The Cryptic Peptides, Prepro-Thyrotropin Releasing Hormone 186-199 and 194-199, Suppress Anterior Pituitary Prolactin Secretion *in vivo* and *in vitro*

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DEDICATION

Dedicated to my parents

Acknowledgements

Chad D. Foradori, Alicia M. Quihuis and Robert J. Handa (University of Arizona College of Medicine – Phoenix), Robert F. McGivern (San Diego State University), and T. John Wu (Uniformed Services University of the Health Sciences) contributed to this thesis and will be listed as co-authors when it is submitted for publication.

ABSTRACT

Prepro-thyrotropin releasing hormone (ppTRH)-176-199 is one of several peptide fragments cleaved during TRH synthesis and has been implicated as a regulator of neuroendocrine function. ppTRH 176-199 has been shown to acutely inhibit the stress-induced rise in ACTH, corticosterone (CORT), and prolactin (PRL) in the rat. The receptor for ppTRH 176-199 currently remains unknown. In this study we sought to characterize the active domain of ppTRH 176-199 and, using *in vivo* and *in vitro* approaches, determine its role in regulating anterior pituitary secretion of PRL.

The 186-199, 194-199, and 186-191 amino acid fragments of ppTRH were administered I.P. to adult male Sprague-Dawley rats 15 min. prior to a 20 min restraint stress to determine the peptide's active moiety in regulating prolactin secretion. Animals were euthanized and plasma was saved for assay of circulating PRL using enzyme immunoassay (EIA). ppTRH 186-199 significantly attenuated the stress-induced rise in prolactin in male rats in a dose-responsive fashion. This effect was mimicked by ppTRH 194-199 but not by ppTRH 186-191. At the highest dose (10 mg/kg BW), ppTRH 194-199 also reduced the stress-induced rise in plasma CORT.

Additionally, *in vitro* studies were performed using the rat growth hormone (GH)/PRL –secreting MMQ cell line. MMQ cells were treated with ppTRH 186-199 and media was assayed for PRL levels. Cells were harvested and examined for changes in PRL mRNA. Within 30 minutes following treatment of estradiol-stimulated MMQ cells with ppTRH 186-199 there was a decrease in media levels of PRL compared to vehicle. Furthermore, in MMQ cells that were primed with 10nM estradiol for 48 hours there was an increase in media PRL levels, which was reduced following ppTRH 186-199 treatment. After 4 hrs of treatment, the inhibitory effect of ppTRH 186-199 on PRL secretion from MMQ cells was only seen on estradiol-stimulated cells. There were no effects of ppTRH 186-199 when examined after 24 hrs of treatment. There were no effects of ppTRH 186-199 or 194-199 of PRL mRNA levels.

These data suggest that the carboxy terminal fragment of preproTRH 178-199 contains all the activity of this ppTRH cryptic peptide for regulation of PRL and corticosterone secretion. This suggests a potential moiety responsible for interaction with the peptide's receptor. The inhibitory effect of ppTRH 186-199 and 194-199 on media PRL levels and not on mRNA synthesis implicates it as an effector of hormone secretion rather than protein synthesis. The short-lived duration of its effects supports a role as

an acute effector of the PRL system. The target receptor of the ppTRH 178-199 fragment remains uncertain. However the use of ppTRH 194-199 as a peptide bait may prove useful in identifying the receptor.

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INTRODUCTION

Thyrotropin releasing hormone (TRH) is a tripeptide amide produced by neuroendocrine neurons of the hypothalamus by derivation from amino acid precursor protein, preproTRH (ppTRH). The TRH peptide is spliced from the precursor protein, yielding five TRH molecules. A number of intervening peptide sequences are produced during the TRH cleavage process. While much remains unknown regarding the functions of these peptides, several have been found to modulate specific neuroendocrine functions. Specifically, PreproTRH 178-199, a 22-amino acid peptide cleaved during TRH synthesis, has been shown to cause attenuation of the stress-induced rise in ACTH, corticosterone, and prolactin in the rat (McGivern, 1997), as well as inhibition of basal and CRH-induced ACTH release by pituitary tumor cells (Redei Apr 1995). The same peptide has also demonstrated suppression of ACTH secretion by human pituitary adenoma cells (Giraldi 2010). PreproTRH 178-199 has also demonstrated a role in regulation of stress behaviors, as it increases physical activity and decreases anxiety-related behaviors in rats. Interestingly, the peptide is expressed less densely in the periventricular nucleus (PVN) and parastrial nucleus (PSN) of Wistar-Kyoto hyper-anxiety rats as compared to normal

