Neurosteroids as Selective Inhibitors of Glycine Receptor Activity: Structure-Activity Relationship Study on Endogenous Androstanes and Androstenes

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The ability of androstane and androstene neurosteroids with modifications at C-17, C-5, and C-3 (compounds 1-9) to influence the functional activity of inhibitory glycine and γ-aminobutyric acid (GABA) receptors was estimated. The glycine- and GABA-induced chloride current (IGly and IGABA) were measured in isolated pyramidal neurons of the rat hippocampus and isolated rat cerebellar Purkinje cells, correspondingly, using the patch-clamp technique. Our results demonstrate that all the nine neurosteroids display similar biological activity, namely, they strongly inhibited IGly and weakly inhibited IGABA. The threshold concentration of neurosteroids inducing effects on IGly was 0.1 µM, and for effects on IGABA was 10–50 µM. Moreover, our compounds accelerated desensitization of the IGly with the IC50 values varying from 0.12 to 0.49 µM and decreased the peak amplitude with IC50 values varying from 16 to 22 µM. Interestingly, our study revealed that only compounds 4 (epiandrosterone) and 8 (dehydroepiandrosterone) were able to cause a significant change in IGABA in 10 µM concentration. Moreover, compounds 3 (testosterone), 5 (epitestosterone), 6 (dihydroandrostenedione), and 9 (etiocholanedione) did not modulate IGABA up to the concentration of 50 µM. Thus, we conclude that compounds 3, 5, 6, and 9 may be identified as selective modulators of IGly. Our results offer new avenues of investigation in the field of drug-like selective modulators of IGly.

Keywords: neurosteroid, GABA receptor, glycine receptor, androstane, androstenone, structure-activity relationship

INTRODUCTION

γ-Aminobutyric acid receptors type A and glycine receptor (GABA_A and GlyR) channels are the major inhibitory ligand-gated ion channels of the central nervous system which mediate both fast synaptic and tonic extrasynaptic inhibition (Lynch, 2009; Ziegler et al., 2009; Yevenes and Zeilhofer, 2011). Disturbance of functional activity of GlyRs and GABA_ARs underlies many neurological disorders. Dysfunction of GABA_ARs leads to channelopathies associated with epilepsy, insomnia, anxiety, and chronic pain (Möhler, 2006). Malfunctions of GlyR have been linked to a range of neurological disorders caused by mutations in genes which encode GlyR subunits, including hyperekplexia (mutations in the GlyR α1-subunit gene) (Lynch, 2004) or autism (mutations in the human GlyR α2-subunit gene) (Dougherty et al., 2013; Zhang et al., 2017). Finally, the α3 GlyRs
have emerged as a promising therapeutic target for chronic pain, as the selective enhancement of the magnitude of the α3 GlyR current has been shown to exhibit analgesic effects in animal models of inflammatory pain (Lynch et al., 2017). In summary, diminished glycineric inhibition (e.g., hyperekplexia, autism) would benefit most from facilitated glycineric inhibition, through positive allostery GlyR modulators. Interestingly, GlyRs modulation also plays a crucial role in synaptogenesis (Ganser and Dallman, 2009), neurite outgrowth (Tapia et al., 2000), or produces neuroprotection against metabolic stress such as oxygen/glucose deprivation (Tanabe et al., 2010). Given these considerations, GlyR-modulating compounds offer great potential for research on novel drug-like compounds.

The function of GlyRs can be modulated by various ligands, including neurosteroids (NS). Neurosteroids are compounds that accumulate in the nervous system independently of the steroidogenic endocrine glands and which can be synthesized de novo in the nervous system from cholesterol or other steroidal precursors imported from peripheral sources (Baulieu, 1998). The steroid numbering, ring letters, stereochemistry and nomenclature is summarized in Figure 1. The biosynthetic pathway (Do Rego et al., 2009) of NS (Figure 2) is triggered by the conversion of cholesterol to pregnenolone (PREG). Then, PREG is converted to progesterone (PROG) and dehydroepiandrosterone (DHEA). Subsequently, PROG is metabolized to 5α- or 5β-dihydroprogesterone, followed by their reduction to 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) or 3α-hydroxy-5β-pregnan-20-one (pregnanolone).

