

RAPID COMMUNICATION

Pregnenolone Sulfate Activates NMDA Receptor Channels**E. ADAMUSOVÁ¹, O. CAIS¹, V. VYKLIČKÝ¹, E. KUDOVÁ², H. CHODOUNSKÁ²,
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Summary

Pregnenolone sulfate (PS), an endogenously occurring neurosteroid, has been shown to modulate the activity of several neurotransmitter-gated channels, including the NMDA receptor (NMDAR). NMDARs are glutamate-gated ion channels involved in excitatory synaptic transmission, synaptic plasticity, and excitotoxicity. In this study, we analyzed the effects of PS on calcium signaling in cultured hippocampal neurons and HEK293 cells expressing NMDAR. The cells were loaded with the Ca²⁺ sensor Fura-2. In agreement with previous electrophysiological experiments, PS potentiated the increases in intracellular Ca²⁺ induced by an exogenous application of glutamate; however, PS also increased intracellular Ca²⁺ in the absence of exogenous NMDA agonist. The agonist-independent effect of PS was induced in all neurons studied and in HEK293 cells expressing GluN1/GluN2A-B receptors in a neurosteroid-specific manner. We conclude that PS is an endogenous NMDA agonist that activates the GluN1/GluN2A-B receptors.

Key wordsNeurosteroids • *N*-methyl-D-aspartate receptor • Pregnenolone sulfate • Calcium imaging • Recombinant receptors**Corresponding author**

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NMDA receptors (NMDARs), a subtype of ionotropic glutamate receptors, are involved in synaptic transmission and plasticity (Citri and Malenka 2008). These receptors are positively and negatively modulated

by neurosteroids. Pregnenolone sulfate (3 α 5 β S) is an endogenous neurosteroid that has a use-dependent effect though voltage-independent inhibitory action on NMDARs (Park-Chung *et al.* 1994, Abdrachmanova *et al.* 2001, Petrovic *et al.* 2005). On the other hand, pregnenolone sulfate (PS), a structurally similar neurosteroid, allosterically potentiates responses mediated by NMDARs (Wu *et al.* 1991, Bowlby 1993, Park-Chung *et al.* 1994). Its action is dependent on the receptor subunit composition and receptor state; i.e., upon receptor activation, the PS affinity is decreased ~50-fold (disuse-dependent action) (Horak *et al.* 2004). At GABA_A receptors, three mechanisms have been described for neurosteroid modulation at physiologically relevant concentrations: potentiation (Zhu and Vicini 1997, Stell *et al.* 2003), direct receptor activation (Majewska *et al.* 1986), and inhibition (Majewska and Schwartz 1987, Wang *et al.* 2002). Even though the binding site(s) by which these effects are mediated has not yet been firmly identified, pharmacological, functional, and molecular studies indicate multiple binding sites (Hosie *et al.* 2007).

All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and with the approval of the Institutional Animal Care and Use Committee. Primary dissociated hippocampal cultures were prepared from 1- to 2-day-old postnatal rats. Animals were decapitated, and the hippocampi were dissected. Trypsin digestion, followed by mechanical dissociation, was used to prepare cell suspension. Single cells were plated at a density of 500,000 cells/cm² on 31-mm or 12-mm polylysine-coated

glass cover slips. Neuronal cultures were maintained in NeurobasalTM-A (Invitrogen, Carlsbad, CA) medium supplemented with glutamine (0.5 mM) and B-27 Serum-Free Supplement (Invitrogen).

HEK293 cells (American Type Culture Collection, ATTC No. CRL1573, Rockville, MD) were cultured in Opti-MEM[®] I (Invitrogen) with 5 % fetal bovine serum at 37 °C and transfected with GluN1/GluN2/GFP plasmids as described previously (Cais *et al.* 2008). Equal amounts (0.3 µg) of cDNAs coding for GluN1, GluN2, and GFP (green fluorescent protein) (pQBI 25, Takara, Japan) were mixed with 0.9 µl of Matra-A Reagent (IBA, Göttingen, Germany) and added to confluent HEK293 cells on a 24-well plate. After trypsinization, the cells were resuspended in Opti-MEM[®] I containing 1 % fetal bovine serum supplemented with 20 mM MgCl₂, 1 mM D,L-2-amino-5-phosphonopentanoic acid, and 3 mM kynurenic acid and plated on 30-mm polylysine-coated glass cover slips. The following gene-encoding NMDAR subunits were used: GluN1-1a (GluN1; GenBank accession no. U08261) (Hollmann *et al.* 1993), GluN2A (GenBank accession no. D13211) (Ishii *et al.* 1993), GluN2B (GenBank accession no. M91562) (Monyer *et al.* 1992). NMDAR subunits were identified with the nomenclature recently recommended by IUPHAR (Collingridge *et al.* 2009).

