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Escalation in high fat intake in a binge eating model differentially engages dopamine neurons of the ventral tegmental area and requires ghrelin signaling

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ABSTRACT

Q3 Binge eating is a behavior observed in a variety of human eating disorders. *Ad libitum* fed rodents daily and time-limited exposed to a high-fat diet (HFD) display robust binge eating events that gradually escalate over the initial accesses. Intake escalation is proposed to be part of the transition from a controlled to a compulsive or loss of control behavior. Here, we used a combination of behavioral and neuroanatomical studies in mice daily and time-limited exposed to HFD to determine the neuronal brain targets that are activated – as indicated by the marker of cellular activation c-Fos – under these circumstances. Also, we used pharmacologically or genetically manipulated mice to study the role of orexin or ghrelin signaling, respectively, in the modulation of this behavior. We found that four daily and time-limited accesses to HFD induce: (i) a robust hyperphagia with an escalating profile, (ii) an activation of different sub-populations of the ventral tegmental area dopamine neurons and accumbens neurons that is, in general, more pronounced than the activation observed after a single HFD consumption event, and (iii) an activation of the hypothalamic orexin neurons, although orexin signaling blockage fails to affect escalation of HFD intake. In addition, we found that ghrelin receptor-deficient mice fail to both escalate the HFD consumption over the successive days of exposure and fully induce activation of the mesolimbic pathway in response to HFD consumption. Current data suggest that the escalation in high fat intake during repeated accesses differentially engages dopamine neurons of the ventral tegmental area and requires ghrelin signaling.

1. Introduction

Binge eating is characterized by the consumption of usually large amounts of food in a discrete period of time, while feeling a sense of loss of control over eating (Perello et al., 2014). This type of behavior is observed in a variety of human eating disorders (i.e. bulimia nervosa, binge eating disorder), in overweight and obese people, and also in non-clinical populations under specific circumstances such as stress (Perello et al., 2014). The etiology of the human binge eating is currently unknown. Thus, several animal models have been developed in order to get insights into the mechanisms regulating this complex behavior (Corwin and Buda-Levin, 2004). *Ad libitum* fed rodents exposed to a palatable food, including a high-fat diet (HFD), display a robust event of hyperphagia (Valdivia et al., 2014). Since it is believed that binge eating episodes in human beings are not driven by metabolic needs, exposure of satiated rodents to palatable stimuli has been extensively used as a model of binge eating (Corwin and Buda-Levin, 2004). Interestingly, several studies using intermittent or daily access to palatable foods have shown that rodents' consumption gradually escalates over the initial accesses until it finally stabilizes (Avena et al., 2008; Bake et al., 2014; Berner et al., 2008; Davis et al., 2007; Lardeux et al., 2013; Rada et al., 2005; Wojnicki et al., 2008). Intake escalation appears to be a critical phenomenon since it has been proposed to mediate the transition from a controlled to a compulsive or loss of control behavior (Goeders et al., 2009). Notably, intake escalation is also observed with drugs of abuse, including cocaine, heroin and amphetamine, among others (Zernig et al., 2007). Despite its potential relevance for understanding these disorders, the molecular substrates controlling the escalation of high-fat intake in rodent binge eating models have not been systematically studied.

Binge eating intake in animals allowed intermittent or daily access to palatable foods presumably involve neuronal circuits

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regulating rewarding aspects of eating (Avena et al., 2008; Bake et al., 2014; Berner et al., 2008; Davis et al., 2007; Lardeux et al., 2013; Rada et al., 2005; Wojnicki et al., 2008). These neuronal circuits include the dopamine neurons of the midbrain ventral tegmental area (VTA) that project to the nucleus accumbens (Acb) in the ventral striatum as well as other areas such as the amygdala, medial prefrontal cortex, hippocampus and hypothalamus (Kenny, 2011). Acute rewarding stimuli activate VTA dopamine neurons, with the consequent dopamine release in the Acb, potently enhancing the drive to obtain palatable foods (Rada et al., 2005; Salamone and Correa, 2012). Still, the contribution of the dopamine system in binge eating behaviors is a controversial issue (Salamone and Correa, 2012). Importantly, neuronal circuits regulating rewarding aspects of eating are sensitive to peripheral factors, including the orexigenic stomach-derived hormone ghrelin (Perelló and Zigman, 2012). Ghrelin is known to enhance the rewarding value of HFD (Perelló and Zigman, 2012); however, a potential role of ghrelin signaling on high-fat binge eating models has not been tested. Here, we used a combination of behavioral and neuroanatomical studies in genetically or pharmacologically manipulated mice in order to determine not only the neuronal targets activated in a binge eating model induced by daily and time-limited access to HFD but also the potential role of orexin and ghrelin signaling in the modulation of this behavior.

