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REVIEW ARTICLE

Calcium-engaged Mechanisms of Nongenomic Action of Neurosteroids

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Abstract: ***Background*:** Neurosteroids form the unique group because of their dual mechanism of action. Classically, they bind to specific intracellular and/or nuclear receptors, and next modify genes transcription. Another mode of action is linked with the rapid effects induced at the plasma membrane level within seconds or milliseconds. The key molecules in neurotransmission are calcium ions, thereby we focus on the recent advances in understanding of complex signaling crosstalk between action of neurosteroids and calcium-engaged events.

***Methods*:** **S**hort-time effects of neurosteroids action have been reviewed for $GABA_A$ receptor complex, glycine receptor, NMDA receptor, AMPA receptor, G protein-coupled receptors and sigma-1 receptor, as well as for several membrane ion channels and plasma membrane enzymes, based on available published research.

***Results*:** The physiological relevance of neurosteroids results from the fact that they can be synthesized and accumulated in the central nervous system, independently from peripheral sources. Fast action of neurosteroids is a prerequisite for genomic effects and these early events can significantly modify intracellular downstream signaling pathways. Since they may exert either positive or negative effects on calcium homeostasis, their role in monitoring of spatio-temporal Ca^{2+} dynamics, and subsequently, Ca^{2+}-dependent physiological processes or initiation of pathological events, is evident.

***Conclusion*:** Neurosteroids and calcium appear to be the integrated elements of signaling systems in neuronal cells under physiological and pathological conditions. A better understanding of cellular and molecular mechanisms of nongenomic, calcium-engaged neurosteroids action could open new ways for therapeutic interventions aimed to restore neuronal function in many neurological and psychiatric diseases.

Keywords: Calcium, neurosteroids, nongenomic action, central nervous system, neuroprotection, neuropathology.

1. INTRODUCTION

Steroid hormones affecting brain functions are called neuroactive steroids [1]. Generally, they are classified according to the site of their synthesis. All steroid hormones synthesized *de novo* from cholesterol inside the brain cells belong to a big group named neurosteroids. These compounds are generated from identical precursor – pregnenolone – by enzymes present in various brain regions (Fig. **1**) [2, 3]. Neurosteroids can exist in the free form or may be converted into sulfate derivatives or fatty acid esters. Sulfate derivatives of two most popular neurosteroids, DHEA and pregnenolone, are even more active than their free forms. Some of typical neurosteroids (*e.g.* DHEAS, PregS, allopregnanolone, androstenedione, THDOC) are specific for various brain cells and their concentration in peripheral tissues is very low [4]. On the other hand, the steroids generated in peripheral glands can also modulate CNS functions, as they are able to cross blood-brain barrier due to their lipophilic nature. Some of them - particularly progesterone or estradiol - are present in the brain and peripheral tissues, and their level in blood can be higher or similar to the level in the brain.

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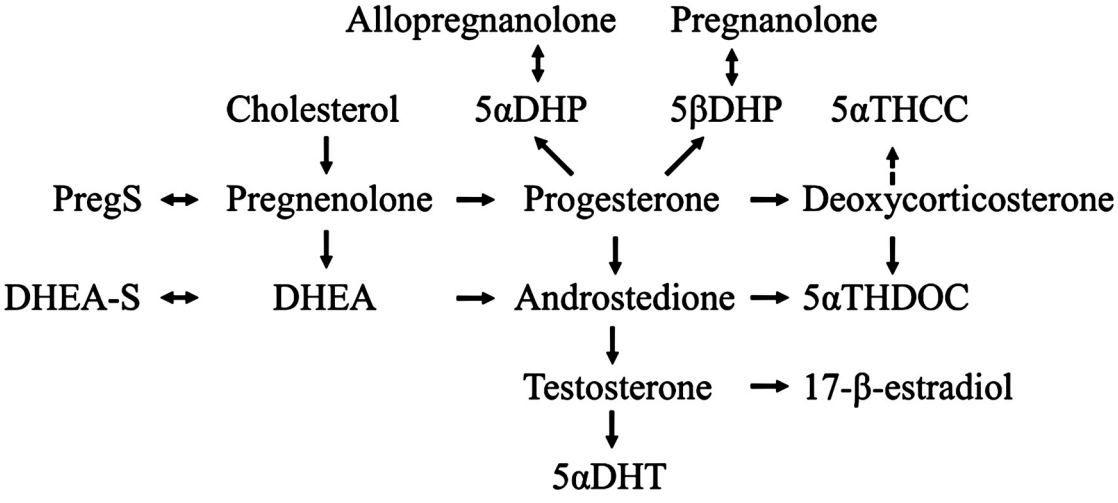

Fig. (1). Schematic illustration of the main pathways of cholesterol conversion into neurosteroids. DHP-dehydropregnenolone, DHT-dihydrotestosterone

Besides classical, genomic action of neurosteroids, their biologically important properties are their fast actions detected within seconds or minutes. In most cases, neurosteroids directly affect the membrane specific receptors, but also interact with the receptors selective for various neurotransmitters and extracellular ligands. Action through classical intracellular receptors requires a time period between minutes and hours and involves transcriptional machinery.

Most neurosteroids do not exhibit high affinity to classical intracellular receptors, thus only a non-classical action is possible. Nongenomic pathways comprise the stimulation of numerous membrane receptors, *i.e.* GABA-, glycine-, NMDA- , AMPA/kainate- , sigma- and G-protein coupled receptors, but also directly activate several type of ion channels (VGCC, TRPC) and enzymes. It should be noted that this fast, non-classical effect appears to be, at least in part, a prerequisite step for further genomic processes. The level of neurosteroids fluctuates throughout life, as well as during pregnancy, stress and aging. Both, genomic and nongenomic activity of neurosteroids may contribute to the pathophysiology of various psychiatric disorders, but may also protect from neurodegenerative insults. Therefore, they are considered as potentially useful in the treatment of diseases such as depression and anxiety.

Calcium ions have been indisputably recognized as a crucial element of complex machinery regulating normal neuronal functions. The physiological effects of changes in Ca^{2+} concentration can be detected after short time (within seconds), but also after longer time period, when the altered gene expression becomes visible. It is now evident that Ca^{2+} concentration in neurons is tightly controlled by multiple mechanisms [5]. Many of them trigger different Ca^{2+}-dependent signaling pathways which may directly or indirectly stimulate downstream effectors, thereby controlling and/or inducing changes in neuronal activities. Moreover, the disturbances in calcium homeostasis have been documented to be associated with neurodegenerative processes and several psychiatric diseases [6, 7]. In this review, we focus on the crosstalk between nongenomic action of neurosteroids and intracellular calcium events.

2. CALCIUM AND NEUROSTEROIDS

2.1. $GABA_A$ Receptors

Among different γ-aminobutyric acid receptors, the type A ($GABA_A$) is regulated by various neurosteroids. It consists of several subunits able to bind different agonists such as benzodiazepines, barbiturates, alcohols, anesthetics and some neuroactive steroids. Most neurosteroids with 3α-hydroxyl group within A-ring (mainly 3α-reduced metabolites of progesterone and deoxycorticosterone, allopregnanolone, 3α,5α-THDOC or androstenediol) are positive allosteric modulators of $GABA_AR$ enhancing GABA-evoked chloride current [8]. This very fast action allows for influx of chloride and thus generation of physiological cell response within milliseconds to seconds. Neurosteroids acting as $GABA_AR$ agonists show similar properties to GABA including anticonvulsant, sedative-hypnotic, analgesic and anxiolytic effects [9]. Based on these observations, several synthetic neurosteroids (Alfaxalone, Minoxolone, Ganaxolone) are frequently used in medicine for premedication and anesthesia.