These compounds and their synthetic analogs are mainly known as potent modulators of GABA_A Rs (Chen et al., 2019) and N-methyl-D-aspartate receptors (NMDARs) (Burnell et al., 2019), respectively. Neurosteroids and their synthetic analogs (neuroactive steroids, NAS) have been extensively studied during the last three decades as they modify neuronal activity and thus brain function via a fast, non-genomic action (Rebas et al., 2017), by acting as allostERIC modulators of various ligand-gated ion channels, including GABA_A Rs and GlyR. In brief, NS and NAS are effective modulators of GABA_A Rs-induced chloride current ($I_{GABA}$) and their modulatory action is dependent on their structure and subtype (for a review, see: Majewska et al., 1988; Wu et al., 1990; Belelli and Lambert, 2005; Korinek et al., 2011; King, 2013; Zorumski et al., 2013). Those that potentiate GABA activity are termed as "potentiating NS" and these include, e.g., allopregnanolone (3α-hydroxy-5α-pregnan-20-one) or pregnanolone (3α-hydroxy-5β-pregnan-20-one) (Park-Chung et al., 1999). The α-configuration at C-3 is extremely important for potentiating steroids, contrasting with a relatively vague requirement for a 3α/3β-configuration for "inhibitory NS" that are referred to as those that antagonize $I_{GABA}$ (Park-Chung et al., 1999). The inhibitory NS incorporate mainly a subclass known as the C-3 sulfated steroids (e.g., pregnenolone sulfate and DHEA sulfate) (Gibbs et al., 2006) or the C-3 hemiester steroids (e.g., pregnanolone hemisuccinate) (Seljeset et al., 2015), although C-3 negative charge is not obligatory for the inhibition (e.g., DHEA). The relevance of configuration or double bond at C-5 for the potentiation/inhibitory action is driven by its combination with α/β-configuration at C-3 (Park-Chung et al., 1999) that define a planar or “bent-shape” of the molecule (Figure 1E). Interestingly, the nature of the group at C-17, concerning inhibition, is less stringent given that 17-acetyl, 17-acetoxy, and 17-keto groups substituted onto a 3β-hydroxy-androst-5-ene retain similar inhibitory activities. On the other hand, 17-acyt, 17-acetoxy, 17-dihydroxy or 17-keto groups substituted onto a 3α-hydroxy-5α-androstan-20-one exhibit markedly various enhancement of $I_{GABA}$ varying up to 9-folds (Park-Chung et al., 1999). For example, the reduction of the C-20 ketone of 3α-hydroxy-5α-pregnane-20-one to its 20α-hydroxy analog greatly decreases the efficacy of potentiation 166% vs. 1373%.

The GlyR-induced chloride current ($I_{Gly}$) has been also shown to be modulated by NS, but the data on potencies are rather limited to compounds with a pregnane skeleton (Figure 1F). Allopregnanolone (Figure 2) enhanced the glycine-induced current of native or recombinant receptors (Weir et al., 2004; Jiang et al., 2006), while Fodor et al. (2006) showed that micromolar concentrations of allopregnanolone blocked GlyRs of native cells. These variances may be ascribed to the difference between neuronal and recombinant GlyRs (Kung et al., 2001). Next, pregnanolone (Figure 2) proved to be an inhibitor of both α1 GlyRs and native cells (Weir et al., 2004; Fodor et al., 2006; Jiang et al., 2006). Finally, 3β-hydroxy-5α-pregnan-20-one and 3β-hydroxy-5β-pregnene-20-one were demonstrated as inactive on both neurons and recombinant α1 receptors (Wu et al., 1990; Weir et al., 2004). Interestingly, PROG exhibited incomplete and non-competitive inhibition of GlyR currents in contrast to the full and competitive inhibition by its sulfated analog (PREG-S) of chick spinal cord (Wu et al., 1997) and selectively inhibited embryonic α2 GlyRs, with no effect on α1 and α1β GlyRs (Maksay et al., 2001). To date, only three androstanate compounds were tested – DHEA sulfate and 3β-hydroxy-5α-androstan-17-one, and 3α-hydroxy-5α-androstan-17-one inhibited $I_{Gly}$ currents in micromolar range on recombinant α1 receptors (Maksay et al., 2001). As such, the biological potential of androstanate and androstene skeletons (Figure 1F) concerning their effect on GlyR remains unknown.

