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

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A R T I C L E   I N F O  
Keywords:  
Medroxyprogesterone-acetate  
GABA-A receptor  
Alpha5 GABA-A receptor  
Neurosteroids  
Dementia  
Positive GABA-AR modulator

A B S T R A C T  
Medroxyprogesterone acetate (MPA) is a progestin widely used in humans as hormone replacement therapy and at other indications. Many progestin metabolites, as the progestosterone 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 α1β2γ2L or α2β3γ2L or α2β3γ2S were created. The MPA metabolites 3x5α-MPA, 3β5α-MPA and 3β5β-MPA were synthesised and purified for electrophysiological patch-clamp measurements with a Dynaflow system. The effects of MPA and tetrahydrodeoxycorticosterone were also studied. None of the studied MPA metabolites affected the responses mediated by α1β2γ2L or α2β3γ2L GABA_A receptors. Contrary, MPA clearly acted both as a positive modulator and as a direct activator of the α2β3γ2L and α2β3γ2S GABA_A receptors. However, in concentrations up to 10 μM, MPA was inactive at the α1β2γ2L 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 (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.

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https://doi.org/10.1016/j.psyneuen.2022.105754  
Received 6 December 2021; Received in revised form 14 February 2022; Accepted 28 March 2022  
Available online 31 March 2022  
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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 a 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 interested to use the same brain region for confirming MPA experiment as the region is very sensitive to GABAergic receptor modulators (Purdy et al., 1990, 1992). THDOC and allopregnanolone bind to the same binding site on the GABA_A receptor (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 (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 3α-hydroxy-A ring 5α 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 of 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 delta4-3keto configuration are first metabolized by a 5α-reduction catalyzed by 5α-reductase (5αR). Thereafter, a 3α-hydroxylation by 3α-hydroxysteroid dehydrogenase (3αHSD) may occur which modifies the steroids to contain a hydroxy group at 3α 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 3α-hydroxy metabolites of MPA were of interest to investigate for effects on the GABA_A receptor subtypes.

The 3αOH A-ring structure in endogenous steroids is important for binding to the GABA_A receptor. Allopregnanolone and tetrahydrodeoxycorticosterone (THDOC) are both 3α5α-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 (Pospiech 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 Umeccrine 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) 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 μm 78 × 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 °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);

3β hydroxy-5α-Medroxyprogrenalone acetate (3β5α-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).

3α hydroxy-5α-Medroxyprogrenalone acetate (3α5α-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).

3β-hydroxy-5β-Medroxyprogrenalone acetate (3β5β-MPA), (400 MHz, CDCl3-d6): δ (ppm) 1.97 (s, 3 H); 2.05 (s, 3 H); 1.08 (s, 3 H); 1.03 (d, 3 H); 0.67 (s, 3 H).

3α-hydroxy-5β-Medroxyprogrenalone acetate (3α5β-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).

3β-hydroxy-Medroxyprogesterone (3β-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).

3α-hydroxy-Medroxyprogesterone (3α-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: 3α5α-MPA : 3β5α-MPA : 3α5β-MPA : 3αβ-MPA, 23:23:45:9.

2.2. Experiment 1: recombinant human GABAA 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 MgCl2, 10 mM HEPES, 1.0 mM EGTA (Sigma Chemical Co., St. Louis, MO, USA), 140 mM Cs-glucuronate (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 Dynaflow® system (Dynaflow Pro II Platform Zeiss Axiovert 25) and the DF-16 Pro II chip (Cellenectron 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 into 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 MΩ 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 Dynaflow® system. The syringe pump flow rate was 26 μl/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 α5β3γ2L GABAA 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 α1β2γ3S and α2β3γ3S are the most common synaptic GABA A receptors. They are exposed to high GABA concentrations (>100 μM) for short periods (ms). Thus, to resemble natural conditions, these receptors were exposed to 30 and 45 μM GABA (EC 3). GABA was applied together with the studied steroid for 40 ms. Studies of the α5β3γ2L receptor was with 0.3 μM GABA together with the studied steroid for 6 s. The responses to 1.0 μM THDOC on HEK-293 α5β3γ2L and α2β3γ3S are shown as reference.

