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Neuroscience Letters 419 (2007) 168–171
Neuroscience Letters
www.elsevier.com/locate/neulet

Effects of the testicular feminization mutation (tfm) of the androgen receptor gene on BSTMPM volume and morphology in rats

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Received 4 October 2006; received in revised form 26 March 2007; accepted 17 April 2007

Abstract

The posteromedial bed nucleus of the stria terminalis (BSTMPM) is an important component of the extended amygdala that is sexually dimorphic in rats. We examined the effect of the testicular feminization mutation (tfm), which renders the androgen receptor (AR) dysfunctional, on BSTMPM volume and average somal area. As expected, we found a significant sex difference in the volume of the BSTMPM, with females having a smaller BSTMPM than wild type males. Size of the BSTMPM in tfm males was also significantly smaller than that of wildtype males, although this difference was significant only on the left side. We found no sex difference in BSTMPM somal areas. These findings support the role of androgen receptors in the sexual differentiation of this nucleus.

Keywords: Sexual differentiation; Sexual dimorphism; Androgen insensitivity; MePD; Bed nucleus of the stria terminalis; BST; Androgen receptor

Mammals display behavioral and morphological characteristics that differ dramatically between males and females. These sexually dimorphic traits are controlled in large part by exposure to gonadal steroids and include aggression, sexual behavior, gonadotropin secretion and integration of olfactory information. The extended amygdala represents one of the most important neural circuits to mediate these functions. Part of this circuitry is the posteromedial nucleus of the bed nucleus of the stria terminalis (BSTMPM). In the rat, this region displays several sexually dimorphic traits, with a higher density of androgen receptor (AR) and significantly larger volume in males than in females [13,15,19]. This sex difference in volume can be reversed by perinatal gonadectomy in males or androgen treatment in females [11]. Furthermore, gonadectomy in adult males also decreases the volume of this nucleus by approximately 28% [16].

The BSTMPM communicates with the posterodorsal medial amygdala (MePD), via bidirectional connections [2,3,5,10] and constitutes an important relay center in the rodent mating neural circuit. The male receives olfactory sensory cues from an estrous female that reach the medial amygdala via the accessory olfactory pathway. The MePD then relays olfactory information to the BSTMPM, which along with the MePD sends substantial projections to the sexually dimorphic nuclei responsible for the initiation of mating behaviors [2,3,22]. In addition to strong bidirectional connections, the MePD and BSTMPM also share similar profiles of protein expression, including the expression of neuropeptides and both ARs and estrogen receptors [1,6,7,12,17,19,23–25].

Much research has focused on determining which gonadal hormones are responsible for modulating the morphology of these sexually dimorphic brain regions. We recently reported that the testicular feminization mutation (tfm) of the *ar* gene in rats, which renders males largely insensitive to androgens, results in MePD volume and soma sizes that are intermediate between wild-type males and females, raising the possibility that ARs have an important organizational role in this nucleus [18]. Based on the close functional and developmental association of the BSTMPM and the MePD, as well as their similarities in hormone responsiveness and AR content, we hypothesized that the BSTMPM of tfm males would also differ significantly in volume and/or neural soma area compared to wild-type males. Garcia-Falgueras et al. [9] previously examined whether BSTMPM volume or neuronal number is different in tfm males as compared to wild type males, but their results were inconclusive. There was no sex difference in BSTMPM volume and neuronal number between male and female control littermates of the tfm rats on a mixed Wistar/Long Evans background, making it

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doi:10.1016/j.neulet.2007.04.033

difficult to interpret the data from tfm males. Thus, this study was performed to re-examine the potential role of AR in morphological sex differences in the BSTMPM of male, female and tfm animals on a pure Long Evans background.

Littermates of wild-type male, female (tfm carriers and non-carriers) and tfm rats of Long Evans background were taken from our colony at Michigan State University. The sires for this colony, for over 20 generations, have been commercially purchased Long Evans males, so it represents a relatively pure Long Evans strain. Brains from many of these same animals were used in a previous study of the MePD [18]. Animals were housed by group, three to a cage, after weaning and kept in a 12:12 light–dark cycle with lights on at 0700 h and off at 1900 h. All animal care and use procedures met standards set by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Michigan State University. Animals were given an overdose of sodium pentobarbital (120 mg/kg) to induce deep anesthesia and transcardially perfused with 0.9% saline, followed by 10% buffered formalin. Genotype was determined by examining the external genitals, presence of nipples and gonads. Despite tfm males having high physiological levels of circulating T [21], they have feminized genitalia with short anogenital distances, nipples but abdominal testes. Brains were removed and placed in 10% buffered formalin for at least 1 month, after which they were placed overnight in 20% phosphate-buffered sucrose (pH 7.4) at 4 °C prior to slicing.

