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ORIGINAL INVESTIGATION

Effects of the increase in neuronal fatty acids availability on food intake and satiety in mice

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Abstract
Rationale Neurons detect free fatty acids (FFAs) availability and use this nutritional status to modulate feeding and control body weight.
Objectives The work is designed to characterize the impact on feeding behavior of either oleic acid (OA) administration (experiment 1) or the inhibition (experiment 2) of the enzyme carnitine palmitoyltransferase-1 (CPT-1). The structure of feeding behavior and satiation time course were examined through the behavioral satiety sequence (BSS) paradigm.
Methods Adult male mice were initially habituated to a palatable diet, then subjected to intracerebroventricular (i.c.v.) infusion of different doses of OA or the CPT-1 inhibitor ST1326. Food intake at different time points, duration, and frequencies of feeding and non-feeding-related behaviors were continuously monitored over 40 min and satiety development profiled according to BSS.
Results Intra-i.c.v. infusion of oleic acid (300 nM) and ST1326 (50 and 75 pM) suppressed food intake. As indicated by the earlier leftward shifting of the normal transition from eating to resting, both strategies similarly accelerated the onset of satiety. The premature onset of satiety resulted in a dose-related fashion with 50 pM of ST1326 producing a marked premature onset than the lower dose. However, at the highest dose injected, the inhibition of CPT-1 disrupted the BSS profile.
Conclusions The increased neuronal availability of FFAs mediates a significant anorectic response which is mirrored by an early occurrence of satiety onset. Besides supporting the role of central nutrient sensing in feeding, the present data demonstrate that the modulation of satiety enhancement can produce appetite suppressant effects within narrow range of neuronal FFAs availability.

Keywords Free fatty acids · Carnitine palmitoyltransferase-1 · Behavioral satiety sequence · Nutrients sensing · Satiety

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Introduction

The rising incidence of obesity and overweight-related risk factors (e.g., diabetes mellitus, insulin resistance) is a serious threat to lifespan and, in spite of the epidemic proportion, the clinical management of these diseases is greatly limited by the paucity of newer and safer anti-obesity drugs (Li and Cheung 2009; Padwal et al. 2004). Among the existing anti-obesity options (Bays 2004; Florentin et al 2008; Padwal and Majumdar 2007), these drugs are broadly classified as appetite suppressants of various origin (e.g., phentermine, dexfenfluramine, sibutramine), inhibitors of fat absorption (e.g., orlistat), energy homeostasis modulators (e.g., catabolic pathways stimulants), and thermogenic agents (e.g., beta3-adrenergic agonists and modulators of transcription factors).

Concerning pharmacotherapies targeting the neural control of energy balance, great expectations have been raised by the drugs targeting the cannabinoids system such as the inverse agonist of the CB1 receptor (SR141716), but the high incidence of side effects has set limitations to its potential clinical use (Li and Cheung 2009). The search for effective and safer new anti-obesity therapies is currently

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exploring different, non-mutually exclusive, strategies such as those that targeting the nervous system may act as appetite suppressants and regulators of eating behavior. One of these potential lines of investigation is offered by the data reporting the thermogenic and anti-obesity effects of the central modulation of fatty acids and nutrient metabolism.

For a long time it was thought that fatty acids could not cross the blood-brain barrier. As consequence, the notion that free fatty acids (FFA) act at neural level as cellular messenger conveying information about peripheral metabolic status and energy availability has been only recently recognized. The idea that, besides to sensing glucose availability, brain cells, and hypothalamic neurons are sensitive to metabolic fuels such as FFA has received substantial confirmation by the observation that the inhibition of fatty acid synthase (FAS) can suppress food intake, decrease body weight and significantly reduce the hypothalamic expression of the orexigenic neuropeptide Y (Kumar et al. 2002; Loftus et al. 2000). This was in line with the view that FFA signaling contributes to encode and transmit the nutritional status of the organism, thus participating to the regulation of energy balance and hypothalamic homeostasis. Hypothalamus comprises lipid-sensitive nuclei such as the arcuate nucleus (ARC) and discrete ARC neurons have been demonstrated to be selectively responsive to the long-chain fatty acids (LCFAs) OA (Wang et al. 2006). A specific linkage between the effects of fatty-acid synthase inhibitors (i.e., C75) and fatty acids metabolite malonyl coenzyme A (malonyl-CoA) does exist. The malonic acid derivative, malonyl-CoA, is an intermediate step of fatty acid biosynthesis and C75-mediated inhibition of FAS induces a significant increase of malonyl-CoA levels (Loftus et al. 2000). In turn, the increase of malonyl-CoA lead to the allosteric inhibition of outer mitochondrial membrane enzyme carnitine palmitoyltransferase-1 (CPT1), thus blocking the translocation of long-chain fatty acyl-coenzyme A (LCFA-CoAs) into the mitochondria and consequently fatty acids β-oxidation. The possibility to reduce food intake and glucose production by CPT1 inhibition further support the hypothesis that the increased neuronal source of LCFAs may convey a signal of nutrient loading able to trigger hypothalamic counter-regulatory responses (Obici et al. 2003). Accordingly, in the last few years several evidence of centrally mediated anorectic effects of hypothalamic carnitine-dependent palmitoyltransferase-1 (CPT-1) inhibition (Obici et al. 2003) as well as of the suppressive effects of LCFA oleic (Obici et al. 2002) on food intake and endogenous glucose production have been accumulating. Recently, also the synthetic derivative of oleic acid, 2-OHOA, has been shown to induce weight loss and decrease food intake after sub-chronic (7 days) peripheral administration in rats (Vögler et al. 2008).

