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Adolescent Corticotropin Releasing Hormone Neuroplasticity in the Rat Bed Nucleus of Stria Terminalis

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The purpose of this study was to find out whether there was an adolescent neurochemical plasticity in the bed nucleus of stria terminalis (BNST), especially the laterodorsal nucleus (BNSTLD) that is involved in stress and anxiety responses. We also aimed to register if there was any difference between the sexes. We chose to investigate the corticotropin-releasing hormone (CRH), which is involved in stress responses. The laterodorsal nucleus has a lot of CRH-expressing neurons, which are GABA-ergic. We observed that there was a preadolescent sexual difference with a higher expression of CRH in females, while in males we found that the expression of CRH increased in the pubertal period. Nevertheless after puberty such a difference disappears. Our results suggest different stress susceptibility between sexes in the preadolescent period, and could explain the inadequacy of stress coping in males during puberty.

Key words: bed nucleus of stria terminalis, corticotropin-releasing hormone, anxiety, puberty, rat

Introduction

The bed nucleus of stria terminalis (BNST) is involved in a wide variety of limbic functions, in particular anxiety responses and addiction [1]. It is a complex structure, and is considered to be part of the extended amygdala, and according to various authors is made up of 12-18 subnuclei [6, 10]. The neurons of the BNST are mainly GABA-ergic, although some glutamate-ergic ones can also be found [11]. The complexity of the structure is further deepened by the co-expression of a variety of neuropeptides [10]. One of the most commonly co-expressed neuropeptide in the BNST is the corticotropin-releasing hormone (CRH). Indeed, the subnucleus with a major CRH expression is the laterodorsal nucleus of BNST (BNSTLD) [5]. The CRH function is to activate the hypothalamic-pituitary-adrenal axis in response to stress [4], albeit the sex and age differences in the CRH expression in the BNST are not well investigated.

Material and Methods

Adult (60 days old; female n=5 and male n=5) and pre-adolescent (20 days old; female n=5 and male n=5) Wistar rats were used for the aim of this study. Pre-adolescent rats were 25-35 g of weight, and adults 230-260 g. The experiments were conducted in accordance with the ethical guidelines of the EU Directive 2010/63/EU for the protection of animals used for scientific purposes and permission from the Bulgarian Food Safety Agency for working with the experimental animals with № 374 was issued. A minimum number of rats were used to reduce the suffering and to fulfill the requirements for a good statistical analysis. The animals were anesthetized intraperitoneally with Pentobarbital (35 mg/kg). After that they were transcardially perfused with phosphate-buffered saline at a volume of approximately 50 ml, which contained heparin at a concentration of 10 UI/ml, followed by 4% solution of formaldehyde at a volume of approximately 1L/kg. The speed of the infusion was 20 ml/min and the pressure was 80-120 mmHg. After the perfusion, the brains were excised, then sectioned, and put in the perfusion fixative for another 8-20 hours at 4°C for postfixation. Each brain was sectioned in two different planes, i.e. in the coronal plane 2 mm posterior to the olfactory bulbs, and through the horizontal fissure. Thereafter they were embedded in paraffin, and sliced on a microtome at a thickness of 6 μm. Then immunohistochemistry was performed, using kit with DAB-detection system (PolyQ Stain 2 step detection system goat anti-mouse/rabbit HRP, Peroxidase quench, DAB kit; quartet GmBH). Briefly, after deparaffinization of the sections, the endogenous peroxidase was blocked with the use of previously mentioned kit for 10 min, followed by antigen retrieval using citrate buffer at 95°C for 20 min, and applying the primary rabbit polyclonal antibody (dilution 1:50, Affinity Biosciences) incubated overnight at 4°C. Then the incubation with the secondary antibody from the kit was performed. The visualization of the reaction was with DAB. All slides were proceeded simultaneously to standardize the results. The ready slides were scanned using Olympus VS-ASW Image Acquisition software. 20 images were taken from the BNSTLD and an average number of 600 neurons were examined. The images were turned black and white, and the intensity of the immunoreaction was obtained using Fiji software, measuring the intensity of the grey (from 0-black, to 255-white). To illustrate the distributional data we used Box and Whiskers plot diagram, where the mean, the 75 and 25 percentile and the highest and lowest measurements are presented. Statistical analysis was performed using T-test for parametric data, which were checked with Kolmogorov-Smirnov test of normality. The differences in the means were considered as statistically important only when the p-value was under 0.05.