2. Methods

2.1. Animals and diets

The study was performed with adult (9–12 weeks old) C57BL/6J wild-type and GHSR-null mice, which fail to express the ghrelin receptor (or GHSR from growth hormone secretagogue receptor). All mice were generated at the animal care facility of the IMBICE. GHSR-null mice were derived from crosses between heterozygous animals back-crossed for more than 10 generations onto a C57BL/6J genetic background (Perello et al., 2010 Zigman et al., 2005). Mice were housed under a 12-h light/dark cycle with food and water available ad libitum. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council, USA. The protocol was approved by the Institutional Animal Care and Use Committee of the IMBICE (approval ID 10-0113). Both regular chow (RC) and HFD were provided by Gepsa (Grupo Pilar, www.gepsa.com). RC pellets provided 2.5 kcal/g energy, and its percent weight composition was as follows: carbohydrate 28.8, proteins 25.5, fat 3.6, fibers 27.4, minerals 8.1 and water content 6.7. HFD pellets were custom-prepared and provided 3.9 kcal/g energy. The percent weight composition of the HFD was as follows: carbohydrate 22.5, proteins 22.8, fat 21.1, fibers 23.0, minerals 5.6 and water content 5.0. For details see (Valdivia et al., 2014).

2.2. Experimental protocols

Mice were single-housed in clean cages three days before the experiment and then assigned into three experimental groups: (i) RC *ad lib* group, which received unlimited access to RC diet and was daily exposed to a pellet of RC in the home cage from 9.00 am to 11.00 am ($n = 14$); (ii) HFD *ad lib* group, which was daily exposed to a pellet of HFD in the home cage from 9.00 am to 11.00 am and also shifted to unlimited access to HFD after the first HFD exposure ($n = 8$); and (iii) daily HFD access group, which received unlimited access to RC during the whole experiment and was daily exposed to a pellet of HFD in the home cage from 9.00 am to 11.00 am. The cumulative food intake was recorded in the 2-h food access. RC *ad lib* and HFD *ad lib* mice received four successive accesses to their diet. Mice with daily and time-limited HFD access were sacrificed at three different time points: 2-h after HFD on day 1 (HFD post-day 1, $n = 10$); (ii) 2-h after HFD on day 4 (HFD post-day 4, $n = 28$); and (iii) right before HFD on day 4 (HFD pre-day 4, $n = 11$). The 22-h RC intake, from 11.00 am to 9.00 am next day, was quantified for some mice of the RC *ad lib* and HFD post-day 4 groups ($n = 11$ and 24, respectively). RC ad libitum fed GHSR-null mice were exposed to HFD or RC at 9.00 am ($n = 13$ and 3, respectively), as described above, and the cumulative food intake was recorded 2-h after food exposure during four consecutive days. In an independent experiment, the orexin 1 receptor antagonist SB-334867 (Tocris, cat. 1960) was used to block orexin 1 receptor signaling. SB-334867 was dissolved to 100 mM in DMSO containing 100 mM of HCl and then injected i.p. in a dose of 5 μg/g body weight in 200 μl of saline. Mice were daily administered with SB-334867 four successive days before HFD intake. In particular, mice were administered with vehicle alone or containing SB-334867 ($n = 10$ and 8, respectively) at 8.30 am and exposed to HFD at 9.00 am. Cumulative food intake was recorded 2-h after food exposure. This dose of SB-334867 was shown to have no effect on intake of freely available food (Perello et al., 2010). At the end of the experiments, mice were anesthetized and perfused with 10% formalin.

2.3. Immunostaining

Brains were coronally cut at 32 μm into three equal series. Immunostaining was performed in brain sections from at least 5 animals per experimental group, as described before (Valdivia et al., 2014). Briefly, sections were pretreated with H_2O_2, treated with blocking solution and incubated with anti-c-Fos antibody (Calbiochem, cat. PC38, 1:30000) for 2 days at 4 °C. Then, sections were incubated with biotinylated donkey anti-rabbit antibody (Vector Laboratories, cat. BA-1000, 1:1500), and with Vectastain Elite ABC kit (Vector Laboratories, cat. PK-6200), according to manufacturer's protocols. Then, visible signal was developed with diaminobenzidine/nickel solution (Sigma–Aldrich, cat. 32750), which generated a black precipitate. Double c-Fos and tyrosine hydroxylase (TH) immunostaining was performed on independent brain series. In this case, sections were incubated with a rabbit anti-TH antibody (Santa Cruz, cat. sc14007, 1:20000) for 48-h after c-Fos immunostaining was completed, and then sequentially incubated with a secondary antibody and the Vectastain Elite ABC kit, as detailed above. Visible signal was developed with diaminobenzidine solution without nickel, generating a brown precipitate. Double c-Fos and orexin immunostaining was performed similarly as described above but using an anti-orexin antibody (Phoenix Pharmaceuticals, cat. H-003-30, 1:20000) instead of the anti-TH antibody. Finally, sections were mounted on glass slides and coverslipped with mounting media. Bright-field images were acquired with a DS-Ri1 Nikon digital camera. Adobe Photoshop CS4 software was used to adjust levels, contrast and brightness.

