Hindbrain noradrenergic input to the hypothalamic PVN mediates the activation of oxytocinergic neurons induced by the satiety factor oleylethanolamide

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Submitted 25 July 2013; accepted in final form 17 September 2013

Romano A, Potes CS, Tempesta B, Cassano T, Cuomo V, Lutz T, Gaetani S. Hindbrain noradrenergic input to the hypothalamic PVN mediates the activation of oxytocinergic neurons induced by the satiety factor oleylethanolamide. Am J Physiol Endocrinol Metab 305: E1266–E1273, 2013. First published September 24, 2013; doi:10.1152/ajpendo.00411.2013.—Oleoylethanolamide (OEA) is a gut-derived endogenous lipid that stimulates vagal fibers to induce satiety. Our previous work has shown that peripherally administered OEA activates c-fos transcription in the nucleus of the solitary tract (NST) and the paraventricular nucleus (PVN), where it enhances oxytocin (OXY) expression. The anorexigenic action of OEA is prevented by the intracerebroventricular administration of a selective OXY receptor antagonist, suggesting a necessary role of OXYergic mediation of OEA’s effect. The NST is the source of direct noradrenergic afferent input to hypothalamic OXY neurons, and therefore, we hypothesized that the activation of this pathway might mediate OEA effects on PVN neurons. To test this hypothesis, we subjected rats to intra-PVN administration of the toxin saporin (DSAP) conjugated to a monoclonal antibody against dopamine-β-hydroxylase (DBH) to destroy hindbrain noradrenergic neurons. In these rats we evaluated the effects of OEA (10 mg/kg, ip) on feeding behavior, on c-Fos and OXY immunoreactivity in the PVN, and on OXY immunoreactivity in the posterior pituitary gland. We found that the DSAP lesion completely prevented OEA’s effects on food intake, on Fos and OXY expression in the PVN, and on OXY immunoreactivity of the posterior pituitary gland; all effects were maintained in sham-operated rats. These results support the hypothesis that noradrenergic NST-PVN projections are involved in the activation of the hypothalamic OXY system, which mediates OEA’s prosatiety action.

acylethanolamides; neuropeptides; noradrenergic projections; nucleus of solitary tract; paraventricular nucleus; gut-brain axis

VISCEROSENSORY SIGNALS FROM THE GUT contribute to build a complex network of neural and hormonal signals that converge in the brain to control feeding behavior and energy balance. The acyl ethanolamides (OEA) appears to play an important role in this network (5, 13). OEA acts as an endogenous lipid mediator of satiety released from the enterocytes upon the intake of dietary fats (7, 23). OEA stimulates local lipid absorption and activates the “gut-brain axis” to prolong the interval to the next meal (8). These effects require the activation of peroxisome proliferator-activated-receptor-α (PPARα), which is expressed largely in the duodenum and jejunum, and the involvement of intact vagal fibers ascending to the nucleus of solitary tract (NST) (6, 20).

Our previous work has shown that, like other anorexigenic gut-derived signals, peripherally administered OEA (10 mg/kg ip) selectively activates c-fos transcription in brain areas involved in the central control of satiety and energy balance, such as the NST in the brainstem and the paraventricular (PVN) and supraoptic (SON) nuclei in the hypothalamus (8, 20). In both hypothalamic nuclei, c-fos mRNA is increased in neurons expressing oxytocin (OXY), whose activation is paralleled by increased OXY mRNA levels, increased peptide immunoreactivity in the posterior pituitary gland, and elevated circulating OXY levels (8), thus suggesting a stimulatory effect of OEA on OXY neurosecretion (21). No effect is observed on arginine/vasopressin (AVP)-expressing neurons or on circulating AVP levels (8, 24).