Wistar rats (Suzuki 2001), demonstrating a phenotypic correlate in its role in inhibition of the stress axis.

Synthesis of TRH precursor peptides and the cellular processing that yields discrete TRH segments has been described in great detail (Nillni 1999). While ppTRH 178-199 is produced by serine protease cleavage in hypothalamus, producing the above described effects, this peptide is further cleaved at the carboxy terminus within the anterior pituitary to yield ppTRH 186-199 (Fig. 1) (Nillni 1999, Romero 2008). The function of this smaller peptide segment is currently unknown, as is the parent moiety of preproTRH 178-199. In this study we aim to determine the actions of preproTRH 186-199 at the pituitary by measuring its affects on plasma PRL, CORT, and ACTH in the stressed rat, and also on PRL release by pituitary cells directly. Furthermore, we evaluate the influence of ppTRH 186-199 on estradiol's prolactotropic effect at the pituitary by priming cells with estradiol prior to treatment. We also aim to determine the terminus of preproTRH 186-199 responsible for activating its receptor. This is done by synthesizing peptides with only the amino- or carboxy- terminus of preproTRH 186-199 conserved, and using them to treat rats and pituitary cells in the same manner.

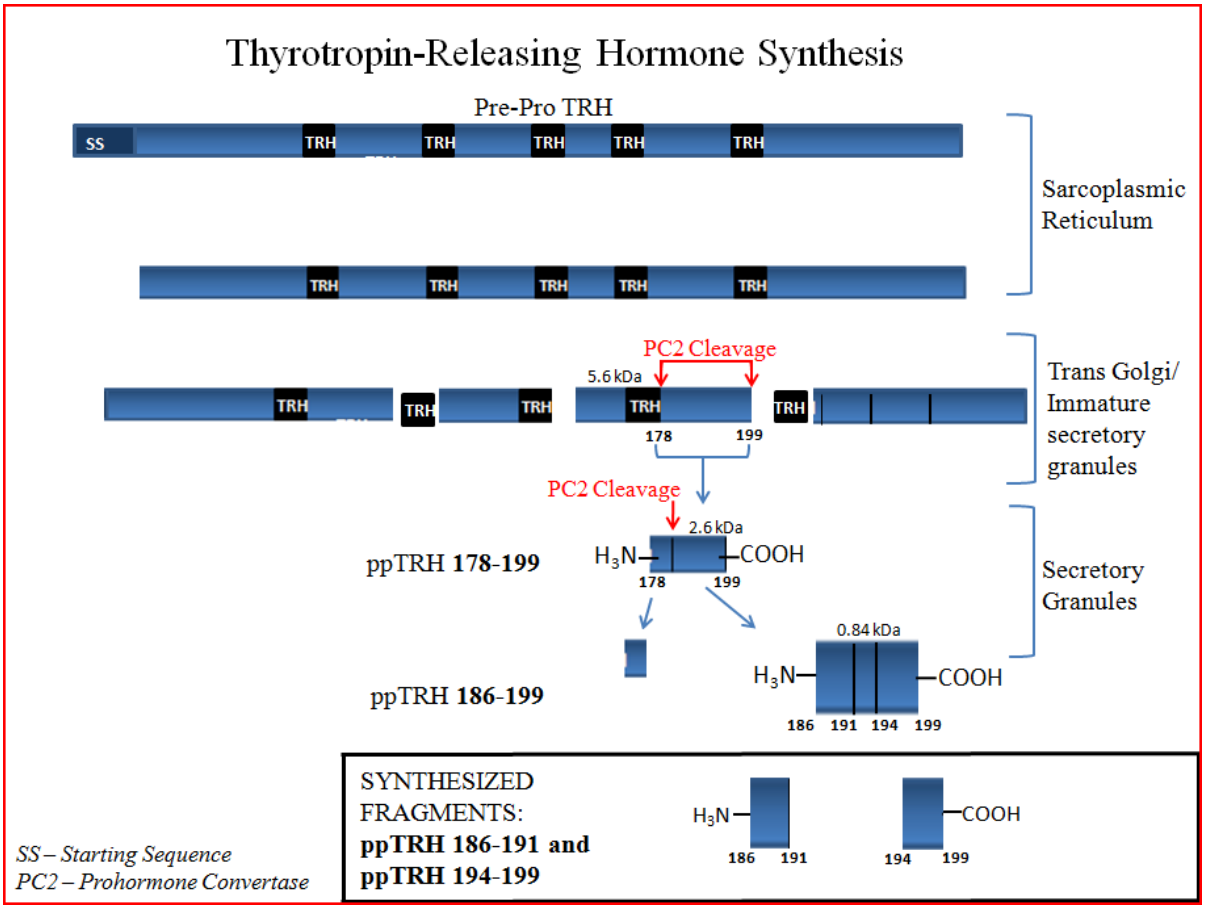

Figure 1. Thyrotropin-Releasing Hormone Synthesis. (a) Serine proteases (Prohormone convertase 1 and 2) cleave active TRH residues, followed by deamination by carboxypeptidase and addition of a polyglutamate tail, yielding active TRH. PC2 cleaves ppTRH 178-199 further at the carboxy terminus to yield ppTRH 186-199. (b) Short six-amino acid peptides (ppTRH 186-191 and 194-199) were synthesized to determine the activity of the amino and carboxy terminus of ppTRH 186-199 on prolactin secretion *in vivo* and *in vitro*.

MATERIALS AND METHODS

In Vivo Studies

Animals