2.4. HEK-293 cells expressing recombinant human GABAA 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 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 α1β2γ3S, α5β3γ2L or α2β3γ3S GABA A receptor subtypes, respectively. The GABA A receptor subunits α1 (308–1727 in NM_000806), β2 (214–1679 in NM_000813), γ2L (290–1785 in NM_198904), α5 (329–1735 in NM_000810), β3 (45–1485 in NM_021912), α2 (199–1571 in NM_000807), γ2S (290–1761 in NM_000816) with introduced Kozak sequences just before the start codons were sub-cloned into the mammalian expression vectors (Invi-trogen); pcDNA3.1(+) for α1 and α5, pcDNA3.1(-) for β2, pcDNA3.1/ Hygro(-) for β2 and β3, and pcDNA3.1/Zeo(-) for γ2S and γ2L. 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 djurförskötselstäta 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 \( \text{O}_2 \) gas) and contained (in mM) as follows:

- 2.0 CaCl\(_2\), 2.0 MgCl\(_2\), 125 NaCl, 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, 2.5 NaCl, 2.5 KCl, 0.5 CaCl\(_2\), 2.5 MgCl\(_2\), 10 HEPES, 10 sucrose, 10 D-glucose. pH was adjusted to 7.4 using NaOH.
- The intracellular solution used for cutting and recovery of brain slices was provided with moderate rate of bubbling for oxygenation (with \( \text{O}_2 \) gas) and contained (in mM): 124 NaCl, 3 KCl, 2.0 CaCl\(_2\), 1.2 MgCl\(_2\), 6 H\(_2\)O, 2 MgSO\(_4\), 10 HEPES, 20 Sucrose, 10 D-glucose. pH was adjusted to 7.4 using NaOH.

A block/part of brain tissue from the rostral part containing the hypothalamic region were prepared and incubated for at least 1 h in incubation solutions at 28 °C. A summary of the procedures is given below.

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 hypothalamic region was dissected. Brain slices (thickness 250 \( \mu \)m) 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 amphoterin-B-perforated patch-clamp technique (Strömbärg 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 (Strömbärg 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 \( \Omega \)M 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 GABAs EC\(_50\)s 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

| GABA\(_A\) receptor | Test compound | MPA conc. (\( \mu \)M) | MPA-induced change in GABA\(_A\)-evoked current (AUC; %) | n | P |
|------------------|--------------|-----------------|---------------------------------|---|---|
| α1(2)/2 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 |
|                  | 3α5β-MPA     | 20              | -59.8                           | 8.9 | -76.9 | -36.2 | 4 | 1 |
|                  | 3αδ-MPA      | 20              | -45.8                           | 5.7 | -57.6 | -34.9 | 4 | 1 |
| α5(3)/2 L        | 3α5α-MPA     | 0.1             | -9.1                            | 2.7 | -28.2 | 0 | 9 | NS |
|                  |              | 0.3             | -4.6                            | 5.7 | -22.6 | + 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 |

* 30 \( \mu \)M with α1(2)/2L GABA\(_A\) receptors and 0.3 \( \mu \)M with α5(3)/2L GABA\(_A\) receptors.
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 test was used to evaluate effects between groups. Each cell (1 and 2) are displayed as mean ± 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 3α- or 3β-hydroxy group in the 3rd position, were tested with patch-clamp recordings for effect at the human GABA_A receptor subtypes α1β2γL and α5β3γL.

None of the 3α-hydroxy-MPA metabolites applied in a concentration of 0.1 – 3.0 μM showed any significant modulation of the GABA response at α1β2γL or at α5β3γL GABA_A receptors (Table 1). A high concentration (20 μM) of 3α-hydroxy-MPA metabolites, tested in four α1β2γL-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 3α-hydroxy-Medroxyprogesterone (3α-OH-MprogA) and 3β-hydroxy-Medroxyprogesterone (3β-OH-MprogA) on GABA-evoked currents mediated by α1β2γL and α5β3γL receptors

The MPA metabolites 3α-OH-MprogA and 3β-OH-MprogA were synthesized and tested for modulatory effects on GABA responses mediated by α1β2γL and α5β3γL GABA_A receptors. 3α-OH-MprogA did not significantly affect the currents evoked by 30 μM GABA at α1β2γL receptors (effect on AUC: -1.0 ± 5.9%, n = 14, for 10 μM 3α-OH-MprogA and 1.3 ± 13.3%, n = 6, for 20 μM 3α-OH-MprogA). Neither did 3β-OH-MprogA significantly affect the currents evoked by 30 μM GABA at the same receptor subtype (effect on AUC: 5.0 ± 3.2%, n = 21, for 10 μM and 0.7 ± 4.1%, n = 5, for 20 μM of 3β-OH-MprogA).

