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VASOPRESSINERGIC NETWORK ABNORMALITIES POTENTIATE CONDITIONED ANXIOUS STATE OF RATS SUBJECTED TO MATERNAL HYPERTHYROIDISM

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Abstract—We have previously reported that a mild maternal hyperthyroidism in rats impairs stress coping of adult offspring. To assess anxiogenesis in this rat model of stress over-reactivity, we used two behavioural tests for unconditional and conditional anxious states: elevated plus maze test (EPM) and Vogel conflict test (VCT). In the latter one, arginine vasopressin (AVP) release was enhanced due to osmotic stress. With the EPM test no differences were observed between maternal hyperthyroid rats (MH) and controls. However, with the VCT, the MH showed increased anxiety-like behaviour. This behavioural difference was abolished by diazepam. Plasma AVP concentration curve as a function of water deprivation (WD) time showed a marked increase, reaching its maximal levels within half the time of controls and another significant difference after VCT. A general increase in Fos expression in hypothalamic supraoptic and paraventricular nuclei (PVN) was observed during WD and after VCT. There was also a significant increase of AVP immunoreactivity in anterior hypothalamic area. A large number of Herring bodies were observed in the AVP containing fibres of MH hypothalamic-neurohypophysial system. Numerous reciprocal synaptic connections between AVP and corticotropin releasing factor containing neurons in MH ventromedial PVN were observed by electron microscopy. These results suggest that a mild maternal hyperthyroidism could induce an aberrant organization in offspring's hypothalamic stress related regions which could mediate the enhanced anxiety seen in this animal model.

Key words: anxiety, Vogel conflict test, arginine vasopressin, corticotropin releasing factor, electron microscopy, synapse.

Thyroid hormones (TH) play an important role in the development of the fetal brain. TH cross the placenta reaching the fetus and it is vital up to the first trimester of pregnancy during which time the fetal thyroid gland is not functional (Vulsma et al., 1989). Deficiency of maternal thyroid function at the beginning of fetal neocorticogenesis alters neuronal migration (Auso et al., 2004) and mild maternal hyperthyroidism in rats leads to an increased dendritic arborization of hippocampus CA3 pyramidal neurons (Zhang et al., 2008). Maternal hyperthyroid rat models have shown that the expression of cytoskeletal proteins is affected, indicating an accelerated neuronal differentiation (Evans et al., 2002).

Wistar rat male offspring of mild hyperthyroid females bred in our laboratory were similar to control animals in a number of physiological parameters when tested in non-stressful conditions. However, their responses to mild acute and sub-chronic stressors were markedly enhanced, leading to an impaired cognitive function and depression-like behaviours (Zhang et al., 2008). However, anxiety-like behaviour measured as unconditioned explorative behaviour showed no difference to controls (Hernandez et al., 2007).

On the other hand, the arginine vasopressin (AVP), also referred as antidiuretic hormone, acts both as a hormone and as a neurochemical. AVP is largely synthesized by hypothalamic magnocellular neurons localized in paraventricular (PVN) and supraoptic (SON) nuclei. Through the hypothalamic–neurohypophysial system (HNS) AVP is transported to the neurolobe of the pituitary gland and further released upon osmoreceptor/baroreceptor activation. Peripherally circulating AVP is responsible for the classic endocrine functions ascribed to this neurohormone (e.g. vasoconstriction, antidiuresis) (Ring, 2005). The central vasopressinergic system includes the sites of AVP synthesis and release, where AVP acts as a neuromodulator/neurotransmitter regulating a variety of CNS-mediated functions (e.g. learning and memory, neuroendocrine reactivity, social behaviours, circadian rhythmicity, thermoregulation, and autonomic function) (Ring, 2005; Caldwell et al., 2008; Frank and Landgraf, 2008). AVP is also critically involved in stress-related anxiogenesis (Millan, 2003; Landgraf, 2005).