In our previous work, a series of pregnanolone derivatives (modulators of NMDA receptors) displayed the effects on the $I_{GABA}$ and $I_{Gly}$ in rat pyramidal hippocampal neurons (Bukanova et al., 2018). Interestingly, we demonstrated that the nature of the substituent at C-3 defines the positive or negative character of $I_{GABA}$. Indeed, pregnanolone glutamate was found to potentiate $I_{GABA}$, while pregnanolone hemisuccinate and pregnanolone hemipimelate inhibited $I_{GABA}$, and all three steroids inhibited $I_{Gly}$. The conversion of the 5β-pregnanolone skeleton into a 5β-androstane skeleton, an analog that lacks the C-17 acetyl moiety, eliminated the effects on both GABA_A Rs and GlyRs.

As mentioned previously, the modulatory effect of NS on GABA_A Rs or GlyRs is a relevant avenue of investigation in neuropharmacology. To understand the structure-activity relationship of NS on $I_{GABA}$ and $I_{Gly}$, further structure-activity relationship studies (SAR) are required. In the present study, we examine the effects of a series of endogenous NS on the GABA- and Gly-induced current in voltage-clamped rat cerebellar Purkinje cells and rat hippocampal neurons, respectively. This series contained 9 natural NS with an androstanate and androstene
FIGURE 1 | (A) Steroid numbering and ring letters; (B) schematic orientation of substituents. When the rings of a steroid are denoted as projections onto the plane of the paper, the α-substituent (hashed bond) lies below and the β-substituent (bold bond) lies above the plane of the paper; (C) explicitly written configuration for all stereocenters of cholesterol; (D) unless implied or stated to the contrary in figures and schemes, the stereochemistry of steroid molecule is simplified. Depicted structure implies that atoms or groups attached at the bridgehead positions 8, 9, 14, and 17 are oriented as shown in formula C (8α,9α,14α). Angular methyles (CH$_3$) at positions 10, 13 are omitted and shown only as bold bonds; (E) a perspective representation of planar 5α-steroid and a bent molecule of 5β-steroid; (F) fundamental names of steroid skeletons relevant to this paper.

FIGURE 2 | Schematic illustration of neurosteroid biosynthesis.

skeleton with variable substituents at C-3, C-5, and C-17 positions (Table 1).

MATERIALS AND METHODS

Cell Preparation

All experiments were conducted per the requirements of the Ministry of Public Health of the Russian Federation and were consistent with the EU directive for Use of Experimental Animals of the European Community. The study was approved by the Ethics Committee of the Scientific Center of Neurology, Protocol No. 2-5/19 of 02.20.19. The cells were isolated from transverse hippocampal slices as described in detail elsewhere (Vorobjev, 1991). Briefly, the slices (200–500 μm) of Wistar rats (11–14 days of age) hippocampus or cerebellum were incubated at room temperature for at least 2 h in a solution containing the following components (in mM): 124 NaCl, 3 KCl, 2 CaCl$_2$, 2 MgSO$_4$, 25 NaHCO$_3$, 1.3 NaH$_2$PO$_4$, 10 D-glucose, pH 7.4. The saline was continuously stirred and bubbled with carbogen (95% O$_2$ + 5% CO$_2$). Single pyramidal neurons from the hippocampal CA3 area or Purkinje cells from sagittal slices of the cerebellum were isolated by a vibrating fused glass pipette with a spherical tip.

