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
(Correspondence should be addressed to L A Arbogast; Email: larbogast@siumed.edu)

Abstract

The progesterone (P_4) 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 P_4 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. P_4 (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 P_4 had no 1 h effect on TH. PP2A activity in the SME was enhanced after P_4 treatment for 1 h on proestrous afternoon without a change in PP2A catalytic subunit quantity, whereas P_4 treatment had no effect on PP2A activity or quantity on proestrous morning. cAMP levels in the SME were unchanged with 1 h P_4 treatment. At 5 h after P_4 treatment, TH activity and phosphorylation state declined coincident with an increase in plasma prolactin in both P_4-treated morning and afternoon groups. PP2A activity in the SME was unchanged in 5 h P_4-treated rat. Our data suggest that P_4 action on tuberoinfundibular dopaminergic (TIDA) neurons involves at least two components. A more rapid (1 h) P_4 effect engaged only on proestrous afternoon likely involves the activation of PP2A. The longer P_4 action on TIDA neurons is evident on both the morning and afternoon of proestrus and may involve a common, as yet unidentified, mechanism.
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Introduction

A preovulatory prolactin surge is evident on proestrous afternoon in rats (Smith *et al.* 1975, Liu & Arbogast 2008). The rising titer of estradiol (E_2) 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 (P_4) on proestrous afternoon augments the magnitude or extends the duration of this 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 P_4 on TIDA neurons contributes to P_4-enhancing effect on the preovulatory prolactin surge. A decrease in TH activity and *Th* mRNA levels occurs concomitantly with the preovulatory P_4 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. P_4, but not E_2, 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 P_4 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,

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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 (Haavik *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 proestrous 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 L-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% I-Block (Tropix, Bedford, MA, USA) in PBS for 1 h to block non-specific 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, Rockland Immunochemicals, Gilbertsville, PA, USA) and Alexa Fluor 680 goat anti-rabbit IgG (1:20 000, A-21109, Invitrogen Corporation) for 40 min, as described previously (Liu & 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.

P_4 and prolactin levels in plasma and cAMP level in the SME detected by RIA

Plasma P_4 concentrations were measured using Coat-A-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., Stroughton, 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 P_4, plasma prolactin, and TH activity data were evaluated by two-way ANOVA. When repeated samples were collected over time to analyze the P_4 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 P_4 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 P_4 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 P_4 (7·5 mg/kg, s.c.) on proestrus to assess P_4 contribution to prolactin secretion on proestrous afternoon (Fig. 1). Rats were ovariectomized at 1200–1230 h on

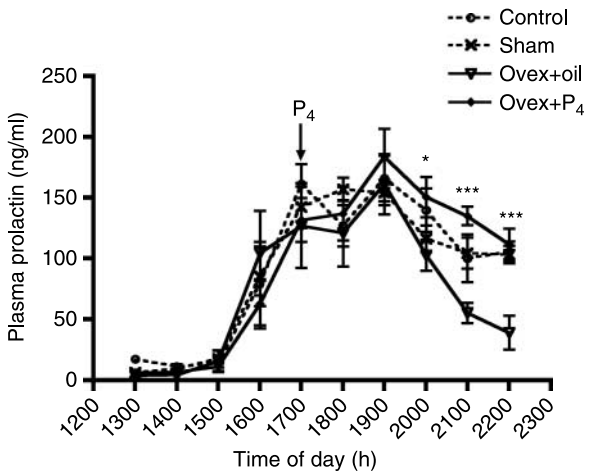

Figure 1 Plasma prolactin levels in control, sham-operated, and ovariectomized rats treated with oil vehicle (1 ml/kg, s.c.) or P_4 (7·5 mg/kg, s.c.) on proestrus. Ovariectomy was performed at 1200–1230 h, and P_4 was injected at 1700 h on proestrous afternoon. Sham operation ($n=7$) did not influence prolactin secretion as compared with that of the control ($n=6$). P_4 administration ($n=9$) in the ovariectomized rats prolonged prolactin secretion at 3–5 h later as compared to that of oil treatment ($n=7$). Each value represents a mean±S.E.M. $^*P<0·05$, $^{***}P<0·001$ versus corresponding oil-treated ovariectomized rats.

