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Estradiol reduces dendritic spine density in the ventral striatum of female Syrian hamsters

Nancy A. Staffend, Caroline M. Loftus, and Robert L. Meisel

Department of Neuroscience, University of Minnesota, 6-145 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455, USA

Abstract

Estradiol affects a variety of brain regions by modulating physiological and cellular functions as well as neuronal morphology. Within the striatum, estradiol is known to induce physiological and molecular changes, yet estradiol's effects on striatal dendritic morphology have not yet been evaluated. Using ballistic delivery of the lipophilic dye DiI to tissue sections, we were able to evaluate estradiol's effects on striatal morphology in female Syrian hamsters. We found that estradiol significantly decreased spine density within the nucleus accumbens core, with no effect in the nucleus accumbens shell or caudate. Interestingly, estradiol treatment caused a significant deconstruction of spines from more to less mature spine subtypes in both the nucleus accumbens core and shell regardless of changes in spine density. These results are significant in that they offer a novel mechanism for estradiol actions on a wide variety of nucleus accumbens functions such as motivation or reward as well as their pathological consequences (e.g. drug addiction).

Keywords

Estradiol; Nucleus accumbens; Caudate; Hippocampus; Spine density; Spine morphology

Introduction

The striatum is not historically thought of as a site of estradiol action, yet there is an expansive literature on physiological and molecular effects of estradiol treatment in this brain region (Morissette et al. 2008). These actions of estradiol impact a number of functional endpoints (Becker 2000; Korol 2004), as well as having consequences for multiple disease states such as Huntington's (Tunez et al. 2006), schizophrenia, Parkinson's (Cyr et al. 2002; Bourque et al. 2009), and drug addiction (Becker and Hu 2008). Cumulatively, these data suggest an important role of estradiol regulation in the striatum.

Within this rich literature, studies evaluating estradiol effects in the striatum have focused largely on synaptic transmission and intracellular signaling and have neglected to evaluate whether estradiol exerts effects on the morphology of striatal neurons. This appears to be a significant omission, as fluctuations in circulating estradiol affect not only neuronal physiology, but also neuronal architecture in a wide variety of brain areas including the CA1 region of the hippocampus, the ventromedial nucleus of the hypothalamus (VMN), the medial nucleus of the amygdala, and the prefrontal cortex (McEwen 2002; Cooke and Woolley 2005). In the CA1 region of the hippocampus, high levels of estradiol increase spine density in pyramidal neurons (Woolley and McEwen 1993). These increases in spine

density are associated with enhanced neuronal excitability (Woolley 1998), and improved cognitive performance in rats (Walf and Frye 2008). In the prefrontal cortex, estradiol treatment significantly increases dendritic spine density of primary cortical neurons in both rats and monkeys (Hao et al. 2006; Chen et al. 2009) and is associated with increased performance on delayed reaction tasks. In the VMN, estradiol treatment results in differential regulation of the nucleus causing increases in spine density of one neuronal population and simultaneous decreases in spine density of another (Calizo and Flanagan-Cato 2000, 2002). These morphological changes are implicated in the expression of female sexual behavior in rats (Calizo and Flanagan-Cato 2000).

To investigate the regulatory role of estradiol on medium spiny neurons of the striatum, including both the caudate nucleus and nucleus accumbens, we took a “DiOlistic approach” (Gan et al. 2000) to evaluate potential changes in spine density or morphology in female Syrian hamsters. Our results demonstrate that estradiol treatment produced a significant decrease in spine density and morphology in the nucleus accumbens, with no effect on dendritic spines in the caudate nucleus. To our knowledge, our results are the first in vivo evidence that estradiol has a regulatory effect on neuronal morphology in the nucleus accumbens.