Current Recordings

Isolated neurons were patch clamped and then lifted into the outflow of the control bath solution. Bath solution flowed
TABLE 1 | Structure-activity relationship study overview for compounds 1-9: their chemical names, structures, $\tau_{\text{des}}$ ($I_{\text{Gly}}$ vs. $I_{\text{GABA}}$) values.

| Cmpd. | Chemical name | Common name | Structure | $\tau_{\text{des}}$ |
|-------|---------------|-------------|-----------|------------------|
| 1     | $17\beta$-Hydroxyandrost-4-en-3-one | Testosterone | ![Structure](image1) | $-67\%$ |
| 2     | Androst-4-en-3,17-dione | Androstenedione | ![Structure](image2) | $-84\%$ |
| 3     | $17\beta$-Hydroxy-5α-androstan-3-one | 5α- Dihydrotestosterone | ![Structure](image3) | $-82\%$ |
| 4     | $3\beta$-Hydroxy-5α-androstan-17-one | Epiandrosterone | ![Structure](image4) | $-64\%$ |
| 5     | $17\alpha$-Hydroxyandrost-4-en-3-one | Epitestosterone | ![Structure](image5) | $-72\%$ |
| 6     | $5\alpha$-Androstane-3,17-dione | Dihydroandrostenedione | ![Structure](image6) | $-72\%$ |
| 7     | $3\beta,17\beta$-Androst-5-ene-3,17-diol | Androstenediol | ![Structure](image7) | $-70\%$ |
| 8     | $3\beta$-Hydroxy-androst-5-en-17-one | Dehydroepiandrosterone (DHEA) | ![Structure](image8) | $-79\%$ |
| 9     | $5\beta$-Androstane-3,17-dione | Etiocholanedione | ![Structure](image9) | $-68\%$ |

The first column gives number of compound, second gives the chemical name, the third column indicates a common name accepted for this compound, and the fourth column shows its structure. The effect of compounds 1-9 at 10 µM concentration on the value of time constant of desensitization ($\tau_{\text{des}}$) of the $I_{\text{Gly}}$ and $I_{\text{GABA}}$ is expressed in the fifth column. $^a$ The effect of compounds 1-9 on the value of time constant of desensitization ($\tau_{\text{des}}$) of the $I_{\text{Gly}}$ and $I_{\text{GABA}}$. $^b$ Compounds did not modulate $I_{\text{GABA}}$ up to the concentration of 10 µM. $^c$ Compounds did not modulate $I_{\text{GABA}}$ up to the concentration of 50 µM.

The solution in the recording pipette contained the following (in mM): 40 CsF, 100 CsCl, 0.5 CaCl₂, 5 EGTA, 3 MgCl₂, 4 NaATP, 5 HEPES, pH 7.3. The composition of the extracellular solution was as follows (in mM): 140 NaCl, 3 KCl, 3 CaCl₂, 3 MgCl₂, 10 D-glucose, 10 HEPES hemisodium, and pH 7.4. Recording of the currents was performed using EPC7 patch-clamp amplifier (HEKA Electronik, Germany). The holding potential was maintained at $-70$ mV. Transmembrane currents were filtered at 3 kHz, stored and analyzed with IBM-PC computer, using homemade software.

**Reagents**

All the drugs used for intracellular and extracellular solutions were purchased from Sigma-Aldrich (United States). Compounds 1-9 are available from Sigma-Aldrich or Carbosynth under the following CAS and catalog numbers: compound 1 (Sigma, CAS 58-22-0, Cat. No. T1500), compound 2 (Sigma, CAS 63-05-8, Cat. No. 46033), compound 3 (Sigma, CAS 521-18-6, Cat. No. A8380), compound 4 (Sigma, CAS 481-29-8, Cat. No. 46033).
Effects of Neurosteroids 1-9 on the $I_{\text{Gly}}$

