Progesterone decreases tyrosine hydroxylase phosphorylation state and increases protein phosphatase 2A activity in the stalk-median eminence on proestrous afternoon

Bin Liu and Lydia A Arbogast

Department of Physiology, Southern Illinois University School of Medicine, Carbondale, Illinois 62901-6523, USA

Abstract

The progesterone (P₄) rise on proestrous afternoon is associated with dephosphorylation of tyrosine hydroxylase (TH) and reduced TH activity in the stalk-median eminence (SME), which contributes to the proestrous prolactin surge in rats. In the present study, we investigated the time course for P₄ effect on TH activity and phosphorylation state, as well as cAMP levels and protein phosphatase 2A (PP2A) activity and quantity, in the SME on proestrous morning and afternoon. P₄ (7.5 mg/kg, s.c.) treatment on proestrous afternoon decreased TH activity and TH phosphorylation state at Ser-31 and Ser-40 within 1 h, whereas morning administration of P₄ had no 1 h effect on TH. PP2A activity in the SME was enhanced after P₄ treatment for 1 h on proestrous afternoon without a change in PP2A catalytic subunit quantity, whereas P₄ treatment had no effect on PP2A activity or quantity on proestrous morning. cAMP levels in the SME were unchanged with 1 h P₄ treatment. At 5 h after P₄ treatment, TH activity and phosphorylation state declined coincident with an increase in plasma prolactin in both P₄-treated morning and afternoon groups. PP2A activity in the SME was unchanged in 5 h P₄-treated rat. Our data suggest that P₄ action on tuberoinfundibular dopaminergic (TIDA) neurons involves at least two components. A more rapid (1 h) P₄ effect engaged only on proestrous afternoon likely involves the activation of PP2A. The longer P₄ action on TIDA neurons is evident on both the morning and afternoon of proestrus and may involve a common, as yet unidentified, mechanism.


Introduction

A preovulatory prolactin surge is evident on proestrous afternoon in rats (Smith et al. 1975, Liu & Arbogast 2008). The rising titer of estradiol (E₂) beginning late on diestrous day 2 and continuing into proestrous morning is essential for the occurrence of the prolactin surge and drives the early phase of the surge (Neill et al. 1971, Arbogast & Ben-Jonathan 1990). The preovulatory rise in progesterone (P₄) on proestrous afternoon augments the magnitude or extends the duration of the prolactin surge (Arbogast & Ben-Jonathan 1990, Arbogast & Voogt 1994, Liu & Arbogast 2008). This proestrous prolactin surge may have a luteolytic role to maintain the estrous cycle (Gaytan et al. 2001).

Dopamine is the major inhibitor of prolactin release from the anterior pituitary gland (Freeman et al. 2000, Ben-Jonathan & Hnasko 2001). Dopamine is released from tuberoinfundibular dopaminergic (TIDA) neurons, which originate in the arcuate nucleus and project to the median eminence. Dopamine synthesis is dependent on the activity of tyrosine hydroxylase (TH), which is the rate-limiting enzyme in the catecholaminergic biosynthetic pathway. In response to a positive stimulus, TH enzyme is rapidly phosphorylated, resulting in increased hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and its instant conversion to dopamine (Haycock & Haycock 1991). TH can be phosphorylated at four serine sites, Ser-8, Ser-19, Ser-31, and Ser-40, in the N-terminal regulatory domain of TH. Each serine site is the target of specific protein kinase(s) and phosphoprotein phosphatase(s), and the phosphorylation state of TH results from the dynamic balance between these opposing actions.

An inhibitory action of P₄ on TIDA neurons contributes to P₄-enhancing effect on the preovulatory prolactin surge. A decrease in TH activity and Th mRNA levels occurs concomitantly with the preovulatory P₄ rise (Arbogast & Ben-Jonathan 1989, Arbogast & Voogt 1994, Liu & Arbogast 2008). Acute ovariectomy on proestrous morning prevents this decline in TH activity and Th mRNA levels. P₄, but not E₂, replacement at the appropriate time restores the decline in TH activity, as well as the increase in circulating prolactin levels (Arbogast & Ben-Jonathan 1990, Arbogast & Voogt 1994). The P₄ rise on proestrous afternoon is associated with decreased phospho–TH at Ser-19, Ser-31, and Ser-40 as early as 1700 h and extending to at least 2200 h (Liu & Arbogast 2008). Ser-40, a target of cAMP-dependent protein kinase,
exhibits the most marked dephosphorylation changes, suggesting that this site may exert the greatest impact on TH activity. In ovariectomized rats primed with E_2, P_4 given early in the morning exerts an inhibitory effect on TIDA neuronal activity (Yen & Pan 1998) and augments prolactin secretion (Caligaris et al. 1974) after 4–5 h. P_4 decreases the number of detectable \( Th \) mRNA-containing cells in the arcuate and periventricular regions between 2 and 8 h after treatment (Morrell et al. 1989). A reduction in the quantity of TH protein was observed within 1 day after P_4 treatment (Wang & Porter 1986).

