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# Characterization of the Thermoregulatory Response to Pituitary Adenylate Cyclase-Activating Polypeptide in Rodents

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**Abstract** Administration of the long form (38 amino acids) of pituitary adenylate cyclase-activating polypeptide (PACAP38) into the central nervous system causes hyperthermia, suggesting that PACAP38 plays a role in the regulation of deep body temperature ($T_b$). In this study, we investigated the thermoregulatory role of PACAP38 in details. First, we infused PACAP38 intracerebroventricularly to rats and measured their $T_b$ and autonomic thermoeffector responses. We found that central PACAP38 infusion caused dose-dependent hyperthermia, which was brought about by increased thermogenesis and tail skin vasoconstriction. Compared to intracerebroventricular administration, systemic (intravenous) infusion of the same dose of PACAP38 caused significantly smaller hyperthermia, indicating a central site of action. We then investigated the thermoregulatory phenotype of mice lacking the *Pacap* gene (*Pacap*$^{-/-}$). Freely moving *Pacap*$^{-/-}$ mice had higher locomotor activity throughout the day and elevated deep $T_b$ during the light phase. When the *Pacap*$^{-/-}$ mice were loosely restrained, their metabolic rate and $T_b$ were lower compared to their wild-type littermates. We conclude that PACAP38 causes hyperthermia via activation of the autonomic cold-defense thermoeffectors through central targets. *Pacap*$^{-/-}$ mice express hyperkinesis, which is presumably a compensatory mechanism, because under restrained conditions, these mice are hypometabolic and hypothermic compared to controls.

**Keywords** PACAP · Hyperthermia · Thermoregulation · Locomotor activity · Autonomic thermoeffectors



## Introduction

The long form of the pituitary adenylate cyclase-activating polypeptide (PACAP) consists of 38 amino acids (PACAP38), and together with its receptors (PAC1 and VPAC1/2 receptors), it is widely expressed both in peripheral organs and in the central nervous system (CNS), explaining its diverse biological functions (for review, see Vaudry et al. 2009). Of note, a shorter form of the peptide (PACAP27) has also been identified (Miyata et al. 1990), but since in most tissues PACAP38 is the predominant form with a concentration ratio of PACAP27:PACAP38 to <1:9 (Vaudry et al. 2000, 2009) and because the effects of the PACAP27 and PACAP38 on cAMP formation (Nowak and Kuba 2002), vascular responses (Lenti et al. 2007), and on body temperature (Seeliger et al. 2010) are similar, the current study focused on the effects of PACAP38. In the CNS, PACAP exerts neurotrophic effects (Vaudry et al. 1999; Njaine et al. 2014) as well as neuroprotective actions in experimental models of local ischemia (Reglodi et al. 2000; Danyadi et al. 2014), Parkinson's disease (Brown et al. 2013, 2014) and viral neurotoxicity (Rozzi et al. 2014). The peptide is known to regulate pituitary hormone secretion (Koves et al. 2014) and improve barrier properties of the endothelial cells in the brain (Wilhelm et al. 2014). On the periphery, PACAP has been shown to have an anti-

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inflammatory role in diabetic kidney damage (Banki et al. 2013, 2014), neurogenic inflammation (Helyes et al. 2007), and contact dermatitis (Kemeny et al. 2010). PACAP has been reported to play an important role in the regulation of numerous homeostatic processes by influencing circadian rhythm (Nagy and Csernus 2007; Racz et al. 2008) and food intake (Hawke et al. 2009; Resch et al. 2011). Since the isolation of PACAP38 by Miyata et al. (1989), a large number of studies have been conducted to identify the role of the peptide in various homeostatic functions, including the regulation of deep body temperature ($T_b$).

Supporting the role of PACAP38 in thermoregulation, the peptide and its receptors are broadly expressed in main thermoregulatory areas of the brain, including the lateral parabrachial area, the preoptic area of the hypothalamus (POA), the dorsomedial nucleus of the hypothalamus, the periaqueductal gray matter, and the nucleus raphe pallidus (Palkovits et al. 1995; Joo et al. 2004; Das et al. 2007). In physiological studies, the injection of PACAP38 into the lateral cerebral ventricle (Pataki et al. 2000, 2003; Hawke et al. 2009), the intrathecal space (Inglott et al. 2011), or onto the ventromedial hypothalamic nucleus (Resch et al. 2011, 2013) caused an increase of $T_b$ in rats and mice. Although elevation of non-shivering thermogenesis (Hawke et al. 2009; Inglott et al. 2011; Resch et al. 2011) and increase of locomotor activity (Resch et al. 2011, 2013) have been shown to contribute to the PACAP38-induced hyperthermia, no study has yet been conducted to investigate simultaneous activation of autonomic thermoeffectors.

The thermoregulatory system operates as a federation of independent thermoeffector loops, in which each loop consists of a sensor, an afferent, and an efferent branch (Romanovsky 2007a). For example, environmental cold activates cutaneous cold receptors and signals from the skin are conveyed to thermoregulatory centers in the brain from where autonomic (thermogenesis, cutaneous vasonconstriction) and behavioral (warmth seeking) cold-defense effectors are driven (Romanovsky 2014). These defense mechanisms can be modulated from peripheral (Almeida et al. 2012) as well as from central sites (Nakamura and Morrison 2008b). A substance such as PACAP38 can act at any element of a thermoeffector loop and cause the same effect: hyperthermia. Since the thermoregulatory response to PACAP38 has not been compared between systemic (outside the blood–brain barrier) and central (into CNS) substance delivery, it cannot be firmly stated whether the primary site of action of PACAP38 is on peripheral afferents, in the brain as proposed by Resch et al. (2011, 2013), in the spinal cord as suggested by Inglott et al. (2011), or on the efferent neural pathway of a thermoeffector (e.g., that of the brown adipose tissue).

