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Original Communication

Rapid non-genomic effect of corticosterone and its two tetrahydrometabolites on the $GABA_A$-receptor

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ABSTRACT

In the present work, the effect of corticosterone, 3α5α-corticosterone and 3α5β-corticosterone on GABA mediated chloride flux were studied in rat cortical microsacs. All three steroids reduce the chloride ion uptake with a maximum of approximately -30%. The reduction induced by corticosterone and 3α5α-corticosterone showed a concentration-response pattern with IC_{50} values of 1.6 nM and 1.0 nM respectively. The reducing effect cannot be seen in the absence of GABA. The GABAergic transmission plays a crucial role in pathophysiology of diseases such as anxiety and in mental disorders such as depression, schizophrenia and drugs of abuse. The pathophysiology of these disorders can to some extent be related to changes in neurosteroid levels and thereby to the modulations of $GABA_A$ receptor function. The well-known $GABA_A$-receptor modulators, allopregnanolone and tetrahydrodeoxycorticosterone, are released during stress and originate from the same branched synthetic pathway as the species-specific glucocorticoid, corticosterone, in rats.

KEYWORDS: allopregnanolone, chloride ion flux, corticosterone, gamma-aminobutyric acid-A receptor, rat, stress

INTRODUCTION

In the last twenty years it has become apparent that stress, changes in neurosteroid levels and the immediate changes in GABAergic transmission play a crucial role in neuropsychiatric conditions such as anxiety disorders, schizophrenia and depression. Two decades ago, observations that acute stress in rats induced changes in the GABAergic transmission provide the association between $GABA_A$ receptor and stress [1-5]. Stress activates the hypothalamic-pituitary-adrenal axis resulting in release of glucocorticoids. All events that elevate activities of physiological systems are defined as stressors [6]. Short-term effects of stress in adult nervous system as well as long-term stress in developing nervous system are involved in the onset of neuropsychiatric conditions such as anxiety disorders, schizophrenia and depression [7]. The GABAergic transmission has become one of the most fascinating fields of research in neuropsychopharmacology since GABA seem to play a crucial role not only in anxiety disorders but also in mental disorders such as depression, schizophrenia and drugs of abuse [8]. The classical stress hormones, glucocorticoids, as well as the progesterone metabolites, allopregnanolone and tetrahydrodeoxycorticosterone (THDOC), are released during stress and produced in the adrenal gland [5, 8-10]. Allopregnanolone can also be synthesized within the central nervous system de novo from cholesterol [6]. As a positive $GABA_A$ receptor modulator, allopregnanolone and THDOC have anxiolytic, analgesic, anticonvulsant, sedative and anaesthetic properties [11-13]. The metabolism of progesterone to corticosterone and further may take different paths. Approximately 45% of

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corticosterone is metabolized into 3α5α-corticosterone and 3α5β-corticosterone [14]. To obtain the two tetrahydrometabolites the $\Delta^{4.5}$ double bond in the A-ring of corticosterone is first reduced. 3α-hydroxysteroid dehydrogenases then further reduce the formed dihydro-metabolites which results in the formation of 3α5α-corticosterone and 3α5β-corticosterone (Figure 1) [15]. These metabolites share the same basic structure with the well known potent positive $GABA_A$ receptor modulators, allopregnanolone and THDOC [5, 9, 11-13]. Glucocorticoids generally bind to intracellular receptors, glucocorticoid receptor and mineralocorticoid receptor [16] and mediate a genomic response [17, 18]. However, during the last decade research has revealed an alternative non-genomic path of action for glucocorticoids and its metabolites [19-24].

The objectives of the present study were to investigate the effects of the rat species-specific glucocorticoid corticosterone and its two tetrahydrometabolites, 3α5α-corticosterone and 3α5β-corticosterone on the GABA mediated chloride ion uptake. Further, to investigate if corticosterone and its metabolites interact with allopregnanolone enhanced GABA mediated chloride ion uptake. Finally, to study if corticosterone and its metabolites modulate the $GABA_A$ receptor functions in the absence of GABA. All experiments were performed in cortical micro sacs from handled adult male rats.