Both PVN and SON OXYergic neurons receive a direct excitatory input arising from the A2 noradrenergic cell group in the NST (17), whereas the A1 cell group of the brainstem appears to provide a direct excitatory input to AVP neurons (22). Moreover, the NST receives dense projections from hypothalamic OXYergic parvocellular neurons. Our previous study demonstrated that the intracerebroventricular (icv) infusion of a selective OXY antagonist prevents OEA’s anorexigenic action but not its ability to selectively induce c-fos expression in the NST. This observation suggests a necessary role of central OXY in mediating the effects of OEA on satiety and indicates that the activation of NST induced by OEA seems to precede the stimulation of OXYergic neurons in the hypothalamus. Based on these considerations, we hypothesized that the activation of the NST-PVN/SON noradrenergic pathway might mediate OEA’s effects on hypothalamic neurons and on eating. To evaluate this hypothesis, we subjected rats to a lesion of A2 noradrenergic neurons originating in the NST by the intra-PVN administration of the toxin saporin (DSAP) conjugated to a monoclonal antibody against dopamine-β-hydroxylase (DBH) (15). In DSAP-lesioned rats, we evaluated the effects of OEA (10 mg/kg ip) on c-fos and OXY immunoreactivity in the PVN, on OXY immunoreactivity in the pituitary gland, and on cumulative food intake; we hypothesized that the absence of the NST-PVN/SON noradrenergic...
pathway would abrogate or at least attenuate such effects of OEA.

MATERIALS AND METHODS

Animals

Thirty-two adult male Wistar rats from Elevage Janvier (Le-Genest-St. Isle, France), weighing ~300 g at the beginning of the experiment, were used. Rats were housed under controlled conditions of temperature and humidity, kept on a 12:12-h light-dark cycle with lights on at 1 AM, and given appropriate veterinary care. Water and standard chow pellets (cat. no. 890 25 W16; Provimi Kliba, Gossau, Switzerland) were available ad libitum unless indicated otherwise. Animals were housed in individual wire mesh cages (50 × 25 × 20 cm) to allow food intake measurements corrected for spillage, and they were accustomed to handling and injections before each experimental procedure.

All procedures were performed in accordance with the EC and Italian guidelines (86/609/EEC; D.Lvo 116/1992) and approved by the Cantonal Veterinary Office, Zurich, Switzerland. All efforts were made to minimize animal suffering and reduce the number of animals used. In particular, to mitigate pain and distress due to the surgical procedures, the following pre- and postoperative treatments were administered to animals: 10 mg/kg po enrofloxacin 1 day before and 2 days after surgery, 10 mg/kg sc enrofloxacin, 20 mg/kg sc prednisolone, and 5 ml sc warm saline at end of surgery (using 3 different injection sites), and 0.05 mg/kg sc buprenorphin if necessary after recovery from anesthesia. To reduce the number of animal used in the study, all of the behavioral and immunohistochemical observations were made on the same animals.

Personnel who cared or performed the procedures on the animals had received appropriate training for these tasks.

Experimental Groups

PVN microinjection. The protocol for neuronal lesions of noradrenergic NST/PVN projections was adapted from previous studies conducted by Rinaman (15). Rats were randomly divided into two groups. The first group received bilateral PVN injections of DSAP (lesioned rats, n = 16; Advanced Targeting Systems, San Diego, CA), and the second group (sham-operated rats, n = 16) received injections of nontargeted DSAP control molecule (IgG-DSAP) that was devoid of any ability to target cells.

All surgeries were done in random order over a period of 3 days. The animals were anesthetized with isoflurane (5% to induce anesthesia and 2.5–3% to maintain anesthesia) and secured in a stereotaxic frame in the flat-skull position. By means of a glass micropipette attached to a pneumatic injector (Picospritzer III; General Valve) and with the aid of a surgical microscope (Carl Zeiss, Feldbach, Switzerland), a total of 84 ng of DSAP or IgG-DSAP dissolved in 400 nl of saline was microinjected bilaterally into the PVN [stereotaxic coordinates: dorsoventral −8 mm, lateralmedial ± 0.4 mm, and antero- posterior −1.88 mm from bregma (11)]. The beveled glass pipette had an inner diameter of 40 μm, and solutions were injected at a pressure of 40 psi, using four consecutive pulses of 10 ms each at 4-min intervals. These injection parameters were chosen according to pilot experiments and resulted in successful and reproducible lesions of the targeted neurons. After surgery and recovery from anesthesia, rats were returned to their home cages.

