

Medroxyprogesterone acetate positively modulates specific GABA_A-receptor subtypes - affecting memory and cognition

Roshni Das^{a,d,1}, Gianna Ragagnin^{b,1}, Jessica Sjöstedt^{b,1}, Maja Johansson^{b,d}, David Haage^{b,c,d}, Michael Druzin^a, Staffan Johansson^a, Torbjörn Bäckström^{b,d,*}

^a Department of Integrative medical biology, Umeå University, SE-901 87 Umeå, Sweden

^b Umeå Neurosteroid Research Center, Department of Clinical sciences, Umeå University, SE-901 85 Umeå, Sweden

^c Department of Nursing Sciences, Mid Sweden University, Sundsvall, Sweden

^d Umeå AB, Norrlands University Hospital Umeå, Building 6 M 4th floor, Sweden

ARTICLE INFO

Keywords:

Medroxyprogesterone-acetate
GABA-A receptor
Alpha5 GABA-A receptor
Neurosteroids
Dementia
Positive GABA-AR modulator

ABSTRACT

Medroxyprogesterone acetate (MPA) is a progestin widely used in humans as hormone replacement therapy and at other indications. Many progestin metabolites, as the progesterone metabolite allopregnanolone, have GABA_A-receptor modulatory effects and are known to affect memory, learning, appetite, and mood. In women, 4 years chronic treatment with MPA doubles the frequency of dementia and in rats, MPA causes cognitive impairment related to the GABAergic system. Activation of the membrane bound GABA_A receptor results in a chloride ion flux that can be studied by whole-cell patch-clamp electrophysiological recordings. The purpose of this study was to clarify the modulatory effects of MPA and specific MPA metabolites, with structures like known GABA_A-receptor modulators, on different GABA_A-receptor subtypes. An additional aim was to verify the results as steroid effects on GABA response in single cells taken from rat hypothalamus. HEK-293 cell-lines permanently expressing the recombinant human GABA_A-receptor subtype $\alpha 1\beta 2\gamma 2L$ or $\alpha 5\beta 3\gamma 2L$ or $\alpha 2\beta 3\gamma 2S$ were created. The MPA metabolites 3 α 5 α -MPA, 3 β 5 α -MPA and 3 β 5 β -MPA were synthesised and purified for electrophysiological patch-clamp measurements with a Dynaflo system. The effects of MPA and tetrahydrodeoxycorticosterone were also studied. None of the studied MPA metabolites affected the responses mediated by $\alpha 1\beta 2\gamma 2L$ or $\alpha 5\beta 3\gamma 2L$ GABA_A receptors. Contrary, MPA clearly acted both as a positive modulator and as a direct activator of the $\alpha 5\beta 3\gamma 2L$ and $\alpha 2\beta 3\gamma 2S$ GABA_A receptors. However, in concentrations up to 10 μ M, MPA was inactive at the $\alpha 1\beta 2\gamma 2L$ GABA_A receptor. In the patch-clamp recordings from dissociated cells of the preoptic area in rats, MPA increased the amplitude of responses to GABA. In addition, MPA alone without added GABA, evoked a current response. In conclusion, MPA acts as a positive modulator of specific GABA_A receptor subtypes expressed in HEK cells and at native GABA receptors in single cells from the hypothalamic preoptic area.

1. Introduction

Medroxyprogesterone acetate (MPA) is widely used by women, both as contraceptive, and as a progestin during hormone therapy. In the year 2004, about 30 million women in the United States were prescribed medications including MPA.

MPA was used in the Women's Health Initiative Memory Study where postmenopausal women received long-term continuous hormone treatment (5–7 years), either with only conjugated equine estrogens (CEE), in women without a uterus (Shumaker et al., 2004), or with CEE plus

MPA (Shumaker et al., 2003). The study was disrupted because of complications, among them the group treated with CEE plus MPA showed an increased risk for development of dementia, mostly Alzheimer's disease (AD) (Shumaker et al., 2003). This increased risk was not shown in the group receiving CEE alone (Shumaker et al., 2004) and the increased dementia frequency was not due to stroke or other cardio-vascular events (Coker et al., 2010). The increased dementia risk in the MPA treated group was interpreted as due to a biological effect of MPA in the brain of the treated patients (Coker et al., 2009). In animal studies, worse performance in learning and memory has been shown

* Corresponding author at: Umeå Neurosteroid Research Center, Department of Clinical sciences, Umeå University, SE-901 85 Umeå, Sweden.

E-mail address: torbjorn.c.backstrom@umu.se (T. Bäckström).

¹ Equally contributed to the paper.

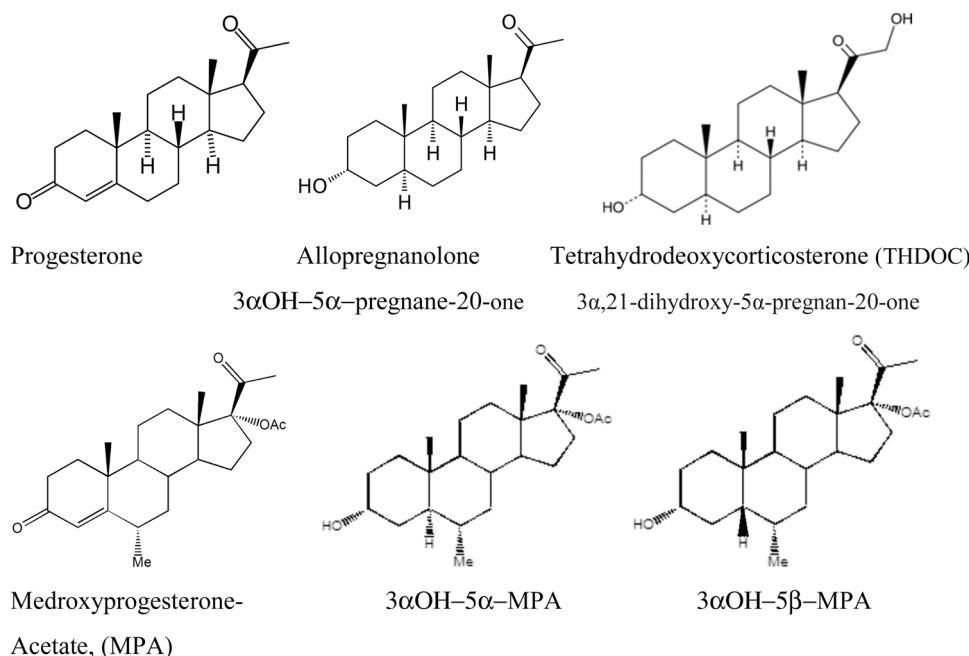


Fig. 1. Chemical structure of steroids discussed in the present paper.

after long-term treatment (6 month) with estradiol plus MPA, or after treatment with only MPA (Braden et al., 2010, 2011; Lowry et al., 2010).

MPA has in addition to the effect related to cognition, also an orexigenic effect and increases the food intake in animals and humans (Steward et al., 2016; Le et al., 2009). MPA has even been used as treatment for cachexia (Simons et al., 1998). We have earlier shown that the neuroactive steroid allopregnanolone has a pronounced effect in hypothalamic cells and it is therefore of interest to use the same brain region for confirming MPA experiment as the region is very sensitive to allopregnanolone with significant effects already at 2 nM (Löfgren et al., 2019). This effect is at least partly mediated by the GABA_A receptor subtype $\alpha 2$ in the hypothalamus and is involved in stimulating hyperphagia. (Holmberg et al., 2018; Bauer et al., 2012).

MPA can induce anesthesia in rats, an effect also seen with progesterone, which has a chemical structure like MPA (Fig. 1) (Meyerson, 1967; Bixo and Bäckström, 1990). The anesthetic effect of progesterone has been attributed to the strong positive GABA_A-receptor modulation by allopregnanolone, a 3 α -hydroxy-A ring 5 α reduced progesterone metabolite. Allopregnanolone is active at the GABA_A receptor, the major inhibitory receptor within the brain, and anesthesia is easily induced by allopregnanolone (Majewska et al., 1986; Norberg et al., 1987). Continuously raised allopregnanolone concentration, as during chronic stress or at frequent repeated injections, enhances the development of AD-like symptoms (decreased memory and learning) in transgenic AD-mice, already after one month's treatment (Bengtsson et al., 2012, 2013; Chen et al., 2011). With 5 months exposure to allopregnanolone, even wild type mice showed a permanent memory impairment and a reduction of the hippocampal volume (Bengtsson et al., 2016).

MPA and progesterone are both metabolized at the A-ring, and several metabolites of MPA have been detected in human plasma and bile, among these are A-ring reduced tetrahydro-MPA variants. Steroids as progesterone with a $\Delta 4$ -3keto configuration are first metabolized by a 5 α -reduction catalyzed by 5 α -reductase (5 α R). Thereafter, a 3 α -hydroxylation by 3 α -hydroxysteroid dehydrogenase (3 α HSD) may occur which modifies the steroids to contain a hydroxy group at 3 α position. Steroid metabolites with this configuration can act as positive modulators of GABA_A receptors (Melcangi et al., 1999; Fukushima et al., 1979; Zhang et al., 2008; Chen et al., 2009). Therefore, the 3 α -hydroxy metabolites of MPA were of interest to investigate for effects on the GABA_A

receptor subtypes.

The 3 α OH A-ring structure in endogenous steroids is important for binding to the GABA_A receptor. Allopregnanolone and tetrahydrodeoxycorticosterone (THDOC) are both 3 α 5 α -reduced-pregnane-steroid metabolites thought to be among the most potent positive GABA_A-receptor modulators (Purdy et al., 1990, 1992). THDOC and allopregnanolone bind to the same binding site on the GABA_A receptor (Hosie et al., 2006) and have similar efficacy and potency at the receptor (Stromberg et al., 2005).