Although the inhibition of fatty acids oxidation has been exploited as a promising option to deal with obesity, insulin resistance, and type 2 diabetes mellitus, no specific knowledge of the impact of central modulation of nutrient abundance on feeding behavior is currently available. Aim of this study is to investigate, by the use of the behavioral satiety sequence (BSS) paradigm, feeding behavior, and food intake of CD-1 mice centrally administered with different doses of either OA (experiment 1) or the reversible CPT-1 inhibitor (R)-*N*-(tetradecylcarbamoyl)-aminocarnitine (ST1326) and its inactive stereoisomer ST1340 (experiment 2). It is increasingly recognized that a detailed analysis of the structure of feeding behavior may provide revealing features of the extent to which pharmacological treatments may induce changes in the balance between hunger and satiety signals (Clifton 2000). Hence, the BSS paradigm was chosen because it is a valuable ethological approach that allows to examine and describe the rodent's natural progression from eating to resting through active grooming (Antin et al. 1975; Blundell and Latham 1979; Halford et al. 1998). In particular, the BSS analysis makes possible to study the relationship that links the progression of eating, locomotion, grooming and resting behaviors, and the specificity of the effects underlying food intake termination and satiety (post-ingestional inhibition of food intake) onset. Moreover, in lack of preservation of the stochastic sequence of the collected behaviors (i.e., disruption of the BSS), the observed changes in food intake may be attributed to mechanisms other than satiety and therefore to factors different from the post-ingestive occurrence of satiety. For this reason, by the analytic examination of the behavioral profile of satiety development, this paradigm provides an important tool to assess the impact of different pharmacological agents on these physiological processes.

The present study explores in two series of experiments the effects of the acute intracerebroventricular (i.c.v.) bolus injection of different doses of OA, ST1326, and ST1340 on both BSS and food intake of a palatable wet mash diet in mice.

Materials and methods

Animals

Subjects were male CD-1 outbred mice purchased from Charles River (Calco, CO, Italy). Animals (weighing 28± 0.5 g on arrival) were individually housed in breeding cages (26.7×20.7×14 cm) placed in a room with a 12:12-h light–dark cycle (light on between 07:00 AM and 07:00 PM), at a constant temperature (22±1°C) and humidity with standard diet (4RF21; Mucedola s.r.l., Milan, Italy) and water freely

available. The 4RF21 is a regular diet for laboratory mice constituted by 66.5% carbohydrates, 21.7% proteins (18.2% plus 3.5% of vegetable and animal proteins, respectively), 0.4% lipids, 0.1% amino acids, 3.2% vitamins and 7.5% forages that provide about 2.668 Kcal/g of energy level. Forty-four and ninety CD-1 mice were used for experiments 1 and 2, respectively. Housing, animal maintenance, and experiments were conducted in accordance with the Council Directive of the European Community (86/EEC) of the Italian D.L. 116 (January 27, 1992) and approved by veterinarian supervision.

Drugs

For experiment 1, and in accordance with previously suggested protocols for intracerebral administration of lipophilic agents (Pitha et al. 1994; Yaksh et al. 1991), different doses of OA (0 nM/1 μl N=12; 0.3 nM/1 μl N=11; 30 nM/1 μl N=11; 300 nM/1 μl N=11) were complexed with the polymer (2-hydroxypropyl)-β-cyclodextrin (CDEX). Both OA and CDEX were purchased from Sigma-Aldrich®. CDEX was also used for ICV vehicle solution. In experiment 2, the CPT-1 reversible inhibitor (R)-*N*-(tetradecylcarbamoyl)-aminocarnitine (ST1326) as well as the inactive stereoisomer ST1340 were diluted in sterile saline solution (0.9% w/v of NaCl) and then intraventricularly delivered at the following doses: 0 pM/1 μl N=22, 25 pM/1 μl N=10, 50 pM/1 μl/side N=19 and 75 pM/1 μl N=20 (ST1326); 50 pM/1 μl N=9 and 75 pM/1 μl N=10 (ST1340).

Diet and habituation procedure

Before starting the i.c.v. OA drug delivery mice were habituated for 7 days to a special high-sweet palatable diet (HS wet mash). During this phase, fresh wet mash was prepared daily and offered for 2 h/day. To minimize diet spillage, wet mash was provided in small opaque plastic beakers (3-cm diameter) mounted on a plastic Petri dish. Wet mash was made up of a mixture of one part ground standard dry powdered food pellets in sweetened distilled water (HS 10% sucrose solution). Powdered food pellets were obtained by the same standard 4RF21 diet (Mucedola s.r.l., Milan, Italy) described above (animals housing). During the wet mash habituation, phase both body weight (g) and food intake (g) were measured daily. The intake of HS wet mash was calculated as the difference between the weight of wet mash-containing food dispenser immediately before the meal presentation and remaining food collected 2-h after. The same procedure was used to determine the intake of HS wet mash during the BSS procedure for each time points considered (see below). The same diet and wet mash habituation procedure were used for both ST1326 and ST1340 i.c.v.-injected mice (experiment 2).

Surgery

Mice were anesthetized with chloral hydrate (500 mg/kg) and placed on a stereotaxic frame equipped with mouse adapter and ear bars (Kopf Instruments). After immobilization of the mouse head, a midline incision was made in the atlanto-occipital membrane, the skull surface cleaned and a single hole was drilled to allow the insertion of a guide cannulae. A stainless steel cannula (5 mm in length; outside diameter, OD, 0.5 mm) was then implanted in the lateral ventricle and fixed to the skull using dental (polycarboxylate) cement. The side of cannula insertion (left vs right ventricle) was alternated during the surgery procedure in order to have, for each side, approximately half of the animals included in each group of treatment. The following coordinates with lambda and bregma in the same horizontal plane were used: anterior to bregma, ±0.0 mm; lateral to midline, ±1 mm; ventral from the dura, −1.2 mm, according to the mouse brain in stereotaxic coordinates (Franklin and Paxinos 1997). Mice were then left in their home cage for a recovery period of 7 days. Chloral hydrate was purchased from Sigma (St. Louis, MO, USA). The surgical procedure for ST (1326 or 1340)-injected mice was the same as for experiment 1.