MATERIALS AND METHODS

Chemicals

[$^{36}Cl^-$] was purchased from Amersham Biotech (Uppsala, Sweden) and GABA (gamma-aminobutyric-acid) from Sigma Chemical Co. (St. Louis, MO, USA). Picrotoxin and Corticosterone (4-pregnene-11β,21-diol-3,21-dione) were purchased from FLUKA Biochemica (Buchs, Switzerland). 3α5α-corticosterone (5α-pregnan-3α,11β,21-triol-20-one) and 3α5β-corticosterone (5β-pregnan-3α,11β,21-triol-20-one) were supplied by Steraloids (Newport, Rhode Island, USA) and BioRad D_C Protein Assay was purchased from BioRad Laboratories (San Francisco, CA, USA). Radioimmunoassay kit (Coat-A-Count) for corticosterone was supplied by Diagnostic Products Corp. Scandinavia AB (Mölndal, Sweden). Other chemicals used were purchased from a local supplier.

Animals

The animals used for this study were adult male Wistar rats (B&K Universal, Sollentuna, Sweden) at a weight between 240-320 g. They were housed four rats per cage and maintained on a rotating dark:light schedule (12:12 h, lights on 07:00-19:00). The surrounding environment of the cage was kept at a regular temperature of 22°C and the animals had free access to water and standard food. All animals in the study were handled, meaning that each rat was picked up from the cage and held for a couple of minutes. The handling procedure was performed every weekday for at least one week before sacrifice by decapitation. Animals were weighed the day before the experiment. All these procedures were made to minimize interference of endogenous steroids in the experiment.

The experiment protocols were approved by the Animals Experimental Ethical Committee (Umeå, Sweden).

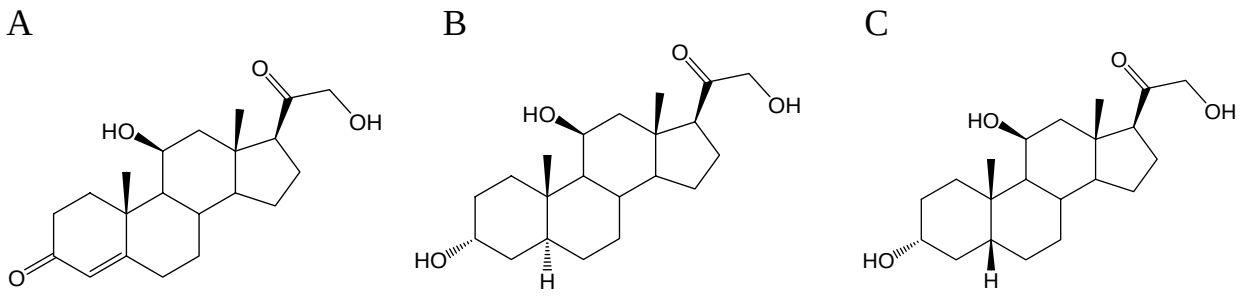

Figure 1. The chemical structure of (A) corticosterone, (B) 3α5α-corticosterone and (C) 3α5β corticosterone.

Chloride ion uptake experiment

Membrane preparation

Cortical membranes (microsacs) from rat brain were prepared as previously described [23, 25, 26]. For details see [23, 26]. Shortly, the brain was dissected immediately after the decapitation and the cerebral cortex was removed. Cortices from two rats were then homogenized in buffer. The two homogenates were diluted with cold buffer to a final volume of 40 ml and then filtered through two layers of 160 µm nylon mesh and washed twice by centrifugation. The pellets were resuspended in cold buffer.

Chloride ion uptake assay

To perform the chloride uptake assay a 12-channel cell harvester (Skatron, Lier, Norway) and 96-well microplates were used.

The three steroids used were dissolved in ethanol and then added to the wells in varying physiologically occurring concentrations. The steroids were then left to evaporate to dryness before adding [$^{36}Cl^-$] (0.5 µCi), buffer, GABA (10µM) and homogenate to each well. On each plate there were also prepared dose-response curves for GABA and controls. The GABA-curves were made by adding different concentrations of GABA, isotope and buffer to the wells. The different controls consisted of wells including isotope and buffer; isotope, buffer and homogenate; isotope, buffer, homogenate and 10 µM GABA; the three steroids in different concentrations, isotope, homogenate and buffer containing picrotoxin at a concentration of 100 µM or the three steroids in different concentrations, isotope, buffer and homogenate.

