached. The temperature was then lowered to 20 °C for 30 min followed by induction with 1 mM IPTG. Growth was continued for 16–18 h at 20 °C, after which time the cells were centrifuged for 10 min at 4 °C and 4000 rpm. The pellets were resuspended in 50 mL buffer A (20 mM Tris, 50 mM NaCl, 1 mM glutamate, pH 8) containing 5 mM $MgSO_4$, 0.5 mM PMSF, 0.3 mM deoxycholic acid, and 16 μM lysozyme, and lysed by sonication. Lysed cells were centrifuged at 12,500 rpm for 30 min at 4 °C, and the supernatant loaded onto a nickel affinity column (~20 mL, Novagen) and rotated overnight at 4 °C. The column was rinsed with 30 mL of 10, 25, 50, 100, and 250 mM, and 1 M imidazole in buffer A. Further purification for the GluN2B S1S2 domain included anion exchange chromatography (AEC, GE Q Sepharose) at both pH 7.2 and 8.0 (buffer A) followed by size exclusion chromatography (SEC, Superdex 75