Materials and methods

Animals

Female Syrian hamsters (Charles Rivers Laboratories, Wilmington, MA) were individually housed in polycarbonate cages and kept under a 14:10 light:dark cycle, with lights out at 13:00 hour. Food and water were available to the animals ad libitum. At 3–5 months of age, 16 female hamsters were bilaterally ovariectomized using Nembutal anesthesia (85 mg/kg, i.p., Abbott Laboratories, Abbott Park, IL). After a 10-day recovery, 10 µg of estradiol benzoate dissolved in 0.1 ml of cottonseed oil ($n = 8$) or 0.1 ml cottonseed oil alone ($n = 8$) was administered to the females s.c. daily for 2 consecutive days. After 1 day, the animals were anesthetized using 0.2 ml Sleepaway (Fort Dodge Animal Health, Fort Dodge, IA) and transcardially perfused with 25 mM phosphate buffered saline (PBS, pH = 7.2) for 3 min at 25 ml/min, followed by 1.5% paraformaldehyde in 25 mM PBS for 20 min. All animal procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the University of Minnesota IACUC.

Tissue preparation

Brains were removed, blocked at the level of the cerebellum, and post-fixed for 1 h in 1.5% paraformaldehyde in PBS. Brains were Vibratome (Lancer Series 1000, St. Louis, MO) sectioned in 300 µm serial, coronal sections, from the level of the nucleus accumbens through the hippocampus. Sections were placed in 25 mM PBS until labeled with DiI.

Preparation of DiI-coated “bullets”

Coating of particles with lipophilic dye DiI was adapted from methods described elsewhere (Gan et al. 2009). Briefly, 2 mg carbocyanine fluorescent DiI (Molecular Probes, Carlsbad, CA) was dissolved in 75 µl methylene chloride. DiI was applied to 90 µg of 1.3 µm tungsten particles (BioRad, Hercules, CA) spread evenly on a glass slide. Tungsten particles were allowed to dry and then were scraped from the slide and collected into 10 ml of 10 mg/ml polyvinylpyrrolidone (PVP; Sigma-Aldrich, St. Louis, MO) dissolved in deionized water. The suspension was sonicated for 10 min with intermittent vortexing. Tefzel tubing (BioRad) was pre-coated with 10 mg/ml PVP and dried under 0.4 LPM nitrogen gas flow. The DiI/PVP suspension was quickly drawn into the Tefzel tubing and allowed to settle for 3 min. The PVP solution was withdrawn slowly from the tubing making certain not to

disturb the DiI coated tungsten. The Tefzel tubing was slowly rotated 360° and dried for 20 min under 0.4 LPM nitrogen gas flow. After drying, the tubing was cut into 1.3 mm segments (bullets) and stored at 4°C with desiccant in the dark until use.

Delivery of DiI-coated tungsten particles

A Helios Gene Gun (BioRad) with a modified barrel (O'Brien et al. 2001) was used for delivery of DiI-coated tungsten particles. A 40 mm spacer was attached to the modified barrel to maintain a consistent placement and distance between the Gene Gun and brain section. A 70 µm nylon mesh filter (Plastok Associates Ltd, Birkenhead Merseyside, UK) was secured at the head of the barrel to prevent large clusters of tungsten particles from reaching the tissue. The Gene Gun was loaded with DiI "bullets". Immediately prior to labeling, PBS was removed from the well containing the sections. DiI-coated particles were delivered at 100 PSI helium pressure and one DiI bullet was shot per brain section. Labeled sections were re-suspended in PBS and dye was allowed to diffuse through neuronal membranes for 24 h in the dark at room temperature. Slices were post-fixed for 1 h in 4% paraformaldehyde in PBS, and then placed in PBS until mounted on Superfrost slides (Brain Research Laboratories, Newton Highlands, MA) using 5% *n*-propyl-gallate in glycerin.