The pooled homogenate was diluted 1:2 and allowed to attain room temperature (22°C) for at least 10 minutes. The addition of the homogenate to the pre prepared wells initiated the reaction which then were terminated after 5 seconds by adding cold buffer containing 100 µM picrotoxin followed by a rapid filtration through glass fiber filters (Whatman GF/C; Millipore, Bedford, MA, USA) under vacuum. The filters were washed with cold buffer containing 100 µM picrotoxin and then left to suspend in scintillation liquid. Finally the filter-bound radioactivity was measured by liquid scintillation spectroscopy (1409 DSA; Wallac, Turku, Finland). Each chloride ion uptake test were performed in quadruples within the same experiment and the data is expressed as the net chloride ion uptake which means the chloride ion uptake in the presence of GABA and the presence or absence of steroids minus the basal chloride ion uptake in the homogenate. The control-value for each steroid was determined by taking the mean of all values of chloride ion uptake for 10 µM GABA in one experiment.

Protein content was determined by the Bio-Rad DC protein assay, a modification of the method of Lowry O.H., 1951 [27]. The mean protein content in the homogenate was 0.77-0.92 mg/ml.

Statistical analysis

All values in Table 1 and Figures 2-4 are presented as means ± SEM. The data analysis of the concentration-response data was fitted to the sigmoid concentration-response equation:

$$Y=E_0+(E_{max} - E_0)/[1 +10^{(\log EC50-X) \times Hill\ slope}]$$

with GraphPad Prism version 3.0 (1999, GraphPad Software Inc., San Diego, CA, USA). To evaluate the chloride ion uptake parameters analysis of variance (ANOVA) the Kruskal Wallis non-parametric test was used. The independent factors were concentration of the substances (GABA, corticosterone, 3α5α-corticosterone and 3α5β-corticosterone). The Mann-Whitney test with Bonferroni adjustment was used to compare the effects between the three steroids. That test was also used when examining whether the separate concentrations of the steroids in presence of GABA and picrotoxin and in absence of GABA, differed from the basal chloride ion uptake. Significance was marked as * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$. For all ANOVA and Mann-Whitney tests the SPSS statistical package version 10.0 (SPSS Inc., Chicago, IL, USA) was used.

RESULTS

Corticosterone, 3α5α-corticosterone and 3α5β-corticosterone reduce 10 µM GABA-mediated chloride flux

1 nM – 100 nM corticosterone, 3α5α-corticosterone and 3α5β-corticosterone in presence of 10 µM

Table 1. Chloride ion uptake under the influence of corticosterone, 3α5α-corticosterone and 3α5β-corticosterone respectively without addition of GABA. The effect of corticosterone, 3α5α-corticosterone and 3α5β-corticosterone in absence of GABA. All data in the table are presented as mean ± SEM CPM value of chloride ion uptake and number of experiments within brackets. The basal chloride ion uptake is the uptake obtained from homogenate without addition of either steroids or GABA.

[Steroid] nM	Chloride ion uptake (CPM)			
	Basal	Corticosterone	3α5α-corticosterone	3α5β-corticosterone
	70.4 ± 0.85 (128)			
1		70.2 ± 4.9 (4)	71.3 ± 2.1 (4)	73.0 ± 5.4 (5)
3		69.8 ± 4.0 (4)	70.2 ± 2.7 (4)	72.5 ± 4.1 (5)
10		75.4 ± 2.1 (4)	77.1 ± 1.0 (4)	72.2 ± 2.6 (5)
30		65.0 ± 2.5 (4)	63.2 ± 3.2 (4)	68.1 ± 3.0 (5)
100		64.1 ± 2.6 (4)	68.2 ± 2.4 (4)	66.9 ± 0.9 (5)
300		74.8 ± 2.3 (4)	74.7 ± 5.2 (4)	72.8 ± 3.2 (5)
1000		68.0 ± 1.5 (4)	70.5 ± 3.4 (4)	70.0 ± 2.8 (5)
3000		71.6 ± 4.4 (4)	66.6 ± 4.4 (4)	77.3 ± 2.0 (5)