Young-adult (60- to 90-day-old) male Sprague–Dawley rats were obtained from Charles Rivers Laboratories (Hollister, CA), housed two per cage in the Arizona State University vivarium, and maintained on a 14-h dark 10-h light schedule (lights on at 0700 h) with *ad libitum* access to food and water. All procedures conformed to the Public Health Service policy on humane care and use of laboratory animals. Experiments were conducted in accordance with IACUC-approved protocols.

Drug Treatment

All three experiments consisted of the following progression: Animals were handled for three days prior to injection to minimize the stress-induced response. Animals were injected i.p. and then returned to the home cage. After ten minutes animals were placed in a plexiglass restraint tube (Plas-Laboratories, Lansing, MI, USA) for ten minutes, followed by immediate decapitation at 20 minutes post-treatment. Trunk blood was collected into ice-chilled tubes containing 0.5 M EDTA (200 μl) and 4 μg/ml Aprotinin (100 μl). Blood was centrifuged at 2,000 RPM for 15 minutes and

plasma was removed and stored at -80°C until assayed for prolactin, ACTH, or corticosterone by radioimmunoassay

All doses of ppTRH peptides were suspended in sterile saline (0.9% NaCl) vehicle. Controls received sterile saline vehicle only.

Experiment 1 utilized ppTRH 186-199 i.p. at doses of 2 mg/kg and 5 mg/kg to two separate groups of rats. Sterile saline vehicle was administered i.p. to the control group.

Experiment 2 utilized ppTRH 186-199 injections at three different doses (2 mg/kg, 5 mg/kg, and 10 mg/kg) to three different groups. All treatments, including vehicle, were administered i.p.

Experiment 3 also utilized ppTRH 186-199, as well as two shorter peptides, six amino acid residues in length. Peptides were synthesized by the peptide synthesis core facility at the Uniformed Services University of the Health Sciences, and purity was checked by HPLC. The ppTRH 194-199 peptide represented the carboxy terminal 6 peptides of the 186-199 fragment, whereas ppTRH 186-191 represented the amino terminus. All treatments, including vehicle, were administered i.p.

