Ph.D. THESIS

Capsaicin and its receptors, neuropeptides: their roles in thermoregulatory and other processes of energy balance.

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1. Introduction

The energy balance and its regulation is a multifactorial, complex process. In mammals, the energy balance is maintained basically by two regulatory circuits, and by their interaction with each other. One of them serves the long-term, while the other the short-term needs of the organism. They are inseparable and – as it will be described later – compose a firm entity.

The long-term regulation is valid for all animal species, it refers to the nutritional status of the body in various stores of metabolazible substrates (such as fat, glycogen, protein). The common variable is, therefore, the bone- and water-free body weight (energy content), which is based on the balance between food intake (energy intake) and metabolic rate (energy expenditure). A positive long-term balance (e.g., large food intake with low metabolic rate) is regarded anabolic, in contrast to the catabolic type negative balance. This long-term regulatory system is manifested via repeated episodic changes of the feeding status, which can initiate or stop food intake through hunger and satiety and can also make intermittent adjustments in metabolic rate according to the actual need. Tonic effects of nutritional status (e.g., obesity or cachexia) set the level for the episodic changes, but satiety and hunger are partly independent of the nutritional status[1,2]. Accordingly, the short-term regulation of food intake (feeding status) does not depend only from the nutritional status, but also from the feeding act itself, that is, hunger and satiety can develop in both obese and cachectic individuals. Based on the aforementioned, it is also obvious that a long-lasting anabolic shift of the balance (e.g., excessive food intake with insufficiently enhanced metabolic rate) will result in obesity, while a catabolic shift (e.g., a pathologically increased metabolic rate with insufficient food intake) will lead to cachexia.

The short-term regulation, in turn, is valid for only for homeothermic species and it refers to caloric (thermal) balance, i.e. to a relationship between heat loss and heat production (metabolic rate), the main autonomic thermoeffectors of thermoregulation. Due to the stability of the described balance, the physiological core body temperature is maintained in a relatively narrow range, within a few tenths of degree Celsius[3,4]. Accordingly, decreased or increased heat loss (e.g. due to ambient heating or cooling) results in a compensatory reduction or elevation of the metabolic rate. Conversely, primary changes in the metabolic rate must be accompanied by commeasurable compensatory changes in heat loss in order to maintain homeothermy and the short-term energy balance. Imbalance can result in either hypothermia or hyperthermia when an exogenous/endogenous heat loss or heat load, respectively, cannot be fully compensated[5]. In contrast to such passive imbalances, primary shifts in the set-point can induce active imbalances of central origin[6]. These may result in either a rise in body temperature, which is called fever, e.g., due to central prostaglandins in rats[7]; or a fall in body temperature called anapyrexia, e.g., due to central serotonin in sheep[4]. Both fever and anapyrexia develop through coordinated changes in thermoregulatory effector mechanisms: in case of fever hypermetabolism with decreased heat loss, in case of anapyrexia hypometabolism with high heat loss. Noteworthy, the set-point based thermoregulatory theory is being questioned nowadays[8]. According to the new concept, which rejects set-point, the thermoregulatory system functions as a federation of independent thermoeffector loops. This concept emphasizes the different activation thresholds controlling a given thermoeffector and explains with this difference the different thermoregulatory responses, which may occur either as coordinated or incoordinated reactions[8,9].

The two regulatory circuits of energy balance are strictly interrelated via the metabolic rate. On the one hand, changes of either the feeding or nutritional status may influence the thermoregulatory status; in this respect the fasting- or starvation-induced hypometabolism and tendency for hypothermia are just as characteristic as the postprandial hypermetabolism and hyperthermia or the diet-induced thermogenesis and thermic effect of feed[10,11]. On the other hand, primary alterations of temperature regulation also modify consumption behavior, e.g., food intake increases in the cold (the excessive heat loss being compensated by high metabolic rate to maintain homeothermy – the high metabolic rate, in turn, is compensated by hyperphagia to secure a relatively standard body weight)[12]. In contrast to cold exposure, febrile illnesses are characterized by concurrent anorexia[13] – even though the fever-induced anorexia is unrelated to body temperature.

Individual factors of the complex system might be influenced selectively, causing functional alteration in either system and invoking secondary alterations in the other one. The regulations of these systems often overlap and the regulatory factors (e.g., neurotransmitters, neural elements) are many times identical or similar.

2. Aims and goals of the study

In the present study we aimed at investigating certain specific – from a physiological point of view principal – details of the thermoregulatory phenomena described in the introduction by using animal models (rats and mice). Among the components of the aforementioned complex energy balance, according to the methodological profile of our laboratory, we studied factors of both the long-term regulatory system (body weight, food intake, metabolic rate) and the short-term regulatory system (body temperature, heat loss, metabolic rate), and their changes under specific environmental conditions. The experimental plans were assigned to two, with each other strictly correlated, projects.

One of the projects was aimed to provide a better understanding for the roles of capsaicin (CAP)-sensitive neural afferents and transient receptor potential vanilloid (TRPV)-1 channels in processes of the complex energy balance: we studied their significance in different thermoregulatory and feeding mechanisms. In this part of the study, we investigated the neural, TRPV1-dependent afferent side of the complex energy balance, specifically whether these factors participate in conveying information from the periphery to the central nervous system in processes of the complex energy balance, and if yes, how.