proestrus to eliminate the endogenous P_4 rise. P_4 was restored by injection at 1700 h. Plasma prolactin levels during the early phase (1600–1900 h) of the prolactin surge were similar in all groups. Circulating prolactin levels in ovariectomized rats were reduced ($P<0·05$) at 2100–2200 h on proestrous evening, as compared to sham-operated rats, whereas P_4 administration to ovariectomized rats sustained prolactin secretion at a value similar to that of control or sham-operated rats. Circulating prolactin levels were higher ($P<0·01$) by 1·5-fold at 3 h (2000 h), 2·4-fold at 4 h (2100 h), and 2·9-fold at 5 h (2200 h) after P_4 administration as compared to that of oil-treated ovariectomized rats. Prolactin secretion was similar in sham-operated and control rats, suggesting that anesthesia and surgical manipulations on mid-day proestrus did not alter the amplitude or duration of the prolactin surge.

Plasma P_4 and prolactin levels in P_4-treated rats

Plasma prolactin levels at 0, 1, 3, and 5 h after P_4 (7·5 mg/kg, s.c.) or vehicle oil (1 ml/kg) administration on proestrous morning and afternoon were examined to assess the time course of P_4 effect on prolactin secretion. Rats included in the afternoon group were ovariectomized at 1200–1400 h on proestrus to eliminate the endogenous P_4 rise. P_4 administered at 0930 or 1700 h on proestrus increased ($P<0·001$) plasma P_4 concentration to 80 ng/ml within 1 h, and this P_4 level was maintained for at least 5 h (Fig. 2A and C). When P_4 was administered on proestrous morning, the increased plasma P_4 level did not alter circulating prolactin levels at 1 h (1030 h) and 3 h (1230 h) after P_4 administration. However, plasma prolactin level was increased ($P<0·001$) 12·2-fold by 5 h (1430 h) after P_4 treatment (Fig. 2B). The profile of circulating prolactin on proestrous afternoon is complex, and P_4 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 P_4 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 P_4-treated rats

TH activity was evaluated by DOPA accumulation in the SME in P_4-treated rats on proestrous morning and afternoon. TH activity was unaltered at 1 h (1030 h) after morning P_4 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). P_4 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 P_4 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). P_4 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 P_4-treated rats

Consistent with TH activity data at 1 h (1030 h) after P_4 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 P_4 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, P_4 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 P_4-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 P_4 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 P_4 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) P_4 treatment on proestrous morning or afternoon.

