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Continuous stress-induced dopamine dysregulation augments PAP-I and PAP-II expression in melanotrophs of the pituitary gland

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ABSTRACT

Under continuous stress (CS) in rats, melanotrophs, the predominant cell-type in the intermediate lobe (IL) of the pituitary, are hyperactivated to secrete α-melanocyte-stimulating hormone and thereafter degenerate. Although these phenomena are drastic, the molecular mechanisms underlying the cellular changes are mostly unknown. In this study, we focused on the pancreatitis-associated protein (PAP) family members of the secretory lectins and characterized their expression in the IL of CS model rats because we had identified two members of this family as up-regulated genes in our previous microarray analysis. RT-PCR and histological studies demonstrated that prominent PAP-I and PAP-II expression was induced in melanotrophs in the early stages of CS, while another family member, PAP-III, was not expressed. We further examined the regulatory mechanisms of PAP-I and PAP-II expression and revealed that both were induced by the decreased dopamine levels in the IL under CS. Because the PAP family members are implicated in cell survival and proliferation, PAP-I and PAP-II secreted from melanotrophs may function to sustain homeostasis of the IL under CS conditions in an autocrine or a paracrine manner.

1. Introduction

Continuous stress (CS) causes defects in physical and mental health [1–3]. We analyzed the influences of CS on various organs using an established rat model for prolonged stress [4–7]. In this model, rats were kept in a cage filled with water to a height of 1.5 cm, which caused partial sleep deprivation and hyperactivation of voluntary locomotor behavior. Our previous studies demonstrated that, under these conditions, the intermediate lobe (IL) of the pituitary showed dramatic changes leading to cell death of melanotrophs [5,6]. Melanotrophs are the predominant cell-type in the IL and secrete α-melanocyte-stimulating hormone (α-MSH). Under CS the melanotrophs are substantially activated and exhibit abnormal morphologies such as a highly developed rough endoplasmic reticulum and Golgi apparatus. Prolonged stress kept the melanotrophs over-activated, and eventually led to their cell death [6]. These dramatic morphological changes and cell death were not observed in other regions of the pituitary or in other organs. Subsequent experiments demonstrated that the CS-induced cellular changes were largely due to suppression of dopaminergic innervation in the IL from the periventricular nucleus of the hypothalamus (A14) [6,8,9]. These studies might be the first to demonstrate the link between CS and cell death. Subsequently, we performed a microarray analysis to explore the molecular basis of the cellular changes, and identified several genes whose expression was altered in the IL under CS [7]. Among these genes, two belonged to the pancreatitis-associated protein (PAP; alternatively called the regenerating gene, Reg) family. Therefore, in this study, we focused on the PAP/Reg family members. The PAP family consists of three members PAP-I, PAP-II and PAP-III. PAP-I is also known as Reg-2 in rat and Reg-IIIβ in mouse, and PAP-II and PAP-III are known as Reg-IIIα and Reg-IIIγ in mouse, respectively [10,11]. Structurally, these proteins belong to the group of small secretory lectins [12–14]. Here, we showed that both PAP-I and PAP-II exhibited significant up-regulation in melanotrophs under CS, and demonstrated that the decreased dopamine level in the IL during CS might trigger this gene induction.

Abbreviations: 1-5d CS, continuous stress for 1–5 days; AL, anterior lobe; α-MSH, alpha-melanocyte stimulating hormone; CS, continuous stress; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; Id, inhibitor of DNA binding/differentiation; IL, intermediate lobe; IPL, intermediate lobe plus posterior lobe; PAP, pancreatitis-associated protein; PB, phosphate buffer; PL, posterior lobe; POMC, proopiomelanocortin; Reg, regenerating gene; TH, tyrosine hydroxylase.

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2. Materials and methods

2.1. Animal model

The stress model has been described previously [4–7]. Briefly, 7-week-old male Sprague–Dawley rats were singly housed and acclimatized to the temperature-, humidity- and light-controlled environment for 1 week. Then rats were randomly assigned to the CS or non-CS group. In the non-CS group, rats were kept in the normal cages. In the CS group, rats were transferred to identical cages filled with water to a height of 1.5 cm at 10.00 h, and sacrificed between 10.00 and 13.00 h after 1, 3 or 5 days (1d CS, 3d CS and 5d CS, respectively). This experiment was approved by the Animal Ethics Committee of Osaka City University (approval numbers 00093, 05049 and 08041).