Drug delivery

Drugs were i.c.v. delivered with mice placed in their home cage. For each subject, a stainless steel injection microneedle (6.2 mm length; OD, 0.25 mm) was connected through a polyethylene tubing to a 2-μl Hamilton syringe and then lowered into the lateral cerebral ventricle (dorsoventral, DV 2.5 mm from bregma). Drugs were administered via an infusion pump and the volume injected was always 1 μl for a 1-min injection length. After drug delivery, the injection needle was left in place for an additional 30 s to allow better diffusion. All doses of both ST1326 and ST1340 were delivered as described for experiment 1.

BSS procedure and food intake assessment after drugs delivery

According to the BSS paradigm both duration (sec) and frequencies of feeding, locomotor activity (horizontal locomotion and rearing), resting (immobility), and grooming (face and body cleansing) were recorded for each subject and separately scored. Because of its documented advantages (Halford et al. 1998), all these behavioral patterns were monitored and observed continuously and not by sampling techniques, which do not allow to reliably assess the transition between different behaviors and clearly identify short (event) and long (state) duration of the observed behaviors. To circumvent the necessity of the

preliminary habituation of animals to the testing environment, in both experiments, BSS-related behaviors were recorded in animal home cages. The evaluation of the BSS was made during the dark cycle, between 07:00 and 08:00 PM, for a total of 40-min test, in a soundproof cubicle equipped with a video-recording camera. The total test duration of 40-min was chosen on the basis of several studies documenting the mean duration of satiety development in rodents (Clifton 2000; Halford et al. 1998), especially in case of non adulterated palatability of the food offered to animals (Ishii et al. 2003). One hour before each behavioral recording session, mice were food deprived, while water remained freely available. Immediately after i.c.v. drug delivery, HS wet mash was made available to animals. Each food container was pre-weighed, and the difference between initial and final weights corresponded to the measure of HS wet mash intake. HS wet mash intake was collected at two (40-min and 14-h) and three (40-min, 2-h, and 14-h) time points for experiments 1 and 2, respectively.

Histological verification

After the completion of experiments 1 and 2, mice were anesthetized with chloral hydrate (500 mg/kg), and an aliquot of 1 μl of methylene blue was injected down the cannulae to verify the diffusion of the injected solution. Then animals were sacrificed by decapitation and brain removal. Later, each brain was coronally cut in two halves along the bregma landmark and the appropriate methylene blue perfusion all through lateral and third ventricles verified. Only animals accurately perfused were included in the experiments.

Statistical analysis

For experiment 1, one-way analysis of variance (ANOVA) was used to analyze the amount of wet mash consumed after 2 and 14 h, respectively. For the BSS, a repeated measure ANOVA was used to analyze duration of observed behavior with drug (4 doses) as between-subject variable and time (subdivided in 8 time bins of 5-min each, from T1 to T8) as within-subject variable. Eating frequencies were also analyzed. For experiment 2, a repeated measure ANOVA was used for the evaluation of the wet mash consumed at 40 min and 2 h. A one way ANOVA was used to evaluate the differences at 14 h. The separate analysis was chosen because of the delay between the first two time points (80 min) and that existing between the second and the third measurements (720 min). Since control animals are supposed to gradually increase their food intake overnight, the large differences between the food intake observed during the early phase of the dark cycle (up to 2 h) and the food consumed when dark cycle is over (after 14 h), may introduce an unwanted source of too large variability into the analysis. Although three measures were taken on the same animals, the last was obtained in a different experimental condition, and pooled together, all the conditions may result in a type II error (i.e., false negative). The duration of the observed behaviors as well as eating frequencies collected by BSS paradigm were analyzed by repeated measure ANOVA with drug (6 doses), as between-subject variable, and time (8 time bins 5 min each, T1-T8) as within-subject variable. For both experiments, post hoc comparisons were carried out by Newman–Keuls test.

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research.

Results

Experiment 1

Food intake

The highest dose of OA (300 nM) administered significantly decreased HS wet mash intake. ANOVA analyses evidenced a significant effect of i.c.v. drug administration after 2 h ($F_{3,41}$=16.36, P<0.0001) and 14 h ($F_{3,41}$=6.75, p<0.001). Post hoc comparison at 2 h further evidenced (Fig. 1) a significant decrease of HS wet mash intake only in mice injected with the higher dose (300 nM) of OA

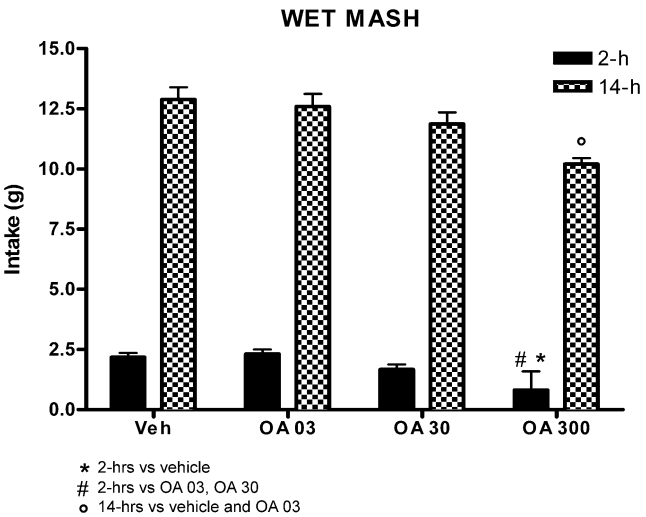

Fig. 1 The figure shows the effects of intra-i.c.v. infusion of different doses of OA on wet mash intake. Vertical bars represent the mean food intake ±SE after 2 and 14 h from OA intracerebral administration. *Significant difference from vehicle and OA 03 nM/1 μl, after 2 and 14 h OA infusion, respectively (p< 0.05 Newman–Keuls test, after significant ANOVA). # Significant, at 2 h, difference from OA 03 nM and OA 30 nM

(Newman–Keuls; $p<0.01$). Likewise, a significant decrease of HS wet mash intake was found at 14 h only in animals injected with 300 nM of OA. On the contrary, no differences in the amount of food eaten were found between vehicle, 03 nM and 30 nM OA-injected mice, whatever time interval considered (2 or 14 h).