In addition to exogenous PACAP38 administration, genetically modified mice lacking the *Pacap* gene have also been utilized to investigate how the absence of PACAP affects deep $T_b$. These studies obtained contradictory results showing that the absence of PACAP in mice lead to increased (Hashimoto et al. 2001) versus unchanged locomotor activity (Adams et al. 2008), as well as to lower (Hashimoto et al. 2009) versus unchanged $T_b$ as compared to controls (Cummings et al. 2008).

In the present study, we characterized the dose-dependency of and the autonomic thermoeffector pattern involved in the response to PACAP38. Then, in a comparative experiment, we addressed the question whether exogenous PACAP38 administration acts primarily through peripheral or central targets in rats. Lastly, as an additional approach to identify the role of PACAP in thermoregulation, we used mice lacking the *Pacap* gene and studied how the absence of PACAP affects circadian changes of their deep $T_b$ and locomotor activity as well as their basal $T_b$ and metabolic rate.

## Materials and Methods

### Animals

The physiological experiments were performed in 40 adult male Wistar rats and 42 adult mice of both sexes. The mice had the *Pacap* gene homozygously either present ($Pacap^{+/+}$) or absent ($Pacap^{-/-}$) due to a targeted disruption (Hashimoto et al. 2001). Generation by a gene-targeting technique, maintenance, and backcrossing of $Pacap^{-/-}$ mice on a CD1 background has been reported previously (Hashimoto et al. 2001, 2009). Animals were housed in temperature-controlled rooms on a 12 h light–dark cycle. Standard rodent chow and tap water were available ad libitum. At the time of the experiments, the rats weighed 331±33 g and the mice weighed 24±2 g.

Rats and mice were extensively handled and then habituated to staying inside wire-mesh cylindrical confiners. The cylindrical confiner prevented the animal from turning around, but allowed for some back-and-forth movements; it was used in the respirometry setup (see the "Experimental Setups" section below).

All procedures were conducted under protocols approved by Institutional Animal Use and Care Committee of the University of Pecs and were in accordance with the directives of the National Ethical Council for Animal Research and those of the European Communities Council (86/609/EEC).

### Surgeries

#### *Mice*

Mice were anesthetized with a ketamine–xylazine cocktail (81.7 and 9.3 mg/kg, respectively, i.p.) and received antibiotic protection (gentamycin, 6 mg/kg, i.m.). During surgery, a mouse was heated with a temperature-controlled heating pad

(model TMP-5a; Supertech Instruments UK Ltd., London, UK) placed under a surgery board.

A mouse designated for an experiment in the telemetry setup was implanted with a miniature telemetry transmitter (G2 E-Mitter series; Mini Mitter, Bend, OR, USA) to record abdominal temperature ($T_{ab}$, a measure of deep $T_b$) and locomotor activity. The device was inserted into the peritoneal cavity via midline laparotomy and fixed to the lateral abdominal wall (right side) with a suture. The surgical wound was sutured in layers. After the surgery, mice were allowed to fully recover for 10 days before data collection started.

*Rats*

Surgeries were performed under ketamine–xylazine (55.6 and 5.5 mg/kg, respectively, i.p.) anesthesia and antibiotic protection (gentamycin, 6 mg/kg, i.m.). Experiments were performed 2 to 4 days after surgery. Each rat was implanted with either an intravenous (i.v.) catheter or an intracerebroventricular (i.c.v.) cannula as described below.

For i.v. catheter implantation, a small longitudinal incision was made on the ventral surface of the neck, left of the trachea. The left jugular vein was exposed, freed from its surrounding connective tissue, and ligated. A silicone catheter (ID 0.5 mm, OD 0.9 mm) filled with heparinized (10 U/ml) saline was passed into the superior vena cava through the jugular vein and secured in place with ligatures. The free end of the catheter was knotted, tunneled under the skin to the nape, and exteriorized. The wound was sutured. The catheter was flushed with heparinized saline (10 U/ml) on the day after the surgery and every other day. This technique was repeatedly used in our earlier studies (Petervari et al. 2005; Garami et al. 2010).

For i.c.v. cannulation, each rat was fixed to a stereotaxic apparatus as carried out in our earlier studies (Petervari et al. 2009, 2010). The scalp was incised over the sagittal suture; the periosteum was excised; the skull was cleaned and dried; two supporting microscrews were driven into the skull; and a small hole was drilled in the skull 1.0 mm antero-posterior from bregma and 1.5 mm lateral from midline. A 22-G steel guide cannula was attached to a plastic tube fitted into a stereotaxic manipulator (Narishige Scientific Instruments Laboratory, Tokyo, Japan), which was used to insert the cannula into the brain through the bone hole. The tip of the cannula was placed within the right lateral ventricle (3.8 mm from dura). The cannula was secured to the supporting microscrews with dental cement and released from the manipulator. The guide cannula was closed by a dummy cannula.

## Experimental Setups

Physiological experiments in unanesthetized animals were conducted in either the respirometry setup or the telemetry setup. The respirometry setup was used (a) to measure the thermoregulatory responses of rats to non-stressful administration of PACAP38 and (b) to assess the basal thermoregulatory parameters of untreated, loosely restrained *Pacap*$^{-/-}$ and *Pacap*$^{+/+}$ mice. The telemetry setup was used only in untreated, freely-moving *Pacap*$^{-/-}$ and *Pacap*$^{+/+}$ mice to record their $T_{ab}$ and locomotor activity over a longer period (24 h) of time.