Postsurgery maintenance. For 6 days after surgery, all rats were given ad libitum access to a palatable liquid diet (chocolate-flavored Ensure; Abbott, Baar ZG, Switzerland) in addition to ad libitum standard chow pellet to facilitate postoperative adequate caloric intake and body weight gain. From the 7th day postsurgery until the end of the study, rats received ad libitum standard chow pellet.

Food intake experiment. Fifteen days after surgery, all rats showed a similar body weight (381 ± 18) and were subjected to the feeding and terminal experiment. The time point of 15 days postsurgery was chosen accordingly to pilot experiments with different rats aimed at assessing the optimal time course of toxin-induced neuronal death.

During the feeding experiment, food was temporary removed for 1 h before the beginning of the dark phase. About 10 min before dark onset, rats received an intraperitoneal (ip) injection of vehicle (saline polyethylene glycol-Tween 80, 90:5:5; vol/vol/vol, 2 ml/kg) or OEA (10 mg/kg). Preweighted standard chow pellets were returned immediately after treatment, and rats were left undisturbed in their home cage for 2 h. At the end of this 2-h period, rats were euthanized as described below. Two-hour cumulative food intake was calculated as the difference between the amount of food given after treatment and remaining after the 2 h of food access, with correction for spillage. The timing schedule, the dosage of OEA, and the route of administration were chosen according to our previous studies (8, 20).

Immunohistochemistry Analysis

At the end of the food intake experiment (after 2 h from OEA administration), all of the animals were deeply anesthetized with pentobarbital sodium (80 mg/kg ip; Kantonssapotheke, Zurich, Switzerland) and transcardially perfused with ice-cold sodium phosphate buffer (0.1 M PBS, pH 7.4) followed by fixative solution containing 4% paraformaldehyde. Fixed brains were removed from the skull, postfixed overnight, and then cryoprotected in 20% sucrose-phosphate buffer (48 h at 4°C). Five series of 20-μm coronal brain sections containing the NST and the PVN (11) were cut on a cryostat (model CM3050S; Leica Nussloch) mounted on positively charged microscope slides (SuperFrost Plus, Menzel, Germany) and stored at −20°C. One series of sections was used for DBH immunostaining, which allowed us to assess the success of the noradrenergic lesion. Sections were rehydrated in PBS (pH 7.4) containing 0.1% Triton X-100 (PBST) and incubated for 2 h in a solution containing 1% BSA (Sigma-Aldrich) in 0.3% PBST, followed by incubation with mouse monoclonal anti-DBH primary antibody (1:2,000) in BSA/PBST, model MAB308, lot LV1390388; Chemicon) overnight at room temperature. The sections were then incubated with biotinylated goat anti-mouse IgG (1:400 in 0.3% PBST, model BA-9200, lot T0206; Vector Laboratories) for 1 h at room temperature. After incubation for 1 h to 1.5 h avidin-biotin complex (Vectastain ABC kit; Vector Laboratories), DBH-labeled cells were stained by incubation in 3,3-diaminobenzidine-tetrahydrochloride (DAB) chromogen solution prepared in PBS (0.05% DAB, 0.009% H2O2; Sigma-Aldrich). The slides were rinsed in PBST, dehydrated in graded alcohol, immersed in xylol, and cover-slipped with Entellan (Merck, Darmstadt, Germany). Brain sections were analyzed by using a microscope equipped with a digital camera (Axioskop; Carl Zeiss, Feldbach, Switzerland). Quantification of the total number of DBH-immunoreactive neurons in the NST per each rat was performed manually at a microscope with a ×20 objective (14); the investigator was blinded to the prior treatment of the respective rats. Criteria for counting a neuron as DBH positive included the presence of brown cytoplasmatic immunoreactivity and a visible nucleus (2). The extent of noradrenergic lesion was calculated as percentage of noradrenergic cells remaining after surgery, being 100% correspondent to the average number of DBH-positive cells in the NST of sham-operated rats. DBH terminal immunolabeling density within the PVN was evaluated to determine the efficiency of DSAP lesion, to confirm the correct localization of the injection site, and to exclude possible mechanical damage of the area due to the surgical procedure. DBH immunolabeling densities within the SON and the A1/C1 region were also evaluated.