Behavioral satiety sequence

At both intermediate and highest doses OA-administered mice (30 nM and 300 nM), the BSS was reallocated (leftward shifted) from T5 (panel A, vehicle group, Fig. 2) to T1–T2 (panel C) and to T2 (panel D), respectively. The occurrence of premature satiety onset is demonstrated by the earlier point of interaction between resting and eating activities or, in other words, by the earlier cessation of feeding behavior. The analysis of the behavioral patterns over the course of the 40-min session showed a significant effect of time for the duration of feeding, locomotion, resting, and grooming. These changes reflected the characteristic over time reduction in active behavior with the parallel increase in resting, which are typically described under BSS test condition. The ANOVA analysis revealed a significant main drug effect for the following behavioral patterns recorded: *eating* ($F_{3,41}=5.66$, $P<0.01$), *grooming* ($F_{3,41}=5.92$, $P<0.01$), *resting* ($F_{3,41}=3.65$, $P<0.01$), *locomotion* ($F_{3,41}=3.65$, $P<0.05$) but not for *rearing*. Conversely, the analysis of the frequency of eating behavior did not reveal a significant effect of treatment. A significant treatment×time interaction has been evidenced for *eating* ($F_{7,287}=1.89$, $P<0.01$), *resting* ($F_{7,287}=2.03$, $P<0.01$) and *locomotion* ($F_{7,287}=1.81$, $P<0.05$), while no significant differences were found for *grooming* and *rearing*. Post-hoc comparisons for each 5-min time bins evidenced that 30-nM and 300-nM OA-injected mice significantly decreased (Newman–Keuls; $p<0.01$) eating behavior during the fourth interval (15–20 min). Moreover, animals injected with 300 nM of OA also significantly decreased their eating behavior during the seventh interval (30–35 min). Grooming behavior was showed to be significantly increased at

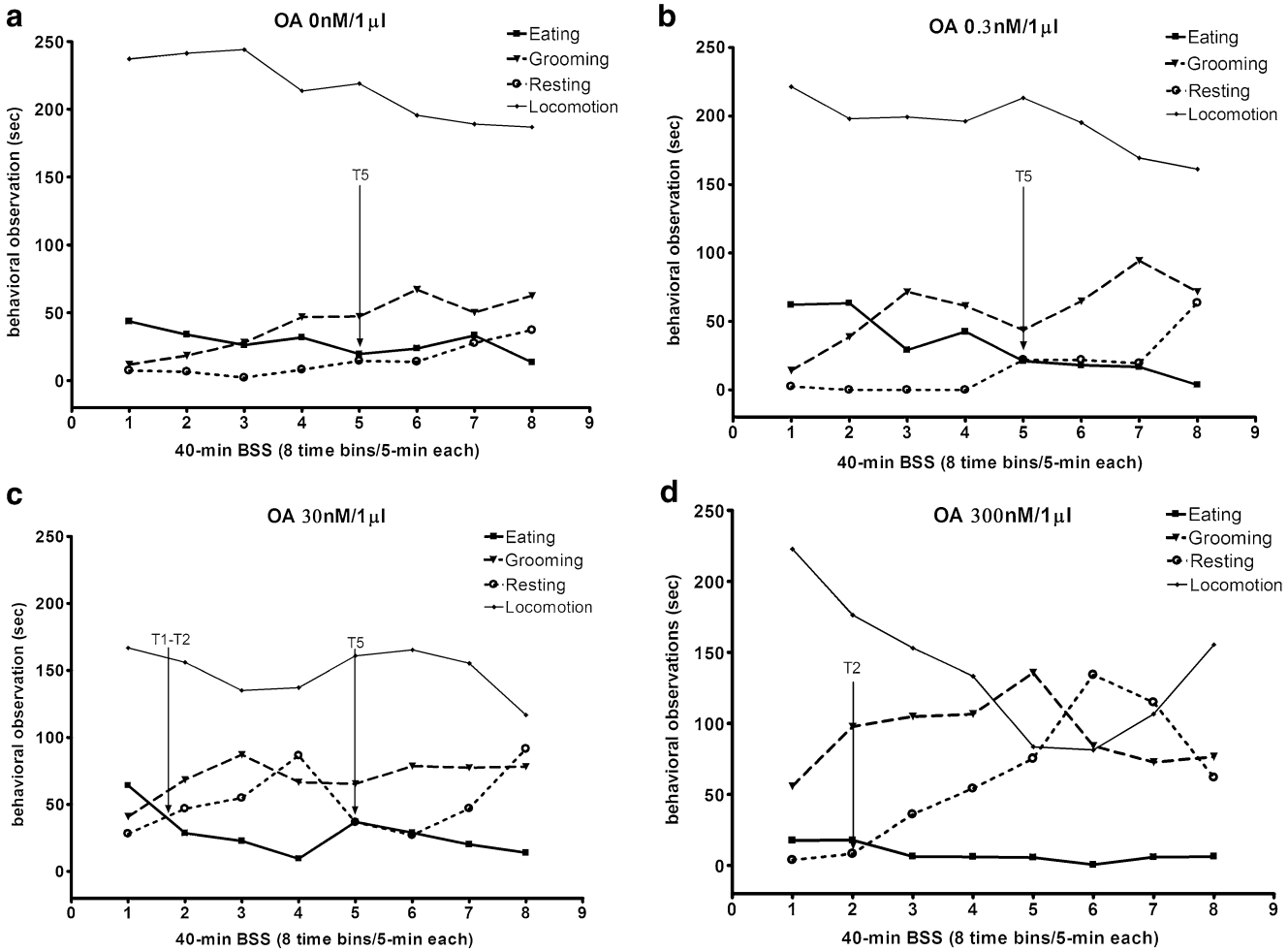

Fig. 2 The figure shows the temporal development of eating, locomotion, grooming, and resting duration. Panels show the results for each group of OA i.c.v-infused mice. Represented on the *X*-axes are 8 time bins of 5 min each for a total of 40 min of behavioral observation

