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Research report

Chronic social stress during puberty enhances tyrosine hydroxylase immunoreactivity within the limbic system in golden hamsters

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Abstract

The present study was carried out to determine the effects of chronic exposure to social stress during puberty on the dopamine system in male golden hamsters. Experimental animals were socially subjugated between postnatal days 28 (P28) and 42. All animals were sacrificed on P46 and their brains processed for immunocytochemistry to tyrosine hydroxylase (TH). A large increase in the number of TH-immunoreactive (TH-ir) neurons was noted within the posterior portion of the medial amygdaloid nucleus and the posterior portion of the medial division of the bed nucleus of the stria terminalis in subjugated animals as compared to controls. This effect appeared to be site-specific as no difference was seen between groups in the periventricular nucleus, another steroid receptor-rich area. The data suggest that these dopamine neurons may play an important role in the behavioral changes associated with chronic social stress during puberty.
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1. Introduction

In male golden hamsters, chronic exposure to social stress during puberty results in unique context-dependent alterations in aggressive behavior [5]. Although subjugated individuals were more likely to avoid intruders of equal size and age, they were also more aggressive towards smaller and younger intruders. These findings were associated with increased serotonin innervation and decreased vasopressin content in the anterior hypothalamus. These results showed a correlation between neurochemical changes and behavioral inhibition towards intruders of equal size and age, as offensive aggression is inhibited by serotonin and facilitated by vasopressin release in the anterior hypothalamus [7,8]. At the same time, however, these findings were not consistent with the increased aggression displayed by subjugated individuals when in the presence of smaller and younger stimuli. It was previously hypothesized that the enhanced aggression in the presence of smaller and younger intruders was associated with alterations within vasopressin and serotonin receptors [5]. However, preliminary experiments failed to support this hypothesis [Ferris and Delville, unpublished observations], suggesting the involvement of another neurotransmitter system.

A recent report by Harrison et al. has implicated dopamine in the control of offensive aggression in hamsters [9]. In this study, male hamsters received chronic injections of low cocaine doses during puberty. When tested for offensive aggression during early adulthood, these animals were particularly aggressive, suggesting a role for dopamine in the regulation of offensive aggression. It possible that stress enhances activity within specific populations of dopamine neurons related to the control of offensive aggression in hamsters. Interestingly, hamsters have dopamine neurons within areas associated with the consummation of offensive aggression. In particular, the medial amygdala, notably the posterodorsal portion of the medial amygdaloid nucleus (MePD), and the bed nucleus of the stria terminalis (BST), notably, the

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posterior portion of the medial division of the bed nucleus of the stria terminalis (BSTPM), have reciprocal connections with the anterior hypothalamus, an area that plays a critical role in the display of aggressive behaviors [6]. Increased neuronal activity within these areas is associated with the consummation of offensive aggression [6]. Also, a population of testosterone-dependent dopamine neurons has been reported in the BSTPM and MePD in hamsters [2,3]. Therefore, the present study is focused on these neurons.

In the present study, we tested the possibility that chronic exposure to stress during puberty affects tyrosine hydroxylase (TH) activity in areas involved in aggressive behavior. TH-immunoreactivity (TH-ir) was compared between previously subjugated individuals and their controls.

2. Materials and methods

2.1. Animals and treatment

Adult male ($n=8$) and adult female ($n=12$) golden hamsters (9–10 weeks old) were purchased from Harlan Sprague–Dawley (Indianapolis, IN, USA). Two months later, the females were mated and became pregnant. Litter size was limited to six pups per litter including males and females. Male pups were weaned on postnatal day 25 (P25) and individually housed in plexiglass cages (8×14×5 inches). All animals were housed in a reversed daylight cycle (14L, 10D, lights on at 9:00 a.m.) and received food and water ad libitum. The animals were distributed evenly between subjugated and control groups according to body weight and litter. Chronic exposure to social subjugation was performed as previously described [5]. Briefly, animals in the experimental group (subjugated, $n=6$) were placed in the home cage of an aggressive adult male daily for 20 min from P28 to P42. This period corresponds to puberty in golden hamsters [16]. During this period, all subjugated animals were observed to ensure that each was exposed to attacks, chases, and bites by the resident adult males. Control animals ($n=6$) were placed in clean empty cages daily for 20 min during this same period of time. All animal manipulations were performed during the second half of the dark phase. The animals were weighed twice weekly between P28 and the end of the experiment. The hamsters were sacrificed by rapid decapitation on P46, and their brains ($n=6$/group) were processed for TH immunocytochemistry. Testes weights were also recorded and compared between groups with a Student's t-test (two-tailed).