Hormone Measurement

Serum prolactin and corticosterone for all three experiments was

measured by enzyme immunoassay, using a Rat Prolactin EIA Kit (Cayman Chemicals, Ann Arbor, MI) and Corticosterone EIA Kit (Cayman Chemical), respectively. ACTH was measured by radioimmunoassay, using an ACTH RIA kit (MP Biomedicals, Santa Ana, CA) according to manufacturer's directions.

In Vitro Studies

The rat pituitary prolactinoma-derived MMQ cell line was purchased from American Type Culture Collection® (Manassus, VA, Catalog No. CRL-10609™) and used for all *in vivo* studies. MMQ cells were plated at 20-50% confluence and treated for two days with 10nM in DMEM with 5% charcoal-stripped fetal bovine serum. The final concentration of ethanol in the media was less than 0.01%. After three days E2-primed groups were treated with 100 nM 17-□-estradiol for ten minutes prior to peptide treatment. Cells were treated with ppTRH 186-199 at 100 nM concentration. Control groups did not receive ppTRH treatment. Starting from time of ppTRH administration, cell media was withdrawn at time-points of 30 min, 2 h, 4 h, 24 h, and 48 h. Cells were harvested at either 24 hours or 48 hours post-treatment. The same protocol was used to examine effects of ppTRH 194-199.

To determine the optimal dose of ppTRH, MMQ cells were treated with several doses of ppTRH 186-199 or 194-199. Cells were treated with peptides at doses of 0.1 nM, 1nM, 10nM, or 100nM, and media for all doses were collected 30 minutes post-treatment.

All media were stored at -20˚ C until assayed for prolactin. Prolactin was measured by enzyme immunoassay (EIA), using a Rat Prolactin EIA Kit (Cayman Chemical, Catalog number 589701), or by radioimmunoassay, using an ^{125}I Rat Prolactin Kit (MP Biomedicals, Catalog number RK-553A101101).

Data Analysis

In Vivo comparisons of serum hormone concentrations were performed using one-way ANOVA and post-hoc Tukey's multiple comparison test. *In Vitro* comparisons of media PRL concentrations with and without E2 priming were performed using two-way ANOVA and post-hoc Bonferonni comparison tests.

RESULTS

***In Vivo* Studies**

ppTRH 186-199 in Rats. Administration of ppTRH 186-199

intraperitoneally to adult male rats 10 minutes prior to restraint stress caused a significant reduction in stress-reactive serum prolactin levels as compared with vehicle, and a trending reduction in corticosterone and ACTH that was not dose-responsive (Fig. 2). Reductions in serum prolactin were dose-responsive, with the 5 and 10 mg/kg doses causing significant reduction.

ppTRH fragments 181-191 and 194-199 in Rats. Administration of the 194-199 fragment, representing the carboxy terminal 6 peptides of ppTRH, at both 10 an 20 mg/kg doses significantly reduced stress-activated serum prolactin and corticosterone (Fig. 3a). Conversely, ppTRH 186-191, representing the amino terminus 6 peptides, had no effect on serum prolactin. Further, the effects of ppTRH 194-199 were comparable to those of ppTRH 186-199. Administration of ppTRH 194-199 at doses of 10 mg/kg and 20 mg/kg caused a significant reduction in plasma corticosterone as compared to vehicle (Fig. 3b), and a reduction in ACTH that was not significant (Fig. 3c).