Early studies considered AVP originating from magnocellular neurons of the HNS as a major modulator of the hypothalamic–pituitary–adrenal (HPA) axis (Antoni, 1993). However, it is now generally accepted that the parvocellular neurons of the PVN trigger corticotropin (ACTH) secretion via the release of corticotropin-releasing factor (CRF) and AVP. As a consequence, less attention was paid to the contribution of the HNS to the HPA axis regulation (Engelmann et al., 2004). The involvement of vasopressinergic magnocellular neurons in the control of the ACTH secretion has been a much-debated issue. This study was designed and carried out with this issue in mind.

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Abbreviations: AHA, anterior hypotalamic area; AT, axon terminal; AVP, arginine vasopressin; CRF, corticotropin releasing factor; DAB, 3,3'-diaminobenzidine; EPM, elevated plus maze test; HNS, hypothalamic–neurohypophysial system; HPA, hypothalamic–pituitary–adrenal axis; MH, maternal hyperthyroid rats; PVCT, post Vogel conflict test; PVN, paraventricular nucleus; PVNlm, PVN lateral magnocellular part; PVNmpd, PVN medial parvocellular part, dorsal zone; SON, supraoptic nucleus; VCT, Vogel conflict test; WD, water deprivation.

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Using an osmotic stressor we aimed to assess the anxiogenesis in maternal hyperthyroid offspring. We combined behavioural characterizations with analysis of plasma AVP variations and the protein product of the immediate early gene *c-fos* (Fos) expression in PVN and SON during the 48 h of water deprivation (WD) and after the conflict test used in this study. Furthermore, AVP immunoreactivity, morphology and synaptic connectivity of AVP containing fibres in PVN and HNS were analyzed under light and electron microscopy.

EXPERIMENTAL PROCEDURES

Animals

Wistar rats were used in this study. All animal procedures were approved by the local bioethical and biosecurity committees in accordance with the principles exposed in the Handbook for the Use of Animals in Neuroscience Research (Society for Neuroscience. Washington, D.C.1991). Animals were housed on a 12-h light schedule in a room with temperature between 20 and 24 °C with adequate ventilation and given access to standard rat chow and water *ad libitum*, unless otherwise specified.

The breeding of the maternal hyperthyroid rats offspring has been described elsewhere (Zhang et al., 2008). Briefly, 32 female rats of postnatal 90 days (P90) were implanted, under general anaesthesia (pentobarbital, Barbithal, Holland de Mexico, S. A. de C. V., 50 mg/kg i.p.), with s.c. Alzet osmotic pumps (Model 2ML4, pumping rate 2.5 μl/h for 28 days, DURECT Corporation, Cupertino, CA, USA) infusing either L-thyroxine or vehicle (n=16). L-thyroxine (T4, Sigma-Aldrich Inc. T2501, St. Louis, MO, USA) dose was 1.5 μg/100 g/d of pre-mating body weight. With this dose, the female rat serum *free* T4 concentration was about 2.26±0.14 ng/dl (n=4) whereas the control females was about 1.34±0.11 ng/dl (n=4), which represents a mild hyperthyroid experimental condition (Varas et al., 2001; Evans et al., 2002). This measurement was made through blood samples obtained from sacrificed rats under general anaesthesia at day 10 of gestation and determined with chemiluminescent microparticle immunoassay (CMIA, ARCHITECT®, Abbott Laboratories, Abbott Park, IL, USA).

After 2 days of recovery from the implantation, female rats were mated with normal males for three consecutive days. The females were then singly housed during the gestational period. The dams gave birth between 19 and 21 days after the 3 day-long mating period and the litter sizes were 11.8±0.82 (mean±SEM) in maternal hyperthyroid rats (MH) group versus 11.6±0.66 for controls. The dams were then housed with their own litter during the lactation period with minimum disturbances. Osmotic pumps were surgically removed from the females on offspring postnatal day 1 (P1) under local anaesthesia (0.2 ml of 1% lidocaine). After the extraction was completed, skin was surgically sutured and dams were returned to their home cages. The sutures were removed after 4 days. All these procedures lasted at most 20 min. We did not observe any abnormalities regarding maternal care.

