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Brain region-dependent alterations in polysialic acid immunoreactivity across the estrous cycle in mice

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Abstract

N-glycosylation is a posttranslational modification that plays significant roles in regulating protein function. One form of N-glycosylation, polysialylation, has been implicated in many processes including learning and memory, addiction, and neurodegenerative disease. Polysialylation appears to be modulated by the estrous cycle in the hypothalamus in rat, but this has not been assessed in other brain regions. To determine if polysialylation was similarly estrous phase-dependent in other neuroanatomical structures, the percent area of polysialic acid (PSA) immunoreactivity in subregions of the medial prefrontal cortex, hippocampus, and nucleus accumbens was assessed in each of the four phases of adult female mice. In this study, we found that PSA immunoreactivity fluctuated across the estrous cycle in a subregion-specific manner. In the prefrontal cortex, PSA immunoreactivity was significantly lower in proestrus phase compared to estrus in the prelimbic cortex, but did not differ across the estrous cycle in the infralimbic cortex. In the hippocampus, PSA immunoreactivity was significantly increased in proestrus compared to metestrus in the CA1 and CA2 and compared to diestrus in CA3, but remain unchanged in the dentate gyrus. PSA immunoreactivity did not vary across the estrous cycle in the nucleus accumbens core or shell. These findings may have implications for estrous cycle-dependent alterations in behavior.

Keywords

N-glycosylation; polysialylation; estrous cycle; prefrontal cortex; nucleus accumbens; hippocampus

Introduction

N-glycosylation is a posttranslational modification involving the addition of a peptide with oligosaccharide side chains to the asparagine residue of a protein (Ohtsubo and Marth, 2006). These glycans can then modulate protein conformation, receptor signaling, activation,

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Declaration of competing interest

The authors declare that they have no competing interests.

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and trafficking, and the strength of protein-protein interactions (Ohtsubo and Marth, 2006). In the brain, N-glycosylation occurs on a variety of receptors, ion channels, and cell adhesion molecules (Scott and Panin, 2014).

The glycan polysialic acid (PSA) is an form of N-glycosylation with great specificity for its limited central nervous system targets, including the cell adhesion molecules neural cell adhesion molecule (NCAM) and synaptic cell adhesion molecule 1 (SynCAM1), and the receptor, neuropilin-2 (Mühlenhoff et al., 2013). NCAM modification has been the most thoroughly characterized. NCAM and SynCAM1 are surface glycoproteins that mediate homophilic and heterophilic cell-cell interactions and heterophilic cell-substrate interactions (Acheson et al., 1991; Fogel et al., 2007; Galuska et al., 2010), such as with extracellular matrix proteins. N-glycosylation appears to regulate cell adhesion, but in opposing directions for NCAM and SynCAM1. While N-glycosylation of NCAM leads to steric repulsion, promoting anti-adhesive properties (Johnson et al., 2005), adhesive strength of SynCAM1 can be either increased or decreased (Fogel et al., 2010).

NCAM and SynCAM1 are expressed by both excitatory and inhibitory neurons and by astrocytes. Their expression is regulated in a brain region-dependent manner from early postnatal development into adulthood (Di Cristo et al., 2007; Niquet et al., 1993; Ribic et al., 2019; Sandau et al., 2011; Seki and Arai, 1993; Thomas et al., 2008). Roles for PSA-NCAM and SynCAM1 include neurogenesis, synaptic plasticity, and modulation of spine density (Cremer et al., 2000; Doengi et al., 2016; Gascon et al., 2010, 2007; Guirado et al., 2014; Muller et al., 1996; Robbins et al., 2010). NCAM and SynCAM1 have both been implicated in learning and memory in males (Barker et al., 2012; Becker et al., 1996; Lopez-Fernandez et al., 2007; Robbins et al., 1999), with much of the work examining the role of N-glycosylated proteins focused on the role of PSA-NCAM in learning and memory in the hippocampus. Cleavage of PSA in the hippocampus via administration of the bacterial enzyme endo-neuraminidase (endo-N) impairs acquisition and expression of spatial memory and reduces contextual freezing following fear conditioning in male rats (Becker et al., 1996; Lopez-Fernandez et al., 2007). In addition, PSA-NCAM expression in the prefrontal cortex has been implicated in extinction of ethanol seeking in male mice (Barker et al., 2012). NCAM and SynCAM1 within the nucleus accumbens are sensitive to stress and drugs of abuse (Bessa et al., 2013; Giza et al., 2013; Kähler et al., 2020; Wang et al., 2020), suggesting a potential role for N-glycosylation in the nucleus accumbens as a mediator of synaptic structure and related behavior. To our knowledge, this has not yet been studied. PSA-NCAM expression also appears to be modulated by stress (Nacher et al., 2004a, 2004b; Pham et al., 2003) and in various neuropsychiatric diseases such as Schizophrenia, depression, and addiction (Barbeau et al., 1995; Gilabert-Juan et al., 2012; Varea et al., 2012; Weber et al., 2006).