DBH immunostaining in PVN, SON, and A1/C1 was measured semiquantitatively in each slice as optical density (OD) using the Scion Image software and considering the averaged OD of nonimmunoreactive regions of the brain for background normalization. ODs were calculated as percentage with respect to sham-operated rats (100%). An animal was considered to be properly lesioned if there
was <50% of the remaining DBH-positive neurons in the NST, <20% OD in the PVN, correct placement of the glass pipette into the targeted area, and no sign of mechanical lesion in the PVN. These criteria were chosen on the basis of previous pilot studies targeting noradrenergic neurons in the NST (15, 16).

A second series of sections containing the PVN was double-stained for c-Fos and OXY to assess their coexpression within the PVN. After rehydration in PBT, sections were incubated in 1.5% normal donkey serum (Jackson ImmunoResearch) in 0.3% PBT for 1 h. Sections were then incubated for 24 h at 4°C with anti-c-fos antibody (1:5,000 dilution, Ab-5 Calbiochem BC38) and anti-OXY antibody (1:1,000 dilution; Chemicon, Temecula, CA) and then rinsed in PBT and exposed to anti-mouse Alexa Fluor 488 (1:400 dilution; Invitrogen) and anti-rabbit Alexa Fluor 555 (1:259 dilution; Invitrogen) for 90 min. After an additional 10-min rinse, the sections were cover-slipped with Entellan (Merck) and captured by using a Leica LSM 710 confocal microscope and Zen 2008 software. Analysis of positive cells was conducted manually by counting separately each c-fos- or OXY-positive cell of the PVN. Coexpression was assessed as the percentage of OXY-positive cells within c-fos-positive neurons.

For each animal, measurements were obtained in at least four consecutive tissue sections containing the desired structure.

OXY immunostaining of the Pituitary Gland

Pituitary glands were collected after transcardial perfusion and cut at the cryostat into 18-μm-thick sagittal sections mounted on positively charged glass slides. Sections were incubated overnight at 4°C with the anti-OXY antibody (1:1,000 dilution; Chemicon) and then incubated at room temperature for 2 h with the anti-mouse Alexa Fluor 488 (1:1,000 dilution; Invitrogen) in the presence of Hoechst 33258 (1:5,000 dilution; Sigma-Aldrich) to detect pituicyte nuclei. The sections were evaluated under a Nikon Eclipse 80i microscope at evaluating c-fos expression. Therefore, cumulative food intake was monitored for only 2 h; thereafter, animals were euthanized. The two-way ANOVA of the 2-h food intake showed a significant effect of treatment (Ftreatment = 7.866, df = 1/27, P = 0.010) with no effect of lesion (Flesion = 0.355, df = 1/27, P = 0.557) and no interaction between the two factors (Finteraction = 1.859, df = 1/27, P = 0.185). In particular, the systemic administration of OEA markedly reduced food intake in sham-operated rats (P = 0.009; Fig. 2) that consumed ~56% less of their controls administered with vehicle. DSAP treatment per se did not affect food intake, as demonstrated by the lack of significant differences between food intake of lesioned and sham-operated rats administered with vehicle (P = 0.615; Fig. 2). As we hypothesized, DSAP lesion completely prevented OEA hypophagic effects, since all rats that underwent DSAP lesion consumed similar amounts of food (P = 0.305). No correlation was found between the slight, albeit not significant, decrease in food intake and the extent of lesion observed in DSAP-lesioned rats treated with OEA (data not shown). Water intake did not differ among all experimental groups (data not shown).

RESULTS

DSAP Administration-Induced Loss of DBH-Immunopositive NST Neurons Projecting to the PVN

DBH immunostaining revealed signs of mechanical lesion in the PVN of three sham-operated rats and an unsuccessful lesion in one rat that received intra-PVN DSAP administration. Therefore, the data sets from these rats were excluded from all further analyses. In the remaining 15 intra-PVN DSAP administered rats, the reduction of DBH immunostaining in the targeted areas reached our criteria for a successful lesion. In particular, the bilateral toxin injections were associated with an average reduction of about ~65% of A2 DBH-positive neurons in the NST (P < 0.001, Fig. 1, A and B) and an average reduction of ~76% of the OD of DBH-positive fibers within the PVN (P < 0.001; Fig. 1, E and F) with respect to those found in the 13 sham-operated rats. In all rats included in the analyses, the placement of the glass pipette into the target area was correct (data not shown).