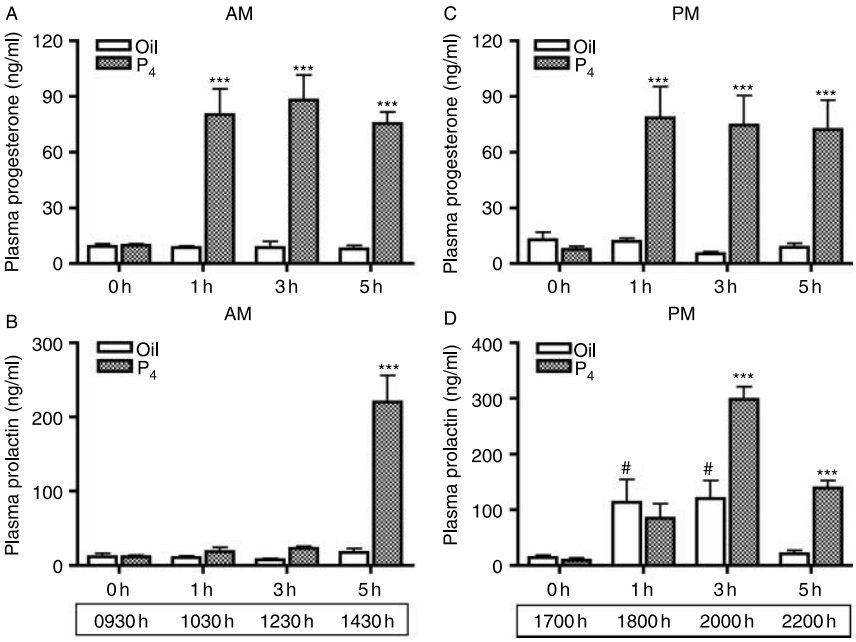

Figure 2 Plasma P_4 (A and C) and prolactin (B and D) levels at 0, 1, 3, and 5 h after morning (A and B) or afternoon (C and D) administration of P_4 (7·5 mg/kg, s.c.) or sesame oil vehicle (1 ml/kg, s.c). For the afternoon treatment groups (C and D), rats were ovariectomized at 1200–1400 h on proestrus. P_4 administered at 0930 h ($n=10$) or 1700 h ($n=8$) on proestrus increased plasma P_4 to 80 ng/ml within 1 h, and P_4 levels remained high for at least 5 h. Oil treatment ($n=6$ for each group) did not affect the plasma P_4 level. After P_4 treatment at 0930 h (B), circulating prolactin levels were unchanged at 1 and 3 h after P_4 treatment, but increased 12·2-fold within 5 h. After P_4 treatment at 1700 h (D), circulating prolactin levels were increased by 2·5-fold at 3 h and 6·6-fold at 5 h after P_4 administration, compared to oil-treated control. Each value represents a mean ± S.E.M. $^{***}P<0·001$ versus corresponding oil-treated control. $^{\#}P<0·05$ versus oil treatment for 0 h.

PP2A activity and PP2Ac protein levels in the SME of P_4-treated rats

PP2A activity in the SME was not altered by P_4 treatment on proestrous morning, but was increased by 22% at 1 h (1800 h) after P_4 administration on proestrous afternoon ($P<0·05$, Fig. 6A 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 P_4 administration on proestrous morning or afternoon (Fig. 6C and D). There were no changes in PP2Ac quantities in the SME at 1 h after P_4 treatment on proestrous morning and afternoon (Fig. 7).

cAMP levels in the SME of rats after P_4 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 P_4 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. P_4 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 P_4 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 P_4 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 P_4 treatment on proestrous afternoon, suggesting that PP2A may induce TH dephosphorylation leading to a decline of TH activity. It is notable that 1 h P_4 treatment on proestrous morning did not alter TH activity, TH phosphorylation state, or PP2A activity. However, P_4 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 P_4 treatment, P_4 had no effect