Given the role of PSA in regulation of key neurocognitive functions, it is critical to extend research on PSA to females. To date, the majority of this research has been conducted exclusively in males and PSA-NCAM expression and function remains minimally characterized in females. The little data that do exist suggest that PSA-NCAM expression may change across the estrous cycle, at least in a subset of brain regions which may be a potential contributor to estrous-associated variations in synaptic plasticity and function.

Specifically, PSA-NCAM immunoreactivity is increased in proestrus relative to metestrus in the hypothalamus (Tan et al., 2009), suggesting that polysialylation is regulated by circulating hormones. However, it is unclear if polysialylation varies across the estrous cycle in other brain regions.

Dendritic spine density is regulated by PSA in males (Guirado et al., 2014), though this is not characterized in females. Spine density varies across the estrous cycle and in response to estradiol administration, but the direction of these effects is brain region dependent with different patterns in the hippocampus, prefrontal cortex, and nucleus accumbens. In the CA1 subregion of hippocampus, spine density decreases from proestrus, when estradiol and progesterone levels are high in rats, to estrus, a low hormonal state (Woolley et al., 1990; Woolley and McEwen, 1993). Estradiol administration also increases dendritic spine density in the prefrontal cortex (Khan et al., 2013; Tuscher et al., 2016), but decreases spine density in the core, but not shell, of the nucleus accumbens (NAc) (Peterson et al., 2015). As spine density is regulated by both PSA and circulating hormones, we hypothesized that PSA expression would vary across the estrous cycle in a brain subregion-dependent manner. A thorough characterization of estrous cycle -associated changes in neuroplasticity-associated protein expression within key neurobiological substrates is critical for our comprehension of neurobehavioral and neurocognitive function in female mice. Thus, we evaluated PSA expression in each phase of the estrous cycle across subregions of the hippocampus, prefrontal cortex, and nucleus accumbens of adult female mice.

Materials and Methods

Subjects

Female C57BL/6J mice (n=27, 9 weeks, Jackson Laboratories) were group housed in same-sex cages in a temperature- and humidity-controlled environment and maintained under a 12h light/dark cycle with *ad libitum* access to food and water. All procedures were approved by the Institutional Animal Use and Care Committee at Drexel University.

Identification of estrous cycle

Mice were allowed to acclimate to the colony room for 1 week prior to handling. Following acclimation, mice were handled and monitored for at least two weeks prior to perfusion. Approximately 1–2 hours prior to perfusion at the same time every day (3 hours into the light cycle), vaginal cytology was used to monitor the phase of estrous cycle of C57BL/6J female mice. Vaginal cells were collected via lavage of the opening of the vaginal canal with saline (McLean et al., 2012). As the method employed for cell collection does not involve touching the vaginal opening or penetrating of the vaginal canal, the likelihood of inducing pseudopregnancy is minimized and we did not observe pseudopregnancy in any of the mice included in the study. The vaginal fluid collected was placed on a clean glass slide. Stage of the estrous cycle was determined by examining smears under a light microscope without staining. Mice were randomly assigned to be perfused in each of the four phases.

The proestrus phase was identified by the presence of primarily nucleated epithelial cells. The estrus phase was identified by the presence of predominantly cornified epithelial cells.

The diestrus phase was identified by the presence of predominantly leucocytes. The metestrus phase was identified by a mixture of nucleated cells, cornified cells, and leucocytes.