Intra-PVN DSAP administration caused a significant reduction (about ~34%) of DBH immunostaining of the A1/C1 region of the brainstem (P < 0.01; Fig. 1, C and D), whereas it did not significantly affect the OD of DBH-positive fibers within the SON (P = 0.2967, Fig. 1, G and H).

DSAP Lesion Attenuated OEA Hypophagic Action

We hypothesized that noradrenergic neurons from the NST are activated by peripherally administered OEA to stimulate OXYergic neurons of the PVN to release OXY and to reduce eating. Therefore, our first aim was to evaluate whether the pharmacological lesion of the noradrenergic NST neurons would prevent OEA anorexigenic action. We followed the same protocol used in our previous studies (8), according to which OEA was administered ip few minutes before dark onset and at a dosage that does not readily allow penetration into the brain but is able to inhibit food intake in free-feeding rats. To minimize the number of animals used in the study, the feeding experiment was carried out in the same rats that were used for all of the immunohistochemical analyses, including those aimed at evaluating c-fos expression. Therefore, cumulative food intake was monitored for only 2 h; thereafter, animals were euthanized. The two-way ANOVA of the 2-h food intake showed a significant effect of treatment (Ftreatment = 7.866, df = 1/27, P = 0.010) with no effect of lesion (Flesion = 0.355, df = 1/27, P = 0.557) and no interaction between the two factors (Finteraction = 1.859, df = 1/27, P = 0.185). In particular, the systemic administration of OEA markedly reduced food intake in sham-operated rats (P = 0.009; Fig. 2) that consumed ~56% less of their controls administered with vehicle. DSAP treatment per se did not affect food intake, as demonstrated by the lack of significant differences between food intake of lesioned and sham-operated rats administered with vehicle (P = 0.615; Fig. 2). As we hypothesized, DSAP lesion completely prevented OEA hypophagic effects, since all rats that underwent DSAP lesion consumed similar amounts of food (P = 0.305). No correlation was found between the slight, albeit not significant, decrease in food intake and the extent of lesion observed in DSAP-lesioned rats treated with OEA (data not shown). Water intake did not differ among all experimental groups (data not shown).

DSAP Lesion Prevented the Effects of OEA on c-fos and OXY Expression in the PVN

Previous studies demonstrated that OEA’s effect on food intake is paralleled by a selective induction of c-fos expression in specific brain areas. These include the PVN, which plays a key role in the modulation of energy balance. Therefore, the second aim of our study was to evaluate whether the DSAP lesion prevents the effects of OEA on c-fos activation in the PVN. We found that c-fos-positive cells were particularly evident in OEA-treated sham-operated rats but not in any other group of rats (Fig. 3A). This observation was confirmed by the
results of the two-way ANOVA performed on the data sets obtained by counting c-fos-positive neurons in the analyzed PVN slice, revealing a significant effect of treatment ($F_{\text{treatment}} = 5.816$, $df = 1/27$, $P = 0.024$) with no effect of the lesion ($F_{\text{lesion}} = 2.962$, $df = 1/27$, $P = 0.098$) and a significant effect of the interaction between the two factors ($F_{\text{interaction}} = 7.620$, $df = 1/27$, $P = 0.011$). In particular, and in keeping with our previous observation (8), OEA treatment significantly induced c-fos expression in the PVN of sham-operated rats ($+511\%$ vs. their vehicle-administered controls, $P = 0.002$) but did not exert any effect in the PVN of lesioned rats ($P = 0.802$; Fig. 3B). DSAP lesion per se did not affect the number of c-fos-positive neurons in lesioned rats ($P = 0.481$; Fig. 3B).