Confocal imaging

A Leica TCS SPE confocal microscope (Leica, Mannheim, Germany) was used to image DiI impregnated cells. DiI was imaged with excitation and emission specified to the manufacturer's spectral characteristics (Molecular Probes, Carlsbad, CA). The complete dendritic profile of each DiI impregnated neuron was captured using a 20× lens and *xy* pixel distribution of 512 × 512 at a frequency of 400 Hz. The neuron was scanned at 1.0 µm increments along the *z*-axis and reconstructed using Leica LAS AF software (Leica) to determine distance from the soma to the branch level of target dendrites prior to dendrite/spine imaging. Dendritic segments of CA1 pyramidal neurons were imaged at a distance of 250–400 µm from the soma (Woolley and McEwen 1993). Dendritic segments of medium spiny neurons from the nucleus accumbens core (NAc core), shell (NAc shell), and caudate/putamen (CPu) were 70–200 µm from the soma (Shen et al. 2009). After distance from soma was determined, magnification was increased to 63× oil immersion. Frame size was maintained at 512 × 512 and optical zoom of 5.61 was utilized to allow for maximum distribution of pixel size (60 nm) to tissue dimensions (60.91 nm) without over sampling. Z stacks of dendritic segments were taken at 0.12 µm steps, with a maximum of 200 steps. Images of three DiI impregnated cells were captured per brain region (CA1, NAc shell, NAc core, CPu) per animal, as well as three high power dendritic segments from each cell, yielding a total of nine dendritic segments per brain region.

Quantitation and analysis of dendritic spine density and spine head morphology

Dendritic Z stacks were reconstructed using the Surpass module of the Imaris software package (version 7.0, Bitplane Inc., St. Paul, MN). Dendritic shafts and spines were manually traced in the *xy* plane using the AutoDepth function of the Filament module. After tracing, accurate reconstruction of the diameter of the dendritic shaft, spine neck and head was made possible using the diameter function with a contrast threshold of 0.7. Spine head classifications of stubby, filapodial, long thin and mushroom were completed through the Classify Spines wizard within the Imaris software package. Criteria for spine head classifications have been described elsewhere (Harris et al. 1992; McKinney et al. 1999).

Data analysis

Spine density was calculated by summing the total number of spines per dendritic segment length and calculating average spine density/10 µm. These values were then averaged to

yield the spine density/10 μm for each animal. A Student t test was used to evaluate statistical differences between treatment groups.

Total spine population and counts of each spine class (stubby, filapodial, long thin, and mushroom) were summed for each treatment group. A χ^2 test was used to determine significant differences in spine morphology.

Results

Changes in dendritic spine density with estradiol treatment

Figure 1 shows a representative medium spiny neuron (MSN) and dendritic segment. Figure 1a illustrates the complete DiI impregnation of a nucleus accumbens core MSN neuron. An image generated from 63 $\times$ oil immersion captures how well DiI is taken up into the fine structures of thin spines (Fig. 1b). Filling of spines and dendrites allows for accurate reconstruction of the dendrite and spine heads using the diameter function of the Filament module in Imaris (Fig. 1c).

In estradiol-treated females, spine density of CA1 pyramidal neurons was significantly increased by 43% ($t_{(12)} = 4.17$, $p < 0.01$, two tailed) with 17.35 ± 2.98 spines/10 μm compared to 12.12 ± 1.45 spines/10 μm in control females (Fig. 2a). The opposite effect was observed in the NAc core (Fig. 2b), where dendritic spine density was significantly decreased ($t_{(12)} = 2.49$, $p < 0.05$, two tailed). No significant change in dendritic spine density was seen with estradiol treatment in either the NAc shell (Fig. 2c) or CPu (Fig. 2d). The apparent basal spine differences between the NAc core and NAc shell are consistent with previous reports (Meredith et al. 1992).

Dendritic spine morphology

Spines were classified into one of four groups: stubby, filapodial, long thin or mushroom. Spine classification provided normative values for the total spine number per treatment group, populations counts for each spine subtype in a given brain region, as well as percentage of total spine population (Table 1).