2.2. Tyrosine hydroxylase immunocytochemistry

Brains were fixed by overnight immersion in 10% acrolein in 0.1 M KPBS buffer (pH 7.2) at 4 °C and later saved in 20% sucrose–KPBS. The brains were then cut into 40-μm thick sections with a freezing rotatory microtome and the sections were saved in a cryoprotectant [17] at −20 °C until labeled by immunocytochemistry. Immunocytochemistry for TH was performed as previously described [11]. First, the sections were pretreated in 1% sodium borohydrite (to remove residual aldehydes) followed by a preincubation in a solution containing 20% normal goat serum, 1% hydrogen peroxide, and 0.3% Triton X-100 (respectively, to block nonspecific labeling, eliminate endogenous peroxidase activity, and permeabilize the tissue). Sections were then incubated in a mouse monoclonal antibody to TH (1:20 000; Sigma, St. Louis, MO, USA), containing 2% normal goat serum and 0.3% Triton X-100 for 48 h at 4 °C. After washing, the sections were incubated in the secondary antibody (biotinylated goat antimouse IgG; 7.5 μg/ml; Vector Labs., Burlingame, CA, USA) followed by a tertiary incubation (Vectastain ABC Elite Kit, Vector). Between incubations, sections were washed in 0.05 M TBS (pH 7.6). Finally, the sections were labeled with diaminobenzidine (DAB, 0.5 mg/ml) and 0.05% hydrogen peroxide. This procedure yields optimal labeling of TH-immunoreactive (TH-ir) neurons, dendrites and axons throughout the brain.

Labeled sections were mounted on gel-coated slides and coverslipped with permount. Later, TH-IR was observed with a Nikon Eclipse E600 microscope. TR-ir neurons were counted with a camera lucida attachment on the microscope. The areas selected for cell counts (Fig. 1) included BSTPM, MePD, and the periventricular hypothalamic nucleus (Pe) at the level of the optic chiasma [13]. Six to twelve measures were taken from consecutive sections on either side of the brain for each area analyzed and each individual. Immunoreactive cells were counted only when their nucleus was clearly visible. The average number of TH-ir cells per count per area and per individual was compared between groups for each selected area with Student's t-tests (two-tailed).

2.3. Dopamine β-hydroxylase immunocytochemistry

Alternate sections were also labeled by immunocytochemistry for dopamine β-hydroxylase (DBH), a key enzyme in the synthesis of norepinephrine from dopamine. This procedure was used to determine whether neurons expressing TH-IR are capable of producing norepinephrine. The sections were labeled following a slightly modified protocol, using a rabbit polyclonal antibody to DBH (1:500; Protos Biotech, New York, NY, USA). Normal goat serum was substituted with normal donkey serum. The secondary antibody was a biotinylated donkey antirabbit IgG (5 μg/ml; Jackson Immunoresearch Labs., West Grove, PA, USA). All solutions were prepared with 0.1 M PBS (pH 7.4) .