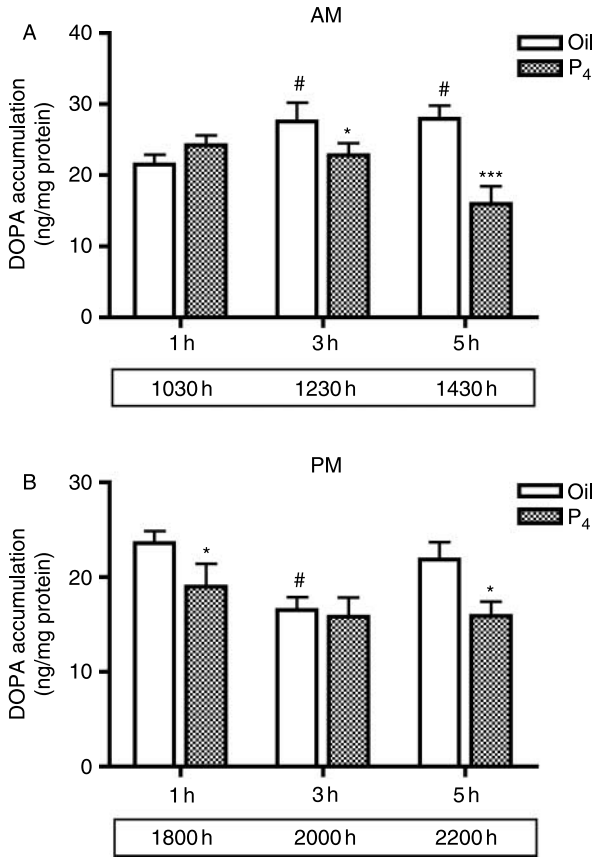

Figure 3 TH activity in the SME of rats at 1, 3, and 5 h after P_4 or oil treatment at 0930 h (A) or 1700 h (B) on proestrus. TH activity in the SME was unaltered at 1 h ($n=8$) after morning P_4 treatment, but was decreased by 16% at 3 h ($n=8$) and 51% at 5 h ($n=9$). TH activity was increased in the 3 h ($n=10$) and 5 h ($n=9$) oil-treated groups compared to the 1 h control ($n=8$). Rats were ovariectomized at 1200–1400 h on proestrus for the afternoon P_4 treatment. P_4 administration at 1700 h decreased TH activity by 17% at 1 h ($n=12$) and by 32% at 5 h ($n=10$) later as compared to the corresponding 1 h control ($n=14$ and $n=10$ respectively). TH activity was reduced in the oil-treated group at 2000 h in the 3 h group, but there was no significant difference between oil- and P_4-treated groups at 3 h ($n=11$ each). Each value represents a mean ± S.E.M. $^{*}P<0{\cdot}05$, $^{***}P<0{\cdot}001$ versus corresponding oil-treated control. $^{\#}P<0{\cdot}05$ versus oil treatment for 1 h.

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 P_4 administration at 0930 h on proestrous morning decreased TH activity and increased serum prolactin level at 5 h after P_4 treatment (Liu & Arbogast 2008). Our current study addressed the time course for P_4's effects on TH activity in the SME and on plasma prolactin levels. On proestrous morning, the inhibitory P_4 effect on TH activity and TH phosphorylation was delayed. It was only 5 h after P_4 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 P_4, given for 3–6 h, can lower TIDA neuronal activity and increase prolactin levels (Beattie *et al.* 1972, Caligaris *et al.* 1974, Babu & Vijayan 1984, Yen & Pan 1998).

Analysis of a P_4 effect on proestrous afternoon is more complex due to hormonal changes during the preovulatory period. Our approach to understand P_4 action has been a classical ablation-replacement approach where rats are intact on proestrous morning to allow for elevated E_2 levels, but ovariectomized in early afternoon to prevent the endogenous P_4 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 proestrous prolactin surge in the acutely ovariectomized rats likely reflects an E_2-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). P_4 replacement to acutely ovariectomized rats amplified and extended the prolactin surge beyond an E_2-dependent component. In contrast to morning P_4 administration, P_4 treatment at 1700 h on proestrous 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 P_4 rise on proestrous 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 proestrous afternoon. Alternatively, this initial decrease in TIDA neuronal activity may set the stage for later P_4-dependent amplification of the prolactin surge. A nonP_4-dependent decline in TH activity in the SME occurred at 2000 h in acutely ovariectomized rats. Thus, although TH activity of both control and P_4-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 every day of the estrous cycle in female rats (Mai *et al.* 1994, Shieh & Pan 1996) and is apparent even in ovariectomized female rats (Lerant & Freeman 1997). Similar to the morning study, P_4 administration induced a significant decline in TH activity and increase in plasma prolactin at 5 h later. The similarity of response with respect to TH activity and TH phosphorylation state suggests that common mechanism(s) may exist on proestrous morning and proestrous afternoon at 5 h after P_4 administration.