Results

By using immunohistochemistry in the BNSTLD, we found that reactivity for CRH in the nucleus of preadolescent females was increased compared to preadolescent males. Thus, expression was also seen in the males although to a lesser extent. The reaction was mainly confined to the perikaryal cytoplasm (**Fig. 1 a, b**). On the other hand, the immunoreaction for CRH in the nucleus of adult rats was evenly expressed in both sexes (**Fig. 1 c, d**). It should also be noted that in the adult males in particular the reaction was much more pronounced than in the younger species of the same sex.

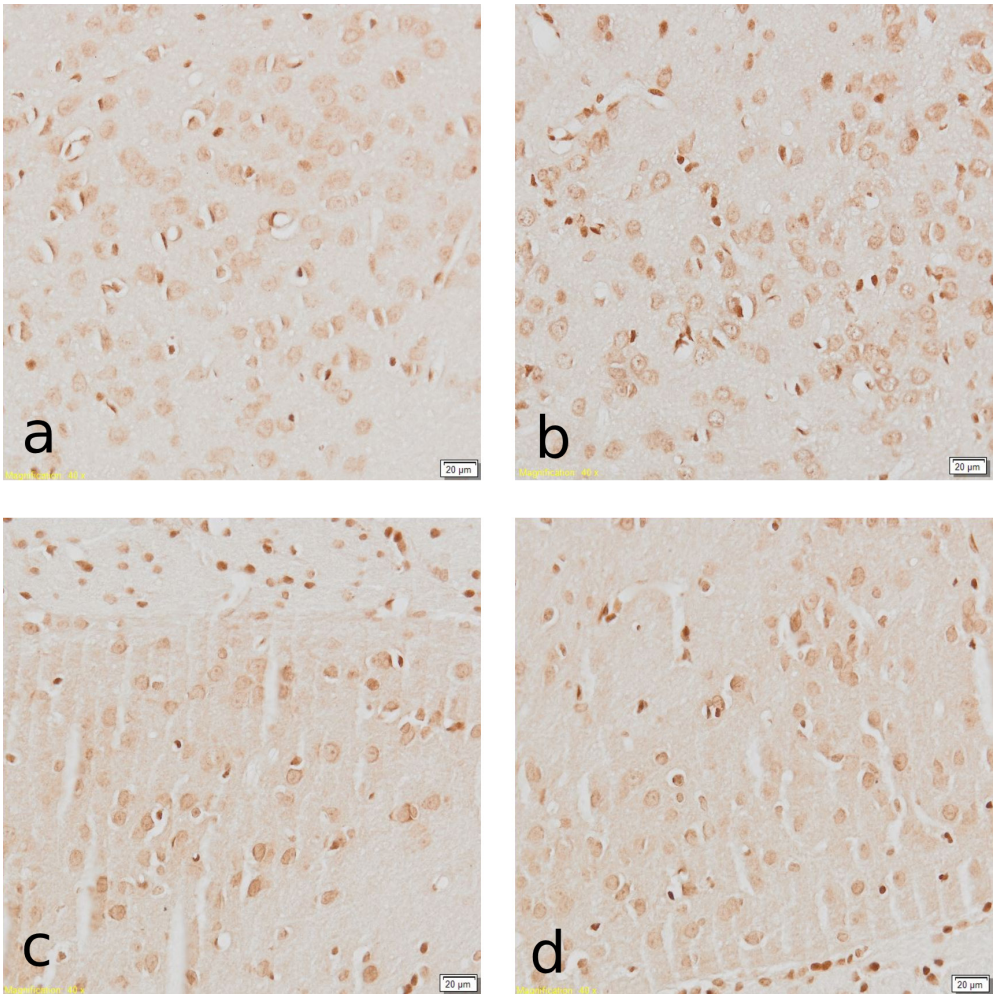

Fig. 1. Photomicrographs of immunohistochemical reaction for CRH at the level of BNSTLD. Images (a) and (b) are sections from preadolescent male and female rats respectively. Images (c) and (d) show sections of adult male and female animals respectively. It is clearly visible that the reaction in preadolescent males is weaker than the preadolescent females and also than adult males. In adults there is no visible sex difference in the CRH expression.