However, it has been established that in some cells, increased chloride current led to membrane depolarization and to activation of L-type of voltage-gated calcium channels [10]. Thereby, GABA may also exhibit non-typical, excitatory effect instead of most popular inhibitory action (Fig. **2**). Such an atypical GABA role has been mainly observed in developing cortical neurons or in other immature CNS cells [11]. In primary cultures of rat cortical neurons, calcium influx induced by GABA was inhibited by PregS, and this effect was concentration-dependent with an IC_{50} of 30 μM. The authors suggested that the PregS site of action could be different from GABA binding site. Although PregS has mixed allosteric GABA-agonistic/antagonistic properties, it is rather considered as excitatory hormone with anxiogenic and proconvulsant action. The inhibition of GABA-induced calcium influx by PregS seems to confirm it [12]. Other

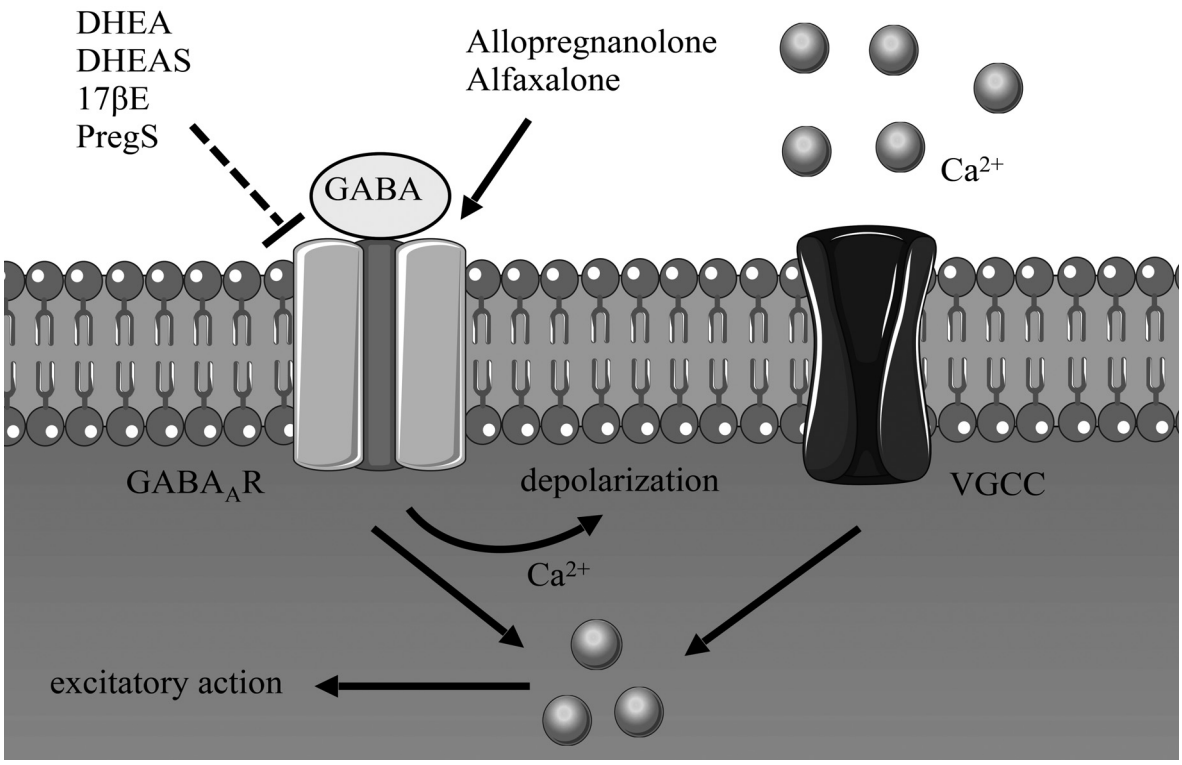

Fig. (2). The effect of neurosteroids on $GABA_A$ receptor-mediated changes in intracellular calcium concentration. $GABA_A$ receptor agonists, allopregnanolone and synthetic neurosteroid Alfaxalone stimulate GABA-evoked membrane depolarization and Ca^{2+} influx. GABA action is attenuated in the presence of DHEA, DHEAS, PregS and 17-β-estradiol. Excitatory function of GABA is characteristic for immature neuronal cells.

excitatory neurosteroids, DHEA and DHEAS, evoked similar effect in primary cultures of rat hippocampal neurons [13]. Interestingly, the inhibitory potency of DHEAS at a concentration of 30 μM was stronger than that of free form of this steroid. All neurosteroids acted *via* nongenomic way, because the effects were observed within 5 minutes.

The experimental data indicate that another typical neurosteroid, allopregnanolone, is able to induce the changes in cellular calcium homeostasis. Excitatory action of this hormone was prevented not only by inhibition of $GABA_AR$, but also by nifedipine, a selective Ca^{2+} channel blocker, suggesting that allopregnanolone can generate membrane depolarization and calcium influx after activation of $GABA_A$ receptor [14, 15]. Similarly, allopregnanolone promoted a rapid, dose-dependent and developmentally regulated increase in intracellular Ca^{2+} concentration in rat embryonic hippocampal neurons *via* a mechanism that requires both, the $GABA_A$ receptor and L-type VGCC [16]. Application of bicuculline and picrotoxin, the competitive antagonists of $GABA_AR$, completely abolished allopregnanolone-induced calcium rise. Excitatory GABA action was also observed in gonadotrope cells of anterior pituitary cells [17]. The allosteric ligand of $GABA_AR$, 5α-pregnane-3α-ol-11, 20-dion, changed cytosolic Ca^{2+} concentration through $GABA_AR$ and L-VGCC in a manner similar to allopregnanolone. This was confirmed using muscimol and nifedipine (as inhibitors of $GABA_AR$ and L-VGCC, respectively). The presence of phaclofen, another specific blocker of $GABA_B$ R, supported the involvement of this receptor in GABA- and neurosteroid-induced calcium influx.

The GABA-induced Ca^{2+} increase can be affected by 17βE, which was found to inhibit Ca^{2+} rise acting on VGCCs in a non-competitive manner [13]. Antagonist of classical estradiol receptor - tamoxifen - had no effect on 17βE modulation of GABA response, pointing out a nongenomic mechanism of action. 17βE also abolished positive modulatory effect of L-type VGCCs agonist, Bay K 8644, suggesting that the mechanism of action involved L-type VGCCs. It is worth of notice that excitatory GABA action comprising membrane depolarization and calcium signaling is characteristic for immature neurons, and excitatory vs. inhibitory GABA effect may serve as a major divergence point in estradiol-mediated sexual differentiation of the brain [18]. Stimulation of GABA-dependent calcium influx by 17βE was demonstrated in neonatal hypothalamic neurons. No changes in GABA-induced intracellular calcium were observed in the presence of α-estradiol, corticosterone and androstenedione [13].

2.2. Glycine Receptor

The glycine receptor (GlyR) is a chloride channel protein forming homo- or heteropentamers assembled from various combinations of α1, α2, α3, α4 or β subunits [19]. Activation of this ionotropic receptor by agonists leads to channel opening allowing for chloride influx and membrane hyper-

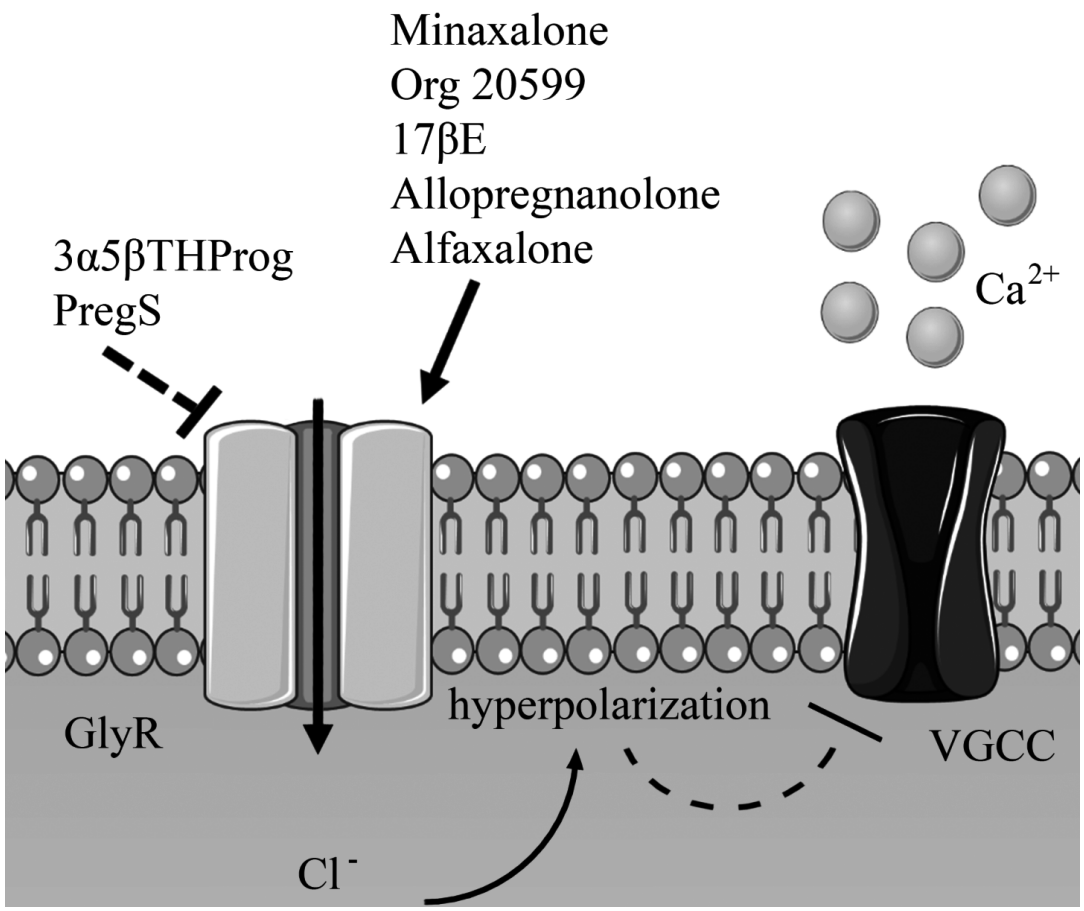

Fig. (3). The effect of neurosteroids on glycine receptor-mediated changes in intracellular calcium concentration. 3α5βTHProg and pregnenolone sulphate inhibit glycine receptor current by direct binding to the receptor. 17βE, allopregnanolone and synthetic neurosteroids activate GlyR and stimulate GlyR-induced membrane hyperpolarization what may inhibit Ca^{2+}-entry through VGCCs.

polarization. Because GlyR belongs to the same group of receptors as $GABA_A$ receptor, it can inhibited by non-competitive inhibitors such as picrotoxin or ginkgolide B, but to a lesser degree than $GABA_AR$ [20].