2.2. Administration of dopamine agonist and antagonist

CS rats were injected intraperitoneally with the dopamine D2 receptor agonist (+)-bromocriptine mesylate (bromocriptine) dissolved in saline containing 0.5% polyethylene sorbitan monolaurate (10 mg/kg) (MP Biomedicals, LLC, Aurora, OH), or vehicle, at 10.00 h on days 0–4, and were sacrificed on day 5. Normal rats were injected intraperitoneally with the dopamine D2 receptor antagonist (−)-sulpiride (sulpiride) dissolved in phosphate-buffered saline (50 mg/kg) (Sigma, St. Louis, MO), or vehicle, at 10.00 h on days 0–3, and were sacrificed at 13.00 h on day 3.

2.3. RT-PCR

The anterior lobe (AL) and the IL plus the posterior lobe (IPL) of the pituitary were obtained from non-CS, 1d CS, 3d CS and 5d CS rats, and frozen in liquid nitrogen. At least six rats were used in each group. Total RNA was purified using the acid guanidine isothiocyanate/phenol/chloroform method and was converted to cDNA using SuperScript II (Invitrogen, Carlsbad, CA). Amplification was performed as follows using primers described in our previous paper [10]: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (23 cycles), PAP-I (30 cycles), PAP-II (30 cycles) and PAP-III (35 cycles), at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The amplified products were electrophoresed on 1.8% agarose gels and stained with ethidium bromide. For statistical analysis, the optical density of the bands was measured using NIH Image (version 1.62f; National Institutes of Health, Bethesda, MD). The intensity of the PAP bands was calculated relative to those of GAPDH.

2.4. In situ hybridization

PCR products for the PAP genes obtained by RT-PCR were subcloned into the pGEM-T easy vector (Promega, Madison, WI). Then, digoxigenin-labeled cRNA probes (Roche Diagnostics, Mannheim, Germany) were transcribed *in vitro* using SP6 or T7 RNA polymerase (Promega) according to the manufacturer's protocol. Whole pituitaries from non-CS and 5d CS rats were quickly removed after decapitation and frozen on powdered dry ice. Then 18-μm-thick sections were cut on a cryostat and thaw-mounted onto 3-aminopropyltriethoxysilane-coated slides. Hybridization, washing and developing were performed according to a previous report [15].

2.5. Immunohistochemistry

Non-CS and 5d CS rats were perfused with Zamboni's fixative (0.1 M phosphate buffer (PB) containing 2% paraformaldehyde and 0.2% picric acid). The pituitary was removed, postfixed in the same fixative for 1 day, and immersed in 0.1 M PB containing 25% sucrose for an additional day. Then 18-μm-thick sections were cut on a cryostat and thaw-mounted onto gelatin-coated slides. Staining was performed as described previously [16,17] using rabbit polyclonal anti-PAP-I [10,18], sheep polyclonal anti-α-MSH (#AB5087; Chemicon, Temecula, CA) and mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (#G3893; Sigma) antibodies. Images were acquired using a confocal microscope (TCS-SP5; Leica, Heidelberg, Germany).

3. Results

3.1. Expression of mRNA for the PAP family members in the pituitary under CS

In our previous study, we performed a microarray analysis using cDNAs from the IL plus the PL (IPL) of CS and non-CS animals, and found that two PAP family mRNAs were up-regulated [7]. Here, we first examined the mRNA expression profiles for all the PAP family members (PAP-I, -II and -III) by RT-PCR using cDNA from the IPL of CS and non-CS animals. PAP-I and PAP-II mRNAs were markedly induced in the IPL at 1d CS, and their expression showed a slight increase thereafter, while PAP-III expression was below the level of detection even with a high number of PCR cycles in the IPL (Fig. 1). This was consistent with the data obtained in our previous microarray analysis, in which up-regulation of PAP-I (described as PAP in the gene list; 3.22-fold up-regulation) and PAP-II (described as Reg-IIIα in the gene list; 2.38-fold up-regulation) was prominent [7]. The mRNAs for all the family members were barely expressed in the AL, except for moderate expression of PAP-I at 5d CS (Fig. 1A).