The aim of the second project was to better understand how the information that already reached the brain from the periphery will be further processed in the central nervous system, thus we studied one of the most important central components in the regulatory processes of the complex energy balance: in rats, we investigated the significance of the “pro-opiomelanocortin – melanocyte-stimulating hormone (MSH) – melanocortin” system via the thermoregulatory and anorexigenic effects of its endogenous agonist, α-MSH. We analyzed the central effects of α-MSH on different thermoregulatory effectors, furthermore, on spontaneous and fasting-induced food intake. We wanted to shed light on the thermoeffectors through which the thermoregulatory effects of the most important catabolic system are brought about. We studied whether intraperitoneal (IP) CAP desensitization influences the effects of centrally administered anabolic and catabolic neuropeptides (neuropeptide Y, NPY and α-MSH, respectively).

3. Materials and methods

3.1. Experimental animals and their housing

In most of our experiments Wistar rats were used. The animals were housed individually in plastic cages containing a small amount of bedding. Standard rodent chow and tap water were available *ad libitum*, except for the experiments with food deprivation. The animal house operated on a 12/12 h light/dark cycle with lights on at 6:00 a.m. The ambient temperature (T_a) was kept at 23-26°C. The animals were adapted to experimental conditions by regular body weight measurements and extensive habituation to the experimental confiners. Occasionally, a rat was used in experiments multiple times (e.g., for comparison of effects of food-deprivation + refeeding + α-MSH), then urethane euthanasia was induced and, if necessary, *post mortem* examination was performed. In experimental tests and interventions, the general rules and special approval of the University of Pécs Ethical Committee for the Protection of Animals in Research, in agreement with national standards (BA 02/2000-13/2006) were strictly observed.

Housing of male Wistar rats and guinea pigs, which were used in experiments performed abroad (Systemic Inflammation Laboratory, Trauma Research, St. Joseph's Hospital and Medical Center, Phoenix, Arizona, USA) did not substantially differ from the abovementioned conditions. These experiments were conducted under protocols approved by the St. Joseph's Hospital Animal Care and Use Committee.

In a series of experiments, C57BL/6 wild type and *Trpv1* knockout (KO) male mice were used. Mice were housed individually in plastic cages kept at a T_a of 26-28°C (thermoneutral), or 23-25°C (subneutral). Light and dark cycles were the same as described above. Except for the experiments, in which food deprivation was applied, standard rodent chow and tap water was available *ad libitum*. The experiments were executed according to the general rules set in the Hungarian law on animals and the experimental protocols were approved by the University of Pécs Ethical Committee for the Protection of Animals in Research (BA 02/2000-13/2006).

3.2. Surgeries

Surgeries were performed under anesthesia induced by ketamine + xylazine (78 + 13 mg/kg, IP). To prevent infections, 2 mg gentamycin was administered IP. The anesthesia was always the same regardless from the type of the surgery [intravenous (IV) cannula implantation, intracerebroventricular (ICV) cannula implantation, perivagal CAP treatment, IP radiotransmitter implantation]. The implanted, closed cannulas were exteriorized at the nape. Most procedures (ICV injection, food deprivation, another surgery, e.g., ICV cannula insertion after IP radiotransmitter implantation, etc.) were executed at least one whole week after the (first) surgery, except the administration of lipopolysaccharide (LPS), which was performed 3-4 days after the surgery.

For the experiments abroad, rats and guinea pigs were implanted with an IV cannula inserted into the right jugular vein 5-7 days before an experiment under ketamine + xylazine + acepromazine (55.6, 5.5, 1.1 mg/kg, respectively, IP) anesthesia. To prevent infections, 1.1 mg/kg enrofloxacine was administered IP. During the same anesthesia, a miniature datalogger (Subcue Dataloggers, Calgary, Canada) was implanted into the abdominal cavity of guinea pigs in order to monitor body temperature.

3.3. Recordings of the metabolic rate, heat loss and core temperature

The metabolic rate of rats was determined by the help of a Kipp-Noyons diaferometer based on the analysis of the gas sample from the air flowing through a metabolic chamber. In some experiments – by utilizing the same principle, i.e. indirect calorimetry – other instruments were used for measurements of the metabolic rate: Oxymax (Columbus Inc., Columbus, OH), Small Animal System (Sable Systems, Las Vegas, NV).

For recording of core temperature (T_c), a copper-constantan thermocouple was inserted 10-cm deep into the colon, while skin temperature (T_s) was measured by a thermocouple attached to the tail skin by tape. To describe heat loss, we used calculation of an index, which was originally introduced by our laboratory and has been used by numerous other groups. This index can be also used in the case of a suddenly changing T_a and it is known as the "heat loss index" (HLI):

$$\mathbf{HLI = T_s – T_a / T_c - T_a}$$

Based on the formula it is obvious that the HLI changes always between 0-1, where low (near-zero) values indicate a decreased and high (close to 1) values indicate an increased state of heat loss.

