จุฬาลงกรณ์มหาวิทยาลัย
ทุนวิจัย
กองทุนรัชดาภิเษกสมโภช

รายงานวิจัย

กลไกการกระตุ้น ERα โดย PPT ต่อพฤติกรรมการกินอาหาร และระดับคอติโค โทรปิน รีลิสซิ่งฮอร์โมนในสมอง ของหนูแรทเพศเมียที่ถูกตัดรังไข่

โดย

สัมพันธ์ ธรรมเจริญ

พฤศจิกายน ๒๕๕๗

กิตติกรรมประกาศ

โครงการวิจัยเรื่อง "กลไกการกระตุ้น ERα โดย PPT ต่อพฤติกรรมการกินอาหารและระดับคอติโค โทรปิน รีลิสซิ่งฮอร์โมนในสมองของใบหนูเพศเมียที่ถูกตัดรังไข่" ได้รับการสนับสนุนทุนวิจัยจาก "กองทุนรัชดาภิเษกสมโภช" จาก จุฬาลงกรณ์มหาวิทยาลัย ประจำปีงบประมาณ 2556 (R004_2556)

คณะผู้วิจัยขอขอบพระคุณหน่วยงานต่างๆ ที่กรุณาให้ความช่วยเหลือด้านอุปกรณ์และเครื่องมือวิจัย เช่น การตัดชิ้นเนื้อเยื่อกแข็ง เครื่องมือถ่ายภาพและวิเคราะห์ตัวอย่าง เครื่องมือย่อยสลายชิ้นเนื้อ ฯลฯ ดังนี้ ภาควิชาเภสัชวิทยาและสรีรวิทยา คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย หน่วยชัณสูตร น.สพ. รชฎ ตันติเลิศเจริญ ภาควิชากายวิภาคศาสตร์ และ ผศ.สพญ.ดร. ศยามณ ศรีสุวัฒนาสกุล ภาควิชาสรีรวิทยา และ รศ.สพญ.ดร. สฤณี กลันทกานนท์ ทองทรง และ รศ. อัจฉรา ธวัชสิน

ลำดับสุดท้ายผู้วิจัยขอขอบพระคุณ ศ.น.สพ.ดร โธมัส ลุทซ์ (Prof. Thomas Lutz) ภาควิชาสรีรวิทยา คณะสัตวแพทยศาสตร์ มหาวิทยาลัยซูริค สวิตเซอร์แลนด์ ซึ่งอนุเคราะห์กรงหนูสำหรับวัดการกินอาหาร และอุปกรณ์กำหนดพิกัดสมองหนูสำหรับการผ่าตัดสอดท่อเข้าสู่สมอง

บทคัดย่อ

ชื่อโครงการ: กลไกการกระตุ้น ERα โดย PPT ต่อพฤติกรรมการกินอาหารและระดับคอติโค โทรปิน รีลิสซิ่งฮอร์โมนในสมองของในหนูเพศเมียที่ถูกตัดรังไข่

ชื่อนักวิจัย: นายสัตวแพทย์ สัมพันธ์ ธรรมเจริญ และ น.ส. ปรมัตถ์ กิจจานุกิจวัฒนา

ภาควิชาสรีรวิทยา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ระยะเวลาโครงการ: 1 กุมภาพันธ์ 2556 ถึง 31 มกราคม 2557

โพรพิลไพราโซลไตรออล (Propyl-pyrazole-triol, PPT) ยับยั้งการกินอาหารอย่างรวดเร็วโดยการกระตุ้นตัวรับฮอร์โมนเอสโตรเจนชนิดแอลฟ่า (estrogen receptor alpha, ERα) ภายในสมอง กลุ่มผู้วิจัยได้เคยรายงานเกี่ยวกับผลการยับยั้งการกินอาหารที่รวดเร็วซึ่งอาจเกี่ยวข้องกับการกระตุ้นเซลล์ประสาทที่สามารถสร้าง คอร์ติโคโทรปิน รีลิ่สซิ่ง ฮอร์โมน (corticotropin releasing hormone, CRH) ที่สมองส่วนพาราเวนตริคูล่านิวเคลียสของไฮโปทาลามัส (paraventricular nucleus of hypothalamus, PVN) โครงการวิจัยฉบับนี้ดำเนินการเพื่อศึกษาความเชื่อมโยงของ CRH ซึ่งเป็นสารสื่อประสาท (neuromediator) และฤทธิ์ยับยั้งการกินอาหารของ PPT ในการทดลองแรกผู้วิจัยแสดงให้เห็นว่าภายใต้สภาวะการทดลองที่ทำการทดลองนี้ PPT ยับยั้งการกินอาหารในหนูพันธุ์วิสต้าเพศเมียที่ถูกตัดรังไข่ดังเช่นที่เคยถูกรายงานโดยมีระยะเวลาการออกฤทธิ์ราว 3 ชั่วโมง หลังการให้สาร และเนื่องจากผู้วิจัยได้เคยรายงานแล้วว่า PPT สามารถกระตุ้นการแสดงออกของโปรตีน ซี ฟอส (c-Fos) ที่บริเวณสมองหลายนิวเคลียส แต่ผลการทดลองดังกล่าวมีความเกี่ยวข้องกับการได้รับอาหาร การทดลองในลำดับต่อมา ผู้วิจัยทำการศึกษาผลของ PPT ต่อการกระตุ้นการแสดงออกของ c-Fos โดยที่ไม่มีการให้อาหาร ผลการทดลองพบว่าจำนวนสัญญาณ c-Fos ที่นับได้จากสมองกลุ่มที่ได้รับ PPT ไม่ต่างจากกลุ่มควบคุมในทุกบริเวณ อย่างไรก็ตามเนื่องจากการให้ PPT ด้วยวิธีการดังกล่าวสามารถลดระดับของ อะดรีโนคอร์ติโคโทรปิน ฮอร์โมน (adrenocorticotropin hormone) ผู้วิจัยจึงให้เห็นผลว่าการกระตุ้น ERα โดย PPT เพียงอย่างเดียวไม่สามารถกระตุ้นการแสดงออกของ c-Fos ในสมองได้ ผู้วิจัยดำเนินการวิจัยในลำดับต่อไปโดยศึกษาระดับของ CRH จากตัวอย่างสมองส่วนนิวเคลียนสต่างๆ ที่สนใจทั้งจากสมองส่วนหน้าและส่วนหลังในช่วงเวลาเดียวกับที่ PPT ยับยั้งการกินอาหาร ผลการทดลองแสดงให้เห็นว่า PPT มิได้มีผลกระทบต่อระดับของ CRH ในทุกนิวเคลียสจากสมองส่วนไฮโปทาลามัส แต่ระดับของ CRH ที่สมองส่วนท้ายบริเวณ นิวเคลียส แทรกทัสโซลิทาเรียส (nucleus tractus solitarius, NTS) จากกลุ่มที่ได้รับ PPT สูงกว่ากลุ่มควบคุม ในลำดับสุดท้ายผู้วิจัยดำเนินการทดลองโดยใช้สารต้านตัวรับ CRH (CRH receptor antagonist, α-Helical CRF (9-41)) ปล่อยเข้าสู่สมองส่วนท้ายโดยวิธีการปล่อยสารผ่านสู่ช่องของสมองลำดับที่ 4 (the 4th cererebroventricular infusion, 4th icv) วัตถุประสงค์ของการทดลองนี้เพื่อประเมินกรณีที่ PPT ทำให้ระดับ CRH ที่สมองส่วนท้ายเพิ่มขึ้นในช่วงเวลาเดียวกับฤทธิ์ที่ทำให้การกินอาหารลดลง เป็นที่น่าเสียใจที่ผลการทดลองพบว่า

การให้ α-Helical CRF (9-41) โดยวิธีการ 4th icv อย่างต่อเนื่องเข้าสู่สมองส่วนท้ายไม่สามารถต้านฤทธิ์ของ PPT ที่มีต่อการกินอาหารได้

จากผลการทดลองทั้งหมดผู้วิจัยสรุปว่า PPT สามารถกระตุ้นเซลล์ประสาทส่วน PVN ได้ การกระตุ้นเซลล์ประสาทดังกล่าวน่าจะทำให้ระดับของ CRH เพิ่มขึ้นที่สมองส่วนท้ายบริเวณ NTS ซึ่งเป็นช่วงเวลาเดียวกับที่ออกฤทธิ์ยับยั้งการกินอาหาร

คำหลัก: พีพีที ซีอาร์เอช หนูเพศเมีย การกินอาหาร

ABSTACT

Project title: The mechanism of ERα activation by PPT on eating behavior in ovariecmtomized rat

Investigator: Dr. Sumpun Thammacharoen and Miss Poramat Kitchanukitwattana
Department of Physiology, Faculty of Veterinary Science, Chulalongkorn University.

Project period: 1 Febuary 2013 to 31 January 2014

Propyl-pyrazole-triol (PPT) inhibits food intake acutely by specifically activating estrogen receptor alpha (ERα) within the brain. We showed previously that the effect of PPT to inhibit eating was rapid and mediated in part by activate corticotropin releasing hormone (CRH) neuron at paraventricular nucleus of hypothalamus (PVN). The current experiments were performed to investigate that CRH is neuromediator participate in eating inhibitory effect of PPT. First, we demonstrated that in our condition PPT decreased eating in ovariectomized female Wistar rat rapidly similar to previous reports. The onset of PPT action was 3 h after treatment. Previously, PPT could activate c-Fos expression in several brain areas. However, the result was in part related with eating paradigm. In the current report, we investigated the expression of c-Fos after PPT treatment without eating. Interestingly, c-Fos immunoreactivity from PPT treated group was not different from control group at any brain nuclei especially at PVN. Because PPT could decrease adrenocorticotorpin hormone in the same experimental paradigm, we argued that an activation ERα by PPT per se couldn't activate neuronal c-Fos expression. We further investigated the concentration of CRH at interested forebrain and hindbrain nuclei during the time that PPT decreased eating. It was surprising that PPT didn't affect CRH level at any hypothalamic nuclei. However, CRH level at hindbrain nucleus tractus solitarius (NTS) from PPT treatment group was significant higher than from vehicle treatment group. Final, we performed an experiment using CRH receptor antagonist, α-Helical CRF (9-41), infused directly to hindbrain by the 4th intracerebroventricular infusion (4th icv). The aim of this experiment was to determine if PPT induced hindbrain CRH involved to eating inhibitory effect. Unfortunately, continuous 4th icv of α-Helical CRF (9-41) failed to eating inhibitory effect of PPT. Taken together; we concluded that as well as the rapid effect of eating behavior, PPT could activate PVN neuron. This activation apparently increased CRH level at hindbrain NTS at the period when PPT eating inhibitory occurred.

Key words: PPT, CRH, female rat, food intake

Table of Content

List of Figures

INTRODUCTION

Estrogens (Blaustein, 2008) are a major group of female sex steroid hormones. The natural forms of estrogens are 17β-estradiol (E_2), estrone (E_1) and estriol (E_3). Estradiol is the main estrogen form because of its high level in the circulation and its potency to activate estrogen receptor (ER) mediated transcription activity. Estradiol has an important role in many physiological functions including, e.g. development, growth and homeostasis. One important action of E_2 and the major focus of this work is its role in the control of eating and body weight (BW) in female animals. These effects are clinically important because it is well accepted that women are more prone to developing severe obesity and eating disorders than are men (Geary, 2001; Klein and Walsh., 2004).

Eating is a basic behavior that is controlled by multiple brain centers. In female, this behavior is in part controlled by E_2. Unlike the effect of E_2 on reproductive behavior, lodosis (Pfaff, 2005), the effect of E_2 on eating has not been work out thoroughly. In general, the effect of E_2 on eating apparently mediated via ERα in the brain. However, the eating effect from both estradiol benzoate (EB) and Propyl-pyrazole-triol (PPT) were proved to have 2 different onsets (Santollo et al., 2007; Thammacharoen et al., 2007), which suggest that they may be mediated via separate pathways. While ERα activated by EB mediates the late onset pathway, the rapid onset by PPT is an alternative bypass pathway. Moreover, the eating inhibitory effect of PPT apparently relates to the corticotropin releasing hormone (CRH) neuron at paraventricular nucleus of hypothalamus (PVN, Thammacharoen et al., 2009). In the current report, we focused our research on the mechanism by which PPT inhibits eating and activates CRH neuron in female rats. The report contains, in the first part, the basic

information on the mechanism of E_2 action, the mechanism of peripheral and central controls of eating behavior and the general knowledge regarding estrogenic control of eating. The second part contains the current experiments including the detail material & methods, results, discussions, conclusion and perspectives.

Mechanisms of estrogens action

All physiological effects of E_2 are mediated by ligand-inducible nuclear transcription factor, ERs. Two ER subtypes, ERα and ERβ have been identified and cloned (Green et al., 1986; Greene et al., 1986; Kuiper et al., 1996). ERs belong to the steroid/thyroid hormone superfamily of nuclear receptors. The receptors contain three domains including: the NH2-terminal A/B domain; the C domain; and the carboxyl terminal D/E/F domain (Nilsson et al., 2001). The NH2-terminal A/B domain encodes a ligand-independent activation function (AF1), a region involved in transcriptional activation and in protein-protein interactions. The highly homologous C domain contains the DNA binding domain (DBD) with two zinc finger structures. This domain has a role in receptor dimerization and target DNA binding. The carboxyl terminal D/E/F domain contains the E/F ligand-binding domain (LBD), which harbors the ligand-dependent activation function (AF2). The overall structure of ER-LBD is composed of 12 helices (H1-H12) and two stranded β-sheets (S1 and S2). After ligand binding, the position of H12 is the key event that permits discrimination between estrogen receptor agonist and antagonist (Brzozowski et al., 1997). The LBD plays an important role in ligand binding, receptor dimerization, nuclear translocation and target gene transcription activation.