Tissue Processing and Immunohistochemistry

Mice were transcardially perfused with 4% paraformaldehyde in each of the four phases of the estrous cycle. Brains were cryoprotected in 30% w/v sucrose and sliced using a cryostat to obtain 40µm coronal sections. Sections were incubated in 1% hydrogen peroxide solution for 1 hour to block endogenous peroxidase activity. Sections were blocked in 5% normal donkey serum for 1 hour followed by incubation with anti-PSA primary antibody (1:1000, Absolute Antibody, mab735, Oxford, UK) at room temperature overnight (18–24 hours), and biotinylated donkey anti-mouse secondary (1:1000, Jackson Labs, West Grove PA, 715–065-151) for 30 minutes. This antibody was selected as it has been well-validated for selectivity to PSA through the use of a variety of methods, including in a knockout mouse model and after enzymatic cleavage of PSA (Galuska et al., 2006; Husmann et al., 1990; Nakayama et al., 1998). Staining was visualized using nickel-enhanced DAB (Vector labs, Burlingame, CA, SK-4100) for 10 minutes. After staining, sections were mounted on plus slides and coverslipped with DPX mounting medium (Election Microscopy Science, Hatfield, PA). To control for any batch-to-batch differences in background staining, all batches contained representation from each phase of the estrous cycle.

To analyze PSA immunoreactivity, 10X images from 3 sections were taken and stitched together using Microsoft Image Composite Editor of slices in the prefrontal cortex (PFC, 1.54mm, 1.70mm, and 1.98mm anterior of Bregma), nucleus accumbens (NAc, 1.10mm, 1.42mm, and 1.70mm anterior of Bregma), and dorsal hippocampus subregions (–1.34mm, –1.82mm, and –2.18mm posterior of Bregma). Due to the density of labeling, all images were analyzed bilaterally as percent area using ImageJ using an automated thresholding approach with manual adjustments for artifacts (Donnelly et al., 2009) under blinded conditions. We have established high interrater reliability with this technique and the blinded and predominantly automated nature of the analysis reduces experimenter bias. Animals were excluded from analysis if any of the three sections for a particular brain region were not available for analysis.

Statistical Analysis

Data were analyzed in GraphPad using one-way ANOVA when variances were equal for comparing percent area of staining in proestrus, estrus, metestrus, and diestrus, followed by Tukey's multiple comparisons test when significant. When variances were not equal as indicated by Brown-Forsythe's test, means were compared using Welch's ANOVA, followed by Dunnett's T3 multiple comparisons test when significant.

Results

Medial Prefrontal Cortex

To determine if estrous cycle impacted PSA expression in the prelimbic and infralimbic cortices, tissue from female mice sacrificed in each phase of the estrous cycle underwent

immunohistochemical analysis and quantification of PSA immunoreactivity (Fig. 1a, 1b). In the prelimbic cortex, a Brown-Forsythe test revealed no significant differences in variance of percent area of PSA [$F(3,19) = 1.658$, $p = 0.2097$]. A one-way ANOVA of percent area of PSA immunoreactivity in the prelimbic cortex revealed a significant difference between groups [$F(3,19) = 4.026$, $p = 0.022$, $\eta^2=0.39$] (Fig. 1c). Post hoc analyses indicated that PSA immunoreactivity was significantly decreased in proestrus compared to estrus ($p = 0.035$). In contrast, in the infralimbic cortex, a Brown-Forsythe test revealed a significant difference in the variances of percent area of PSA immunoreactivity [$F(3,19) = 4.141$, $p = 0.048$, $\eta^2=0.40$] (Fig. 1d). Post hoc analyses revealed the variance in diestrus was significantly less than the variance in proestrus ($p=0.0019$) and metestrus ($p=0.0078$). Welch's ANOVA revealed no significant differences in mean PSA immunoreactivity across groups [$W(3,9) = 2.886$, $p = 0.0970$].

Hippocampus

To determine if estrous cycle impacted PSA expression in the CA1, CA2, CA3 and dentate gyrus subregions of the hippocampus, PSA immunoreactivity was quantified separately for each subregion in each of the four phases (Fig. 2a, 2b). In the CA1, A Brown-Forsythe test revealed a significant difference in the variances of percent area of PSA [$F(3,23) = 3.303$, $p = 0.0382$, $\eta^2=0.32$] (Fig. 2c). Post hoc analyses revealed the variance in diestrus was significantly greater than the variance in proestrus ($p=0.0011$) and metestrus ($p=0.0416$) and the variance in estrus is significantly greater than the variance in proestrus ($p=0.0184$). Welch's ANOVA revealed a significant difference in mean between groups in [$W(3,11) = 6.923$, $p = 0.0068$]. Post hoc analyses indicated that PSA immunoreactivity was significantly increased in proestrus compared to metestrus ($p = 0.0407$).