The calcium-sensitive fluorescent dye fura-2AM (Molecular Probes, Eugene, OR) was used to determine transient alterations in intracellular calcium concentration ([Ca²⁺]_i). Cells (HEK293 cells 24-48 hours after the end of transfection or primary hippocampal neurons cultured for 5-8 days) were incubated in 5 µM fura-2AM for 60 min at 37 °C in the presence of extracellular solution (ECS) containing (in mM) 160 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, and 2 CaCl₂ (pH-adjusted to 7.3 with NaOH) supplemented with 1 mM MgCl₂, 0.02 % pluronic acid, and 25 µM probenecid. Cells were then washed three times with Mg²⁺-containing ECS and incubated for an additional 30 min before imaging. Fura-2 was excited alternately at 340 and 380 nm and an image of the emission from each excitation wavelength was collected using fluorescence imaging system Cell[^]R (Olympus) consisting of an inverted microscope Olympus IX 81 equipped with a cooled CCD camera Hamamatsu Orca-ER (Hamamatsu Photonics K.K., Shizuoka, Japan) and Polychromator V (TILL Photonics, Munich, Germany). The following filters were used: excitation filter SP410, emission filter LP440, and dichroic mirror DCLP410 (Olympus). All cells within the field of view

were analyzed for each experiment. The average number of cells imaged per field of view was 15±3. The cells in the experimental chamber were perfused with Mg²⁺-containing ECS at the rate of 0.1 ml/min at room temperature. Glycine (20 µM), an NMDAR co-agonist, was routinely used. TTX (0.5 µM) was used in experiments on cultured hippocampal neurons. 20-oxopregn-5-en-3β-yl sulfate (PS; pregnenolone sulfate) solutions were made from freshly prepared 30 mM stock in dimethyl sulfoxide (DMSO). The same concentration of DMSO (1 %) was maintained in the test and control extracellular solutions.

Fluorescence intensity was measured in cell bodies and expressed as fluorescence ratio $F_{340/380}(t) = F_{340}/F_{380}$, where t is time and F_{340} and F_{380} are the fluorescence intensities measured at 340 and 380 nm, respectively. Relative change in the fluorescence (ΔF) induced by drug treatment was calculated from $\Delta F = F_{340/380}(test) - F_{340/380}(control)$, where $F_{340/380}(test)$ is the maximal fluorescence intensity achieved during test solution application (usually 1-2 min) and $F_{340/380}(control)$ is the fluorescence intensity before the start of test solution application. For qualitative evaluation of the drug action, increases in ΔF greater than 0.1 were considered to be relevant changes. This criterion was chosen as mean + SD of ΔF induced by application of control ECS containing 1 % DMSO, 20 µM glycine, 2 mM Ca²⁺, and no added Mg²⁺ in GFP/GluN1/GluN2B-transfected cultures and assessed in GFP-positive HEK293 cells ($\Delta F=0.06\pm 0.04$; $n=27$) (see below).

The drugs were purchased from Sigma (St. Louis, MO) or Tocris Cookson Ltd. (Avonmouth, UK). The neurosteroid PS was synthesized by H.C. according to a previously published method (Arnostova *et al.* 1992) and their purity (>98 %) was repeatedly tested by nuclear magnetic resonance, high performance liquid chromatography, thin-layer chromatography, and elemental analyses. Results are presented as mean ± SEM, with n equal to the number of cells studied; for the statistical comparison of groups, the Mann-Whitney Rank Sum Test was used. $P<0.05$ was used to determine the significance.