It is well accepted to date that the two basic mechanisms of ER-mediated E_2 actions involve genomic and non-genomic effects. Both mechanisms depend on the

binding structure of E_2 to ER. For the ER dependent gene transcription, the receptor is localized predominantly in the nucleus, the nuclear ER (nER) (King and Greene., 1984; Welshons, 1984; Kawata et al., 2001). In addition, the plasma membrane associated ER (mER) has been shown to mediate the rapid non-genomic actions of estrogen (Mendelsohn, 2000b; Milner et al., 2005; Pedram et al., 2006; Pietras and Marquez-Garban, 2007; Ronnekleiv et al., 2007; Song, 2007; Toran-Allerand, 2004; Vasudevan et al., 2005).

For the genomic action, the receptor is dissociated from the chaperone protein, phosphorylated and dimerized after the binding of the ligand. The ligand-ER complex stimulates the target gene by either direct or indirect initiation of transcription. The direct binding of the complex to the estrogen response element (ERE) activates specific gene transcription. In the indirect activation of transcription, the ligand-ER complex does not bind directly to DNA, but tethers with another transcriptional activator to promote gene expression. Many transcriptional factors have been shown to interact with ER via the indirect mechanism i.e. NFκB (Kalaitzidis et al., 2005), Sp1 (Safe, 2001) and AP-1 (Kushner et al., 2000). Moreover, the ER itself can be activated by mechanisms independent of ligand binding. Many signaling pathways can modulate ER though phosphorylation via regulators of the phosphorylation state (PKA or PKC) i.e. extracellular signals (peptides growth factors, cytokines or neurotransmitters) and cell cycle regulators (Nilson et al., 2001). The DBD, AF1 and AF2 domains of ERs are all responsible for the activation of transcription via the genomic action of ER. While DBD specifically binds with ERE (Klinge, 2001), AF1 and AF2 synergistically activate transcription by recruiting the basic transcriptional machinery and several coregulatory proteins. The coregulator proteins recruited by AF1 and AF2 can be subdivided into coactivators and corepressors (Hall and McDonnell, 2005; Perissi and Rosenfeld,

2005). The detailed mechanisms by which AF1 and AF2 recruit the coregulator proteins and initiate the transcription are different (Lavery and McEwan, 2005; Pike, 2006; Warnmark et al., 2003). The role of AF1 and AF2 on ER activated transcription has been revealed by many estrogen agonists/antagonists and by different cells and promoter contexts. First, different cells that were transfected with the same promoter appeared to have different degrees of transcription after estradiol treatment. Second, different promoters that were transfected to the same cell also showed differences in transcription activity. Tamoxifen, acting as an AF2 domain blocker, has an estrogen antagonistic effect on the gene that requires only the AF2 domain for ER-mediated transcription. In contrast, tamoxifen has a partial agonist effect on genes where AF2 is not required. In addition, the AF1 of ERα appears to have stronger activity than ERβ (Delaunay et al., 2000; Tzukerman et al., 1994). Collectively, this information suggest that the genomic effects of E_2 and estrogen agonists/antagonists depend partly on the interaction among AF domains of ER, cell types and promoter contexts of estrogen responsive genes (Delaunay et al., 2000; McDonnell et al., 1995; Tzukerman et al., 1994).

Another pathway of E_2 dependent ER actions involves rapid effects that cannot be attributed to genomic actions. This is the so-called non-genomic pathway. E_2 is able to evoke fast responses in many tissues, within seconds to minutes after ligand binding. Many intracellular signaling cascades have been shown to be responsible for these rapid effects. These include e.g. the activation of ion channels, the MAPK pathway; the CREB pathway, the phosphatidylinositol 3-kinase (PI3K)/Akt pathways; the G-protein coupled receptor (cAMP and intracellular calcium); and the nitric oxide pathway (Collins and Webb, 1999; Mendelsohn, 2000b; Pietras and Marquez-Garban, 2007; Ronnekleiv et al., 2007; Vasudevan et al., 2005). The mERs appear to mediate these

rapid effects. These mERs probably share a common origin with nuclear ERs (Pietras and Marquez-Garban, 2007). Both ERα and ERβ and a novel ER (ERX) have been identified at the membrane (Chambliss et al., 2002; Kelly and Ronnekleiv, 2008; Milner et al., 2005; Pedram et al., 2006; Pietras and Marquez-Garban, 2007; Song, 2007; Toran-Alland et al., 2002). It should be noted here that E_2 can activate intracellular signaling independent of mER. The G protein coupled receptor 30 (GPR30) was reported to bind E_2 but the biological function which is mediated by GPR30 has yet to be investigated (Filardo and Thomas, 2005; Funakoshi et al., 2006; Pedram et al., 2006; Prossnitz et al., 2008; Revankar et al., 2005). Evidence for an important functional role of the non-genomic ER pathway has been provided for many different tissues including the reproductive system (Luconi et al., 2004), cardiovascular system (Fu and Simoncini, 2007; Leung et al., 2007; Mendelsohn, 2000a; Fu and Simoncini, 2007) and central nervous system (Behl, 2002; Kelly and Ronnekleiv, 2008; McEwen et al., 2001; Ronnekleiv et al., 2007). In the brain, the rapid non-genomic ER pathway appears to involve mechanisms of neuroprotection and aging (Behl, 2002; Garcia-Sugura et al., 2007; Mendez et al., 2005), reproduction (Vasudevan et al., 2005; Kow and Pfaff, 2004) and eating behavior (Asarian, 2006; Arbogast, 2007; Dagnault and Richard, 1997; Liang et al., 2002; Gao et al., 2007). Despite these reports, it is still difficult to dissociate the role of genomic and non-genomic pathways of E_2 for a specific behavior or brain function. The lordosis behavior in female rats is one example of influence by both pathways (Kow and Pfaff, 2004). It was first suggested that lordosis is a behavior which requires the genomic action of E_2 (Parsons et al., 1982). Later, using the bovine serum albumin conjugated E_2 (E_2-BSA) which acts only on the membrane and the couple treatments paradiagm; Kow and Pfaff (2004) demonstrated that the first treatment with E_2-BSA potentiates the second treatment with E_2 on lodosis

score. The result suggested that rapid non-genomic ER action potentates the genomic ER action of lordosis.

Estrogens and ER ligands have diverse effects in many organs. Besides their physiological functions, estrogens are also involved in many pathophysiological processes, e.g. cancer, osteopenia, menopause syndromes, and brain and psychological disorders. Based on the variety of ERs dependent mechanisms reviewed above, it is not surprising that one steroid can influence many different biological functions and diseases. The simple model that determines the outcome of action of E_2 and ERs dependent mechanisms involves three fundamental factors: the spatio-temporal expression of both ER subtypes (Laflamme et al., 1998; Milner et al., 2001; Milner et al., 2005; Mitra et al., 2003; Schlenker and Hansen, 2006; Shughrue et al., 1997; Toran-Alland et al., 2002); the nature of the cell types (the coregulator molecules and promoter context; Lavery and McEwan, 2005; Pike 2006; Warnmark et al, 2003); and the type of ligands (E_2 or SERMs; Osborne et al., 2000). It is therefore crucial to identify all these factors in order to understand E_2 effect on behavior, e.g. eating behavior.

Food intake control mechanisms

The biological goals of eating behavior are to provide energy and necessary nutrients to the body. The pattern of eating is generally characterized by distinct meals or eating bouts that are distributed over the course of a day. Meal pattern varies between species and also between individuals. Daily food intake (FI) depends on meal frequency and meal size. Eating behavior is controlled by two fundamental factors: internal controls and external stimuli (i.e. pleasure of food, social system, predation, reproduction etc). The internal control mechanisms of meals can be considered into

four categories. These are signals for the initiation of eating; signals for maintaining eating during a meal; signals that terminate eating; and signals that maintain the intermeal interval. Animal starts to eat (meal initiation) when they are hungry. At this state, animals are more sensitive to a variety of food stimuli including the signals from the olfactory, visual and gustatory systems. However, the mechanisms of meal initiation themselves are still not clear. Meal initiation has been demonstrated to correlate with the concentration of metabolites (glucose and fatty acids), metabolic rate and body temperature (Even and Nicolaidis, 1985; De Vries et al., 1993). At least in a series of experiments, a premeal reduction of glucose was demonstrated a few minutes prior to a spontaneous meal (Campfield and Smith, 2003). However, eating also occurs even in a state of ample energy balance and without external cues. During a meal (meal maintenance), the presence of food in the GI tract produces a set of mechanical and chemical signals. The balance of positive feedback (pleasure) and negative feedback (satiation) signals determines the size of a meal and the rate of eating. While pleasure from food facilitates eating, satiation promotes meal termination, thereby limits meal size. The postprandial feeling and behavior that affects the interval to the next meal is referred to as satiety.

One characteristic of eating behavior is that animals, and obviously humans as well, select foods preferentially when food choices are ample, instead of having the same menu every day. This suggests that the internal control systems contain not only homeostatic but also hedonic components (Saper et al., 2002; Berthoud, 2004). Both mechanisms participate in the decision about what kind and how much food an animal eats. While homeostatic controls maintain normal energy and nutrient supplies to the body, hedonic controls of eating have specific characteristics that can overpower the homeostatic controls and result in eating behavior at any times and even at excessively

high levels. These internal controls of eating behavior include the interplay between peripheral sensing and signaling systems (sensory organs, gastrointestinal tract and adipose tissue), and central integration (the brain). The hedonic components receive signals mainly via sensory organs, as well as from previous experiences with food that have been memorized and learned. This component plays a role in food rewarding aspect (Berridge and Robinson, 2003). In the homeostatic control of eating, peripheral signals could be classified into **"short and long term control mechanisms"**. In the short term control of eating, GI tract translates the signals (both volume and nutrient) from ingested food into hormonal (Chaudhri et al., 2006; Cummings and Overduin, 2007) or neuronal signals (Marty et al., 2007; Thaler and Cumming, 2008). In addition, some nutrients can work directly as signals to control eating behavior (Levin et al., 2004; Marty et al., 2007). The long-term control involves somewhat different properties. Adipose tissue and pancreas (adiposity signals) provide tonic signals for maintaining homeostasis to match the energy input and expenditure (Woods et al., 2000). Another peripheral signal that control eating depends on the cyclic pattern reproductive cycle especially in female. It is well known that female animal eat less during the estrous phase of ovarian cycle and this behavior is mediate mainly by E_2. The estrogenic effect on FI in female rat is the main interested of the current work and will be introduced in detail in "**Estrogenic control of food intake**".

Estrogenic control of food intake

Eating behavior shows specific gender-related differences between males and females. A clear phenotypic difference between intact males and females is that females show a cyclic pattern of eating while males do not (Asarian and Geary, 2006). During the peri-ovulatory phase of the estrous cycle, female dog, pig, rat, monkey and woman

decrease their daily intake (Czaja and Goy, 1975; Eckel et al., 2000; Friend, 1971; Gong et al., 1989; Houpt et al., 1979; Lyons et al., 1989). This phenomenon has been studied most extensively in rats which typically have a four or five day cycle (Fig1). The reduction of FI usually occurs during the night of the estrus. This is preceded by an increased plasma E_2 concentration during proestrus (Fig1). The reduction of FI is due to a decrease in meal size with a partially compensatory increase in meal frequency (Asarian and Geary, 2002). FI then returns to baseline in the subsequent diestrus. An ovariectomy removes the major source of E_2 in females. Ovariectomized (OVX) rats have dramatically decreased levels of plasma E_2 and gradually increase daily FI and BW. The increase in daily intake in OVX rats is due to an increase in meal size while meal frequency decreases (Asarian and Geary, 2002). Daily FI in OVX rats is generally higher than in intact rats at all stages of the estrous cycle. This suggests two functional components of E_2's effect on eating. The first is a tonic inhibition by E_2, which is revealed by an increase in the basal level of eating after OVX. The second is a phasic inhibition by E_2, which is the absence of the cyclic decrease in eating after OVX. Replacement with a physiological dose of EB but not progesterone reverses the effect of OVX on FI and BW in rats. Administration of EB in the middle of the light phase increased plasma E_2 in the first night after injection, which corresponds to the increase of plasma E_2 during proestrus in intact rats. Rats eat less in the second night after EB injection, which corresponds to the decrease in FI during the night of estrus in intact rats (Asarian and Geary, 2002). The effect of exogenous E_2 on FI again occurs mainly via a change on meal size. Meal size is decreased after replacement, while meal frequency usually partially compensates by increasing (Asarian and Geary, 2002).