Amino acid glycine, along with GABA, is the primary fast inhibitory neurotransmitter in the CNS. In addition, it may exert positive modulatory action on glutamate *via* its co-agonist site on the NMDA receptors, but most of glycine effects are produced through specific ionotropic glycine receptor localized at the post synaptic membranes. GlyR is selectively blocked by the high-affinity competitive antagonist strychnine [20]. Three isoforms of GlyR can also bind agonists like taurine, anesthetics, glutamate, ethanol, divalent cations such as zinc and nickel (all having activating properties) but also compounds with mixed agonist/antagonist properties including ivermectin, endocannabinoids and tropeines [21, 22]. GlyR is thought to participate in alcohol addiction and, therefore, is a common target for drugs preventing alcohol relapse [22].

The process of glycine release into the synaptic cleft is Ca^{2+}-dependent and results in the activation of postsynaptic GlyRs, thereby increasing the chloride conductance of the postsynaptic cell [19]. On the other hand, the activation of glycine receptor can evoke changes in intracellular calcium. It has been demonstrated that potentiation of glycine-evoked current by intracellular calcium observed in spinal cord neurons and HEK cells was dependent on Ca^{2+} entry through NMDA, AMPA or VGCC channels but did not involve Ca^{2+}-dependent phosphorylation or G-protein activation [23].

GlyR has also been reported to permeate Na^+ and K^+ [20]. Although glycine is a major inhibitory neurotransmitter in the adult CNS, its excitatory action resulting in membrane depolarization was reported during embryonic development [24]. Moreover, glycine increased cytosolic Ca^{2+} level in neocortex at embryonic day 13 (E13) of C57_Bl6 mouse embryos and this effect was completely abolished in the presence of strychnine. Activation of GlyR initiated the reaction cascade involving:*(i)* stimulation of Na^+ channel, *(ii)* activation of Na^+/Ca^{2+} exchanger and *(iii)* Ca^{2+} influx with subsequent Ca^{2+}- dependent exocytosis and glutamate secretion [24]. The final output was autocrine or paracrine activation of NMDA and AMPA receptors. The other mechanism of glycine-evoked calcium influx involves membrane depolarization and subsequent activation of VGCC [25].

Most of the endogenous neurosteroids exhibit weak modulatory action on glycine receptors with the exception of 3α,5β-THPROG and pregnenolone sulphate having

significant inhibitory effect [21]. PregS appeared to be more potent toward α1 than α2 subunit-containing receptors and can be used to distinguish between these two isoforms [19]. Pregnenolone has also been shown to inhibit GlyR (Fig. **3**). It decreased the glycine-induced current in a dose-dependent manner in neurons isolated from rat spinal dorsal horn [26]. By contrast, allopregnanolone and synthetic neurosteroids, such as alphaxalone, Org20599 and minaxalone, seem to be powerful effectors of GlyRα1 and α3, and potentiate GlyR currents [21, 27]. Interaction of these neurosteroids with glycine receptor was direct and voltage-independent [26]. Stimulatory effect of 17β-estradiol on glycine-evoked current was confirmed in cultured rat hippocampal and spinal dorsal horn neurons. Using staurosporine, tamoxifen and G-protein modulators, the authors excluded the genomic mechanism of steroid action and suggested a direct action of estradiol on GlyR. However, the site of estradiol binding seems to be different from pregnenolone binding site [28].

2.3. NMDA Receptors

Exposure of neurons to the excitatory neurotransmitter glutamate causes an increase in the concentration of intracellular Ca^{2+} and activates the selected calcium-dependent signaling pathways. However, over-activation of the receptors and subsequent Ca^{2+} overload may initiate the process of excitotoxic cell death. There are four pharmacologically distinct ionotropic glutamate receptors that differ by their sensitivity to the selective agonists. Based on structural features, they have been grouped into distinct classes – NMDA receptors, AMPA receptors, kainate receptors and delta receptors [29, 30]. Except for the last one, neurosteroids have been shown to regulate the activity of these receptors and both, positive and negative modulation was observed suggesting different ways of neurosteroid action.

NMDA receptors are heteromeric complexes permeable for calcium and composed of four transmembrane subunits represented by three subtypes: NR1 (GluN1), NR2 (GluN2A, B, C and D) and NR3 (GluN3A and B). The first report describing the action of pregnenolone sulfate on NMDA receptors was published 25 years ago [31]. Since then, PregS has become one of the most widely examined neurosteroids. It is now well documented that its action strongly depends on NMDA subunit composition, as well as PregS concentration. The inhibition of NMDA receptors by neurosteroids involves binding to the extracellular ion channel entry, but the efficacy of inhibition depends on the receptor activation state

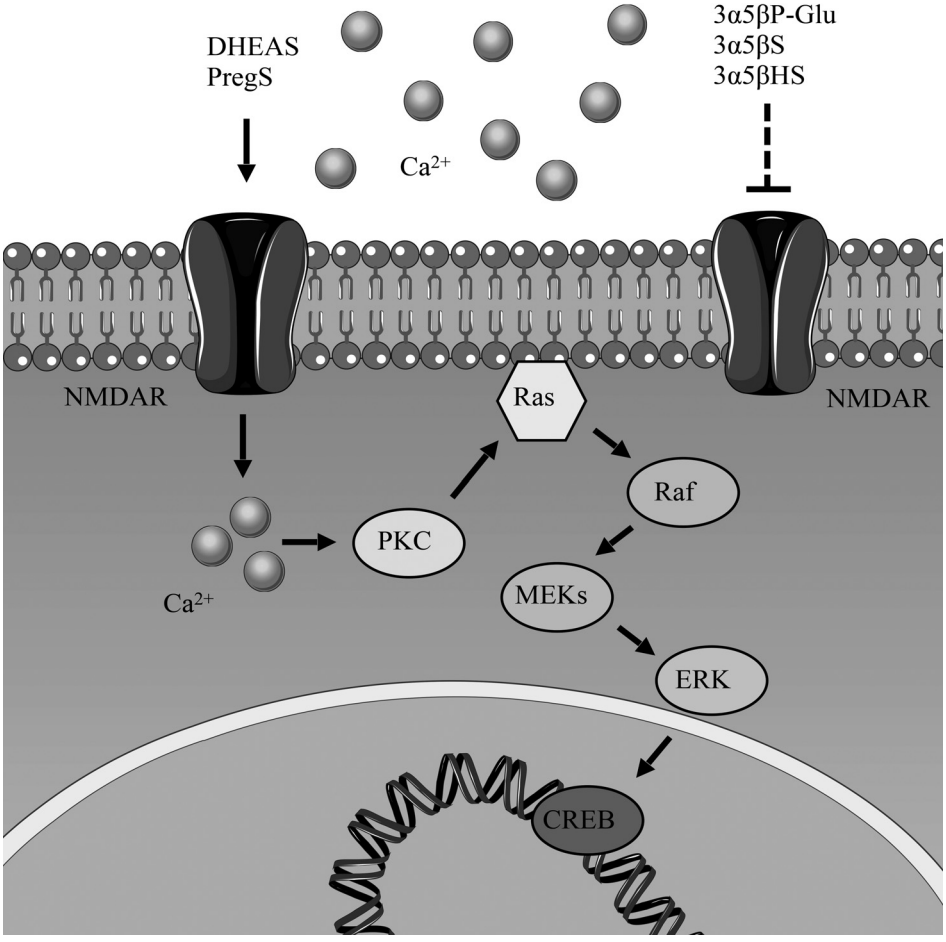

Fig. (4). Modulation of NMDA receptor by neurosteroids. Sulfate derivatives of DHEA and Preg bind to NMDA receptor promoting Ca^{2+} influx and subsequently activate intracellular signaling cascades involving PKC, Ras, Raf, MEKs, ERK and CREB pathways. 3α5β-pregnanolone glutamate, pregnanolone sulfate (3α5βS) and its hemisuccinate derivative (3α5βHS) act as direct antagonists and block NMDA receptor activity.

and a proper channel structure conformation [32]. Preg is neutral and can permeate the membrane easily, while a sulfate derivative - PregS is negatively charged and can be compartmentalized intracellularly.