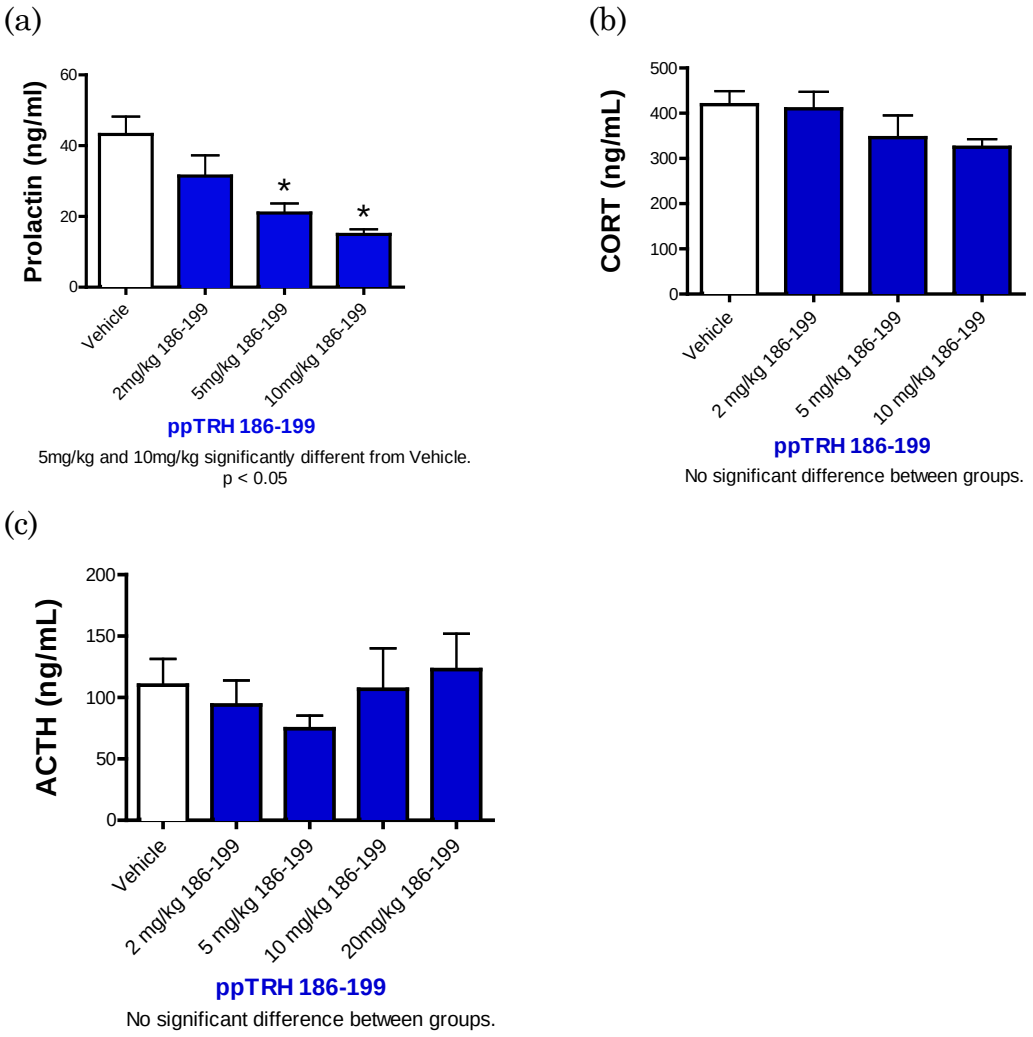

Figure 2. Intraperitoneal injection of ppTRH 186-199 reduces stress-reactive plasma levels of PRL, CORT, and ACTH in the rat. Samples taken at 20 minutes post-treatment, and following 10 minutes of restraint stress. (a) ppTRH 186-199 significantly reduced the stress-induced rise in rat serum PRL in a dose-dependent fashion (2, 5, and 10 mg/kg) ($p<0.05$). (b) ppTRH caused a trending reduction in plasma CORT in the stressed rat (ns). (c) ppTRH caused a trending reduction in plasma ACTH in the stressed rat (ns).