Animals equipped with thermocouples (sometimes cannula-extensions) or implanted with a miniature datalogger were placed into experimental confiners, which made them unable to turn around, but allowed for free back-and-forth movements. Due to the preceding extensive habituation the animals were not stressed under the experimental conditions: in our experience, their T_c was only slightly different (with a maximum of few tenths of a centigrade higher) from their freely-moving counterparts, in which T_c was recorded by telemetry at the same time interval of the day. In the case of metabolic rate recordings, the animals in the experimental confiners were placed into an open-circuit metabolic chamber, the T_a of which was maintained (or changed, if necessary) by a water bath or by an incubator chamber. The extensions of cannulas and the thermocouples were passed through a sealable port of the hermetically sealed (air-ventilated) metabolic chamber: through the cannulas drugs could be administered during the recordings in a non-stressful manner without disturbing the animals.

In a separate set of experiments, T_c and general locomotor activity of the animals were continuously monitored by telemetry. This method did not allow us to measure metabolic rate and T_s, but enabled us to perform recordings in freely-moving animals for an extended period of time (sometimes several weeks). The signal from the surgically implanted IP radiotelemetric transmitter (ER-4000 model VMFH, Minimitter, Sunriver, OR) were collected by a metal-plate-like antenna beneath the plastic cage of the animal and then stored on a computer. With this method we were also able to analyze the circadian changes in T_c (and general locomotor activity).

3.4. Induction of fever, intravenous drug administration, cannulas

To induce fever, we used bacterial LPS [*E. Coli* (Sigma-Aldrich)], which was administered at a dose of 10 μg/kg through a pp10 polyethylene (Portex), or silicone (Baxter) cannula pre-implanted into the jugular vein. The IV cannula was implanted 3-4 days before an experiment. At the time of LPS administration, the unanesthetized animals were in sealed metabolic chambers, the pyrogen was dissolved in 0.9% NaCl, and then 0.5 ml of the solution was injected through an extension of the IV cannula unnoticeable for the animal (control animals were treated with 0.5 ml of pyrogen-free 0.9% NaCl solution).

Similar IV cannulas were used for administration of the TRPV1 antagonists AMG517 (100 μg/kg), capsazepine (CPZ; 65.5 μmol/kg), and their vehicles. In these

experiments, the substances were infused with a rate of 167 µl/min over 2 minutes through similar extensions as described above.

3.5. Methods of intracerebroventricular injection and infusion

The effects of central administration of α-MSH were studied after ICV injection of the drug. A 22-gauge stainless-steel guide cannula was implanted into the right lateral cerebral ventricle of anaesthetized rats with the following stereotaxic coordinates: A = -1.0 mm, L = 1.5 mm, V = 3.8 mm. Through an insertable inner 28-gauge injector needle α-MSH (2, 5, or 10 µg), NPY (2 µg) or vehicle was administered in a volume of 5 µl not more than once per week. The injection was performed through a pp10 extension of the injector, thus without disturbing the animal.

3.6. Abdominal, perivagal desensitization (CAP pretreatments)

To achieve local, intra-abdominal desensitization, IP CAP (Sigma-Aldrich) pretreatment was used. CAP (dissolved in 96% ethanol, and then diluted to contain 10% ethanol, 10% Tween-80 and 0.9% NaCl at different CAP concentrations) was injected into the abdominal cavity fractionally, in two small doses (2 + 3 mg/kg, totaling 5 mg/kg) at 9:00 a.m. and 3:00 p.m. This treatment damaged only the abdominal afferents[14,15], and the effect was reliable for a few (2-3) week, but starting from approximately the 6th week it gradually relapsed. Mainly, the damage of the chemo- and mechanosensitive elements was important, because physiological importance of the abdominal heat-sensitive nerve terminals is negligible, thus their impairment probably has no physiological consequences – such treatment had no systemic effects.

In case of perineural CAP desensitization, a 3-4 cm midline laparotomy was made, immediately below the diaphragm a 3-4 mm wide strip of cotton wool was introduced to surround the anterior and posterior trunks of the abdominal vagus that was released from the esophagus. The cotton wool was isolated from the surrounding tissues by a small sheet of polyethylene, and then wetted with freshly prepared solution 1% solution of CAP. This wrapping was kept in place for 20 minutes, before it was removed carefully so as not to soil other tissues or damage the vagus nerve mechanically. Following removal of the cotton wool, the operation area was carefully rinsed with 0.9% NaCl; after absorbing the excess fluid and polyethylene sheet, the surgical wound was sutured in layers. Control animals were treated with CAP-free vehicle.

3.7. Food deprivation and refeeding, measurements of the food intake and body weight

Food deprivation started at 9:00 a.m. and it lasted for either 120-h or 48-h (sometimes 24-h), whilst water remained available. During fasting rats were kept in their home cages, their body weights were measured regularly. In a series of experiments, we studied the metabolic state (basal metabolic rate and T_c) of food-deprived rats in metabolic chambers at different stages of fasting. In another group of animals, the fasting-induced daily loss of body weight was investigated. After refeeding, rats were allowed to freely move around in their home cages and their gain of body weight was measured every 30 min for 3 h, which (apart from the simultaneous weight gain by water intake and weight loss due to urination and defecation) strongly correlates to the dynamics of food intake. We also measured the 3-h amount of rodent chow consumption, additionally in some cases the changes of food intake and body weight in the following 21 h (to calculate 24-h values).