The aim of this study is to clarify if tetrahydro-MPA metabolites or MPA itself affect the currents mediated by different GABA_A receptor subtypes.

2. Materials and methods

2.1. Synthesis of MPA metabolites

The syntheses of the medroxyprogesterone acetate metabolites were made at Umecrine AB as described below. 300 mg (0.78 mmol) of MPA was dissolved in 150 ml ethanol (95%, Kemetyl), 0.45 ml of glacial acetic acid (Merck, Sigma-Aldrich), and 5% Rhodium on carbon catalyst (5%, Alfa Aesar) were added. A stream of hydrogen gas from a separate flask with sodium borohydride (Fisher) in methanol (pro analysis (p.a.), Merck, Sigma-Aldrich) was bubbling overnight in the MPA solution under stirring. Thin-layer chromatography (TLC) (detection with UV light and/or vanillin stain) monitored the reaction mixture. At completion, the mixture was filtered, the solvent evaporated in vacuo, and the white residue extracted with dichloromethane (Sigma) and water. The organic phase was washed with aqueous sodium bicarbonate (Merck, Sigma-Aldrich) yielding 290 mg of a white solid material.

Thus, MPA was hydrogenated with the use of Rhodium-catalyzed reactions, leading to the formation of four isomers of MPA. The synthesized compounds were purified by use of preparative high pressure liquid chromatography (HPLC), Waters: a 1515 Isocratic Pump, 717 plus Auto-sampler equipped with a 1500 Column Heater, a Symmetry-Prep C18 7 μ m 78 \times 300 mm column, 2487 Dual λ Absorbance Detector, and Fraction Collector II. The detector output was recorded on a PC-based Waters Breeze Chromatography Software (version 3.20). The conditions used were T (column) 45 $^{\circ}$ C; detection at 206 nm; flow rate:

4.0 ml/min. eluents: methanol: water, 70:30, V/V.

Synthesized products were identified by nuclear magnetic resonance (NMR) (Bruker 400 MHz);

3 β hydroxy-5 α -Medroxyprogesterone acetate (3 β 5 α -MPA), (400 MHz, CDCl₃-d₆): δ (ppm) 3.56 (m, 1 H); 2.13 (s, 3 H); 2.06 (s, 3 H); 0.86 (s, 3 H); 0.85 (d, 3 H); 0.65 (s, 3 H).

3 α hydroxy-5 α - Medroxyprogesterone acetate (3 α 5 α -MPA), (400 MHz, CDCl₃-d₆): δ (ppm) 4.11 (m, 1 H); 2.14 (s, 3 H); 2.05 (s, 3 H); 0.83 (s, 3 H); 0.82 (d, 3 H); 0.65 (s, 3 H).

3 β hydroxy-5 β - Medroxyprogesterone acetate (3 β 5 β -MPA), (400 MHz, CDCl₃-d₆): δ (ppm) 4.19 (m, 1 H); 2.16 (s, 3 H); 2.06 (s, 3 H); 0.99 (s, 3 H); 0.84 (d, 3 H); 0.64 (s, 3 H).

3 α hydroxy-5 β - Medroxyprogesterone acetate (3 α 5 β -MPA), (400 MHz, CDCl₃-d₆): δ (ppm) 3.74 (m, 1 H); 2.15 (s, 3 H); 2.05 (s, 3 H); 0.98 (s, 3 H); 0.82 (d, 3 H); 0.64 (s, 3 H).

3 β -hydroxy-Medroxyprogesterone (3 β -OH-MprogA): (400 MHz, CDCl₃-d₆): δ (ppm) 5.31 (s, 1 H); 4.20 (m, 1 H); 2.94 (m, 1 H); 2.11 (s, 3 H); 2.05 (s, 3 H); 1.08 (s, 3 H); 1.03 (d, 3 H); 0.67 (s, 3 H).

3 α -hydroxy-Medroxyprogesterone (3 α -OH-MprogA) OH-MPA: (400 MHz, CDCl₃-d₆): δ (ppm) 5.44 (d, 1 H); 4.38 (m, 1 H); 2.94 (m, 1 H); 2.12 (s, 3 H); 2.05 (s, 3 H); 1.19 (s, 3 H); 1.02 (d, 3 H); 0.68 (s, 3 H).

Spectra of the crude reaction mixture showed the following proportions among reduced products: 3 α 5 α -MPA : 3 β 5 α -MPA:3 β 5 β -MPA:3 α 5 β -MPA, 23:23:45:9.

2.2. Experiment 1: recombinant human GABA_A receptors expressed in HEK-293 cells, electrophysiological recordings

2.2.1. Solutions and instruments used for recordings

The extracellular solution (EC) used for recording contained: 137 mM NaCl and 1.0 mM CaCl₂ (both from Merck, Darmstadt, Germany), 5.0 mM KCl and 1.2 mM MgCl₂ (both from Scharlau, Barcelona, Spain), 10 mM HEPES (Saveen Werner AB, Limhamn, Sweden), and 10 mM glucose (VWR International, Poole, England). pH was adjusted to 7.4 with 1 M NaOH (Eka Chemicals AB, Bohus, Sweden). The intracellular solution (IC) contained: 3.0 mM NaCl, 1.2 mM MgCl₂, 10 mM HEPES 1.0 mM EGTA (Sigma Chemical Co., St. Louis, MO, USA), 140 mM Cs-gluconate (synthesized by Dr. Ragagnin). pH was adjusted to 7.2 with 1 M CsOH (Sigma Chemical Co.).

GABA (Sigma Chemical Co.) was dissolved in EC-solution, while THDOC (Sigma Chemical Co.), MPA and MPA-metabolites were dissolved in DMSO (Sigma Chemical Co., 6 mM stock concentration). During all electrophysiological measurements, the DMSO concentration was 0.1%. Whole-cell voltage-clamp recordings were made with the Dynaflo[®] system (Dynaflo Pro II Platform Zeiss Axiovert 25) and the DF-16 Pro II chip (Celletricon AB, Göteborg, Sweden) that allows rapid solution exchanges. The patch-clamp instruments used were an Axon-patch 200B amplifier, a Digidata 1322 A digitizer and pCLAMP software (Axon Instruments, Foster City, CA, USA).

2.3. Electrophysiological conditions

Whole-cell currents from Human embryonic kidney (HEK-293) cells permanently transfected to constitutively express recombinant human GABA_A receptors (see below) were recorded with the whole-cell patch technique at room temperature (21–23 °C). The glass pipettes used had a resistance of 2–6 M Ω when filled with IC solution. After compensation for liquid-junction potential, a steady holding potential of –17 mV was used in all experiments. The cells were added to the chip and kept in EC solution. EC solution with or without steroids and GABA were applied by the Dynaflo[®] system. The syringe pump flow rate was 26 μ l/min. The standard study protocol included cell exposure to THDOC, MPA or MPA metabolites for 20 s before GABA was applied. Thereafter followed a washout period of 2 min in EC solution to completely remove studied steroids before the next application. The human GABA_A receptor

subtypes studied are endogenously found in different compartments of the neurons. Thus, the α 5 β 3 γ 2 L GABA_A receptor is typically found outside the synapse, i.e. extra-synaptic (Belelli and Lambert, 2005). Such receptors are activated by lower GABA concentrations during a longer period than receptors located in the synapse. The α 1 β 2 γ 2 L and α 2 β 3 γ 2 S are the most common synaptic GABA_A receptors. They are exposed to high GABA concentrations (> 100 μ M) for short periods (ms). Thus, to resemble natural conditions, these receptors were exposed to 30 and 45 μ M GABA (EC₇₅). GABA was applied together with the studied steroid for 40 ms. Studies of the α 5 β 3 γ 2 L receptor was with 0.3 μ M GABA together with the studied steroid for 6 s. The responses to 1.0 μ M THDOC on HEK-293 α 5 β 3 γ 2 L and α 2 β 3 γ 2 S are shown as reference.

2.4. HEK-293 cells expressing recombinant human GABA_A receptors

The effect of THDOC, MPA or MPA metabolites was measured as the area under the curve (AUC) of evoked currents (corresponding to charge transfer). AUC was analysed to include both potential effects on the peak amplitude and on the desensitization and deactivation phases. The AUC was measured semi-manually by using cursors and the curve-fitting procedures of the pCLAMP software. Direct activation of the GABA_A receptor by THDOC or MPA (in the absence of applied GABA) was measured as change in steady-state current. Steroid effects on AUC were normalized to control in order to avoid effects of inter- and intracellular (e.g. due to slow drift) variation in measured parameters.

HEK-293 cells were permanently transfected with cDNA to constitutively express the human α 1 β 2 γ 2L, α 5 β 3 γ 2L or α 2 β 3 γ 2S GABA_A receptor subtypes, respectively. The GABA_A receptor subunits α 1 (308–1727 in NM_000806), β 2 (214–1679 in NM_000813), γ 2L (290–1785 in NM_198904), α 5 (329–1735 in NM_000810), β 3 (45–1485 in NM_021912), α 2 (199–1571 in NM_000807), γ 2S (290–1761 in NM_000816) with introduced Kozac sequences just before the start codons were sub-cloned into the mammalian expression vectors (Invitrogen); pcDNA3.1(+) for α 1 and α 5, pcDNA3.1(-) for α 2, pcDNA3.1/Hygro(-) for β 2 and β 3, and pcDNA3.1/Zeo(+) for γ 2S and γ 2L. A HEK-293 cell line stably expressing the three GABA_A-receptor subunits was produced by transfection of the subunits one at a time. The transfections were followed by selection with the appropriate antibiotics, cell separation with the use of subunit-specific antibodies, and production of single cell colonies. Produced cell lines were analyzed with immunocytochemistry for the GABA_A receptor subunits, followed by selection of a suitable cell line showing good reactivity towards GABA and THDOC in electrophysiological recordings. For electrophysiological experiments, HEK-cell pellets were resuspended in 2–3 ml EC-solution and kept at 37 °C for 15–30 min. The cells were then resuspended and added to the chip bath.