The concerted dephosphorylation of TH at Ser-19, Ser-31, and Ser-40 induced by endogenous and exogenous P_4 administration (Liu & Arbogast 2008) supports the notion that a common phosphatase mechanism may be involved. Protein phosphatase 2A (PP2A) acts on these serine sites (Havik et al. 1989, Berresheim & Kuhn 1994) and thus is a potential mediator. The objective for this study was to examine the time course for P_4 effect on plasma prolactin levels, TH activity, and TH phosphorylation state in the stalk–median eminence (SME) during proestrous morning and afternoon. These data will provide insight into the mechanism(s) that may be involved in the endogenous P_4 action on TIDA neurons on proestrus. We also evaluated the effect of P_4 administration on PP2A and cAMP levels in the SME of rats on proestrous morning and afternoon, to explore the cellular mechanism underlying P_4 modulation of TIDA neurons.

Materials and Methods

Animals and experimental groups

Adult female (200–250 g) Sprague–Dawley rats were obtained from Charles River (Raleigh, NC, USA). Rats were housed under controlled temperature and lighting (lights on from 0700 to 2100 h) and supplied with food and water ad libitum. Estrous cycles were followed by daily vaginal lavage, and only those displaying at least three consecutive 4-day estrous cycles were used. Experiments were performed in the rats on diestrus-2 and/or proestrus. All animal experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Southern Illinois University at Carbondale.

Some rats were implanted with a chronic jugular cannula on diestrus-2 under isoflurane anesthesia. Where indicated, ovariectomy was performed under isoflurane anesthesia between 1200 and 1400 h on proestrus. Rats were treated with P_4 (7.5 mg/kg, s.c.) or oil (1 ml/kg) at 0930 h or at 1700 h on proestrus. This dose for P_4 decreased \( Th \) mRNA level and enzyme activity in the TIDA system in our previous studies (Arbogast & Ben-Jonathan 1990, Arbogast & Voogt 1994, Liu & Arbogast 2008). To examine the effect of ovariectomy and P_4 replacement on prolactin secretion in proestrus rats, blood samples (0–25 ml) were collected from 1300 to 2200 h at 1 h interval in normal cycling rats, sham-operated rats, and ovariectomized (1200–1230 h) rats treated with oil vehicle or P_4 at 1700 h. To evaluate the time course for exogenous P_4 regulation of plasma prolactin and compare the different effects between proestrous morning and afternoon, blood samples (0-6 ml) were collected at 0, 1, 3, and 5 h after treatment, i.e. at 0930, 1030, 1230, and 1430 h after proestrous morning injections or at 1700, 1800, 2000, and 2200 h after proestrous afternoon injections. Blood was centrifuged at 10 000 g for 5 min, and plasma was stored at −20 °C for subsequent determination of P_4 and/or prolactin levels. To examine P_4 effects on TH activity, TH phosphorylation state, cAMP level, PP2A enzyme activity, and protein levels in the SME, groups of rats were treated with P_4 or oil and killed at 1, 3, or 5 h after treatment. SME tissue was dissected with fine scissors using a dissecting microscope and frozen immediately on dry ice. The tissue was then stored at −80 °C until analysis for TH activity by HPLC, phospho-TH, and catalytic subunit of PP2A protein level by western blot, PP2A activity assay, and cAMP level by RIA within 1 week.

Estimation of TH activity by HPLC

DOPA accumulation in the SME was used as an index of TH activity. Briefly, rats were injected with \( m \)-hydroxybenzylhydrazine dihydrochloride (NSD 1015; 100 mg/kg, i.p.), an \( \alpha \)-aromatic amino acid decarboxylase inhibitor, and decapitated 30 min thereafter. The dissected SME tissue was homogenized by sonication in 250 μl 0·1 M perchloric acid and centrifuged at 13 000 g for 15 min. DOPA content in the supernatant was determined by HPLC with electrochemical detection, as described previously (Arbogast & Voogt 1991, 1994, Liu & Arbogast 2008). The pellet was solubilized in 0·5 M sodium hydroxide and analyzed for protein content with the Bio-Rad Protein Assay (Bio-Rad Laboratories). Tissue DOPA levels were normalized to protein contents.