In a further series of experiments, instead of standard rodent chow pellets the rats were adapted to powdered rodent chow and their food intake was continuously measured as cumulative-powdered-chow consumption by a Feed-Scale system

(Columbus Inc., Columbus, OH) for 3 h, occasionally for further 21 h at regular time intervals. The collected data were stored on a computer. To assess spontaneous food intake, recordings started at 6:00 p.m., i.e. at the beginning of the active, dark cycle, while fasting-induced refeeding was investigated in experiments beginning at 9:00 a.m.

In the mice experiments, food deprivation started at 9:00 a.m. at least 1 week after the implantation of the IP radiotransmitter, and then lasted either for 48 h in case of a subneutral T_a, or for 72 h in case of a thermoneutral T_a. The longer (72-h) fasting allowed for longer observation of the changes in the animals' energetics without significantly influencing the extent of fall in their body weight.

3.8. Statistical analyses

Based on the design of the actual experiment, for statistical analyses ANOVA repeated measures, one-way ANOVA with *post hoc* test, or Student t-test was used, as appropriate. All results are presented as means ± S.E.M.

4. Results

4.1. Roles of CAP-sensitive abdominal vagal afferent fibers in endotoxin-induced polyphasic fever

IV injection of the vehicle was without effect in perineurally or IP CAP pretreated, as well as in sham-operated or IP vehicle pretreated animals. In sham-operated rats, the febrile response to LPS was similar to the usual triphasic fever course in control (IP vehicle treated) animals: it started about 35 min after LPS injection and exhibited the characteristic three phases. In rats with perineural CAP treatment, the fever course following a similar LPS injection differed from the fever course of sham-operated animals in that the third febrile phase appeared to be attenuated (although this was not statistically significant). However, the beginnings of the febrile response remained unaltered. This response was different from that seen in rats with IP CAP pretreatment: in IP desensitized rats, mainly the first phase was delayed and inhibited, later parts of the febrile response were not affected.

4.2. Roles of CAP-sensitive abdominal afferents in the energetic processes during food intake and food deprivation

An IP desensitized status, *per se*, did not result either in lasting enhancement, or in lasting suppression of daily food intake, nor in altered rate of body weight gain: both the daily food intakes and daily weight gains were similar in control and IP CAP pretreated rats. A small number of rats exhibited a minor enhancement in the rate of body weight rise for one or two days after CAP treatment.

Food deprivation for 48 h resulted in 7.3 ± 0.6 vs 8.6 ± 0.4% loss of body weight in control vs CAP desensitized rats; 120-h food deprivation caused 15.9 ± 1.0 vs 18.9 ± 0.8% loss, respectively, i.e., almost 20% greater fall in desensitized animals, significantly larger ($p < 0.05$, t-test) than in controls. In either group, hardly more than half of the lost body weight was regained on the first day of refeeding; desensitized rats regained more of their weight loss during this day.

In a different group of animals, we investigated resting daytime T_c and metabolic rate in control and desensitized animals prior to and at the end of a 48-h or 120-h food deprivation period, as well as 1-day after the long deprivation. Both metabolic rate and T_c declined significantly by the late (but not early) part of the fasting period, however no significant differences were observed between the control and desensitized groups.

In another two animal groups, we continuously measured the metabolic rate and T_c for ca. 10 days and nights, which time period also included the 120-h food deprivation. In order to better observe the fasting-induced hypometabolism, measurements of metabolic rate in these experiments were conducted at a subneutral (20°C) T_a. In accordance with our results above, fasting induced a gradual fall in the day-time (inactive period) and night-time (active period) metabolic rate both in control and desensitized rats. With the applied conditions, fasting-induced falls of the metabolic rate were significantly more pronounced in the control group than in desensitized animals on the 5^{th} day of food deprivation. The respiratory quotient decreased in all rats from pre-fasting values (0.97 ± 0.01 at night and 0.91 ± 0.02 during day-time) to 0.71 ± 0.02 (with negligible day/night differences), suggesting predominant fat utilization during food deprivation, but no significant differences were observed between the control and desensitized groups.

Next, in freely-moving animals we have found that fasting also induced a gradual fall in the T_c of control rats, first affecting only the day-time, from the third day also the night-time T_c-s. In desensitized animals the T_c fall at day-time was minimal and fluctuating, while the night-time T_c remained normal until the last day of fasting. The difference between the two groups was significant. Under these subneutral conditions, both the night-time maxima and the day-time minima values of T_c were significantly lower in control rats than in IP CAP desensitized animals in the late phase of food deprivation (4-5^{th} days).

Upon refeeding after 48-h food deprivation, the IP CAP desensitized rats ate more than their control counterparts (6.2 ± 0.6 vs 4.4 ± 0.3 g, $p < 0.05$, t-test) during the first 3 h of refeeding, while in the subsequent 21-h period the food intake was smaller in the desensitized group than in controls, as if compensating for the earlier overfeeding. Similar results were observed after 120-h fasting. It is striking that, despite consuming less during this 21-h period, the body weight rise was still highly significantly greater in desensitized rats, suggesting delayed passage of the food and water consumed. Similar results were found in the case of 120-h fasting. In contrast to the early (first 3-h) period, in this period the food intake and body weight changes did not run parallel in either group.