2.5. Experiment 2: dissociated cells from the preoptic area in rats: electrophysiological recordings

2.5.1. Animals

Male Sprague-Dawley rats (n = 19, weight < 130 g), bred at Umeå Centre for Comparative Biology (UCCB) Umeå University, Umeå, Sweden were used. They were housed two or four rats per cage. All housing was at constant temperature, 22 °C, and with artificial daylight. Rats had free access to water and standard food. The regional ethics committee for animal research (“Umeå djurförsöksetiska nämnd”; approval no. A44–2018) approved the experimental protocols. All efforts were made to minimize the number of animals used and their suffering, and all procedures including animals were conducted in accordance with the ethical guidelines set by the European Union.

2.5.2. Drugs and chemicals

Medroxyprogesterone (MPA), GABA, Picrotoxin and amphotericin B were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals used were purchased from a local supplier.

Electrophysiological experiments:

The methods used for electrophysiological recording and cell preparation have been described in detail elsewhere (Haage and Johansson, 1999). A summary of the procedures is given below.

2.5.3. Solutions

The incubation solution used for cutting and recovery of brain slices were provided with moderate rate of bubbling for oxygenation (with O₂ gas) and contained (in mM) as follows: "Recovery solution": 124 NaCl, 3 KCl, 2.0 CaCl₂•H₂O, 6 MgCl₂•6 H₂O, 2 MgSO₄, 10 HEPES, 20 Sucrose, 10 D-glucose. pH was adjusted to 7.4 using NaOH. "Cutting solution": 87 NaCl, 2.5 KCl, 0.5 CaCl₂•H₂O, 7 MgCl₂•6 H₂O, 10 HEPES, 70 sucrose, 25 D-glucose. pH was adjusted to 7.4 using NaOH. The intracellular solution used for filling the pipettes contained (in mM): 140 Cs-acetate, 3.0 Na-acetate, 1.2 MgCl₂•6 H₂O, 1 EGTA, 10 HEPES, and 10 sucrose. pH was adjusted to 7.2 with CsOH. Amphotericin B was dissolved in DMSO and added to a final concentration of 10–12 µl / 2 ml of intracellular solution. The extracellular (EC) solution used during recordings contained (in mM): 116.6 NaCl, 23.4 Na-acetate, 3.0 KCl, 1.5 CaCl₂•H₂O, 1.2 MgCl₂•6 H₂O, 10 HEPES, and 10 D-glucose. pH was adjusted with NaOH to 7.4. MPA was dissolved by sonification in EC solution with DMSO as solvent. The concentration of MPA in patch-clamp experiments were 10 µM.

2.5.4. Cell preparation

The rats were killed by decapitation without anesthesia. The brain was quickly removed and placed in ice-cold incubation solution (see above). A block/part of brain tissue from the rostral part containing the hypothalamus was dissected. Brain slices (thickness 250 µm) from the hypothalamic region were prepared and incubated for at least 1 h in oxygenated incubation solutions at 28 °C. Single cells with adhering synaptic nerve terminals from the medial preoptic area were isolated by vibrodissociation (Vorobjev, 1991) using a thin glass rod (~0.5 mm in diameter). The cell bodies of obtained neurons were round / oval, some with parts of dendrites attached.

2.5.5. Electrophysiological recordings

The amphotericin-B-perforated patch-clamp technique (Strömberg et al., 2009) was used to record whole-cell currents from the post-synaptic neurons under voltage-clamp conditions. In the perforated patch technique, the internal cellular components of the cell are preserved while still allowing control over internal Na⁺, K⁺, and Cl[−] concentrations (Strömberg et al., 2009). The steady voltages used for recording from preoptic neurons were (after correction for liquid-junction potential) −51 mV and −11 mV, the former implying a voltage close to the resting potential and in this respect physiological, while the latter voltage was used to improve the signal-to-noise ratio as a consequence of the larger driving force for the GABA-evoked Cl[−] currents. (The equilibrium potential for Cl[−] in these recordings was −100 mV.) Haage and Johansson (1999) describe the techniques used in

detail. Cells were bathed in an EC solution and the patch pipettes were filled with intracellular solution (IC). EC-solutions, with or without test substances, were applied by a gravity-fed fast perfusion system controlled by solenoid valves. All patch-clamp experiments were performed at room temperature (21 – 23 °C). Borosilicate glass pipettes (GC150, Clark Electromedical Instruments, Pangbourne, UK), with a resistance of 3–4 MΩ when filled with intracellular solution and immersed in extracellular solution, were used.

2.5.6. Protocol and analysis

A standard protocol was used for the experiments. Firstly, the current was recorded in EC solution for 30 – 60 s, followed by a 60 – 300 s test period with or without GABA alone, MPA alone, GABA + MPA or GABA + MPA + picrotoxin. The aim in this study was to use a GABA concentration as in natural situation. *In vivo*, the intra-synaptic receptors are exposed to a much higher GABA concentration than the extra-synaptic receptors. GABA_A receptors α1 and α2 are considered intra-synaptic and are exposed to high GABA concentrations while α5 receptor is considered extra-synaptic and is thus exposed to much lower GABA concentrations. In addition, we used GABA_A EC₇₅ of the receptor subtype to be able to detect both positive and negative modulation by MPA. We have therefore used different GABA concentrations depending on the receptor subtype.

To allow for near steady-state conditions, the first 30 s interval of the test period was not used for analysis. The test period was followed by a 2 – 4 min washout period in EC solution at steady rate during recording. The protocol was applied repeatedly for recordings from individual cells. The time-course and amplitude of the evoked currents were measured semi-manually by using cursors and were fitted to an exponential decay curve by the curve-fitting routine of the pCLAMP software (versions 7 – 9; Axon Instruments, Foster City, CA, USA). The peak amplitudes of the currents were analyzed for events of amplitudes ~1.5 times the peak-to-peak noise (in the solution with the largest noise and with lowpass Bessel filtering at 2.0 kHz). To avoid intra- and intercellular variations in the time-course and peak amplitude, all events recorded in the presence or absence of MPA in the individual cells were indicated relative to a control. Finally, all the relative values from the recorded cells were pooled and subjected to statistical analysis, Origin and SPSS (IBM statistical package for the social sciences) software were used. To quantify the effect of MPA, measures of time constants, amplitudes, and area under the curve from each cell were expressed relative to the mean values under control conditions.

2.6. Statistical analysis

In experiment 1, SPSS was used for statistical analyses and GraphPad Prism, (GraphPad Software Inc., San Diego, CA, USA) was used for making illustrations. Area under the curve (AUC), peak amplitude and decay time constant (tau) were measured.

All values obtained from recordings in a certain condition were

Table 1

Modulatory effects of 3α/β-hydroxy-MPA metabolites on area under the curve (AUC) of GABA-evoked current responses calculates as % changes from GABA evoked current. Minus indicates inhibition and + indicates positive modulation. Nonparametric statistics were used.

GABA _A receptor	Test compound	MPA conc. (µM)	MPA-induced change in GABA _A -evoked current (AUC; %)				n	P
			Mean	SEM	Min	Max		
α1β2γ2 L	3α5α-MPA	0.1	-29.2	10.2	-56.4	+ 2.7	7	NS
		1.0	-4.8	5.1	-34.2	+ 23.4	11	NS
		3.0	-18.2	13.9	-68.7	+ 49.2	11	NS
	3β5α-MPA	20	-59.8	8.9	-76.9	-36.2	4	\$
	3β5β-MPA	20	-45.8	5.7	-57.6	-34.9	4	\$
α5β3γ2 L	3α5α-MPA	0.1	-9.1	2.7	-28.2	0	9	NS
		0.3	-4.6	5.7	-23.6	+ 23.8	9	NS
		1.0	-4.6	6.9	-28.2	+ 17.5	6	NS
		3.0	-5.0	5.6	-23.3	+ 19.3	7	NS

^a 30 µM with α1β2γ2L GABA_A receptors and 0.3 µM with α5β3γ2L GABA_A receptors.

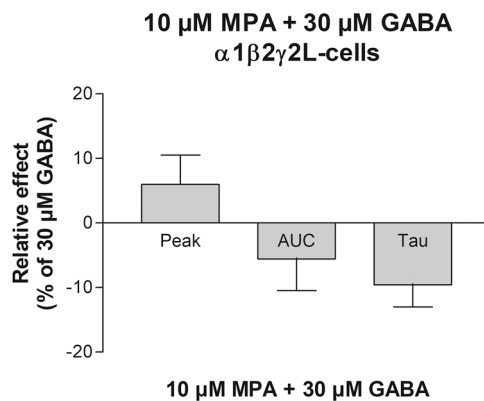


Fig. 2. Effects of MPA on responses to 30 μ M GABA applied to HEK-293 cells expressing human $\alpha 1\beta 2\gamma 2$ L GABA_A receptors ($n = 11$). None of the measured relative effects are significantly different from 0.

pooled and utilized in the statistical analysis. Kruskal Wallis non-parametric test was used to evaluate effects between groups. Each cell had its own control and non-parametric Friedman's test, equivalent to Analysis of variance (ANOVA), with repeated measures were used for comparisons within the cells. The independent factors were concentration of the substances (GABA, MPA, MPA-metabolite or THDOC). The analysis was followed ad hoc by the paired non-parametric Wilcoxon Signed Ranks Test to evaluate effects of each concentration separately. In experiment 2, peak amplitude and tau were measured, the non-parametric Friedman test, Wilcoxon Sign Rank Test and Mann-Whitney U test with Bonferroni adjustment were used to compare the MPA effect with the control value. All values in the text (for experiment 1 and 2) are displayed as mean \pm S.E.M. Significance is marked as follows, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. NS = non-significant, n = number of events pooled from five to twelve cells.

