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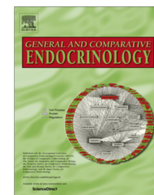

Expression of stress hormones AVP and CRH in the hypothalamus of *Mus musculus* following water and food deprivation

Arun Kumar Yadawa, Chandra Mohini Chaturvedi *

Department of Zoology, Banaras Hindu University, Varanasi 221005, India

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ABSTRACT

Neurohypophyseal hormone, arginine vasopressin (AVP), in addition to acting as antidiuretic hormone is also considered to be stress hormone like hypothalamic corticotropin-releasing hormone (CRH). Present study was designed to investigate the relative response of these stress hormones during water and food deprivation. In this study, male laboratory mice of Swiss strain were divided in 5 groups, control – provided water and food *ad libitum*, two experimental groups water deprived for 2 and 4 days respectively (WD2 and WD4) and another two groups food deprived for 2 and 4 days respectively (FD2 and FD4). Results indicate an increased expression of AVP mRNA as well as peptide in the hypothalamus of WD2 mice and the expression was further upregulated after 4 days of water deprivation but the expression of CRH remained unchanged compare to their respective controls. On the other hand no change was observed in the expression of hypothalamic AVP mRNA while AVP peptide increased significantly in FD2 and FD4 mice compare to control. Further, the expression of CRH mRNA although increased in hypothalamus of both FD2 and FD4 mice, the immunofluorescent staining shows decreased expression of CRH in PVN of food deprived mice. Based on these findings it is concluded that since during osmotic stress only AVP expression is upregulated but during metabolic stress i.e. food deprivation transcription and translation of both the stress hormones are differentially regulated. Further, it is suggested that role of AVP and CRH may be stress specific.

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1. Introduction

Arginine vasopressin (AVP) is a nine amino acid neurohormone synthesized by both parvocellular and magnocellular neurons of hypothalamus. AVP exhibits three main physiological roles: regulation of water homeostasis, vascular constriction and control of ACTH secretion from pituitary via its different receptor subtypes belonging to G protein coupled receptor family (Murat et al., 2012). The physiological role of AVP is mediated by three specific receptor subtypes. The V2 receptors expressed in kidney, are positively coupled to adenylyl cyclase (AC) and mediate water reabsorption in the kidney tubules, V1a isoform induces vascular constriction via phospholipase C (PLC) activation (Barberis et al., 1999) and the V1b receptor, discovered last in the pituitary gland, is also coupled to the PLC pathway and has a well-known effect on ACTH secretion. The magnocellular neurons project through median eminence to neuronal lobe of hypophysis being involved in water balance. It has been reported that AVP is a key regulator of the HPA axis and has a strong impact on the stress related behaviors (Zelena and Jain, 2010). AVP has been reported to be involved in modulating social behavior in human and rodents (Young and Wang, 2004). The expression of isoform of AVP in birds (AVT) increased in water deprived domestic fowl and Japanese quail. It has been reported that osmotic stress not only upregulates the expression of the AVT gene in existing neurons but also recruits many more neurons to increase the rate of AVT synthesis and secretion in chicken and Japanese quail (Chaturvedi et al., 1996, 2000; Seth et al., 2004; Singh and Chaturvedi, 2006).

Corticotropin-releasing hormone (CRH), a 41 amino acid neuropeptide is involved in the control of stress-related behaviors (Vale et al., 1981; Bale and Vale, 2004; Keck, 2006). CRH is present in the nerve cell bodies in and near the dorsomedial parvicellular division of the paraventricular nucleus (PVN) of the hypothalamus. Besides PVN, CRH is also expressed in amygdala and bed nucleus of stria terminalis and increases in response to stress (Chalmers et al., 1995). CRH is required for adrenal response to different stressors (Jacobson et al., 2000) including fasting (Jeong et al., 2004). The tissue distribution of the type 1 and 2 receptors of CRH varies considerably (Van et al., 2000; Bale and Vale, 2004). CRH-R1 is expressed throughout the brain, being concentrated on anterior pituitary

* Corresponding author at: Molecular Neuroendocrinology Lab, Department of Zoology, Banaras Hindu University, Varanasi 221005, India.
E-mail address: cmcbhu@gmail.com (C.M. Chaturvedi).

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corticotrophs. CRH-R2α is expressed exclusively in the brain. CRH-R2β is expressed primarily in the periphery, with the highest levels found in heart and skeletal muscle. CRH-R1 mediates the actions of CRH at the corticotroph as well as some aspects of the behavior stress-response, including fear and anxiety. CRH-R2 may be involved in vasodilation and blood pressure control, consistent with its anatomic localization. CRH secreted by postganglionic sympathetic neurons at inflammatory sites has proinflammatory properties, one of its key roles in the degranulation of mast cells (Elenkov et al., 1999; Chrousos et al., 1998).

AVP mRNA levels in the PVN of rat was increased after water deprivation, whereas there was slight decrease in food deprived rat. CRH mRNA in the PVN was reduced after food and water deprivation in rat (Kiss et al., 1994). This is also suggested that CRH and AVP might work together to stimulate the secretion of pituitary ACTH (DeBold et al., 1984). Food deprivation and refeeding for 48 h influenced the hypothalamic monoamine oxidase activity and antioxidant enzyme (SOD and catalase) activities (Cvijic et al., 2000). In view of the fact that receptors of both these peptides are present on pituitary corticotrophs, present study was undertaken to compare their role as stress hormone during both osmotic (water deprivation) and metabolic stress (food deprivation).