Modulatory effect of PregS has been observed from picomolar to micromolar concentrations indicating the multiple mechanisms of action (Fig. **4**). Potentiation by PregS is believed to be exerted allosterically by its binding to a specific site on the NMDA receptor [33]. At micromolar range PregS significantly enhanced the activity of NMDAR containing GluN2A and GluN2B subunits, but exhibited lower efficacy on receptors composed from GluN2C and GluN2D variants [34-37]. The direct action of PregS on NMDA receptors was shown to alter channel opening time and to increase the receptor desensitization [33, 34]. Moreover, in the hippocampal dentate gyrus of adult rats, PregS activated Src tyrosine kinases which are important regulators of NMDAR [38, 39]. In hippocampal slices, PregS induced an acute increase in the NR2B tyrosine phosphorylation enhancing Ca^{2+} influx through NMDAR, followed by an activation of ERK/CREB signaling cascade, which is crucial for the LTP [40]. The potentiation of NMDA-induced currents by PregS was also modulated by the phosphorylation state of the receptors monitored by serine/threonine kinases [41]. An interesting, potentially physiological role of PregS has been reported in striatal synaptic terminals, where it modulated dopamine release at concentrations in the low pM range [42].

PregS acting as a positive allosteric modulator of NMDAR increased inward currents and cytoplasmic free Ca^{2+}, whereas another endogenous steroid 3α5βS(20-oxo-5β-pregnan-3α-yl sulfate), despite the structural similarity, acted as a negative modulator and inhibited NMDA-stimulated Ca^{2+} increase [43]. Although PregS and 3α5βS are structurally related, they do not interact competitively, arguing that their respective positive and negative modulatory effects are exerted through different sites or associated with NMDA receptors [43]. Interestingly, a synthetic analog of the 3α5βS - 3α5βHS (a hemisuccinate derivative) inhibited NMDA-induced currents *in vitro* and *in vivo*, showing a neuro-protective activity. 3α5βS was a two times more potent inhibitor of responses mediated by GluN1/GluN2C-D receptors than those mediated by GluN1/GluN2A-B [44]. Both steroids may be potentially useful as the therapeutic agents for the treatment of neurodegenerative diseases initiated by over-activation of NMDA receptors. Another synthetic NMDAR antagonist derived from naturally occurring neurosteroids - 3α5β-pregnanolone glutamate (3α5βP-Glu) that is able to cross the blood-brain barrier, has been shown to preferentially inhibit activated NMDAR and to reduce an excitotoxic damage of rat brain tissue [45-47]. Recently, a series of structural analogs of NMDAR inhibitors have been developed, *i.e*. pregnanolone hemipimelate or new class of amide-based inhibitors, which appear to be promising therapeutic drugs for neuroprotection [48-50].

Some endogenous pregnenolone metabolites synthesized in the nervous tissue *i.e*. allopregnanolone, pregnanolone, epipregnanolone and etiocholanone can be sulfated in the C3-position. These derivatives are also able to modulate local neuronal activity. NMDA-induced excitotoxicity was diminished by allopregnanolone in several models including hippocampal neurons, rat embryonic cerebral cortical neurons and human NT2 neurons [51-53]. Furthermore, P19-N differentiated neurons treated with allopregnanolone were protected from NMDA-induced apoptotic cell death, preserving cytochrome *c* in the mitochondrion and Bax in the cytoplasm [54]. Another study has evidenced that allopregnanolone and DHEA exerted their neuroprotective effects on P19-derived neurons through the PI3K/Akt signaling pathway [55]. These data highlight the neurosteroid inhibitory sites on NMDA receptors as a valuable therapeutic target against excitotoxic pathologies including acute and chronic neurodegeneration.

Dehydroepiandrosterone (DHEA) and its sulfate ester (DHEAS) are the most abundant steroid hormones and DHEA was described as the first neurosteroid produced in the brain. Both forms have been shown to participate in multiple events in the brain, including protection of hippocampal neurons from glutamate-induced neurotoxicity and ischemia [56-58]. DHEAS level in the plasma is about 100-fold higher than DHEA. It also has longer half-life. Moreover, DHEAS concentration in the brain is higher than in peripheral system [59]. DHEAS has been shown to act as a positive modulator of NMDA receptors and facilitate glutamatergic neuro-transmission through central sigma receptors [60, 61]. In mouse neocortical neuronal cultures, DHEAS potentiated intracellular Ca^{2+} fluxes mediated by NMDAR channels [62]. In opposite to PregS, DHEAS potentiated the NMDA-evoked catecholaminergic release and firing activity of CA3 hippocampal neurons [63, 64]. In rat CA1 pyramidal neurons treated with DHEAS, NMDA-induced intracellular Ca^{2+} transients were significantly potentiated [39]. Subsequent activation of ERK2, a downstream effector of Src family kinases, was required for DHEAS-facilitated LTP.

2.4. AMPA/Kainate Receptors

Few studies have been conducted to describe neurosteroid action on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors. However, some sulfated steroids, such as PregS, pregnanolone sulfate and pregnenolone hemisuccinate were shown to inhibit GluA1 and GluA3 AMPA receptors and GluK2 kainate receptors [65-67].

AMPA receptors are represented by four subunits termed GluA1–4, whereas kainate receptors (KARs) are multimeric structures and possess five subunits named GluK1-GluK5 [48, 68]. Sulfated steroid-mediated inhibition of AMPAR appears to be voltage independent and noncompetitive, reducing agonist efficacy, but not potency [69, 70]. PregS at micromolar concentrations induced an increase in AMPA receptor-mediated miniature excitatory postsynaptic current frequency in rat cerebellar slices, suggesting its participation in development of cerebellar Purkinje cells [71].

Recently, the ability of PregS to bind the AMPAR amino terminal domain has been reported thus supporting the allosteric modulation AMPAR signaling [72]. An interesting model proposed by Valenzuela *et al*. [73] suggested that PregS- induced presynaptic Ca^{2+} influx enhanced presynaptic glutamate release and promoted insertion of AMPARs to the postsynaptic membrane, thereby activating silent synapses.

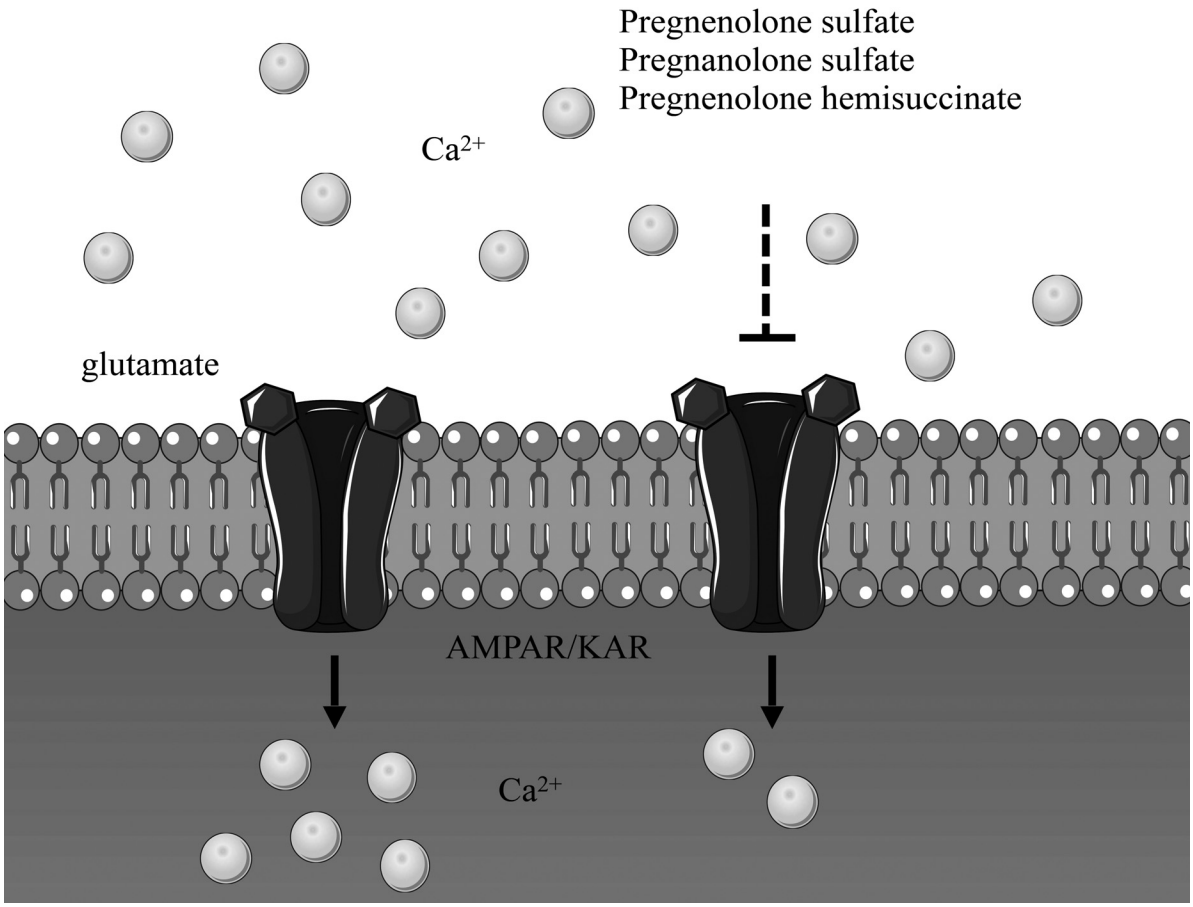

Fig. (5). Regulation of Ca^{2+} influx through AMPA /kainate receptors by neurosteroids. Pregnanolone sulfate, pregnenolone sulfate and its hemisuccinate derivative reduce glutamate-induced Ca^{2+} influx.