Upon refeeding, rats that had fasted longer and had lost more weight did not eat either faster or more than those rats that had fasted for a shorter period only. The fractional weight gains of longer-fasting animals (expressed either in grams or in % of initial weight) were not larger, as compared with the shorter-fasted ones, and the 3-h cumulative gains were similar in the two fasting groups. However, after either shorter or longer fasting, the body weights increased more rapidly in CAP desensitized than in control rats, indicating that immediately upon refeeding the desensitized animals ate more than the controls. Still, satiety was always reached (eating stopped) before regaining all of the lost body weight, i.e. satiety depended on the amount of consumed food (and resultant stretch of the stomach) rather than on the extent of regained active weight.

Fractional analysis of the regained weight within the 3-h refeeding interval after a 48-h deprivation revealed most weight gain to occur during the first 30-min period both in desensitized and in control animals. For this period, a significant difference could be demonstrated between the two groups: desensitized rats ate more. Further weight gains at later periods were much smaller, without statistically significant differences between the groups. Although the initial difference was maintained for the rest of the 3-h period, the difference between cumulative weight gains of desensitized and control rats (6.0 ± 0.8 and 5.0 ± 0.5%, respectively) failed to reach the level of statistical significance,

despite that the 3-h cumulative food intake values were different. In case of longer food deprivation, the pattern was similar, except that the early enhancement of food intake upon refeeding was somewhat smaller, but the effect lasted longer both in desensitized and control rats. In the longer fasting animals, the differences between weight gains of desensitized and control rats were statistically significant not only in the first but also in the second 30-min fraction, while the cumulative weight gains also proved to be significantly larger in desensitized than in control animals (6.3 ± 0.5 vs 4.3 ± 0.5%, respectively, $p < 0.05$, t-test). Apparently, desensitization may induce an increase in the first meal, rather than in total mean consumption or in feeding time after fasting.

4.3. Role of the TRPV1 channel in food deprivation

The overall response of T_c to 48-h-long or 72-h-long complete fasting carried out at a cool or a thermoneutral T_a, respectively, consisted of a progressive fall of day minima with maintenance of night maxima at or close to pre-fasting values. Locomotor activity showed parallel changes to T_c both during day-time and night-time.

As compared to *Trpv1* KO mice, wild type mice responded to 48-h fasting with a more pronounced day-time hypothermia, which further progressed when fasting was extended to 72 h. In *Trpv1* KO mice, the second peak value of locomotor activity – recorded on the 2nd day of fasting – was significantly higher than the first peak ($p < 0.05$, HSD-test). This progressive rise in locomotor activity may have contributed to the ability of *Trpv1* KO mice to reach normal T_c even on the third night of fasting. Food deprivation resulted in the enhancement of night-time locomotor activity also in wild type mice, but this enhancement did not grow further on the second, or third day of fasting.

Analysis of difference in locomotor activity between the two genotypes revealed that in *Trpv1* KO mice the rising phases of T_c and activity occurred around the same time just before the start of the dark period. In wild type mice, however, there occurred a progressive advance in the appearance of rises in T_c and activity well before the start of the dark period of the day. In *Trpv1* KO mice, mean duration of daily cycles of T_c were 24-25 h in the fed state and 24 h during fasting. In wild type mice, these values were 23-25 vs 17 h before food deprivation and during fasting, respectively.

Upon refeeding, immediately after the return of food to the mice, T_c started to rise and reached normal values independent from the actual T_c observed at the last morning of fasting. The speed of the rise in T_c was very rapid in both genotypes of mice and reached normothermia within 30-50 minutes. In neither type of mice did increased activity accompany the sharp rise in T_c, indicating that a markedly increased heat production can be behind this phenomenon.

4.4. Role of TRPV1 channels in maintaining normal body temperature

In a thermoneutral environment (T_a = 26 °C), IV infusion of 100 μg/kg of AMG517 (selective TRPV1 antagonist) caused significant rise of T_c in rats, while infusion of its vehicle was without any effect.

Due to the thermoneutral environment, the partially restrained animals were able to utilize both physiological autonomic thermoeffectors (i.e. heat loss and thermogenesis), thus allowing us to investigate the thermoregulatory mechanisms of the hyperthermia. We have found that simultaneously with the rise in T_c, AMG517 elicited a decrease in heat loss and an increase in metabolic rate, while its vehicle had no effect on either thermoeffector.

4.5. Role for the inhibition of proton activation in the TRPV1 antagonist-induced hyperthermia

In guinea pigs, IV infusion of 65.5 μmol/kg of CPZ significantly increased T_c by 0.5 ± 0.1°C ($p < 0.005$). When the same dose of CPZ (65,5 μmol/kg), was infused to rats IV, we did not record any changes in either their T_c or HLI. The latter is important because vasoconstriction of the cutaneous vessels is the first autonomic cold-defense effector to be recruited in a response, therefore, it is the most sensitive parameter of the TRPV1 antagonist-induced hyperthermic reaction.

4.6. Coordinated energetic effects of centrally administered α-MSH

In three-month-old rats, there was a significant suppression of spontaneous food intake in the first 4 hours of the active, dark phase after central injection of α-MSH. Food intake of the α-MSH treated animals did not catch up to that of controls in the following 24 hours either. The 2 and 5 μg doses were equally effective, while in the case of 10 μg α-MSH, the suppression was somewhat more pronounced.