2.4. Quantitative neuroanatomical analysis

Blind quantitative analysis was performed independently by two observers under the same optical conditions. Quantitative analysis was performed in sections between bregma 0.86 and 1.18 mm for the Acb, between bregma −1.22 and −1.94 mm for the lateral hypothalamic area (LHA), and between bregma −3.28 and −3.92 mm for the VTA. For the analysis, the Acb was subdivided into medial Acb shell (MAcbSh), lateral Acb shell (LAcbSh) and Acb core (AcbC) while the VTA was subdivided into paranigral (PN), parabrachial pigmented (PBP) and interfascicular (IF) subnuclei, according to previous descriptions (Ikemoto, 2007; Kelley, 2004). Anatomical limits of each brain region were identified using a mouse brain atlas (Paxinos and Franklin, 2001). The number

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of c-Fos-immunoreactive (IR) cells was quantified in each side of all sub-regions of the Acb and in the whole LHA of a complete series of each animal. The numbers of double-labeled c-Fos/TH or c-Fos/orexin neurons were quantified in all sub-regions of the VTA or in each side of the whole LHA, respectively, of a complete series of each animal. TH and orexin immuno-staining were confined to the perikarya, thus allowing the easy visualization of the c-Fos-positive nuclei. Data are expressed as the total number of IR cells (or cell nuclei) in each area and were calculated from the formula of Konigsmark, where the total cells are equal to the number of cells counted multiplied by the total number of sections through the nucleus and divided by the number of sections in which cells were counted (Konigsmark, 1970). For wild-type and GHSR-null mice, total number of TH-IR neurons in each sub-region of the VTA and total number of LHA orexin-IR neurons were also quantified using the same strategy. All quantitative neuroanatomical analyses were corrected for double counting, according to the method of Abercrombie, as we have done in the past (Valdivia et al., 2014). In particular, the number of total cells or nuclei was multiplied by a correction factor equal to the ratio of the section's thickness divided by the sum of the section's thickness plus the mean diameter of the positive cells or nuclei. For this, the diameter of at least 40 positive cells or nuclei was quantified in each brain area and experimental group using the software ImageJ.

2.5. Anticipatory locomotor activity assessment

To register mouse activity, home cages ($19 \times 28 \times 13$ cm) were placed in a ventilated and acoustically-isolated monitoring box ($55 \times 35 \times 90$ cm) equipped with an overhead camera and dimmable LED illumination. An independent set of mice were habituated to the recording environment by placing them in the monitoring box during 90 min on two consecutive days before the experiment. The experimental procedure consisted in 150-min sessions in 4 consecutive days from 8.30 am to 11.00 am. Every morning, mice were placed in the monitoring box, recorded for 30 min and then given access to RC ($n = 6$) or HFD ($n = 6$) for 120 min. For the analysis, the videos of the first 30 min of activity inside the box were converted to 5 frames per second rate and analyzed with the open source SwisTrack software. Briefly, video frames were color thresholded to generate a binary image, and then eroded and dilated to allow for better mouse tracking using blob detection and nearest neighbor tracking algorithm. Video track coordinates were scale- and curvature-corrected using a linear mapping of a multi-point calibration pattern previously recorded. Exported trajectories were further analyzed in Microsoft Excel to calculate travelled distance and mean velocity in 5-min bins. As a positive control for the procedure, anticipatory activity was assessed in an extra set of calorie restricted mice ($n = 6$), which had overnight access to an amount of RC corresponding to 70% of their average daily food intake and anticipatory locomotor activity to RC or HFD ($n = 3$, each) was assessed 30 min before the feeding period and after one week of habituation to the calorie restriction protocol.

2.6. Statistical analyses

Data were expressed as mean ± SEM. No significant differences were observed between the two groups of wild-type animals (those from crosses between wild-type mice vs. wild-type littermates of the GHSR-null mice) in any measures taken, and thus their data were pooled for the analysis. Equality of variance was analyzed using Bartlett's or Levene's tests. When variances were equal, one-way ANOVA followed by the Newman–Keuls test was used. When variances significantly differed, one-way ANOVA followed by the Games–Howell test was used. *T*-test was performed in order to compare data from vehicle- vs. SB-334867-treated mice. Two-way ANOVA followed by the Bonferroni test was used in order to compare data from wild-type and GHSR-null mice. Differences were considered significant when $p < 0.05$.