E_2 is generally thought to act in the brain to inhibit feeding (Butera et al., 1993; Geary et al., 1996; Rivera and Eckel., 2010). Various experiments have shown that

microinjection of E_2 into various sites of the brain, especially into various hypothalamic nuclei, decreased FI in rats. E_2 implantation into the ventromedial hypothalamus (VMH, Wade and Zucker, 1970; Nunes et al., 1980), the medial preoptica area (MPA) (Dagnault and Richard, 1997) or the PVN (Palmer and Gray, 1986; Butera and Beikirch, 1989) reduced FI in rats. However, the exact site(s) where E_2 mediates its action on FI is still not clear for because of inconsistent results from above studies. Palmer and Gray (1986) failed to reproduce the effect on FI of E_2 implantation into the VMH as originally reported by Wade and Zucker (1970). Furthermore, Butera and Beikirch (1989) found that only PVN implantation (not VMH and MPA) of E_2 reduced 3-d FI in OVX rats. Further, peripheral E_2 treatment has been shown to decrease FI and BW in mice with a specific ER-α knockdown in the VMH, strongly suggesting that the VMH may not be required for the control of feeding and BW by E_2 (Musatov et al., 2006). The role of the PVN has been questioned as well because bilateral PVN lesions did not abolish the effect of E_2 on FI in OVX rats (Dagnault and Richard, 1994). This was corroborated by a study published by Hrupka et al. (2002) suggesting that the action of E_2 in the PVN is not sufficient to account for the estrogenic control of FI. However, information from some of c-Fos studies appears to support that the PVN region may be one of the potential sites for the estrogenic inhibition of eating. E_2 increased c-Fos in different paradigms related to cholecystokinin (CCK) satiation at several brain regions (Eckel and Geary, 2001; Eckel et al., 2002; Asarian and Geary, 2007). Eating induced c-Fos was first demonstrated to be enhanced by E_2 in the nucleus tractus solitarius (NTS), PVN and the central amygdala (CeA) (Eckel and Geary, 2001). The same c-Fos pattern and sites could also be observed when rats were treated with exogenous CCK and E_2 (Eckel et al., 2002). It was demonstrated later that E_2 treatment in rats with intraduodenal lipid infusion (a secretagogue of intestinal CCK) had significant higher

c-Fos in caudal NTS than in control rats; this result could not be observed in PVN (Asarian and Geary, 2007). The results on c-Fos studies suggest that at the hindbrain NTS may all involve in E_2 enhanced CCK's satiation either from eating and exogenous CCK models, however only the caudal NTS is the area where E_2 enhances intraduodenum lipid induced-cFos. We showed the results suggesting the possibility that NTS is sufficient for mediating the estrogenic effect on FI (Thammacharoen et al., 2008). We demonstrated first, that the spreading of E_2 was very limited and affected only the dorsal but not to the ventral part of the NTS. Importantly, E_2 spreading was not observed in the forebrain either. Second, FI in OVX rats with EB applied to NTS was lower than in control OVX rats. Third, with the same paradigm we demonstrated that CCK activated c-Fos only at the NTS but not at any forebrain nuclei and the CCK induced c-Fos at cNTS was colocalized with ERα-expressing neurons. These results suggested that E_2 acts on ERα-expressing neurons at the NTS, especially at its caudal part. Taken together, both hypothalamic nuclei and hindbrain NTS appeared to be the target area for eating inhibitory effect of E_2.

Estrogenic inhibition of eating is partly due to a modulation of the peripheral feedback controls of eating. E_2 increases the potency of gastrointestinal satiation hormones like CCK (Geary, 2001) and glucagon (Asarian and Geary, 1999), and decreases the potency of ghrelin which is a gastric orexigenic hormone (Clegg et al., 2007). The most extensive studies on the estrogenic modulation of peripheral signals came from the studies of an E_2's effect to increase CCK satiation (Geary, 2001 and Asarian and Geary, 2006). Exogenous E_2 enhanced exogenous CCK's satiation effect (Butera et al., 1993; Geary et al., 1994; Linden et al., 1990). Later, it has been shown that the CCK-1 antagonist (devazepide) increased FI only during the day of estrus in female rats. This suggests that endogenous CCK action also changes across the estrus

cycle (Eckel and Geary, 1999). In OVX rats, endogenous CCK satiation is also enhanced by exogenous E_2 (Asarian and Geary, 1999; Asarian and Geary, 2007). It is clear from the above information that endogenous and exogenous E_2 seem to modulate the satiation effect of both exogenous and endogenous CCK. The estrogenic inhibition of eating may also be mediated by the interaction with other peripheral feedback controls of eating, especially adiposity signals. It has been shown that female rats were more sensitive to leptin than male rats (Clegg et al., 2003). Later, the same group demonstrated that E_2 increased leptin effect in female rat (Clegg et al, 2006). However, some evidences reported the contrary results. First, importantly, it was also demonstrated contrary that leptin sensitivity does not change in both intact and OVX rats (Pelleymounter et al., 1999; Chen and Heiman, 2001). Second, plasma leptin was not changed before the onset of obesity after OVX, and leptin levels did not change when corrected by fat mass either in OVX or E_2 replacement (Pelleymounter et al., 1999). Third, female OVX ob/ob and db/db mice still respond to E_2 replacement as in sham control (Gao et al., 2007; Shimomura et al., 2002). Based on above information, it seems that an interaction of E_2 and leptin to control FI and BW needs further investigations with an appropriate experimental design.

Estradiol appears to affect FI and BW through the stimulation of ERα rather than ERβ. Firstly, ERα receptor knockout (αERKO) mice were higher BW than wild type whereas BW of ERβ receptor knockout (βERKO) mice did not differ from wild type (Couse and Korach, 1999). Secondly, E_2 produced its effects on BW and FI in wild types and βERKO mice, but had no effects in αERKO mice (Geary et al., 2001; Geary, 2004). Finally, OVX rats treated with specific ERα but not ERβ agonists decreased FI (Roesch, 2006; Santollo et al., 2007; Thammacharoen et al., 2007) and the same ERα agonist produced no effect on FI in αERKO mice (Thammacharoen et al., 2009).

While it has been established that the estrogenic inhibition of FI appears to be an activation of ERα, it remains unclear which downstream genomic or non-genomic pathways are responsible for eating inhibitory effect. The coupling of plasma E_2 and behavior outcome that can be observed in intact rats and in the OVX rats after cyclic EB replacement suggested that the physiologic effect of E_2 on eating needs time to develop. In addition, direct administration of a protein synthesis inhibitor (e.g. anisomycin) blocked E_2's effect on eating (Butera et al., 1993). This suggested that the effect is mediated partly via the genomic effect of ER action. Some evidences however support the role of non-genomic ER action on FI. First, E_2 administration to the brain at pharmacological doses decreased FI shortly after treatment (Dagnault and Richard, 1997; Gao et al., 2007). However, Liang et al. (2002) demonstrated no acute effect of central administration of E_2 into brain on eating. Second, the ERα agonist PPT, injected subcutaneously, produced an inhibitory effect on FI within 4 hours (Santollo et al., 2007; Thammacharoen et al., 2007). The onset of PPT to decrease FI was faster than what can be observed after peripheral E_2 replacement. An example that has been shown previously about a participation of both genomic and non-genomic E_2 actions is the lordosis behavior in female rats. Lordosis is one of the sexual behaviors that require genomic action of ER. It has been shown later that a non-genomic E_2 action enhances the genomic action of ER on lordosis behavior (Kow and Pfaff, 2004). Based on the above information, it seems plausible that both genomic and non-genomic E_2 actions may contribute to the estrogenic inhibition of FI. However, further experiments need to be conducted to test directly whether and how the genomic and non-genomic actions of ER could participate on the estrogenic inhibition of FI.

Another interesting aspect of the mechanism by which PPT modulate eating behavior was that PPT specific activated ERα to inhibit eating and the downstream

mechanism appear to related with corticotropin releasing hormone (CRH) neuron at the paraventricular nucleus of hypothalamus (PVN; Thammacharoen et al., 2009). In light of our previous information, the current experiment strengthen the role of CRH on the eating inhibitory effect of PPT. PPT activated CRH neuron at the PVN and increased CRH level at the hindbrain NTS.

MATERIALS & METHODS

Animals and housing condition

Female Wistar rats (National Laboratory Animal Care, Mahidol University) weighing around 250-300 g were housed individually in hanging cages with stainless steel wire-mesh floors (33X18X20 cm) in a room maintained at 22±2 °C with 12:12 light: dark cycle (light on 0000h, unless otherwise states). All rats had ad libitum to pelleted standard chow (#082, Perfect Companion Group Ltd., Samutrprakarn, Thailand) and tap water. Rats were adapted to the housing condition for at least 1 week before starting experiment. Daily FI (± 0.1 g corrected for spillage) and BW (± 1 g) were measured throughout the experimental period. All procedures were performed according to the ethical principles and guidelines for the use of animals for scientific purposes from the National Research Council of Thailand and were approved by the animal used committee, Faculty of Veterinary Science, Chulalongkorn University.

Ovariectomy

In the current project, intact female rats were ovariectomized at different time point depending on the experiment. Rats were anesthetized with isoflurane (2.5-3%, Minrad, Inc, USA) and bilaterally ovariectomized using an intraabdominal approach (Thammacharoen et al., 2008). Immediately after surgery, rat was subcutaneously injected with enrofloxacin (2.5-5.0 mg/kg iv; Bayer Korea Ltd., Korea) for antibiotic prophylaxis. Ibuprofen (Reckitt Benckiser, Inc., UK) was given once orally (15 mg/kg po) and via drinking water at the concentration of 12 mg/100ml for 4 days to minimize post-surgical pain.

Brain perfusion & Immunohistochemistry for cFos

In the experiment 2a, the expression of cFos was investigated after EB, PPT or control treatment in 10 hour fasted OVX rats. On the experimental day, rats were transcardially perfused after 90 min injection with EB, PPT or control. Briefly, rat was deeply anesthetized with sodium pentobarbital intraperitoneally and transcardially perfused with ice-cold phosphate buffer [PB, 0.1 M (pH 7.4)] followed by 4% paraformaldehyde in 0.1 m PB. The brains were removed, postfixed at 4 °C in the paraformaldehyde perfusion solution for 2 h and in 20% sucrose in 0.1 M PB for 2 d. Brain were cut into 40 μm sections on a cryostat (CM1800, Leica instrument GmbH., Germany). Sets of each fifth hindbrain [~17 to 11 mm posterior to bregma (Paxions and Watson., 1998)] and forebrain [~ 0.9 to 3.6 mm posterior to bregma (Paxions and Watson., 1998)] sections were stored in cryoprotectant solution (a 4:3:3 mixture of 0.1 m PB, ethylene glycol, and glycerol; Sigma) at -20 °C.

Immunohistochemistry staining (IHC) of cFos from the brain section was performed using our previous protocol (Thammacharoen et al., 2008). Briefly, free-floating brain sections incubated for 10 min each in 0.5% H_2O_2 solutions. After 3 times washed with 0.1 M PB, the blocking and detecting process were done with 1 h incubation in 1% normal goat serum in 0.1 m PB 0.3% Triton X-100, and then overnight with rabbit polyclonal cFos antibody (Ab5, 1:10,000; EMD chemical, Inc., CA, USA). Sections were then washed and incubated with biotinylated antirabbit goat antibody (1:300; BA1000, Vector laboratory, CA) and avidin-biotin complex (1:300; PK-6100, Vector laboratory, CA), for 1 h each. ABC-cFos complex was visualized with DAB peroxidase complex reaction (SK-4105, Vector laboratory, CA, USA). Finally, sections were mounted on gelatinized microscope slides, coverslipped, dried, and digitally imaged. The numbers of ERα positive neurons were counted within the following areas

of interest using templates based on the atlas of Paxinos and Watson (1998): NTS (NTS subregion nomenclature is our own (Thammacharoen et al., 2008); locations are millimeters caudal to bregma), caudal NTS (cNTS: about 14.1–14.4 mm); subpostremal NTS (spNTS; about 13.7–14.0 mm); POA (0.4 mm posterior to bregma), Arc (2.3 mm posterior to bregma) and VMH (2.3 mm posterior to bregma). Cells were considered cFos positive if their nuclei contained punctate brown-black immunolabeling and were counted using constant minimum and maximum OD and object size criteria, which were validated simultaneously with visual counts.

Brain microdissection and CRH measurement

In the experiment 2b, we investigated the effect of PPT on brain CRH. Palkovit's microdissection technique was used to isolate the interested nuclei from frozen brain with modification (Plamondon and Merali.,. 1997). Briefly, the individual brain was freshly removed from the skull and immediately frozen under -80 °C dry ice. An individual nucleus isolation was sampled using needle biopsy (i.d. 1 mm and 0.5 mm) and serial section technique with cryostat (CM1800, Leica instrument GmbH., Germany). With the atlas of rat brain (Paxinos and Watson., 1998), frozen brain was adjusted for both horizontal and vertical planes before pre-sampling cut. The range where nuclei were sampled from frozen forebrain and hindbrain were -0.3 to -3.6 mm and -14.6 to -13.6 mm from bregma. Serial sections of frozen forebrain were cut until the beginning of target nucleus and the sampling was performed at the range as follows: medial preoptic nucleus (MPO, -0.3 to -1.3 mm bregma), paraventricular nucleus (PVN, -1.3 to -1.88 mm bregma), ventromedial hypothalamic nucleus (VMH, -2.12 to -3.6 mm bregma), medial eminence and arcuate nucleus of hypothalamus (Me and Arc, -2.12 to -3.3 mm bregma), central amygdala nucleus (CeA, -1.6 to -3.14 mm bregma).

Serial sections of frozen hindbrain were cut until the beginning of caudal NTS (-14.6) and the sampling was collected. Frozen sample was put into pre-weighting microtube, weighed and immersed in 250 µl 0.5 M acetic acid. The samples were boiled for 10 min, homogenized and centrifuged (2710 xg 10 min). The supernatant were then collected, dried and stored in -80 °C for CRH measurement.

CRH EIA kits (EK-019-06, Phoenix Pharmaceuticals, Inc., Belmont, CA, USA) were used to measured brain CRH protein according to the manufacturer's protocol. The linear range of this kit was 0.33-3.73 ng/ml. Intra- and inter-assay variability were 7.2 and 6.9 %, respectively. The quantitative level of brain CRH was expressed as pg/mg wet weight.