KARs are distributed throughout the brain, but unlike AMPA and NMDA receptors, they act principally as modulators of synaptic transmission and neuronal excitability (Fig. **5**). Glutamate release activates presynaptic KARs, thus potentiating calcium signal which, in part, also originates from stimulation of Ca^{2+} release from internal stores [74-76]. The dose-response study demonstrated that PregS reduced the AMPA/kainate receptor-mediated maximum current response to kainate application without affecting the EC_{50}, indicating clearly that the mechanism of action was noncompetitive [65]. In cultured neurons, PregS and 3α5βS were shown to inhibit AMPA/kainate receptors decreasing the current response induced by AMPA and kainate [31]. Similarly, recombinant AMPA receptors were equally inhibited by PregS and pregnenolone hemisuccinate, but kainate receptors were less sensitive to 3α5βHS than to PregS. Moreover, pregnenolone hemisuccinate was reported to have no significant effect on AMPA-induced currents in cultured cortical neurons whereas PregS acted as an inhibitor [66]. Synaptic transmission mediated by AMPA receptors is also affected by sulfated steroids. However, the underlying mechanism of this action seems to involve indirect modulation of receptor activity. PregS application increased the frequency of AMPAR-mediated mEPSCs in cultured hippocampal neurons, as well as in acute hippocampal slices of P3-4 rats [77, 78].

2.5. Sigma-1 Receptor

The intriguing functional proteins, which mediate signal transmission between neurosteroids and calcium, are sigma receptors (sigma-1, σ1R and sigma-2, σ2R). They are expressed in many different tissues including central nervous system, especially in neurons, microglia and astrocytes [79]. These receptors belong to a class of protein that can modulate various Ca^{2+}-dependent physiological processes, as well as some pathological mechanisms related to neuro-degeneration or development of some drug addiction [80]. For that reason they are a potential target for pharmacological interventions. However, it should be noted that σ1R ligands may be involved in neuroprotection, not only by regulation of intracellular calcium homeostasis, but owing to reduction of ROS accumulation [81, 82].

At a molecular level, sigma receptors are associated with inositol triphosphate receptor and can form complexes distributed mainly in endoplasmic reticulum membrane, as well as in plasma, nuclear and mitochondrial membranes [83]. Interaction of σ1R sites with neurosteroids is now considered as ligand-regulated molecular chaperones, modulating intracellular calcium signaling (Fig. **6**). Formation of σ1R/agonists complex caused translocation of the receptor protein from endoplasmic reticulum to the cell membrane, where the receptor can regulate the activity of voltage- and

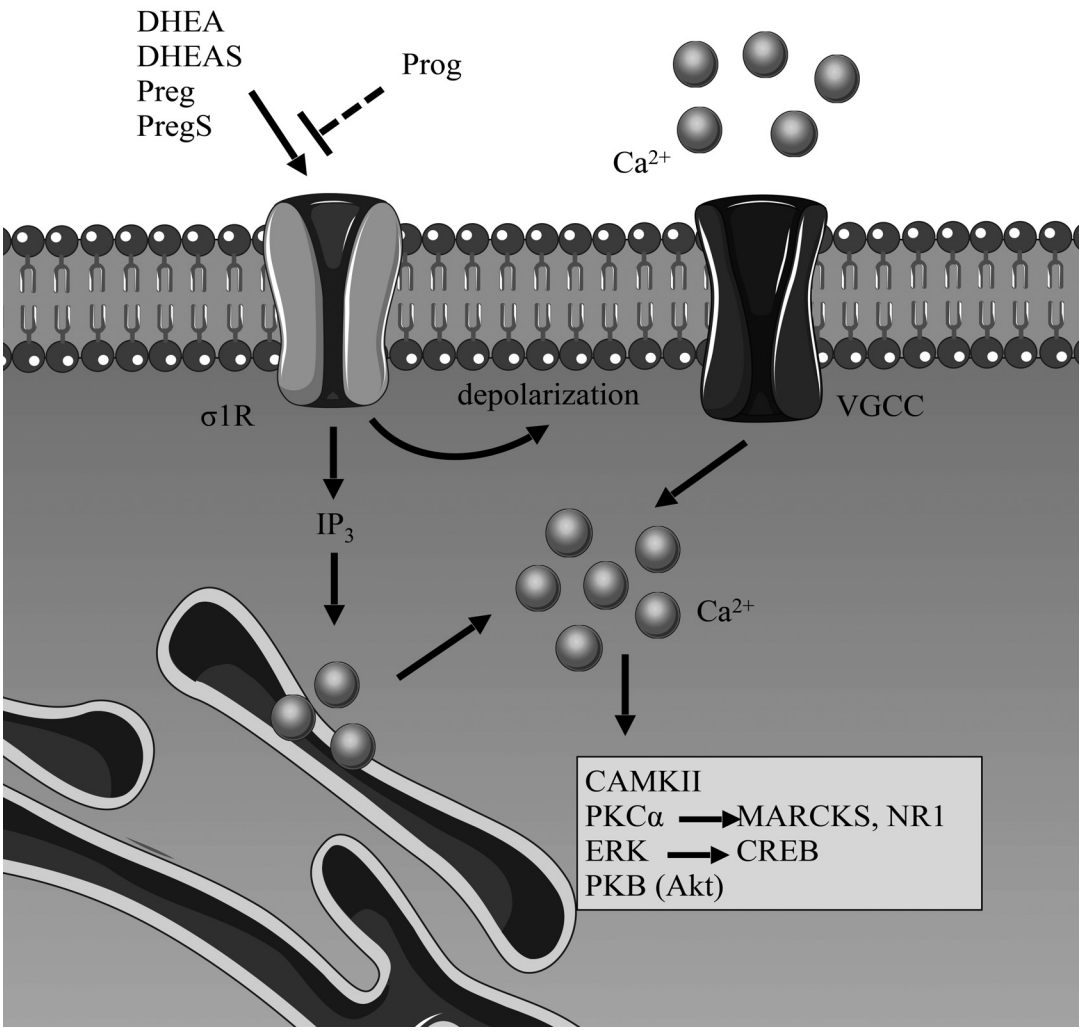

Fig. (6). Neurosteroids-mediated regulation of sigma-1-receptor. DHEA, Preg and their sulfate derivatives act as direct positive modulators of sigma-1 receptor. Activation of this receptor initiates Ca^{2+} influx through VGCC or Ca^{2+} release from endoplasmic reticulum *via* IP_3 receptors. Increased Ca^{2+} can stimulate intracellular signaling pathways involving PKC, CaMKII, ERK and Akt. Progesterone does not act alone but it abolishes the effect of other neurosteroids.

ligand-gated ion channels [84, 85]. Under physiological conditions, DHEA and Preg acted as positive modulators of this receptor, whereas progesterone was a potent antagonist [61]. Activation of σ1R and increased Ca^{2+} level may trigger downstream signaling pathways mediated by calcium-dependent proteins, including a group of protein kinases.

Stimulation of the sigma-1 receptor by DHEA has been shown to improve cognitive function by activating CaMKII, PKC and ERK in olfactory bulbectomized mouse hippocampus [86]. Further study has revealed that DHEA acting by σ1R was able to improve LTP in mice subgranular zone of the hippocampal dentate gyrus [87]. DHEA increased the phosphorylation level of CaMKII, PKCα and ERK in hippocampal CA1 region, as well as phosphorylation of their substrates *e.g*. MARCKS and NR1 by PKCα, and CREB protein by ERK. Because PKC is essential for LTP induction and phosphorylation of NR1 by PKC is necessary for regulation of NMDAR function, σ1R activation by DHEA and stimulation of PKC may improve memory-related behaviors. In ischemic mice, DHEA-stimulated σ1R prevented neuronal cell death by activation of CaMKII, thereby recovering cognitive deficits, and this effect was blocked in the presence of σ1R antagonist, NE-100 [88].

The participation of sigma receptor ligands and some neurosteroids in bradykinin-induced intracellular Ca^{2+} changes was investigated using SH-SY5Y neuroblastoma cells [89]. Bradykinin alone caused the transient rises in Ca^{2+} level, and the presence of Pregor DHEA enhanced this effect after 10 min of incubation. Co-incubation with haloperidol, a blocker of σ1R, eliminated the effect of Preg. Interestingly, whereas Prog alone did not alter bradykinin-induced Ca^{2+} increase, its co-application with Preg or DHEA abolished enhancing effects of both steroids.