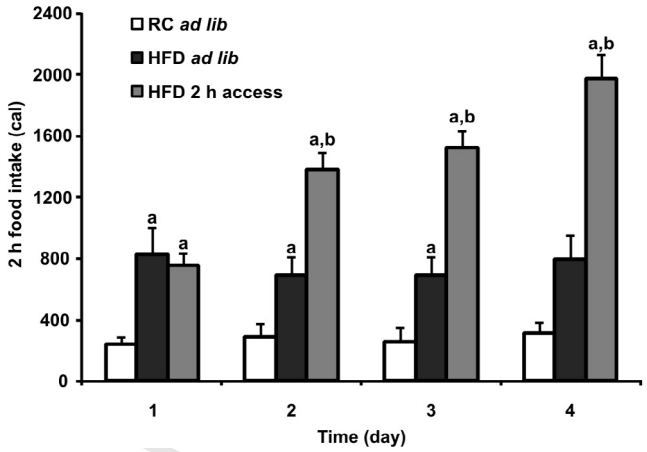

Fig. 1. Daily and time-limited access to HFD in mice induces events of hyperphagia with an escalating profile.
Panel shows 2-h food intake in mice with access to RC ad libitum, HFD ad libitum or daily and time-limited access to HFD. Values are the mean ± SEM, and were compared by one-way ANOVA. $n = 9$–29 per group. a, $P < 0.05$ vs. RC ad lib group at the same day. b, $P < 0.05$ vs. same group at day 1.

3. Results

3.1. Daily and time-limited access to HFD induces binge eating events with an escalating profile

Initially, food intake for experimental groups was measured (Fig. 1). RC *ad lib* group ate similar amounts of food among days when 2-h daily exposed to a pellet of RC [$F(3,52) = 0.06$, $P = 0.9799$]. Mice 2-h exposed to HFD ate significantly higher amounts of this food the first day of exposure, as compared to RC *ad lib* group [$F(2,47) = 9.34$, $P = 0.0004$]. For the successive days, 2-h food consumption during the HFD exposure events remained unchanged in the HFD *ad lib* group, as compared to their own food intake on day 1 [$F(3,28) = 0.67$, $P = 0.5749$]. Mice with 2-h daily access to HFD displayed a significant escalating profile of HFD consumption for the successive days [$F(3,108) = 19.74$, $P < 0.0001$]. Notably, RC intake during the rest of the day was not affected by the daily event of HFD consumption. Mice *ad lib* fed with RC ate 9.27 ± 0.31, 8.56 ± 0.55 and 9.17 ± 0.31 kcal of RC the 22 h after each daily event of RC consumption [$F(2,30) = 0.10$, $P = 0.9068$]. Mice with 2-h daily access to HFD ate 8.68 ± 0.30, 8.49 ± 0.49 and 8.53 ± 0.43 kcal of RC the 22 h after each event of HFD consumption [$F(2,69) = 1.40$, $P = 0.2537$]. Mean body weight of all experimental groups was not significantly changed throughout the experiment (not shown). The total distance traveled in the 30-min before eating events was also unchanged in mice with daily access to RC or HFD for 4 days. The total distance traveled in anticipation to the first HFD consumption event, which was 35.9 ± 4.8 m, did not differ from the total distance traveled in anticipation to HFD consumption events on day 2–4 that were 34.6 ± 4.6, 42.9 ± 6.0 and 36.8 ± 5.3 m, respectively [$F(3,11) = 0.40$, $P = 0.7567$]. It is important to stress that the anticipatory locomotor activity was confirmed to increase in calorie restricted mice daily exposed to HFD, as previously shown (Merrer and Stephens, 2006). On the first day, the total distance traveled in anticipation to food for calorie restricted mice was 28.1 ± 2.6 m. The total distance traveled in anticipation to RC consumption events did not differ among days 2–4, when it was 28.1 ± 2.6, 31.4 ± 0.9, 30.8 ± 3.4 and 33.2 ± 0.2 m, respectively [$F(3,11) = 0.54$, $P = 0.6634$]. However, the total distance