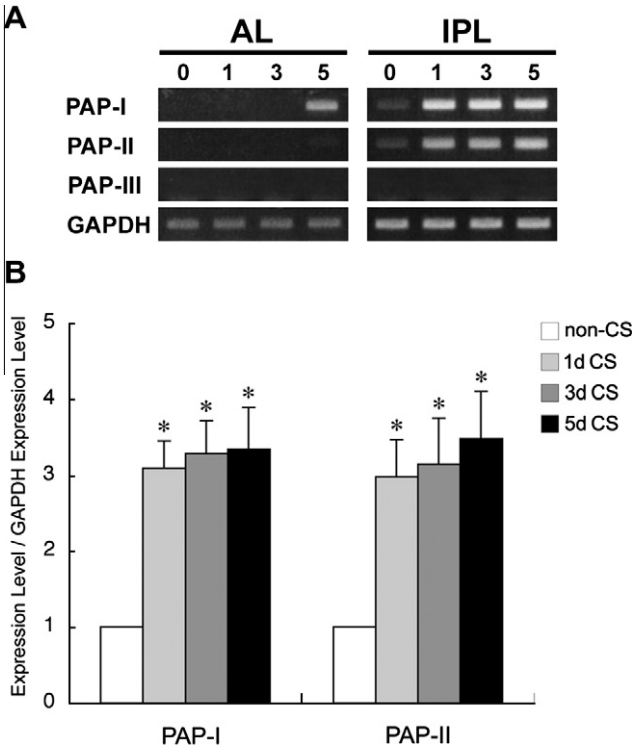

Fig. 1. Induction of PAP-I and PAP-II mRNA expression in the IPL of the pituitary under CS. (A) mRNA expression of the PAP family members in the pituitary of CS rats. Pituitaries obtained from non-CS (0), 1, 3 and 5 d CS rats were separated into the AL and IPL. The expression of PAP-I, PAP-II and PAP-III was examined by RT-PCR. GAPDH was used as an internal control. (B) Semi-quantification of PAP-I and PAP-II expression levels in the IPL. The data are expressed as fold intensity compared with the non-CS bands. Error bars indicate the SD. Statistically significant differences by the paired *t*-test, $^{*}P < 0.0005$.