The data in this study support and extend our previous observation (Arbogast & Ben-Jonathan 1990, Arbogast & Voogt 1994) that P_4 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 P_4 administration recruits nondopaminergic

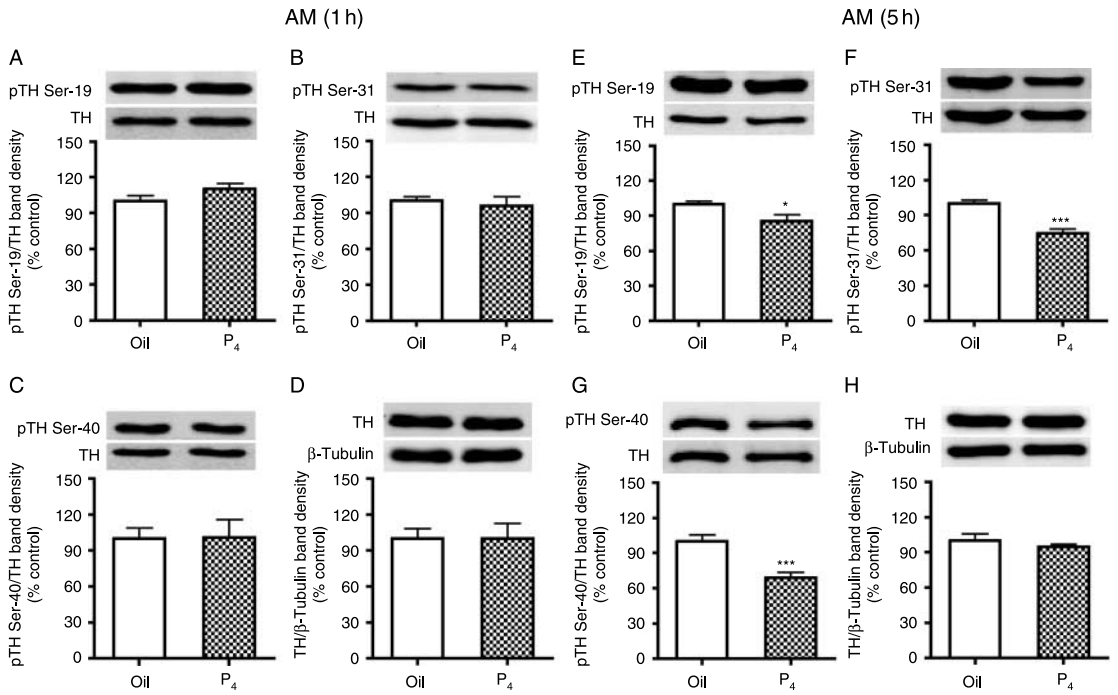

Figure 4 Phosphorylation state of TH at Ser-19, Ser-31, and Ser-40 within 1 h (A–D) or 5 h (E–H) after P_4 or oil treatment on proestrous morning. A representative immunoblot for each phosphorylation site of TH or TH protein is displayed on top of the bar graph. Similar to TH activity data, there were no differences in the phosphorylation state of TH at Ser-19, Ser-31, and Ser-40 within 1 h after P_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 β-tubulin values. Each value represents a mean±S.E.M. of determinations of seven to eight rats. $^{*}P<0{\cdot}05$, $^{***}P<0{\cdot}001$ versus oil treatment.

factors. However, a recent study (Kennett *et al.* 2009) indicates that while an oxytocin antagonist blocks the E_2-induced prolactin surge, the oxytocin antagonist does not block the E_2+P_4-induced prolactin surge. These data suggest that P_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_2 decreases pituitary sensitivity to dopamine, whereas P_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 phosphatase activity in adrenal extracts, and PP2C accounts for 10% of the phosphatase 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_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