In the respirometry setup, a rat or mouse equipped with copper–constantan thermocouples (Omega Engineering, Stamford, CT, USA) to measure colonic ($T_c$), and tail skin temperature ($T_{sk}$) was placed in a confiner. The colonic thermocouple was inserted 10 or 3 cm beyond the anal sphincter in rats and mice, respectively, and fixed to the base of the tail with a loop of adhesive tape. The skin thermocouple was positioned on the lateral surface of the tail (at the boundary of the proximal and middle thirds) and insulated from the environment with tape. The thermocouples were plugged into a data logger (Cole-Parmer, Vernon Hills, IL, USA). Then, each animal in its confiner was transferred to a Plexiglas chamber of the four-chamber open-circuit calorimeter integrated system (Oxymax Equal Flow, Columbus Instruments, Columbus, OH, USA). The chamber was sealed, submerged into a temperature-controlled water bath, and continuously ventilated with room air (1,000 and 200 ml/min for rats and mice, respectively). The fractional concentration of oxygen was measured in the air entering and exiting the chamber, and the rate of oxygen consumption ($VO_2$) was calculated according to the manufacturer's instructions using the Oxymax Windows software (v3.1). When present, the venous catheter was connected to a polyethylene-50 extension filled with the drug of interest. When the animal had an i.c.v. cannula, a needle injector was fitted into the guide cannula and connected to a polyethylene extension (ID 0.28 mm, OD 0.61 mm). The extension was passed through a port of the chamber and connected to a syringe. All experiments were conducted at an ambient temperature ($T_a$) of 28.0 °C or 31.0 °C, which is thermoneutral for rats and mice, respectively, in this setup (Balasko et al. 2010; de Oliveira et al. 2014).

In the telemetry setup, mice were studied inside their home cages. Telemetry receivers (model ER-4000; Mini Mitter) were positioned in a temperature-controlled room, and the home cages of mice were placed on top of the receivers. In this setup, a $T_a$ of 27.0 °C was used, which is near the lower end of the thermoneutral zone for mice (Kanizsai et al. 2009). The mouse was preimplanted with a telemetry transmitter to measure $T_{ab}$ and locomotor activity. The latter has been shown to play an important thermoregulatory role in small rodents such as rats and mice (Mount and Willmott 1967; Brown et al. 1991; Weinert and Waterhouse 1998). A similar method was also used to detect small differences in the thermoregulatory phenotype between transient receptor potential vanilloid-1 (TRPV1) channel knockout and control mice (Kanizsai et al. 2009; Garami et al. 2011).

### Substance Administration

PACAP38 was synthesized at the University of Szeged as described in details elsewhere (Gasz et al. 2006). Lyophilized aliquots of PACAP38 were stored at 4 °C. On the day of the experiment, an aliquot was dissolved in saline to give a working solution of PACAP38 at 0.3, 0.6, or 6 mg/ml. For the i.v. drug administration, the 0.3 mg/ml working solution was infused to rats at a rate of 87 μl/min/kg (~29 μl/min/rat) for 4 min to deliver a final dose of PACAP38 at 100 μg/kg (~33 μg/rat). For i.c.v. drug administration, 5 μl of the 0.6 or 6 mg/ml working solutions were infused over a 3-min time period to deliver PACAP38 at doses of 10 and 100 μg/kg (~3.3 and 33 μg/rat), respectively. Control animals were infused with saline.

### Immunocytochemistry for c-Fos

The labeling was performed as published earlier (Gaszner et al. 2012). Briefly, *Pacap*$^{-/-}$ and *Pacap*$^{+/+}$ mice were injected within a time period of 2 min with i.p. administered Nembutal (sodium-pentobarbital; 100 mg/kg body weight; Sanofi, Budapest, Hungary). All mice became unconscious within 2 min. Then, they were transcardially perfused with 25 ml of 0.1 M sodium phosphate-buffered saline (PBS; pH 7.4) for 2 min, followed by perfusion with 150 ml of ice-cold 4 % paraformaldehyde in 0.2 M Millonig sodium phosphate buffer (pH 7.4), for 20 min. Brains were removed and post-fixed for 24 h. Coronal sections (30 μm) were prepared on vibratome (Lancer, Ted Pella Inc., Redding, CA, USA) and stored in anti-freeze solution at−20 °C. For free-floating diaminobenzidine (DAB; Sigma Chemical, Zwijndrecht, The Netherlands) immunocytochemistry, sections were washed 6×10 min in 0.1 M PBS. Consecutively, sections were incubated in 0.5 % Triton X-100 (Sigma Chemical), then in a blocking buffer of 2 % normal goat serum (NGS, Jackson Immunoresearch Europe Ltd., Suffolk, UK) in PBS for 30 min. Sections were transferred into an antiserum solution (1:500) raised against c-Fos (Santa Cruz Biotechnology Inc., sc-52, Santa Cruz, CA, USA). After 3×10 min washes, sections were treated with biotinylated goat anti-rabbit IgG, (1:200) containing 2 % NGS, for 2 h at 20 °C. After a rinse in cold PBS, sections were placed into avidin–biotin-complex solution (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA), for 1 h at 20 °C, followed by PBS for 3×10 min washes. The immunoreaction was visualized using 0.02 % DAB in Tris buffer with 0.00003 % $H_2O_2$, for 10 min. The reaction was observed under microscope and stopped with PBS. After washes, sections were mounted on gelatin-coated slides, dried, cleared by 2×10 min xylene treatment, coverslipped with DePex (Fluka, Heidelberg, Germany), and studied with a Nikon Microphot FXA microscope and Spot RT color digital camera (Nikon, Tokyo, Japan).

The polyclonal c-Fos antiserum (Santa Cruz Biotechnology Inc., sc-52, Santa Cruz, CA, USA) had been generated against the 3–16 amino acid c-Fos peptide fragment of human origin. Preadsorption with 0.1, 1, and 10 μg synthetic c-Fos blocking peptide (Santa Cruz Biotechnology Inc., sc-52 P, Santa Cruz, CA, USA); moreover, omission or replacement of the c-Fos serum by nonimmune rabbit serum effectively prevented the staining. The cross reactivity of this antiserum with other Fos-related proteins was excluded earlier by Ryabinin et al. (1999). Western blot analysis support the specificity of the antibody used (for details see supplier's web site: http://http://datasheets.scbt.com/sc-52.pdf).