Each brain was scored along the left cortex to mark laterality, blocked at the cerebellum and olfactory tubercle, and coronally sectioned on a freezing sliding microtome set to 40 μm. Sections were collected into a phosphate buffer (0.1 M PO_4, 1% gelatin, 0.3% Triton X-100; pH 7.4); every third section was mounted onto gel-subbed glass slides, with a random start from the first series of each brain to ensure that every section had an equal probability of being chosen for sampling. Missing sections due to damage were replaced by one of the two remaining sections within that interval, as determined by a coin flip, or a space was left on the slide. After being allowed to air dry, mounted tissue was stained with thionin for Nissl substance, and coverslipped with Permount.

The volume of the BSTMPM was measured by an investigator blind to the group status of the animals. BSTMPM boundaries were determined following nomenclature from a standard rat atlas (Paxinos and Watson, 1998). It is located in coronal sections below the lateral ventricle and lateral to the third ventricle and the fornix (Fig. 1).

StereoInvestigator software (Microbrightfield, Colchester, VT) was used to estimate the volume of the BSTMPM and average soma size of BSTMPM neurons for both brain hemispheres. Images were captured by a digital camera and displayed on a computer screen, allowing the BSTMPM or its neurons to be traced on the monitor using a computer mouse. BSTMPM area was traced throughout its rostrocaudal extent using a 5× objective, while BSTMPM neuronal somata were traced using a 100× oil objective. An average of 27 somata were measured from each hemisphere of each successive section containing the BSTMPM (sampling a total of 13–182 neurons/hemisphere).

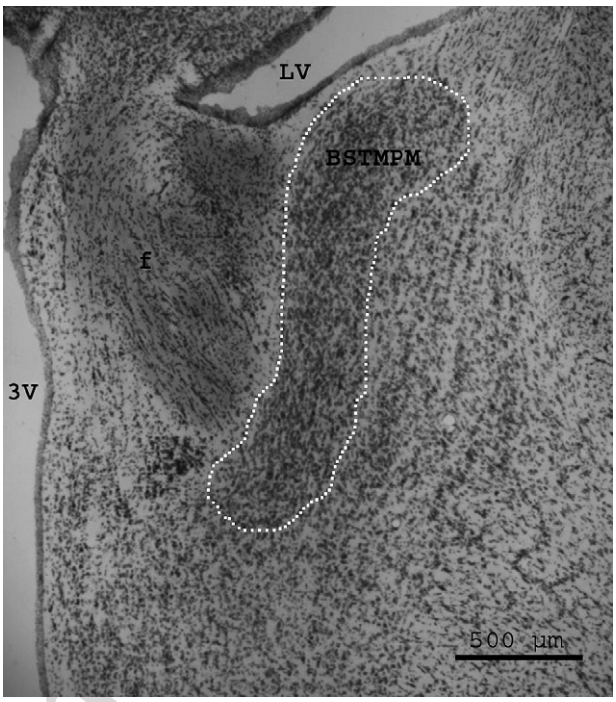

Fig. 1. Representative photomicrograph showing the right BSTMPM in a Nissl-stained cross section of brain of a wild-type male. The BSTMPM is located right below the lateral ventricle (LV) and lateral to the third ventricle (3V) and the fornix (f).

Neurons were randomly selected by the StereoInvestigator software using the optical fractionator probe and were identified by the presence of a distinct Nissl-stained cytoplasm and nucleolus. Soma measures were then averaged within each hemisphere yielding a mean soma size per hemisphere for each animal. Left and right BSTMPM volumes for each animal were calculated by multiplying the total cross sectional area by the sampling interval (3) and section thickness (40 μm).

For BSTMPM volumes, groups were compared using a mixed design ANOVA with genotype (male, $N = 8$; tfm male, $N = 14$; female, $N = 18$) as an independent variable and laterality (left versus right) as the repeated measure. A separate ANOVA was used to compare average somal area between groups (male, $N = 8$; tfm, $N = 14$; female, $N = 17$), again using laterality as a repeated measure. A Tukey post hoc test was used to identify significant sources of variance.

The two-way ANOVA for volume indicated a main effect of laterality ($p = 0.001$) with the left BSTMPM being larger than the right (Fig. 2). There was also a main effect of genotype ($p < 0.001$) but no significant interaction ($p = 0.87$). BSTMPM bilateral volume was greater in males than in either females ($p < 0.001$) or tfm males ($p = 0.007$). Further, probing revealed that BSTMPM volume in wild type males was significantly larger than in females in both the left ($p < 0.001$) and right ($p = 0.004$) hemispheres, but was significantly larger than tfm males ($p = 0.002$) only on the left side. Moreover, BSTMPM volume in tfm males was not significantly different from females on either side, although there was a trend toward significance ($p = 0.58$) in the right hemisphere. For average somal area, there were no main effects of genotype or laterality and no interaction,