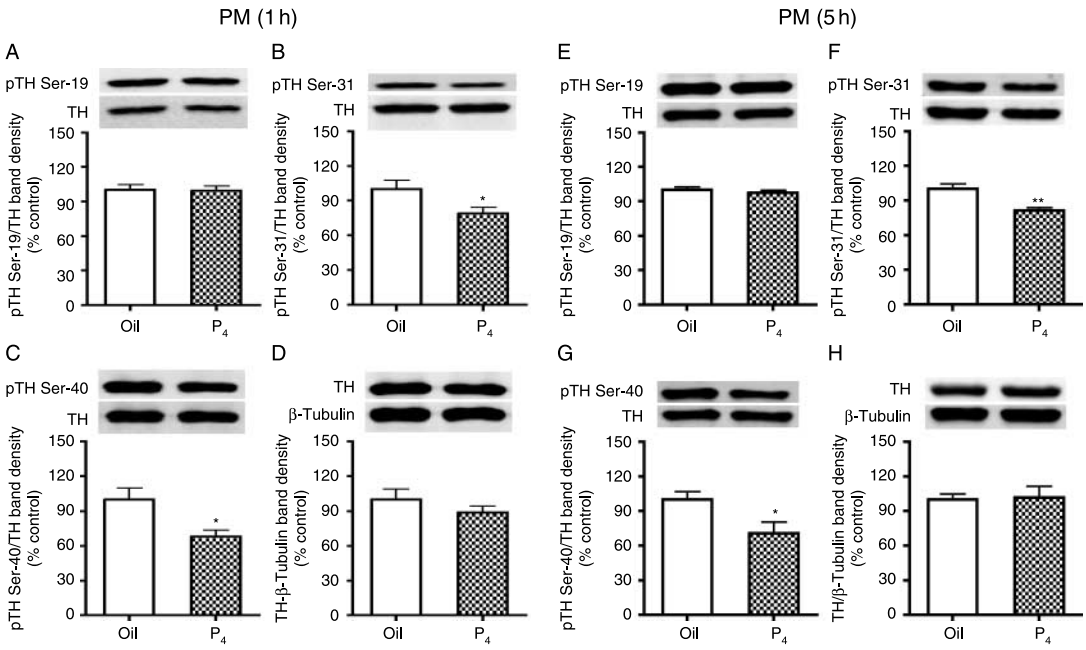

Figure 5 Phosphorylation state of TH at Ser-19, Ser-31, and Ser-40 at 1 h (A–D) or 5 h (E–H) after P_4 or oil treatment on proestrous afternoon. A representative immunoblot for each phosphorylation site of TH, TH protein, or β-tubulin is displayed on top of the bar graph. In contrast to morning administration, P_4 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 P_4- 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 (P_4-treated) rats. $^*P<0{\cdot}05$, $^{**}P<0{\cdot}01$ 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 P_4-responsive cells or terminals in the SME. A role for a phosphatase being involved in P_4 action is further supported by the fact that okadaic acid, a PP2A and PP1 inhibitor, reversed the P_4-dependent decrease in radiolabeled phosphate incorporation into TH protein in hypothalamic cells *in vitro* (Arbogast & Voogt 2002). P_4 did not alter PP2Ac protein level in the SME at 1 h after P_4 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 P_4 stimulates PP2A activity.

While our data support increased PP2A activity associated with the early proestrous afternoon-specific component of P_4 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 P_4 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 P_4 was similar to control values on proestrous morning and afternoon. Our previous data suggested a role for phosphatase involvement in P_4 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 P_4-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.

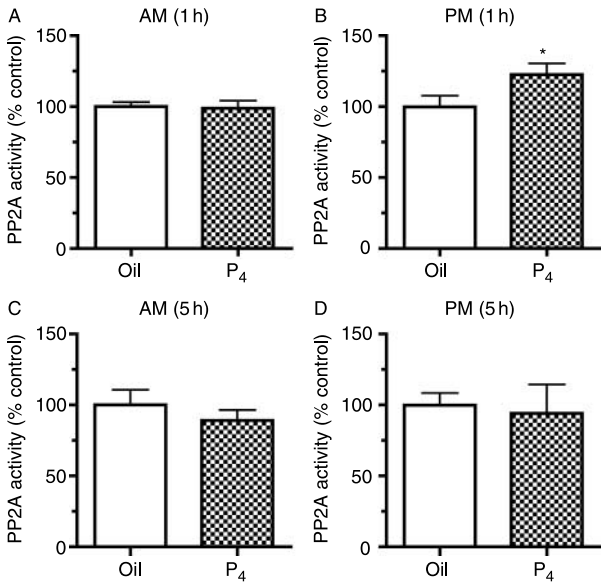

Figure 6 PP2A activity in the SME of rats regulated by P_4 treatment for 1 h (A and B) or 5 h (C and D) on proestrous morning (A and C) and afternoon (B and D). P_4 administration at 0930 h did not alter PP2A activity in the SME of rats at 1 h (A, $n=9$) and 5 h (C, $n=6$) after the treatment on proestrous morning. However, afternoon P_4 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. *$P<0{\cdot}05$ versus oil-treated control.

