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Ultrastructural analysis of sex differences in nucleus accumbens synaptic connectivity

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Abstract

Despite robust sex differences in behavioral responses to drugs of abuse, relatively little is known about structural sex differences in synaptic connectivity of reward circuits such as in the nucleus accumbens (NAc). Previously, we showed that distal dendritic spine density on medium spiny neurons in the NAc is higher in females than males, suggesting that sex differences in NAc excitatory synapses could play a role in differential behavioral responses to drugs. In the current study, we used electron microscopy and stereological counting methods to evaluate dendritic spine and shaft synapses, as well as tyrosine hydroxylase-immunoreactive (TH-IR) profiles, in the NAc core of male and female rats. We found an unanticipated rostral-caudal gradient in spine synapse density in females but not males, resulting in a sex difference favoring females in the caudal NAc core. The volume of the NAc was not different between males and females. We also found that the percentage of spines with large spine heads was greater in females in the rostral core. The density of shaft synapses was low compared to spine synapses, and sex differences were minor. The density of TH-IR profiles was not different between males and females, but females had a higher proportion of spines with large heads near TH suggesting a potential sex difference in dopaminergic modulation of large spine synapses. These findings underscore the importance of including both males and females in studies of reward circuitry, and of considering variation along the rostral-caudal axis of the NAc in future studies.

Keywords

Electron microscopy; Stereology; Synapse; Dendritic spine; Glutamate; Dopamine

Introduction

The nucleus accumbens (NAc) is important in the neural circuitry of reward (Koob and Volkow 2010). The principal neurons in the NAc, medium spiny neurons (MSNs), receive glutamatergic inputs from the prefrontal cortex, hippocampus, amygdala, and thalamus, as well as dopaminergic inputs from the ventral tegmental area (Zahm 2000). Repeated exposure to drugs of abuse produces plasticity of glutamatergic inputs to MSNs that may be important in addiction (Russo et al. 2010). For example, repeated exposure to psychostimulants increases MSN dendritic spine density (Robinson and Kolb 1999a, b;

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Wissman et al. 2011), intrinsic excitability (Kourrich and Thomas 2009), and synaptic plasticity (Martin et al. 2006). Interestingly, in both humans and animals, females show stronger behavioral responses to addictive drugs and increased sensitization to repeated drug exposure (Carroll and Anker 2010). Baseline sex differences in synapse structure and function in the NAc may contribute to behavioral sex differences in response to drugs.

Previously, we used confocal microscopy of DiI-labeled dendrites to show that MSNs in the NAc of drug-naïve females have higher dendritic spine density and more large-headed spines compared to males; MSN total dendritic length and branching were not different between males and females (Forlano and Woolley 2010). These findings were an initial indication that NAc synaptic connectivity might differ between the sexes. Two aspects of our previous study limit this interpretation, however. First, our analysis of dendritic spine density focused specifically on distal dendrites, based on evidence that drug-induced synaptic plasticity is concentrated distally (Li et al. 2003). Second, owing to use of light microscopy, we could not detect synapses formed on dendritic shafts. Thus, the goal of the current study was to compare synapses at the ultrastructural level in the NAc of males versus females. In addition, because interaction between dopaminergic and glutamatergic inputs in the NAc could be particularly important for drug-induced locomotor activation (David et al. 2004), which differs in males and females (van Haaren and Meyer 1991), we also examined the spatial relationship between tyrosine hydroxylase-immunoreactive (TH-IR) fibers and dendritic spine synapses in both sexes. We focused on the core subregion of the NAc because the sex difference in dendritic spine density is greater in the core than the shell (Forlano and Woolley 2010).

Methods

Animals

All procedures were performed in accordance with the National Institutes of Health *Guide to the Care of Laboratory Animals* and were approved by the Northwestern University Animal Care and Use Committee. Adult male ($n = 5$) and female ($n = 5$) Sprague–Dawley rats (Harlan, Indianapolis, IN), 60- to 67-day old, were separated by sex and kept under a 12-h light: dark cycle with soy-free rat chow and water freely available. Estrous cycles in females were monitored via vaginal lavage, and all females were sacrificed on the afternoon of proestrus when circulating estradiol levels are highest (Smith et al. 1975) to facilitate comparison with Forlano and Woolley (2010).