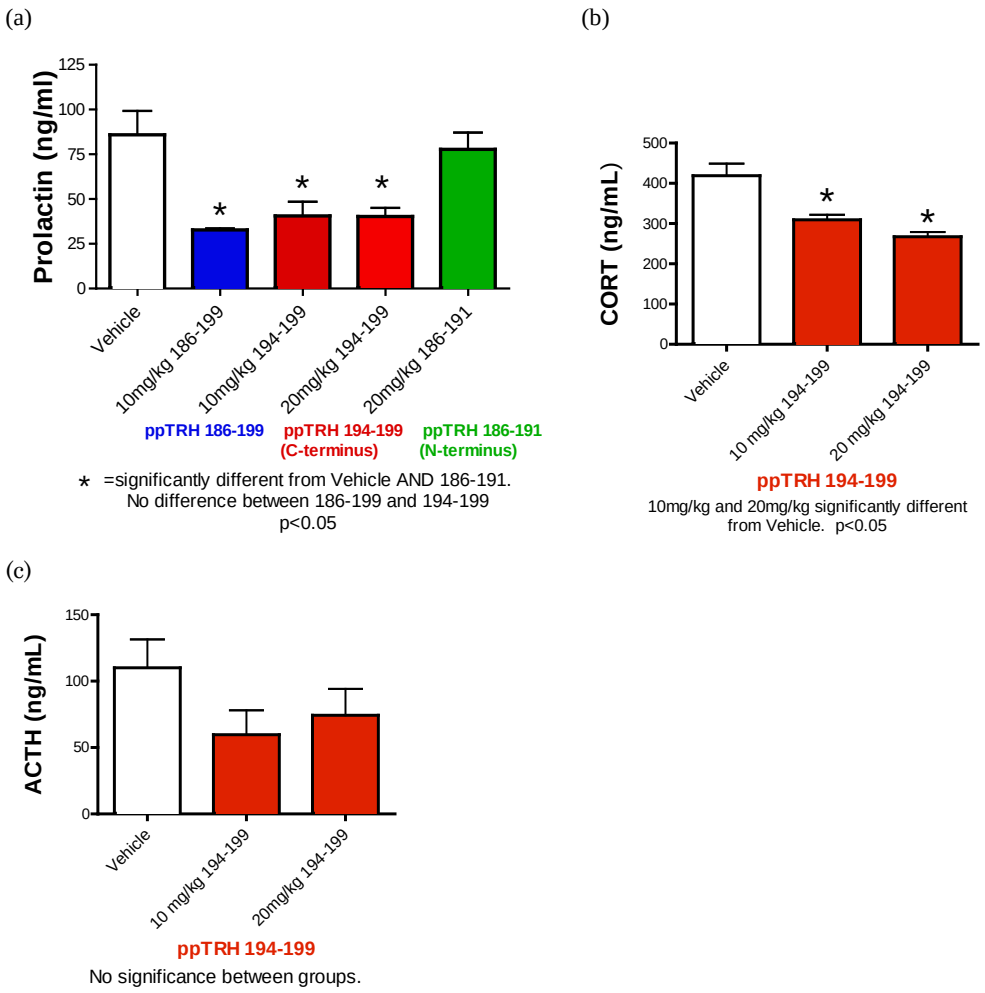

Figure 3. Intraperitoneal injection of ppTRH 194-199 reduces stress-reactive plasma levels of PRL and CORT in the stressed rat, while ppTRH 186-191 does not. Samples taken at 20 minutes post-treatment, and following 10 minutes of restraint stress. (a) ppTRH 194-199 significanly reduced the stress-induced rise in PRL (at 10 and 20 mg/kg) ($p<0.05$). The reduction caused by ppTRH 194-199 was comparable to that caused by ppTRH 186-199 (ns). ppTRH 186-191 did not create a reduction in plasma PRL. (b) ppTRH 194-199 significantly reduced the stress-induced rise in CORT (at 10 and 20 mg/kg) ($p<0.05$). (c) ppTRH 194-199 caused a trending reduction in ACTH (at 10 and 20 mg/kg) in the stressed rat.