Short (600–1000 ms) application of 100 µM glycine on pyramidal neurons of rat hippocampus evoked $I_{\text{Gly}}$, which amplitude and kinetics were dependent on glycine concentration with an EC$_{50}$ value of 90 ± 7 µM. An average value of the reversal potential of $I_{\text{Gly}}$ was −9.6 ± 0.8 mV matched well the chloride reversal potential calculated for the chloride concentrations used (−9.5 mV, not shown). We used agonist concentration of 100 µM that was near EC$_{50}$, because it allow to achieve stable current with well visible and measurable either suppressive or augmenting effect. All 9 compounds caused a similar effect on the $I_{\text{Gly}}$, which consisted of two components: acceleration of desensitization and decrease in peak amplitude. The effects were reversible upon washout during 1–2 min. The effect of desensitization acceleration developed at significantly lower concentrations of NS than the effect of peak amplitude suppression. Noteworthy, the threshold concentration of NS for initiating the effect of desensitization acceleration was 0.1 µM, while the threshold concentration of the same compounds for developing the effect of the peak amplitude reduction was 10 µM. A representative effect of NS on $I_{\text{Gly}}$ of one cell is shown in Figure 3A. Compound 1 in low concentrations of 0.1 and 1 µM accelerated desensitization without effect on the peak amplitude, while at a concentration of 10 and 100 µM it causes two effects: acceleration of desensitization and a decrease in peak amplitude. The effects of the remaining eight NS on the $I_{\text{Gly}}$ did not differ significantly from the testosterone effect (for details, see Figures 3B,C and Table 2). When co-applied with glycine, NS at concentration 0.1 µM barely affected the $I_{\text{Gly}}$ peak amplitude but decreased the time constant of $I_{\text{Gly}}$ desensitization ($\tau_{\text{des}}$) by 27–35% ($P < 0.01$ or $P < 0.05$). On the contrary, when applied at a concentration of 10 µM, NS accelerated desensitization by 67–82% ($P < 0.01$) and reduced the peak current amplitude by 18–25% ($P < 0.01$ or $P < 0.05$). Figure 4 shows the concentration dependence of the NS effect on the normalized peak amplitude (Figure 4A) and normalized $\tau_{\text{des}}$ of the $I_{\text{Gly}}$ (Figure 4C). An increase in the concentration of NS up to 100 µM caused a decrease in the peak amplitude of the $I_{\text{Gly}}$ by 45–70% with the IC$_{50}$ values of 16–22 µM (Figure 4B and Table 3). Maximal decrease (70–90%) of the $\tau_{\text{des}}$ can be observed in the presence of 10 µM of NS. The IC$_{50}$ values for the effect on the $\tau_{\text{des}}$ are in the range of 0.12–0.49 µM (Figures 4C,D and Table 3), which are two orders of magnitude lower than the IC$_{50}$ values for the effect on peak amplitude.

Effects of Compounds 1-9 on the $I_{\text{GABA}}$

The brief application of GABA for 600–1000 ms on isolated Purkinje cells evoked a chloride current ($I_{\text{GABA}}$) with an amplitude-dependent on GABA concentration with an EC$_{50}$ value of 7.5 ± 2.9 µM. The specific antagonist of GABA$_{A}$ receptors bicuculline (3 µM) reversibly blocked the current (data not shown), which allows us to classify the receptors as GABA$_{A}$ type. We studied $I_{\text{GABA}}$ evoked by 5 µM of GABA. Figure 5 shows the effects of NS on $I_{\text{GABA}}$. Our experiments demonstrate that GABA$_{A}$Rs are much less sensitive to the studied NS than sensitive, whilst neuronal GABA$_{A}$Rs are weakly sensitive to tested compounds 1-9.
GlyRs. The addition of compounds 1, 2, 3, 5, 7, 9 to the applicator pipette at a concentration of 0.1–10 μM did not change either the peak amplitude or the rate of decay of $I_{GABA}$. Only two out of nine compounds – compounds 4 and 8 – in 10 μM concentration were able to cause a significant change in $I_{GABA}$, which consisted of the acceleration of decay (Figures 5A,B and Table 4). When the concentration of the tested compound was increased up to 50 μM, compounds 3, 5, 6, and 9 remained inactive. In contrast, compounds 1, 2, 4, 7, and 8 at 50 μM concentration showed an inhibitory effect with a decrease in the peak amplitude of the current by 14–25% ($P < 0.01$ or $P < 0.05$) and the acceleration of its decay by 23–45% ($P < 0.01$) (Figure 5 and Table 4). Figure 6 shows a comparison of the effects of compounds 1-9 on the $I_{Gly}$ and the $I_{GABA}$. Our results demonstrate that tested NS in the concentration of 10 μM cause strong action on $I_{Gly}$ and weak action on $I_{GABA}$.