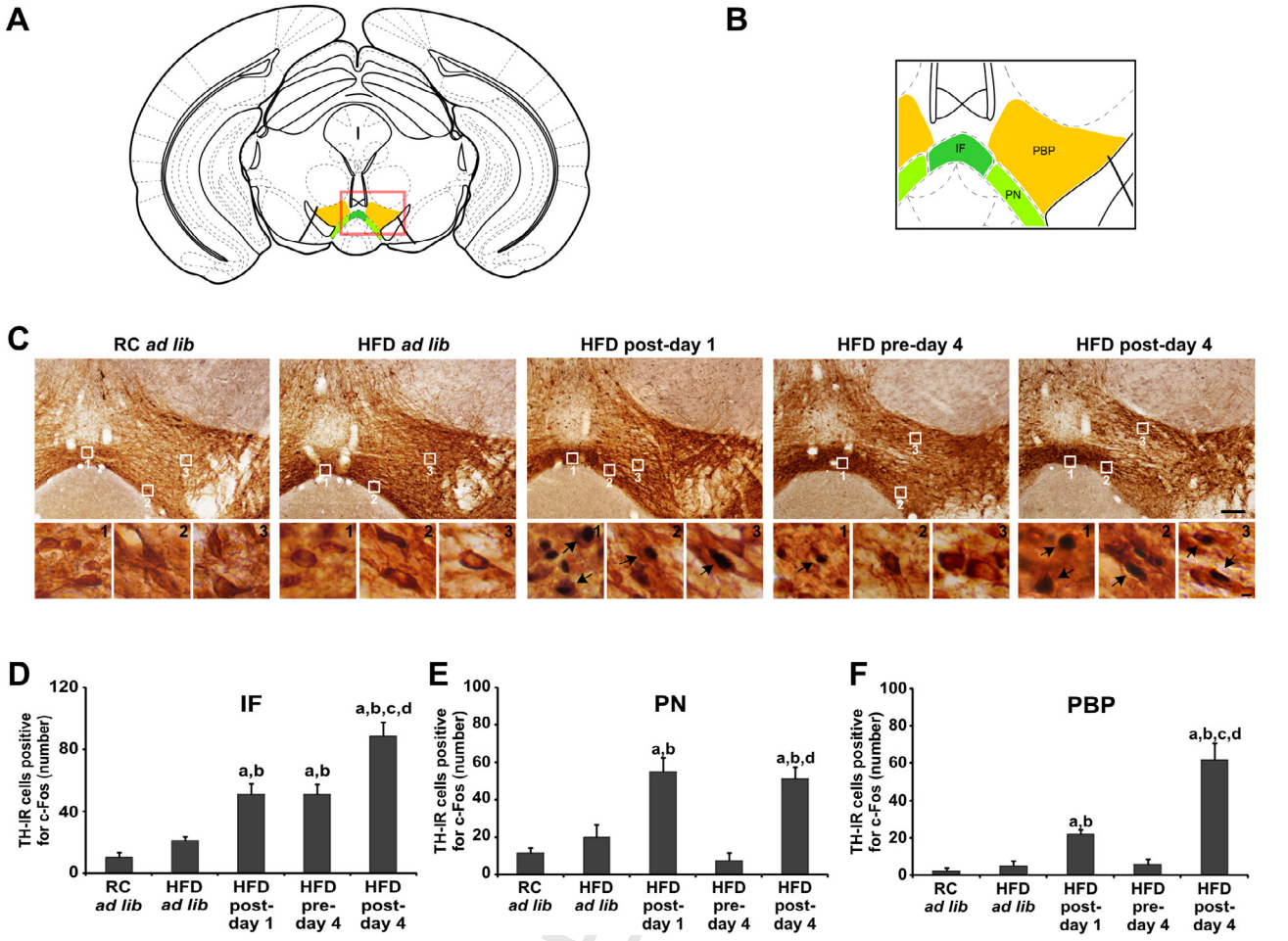

Fig. 2. Daily and time-limited access to HFD differentially induces c-Fos in TH-IR neurons of VTA sub-regions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Mice of RC *ad lib* or HFD *ad lib* groups were sacrificed 2 h after food consumption on day 4 while mice with daily and time-limited access to HFD were sacrificed: 2 h after HFD on day 1 (HFD post-day 1), right before HFD on day 4 (HFD pre-day 4) or 2 h after HFD on day 4 (HFD post-day 4). Panel A and B show lower and higher magnification, respectively, diagrams of representative reference atlas levels highlighting the subdivisions of the ventral tegmental area (VTA): paranigral nucleus (PN, light green), parabrachial pigmented area (PBP, orange) and interfascicular nucleus (IF, dark green). Panel C shows representative low and high magnification photomicrographs of brain sections containing the VTA and subjected to double immunohistochemistry using anti-TH (brown staining) and anti-c-Fos (black staining) antibodies in all experimental groups. For each experimental group, upper panel shows low magnification images and bottom panels show high magnification images of the areas marked in low magnification images. High magnification images show the IF (insert 1), the PN (insert 2) and PBP (insert 3) sub-regions of the VTA. Arrows point to dual-labeled cells. Scale bars, 200 μm (low magnification), 20 μm (high magnification). Bottom panels show the number of TH-IR cells positive for c-Fos staining in the IF (D), PN (E) and PBP (F) sub-regions of the VTA for each experimental group. Values are the mean ± SEM, and were compared by one-way ANOVA. $n = 5–14$ per group. a, $P < 0.05$ vs. RC *ad lib* group. b, $P < 0.05$ vs. HFD *ad lib* group. c, $P < 0.05$ vs. HFD post-day 1 group. d, $P < 0.05$ vs. HFD pre-day 4 group.

traveled in anticipation to HFD consumption events for calorie restricted mice was significantly increased on days 2–4, when animals traveled 55.8 ± 4.2, 53.3 ± 7.2 and 52.2 ± 3.0 m, respectively ([$F(3,11) = 12.87$, $P = 0.0006$], and $P < 0.01$ for all days vs. day 1).