Previously, we demonstrated that in normal rats systemically treated with OEA, c-fos is activated in a subpopulation of PVN neurons that includes mostly, albeit not exclusively, OXYergic neurons. In these neurons, OEA treatment increases peptide expression and peptide release at both the somatodendritic and terminal levels (8). On the basis of these observations, our next step was to evaluate whether the DSAP lesion would prevent OEA’s effects not only on c-fos induction but also on OXY expression in PVN neurons. The two-way ANOVA of the
number of OXY-positive neurons observed in each brain slice indicated that OEA treatment affected OXY expression differently in the PVN of sham-operated and lesioned rats, with a significant effect of treatment (F \(_{\text{treatment}} = 23.159, \text{df} = 1/27, P < 0.001\)), lesion (F \(_{\text{lesion}} = 17.523, \text{df} = 1/27, P < 0.001\)), and interaction (F \(_{\text{interaction}} = 22.315, \text{df} = 1/27, P < 0.001\)). In particular, OXY immunolabeling revealed a strong signal in the PVN of sham-operated rats treated with OEA administered with vehicle (Tukey's test; n = 6–7/sham-operated groups, n = 6–9/DSAP-lesioned groups).

The evidence supporting this conclusion was collected in rats that were subjected to bilateral intra-PVN microinjections of the immunotoxin saporin, which was linked to an antibody against DBH and allowed us to destroy noradrenergic neurons with cell bodies in the NST and with terminals reaching the PVN. DBH is the key enzyme involved in the conversion of dopamine to noradrenaline and is considered a marker for noradrenergic neurons. DSAP binds to vesicular DBH, is internalized during vesicle endocytosis, and is retrogradely transported. Upon reaching the cell body, the toxin inactivates ribosomes to interrupt protein synthesis. Such effects produce cell death of noradrenergic neurons within 1–2 wk (2). The neurochemical specificity of DSAP as a noradrenergic lesioning agent has been demonstrated in several reports and is broadly accepted (1, 10, 14, 15, 18, 19). In the present study, DSAP induced loss of the majority of DBH-positive neurons in the NST, specifically in the region corresponding to the location of the A2 cell group that projects directly to OXYergic neurons of the PVN (22). This loss was paralleled by the marked ablation of DBH-labeled fibers and terminals throughout the PVN. Moreover, as reported previously by other authors using a similar experimental protocol, DSAP infusion also caused a significant decrease in DBH immunostaining in the A1/C1 region of the brainstem (18), albeit to a lesser extent compared with the effect in the NST. Interestingly, the toxin did not alter DBH expression in the SON, which receives also noradrenergic fibers from the brainstem, thus possibly suggesting that there are distinct subpopulations of adrenergic neurons that were not affected by retrograde lesion. We believe that the protocol used to inject DSAP into the PVN allowed us to sensibly limit the extent of the diffusion area and contributed to obtain marked ablation effects in the noradrenergic fibers reaching the PVN, with likely only a partial destruction of other noradrenergic cell populations such as the A1/C1. However, the possible contribution of these other cell groups in mediating OEA effects on hypothalamic neurons cannot be completely ruled out.

In DSAP-lesioned rats, all the neurobehavioral effects of OEA that were observed in intact rats were markedly attenuated. In particular, the DSAP lesion, which did not affect food intake per se, significantly prevented the anorexigenic effect of OEA that remained evident in sham-operated rats. A certain degree of variability in the effects of OEA on food intake was observed in DSAP lesioned rats, although it did not correlate with the variability of the extent of the lesion in their brains. One limitation of our study is that we investigated only a single

**DISCUSSION**

The results of the present study support our hypothesis that noradrenergic projections from the NST to the PVN are a necessary component of the circuit responsible for the stimulation of hypothalamic OXY neurons, which sustain the inhibition of food intake induced by peripheral OEA administration.

As the last step of our study, we investigated whether the DSAP lesion would prevent the effects of OEA on OXY immunoactivity of the posterior pituitary gland (Fig. 4A) that we observed previously (8). The results of the two-way ANOVA of the OD measured in the pituitary glands that were immunostained for OXY revealed a significant effect of treatment (F \(_{\text{treatment}} = 7.279, \text{df} = 1/13, P = 0.022\)) but no effect of lesion (F \(_{\text{lesion}} = 2.345, \text{df} = 1/13, P = 0.157\)) or their interaction (F \(_{\text{interaction}} = 4.472, \text{df} = 1/13, P = 0.061\)). The multiple comparisons demonstrated that OEA caused a significant increase in OXY immunostaining in the posterior pituitary gland of sham-operated rats (P = 0.007) but not of lesioned rats (P = 0.689), which did not differ from vehicle-administered sham-operated rats per se (P = 0.708; Fig. 4B).