Western blot for phospho-TH and catalytic subunit of PP2A protein

SME tissue was sonicated in 35 μl homogenization buffer and centrifuged at 12 000 g for 15 min, as previously described (Liu & Arbogast 2008). A 2·5 μl aliquot of the supernatant was used for protein content determination using the Bio–Rad Protein Assay. An equivalent amount of Laemmli sample buffer (Sigma Aldrich Chemical Co.) containing 5% 2-mercaptoethanol was added to each supernatant, and samples were heated to 95 °C for 4 min. Equal amounts of protein (10–25 μg) from each experimental sample were loaded to individual wells on an 8% polyacrylamide gel. Gels were calibrated with molecular weight standards between 49 and 211 kDa. The proteins were separated by SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane. For measuring the phosphorylation
state of TH, the membranes were incubated in 0.2% 1-Block (Tropix, Bedford, MA, USA) in PBS for 1 h to block nonspecific binding, and then immunoblotted using one of the following antisera combinations diluted in 1% BSA–PBS-T (0-05% Tween–20 in PBS) overnight at 4°C: 1) rabbit anti-phospho-TH at Ser-19 (1:2500, 36-9800, Zymed Laboratories, South San Francisco, CA, USA) and mouse anti-TH (1:3000, MAB 318, Chemicon International, Temecula, CA, USA); 2) rabbit anti-phospho-TH at Ser-31 (1:2500, 36-9900, Zymed Laboratories) and mouse anti-TH (1:3000); 3) rabbit anti-phospho-TH at Ser-40 (1:2500, 36-8600, Zymed Laboratories) and mouse anti-TH (1:3000); 4) rabbit anti-TH (1:3000, AB152, Chemicon International) and mouse anti-β-tubulin (1:4000, Upstate Biotechnology, Temecula, CA, USA). For determining catalytic subunit of PP2A (PP2Ac) levels in the SME, the membrane was incubated with rabbit anti-PP2Ac (1:2500, 06-222, Upstate Biotechnology) and mouse anti-β-tubulin (1:4000). After incubation with primary antisera, membranes were washed in PBS-T and incubated with both IRDye 800 Conjugated Affinity Purified anti-mouse IgG (1:20 000, 610-132-003, Invitrogen Corporation) for 40 min, as described previously (Li & Arbogast 2008). The respective proteins were detected with Odyssey infrared imaging system (LI-COR, Biosciences, Lincoln, NE, USA). Protein band intensities were quantified using the associated ArrayPro Analyzer 4.5 Software. All samples for each experiment were included on two blots, and control samples on each blot were averaged and data were expressed as percent control.

PP2A activity assay

PP2A activity assay was carried out using the Serine/Threonine Phosphatase Assay System (Promega) with minor modifications. Briefly, SME tissue was homogenized in 50 μl ice-cold lysis buffer (25 mM Tris–HCl, pH 7.5, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 10 mM β-mercaptoethanol). The homogenate was centrifuged for 1 h at 40 000 g to remove particulate matter. Sephadex columns were used to remove free phosphate from the supernatant. Protein concentrations in the phosphate-free tissue lysate were determined using the Bio-Rad Protein Assay. PP2A activity was determined by measuring the generation of free phosphate from the phosphopeptide RRA(pT)VA using the molybdate–malachite green–phosphate complex assay as described by the manufacturer. For a control experiment, PP2A assay buffer containing no substrate or 10 μM okadaic acid was used for the enzyme reaction. After a 20-min incubation at 30°C, the reaction was terminated by adding 50 μl molybdate dye/additive mixture, and the 96-well plate was incubated at room temperature for 15 min. The PP2A activity was quantified by measuring the optical density at 630 nm using a BioTek Synergy 2 microplate reader (Fisher Scientific, St Louis, MO, USA). The optical density value was quantified using the associated Gen5 software, and adjusted by the optical density obtained from the no substrate control samples. The amount of phosphate released in the reaction was calculated from a curve of phosphate standards run in parallel. The effective range for the detection of phosphate released in this assay is 100–4000 pmol phosphate.

PP4 and prolactin levels in plasma and cAMP level in the SME detected by RIA

Plasma P4 concentrations were measured using CoatA-Count kit (Diagnostic Products Corporation, Los Angeles, CA, USA) with a sensitivity of 0-02 ng/ml. The intra- and inter-assay coefficients of variation (CV) were 6.5 and 11-9% respectively. Plasma prolactin levels were assessed using a RIA kit provided by Dr Albert Parlow and the National Hormone and Pituitary Program (Harbor–UCLA Medical Center, CA, USA). Prolactin RP-3 was used as a reference preparation and the limit of sensitivity for the assay was 0-25 ng/ml. The intra- and inter-assay CV were 7-6 and 9-3% respectively. cAMP level in the SME tissue was measured by the cAMP RIA kit (Biomedical Technologies Inc., Stoughton, MA, USA) with minor modifications. SME tissue was extracted by homogenization in ice-cold 3% perchloric acid. The supernatant was collected after centrifugation, and small drops of a chilled 30% (w/v) solution of potassium bicarbonate were then added into the supernatant. After a second centrifugation, the supernatant was collected for the cAMP assay. The limit of sensitivity for cAMP assay was 1 pmol/ml or 0-005 pmol/tube.