Our recent experiments have shown that the efficacy of PS modulation of NMDARs is affected by intracellular factors (Petrovic *et al.* 2009). To study the effect of neurosteroids on NMDARs under conditions of relatively intact cells, calcium imaging technique was used. Figure 1A shows the results of experiments in which the effect of PS on glutamate-induced change in

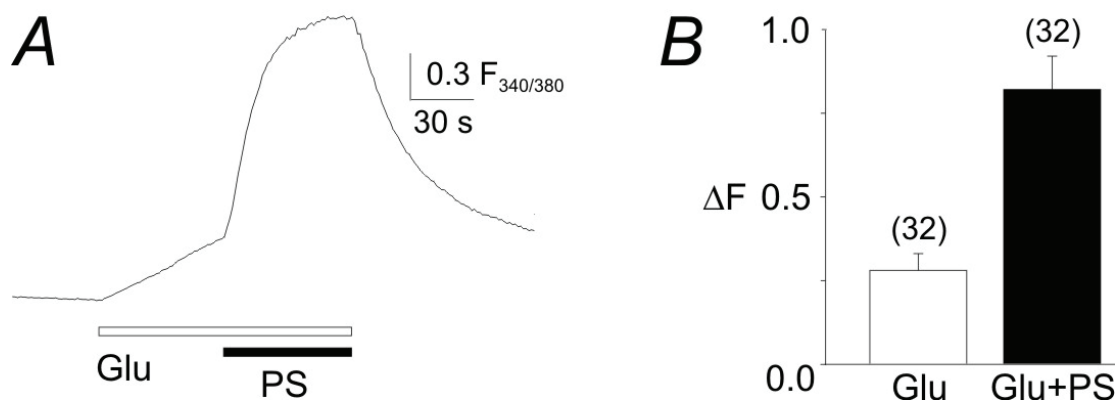


Fig. 1. Agonist-dependent effect of PS. **A.** A change in the fluorescence ratio ($F_{340/380}$) in a GFP-positive HEK293 cell in a culture transfected by GFP, GluN1, and GluN2B. The response was induced by 0.3 μM glutamate (the duration is indicated by open bar) and its co-application with 300 μM PS (indicated by filled bar). Glutamate and the neurosteroid were applied in the ECS containing 1 % DMSO, 20 μM glycine, 2 mM Ca^{2+} , and no added Mg^{2+} . **B.** Bar graph summarizes the relative changes in the fluorescence (ΔF) induced by 0.3 μM glutamate (Glu) and its co-application with 300 μM PS (Glu + PS). The relative changes in the fluorescence induced by glutamate application were calculated from $\Delta F = F_{\text{Glu}} - F_0$, where F_0 is the fluorescence intensity ratio at 340 and 380 nm before the start of test solution application and F_{Glu} is the fluorescence ratio at the end of glutamate application. In the case of glutamate and PS, co-application the fluorescence was calculated from $\Delta F = F_{(\text{Glu}+\text{PS})} - F_0$, where $F_{(\text{Glu}+\text{PS})}$ is the fluorescence ratio at the end of glutamate and PS co-application. Error bars represent mean \pm SEM, and the number of cells studied is indicated in parentheses. There is a statistically significant difference between ΔF induced by glutamate and its co-application with PS (Mann-Whitney: $P=0.002$).

fluorescence (ΔF) was assessed. In the continuous presence of 20 μM glycine, application of 0.3 μM glutamate in ECS (with no added Mg^{2+}) for 60 s induced a change in fluorescence ($\Delta F=0.28\pm 0.05$; $n=32$) (Fig. 1A,B) in GFP/GluN1/GluN2B-transfected cultures of HEK293 cells. This response was significantly enhanced when PS (300 μM) was co-applied with glutamate for an additional 60 s ($\Delta F=0.82\pm 0.1$ ($n=32$); Mann-Whitney: $P=0.002$). Surprisingly, in control experiments the application of PS (300 μM) in the absence of glutamate also induced a change in the fluorescence of GFP-positive HEK-293 cells (Fig. 2A,C). The effect of PS on agonist-induced NMDAR responses is consistent with the effect of this steroid on current responses recorded from both native and recombinant receptors using patch clamp technique (Bowlby 1993, Weaver *et al.* 1998, Horak *et al.* 2004); however, $[\text{Ca}^{2+}]_i$ rise induced by PS application in the absence of added NMDAR agonist has not yet been characterized.

