Adolescent Changes in Hindbrain Noradrenergic A2 Neurons in Male Rats

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Short Title: Adolescent Changes in A2 Neurons

ABSTRACT

During adolescence, the increased susceptibility to stress-related dysfunctions (e.g., anxiety, drug use, obesity) may be influenced by changes in the hormonal stress response mediated by the hypothalamic-pituitary-adrenal (HPA) axis. We have previously reported that restraint stress leads to significantly prolonged HPA responses in pre-adolescent compared to adult rats. Further, pre-adolescent animals exposed to restraint show greater levels of neural activation than adults in the paraventricular nucleus of the hypothalamus (PVN), a key nucleus integrating information from brain regions that coordinate HPA responses. Here, we examined the potential contribution of the noradrenergic A2 region of the nucleus of the solitary tract (NST) as a contributor to these age-dependent shifts in HPA reactivity. Specifically, we used double-labeled immunohistochemistry for FOS and dopamine- β -hydroxylase (D β H) to measure cellular activation and noradrenergic cells, respectively, before or after restraint stress in pre-adolescent (30 days old) and adult (70 days old) male rats. We also measured the density of D β H-immunoreactive fibers in the PVN as an index of noradrenergic inputs to this area. We found that pre-adolescent animals have a greater number of D β H-positive cells in the A2 region compared to adults, yet the number and percentage of double-labeled D β H/FOS cells were similar between these two ages. We found no differences between the ages in the staining intensity of D β H-immunoreactive fibers in the PVN. These data indicate there are adolescent-related changes in the number of noradrenergic cells in the A2 region, but no clear association between the increased stress reactivity prior to pubertal maturation and activation of A2 noradrenergic afferents to the PVN.

Keywords: adolescence, dopamine-beta-hydroxylase, nucleus of the solitary tract, puberty, restraint stress

1. Introduction

Adolescent development is marked by significant changes in hypothalamic-pituitary-adrenal (HPA) axis stress-induced hormonal reactivity in both human and non-human animals (Dahl and Gunnar, 2009; Hostinar and Gunnar, 2013; McCormick and Mathews, 2007; McCormick et al., 2010; Romeo, 2010a; Romeo, 2010b; Romeo, 2013). For instance, stressors with both physiological and psychological components, such as restraint (Hall and Romeo, 2014; Minhas et al., 2016; Romeo et al., 2004a; Romeo et al., 2004b; Romeo et al., 2006b), hypoxia (Goldman et al., 1973; Vazquez and Akil, 1993), and intermittent foot shock (Goldman et al., 1973), result in prepubertal males and females exhibiting significantly greater and more prolonged adrenocorticotrophic hormone (ACTH) and corticosterone responses compared to adults. Though the exact mechanisms that mediate this heightened hormonal reactivity prior to pubertal maturation are unclear, it appears that both peripheral and central factors play a role (Romeo et al., 2016). For example, the adrenal glands of prepubertal rats produce higher levels of corticosterone and display greater sensitivity to ACTH than those of adults (Foilb et al., 2011; Romeo et al., 2014). In the brain, the paraventricular nucleus of the hypothalamus (PVN) shows higher levels of neuronal activation following restraint stress in prepubertal compared to adult animals (Lui et al., 2012; Romeo et al., 2006a; Viau et al., 2005), particularly within the corticotrophic-releasing hormone (CRH)-containing cells in the parvocellular region of the PVN (Romeo et al., 2006a). Importantly, it is this parvocellular portion of the PVN that is a critical integrator of

information from limbic, hypothalamic, and brainstem regions that coordinates the HPA hormonal response (Herman et al., 2003; Ulrich-Lai and Herman, 2009).

Converging lines of research have drawn attention to the importance of the noradrenergic-containing A2 region of the nucleus of the solitary tract (NST) in HPA-mediated hormonal responses following a variety of physical and/or psychological stressors (Kvetnansky et al., 2009; Rinaman, 2011). First, the A2 region sends direct noradrenergic afferent projections to the parvocellular PVN (Cunningham and Sawchenko, 1988; Cunningham et al., 1990; Sawchenko and Swanson, 1981; Sawchenko and Swanson, 1982). Second, acute stressors lead to increased neuronal activation, as indexed by FOS, in noradrenergic-containing cells in this region (Crane et al., 2005; Dayas et al., 2001a; Dayas et al., 2001b; Dayas et al., 2004; Pezzone et al., 1993). Finally, lesions of noradrenergic neurons in the A2 region significantly attenuate the rise of plasma corticosterone levels following restraint or ether inhalation stress (Bundzikova-Osacka et al., 2015; Gaillet et al., 1993). Though the A2 region of the NST is not the only direct afferent driving stress-induced HPA reactivity (Herman et al., 2003), these studies suggest that pubertal changes in NST function may in part mediate the differential PVN and hormonal responses observed in pre-adolescent and adult animals.