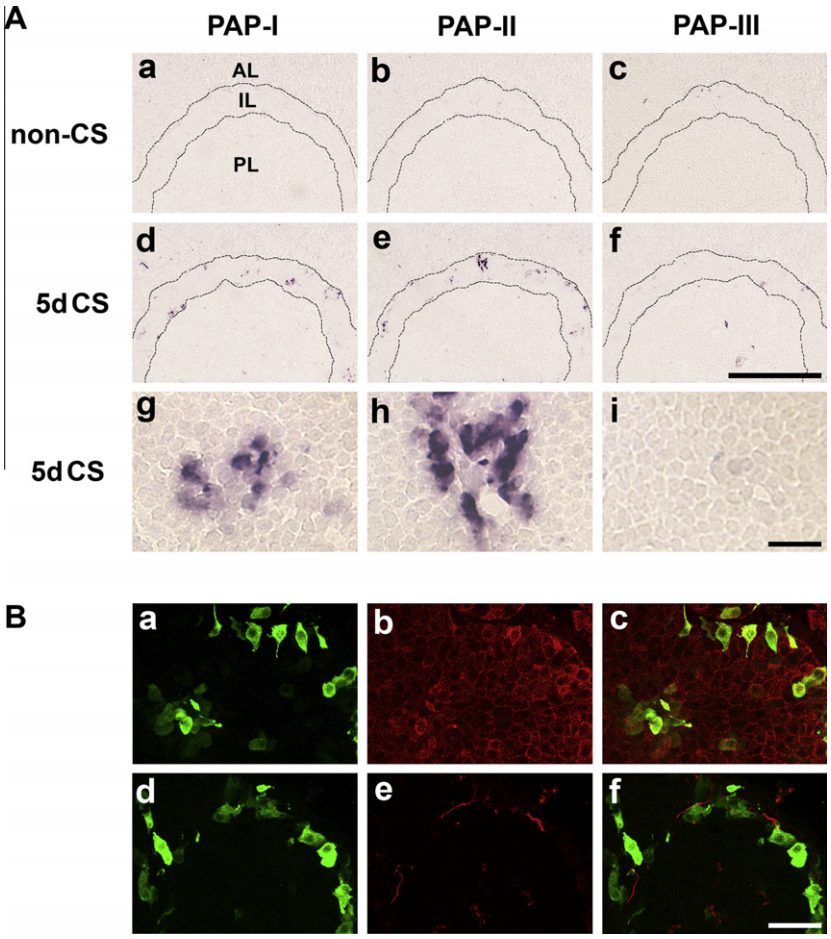

Fig. 2. Induction of PAP-I and PAP-II in melanotrophs of the IL under CS. (A) *In situ* hybridization performed with whole pituitary sections from non-CS (a–c) and 5d CS (d–f) rats. Higher magnification images of the IL of the 5d CS rats are shown (g–i). Dashed lines indicate the boundaries between the lobes. Scale bars = 500 μm (a–f) or 50 μm (g–i). (B) Using anti-PAP-I antibody (a and d, green) and either anti-α-MSH antibody (b, red) or anti-GFAP antibody (e, red), the cell-types expressing PAP-I were examined. Merged images are shown in the right-hand column (c and f). Note that all PAP-I-positive cells are simultaneously labeled by α-MSH, whereas they do not co-localize with GFAP immunoreactivity. Scale bar = 50 μm. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

3.2. PAP-I and PAP-II expression in melanotrophs of the IL under CS

Next, we performed *in situ* hybridization to determine whether PAP-I and PAP-II mRNAs were expressed in the IL or the PL. Intense signals for PAP-I and PAP-II were observed in the IL under CS (Fig. 2Aa, b, d and e); higher magnification showed that PAP-I and PAP-II were expressed in some melanotrophs (Fig. 2Ag and h) [19]. Concordant with the RT-PCR results, PAP-III signals were barely observed throughout the section (Fig. 2Ac, f and i). We then performed immunohistochemical double-staining using anti-PAP-I antibody, and confirmed that all PAP-I immunoreactivity was observed in α-MSH-positive melanotrophs, but that not all α-MSH-positive melanotrophs expressed PAP-I (Fig. 2Ba–c). The morphology of the PAP-I-expressing cells was round or polygonal, and was distinct from that of the GFAP-positive glia-like cells, which typically exhibit an elongated shape (Fig. 2Bd–f) [19].

3.3. Dopaminergic regulation of PAP-I and PAP-II expression in the IL under CS

Our previous studies demonstrated that dopaminergic innervation of the IL from the hypothalamus was suppressed under CS, and that application of dopamine agonist or antagonist to melanotrophs altered the expression of certain genes, including the pro-opiomelanocortin (POMC) gene that encodes α-MSH [6,7]. We therefore examined the possibility that a decrease in dopamine level in the IL under CS altered the expression of PAP-I and PAP-II. We chose the dopamine D2 receptor-specific agonist bromocriptine, because the IL predominantly expresses D2 receptors [20]. We injected bromocriptine into CS rats and examined changes in PAP-I and PAP-II expression (Fig. 3A and B). Bromocriptine suppressed the induction of PAP-I and PAP-II mRNAs in CS rats by 36.6% and 28.2%, respectively. Conversely, we hypothesized that dopamine suppression in the IL in non-CS rats might induce PAP-I and PAP-II expression, and examined this possibility using the dopamine D2 receptor antagonist sulpiride (Fig. 3C and D). When sulpiride was administered to normal rats, PAP-I and PAP-II expression was induced in the IPL (PAP-I: 3.09 ± 0.42-fold; PAP-II: 3.16 ± 0.46-fold up-regulation). Collectively, these results indicate that dopamine suppression may induce the expression of PAP-I and PAP-II in the IL under CS conditions.

4. Discussion

In the present study, we demonstrated that expression of both PAP-I and PAP-II was specifically induced in a group of melanotrophs in CS conditions, and that dopamine dysregulation, presumably caused by the CS, is responsible for this induction.

