Hormones and Behavior 56 (2009) 348–353

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Effects of environmental stress on mRNA and protein expression levels of steroid 5α-Reductase isozymes in adult rat brain

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ARTICLE INFO

Article history:
Received 22 April 2009
Revised 7 July 2009
Accepted 7 July 2009
Available online 15 July 2009

Keywords:
5α-Reductase isozymes
mRNA levels
3α,5α-reduced neurosteroid
Environmental stress
Prefrontal cortex
Adult rat

ABSTRACT

Environmental stress conditions are important factors in human health and should be considered in the development of appropriate health policies, since they have been associated with psychological disorders and even with death. A link between stress and changes in 3α,5α-reduced neurosteroids has been reported. Steroid 5α-Reductase (5α-R) is the rate-limiting enzyme in the biosynthesis of 3α,5α-reduced neurosteroids. Using reverse transcription-polymerase chain reaction and immunohistochemistry, 5α-R isozymes (5α-R1 and 5α-R2) mRNA and protein levels were detected in prefrontal cortex of male and female rats after they underwent environmental stresses, i.e., excessive heat, artificial light, and the sensation of immobility in a small space, similar to those found in common workplace situations. Results showed significantly higher 5α-R2 mRNA and protein levels in environmentally-stressed versus control rats. Interestingly, a sexual dimorphism in 5α-R1 mRNA and protein levels was observed after environmental stress, with an increase in males and a decrease in females. This fact might explain gender differences in the incidence of some type of minor depression.

Introduction

An association between acute stress and changes in neurosteroid concentrations was first detected by Purdy et al. (1991), who showed that physical stress (e.g., forced swimming) induced a time-dependent increase in the amount of progesterone and its 3α,5α-reduced metabolite (allopregnanolone) in rat plasma and brain. Thereafter, numerous authors reported modifications in plasma and brain neurosteroids in rodents under different stress situations (Biggio et al., 1996, 2000), differentiating between the effects of acute and chronic stress (Barbaccia et al., 2001). Likewise, studies in humans have shown that acute stress situations (e.g., acute alcohol intoxication) increase plasma levels of 3α,5α-reduced neurosteroid allopregnanolone in male and female adolescents (Torres and Ortega, 2003a, 2004a).

3α,5α-reduced neurosteroids are synthesized from both progesterone and deoxycorticosterone (DOC) by the steroidogenic enzymes 5α-Reductase (5α-R) and 3α-hydroxysteroid dehydrogenase (3α-HSD), with the former being the rate-limiting enzyme of the reaction. 5α-R enzyme is expressed as two isozymes, 5α-R1 and 5α-R2, which are both present in various brain regions, including cerebral cortex (Agís-Balboa et al., 2006; Sánchez et al., 2005, 2006; Torres and Ortega, 2003b, 2006). It has also been reported that stress situations might increase brain tetrahydrodeoxycorticosterone (THDOC) levels (Barbaccia et al., 2001) and allopregnanolone (Purdy et al., 1991).

Central GABAergic transmission plays a key role in controlling emotional state and in regulating reactivity to rapid changes in environmental conditions that may lead to anxiety (Barbaccia et al., 2001). Thus, stress situations modified $GABA_A$-R function in rat cerebral cortex (Biggio et al., 1981, 1987; Biggio, 1983; Concas et al., 1985, 1987). Benzodiazepines and their congeners, which act as positive allosteric modulators of the $GABA_A$-R complex, relieve anxiety in humans, and may participate in the endogenous response to stress (Haefely, 1994). 3α,5α-reduced neurosteroids and benzodiazepines allosterically modulate $GABA_A$-R function via different binding sites (Paul and Purdy, 1992; Majewska, 1992; Mellon et al., 2001; Akk et al., 2007) and may, therefore, play a role in this response.

Determination of neurosteroids in brain under stressful conditions may improve our understanding of the role of neurosteroids against stress and assist the development of novel steroidal therapeutic agents for psychotic or mental disorders (Higashi et al., 2005). Since 3α,5α-reduced neurosteroid levels in the brain are modified after some stress situations (Purdy et al., 1991; Biggio et al., 1996, 2000), it can be hypothesized that 5α-R1, 5α-R2 or both may also be modified in the stress situations.

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0018-506X/$ – see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.yhbeh.2009.07.001

Environmental stress conditions are important factors in human health and should be considered in the development of appropriate health policies, since they have been associated with psychological disorders and even death (Rainham and Smoyer-Tomic, 2003; Tanaka, 2007; Woo and Postolache, 2008). The prefrontal cortex plays an important role in higher cognitive processes, and in the regulation of stress-induced hypothalamic–pituitary–adrenal (HPA) activity (Diorio et al., 1993; Sullivan and Gratton, 1999; Radley et al., 2006a,b).

With the aim of exploring at molecular level the effects of environmental stress situations in mammals, the objective of the present study was to evaluate mRNA and expression levels of both 5α-R isozymes in the prefrontal cortex of male and female rats in a model of environmental stress conditions, i.e., excessive heat, artificial light, and the sensation of immobility in a small space, which emulate common daily stress workplace situations (Woo and Postolache, 2008).

Materials and methods

Animals and stress procedure

Adult male ($n=10$) and female ($n=10$) Wistar rats weighing 260–280 g and 180–200 g, respectively, were housed in an air-conditioned room with fluorescent lights on from 7.00 to 19.00 h, and were given standard laboratory pellet chow and water *ad lib*. Experiments were made in strict accordance with the NIH guide for the "Care and Use of Laboratory Animals".