In CA1 pyramidal neurons, estradiol treatment uniformly increased all spine subtypes, resulting in the significant increase in spine density observed. This global increase in all spine subtypes occurred without significantly altering the proportions of any given spine subtype in relationship to the total spine population (Table 1). In the NAc core, the percentages of long, thin, filapodial and stubby spine subtypes were significantly different between estradiol treated and control groups (Fig. 3a). With estradiol treatment, long thin spines constituted a smaller percentage of total spine population than in the control group ($\chi^2_{(1)} = 32.41$, $p < 0.0001$, two-tailed; Fig. 3a). The opposite relationship (Fig. 3a) was seen with filapodial ($\chi^2_{(1)} = 7.57$, $p < 0.01$, two-tailed) and stubby spines ($\chi^2_{(1)} = 20.23$, $p < 0.0001$, two-tailed), as these spine subtypes constituted greater percentages of the total spine population in the estradiol treated group when compared with the control group.

Despite the fact that no change in spine density was observed in the NAc shell, significant differences in long thin and stubby spine morphology were observed. Similar to the changes in the NAc core, estradiol treatment resulted in a significant decrease in the proportion of long thin spines in the total population ($\chi^2_{(1)} = 20.68$, $p < 0.0001$, two-tailed; Fig. 3b). Also, the percentage of total spines constituted by the stubby spine subtype was significantly higher in the estradiol treated group as compared to controls ($\chi^2_{(1)} = 30.27$, $p < 0.0001$, two-

tailed). No significant differences in spine morphology were observed in the CPu (data not shown).

Discussion

The goal of this experiment was to evaluate the effects of estradiol treatment on spine morphology in the striatum. We believe this is the first report of estradiol effects on dendritic architecture and spine morphology in the nucleus accumbens.

Validation of DiOlistic approach

As a positive control for estradiol effects on striatal neurons, we examined changes in CA1 pyramidal neuron spine density following estradiol treatment. This effect of estradiol treatment on CA1 pyramidal neurons has been well-established in female rats using Golgi staining techniques (Woolley et al. 1990; Woolley and McEwen 1993). Indeed, in our female hamsters, estradiol yielded similar increases in dendritic spine density. Our normative data on spine density in the nucleus accumbens compared favorably to those of Shen et al. (2009), who used the same DiOlistic approach.

Estradiol effects on medium spiny neurons

Our results demonstrate that estradiol treatment impacts spine density and morphology of medium spiny neurons in the nucleus accumbens, with some differences in the response of neurons in the shell and core regions. There are multiple reports of estradiol's ability to modulate spine density in several brain areas, including the hippocampus (Woolley et al. 1990; Woolley and McEwen 1993), prefrontal cortex (Hao et al. 2006), medial nucleus of the amygdala (de Castilhos et al. 2008), and ventromedial nucleus of the hypothalamus (Frankfurt et al. 1990; Calizo and Flanagan-Cato 2000, 2002). We can now add the nucleus accumbens to that growing list. Initially, we were surprised by the decrease in spine density in the core of the nucleus accumbens. However, there is precedent from studies of the ventromedial nucleus of the hypothalamus (Calizo and Flanagan-Cato 2000, 2002), where estradiol decreased dendritic spine density selectively in neurons projecting to the midbrain central gray.

Estradiol also altered the morphological distribution of spine subtypes within the nucleus accumbens core and shell. Spine morphology is a reflection of spine maturity, stability and excitability (Hering and Sheng 2001; Wang and Zhou 2010). One common nomenclature classifies spines into four main categories in order of increasing maturity: stubby, filopodial, long thin and mushroom. With maturity of the spines comes an associated stability and functionality. As postsynaptic densities are proportional to the number of docked vesicles available for release presynaptically (Schikorski and Stevens 1997), larger spine heads have a greater potential contribution to the synaptic excitability of the cell. In the core and shell of the accumbens, we observed a "deconstruction" of spines from more to less mature offering the possibility that estradiol treatment decreases synaptic excitability of both the core and shell medium spiny neurons independent of changes in spine density.