Statistical analysis

Plasma P4, plasma prolactin, and TH activity data were evaluated by two-way ANOVA. When repeated samples were collected over time to analyze the P4 effect on the proestrous prolactin surge, plasma prolactin data were analyzed by a split-plot ANOVA. Multiple comparisons were made with Fisher’s least significant procedures. TH phosphorylation states, PP2A activity, PP2Ac protein, and cAMP levels in the SME between P4 and oil vehicle-treated rats were compared using Student’s t-test. P<0.05 was considered statistically significant difference.

Results

Effect of ovariectomy and P4 replacement on the proestrous prolactin surge

Plasma prolactin levels were evaluated in control, sham-operated, and ovariectomized rats treated with oil vehicle (1 ml/kg, s.c.) or P4 (7.5 mg/kg, s.c.) on proestrus to assess P4 contribution to prolactin secretion on proestrous afternoon (Fig. 1). Rats were ovariectomized at 1200–1230 h on
Plasma P₄ level did not alter circulating prolactin levels at 1 h (1030 h) and 3 h (1230 h) after P₄ administration. However, plasma prolactin level was increased (P<0.001) 12-2-fold by 5 h (1430 h) after P₄ treatment (Fig. 2B). The profile of circulating prolactin on proestrous afternoon is complex, and P₄ treatment augmented (P<0.001) the prolactin surge at 3 h (2000 h) and extended (P<0.001) the prolactin surge at 5 h (2200 h) after P₄ treatment at 1700 h. Notably, circulating prolactin levels were increased (P<0.05) by 2-5- and 6-6-fold at 3 h (2000 h) and 5 h (2200 h) respectively, compared with the oil-treated control (Fig. 2D).

**TH activity in the SME of P₄-treated rats**

TH activity was evaluated by DOPA accumulation in the SME in P₄-treated rats on proestrous morning and afternoon. TH activity was unaltered at 1 h (1030 h) after morning P₄ treatment, but was decreased (P<0.05) by 16% at 3 h (1230 h) and 51% at 5 h (1430 h), compared with the oil-treated control. TH activity in the SME of vehicle oil-treated rats at 1230 and 1430 h was higher (P<0.05) than that at 1030 h (Fig. 3A). P₄ administration at 1700 h on proestrous afternoon exhibited a different profile. TH activity was decreased (P<0.05) by 17% at 1 h (1800 h) and by 32% at 5 h (2200 h) after the afternoon P₄ administration. TH activity was lower (P<0.05) in the oil-treated group at 3 h (2000 h) as compared with the values at 1 h (1800 h) and 5 h (2200 h). P₄ did not alter TH activity in the SME at 3 h (2000 h) after the afternoon treatment as compared with oil-treated control group (Fig. 3B).

**Phosphorylation state of TH in the SME of P₄-treated rats**

Consistent with TH activity data at 1 h (1030 h) after P₄ treatment on proestrous morning, there were no differences in the phosphorylation state of TH at Ser-19, Ser-31, and Ser-40 at this time (Fig. 4A–D). However, at 5 h (1430 h) after morning P₄ treatment, SME phospho-TH signals at Ser-19, Ser-31, and Ser-40 were decreased by 14% (P<0.05), 26% (P<0.001), and 31% (P<0.001) respectively (Fig. 4E–H). In contrast to morning administration, P₄ administration at 1700 h decreased (P<0.05) phospho-TH signals at Ser-31 by 21% and Ser-40 by 32% within 1 h (1800 h), which suggests relatively rapid P₄-dependent dephosphorylation of TH unique to proestrous afternoon (Fig. 5B and C). The phospho-TH signals at Ser-31 and Ser-40 remained reduced by 19% (P<0.05) and 29% (P<0.01) respectively, at 5 h (2200 h) after afternoon P₄ administration (Fig. 5F and G). Phospho-TH at Ser-19 (Fig. 5A and E) was not altered at 1 h (1800 h) or 5 h (2200 h) after P₄ treatment on proestrous afternoon. TH protein quantity (Figs 4D and H, 5D and H) and β-tubulin levels in the SME were not altered by 1 h (1030 or 1800 h) or 5 h (1430 or 2200 h) P₄ treatment on proestrous morning or afternoon.
PP2A activity and PP2Ac protein levels in the SME of P4-treated rats

PP2A activity in the SME was not altered by P4 treatment on proestrous morning, but was increased by 22% at 1 h (1800 h) after P4 administration on proestrous afternoon ($P < 0.05$, Fig. 6 A and B). Okadaic acid (10 μM), a PP2A inhibitor, completely abolished the phosphatase activity in the SME tissue (data not shown). In contrast to the 1 h afternoon data, PP2A activity was not altered at 5 h after P4 administration on proestrous morning or afternoon (Fig. 6 C and D). There were no changes in PP2Ac quantities in the SME at 1 h after P4 treatment on proestrous morning and afternoon (Fig. 7).

cAMP levels in the SME of rats after P4 administration

Since phosphorylation of TH at Ser-40 site can be activated through cAMP-dependent protein kinase and Ser-40 exhibited the most marked dephosphorylation in our study, we examined cAMP levels in the SME after P4 treatment on proestrous morning and afternoon ($n = 6$ for each group). cAMP levels in the SME of oil-treated control groups were 200.64 ± 18.13 and 202.70 ± 17.85 pmol/mg protein at 1030 and 1800 h respectively. P4 administration for 1 h did not change cAMP levels in the SME of rats on proestrous morning and afternoon. cAMP levels at 1 h after P4 treatment were 195.18 ± 13.27 and 201.70 ± 10.48 pmol/mg protein at 1030 and 1800 h respectively.