The PAP/Reg family comprises small secretory proteins structurally classified as calcium-dependent lectins [12–14]. Although

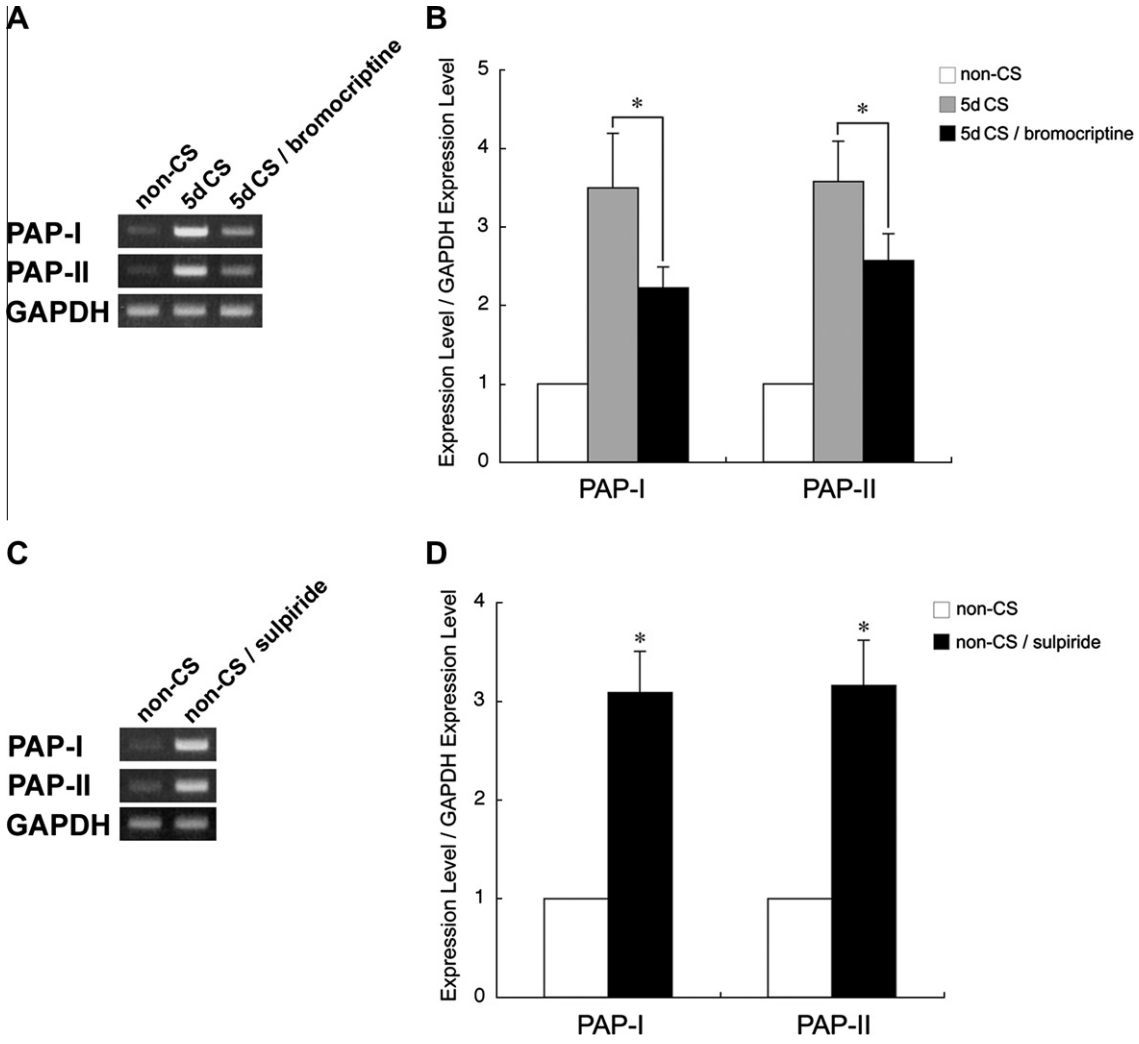

Fig. 3. Dopamine regulates PAP-I and PAP-II mRNA expression in the IPL. (A) mRNA expression of PAP-I and PAP-II in the IPL of CS rats in the presence or absence of bromocriptine. IPLs isolated from non-CS and CS rats intraperitoneally injected with vehicle or bromocriptine every 24 h for 5 days were analyzed by RT-PCR. GAPDH was used as an internal control. (B) Semi-quantification of the mRNA expression levels. The data are expressed as fold intensity compared with the non-CS bands. Error bars indicate the SD. Statistically significant differences by the paired *t*-test, $^{*}P < 0.005$. (C) mRNA expression of PAP-I and PAP-II in the IPL of normal rats (non-CS) in the presence or absence of sulpiride. IPLs isolated from normal rats intraperitoneally injected with vehicle or sulpiride every 24 h for 3 days were analyzed by RT-PCR. GAPDH was used as an internal control. (D) Semi-quantification of mRNA expression levels. The data are expressed as fold intensity compared with the bands from vehicle-injected rats. Error bars indicate the SD. Statistically significant differences by the paired *t*-test, $^{*}P < 0.0005$.