### Microscopy, Digital Imaging, and Morphometry

Per animal, the cell counts positive for c-Fos were determined in five serial sections, each interspaced by 60 μm in the median preoptic nucleus (MnPO) and medial preoptic area (MPO) according to Paxinos and Franklin (2004) atlas. Cell counting was carried out on non-edited digital images using ImageJ software (version 1.37, NIH, Bethesda, MD, USA). Quantitation was performed in a double-blind setup by a colleague who is an expert in the rodent neuroanatomy, but was blinded to the identity of preparations. Two representative digital images were grayscaled and contrasted using Photoshop software (Adobe, San Jose, CA, USA) for publication purposes.

### Data Processing and Analysis

Data on $T_c$, $T_{ab}$, heat loss index (*HLI*), and $VO_2$ were compared by two-way ANOVA followed by Fisher's LSD *post hoc* tests, as appropriate. The *HLI* was calculated as:

$$HLI = \frac{T_{sk} - T_a}{T_c - T_a}.$$

The *HLI* changes between 0 (maximum heat conservation due to skin vasoconstriction) and 1 (theoretical maximum heat loss due to skin vasodilation; Romanovsky et al. 2002). Numbers of the c-Fos-positive cells were compared by Student's two sample *t* test (alpha = 5 %). For statistical analysis, Sigmaplot

11.0 (Systat Software, San Jose, CA, USA) software was used. All data are reported as mean±SE.

## Results

### Characteristics of the Thermoregulatory Response to Central (i.c.v.) PACAP38 Administration

To characterize the thermoregulatory effect of PACAP38 in details, we infused 10 or 100 μg/kg of the peptide (or saline) into the lateral cerebral ventricle of rats and recorded their $T_c$, $T_{sk}$, and $VO_2$ in the respirometry setup. In all rats studied, infusion of saline did not have any influence on $T_c$, *HLI*, and $VO_2$ (Fig. 1). On the contrary, both of the applied doses of PACAP38 caused a marked rise in the $T_c$ starting already at 10 min after the injection ($p<0.001$ for both; Fig. 1). The magnitude of the PACAP38-induced hyperthermia was dose-dependent with a maximal $T_c$ change of 2.0±0.3 °C and 1.4±0.3 °C at the dose of 100 and 10 μg/kg, respectively ($p<0.001$ for both). Statistical analysis also revealed significant difference ($p<0.001$) between the effects of the 10 vs. 100 μg/kg dose of PACAP38 on $T_c$. The hyperthermic response to intrabrain administration of PACAP38 is in harmony with earlier reports on the effect of PACAP38 injection on $T_b$ in rats (Pataki et al. 2000, 2003; Resch et al. 2011, 2013).

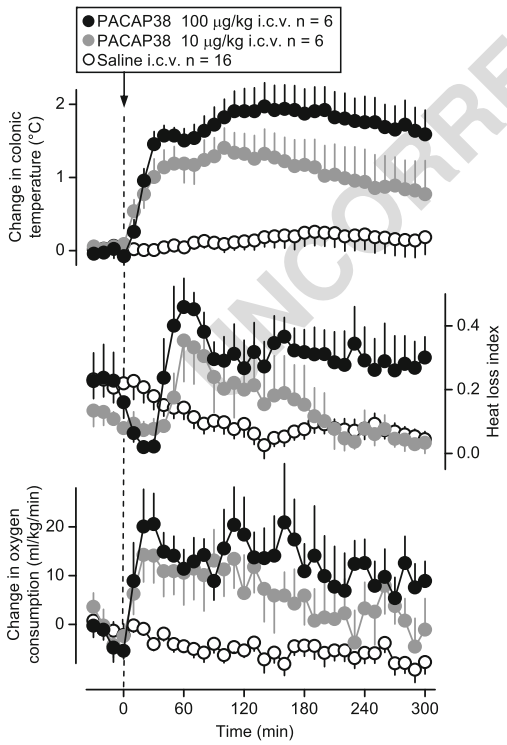


**Fig. 1** Thermoeffector and $T_c$ responses of rats to different doses (10 and 100 μg/kg) of PACAP38 or saline administered i.c.v. The changes of $T_c$ (a measure of deep $T_b$) are shown in the *upper panel*; alterations in the activity of the two main autonomic thermoeffectors, *HLI* and $VO_2$, are depicted in the *middle* and *lower panel*, respectively. These experiments were performed in the respirometry setup at a $T_a$ of 28 °C. Numbers of animals in the corresponding groups are indicated in the figure

In the case of the 100 μg/kg dose, the development of the hyperthermia was preceded by significant tail skin vasoconstriction (as indicated by a decreased *HLI*; $p<0.05$). This is a novel finding of our study, and to our knowledge, the first to report cutaneous vasomotor responses to i.c.v. PACAP38 in conscious rats. It indicates that the thermoregulatory (constrictor) effect of PACAP38 on the cutaneous vascular tone is different from its direct (dilator) effect on skin vessels, which was shown earlier in small rodents (Absood et al. 1992; Tsueshita et al. 2002). The initial drop of *HLI* lasted for ~40 min, and then, it was followed by a pronounced elevation of *HLI* due to tail skin vasodilation, which remained significantly ($p<0.05$) higher than the *HLI* of saline-treated rats until the end of the experiment in accordance with the vasodilatory effect of PACAP38 reported earlier (Absood et al. 1992; Tsueshita et al. 2002). Rats treated with 10 μg/kg PACAP38 i.c.v. had low *HLI* already before substance administration; thus, the initial drop of *HLI* in this group could not be observed, however, ~50 min after drug infusion *HLI* increased above baseline levels and became higher than that of controls ($p<0.05$), although the magnitude and the duration of *HLI* elevation were smaller than those observed at 100 μg/kg.

Similarly to $T_c$, the $VO_2$ of the rats increased already at 10 min after i.c.v. PACAP38 administration as compared to saline-treated animals in a dose-dependent manner. It reached a maximal rise of 21±6 and 14±6 ml/kg/min at 100 and 10 μg/kg, respectively ($p<0.001$ for both). This finding is in harmony with previous studies, in which PACAP38 elevated the metabolic rate (Hawke et al. 2009; Inglott et al. 2011; Resch et al. 2011). In addition, our results demonstrate that simultaneous immediate activation of both autonomic cold-defense thermoeffectors (cutaneous vasoconstriction and brown adipose tissue thermogenesis) contribute to the development of hyperthermia in response to PACAP38.