3. Results

3.1. MPA metabolites evaluated with different GABA_A receptor subtypes

MPA metabolites with A-ring structures identical to those in compounds known to affect the GABA_A receptor, i.e. a 3 α - or 3 β -hydroxyl group in the 3rd position, were tested with patch-clamp recordings for effect at the human GABA_A receptor subtypes $\alpha 1\beta 2\gamma 2$ L and $\alpha 5\beta 3\gamma 2$ L.

§ No statistical testing made, due to few observations.

NS = not significant.

None of the 3 α -hydroxy-MPA metabolites applied in a concentration of 0.1 – 3.0 μ M showed any significant modulation of the GABA response at $\alpha 1\beta 2\gamma 2$ L or at $\alpha 5\beta 3\gamma 2$ L GABA_A receptors (Table 1). A high concentration (20 μ M) of 3 β -hydroxy-MPA metabolites, tested in four $\alpha 1\beta 2\gamma 2$ L-expressing HEK-cells, reduced the GABA-evoked current in all cells tested (Table 1), but due to the low number of cells tested with this concentration, no statistical analysis was made.

3.2. Effects of 3 α -hydroxy-Medroxyprogesterone (3 α -OH-MproGA) and 3 β -hydroxy-Medroxyprogesterone (3 β -OH-MproGA) on GABA-evoked currents mediated by $\alpha 1\beta 2\gamma 2$ L and $\alpha 5\beta 3\gamma 2$ L receptors

The MPA metabolites 3 α -OH-MproGA and 3 β -OH-MproGA were synthesized and tested for modulatory effects on GABA responses mediated by $\alpha 1\beta 2\gamma 2$ L and $\alpha 5\beta 3\gamma 2$ L GABA_A receptors. 3 α -OH-MproGA did not significantly affect the currents evoked by 30 μ M GABA at $\alpha 1\beta 2\gamma 2$ L receptors (effect on AUC: $-1.0 \pm 5.9\%$, $n = 14$, for 10 μ M 3 α -OH-MproGA and $1.3 \pm 13.3\%$, $n = 6$, for 20 μ M 3 α -OH-MproGA). Neither did 3 β -OH-MproGA significantly affect the currents evoked by 30 μ M GABA at the same receptor subtype (effect on AUC: $5.0 \pm 3.2\%$, $n = 21$, for 10 μ M and $0.7 \pm 4.1\%$, $n = 5$, for 20 μ M of 3 β -OH-MproGA).

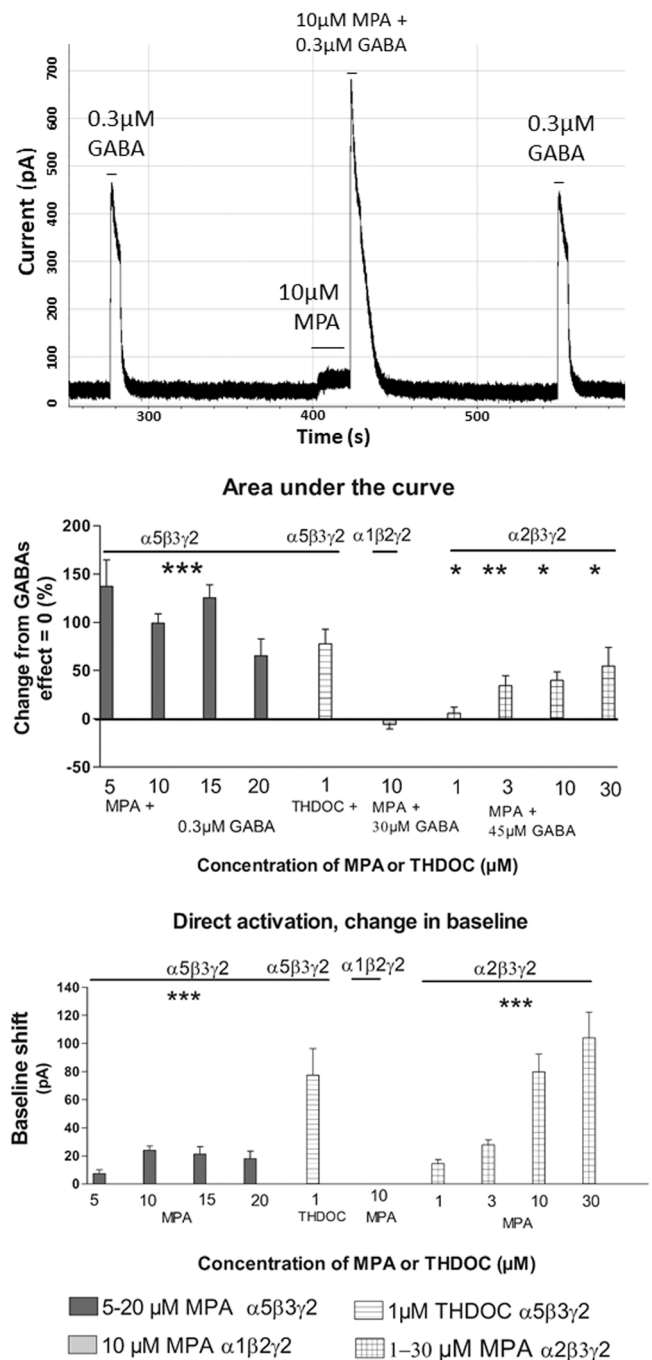


Fig. 3. Effects of MPA and THDOC on GABA-evoked currents in HEK-293 cells permanently expressing human GABA_A receptors. Top panel shows currents from a single $\alpha 5\beta 3\gamma 2$ L-expressing cell at application of GABA alone, MPA alone and GABA + MPA. Middle panel, relative % increase in AUC in comparison with GABA alone (set as 0) at addition of indicated concentrations (in μ M) of MPA and THDOC to GABA (0.3 μ M at $\alpha 5\beta 3\gamma 2$ L and 45 μ M at $\alpha 2\beta 3\gamma 2$ S and 30 μ M $\alpha 1\beta 2\gamma 2$ L receptors). Number of cells in $\alpha 5\beta 3\gamma 2$ L (5 μ M: $n = 4$; 10–20 μ M: $n = 6–11$), $\alpha 2\beta 3\gamma 2$ S ($n = 8$) and $\alpha 1\beta 2\gamma 2$ L (10 μ M: $n = 11$). Bottom panel, current evoked (“direct activation”) by MPA or THDOC alone same number of cells as in middle panel. Significance compared to GABA alone $* = p < 0.02$, $** = p < 0.01$, $*** = p < 0.001$.

At $\alpha 5\beta 3\gamma 2$ L GABA_A receptors, neither 10 μ M 3 α -OH-MproGA nor 10 μ M 3 β -OH-MproGA affected the currents evoked by 0.3 μ M GABA at $\alpha 5\beta 3\gamma 2$ L GABA_A receptors (effects on AUC: $-14.1 \pm 8.5\%$ and $-16.2 \pm 13.3\%$, $n = 4$ respectively). The possible effect of 10 μ M 3 α -OH-MproGA on the currents evoked by 200 nM THDOC + 30 μ M GABA in

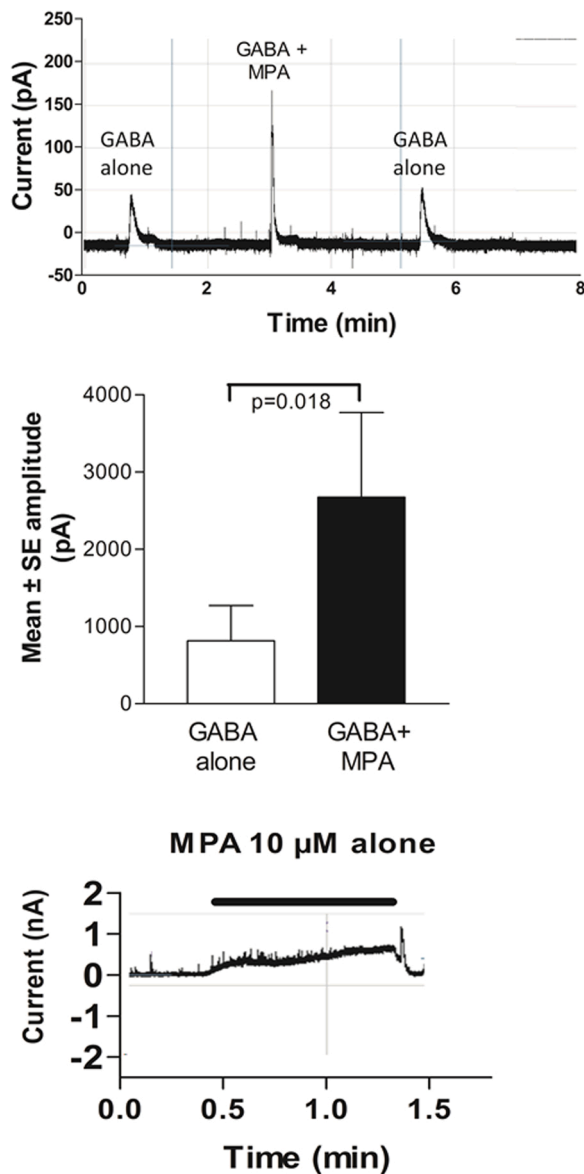


Fig. 4. Top panel: Current recording in one hypothalamic cell at -51 mV during application of GABA alone ($100 \mu\text{M}$) and GABA ($100 \mu\text{M}$) + MPA ($10 \mu\text{M}$).