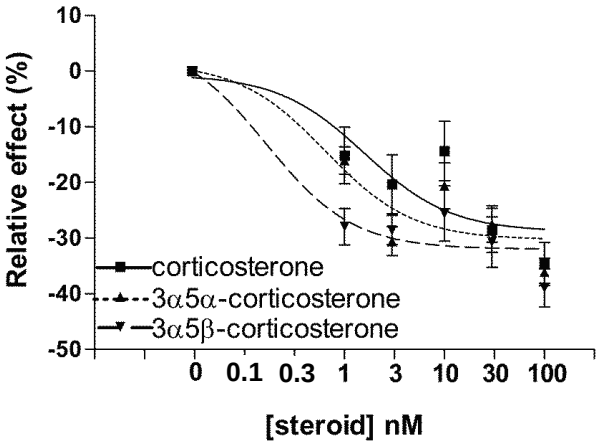

Figure 2. Corticosterone, 3α5α-corticosterone and 3α5β-corticosterone, at low concentrations, reduce 10 μM GABA-mediated chloride ion flux in a sigmoidal concentration-response manner. Each point is calculated as mean ± SEM percentage chloride ion uptake of control value (0%). The control value is the chloride ion uptake induced by 10 μM GABA and no steroids.

GABA was studied. Both corticosterone and its metabolites significantly reduced the effect of 10 μM GABA in a concentration dependent manner; corticosterone ($P \leq 0.001$, $n = 11$), 3α5α-corticosterone ($P \leq 0.001$, $n = 11$) and 3α5β-corticosterone ($P \leq 0.001$, $n = 9$) (Figure 2). Already at 1 nM, corticosterone, 3α5α-corticosterone and 3α5β-corticosterone significantly reduced the uptake by -15 ± 5.1% ($P = 0.021$, *n = 11*), -16 ± 2.5% ($P = 0.003$, *n = 11*) and -28 ± 3.3% ($P = 0.008$, *n = 9*) respectively. The effect of 3α5β-corticosterone significantly differed from the effect of corticosterone ($P = 0.039$). The IC_{50} values for the best fitted sigmoidal curves for corticosterone, 3α5α-corticosterone and 3α5β-corticosterone induced GABA-mediated chloride ion flux were calculated to 1.6 nM, 0.6 nM and 0.2 nM. The I_{max} for corticosterone, 3α5α- and 3α5β-corticosterone are -29%, -30% and -32% respectively.

Interactions between corticosterone, 3α5α-corticosterone and 3α5β-corticosterone and the $GABA_A$ active neurosteroid allopregnanolone

The $GABA_A$ active neurosteroid allopregnanolone is released in brain during different conditions. Therefore, it is of interest to study the interactions between allopregnanolone and corticosterone, 3α5α-corticosterone and 3α5β-corticosterone on 10 μM GABA-mediated chloride ion flux. The effect on 10 μM in presence of 200 nM or 1 μM allopregnanolone and 3-100 nM corticosterone, 3α5α-corticosterone and 3α5β-corticosterone respectively was studied. 200 nM allopregnanolone significantly reduce the effect of corticosterone by