The purpose of the present experiment was to examine the number of single- and double-labeled dopamine-beta-hydroxylase (D β H)- and FOS-immunopositive cells in the A2 region of the NST in prepubertal and adult male rats before, immediately after, or 40 min following a 30 min session of restraint stress. D β H and FOS were used as markers of norepinephrine-producing cells and neural activation,

respectively. An additional goal of this experiment was to assess the staining intensity of D β H-immunopositive fibers in the parvocellular PVN as an index of potential age- and stress-dependent changes in noradrenergic innervation to this region. This semi-quantitative method has been used previously to measure alterations in the amount of noradrenergic input to the PVN following noradrenergic-specific lesions of the A2 region of the NST (Bundzikova-Osacka et al., 2015). Based on the positive relationship between stress-induced activity of the NST and the HPA-mediated hormonal response, we hypothesized that pre-adolescent males would show greater numbers of double-labeled D β H- and FOS-positive cells than adults in the A2 region of the NST following restraint stress. Furthermore, we hypothesized that pre-adolescent males would have higher levels of D β H immunoreactive fiber densities in the PVN compared to adults.

2. Results

2.1. Experiment 1: D β H- and FOS-positive cells in the A2 Region of the NST

A two-way ANOVA revealed a significant main effect of age on the number of D β H-positive cells in the A2 region ($F(1,38) = 14.07, P < 0.05$), with pre-adolescent animals showing a greater number of D β H-positive cells compared to adults, regardless of the stress time point (Figure 1A). There was no significant main effect of stress or interaction between age and stress on the number of single-labeled D β H-positive cells. A two-way ANOVA revealed a significant main effect of time on the number of FOS-immunoreactive cells in the A2 region ($F(2,38) = 4.03, P < 0.05$), such that pre-adolescent and adult animals show a greater number of FOS-positive

cells 40 min after the termination of the 30 min restraint sessions (Figure 1B). There was no significant main effect of age or interaction between age and stress on the number of single-labeled FOS-positive cells. Finally, a two-way ANOVA revealed a main effect of time on both the number and percentage of D β H/FOS double-labeled cells in the A2 region ($F(2,38) = 4.04$ and 3.07 , respectively, $P < 0.05$). More specifically, both pre-adolescent and adult animals had a greater number and percentage of D β H/FOS cells 40 min following the stressor than either before or immediately after the 30 min restraint stress session (Figure 1C and 1D). Figure 2 A and B provide a representative photomicrograph of the single- and double-labeled D β H and FOS-positive cells of the A2 region of the NST of a pre-adolescent male 40 min after the 30 min session of restraint stress had been terminated.

2.2. Experiment 2: D β H RODs in PVN

For D β H RODs in the parvocellular PVN, a two-way ANOVA showed a significant main effect of time ($F(2,38) = 3.21$, $P < 0.05$) such that pre-adolescent and adult males 40 min after exposure to a 30 min session of restraint had higher D β H immunoreactive RODs in the PVN compared to the pre-adolescent and adult males either before or immediately after termination of the restraint stress (Figure 3). Figure 4 A-D provides representative photomicrographs of the D β H immunoreactivity in the PVN of a pre-adolescent male before or 40 min after the 30 min session of restraint stress had been terminated and the approximate areas that the immunoreactive RODs were measured (Figure 4).

3. Discussion

These data describe an adolescent-related decrease in the number of D β H-containing cells in the A2 region of the NST. These data also show that despite pre-adolescent animals demonstrating significantly greater ACTH and corticosterone hormonal responses compared to adults after restraint stress (Hall and Romeo, 2014; Minhas et al., 2016; Romeo et al., 2004a; Romeo et al., 2004b; Romeo et al., 2006b), animals at both ages have similar levels of restraint-induced cellular activation in this noradrenergic cell group. Further, we report that pre-adolescent and adult males have similar levels of D β H immunoreactivity in the parvocellular PVN, suggesting comparable noradrenergic inputs to the PVN before and after adolescence, a result consistent with a previously published report showing similar levels of noradrenergic innervation of the PVN in weanling and adult rats (Rinaman, 2001). Though these data collectively do not support our initial hypothesis of greater stress-induced noradrenergic activation in the NST prior to adolescence, these data do suggest adolescent-dependent changes in the A2 region of the NST.

