

Title: Neurosteroid modulation of ionotropic glutamate receptors and excitatory synaptic transmission

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Summary

Ionotropic glutamate receptors function can be affected by neurosteroids, both positively and negatively. N-methyl-D-aspartate (NMDA) receptor responses to exogenously applied glutamate are potentiated or inhibited (depending on the receptor subunit composition) by pregnenolone sulphate (PS) and inhibited by pregnanolone sulphate (3 α 5 β S). While PS effect is most pronounced when its application precedes that of glutamate, 3 α 5 β S only binds to receptors already activated. Synaptically activated NMDA receptors are inhibited by 3 α 5 β S, though to a lesser extent than those tonically activated by exogenous glutamate. PS, on the other hand, shows virtually no effect on any of the models of synaptically activated NMDA receptors. The site of neurosteroid action at the receptor molecule has not yet been identified, however, the experiments indicate that there are at least two distinct extracellularly located binding sites for PS mediating its potentiating and inhibitory effects respectively. Experiments with chimeric receptors revealed the importance of the extracellular loop connecting the third and the fourth transmembrane domain of the receptor NR2 subunit for the neurosteroid action. α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate receptors are inhibited by both PS and 3 α 5 β S. These neurosteroids also affect AMPA receptors-mediated synaptic transmission, however, in a rather indirect way, through presynaptically located targets of action.

Key words: N-methyl-D-aspartate receptor • AMPA receptor • glutamate receptor • EPSC
• neurosteroid • allosteric modulation

Introduction

Steroid hormones can easily cross the blood-brain barrier and function at the genomic level to produce changes in mood and behaviour. These effects occur relatively slowly (taking from minutes to hours) (McEwen 1991). In addition, certain steroids can produce immediate changes (within seconds or even milliseconds) in neuronal excitability, which suggests a different way of functioning. Even though this rapid effect of these neuroactive compounds was observed more than half a century ago (Selye 1941), the molecular mechanism of their action remained unknown for a long time. It was only in the 1980s that the first study on the effects of steroids on GABA_A receptors appeared (Harrison and Simmonds 1984), followed by works on modulation of the other types of ligand-gated ion channels such as glutamate, glycine or nicotinic acetylcholine receptors (Wu *et al.* 1990, 1991, Bullock *et al.* 1997); for review see (Rupprecht and Holsboer 1999). At about the same time it was shown that the neuroactive steroids are synthesized directly in the nervous tissue (Corpechot *et al.* 1981), hence the term neurosteroids. Evidence has been provided for synthesis *de novo* from cholesterol or *in situ* from steroidal precursors imported from peripheral sources (Baulieu 1998).

The modulatory effect of neurosteroids on the activity of ligand-gated ion channels was suggested to play a role in a range of physiological processes such as learning, aging and stress as well as certain neuropsychiatric disorders (Flood and Roberts 1988, Nasman *et al.* 1991, Grobin *et al.* 1992, Vallee *et al.* 1997); for review see (Gasior *et al.* 1999). Therefore, full understanding of the biochemical pathways, mechanisms of function, and receptor binding sites of these compounds can be crucial for new drug design and therapeutic procedures.

The mechanism of neurosteroid action at GABA_A receptors was reviewed recently (Belelli *et al.* 2006, Herd *et al.* 2007, Hosie *et al.* 2007, Morrow 2007). The aim of this review is to summarize current knowledge of the molecular mechanism of the effect of neurosteroids on NMDA and AMPA/kainate receptors and the synaptic transmission mediated by these receptors.

1. Neurosteroid modulation of NMDA receptors

1.1. Molecular mechanism of neurosteroid action with positive allosteric effect

The first observation of a neurosteroid action on NMDA receptors was published by (Wu *et al.* 1991). Cell-cultured chick spinal cord neurons exhibited a more than 200 % increase of response to NMDA when 300 μ M pregnenolone sulfate (PS) was present before and during the NMDA application. This finding was later confirmed by numerous studies of cells with native as well as recombinant NMDA receptors (Bowlby 1993, Park-Chung *et al.* 1997, Ceccon *et al.* 2001, Malayev *et al.* 2002, Horak *et al.* 2004).