the highest dose OA-injected mice throughout the first (0–5 min) as well as the second (5–10 min), third (10–15 min), and fifth intervals (20–25 min). Resting behavior appeared increased along the course of the first (0–5 min) and fourth intervals (15–20 min) in mice treated with the intermediate (30 nM) dose of OA. Likewise, resting resulted in an increase at the highest dose OA-injected mice but only during the sixth interval (25–30 min). Locomotor activity was reduced during the first three intervals (0–15 min) in mice injected with 30 nM OA and during the fifth (20–25 min) and sixth intervals (25–30 min) in mice treated with 300 nM.

Experiment 2

Food intake

HS wet mash intake was significantly decreased by the i.c.v. administration of both intermediate and highest doses (50 pM and 75 pM) of ST1326. Such decrease in food intake was evidenced after 40-min and 2-h from the start of BSS and resulted significantly reduced also in comparison with the animals administered with the same doses of ST1340 (Fig. 3). The ANOVA analysis revealed a significant main drug effect that affected HS wet mash food intake ($F_{5,80}$=32.18, P<0.0001), a significant time effect ($F_{1,80}$=488.92, P<0.0001) as well as a significant treatment× time interaction ($F_{5,80}$=3.44, P<0.007). Post hoc comparison further revealed a significant decrease of food intake which was detected for the doses of 50 and 75 pM after 40 min and 2 h from the ST1326 administration, while none of these doses affected wet mash intake at 14 h. Conversely, the lower dose of ST1326 (25 pM) never affected the amount of food eaten, whatever the time interval considered. Moreover, ST1340 treatment was always ineffective, and no statistical differences in comparison to vehicle or to ST1326-injected mice were found.

Behavioral satiety sequence

At both lower and intermediate dose-administered mice (25 pM and 50 pM), the BSS was reallocated (leftward shifted) from T4 (panel A, vehicle group, Fig. 4) to T3 (panel B, 25 pM) and to T1-T2 (panel C, 50 pM), respectively. The occurrence of premature satiety onset is demonstrated by the earlier point of interaction between resting and eating. Conversely, at the highest dose injected (75 pM), ST1326 induced a disruption of the normal occurrence of BSS development (panel D, Fig. 4). Repeated measures ANOVA evidenced a significant treatment effect for the following behavioral categories: *eating* ($F_{5,53}$= 16.00, P<0.0001) and *resting* ($F_{5,53}$=3.13, P<0.01). On the contrary, *grooming* and *locomotion* were not affected by ST1326 and ST1340 treatment. Moreover, a significant treatment×time interaction was found for the duration of *eating* ($F_{35,371}$=4.42, P<0.0001), *resting* ($F_{35,371}$=1.83, P< 0.01) and *grooming* ($F_{35,371}$=1.59, P<0.05) behaviors. No effects were found for *locomotion*. Post hoc comparison further revealed that mice injected with 50 pM and 75 pM of ST1326 significantly decreased the duration of eating behavior during the first two intervals (0–10 min) in comparison to those injected with either corresponding doses of ST1340 or vehicle (Newman–Keuls; p<0.01). Mice infused with ST1340 were never significantly different from vehicle infusion group, whatever the dose administered. As shown by the leftward shifting toward earlier intervals

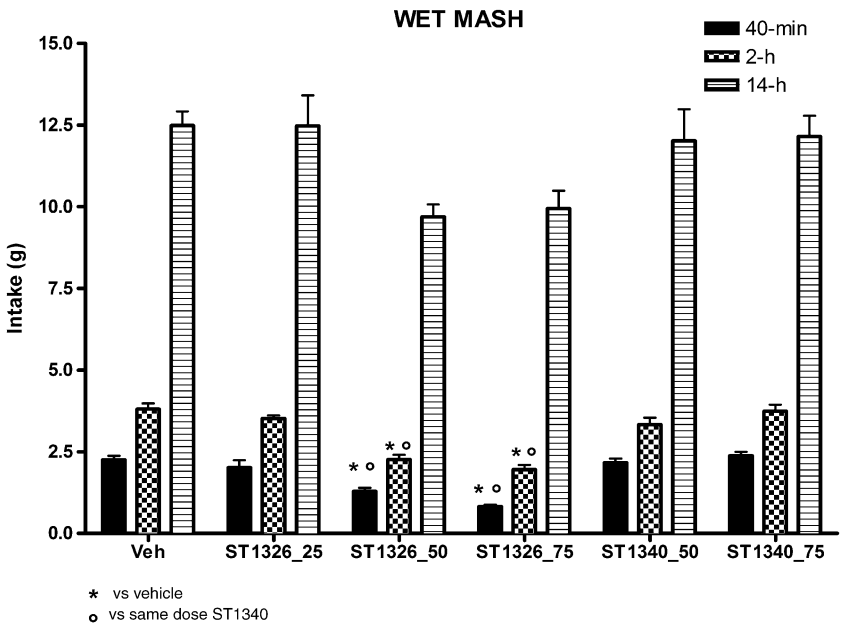

Fig. 3 The figure shows the effects of intra-i.c.v. infusion of different doses (25 pM/1 µl, 50 pM/1 µl, 75 pM/1 µl) of ST1326 and ST1340 (50 pM/1 µl, 75 pM/1 µl) on wet mash intake. Vertical bars represent the mean food intake ±SE after 40 min, 2 and 14 h from ST1326 and ST1340 intracerebral infusion. *Significant difference of doses of ST1326 and ST1340 from vehicle (p<0.05 Newman–Keuls test, after significant ANOVA). Significant difference of 50 pM and 75 pM doses of ST1326 from the same doses of ST1340