In the CA2 subregion, a Brown-Forsythe test revealed a significant difference in the variances of percent area of PSA immunoreactivity [$F(3,23) = 3.272$, $p = 0.0394$, $\eta^2=0.22$] (Fig. 2d). Post hoc analyses revealed the variance in diestrus was significantly greater than the variance in proestrus ($p=0.0018$) and metestrus ($p=0.0381$) and the variance in estrus is significantly greater than the variance in proestrus ($p=0.0101$). Welch's ANOVA revealed a significant difference in mean between groups [$W(3,11) = 5.747$, $p = 0.0126$]. Post hoc analyses indicated that PSA immunoreactivity was significantly increased in proestrus compared to metestrus ($p = 0.0321$).

In the CA3, variance was not estrous phase-dependent [$F(3,23) = 2.446$, $p = 0.0896$, $\eta^2=0.30$]. A one-way ANOVA revealed a significant difference among groups [$F(3,23) = 3.236$, $p = 0.0408$] (Fig. 2e). Post hoc analyses revealed that PSA immunoreactivity was significantly greater in proestrus compared to diestrus ($p = 0.0268$).

For the dentate gyrus, a Brown-Forsythe test revealed a significant difference in the variances of percent area of PSA immunoreactivity [$F(3,23) = 4.262$, $p = 0.0156$, $\eta^2=0.25$] (Fig. 2f). Post hoc analyses revealed the variance in diestrus was significantly greater than the variance in proestrus ($p=0.0136$) and metestrus ($p=0.0443$). Welch's ANOVA revealed no significant difference among groups [$W(3,12) = 3.105$, $p = 0.0675$].

Nucleus accumbens

To determine if estrous cycle impacted PSA expression in the core and shell of the nucleus accumbens, PSA immunoreactivity was analyzed in each of the four phases for both subregions (Fig. 3a, 3b). A Brown-Forsythe test revealed no significant differences in variance in either nucleus accumbens core [$F(3,19) = 0.6314$, $p = 0.6038$] or shell [$F(3,19) = 0.3874$, $p = 0.7633$]. A one way ANOVA of percent area of PSA immunoreactivity in the nucleus accumbens shell [$F(3,19) = 0.936$, $p = 0.442$, $\eta^2=0.13$] (Fig. 3c) and core [$F(3,19) = 0.979$, $p = 0.423$, $\eta^2=0.13$] (Fig. 3d) revealed no difference among groups.

Discussion

Our findings demonstrate that PSA immunoreactivity varied across the estrous cycle in a brain subregion-specific manner, with opposing effects in discrete structures (Fig. 4). We found that PSA immunoreactivity was significantly higher in estrus compared to proestrus in the prelimbic cortex, but was significantly lower in metestrus compared to proestrus in CA1 and CA2 and in diestrus compared to proestrus in CA3. In contrast, we observed no estrous cycle-dependent changes in PSA immunoreactivity in the nucleus accumbens, dentate gyrus, and infralimbic cortex.

In the PFC, PSA immunoreactivity varied across the estrous phase in the prelimbic cortex, but not the infralimbic cortex. The prelimbic and infralimbic cortices play distinct roles in learning and memory (Ashwell and Ito, 2014; Sierra-Mercado et al., 2011) and project to distinct brain regions (Vertes, 2004). This increase in PSA expression in during estrus may have implications for behaviors dependent on the prelimbic cortex. For example, prelimbic cortex projections to the nucleus accumbens core appear to drive drug-primed reinstatement of cocaine seeking (McFarland et al., 2003), a behavior which is augmented in estrus females (Kippin et al., 2005).

In the hippocampus, PSA immunoreactivity varied across the estrous phase in CA1, CA2, and CA3. Given that estrous cycle-dependent alterations in spine density appear to be specific to CA1 (Woolley et al., 1990), we did not expect to observe similar alterations in CA2 and CA3. As depletion of PSA is associated with an increase in spine density and spine density in the CA1 is higher in proestrus compared to estrus (Woolley et al., 1990), contrary to our findings, we anticipated that PSA would be lower in proestrus in CA1. However, the study investigating PSA depletion was examining spine density on interneurons of the CA1, while estrous cycle effects on spine density have been characterized in pyramidal neurons. Thus, our findings may indicate that PSA regulation of spine density is distinct in differing neuronal subtypes. Alternatively, our findings identify modest changes in PSA expression across the estrous cycle, and it is possible that the impact of physiologically relevant fluctuations in PSA expression impact spine density differently from enzymatic depletion of PSA. Estrous cycle is also known to regulate spine morphology of pyramidal neurons, with mushroom type spines predominating in proestrus and thin type spines predominating in estrus (González-Burgos et al., 2005). Thus, it is possible that the decrease in PSA during estrus may be associated with the increased prevalence of immature, thin-type spines. These estrous cycle-related alterations in PSA immunoreactivity in the hippocampus may have implications for learning and memory. For example, as polysialylation of NCAM in the