In Vitro Studies

ppTRH 186-199 in cells with and without Estradiol priming. When MMQ cells were pre-treated with estradiol (E2), there was a significant increase in cell media prolactin for up to 48 hours post-treatment. Estradiol's effect was mitigated by administration of ppTRH 186-199, as dually treated groups exhibited a significant reduction in media prolactin as compared to groups treated only with E2. This effect occurred at 30 minutes and two hours following treatment with ppTRH 186-199, and became nonsignificant after four hours. No significant effect on media prolactin was induced by ppTRH 186-199 in cells not pre-treated with E2 (Fig. 4).

No difference was found in the quantity of PRL mRNA between homogenized MMQ cells treated with ppTRH 186-199 or 194-199 and/or E2 and untreated cells.

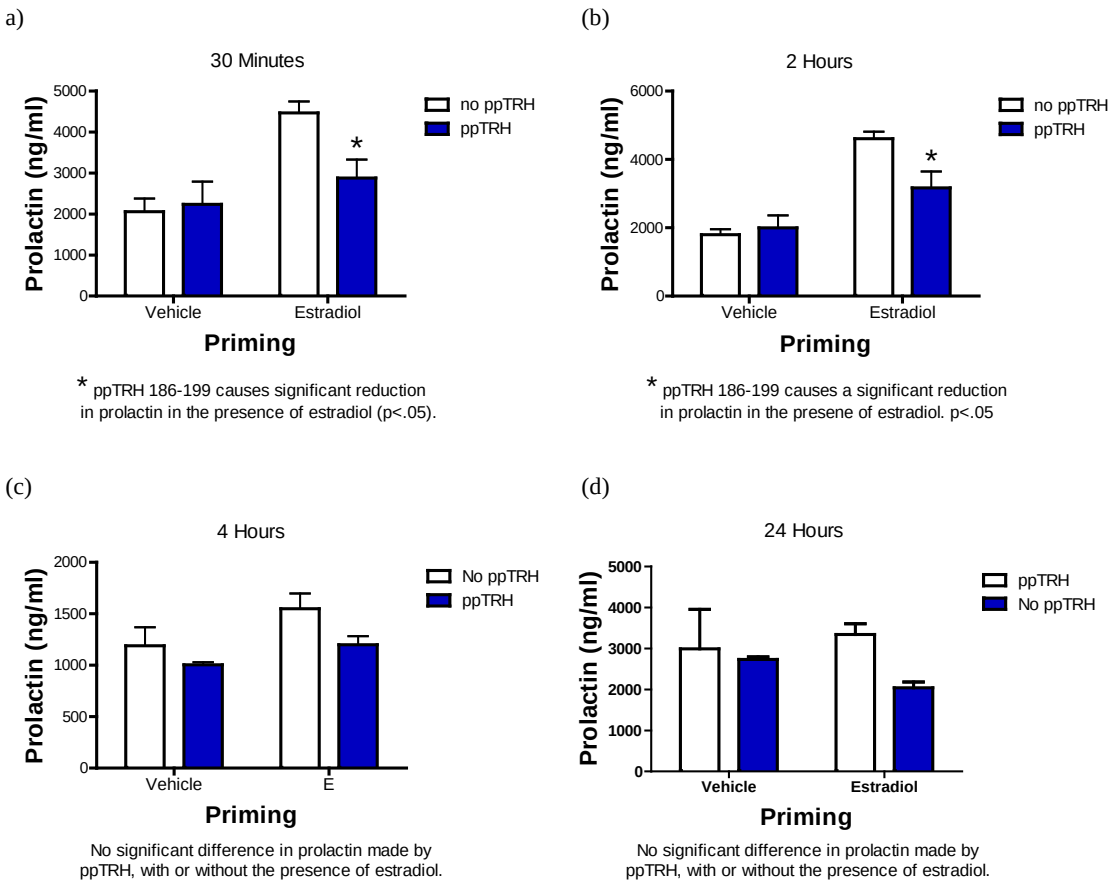

Figure 4. E2 administration induced an increase in PRL secretion for up to 48 hrs, which was reduced by ppTRH 186-199 (a) at 30 min and (b) 2 hrs after treatment with ppTRH 186-199. (c) This effect became insignificant by 4 hours (d) and was absent by 24 hrs.