Several studies aimed to find the functional group of PS responsible for potentiation. Unlike the strong potentiating effect of PS, pregnenolone does not affect NMDA receptor responses (Weaver *et al.* 2000). Thus, the sulfate group at carbon C3 is important for the potentiating effect (see Fig. 1A). However, the potentiating effect is maintained if the sulfate group is replaced by another negatively charged group, e.g. hemioxalate, hemisuccinate or hemiglutarate (Park-Chung *et al.* 1997, Weaver *et al.* 2000). The double bond between C5 and C6 is also of essential importance. Its absence, accompanied by 5 β arrangement of chiral C5 in pregnanolon sulfate (3 α 5 β S), changes the shape of the neurosteroid molecule (see Fig. 1A). While the PS molecule is roughly planar, the 3 α 5 β S molecule has a hooked shape, which results in the complete reversal of the potentiating effect, making 3 α 5 β S a strong inhibitor of

NMDA receptors (Park-Chung *et al.* 1994, Weaver *et al.* 2000, Petrovic *et al.* 2005). The effect of neurosteroids with a potentiating and inhibitory effect seems to be mediated by independent binding sites located at the extracellular domain of the NMDA receptor (Park-Chung *et al.* 1997, Horak *et al.* 2006).

To say that NMDA receptors are potentiated by PS is to simplify a more complex phenomenon. Electrophysiological measurements of recombinant NMDA receptors expressed in *Xenopus laevis* oocytes and HEK-293 cells revealed that receptors containing NR1/NR2A and NR1/NR2B subunits are potentiated by PS, whereas NR1/NR2C and NR1/NR2D receptors are inhibited by PS (Ceccon *et al.* 2001, Malayev *et al.* 2002, Horak *et al.* 2004, Horak *et al.* 2006). This fact was utilized to localize the amino-acid region which is crucial for PS potentiation (Jang *et al.* 2004, Horak *et al.* 2006). Chimeras of NR2 subunits assembled from fragments of NR2A or NR2B (potentiated by PS) and NR2C or NR2D subunits (less affected by PS) were used to infer the subunit domain important for PS action at NMDA receptors. The studies from both laboratories indicate the importance of the extracellular loop spanning the third and fourth transmembrane domains and the fourth transmembrane domain for the potentiating effect of PS. (Jang *et al.* 2004) further focused on point mutations of amino acid residues within this region. Surprisingly, out of 15 mutated NR2B subunits only the substitution of NR2B glutamine by NR2D lysine at residue 812 significantly reduced the response to PS. This residue may be directly involved in PS binding; however, other explanations must be considered. The results of experiments of (Horak *et al.* 2006) show that the low degree of PS-induced potentiation of receptors containing NR2C-D subunits resides in low efficacy rather than low PS affinity (which is actually higher for these subunits). This indicates that the M3-M4 loop may be involved only in signal transduction rather than in direct PS binding. High-resolution crystal structure data and further mutagenesis studies are necessary to identify the PS binding site at the NMDA receptor.

Exploration of the molecular mechanisms responsible for the potentiating effect of PS indicates that this neurosteroid increases the probability of NMDA receptor channel opening. At a saturating concentration of PS it results in approximately 4-fold potentiation of responses of receptors composed of NR1/NR2A and NR1/NR2B subunit combinations (Horak *et al.* 2004, Horak *et al.* 2006). Surprisingly, the effect of PS is robust when it is pre-applied before the agonist but much weaker if the PS is co-applied with the agonist (see Fig. 1A) (Horak *et al.* 2004). The results of electrophysiological experiments indicate that this is due to an allosteric coupling of glutamate and PS binding sites, resulting in a decrease in the affinity of the receptor to PS after agonist-induced activation.

Not only does PS potentiate NMDA (NR1/NR2A and NR1/NR2B) receptor response to the agonist but it also exhibits a weaker inhibitory effect, which is usually masked by much stronger potentiation (Horak *et al.* 2004, Horak *et al.* 2006). The inhibitory effect becomes apparent, for instance, in experiments when the solution with PS is swiftly washed away from the cultured cells. Instead of the gradual decrease of the current conducted by the ion channels, the current first rises temporarily and then it falls. The temporary rise is due to the faster unbinding of PS from the inhibitory binding site, allowing the receptors to be fully potentiated until PS also unbinds from the potentiating binding site. The ability of PS to bind simultaneously to potentiating and inhibitory binding sites was observed in the NMDA receptors of all subunit compositions (Horak *et al.* 2006). The overall effect of PS (potentiation of NR1/NR2A and NR1/NR2B, inhibition of NR1/NR2C and NR1/NR2D) is the result of different affinities of PS towards these binding sites and the subunit-dependent efficiency of potentiation/inhibition from these binding sites. Simultaneous occupation of potentiating and inhibitory binding sites was also reported in the case of neurosteroid modulation of GABA receptors (Park-Chung *et al.* 1999).