### DISCUSSION

In the present study, we examined the effects of a series of endogenous NS on the GABA- and Gly-induced current in rat central neurons. It is known that NS modulate GABA$_A$Rs and GlyRs functions in subunit-specific manner (Maksay et al., 2001; Belelli and Lambert, 2005) and this has implications for native receptors that may differentiate throughout development. We used in our experiments Wistar rats at 11–14 days of age where GlyRs and GABA$_A$Rs were studied in pyramidal hippocampal neurons and cerebellar Purkinje cells, accordingly. Literature
data indicate that starting from the second postnatal week, the subunit composition of GlyR in the hippocampal neurons (Aroeira et al., 2011) and GABA_A R in the Purkinje cells of the cerebellum (Laurie et al., 1992) is close to that in the brain of adult animals. Extrasynaptic GlyRs with different subunit composition are described in pyramidal hippocampal neurons. There may be either heteromeric receptors with α (1, 2, or 3) and β subunits, or homomeric ones with multiple α subunits (for review, see Keck and White, 2009; Xu and Gong, 2010). The major adult isoform of GABA_A Rs in Purkinje cells was shown to be composed of α_1β_2γ_2.
subunits and with a subunit stoichiometry of 2:2:1 (Pirker et al., 2000; Sieghart and Savić, 2018).

The series of steroids we studied included endogenous androstane and androstene NS (compounds 1-9) with variable substituents at positions C-3, C-5, and C-17 (Table 1). In brief, compounds 1, 3, 7, and 5 bear 17β- and 17α-hydroxyl groups, respectively. Compounds 2, 4, 6, 8, and 9 have a carbonyl group at C-17. Compounds 1, 2, 5, 7, and 8 have a double bond in their skeleton and as such belong to a family of androstene steroids. Oppositely, compounds 3, 4, 6 (5α-H), and 9 (5β-H) are fully saturated androstanes. The results of our study show that biological activity is similar for all compounds.

In summary, compounds 1-9 at a concentration up to 10 µM strongly affected $I_{\text{Gly}}$ and had weak action on $I_{\text{GABA}}$. The effect of NS on $I_{\text{Gly}}$ contained two components: a decrease in peak amplitude and an acceleration of decay. The effect of NS on $I_{\text{Gly}}$ decay and the associated decrease in time constant of desensitization ($\tau_{\text{des}}$) was 2–3 times stronger than on the peak of $I_{\text{Gly}}$. Such a different regulation of these two $I_{\text{Gly}}$ parameters by NS suggests the existence of two independent mechanisms of their action on GlyRs, one of which regulates the peak amplitude, and the second – the desensitization process. This assumption is supported by our previous research (Bukanova et al., 2018), where it was shown that these two effects of NS afford different outcome with increasing glycine concentration. Namely, the effect on the peak amplitude of $I_{\text{Gly}}$ disappeared and the acceleration of desensitization remained. The fact that peak inhibition is reduced at higher agonist concentration suggest that inhibiting drugs act as competitive inhibitors of agonist

![Figure 5](image-url)

FIGURE 5 | The effects of compounds 1-9 on $I_{\text{GABA}}$ of cerebellar Purkinje cells. (A) Representative traces of $I_{\text{GABA}}$ induced by 600 ms application of 5 µM GABA, obtained in control and the presence of 10 and 50 µM of compound 4 (epiandrosterone) (left), or compound 1 (testosterone) (right). (B) Mean ± SEM of the normalized values of the time constant of desensitization ($\tau_{\text{des}}$) of $I_{\text{GABA}}$ in the presence of 10 µM of compounds 1-9. (C) Mean ± SEM of the normalized values of the peak amplitude of $I_{\text{GABA}}$ in the presence of 50 µM of compounds 1-9. Probability levels were estimated with ANOVA-test using Dunnett’s multiple comparison test.