3.2. Daily and time-limited access to HFD activates the mesolimbic pathway

In order to map brain areas responsive in these experimental conditions, brain sections were processed for immunostaining. Mice of the RC *ad lib* and HFD *ad lib* groups were sacrificed after food consumption on day 4, and mice with daily HFD access were sacrificed at three different time points: HFD post-day 1, HFD pre-day 4 or HFD post-day 4. Quantitative analysis of TH-IR VTA neurons positive for c-Fos was performed within the different sub-regions of the VTA (Fig. 2A–C), and significant differences were found in the IF [$F(4,43) = 23.27$, $P < 0.0001$, Fig. 2D], the PN [$F(4,43) = 13.12$, $P < 0.0001$, Fig. 2E], and the PBP [$F(4,43) = 22.16$, $P < 0.0001$, Fig. 2F]. In the IF, the number of TH-IR neurons positive for c-Fos of mice with daily and time-limited access to HFD was significantly higher as compared to the values found in both the RC *ad lib* and HFD *ad lib* groups. Notably, the number of TH-IR neurons positive for c-Fos in the IF of the HFD post-day 4 group was significantly higher than the values found in the HFD post-day 1 and HFD pre-day 4 groups, which were not different between them. In the PN, similar numbers of TH-IR neurons positive for c-Fos were found in the HFD post-day 1 and HFD post-day 4 groups and both values were significantly higher as compared to the values found in the other groups. In the PBP, the number of TH-IR neurons positive for c-Fos of the HFD post-day 1 or HFD post-day 4 groups was significantly higher as compared to the values found in the other groups, having the HFD post-day 4 group significantly higher number of TH-IR neurons positive for c-Fos than the HFD post-day 1 group. Quantitative analysis of c-Fos-IR cells in the Acb sub-regions (Fig. 3A–C) also showed significant differences in the MAcbSh [$F(4,37) = 11.50$, $P < 0.0001$, Fig. 3D], the LAcbSh [$F(4,37) = 12.49$, $P < 0.0001$, Fig. 3E] and the AcbC [$F(4,37) = 33.04$, $P < 0.0001$, Fig.3F]. In the MAcbSh, the number of c-Fos-IR cells of mice with daily and time-limited access