The fourth ventricle cannulation, infusion and verification

The fourth ventricle intracerebroventricular cannulation (the 4th icv) was operated to study the hindbrain infusion of CRH antagonist (α-Helical CRF (9-41): C2917, Sigma-aldrich Co., MO, USA) on FI effect of PPT (experiment 3). Seven days after OVX, rats were anesthetized with intraperitoneally pentobarbital sodium (50 mg/kg ip, Nembutal®, Ceva Santa Animal, France). A guide cannula (22 G, PlasticsOne, Roanoke, VA, USA) was stereotaxically positioned into the fourth ventricle. The cannula tip was placed 3.5 mm posterior to the interaural line, 1.4 mm lateral to midline and 6.2 mm ventral to the skull surface (Blevins et al., 2004). The cannula was fixed to the skull with stainless steel mounting screw and dental cement. The guided cannula was attached to the osmotic pump (Alzet Model 1002, reservoir volume 100 µl) contained 0.16 µg/µl C2917. The C2917 concentration was used because of the ability to antagonize BDNF anorectic effect (Toriya et al., 2010). After

surgery, rat was received antibiotic and analgesic as described previous. FI (± 0.1 g, corrected for spillage) and BW (± 1 g) were measured daily throughout the experimental period.

At the end of experiment, all rats were killed by intravenously injection with the high dose of pentobarbital sodium (65 mg/kg ip, Nembutal®, Ceva Santa Animal, France). To verify the cannula placement in the second experiment, 5 µl of Evans blue was slowly injected through the ICV cannula. After the cannula was carefully removed, the brain was isolated and frozen. The frozen brain was sectioned to confirm cannula tip and dye diffusion.

Experiment 1: The acute effect of PPT on food intake in OVX rats

The first experiment (experiment 1a) aimed at demonstrating the rapid effect of PPT on FI and BW in OVX female rat. In this experiment, PPT (Tocris cookson Ltd, Bristol, UK) was dissolved in sesame oil (Sigma-aldrich Co., MO, USA). All rats were ovariectomized after 1 week of adaptation to the housing condition and management. Four days after OVX all rats were injected subcutaneously with 2 µg/rat estradiol benzoate (EB). This injection was done to synchronize the estrogenic property of all rats until surgical recovery (Thammacharoen et al., 2009). Four days after the first EB injection rats were divided into 3 groups of 7 animals each. On this test day, 0.1 ml of PPT (75 and 100 µg) or vehicle (sesame oil) was subcutaneously injected at the onset of dark phase (1300). Food intake was measured at 3, 6 and 24 hours after injection. Because PPT effect on FI in the experiment 1a was later than that had been reported (Thammacharoen et al., 2007 and Santollo et al., 2007) and because we need to see the effect of PPT during early period of dark phase, we do another experiment by changing the injection time from the onset of dark phase to the midlight phase (0700). OVX rats

from experiment 1a were used in this additional experiment. Rats were observed for daily FI and body weight for 8 days. On day 4 of this period, all rats were injected with 2 μg of EB. On the experimental day (day 8), 0.1 ml of PPT (100 μg) or vehicle (sesame oil) was subcutaneously injected at the midlight phase (0700). Food intake was measured at the period of 5 h before dark onset (0730-1230) and at the period of 3, 6 and 24 hours after dark onset.

We have performed the separated experiment (experiment 1b) of PPT and EB effect on FI to weather the discrepancy effect of PPT from experiment 1a was due to the PPT preparation processes. Instead of dissolved in sesame oil, PPT was dissolved with dimethyl sulfoxide (DMSO, Fisher scientific, UK). In this experiment, a group of 8 rats received subcutaneous injection of PPT (100 μg), EB (2 μg) or vehicles at onset of dark phase (1300) according to the crossover design, with 5 days between trial. At the day of injection, food intake was measured at 3, 6 and 24 hours. In addition, daily FI was measured everyday throughout the experimental period. This EB injection was done according to our previous model showed that EB treatment could mimic both plasma estradiol and eating behavior in intact female rat (Asarian and Geary., 2002). Rats weighed 26s7 ± 11.5 g at the beginning of testing and 275 ± 10.7 g at the ending of experiment.

Experiment 2: The effect of PPT on brain cFos and CRH

The second experiment was performed to investigate the effect of peripheral PPT injection on cFos expression and CRH concentration in the brain. To investigate PPT induced cFos expression in the brain (experiment 2a), OVX rats were trained to fast 10 hours (from 0700 to 1700) before injection at the onset of dark phase (1700). This training period aim at reducing the interference of ingestion to and the background

of cFos in the brain. On the experimental day, OVX rats received subcutaneously injection with PPT or vehicle control at dark onset. After injection, rats were left in their cages without food for 90 min. Rats were then deeply anesthetized and perfused to collect brain for cFos immunohistochemistry staining as described above.

To investigate PPT injection on brain CRH (experiment 2b), the experimental paradigm was performed according to the experiment 1b. Briefly, OVX rats received subcutaneously injection with PPT or vehicle control at dark onset. Food cups were provided to the cage immediately after injection was finished. Rats were allowed to eat for 3 hours. This time point was selected according to the effect of PPT on FI in the experiment 1b. Rat was decapitated with guillotine. Trunk blood was collected immediately and their brains was quickly removed, separated fore and hindbrain, at the posterior end of cerebral cortex (approximately 8 mm. posterior to bregma), and kept under -80 °C until CRH measurement as described previously. Plasma from trunk blood sample was separated and kept at -20 °C until adrenocorticotropic hormone (ACTH) measurement using chemiluminescent immunoassay (LKAC1, Immulite 1000 systems, Siemens, USA) with intraassay coefficient of variation of 5.08%.

Experiment 3: The fourth ventricle continuous infusion of CRH antagonist on PPT effect on food intake

The third experiment was performed to investigate the effect of hindbrain CRH receptor (CRHR) blockage on the PPT effect of FI via the 4th icv CRH antagonist (α-Helical CRF (9-41): C2917, Sigma-aldrich Co., MO, USA). The infusion was done over a period of 14 days as depicted in Fig 1. OVX rat (n = 9 per group) was cannulated directly to the 4th ventricle and connected with the OP contained either C2917 or vehicle

(normal saline). This yielded 2 treatment hindbrain infusion groups; C2917 and control. Rats were allowed 5 days to recover from surgery. At the onset dark phase (1300) of day 6 postsurgery, half the rats from each group was injected subcutaneously with PPT, another half was injected with DMSO as vehicle control. Injections were reversed on day 11 postsurgery. With this within-subject design, each group received single injection of PPT. FI and BW were monitored throughout the experimental period.

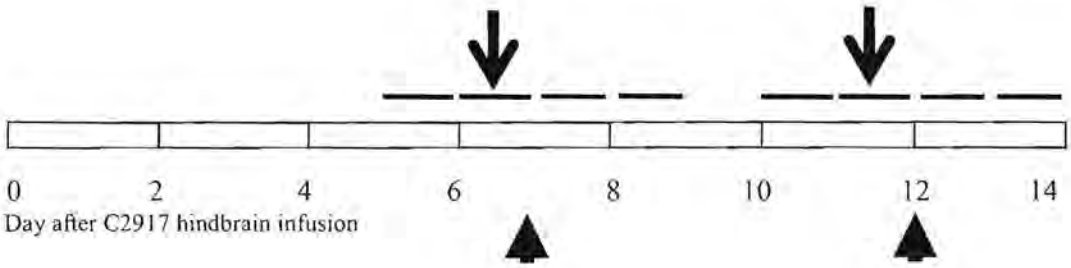

Figure 1 Design for experiment 3. Continuous C2917 hindbrain infusion used to test if C2917 could attenuate the eating inhibitory effect of PPT. Rat was performed 4th icv and started C2917 infusion at day 0. According to the 4-day cycle treatment of EB (Asarian and Geary., 2006), Either PPT or vehicle was administrated on day 6 and 11 of C2917 infusion period. This injection time supposed to be the second day of treatment cycle (arrows that indicate on the second broken line). PPT effects on food intake were expected on the night after injection (arrow heads).

Statistical analysis

Data from the experiment that contain either multiple time points or 2 factors were analyzed using one way or two way analysis of variance (ANOVA) followed by Bonferroni posttest. Data of two experimental groups were compared with student t test. Significant main effects were followed up with pair wise comparisons using Bonferroni posttest. All data were presented as mean ± SEM.

RESULTS

Experiment 1: The acute effect of PPT on food intake in OVX rats

In experiment 1a, injection of PPT (both 75 and 100 μg in sesame oil) during the onset of dark phase tend to decrease 24 hr FI (Fig 2a, $F_{2,18}$= 2.71, P=0.09). However, PPT failed to decrease FI at 3 and 6 hr after injection (Fig 2a, $F_{2,18}$= 0.57 and 0.93, P>0.05 respectively). When the injection (PPT 100 μg in sesame oil) was done at the mid light phase, FI from the first 5 h (before dark onset) and from 3 h after dark onset were not significantly difference (Fig2b, t_{18} = 0.11 and 1.17, P>0.05 respectively). Importantly, there were significantly different in FI between control and PPT treatment groups at 6 hr after dark phase and 24 hr later (Fig2b, t_{18} = 3.70 and 3.33, P<0.05 respectively).

Further, the additional experiment (experiment 1b) was performed to investigate whether the delay effect of PPT injection in experiment 1a was due to the processes of PPT preparation. In this experiment, we also added EB injection group (2 μg/rat sc) as an additional control. When PPT was dissolved in DMSO and the injection (vehicle, PPT or EB) was done at the onset of dark phase, PPT ,but not EB, decreased FI at 3-6 and 24 hr after injection (Fig3a, $F_{2,21}$= 6.37 and 6.26, P<0.05 respectively). On the second day after injection (day3 of the experiment), daily FI from PPT and EB injected groups were lower than from vehicles treatment group (Fig3b, t_{14}= 3.04 and 3.03, P<0.05 respectively). Both PPT and EB treatment in the current experiment had no effect on body weight across experimental period (Fig3c, $F_{2,63}$= 3.12, P>0.05).

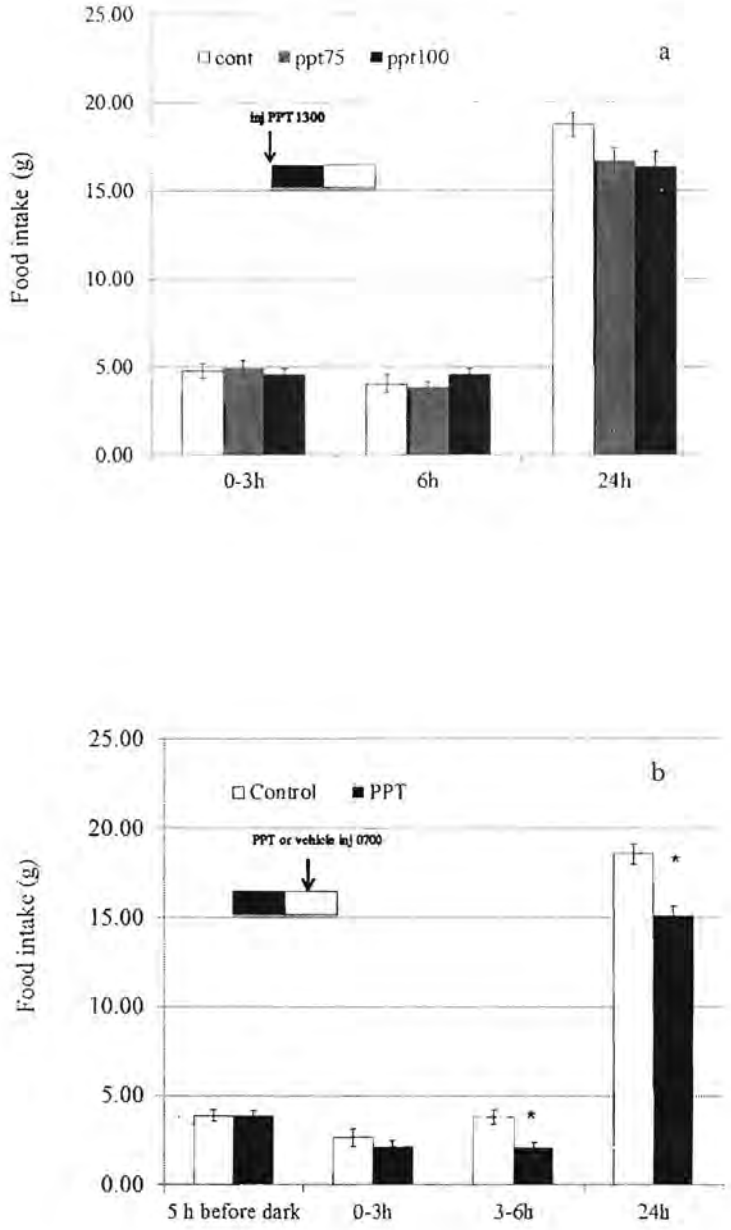

Fig 2 Both 75 and 100 μg/rat of PPT (dissolved in sesame oil) failed to decreased food intake when injection was done at the onset of dark (a) However, when the injection was done at mid-light phase PPT (at 100 μg/rat) decreased food intake significantly at 3-6 h after dark onset (b) * significant lower food intake, $P<0.05$.