For studies with fasting-induced refeeding, we used the 5 μg dose. We found that during the refeeding following 24-h food deprivation, food intake of rats treated centrally with α-MSH was significantly reduced as compared to vehicle-treated rats.

To clarify the presence of a coordinated catabolic effect, we also registered the α-MSH-induced thermal effects, and then we compared the time dynamics of the thermoregulatory effects to the abovementioned effects on food intake. We found that ICV injection of 5 μg α-MSH at the lower end of the thermoneutral zone, or below that (T_a = 25°C, or lower) resulted in an immediate increase in T_c. In a different series of experiments, on a similar time scale, the same dose of α-MSH caused a significant suppression of food intake upon refeeding following 24-h fasting as compared to controls.

4.7. The roles of CAP-sensitive abdominal afferents in the effects of centrally administered NPY and α-MSH on food intake

ICV injection of 2 μg NPY induced feeding in otherwise satiated animals. However, in contrast to the data on fasting-induced hyperphagia and weight regain, neither the food intake, nor the cumulative/fractional weight gains induced by such NPY-administration were significantly greater in desensitized animals than in non-desensitized controls. Interestingly, the daytime overfeeding for a 3-h period was followed by a "compensatory" attenuation of food intake in the next 21-h period (as compared with desensitized rats injected with vehicle), but there was no difference between desensitized and non-desensitized animals. The early NPY-effects were qualitatively similar as the effects of 48-h fasting, but the absolute amount of consumed food was much smaller. Due to the hypophagia, the 24-h changes of body weight were different: in contrast to fasting rats, the body weight of NPY-treated animals effectively decreased.

In accordance with our previous findings in the present study, ICV injection of α-MSH significantly reduced the fasting-induced food intake during refeeding in both desensitized and non-desensitized control animals. The magnitude of the anorexigenic effect showed no significant difference between the IP CAP desensitized and control groups.

5. Discussion

5.1. CAP-sensitive abdominal vagal afferents and fever

Based on our results described in 4.1. we can conclude: the effects of IP and perivagal CAP desensitization on LPS-induced fever are different, inasmuch the latter does not attenuate the first phase of the polyphasic fever. This dissimilarity provides a certain proof for the opinion that CAP-sensitive afferent vagal fibers do not participate in the early febrile response, furthermore that IP CAP exerts its fever-modifying effect by non-vagal mechanisms. Based on earlier results from our laboratory, it is known that both IP and perineural CAP treatment influences the postprandial hyperthermia, which confirms that vagal afferent fibers are, indeed, involved in thermoregulatory responses to events in the abdominal cavity, but bacterial endotoxin appears to act in a different way, not using the vagal afferent nerves, for example through mechanisms carried out in the liver[16,17].

We can conclude that vagally conveyed chemonociceptive and mechanical information may originate from substances/factors (nutrients, gastrointestinal hormones, osmotic effects, stretch, etc.) that are thought to be important in non-febrile manifestations of thermoregulation and energy balance. These non-febrile changes may be important in the metabolic/thermal adaptation to the feeding status (e.g., fasting) and to other effects influencing the gastrointestinal system. In contrast, in the pathogenesis of fever, vagal afferent functions play, at most, a minor role.

5.2. CAP-sensitive abdominal vagal afferents: food intake and fasting

From the difference in the fasting-induced food intake between IP CAP desensitized and control animals – CAP treated rats developed a more pronounced hyperphagia and weight gain – we can draw the conclusion: damage of strictly local and afferent fibers is able to attenuate satiety. Since such desensitization does not affect physiological thermoregualtion[14,15], the effect can not be explained by temperature abnormalities. The fact, that at a later time CAP desensitized animals also expressed satiety suggests that the abdominally – probably vagally – carried signals (from stimulation of either stretch- or chemoreceptors of the gastrointestinal/hepatic system) are likely to influence meal size rather than the duration of feeding or the total amount of consumed food and that the CAP-sensitive processes are not exclusive.

CAP-induced damage of afferent abdominal fibers, *per se*, did not cause and did not prevent hunger, but attenuated satiety. Comparison of spontaneous courses of body weight gain in control and desensitized groups suggests that a deficiency of vagal satiety signals may have, at most, a moderate and transient influence on food intake regulation. It is possible that in the course of spontaneous feeding the meal size remains larger and the overeating episodes are compensated by a decreased meal frequency in CAP-treated animals, but the present experiments cannot provide convincing data for or against such suggestion.

Upon refeeding, in desensitized rats the 3-h period of marked hyperphagia was followed by a relative “compensatory” hypophagic 21-h period. Presumably, the hypophagia can be explained by an unusual stretch applied at an unusual time, which initiated strong and lasting satiety signals[18].

Interestingly, after longer period of food deprivation the food intake in the first periods of refeeding did not exceed the corresponding values observed in response to shorter fasting in non-desensitized rats. This was remarkably in contrast with the observations that in many other cases – e.g., following an ICV administration of large NPY or orexin doses – much greater fractional and cumulative weight gains were

possible[19,20]. Therefore, the meal size at refeeding was probably not mechanically limited but it appeared to be regulated by hunger and satiety signals. Thus, the present findings might indicate that longer food deprivation did not induce more severe hunger sensation than that developing already during the first 48-h of fasting.