An interesting function of PregS has been assessed in adult mice hippocampal dentate gyrus, where the steroid was shown to enhance the survival of newborn neurons through the potentiation of synaptic input activity [90]. PregS-induced presynaptic potentiation required a co-activation of α7nAChR (α7 nicotinic acetylcholine receptor), NMDAR and σ1R, which were critical to keep the enhanced neurons

survival in NMDAR-dependent way. Cooperation of σ1R with NMDA receptor initiated by neurosteroids appears to be more universal process, since intrathecally administered DHEAS has been found to significantly potentiate NMDA-induced spontaneous pain behaviours in mice [91]. This effect was mediated by the activation of spinal sigma-1 receptors with subsequent PKC and PKA-dependent phosphorylation of the NMDA receptor subunit NR1.

2.6. G Protein-Coupled Receptors

G protein-coupled receptors family comprises of receptors regulating many cellular processes including neurotransmitter release and the function of ion channels [92]. Various extracellular factors can reduce intracellular Ca^{2+} concentration through G protein-dependent mechanism and associated calcium channels [92, 93]. The possible mechanisms of GPCR-mediated action are summarized in Fig. (**7**). It has been demonstrated that Preg, PregS, and THCC inhibited calcium channels current in pyramidal neurons obtained from adult guinea pig hippocampal CA1 region. The participation of G-protein coupled receptor was confirmed by using pertussis toxin (PTX) and GDP-β-S, the inhibitors of G-protein mediated signaling. In neurons from PTX-treated animals or in cells injected with GDP-β-S, the effect of neurosteroids was abolished. Additionally, authors verified the involvement of N-Type Ca^{2+} channels in steroid action by applying specific VGCC blockers. These studies also showed that mechanism of steroid action was associated with activation of PKC [94].

The membrane-located G-protein-coupled estrogen receptor (GPER) has been identified recently [95]. GPER (formerly GPR30) is expressed in various tissues including brain and blood vessels. Extracellular (but not intracellular) 17-βE administration increased cytosolic Ca^{2+} and simultaneous treatment with tamoxifen, selective blocker of classical estrogen receptors, did not change steroid-evoked calcium rise in brain microvascular endothelial cells [96]. Similarly, rise in Ca^{2+} was observed when G-1, a GPER agonist, was used and this effect was reduced by PKA antagonist H-89. The effect of hormone action was also abolished in the presence of L-type VGCC blocker suggesting GPER-dependent activation of PKA and subsequent opening of L-VGCC.

In cortical neurons, PregS action was linked with PLC activation. This involved the generation of diacylglycerol and IP_3 leading to Ca^{2+} release from intracellular stores, independently from NMDA receptor activation. PregS-induced potentiation was diminished in the presence of PTX confirming the involvement of G-protein in PregS action [97].

Another important mechanism of steroid action involves metabotropic glutamate receptors (mGluR), which belong to the GPCRs, and are coupled to the variety of second messenger systems. A family of mGlu receptors consists of 8 members organized in three subfamilies [98]. The group I (mGluR1 and mGluR5) activates PLC by G_q protein resulting in generation of DAG and IP_3. Group II (mGluR2 and

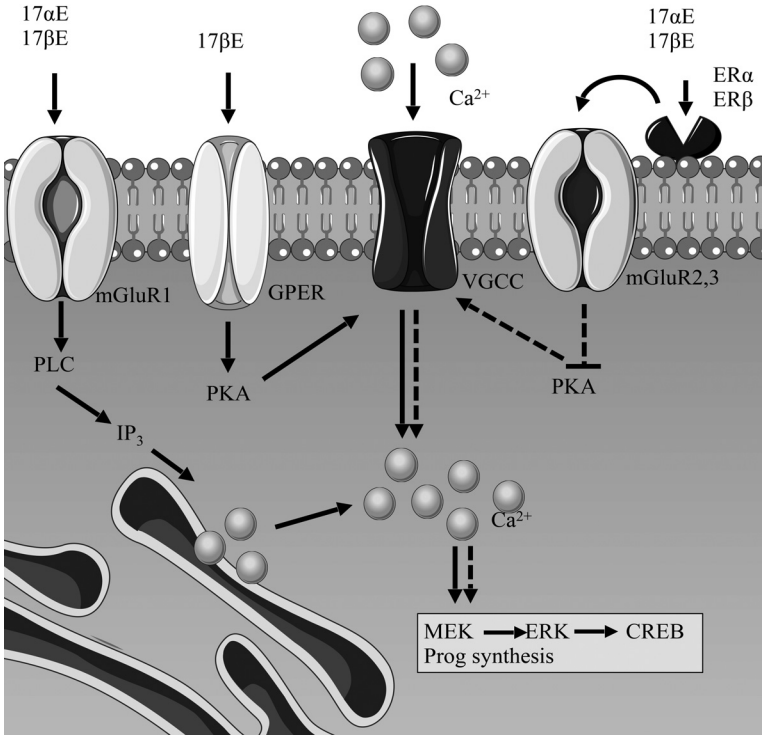

Fig. (7). 17-β-estradiol effect on G-protein coupled receptors. 17-βE regulates intracellular Ca^{2+} by its release from endoplasmic reticulum (precluded by activation of mGluR1, PLC and IP_3 receptor) and/or by modulation of VGCC-dependent influx associated with G-protein-coupled estrogen receptor, metabotropic glutamate receptor type 2,3 and membrane estrogen receptors ERα or ERβ. Increased Ca^{2+} activates the kinase cascade pathways and CREB phosphorylation or stimulates progesterone synthesis.

mGluR3) and group III (mGluR4, mGluR6, mGluR7, mGluR8) are linked to Gi/Go [99]. mGluR are localized in many tissues and organs including central nervous system and can be detected in both presynaptic and postsynaptic cells [100].

A number of studies have shown a modulatory effect of estradiol and their membrane-localized receptors - ERα and ERβ - on mGluR functioning. For example, estradiol acting through mGluR1 initiated signaling pathways *via* PKC, IP3 and MEK and finally led to CREB phosphorylation [98]. On the other hand, activation of mGluR2 and/or mGluR3 through ERα or ERβ diminished cAMP concentration and reduced PKA activity in hippocampal pyramidal neurons. Activation of these receptors resulted in dephosphorylation of L-type VGCC and reduction of VGCC-mediated CREB phosphorylation [101]. CREB phosphorylation level could be also regulated by membrane ERα linked with mGluR5 with subsequent activation of MAP kinase [102]. In the same study both estrogen receptors activated mGluR3 to attenuate L-type VGCC-dependent CREB signaling. The mGluR5 also participated in the regulation of synaptic transmission by DHEAS in hippocampal dentate gyrus [103]. Short-term potentiation evoked by DHEAS was abolished by mGluR antagonist (MPEP) or by inhibitors of ryanodine receptors (ryanodine and ruthenium red) suggesting the contribution of mGluR5-RyR cascade to postsynaptic neuron functioning.

Among diverse processes modified by estradiol in the brain, Ca^{2+}-regulated synthesis of progesterone in astrocytes appears to be particularly interesting. Mechanism of estradiol action involves activation of PKC followed by generation of IP_3 and finally, Ca^{2+} release from intracellular stores. Progesterone synthesis was stimulated not only by 17-β-E, but also by 17α form [104]. This process required mGluR1, because the presence of selective mGluR1 blocker prevented Ca^{2+} increase. Moreover, binding of estradiol to its receptor promoted transactivation of mGluR, initiating signaling without the requirement of glutamate [105]. In dorsal root ganglia, estradiol inhibited ATP-mediated calcium influx that engaged both, ERα and mGluR [101]. Opening of ATP-dependent P2X receptor led to membrane depolarization and calcium influx *via* VGCC. Inhibition of P2X receptor required the interaction of membrane ERα with mGluR2/3, as was demonstrated by a lack of response in ERα knockout mice.

A close cooperation of mGlu receptors and neurosteroids appears to be a widespread mechanism regulating neuronal activity but the presence of a different set of mGluRs as well as diversity of structurally distinct neurosteroids make their action more complex.

2.7. Voltage-Gated Calcium Channels

Regulation by neurosteroids has also been reported for members of voltage-gated calcium channels family (VGCC). The first work on modulation of VGCC by neurosteroids was conducted in the early 1990s. Several reports demonstrated that among four main types of VGCC known as L-, N-, P/Q- and R, the activity of L-type seems to be highly sensitive to neurosteroid action.