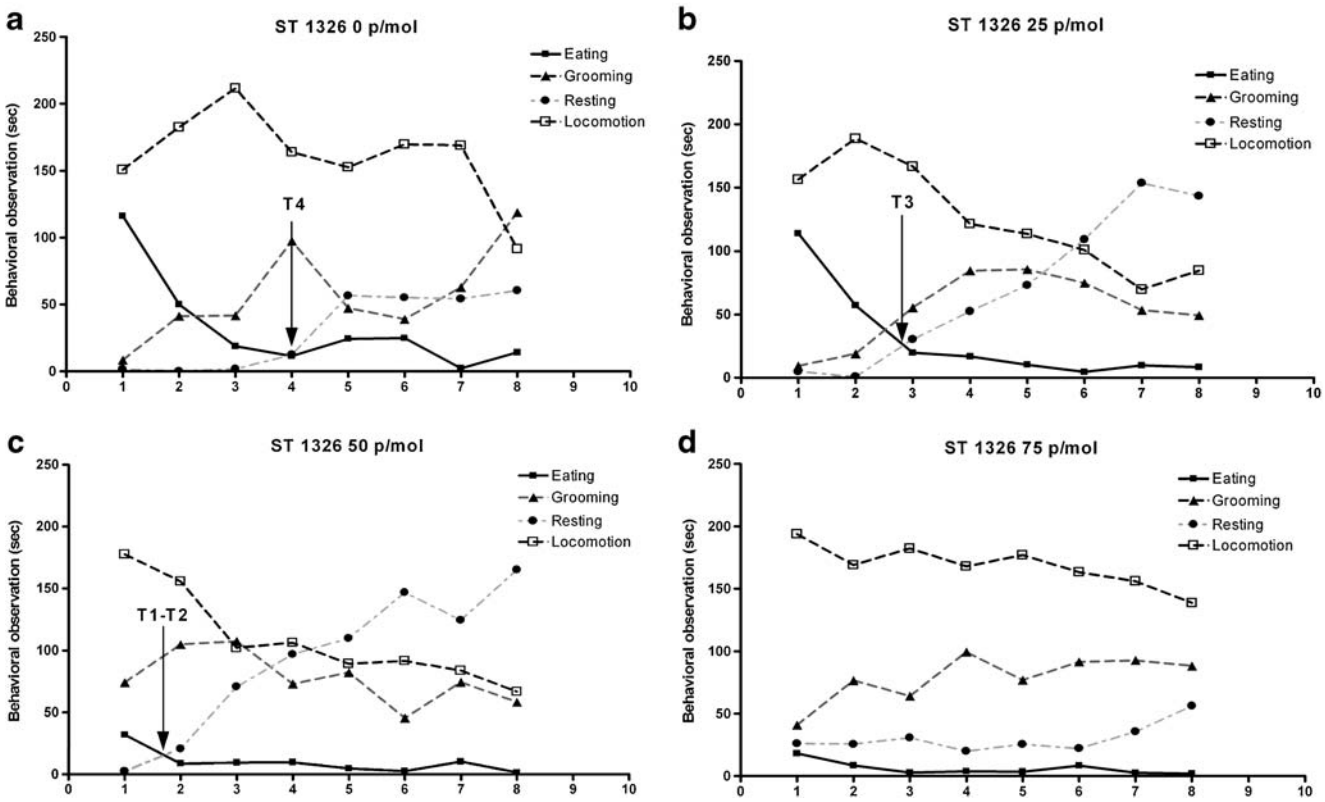

Fig. 4 The figure shows the temporal development of eating, locomotion, grooming, and resting duration. Panels show the results for each group of ST1326 i.c.v-infused mice. Represented on the *X*-axes are 8 time bins of 5 min each for a total of 40 min of behavioral observation

(Fig. 4), both 25 pM and 50 pM of ST1326 induced a premature satiety onset so that eating activity resulted quickly replaced by resting. The effects of ST1326 infusion on satiety onset were produced in a dose-dependent fashion. Indeed, while 25 pM shifted of about 5 min the satiety occurrence (from T4 to T3), the higher dose further highlighted the premature onset of satiety (which occurred between T1 and T2). As compared to vehicle and the equivalent dose of ST1340, mice injected with the intermediate dose (50 pM) of ST1326 significantly increased grooming activity during the first two intervals (0–10 min) of behavioral observation (Newman–Keuls; $p<0.01$). Conversely, no significant differences among vehicle, ST1340 and ST1326 were found for resting duration. Concerning eating frequency, the ANOVA analyses evidenced significant treatment ($F_{5,53}=3.15$, $P<0.014$) and time effect ($F_{7,371}=66.15$, $P<0.0001$) but no significant treatment×time interaction.