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**Table 4** The inhibitory effect of the tested neurosteroids on the peak amplitude ($I_{\text{peak}}$) and time constant of desensitization ($\tau_{\text{des}}$) of the $I_{\text{GABA}}$.

| Cmpd. | $\frac{\tau_{\text{des}}}{\tau_{\text{des}}}$ control | $P$-value | n | $\frac{I_{\text{peak}}}{I_{\text{peak}}}$ control | $50 \mu M$ of steroid | $P$-value | n |
|-------|-----------------|------------|---|-----------------|----------------|------------|---|
| 1     | 0.99 ± 0.02     | 0.8273     | 5 | 0.86 ± 0.03     | 0.0040        | 8          |
| 2     | 0.98 ± 0.02     | 0.4625     | 6 | 0.77 ± 0.02     | 0.0001        | 7          |
| 3     | 0.98 ± 0.02     | 0.5264     | 6 | 0.95 ± 0.03     | 0.2608        | 6          |
| 4     | 0.80 ± 0.03     | 0.0014     | 6 | 0.75 ± 0.03     | 0.0001        | 7          |
| 5     | 0.97 ± 0.03     | 0.4228     | 6 | 1.12 ± 0.08     | 0.0269        | 8          |
| 6     | 0.99 ± 0.02     | 0.8273     | 5 | 0.92 ± 0.04     | 0.0026        | 7          |
| 7     | 0.98 ± 0.02     | 0.4626     | 5 | 0.77 ± 0.06     | 0.0055        | 7          |
| 8     | 0.88 ± 0.05     | 0.0265     | 5 | 0.75 ± 0.04     | 0.0005        | 8          |
| 9     | 0.97 ± 0.03     | 0.4228     | 5 | 0.99 ± 0.01     | 0.5690        | 8          |

Mean ± SEM of the normalized values of the $\tau_{\text{des}}$ and $I_{\text{peak}}$ of the $I_{\text{GABA}}$ are shown. All comparisons with control value were made with unpaired Student’s t-test. Significance level of $P = 0.05$. n- the number of cells used.
binding or that the inhibitors preferentially bind to resting states of the receptor (Li et al., 2007). However, the effect of NS on desensitization is insensitive to agonist concentration and therefore requires other explanations. In our opinion, the acceleration of the $I_{GABAA}$ decay can be explained by the slow block of the open channel or/and the acceleration of the desensitization gate (Gielen et al., 2015). Other authors (Borovska et al., 2012; Vyklicky et al., 2016) described the acceleration of the decay of NMDA current under the influence of NS and explain this effect by slow NS diffusion to the site of action at the extracellular vestibule of the NMDAR. At present, we cannot give preference to any of these assumptions regarding the mechanisms for accelerating the desensitization of $I_{Gly}$ under the influence of NS. This remains to be elucidated.

Interestingly, in the literature, we have not found any indications of the ability of NS to accelerate the desensitization of $I_{Gly}$. The published studies of the action of steroids on $I_{Gly}$ were performed on recombinant GlyRs expressed in frog oocytes (Maksay et al., 2001), a chicken spinal neuron culture (Wu et al., 1990), and a rat hippocampal and spinal neuron culture (Jiang et al., 2009). In all of the described models available in the literature, the authors describe a decrease in the $I_{Gly}$ peak amplitude under the influence of NS. The reason for this contradiction may be due to the features of the methodological approach. We use short (600–1000 ms) co-application of glycine and NS, while other authors used 10–30 s pre-application of the NS followed by 10–15 s application of glycine along with the NS. It is possible that the prolonged exposure of NS to the nerve cell leads to a change in properties of the structures responsible for the desensitization of the GlyRs. However, this issue requires special research. The $IC_{50}$ values for the effect of compounds 1-9 on the $\tau_{des}$ of $I_{Gly}$ were in the range of 0.12–0.49 µM, and on the peak amplitude – in the range of 16–22 µM. Our results are consistent with data from other authors who studied the effects of androsta(e)ne steroids with substitutions at C-17 on GlyRs. Maksay et al. (2001) showed that DHEA sulfate inhibits the recombinant GlyRs expressed in frog oocytes with an $IC_{50}$ value of 2.5–6.3 µM.