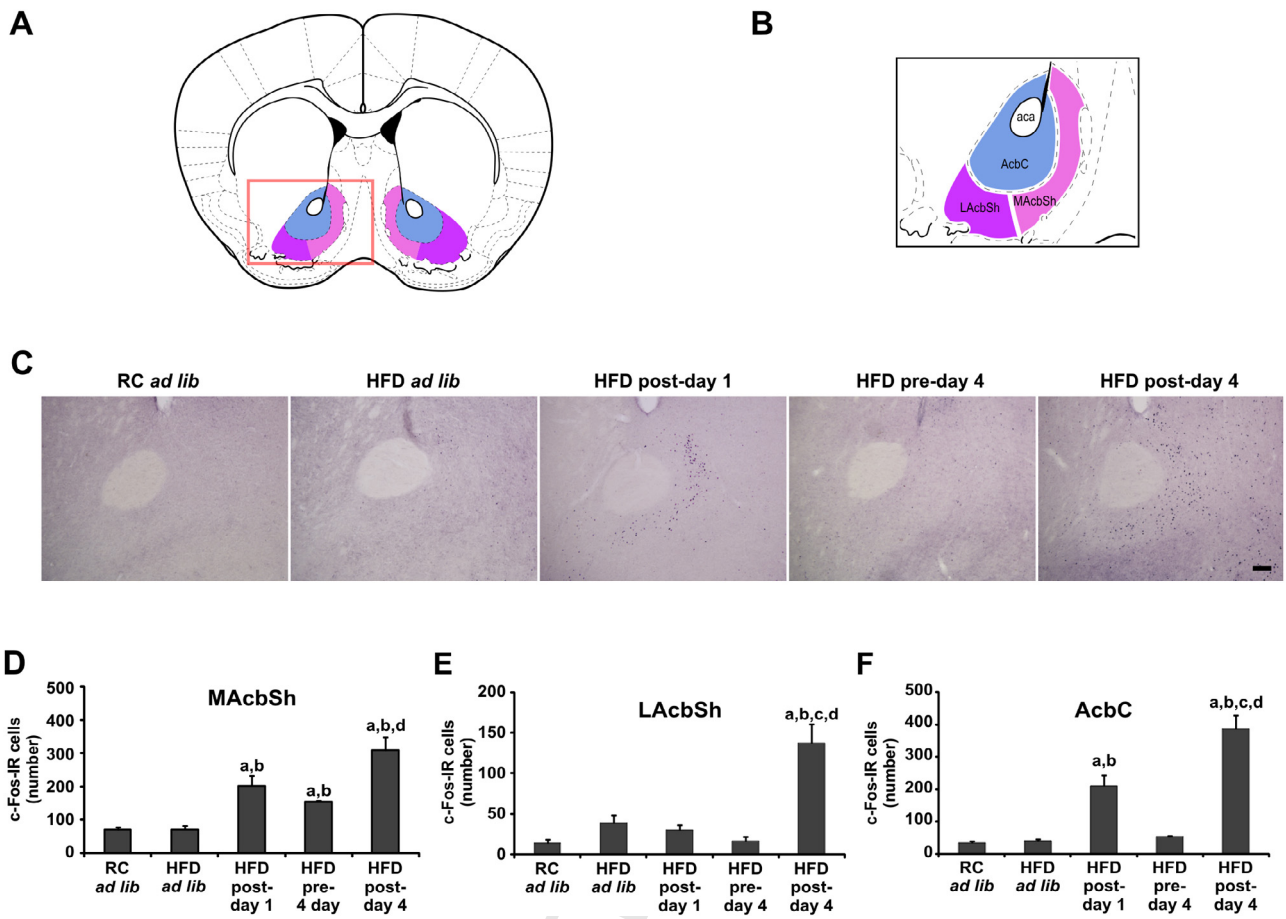

Fig. 3. Daily and time-limited access to HFD differentially induces c-Fos in the different Acb sub-regions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Panel A and B show lower and higher magnification, respectively, diagrams of representative reference atlas levels highlighting the subdivisions of the nucleus accumbens (Acb). Nucleus accumbens core (AcbC, blue), medial and lateral nucleus accumbens shell (MAcbSh and LAcbSh, light and dark purple, respectively). aca: anterior part of the anterior commissure. Panel Cshows representative microphotographs of the Acb region in brain sections subjected to c-Fos (black signal) immuno-staining ofall experimental groups. Scale bar: 100 μm. Bottom panels show thenumber of c-Fos-IR neurons in the MAcbSh (D), LAcbSh (E) and AcbC (F) sub-regions of the Acb of all experimental groups. Values are the mean ± SEM, and were compared by one-way ANOVA. $n = 5–11$ per group. a, $P < 0.05$ vs. RC *ad lib* group. b, $P < 0.05$ vs. HFD *ad lib* group. c, $P < 0.05$ vs. HFD post-day 1 group. d, $P < 0.05$ vs. HFD pre-day 4 group.

to HFD was significantly higher than the number found in both the RC *ad lib* and HFD *ad lib* groups; the HFD post-day 4 group displayed higher number of c-Fos-IR cells than the HFD post-day 1 group, but this difference did not reach statistical significance. In the LAcbSh, a significant increase in the number of c-Fos-IR cells was found in the HFD post-day 4 group, as compared to the other groups. In the AcbC, the numbers of c-Fos-IR cells of the HFD post-day 1 and HFD post-day 4 groups were significantly higher than the values found in the other groups, having the HFD post-day 4 group significantly higher number of c-Fos-IR cells than the HFD post-day 1 group.

3.3. Daily and time-limited access to HFD increases c-Fos expression in LHA orexin-IR neurons

Because LHA orexin neurons are involved in HFD consumption (Thompson and Borgland, 2011), c-Fos-IR cells and orexin-IR cells positive for c-Fos were quantified in the LHA of all experimental groups (Fig. 4A–C), and significant differences were detected for both analyses ([$F(4,22) = 24.02$, $P < 0.0001$] and [$F(4,22) = 10.44$, $P < 0.0001$], respectively). The number of c-Fos-IR cells in LHA was significantly higher in the HFD post-day 1 and HFD post-day 4 groups, as compared to the numbers found in the other experimental groups; still, the number of c-Fos-IR cells in the HFD post-day 4 group was significantly higher among these groups (Fig. 4D). In terms of the number of orexin-IR cells positive for c-Fos, a significant increase was found in both the HFD post-day 1 and HFD post-day 4 groups, as compared to the values found in the others experimental groups (Fig. 4E). The distribution of the orexin-IR neurons positive for c-Fos did not show any particular topography within the LHA.

3.4. Pharmacologic orexin signaling blockage fails to affect both escalation of HFD consumption and c-Fos induction in the mesolimbic pathway

In order to determine if orexin 1 receptor signaling is required for escalation of HFD consumption in this mouse model, SB-334867 was daily administered to ad libitum fed mice that were subsequently exposed to HFD. Both vehicle- and SB-334867-treated mice displayed a significant escalating profile of HFD consumption for the successive days ([$F(3,36) = 7.06$, $P = 0.0007$] and [$F(3,28) = 12.64$, $P < 0.0001$], respectively). The SB-334867 treatment significantly reduced 2-h HFD intake the first day of HFD exposure ($t(16) = 3.46$, $P = 0.0032$). However, 2-h HFD consumption on the experimental days 2–4 showed the same magnitude as observed for the vehicle-treated group (Fig. 5A). The 22-h RC intake was not affected through the experiment for any group (Fig. 5B). Quantitative neuroanatomical analysis of vehicle- and SB-334867-treated mice