DISCUSSION

The aim of this study was to examine components of the ppTRH 176-199 peptide segment by means of its successor, ppTRH 186-199, in modulating secretion of PRL, CORT, and CRH, to define the general site of action of ppTRH 186-199 in the hypothalamic-pituitary axis, and to identify the peptide's active terminus. We examined the effects of preproTRH 178-199 derivative, ppTRH 186-199, and its fragments, preproTRH 186-191 and 194-199, on the secretion of prolactin using both *in vivo* and *in vitro* approaches. Our results demonstrate that ppTRH 186-199 and its carboxy terminal fragment, ppTRH 194-199, share the activity of ppTRH 178-199 (CRIF (Redei Aug 1995)) in reducing the stress-reactive rises in PRL and CORT *in vivo*. ppTRH 186-199 also demonstrates direct inhibition of PRL secretion by anterior pituitary cells, showing that the action of the peptide occurs at the level of the pituitary.

PRL secretion was inhibited by preproTRH 186-199 both *in vivo* and *in vitro*. ppTRH 186-199 reduced plasma circulating PRL in the stressed male rat. McGivern et al. in 1997 described marked reductions in stress-induced plasma elevations of PRL, CORT, and ACTH in direct response to ppTRH 178-199 administration. These

findings together suggest that ppTRH 186-199 causes a reduction in the stress-reactive rise in plasma PRL. Reductions in PRL secretion by the MMQ rat anterior pituitary cell line, which possesses no dopaminergic nor dopamine-receptive activity, indicates a direct effect of the peptide on the pituitary cell, as opposed to an indirect pathway, involving additional neuroendocrine mediators. These findings contrast with prior studies, reporting ppTRH 178-199, as well as breakdown products ppTRH 178-185 and ppTRH 186-199, working as PRL secretagogues in primary pituitary culture (Nillni 1999). The reason underlying this discrepancy is unclear.

The smaller, synthesized peptide fragment, ppTRH 194-199, showed the same inhibition of stress-reactive PRL rise *in vivo,* while ppTRH 186-191 demonstrated none of this activity, demonstrating the necessity of the carboxy terminus for ppTRH 176-199 (CRIF) or 186-199 to exert inhibitory actions. It is worthy to note that prior studies have been unable to demonstrate ACTH inhibition by ppTRH 178-199 in pituitary culture (Nicholson 1996).

ppTRH 186-199 and 194-199 also demonstrated suppression of CORT secretion in the rat, supporting the role of ppTRH 178-199 as a corticotropin-release inhibiting factor. Suppression of ACTH release

by ppTRH 178-199 was first demonstrated by Redei et al in 1995, defining the peptide as endogenous CRIF. Our findings demonstrate that ppTRH 186-199, produced by PC2 cleavage within the pituitary (Nillni 1999), shares this inhibitory activity.

No changes in plasma ACTH were noted in response to ppTRH 186-199, as compared to the study using ppTRH 176-199 by Redei in 1995. It is possible that a reduction in plasma ACTH occurred earlier than the time of blood sampling, which was performed at 20 minutes post-treatment in this study. Similar *in vivo* studies with blood sampled at an earlier time after ppTRH treatment would be helpful in better defining the location(s) of peptide action along the H-P axis.

The receptor-mediated mechanism of action of the peptide is currently unknown. An orphan member of the GPCR101 (g-protein coupled receptor) family is a potential candidate that is highly expressed in the hypothalamus, and known to play a role in central energy balance (Nilaweera 2006). Recent studies have suggested the receptor has regulatory functions during late gestation and lactation, as it upregulates markedly in the PVN and SON during these times (Kanishka 2008), suggesting a potential role in the mediation of PRL

release. Studies that attempt to demonstrate co-localization of ppTRH 176-199 or 186-199 with the GPCR101 receptor would be useful.

In summary, our studies have identified the carboxy end of ppTRH 178-199 as exhibiting all of the effects of this cryptic peptide in regulation of PRL and CORT secretion, interacting directly with an unknown receptor at the anterior pituitary.

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