Before characterizing the effect of PS in detail, we performed a series of control experiments. These indicated that a majority (65-81 %, depending on the transfection batch) of GFP-positive HEK293 cells in GFP/GluN1/GluN2B-transfected cultures were sensitive to 1 μM glutamate applied in ECS containing no added Mg^{2+} , while only a small fraction (<7 %) of GFP-negative HEK293 cells in GFP/GluN1/GluN2B-transfected cultures and HEK293 cells in non-transfected cultures responded to glutamate by a change in ΔF . This

agrees well with the results of our electrophysiological experiments showing a high variability in the amplitude of glutamate-induced responses, with a small fraction of GFP-positive HEK293 cells not responding even to a high glutamate concentration. To reduce the variability in the data, we analysed the PS responses in GFP-positive HEK293 cells in GFP/GluN1/GluN2-transfected cultures that were also sensitive to 1 μM glutamate (GG-cells). Glutamate non-responding GFP-positive HEK293 cells were excluded from the analysis. Critical ΔF ($\Delta F_{\text{Critical}}$), used to determine glutamate sensitivity, was defined as mean + SD of the increase in fluorescence ($\Delta F=0.06\pm 0.04$; $n=27$) after application of control ECS with no added Mg^{2+} in the GG-cell in the GFP/GluN1/GluN2B-transfected cultures (see Fig. 2B,F).

On average, 64 % ($n=31$) of GG-cells in GFP/GluN1/GluN2B transfected cultures (the average number of PS-sensitive cells ranged from 62 % to 73 % of GFP-positive HEK293 cells in 8 different transfections) were sensitive to 300 μM PS (PS-induced $\Delta F \geq \Delta F_{\text{Critical}}$; $\Delta F_{\text{Critical}}=0.1$) applied in ECS with no added Mg^{2+} ; these cells responded on average with a mean increase in fluorescence of $\Delta F=0.6\pm 0.1$ ($n=31$). Following PS application, the cells were allowed to recover (in ECS containing 2 mM Mg^{2+}) for ~6 min and subsequently exposed to 1 μM glutamate (Fig. 2C). The glutamate-sensitive cells responded with an increase in fluorescence of $\Delta F=1.3\pm 0.1$ ($n=31$) (Fig. 2F).