Recently, it has been reported that PS affects NMDA receptors even at nanomolar concentrations. (Johansson *et al.* 2007) studied the binding of ifenprodil (the specific inhibitor of NR2B subunit) to NR1/NR2B receptors. They found that at nanomolar concentrations, PS does not alter the response to glutamate but influences the extent of ifenprodil inhibition of the receptor. This is therefore probably another phenomenon, which is distinct from the potentiating and inhibitory phenomena of PS described above (Johansson *et al.* 2007).

1.2. *Pregnenolone sulfate action on synaptically activated NMDA receptors*

Surprisingly, although PS has a strong potentiating effect on recombinant and native (extrasynaptic) receptors activated by exogenous agonist application, it produces virtually no effect on the amplitude of excitatory postsynaptic currents mediated by NMDA receptors (NMDA receptor-mediated EPSCs) in hippocampus (Partridge and Valenzuela 2001), dentate gyrus (Chen and Sokabe 2005), spinal cord (Abdrachmanova *et al.* 2001) and cultured hippocampal neurons (Meyer *et al.* 2002). The reason for this is not clear, but possible explanations are site-specific differences in subunit composition, a developmental switch in subunit gene expression, an association with various sets of sub-membrane proteins or a regulatory change in receptor function – for example, by phosphorylation as the universal process. Some of these possibilities are discussed below.

It has been recently reported that in the hippocampal dentate gyrus of adult rats, the effect of PS on synaptic responses depends not only on the direct action of PS on NMDA receptors, but also on the activation of tyrosine kinases in the postsynaptic cell (Chen *et al.* 2007) – more specifically, on src-kinases, which, by themselves, are important activators of NMDA receptor activity (Salter and Kalia 2004). One possible mechanism for achieving this PS action could involve the activation of src-kinases in a round-about manner, through

activation of sigma-1 receptors, similar to what has been shown for the effect of dehydroepiandrosterone sulfate, another neurosteroid that activates src-kinases through the activation of sigma-1 receptor (Chen *et al.* 2006, Li *et al.* 2006).

In addition, PS action is not necessarily limited to the postsynaptic cell but could involve the presynaptic terminal as well. PS facilitates both evoked and spontaneous glutamate release in cultured neonatal hippocampal neurons. This can be concluded from the effects of PS on changes in neurotransmitter release, which is represented by paired-pulse facilitation and/or change in frequency of miniature EPSCs (Meyer *et al.* 2002). On the other hand, opposite results were obtained when the studies with PS were performed on hippocampal neurons from adult animals (Partridge and Valenzuela 2001).

These findings can be reconciled by assuming that PS acts via presynaptic NMDA receptors, but that the developmental switch in the subunit type changes the overall probability of glutamate release (Mameli *et al.* 2005). Similar age-dependency exists in the rat calyx of Held, because in younger animals (postnatal day 7-9), PS applied at a concentration of 100 μ M increases the frequency of miniature EPSCs and strongly potentiates evoked EPSCs. The latter effect becomes inconsistent, sometimes even inhibitory, on older (postnatal day 13) animals. In addition, it was found that the presynaptic effect of PS in this model is mediated by still another type of channel, namely, by activation of voltage-gated calcium channels (Hige *et al.* 2006). Importantly, it was found that the intracellular downstream signal transduction pathways appear to involve adenylyl cyclase and protein kinase A on the one hand and release of calcium from intracellular stores coupled to activation of protein kinase C on the other (Dong *et al.* 2005). This, however, does not seem to be the universal mechanism, because in the calyx of Held (Hige *et al.* 2006), the PS effect depended on neither of these protein kinases (PKA and PKC). Also, different findings come from the hippocampus, where PS action was dependent exclusively on extracellular calcium (Meyer *et al.* 2002).

Adding to the complexity is the possibility that it is not only receptors themselves that need to be phosphorylated, but that the phosphorylation of other proteins associated with them in the synapse can also influence the activity of the whole complex, as is the case for GABA_A receptors (Kannenbergs *et al.* 1997).