**Fig. 2.** DSAP lesion prevented the anorexigenic effects of oleylthanolamide (OEA). Two hours of cumulative food intake (normalized to body weight, i.e., g/kg) observed in sham-operated and lesioned rats after the intraperitoneal administration of vehicle (open bars) or OEA (10 mg/kg; black bars). Data are expressed as means ± SE. *P < 0.05 vs. sham-operated rats administered with vehicle (Tukey’s test; n = 6–7/sham-operated groups, n = 6–9/DSAP-lesioned groups).
dose of OEA (10 mg/kg ip). It would be beneficial in the future to perform a dose-response study to evaluate the ED50 of OEA in lesioned or sham animals. At present, we cannot exclude the possibility that a higher dose of OEA would have also reduced eating in the lesioned rats. However, because in our previous studies (9, 20) we showed that higher doses of OEA may cause nonspecific side effects (i.e., reduction of water intake and motor behavior), we decided to perform our experiments with one single dose only that produced the biggest effect in our previous work. Moreover, a similar failure to respond to the hypophagic action of the same dosage of OEA was observed previously in rats deprived of afferent fibers ascending from

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**Fig. 3.** DSAP lesion prevented the neurochemical effects of OEA in the PVN. **A**: representative fluorescent photomicrographs (×20 magnification, scale bar = 20 μm) of c-fos immunostaining (red), oxytocin (OXY) immunostaining (green), and double c-fos/OXY immunostaining (red/green) of the PVN (anteroposteriority between –1.88 and –2.12 mm from bregma) collected from sham-operated and lesioned rats 2 h after the administration of vehicle (veh) or OEA (10 mg/kg ip). **B** and **C**: average no. of c-fos-positive (**B**) and OXY-positive cells (**C**) per PVN section collected from sham-operated and lesioned rats treated with either veh (open bars) or OEA (black bars). **D**: average %/PVN section of c-fos-positive cells coexpressing OXY measured in sham-operated and lesioned rats after veh (open bars) or OEA treatment (black bars). Data are expressed as means ± SE. *P < 0.05 and ***P < 0.001 vs. sham-operated/veh (Tukey’s test; n = 6–7/sham-operated groups; n = 6–9/DSAP-lesioned groups).
the upper gut (20), in mice genetically lacking PPARα receptors (6), and in rats previously administered with a selective OXY antagonist into the third ventricle (8).

A further limitation in the interpretation of our results might derive from the limited time window (2 h) considered to evaluate OEA effects on food intake. Possible delayed effects of OEA on feeding behavior of DSAP-lesioned rats cannot be ruled out. A kinetic/time study is beyond the scope of the current study but might be useful to confirm our hypothesis in the future. However, in all of our previous studies on the long-term effects of OEA on feeding behavior, we always observed that food intake was inhibited within the first few hours following OEA administration at the dark onset without detecting any compensatory hyperphagia later on (6, 9).

It is well known from previous work that OEA treatment at dark onset is associated with the activation of c-fos and OXY gene transcription in the PVN and SON of free-feeding rats (8, 20), showing its maximum effects 1 h after administration and at the same dosage used in the present study (8). This effect was paralleled by an increase in OXY immunoreactivity of the posterior pituitary gland and of plasma observed 2 h after OEA treatment. This suggests a stimulatory effect of OEA on OXY neurosecretion from nerve terminals of OXY magnocellular neurons of the PVN and SON that through the neurohypophysis release OXY into the blood stream. Such a hypothesis has been confirmed recently (21).