hippocampus appears to be an important contributor to fear conditioning in males (Lopez-Fernandez et al., 2007), it may be possible that estrous cycle-dependent alterations in fear conditioning (Markus and Zecevic, 1997; Milad et al., 2009) may be, in part, mediated by alterations in PSA expression. Notably, much of this work has been performed in rats in which hormone fluctuation patterns may be distinct from those observed in mice (Bastida et al., 2005; Bertolin and Murphy, 2014; Butts et al., 2010; Fata et al., 2001; McLean et al., 2012; Walmer et al., 1992), thus it will be important to determine how these findings generalize across species.

Further, targets of PSA are also expressed on other cell types, including astrocytes (Miñana et al., 1998; Sandau et al., 2011). Sialylation of astrocytic proteins is critical for activity-dependent changes in astrocyte morphology (Theodosios et al., 1999). Astrocytic volume in the CA1 is increased during estrus, which is hypothesized to compensate for the loss of dendritic spines observed in estrus (Klintsova et al., 1995) and mirrors the pattern of PSA immunoreactivity we observed in CA1. NCAM expression is also increased in reactive astrocytes in the hippocampus (Niquet et al., 1993) and astrocyte reactivity is regulated by the estrous cycle in the hippocampus (Arias et al., 2009; Struble et al., 2006). Thus, PSA expression of astrocytes may be contributing to the estrous cycle-related changes in total PSA immunoreactivity in the hippocampus. As hippocampal astrocytes actively participate in learning and memory (Adamsky et al., 2018; Bracchi-Ricard et al., 2008; Habbas et al., 2015; Suzuki et al., 2011), alterations in astrocyte PSA expression may also contribute to estrous cycle-dependent changes in behavior.

PSA expression did not vary across the estrous cycle in the dentate gyrus. PSA-NCAM expression in the dentate gyrus appears to be modulated by stressors such as chronic restraint stress or drug use in males (Ma Kowiak et al., 2005; Pham et al., 2003; Weber et al., 2006). Thus, it is possible that estrous cycle-related alterations in PSA in the dentate gyrus may be uncovered under conditions in which stressors have been introduced, though this was not assessed in the current study.

In addition to significant differences in the mean immunoreactivity across the estrous cycle, we also observed significant differences in the variance in the infralimbic cortex and CA1, CA2, and dentate gyrus. These differences in range of expression may reflect differences in hormonal fluctuations, particularly in the case of the infralimbic cortex where there is a wide range of values in the proestrus phase. Alternatively, these differences in variance may indicate that only a subset of females are sensitive to changes in hormone level-induced alterations in PSA expression.

In contrast to the PFC and hippocampus, we did not observe estrous cycle-related differences in PSA immunoreactivity in either the shell or the core of the nucleus accumbens. To our knowledge, previous work has not investigated the relationship between polysialylation and synaptic plasticity in the nucleus accumbens. However, this brain region is known to be regulated by estradiol. Neuronal excitability in the nucleus accumbens is modulated by the estrous cycle (Proaño et al., 2018) and estradiol administration modulates spine density of medium spiny neurons in the nucleus accumbens core (Peterson et al., 2015). Thus, polysialylation may not be a major contributor to these estrous cycle-dependent

alterations in excitability and spine density in the accumbens. Alternatively, as our findings did not differentiate between neuronal and glial expression of PSA, this lack of change in total PSA immunoreactivity does not preclude the possibility of cell type-specific alterations. Further, differential effects of estrous cycle on polysialylation of either NCAM or SynCAM1 may be masked by measuring total PSA.