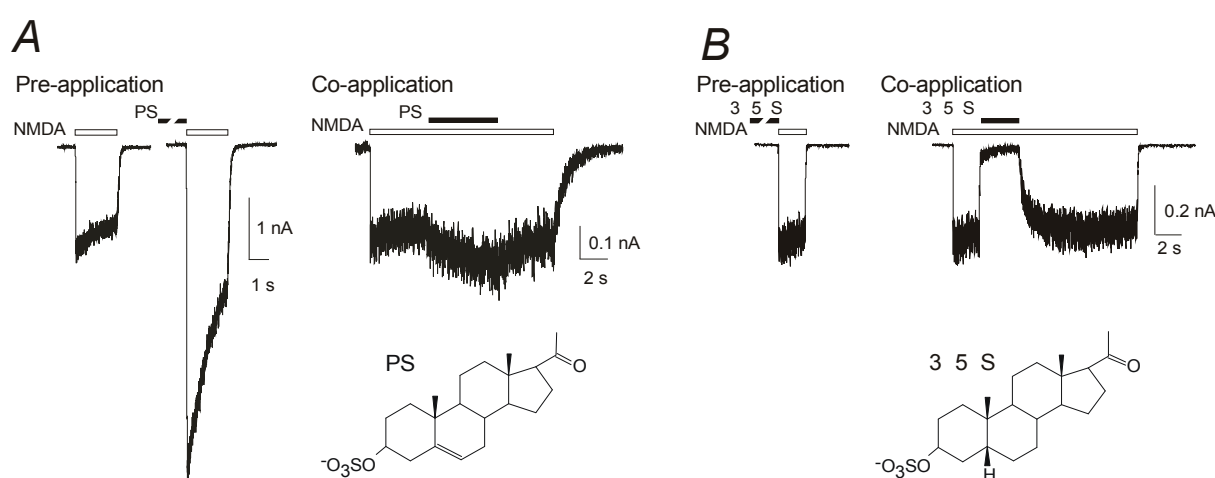


Figure 1

Disuse-dependent and use-dependent effect of neurosteroids on NMDA receptor activity. **(A)** Effects of PS on NR1-1a/NR2B NMDA receptors expressed in HEK cells. Pre-application of PS (300 μ M) produces several-fold increase in responses evoked by NMDA (100 μ M). Simultaneous application of PS (100 μ M) and the agonist produces little effect. Inset shows structure of pregnenolone sulfate (PS). **(B)** Effects of 3 α 5 β S (300 μ M) on NR1-1a/NR2B NMDA receptors expressed in HEK cells. Pre-application of 300 μ M 3 α 5 β S produces no effect on the time-course and amplitude of the subsequent response to NMDA (100 μ M). Simultaneous co-application of NMDA (100 μ M) and 3 α 5 β S produces almost complete inhibition. Inset shows structure of pregnanolone sulfate (3 α 5 β S).

1.3. *Molecular mechanism of neurosteroid action with negative allosteric effect*

Pregnanolone sulfate ($3\alpha5\beta\text{S}$) is a neurosteroid naturally occurring in the mammalian central nervous system. In contrast to PS, it has an inhibitory effect on responses mediated by NMDA receptors (Park-Chung *et al.* 1994). It differs from pregnenolone sulfate (PS) by just a single unsaturated bond (see Fig. 1B). Sulfate moiety has been shown to be essential for the neurosteroid efficacy since unsulfated derivative ($3\alpha5\beta$) has no effect on responses mediated by NMDA receptors (Park-Chung *et al.* 1994). Several lines of evidence indicate that the action of $3\alpha5\beta\text{S}$ on NMDA receptors is noncompetitive and that it acts through a site distinct from the NMDA recognition site (Park-Chung *et al.* 1994).

The binding of $3\alpha5\beta\text{S}$ to its sites at the NMDA receptors is strongly dependent on receptor activation – the neurosteroid binds and unbinds in the presence of NMDA receptor channel agonists (NMDA or glutamate) while in the absence of these agonists the neurosteroid is unable to bind to the receptor (see Fig. 1B) (Petrovic *et al.* 2005). This use-dependency is typical also for another group of compounds – the NMDA receptor channel blockers such as Mg^{2+} , ketamine, memantine and MK-801, – which, in contrast to $3\alpha5\beta\text{S}$, exhibit a voltage-dependent block of NMDA receptor channels (Park-Chung *et al.* 1994, Abdrachmanova *et al.* 2001). This difference strongly suggests that the $3\alpha5\beta\text{S}$ binding site is located outside the ion channel pore and therefore must be different from the site of ion channel blockers.