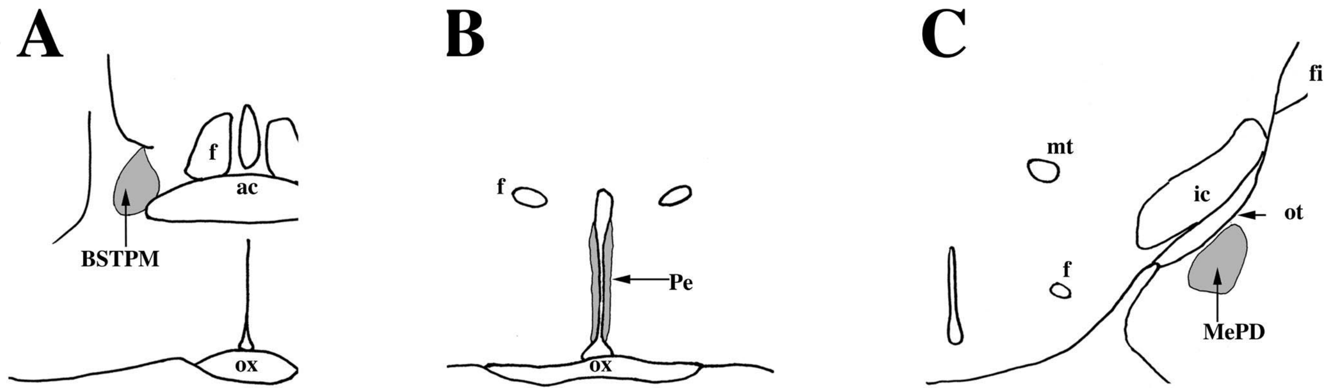

Fig. 1. Camera lucida drawings showing the location of the areas quantified (shaded areas) for TH-IR. These areas include the posterior portion of the medial division of the bed nucleus of the stria terminalis (BSTPM, A), periventricular nucleus of the hypothalamus (Pe, B), and the posterodorsal portion of the medial amygdaloid nucleus (MePD, C); ac, anterior commissure; ic, internal capsule; f, fornix; fi, fimbria of hippocampus; mt, mammillothalamic tract; ot, optic tract; ox, optic chiasma.

3. Results

As the animals were weaned on P25, the average body weights were 43.0 g and 41.0 g in the subjugated and control groups, respectively. By the end of the experiment, all animals had grown substantially. However, subjugated animals were slightly heavier (8%) than the controls (91.5±9.0 g and 84.2±7.4 g, respectively). The difference was not statistically significant [$t(10)=1.53$, $P>0.1$]. A similar difference was previously observed in chronically subjugated hamsters [5]. In addition, testicular weights were also compared between groups. The testes averaged 2.4±0.4 g in subjugated animals and 2.3±0.4 g in control animals. The differences were not statistically significant [$t(10)=0.04$, $P>0.1$].

While observing the distribution of TH-IR, we noted strong differences in the amygdala and the BST. The distribution of TH-ir neurons in this study was similar to previously described distributions of dopamine neurons in hamsters [2,3], including a population of cells located within the amygdala and BST. The populations of TH-ir cells were particularly prominent in subjugated individuals and consisted of clusters of 10–15 TH-ir neurons per area in both the BSTPM and MePD (Figs. 2 and 3). In contrast, only 1–3 TH-ir neurons per area were counted in control individuals. These differences were statistically significant for each area [BSTPM: $t(10)=8.65$, $P<0.001$; MePD: $t(10)=8.37$, $P<0.001$]. In order to determine the extent of the differences in numbers of TH-ir cells between the groups, we also compared cell counts in a neighboring neuroendocrine population—the Pe. In this population, the average number of TH-ir neurons per area ranged between 70 and 80 (Fig. 3). These differences were not statistically significant [$t(10)=0.68$, $P>0.1$].

The immunocytochemical procedure for DBH resulted in immunoreactive innervation throughout the hypothalamus, preoptic area, central amygdala, posterointermediate and posterolateral divisions of the BST. Immunoreactive neurons were clearly visible within the locus coeruleus and its subnuclei. However, only a few DBH-IR fibers were seen within the MePD and the BSTPM. No DBH-IR neurons were observed within these two nuclei in subjugated animals.

4. Discussion

The present data show that chronic exposure to social stress during puberty has site-specific effects on dopaminergic neurons. Up to five times more TH-IR neurons were counted within the MePD and the BSTPM of subjugated animals as compared to the controls. However, no significant differences between groups were observed in the Pe, another neuroendocrine region rich in gonadal steroid receptors [12,18,19]. These differences were very reliable. Additional sections were also labeled for TH immunocytochemistry. These sections had been preserved for several years in cryoprotectant and originated from a previous study testing the effects of chronic social stress during puberty [5]. Although the number of remaining sections was not large enough to allow for suitable quantification, similar differences in TH-IR were observed between subjugated and control animals in the available sample. These observations suggest that the increases in the number of TH-IR neurons in the MePD and the BSTPM are consistent results of chronic social stress during adolescence.