In addition to its roles in healthy brain function, polysialylation has also been implicated in neurodegenerative disease and neuropsychiatric disorders including substance use disorders, and may have a role in other diseases with neurocognitive impairments such as HIV (Barbeau et al., 1995; Brennaman and Maness, 2010; El Maarouf et al., 2006; Gilbert-Juan et al., 2012; Poluektova et al., 2005; Sato et al., 2016; Schnaar et al., 2014; Varea et al., 2012; Weber et al., 2006). As the prevalence, symptoms, and long term outcomes of neuropsychiatric and neurodegenerative disorders are frequently sex-dependent (Altemus et al., 2014; Giacometti and Barker, 2020; Ullah et al., 2019), we believe these data will have implications for our understanding of the neurobiological basis of sex differences in risk and prognosis of neuropsychiatric and neurodegenerative disease.

Together, these data suggest that N-glycosylation may be regulated by the estrous cycle in a brain region-specific manner. These alterations are likely driven by circulating estradiol, as all alterations occurred in proestrus, a phase characterized by high levels of estradiol, relative to low estradiol phases. However, it is possible that increases in progesterone during the diestrus phase may contribute to alterations during this phase and subsequent studies should interrogate the independent and interactive roles of progesterone and estradiol. Interestingly, the direction of changes in proestrus relative to other phases was not unidirectional, suggesting that the relationship between estradiol and PSA expression may be cell type- or brain region-specific. This estrous cycle-dependent regulation will likely have implications for estrous cycle-dependent behavioral changes. Further, naturally occurring cyclicity in PSA expression may relate to periods of heightened vulnerability to stress-, infection-, or drug-induced alterations in neural plasticity and function across the estrous cycle, which will inform understanding of the neurobiological consequences of these insults.

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Highlights

- Estrous cycle modulates total polysialic acid immunoreactivity as well as variance
- PSA immunoreactivity was modulated in the prelimbic PFC and hippocampal subregions
- Estrous cycle did not modulate polysialic acid immunoreactivity in nucleus accumbens

Fig. 1. Estrous Cycle-Dependent PSA immunoreactivity in the Medial Prefrontal Cortex
 (a) Schematics of locations of prelimbic and infralimbic cortices. (b) Representative images of PSA immunoreactivity in the medial PFC in proestrus, estrus, metestrus, and diestrus. (c) Percent area of PSA immunoreactivity in the prelimbic cortex in each phase of the estrous cycle. (d) Percent area of PSA immunoreactivity in the infralimbic cortex in each phase of the estrous cycle. Dashed line represents median value, dotted lines represent quartiles, P* mean percent area $p < 0.05$ vs proestrus, P† variance $p < 0.05$ vs proestrus, M\$ variance $p < 0.05$ vs metestrus

Fig. 2. Estrous Cycle-Dependent PSA immunoreactivity in the Hippocampus

(a) Schematics of locations of CA1, CA2, CA3, and dentate gyrus. (b) Representative images of PSA immunoreactivity in the hippocampus in proestrus, estrus, metestrus, and diestrus. (c) Percent area of PSA immunoreactivity in the CA1 in each phase of the estrous cycle. (d) Percent area of PSA immunoreactivity in the CA2 in each phase of the estrous cycle. (e) Percent area of PSA immunoreactivity in the CA3 in each phase of the estrous cycle. (f) Percent area of PSA immunoreactivity in the dentate gyrus in each phase of the estrous cycle. Dashed line represents median value, dotted lines represent quartiles, P* mean

percent area $p < 0.05$ vs proestrus, $P^{\dagger}$ variance $p < 0.05$ vs proestrus, $M^{\S}$ variance $p < 0.05$ vs metestrus

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Fig. 3. Estrous Cycle-Dependent PSA immunoreactivity in the Nucleus Accumbens
 (a) Schematics of locations of core and shell of nucleus accumbens. (b) Representative images of PSA immunoreactivity in the nucleus accumbens in proestrus, estrus, metestrus, and diestrus. (c) Percent area of PSA immunoreactivity in the nucleus accumbens shell in each phase of the estrous cycle. (d) Percent area of PSA immunoreactivity in the nucleus accumbens core in each phase of the estrous cycle. Dashed line represents median value, dotted lines represent quartiles

Fig. 4. Summary of estrous cycle-dependent PSA expression in the female mouse brain
 Solid lines represent approximate changes in mean across each phase for each brain subregion. Lighter shaded area represents changes in variance across the estrous cycle. Progesterone and estradiol curve schematics are based on McLean et al 2012. P* mean percent area $p < 0.05$ vs proestrus, P† variance $p < 0.05$ vs proestrus, M\$ variance $p < 0.05$ vs metestrus