To check the statistical significance, we performed an analysis. The mean and the standard deviation of the expression of CRH (mean grey values) in the BNSTLD of the preadolescent male and female, and adult male and female rats were as follows: 193.59±3.71; 180.86±4.13; 182.06±6.54; 181.34±5.71 (**Fig. 2**). After the statistical analysis the following conclusions were made: the expression of CRH in the BNSTLD was higher in adult than in preadolescent rats, but only in males (**Fig. 3**). Also, the expression in preadolescent rats was higher in female than male rats, albeit in the adult rats we found no difference between sexes. The statistical data and measurements are presented in Table 1.

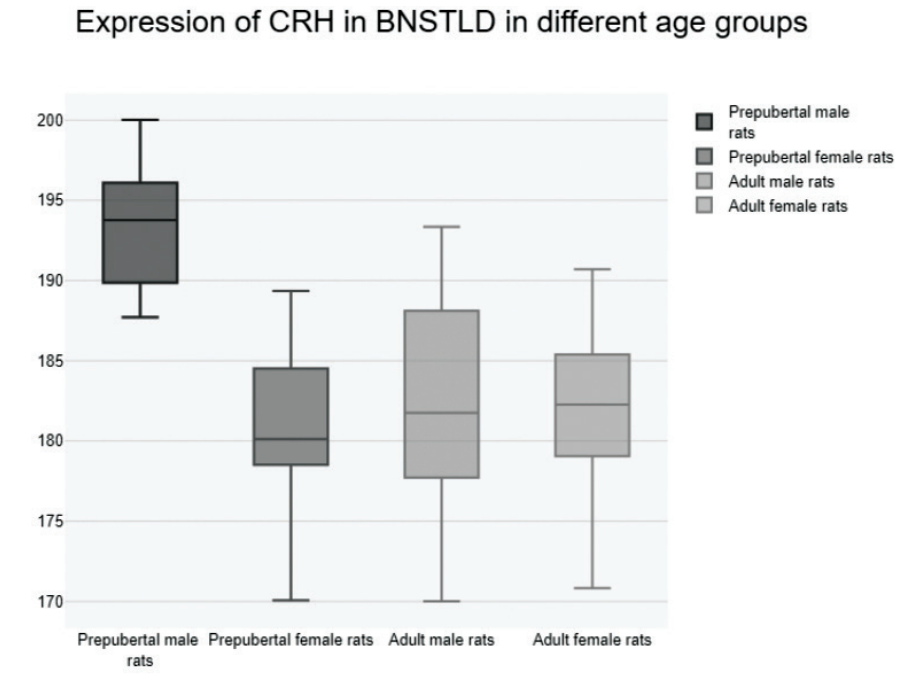

Fig. 2. Box and whiskers plot diagram representing the obtained data with the means, 25th and 75th percentile in the different age groups. On the vertical line the intensity of grey is shown (from 0-black to 255-white). There is statistical difference between preadolescent males and females and also between preadolescent males and adult males.

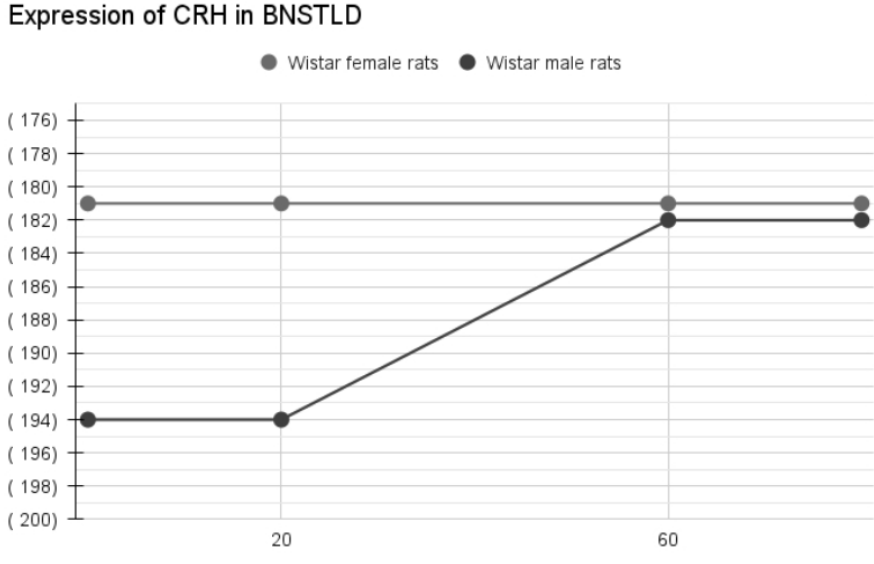

Fig. 3. Linear graph representing the periadolescent dynamics in CRH expression in BNSTLD. There is an obvious pubertal increase in CRH expression only in males, therefore the preadolescent sexual difference disappears after puberty.