Hunger-related signals (and not simply lack of satiety signals) could normally contribute not only to food intake after fasting but also to the metabolic suppression and fall of T_c during fasting (as an inverse of postprandial hypermetabolism)[21,22]. However, the vagal damage by CAP desensitization could possibly result in defective metabolic adjustment to fasting and to a lack of appropriate hypometabolism. In fasting, resting metabolic rate starts to decrease characteristically at phase 2 starvation[23], i.e. relatively late – suppression of this process in desensitized rats might explain a gradual enhancement of weight loss during food deprivation.

In fasting rats the concurrent hypometabolism and tendency for hyperphagia point to an anabolic pattern of energy status. As in control rats, in IP CAP desensitized animals fasting elicited an anabolic regulation, the hyperphagia upon refeeding was, indeed, more pronounced. A hypothermia also developed, as measured by indirect calorimetry hypometabolism was present as well. By 24-h continuous registration in freely-moving rats, we were able to demonstrate the significantly smaller fasting hypometabolism and hypothermia of IP CAP desensitized animals in the late phase of fasting at a subneutral T_a. The defective hypometabolism (or abnormal relative hypermetabolism), in turn, allows the maintenance of a relatively high T_c, furthermore it can contribute to an enhanced weight loss of IP CAP desensitized animals during food deprivation.

In conclusion, in IP CAP desensitized rats the fall in body weight in the course of food deprivation is aggravated, suggesting a role for fasting-induced hunger signals (which normally might also cause hypometabolism) that are conveyed by vagal afferent fibers. At the same time, upon refeeding desensitized rats ate more and regained more of the lost weight, suggesting defective function of vagally transmitted satiety signals, which should exert a negative feedback action. The vagal satiety signals appear to act on the short-term only, and they influence meal size rather than total food intake; on the long-term both the food intake and the weight gain rate are similar in desensitized and control animals.

5.3. The TRPV1 channel and food deprivation

The difference between the responses of *Trpv1* KO mice and the wild type ones were twofold: on the one hand, the extent of decrease in T_c on fasting was significantly greater in wild type mice, on the other hand, during fasting there appeared an advance in the circadian rises of T_c and activity in wild type mice well ahead of the dark period. The latter can be explained as a sign of resetting of the circadian pacemaker caused by the anticipatory activity otherwise occurring in connection with food intake. In the present experiment the phase-advance caused by fasting occurred in spite of the maintenance of the 12-12 hour light-darkness schedule. It can be assumed that the rapidly developing energetic insufficiency induced by complete fasting might have induced a strong speeding up of the need for food and thus masking the effect of the main pacemaker stimulus that is the darkness cue. The attenuated hypothermia of *Trpv1* KO mice during food deprivation agrees with the compromised energetic adaptation ability of fasting IP CAP desensitized rats (i.e. local abdominal loss of functioning TRPV1 channels). During the inactive period, the characteristic fall in T_c was more pronounced in wild type mice than in their *Trpv1* KO counterparts. This indicates that the TRPV1 channel can influence the energetic adaptation to fasting – in the lack of the channel, the hypometabolic periods are attenuated.

Based on the fasting-induced – to a different extent in both genotypes occurring – daytime hypothermia, we can conclude that our results described in 4.3. support those of others regarding the food deprivation induced energetic changes, furthermore they agree with our findings with fasting IP CAP desensitized animals via the function of TRPV1 channels in food deprivation.

5.4. The TRPV1 channel and normal thermoregulation

By studying the AMG517-induced hyperthermia, we found that both autonomic cold-defense thermoeffectors (heat loss, metabolic rate) participate in the rise of T_c. In accordance with a coordinated thermoregulatory response, at a thermoneutral T_a, the hyperthermia is brought about by a decrease of heat loss and a simultaneous increase of the metabolic rate. These results exclude a direct effect on a specific thermoeffector, instead they suggest an involvement of the afferent or central sites of body temperature regulation. The antagonist-induced changes indicate that the substance evoked its – T_c-increasing – effect via an inhibition of a tonically activated regulatory system[15,24].

Our results suggest that the TRPV1 channel is tonically activated *in vivo*. Through its tonically activated state, TRPV1 continuously inhibits the autonomic cold-defense thermoeffectors (heat conservation, thermogenesis), thus maintaining T_c in its physiological range. By blockade of this tonic activation, i.e. by administration of a TRPV1 antagonist, the heat conservatory and thermogenic processes will be released from their inhibition, thus leading to hyperthermia. This phenomenon could have also contributed to an attenuated fasting-induced hypometabolism (relative hypermetabolism) in IP CAP desensitized rats.

5.5. Factors responsible for tonic activation of the TRPV1 channel

When we infused the same dose of CPZ to guinea pigs and rats, we found that, while in guinea pigs this TRPV1 antagonist caused significant hyperthermia, in rats it had no thermoregulatory effects. By comparing the thermal effects of CPZ in these two species, we investigated the role of proton in maintaining the tonic activation of TRPV1 channels *in vivo*.