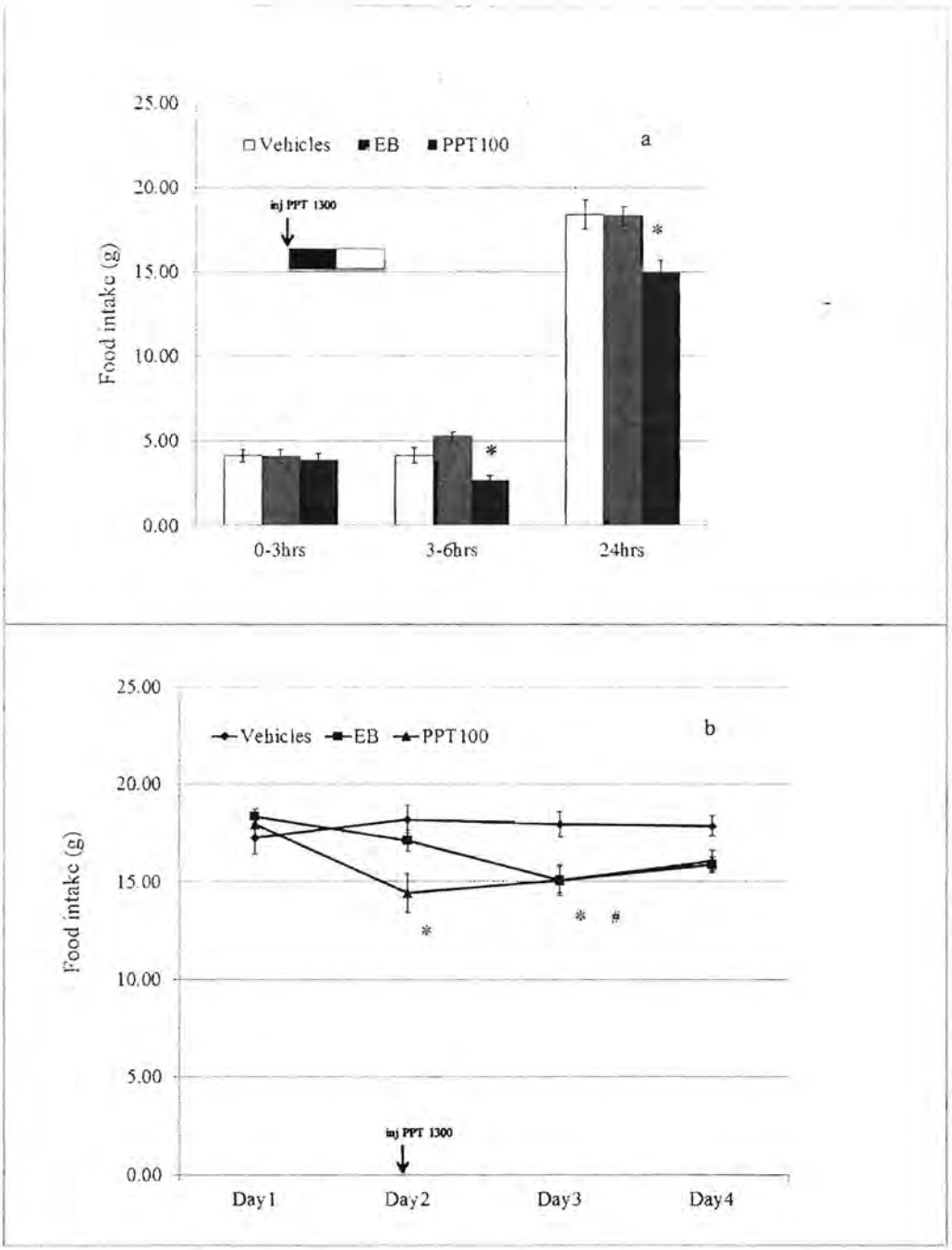

Fig 3 When PPT was dissolved in DMSO and the injection was done at the beginning of dark onset, PPT (100 μg/rat) but not EB decreased food intake 3 and 24 h after treatment (a), PPT and EB decreased food intake significantly at day 3 of treatment cycle (b). * Significantly lower food intake in PPT treated group, $P<0.05$; # Significance lower food intake in EB treated group.

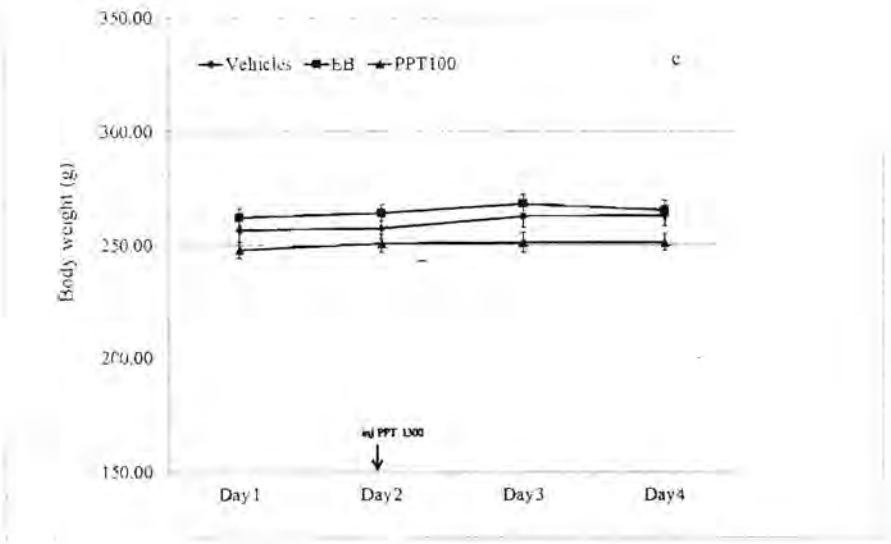

Fig 3c PPT and EB didn't affect body weight across the experiment period

Experiment 2: The effect of PPT on brain cFos and CRH

The effect of PPT on neuronal activation was investigated from both fore- and hindbrain. There were no significant different of cFos immunoreactive cells between control and PPT treatment groups from any forebrain nuclei including: PVN, Arc, MPA, VMH and CeA (Fig 4, t_{14}= 0.57, 0.09, 0.11, 0.88 and 0.09 respectively, P>0.05). Because the number of cFos immunoreactive cells from hindbrain NTS were sparse, an analysis of cFos from this area couldn't be done.

The effect of PPT on brain CRH was investigated at the same period when PPT produced its effect on FI, 3 hr after injection. In the forebrain, PPT couldn't change brain CRH at both PVN and ME/Arc (Fig5, t_9= 1.38 and t_{14}= 1.0, P>0.05 respectively). PPT couldn't change brain CRH at MPA, VMH and CeA as well (Fig5, t_{13}= 0.60, t_{11}= 0.52 and t_{13}= 1.67, P>0.05 respectively). However, brain CRH at NTS from PPT treatment group was higher than from vehicle group (Fig5, t_{11}= 3.06, P<0.05). In this experiment, trunk blood was collected at the same time point to measure plasma ACTH.

There was lower plasma concentration of ACTH in PPT treated group (42.66 ± 3.24 pg/ml) than in control group (57.38 ± 3.814 pg/ml; $t_{14} = 2.94$, $P<0.05$).

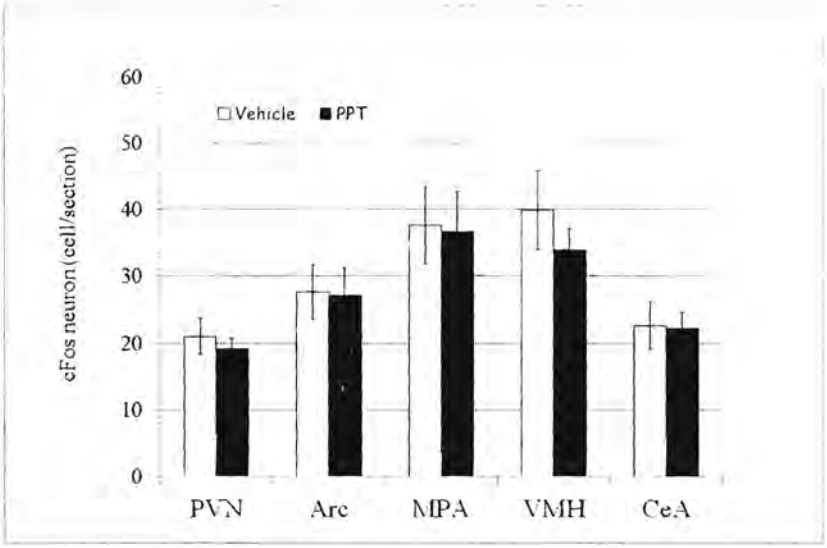

Fig 4 The effect of PPT on c-Fos expression at hypothalamic nuclei and central amygdala nucleus. PPT didn't affect c-Fos expression at any brain nuclei.

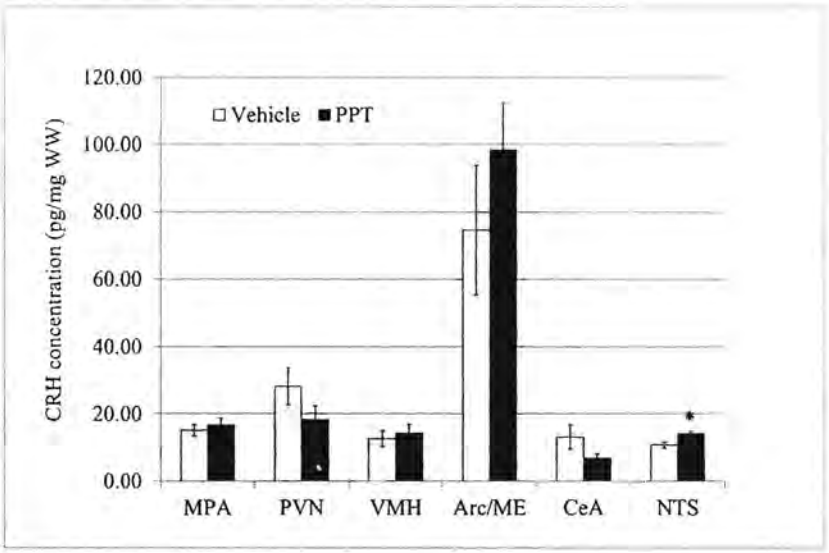

Fig 5 The effect of PPT on brain CRH concentration (pg/ mg tissue wet weight). PPT treatment didn't influence brain CRH at any hypothalamic and central amygdala nuclei. However, there was significant higher in CRH level in PPT treated group at hindbrain NTS. * significant difference of CRH level, $P<0.05$.

Experiment 3 The fourth ventricle continuous infusion of CRH antagonist on PPT effect on food intake

Because PPT effect on FI was associated to an increase in brain CRH at NTS area, the third experiment was performed to investigate whether infusion of CRH antagonist (C2917) to this area via the 4th icv could attenuate PPT effect on FI. In this experiment, PPT decreased 24 hr FI after injection (Fig6c, $F_{1,16}$ = 12.87, P<0.05) but not FI from 0-6 h after injection (Fig6a and 6b, $F_{1,16}$= 0.74 and 2.54, P>0.05). However, infusion of C2917 to hindbrain couldn't attenuate the effect of PPT on 24 hr FI (Fig6c, t_8= 0.28, P>0.05).

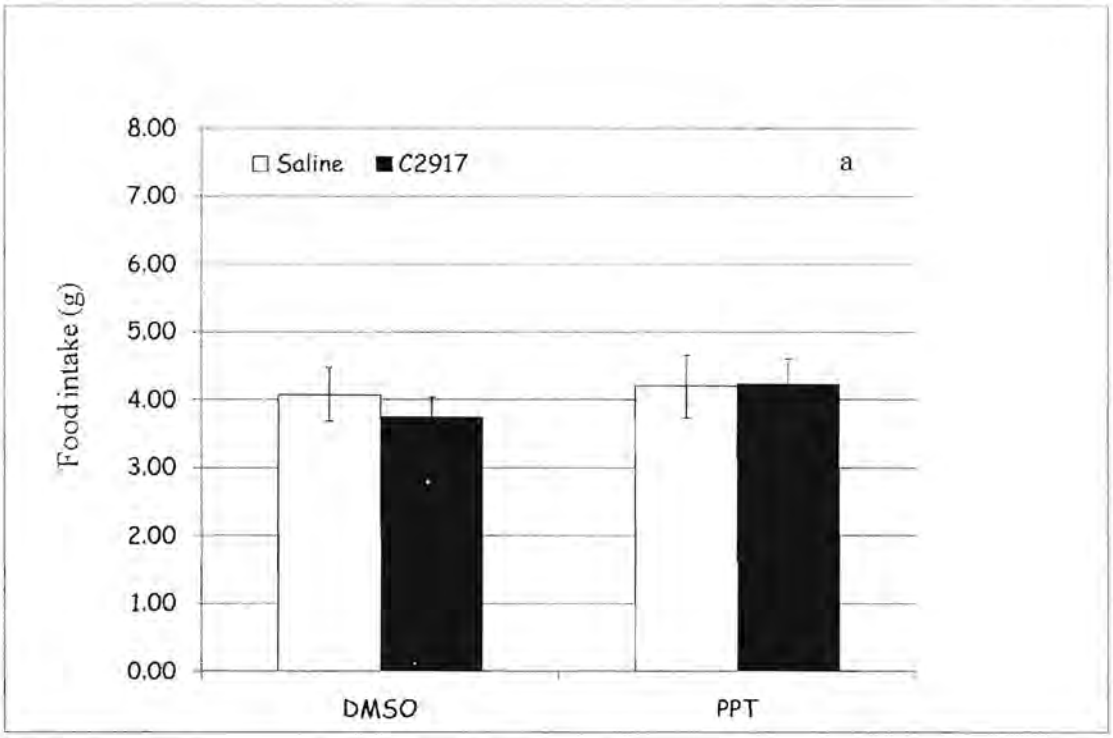

Fig 6a Effect of C2917 on PPT eating inhibitory effect. PPT and continuous infusion of C2917 had no effect on 3 h food intake (a).