Kinetic experiments were used to assess the molecular mechanisms by which $3\alpha5\beta\text{S}$ inhibits NMDA receptors. The results indicate that the main effect of $3\alpha5\beta\text{S}$ is to reduce the probability of NMDA receptor ion channel opening. In addition, the results of single-channel analysis have shown that the relative degree of inhibition is similar to that observed on the

whole-cell NMDA receptor responses. In the presence of neurosteroid, the frequency of single NMDA receptor channel openings was reduced and the mean open time of NMDA receptor channel openings shortened. In contrast, no significant difference was observed in the distribution of NMDA receptor channel amplitudes. These results indicate that the neurosteroid mainly affects the rate constant of channel opening rather than of channel closing (Petrovic *et al.* 2005).

Single channel analysis of $3\alpha 5\beta S$ action on the NMDA receptor channel activity induced in outside-out patches isolated from spinal cord motoneurons revealed effects which were dependent on the single channel conductance. This indicates that the inhibitory action of the neurosteroid may depend on the receptor subunit composition (Abdrachmanova *et al.* 2001). The subunit preference of $3\alpha 5\beta S$ action at NMDA receptors was confirmed by using recombinant receptors, showing that this neurosteroid has lower potency on receptors containing NR2A-B subunits than on those containing NR1/NR2C-D subunits. However, it is relatively independent of the type of NR1 subunit (Malayev *et al.* 2002, Petrovic *et al.* 2005). The experiments with chimeric NR2A-NR2C receptors indicate the importance of the M3-M4 loop of the NR2 subunit for the $3\alpha 5\beta S$ effect (Petrovic *et al.* 2005).

1.4. $3\alpha 5\beta S$ action on synaptically activated NMDA receptors

The effects of $3\alpha 5\beta S$ on the NMDA receptor-mediated EPSCs were studied in motoneurons in spinal cord slices and neocortical layer II/III pyramidal neurons (Abdrachmanova *et al.* 2001, Petrovic *et al.* 2005). Surprisingly, despite the similar experimental approaches and species used, $3\alpha 5\beta S$ (100 μM) had no effect on either the amplitude or the deactivation kinetics of NMDA receptor-mediated EPSCs recorded from spinal cord motoneurons (Abdrachmanova *et al.* 2001); however, it reduced the amplitude of

NMDA receptor-mediated EPSCs recorded from neocortical layer II/III neurons (Petrovic *et al.* 2005). Several factors may account for the lower effect of $3\alpha,5\beta$ S on NMDA receptors activated during synaptic transmission and those activated by exogenous agonist application. One of them may be related to the use-dependent action of $3\alpha,5\beta$ S. In the case of tonically activated NMDA receptors, $3\alpha,5\beta$ S-induced inhibition will reach a steady-state within several seconds; however, the relatively slow rate constant of neurosteroid binding and its use-dependency indicate that during brief NMDA receptor activation the neurosteroid will not reach equilibrium. Therefore, it affects synaptic receptors that are phasically activated by glutamate less than those tonically activated by prolonged agonist activation. This mechanism was sufficient to explain the differences in the degree of $3\alpha,5\beta$ S-induced inhibition of NMDA receptors activated during synaptic transmission and those tonically activated in neocortical pyramidal neurons (Petrovic *et al.* 2005). At this stage, our knowledge of the mechanism of $3\alpha,5\beta$ S action at synaptically activated NMDA receptors is limited. It is likely that some endogenous factors control the receptor sensitivity to the inhibitory neurosteroids. This may be the cause of the relative insensitivity of synaptic NMDA receptors to $3\alpha,5\beta$ S.

2. Neurosteroid modulation of AMPA/kainate receptors

The neurosteroid modulation of AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate) and kainate receptors (two distinct subclasses of ionotropic glutamate receptors often grouped together as “non-NMDA”) has been studied less intensively than the neurosteroid modulation of NMDA receptors but some common features, as well as differences, have nonetheless been discovered. The function of all subclasses of ionotropic glutamate receptors is affected by PS and $3\alpha,5\beta$ S but not by their nonsulfated analogues (Park-Chung *et al.* 1994, Shirakawa *et al.* 2005). However, unlike their effect on NMDA