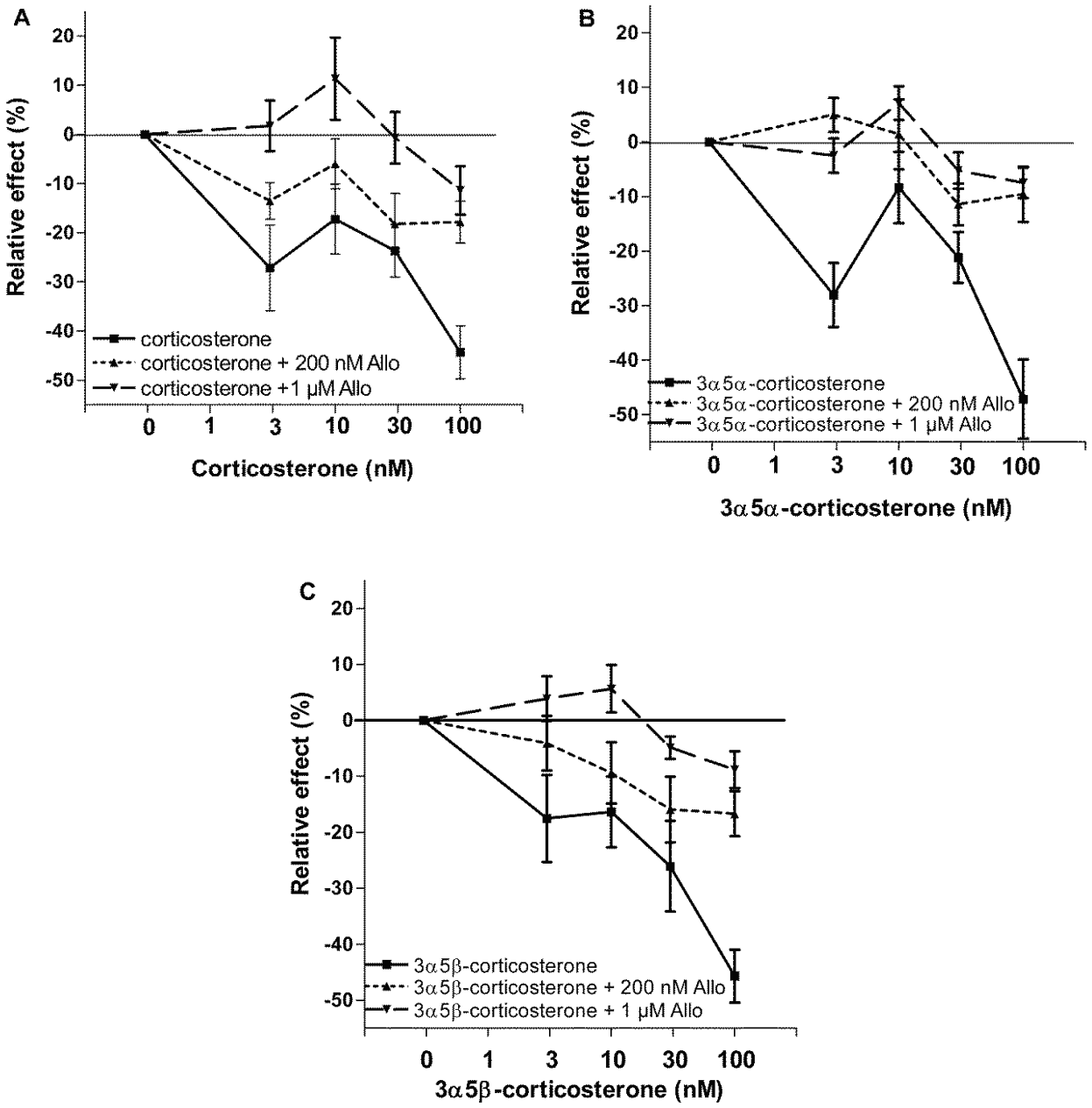

Figure 3. Interactions between allopregnanolone and (A) corticosterone, (B) 3α5α-corticosterone and (C) 3α5β-corticosterone in presence of 10 µM GABA. Each point is calculated as mean ± SEM percentage chloride ion uptake of control value (0%). The control value is the chloride ion uptake induced by 10 µM GABA and 0/200 nM/1µM allopregnanolone respectively.

approximately 50% (P = 0.003) while 1 µM allopregnanolone completely blocked the effect of 3-100 nM corticosterone (P ≤ 0.001) (Figure 3A). The blocking effect of 1 µM allopregnanolone significantly differ from the reducing effect of 200 nM on 3 - 100 nM corticosterone (P = 0.003).

The effect of 3α5α-corticosterone on 10 µM GABA mediated chloride flux was blocked both by 200 nM and 1 µM allopregnanolone (P ≤ 0.001) (Figure 3B). There are no difference between the effect of 200 nM and 1 µM allopregnanolone on 3 -100 nM 3α5α-corticosterone.

The effect of 3α5β-corticosterone is significant reduced by 200 nM allopregnanolone (P = 0.002) and blocked by 1μM allopregnanolone, except for the highest tested concentrations, (P ≤ 0.001) (Figure 3C).

Corticosterone, 3α5α-corticosterone and 3α5β-corticosterone had no effect in presence of picrotoxin or in absence of GABA

To investigate if the effect of the three tested steroids (1nM – 10 μM) in presence of 10 μM GABA is mediated through the $GABA_A$-receptor, picrotoxin was added to a final concentration of 100 μM in each well. In presence of picrotoxin, corticosterone, 3α5α-corticosterone and 3α5β-corticosterone had no effect on GABA mediated chloride ion flux compared to basal values (Figure 4). Basal value is the chloride ion uptake for homogenate without GABA or steroid added.

Corticosterone, 3α5α-corticosterone and 3α5β-corticosterone were also studied in absence of GABA (Table 1). None of the separate concentrations of corticosterone, 3α5α-corticosterone and 3α5β-corticosterone differed significantly from basal chloride ion uptake, except for 10 μM 3α5β-corticosterone where an increase of uptake from about 70 to 80 CPM was seen (P ≤ 0.05, *n* = 5).