Middle panel: peak current amplitude (mean \pm SEM) at -11 mV and application of GABA alone ($100 \mu\text{M}$) and GABA ($100 \mu\text{M}$) + MPA ($10 \mu\text{M}$), $n = 7$.

Bottom panel: Current recording at -11 mV showing direct effect (baseline shift) of $10 \mu\text{M}$ MPA alone on a dissociated preoptic neuron.

$\alpha 1\beta 2\gamma 2$ L receptors was also studied, but no significant effect (AUC: $-7.5 \pm 5.1\%$, $n = 5$) was observed. The effect of THDOC by itself is shown in Fig. 3.

3.3. Effects of MPA and THDOC on different GABAA receptor subtypes

3.3.1. GABAA receptor $\alpha 1\beta 2\gamma 2$ L

Mexdroxyprogesterone acetate was tested up to $10 \mu\text{M}$ in the most common type of GABAA receptors, $\alpha 1\beta 2\gamma 2$ L. MPA showed no significant effect on peak amplitude, area under the curve (AUC) or decay time constant (τ), of the current evoked by $30 \mu\text{M}$ GABA (Fig. 2, $n = 11$). As expected, THDOC acted as a strong dose-dependent positive modulator of the $\alpha 1\beta 2\gamma 2$ L receptor subtype. Significant positive modulation of the $30 \mu\text{M}$ GABA-mediated current was shown for AUC by 100 nM ($+77.7 \pm 12.9\%$; $p < 0.001$), 300 nM ($+103.9 \pm 32.4\%$; $p < 0.001$) and $1.0 \mu\text{M}$

($117 \pm 24.3\%$; $p < 0.01$) THDOC ($n = 6-8$ cells/concentration of THDOC).

3.3.2. MPA effects on $\alpha 5\beta 3\gamma 2$ L and $\alpha 2\beta 3\gamma 2$ S GABAA receptors

When MPA was tested together with GABA at the $\alpha 5\beta 3\gamma 2$ L GABAA receptor, the results clearly show that MPA is a positive modulator at this receptor subtype. As shown in Fig. 3 top panel, $10 \mu\text{M}$ MPA potentiated the effect of GABA causing both an increased peak amplitude and a prolonged current, with increased deactivation time constant (τ). It is also clear that $10 \mu\text{M}$ MPA induces a current in the absence of GABA, here hypothetically interpreted as due to a direct activation of $\alpha 5\beta 3\gamma 2$ L GABAA receptors by MPA. At all concentrations tested ($5-20 \mu\text{M}$), MPA significantly increased the current responses compared to GABA at $\alpha 5\beta 3\gamma 2$ L and $\alpha 2\beta 3\gamma 2$ S receptors respectively (Fig. 3, middle panel). A significant current change (baseline shift) induced by MPA alone (presumed “direct activation”) was seen at all concentrations tested ($5-20 \mu\text{M}$, Fig. 3, bottom panel). The effects of MPA are like the effects caused by THDOC (shown for reference in Fig. 3) at receptor subtype $\alpha 5\beta 3\gamma 2$ L, but THDOC has a higher potency. As reflected by the increased AUC, MPA increased the deactivation time constant (τ) in a concentration-dependent manner up to $15 \mu\text{M}$ at $\alpha 5\beta 3\gamma 2$ L GABAA receptor subunits ($p < 0.05$ to $p < 0.001$, $n = 5-10$) whereas $20 \mu\text{M}$ MPA slightly reduced the time constant. $10 \mu\text{M}$ MPA had no effect on $\alpha 1\beta 2\gamma 2$ L-expressing cells ($n = 5$). At $\alpha 2\beta 3\gamma 2$ S GABAA receptors, MPA ($1.0-30 \mu\text{M}$) enhanced the responses to GABA ($45 \mu\text{M}$, EC_{75}) (Fig. 3).

The positive control THDOC ($1.0 \mu\text{M}$) also significantly affected the GABA-evoked current (AUC) in $\alpha 2\beta 3\gamma 2$ S-expressing cells as well as evoked a current change (“direct activation”) in the absence of GABA when applied to the same cells (data not shown).

3.4. MPA effects on dissociated neurons from the rat preoptic area in hypothalamus

3.4.1. Effects by MPA on GABA-evoked currents in dissociated neurons from the rat preoptic area

The effect of MPA on GABA-evoked currents was tested at a steady holding potential of -51 mV (12 cells) and -11 mV (7 cells). Compensation for liquid junction potential was maintained. At both holding potentials, MPA increased the amplitude of GABA-evoked current (-51 mV: 343% of control, $p = 0.011$, Friedman test, Fig. 4, top; -11 mV: 229% of control, $p = 0.018$, Wilcoxon test, Fig. 4, middle). The effect of MPA was reversible (Fig. 4, top). In the ad hoc test of recordings at -51 mV holding potential, co-application of MPA ($10 \mu\text{M}$) and GABA ($100 \mu\text{M}$) resulted in higher response current amplitude compared to that at GABA alone before ($p = 0.008$) and GABA alone after ($p = 0.034$) co-application of GABA and MPA.

3.4.2. Effect by MPA on baseline current suggesting direct activation

At -51 mV holding potential, MPA alone did not evoke any significant baseline shift (presumed “direct activation” of the GABAA receptor). However, at -11 mV, where the driving force for Cl^- currents through GABAA receptors is larger, MPA alone did induce a baseline shift in 6 out of 13 cells (46% of tested cells) (Fig. 4, bottom shows traces in a single cell).

3.4.3. Effects of MPA on decay of GABA-evoked currents in dissociated cells from rat preoptic area

MPA did neither significantly affect the desensitization (in the presence of GABA) nor the deactivation (after the end of GABA application) time constants. At a steady holding potential of -11 mV, the desensitization time constant in GABA alone was 2599 ± 427 ms (mean \pm SE) and in GABA + MPA it was 3457 ± 1646 ms ($n = 5$). At -51 mV steady holding potential, the corresponding values were 5054 ± 708 ms, $n = 7$, for GABA alone and 3602 ± 773 ms, $n = 7$, for GABA + MPA (not significantly different, Wilcoxon’s signed rank test).

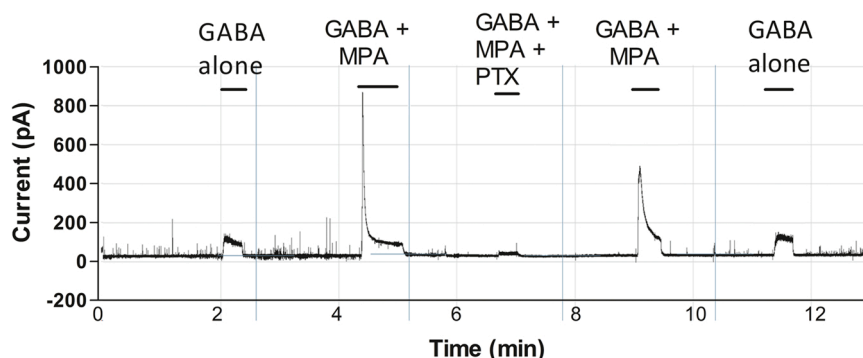


Fig. 5. Current recording at -11 mV during application of GABA ($100 \mu\text{M}$) alone, GABA ($100 \mu\text{M}$) + MPA ($10 \mu\text{M}$) and picrotoxin (PTX, $200 \mu\text{M}$). Note that a major fraction of the GABA-evoked current is blocked by PTX.

3.4.4. Effects of picrotoxin on MPA-induced current changes

We tested the effect of picrotoxin (PTX, a blocker of the chloride channel in the GABA_A receptor) to confirm that MPA was indeed acting at the GABA_A receptor in preoptic neurons. In all eight cells tested, PTX ($200 \mu\text{M}$) blocked 94.5% of the current response to co-application of GABA ($100 \mu\text{M}$) and MPA ($10 \mu\text{M}$) (Fig. 5), suggesting that the additional current evoked by adding MPA to GABA is indeed mediated by GABA_A receptors.

4. Discussion

In the results above, we have four new findings: 1). MPA directly, in the absence of GABA, evokes a current in cells expressing $\alpha 5\beta 3\gamma 2\text{L}$ and $\alpha 2\beta 3\gamma 2\text{S}$ GABA_A-receptor subtypes but not in those expressing the $\alpha 1\beta 2\gamma 2\text{L}$ subtype, 2). MPA is a positive GABA_A-receptor modulator. 3). MPA is a selective positive GABA_A-receptor modulator, as it does not affect the $\alpha 1\beta 2\gamma 2\text{L}$ GABA_A receptor subtype. Thus, the MPA effect on the GABA_A receptor depends on the α subunit, and 4). Surprisingly, MPA's $3\alpha\text{-OH}$ metabolites do not affect the investigated GABA_A receptors, in spite of their $3\alpha\text{-OH}$ structure, a similar A-ring steroid structure as of e. g., the potent GABA_A-receptor modulator allopregnanolone.

The current induced by MPA alone, similar to what is often termed “direct activation” of GABA_A receptors by steroids (Chen et al., 2019), may in principle be due either to MPA-induced activation, i.e., opening, of closed GABA_A receptors, but may also be due to potentiation of GABA_A receptors that are open in the absence of externally applied GABA (Birnr et al., 2000). Indeed, PTX-sensitive GABA_A receptors have been shown to account for a major fraction of the resting Cl^- conductance in preoptic neurons (Yelhekar et al., 2017).