That OXY neurosecretion is critically involved in OEA anorexigenic action was suggested by our previous observation that the infusion of the selective OXY receptor antagonist L-368,899 into the third brain ventricle can prevent the satiety effect of OEA (8). However, where PVN OXY-mediated outputs are going to control for food intake suppression following OEA treatment has remained unexplored. Here, we show that the DSAP lesion significantly reduced the ability of OEA to activate OXYergic neurons in the PVN and increase OXY content in the posterior pituitary gland, thus possibly suppressing its effects on OXY neurosecretion. Although such a conclusion is drawn from a semiquantitative analysis of immunohistochemical data that does not represent the most appropriate methodological approach to investigate oxytocin neurosecretion, this hypothesis is supported by our recent results obtained by brain microdialysis and immunohistochemistry experiments (21). In particular, following an experimental protocol very similar to the procedure adopted in this study, we showed that OEA treatment can stimulate oxytocin neurosecretion from the PVN and enhance oxytocin expression at both axonal and somatodendritic levels of hypothalamic neurons and that both techniques allow for the detection of a maximum effect 2 h after OEA administration (21).

The findings of the present study further support the hypothesis that PVN OXYergic neurons mediate OEA’s action on feeding behavior and demonstrate that intact NST/PVN noradrenergic fibers play a necessary role. That OXY neurons in the PVN receive noradrenergic excitatory inputs from the NST was demonstrated previously (22). Moreover, the finding by Serrano et al. (24) that peripherally administered OEA increased hypothalamic noradrenaline concentration suggests the hypothesis that OEA might stimulate OXY neurosecretion from PVN by increasing noradrenaline release from NST afferents sensitive to our DSAP lesion protocol.

The NST is the primary central nervous system (CNS) site for a variety of gastrointestinal mechanical, nutrient chemical, and vagal signals that are able to control feeding behavior. Second-order NST neurons do not merely relay to higher brain centers, but they also incorporate afferent information with activity in local circuits and upstream efferent signals to control homeostasis. The local circuits include OXY fibers descending from the PVN (mostly from parvocellular neurons). It has been shown that OXY released from PVN/NST fibers can enhance visceral afferent transmission to the NST acting via both presynaptic and postsynaptic mechanisms (12). This system is involved in the NST integration of adiposity signals that act centrally (i.e., leptin) with peripheral meal-related negative feedback signals (such as cholecystokinin) (4).

With respect to the possible involvement of the same mechanism in mediating the effects of OEA on NST neurons, we demonstrated previously that infusion of the L-368,899 into the third brain ventricle prevented OEA’s effects on feeding without blocking c-fos activation in the NST (8). This finding does not support the hypothesis that OXYergic parvocellular projections might contribute to the NST signals but rather would suggest that the PVN involvement is downstream to the NST activation. This view is also supported by the present findings. However, further studies should address directly whether OXY...
transmission might control the activation of NST neurons induced by OEA and provide further insights on the mechanism by which the OEA signal reaches the CNS. So far, all evidence combined suggests an involvement of vagal fibers ascending form the first tract of the small intestine and reaching the NST. Second-order noradrenergic neurons from this nucleus might then project the signal to the hypothalamus.

Unveiling the complex network of peripheral and central mechanisms regulated by OEA might open new perspectives on the role played by this lipid mediator in the control of satiety and energy homeostasis, thus possibly paving the way for a better understanding of eating disorders and obesity pathogenesis.

ACKNOWLEDGMENTS

We thank Drs. Loredana Asarian and Christina Boyle from the University of Zurich for their suggestions during the development of this study. Moreover, we thank Fiona Bragger, Dr. Katrin Abegg, Annika Donauer, Tito Borner, and Claudia Liberini from the University of Zurich for their technical help.

GRANTS

This study was supported by the University of Zurich and by the Italian Ministry for Education, University and Research (PRIN 2009ESX7T3 and FIR RBF12DEL, 003), which had no further role in study design, the collection, analysis, and interpretation of data, the writing of the report, or the decision to submit the article for publication.

DISCLOSURES

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

A.R., C.S.P., and B.T. performed the experiments; A.R., B.T., and T.C. analyzed the data; A.R., V.C., T.L., and S.G. interpreted the results of the experiments; A.R. prepared the figures; A.R. drafted the manuscript; A.R., B.T., T.C., V.C., T.L., and S.G. approved the final version of the manuscript; V.C., T.L., and S.G. contributed to the conception and design of the research; T.L. and S.G. edited and revised the manuscript.

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