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CORTICOSTERONE INDUCED MORPHOLOGICAL CHANGES OF HIPPOCAMPAL AND AMYGDALOID CELL LINES ARE DEPENDENT ON 5-HT$_7$ RECEPTOR RELATED SIGNAL PATHWAY

Y. XU,[a,b] C. ZHANG,[a,b] R. WANG,[c] S. S. GOVINDARAJAN,[a] P. A. BARISH,[a] M. M. VERNON,[a] C. FU,[d] A. P. ACHARYA,[a] L. CHEN,[e] E. BOYKIN,[a] J. YU,[e] J. PAN,[e] J. M. O'DONNELL[b]* AND W. O. OGLE[a]*

[a]*Crayton Pruitt Family Department of Biomedical Engineering and Evelyn F. & William L. Mcknight Brain Institute, University of Florida, Gainesville, FL 32611, USA*

[b]*Departments of Behavioral Medicine and Psychiatry and Neurobiology and Anatomy, West Virginia University Health Sciences Center, Morgantown, WV 26506, USA*

[c]*Program of Pharmacology, Weill Graduate School of Medical Science, Cornell University, New York, NY 10065, USA*

[d]*Department of Neuroscience and Evelyn F. & William L. Mcknight Brain Institute, University of Florida, Gainesville, FL 32611, USA*

[e]*Institute of Experimental Neurobiology, Wenzhou Medical College, Wenzhou, 325021, China*

Abstract—Stress is an unavoidable life experience. It induces mood, cognitive dysfunction and plasticity changes in chronically stressed individuals. Among the various brain regions that have been studied, the hippocampus and amygdala have been observed to have different roles in controlling the limbic-hypothalamic-pituitary-adrenal axis (limbic-HPA axis). This study investigated how the stress hormone corticosterone (CORT) affects neuronal cells. The first aim is to test whether administration of CORT to hippocampal and amygdaloid cell lines induces different changes in the 5-HT receptor subtypes. The second goal is to determine whether stress induced morphological changes in these two cell lines were involved in the 5-HT receptor subtypes expression. We now show that 5-HT$_7$ receptor mRNA levels were significantly upregulated in HT-22 cells, but downregulated in AR-5 cells by exposure to a physiologically relevant level of CORT (50 μM) for 24 h, which was later confirmed by primary hippocampal and amygdaloid neuron cultures. Additionally, pretreatment of cells with 5-HT$_7$ antagonist SB-269970 or agonist LP-44 reversed CORT induced cell lesion in a dose-dependent manner. Moreover, CORT induced different changes in neurite length, number of neurites and soma size in HT-22 and AR-5 cells were also reversed by pretreatment with either SB-269970 or LP-44. The different effects of 5-HT$_7$ receptors on cell lines were observed in two members of the Rho family small GTPase expression: the Cdc-42 and RhoA. These observed results support the hypothesis that 5-HT may differentially modulate neuronal morphology in the hippocampus and amygdala depending on the expression levels of the 5-HT receptor subtypes during stress hormone insults. **© 2011 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: corticosterone, 5-HT, 5-HT$_7$ receptor, agonist, antagonist, neural morphology.

Stress may be described as any environmental change, either internal or external, that disturbs the maintenance of homeostasis (Leonard, 2005). The stress response is to maintain homeostasis, which includes a series of physiological reactions such as modulation of neuroplasticity (limbic system), endocrine activation (especially of the HPA axis), and cardiovascular changes (Sapolsky, 2003). The central feature of the limbic-HPA stress response is the synthesis and secretion of glucocorticoids from the adrenal cortex. However, excessive stress hormone levels often trigger the onset of major depression and Alzheimer's disease (AD), which is associated with a decreased sensitivity of the HPA axis to feedback inhibition by cortisol in primates or corticosterone (CORT) in rodents. In addition to the HPA axis, neuronal systems within the brain including the monoaminergic systems and by greater contribution the 5-HT containing neurons, play critical roles in stress-related disorders (Xu et al., 2006; Lanfumey et al., 2008). Numerous studies have demonstrated the existence of reciprocal interactions between the 5-HT system and HPA axis in stress related depression (Kitamura et al., 2002; Lanzenberger et al., 2010). 5-HT, when bound to its receptors, is involved in regulating various aspects of brain development, including cell proliferation, differentiation, neurite outgrowth and synapse formation (Azmitia, 2001; Hayashi et al., 2010). Chronic stress induces changes in hippocampal 5-HT and its receptors, which may lead to increased vulnerability to depression, anxiety and disturbance in cognitive processes and neuroplasticity (Dwivedi et al., 2005; Trajkovska et al., 2009). The 5-HT$_7$ receptor is a relatively recently discovered receptor from among the 14 subtypes of 5-HT$_7$ receptors with seven families (5-HT$_{1–7}$). Our preliminary gene microarray data suggest that the differences in 5-HT$_7$ receptor gene expressions between neurons of the hippocampus and the amygdala under conditions of chronic stress in rats (unpublished observation by William O. Ogle et al.). But the functional relevance of the receptor in the different brain regions during stress is not fully understood.