The pubertal decrease in D β H-positive cells in the NST was unexpected, and we are unclear what mechanism(s) may account for these age-related differences. It is possible that expression of D β H decreases following pubertal maturation in the A2 region, and thus the detectability of the cells decreases as well. Additional experiments assessing potential pubertal changes in D β H mRNA may shed on this possibility. The changes in the number of D β H-positive cells before and after pubertal development may be related to potential increases in cell death in this region of the NST during puberty. There are precedents in the literature for pubertal

declines in cell proliferation in the male hippocampus (Ho et al., 2012) and decreases in the number of neurons in the amygdala and medial prefrontal cortex of male and female rats (Markham et al., 2007; Rubinow and Juraska, 2009; Willing and Juraska, 2015). Future experiments will need to further examine this pubertal-related difference in D β H-positive cell number in the A2 region and the processes responsible for the change.

The significant restraint stress-induced increase in the number of FOS single- and FOS/D β H double-labeled cells in the NST are in agreement with previously published experiments in adult rats (Crane et al., 2005; Dayas et al., 2001a; Dayas et al., 2001b; Dayas et al., 2004; Pezzone et al., 1993). However, the similar number and percentage of cells double-labeled with FOS and D β H suggests little association between the prolonged stress-induced HPA response in prepubertal compared to adult animals and changes in noradrenergic activity in the NST afferents to the PVN. It is important to note that not all A2 noradrenergic neurons project to the PVN, and thus we may have missed a differential response within a more specific population of noradrenergic cells in this region. Studies using a combination of both D β H and FOS labels in the NST with a retrograde tracer from the PVN, for instance, would help address this question.

A recent study utilized the intensity of D β H immunoreactivity in the PVN as an index of the fiber density from noradrenergic NST afferents (Bundzikova-Osacka et al., 2015). In particular, Bundzikova-Osacka *et al.* reported that 6-hydroxydopamine (6-OHDA) lesion of the A2 region of the NST, which specifically targets the PVN projecting noradrenergic neurons, resulted in both a significant decrease in the

number of D β H-positive cells in the A2 region as well as a significant decrease in D β H-immunoreactive fibers in the PVN (Bundzikova-Osacka et al., 2015). Though we did not find support for our hypothesis that pre-adolescent animals would have greater levels of D β H staining in the PVN than adults, we did find that stress exposure in both pre-adolescent and adult animals resulted in higher D β H densities in the PVN. This effect of stress on D β H staining intensity is possibly due to stress-induced increases in D β H expression in the A2 projection neurons, as has been noted previously in male rats (Mravec et al., 2015), and not stress-induced changes in the innervation of the PVN *per se*. Regardless, this change in D β H staining is independent of the pubertal decrease in the number of D β H-positive cells in the A2 region of the NST reported here and the heightened stress-induced hormonal responses observed prior to puberty reported previously (Foilb et al., 2011; Goldman et al., 1973; Hall and Romeo, 2014; Minhas et al., 2016; Romeo et al., 2004a; Romeo et al., 2004b; Romeo et al., 2006b; Romeo et al., 2014; Vazquez and Akil, 1993).

Our data indicate that the increased hormonal stress reactivity exhibited by pre-adolescent animals following restraint stress is not associated with differences in neural activation of the D β H-containing cells in the A2 region of the NST. However, a substantial sub-population of neurons within this region also express vesicular glutamate transporters (Stornetta et al., 2002), a marker of glutamatergic cells, thus suggesting these neurons are co-releasing both norepinephrine and glutamate into the PVN upon stimulation. Given the known role of glutamate in activation of the

PVN and the stress-induced hormonal response (Busnardo et al., 2013; Ziegler and Herman, 2000), it is possible that, with additional phenotyping of these cells, age-dependent differences in neural activation patterns within the A2 region may emerge.

In conclusion, our data show pubertal decreases in the number of D β H-containing cells in the A2 region of the NST. Moreover, despite increased restraint stress-induced hormonal reactivity prior to puberty, the D β H-positive cells in the A2 region display similar levels of cellular activation following restraint stress in both pre-adolescent and adult male rats. These studies are the first steps in establishing the contribution of the NST, and other PVN afferents, in adolescent-related changes in HPA stress reactivity and the mechanisms through which these PVN afferents mediate this developmental shift in neuroendocrine function.