### Investigation of the Thermoregulatory Response to Systemic (i.v.) PACAP38 Administration

The dose-dependent hyperthermia in response to PACAP38 injection either into the lateral ventricle (current study; Pataki et al. 2000, 2003) or into the hypothalamus (Resch et al. 2011, 2013) suggests that the site of action for PACAP38 is located in the CNS, but one can not rule out the possibility that intrabrain PACAP38 administration acts on central elements of a thermoregulatory loop, which receives its afferentation from the periphery and through central neural structures innervates the corresponding thermoeffector. Such scenario is plausible based on the modern concept of thermoregulation, according to which deep $T_b$ is controlled by a federation of independent loops of thermoeffectors (for review, see

Romanovsky 2007b). In such a loop, activation of the afferent pathway upstream from the central nuclei can evoke equal effects as its activation at any, for example central, part of the loop.

To study the possibility of a peripheral site of action for PACAP38 in a comparative experiment, we investigated whether the same (100 μg/kg) dose of PACAP38 that caused pronounced hyperthermia when injected i.c.v. has a similar effect on deep $T_b$ in the case of a systemic (i.v.) administration. Infusion of saline did not have thermoregulatory effects in the rats studied (Fig. 2). When PACAP38 at 100 μg/kg was infused i.v., it caused a slight, but significant ($p<0.05$) rise of $T_c$. Both the maximum $T_c$ elevation (~0.3 °C) and the duration (60 min) of the hyperthermic response to systemic PACAP38 infusion were markedly less than what i.c.v. administration of the same dose evoked ($p<0.05$). One can argue that neurons in the CNS were exposed to higher local concentrations of PACAP38 than neurons on the periphery after infusion of the same dose into the two compartments, but the differences in local concentrations are unlikely to account for the substantial differences observed in the $T_c$ response. Importantly, the response to PACAP38 at 100 μg/kg i.v. was substantially smaller both in magnitude (~5-fold lesser) and in duration (~2 times shorter) than the effect of a tenfold smaller dose (10 μg/kg) delivered i.c.v. (Fig. 1). Similarly to what we observed after i.c.v. drug delivery, in the case of the i.v. infusion of PACAP38, the hyperthermia was also brought about by a decreased heat loss and an increased $VO_2$, although activity of both thermoeffectors changed to a much lesser extent than after i.c.v. delivery (Fig. 2). The result that even a tenfold lower dose of PACAP38 caused much stronger hyperthermia after i.c.v. administration compared to i.v. delivery, unequivocally shows that the site of action for the thermoregulatory response to PACAP38 is situated within the CNS.

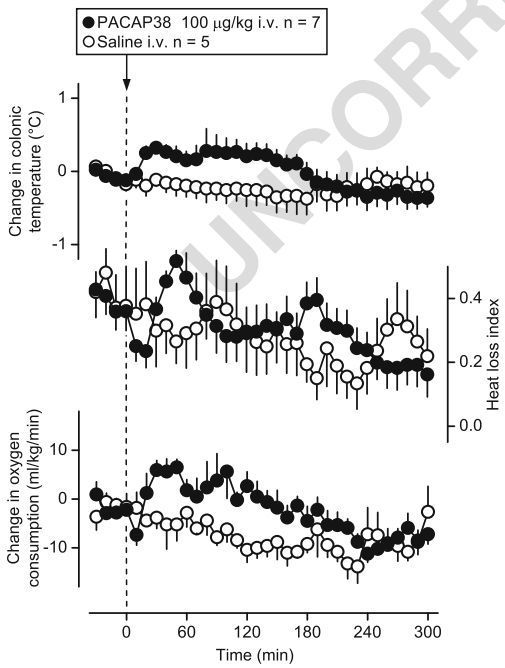


**Fig. 2** The thermoregulatory response of rats to PACAP38 (100 μg/kg) or saline administered i.v. Changes of $T_c$ (*upper panel*), *HLI* (*middle panel*), and $VO_2$ (*bottom panel*) are shown with the same scale intervals as in Fig. 1. The experimental conditions were also identical to those described in Fig. 1 (respirometry setup, $T_a$ of 28 °C). Numbers of animals in the corresponding groups are indicated in the figure

## Thermoregulatory Characteristics of *Pacap*$^{-/-}$ Mice

After we characterized the thermoregulatory response to exogenous PACAP38 administration, we wanted to know how the absence of PACAP affects deep $T_b$.

First, we studied the circadian changes of $T_{ab}$ and locomotor activity in freely moving *Pacap*$^{-/-}$ and *Pacap*$^{+/+}$ mice (Fig. 3a). Representing the characteristic circadian rhythm of rodents, mice of both genotypes had lower $T_{ab}$ and activity levels during the light (inactive) phase than during the dark (active) phase. In accordance with the study by Hashimoto et al. (2001), we found that *Pacap*$^{-/-}$ mice were more active than their wild-type littermates during both the light and the dark phase of the day ($p<0.001$). During most of the light phase (between 5 a.m. and 3 p.m.), the increased locomotor activity resulted in a moderately higher $T_{ab}$ in the *Pacap*$^{-/-}$ mice compared to controls ($p<0.05$); but in the night, there was no significant difference in $T_{ab}$ between the genotypes (Fig. 3a). Similar results on the effect of hyperactivity on deep $T_b$ were also demonstrated in chicken (Aschoff and von Saint-Paul 1973) and in mice (Weinert and Waterhouse 1998, 1999), showing that elevated locomotor activity resulted in higher $T_b$ during the inactive phase, but not during the active phase. It can be assumed that the different light–dark influence of locomotor activity on deep $T_b$ can originate from the circadian changes of cutaneous vasodilation, thus heat loss mechanisms (Weinert and Waterhouse 1998).