The hippocampus and amygdala are two essential components of the neural circuitry mediating stress responses.

*Corresponding author. W. O. Ogle, Tel: +1-352-273-5877; fax: +1-352-392-9791 or J. M. O'Donnell, Tel: +1-304-293-2433; fax: +1-304-293-7038.
E-mail address: wogle@bme.ufl.edu (W. O. Ogle) or jodonnell@hsc.wvu.edu (J. M. O'Donnell).

Abbreviations: CORT, corticosterone; DMEM, Dulbecco's modified Eagle's medium; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenal; LP-44, ((4-[2-(Methylthio)phenyl]-N-(1,2,3,4-tetrahydro-1-naphthalenyl)-1-piperazinehexanamide); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; SB-269970, ((2R)-1-[(3-Hydroxyphenyl)sulfonyl]-2-(2-(4-methyl-1-piperidinyl)ethyl)pyrrolidine.

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The role of hippocampus is critical in controlling the limbic-HPA axis, mood and memory through inhibitory inputs. Changes within this structure, such as synaptic loss and atrophy, are known to be involved in prolonged elevated glucocorticoid levels, major depression and cognitive impairment (Lupien et al., 1998). The amygdala is responsible for detecting an environmental stressor and controls the expression of the fear reaction, including behavioral, autonomic and endocrine responses. This process operates through downstream areas, such as hypothalamus and central gray, which in turn regulate the secretion of neurotransmitters, corticotropin-releasing hormone (CRH) and glucocorticoids (Fanselow and Poulos, 2005; Rodrigues et al., 2009). However, recent studies show that enhanced hippocampal input would suppress the HPA axis, while enhanced amygdaloid input produces an opposite effect (Vyas et al., 2002). In rodents, CORT reduces hippocampal response to 5-HT, contributing to the onset of the symptoms of depression in predisposed individuals (Joels et al., 2004). However, activation of the amygdala leads to an increase in arousal and vigilance as a component of the fear reaction in response to stress, which results in the release of neurotransmitters (5-HT, noradrenaline and dopamine) and changes in the expression of their receptor subtypes (Leonard, 2005; LeDoux, 2007).

Given the potential contrast that stress hormones exert at the behavioral level and neuroendocrine regulation effects on the hippocampus and amygdala, the importance of studying the underlying molecular mechanisms arises. Therefore, the present study was designed to investigate the morphological changes of hippocampal and amygdaloid cell lines under CORT exposure, in conjunction with its relation to the 5-HT system and the receptor subtypes dependent pathways.