Discussion

Several lines of evidence support the idea of a link between fatty acid synthesis, feeding behavior, and energy balance. Energy-related signals are conveyed to the central nervous system and then integrated by distinct hypothalamic nuclei, which provide a fine regulation of metabolic needs. According to this view, different reports posit that fatty acid-related signals contribute to the modulation of hypothalamic control of homeostasis and feeding (Loftus et al. 2000; Obici et al. 2003; Pocai et al. 2006). Hence, the regulation of the different steps of fatty acid synthesis is one means by which hypothalamus can modulate energy homeostasis and balance body weight and food intake. Fatty acid translocation into mitochondria and therefore inhibition of fatty acid oxidation can be suppressed either indirectly, by increasing malonyl-CoA levels (e.g., by FAS inhibitors) or by the direct inhibition of the catalytic activity of CPT-1. The reversible CPT-1 inhibitor ST1326 has been identified as possible candidate as antiketotic and antidiabetic agents in vivo (Giannessi et al. 2003), with a marked inhibitory selectivity in isolated rat mitochondria which was shown to be greater for the liver (L-CPT-1) than for the muscle (M-CPT-1) isoform. Besides the importance of this selectivity in vitro (IC_{50} 1.1 μM, liver vs IC_{50} 43.4 μM, heart) for the reducing risk of cardiac muscle hypertrophy, a pronounced decrease of serum glucose levels after oral administration of ST1326 in db/db mice was in parallel shown (Giannessi et al. 2003). This is consistent with the reported effects of central ST1326 administration on endogenous glucose production (Obici et

al. 2003), further supporting the view that the inhibition of LCFAS import into the mitochondria may be an interesting strategy for the management of type 2 diabetes mellitus.

However, despite the great deal of interest toward the sensing energy mechanisms involved in the maintenance of metabolic balance, there is still a gap of knowledge concerning the behavioral specificity of agents with modulatory action on fatty acid biosynthetic pathway. To deal with this issue, this study investigated the hypothesis that these compounds can act as an appetite suppressant in a specific way. BSS formal definition (Antin et al. 1975) stemmed out from the observation that food ingestion is usually followed by a series of activities (locomotion, grooming) before resting prevails. If such stochastic structure is preserved, then the drug-dependent reduction of food intake can be assumed as a physiological (post-ingestive) mechanism of satiety. A "truly" anorectic should indeed preserve the BSS structure, so to rule out the possibility that anorexia is induced by unselective mechanisms (Halford et al. 1998), such as induction of nausea (e.g., lithium chloride), alteration of palatability (e.g., quinine), sedation (e.g., MK-212), or hyperlocomotion (e.g., D-amphetamine).

The present data show that the increased neuronal availability of fatty acids induced a satiety sequence that strongly resembles to the action described for other anorectic agents such as cholecystokinin (Dourish et al. 1989) or 5-HT2 receptor agonists (Kitchener and Dourish 1994). Specifically, the results of these experiments are consistent with the concept that increasing cytosolic levels of LCFAs (by i.c.v. OA infusion) or limiting the entry of fatty acids into the mitochondria (by the inhibition of CPT-1 activity) produce both powerful effects in decreasing food intake (Figs. 1 and 3). As far as we know, the impact of pharmacological modulation of fatty acid synthesis on the process of satiety development has never been investigated. This study demonstrated for the first time that both 30 nM and 300 nM of OA central infusion were able to induce an anticipation of the temporal development of satiation process (Fig. 2) as evidenced by the shift to the left of the crossing point between resting and eating activities, in comparison to vehicle. Indeed, satiety is demonstrated by the cessation of feeding behavior as well as by the concomitant starting of non feeding-related behaviors.

Nevertheless, despite the whole picture of the BSS and food intake reduction are consistent to the effects produced by anorectic agents, 30 nM and 300 nM OA infusion also temporarily reduced the duration of locomotion and increased grooming (intervals I–III, and V, 300 nM dose only). However, in 300 nM-injected mice, locomotion did not change during the first three intervals, and a competing interaction between grooming and locomotion may be hypothesized to occur only in the fifth interval. Because satiety appeared earlier (during the second interval, Fig. 2), the possibility that a competing action between locomotion and grooming would have affected feeding behavior can be ruled out. While the significant increase of grooming during the fifth interval is reminiscent of fluoxetine-induced increase of post-prandial grooming (Willner et al. 1990), the augmentation of grooming during the first three intervals may have prevented the expression of feeding behavior. This leaves open the possibility that non-specific responses, particularly a competing grooming activity, may have contributed to anticipate feeding reduction, satiety occurrence, and the decrease of food intake found 2 h after the highest dose of OA infusion. On the other hand, grooming was not affected by the infusion of the intermediate dose of 30 nM. Thus, at this dose, the OA-mediated satiety was totally devoid of potential competing behaviors. Furthermore, it should be noted that eating frequencies were never influenced by OA infusion. This suggests that, under the action of OA, the eating behavior was displayed as a continuous and non-disorganized activity, poorly influenced by potential contrasting activities. Moreover, since the duration of resting periods was increased by the 300-nM dose only during the VI interval (much later than satiety emergence), whereas the increase observed at 30 nM appeared at intervals (I and IV) different from the point (T1–T2) during which eating decreased and satiety occurred, the likelihood of OA-induced sedation can be ruled out for both doses.

In the second experiment, the lack of behavioral disruption of non-feeding behaviors matches with the preservation of feeding behavioral structure, thus indicating a specific interference of 50-pM ST1326 with satiety development (Fig. 4). Conversely, at the higher dose injected, ST1326 induced a disruption of the temporal occurrence of the different behavioral parameters observed. As recognizable in control as well as in ST1340-infused mice, all the behaviors are in co-dependent relationship, so that the relative incidence of each behavioral pattern is displayed in a temporally related fashion. Both 25 and 50 pM of ST1326 produced a marked leftward shifting which is matched by the concomitant anticipation of food intake suppression and increase in resting. Moreover, such effect was dose-dependent (Fig. 4) with the temporal anticipation of the satiety peak shifted either to interval 3 (T3) or between intervals 1 and 2 (T1-T2) for 25 and 50 pM, respectively. At these doses, the inhibition of CPT-1 was able to affect the temporal profile of feeding but leaving unaffected the basic structure of the behavioral sequence. This suggests that CPT-1 inhibition may act as an appetite suppressant agent, capable to speed up the whole temporal pattern of satiety sequence with ST1326-infused animals reaching satiation (meal termination) faster than the control group. Given the OA and ST1326-mediated anorectic effects found in this study, these finding

evidenced the potential of LCFAs extracellular accumulation as anti-obesity strategy. Because of the lack of alteration of locomotion and grooming activities, the ST1326 infusion has demonstrated to possess a major reliability as anorexigenic agent.