We and others have earlier shown that positive GABA_A receptor modulating steroids have negative effects on memory and learning (Johansson et al., 2002; Vallee et al., 2001). The findings of the present study implicate that MPA might influence memory and cognitive processes via positive modulation of specific GABA_A receptor subtypes, such as those investigated in this study, $\alpha 5\beta 3\gamma 2\text{L}$ and $\alpha 2\beta 3\gamma 2\text{S}$. Our hypothesis was that especially the $3\alpha\text{-hydroxy-5}\alpha\text{-reduced}$ MPA-metabolite should be active at GABA_A receptors, as steroid compounds with a similar $3\alpha\text{-OH}$ structure have positive modulatory effects at GABA_A receptors but not steroids with a ketone at 3-position (Hogenkamp et al., 1997). To our surprise, $3\alpha 5\alpha\text{-MPA}$ or $3\alpha\text{-OH-MproGA}$ did not modulate the $\alpha 1\beta 2\gamma 2\text{L}$ - or the $\alpha 5\beta 3\gamma 2\text{L}$ - GABA_A receptors, while MPA itself, which have a ketone at the 3-position, was found to be a positive modulator of the GABA_A receptor subtypes $\alpha 5\beta 3\gamma 2\text{L}$ and $\alpha 2\beta 3\gamma 2\text{S}$.

As shown here, MPA itself acts as a positive GABA_A-receptor modulator and “direct activator” (see above) at specific GABA_A-receptor subtypes. MPA has been shown to inhibit learning and memory (Braden et al., 2010, 2011). This is interesting as continuous long-term exposure to low stress concentrations of another positive GABA_A receptor-modulating steroid, allopregnanolone, permanently

deteriorates memory and learning in transgenic Alzheimer mice (Bengtsson et al., 2012, 2013) and, after a longer exposure (5 months), also in wild type mice (Bengtsson et al., 2016).

However, there are also results showing a positive memory enhancing effect of allopregnanolone when given in high dosages intermittent, once per week, to transgenic AD mice (Chen et al., 2011; Singh et al., 2012). However, when the allopregnanolone injections were given with short interval, that is 3 times/week, a deteriorating effect is noted in transgenic AD mice (Chen et al., 2011) similar to what is noted after continuous exposure in AD-mice. This issue has been described in a review paper where the dual effect of allopregnanolone is discussed in greater detail (Bengtsson et al., 2020).

There is also a vast literature on effects of neuroactive steroids in the brain and a deep review of that literature is out of the scope of this paper. Especially the effect of allopregnanolone has been studied concerning mood, depression, cognition, food intake and appetite with studies in animals and humans. Some of the results are reviewed in these papers (Bengtsson et al., 2020; Bäckström et al., 2011, 2021; Rasmusson et al., 2021; Bortolato et al., 2021; Yilmaz et al., 2019; Holmberg et al., 2018).

The GABA_A receptor subtypes shown to be modulated by MPA were $\alpha 5\beta 3\gamma 2\text{L}$ and $\alpha 2\beta 3\gamma 2\text{S}$. To our knowledge, the effect of MPA on these receptor subtypes has not been described earlier. MPA has been reported not to affect recombinant rat $\alpha 1\beta 3\gamma 2$ GABA_A receptors expressed in *Xenopus laevis* oocytes (Belelli and Herd, 2003), as supported in the present study by the lack of MPA effect at the recombinant human $\alpha 1\beta 2\gamma 2$ GABA_A receptors. In the $\alpha 5\beta 3\gamma 2\text{L}$ receptor subtype, there was an increased deactivation time constant, but no effect was noted in the $\alpha 1\beta 2\gamma 2$ GABA_A receptors. Both in the recombinant human receptors and in dissociated cells from rat hypothalamus, MPA showed a “direct activation” effect. In the $\alpha 2\beta 3\gamma 2\text{S}$ GABA_A receptor this effect was concentration dependent. In the $\alpha 5\beta 3\gamma 2\text{L}$ subtype, $10 \mu\text{M}$ MPA gave a maximal effect, likely due to saturation.

In the dissociated hypothalamic neurons, the modulatory effect of MPA was mainly noted as a potentiation of the GABA-evoked current amplitude and not as a large effect on the deactivation time constant. We have not measured the receptor subtype content of the hypothalamic cells studied and thus the GABA_A receptor subtype composition in the investigated cells is unknown. In the recombinant cells, the GABA_A receptor subtypes are well defined and minimal contribution of unknown GABA-evoked current mediators is expected. In the dissociated neurons studied, however MPA effects could hypothetically be mediated by unknown channels and receptor types. Thus, we tested the effect of PTX, a well-known GABA_A-receptor channel blocker. The blocking effect of PTX on the majority of current evoked by GABA + MPA suggests that the additional current evoked by adding MPA to GABA is indeed mediated by GABA_A receptors.

Other possible effects of MPA on GABA_A receptors via indirect mechanisms has been reported in the literature. *In vitro*, MPA has been shown to be an inhibitor of the enzyme involved in both synthesis and

degradation of allopregnanolone (Penning et al., 1985). In the rat dentate gyrus of the hippocampal area, MPA inhibits the degradation of allopregnanolone and thereby claimed to enhance GABA_A-receptor mediated inhibitory neurotransmission (Belelli and Herd, 2003; Bernardi et al., 2006). In the rat spinal cord, on the contrary, MPA is shown to reduce the endogenous concentration of allopregnanolone and decrease the effect on the GABA_A receptor (Meyer et al., 2008). The results in these studies show disparate effects of MPA via action on enzymes that either increase or decrease concentration of allopregnanolone. It is thus unclear how MPA affects endogenous allopregnanolone concentrations and to what extent in vivo effects of MPA can be attributed to allopregnanolone.

The GABA_A-receptor subtypes studied here were chosen for several reasons. The $\alpha 1$ subunit containing GABA_A receptor is the most common receptor subtype in the brain and is an intrasynaptic receptor with rapid response (Belelli and Lambert, 2005; Olsen and Sieghart, 2008; Ghit et al., 2021). The $\alpha 1$ subunit is thought to be involved in sedation and anesthesia and, $3\alpha 5\alpha$ -reduced progesterone metabolites are potent positive modulators of GABA_A receptors with sedative effect (Norberg et al., 1987), but as MPA can induce anesthesia, the $\alpha 1$ subunit was an obvious choice (Meyerson, 1967). GABA_A receptors including the $\alpha 5$ subunit are mainly found in the hippocampus, have been implicated in memory and learning and are discussed in relation to dementia development (Shumaker et al., 2003; Johansson et al., 2002; Collinson et al., 2002; Maubach et al., 2003; Birzniece et al., 2006). MPA is also known to induce negative mood, and $\alpha 2$ subunit containing GABA_A receptors are present in the amygdala and seem to be related to disturbed mood (Löw et al., 2000; Korpi et al., 2002).

The enzymes $5\alpha R$ and $3\alpha HSD$ are present in the brain and the metabolism of MPA into GABA_A-receptor-active metabolites seems a possible explanation to MPA-induced changes seen in women (Shumaker et al., 2003) and in rat (Meyerson et al., 1967). However, as $3\alpha 5\alpha$ -MPA in this study was devoid positive modulating activity at GABA_A receptors this hypothesis is not supported. Rather, it seems likely that unmetabolized MPA directly induced the observed effects.

It is well known that progesterone can be metabolized in the brain. Less is known about the metabolism of MPA in the brain. The metabolism of MPA in the liver is mainly via hydroxylation, at positions C6 β , C21, C2 β , and C1 β , mediated primarily via CYP3A4, (Kuhl, 2005). But it has been found that the major hepatic, adrenal and gonadal CYP isozymes contribute very little to the overall steroid metabolism in brain (Miksys et al., 2004). However, 3-hydroxy and 5-dihydro and 3,5-tetrahydro metabolites of MPA are also formed in the brain. (Kuhl, 2001; Stanczyk and Bhavnani, 2015).

MPA concentration in plasma has been determined by liquid chromatography-atmospheric-pressure ionization tandem mass spectrometry (LC-APCI-MS/MS). MPA given subcutaneously with Alzet osmotic pumps gave a plasma concentration of 6.67 ± 1.08 ng/ml = 21 nM (Braden et al., 2010). The concentration of allopregnanolone needed to activate GABA_A receptors in the hypothalamus of the rat is as low as 2 nM (Löfgren et al., 2019). However, as seen in the results above, THDOC is more potent than MPA and a higher concentration of MPA was required for a similar effect. However, a continuous concentration of 21 nM MPA for 66 days gives an impairment of memory and learning in rats (Braden et al., 2010).

The present experiments on dissociated neurons from rat hypothalamus confirm the effect of MPA as a positive modulator of PTX-sensitive GABA_A receptors. MPA also likely has a direct activating effect alone as seen at least in some of the cells analysed. We do not know the receptor subtypes in the hypothalamic cells studied, but we assume that some of these cells may contain the $\alpha 1$ and do not have $\alpha 5$ or $\alpha 2$ receptor subunits as $\alpha 1$ is highly expressed in hypothalamus (Korpi et al., 2002; Wisden et al., 1992).

Surprisingly, the results from the hypothalamic neurons show that MPA have different pharmacokinetic properties compared to allopregnanolone. MPA mainly affects the amplitude of GABA-evoked currents

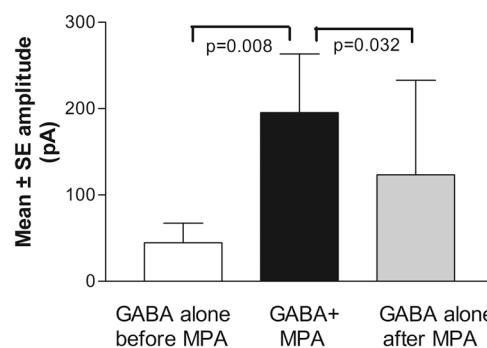


Fig. A1. Mean \pm SE amplitude, during recording at -51 mV, at application of GABA alone (100 μ M) and GABA + medroxyprogesterone (100 μ M + 10 μ M), $n = 12$.

while allopregnanolone and THDOC mainly affects the deactivation time constant of GABA-evoked currents in the human recombinant GABA_A receptors as well as the decay time constant of miniature inhibitory postsynaptic currents (Haage and Johansson, 1999). Our expectation was that MPA would prolong the effect of GABA on the GABA_A receptors, e.g. like the neurosteroids allopregnanolone and THDOC, but MPA did not. The different effects of MPA and of allopregnanolone on GABA_A-receptor kinetics will be investigated in more detail in forthcoming studies.