The TRPV1 channel can be activated by heat, protons, and molecular ligands[25], the latter commonly summarized as vanilloids including the aforementioned CAP, resiniferatoxin and endovanilloids. If inhibition of the proton activation mode plays an important role in TRPV1-antagonist-induced hyperthermia, or in other words, in the maintenance of the tonically activated state of the TRPV1 channel *in vivo*, then it can be expected that guinea pigs will be much more sensitive to the hyperthermic effect of CPZ than rats. This is based on the fact that in rats CPZ does not block the proton activation mode of the TRPV1 channel, in this mode the 50% inhibitory concentration of CPZ > 40000 nM[26], on the contrary, in guinea pigs CPZ inhibits the activation of TRPV1 channels by protons relatively strongly (50% inhibitory concentration = 355 nM)[27]. It is important to mention that CPZ also blocks the heat and vanilloid activation modes both in rats and guinea pigs[26,27].

In accordance with our expectations, we found that in guinea pigs, in which species CPZ blocks the proton activation mode, it caused hyperthermia, while in rats, in which species CPZ does not block the proton activation mode, it was thermally ineffective. We can conclude that blockade of the proton activation mode of the TRPV1 channel is crucial in TRPV1 antagonist-induced hyperthermia, which is in harmony with earlier findings of other authors[28]. Therefore, these findings suggest that it is the proton activation mode that is responsible for the tonically activated state of the TRPV1 channel *in vivo*.

5.6. Role α-MSH in energetic processes

Exogenous, centrally injected α-MSH reduced both the spontaneous and fasting-induced food intake in adult rats. This finding is in accordance with earlier reports by other groups[29,30]. The daytime spontaneous food intake was unaffected by the substance, the reason for which is that spontaneous food intake during the inactive, light phase is, *a priori*, minimal in rats[31], and its significant further decrease was not detectable with the methods of the present study. In contrast to this, both the spontaneous food intake during the active, dark phase, which is substantial in rodents[31], and the fasting-induced food intake during the light phase were significantly reduced by the substance in accordance with the related literature.

At the lower end of the thermoneutral zone, centrally administered α-MSH caused a significant rise of T_c as compared to controls. By comparing the time dynamics of the thermoregulatory and anorexigenic effects, we unequivocally demonstrated the occurrence of a coordinated (catabolic) energetic reaction, which is in accordance with previous data in the literature[29,32]. The hyperthermia found in the present study finds support in an earlier report[33], but contradicts the results of another study, in which antipyretic effects were found[34]. It is important to note, that in our study, in some animals the latency and the extent of the hyperthermic response substantially differed from those of the average. Presumably, the various dominance of peripheral core- and skin temperature signals of the actual thermoregulatory status could explain these contradictory phenomena.

In summary, by using young adult rats, we demonstrated the coordinated catabolic energetic (simultaneous anorexigenic and hyperthermic) effects induced by central administration of α-MSH, furthermore we determined the optimal dose used in the rest of the experiments in our study.

5.7. CAP-sensitive abdominal afferents and the (feeding) energetic response to NPY and α-MSH

In our experiments we successfully reproduced the acute hyperphagic effect[35] and the subacute hypophagic effect[20] following NPY administration. We have found that IP CAP desensitized rats responded normally (indifferently from their vehicle treated counterparts) to central administration of exogenous NPY and α-MSH. We can conclude that IP CAP desensitization does not impair the central elements of the complex energetic regulation: in accordance with our expectations IP CAP treatment causes damages exclusively in the abdominal afferentation[14,15]. The lack of vagal satiety signals did not influence the central NPY-, and α-MSH-induced – from the literature well known hyperphagic[20,35], and hypophagic[29,30] – feeding response. In summary, these results suggest that in the development and maintenance of satiety/hunger other (non-vagal) signals also play an important role.

6. Summary

1. CAP-sensitive abdominal vagal afferents play no role in the development of the LPS-induced polyphasic fever response, the effects of IP CAP desensitization are not mediated by the vagal nerve.

2. IP CAP desensitization exaggerates the fasting-induced weight loss, nevertheless it enhances the early weight gain upon fasting-induced refeeding, which suggest the existence of both vagal hunger and vagal satiety signals.

3. In *Trpv1* KO mice, the fasting-induced hypothermia is attenuated compared to their wild type counterparts, furthermore during food deprivation their circadian T_c and activity rhythms are shifted. The attenuated hypothermia is in accordance with our results with IP CAP desensitization.

4. The TRPV1 antagonist AMG517 causes hyperthermia, which – as a coordinated thermoregulatory reaction – is the result of a decrease in heat loss and an increase in heat production. The TRPV1 channel is tonically activated *in vivo*, continuously inhibiting the autonomic cold-defense thermoeffectors (heat conservation, thermogenesis), thus maintaining the physiological deep body temperature.

5. Blockade of the proton activation mode of the TRPV1 channel is undoubtedly needed for the occurrence of the hyperthermic effect of CPZ. Hyperthermia develops only in that case (species), in which CPZ blocks the activation of TRPV1 channels by protons (e.g. in guinea pigs). This suggests that protons are responsible for maintaining the tonic activation of the TRPV1 channel *in vivo*.

6. ICV administered α-MSH causes a dose-dependent suppression in the fasting-induced food intake upon refeeding and in the spontaneous nighttime food intake, parallel it increases T_c with similar time dynamics, which is a coordinated catabolic energetic reaction.

7. The lack of vagally conveyed hunger and satiety signals has no influence on the feeding response to centrally administered NPY or α-MSH.

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