2. Materials and methods

2.1. Experimental animals

Sexually mature 10 week old Swiss strain male mice, *Mus musculus*, obtained from the mice colony of Central Animal House, Institute of Medical Sciences, Banaras Hindu University. Mice were maintained under hygienic conditions in a photoperiodically controlled (LD 12:12) and well ventilated room (25 ± 3 °C). Mice of all the groups were kept separately in polypropylene cages with dry rice husk as the bedding material and were supplied with food (standard rodent food pellets supplied by Pashu Aahar Kendra; Varanasi) and tap water *ad libitum*.

2.2. Experimental design

Male laboratory mice weighing 25 ± 2 g were divided in 5 groups ($n = 10$ in each group). (i) Control, provided food and water *ad libitum*, (ii) water deprived for 2 days (WD 2), (iii) water deprived for 4 days (WD 4), (iv) food deprived for 2 days (FD 2) and (v) food deprived for 4 days (FD 4). At the end of food and water deprivation period, mice of all the groups were weighed and sacrificed by decapitation. Blood was collected in microcentrifuge tubes and serum was isolated for the ELISA of corticosterone ($n = 5$ from each group). Brain of five mice in each group were dissected out, hypothalamus was separated, processed for extraction of total RNA for reverse transcriptase-PCR for AVP, CRH and β-actin. Brains of remaining five mice were fixed in 4% PFA for Immunohistochemistry (IHC)/Immunofluorescence after the whole body perfusion.

The experiment was conducted in accordance with Institutional practice and within the framework of the revised Animals (scientific procedures) Act of 2002 of the Govt. of India on Animal Welfare.

2.3. RNA extraction

The total RNA from hypothalami of the mice of various groups was extracted using TRIzol Reagent (Ambion, life technologies) according to its user manual, dissolved in DEPC – treated water and quantified using spectrophotometer.

2.4. cDNA synthesis

cDNA was synthesized from 10 μg of total RNA using random hexamer primer reverse transcriptase. In brief, 10 μg of RNA was mixed with 200 ng of random hexamer in DEPC – treated water in the reaction volume of 11 μl. It was incubated at 70 °C for 5 min. Thereafter the following components were added in the order indicated: 5× Reaction buffer-4.0 μl, dNTP mix (2.5 mM each) – 2.0 μl, RNase inhibitor – 0.5 μl, DEPC treated water – 1.5 μl.

The tubes containing reaction mixture were incubated for 5 min at 25 °C and 1.0 μl of M-MuLV reverse transcriptase (RevertAid, 200 units) was added. Further, the tubes were incubated for 10 min at 25 °C and then at 42 °C for 1 h. The reaction was terminated by heating at 70 °C for 10 min, and after chilling, the tubes were stored at −20 °C to be used directly for the PCR reaction.

2.5. Polymerase chain reaction

Specific oligonucleotides used for the analysis of AVP (forward primer 5′-ACT ATG CAC GAC TTC GGG TG-3′, Reverse primer 5′-AGT CCG TGG ATT CTG CCA AG-3′), CRH (Forward primer 5′-GTA CAG AGG AAA GCC CAG GAC-3′, Reverse primer 5′-TTC TTG AGG GGT GGC TAG GA-3′) and β-actin (Forward primer 5′-ATC GTG GGC CGC TCT AGG CAC C-3′ Reverse primer 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′) were synthesized by mwg – operon, Bangalore, India. The PCR were performed in 25 μl reaction mixture containing 2 μl cDNA, 10× Taq polymerase buffer, 2.5 mM of each dNTP (Biobharati, India), 1.0 unit of Taq DNA polymerase (Biobharati, India), and 10 pmol of appropriate primers. The reactions were carried out in T 100™ BioRad Thermal cycler. The samples were denatured at 94 °C for 5 min and amplified using different conditions for AVP (denaturation at 94 °C for 45 s, primer annealing at 62 °C for 45 s elongation at 72 °C for 1 min and no. of cycles – 35); CRH (denaturation at 94 °C for 45 s, primer annealing at 64 °C for 40 s elongation at 72 °C for 1 min and no. of cycles – 35) and β actin (denaturation at 94 °C for 30 s, primer annealing at 57 °C for 30 s elongation at 72 °C for 30 s and no. of cycles – 30). The resultant PCR products were electrophoresed on 2% agarose gel containing ethidium bromide. The resulting gel bands were visualized using UV – transilluminator Alpha Digi doc from Alpha Innotech (India) and photographed by camera Olympus (USA). The expression of AVP and CRH were expressed as percent band intensity relative to that of β actin.

2.6. Immunohistochemistry and Immunofluorescence

Following whole body perfusion brains were excised and fixed for 24 h in 4% (w/v) paraformaldehyde, dehydrated and embedded in paraffin. Tissue blocks were prepared and 8 μm thick coronal sections of brain were cut on a microtome (LEICA) and collected on gelatin-coated slides. Immunohistochemistry was performed in the brain sections for AVP and CRH as described by Singh and Chaturvedi (2014), with some modifications. In brief, the brain sections were deparaffinized, rehydrated in graded series of ethanol and rinsed in PBS and then immersed in 0.3% hydrogen peroxide for 20 min at room temperature. They were preincubated in 10% normal goat serum for 1 h and subsequently incubated in rabbit anti-AVP antibody (Merck Millipore, 1:1000) and anti – CRH antibody (Proteintech, USA, 1:100) separately for overnight at 4 °C. The second incubation with Vectastain Avidin–Biotin complex Kit and 2^0 antibody (Vector Laboratories, Burlingame, CA, USA), were visualized with 0.025% 3,3-diaminobenzidine (Sigma) in PBS containing 0.02% H_2O_2. The slides were washed with PBS, dehydrated through graded series of ethanol, cleaned in xylene and mounted using DPX. Slides were viewed under Carl Zeiss Axioskop 2 Plus microscope.

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