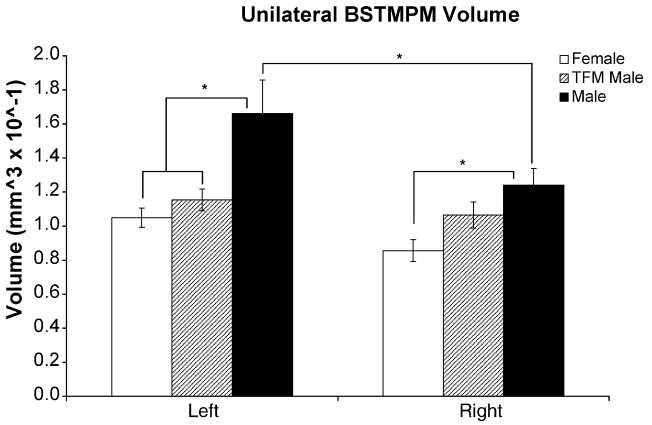

Fig. 2. Mean unilateral BSTMPM volumes. Analysis of variance revealed a main effect of laterality and of genotype, with no interaction. Post hoc tests revealed that male volumes were significantly larger than females ($p < 0.001$) and tfm males ($p < 0.002$) in the left hemisphere. In the right hemisphere, male volumes were larger than females ($p = 0.007$) but not tfm males. tfm Males did not significantly differ from females on any measure. Although the mean volume of the BSTMPM was greater on the left than the right in all these groups, this laterality was statistically significant only in males ($p < 0.001$).

indicating that the size of neuronal somata in the BSTMPM is neither sexually differentiated, nor influenced by this particular mutation in the *ar* gene (Table 1).

The goal of the present experiment was to test the hypothesis that ARs play a role in determining a sex difference in the volume of the BNST using the tfm model. Our results support this hypothesis. A previous report found no differences in volume between males and their littermate tfm males, which was attributed to a lack of organizational effects of androgen in this region [9]. However, this discrepancy may have resulted from differences in our rat strains. In the previous study, the original Long Evans tfm carrier or wild type females were crossed with Wistar rats, resulting in animals that were approximately 50% Long Evans and 50% Wistar. Interestingly, sex differences in BSTMPM volume and neuronal number were observed in males and females from wild-type dams, but not in offspring from tfm carrier dams. While these results suggest the intriguing possibility that the genotype of the dams (i.e., whether they carry the *tfm* allele or not) may influence how robustly sex differences in the brains of their offspring are expressed, these results provide no clear answer about the potential role ARs play in sexual differentiation of the BSTMPM.

Our data are consistent with previous studies in supporting a high degree of functional and anatomical continuity within the extended amygdala, with the MePD and the BSTMPM showing comparable sex differences in volume, and similar responses to the *tfm* allele. Wild-type males had BSTMPM volumes 52% larger than wild-type females and 31% larger than tfm males supporting a role for AR in regulating the morphology of this nucleus. Similar findings were reported for the rat MePD [18]. However, aromatase expression in the MePD appears to be independent of AR, given that tfm males have aromatase mRNA levels comparable to wild-type males. Such is not the case for the BSTMPM, which displays lower levels of aromatase activity in tfm males compared to wild-type males [20,21]. Thus, it is possible that AR in this nucleus might be important in controlling the conversion of testosterone to estradiol. In both cases, however, it is not known when during the animal's lifespan ARs might be exerting this influence. Another notable difference between the two nuclei is that MePD soma size was sexually dimorphic, with tfm males displaying intermediate values, while no comparable differences were found in neuronal somata in the BSTMPM. Thus, it is clear that sex differences in regional volume need not reflect sex differences in neuronal soma size.

Table 1
Mean BSTMPM neuronal somal areas for each experimental group (μm^2) with standard errors of the mean

	Bilateral	Left	Right
Males	146.87 ± 3.89	146.17 ± 4.26	147.54 ± 4.79
tfm Males	143.23 ± 2.07	143.53 ± 2.04	142.32 ± 2.06
Females	140.34 ± 1.91	139.79 ± 2.79	142.01 ± 1.89

ANOVA did not reveal any group differences or laterality in this measure.

The observed lateralization of the BSTMPM warrants further investigation, especially given the fact that an opposite pattern of lateralization has been observed in the MePD. While BSTMPM neuronal number was not quantified in the present study, it is possible that factors other than number and size of neurons might account for the observed differences in volume. For example, volume occupied by dendritic shafts and glia has recently been reported to fully account for the sex difference in overall volume of the left MePD of prepubertal rats [4]. More studies are needed to address this question in the BSTMPM.

These results indicate that ARs play an important role in sexual differentiation of the BSTMPM. When compared to wild-type males, the BSTMPM of tfm males was significantly smaller, and this difference was most apparent in the left hemisphere. These findings complement previous reports that the *tfm* allele of the AR leads to dramatic changes in both behavior and brain morphology of XY individuals [8,14]. Furthermore, these and previously published results from our lab [18] support the role of ARs in regulating sex differences in two very important nodes in the male mating neural circuit, the MePD and the BSTMPM.

Acknowledgements

Thanks to Brittany Dugger for her assistance with stereological procedures and to David Swender for his assistance with the histology. This work was supported by NIH NS045195.

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