By contrast, BSS appeared disrupted by 75-pM infusion, and no transition points between feeding and resting periods were detected (Fig. 4). As such, this dose of ST1326 did not elicit selective effects on satiety progression. In other terms, at the highest dose, the reduced food intake is not conceivable as the result of the modulation of satiety development. Indeed, the progression of satiety sequence appeared perturbated by the sustained locomotor and grooming activities (Fig. 4). The locomotor activity did not show the biphasic profile (e.g., an enhanced initial motility followed by a partial resting) evidenced in control as well as in lower dose ST1326-injected mice. Although speculative, it is tempting to consider the potential effects produced by LCFAs on dopamine (DA) neurotransmission. LCFAs may act as diffusible messengers, and arachidonic acid (AA) has been shown to inhibit DA reuptake and increase DA content in synaptosomes from rat striatum (L'hirondel et al. 1995). Moreover, AA has been shown to produce an inhibitory effect on DA transporter activity in cells expressing the human DA transporter (Zhang and Reith 1996), thus enhancing extracellular DA concentration. The *N*-methyl-D-aspartate-evoked presynaptic DA release in striatal neurons has been shown to be under the facilitatory control of endogenously formed AA (L'hirondel et al. 1999). The degree of unsaturation of hydrocarbon tails appears to determine, in a concentration-dependent fashion, the relative potency in reducing DA uptake, with AA producing more inhibition that saturated acids (i.e., arachidic and stearic acids) and oleic and linoleic acids (cis-unsaturated acids) also producing important inhibition as compared to vehicle incubation in cellular line (HEK-293) expressing the DA transporter (Chen et al. 2003). In striatum-derived cell lines (X61) expressing a dopaminergic phenotype, the exposure to a variety of LCFAs and, particularly OA and AA, produced a linear and time-dependent positive stimulation of the cellular DA content (Heller et al. 2005). Thus, DA neurotransmission may be affected by fatty acid pathway and multiple LCFAs have been shown to be able to increase the extrasynaptic DA content. Although not investigated in this study, it is tempting to hypothesize that under higher (dose-dependent) inhibition of CPT-1 catalytic activity, the translocation of fatty acids across mitochondrial membranes is further reduced, and the accumulation of LCFAs might affect (namely facilitate) DA neurotransmission.

It is conceivable that, outside of a narrow range of CPT-1 inhibition, the disruption of satiety sequence, which occurred at the highest dose, may be the product of competing behaviors and not the result of the subtle regulation of the cellular energy balance, which appears necessary to the modulation of satiety time course. Increase in DA release, especially at striatal level (e.g., nucleus accumbens), may contribute to modulate approach responses (to food as well) and orientation towards incentive stimuli (Koob 1996). Modulation of DA transmission may alter the behavioral patterns displayed by the animals in their interaction with the environment. Consummatory and exploratory responses may interact with competitive tendencies and not only locomotion can actively compete with feeding. The facilitation of a sustained level of investigatory responses (outer- and self-directed) across the entire duration of BSS testing, might have competed with consummatory tendencies and therefore with eating motivation. Whilst in controls as well as in lower and intermediate doses, ST1326-injected mice feeding and exploratory drives coexisted during the first phases of BSS; in animals treated with the higher dose, the immediate exhibition of investigatory responses (grooming, locomotion) seemed to overcome the motivation to eat. It should be reminded that DA-releasing drugs (e.g., amphetamine) disrupt BSS (Halford et al. 1998). Thus, unlike from the lower doses, the BSS profile of 75-pM ST1326 infusion is not consistent with a selective effect on feeding behavior.

Collectively, the effects of ST1326 infusion on BSS are consistent with a role of CPT-1 as energy-sensing device. Thus, the blockade of this malonyl-CoA target during fatty acid synthesis seems to mimic a status of energy surplus, which is interpreted as satiety and opportunely translated into a significant decrease of food intake. Notably, this study further demonstrates that the hypophagic effects of ST1326 on food intake are compatible with the preservation of the physiological stochastic structure of BSS which, in turn, can be taken to signify that alterations in feeding are the product of a specific action on post-ingestive mechanisms of satiety. In recent years, BSS analysis has helped to elucidate the fundamental differences between DA-based anorexigenic drugs (e.g., amphetamine) and the hypophagic effects obtained by the manipulation of serotonin transmission. In opposition to amphetamine-mediated anorexia (Blundell et al. 1976), many influential studies have underlined the non-disruptive effects and the modulatory action on appetite control of both selective serotonin reuptake inhibitors (Simansky and Vaidya 1990; Willner et al. 1990) and 5-HT_{1B} and 5-HT_{2C} receptor subtypes agonists (Dalton et al. 2006; Hewitt et al. 2002; López-Alonso et al. 2007) on the BSS structure.

More recently, the BSS screening of feeding behavior has provided a helpful technique for the characterization of novel potential anti-obesity agents as, for instance, for the drugs acting as orexin-1 selective antagonists (Ishii et al. 2005; Rodgers et al. 2001). Together, these findings support

the idea that acting at the level of the intermediates involved in the control of energy sensing, such as on the gatekeeper function of FFA oxidation played by CPT-1, may represent a physiologically relevant way to design new anti-obesity strategies. Moreover, these data demonstrate that the modulation of neuronal availability of fatty acids is a highly sensitive mechanism that may enhance satiety progression only within a narrow range of cytosolic nutrients concentration.

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