An additional analysis was performed on the

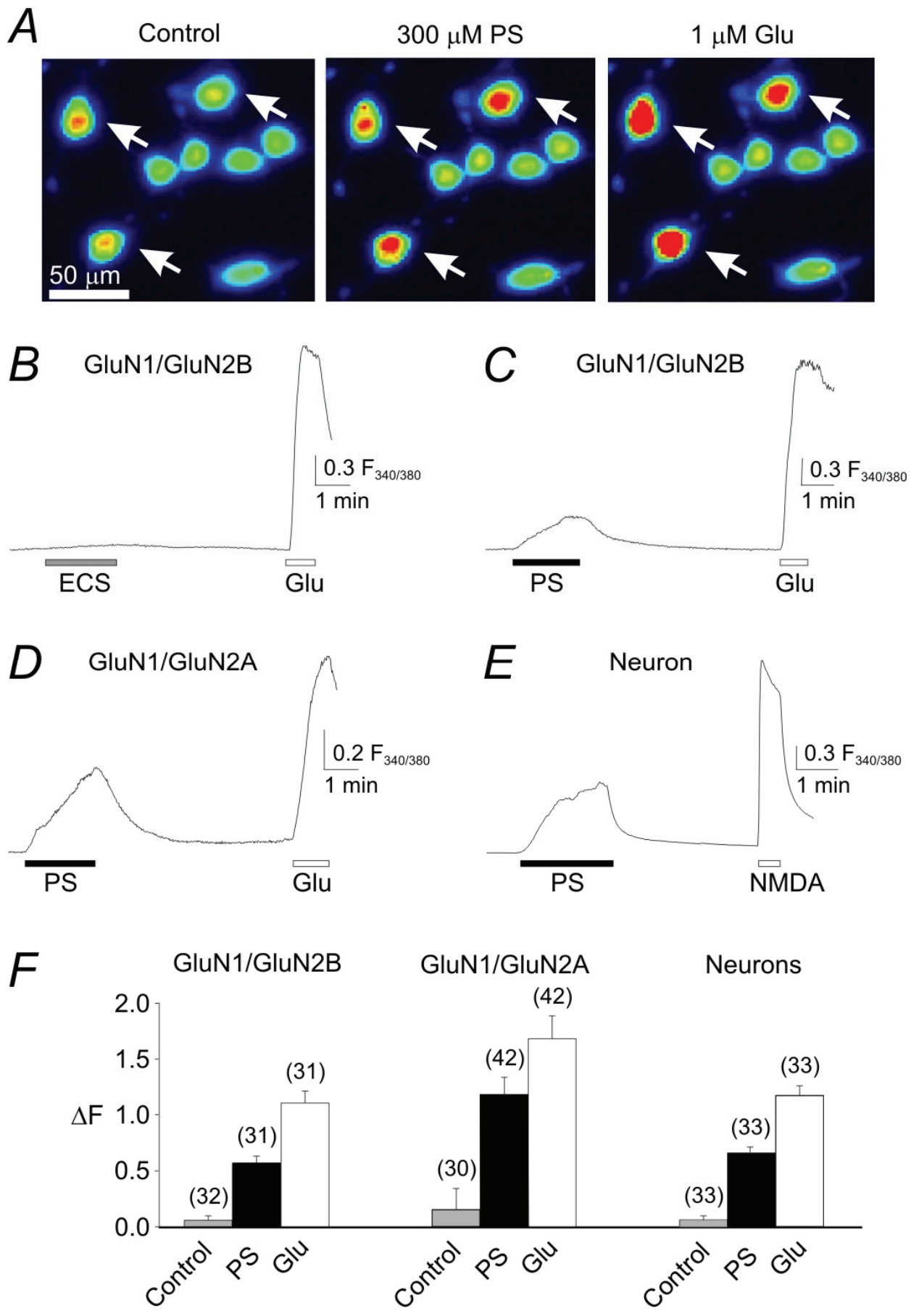


Fig. 2. Agonist-independent effect of PS. **A.** Changes in the fluorescence ratio at 340 and 380 nm are indicated in pseudo-colors (fluorescence intensity increases in the order blue-green-yellow-red). The recordings were made in the presence of ECS (no added Mg^{2+} , Control) and ECS supplemented with PS (300 μM) and glutamate (1 μM). Note that only the GFP-positive cells (indicated by arrows) were glutamate- and PS-sensitive. The figure shows a typical $F_{340/380}$ during application of ECS containing 1 % DMSO, 20 μM glycine, 2 mM Ca^{2+} , and no added Mg^{2+} (ECS) on a GFP-positive HEK293 in culture transfected with GFP/GluN1/GluN2B (**B**), this solution containing 300 μM PS (PS) (**C**), response to PS in GFP-positive HEK293 in cultures transfected with GFP/GluN1/GluN2A (**D**), and response to PS in cultured hippocampal neuron (**E**). The cells sensitive to PS were also sensitive to 1 μM glutamate (the duration is indicated by open bar). Records shown in B-E are from different cultures. **F.** Bar graph summarizing the relative changes in the fluorescence (ΔF) induced by ECS containing no Mg^{2+} applied for 2 min in GFP-positive HEK293 cells in the cultures transfected with GFP/GluN1/GluN2B (Control) and by 300 μM PS (PS) applied for 2 min and the maximal response to 1 μM glutamate (Glu) in GFP-positive HEK293 cells in cultures transfected by GFP/GluN1/GluN2B (GluN1/GluN2B), GFP/GluN1/GluN2A (GluN1/GluN2A), and cultured hippocampal neurons (Neurons). Error bars represent mean \pm SEM, and the number of cells is indicated in parentheses. There is a significant difference (Mann-Whitney: $P < 0.001$) between ΔF induced by ECS and PS in GFP-positive HEK293 cells in the cultures transfected by GFP/GluN1/GluN2B, GFP/GluN1/GluN2A, and neurons.

fluorescence intensity of GG-cells insensitive to 300 μM PS. The value of the glutamate-induced increase in ΔF was 2.3-fold lower in PS-insensitive cells than in PS-sensitive cells. These data indicate that the differences in the sensitivity of the cells to PS and glutamate likely reflect the differences in the density of NMDAR channels in the cytoplasmic membranes of HEK293 cells. The recordings in Figure 2D,E indicate that GG-cells in the cultures transfected with GFP/GluN1/GluN2A and cultured hippocampal neurons responded to 300 μM PS with a ΔF that was similar to that observed in GG-cells transfected with GFP/GluN1/GluN2B (Fig. 2E). In the GFP/GluN1/GluN2A-transfected cultures, 69 % of GG-cells (29 cells analyzed) were sensitive to 300 μM PS and 100 % of neurons (69 neurons analyzed) were sensitive to 300 μM PS.

We conclude that, in addition to the PS-induced allosteric modulatory effect, this endogenous sulfated steroid can directly gate NMDAR channels. Understanding the molecular mechanism of the steroid modulation of NMDAR channel function is important for understanding the conditions under which endogenous

steroids modulate neuronal excitability. In addition, the evaluation of the action of sulfated steroids as probes of receptor behavior may help to assess their potential clinical utility.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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