Electron microscopy

The preparation of tissue for EM was performed as described previously (Hart et al. 2007). Animals were deeply anesthetized with 80 mg/kg sodium pentobarbital (i.p.) and transcardially perfused with 2.5% paraformaldehyde/0.5% glutaraldehyde in PB. Brains were removed and postfixed in the same solution overnight at 4°C, cryoprotected with 30% of sucrose in PB and then cut on a freezing microtome (Leica) into four series of coronal sections (40–80 μ m) including the NAc, from Bregma 1.08–1.92. One 80 μ m series was immunostained for TH as previously described (Forlano and Woolley 2010) using a rabbit anti-TH primary antiserum (1:2000, Chemicon), goat anti-rabbit secondary (1:800, Vector Laboratories), and avidin–biotin horseradish peroxidase amplification (Vectastain Elite ABC kit), visualized with 0.025% diaminobenzidine and 0.01% hydrogen peroxide.

Two series of 80 μ m sections (immunolabeled and not immunolabeled) were stained with 1% osmium tetroxide and flat embedded in Eponate resin (both from Ted Pella). The flat-embedded tissue from each animal was coded to ensure the experimenter was blind to the sex of each brain during all phases of imaging and analysis. Boundaries of the NAc core

were identified based on Paxinos and Watson 2005 5th edition atlas (2005). Tissue blocks of 0.7–2.0 mm² containing the NAc core were dissected and mounted on BEEM capsules. Series of at least 15 ultrathin (~70 nm) sections from 2–4 blocks per animal were cut using a Reichert Ultracut S ultramicrotome (Leica), collected onto formvar-coated slot grids, stained with 3% uranyl acetate, followed by 2.66% Reynold's lead citrate, and imaged with a JEOL 1230 transmission electron microscope equipped with a CCD camera. A low magnification image (400×) was taken of the first section from each block and used to select 4 areas per block by a random systematic approach. Images of each selected area were digitally captured at 20,000× through the series of sections. All analyses were done on the same series of sections.

Synapse density analysis

Synapse density was estimated using the physical disector approach (Mouton et al. 2002). Briefly, a 5.9 × 5.9 μm counting frame was applied to high magnification images taken from each of 15 serial sections per area. The first section in the series was designated as the reference section, and the second as the look-up section. Any synapse present in the reference section but absent in the look-up section was counted. Then, the second section became the reference section and the third was the look-up section, and so forth through all 15 sections. Synapses were counted in both directions for each series (15th section as the reference section, 14th as the look-up section, etc.) and values for the two counts were averaged for each area. Section thickness was measured using the cylindrical diameters method (Fiala and Harris 2001) and synapse density was calculated as the number of synapses present in the volume of each series. In addition, each counted synapse was classified as being located on a dendritic spine head, dendritic shaft, or soma, and as being asymmetric or symmetric. Asymmetric synapses were identified by asymmetric pre- and post-synaptic densities and the presence of round vesicles within 50 nm of the pre-synaptic membrane. Symmetric synapses were identified by symmetric pre- and post-synaptic densities, a synaptic cleft, parallel pre- and post-synaptic membranes, and the presence of vesicles within 50 nm of the pre-synaptic membrane. The total number of images analyzed was 1,080 (female) and 1,075 (male).

Spine size analysis

Spines were categorized as having small (<0.6 μm), large (0.6–0.9 μm), or giant (>0.9 μm) spine heads (Forlano and Woolley 2010), according to measurement along an axis parallel to their synapse in the section in which head diameter was largest.

TH analysis

A subset of the images analyzed for synapse density (720 females, and 835 males) was from tissue immunostained for TH. TH-IR profiles were identified based on containing electron-dense DAB reaction product clearly visible in two or more consecutive images, and were counted. In addition, synapses within 250 nm or 251–500 nm of a TH-IR profile were noted. Control tissue that was processed without primary antibody showed no non-specific secondary antibody staining.