Discussion

The major finding of this study is that P4 suppressed TH activity and TH phosphorylation state in TIDA neurons within 1 h on proestrous afternoon. These changes in TH phosphorylation at Ser-40 and Ser-31 coincided with increased PP2A activity in the SME at 1 h after P4 treatment on proestrous afternoon, suggesting that PP2A may induce TH dephosphorylation leading to a decline of TH activity. It is notable that 1 h P4 treatment on proestrous morning did not alter TH activity, TH phosphorylation state, or PP2A activity. However, P4 treatment at 0930 h on proestrous morning and at 1700 h on proestrous afternoon suppressed TH activity and TH phosphorylation state in TIDA neurons 5 h later, suggesting that a common mechanism may be engaged at the 5 h time point. In contrast to an afternoon-specific effect on PP2A after 1 h P4 treatment, P4 had no effect...
on PP2A activity in the SME on proestrous morning and afternoon at 5 h, suggesting that additional factor(s) may arise to support TH dephosphorylation.

We previously reported that exogenous P4 administration at 0930 h on proestrous morning decreased TH activity and increased serum prolactin level at 5 h after P4 treatment (Liu & Arbogast 2008). Our current study addressed the time course for P4's effects on TH activity in the SME and on plasma prolactin levels. On proestrous morning, the inhibitory P4 effect on TH activity and TH phosphorylation was delayed. It was only 5 h after P4 treatment that a marked change in TH activity and TH phosphorylation was observed, which correlated with a 12.2-fold rise in plasma prolactin levels. These data are in agreement with earlier studies, which have shown that P4, given for 3–6 h, can lower TIDA neuronal activity and increase prolactin levels (Beattie et al. 1972, Calgaris et al. 1974, Babu & Vijayan 1984, Yen & Pan 1998).

Analysis of a P4 effect on proestrus afternoon is more complex due to hormonal changes during the preovulatory period. Our approach to understand P4 action has been a classical ablation-replacement approach where rats are intact on proestrus morning to allow for elevated E2 levels, but ovariectomized in early afternoon to prevent the endogenous P4 rise. As in our previous studies using this paradigm (Arbogast & Ben-Jonathan 1989, 1990, Arbogast & Voogt 1994), acutely ovariectomized rats exhibited a blunted or truncated prolactin surge. This early phase of the proestrus prolactin surge in the acutely ovariectomized rats likely reflects an E2-dependent component of the prolactin surge driven by a prolactin-releasing factor(s) input, rather than decreased dopaminergic tone (Neill et al. 1971, Samson et al. 1986, Murai et al. 1989, Arbogast & Ben-Jonathan 1990, Kennett et al. 2009). P4 replacement to acutely ovariectomized rats amplified and extended the prolactin surge beyond an E2-dependent component. In contrast to morning P4 administration, P4 treatment at 1700 h on proestrus afternoon decreased TH activity within 1 h, although the decrease in TH activity did not functionally affect plasma prolactin levels at the onset of the surge. It is notable that a similar decrease in TH activity occurs concomitantly with the P4 rise on proestrus afternoon in intact animals and thus likely has physiological relevance (Liu & Arbogast 2008).

This decrease in TH activity may be related to other hormonal events, which occur during this same time period on proestrus afternoon. Alternatively, this initial decrease in TIDA neuronal activity may set the stage for later P4-dependent amplification of the prolactin surge. A non-P4-dependent decline in TH activity in the SME occurred at 2000 h in acutely ovariectomized rats. Thus, although TH activity of both control and P4-treated rats was lower than pre-surge levels, there was no significant difference between the two groups. This decrease may represent an endogenous rhythm in TIDA neuronal activity that occurs on proestrus afternoon at 5 h after P4 administration.

The data in this study support and extend our previous observation (Arbogast & Ben-Jonathan 1990, Arbogast & Voogt 1994) that P4 plays an important role in extending the duration of the prolactin surge on proestrus and indicates a contribution of decreased TIDA neuronal activity. It is not clear whether P4 administration recruits nondopaminergic...
factors. However, a recent study (Kennett et al. 2009) indicates that while an oxytocin antagonist blocks the E\textsubscript{2}-induced prolactin surge, the oxytocin antagonist does not block the E\textsubscript{2}P\textsubscript{4}-induced prolactin surge. These data suggest that P\textsubscript{4} is acting through a mechanism independent of the prolactin-releasing activity of oxytocin. A change in pituitary sensitivity to dopamine cannot be discounted, since dynamic changes in anterior pituitary dopamine receptors and responsiveness to dopamine occur during the day of proestrus (Heiman & Ben-Jonathan 1982, Pasqualini et al. 1984, Brandi et al. 1990). Indeed, E\textsubscript{2} decreases pituitary sensitivity to dopamine, whereas P\textsubscript{4} acts antagonistically to restore dopamine responsiveness (Bression et al. 1985, Pasqualini et al. 1986).

