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# Developmental changes in the hypothalamic mRNA expression levels of PACAP and its receptor PAC1 and their sensitivity to fasting in male and female rats

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ABSTRACT

The actions and responses of hypothalamic appetite regulatory and factors change markedly during the neonatal to pre-pubertal period. Pituitary adenylate cyclase-activating polypeptide (PACAP) has been found to play pivotal roles in the regulation of metabolic and nutritional status through its specific receptor PAC1. PACAP/PAC1 have anorectic roles, and their functions are regulated by leptin in adulthood. In the present study, we showed that hypothalamic PACAP mRNA expression decreases during the neonatal to pre-pubertal period (from postnatal day 10–30) in both male and female rats. During this period, hypothalamic PACAP mRNA expression was not affected by 24 h fasting in either sex, while the serum leptin levels (leptin is a positive regulator of hypothalamic PACAP expression in adulthood) of both sexes were decreased by fasting. On the other hand, hypothalamic PAC1 mRNA expression did not change during the neonatal to pre-pubertal period in either sex; however, its levels were consistently higher in males than in females. Hypothalamic PAC1 mRNA expression was decreased by 24 h fasting in males, but no such changes were observed in females. These results indicate while hypothalamic PACAP expression is sensitive to a negative energy state and the serum leptin level in adulthood, no such relationships are seen in the pre-pubertal period. In addition, we speculate that differences in the gonadal steroidal milieu might induce sexual dimorphism in the basal hypothalamic PAC1 mRNA level and its response to fasting. The mechanisms responsible for and the physiological effects of such changes in hypothalamic PACAP and PAC1 expression during the developmental period remain to be clarified.



## 1. Introduction

Many physiological functions develop rapidly during the neonatal to pre-pubertal period. In particular, the actions and responses of hypothalamic appetite-regulating factors change markedly during this period in order to maintain an appropriate metabolic and nutritional state (Iwasa et al., 2015). Our previous studies have shown that the basal expression levels of some hypothalamic orexigenic and anorexigenic factors and their responses to fasting change during the neonatal to pre-pubertal period and that these changes differ between males and females (Iwasa et al., 2014, 2015). These results indicate that the roles of such hypothalamic factors in the regulation of metabolic and nutritional status during the neonatal to juvenile period might differ between the sexes.

Pituitary adenylate cyclase-activating polypeptide (PACAP), which was originally identified in the ovine hypothalamus (Miyata et al., 1989), has been found to play pivotal roles in the regulation of metabolic and nutritional status through its specific receptor PAC1 (Morley et al., 1992; Chance et al., 1995; Moro and Lerner, 1997; Adams et al., 2008). The central injection of PACAP suppresses appetite and feeding (Morley et al., 1992; Chance et al., 1995), and increases core body temperature and locomotor activity (Resch et al., 2011). In addition, PACAP knockout mice exhibit marked reductions in core body temperature (Gray et al., 2002), and PAC1-deficient mice develop hyperinsulinemia in fed conditions (James et al., 2000). The effects of PACAP on energy homeostasis are mainly mediated by two hypothalamic nuclei, the ventromedial nucleus (VMN) and the paraventricular nucleus (PVN) (Resch et al., 2013). Extremely high PACAP mRNA expression is seen in the hypothala-

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mic VMN, and the PACAP-positive neurons in the VMN project into the PVN. PACAP-positive neurons from other brain sites, including the extrahypothalamic area, also project into the VMN and PVN; i.e., the VMN receives such projections from the medial amygdala and lateral parabrachial nucleus (LPB), whereas the PVN receives such projections from the bed nucleus of the stria terminalis and the LPB (Resch et al., 2013). Although the injection of PACAP into the PVN or VMN results in reduced overall food intake, meal patterns are affected by the injection of PACAP into the PVN, but not the VMN (Resch et al., 2013). On the other hand, the injection of PACAP into the VMN increases core body temperature and locomotor activity, but no such effects are seen after the injection of PACAP into the PVN (Resch et al., 2013). PAC1 mRNA expression is widely distributed, with abundant expression seen in the hypothalamic nuclei, and the co-administration of a PAC1 antagonist into the PVN and VMN abrogated the effects of the injection of PACAP into these sites (Resch et al., 2013). These results indicate that PACAP signaling within the PVN induces hypophagia, while PACAP signaling within the VMN stimulates energy expenditure.

Recently, it has been reported that hypothalamic PACAP in the VMN is a target of central leptin signaling. For example, the anorectic actions of leptin are abolished in PAC1-deficient mice (Vu et al., 2015) and PACAP knockout mice (Tanida et al., 2013). In addition, PACAP mRNA expression in the VMN is reduced in leptin knockout mice and increased by the administration of leptin (Hawke et al., 2009). It has also been reported that PACAP mRNA expression fell during fasting, but rose after the administration of a high-fat diet (Takaki et al., 1992; Yokota et al., 1993). We speculate that, as has been found for other factors, the basal hypothalamic expression levels of PACAP and PAC1 and their responses to fasting change during development because the serum leptin level varies markedly during this period. In the present study, the developmental changes in hypothalamic PACAP and PAC1 mRNA expression and the serum leptin level were evaluated. In addition, the changes in the sensitivities of these factors to fasting that occur during the neonatal to pre-pubertal period were examined.