Nucleus accumbens volume and synapse number

To convert synapse density measurements into estimates of total synapse number, we estimated the volume of the NAc core using Cavalieri's principle. The NAc core in flat-embedded sections was traced at 4× on a brightfield microscope equipped with a drawing tube. These traces were scanned and area measurements were obtained and calibrated with ImageJ (NIH, Bethesda, MD). Volumes of each core were calculated using the formula for the volume of a cone frustum (Smith et al. 1997). Synapse density values for each animal

were multiplied by bilateral core volume for the same animal to estimate total synapse number per brain for the NAc core. In addition, to corroborate results with flat-embedded tissue, we also reconstructed NAc core volumes from a separate set of nissl-stained male ($n = 4$) and female ($n = 4$) brains from a previous study (Sato et al. 2011).

Statistics and data display

Means were calculated for each block and each animal and data are presented as mean $\pm$ SEM with n as the number of animals. Coefficients of error (CEs) for synapse counts within tissue blocks were calculated according to the formula presented by Keuker et al. (2001) to test for appropriate sampling density. CEs for asymmetric spine synapses ranged from 0.077 to 0.102, with similar averages in females (0.083) and males (0.085). CEs for symmetric and asymmetric shaft synapses were higher owing to the very low density of these synapse types. Average CEs for symmetric synapses were 0.40 in females and 0.42 in males, and average CEs for asymmetric shaft synapses were 0.48 in females and 0.41 in males. SPSS software (IBM Corporation, Somers, NY) was used to perform two-way ANOVA, Tukey's post-hoc tests, or t tests as appropriate. EM images were viewed in Photoshop (Adobe Systems, San Jose, CA), cropped, and levels adjusted to enhance contrast when necessary.

Results

Dendritic spine synapses

Building from our previous light microscopic studies of dendritic spines (Forlano and Woolley 2010; Wissman et al. 2011), we focused initially on synapses on dendritic spine heads (Fig. 1a). Surprisingly, male and female spine synapse densities were not significantly different overall (t test, $p = 0.29$); females had 1.18 ± 0.067 spine synapses/ μm^3 , while males had 1.10 ± 0.016 spine synapses/ μm^3 . However, plotting the data broken down by the rostro-caudal level from which each tissue block was taken revealed a gradient in synapse density apparent in females (Pearson correlation: $R = 0.79$, $p < 0.001$; Fig. 1b) but not males ($R = 0.05$, $p = 0.85$; Fig. 1c). To further test this, we divided the sampled tissue blocks into rostral (Bregma 2.0–1.56) and caudal (Bregma 1.44–1.0) levels in both sexes (Fig. 1d). A 2-way repeated measures ANOVA showed a main effect of rostro-caudal location ($F = 9.57$, $p = 0.021$) and a significant interaction between sex and location ($F = 10.76$; $p = 0.017$). Post-hoc Tukey tests showed significantly higher spine synapse density in females compared to males in caudal ($p = 0.009$) but not rostral ($p = 0.23$) blocks, and that spine synapse density was higher in caudal than rostral blocks in females ($p = 0.004$) but not males ($p = 0.89$). Caudal spine synapse density in females was 1.29 ± 0.07 spine synapses/ μm^3 compared to 1.10 ± 0.03 in males, an 18% difference. In contrast, rostral spine synapse density was not significantly different between males and females (0.98 ± 0.036 in females compared to 1.10 ± 0.036 in males). Thus, females have a higher density of spine synapses than males in the caudal but not rostral NAc core.

To investigate whether the sex difference in spine synapse density translates into a difference in spine synapse number, we measured the volume of the NAc core and estimated spine synapse number for each animal. This showed that NAc core volumes did not differ between males and females, either considering the core as a whole (females: 3.86 ± 0.37 mm^3 , males: 3.77 ± 0.36 mm^3 ; t test: $p = 0.81$) or broken down into rostral (females: 1.58 ± 0.12 mm^3 , males: 1.67 ± 0.16 mm^3 ; t test: $p = 0.67$) and caudal (females: 2.27 ± 0.078 mm^3 , males: 2.10 ± 0.12 mm^3 ; t test: $p = 0.24$) levels. A separate analysis of NAc core volumes in nissl-stained tissue from 4 male and 4 female control rats from a previous study (Sato et al. 2011) confirmed no sex difference in NAc core volume (3.69 ± 0.39 mm^3 in females, 3.36 ± 0.46 mm^3 in males; t test: $p = 0.31$). We then estimated spine synapse number by multiplying synapse density for each animal by the bilateral core volume for that animal,