TH activation is regulated by long-term induction, including transcriptional regulation, alternative RNA splicing, RNA stabilization, and translational regulation, and short-term activation through phosphorylation/dephosphorylation of the enzyme (Goldstein 2000). There are four serine sites (Ser-8, Ser-19, Ser-31, and Ser-40) in the N-terminal regulatory region of TH (Dunkley et al. 2004). The phosphorylation state of TH at any time is determined by interplay of protein kinases and phosphoprotein phosphatases. Ser-40 can be phosphorylated by a range of protein kinases, including protein kinase A, protein kinase C, calcium- and calmodulin-stimulated protein kinase II, protein kinase G, MAPK-activated protein kinases 1 and 2, p38-regulated/activated kinase, and mitogen- and stress-activated protein kinase 1 (Dunkley et al. 2004). The kinases able to phosphorylate Ser-19 are calcium- and calmodulin-stimulated protein kinase II, p38-regulated/activated kinase, and MAPK-activated protein kinase 2. ERK-1 and ERK-2, as well as cyclin-dependent kinase 5, phosphorylate TH at Ser-31 (Dunkley et al. 2004, Kansy et al. 2004). Although less is known about phosphatase action on TH, PP2A and PP2C dephosphorylate TH at Ser-19, Ser-31, and/or Ser-40 (Dunkley et al. 2004). Indeed, PP2A is responsible for \~90\% of Ser-40 and Ser-19 phoshosphate activity in adrenal extracts, and PP2C accounts for 10\% of the phoshpomerase activity of Ser-40 and Ser-19 (Haavik et al. 1989). Similar results were found in the extracts from rat brain for Ser-40 (Berresheim & Kuhn 1994, Bevilaqua et al. 2003). PP2A, but not PP2C, is able to dephosphorylate Ser-31 in both PC12 cells and bovine adrenal chromaffin cells (Haycock 1990, Leal et al. 2002). PP2A consists of a heterotrimer of a catalytic (C) subunit, a scaffolding A subunit, and a variable regulatory B subunit (Dobrowsky et al. 1993, Kamibayashi et al. 1994).

P\textsubscript{4} treatment administered on proestrous afternoon caused dephosphorylation of TH at Ser-40 and Ser-31 as early as 1 h post-treatment. A similar decrease in radiolabeled phosphate

Figure 4  Phosphorylation state of TH at Ser-19, Ser-31, and Ser-40 within 1 h (A–D) or 5 h (E–H) after P\textsubscript{4} or oil treatment on proestrous morning. A representative immunoblot for each phosphorylation site of TH or TH protein is displayed on top of the bar graph. Similar to TH activity data, there were no differences in the phosphorylation state of TH at Ser-19, Ser-31, and Ser-40 within 1 h after P\textsubscript{4} treatment at 0930 h on proestrus. However, TH phosphorylation at Ser-19, Ser-31, and Ser-40 decreased by 14, 26, and 31\% at 5 h later respectively. Phospho-TH values were individually normalized to respective TH values. TH values were individually normalized to respective \&-tubulin values. Each value represents a mean \pm S.E.M. of determinations of seven to eight rats. *P<0.05, **P<0.001 versus oil treatment.
incorporation into TH protein within 1 h was observed in hypothalamic cells in vitro (Arbogast & Voogt 2002), although the specific serine sites were not identified in this earlier study. The concerted dephosphorylation of regulatory serines supports the notion of a common phosphatase mechanism. Indeed, the activity of PP2A, which is a major phosphatase for TH (Haavik et al. 1989, Dunkley et al. 2004), was increased coincident with the acute afternoon-specific dephosphorylation of TH at Ser-31 and Ser-40. The increase in PP2A activity was observed in SME tissue, which contains a mixed population of nerve terminals as well as cell bodies for various glial and neuronal cells. Thus, the 22% increase in total PP2A activity may actually reflect a more marked increase in P4-responsive cells or terminals in the SME. A role for a phosphatase being involved in P4 action is further supported by the fact that okadaic acid, a PP2A and PP1 inhibitor, reversed the P4-dependent decrease in radiolabeled phosphate incorporation into TH protein in hypothalamic cells in vitro (Arbogast & Voogt 2002). P4 did not alter PP2Ac protein level in the SME at 1 h after P4 administration, suggesting activation of existing PP2Ac enzyme rather than production of new PP2Ac enzyme protein. Further studies are required to identify the mechanism by which P4 stimulates PP2A activity.