Within the shell and core of the nucleus accumbens, subpopulations of medium spiny neurons exhibit differences in dendritic morphology and spine densities (Meredith et al. 1992). Because the DiI technique yields a small number of labeled neurons, there is always the fear that treatment differences in dendritic morphology result from sampling phenotypically different sets of neurons. Having said that, the dispersion pattern with ballistic delivery of DiI should not produce a selection bias in labeling of medium spiny neurons. Therefore, it seems unlikely that the effects of estradiol on spine density in the core of the nucleus accumbens and on spine morphology in both the core and shell are an artifact

of systematic labeling of different neuronal subtypes between the hormone treatment groups. Still, it would be a valuable addition to this technique to consistently characterize neuronal phenotypes of the DiI 1 labeled neurons.

Estradiol modulation of the striatum: direct and indirect actions

Decreases in spine density and destabilization of mature spines could result from estradiol acting directly on the medium spiny neurons within the nucleus accumbens or through indirect modulation via inputs from various afferent brain regions. It is well established that estradiol can exert direct effects in the striatum. Mermelstein and colleagues (1996) demonstrated that estradiol has the ability to reduce L-type Ca^{2+} currents of striatal neurons within seconds of application, an effect determined to be mediated through membrane bound estrogen receptors (ERs). Recent work indicates that these rapid effects are a result of ERs coupling to and activating mGluR3 signaling (Grove-Strawser et al. 2010) in a way that could affect postsynaptic EPSPs (Anwyl 1999). There are also reports of presynaptic localization of mGluR3 receptors in the striatum (Tamaru et al. 2001), along with physiological evidence that activation of these receptors results in reduced presynaptic glutamate release (Schoepp 2001; Ferraguti and Shigemoto 2006). Estradiol can modulate presynaptic glutamate release (Szego et al. 2010) though whether this is through ER coupling to mGluR3 is not known. The limitations of this as a sole mechanism of estradiol action on medium spiny neurons are the common distributions of ERs (Skynner et al. 1999; Donahue et al. 2000) and membrane interactions with mGluR3 receptors (Grove-Strawser et al. 2010) across the dorsal and ventral striatum. Given the discrepant actions of estradiol on spine density between these striatal subdivisions, it is unlikely that a direct mechanism is the sole action of estradiol on these neurons.

In both the hippocampus and ventromedial hypothalamus, estradiol acts on neurons secondary to those in which spine density changes are noted (Woolley and Cohen 2002). Here too, modulation of afferent signaling may play a role in the changes in dendritic spine density in the nucleus accumbens and help explain the differential effects seen across the dorsal and ventral striatum. The nucleus accumbens receives strong glutamatergic input from both the prefrontal cortex (Sesack et al. 1989) and hippocampus (Kelley and Domesick 1982). Many of these afferents synapse onto nucleus accumbens medium spiny neurons, which possess bistable resting membrane potentials (O'Donnell and Grace 1995). Hippocampally derived excitatory inputs are necessary to drive these neurons into their upstate (O'Donnell and Grace 1995). Further, it is only when these neurons are in their upstate that stimulation from the prefrontal cortex can elicit an action potential in nucleus accumbens medium spiny neurons (O'Donnell and Grace 1995). Within this scheme, estradiol may impact medium spiny neurons to create a quiescent state which is reflected by a down regulation of spines. Our working hypothesis is that estradiol is exerting its effects both directly and indirectly: directly by decreasing the synaptic excitability of nucleus accumbens neurons and indirectly by modulating the integrated function of the prefrontal cortex–hippocampus–nucleus accumbens circuit as a whole.

Functional implications

Our results are significant because they contribute a new piece to the complex puzzle of estradiol regulation of neuronal physiology. In the intact female, cyclical release of estradiol induces a fertile period and primes the female to become receptive to reproductive behaviors. Our data offer the possibility that part of this neuronal priming is down-regulation of accumbal excitability through destabilization of mature spines and decreases in spine density.