## 2. Materials and methods

### 2.1. Animals

Pregnant Sprague-Dawley rats were purchased (Charles River Japan Inc., Tokyo, Japan) and housed under controlled lighting (14 h light, 10 h dark) and temperature (24 °C) conditions. The day on which the pups were born was defined as postnatal day 1. Twelve pups were randomly assigned to each dam on postnatal day 2. The rats were weaned at postnatal day 21 and housed at three or four per cage. Rats of both sexes were randomly selected from each dam on postnatal days 10, 20, and 30, and divided into the fed and fasting groups (n = 8 per group). The rats in the fasting groups were subjected to 24 h maternal (postnatal day 10 and 20) or food (postnatal day 30) deprivation. Twenty-four hours later (between 0900 and 1000), the rats' brains and serum were collected by decapitation and stored at −80° C and −20° C, respectively. All animal experiments were conducted in accordance with the ethical standards of the animal care and use committee of the University of Tokushima.

### 2.2. Hormone assay and quantitative real-time polymerase chain reaction

The rats' serum leptin levels were measured using a 125I-radioimmunoassay (RIA) kit (multi-species leptin RIA kit, Linco Research Inc., MO, USA). Whole hypothalamic explants were dissected from the frozen brains, as described previously (Iwasa et al., 2015). Total RNA was isolated using a TRIzol® reagent kit (Invitrogen Co., Carlsbad, CA, USA) and an RNeasy mini kit® (Qiagen GmBh, Hilden, Germany). Five μg of total RNA were used for the cDNA synthesis. cDNA was synthesized with oligo (deoxythymidine) primers at 50 °C using the SuperScript III first-strand synthesis system for the real-time polymerase chain reaction (PCR; Invitrogen Co.). The PCR analysis was performed using the StepOnePlus™ real-time PCR system (PE Applied Biosystems, Foster City, CA, USA). Standard curves, which were generated by serially diluting an abundant sample at least 4 times, were used for the relative quantification of each mRNA expression level. The mRNA expression levels of PACAP and PAC1 were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following forward and reverse primers were used: PACAP: F: 5′- CAT GTG TAG CGG AGC AAG GTT −3′, R: 5′- GTC TTG CAG CGG GTT TCC −3′; PAC1: F: 5′- GGT GCT TGA AGT CCA TAG TG −3′, R: 5′- CTT GTA CAG AAG CTG CAG TC −3′; GAPDH: F: 5′- ATG GCA CAG TCA AGG CTG AGA −3′, R: 5′- CGC TCC TGG AAG ATG GTG AT- 3′. The PCR cycling conditions were as follows: initial denaturation and enzyme activation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s; annealing at 56 °C for 30 s (PAC1), 64 °C for 30 s (PACAP), or 65 °C for 30 s (GAPDH); and extension at 72 °C for 1 min. There are three isoforms of PAC1 (Zhou et al., 1999), and the PAC1 primer used in this study was able to amplify all of these isoforms (Zhou et al., 1999; Basille et al., 2000). In this study, GAPDH mRNA expression was not affected by development or fasting in either sex. In addition, its expression did not differ between the sexes at any of the examined ages.

### 2.3. Statistical analyses

All data are presented as mean ± SEM values. The statistical analyses were performed using one-way or two-way analysis of variance (ANOVA), or the Kruskal-Wallis test together with the Tukey-Kramer or Steel-Dwass post-hoc test for comparisons between the age groups. The Student's *t* test or Mann–Whitney *U* test were used for comparisons between the sexes, or between the rats kept under the fed and fasted conditions. Statistical significance was defined as $P<0.05$.

## 3. Results

### 3.1. Developmental changes in hypothalamic PACAP and PAC1 mRNA expression and the serum leptin level under the fed conditions in male and female rats

Under the fed conditions, the hypothalamic PACAP mRNA expression level differed significantly among the examined age groups in both the male (one-way ANOVA; $F(3,47)=32.9$, $P<0.01$) and female rats (Kruskal-Wallis; df = 2, H = 18.5, $P<0.01$) (Fig. 1A). Hypothalamic PACAP mRNA expression fell with aging in both the male and female rats, but no difference in hypothalamic PACAP mRNA expression was detected between the sexes in any age group. The hypothalamic PAC1 mRNA expression level differed significantly among the examined age groups in the male rats (Kruskal-Wallis; df = 2, H = 10.4, $P<0.01$); however, post-hoc analyses did not find any significant differences among the age groups (Fig. 1B). On the other hand, hypothalamic PAC1 mRNA expression did not differ among the examined age groups in the females (Fig. 1B). Hypothalamic PAC1 mRNA expression was significantly higher in the males than in the females in all age groups (Fig. 1B). The serum leptin level differed significantly among the age groups in both the males (one-way ANOVA; $F(3,47)=22.4$, $P<0.01$) and females (one-way ANOVA; $F(3,47)=13.4$, $P<0.01$) (Fig. 1C). The serum leptin levels recorded on postnatal day 10 were higher than those seen on postnatal days 20 and 30 in both the males and