5. Conclusions

These results show that MPA directly affects $\alpha 5$ - and $\alpha 2$ -subunit-containing GABA_A receptors and positively modulates the effect of GABA on such receptor subtypes. MPA does not affect the $\alpha 1\beta 2\gamma 2L$ GABA_A-receptor subtype. Unexpectedly, MPA's 3α -OH metabolites did not affect the studied receptors, rather the effects were ascribed to MPA as such. These findings imply that MPA is also likely to directly influence memory and cognitive processes while MPA's 3α -OH metabolites, not effective as GABA_A receptor modulators, are less likely to modulate memory function.

Conflict of Interest

This study is supported by an EU-grant, H2020 project number 721802, to Umeçrine via the consortium SYNDEGEN for RD, Umeçrine AB, Umeçrine Cognition AB, and Umeå University Medical Faculty (Karin and Harald Silvander fund). TB has shares in Umeçrine AB. JS, DH and GR has been employed by Umeçrine AB and MJ by Umeçrine Cognition AB. Other investigators have no conflict of interest.

Acknowledgements

This study is supported by an EU-grant, H2020 project number 721802, to Umeçrine via the consortium SYNDEGEN for RD, Umeçrine AB, Umeçrine Cognition AB, and Umeå University Medical Faculty (Karin and Harald Silvander fund). TB has shares in Umeçrine AB. JS, DH, RD and GR has been employed by Umeçrine AB and MJ by Umeçrine Cognition AB.

Appendix

see Fig. A1.

References

- Bäckström, T., Das, R., Bixo, M., 2021. Positive GABA_A receptor modulating steroids and their antagonists – implications for clinical treatments. *J. Neuroendocrinol.* <https://doi.org/10.1111/jne.13013>.

- Bäckström, T., Haage, D., Löfgren, M., Johansson, I.M., Strömberg, J., Nyberg, S., Andréen, L., Ossewaarde, L., Wingen, G.A., Turkmen, S., Bengtsson, S.K., 2011. Paradoxical effects of GABA-A modulators may explain sex steroid induced negative mood symptoms in some persons. *Neuroscience* 191, 46–54. Sep 15.
- Bauer, L.O., Yang, B.-Z., Houston, R.J., Kranzler, H.R., Gelernter, J., 2012. *GABRA2* genotype, impulsivity, and body mass. *Am. J. Addict.* 21 (5), 404–410.
- Belelli, D., Herd, M.B., 2003. The contraceptive agent Provera enhances GABAA receptor-mediated inhibitory neurotransmission in the rat hippocampus: Evidence for endogenous neurosteroids? *J. Neurosci.* 23 (31), 10013–10020.
- Belelli, D., Lambert, J.J., 2005. Neurosteroids: endogenous regulators of the GABA(A) receptor. *Nat. Rev. Neurosci.* 6 (7), 565–575.
- Bengtsson, S., Bäckström, T., Brinton, R., Irwin, R., Johansson, M., Sjöstedt, J., Wang, M., 2020. GABA-A receptor modulating steroids in acute and chronic stress; relevance for cognition and dementia. *Neurobiol. Stress* 12 (100206), 1–20. <https://doi.org/10.1016/j.ynstr.2019.100206>.
- Bengtsson, S.K., Johansson, M., Bäckström, T., 2016. Long-term continuous allopregnanolone elevation causes memory decline and hippocampus shrinkage, in female wildtype B6 mice. *Horm. Behav.* 78, 160–167.
- Bengtsson, S.K., Johansson, M., Bäckström, T., Nitsch, R.M., Wang, M., 2013. Brief but chronic increase in allopregnanolone cause accelerated AD pathology differently in two mouse models. *Curr. Alzheimer Res.* 10 (1), 38–47.
- Bengtsson, S.K., Johansson, M., Bäckström, T., 2012. Chronic allopregnanolone treatment accelerates alzheimer's disease development in $\Delta\text{PppSwePSEN1}\Delta\text{E9}$ mice. *J. Alzheimer's S. Dis.* 31 (1), 71–84.
- Bernardi, F., Pluchino, N., Pieri, M., Begliuomini, S., Lenzi, E., Puccetti, S., Casarosa, E., Luisi, M., Riccardo Genazzani, A., 2006. Progesterone and medroxyprogesterone acetate effects on central and peripheral allopregnanolone and beta-endorphin levels. *Neuroendocrinology* 83, 348–359.
- Birnir, B., Everitt, A.B., Lim, M.S., Gage, P.W., 2000. Spontaneously opening GABA(a) channels in CA1 pyramidal neurones of rat hippocampus. *J. Membr. Biol.* 174 (1), 21–29 <https://doi.org/10.1007/s002320001028>.
- Birnie, V., Bäckström, T., Johansson, I.M., Lindblad, C., Lundgren, P., Löfgren, M., et al., 2006. Neuroactive steroid effects on cognitive functions with a focus on the serotonin and GABA systems. *Brain Res. Rev.* 51 (2), 212–239.
- Bixo, M., Bäckström, T., 1990. Regional distribution of progesterone and 5 alpha-pregnane-3, 20-dione in rat brain during progesterone-induced "anesthesia". *Psychoneuroendocrinology* 15, 159–162.
- Bortolotto, M., Coffey, B.J., Gabbay, V., Scheggi, S., 2021. Allopregnanolone: the missing link to explain the effects of stress on sex exacerbation? *Jul 30 J. Neuroendocr.*, e13022. <https://doi.org/10.1111/jne.13022>.
- Braden, B., Garcia, A., Mennenga, S., Prokai, L., Villa, S., Acosta, J., Lefort, N., Simard, A., Bimonte-Nelson, H., 2011. Cognitive-impairing effects of medroxyprogesterone acetate in the rat: independent and interactive effects across time. *Psychopharmacology* 218, 405–418.
- Braden, B., Talboom, J.S., Crain, I.D., Simard, A.R., Lukas, R.J., Prokai, L., Scheldrup, M. R., Bowman, B.L., Bimonte-Nelson, H.A., 2010. Medroxyprogesterone acetate impairs memory and alters the GABAergic system in aged surgically menopausal rats. *Neurobiol. Learn. Mem.* 93, 444–453.
- Chen, J., Zhang, J.-W., Yang, L., Li, W., 2009. Structure elucidation of major metabolites from medroxyprogesterone acetate by P450. *Chem. Pharm. Bull.* 57 (8), 835–839.
- Chen, S., Wang, J.M., Irwin, R.W., Yao, J., Liu, L., Brinton, R.D., 2011. Allopregnanolone promotes regeneration and reduces β -amyloid burden in a preclinical model of Alzheimer's disease. *PLoS One* 6, e24293.
- Chen, S., Wang, J.M., Irwin, R.W., Yao, J., Liu, L., Brinton, R.D., 2011. Allopregnanolone promotes regeneration and reduces β -amyloid burden in a preclinical model of Alzheimer's disease. *PLoS One* 6 (8), e24293.
- Chen, Z.-W., Bracamontes, J.R., Budelier, M.M., Germann, A.L., Shin, D.J., Kathiresan, K., Qian, M.-X., Manion, B., Wayland, W., Cheng, L., Reichert, D.E., Akk, G., Covey, D.F., Evers, A.S., 2019. Multiple functional neurosteroid binding sites on GABAA receptors. *PLoS Biol.* 17 (3), e3000157 <https://doi.org/10.1371/journal.pbio.3000157>.
- Coker, L.H., Espeland, M.A., Rapp, S.R., Legault, C., Resnick, S.M., Hogan, P., Gaussoin, S., Dailey, M., Shumaker, S.A., 2010. Postmenopausal hormone therapy and cognitive outcomes: the women's health initiative memory study (WHIMS). *J. Steroid Biochem. Mol. Biol.* 118, 304–310.
- Coker, L.H., Hogan, P.E., Bryan, N.R., Kuller, L.H., Margolis, K.L., Bettermann, K., Wallace, R.B., Lao, Z., Freeman, R., Stefanick, M.L., Shumaker, S.A., 2009. Postmenopausal hormone therapy and subclinical cerebrovascular disease. *Neurology* 72, 125–134.
- Collinson, N., Kuenzi, F.M., Jarolimek, W., Maubach, K.A., Cothliff, R., Sur, C., et al., 2002. Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the alpha 5 subunit of the GABAA receptor. *J. Neurosci.* 22 (13), 5572–5580.
- Fukushima, D.K., Levin, J., Liang, J.S., Smulowitz, M., 1979. Isolation and partial synthesis of a new metabolite of medroxyprogesterone acetate. *Steroids* 34 (1), 57–72.
- Ghit, A., Assal, D., Al-Shami, A.S., Hussein, D.E.E., 2021. GABAA receptors: structure, function, pharmacology, and related disorders. *J. Genet. Eng. Biotechnol.* 19, 123. <https://doi.org/10.1186/s43141-021-00224-0>.
- Haage, D., Johansson, S., 1999. Neurosteroid modulation of synaptic and GABA-evoked currents in neurons from the rat medial preoptic nucleus. *J. Neurophysiol.* 82 (1), 143–151 (Jul).
- Hogenkamp, D.J., Hasan Tahir, S., Hawkinson, J.E., Upasani, R.B., Alauddin, M., Kimbrough, C.L., Acosta-Burrue, M., Whittemore, W.R., Woodward, R.M., Lan, N.C., Gee, K.W., Bolger, M.B., 1997. J. Synthesis and in vitro activity of β -substituted-3 α -hydroxypregnan-20-ones: allosteric modulators of the GABAA receptor. *Med. Chem.* 40, 61–72.
- Holmberg, E., Sjöstedt, J., Malinina, E., Johansson, M., Turkmen, S., Ragagnin, G., Lundqvist, A., Löfgren, M., Jaukkuri, L., Bixo, M., Bäckström, T., 2018. Allopregnanolone involvement in feeding regulation, overeating and obesity. *Front. Neuroendocrinol.* 48, 70–77. <https://doi.org/10.1016/j.ynfe.2017.07.002>.
- Hosie, A.M., Wilkins, M.E., da Silva, H.M., Smart, T.G., 2006. Endogenous neurosteroids regulate GABAA receptors through two discrete transmembrane sites. *Nature* 444, 486–489.
- Johansson, I.M., Birnie, V., Lindblad, C., Olsson, T., Bäckström, T., 2002. Allopregnanolone inhibits learning in the Morris water maze. *Brain Res* 934 (2), 125–131.
- Korpi, E.R., Grunder, G., Luddens, H., 2002. Drug interactions at GABA(A) receptors. *Prog. Neurobiol.* 67 (2), 113–159.
- Kuhl, H., 2001. Pharmacology of progestogens. Basic aspects – progesterone derivatives. *Menopause Rev.* 6, 9–16.
- Kuhl, H., 2005. Pharmacology of estrogens and progestogens: influence of different routes of administration (PDF). *Climacteric* 8 (Suppl 1), 3–63. <https://doi.org/10.1080/13697130500148875>. PMID 16112947. S2CID 24616324.
- Le, Y.-C.L., Rahman, M., Berenson, A.B., 2009. Early weight gain predicting later weight gain among depot medroxyprogesterone acetate users. *Obstet. Gynecol.* 114 (2 Pt 1), 279–284 (August).
- Löfgren, M., Holmberg, E., Bäckström, T., Eggecioglu, E., Dickson, S.L., 2019. The additive effect of allopregnanolone on ghrelin's orexigenic effect in rats (Aug). *Neuropeptides* 76, 101937. <https://doi.org/10.1016/j.npep.2019.101937>.
- Löw, K., Crestani, F., Keist, R., Benke, D., Brunig, I., Benson, J.A., Fritschy, J.M., Rulicke, T., Bluethmann, H., Möhler, H., Rudolph, U., 2000. Molecular and neuronal substrate for the selective attenuation of anxiety. *Science* 290, 131–134.
- Lowry, N.C., Pardon, L.P., Yates, M.A., Juraska, J.A., 2010. Effects of long-term treatment with 17 β -estradiol and medroxyprogesterone acetate on water maze performance in middle-aged female rats. *Horm. Behav.* 58, 200–207.
- Majewska, M.D., Harrison, N.L., Schwartz, R.D., Baker, J.L., Paul, S.M., 1986. Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science* 232, 1004–1007.
- Maubach, K., 2003. GABA(A) receptor subtype selective cognition enhancers. *Curr. Drug Targets CNS Neurol. Disord.* 2 (4), 233–239.
- Melcangi, R.C., Magnaghi, V., Martini, L., 1999. Steroid metabolism and effects in central and peripheral glial cells. *J. Neurobiol.* 40 (4), 471–483.
- Meyerson, B., 1967. Relationship between the anesthetic and gestagenic action and estrous behavior-inducing activity of different progestins. *Endocrinology* 81, 369–374.
- Miksys, S., Tyndale, R.F., 2004. The unique regulation of brain cytochrome P450 2 (CYP2) family enzymes by drugs and genetics. *Drug Metab. Rev.* 36 (2), 313–333 (May).
- Norberg, L., Wahlstrom, G., Bäckström, T., 1987. The anaesthetic potency of 3 alpha-hydroxy-5 alpha-pregnan-20-one and 3 alpha-hydroxy-5 beta-pregnan-20-one determined with an intravenous EEG-threshold method in male rats. *Pharmacol. Toxicol.* 1987 (61), 42–47.
- Olsen, R.W., Sieghart, W., 2008. International Union of Pharmacology. LXX. Subtypes of γ -aminobutyric acid A receptors: classification on the basis of subunit composition, pharmacology, and function. *Pharmacol. Rev.* 60, 243–260. <https://doi.org/10.1124/pr.108.00505>.
- Paul, S.M., Purdy, R.H., 1992. Neuroactive steroids. *Faseb J.* 6 (6), 2311–2322.
- Penning, T.M., Sharp, R.B., Krieger, N.R., 1985. Purification and properties of 3alpha-Hydroxysteroid Dehydrogenase from rat brain cytosol, inhibition by nonsteroidal anti-inflammatory drugs and progestins. *J. Biol. Chem.* 260, 15266–15272.
- Purdy, R.H., Morrow, A.L., Blinn, J.R., Paul, S.M., 1990. Synthesis, metabolism, and pharmacological activity of 3 alpha-hydroxy steroids which potentiate GABA-receptor-mediated chloride ion uptake in rat cerebral cortical synaptosomes. *J. Med. Chem.* 33 (6), 1572–1581.
- Rasmusson, A.M., Pineles, S.L., Brown, K.D., Pinna, G., 2021. A role for deficits in GABAergic neurosteroids and their metabolites with NMDA receptor antagonist activity in the pathophysiology of posttraumatic stress disorder. *Nov 7 J. Neuroendocr.*, e13062. <https://doi.org/10.1111/jne.13062>.
- Shumaker, S.A., Legault, C., Kuller, L., Rapp, S.R., Thal, L., Lane, D.S., Fillit, H., Stefanick, M.L., Hendrix, S.L., Lewis, C.E., Masaki, K., Coker, L.H., 2004. Conjugated equine estrogens and incidence of probable dementia and mild cognitive impairment in postmenopausal women. *JAMA* 291, 2947–2958.
- Shumaker, S.A., Legault, C., Rapp, S.R., Thal, L., Wallace, R.B., Ockene, J.K., Hendrix, S. L., Jones III, B.N., Assaf, A.R., Jacksson, R.D., Wassertheil-Smolser, S., Wactawski-Wende, J., 2003. Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women The Women's Health Initiative Memory Study: a randomized controlled trial. *JAMA* 289 (20), 2651–2662. May 28.
- Simons, J.P.F.H.A., Schols, A.M.W.J., Hoefnagels, J.M.J., Westerterp, K.R., ten Velde, G. P.M., Wouters, E.F.M., 1998. Effects of medroxyprogesterone acetate on food intake, body composition, and resting energy expenditure in patients with advanced, nonhormone-sensitive. *Cancer* 82, 553–560.
- Singh, C., Liu, L., Wang, J.M., Irwin, R.W., Yao, J., Chen, S., et al., 2012. Allopregnanolone restores hippocampal-dependent learning and memory and neural progenitor survival in aging 3xTgAD and nonTg mice. *Neurobiol. Aging* 33 (8), 1493–1506.
- Stanczyk, F.Z., Bhavnani, B.R., 2015. Use of medroxyprogesterone acetate for hormone therapy in postmenopausal women: Is it safe? *J. Steroid Biochem. Mol. Biol.* 153 (151–159) <https://doi.org/10.1016/j.jsbmb.2015.08.013>. PMID 26291834. S2CID 23985966.
- Steward, R.G., Bateman, L.A., Cris Slentz, C., Stanczyk, F.Z., Price, T.M., 2016. The impact of short-term depot-medroxyprogesterone acetate treatment on resting metabolic rate. *Contraception* 93, 317–322.