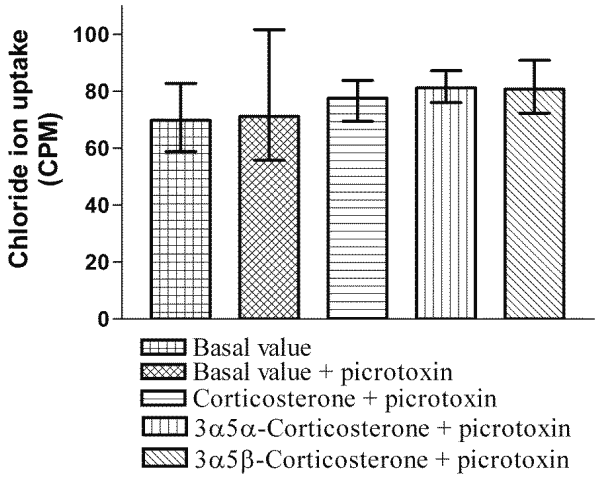

Figure 4. The effect of corticosterone, 3α5α-corticosterone and 3α5β-corticosterone in presence of 100 μM picrotoxin. All values are presented as the mean ± SEM CPM value of chloride ion uptake. The basal uptake is the chloride ion uptake in homogenate without addition of steroids, GABA or picrotoxin.

DISCUSSION

Here, we report that the species-specific glucocorticoids in rats have a direct effect on GABAergic transmission via the $GABA_A$-receptor. Corticosterone and the 3α5α- and 3α5β-metabolite reduce the 10 μM GABA mediated chloride flux in a concentration dependent manner. The stress steroids are potent on the $GABA_A$ receptor with an IC_{50} value range of 0.2-1.6 nM and the maximal effect of about -30%.

During an acute stress situation, not only glucocorticoids but also the levels of neuroactive steroids allopregnanolone and THDOC are increased [5, 28-30]. Allopregnanolone levels increase as much as 8-fold after acute stress [5] and the levels of THDOC increase from 1-5 nM to 15-30 nM [31]. Earlier, we have seen that the human cortisol metabolite interacts with allopregnanolone, give a synergistic effect [23]. It was of interest to study the interaction between corticosterone and its metabolites and allopregnanolone. However, in this study we could not find any synergistic effect of corticosterone or metabolites and allopregnanolone. Instead, in presence of allopregnanolone the reducing effect of corticosterone and its metabolites was reduced or even blocked.

The opposite effect of corticosterone and its metabolites compare to allopregnanolone and THDOC may function to maintain the balance between excitation and inhibition following an acute stress episode. Binding studies have shown that there is a decrease in GABAergic function 10 minutes after acute stress [32]. Further, 30 minutes after acute stress resulted in an increase in $GABA_A$ receptor δ subunit expression and moreover an increase in tonic GABAergic inhibition assessed by whole-cell patch clamp recording (see review [29]). Both allopregnanolone [33] and THDOC in higher concentration activate the $GABA_A$ receptor in the absence of GABA [34]. Corticosterone and its metabolites, on the other hand, cannot activate the $GABA_A$ receptor in the absence of GABA. The reducing effect by corticosterone and its metabolites is only seen in presence of GABA.

The glucocorticoid action on the GABA-system has been suggested to act presynaptic rather than

postsynaptic [35], whereas others claim that there is a post synaptic action but not via that $GABA_A$ receptor [36]. We show that the effect of corticosterone and the 3α5α- / 3α5β-metabolites is blocked by the $GABA_A$ receptor specific blocker, picrotoxin. Thus, the effect of corticosterone and the metabolites in our study suggests to be mediated through the $GABA_A$ receptor.

CONCLUSIONS

Corticosterone and its two metabolites 3α5α- and 3α5β-metabolites reduce the GABA mediated chloride flux in contrast to allopregnanolone and THDOC that are potent positive GABA modulators. The opposite effect of stress steroids on the $GABA_A$ receptor may be a compensatory mechanism to cope with stress.

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ABBREVIATIONS

CPM, counts per minute; GABA, γ-amino-butyric acid; IC_{50}, the concentrations that produce 50% inhibition; SEM, Standard Error of Mean; THDOC, tetrahydrodeoxycorticosterone

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