While our data support increased PP2A activity associated with the early proestrous afternoon-specific component of P4 action on TIDA neurons, our data indicate a differential mechanism between the 1 and 5 h time point. The 5 h mechanism was engaged on both the morning and afternoon of proestrus and was associated with increased prolactin secretion. Our current data confirm our previous study (Liu & Arbogast 2008) that P4 administered on proestrous morning induced dephosphorylation of TH at Ser-40, Ser-31, and Ser-19 after 5 h and extend this finding to proestrous afternoon. It was somewhat surprising that PP2A activity at 5 h after P4 was similar to control values on proestrous morning and afternoon. Our previous data suggested a role for phosphatase involvement in P4 action since okadaic acid, a PP2A and PP1 inhibitor, reverses the dephosphorylation at 2200 h on proestrus (Arbogast & Voogt 1994). However, the lack of change in PP2A activity does not support its involvement in TH dephosphorylation. Additional studies will be required to identify a P4-induced mechanism for the 5 h time point. It may be that another phosphatase is recruited for this later time, the kinase component(s) of the signaling cascade are down-regulated, or the initial dephosphorylation of TH induces a conformational change that stabilizes dephosphorylated TH.

**Figure 5** Phosphorylation state of TH at Ser-19, Ser-31, and Ser-40 at 1 h (A–D) or 5 h (E–H) after P4 or oil treatment on proestrous afternoon. A representative immunoblot for each phosphorylation site of TH, TH protein, or β-tubulin is displayed on top of the bar graph. In contrast to morning administration, P4 treatment at 1700 h significantly decreased TH phosphorylation at Ser-31 by 19–21% and Ser-40 by 29–32% at 5 and 1 h respectively. There were no significant differences between the P4- and oil-treated groups for Ser-19 phosphorylation, TH protein, and β-tubulin levels. Phospho-TH values were individually normalized to respective TH values. TH values were individually normalized to respective β-tubulin values. Each value represents a mean ± S.E.M. of determinations from seven (oil-treated) to eight (P4-treated) rats. *P<0.05, **P<0.01 versus oil treatment.
Exogenous P4 administration on proestrous afternoon caused dephosphorylation of TH at Ser-40 and Ser-31, but not Ser-19. Phosphorylation of TH at Ser-40 increases enzyme activity up to 20-fold and appears to be the main mechanism for short-term TH activation (Daubner et al. 1992, Dunkley et al. 2004), whereas Ser-31 phosphorylation produces a less than twofold increase in TH activity (Haycock et al. 1992). Ser-19 phosphorylation does not directly affect TH activity, but it may potentiate TH phosphorylation at Ser-40 and subsequent TH activation by binding to the 14:3:3 protein or by hierarchical phosphorylation (Haycock et al. 1998, Bevilaqua et al. 2001, Toska et al. 2002, Bobrovskaya et al. 2004, Dunkley et al. 2004). In the current and previous studies (Liu & Arbogast 2008), Ser-40 site exhibited the most marked dephosphorylation change at 5 h after P4 treatment on proestrous morning and at 1 and 5 h after P4 treatment on proestrous afternoon. These data suggest that the Ser-40 site, which is a critical target for cAMP-dependent protein kinase A, may exert the greatest impact on TH activity. To explore whether down-regulation of the cAMP-signaling pathway may account for TH dephosphorylation at Ser-40 in the SME induced by P4, we examined cAMP levels in the SME tissue. However, no change of cAMP concentration was observed at 1 h after P4 treatment on proestrous morning and afternoon. The mechanism(s) underlying P4-induced dephosphorylation at Ser-40 and other serine sites need further investigation.

P4 regulation of TH activity and prolactin secretion requires previous or concomitant treatment with E2 (Beattie et al. 1972, Gonzalez et al. 1989, Morrell et al. 1989, Arbogast & Ben-Jonathan 1990, Arbogast & Voogt 2002), and E2 stimulates P4 receptor expression (Kraus et al. 1994, Shughrue et al. 1997, Scott et al. 2002). Classical nuclear P4 receptors are found in dopaminergic neurons of hypothalamus in rats (Sar 1988, Fox et al. 1990, Lonstein & Blaustein 2004). This colocalization of P4 receptor and TH suggests that P4 may act directly on dopaminergic neurons in the hypothalamus. Further support for a direct P4 action within the hypothalamus is provided by an acute P4-inhibitory effect on TH activity in isolated hypothalamic cells (Arbogast & Voogt 2002). Our data support the notion that there are at least two components to P4 action on TIDA neurons with respect to dopamine synthesis. It is not clear which P4 receptor subtypes mediate these actions. The classical nuclear P4 receptor may act as a transcription factor in the nucleus or modulate intracellular signaling pathways outside the nucleus, whereas membrane P4 receptors may rapidly activate intracellular signaling pathway (Mani 2006, Thomas 2008). The more rapid P4 component is apparent on proestrous afternoon in this study and in E2-treated hypothalamic cells in vitro (Arbogast & Voogt 2002), and involves actions on cytoplasmic proteins, TH, and PP2A. The timing and cellular localization for the 1 h P4 effect are consistent with either P4 membrane receptor or classical P4 receptor in the cytoplasm, but does not preclude a P4-induced transcriptional change. The 5 h P4 effect observed on both proestrous morning and afternoon involves both a decrease in TH phosphorylation as shown in this study as well as suppression of Th mRNA levels by 2200 h on proestrus (Arbogast & Voogt 1994). The change in Th mRNA levels suggests that the classical nuclear P4 receptor may mediate at least part of this later component, although a dephosphorylation action on TH protein would indicate some action within the cytoplasm as well.