4. Experimental Procedure

4.1. Animals and housing

Male Sprague-Dawley rats were obtained from our breeding colony at Barnard College, weaned at 21 days of age, and housed two per cage in clear polycarbonate cages (45 x 25 x 20 cm) with wood chip bedding. All animals were maintained on a 12 h light-dark schedule (lights on at 0900 h). All animals had *ad libitum* access to food and water and the animal room was maintained at 21 $\pm$ 2°C. All procedures were carried out in accordance with the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of Columbia University.

4.2. Experimental design

Two experiments were conducted. Experiment 1 measured the number and activation of noradrenergic cells in the A2 region of the NST using double-labeled immunohistochemistry for D β H and FOS in pre-adolescent (30 days of age) and adult (70 days of age) male rats before and after a 30 min session of restraint stress.

Experiment 2 examined D β H immunohistochemical staining intensity in the parvocellular region of the PVN in the same cohort of animals used in Experiment 1.

To procure the brain tissue for these experiments, pre-adolescent and adult rats were weighed and transcardially perfused before (basal), immediately after the stressor (Time 0), or 40 min following termination of the stressor (n = 8 per age and time point). These time points were chosen for analysis because of the differential stress-induced hormonal response exhibited during these post-stress time points and time for increases in FOS immunoreactivity to be detected (Foillb et al., 2011; Lui et al., 2012; Romeo et al., 2004a; Romeo et al., 2006a; Romeo et al., 2006b). For perfusions, animals were given an overdose of ketamine and xylazine (80 and 5 mg/kg, respectively) and perfused with 0.9% heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were post-fixed in 4% paraformaldehyde for 24 h and then stored in 20% sucrose at 4°C until they were sectioned on a cryostat in the coronal plane at a thickness of 40 μ m and processed for immunohistochemistry (see below). In an effort to reduce variability in staining intensity, tissue sections from all the experimental groups were processed simultaneously within an experiment.

4.3. Immunohistochemistry

In Experiment 1, for the double-labeled D β H and FOS immunohistochemistry, free-floating hindbrain sections that contained the A2 regions of the NST (corresponding to plates 157-160 of a standard rat atlas (Paxinos and Watson, 2005)) were washed in 0.1 M PB and incubated for 10 minutes in 0.05% hydrogen peroxide (H₂O₂) in 0.1 M phosphate buffered saline (PBS). Sections were then washed in 0.1 M PB with 0.1% Triton X-100 (PBT), blocked in 2% normal goat serum (NGS) in PBT for 1h at room temperature, and then incubated in rabbit anti-FOS (1:20,000; sc-52; Santa Cruz Biotechnology, Inc., Dallas, TX) in PBT for 24 hours at 4°C. Sections were then exposed to a secondary antibody (goat anti-rabbit; 1:200, Vector Laboratories, Burlingame, CA) in PBT for 1 hour and then incubated in avidin-biotin horseradish peroxidase complex (1:250; Vectastain ABC Kit, Vector Laboratories) in PBT for 1 hour, both at room temperature. The horseradish peroxidase was visualized with 0.02% nickel-enhance 3,3'diaminobenzidine in a 3 M sodium acetate buffer containing 0.05% H₂O₂. Sections were then washed in PBS and then incubated in mouse anti-D β H (1:2,000; MAB308; Millipore, Billerica, MA) in PBT for 24 hours at 4°C. Sections were then exposed to secondary antibody (goat anti-mouse, 1:200) in PBT for 1 hour and then incubated in avidin-biotin horseradish peroxidase complex (1:250) in PBT for 1 hour, both at room temperature. The horseradish peroxidase was visualized with 0.02% 3,3'diaminobenzidine (without nickel enhancement) in a 3 M sodium acetate buffer containing 0.05% H₂O₂. Sections were washed in PBS, mounted on to Fisher Brand Plus slides (Fisher Scientific), dried, dehydrated in increasing concentrations of alcohol (70%, 95%, and

100%), cleared in xylenes, and coverslipped with DPX Mountant (Sigma-Aldrich, St. Louis, MO).

In Experiment 2, for single-label D β H immunohistochemistry, free-floating sections containing the PVN (corresponding to plates 47-48 of a standard rat atlas; Paxinos and Watson, 2005) were washed in 0.1 M PB and incubated for 10 minutes in 0.05% H₂O₂ PBS. Sections were then washed in 0.1 M PBT, blocked in 2% NGS in PBT for 24 hours at 4°C. Sections were incubated in mouse anti-D β H (1:2,000; MAB308; Millipore, Billerica, MA) in PBT for 24 hours at 4°C. Sections were then exposed to secondary antibody (goat anti-mouse; 1:200) in PBT for 1 hour and then incubated in avidin-biotin horseradish peroxidase complex (1:250) in PBT for 1 hour, both at room temperature. The horseradish peroxidase was visualized with 0.02% 3,3'-diaminobenzidine in a 3 M sodium acetate buffer containing 0.05% H₂O₂. Sections were washed, mounted on to Fisher Brand Plus slides, dried, dehydrated in increasing concentrations of alcohol, cleared in xylenes, and coverslipped with DPX Mountant.