The rats were kept in a restrainer stressor device three times daily (9 am, 1 pm, and 5 pm) for 45 min during 1 week using the Ward (1972) paradigm: restrain, light 2500 lx, and heat 32 °C, in accordance with our previous reports (Pérez-Laso et al., 2008; Rodriguez Martin et al., 1998). At 15 min after the last stress session, rats were euthanized by decapitation to avoid possible adverse effects of anesthesia. Blood samples were collected in heparinized tubes. The blood was centrifuged at 800 g for 10 min. The plasma was separated and stored at −80 °C until hormonal measurements were performed. The brain was removed, frozen in liquid nitrogen, and stored at −80 °C until analysis. The dissection of prefrontal cortex areas was assessed with reference to the Atlas of Paxinos and Watson (1986). One thick slice of brain was cut, demarcated by two coronal planes corresponding to the anterior/posterior position of Plates 7 (bregma 3.70 mm) and 10 (bregma 2.2 mm). Right and left hemispheres were divided by a sagittal cut, and the dorsal anterior cingulate cortex (ACd) and prelimbic (PL) and infralimbic (IL) areas were then removed, using the shape of the subcortical white matter as the primary landmark.

Hormone assays

Plasma ACTH levels were measured with an immunoradiometric kit (CIS Biointernational, Gif-Sur Yvette Cedex, France). Plasma corticosterone levels were measured by RIA using an ICN kit (Biomedical Inc., Costa Mesa, CA) without modifications. Plasma progesterone levels were measured by RIA using a Sorin Biomedical Diagnostic kit (Vercelli, Italy) without modifications. All samples were assayed in duplicate and in the same assay. Intra-assay coefficients of variation for ACTH, corticosterone, and progesterone were 3.4%; 4.4%, and 5.6%, respectively.

Plasma testosterone (T) concentrations were measured by RIA using commercial DiaSorin (Vercelli, Italy) kit without modification. The intra- and inter-assay coefficients of variation were 7.6% and 12.0%, respectively, and the sensitivity was 0.05 ng/ml.

Plasma estradiol (E2) concentrations were measured by RIA using commercial DiaSorin (Vercelli, Italy) kit without modification. The intra- and inter-assay coefficients of variation were 4.8% and 9.5%, respectively, and the sensitivity was 12 pg/ml.

Oligonucleotides used for amplifications

Sequences of rat 5α-R (EC 1.3.99.5) isozymes were obtained from GeneBank® (J05035, M95058), and the sequence of plasmid pEGFP-C1 was obtained from the Clontech web page. These sequences were used to design the primer pairs. All forward primers were end-labeled with 6-carboxy-fluorescein (6-FAM). Oligonucleotides were synthesized by PE-Applied Biosystems (Warrington, UK). Primer sequences (5′–3′) and PCR product sizes were as previously described (Torres and Ortega, 2004b).

Construction of internal standard template

Two synthetic internal standard (IS) DNAs of 300-bp were synthesized from the sequence of plasmid pEGFP-C1 (Clontech, Palo Alto, CA) following Torres and Ortega (2004b). Briefly, both competitive molecules, IS-1 (competitor DNA of 5α-R1) and IS-2 (competitor DNA of 5α-R2), were obtained after two consecutive amplifications from pEGFP-C1, with 5′ and 3′ ends modified to contain the same nucleotide sequences as *srd5a1* or *srd5a2* genes.

Reverse transcription-polymerase chain reaction

Total RNA was extracted from 25 mg of rat prefrontal cortex tissues by acid-guanidinium thiocyanate-phenol-chloroform (Chomczynski and Sacchi, 1987). The RNA was resuspended in diethyl pyrocarbonate (DEPC)-treated water and spectrophotometrically quantitated for analysis. First-strand cDNA was carried out according to Torres and Ortega (2004b). The PCR profile was: denaturing, 94 °C for 30 s; annealing, 55 °C for 30 s; and extension, 72 °C for 30 s. The number of cycles was 35 in all cases. PCR was carried out in a Perkin-Elmer 2400 Thermal Cycler.

The amount of mRNA was expressed as the number of mRNA copies per μg of total RNA. After cDNA was generated from the total RNA by RT reaction, it was co-amplified in the presence of decreasing amounts of competitive DNA (64×10^6–0.5×10^6 molecules). 5α-R cDNAs and the competitive standard DNAs were co-amplified using the same pair of primers. With decreasing amounts of competitive DNA, the relative intensity of amplified product of target DNA increased. The ratio of fluorescence of 5α-R1/IS-1 and 5α-R2/IS-2 were plotted against the amount of competitive DNAs IS-1 and IS-2 respectively.

Analysis of PCR products

A CE system with LIF detection (ABIPRISM 310 Genetic Analyzer, Applied Biosystem) was used to characterize RT-PCR products. Capillary electrophoresis conditions were as previously reported (Torres and Ortega, 2004b).

Fluorescence ratios of both 5α-R/IS were plotted against the amount of the appropriate competitive DNA, and the concentration of target DNA in the sample was calculated following Torres and Ortega (2004b).

Immunohistochemical analysis

Microsomal expression of 5α-R isozymes was characterized by immunohistochemistry. Briefly, rat frontal central nervous system biopsies were fixed in 10% buffered formalin and embedded in paraffin following standard procedures. Antibodies against 5α-R1 and 5α-R2 (sc-20399 and sc-20659, Santa Cruz Biotechnology, CA, USA) were optimized in the liver and prostate (organs that express mainly 5α-R1 and 5α-R2, respectively) as positive control tissues. Sections (4 μm) were dewaxed, and the epitope was recovered in a PT module (Thermo Scientific, CA, USA) and incubated for 60 min at room temperature with primary antibodies diluted 1:50 (5α-R1) or 1:25