The increase in TH-IR following social subjugation of male hamsters during puberty could possibly reflect an increase in dopamine synthesis and release. Such observation has been previously reported in stressed rats. Several studies show that chronic exposure to stress enhances TH expression [1,14,15] and dopamine release [10]. For instance, a 20–50% increase in the amount of TH-IR was observed in homogenized samples of the VTA following repeatedly stressed Fisher but not Sprague–Dawley rats

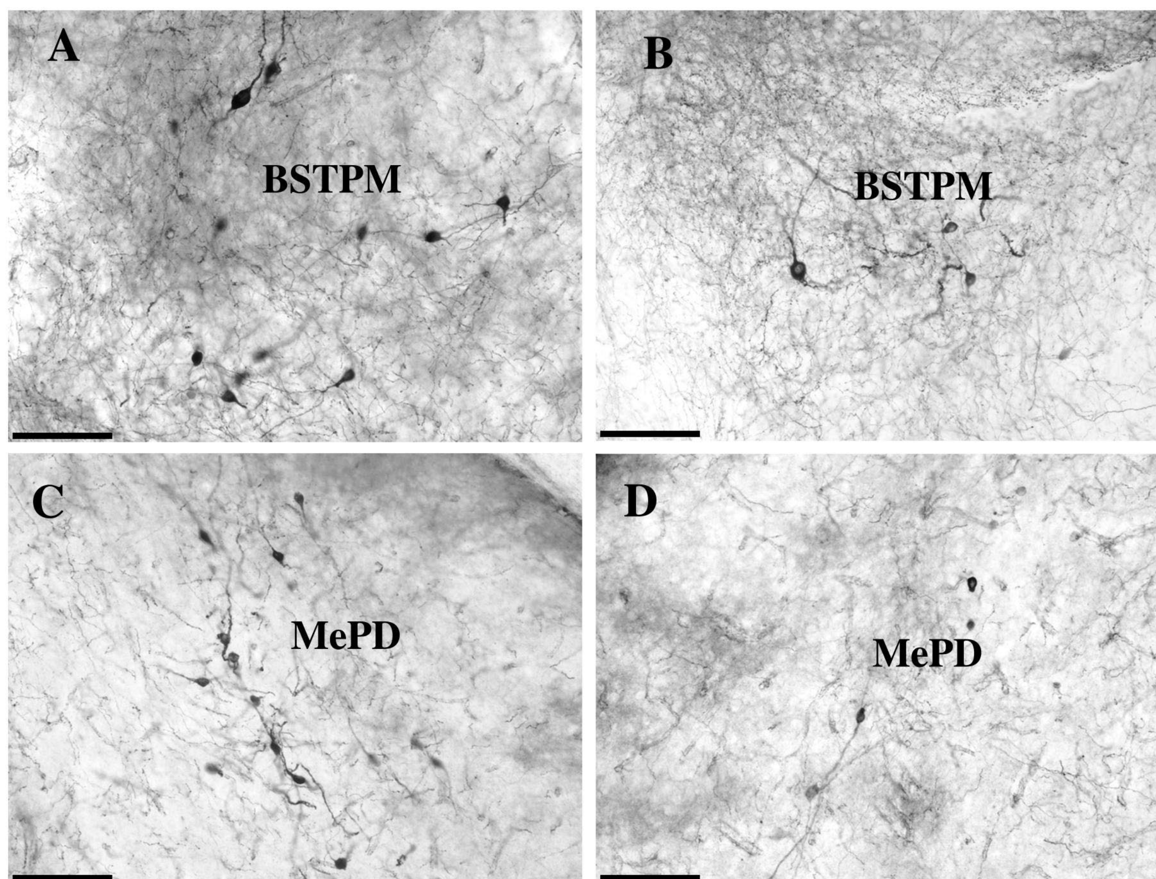

Fig. 2. Photomicrographs showing TH-IR within the posterior portion of the medial division of the bed nucleus of the stria terminalis (BSTPM, A and B) and posterodorsal portion of the medial amygdaloid nucleus (MePD, C and D) in previously subjugated male golden hamsters (A and C) and their controls (B and D). Scale bars: 100 μm.