DISCUSSION

Estrogens inhibit eating in female animals by activating ER in the brain. It has been shown previously that activation ER by specific ERα agonist, PPT, decreases FI faster than by EB (Santollo et al., 2007 and Thammacharoen et al., 2007) and the effect apparently mediate ERα activated CRH neuronal activation (Thammacharoen et al., 2009). The current results confirmed and extend previous knowledge that anomalously PPT's eating inhibitory effect apparently mediate by the activation of brain CRH neurons. New finding here is that PPT activated brain CRH level at hindbrain NTS.

Instead of the rapidly PPT's eating inhibitory effect, we got an unclear effect of PPT on FI. It has been shown by 2 separated research groups that the onset of PPT effect on FI was approximately 2 to 6 hr (Santollo et al., 2007 and Thammacharoen et al., 2007). In the first experiment (experiment 1a), not only PPT produced unclear effect on 24 hr FI, but PPT failed to decrease in FI during 3-6 h after injection (Fig 2a). When the injection was done earlier (Fig2b), the onset of PPT (approximately 9 h after treatment) was later than that was reported previously (Santollo et al., 2007 and Thammacharoen et al., 2007). This unexpected result made us to recheck our conditions and PPT preparation. The only difference method that may be the cause of unexpected result of PPT is the preparation processes. Previously, PPT was dissolved in sesame oil under mild heat condition (not exceed 60 °C for 10 min, Thammacharoen et al., 2007 and Thammacharoen et al., 2009). However in experiment 1a, PPT was inadvertently left under heat for more than 30 min. Because this problem was not our research objective, we performed another experiment using DMSO as PPT solvent (Santollo et al., 2007). It was clear in experiment 1b that when injection was done at the onset of dark phase PPT dissolved in DMSO, but not EB, decreased FI with the onset of 3 h.

The later experiments were then used this condition to investigate the possible mechanism of PPT effect on FI.

Previously, we demonstrated that PPT activated c-Fos expression at PVN, CeA and hindbrain NTS (Thammacharoen et al., 2009). However, the results were derived from the condition that the animal accessed to food before sacrifice. In the current experiment, we aimed to investigate the effect of PPT alone to activate brain c-Fos expression. The results indicated that when the animals didn't access to food PPT didn't affect the number of c-Fos at any brain nuclei. It was well accepted that estradiol and PPT could potentiate neuronal activation in several eating induced cFos paradigms (Eckel and Geary., 2001; Ekcel et al., 2002; Asarian and Geary., 2007; Thammacharoen et al., 2009; Chi et al., 2011;). However, it is clear from the current results that an activation of ERα by PPT per se couldn't induce c-Fos expression. Because PPT decreased plasma ACTH (see below discussion), it suggested that PPT should at least influence some stress related nuclei. All in all, we argued 2 possible explanations that without eating the activation of ERα by PPT didn't induce c-Fos expression. Second, c-Fos immunohistochemistry technique is unable to indicate the brain site(s) that is stimulated after PPT per se.

Brain CRH appears to play the role not only in eating related stress condition but also in normal eating condition (Richard., 1993; Smagin et al., 1998; Heinrichs and Richards., 1999; Richards et al., 2002). Estradiol has been shown to modulate CRH expression in both hypothalamic and extrahypothalamic area (Bohler et al., 1990; Pelletier et al., 2007; Jasnow et al., 2006; Broad et al., 1995). The hypothalamic CRH mRNA expression increased just before the surge of LH during estrous cycle (Bohler et al., 1990). Likewise, the hypothalamic CRH mRNA expression decreased in OVX female rat and E2 replacement could restore the OVX effect within 12 hr (Pelletier et

al., 2007). The hypothesis that brain CRH participates in eating inhibitory effect of ER activation was first proposed by using CRH receptor antagonist in exogenous E_2 treatment (Dagnault et al., 1993). The potential mechanism of brain CRH on eating inhibitory effect of ER activation was highlighted later by our results demonstrating that the eating inhibitory effect of PPT was associated with activation CRH neuron at the hypothalamic PVN (Thammacharoen et al., 2009). We demonstrated further in the current experiment that PPT which produced rapid effect on FI increased CRH at hindbrain NTS but not at any forebrain nuclei. Two possible sources of CRH were apparently accounted for an increase in CRH at hindbrain NTS. First, it is well accepted that CRH neuron from PVN as the HPA axis provides efferent outflow mainly to ME. There was evidence that CRH neuron, especially at autonomic part from this area, provides efferent fiber the NTS as well (Swanson and Kuypers., 1980; Palkovits., 1999; Aguilera and Liu., 2012;). Second, there was also CRH producing neuron located locally at the hindbrain NTS (Merchanthaler., 1984; Morin et al., 1999; Swanson and Kuypers., 1980). Previously, the hindbrain CRH has been shown to participate in CCK and leptin induced anorexia (Blevins et al., 2003; Uehara et al., 1998). Because we showed in the first evidence that PPT activated CRH neuron at the PVN (Thammacharoen et al., 2009), an increase in CRH at the hindbrain NTS appeared to came from the fiber of CRH neuron at PVN that innervated NTS. However, our current results could not exclude the possibility that PPT activated CRH neuron located at the NTS. The mechanism by which brain CRH inhibits eating has been focused at hypothalamic and limbic area (Krahn et al., 1988; Benoit et al., 2000; Ciccocioppo et al., 2003). Paraventricular nucleus of hypothalamus appeared to be an important site of eating inhibitory effect of CRH (Krahn et al., 1988; Arase et al., 1989; Benoit et al., 2000). However, the activation of CRH receptor at the caudal brainstem has been

demonstrated to decrease FI as well (Grill et al., 2000). Moreover, infusion of hindbrain CRHR antagonist could attenuate the anorectic effect of restraint stress (Miragaya et al., 2008). Taken together, both PVN and hindbrain NTS (see Grill et al., 2000) could be the area where CRH mediate inhibitory effect on FI. By demonstrating that PPT increased CRH level only at NTS but not at PVN, the current experiment provided the mechanism for the eating inhibitory effect of PPT that apparently related to increase in CRH at hindbrain NTS.

We showed in the current experiment that plasma ACTH from PPT treated animals was significantly lower than from control animals. The effect of ER activation on HPA axis was investigated extensively (Bao et al., 2008; Weiser and Handa., 2009; Young and Korszun., 2010). In female rat, the effect of E_2 to modulate stress induced plasma ACTH has been shown to depend on dose and preparation of exogenous E_2. Basically, chronic E_2 treatment, but not endogenous rising of E2 (Atkinson and Waddell., 1997), has been shown to increase diurnal plasma ACTH and abolish the effect of dexamethasone to suppress ACTH (Viau et al., 1991; Redei et al., 1994; Young et al., 2001; Figueiredo et al., 2006; Dayas et al., 2000). The present results demonstrated that PPT which is pure ERα agonist apparently suppressed plasma ACTH, HPA axis, within short period after treatment (3 h). Because our current PPT effect was faster than and the condition was different from that has been report, it is difficult at this stage to discuss the mechanism by which PPT acutely decreases plasma ACTH. Moreover, it is remained to be investigated whether or not PPT injection within this period influences behavioral responses to stress?

If the eating inhibitory effect of PPT could be mediated via hindbrain CRH, we probably could antagonize this effect using CRHR antagonist infused direct to

hindbrain by 4th icv. Unfortunately, we demonstrated in the current experiment that hindbrain infusion of α-Helical CRF (9-41) fail to attenuate the eating inhibitory effect of PPT. Alpha-Helical CRF (9-41), a non-selective competitive antagonist of CRHR, has been shown to antagonize stress induced anorexia in several paradigms (Hotta et al., 1999; Miragaya et al., 2008). In addition, the substance also antagonized the inhibitory effect of E2 treatment (Dagnault and Richard., 1997). Previously, the antagonized property of α-Helical CRF (9-41) has been shown to depend on the dose and the ratio of α-Helical CRF (9-41) and CRH (Baram et al., 1996; Gert et al., 1998; Miragaya et al., 2008). Moreover, the antagonized effect of α-Helical CRF (9-41), as well as others CRHR antagonists, to stress induced anorexia was demonstrated using acute infusion (Hotta et al., 1999; Miragaya et al., 2008; more AS30). The failure of continuous infusion of α-Helical CRF (9-41) to antagonize PPT eating inhibitory effect probably came from such reasons. However, it should be noted at this point that the similar dose of α-Helical CRF (9-41) continuous infusion have been reported to antagonize effect of BDNF on FI (Toriya et al., 2010). Since the current results couldn't indicate the potential role of hindbrain CRH with the inhibitory effect of PPT, the additional experiments need to be performed to test this hypothesis.

CONCLUSION & PERSPECTIVES

We report here the potential physiological mechanism of brain ERα activation that produces rapid onset on eating. PPT decreased eating and plasma ACTH while increased hindbrain NTS CRH concentration. Our speculation is that the rapid eating inhibitory effect of PPT appears to mediate in part by activate the specific population of CRH neurons at the PVN that provide the efferent fiber to hindbrain NTS. However, if this is true, it should be kept in mind that one should find the ways and doses to antagonize PPT effect using CRHR antagonists directly to hindbrain. Perhaps an acute administration of C2917 before PPT treatment is the most relevant experiment. The conclusions described here present a number of challenged questions that need for further experiments. First, if PPT increased hindbrain CRH at the similar time of eating inhibition, whether E_2 increased hindbrain CRH at the second night after treatment as well? Second, since the CRH neurons located at the PVN have been well characterize, the investigation of CRH population at the PVN activated by PPT needs to be identified. Together with this question, it is important to identify to ERα positive neuron that is the target of PPT. Although it is difficult to identify the activated neuron using PPT induced c-Fos paradigm, we still think that the tracing experiments aimed to identify the neural connection from CRH neuron at PVN to NTS. Finally, not only PPT produces rapid effect on food intake, we showed in the current experiment that PPT decreased plasma ACTH rapidly as well. The result suggested that PPT could modulate HPA axis in different mechanisms as E_2. This idea paves the way for the future experiments of ER activation and stress responses.

REFERENCES

Aguilera G, Liu Y. 2012. The molecular physiology of CRH neurons. Front Neuroendocrinol. 33(1). 67-84.

Arase K, Shargill NS, Bray GA. 1989. Effects of intraventricular infusion of corticotropin-releasing factor on VMH-lesioned obese rats. Am J Physiol.256(3 Pt 2). R751-756.

Arbogast LA. 2008. Estrogen genomic and membrane actions at an intersection. Trends Endocrinol Metab. 19(1). 1-2.

Asarian L. 2006. Membrane estrogen receptors and energy homeostasis. The Journal of Neuroscience. 26(44). 11255-11256.

Asarian L and Geary N. 1999. Cyclic estradiol treatment phasically potentiates endogenous cholecystokinin's satiating action in ovariectomized rats. Peptides. 20. 445-450.

Asarian L and Geary N. 2002. Cyclic estradiol treatment normalized body weight and restores physiological patterns of spontaneous feeding and sexual receptivity. Horm Behav. 42. 461-471.

Asarian L and Geary N. 2006. Modulation of appetite by gonadal steroid hormones. Phil. Trans. R. Soc. B. 361. 1251-1263.

Asarian L and Geary N. 2007. Estradiol Enhances Cholecystokinin-Dependent Lipid-Induced Satiation and Activates Estrogen Receptor-{alpha}-Expressing Cells in the Nucleus Tractus Solitarius of Ovariectomized Rats. Endocrinology. 148(12). 5656-566.

Atkinson HC, Waddell BJ. 1997. Circadian variation in basal plasma corticosterone and adrenocorticotropin in the rat: sexual dimorphism and changes across the estrous cycle. Endocrinology. 138(9). 3842-3848.

Bao AM, Meynen G, Swaab DF. 2008. The stress system in depression and neurodegeneration: focus on the human hypothalamus. Brain Res Rev. 57(2). 531-53.

Baram TZ, Koutsoukos Y, Schultz L, Rivier J. 1996. The effect of 'Astressin', a novel antagonist of corticotropin releasing hormone (CRH), on CRH-induced seizures in the infant rat: comparison with two other antagonists. Mol Psychiatry. 1(3). 223-226.

Behl C. 2002. Oestrogen as a neuroprotective hormone. Nat Rev Neurosci. 3(6). 433-442.

Benoit SC, Thiele TE, Heinrichs SC, Rushing PA, Blake KA, Steeley RJ. 2000. Comparison of central administration of corticotropin-releasing hormone and urocortin on food intake, conditioned taste aversion, and c-Fos expression. Peptides. 21(3). 345-351.

Berridge KC and Robinson TE. 2003. Parsing reward. Trends Neurosci. 26(9). 507-513.

Berthoud HR. 2004. Mind versus metabolism in the control of food intake and energy balance. Physiology & Behavior. 81. 781-793.

Blaustein JD. 2008. An estrogen by any other name. Endocrinology. 149(6). 2697-2698.

Blevins JE, Eakin TJ, Murphy JA, Schwartz MW and Baskin DG. 2003. Oxytocin innervation of caudal brainstem nuclei activated by cholecystokinin. Brain research. 993. 30-41.

Blevins JE, Schwartz MW, Baskin DG. 2004. Evidence that paraventricular nucleus oxytocin neurons link hypothalamic leptin action to caudal brain stem nuclei controlling meal size. Am J Physiol Regul Integr Comp Physiol. 287(1):R87-96.