At α5β3γL GABA_A receptors, neither 10 μM 3α-OH-MprogA nor 10 μM 3β-OH-MprogA affected the currents evoked by 0.3 μM GABA at α5β3γL GABA_A receptors (effects on AUC: -14.1 ± 8.5% and -16.2 ± 13.3%, n = 4 respectively). The possible effect of 10 μM 3α-OH-MprogA on the currents evoked by 200 nM THDOC + 30 μM GABA in

Fig. 2. Effects of MPA on responses to 30 μM GABA applied to HEK-293 cells expressing human α1β2γL GABA_A receptors (n = 11). None of the measured relative effects are significantly different from 0.

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 α5β3γL-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 μM) of MPA and THDOC to GABA (0.3 μM at α5β3γL and 45 μM at α1β2γL (10 μM: n = 11). Bottom panel, current evoked (“direct activation”) by MPA or THDOC alone as a number of cells as in middle panel. Significance compared to GABA alone % = p < 0.02, *p < 0.01, **p < 0.001.

At α5β3γL GABA_A receptors, neither 10 μM 3α-OH-MprogA nor 10 μM 3β-OH-MprogA affected the currents evoked by 0.3 μM GABA at α5β3γL GABA_A receptors (effects on AUC: -14.1 ± 8.5% and -16.2 ± 13.3%, n = 4 respectively). The possible effect of 10 μM 3α-OH-MprogA on the currents evoked by 200 nM THDOC + 30 μM GABA in
3.3. Effects of MPA and THDOC on different GABAA receptor subtypes

3.3.1. GABA<sub>A</sub> receptor α1β2γ2L

Methoxyprogesterone acetate was tested up to 10 μM in the most common type of GABA<sub>A</sub> receptors, α1β2γ2L. MPA showed no significant effect on peak amplitude, area under the curve (AUC) or decay time constant (tau), of the current evoked by 30 μM GABA (Fig. 2, n = 11). As expected, THDOC acted as a strong dose-dependent positive modulator of the α1β2γ2L receptor subtype. Significant positive modulation of the 30 μM GABA-mediated current was shown for AUC by 100 nM (+77.7 ± 12.9%; p < 0.001), 300 nM (+103.9 ± 32.4%; p < 0.001) and 1.0 μM (117 ± 24.3%; p < 0.001) THDOC (n = 6–8 cells/concentration of THDOC).

3.3.2. MPA effects on α5β3γ2L and α2β3γ2S GABAA receptors

When MPA was tested together with GABA at the α5β3γ2L GABAA receptor, the results clearly show that MPA is a positive modulator at this receptor subtype. As shown in Fig. 3 top panel, 10 μM MPA potentiated the effect of GABA causing both an increased peak amplitude and a prolonged current, with increased deactivation time constant (Tau). It is also clear that 10 μM MPA induces a current in the absence of GABA, here hypothetically interpreted as due to a direct activation of α5β3γ2L GABAA receptors by MPA. At all concentrations tested (5–20 μM), MPA significantly increased the current responses compared to GABA at α5β3γ2L and α2β3γ2S 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 μM, Fig. 3, bottom panel). The effects of MPA are like the effects caused by THDOC (shown for reference in Fig. 3) at receptor subtype α5β3γ2L, but THDOC has a higher potency. As reflected by the increased AUC, MPA increased the deactivation time constant (Tau) in a concentration-dependent manner up to 15 μM at α5β3γ2L GABAA receptor subunits (p < 0.05 to p < 0.001, n = 5–10) whereas 20 μM MPA slightly reduced the time constant. 10 μM MPA had no effect on α1β2γ2L-expressing cells (n = 5). At α2β3γ2S GABAA receptors, MPA (1.0–30 μM) enhanced the responses to GABA (45 μM, EC<sub>50</sub>) (Fig. 3).