- Stromberg, J., Bäckström, T., Lundgren, P., 2005. Rapid non-genomic effect of glucocorticoid metabolites and neurosteroids on the gamma-aminobutyric acid-A receptor. *Eur. J. Neurosci.* Vol 21, 2083–2088.
- Strömberg, J., Lundgren, P., Taube, M., Bäckström, T., Wang, M., Haage, D., 2009. The effect of the neuroactive steroid 5 β -pregnane-3 β , 20(R)-diol on the time course of GABA evoked currents is different to that of pregnenolone sulphate. *Eur. J. Pharmacol.* 605, 78–86. <https://doi.org/10.1016/j.ejphar.2008.12.038>.
- Vallee, M., Mayo, W., Koob, G.F., Le Moal, M., 2001. Neurosteroids in learning and memory processes. *Int Rev. Neurobiol.* 46, 273–320.
- Vorobjev, V., 1991. Vibrodissociation of sliced mammalian nervous tissue. *J. Neurosci. Methods* 38, 145–150.
- Wisden, W., Laurie, D.J., Monyer, H., Seeburg, P.H., 1992. The distribution of 13 GABAA receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J. Neurosci.* 12, 1040–1062.
- Yelhekar TD, Druzin M., Johansson S. Contribution of Resting Conductance, GABAA-Receptor Mediated Miniature Synaptic Currents and Neurosteroid to Chloride Homeostasis in Central Neurons. *eNeuro.* 2017 Mar-Apr; 4(2): ENEURO.0019–17.2017.
- Yilmaz, C., Karali, K., Fodelianaki, G., Gravanis, A., Chavakis, T., Charalampopoulos, I., Alexaki, V.I., 2019. Neurosteroids as regulators of neuroinflammation (Oct). *Front Neuroendocr.* 55, 100788. <https://doi.org/10.1016/j.yfrne.2019.100788>.
- Zhang, J.W., Liu, Y., Zhao, J.-Y., Wang, L.-M., Ge, G.-B., Gao, Y., Li, W., Liu, H.-T., Liu, H.-X., Zhang, Y.-Y., Sun, J., Yang, L., 2008. Metabolic profiling and cytochrome P450 reaction phenotyping of medroxyprogesterone acetate. *Drug Metab. Dispos.* 36 (11), 2292–2298.