We know that the nucleus accumbens is part of a circuit that modulates the motivational components of female sexual behavior (Meisel and Mullins 2006). The question then is how does a reduction in spine density relate to an increase in sexual motivation? One answer to this question comes from the literature on the modulation of neuronal responsiveness in the nucleus accumbens by drugs of abuse. For example, during periods of acute cocaine withdrawal or following a cocaine challenge after a period of prolonged withdrawal there is decreased synaptic excitability (as measured by changes in AMPA and NMDA mediated currents) of medium spiny neurons in the core of the nucleus accumbens (Kourrich et al. 2007). Both acute withdrawal and cocaine challenges produce an increase in self-administration of cocaine (Shaham et al. 2003) suggesting that the reduced excitability in the core of the nucleus accumbens increases the motivation for drug seeking. If, as our spine data indicate, estradiol produces an analogous decrease in nucleus accumbens synaptic excitability in the natural state, this may provide a mechanistic basis for our observed increases in sexual motivation (Bradley et al. 2005; Meisel and Mullins 2006).

Unlike drugs, such as cocaine, which produce persistent (i.e., sensitized) alterations in the nucleus accumbens (Robinson and Kolb 2004), our reading of the literature is that estradiol results in more transient effects on the nucleus accumbens (Hedges et al. 2009). These transient effects of estradiol are matched by surprisingly rapid modifications of spines, which may occur within several hours of estradiol exposure (Mukai et al. 2007). It is clear that estradiol treatment has profound effects on synaptic architecture and excitability of medium spiny neurons in the nucleus accumbens. Further, these decreases in spine density and destabilization of spines identify a novel action of estradiol in this region with potential as a therapeutic target for the treatment of addiction.

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Fig. 1. Image of a representative DiI-labeled medium spiny neuron. **a** Low power fluorescent picture of a nucleus accumbens core medium spiny neuron. *Scale bar* is 20 μm . **b** DiI-labeled dendritic segment. *Scale bar* is 5 μm . **c** Computer rendering of the dendritic segment shown in **b**

Fig. 2.

Estradiol treatment increases spine density within the nucleus accumbens core. **a** CA1 pyramidal neurons treated with estradiol served as an internal positive control. Estradiol treatment significantly increased spine density within CA1 pyramidal neurons of the hippocampus. $**p < 0.01$. **b** Estradiol treatment significantly decreased spine density within NAc core medium spiny neurons. $*p < 0.05$. **c, d** Estradiol had no significant effect on the spine density of NAc shell or caudate medium spiny neurons

Fig. 3.

Estradiol treatment results in a deconstruction of mature spines in the nucleus accumbens. **a** Estradiol treatment significantly reduces the number of long thin spines ($***p < 0.0001$) and increases the number of filapodial ($**p < 0.01$) and stubby ($***p < 0.0001$) spines of medium spiny neurons in the NAc core. **b** Estradiol significantly decreases the number of long thin spines ($***p < 0.0001$) and increases the number of stubby spines ($***p < 0.0001$) in the NAc shell

Table 1

Spine classification: spines were classified into stubby, filapodial, long thin, or mushroom based on their length and diameters of the head and shaft for the CA1 region of the hippocampus, nucleus accumbens shell (NAc shell), nucleus accumbens core (NAc core), and caudate/putamen (CPu)

Brain region	Stubby		Filapodial		Long thin		Mushroom		Total estradiol	Total oil
	Estradiol	Oil	Estradiol	Oil	Estradiol	Oil	Estradiol	Oil		
CA1	161	125	981	788	324	225	90	75	1,556	1,231
CA1 %total (%)	10.4	10.1	63.1	64.0	20.8	18.3	5.8	6.1		
NAc shell	191	107	603	641	291	409	159	140	1,244	1,297
NAc shell %total (%)	15.4	8.2	48.8	49.4	23.4	31.5	12.8	10.8		
NAc core	130	71	897	851	403	570	135	132	1,565	1,624
NAc core %total (%)	8.3	4.4	57.3	52.4	25.8	35.1	8.6	8.1		
CPu	116	110	658	708	257	302	114	112	1,145	1,232
CPu %total (%)	10.1	8.9	57.5	57.5	22.5	24.5	10.0	9.1		