The positive control THDOC (1.0 μM) also significantly affected the GABA-evoked current (AUC in α2β3γ2S-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 μM) and GABA (100 μM) 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<sup>−</sup> 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 not 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 ± 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).
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 μM) blocked 94.5% of the current response to co-application of GABA (100 μM) and MPA (10 μM) (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 α5β3γ2L and α2β3γ2S GABA_A-receptor subtypes but not in those expressing the α1β2γ2L 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 α1β2γ2L GABA_A receptor subtype. Thus, the MPA effect on the GABA_A receptor depends on the α subunit, and 4). Surprisingly, MPA’s 3α-OH metabolites do not affect the investigated GABA_A receptors, in spite of their 3α-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 (Birnir 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 (Yelshekar 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 modulation of specific GABA_A receptor subtypes, such as those investigated in this study, α5β3γ2L and α2β3γ2S. Our hypothesis was that especially the 3α-hydroxy-5α-reduced MPA-metabolite should be active at GABA_A receptors, as steroid compounds with a similar 3α-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, 3αS,5α-MPA or 3α-OH-MprogA did not modulate the α1β2γ2L- or the α5β3γ2L-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 α5β3γ2L and α2β3γ2S.

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 intermittently, 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 were 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; Bäckstrom et al., 2011, 2021; Rasmussen 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 α5β3γ2L and α2β3γ2S. 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 α1β2γ2 GABA_A receptors expressed in Xenopus laevis oocytes (Belelli and Herd, 2005), as supported in the present study by the lack of MPA effect at the recombinant human α1β2γ2 GABA_A receptors. In the α5β3γ2L receptor subtype, there was an increased activation time constant, but no effect was noted in the α1β2γ2 GABA_A receptors. Both in the recombinant human receptors and in dissociated cells from rat hypothalamus, MPA showed a “direct activation” effect. In the α2β3γ2S GABA_A receptor this effect was concentration dependent. In the α5β3γ2L subtype, 10 μM 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<sub>α</sub>-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<sub>α</sub> receptor (Meyer, 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<sub>α</sub>-receptor subtypes studied here were chosen for several reasons. The α1 subunit containing GABA<sub>α</sub> receptor is the most common receptor subtype in the brain and is an intrasynaptic receptor with rapid response (Belelli, and Lambert, 2005; Olesen and Sieghart, 2008; Ghit et al., 2021). The α1 subunit is thought to be involved in sedation and anesthesia and, 3α5α-reduced progesterone metabolites are potent positive modulators of GABA<sub>α</sub> receptors with sedative effect (Norberg et al., 1987), but as MPA can induce anesthesia, the α1 subunit was an obvious choice (Meyerson, 1967). GABA<sub>α</sub> receptors including the α5 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; Biziricze et al., 2006). MPA is also known to induce negative mood, and α2 subunit containing GABA<sub>α</sub> receptors are present in the amygdala and seem to be related to disturbed mood ( Löw et al., 2000; Korpi et al., 2002).

The enzymes 5αR and 3αHSD are present in the brain and the metabolism of MPA into GABA<sub>α</sub>-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 3α5α-MPA in this study was devoid positive modulating activity at GABA<sub>α</sub> 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 C6α, C21, C2β, and C1β, 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<sub>α</sub> receptors in the hypothalamus of the rat is as low as 2 nM (Löfqren 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<sub>α</sub> 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 α1 and do not have α5 or α2 receptor subunits as α1 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 while allopregnanolone and THDOC mainly affects the deactivation time constant of GABA-evoked currents in the human recombinant GABA<sub>α</sub> 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<sub>α</sub> receptors, e.g. like the neurosteroids allopregnanolone and THDOC, but MPA did not. The different effects of MPA and of allopregnanolone on GABA<sub>α</sub>-receptor kinetics will be investigated in more detail in forthcoming studies.

5. Conclusions

These results show that MPA directly affects α5- and α2-subunit-containing GABA<sub>α</sub> receptors and positively modulates the effect of GABA on such receptor subtypes. MPA does not affect the α1β2γ2L MABA<sub>α</sub>-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<sub>α</sub> 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 Umeå via the consortium SYndeGen for RD, Umeå AB, Umecrine Cognition AB, and Umeå University Medical Faculty (Karin and Harald Silvander fund). TB has shares in Umeå AB. JS, DH and GR has been employed by Umeå AB and MJ by Umeå Cognition AB. Other investigators have no conflict of interest.

Acknowledgements

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Appendix

see Fig. A1.

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