which showed that spine synapse number followed the same pattern as spine synapse density. Estimated spine synapse number was $4.53 \times 10^9 \pm 2.97 \times 10^6$ overall in females and $4.14 \times 10^9 \pm 1.74 \times 10^6$ in males (t test: $p = 0.29$). In the caudal core, estimated spine synapse number was significantly higher in females ($2.94 \times 10^9 \pm 2.01 \times 10^7$) compared to males ($2.31 \times 10^9 \pm 1.75 \times 10^7$; t test: $p = 0.044$), whereas in the rostral core, spine synapse number was not significantly different (females: $1.61 \times 10^9 \pm 1.50 \times 10^7$, males: $1.75 \times 10^9 \pm 2.00 \times 10^7$; t test: $p = 0.59$).

Our previous study also showed a sex difference in spine head morphology, with females having a higher proportion of spines with large or giant heads. To investigate this at the EM level, we measured the diameter of spine heads, at their widest point, and classified each spine as giant ($>0.9 \mu\text{m}$), large ($0.6\text{--}0.9 \mu\text{m}$) or small ($<0.6 \mu\text{m}$; Fig. 1a). Because results were the same for large and giant spines considered separately, we combined them for further analyses. Also, to control for the sex difference in overall spine synapse density in caudal areas, we expressed spine head size data as the percentage of all spines in each area that were large/giant. Considering all rostro-caudal levels together, there was no overall sex difference in percentage of spines that were large or giant (t test, $p = 0.21$). In females, $15.3 \pm 0.84\%$ of the spines were large/giant, compared to $13.5 \pm 1.03\%$ in males. However, when we broke the data into rostral versus caudal blocks (Fig. 1e), there was a significant interaction between sex and location (2-way repeated measures ANOVA, $F = 6.02$, $p = 0.049$; no significant main effects, both $p > 0.1$). Tukey's post-hoc tests showed that females had a higher percentage of large and giant spines than males in rostral ($p = 0.032$) but not caudal blocks ($p = 0.97$). The percentages of large and giant spines in rostral blocks were 16.6 ± 0.5 in females and 12.6 ± 0.6 in males, a 32% difference; whereas in caudal blocks the percentages were essentially identical: 14.6 ± 1.3 in females and 14.7 ± 1.5 in males.

Dendritic shaft synapses

In addition to spine synapses, NAc MSNs receive both asymmetric (excitatory) and symmetric (inhibitory) synaptic inputs on dendritic shafts, although at a ~tenfold lower density than spine synapses (Fig. 2a, b). There were no significant sex differences in shaft synapse density or number. The density of asymmetric shaft synapses was 0.05 ± 0.004 synapses/ μm^3 in females and 0.06 ± 0.004 in males, a statistical trend favoring males (t test, $p = 0.076$). We also investigated a rostro-caudal location effect as we did with spine synapses (Fig. 2c). A 2-way repeated measures ANOVA showed a main effect of sex ($F = 6.58$, $p = 0.025$), with trends toward an effect of location ($F = 3.98$, $p = 0.093$) and an interaction between sex and location ($F = 4.273$, $p = 0.084$). Males had slightly higher density of asymmetric shaft synapses in the rostral areas (0.083 ± 0.01 synapses/ μm^3) compared to females (0.047 ± 0.01 synapses/ μm^3), whereas in caudal blocks the density was similar between males (0.046 ± 0.002 synapses/ μm^3) and females (0.050 ± 0.004 synapses/ μm^3). Because asymmetric shaft synapses were so infrequent compared with spine synapses, however, the density of spine and shaft asymmetric synapses combined was nearly identical to that for spine synapses alone (data not shown). Using volume estimates to calculate asymmetric shaft synapse number, there was no sex difference with females having $1.91 \times 10^8 \pm 1.91 \times 10^7$ synapses per bilateral core and males having $2.30 \times 10^8 \pm 1.68 \times 10^7$ (t test, $p = 0.163$). Similar to asymmetric shaft synapses, there was no difference between males and females in the density (males: 0.06 ± 0.006 synapses/ μm^3 , females: 0.07 ± 0.005 synapses/ μm^3 ; t test, $p = 0.22$) or number (males: $2.13 \times 10^8 \pm 1.58 \times 10^7$, females: $2.64 \times 10^8 \pm 2.12 \times 10^7$; t test, $p = 0.092$) of symmetric shaft synapses. Breaking the data down into rostral versus caudal blocks also showed no significant effects on symmetric synapses (2-way repeated measures ANOVA, all $p > 0.3$; Fig. 2d).