The effects of Preg, PregS and Prog on VGCC were examined in acutely isolated adult guinea-pig hippocampal CA1 neurons using the whole-cell patch clamp technique [106]. Whereas progesterone had no effect on Ca^{2+} current, pregnenolone and its sulfate derivative applied at low micromolar concentrations slowed down calcium current, also in the presence of 10 μM picrotoxin, the inhibitor of $GABA_A$ receptor. The specific, rapid action of PregS on VGCC has been confirmed in a clonal pituitary cell line – GH3 [107]. However, the increase in Ca^{2+} influx was observed at 30 μM PregS and this effect was markedly blocked by nicardipine and methoxyverapamil suggesting the principal contribution of L-type VGCC. Using optical recording technique in rat hippocampal slices stained with voltage-sensitive dyes it was shown that application of PregS to the bath solution resulted in an acute augmentation of excitatory postsynaptic potential (EPSP) in a dose-dependent manner [108]. The presynaptic effect of PregS was partially attenuated by nifedipine, an inhibitor of L-type VGCC. Moreover, this neurosteroid sensitized presynaptic α7nACh receptors and led to activation of L-type VGCC to increase the presynaptic glutamate release. Facilitation of glutamatergic transmission by PregS has also been reported in the study on a giant axosomatic synapse in the auditory brainstem slices (calyx of Held) [109]. The neurotransmission was blocked by PregS scavenger - 2-hydroxypropyl-β-cyclodextrin applied extracellularly. This led to the conclusion that PregS may directly modulate VGCCs acting on their extracellular domain, thus enhancing neurotransmitter release.

Contrary, another Preg derivative – allopregnanolone, was reported to reduce PKA activation (a necessary upstream event) and presynaptic glutamate release in rat medial prefrontal cortex [110]. This was verified by using L-type calcium channel antagonists, verapamil and nimodipine, which blocked the effect of allopregnanolone. Interestingly, no similar effect was observed for unstimulated presynaptic terminals, however after stimulation, allopregnanolone inhibited the glutamate release. This could explain the possible antipsychotic effect of allopregnanolone.

Some interesting data related to neurosteroid action on VGCC have been obtained using rat hippocampal slices. Accordingly, PregS selectively facilitated the induction of slow-developing LTP in response to high-frequency afferent stimulation (100 Hz), which was dependent on functional L-type VGCC and sigma-receptors [111]. In addition, PregS promoted further increase in presynaptic function downstream to LTP induction. Further experiments revealed more sophisticated modulation of LTP by showing that the transient elevation required a sustained activation of ERK2 in a L-type VGCC dependent manner [112].

VGCCs appear to be a sensitive target not only for PregS, but also for a rapid, nongenomic action which has been reported in the presence of estrogens (Fig. **8**). Surprisingly, these hormones have been shown to exert the opposite effects. Using whole-cell patch-clamp technique, a dose-dependent reduction of electrical activity in rat dorsal root ganglion neurons (DRG) has been documented after application of 17-βE. Similar level of inhibition was produced by bovine

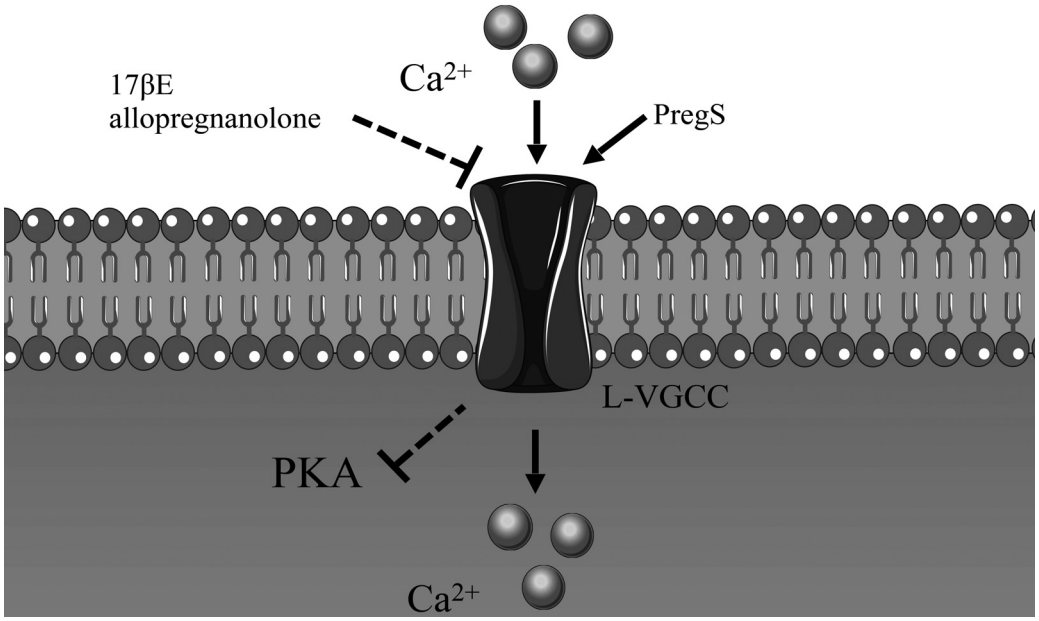

Fig. (8). Regulation of L-type VGCC by neurosteroids. PregS by direct interaction with extracellular domain of L-type VGCC can increase Ca^{2+} influx in a concentration-dependent manner, but 17-β-estradiol exerts inhibitory effect. Allopregnanolone modifies L-type VGCC function acting on PKA-mediated signaling pathway.

serum albumin-conjugated estradiol, which does not diffuse through the plasma membrane [113]. The control experiments with selective blockers revealed that 17-βE acted mainly on L- and N-type VGCC-generated Ca^{2+} currents. Estrogen may also act on primary afferent neurons, whose cell bodies are located within the dorsal root ganglia. The changes in intracellular Ca^{2+} due to the activation of purinergic 2X receptors and VGCCs, were blocked after short-time (5 min) incubation with 100 nM 17-βE [114]. Co-administration of the blockers for VGCCs – nifedipine for L-, omega-conotoxin for N- and omega-agatoxin IVA for P-type channels, attenuated the ATP-induced Ca^{2+} influx but also revealed that estradiol primarily blocked L-type VGCC. More details related to the mechanisms of 17-βE action became available after analysis of its neurotrophic and neuroprotective responses in hippocampal and cortical neurons. 17-βE triggered rapid Ca^{2+} influx in hippocampal neurons with subsequent activation of Src/ERK and CREB cascades, followed by an up-regulation of Bcl-2 expression [115] which was, in turn, blocked by 10 μM nifedipine. Neuroprotective effect of 17-βE has also been demonstrated in primary cultures of Sprague-Dawley rat retinal cells after hydrogen peroxide-induced apoptosis [116]. The transient Ca^{2+} increase induced by 10 μM 17-βE treatment for 0.5 h was mediated by the PI3K and gated by the L-type VGCC.

The neuroprotection, neuronal development, modulation of synaptic plasticity and memory formation may be attributed to estrogen action on L-type VGCC. The results of electrophysiological studies done after administration of extremely low estrogen concentrations showed a rapid Ca^{2+} increase in hippocampal neurons and slices, as well as in HEK-293 cells transfected with neuronal L-type VGCC [117]. Estrogen was shown to directly interact with VGCC and the downstream effects were independent from estrogen receptors.

The action of estrogens appears to be more complex, as their effects can be also mediated by membrane-located estrogen receptors what has been recently reviewed by Vega *et al*. [118].

2.8. Transient Receptor Potential Channels

Another group of membrane targets for neurosteroids includes a family of transient receptor potential channels (TRPC) mediating the influx of cations across cell membranes and thus playing an important role in cellular signaling. TRP channels share a common structure with six transmembrane domains and a re-entrant P-loop, which forms the pore of the channel [119].

The TRP channels superfamily has approximately 30 members and consists of seven subfamilies which are named TRPA (ankyrin), TRPCn (canonical), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin), and TRPV (vanilloid) and TRPN (no mechanopotential), based on the sequence homology [120, 121]. In the brain, TRP expression has been detected in neurons, astrocytes, oligodendrocytes, microglia and ependymal cells as well as in the cerebral vascular endothelium and smooth muscles [122]. TRP channels regulate intracellular Ca^{2+} concentration acting as calcium-permeable channels in the plasma membrane or *via* modulation of driving force for Ca^{2+} entry [123].