As mentioned previously, GlyR-modulating compounds offer great potential for research on novel drug-like compounds. However, their parallel effect on $GABA_A$R might be a disadvantage from the pharmacological perspective. Therefore, the discovery of a selective steroidal modulator of GlyR is a challenging task that has not been, according to our knowledge, described previously in the literature. Here, we demonstrate that the addition of compounds 3, 5, 6, and 9 at a concentration of 0.1–50 µM did not change either the peak amplitude or the rate of desensitization of $I_{GABAA}$ in isolated Purkinje cells. In contrast, compounds 1, 2, 4, 7, and 8 at 50 µM concentration showed an inhibitory effect with a decrease in the peak amplitude of the current by 14–25% ($P < 0.01$ or $P < 0.05$) and the acceleration of its desensitization by 23–45% ($P < 0.01$). We conclude that compounds 3, 5, 6, and 9 are selective modulators of $I_{Gly}$. Their structures, however, do bear similar structural features to those that were able to affect $I_{GABAA}$. Therefore, establishing a pharmacophore from these results would be highly speculative. The data from the literature clearly indicate that a combination of C-3 and C-5 stereochemistry or the presence of double bond (4-ene/5-ene) of a steroid skeleton direct the effect on GlyRs and $GABA_A$Rs activity (Park-Chung et al., 1999; Maksay et al., 2001; Fodor et al., 2006). Unfortunately, a simple additive approach cannot define pharmacophore for the desired combination of activity on one or both receptors. It is important to highlight that saturated 5α-H and unsaturated (4-ene/5-ene) steroidal skeletons possess a planar shape of the molecule, while the 5β-H skeleton is a “bent” structure. The global shape of the molecule is then significantly affected by the stereochemistry of the C-3 substituent. Note, that the
3α-hydroxy group of the planar 5α-H skeleton is axial, whereas the 3α-hydroxy group of the bent 5β-H skeleton is equatorial. Next, in case the substituent at C-3 is a carbonyl group, its location is in between axial and equatorial configuration. Finally, the nature of the modulatory effect seems to be defined by the substituent at position C-17. Taken together with the previously mentioned facts, we believe that we cannot define a pharmacophore for NS that would afford its modulatory action. Rather, a delicate balance of structural features at positions C-3, C-5, and C-17 could manage this extremely challenging task. The results of our unique study confirm this hypothesis. Our discovery of steroidal selective modulators of $\text{J}_{\text{Gly}}$ provides a great potential for further structure-activity relationship studies affording novel compounds. Moreover, such research could lead to the identification of structural requirements of giving active compounds.

**DATA AVAILABILITY STATEMENT**

All datasets generated for this study are included in the article-supplementary material.

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**ETHICS STATEMENT**

The study was approved by the Ethics Committee of the Scientific Center of Neurology, Protocol No. 2-5/19 of 02.20.19.

**AUTHOR CONTRIBUTIONS**

JB conducted experiments to study the effects of neurosteroids on GABA- and glycine-activated current in rat neurons. ES wrote a physiological part of the manuscript. EK prepared compounds 6-9 as described in Materials and Methods section and wrote a chemical part of the manuscript.

**FUNDING**

This work was supported by Technology Agency of the Czech Republic: Czech National Centres of Competence, project “PerMed” Personalized Medicine – Diagnostics and Therapy TN0100013, and ERDF/ESF Project “PharmaBrain,” Grant CZ.02.1.01/0.0/0.0/16_025/0007444 and by Academy of Sciences of the Czech Republic (AS CR) – grant RVO 61388963.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.