TH-immunoreactive profiles

A subset of the sections used for synapse density analysis was immunostained for TH. As shown previously (Bouyer et al. 1984), TH labeling was present in numerous axons and varicosities within the NAc (Fig. 3a–d). Consistent with light microscopy (Forlano and Woolley 2010), there was no difference in the density of TH-IR profiles overall between males (0.30 ± 0.02 profiles/ μm^3) and females (0.37 ± 0.05 profiles/ μm^3 ; Fig. 3e; *t* test, $p = 0.21$). To investigate a sex difference in potential dopamine–glutamate interactions, we counted the number of synapses within 250 nm of each TH-IR profile (Fig. 3a–d) and we combined this with analysis of large/giant spines. Similar to the spine head size analysis, we expressed these counts as a percentage of total synapses near TH-IR profiles to control for sex differences in synapse density. The overall percentage of synapses near TH-IR profiles was not significantly different ($41.1 \pm 9.5\%$ in females, $26.9 \pm 5.3\%$ in males; *t* test, $p = 0.231$) and there were no significant location effects (2-way repeated measures ANOVA, all $p > 0.18$).

Interestingly, however, we observed a relatively high proportion of large and giant spines near TH-IR profiles, particularly in females (Fig. 3a–d). In females, $19.5 \pm 6.3\%$ of the large/giant spines were located within 250 nm of a THIR profile (a density of 0.03 spines/ μm^3), while males had only $4.6 \pm 2.3\%$ (a density of 0.008 spines/ μm^3); this difference was statistically significant (*t* test, $p = 0.005$; Fig. 3f). The sex difference in the proportion of large/giant spines near TH was particularly noticeable in caudal blocks, where females had $20.8 \pm 9.8\%$ of their large and giant spines near TH profiles, and males had only $1.3 \pm 1.3\%$. A 2-way repeated measures ANOVA showed a main effect of sex ($F = 8.28$, $p = 0.02$), a strong trend toward an effect of rostral versus caudal levels ($F = 6.34$, $p = 0.053$), and a significant interaction ($F = 20.7$, $p = 0.006$). Tukey's post-hoc tests showed that the rostro-caudal difference was significant in females ($p = 0.005$) but not males ($p = 0.18$), and the sex difference was present only in caudal blocks ($p < 0.001$). Results were similar for spines 251–500 nm from TH, but differences were not statistically significant (data not shown). These differences indicate that, although the percentage of large/giant spines is relatively higher in females in the rostral NAc, it is in the caudal NAc that these synapses are more likely to be modulated by dopaminergic inputs in females than in males.

Discussion

In this study, we performed a quantitative ultrastructural analysis of synapses in the NAc core of male and female rats in an effort to understand the differences in synaptic connectivity that could contribute to females' greater responsiveness to psychostimulants (Becker and Hu 2008). Although spine synapse density was not different between males and females considering the core as a whole, we found an unanticipated rostro-caudal gradient in females, with lower spine synapse density rostrally and higher spine synapse density in caudal areas. In contrast, males' spine synapse density was consistent throughout rostro-caudal levels. Because regional volume of the NAc did not differ between males and females, the rostro-caudal gradient in spine synapse density in females resulted in a sex difference in spine synapse number in the caudal core with females having a greater number of spine synapses than males. We also found a sex difference favoring females in spine head size in rostral areas. TH labeling did not differ between the sexes, but the percentage of large and giant spines near TH-IR profiles was higher in females, particularly in the caudal areas.

Our EM findings on spine synapses here corroborate our previous suggestion that the sex difference in spine density seen with light microscopy is stronger in the distal dendrites rather than being consistent throughout the dendritic tree (Forlano and Woolley 2010). Most studies of dendritic morphology in NAc MSNs have focused on distal dendritic segments (e.g., Robinson and Kolb 1999a; Lee et al. 2006; Dobi et al. 2011), particularly since Li et

al. (2003) reported that only the distal dendrites show psychostimulant-induced increases in spine density. In our previous study, we found a sex difference in spine density when we limited our analysis to thin (presumably distal) dendritic segments, but analysis of thick (presumably proximal) dendrites showed no sex difference. The lack of an overall sex difference in spine synapse density quantified with systematic random sampling at the EM level, which does not distinguish distal and proximal dendrites, supports the idea that sex differences in spine density are likely to be concentrated in distal dendrites. This highlights the fact that analysis of distal segments alone can be misleading about the overall pattern of excitatory synaptic input to MSNs.