Table 1. Statistical data from measurements of intensity of CRH imminoexpression of corticotropin-releasing hormone (CRH) in laterodorsal nucleus of bed nucleus of stria terminalis (BNSTLD).

Experimental group	Mean	Standard deviation	Kolmogorov-Smirnov-criteria
Pre-juvenile male	193.5992	3.71	0.11437
Pre-juvenile female	180.86063	4.13	0.12447
Adult male	182.05597	6.54	0.1067
Adult female	181.3364	5.71	0.10833

Compared groups	T-test value	P – value
Preadolescent male / Adult male	8.40813	<0.00001
Preadolescent female / Adult female	-0.36993	0.712781
Preadolescent male / Preadolescent female	7.70192	<0.00001
Adult male / Adult female	0.45408	0.65147

Discussion

BNST is the second major location of CRH-expressing neurons in the CNS after the hypothalamus [5] in rats. Unlike the latter, in the BNST the CRH-expressing neurons are GABA-ergic [5]. They are mainly located in the BNSTLD [3]. It is well documented that overexpression of CRH in these neurons is present in chronic stress [3, 12]. These cells are also implicated in reward responses since they send efferents to the ventral tegmental area [7]. Moreover, early maternal separation is involved in the overexpression of CRH [9]. Yu et al. [17] proved that CRH is overexpressed in the rat BNST after pain stimulation in a sex-specific manner. The study demonstrates that certain sex-specific peculiarities may exist in the CRH expression. Besides there is evidence for the different CRH response in males and females after stress [2]. The same review shows that females have a more increased anxiety-like behaviour after CRH-exposure than males. It should be noted that most of the studies, however, were performed in adult animals. Nonetheless, it is an emerging fact that the adolescent period is the second major period for neuroplasticity in the brain after the neonatal period [13] and it is also a period marked with significant behavioural changes [8]. Thus, we decided to check the periadolescent neuroplasticity in these neurons. Viau et al. [16] found out that various CRH-expressing sites in the rat brain are influenced differently by sex and age. The authors report age-related increased CRH mRNA expression in females in the paraventricular hypothalamic nucleus, while the same can only be observed in the central amygdala in males. Being part of the extended amygdala, BNSTLD is greatly associated with the central amygdaloid nucleus, classified as a central or lateral extended amygdala [6]. Therefore it is not surprising that our results are identical to the literature. Sterrenburg et al. [14] proved that there

is no sex difference in the CRH expression in BNSTLD in adult rats. In addition, early life stress can induce adult overexpression of CRH [16], so hyper-responsiveness to stress in females, proved in other brain areas [15], leads us to the conclusion that the preadolescent overexpression of CRH in female rats causes hypersensitivity to stress before puberty. Such hypersensitivity could have long-term effects. In addition, the CRH pubertal changes in males may impact on their stress coping during the adolescent period. Further experiments are needed in order to clarify the specific role of CRH before and during puberty, and how this influences stress susceptibility and coping in adult life.