receptors, the effect of both PS and $3\alpha5\beta\text{S}$ on AMPA/kainate receptors is inhibitory: 100 μM PS inhibits the current responses of chick spinal cord neurons induced by AMPA and kainate by 29% and 25%, respectively (Wu *et al.* 1991); similarly, 100 μM $3\alpha5\beta\text{S}$ causes 29% and 37% inhibition (Park-Chung *et al.* 1994, Shirakawa *et al.* 2005) as opposed to 70% inhibition of NMDAR responses (Petrovic *et al.* 2005). These findings were confirmed by utilizing recombinant AMPA (GluR1 or GluR3) and kainate (GluR6) receptors expressed in *Xenopus* oocytes (Yaghoubi *et al.* 1998). The dose-response curves in this study further demonstrated that PS reduces the AMPA/kainate receptor-mediated maximum current response to kainate application without affecting the EC_{50} of this agonist, indicating clearly that the mechanism of this neurosteroid action is noncompetitive. The independence of binding sites for glutamate and PS (or $3\alpha5\beta\text{S}$) has also been reported in a study using intrinsic fluorescence spectroscopy to identify the neurosteroid binding site on the S1S2 domain of the GluR2 subunit (Spivak *et al.* 2004).

The C-3 sulfate group of PS and $3\alpha5\beta\text{S}$ can be replaced with a hemisuccinate group without losing the NMDA receptor-modulating abilities of the steroid (Weaver *et al.* 2000). Similarly, recombinant AMPA receptors (GluR1 and GluR3 homomers) are equally inhibited by PS and pregnenolone hemisuccinate (PHS) (Yaghoubi *et al.* 1998). On the other hand, kainate receptors (GluR6) are inhibited substantially less by PHS than by PS (15% vs. 42%) (Yaghoubi *et al.* 1998). Moreover, pregnenolone hemisuccinate was reported to have no significant effect on AMPA cytotoxicity in rat cortical slice cultures or AMPA-induced currents in cultured cortical neurons, whereas PS acted as an inhibitor in both cases (Park-Chung *et al.* 1994, Shirakawa *et al.* 2005). Therefore it seems that the presence of negatively charged group maintains the modulatory effect, however the effect is less pronounced.

Synaptic transmission mediated by AMPA receptors is also affected by sulfated steroids. The underlying mechanism of this action, however, seems to be indirect rather than

directly influencing the properties of the receptors. PS application causes an increase in the frequency of AMPA receptor-mediated mEPSCs in cultured hippocampal neurons (Meyer *et al.* 2002), as well as in acute hippocampal slices of P3-4 rats (Mameli *et al.* 2005). However, the mEPSC amplitude is unaffected by PS in both cases, supporting the hypothesis that the site of the steroid action is presynaptic (as discussed in 2.2) – namely, the presynaptic NMDA receptors, since the effect of PS in the slices (though not in the cultivated neurons) is Ca^{2+} - and NMDA receptor-dependent. Moreover, in the slices, a delayed increase in evoked AMPA EPSCs amplitude has been reported (Mameli *et al.* 2005). This action is postsynaptically localized and again Ca^{2+} - and NMDA receptor-dependent. A model has been proposed in which this PS-induced enhancement of AMPA currents is caused by insertion of AMPA receptors in the postsynaptically silent synapses (Mameli *et al.* 2005); this resembles the well-established model of long-term potentiation.

Conclusions

In this review, we present a summary of the effects of two endogenous neurosteroids – pregnenolone sulfate and pregnanolone sulfate – on NMDA receptors. We show that structural and sterical differences between these two substances account for their radically different properties, as summarized in Table 1. Importantly, their action depends also on the timing of their application (relative to the application of the agonist). Still, results from transfected cells and cultured neurons significantly differ from those registered *in vivo*, when only synaptic receptors are activated. The reasons for this are still unclear, but present efforts in our laboratory are directed at solving this issue.

Table 1

Neurosteroid	Effect	Binding	Subunit preference action	Effect on amplitude of EPSC
PS	Potentiating and inhibitory	Disuse-dependent	NR2A-B	No effect
3 α 5 β S	Inhibitory	Use-dependent	NR2C-D	Diminution

In recent years, we have witnessed a surge of interest in neurosteroids. Several lines of research emerge: defining the molecular mechanism of neurosteroid action on various ionotropic receptors, the cellular regulatory mechanisms of neurosteroid effects, an integrative approach to neurosteroid function in various systems within CNS and, most importantly, the possibility of the practical use of the above-mentioned findings in clinical setting. In vivo results are promising, but the final goal is still very distant. Combined with the research in the pathophysiology of neurodegenerative diseases (and particularly the role of NMDA receptors in many of these), neurosteroids will hopefully bring new therapeutical options in the years to come.

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