Comparing EM analysis of sex differences in NAc core synapses with electrophysiological analysis of synaptic input to core MSNs in males versus females raises some interesting issues. Recently, we found that the frequency of miniature excitatory post-synaptic currents (mEPSCs) in core MSNs is higher in females than males (Wissman et al. 2011). In the absence of a sex difference in paired-pulse ratio of evoked EPSCs, which we confirmed, the greater mEPSC frequency in females indicates that females have more excitatory synapses per MSN than males do. How can this be reconciled with the lack of overall sex difference in asymmetric synapse density/number in the current study? The answer may lie in a combination of the female-specific rostro-caudal gradient in spine synapse density and the observation that females have more large spines in the rostral NAc. EM and electrophysiological data are well matched in the caudal NAc, where females have higher synapse numbers by both measures. In the rostral NAc, however, the data match less well. One possible explanation is that the higher proportion of large/giant spines in rostral areas in females reflects greater strength of those synapses, which could increase their detection in electrophysiological recordings. Though this has not been studied specifically in MSNs, in other cell types such as hippocampal pyramidal neurons, it is well known that larger dendritic spines contain stronger synapses with more AMPA type glutamate receptors (Matsuzaki et al. 2004; Nicholson et al. 2006). It is also known that distal synapses in hippocampus contain more AMPA receptors and that this boosts their synaptic strength making distal synapses more detectable in recordings at the soma, termed distance-dependent scaling (Magee and Cook 2000; Smith et al. 2004). Though speculative at this point, it is possible that more large/giant spines in females and/or distance-dependent scaling of synaptic strength underlie greater mEPSC frequency in rostral MSNs in females. More detailed electrophysiological studies, for example with two-photon uncaging of glutamate at individual spines as has been done in the hippocampus, will be required to test these hypotheses.

The rostro-caudal gradient in spine synapse density we observed specifically in females highlights the importance of partitioning the NAc and of considering both sexes in future studies. There have been few previous reports of rostro-caudal differences in the NAc core. Anatomically, the core and shell each have a heterogeneous distribution of afferent and efferent projections (Berendse et al. 1992; Brog et al. 1993; Usuda et al. 1998; Todtenkopf and Stellar 2000), but compartmentalization of the core has been investigated primarily in terms of patch versus matrix territories rather than along the rostro-caudal axis (Wright and Groenewegen 1995). Several groups have demonstrated rostro-caudal variations in the functional output of the shell (Heidbreder et al. 1999; Reynolds and Berridge 2003; Gill and Grace 2011). However, studies that include the core in their analysis tend to treat it as a single unit (Heidbreder and Feldon 1998; Heidbreder et al. 1999). Additionally, these studies were performed in males only. Alcantara et al. (2011) recently reported a stereological EM analysis of NAc core and shell synapses in female rats in response to chronic cocaine or morphine; their core spine synapse density data in controls match well with our averaged data for females. However, this study was focused on a more limited rostro-caudal level of the NAc (Bregma 1.2, which falls into our caudal category). The functional significance of

the rostro-caudal synapse density gradient in females and its relevance to sex differences in response to drugs will be an important topic for future studies. As the core is thought to be involved in drug-induced locomotor activity (David et al. 2004), which is greater in females (Hu and Becker 2003; Wissman et al. 2011), distal spine synapses in the caudal part of the core may play a key role in the behavioral sex differences in response to psychostimulants.