Next, we measured the basal $T_c$ and $VO_2$ in loosely restrained *Pacap*$^{-/-}$ and *Pacap*$^{+/+}$ mice (Fig. 3b). These experiments were performed in the respirometry setup (see the "Materials and Methods" section), so we could minimize the influence of locomotor activity on $T_c$ and $VO_2$. We recorded the basal thermoregulatory parameters for 60 min starting from 11 a.m. because this time period corresponded to the biggest difference in deep $T_b$ between freely moving *Pacap*$^{-/-}$ and *Pacap*$^{+/+}$ mice (Fig. 3a). As shown in Fig. 3b, the basal $VO_2$ was significantly lower in *Pacap*$^{-/-}$ mice as compared to controls throughout the experiment ($p<0.001$). As a consequence of their hypometabolism, $T_c$ of the *Pacap*$^{-/-}$ mice was also slightly lower than that of controls ($p<0.01$).

To assess which neurons are responsible for maintaining the reduced resting metabolic rate in *Pacap*$^{-/-}$ mice, we measured expression of the inducible transcription factor c-Fos, a marker of neuronal activation (Sagar et al. 1988), in the MnPO

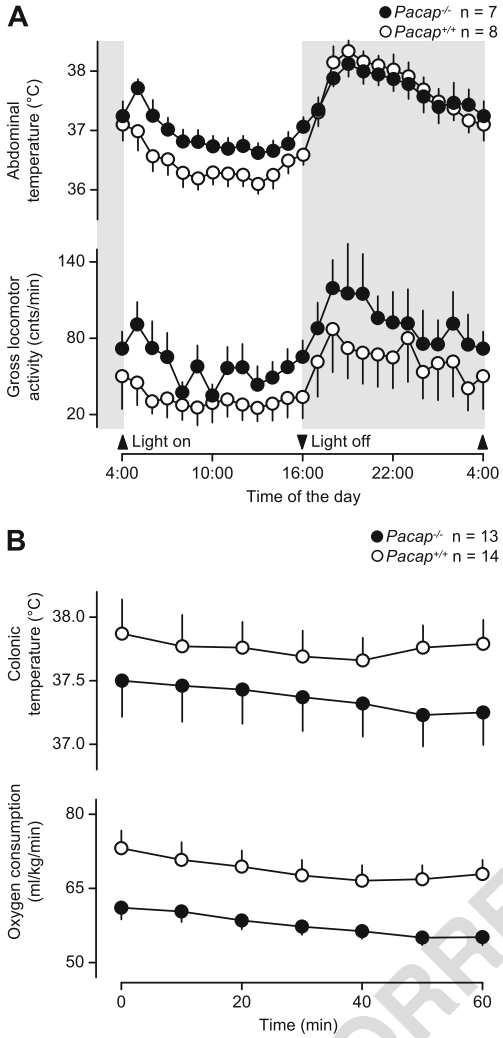


**Fig. 3** The thermoregulatory phenotype of *Pacap*$^{-/-}$ and *Pacap*$^{+/+}$ mice. **a** Circadian changes of $T_{ab}$ and locomotor activity in freely moving *Pacap*$^{-/-}$ and *Pacap*$^{+/+}$ mice. These experiments were performed in the telemetry setup at a $T_a$ of 27 °C. **b** Basal $T_c$ and $VO_2$ of loosely restrained *Pacap*$^{-/-}$ and *Pacap*$^{+/+}$ mice. Recordings of $T_c$ and $VO_2$ were performed between 11 a.m. and 12 p.m. in the respirometry setup at a $T_a$ of 31 °C. Numbers of animals in the corresponding groups are indicated in the figure

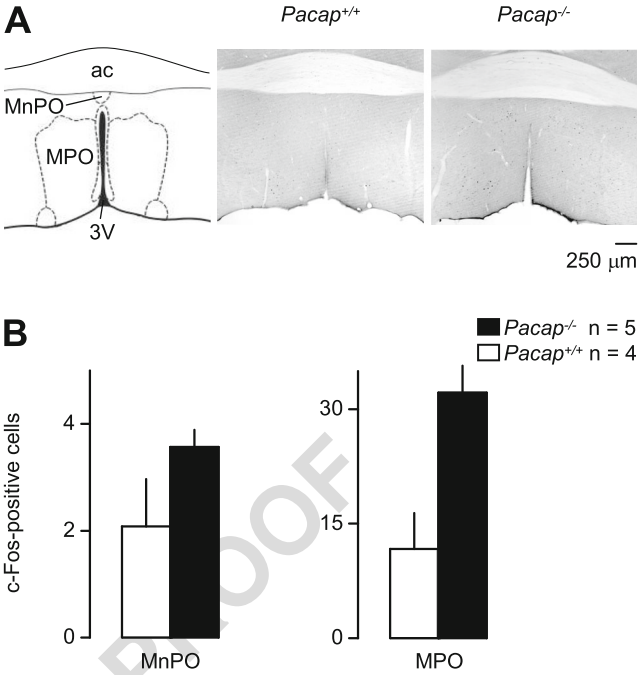


**Fig. 4** The expression of c-Fos positive cells in the MnPO and MPO of *Pacap*$^{-/-}$ and *Pacap*$^{+/+}$ mice. **a** Schematic drawing from Paxinos and Watson (2004) atlas and representative photomicrographs of coronal sections from the MnPO and MPO at the anterior–posterior coordinate of 0.14 mm from Bregma. The anterior commissure (ac) and the third ventricle (3 V) are shown as landmarks. **b** Quantitative analyses of c-Fos immunoreactive cells in the MnPO and MPO. Numbers of animals in the corresponding groups are indicated in the figure



and MPO (Fig. 4a). It is well established that neurons in these brain areas are involved in the regulation of thermogenesis (Nakamura and Morrison 2008b; Romanovsky et al. 2009). In the MnPO, we found no statistical difference in the number of c-Fos-positive cells between *Pacap*$^{-/-}$ and *Pacap*$^{+/+}$ mice; however, c-Fos expression was nearly three times higher ($p<0.05$) in the MPO of the *Pacap*$^{-/-}$ mice as compared to their wild-type littermates (Fig. 4b). It has been shown that GABAergic neurons in the MPO tonically suppress BAT thermogenesis (Osaka 2004) and can be regarded as the first effector neurons of the thermoregulatory loops controlling autonomic thermoeffectors (Romanovsky et al. 2009), therefore, our current findings in *Pacap*$^{-/-}$ mice suggest that the absence of PACAP results in an increased activation of the inhibitory MPO neurons leading to more pronounced suppression of thermogenesis.