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References

1. **Avery, S. N., J. A. Clauss, J. U. Blackford**. The Human BNST: Functional Role in Anxiety and Addiction. – *Neuropsychopharmacology,* **41(1),** 2016, 126-41.
2. **Bangasser, D. A., K. R. Wiersielis.** Sex differences in stress responses: a critical role for corticotropin-releasing factor. – *Hormones (Athens),* **17(1),** 2018, 5-13.
3. **Beyeler, A., J. Dabrowska.** Neuronal diversity of the amygdala and the bed nucleus of the stria terminalis. - *Handb. Behav. Neurosci*. **26,** 2020, 63-100.
4. **Caruso, A., A. Gaetano, S. Scaccianoce.** Corticotropin-Releasing Hormone: Biology and Therapeutic Opportunities. – *Biology (Basel),* **11(12),** 2022, 1785.
5. **Dabrowska, J., R. Hazra, J. D. Guo, S. Dewitt, D. G. Rainnie**. Central CRF neurons are not created equal: phenotypic differences in CRF-containing neurons of the rat paraventricular hypothalamus and the bed nucleus of the stria terminalis. – *Front. Neurosci.* **7**, 2013, 156.
6. **De Olmos, J. S., C. A. Beltramino, G. Alheid.** Amygdala and extended amygdala of the Rat: A cytoarchitectonical, fibroarchitectonical, and chemoarchitectonical survey. In: *The rat nervous system* (Ed. G. Paxinos) 3th ed., London, Elsevie Academis Press, 2004, 509-605.
7. **Dedic, N., C. Kühne, M. Jakovcevski, J. Hartmann, A. J. Genewsky, K. S. Gomes, E. Anderzhanova, M. L. Pöhlmann, S. Chang, A. Kolarz, A. M. Vogl, J. Dine, M. W. Metzger, B. Schmid, R. C. Almada, K. J. Ressler, C. T. Wotjak, V. Grinevich, A. Chen, M. V. Schmidt, W. Wurst, D. Refojo, J. M. Deussing.** Chronic CRH depletion from GABAergic, long-range projection neurons in the extended amygdala reduces dopamine release and increases anxiety. – *Nat. Neurosci*. **21(6),** 2018, 803-807.
8. **Holland-Hall, C., G. Burstein.** Adolescent physical and social development. In: *Nelson's textbook of paediatrics* (Eds. Kliegman R. M., B. F. Stanton, J. W. St Geme III, N. F. Schor, R. E. Behrman) 20th ed, Philadelphia, Elsevier, 2016; 926-931.
9. **Hu, P., I. Maita, M. L. Phan, E. Gu, C. Kwok, A. Dieterich, M. M. Gergues, C. N. Yohn, Y. Wang, J. N. Zhou, X. R. Qi, D. F. Swaab, Z. P. Pang, P. J. Lucassen, T. A. Roepke, B. A. Samuels.** Early-life stress alters affective behaviors in adult mice through persistent activation of CRH-BDNF signaling in the oval bed nucleus of the stria terminalis. – *Transl. Psychiatry,* **10(1),** 2020, 396.
10. **Lebow, M. A., A. Chen.** Overshadowed by the amygdala: the bed nucleus of the striaterminalis emerges as key to psychiatric disorders. – *Mol. Psychiatry*, **21**, 2016, 450-463.
11. **Nguyen, A. Q., J. A. Dela Cruz, Y. Sun, T. C. Holmes, X. Xu**. Genetic cell targeting uncovers specific neuronal types and distinct subregions in the bed nucleus of the stria terminalis. – *J. Comp. Neurol.,* **524(12),** 2016, 2379-2399.

13. **Olivera-Pasilio, V., J. Dabrowska**. Oxytocin Promotes Accurate Fear Discrimination and Adaptive Defensive Behaviors. – *Front. Neurosci*., **14,** 2020, 583878.
14. **Schulz, K. M., H. A. Molenda-Figueira, C. L. Sisk.** Back to the future: The organizational-activational hypothesis adapted to puberty and adolescence. – *Horm. Behav*., **55(5),** 2009, 597-604.
15. **Sterrenburg, L., B. Gaszner, J. Boerrigter, L. Santbergen, M. Bramini, E. W. Roubos, B. W. Peeters, T. Kozicz.** Sex-dependent and differential responses to acute restraint stress of corticotropin-releasing factor-producing neurons in the rat paraventricular nucleus, central amygdala, and bed nucleus of the stria terminalis. – *J. Neurosci. Res.*, **90**, 2012, 179-192.
16. **Toledo-Rodriguez, M., C. Sandi**. Stress before puberty exerts a sex- and age-related impact on auditory and contextual fear conditioning in the rat. – *Neural. Plast*., **2007**, 2007, 71203.
17. **Viau, V., B. Bingham, J. Davis, P. Lee, M. Wong.** Gender and puberty interact on the stress-induced activation of parvocellular neurosecretory neurons and corticotropin-releasing hormone messenger ribonucleic acid expression in the rat. – *Endocrinol*ogy, **146(1),** 2005, 137-46
18. **Yu, W., C. M. Caira, N. Del R Rivera Sanchez, G.A. Moseley, T. L. Kash**. Corticotropin-releasing factor neurons in the bed nucleus of the stria terminalis exhibit sex-specific pain encoding in mice. – *Sci. Rep*., ***11****(1),* 2021, 12500.