EXPERIMENTAL PROCEDURES

Materials

The HT-22 cells used in this study were a generous gift from Dr. David Schubert (The Salk Institute for Biological Studies, La Jolla, CA, USA) (Li et al., 1997). The AR-5 cells were kindly provided by Dr. Rosalie Uht (University of North Texas Health Science Center, Fort Worth, TX, USA) (Lalmansingh and Uht, 2008). The culture plates were acquired from Nunc (A/S, Roskilde, Denmark). The DMEM/F12 media were obtained from Hyclone (Logan, UT, USA). The NeuroBasal medium, fetal bovine serum (FBS) and N2 nutrient supplement were obtained from Invitrogen (Carlsbad, CA, USA). The CORT and 5-HT were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was obtained from Biotium, Inc. (Hayward, CA, USA). The 5-HT_{1A} receptor antagonist, NAN-190 (1-(2-methoxyphenyl)-4[-(2-phthalimido)butyl]piperazine), 5-HT_7 receptor agonist, LP-44 ((4-[2-(Methylthio)phenyl]-N-(1,2,3,4-tetrahydro-1-naphthalenyl)-1-piperazinehexanamide)) and antagonist SB-269970 ((2R)-1-[(3-Hydroxyphenyl)sulfonyl]-2-(2-(4-methyl-1-piperidinyl)ethyl)pyrrolidine) were purchased from Tocris (Avonmouth, UK). Other routine cell culture supplies and reagents were purchased from Sigma, Invitrogen or Fisher Scientific.

Animals

Pregnant SD rats (18 days pregnant, 500 g) were obtained from the Animal Center of University of Florida and fetal pups were used for *in vitro* studies. Experimental procedures were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and the University of Florida Institutional Animal Care and Use of Laboratory Animals (IACUC) guidelines.

Cell cultures

The HT-22 cells were maintained in DMEM, supplemented with 10% FBS, cultured at 37 °C in 5% CO_2, and differentiated in NeuroBasal medium containing 1× N2 supplement for 12 h before treatment. The AR-5 cells were cultured in DMEM/F12 media as described previously (Lalmansingh and Uht, 2008) and differentiated in identical culture conditions as the HT-22 cells. Both cell types were plated at 5×10^4 cells/ml for cell morphology tests, 1×10^5 cells/ml for MTT experiments and mRNA extraction. All treatments with CORT, 5-HT_7 antagonist or agonist were performed in the differentiation media.

Primary hippocampal and amygdaloid cultures

Pregnant (18 days) SD rats were anesthetized with 1 ml/kg of a mixture consisting of 100 mg/kg ketamine, 10 mg/kg acepromazine and 100 mg/kg xylazine. The uterus was carefully separated from the abdominal viscera, the fetus was decapitated and its brains dissected with the aid of a light microscope. Hippocampi or amygdalae was chopped into fine pieces and cells harvested from a homogenized pool of 10 pup brains. Cells were plated at a density of 2×10^5 cells/ml on poly-L-lysine-coated culture plates (Nunc A/S, Roskilde, Denmark). Cultures were maintained in DMEM in a humidified incubator in an atmosphere of 10% CO_2 at 37 °C. After 3 days, the DMEM solution was replaced with DMEM containing 1% cytosine arabinoside (ARC). 2 days later, the solution was replaced with DMEM and the cells were cultured for an additional 7 days before use.

Drug treatment

CORT, 5-HT_7 receptor antagonist SB-269970 and agonist LP-44 were dissolved in dimethyl sulfoxide (DMSO), whereas NAN-190 and RU486 were dissolved in ethanol and the total vehicle administered did not exceed 0.1% of the total volume of the cell culture wells. The SB-269970 and LP-44 were added 2 h before and RU486 was administered 30 min before CORT application (doses and treatment schedules are described in the results section for each experiment). Cells were pretreated with NAN-190 (1 μM) for 30 min wherever LP-44 was used.

The preliminary data showed that incubation with 5, 10 and 20 μM 5-HT for 2 days did not affect HT-22 and AR-5 cells survival, which was consistent with the previous study (Dooley et al., 1997). Therefore, we used 5-HT at a concentration of 5 μM in the cell and primary culture media during treatment with drugs throughout this study.

Cell viability

Cell viability was assessed by MTT assay based on the manufacturers protocol. Briefly, 12 h after differentiation on 96-well plates, cells were treated with CORT and/or other reagents at different concentrations and incubation time. Following the indicated treatments, 10 μM of MTT solution was added to each well and incubated for another 3 h. Then, dark blue formazan crystals formed in intact cells dissolved with 200 μl DMSO/well and a Synergy Multi-mode Microplate Reader recorded the absorbance at 595 nm (BioTek, Winnoski, VT, USA).

mRNA extraction and real-time reverse transcriptase (RT)-PCR

Cultures were washed and total cellular RNA was isolated with TriZOL reagent (TriZOL®Invitrogen, Carlsbad, CA, USA) accord-

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