Bohler HC Jr, Zoeller RT, King JC, Rubin BS, Weber R, Merriam GR. 1990. Corticotropin releasing hormone mRNA is elevated on the afternoon of proestrus in the parvocellular paraventricular nuclei of the female rat. Brain Res Mol Brain Res. 8(3). 259-262.

Broad KD, Keverne EB, Kendrick KM. 1995. Corticotrophin releasing factor mRNA expression in the sheep brain during pregnancy, parturition and lactation and following exogenous progesterone and oestrogen treatment. Brain Res Mol Brain Res. 29(2). 310-316.

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA and Carlquist M. 1997. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature. 389(6652). 753-758.

Butera PC and Beikirch RJ. 1989.Central implants of dilute estradiol: independent effects on ingestive and reporductive behaviors of ovariectomized rats. Brain Res. 491. 266-273.

Butera PC, Campbell RB and Bradway DM. 1993. Antagonism of estrogenic effects on feeding behavior by central implants of anisomycin. Brain research. 624. 354-356.

Campfield LA and Smith FJ. 2003. Blood glucose dynamics and control of meal initiation: A pattern detection and recognition theory. Physiol rev. 83. 25-58.

Chambliss KL, Yuhanna IS, Anderson RG, Mendelsohn ME and Shaul PW. 2002. ERbeta has nongenomic action in caveolae. Mol Endocrinol. 16(5). 938-946.

Chaudri O, Small C and Bloom S. 2006. Gastrointestinal hormones regulating appetite. Phil Trans of The Royal Soc B. 361(1471). 1187-1209.

Chen Y and Heiman ML. 2001. Increased weight gain after ovariectomy is not a consequence of leptin resistance. Am J Physiol Endocrinol Metab. 280(2). E315-E322.

Chi JH, Narita K, Ichimaru T, Murata T. 2011. Estrogen Increases c-Fos expression in the paraventricular nucleus along with its anorexic effect in developing rats. J Reprod Dev. 57(3). 365-372.

Ciccocioppo R, Fedeli A, Economidou D, Policani F, Weiss F, Massi M. 2003. The bed nucleus is a neuroanatomical substrate for the anorectic effect of corticotropin-releasing factor and for its reversal by nociceptin/orphanin FQ. J Neurosci. 23(28). 9445-9451.

Clegg DJ, Riedy CA, Blake Smith KA, Benoit SC and Woods SC. 2003. Differential sensitivity to central leptin and insulin in male and female rats. Diabetes. 52. 682-687.

Clegg DJ, Brown LM, Woods SC and Benoit SC. 2006. Gonadal hormones determine sensitivity to central leptin and insulin. Diabetes. 55. 978-987.

Clegg DJ, Brown LM, Zigman JM, Kemp CJ, Strader AD, Benoit SC, Woods SC, Mangiaracina M and Geary N. 2007. Estradiol-dependent decrease in the orexigenic potency of ghrelin in female rats. Diabetes. 56(4). 1051-1058.

Collins P and Webb C. 1999. Estrogen hits the surface. Nat Med. 5(10). 1130-1131.

Couse JF and Korach K. 1999. Estrogen receptors null mice: What have we learned and where will they lead us? Endocrine rev. 20(3). 358-417.

Cummings DE and Overduin J. 2007. Gastrointestinal regulation of food intake. J Clin Invest. 117(1). 13-23.

Czaja JA and Goy RW. 1975. Ovarian hormones and food intake in female guinea pigs and rhesus monkeys. Horm Behav. 6(4). 329-349.

Dagnault A, Ouerghi D, Richard D. 1993. Treatment with alpha-helical-CRF(9-41) prevents the anorectic effect of 17-beta-estradiol. Brain Res Bull. 32(6):689-92.

Dagnault A and Richard D. 1994. Lesion of hypothalamic paraventricular nuclei do not prevent the effect of estrogen on energy and fat balance. Am J Physiol. 267. E32-E38.

Dagnault A and Richard D. 1997. Involvement of the medial preoptic area in the anorectic action of estrogen. Am J Physiol. 272. R311-317.

Dayas CV, Xu Y, Buller KM, Day TA. 2000. Effects of chronic oestrogen replacement on stress-induced activation of hypothalamic-pituitary-adrenal axis control pathways. J Neuroendocrinol. 12(8). 784-794.

De Vries J, Strubbe JH, Wildering WC, Gorter JA and Prins AJ. 1993. Patterns of body temperature during feeding in rats under varying ambient temperatures. Physiology & Behavior. 53(2). 229-235.

Delaunay F, Pettersson K, Tujague M and Gustafsson JA. 2000. Functional differences between the amino-terminal domains of estrogen receptors alpha and beta. Mol Pharmacol. 58(3). 584-590.

Eckel LA and Geary N. 1999. Endogenous cholecystokinin's satiating action increase during estrus in female rats. Peptides. 20. 451-456.

Eckel LA, Houpt TA and Geary N. 2000. Spontaneous meal patterns in female rats with and without access to running wheels. Physiology &Behavior. 70. 397-405.

Eckel LA and Geary N. 2001. Estrogen treatment increase feeding-induced c-Fos expression in the brain of ovariectomized rats. Am J Physiol. 281. R738-46.

Eckel LA, Houpt TA, and Geary N. 2002. Estradiol replacement increases CCK-induced c-Fos expression in the brains of ovariectomized rats. Am J Physiol. 283. R1378-85.

Even P and Nicolaidis S. 1985. Spontaneous and 2DG induced metabolic changes and feeding: the ischymetric hypothesis. Brain Res Bull. 15(4). 429-35.

Figueiredo HF, Ulrich-Lai YM, Choi DC, Herman JP. 2006. Estrogen potentiates adrenocortical responses to stress in female rats. Am J Physiol Endocrinol Metab. 292(4). E1173- E1182.

Filardo EJ and Thomas P. 2005. GPR30: a seven-transmembrane-spanning estrogen receptor that triggers EGF release. Trends Endocrinol Metab. 16(8). 362-367.

Friend DW. 1971. Self-selection of feeds and water by swine during pregnancy and lactation. J Anim Sci. 32(4). 658-666.

Fu XD and Simoncini T. 2007. Non-genomic sex steroid actions in the vascular system. Semin Reprod Med. 25(3).178-186.

Funakoshi T, Yanai A, Shinoda K, Kawano MM and Mizukami Y. 2006. G protein-coupled receptor 30 is an estrogen receptor in the plasma membrane. Biochem Biophys Res Commun. 346(3). 904-910.

Gao Q, Mezei G, Nie Y, Rao Y, Choi CS, Bechmann I, Leranth C, Toran-Allerand D, Priest CA, Roberts JL, Gao X-B, Mobbs C, Shulman GI, Diano S and Horvath TL. 2007. Anorectic estrogen mimics leptin's effect on the rewiring of

melanocortin cells and Stat3 signaling in obese animals. Nature Medicine. 13. (1). 89-94.

Garcia-Segura LM, Diz-Chaves Y, Perez-Martin M and Darnaudéry M. 2007. Estradiol, insulin-like growth factor-I and brain aging. Psychoneuroendocrinology. Suppl 1.S57-61.

Geary N. 2001. Estrogen, CCK, and satiation. Peptides. 22. 1251-1263.

Geary N. 2004. The estrogenic inhibition of eating, In: Handbook of behavioural neurobiology, 2nd edit volume 14, Stricker EM and Woods SC, editors. NY: Kluwer academic. 307-345.

Geary N, Trace D, McEwen B and Smith GP. 1994. Cyclic estradiol replacement increases the satiety effect of CCK-8 in ovariectomized rats. Physiology & Behavior. 56(2). 281-289.

Geary N, Smith GP and Corp FS. 1996. The increased satiating potency of CCK-8 by estradiol is not mediated by upregulation of NTS CCK receptors. Brain Research. 179. 179-186.

Geary N, Asarian L, Korach KS, Pfaff DW and Ogawa S. 2001. Deficits in E2-dependent control of feeding, weight gain, and cholecystokinin satiation in ER-alpha null mice. Endocrinology. 142(11). 4751-4757.

Gerth A, Hatalski CG, Avishai-Eliner S, Baram TZ. 1998. Corticotropin releasing hormone antagonist does not prevent adrenalectomy-induced apoptosis in the dentate gyrus of the rat hippocampus. Stress. 2(3). 159-169.

Gong EJ, Garrel D and Calloway DH. 1989. Menstrual cycle and voluntary food intake. Am J Clin Nutr. 49(2). 252-258.

Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P and Chambon P. 1986. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature. 320(6058). 134-139.

Greene GL, Gilna P, Waterfield M, Baker A, Hort Y and Shine J. 1986. Sequence and expression of human estrogen receptor complementary DNA. Science. 231(4742). 1150-1154.

Grill HJ, Markison S, Ginsberg A, Kaplan JM. 2000. Long-term effects on feeding and body weight after stimulation of forebrain or hindbrain CRH receptors with urocortin. Brain Res. 867(1-2). 19-28.

Hall JM and McDonnell DP. 2005. Coregulators in nuclear estrogen receptor action: from concept to therapeutic targeting. Mol Interv. 5(6). 343-357.

Heinrichs SC, Richard D. 1999. The role of corticotropin-releasing factor and urocortin in the modulation of ingestive behavior. Neuropeptides. 33(5). 350-359.

Hotta M, Shibasaki T, Arai K, Demura H. 1999. Corticotropin-releasing factor receptor type 1 mediates emotional stress-induced inhibition of food intake and behavioral changes in rats. Brain Res. 823(1-2). 221-225.

Houpt KA, Coren B, Hintz HF and Hilderbrant JE. 1979. Effect of sex and reproductive status on sucrose preference, food intake, and body weight of dogs. J Am Vet Med Assoc. 174(10). 1083-1085.

Hrupka BJ, Smith GP and Geary N. 2002. Hypothalamic implants of dilute estrogen fail to reduce feeding in ovariectomized rats. Physiology &Behavio. 77. 233-241.

Jasnow AM, Schulkin J, Pfaff DW. 2006. Estrogen facilitates fear conditioning and increases corticotropin-releasing hormone mRNA expression in the central amygdala in female mice. Horm Behav. 49(2). 197-205.

Kalaitzidis D and Gilmore TD.2005. Transcription factor cross-talk: the estrogen receptor and NF-kappaB. Trends Endocrinol. Metab. 16(2). 46-52.

Kawata M, Matsuda K, Nishi M, Ogawa H and Ochiai I. 2001. Intracellular dynamics of steroid hormone receptor. Neurosci Res. 40(3). 197-203.

Kelly MJ and Rønnekleiv OK. 2008. Membrane-initiated estrogen signaling in hypothalamic neurons. Mol Cell Endocrinol. 290(1-2). 14-23.

King WJ and Greene GL. 1984. Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature. 307(5953). 745-747.

Klein DA and Walsh BT. 2004. Eating disorders: clinical features and pathophysiology. Physiol & Behavior. 81(2). 359-374.

Klinge CM. 2001. Estrogen receptor interaction with estrogen response elements. Nucleic acids Research. 29(14). 2905-2919.

Kow LM and Pfaff DW. 2004. The membrane actions of estrogens can potentiate their lordosis behavior-facilitating genomic actions. Proc Natl Acad Sci U S A. 101(33). 12354-12357.

Krahn DD, Gosnell BA, Levine AS, Morley JE. 1988. Behavioral effects of corticotropin-releasing factor: localization and characterization of central effects. Brain Res. 443(1-2). 63-69.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S and Gustafsson JA. 1996. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A. 93(12). 5925-5930.

Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM and Webb P. 2000. Estrogen receptor pathways to AP-1. J. Steroid Biochem. Mol. Biol. 74(5). 311-317.

Laflamme N, Nappi RE, Drolet G, Labric C and Rivest S. 1998. Expression and neuropeptidergic characterization of estrogen receptors (Eralpha and ERbeta) throughout the rat brain: anatomical evidence of distinct roles of each subtype. J Neurobiol. 36(3). 357-378.

Lavery DN and McEwan IJ. 2005. Structure and function of steroid receptor AF1 transactivation domains: induction of active conformations. Biochem. J. 391(Pt 3). 449-464.

Leung SW, Teoh H, Keung W and Man RY. 2007. Non-genomic vascular actions of female sex hormones: physiological implications and signalling pathways. Clin Exp Pharmacol Physiol. 34(8). 822-826.

Levin BE, Routh VH, Kang L, Sanders NM and Dunn-Meynell AA. 2004. Neuronal glucosensing: what do we know after 50 years? Diabetes. 53(10). 2521-2528.

Liang Y-Q, Akishita M, Kim S, Ako J, Hashimoto M, Iijima K, Ohike Y, Watanabe T, Sudoh N, Toba K, Yoshizumi M and Ouchi Y. 2002. Estrogen receptor β is involved in the anorectic action of estrogen. Int J of Obesity. 26. 1103-1109.

Linden A, Uvnas-Moberg K, Forsberg G, Bednar I and Sodersten P. 1990. Involvement of cholecystokinin in food intake: III. Oestradiol potentiates the inhibitory effect of cholecystokinin octapeptide on food intake in ovariectomized rats. Journal of Neuroendocrinology. 2. 797-801.

Luconi M, Francavilla F, Porazzi I, Macerola B, Forti G and Baldi E. 2004. Human spermatozoa as a model for studying membrane receptors mediating rapid nongenomic effects of progesterone and estrogens. Steroids. 69(8-9). 553-559.

Lyons PM, Truswell AS, Mira M, Vizzard J and Abraham SF. 1989. Reduction of food intake in the ovulatory phase of the menstrual cycle. Am J Clin Nutr. 49(6). 1164-1168.