[14]. Following chronic exposure to stress, analysis of homogenized punch-samples showed a 2-fold increase in the expression of TH mRNA in the VTA. Individuals exposed to 7–14 days of isolation stress showed increased TH mRNA expression in neurons within the VTA (35%) and SN (50%) as compared to non-stressed controls [1]. Interestingly, stress has also been associated with increased dopamine release, as rats chronically exposed to forced swim tests displayed a 400% increase in DA release [10]. These data suggest that the site-specific increase in TH-IR expression in the brain of hamsters is also associated with enhanced dopamine synthesis and release. This possibility will be tested in further experiments.

These two populations of TH-ir neurons within the BST and amygdala have been previously observed in golden hamsters [2,3]. In these reports, these TH-ir neurons were found to contain dopamine. These authors also tested for the presence of DBH in these areas to further characterize the neurochemical nature of these cells. They found no DBH-immunoreactive (DBH-ir) neurons in the BST and amygdala, suggesting that these TH-ir neurons are dopaminergic. We made similar observations. As in previous studies [3], no DBH-IR neurons were found within the BST and amygdala of socially subjugated hamsters. In addition, these two populations of TH-IR neurons were also characterized as containing androgen receptors and dependent on testosterone treatment [2]. Therefore, it is possible that chronic exposure to stress further induces TH-IR expression within these two populations during puberty.

The areas in which increased TH-IR were seen are of particular interest. The MePD and BSTPM are interconnected [4]. These two nuclei also have reciprocal connections with the anterior hypothalamus [6], a critical site for the control of aggressive behavior in golden hamsters [7,8]. This is especially interesting, since both of these areas show enhanced neuronal activity associated with the consummation of aggressive behavior in golden hamsters [6]. As the BSTPM and MePD are included within a neural network associated with the control of aggression, neurochemical changes within these areas could have a behavioral significance. Chronic treatment with cocaine during puberty enhances aggressive behavior in golden hamsters [9]. The possible relationship between enhanced

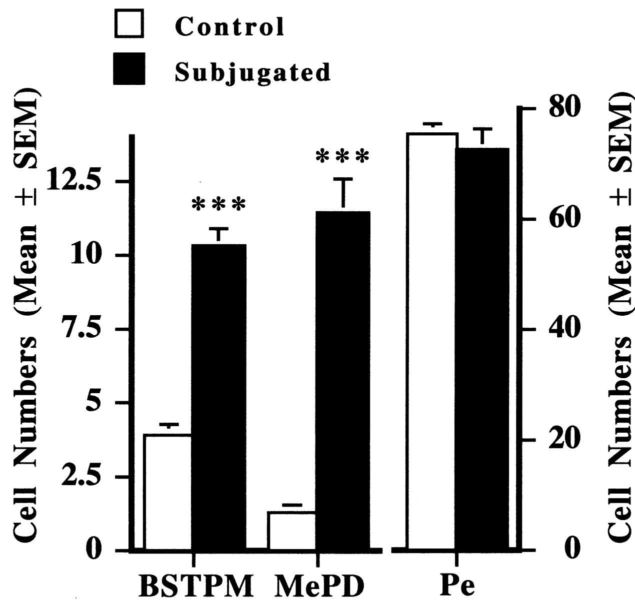

Fig. 3. Comparison of TH-IR neuron counts within the posterior portion of the medial division of the bed nucleus of the stria terminalis (BSTPM), posterodorsal portion of the medial amygdaloid nucleus (MePD), and periventricular hypothalamic nucleus (Pe) in previously subjugated male golden hamsters and their controls. Cell counts are expressed as the average number of TH-IR neurons per count per area. ***, $P<0.001$, Student's *t*-test.

TH-IR in the MePD and BSTPM and changes in aggressive behavior resulting from chronic stress exposure will be addressed in future studies.

Acknowledgements

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