their expression is almost absent in most normal tissues except for the gastrointestinal tract [21], their expression is immediately induced in several tissues in response to stimuli such as inflammation and injury [10,22–27]. Likewise, in the present study, we demonstrated dramatic induction of PAP-I and PAP-II expression in melanotrophs from the early stages of CS (Figs. 1 and 2). However, this induced expression was restricted to a minor population (~5%) of melanotrophs in the IL. This suggests that there are several different subpopulations of melanotrophs. The rat IL comprises many small lobes, which are composed almost homogeneously of hormone-secreting cells (the melanotrophs) and a few glia-like cells located in the border regions of the small lobe [19]. Both PAP-I- and PAP-II-expressing cells tend to be located near the margins of the small lobes (Fig. 2B), whereas, under CS, melanotroph degeneration primarily occurs in the center of the small lobes [6] (Ogawa et al., unpublished data). This may suggest that the PAP-I- and PAP-II-expressing cells are not the initial degenerating cells. Although we could not examine whether PAP-I and PAP-II were expressed in the same or distinct cells, the number of positive cells and their localization in the IL were relatively similar. In addition to the IL, expression of PAP-I alone was observed in the AL at late stages of CS only (Fig. 1A). The PAP-I-positive cells in the AL may be somatotrophs, because PAP-I expression has been previously demonstrated in primary cultured somatotrophs [28,29]. In contrast, PAP-III expression was almost absent throughout the pituitary even under CS (Figs. 1A and 2A). It is intriguing that a combination of PAP-I and PAP-III expression has been commonly observed in the central nervous system after injury [17,18], while the combination was PAP-I with PAP-II in the pituitary under CS conditions. The particular family member(s) expressed in response to diverse stimuli appears to vary among organs [10,27].

The pituitary gland is regulated by multiple hypothalamic dopaminergic systems. Dopaminergic neurons in the arcuate nucleus (A12) mainly regulate cells in the AL *via* portal vessels (the tuberoinfundibular dopamine system), and some dopamine neurons located in rostral A12 and A14 are thought to project to the PL and IL, respectively [8]. Our previous study demonstrated that CS stimuli suppressed the expression of tyrosine hydroxylase (TH) mRNA in the dopaminergic neurons located in A14 and projecting to the IL, and thereby the TH-immunoreactivity of nerve

fivers in the IL decreased dramatically [6]. On the other hand, it was demonstrated that dopamine antagonists induced the production and secretion of α-MSH in mammalian melanotrophs [30,31]. Collectively, hypothalamic dopamine could be one of the strong modulators of melanotrophs. In this regard, it is highly likely that changes in dopaminergic innervation altered the expression of PAP-I and PAP-II (Fig. 3). Consistently, the expression of other genes in melanotrophs is known to be regulated by the dopamine level [7]. We previously revealed that reduction of the dopamine level in the IL triggered expression of the inhibitor of DNA binding/differentiation (Id) family of transcription factors in melanotrophs under CS [7].

Several functions for the PAP family members have been proposed in various models such as inflammation and injury [32–35]. Under CS, abnormal over-dilation of the endoplasmic reticulum and Golgi enlargement were observed in melanotrophs, and eventually some of the cells degenerated [6]. PAP-I has been reported to act as an anti-apoptotic or survival factor in several cells such as pancreatic acinar cells [36–38], hepatocytes [39,40] and neurons [41,42]. Therefore, it is likely that PAP-I and PAP-II function to prevent melanotrophs from death in an autocrine or paracrine manner. Alternatively, PAP-I and PAP-II may be involved in the proliferation of melanotrophs. This is because that although some melanotrophs degenerate under CS [6], their proliferation is also promoted in the IL (Ogawa et al., unpublished data). A proliferative effect for PAP-I and PAP-II was demonstrated [24,39,43]; therefore, it is possible that secreted PAP-I and PAP-II promote melanotroph proliferation to compensate for the cell loss. Further analyses using knockout mice, for instance, are required to elucidate the precise functions of PAP-I and PAP-II in the IL under CS.

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