Marty N, Dallaporta M and Thorens B. 2007. Brain glucose sensing, counterregulation, and energy homeostasis. Physiology (Bethesda). 22. 241-251.

McDonnell DP, Clemm DL, Hermann T, Goldman ME and Pike JW. 1995. Analysis of estrogen receptor function in vitro reveals three distinct classes of antiestrogens. Mol Endocrinol. 9(6). 659-669.

McEwan BS, Akama K, Alves S, Brake WG, Bulloch K, Lee S, Li C, Yuen G and Milner TA. 2001. Tracking the estrogen receptor in neurons: implications for estrogen-induced synapse formation. Proc Natl Acad Sci U S A. 98(13). 7093-7100.

Mendelsohn ME. 2000a. Mechanisms of estrogen action in the cardiovascular system. J Steroid Biochem Mol Biol. 74(5). 337-343.

Mendelsohn ME. 2000b. Nongenomic, ER-mediated activation of endothelial nitric oxide synthase: how does it work? What does it mean? Circ Res. 87(11). 956-960.

Mendez P, Azcoitia I and Garcia-Segura LM. 2005. Interdependence of oestrogen and insulin-like growth factor-I in the brain: potential for analysing neuroprotective mechanisms. J Endocrinol. 185(1). 11-17.

Merchenthaler I. 1984. Corticotropin releasing factor (CRF)-like immunoreactivity in the rat central nervous system. Extrahypothalamic distribution. Peptides. 5 Suppl 1. 53-69.

Milner TA, McEwen BS, Hayashi S, Li CJ, Reagan LP and Alves SE. 2001. Ultrastructural evidence that hippocampal alpha estrogen receptors are located at extranuclear sites. J Comp Neurol. 429(3). 355-371.

Milner TA, Ayoola K, Drake CT, Herrick SP, Tabori NE, McEwen BS, Warrier S and Alves SE.2005. Ultrastructural localization of estrogen receptor beta

immunoreactivity in the rat hippocampal formation. J Comp Neurol. 491(2). 81-95.

Miragaya JR, Harris RB. Antagonism of corticotrophin-releasing factor receptors in the fourth ventricle modifies responses to mild but not restraint stress. 2008. Am J Physiol Regul Integr Comp Physiol. 295(2). R404-416.

Mitra SW, Hoskin E, Yudlovitz J, Pear L, Wilkinson HA, Hayashi S, Pfaff DW, Ogawa S, Rohrer SP, Schaeffer JM, McEwen BS and Alves SE. 2003. Immunolocalization of estrogen receptor β in the mouse brain: comparison with estrogen receptor α. Endocrinology. 144(5). 2055-2067.

Morin SM, Ling N, Liu XJ, Kahl SD, Gehlert DR. 1999. Differential distribution of urocortin- and corticotropin-releasing factor-like immunoreactivities in the rat brain. Neuroscience. 92(1). 281-291.

Musatov S, Chen W, Pfaff DW, Kaplitt MG and Ogawa S. 2006. RNAi-mediated silencing of estrogen receptor α in the ventromedial nucleus of hypothalamus abolishes female sexual behaviors. Proc Natl Acad Sci U S A. 103(27). 10456-10460.

Nilsson S, Mäkelä S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M and Gustafsson JA. 2001. Mechanisms of estrogen action. Physiol Rev. 81(4). 1535-1565.

Nunez AA, Gray JM and Wade GN. 1980. Food intake and adipose tissue lipoprotein lipase activity after hypothalamic estradiol benzoate implants in rats. Physiology & Behavior. 25. 595-598.

Osborne CK, Zhao H, Fuqua SA. 2000. Selective estrogen receptor modulators: structure, function, and clinical use. J Clin Oncol. 18(17). 3172-3186.

Palkovits M. 1999. Interconnections between the neuroendocrine hypothalamus and the central autonomic system. Geoffrey Harris Memorial Lecture, Kitakyushu, Japan, October 1998. Front Neuroendocrinol. 20(4). 270-295.

Palmaer K and Gray JM. 1986. Central vs. peripheral effects of estradiol on food intake and lipoprotein lipase activity in ovariectomized rats. Physiology & Behavior. 37. 187-189.

Parsons B, Rainbow TC, Pfaff DW and McEwen BS. 1982. Hypothalamic protein synthesis essential for the activation of the lordosis reflex in the female rat. Endocrinology. 110(2). 620-624.

Paxinos and Watson. 1998. The rat brain in stereotaxic coordination, 4th edition. Academic Press In, USA. 474PP.

Pedram A, Razandi M and Levin ER. 2006. Nature of functional estrogen receptors at the plasma membrane. Mol Endocrinol. 20(9). 1996-2009.

Pelletier G, Li S, Luu-The V, Labrie F. 2007. Oestrogenic regulation of pro-opiomelanocortin, neuropeptide Y and corticotrophin-releasing hormone mRNAs in mouse hypothalamus. J Neuroendocrinol. 19(6). 426-331.

Pelleymounter MA, Beker MB and McCaleb M. 1999. Does estradiol mediate leptin's effect on adiposity and body weight? Am. J. Physiol. 276. E955-E963.

Perissi V and Rosenfeld MG. 2005. Controlling nuclear receptors: the circular logic of cofactor cycles. Nat Rev Mol Cell Biol. 6(7). 542-554.

Pfaff D. 2005. Hormone-driven mechanisms in the central nervous system facilitate the analysis of mammalian behaviours. J Endocrinol. 184(3). 447-453.

Pietras RJ and Márquez-Garbán DC. 2007. Membrane-associated estrogen receptor signaling pathways in human cancers. Clin Cancer Res. 13(16). 4672-6.

Pike AC. 2006. Lessons learnt from structural studies of the oestrogen receptor. Best Pract Res Clin Endocrinol Metab. 20(1).1-14.

Plamondon H, Merali Z. 1997. Regulation of ingestion by CRF and bombesin-like peptides: distinct meal-related peptide level changes. Am J Physiol. 272(1 Pt 2). R268-R274.

Prossnitz ER, Arterburn JB, Smith HO, Oprea TI, Sklar LA and Hathaway HJ. 2008. Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. Annu Rev Physiol. 70. 165-90.

Redei E, Li L, Halasz I, McGivern RF, Aird F. 1994. Fast glucocorticoid feedback inhibition of ACTH secretion in the ovariectomized rat: effect of chronic estrogen and progesterone. Neuroendocrinology. 60(2). 113-123.

Revankar CM, Cimino DF, Sklar LA, Arterburn JB and Prossnitz ER. 2005. A transmembrane intracellular estrogen receptor mediates rapid cell signaling.Science. 307(5715). 1625-1630.

Richard D. 1993. Involvement of corticotropin-releasing factor in the control of food intake and energy expenditure. Ann N Y Acad Sci. 697. 155-172.

Richard D, Lin Q, Timofeeva E. 2002. The corticotropin-releasing factor family of peptides and CRF receptors: their roles in the regulation of energy balance. Eur J Pharmacol. 440(2-3). 189-197.

Rivera HM and Eckel LA. 2010. Activation of central, but not peripheral, estrogen receptor is necessary for estradiol's anorexigenic effect in ovariectomized rats. Endocrinology. 151 (12). 5680-5688.

Roesch DM. 2006. Effects of selective estrogen receptor agonists on food intake and body weight gain in rats. Physiology & Behavior. 87. 39-44.

Ronnekleiv OK, Malyala A and Kelly MJ. 2007. Membrane-initiated signaling of estrogen in the brain. Semin Reprod Med. 25(3). 165-177.

Safe S. 2001. Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. Vitam Horm. 62. 231-52.

Santollo J, Wiley MD and Eckel LA. 2007. Acute activation of ER{alpha} decreases food intake, meal size, and body weight in ovariectomized rats. Am J Physiol Regul Integr Comp Physiol. 293(6). R2194- R2201.

Saper CB, Chou TC and Elmquist JK. 2002. The need to feed: homeostatic and hedonic control of eating. Neuron. 36(2). 199-211.

Schlenker EH and Hansen SN. 2006. Sex-specific densities of estrogen receptors alpha and beta in the subnuclei of the nucleus tractus solitarius, hypoglossal nucleus and dorsal vagal motor nucleus weanling rats. Brain Res.1123(1).89-100.

Shimomura K, Shimizu H, Tsuchiya T, Abe Y, Uehara Y and Mori M. 2002. Is leptin a key factor which develops obesity by ovariectomy? Endocr J. 49(4). 417-423.

Shughrue PJ, Lane MV and Merchenthaler I. 1997. Comparative distribution of estrogen receptor-α and –β mRNA in the rat central nervous system. The J. of Comp Neuro. 388. 507-525.

Shughrue PJ, Scrimo PJ, Merchenthaler I. 1998. Evidence for the colocalization of estrogen receptor-beta mRNA and estrogen receptor-alpha immunoreactivity in neurons of the rat forebrain. Endocrinology. 139(12). 5267-5270.

Smagin GN, Howell LA, Ryan DH, De Souza EB, Harris RB. 1998. The role of CRF2 receptors in corticotropin-releasing factor- and urocortin-induced anorexia. Neuroreport. 9(7). 1601-1606.

Song RX. 2007. Membrane-initiated steroid signaling action of estrogen and breast cancer. Semin Reprod Med. 25(3). 187-197.

Swanson LW, Kuypers HG. 1980. The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods. J Comp Neurol. 194(3). 555-570.

Thaler JP and Cummings DE. 2008. Metabolism: food alert. Nature. 452(7190). 941-942.

Thammacharoen S, T A Lutz, N Geary and L Asarian. 2007. Anomalously rapid effect of the estrogen receptor-α agonist PPT on food intake in ovariectomized rats. Appetite. 49 (1). 334.

Thammacharoen S, T A Lutz, N Geary and L Asarian. 2008. Hindbrain administraion of estradiol inhibit feeding and activate ERα-expressing cells in the NTS of ovariectomized rats. Endocrinology. Endocrinology. 149(4). 1609-1617.

Thammacharoen S, T A Lutz, N Geary and L Asarian. 2009. Divergent effects of estradiol and the estrogen receptor-α agonist PPT on eating and activation of PVN CRH neurons in ovariectomized rats and mice. Brain research. 1268. 88-96.

Toran-Allerand CD. 2004. A plethora of estrogen receptors in the brain: where will it end? Endocrinology. 145(3). 1069-1074.

Toran-Allerand CD, Guan X, MacLusky NJ, Horvath TL, Diano S, Singh M, Connolly ES Jr, Nethrapalli IS and Tinnikov AA. 2002. ER-X: a novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. J Neurosci. 22(19). 8391-8401.

Toriya M, Maekawa F, Maejima Y, Onaka T, Fujiwara K, Nakagawa T, Nakata M, Yada T. 2010. Long-term infusion of brain-derived neurotrophic factor reduces food intake and body weight via a corticotrophin-releasing hormone pathway in

the paraventricular nucleus of the hypothalamus. J Neuroendocrinol. 22(9). 987-995.

Tzukerman MT, Esty A, Santiso-Mere D, Danielian P, Parker MG, Stein RB, Pike JW and McDonnell DP. 1994. Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. Mol Endocrinol. 8(1). 21-30.

Uehara Y, Shimizu H, Ohtani K, Sato N, Mori M. 1998. Hypothalamic corticotropin-releasing hormone is a mediator of the anorexigenic effect of leptin. Diabetes. 47(6). 890-893.

Vasudevan N, Kow LM and Pfaff D. 2005. Integration of steroid hormone initiated membrane action to genomic function in the brain. Steroids. 70(5-7). 388-396.

Viau V, Meaney MJ. 1991. Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. Endocrinology. 129(5). 2503-2511.

Wade GN and Zucker I. 1970. Modulation of food intake and locomotor activity in female rats by diencephalic hormone implants. J Comp Physiol Psychol. 72(2). 328-336.

Warnmark A, Treuter E, Wright AP and Gustafsson JA. 2003. Activation functions 1 and 2 of nuclear receptors: molecular strategies for transcriptional activation. Mol Endocrinol. 17(10).1901-1909.

Weiser MJ, Handa RJ. 2009. Estrogen impairs glucocorticoid dependent negative feedback on the hypothalamic-pituitary-adrenal axis via estrogen receptor alpha within the hypothalamus. Neuroscience. 159(2):883-895.

Welshons WV, Lieberman ME and Gorski J. 1984. Nuclear localization of unoccupied oestrogen receptors. Nature. 307(5953). 747-749.

Woods SC, Schwartz MW, Baskin DG and Seeley RJ. 2000. Food intake and the regulation of body weight. Annu Rev Psychol. 51. 255-277.

Young E, Korszun A. 2010. Sex, trauma, stress hormones and depression. Mol Psychiatry. 15(1). 23-28.

Young EA, Altemus M, Parkison V, Shastry S. 2001. Effects of estrogen antagonists and agonists on the ACTH response to restraint stress in female rats. Neuropsychopharmacology. 25(6). 881-891.

การเผยแพร่ผลงานวิจัย

- ผลการทดลองส่วนหนึ่งได้ถูกนำเสนอในการประชุมวิชาการนานาชาติ "ศรีนครินทรวิโรฒวิชาการ" ครั้งที่ 7 วันที่ 1-2 เมษายน พ.ศ. 2556 มหาวิทยาลัยศรีนครินทรวิโรฒ ภายใต้ชื่อเรื่อง "ผลของโพรพิลไพราโซลไตรออล (พีพีที) ต่อการควบคุมการกินอาหารที่สมองในหนูเพศเมีย"
- ผลการทดลองทั้งหมดกำลังอยู่ระหว่างดำเนินการเตรียมต้นฉบับเพื่อส่งตีพิมพ์เผยแพร่