Several studies showed the modulation of TRP channels by neurosteroids. Most of them focused, however, on PregS, and only a few TRP channels have been characterized in details [124]. For example, in acutely isolated hippocampal dentate gyrus hilar neurons PregS significantly increased spontaneous excitatory postsynaptic current (sEPSC) frequency in a concentration-dependent manner, without affecting the current amplitude [125]. Presynaptic action of PregS increased the probability of spontaneous glutamate release but this

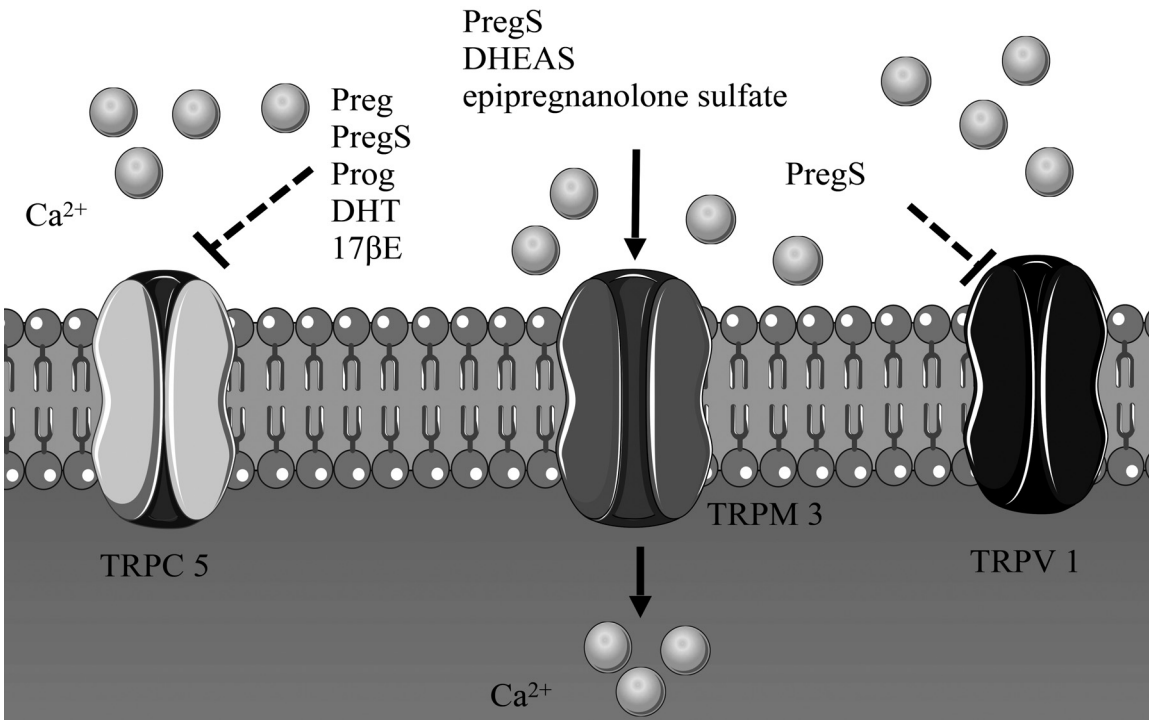

Fig. (9). Modulation of transient receptor potential channels by neurosteroids. The TRPM type 3 is directly affected by sulfate derivatives of pregnenolone, DHEA and epipregnanolone causing intracellular Ca^{2+} increase. Calcium influx through other transient receptor potential channels like TRPC 5 or TRPV1 can be inhibited in the presence of Preg, PregS, Prog, DHT and 17-βE.

process was completely blocked by TRP channel blockers. It appears that PregS increased glutamate release *via* presynaptic "calcium induce – calcium release" mechanism, which was triggered by the influx of Ca^{2+} through presynaptic TRP channels, most likely the subtype C (Fig. **9**).

In acutely isolated rat medullary dorsal horn neurons PregS significantly intensified the frequency of glycinergic spontaneous miniature inhibitory postsynaptic currents (mIPSCs) in a concentration-dependent manner, and this effect was completely attenuated in the presence of general TRP channel blockers - SKF96365 (specific for TRPC3, TRPC5, TRPC6, and TRPC7), ruthenium red and La^{3+} [126]. In that case, PregS acted probably on the presynaptic TRP channels triggering Ca^{2+} influx and increasing calcium in glycinergic nerve terminals. Further experiments on TRPC5-expressing HEK 293 cells showed that the TRPC5 channel currents were strongly suppressed by Preg, PregS, Prog, dihydrotestosterone and 17-βE, although to a different extent [127]. These results strongly suggest that neurosteroids could serve as direct and reversible negative modulators of TRPC5 channel activity at the membrane.

The effects of neurosteroids on melastatin type 3 receptor have been intensively studied not only in neuronal cells. TRPM3 channel that is expressed in the developing rat cerebellar cortex and at glutamatergic synapses in neonatal Purkinje cells, has been stimulated with micromolar concentrations of PregS, DHEAS and epipregnanolone sulfate [128-130]. It has also been established that TRPM3 is activated when PregS binds to a specific, chirally selective binding site on a protein, but cannot be activated by a non-specific membrane effect [131, 132].

The activity of TRPV1 receptor, which is also known as capsaicin receptor and is expressed predominantly by sensory neurons, has been reported to be modulated by neurosteroids as well. In rat dorsal root ganglion neurons, PregS at concentration of 50 μM rapidly and reversibly inhibited the spontaneous excitatory postsynaptic currents induced by 100 nM capsaicin in a non-competitive, but concentration-dependent manner [133].

2.9. Plasma Membrane Calcium Pump

The concentration of calcium ions must be precisely controlled to keep an appropriate gradient across the membrane. Inside the cell, free Ca^{2+} concentration is below 100 nM, whereas it is 10^4 times higher outside [134]. Therefore, a little increase in cytoplasmic Ca^{2+} can exert a huge effect on neuronal activity, even despite spatial restriction of calcium signal to functional microdomains. There are several mechanisms that operate to increase Ca^{2+}, as well as several other responsible for Ca^{2+} removal to extracellular milieu or its sequestration to intracellular stores. Plasma membrane Ca^{2+}-ATPase (PMCA) is an enzyme with the highest affinity for calcium and plays a prominent role in restoration of basal intracellular Ca^{2+} [135]. PMCA not only keeps calcium transients and local Ca^{2+} concentration under tight control, but also acts as the first line of defense from calcium overload. PMCA exists in four isoforms. PMCA1 and PMCA4 are ubiquitous, while PMCA2 and PMCA3 are specific for excitable cells [136].

It is well documented that neurosteroids are capable to modify neuronal function *via* the mechanisms using a nongenomic way. The nerve cells appear to be under

permanent influence of steroids, including regulation of calcium homeostasis. Our first observation regarding the effect of estradiol and pregnenolone sulfate on synaptosomal PMCA activity was reported more than 20 years ago [137]. In the next few years, we demonstrated that the action of neurosteroids on PMCA seems to be more complex and may involve both genomic and nongenomic effect. Thus, we subsequently evaluated whether PMCA purified from synaptosomal membranes of rat cortex could be directly regulated by neurosteroids - PregS, DHEAS, testosterone, and 17-βE at concentrations ranging from nM to μM [138]. Our results have shown a dose-dependent activation of the enzyme at physiologically relevant concentrations of examined hormones. However, higher steroid concentrations produced differential mode of regulation. Testosterone increased Ca^{2+}-ATPase activity in a gradual manner, 17-β-estradiol provided no further enhancement, but PregS and DHEAS decreased the activity of the enzyme. These data suggest structure-specific and concentration-dependent effect of different hormones on PMCA activity. Further experiments additionally confirmed that neurosteroids can also modulate ATP-powered Ca^{2+} transport in neuronal membranes, erythrocyte ghosts and PC12 cell membranes, but their efficiency was dependent on PMCA isoform composition [139, 140]. Thus, neurosteroids could be potentially useful as therapeutic agents when elongated calcium signal is needed to initiate proper neurotransmission or when Ca^{2+} signal should be terminated. Moreover, this indicates the promising role of neurosteroids in restoration of Ca^{2+} homeostasis under some pathological conditions.

CONCLUSION

The available data largely support the view that neurosteroids and calcium are integrated elements of signaling systems in neuronal cells. Although neurosteroids may exert either positive or negative effects on calcium homeostasis, their role in the regulation of spatiotemporal Ca^{2+} dynamics, and subsequently, Ca^{2+}-dependent physiological or pathological processes, is evident. A better understanding of cellular and molecular mechanisms of nongenomic, calcium-engaged neurosteroids action could open new ways for therapeutic interventions aimed to restore neuronal function in many neurological and psychiatric diseases.

LIST OF ABBREVIATIONS

Akt	=	protein kinase B
AMPA	=	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CaMK II	=	Ca^{2+}/calmodulin-dependent protein kinase II
CREB	=	cyclic-AMP response element binding protein
DHEA	=	dehydroepiandrosterone
DHEAS	=	dehydroepiandrosterone sulfate
DHT	=	dihydrotestosterone
ERK 1/2	=	extracellular signal-regulated kinase 1/2
17βE	=	17-β-estradiol
GABA	=	γ-aminobutyric acid
$GABA_A R$	=	$GABA_A$ receptor
GlyR	=	glycine receptor
GPCR	=	G-protein coupled receptor
IP_3	=	inositol triphosphate
KAR	=	kainate receptor
LTP	=	long term potentiation
NMDA	=	N-methyl-D-aspartate
PI3K	=	phosphatidylinositol-3-kinase
PLC	=	phospholipase C
Preg	=	pregnenolone
PregS	=	pregnenolone sulfate
Prog	=	progesterone
PKA	=	protein kinase A
PKC	=	protein kinase C
PMCA	=	plasma membrane Ca^{2+}-ATPase
3α5βS	=	pregnanolone sulphate
3α5βHS	=	3α5βS hemisuccinate derivative
σ1R	=	sigma-1 receptor
σ2R	=	sigma-2 receptor
THCC	=	allotetrahydrocorticosterone
THDOC	=	tetrahydrodeoxycorticosterone
TRPC	=	transient receptor potential channels
VGCC	=	voltage-gated calcium channels

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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