## Discussion

Our findings clearly demonstrate that central, rather than peripheral mechanisms are involved in the hyperthermia-inducing effect of PACAP38. Although PACAP38-induced hyperthermia has been studied earlier in small rodents, based on data currently available from those experiments, no firm conclusion could be drawn about the site of the thermoregulatory action of PACAP38. In previous studies, PACAP38 was administered only into the CNS (Pataki et al. 2000, 2003; Hawke et al. 2009; Inglott et al. 2011; Resch et al. 2011, 2013) and evoked prominent thermoregulatory responses, suggesting a central mediation of the effect. However, taken into account the organization of the thermoregulatory system, which consists of independently operating thermoeffector loops (Romanovsky 2007a, b), the interpretation of the results obtained with one single injection of a substance into the CNS can be misleading, because it can not be excluded that activation of an upstream or downstream peripheral structure of the same thermoregulatory pathway could cause the same or

similar effect. For example, Inglott et al. (2011) found that intrathecally administered PACAP38 exerts sympathoexcitation even after spinal transection and the authors concluded that the effect is, therefore, evoked from the spinal cord itself. Based on the functional architecture of the thermoregulatory system, however, the results by Inglott et al. (2011) can also be explained by a direct effect of PACAP38 on efferent spinal structures (most likely the intermediolateral column) of the same thermoregulatory loop, which can be also activated upstream in the brain by i.c.v. (current study; Pataki et al. 2000, 2003) or by intrahypothalamic infusions (Resch et al. 2011, 2013) and the activation of which leads to equivalent effects regardless of the order of the neuron being activated in the loop. Supporting a peripheral site of action, PACAP38 and its receptor have been shown to be widely expressed on sensory neurons in the periphery (for review, see Mulder et al. 1999; Vaudry et al. 2000) and in the enteric nervous system (Miampamba et al. 2002). In the present study, we compared the effects of central and systemic administration, and showed that the same or even a tenfold lower dose of PACAP38 evokes stronger hyperthermia, when given i.c.v. than when delivered i.v. This result unequivocally supports the central mediation hypothesis. The slight increase of $T_c$ in response to peripherally infused PACAP38 could be attributed to its penetration of the blood–brain barrier (Banks et al. 1993; Nonaka et al. 2002). To our knowledge, this is the first report in which the central and peripheral thermoregulatory responses to PACAP38 were compared under identical experimental conditions.

We also studied the characteristics of the thermoregulatory response to PACAP38 and found that the hyperthermia started to develop promptly: already 10 min after drug administration, we could detect the activation of thermoeffectors and a slight increase of $T_c$. This is a novel finding of the study as in all of the earlier studies investigating the thermal effect of PACAP38, $T_b$ was recorded hourly and the substance was administered in a stressful manner, thus the developing stress-induced hyperthermia, which was also present in the vehicle-treated animals masked the early phase of the response. We found that PACAP38 administration resulted in the simultaneous activation of non-shivering thermogenesis and cutaneous vasoconstriction, which are the two principal autonomic cold-defense thermoeffectors (Romanovsky 2007a). It has to be noted that the measured increase in $VO_2$ can theoretically originate from elevation of both shivering and non-shivering thermogenesis; but in small rodents, non-shivering thermogenesis is the primary source of heat production (for review, see Cannon and Nedergaard 2004). The initial skin vasoconstriction seems to contradict the reported vasodilatory effect of PACAP38 (Absood et al. 1992; Tsueshita et al. 2002), but this contradiction can be resolved by considering that in the current study PACAP38 was delivered into the lateral ventricle of the brain, from where it can broadly access the POA, where neurons of the thermoeffector pathway for tail skin vasomotor tone are situated (Nakamura and Morrison 2008a, b). Therefore, it is plausible that PACAP38 acted on central thermoregulatory elements resulting in skin vasoconstriction, but when the peptide spread to more distant (non-thermoregulatory) areas, it caused vasodilatory effect, which was also observed in the current study ~40 min after PACAP38 injection (Fig. 1). Although the later occurring cutaneous vasodilation lasted longer than the initial skin vasonconstriction, from a thermoregulatory point of view, the initial decrease of heat loss is equally important, as it was present during the developmental phase of PACAP38-induced hyperthermia, thus contributed to the rise of deep $T_b$.

Since neurons of the thermoeffector loop for non-shivering thermogenesis are also located in the POA (for review, see Romanovsky et al. 2009), it is tempting to assume that the site of the hyperthermic effect of PACAP38 is in the POA on neurons, which belong to the common part of the thermoeffector pathways for non-shivering thermogenesis and cutaneous vasoconstriction. Supporting this hypothesis, the PAC1 receptor, which has been shown to be involved in mediation of the hyperthermic effect of PACAP38 (Tachibana et al. 2007; Resch et al. 2013), is abundantly expressed in the MnPO of the POA (Joo et al. 2004), where GABAergic neurons controlling autonomic cold-defense thermoeffectors can be found (Nakamura and Morrison 2008a). In recent studies from Resch et al. (2011, 2013), it has been proposed that the hyperthermic and hypermetabolic effects of PACAP38 are mediated by neurons in the hypothalamic ventromedial nucleus and possibly in the lateral parabrachial nucleus. Our hypothesis is also in harmony with these results, because in the cold-activated pathway glutamatergic neurons from the lateral parabrachial nucleus project to GABAergic neurons in the MnPO (Nakamura and Morrison 2008b), which in turn, are connected to neurons of the hypothalamic ventromedial nucleus (Imai-Matsumura et al. 1988; Thornhill et al. 1994).