Figure 6 PP2A activity in the SME of rats regulated by P4 treatment for 1 h (A and B) or 5 h (C and D) on proestrous morning (A and C) and afternoon (B and D). P4 administration at 0930 h did not alter PP2A activity in the SME of rats at 1 h (A, n=9) and 5 h (C, n=6) after the treatment on proestrous morning. However, afternoon P4 treatment significantly increased PP2A activity in the SME by 22% at 1 h (B, n=9), but not 5 h (D, n=6) later. Data are expressed as the mean percent change relative to oil-treated control ± S.E.M.

*P<0.05 versus oil-treated control.

Figure 7 Catalytic subunit of PP2A (PP2Ac) level in the SME of rats at 1 h after P4 or oil treatment on proestrous morning and afternoon. A representative immunoblot for SME tissue reacted with PP2Ac and β-tubulin antibodies was displayed on top of the bar graph. PP2Ac values were individually normalized to respective β-tubulin values. Data are expressed as the mean percent change relative to oil-treated control ± S.E.M., of determinations from six rats. No statistically significant difference was observed between the P4- and oil-treated rats on proestrous morning and afternoon.
The expression pattern for P₄ receptor subtypes in the brain during the proestrous day may provide some clues as to which receptors mediate the components of P₄ action on TIDA neurons during proestrus. Indeed, P₄ receptor B, membrane P₄ receptor α, and membrane P₄ receptor β mRNA expression are increased on proestrous afternoon and reach the highest levels coincident with the preovulatory P₄ rise (Liu & Arbogast 2009). Further investigation is needed to determine P₄ receptor subtypes involved in P₄ action on TIDA neurons and related transcription and nontranscription-signaling mechanisms.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was support by NIH grants HD045805 and HD048925 to LAA.

Acknowledgements
We would like to thank Dr A F Parlow, Harbor-UCLA Medical Center, and the National Hormone and Peptide Program, NIDDK, for providing prolactin RIA reagents.

References
Arbogast LA & Ben-Jonathan N 1989 Tyrosine hydroxylase in the stalk-median eminence and posterior pituitary is inactivated only during the plateau phase of the preovulatory prolactin surge. Endocrinology 125 667–674.
Arbogast LA & Voogt JL 1991 Hyperprolactinemia increases and hypoprolactinemia decreases tyrosine hydroxylase messenger ribonucleic acid levels in the arcuate nuclei, but not the substantia nigra or zona incerta. Endocrinology 128 997–1005.
Ben-Jonathan N & Hnasko R 2001 Dopamine as a prolactin (PRL) inhibitor. Endocrine Reviews 22 724–763.
Bevilaqua LR, Graham ME, Dunkley PR, von Nagy-Felsobuki EI & Dickson PW 2001 Phosphorylation of Ser(19) alters the conformation of tyrosine hydroxylase to increase the rate of phosphorylation of Ser(40). Journal of Biological Chemistry 276 40411–40416.
Bobrovskaya L, Dunkley PR & Dickson PW 2004 Phosphorylation of Ser19 increases both Ser40 phosphorylation and enzyme activity of tyrosine hydroxylase in intact cells. Journal of Neurochemistry 90 857–864.


www.endocrinology-journals.org

Downloaded from Bioscientifica.com at 03/03/2022 03:34:50PM via free access


Mura I, Keichlin S & Ben-Jonathan N 1989 The peak phase of the proestrous prolactin surge is blocked by either posterior pituitary lobectomy or antiserum to vasoactive intestinal peptide. Endocrinology 124 1050–1055.

Neill JD, Freeman ME & Tilson SA 1971 Control of the proestrus surge of prolactin and luteinizing hormone secretion by estrogens in the rat. Endocrinology 89 1448–1453.


Thomas P 2008 Characteristics of membrane progestin receptor alpha (mPRalpha) and progesterone membrane receptor component 1 (PGMRC1) and their roles in mediating rapid progestin actions. Frontiers in Neuroendocrinology 29 292–312.


Received in final form 22 November 2009
Accepted 27 November 2009
Made available online as an Accepted Preprint 27 November 2009