4.4. Cell Number and Density Quantifications

The areal densities (cells per unit area) of D β H- and FOS-positive cell numbers were quantified in the caudal A2 region of the NST. Specifically, only sections from the caudal NST where noradrenergic cells are highly concentrated and non-overlapping with epinephrine-containing cells (approximately Bregma -15.00mm to -14.50mm; Paxinos and Watson, 2005)). Two sections, separated by 120 μ m and anatomically matched across subjects, were used for cell counting. Immunopositive

cells were counted from images captured with a Zeiss Axiovert 200 microscope under a 20X objective (Carl Zeiss, Thornwood, NY). Using a superimposed grid on each image, bilateral counts were made for each section and averaged to compute the mean number of FOS- and/or D β H-positive cells per 15,625 μm^2 .

For D β H immunoreactive densities, two sections containing the PVN, separated by 120 μm and anatomically matched across subjects, were analyzed from images captured with a Zeiss Axiovert 200 microscope under a 10X objective. ImageJ was used to measure relative optical densities (RODs) by placing an oval tool over the medial parvocellular area of the PVN (Sawchenko et al., 2000) and subtracting the ROD of the background from an area outside and lateral to the PVN (see Figure 4 for approximately placement of imaging tool). This circumscribed area of the PVN was measured as it has the majority of the noradrenergic input specifically from the A2 region of the NST (Cunningham and Sawchenko, 1988). Bilateral density measures were made for each section and averaged to compute the mean ROD of D β H staining in the PVN.

4.5. Statistical Analyses

All data are presented as the mean $\pm$ standard error of the mean (S.E.M.). Two-way (age X time point) ANOVAs were used to analyze D β H- and FOS- immunoreactive single- and double-labeled cells as well as the D β H immunoreactive RODs in the PVN. Significant main effects and interactions were further analyzed with Tukey's Honestly Significant Difference tests. Differences were considered

significant when $P < 0.05$. All statistical analyses were performed using GraphPad Prism software (version 5.04).

Acknowledgements

We would like to thank Page Buchanan for excellent animal care. This work was supported in part from a grant from the National Science Foundation IOS-1456577 (to R.D.R.).

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Figure Legends

Figure 1. Mean ($\pm$ SEM) number of D β H- (A), FOS- (B), and D β H/FOS- (C) labeled cells / $15,625\mu\text{m}^2$ and percent D β H/FOS-labeled cells (D) in the A2 region of the NST in pre-adolescent (30 days of age) and adult (70 days of age) male rats before (basal), immediately after a 30 min session of restraint stress, or 40 min after the restraint session was terminated. Bars and groups of bars that share a letter are not significantly different from one another.

Figure 2. Representative photomicrograph of D β H- and FOS-positive cells in the A2 region of the NST in a pre-adolescent (30 days of age) male 40 min after exposure to a 30 min session of restraint stress (A). Panel B provides a higher magnification image of the cells in Panel A (represented by the square box) with white arrows, black arrows, and black arrowheads indicating D β H single-labeled cells, FOS single-labeled cells, and D β H and FOS double-labeled cells, respectively. Scale bar in each panel = $50\mu\text{m}$.

Figure 3. Mean ($\pm$ SEM) relative optical density (ROD) of D β H immunoreactivity in the PVN of pre-adolescent (30 days of age) and adult (70 days of age) male rats before (basal), immediately after a 30 min session of restraint stress, or 40 min after the restraint session was terminated. Groups of bars that share a letter are not significantly different from one another.

Figure 4. Representative photomicrographs of D β H immunoreactivity in the PVN in a pre-adolescent (30 days of age) male before (basal; A) or 40 min after exposure to a 30 min session of restraint stress (B). Panels C and D provide a higher magnification of the images in Panels A and B, respectively, with a black and gray oval representing the approximate area of ROD measurement for specific and background signal, respectively. Scale bar for Panels A and B = 200 μ m and for Panels C and D = 50 μ m.

Figure 1

Figure 2

Figure 3

Figure 4