As an alternative approach to study the role of PACAP in thermoregulation, we investigated deep $T_b$ and locomotor activity of *Pacap*$^{-/-}$ mice and found that these mice were hyperactive throughout the day and hyperthermic during the light phase as compared to controls. The hyperactivity of *Pacap*$^{-/-}$ mice was also observed in an earlier study (Hashimoto et al. 2001) and, although Adams et al. (2008) reported no alteration in the locomotor activity of the *Pacap*$^{-/-}$ mice compared to controls, in their study during the three consecutive nights of the experiments the number of beam breaks in case of the *Pacap*$^{-/-}$ mice exceeded by ~2,000 (i.e., by ~50 %) that of controls at certain time points. It is a novel finding of the present study that in our experiments, the increased activity of the *Pacap*$^{-/-}$ mice resulted in elevated

$T_b$ during the light phase of the day, in which phase locomotor activity correlates strongly with $T_b$ (Weinert and Waterhouse 1998, 1999). In contrast to our findings, in the study by Hashimoto et al. (2009) *Pacap*$^{-/-}$ mice had lower $T_b$ during the night than controls, but those experiments were conducted at a $T_a$ of 23 °C, which could be presumably below the thermoneutral zone of mice. As it has been repeatedly shown that cold-defense responses of *Pacap*$^{-/-}$ mice are impaired compared with controls (Gray et al. 2002; Adams et al. 2008; Cummings et al. 2008), it can be assumed that the different influence of a chronic, mild cold exposure on the $T_b$ of *Pacap*$^{-/-}$ and *Pacap*$^{+/+}$ mice could contribute to the observed lower $T_b$ in the *Pacap*$^{-/-}$ mice. Indeed, inadequate heat production and lower $T_b$ in *Pacap*$^{-/-}$ mice were observed in response to chronic, mild (21 °C) cold exposure in the study by Gray et al. (2002). In an earlier study by Cummings et al. (2008), the $T_b$ of *Pacap*$^{-/-}$ mice did not significantly differ from that of controls, however, in that study the authors measured rectal temperature in previously decapitated mice, which method is not sensitive enough to detect small (especially locomotion-induced) differences in $T_b$. Locomotor activity is widely viewed as a thermoregulatory effector in mice (Kanizsai et al. 2009; Szentirmai et al. 2010; Garami et al. 2011) and our findings suggest that freely moving *Pacap*$^{-/-}$ mice utilize locomotor activity as a thermoeffector to maintain an elevated $T_b$ during the light phase and normal $T_b$ during the night phase of the day. It has to be mentioned that the increased locomotor activity of the *Pacap*$^{-/-}$ mice could possibly also originate from distinct mechanisms, which are independent from thermoregulation.

We then asked whether the basal daytime $T_b$ of loosely restrained *Pacap*$^{-/-}$ mice (i.e., those that can not use locomotion as a thermoeffector) also differs from their wild-type littermates. In contrast to our results in freely moving mice, when restrained, *Pacap*$^{-/-}$ mice were hypometabolic and had lower $T_b$ than controls. The decreased metabolic rate and $T_b$ in the absence of PACAP is in harmony with our results demonstrating the hypermetabolic and hyperthermic effect of PACAP38 injection in rats. When we measured the expression of c-Fos positive cells in the POA of the mice, we found that the number of c-Fos positive cells in the MPO was markedly higher in *Pacap*$^{-/-}$ mice than in controls, suggesting that the absence of PACAP results in an increased activation of MPO neurons. Since GABAergic neurons in the MPO tonically suppress thermogenesis (Osaka 2004), we propose that in *Pacap*$^{-/-}$ mice inhibitory MPO neurons are more activated and this results in an enhanced suppression of thermogenesis. This hypothesis is also in harmony with the proposed action of PACAP38 injection on GABAergic MnPO neurons (see above), because activation of these neurons results in an increased inhibition of the inhibitory MPO neurons, which leads to elevated metabolic rate and hyperthermia. Although alternate explanations are also plausible, it can be assumed that the absence of PACAP38 results in a lower resting metabolic rate (and $T_b$) and as a compensatory mechanism for the hypometabolism, *Pacap*$^{-/-}$ mice become hyperkinetic to maintain normal (or even higher) $T_b$. Interestingly, a similarly altered thermoeffector pattern (hypometabolism and hyperkinesis) was observed in our recent study with mice lacking the TRPV1 channel (Garami et al. 2011). The similar thermoregulatory consequences of the absence of PACAP and TRPV1 can be explained with the alteration of the same neural pathways as PACAP38 is released from activated capsaicin-sensitive (i.e., TRPV1-expressing) neural afferents into the systemic circulation (Helyes et al. 2007). Although the exact molecular and neuronal mechanisms involved in the development of the observed thermoregulatory phenotype of *Pacap*$^{-/-}$ mice need to be further investigated, an involvement of altered biochemical processes in the CNS of *Pacap*$^{-/-}$ mice can be suspected (Maasz et al. 2014).

In conclusion, we showed in a straightforward comparative experiment that PACAP38 causes hyperthermia by acting on targets within the CNS. The PACAP38-induced hyperthermia is brought about through the simultaneous activation of both autonomic cold-defense effectors: elevation of non-shivering thermogenesis and cutaneous vasoconstriction. We hypothesize that GABAergic neurons within the MnPO are involved in mediation of thermoregulatory response to PACAP38. The absence of PACAP results in hyperkinesis and daytime hyperthermia in freely-moving *Pacap*$^{-/-}$ mice through mechanisms which need to be clarified, but an involvement of TRPV1 and altered central biochemical processes can be suspected. The increased locomotor activity is presumably a compensatory mechanism for the hypometabolism and hypothermia, which is present under resting conditions in the absence of PACAP.

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Q4

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