Dopaminergic input can have strong effects on glutamatergic synapses in the NAc (reviewed by Kauer and Malenka 2007). Dopaminergic terminals in the core, particularly on distal dendrites, are often located adjacent to spines (Totterdell and Smith 1989) and eliminating dopaminergic input to the NAc by 6-OH-DA lesions reduces spine density in the core (Meredith et al. 1999). Although we found no sex difference in the density of TH-IR fibers in the NAc core, there was a closer association between large spines and TH-IR profiles in females, particularly in caudal areas. The more frequent proximity of large spines to TH-IR profiles in females may confer an increased susceptibility to dopamine–glutamate interactions at large spines in females. Alternatively, dopamine may influence spine head size. D1 receptor activation leads to phosphorylation of Rap1GTPase activating protein, which is enriched in the striatum and NAc and regulates Rap1 activity (McAvoy et al. 2009), and Rap1 is known to regulate dendritic spine morphology (Xie et al. 2005). Despite the lack of sex difference in density of TH-IR profiles, there is strong evidence for functional sex differences in dopamine release in the NAc, as well as for effects of estradiol on dopamine release (reviewed by Becker 1999; Becker and Hu 2008). These interactions may contribute to behavioral sex differences in response to psychostimulants, which depend in part on circulating estradiol (Hu and Becker 2003). Our females were sacrificed during proestrus, when circulating estradiol levels are highest (Smith et al. 1975) and when psychostimulant-induced dopamine release is greatest (Becker and Cha 1989; Becker 1990). It will be useful to determine to what extent the sex difference in large spine percentages and proximity to TH are controlled by circulating gonadal steroid levels versus long-term organizational effects. For example, it is possible that ovariectomized females or females sacrificed at a low estradiol point in the estrous cycle would show a similar proportion of large spines as males indicating that estradiol-driven differences, possibly in dopamine release, control spine head size. If spine head size is related to synaptic strength in the NAc, as it is in other brain areas, this may be one mechanism by which estradiol influences drug-related behaviors that depend upon the NAc.

In summary, our analyses show sex differences in the rostro-caudal patterns of spine synapse density and number, spine head size, and the potential for dopamine modulation of large spines in the NAc. Despite the facts that human and animal studies show that females respond more strongly to psychostimulants (Becker and Hu 2008) and that the proportion of young women taking stimulants is on the rise (SAMHSA 2010), most pre-clinical studies of reward circuitry and mechanisms of addiction are performed only in males. Further studies of the interaction between dopaminergic and glutamatergic inputs to the NAc and their potential to modulate behavioral responses to drugs of abuse should include both males and females, as well as take into account subregional variation in reward circuits, including along the rostro-caudal axis.

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Fig. 1. Sex-specific rostro-caudal gradient in dendritic spine synapses

a Representative electron micrograph from the NAc core showing small (*S*, $<0.6 \mu\text{m}$ diameter), large (*L*, $0.6\text{--}0.9 \mu\text{m}$) and giant (*G*, $>0.9 \mu\text{m}$) spine heads and an asymmetric synapse on a dendritic shaft (*D*). Scale bar 500 nm. **b, c** Spine synapse density in females (**b**) and males (**c**) broken down by rostro-caudal level. Each dot represents the average spine synapse density in areas analyzed from one tissue block. **d** Spine synapse density in rostral versus caudal halves of the NAc core in females and males (mean $\pm$ SEM, $n = 5$; * indicates $p < 0.05$ Tukey post-hoc test). **e** Percentage of spine heads that were large or giant in rostral

versus caudal halves of the NAc core in females and males (mean + SEM, $n = 5$; * indicates $p < 0.05$ Tukey post-hoc test)

Fig. 2. Little sex difference in shaft synapse density

a Representative electron micrograph showing an asymmetric shaft synapse (arrowhead). **b** Asymmetric shaft synapse density in rostral versus caudal halves of the NAc core in females and males (mean + SEM, $n = 5$; main effect of sex with a 2-way repeated measures ANOVA, $p = 0.025$). **c** Representative electron micrograph showing a symmetric shaft synapse (arrowhead). Scale bar 500 nm, also applies to (a). **d** Symmetric synapse density in rostral versus caudal halves of the NAc core in females and males (mean + SEM, $n = 5$)

Fig. 3. TH-IR profiles are not sexually dimorphic, but females have more large-headed spines near TH-IR profiles. (a–d) Representative serial electron micrographs of sections stained for TH (arrowheads). This TH-IR profile is near a giant spine head (G). Scale bar 500 nm, applies to all images. e Density of TH-IR profiles in female and male NAc cores (mean + SEM, $n = 5$). f Percentage of large and giant spine heads within 250 nm of a TH-IR profile in female and male NAc cores (mean + SEM, $n = 5$; *indicates $p < 0.005$, t test)