Exogenous P_4 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

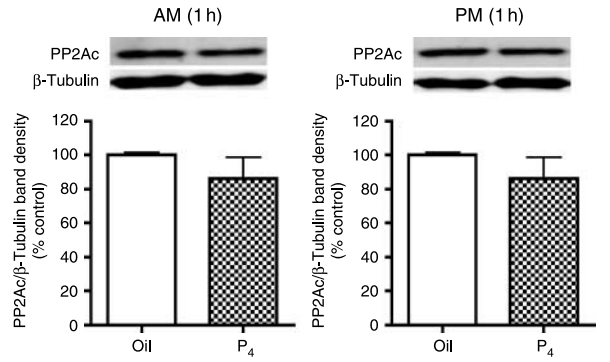

Figure 7 Catalytic subunit of PP2A (PP2Ac) level in the SME of rats at 1 h after P_4 or oil treatment on proestrous morning and afternoon. A representative immunoblot for SME tissue reacted with PP2Ac and β-tubulin antibodies was displayed on top of the bar graph. PP2Ac values were individually normalized 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 P_4- and oil-treated rats on proestrous morning and afternoon.

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 P_4 treatment on proestrous morning and at 1 and 5 h after P_4 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 P_4, we examined cAMP levels in the SME tissue. However, no change of cAMP concentration was observed at 1 h after P_4 treatment on proestrous morning and afternoon. The mechanism(s) underlying P_4-induced dephosphorylation at Ser-40 and other serine sites need further investigation.

P_4 regulation of TH activity and prolactin secretion requires previous or concomitant treatment with E_2 (Beattie *et al.* 1972, Gonzalez *et al.* 1989, Morrell *et al.* 1989, Arbogast & Ben-Jonathan 1990, Arbogast & Voogt 2002), and E_2 stimulates P_4 receptor expression (Kraus *et al.* 1994, Shughrue *et al.* 1997, Scott *et al.* 2002). Classical nuclear P_4 receptors are found in dopaminergic neurons of hypothalamus in rats (Sar 1988, Fox *et al.* 1990, Lonstein & Blaustein 2004). This colocalization of P_4 receptor and TH suggests that P_4 may act directly on dopaminergic neurons in the hypothalamus. Further support for a direct P_4 action within the hypothalamus is provided by an acute P_4-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 P_4 action on TIDA neurons with respect to dopamine synthesis. It is not clear which P_4 receptor subtypes mediate these actions. The classical nuclear P_4 receptor may act as a transcription factor in the nucleus or modulate intracellular signaling pathways outside the nucleus, whereas membrane P_4 receptors may rapidly activate intracellular signaling pathway (Mani 2006, Thomas 2008). The more rapid P_4 component is apparent on proestrous afternoon in this study and in E_2-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 P_4 effect are consistent with either P_4 membrane receptor or classical P_4 receptor in the cytoplasm, but does not preclude a P_4-induced transcriptional change. The 5 h P_4 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 P_4 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.

The expression pattern for P_4 receptor subtypes in the brain during the proestrous day may provide some clues as to which receptors mediate the components of P_4 action on TIDA neurons during proestrus. Indeed, P_4 receptor B, membrane P_4 receptor α, and membrane P_4 receptor β mRNA expression are increased on proestrous afternoon and reach the highest levels coincident with the preovulatory P_4 rise (Liu & Arbogast 2009). Further investigation is needed to determine P_4 receptor subtypes involved in P_4 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.

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