On P30, 96 offspring male rats were chosen from both maternal hyperthyroid and control litters (n=12) to form MH experimental group and control. Four male offspring from each litter were separated from their dam and housed together in one standard rat Plexiglas cage. Each sibling was designated to a different experiment except for plasma AVP concentration measurement. In this latter one, two siblings were chosen to form the group of 24 rats but they were allocated in different time points. Spontaneous locomotor activity, body weight, glycaemia and serum T4 level were tested on P90, as previously described (Zhang et al., 2008) and showed no significant differences between MH and controls.

Experimental design

Experimental procedures were performed on male offspring rats in young adult stage (starting on P100, body weight 350±10 g). Two sets of experiments were carried out separately. (1) Behavioural tests to assess the anxiogenesis threshold, plasma AVP levels and Fos expression during WD and Vogel conflict test (VCT) proper of MH compared to control. (2) Immunohistochemical and morphological characterizations of AVP labelled fibres in the anterior hypothalamus from MH and control rats under basal conditions by light and electron microscopy.

For the first set of experiments, we used the elevated plus maze test to assess the natural (unconditioned) exploratory behaviour of MH, compared to control subjects, to set a baseline for anxious state, and the VCT as both an osmotic stressor and a measure of trained (conditioned) anxious state (Vogel et al., 1971; Millan, 2003; Millan and Brocco, 2003).

Elevated plus maze test (EPM)

We used EPM to assess first the unconditioned acute anxious state. This experimental procedure was done 1 week before the VCT. The maze was made of wood, consisted of a plus-shaped platform elevated 50 cm above the floor with two closed arms (50×10×40 cm^3) and two open arms (50×10 cm^2) surrounded by an upwards-protruding edge of 0.5 cm connecting the central square of 10×10 cm^2. This latter measure prevents the rat falling accidentally without jeopardizing the elemental features of the setting, hence enhances the efficacy of the test. The EPM was lit with dim red light and monitored by CCTV. The maze was cleaned with water containing a detergent and dried before each trial.

Prior to the EPM test, rats were exposed to a standard open-field box (40×40×40 cm^3 made of wood (Pierce and Kalivas, 2007)) during 5 min for three consecutive days before and immediately previous to the EPM test. This procedure was made to increase the likelihood of entering the open arm of the maze, thus increasing the sensitivity of the test (Walf and Frye, 2007). Rats from each experimental group underwent the EPM test during their early activity period. The test started by placing the rat in the centre of the maze heading to an open arm and then left for free exploratory activity for 5 min. The percentages of both time spent on the open arms and the number of entries to the open arm in relation to the total number of entries (closed arms plus open arms) were analyzed as a measure of unconditioned acute anxious state (exploration vs. avoidance).

Vogel conflict test (VCT)

We used VCT (File et al., 2004) to assess the conditioned acute anxious state. VCT involves two main steps: WD for 48 h and food deprivation for the latter 24 h, and the conflict test proper. WD as long as 72 h, is well tolerated by rats with weight loss in an acceptable range (approximately 11%) and no apparent loss of physical vigour (Rowland, 2007). Completing the WD period, thirsty rats were exposed to a mild and intermittent electrical shock via a water bottle. This procedure incorporates an element of conflict, whereby the subject experiences opposing and concomitant tendencies of desire (drinking behaviour for reward) and of fear (avoidance of a potentially aversive stimulus). An indicator of a conditioned anxious state is when fear prevails in this conflict, where no genuine risk is present (Millan and Brocco, 2003). We chose the number of shocks received as our operational parameter for anxious state assessment, according to File et al. (2004).

Before the VCT, the experimental subjects were habituated by staying in the conflict-chamber described below, without current application for 30 min each day during four consecutive days. The conflict chamber consisted in a clear Plexiglas cage (20×30×20 cm^3) with a metal floor and lattice lid, a water bottle with stainless steel drinking spout, a constant current shock gen-