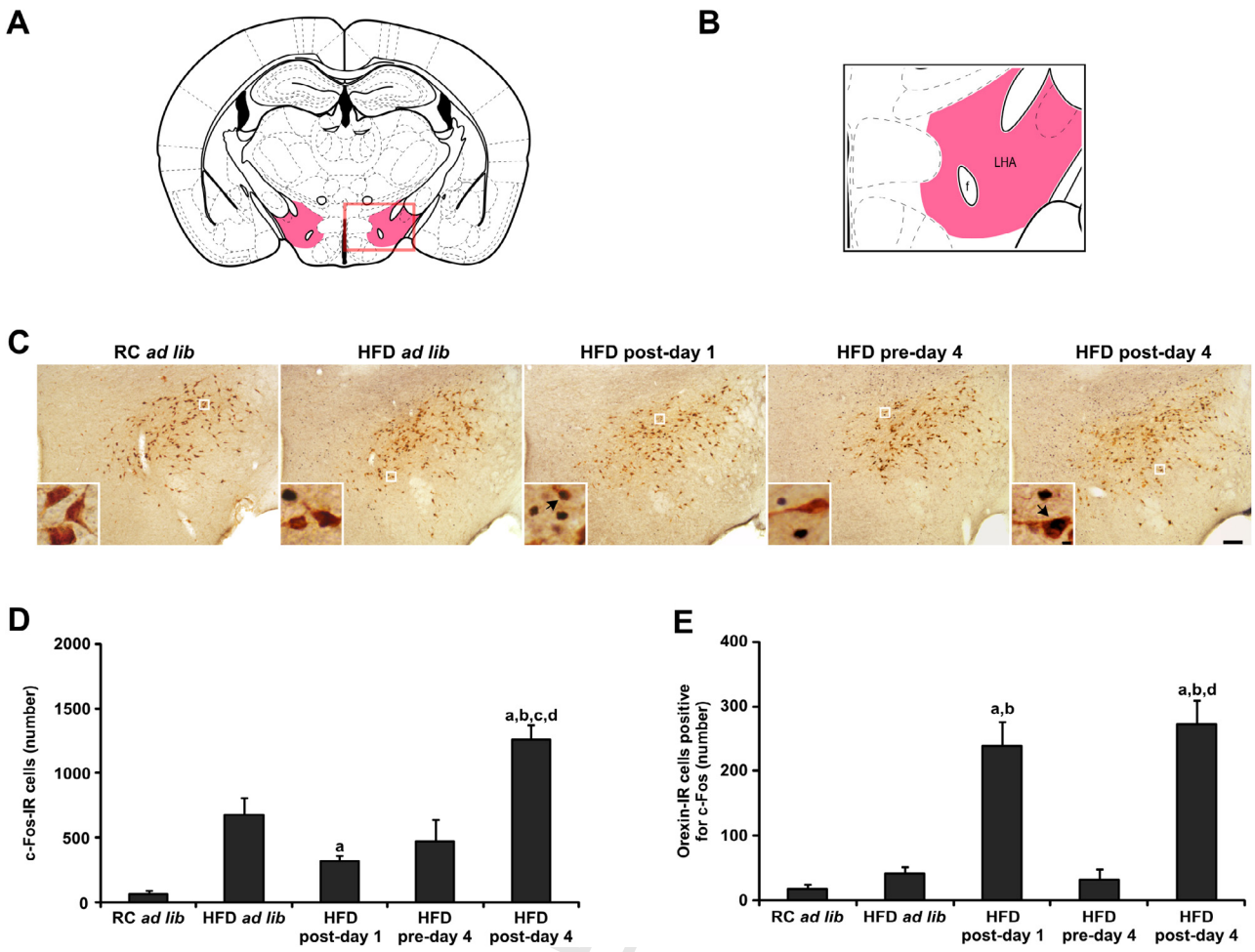

Fig. 4. HFD consumption activates c-Fos in orexin-IR and non-orexin-IR neurons of the LHA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Panel A and B show lower and higher magnification, respectively, diagrams of representative reference atlas levels highlighting lateral hypothalamic area (LHA, pink). f: fornix. Panel C shows representative photomicrographs of brain sections subjected to double immunohistochemistry using anti-orexin (brown staining) and anti-c-Fos (black staining) antibodies in samples from all experimental groups. Inserts show the areas marked in low magnification images at higher magnification. Arrows point to dual-labeled cells. Scale bars, 200 μm (low magnification), 20 μm (high magnification). Bottom panels show the number of c-Fos-IR neurons (D) and the number of orexin-IR cells positive for c-Fos (E) in the LHA of all the experimental groups. Values are the mean ± SEM and were compared by one-way ANOVA. $n = 5–6$ per group. a, $P < 0.05$ vs. RC *ad lib* group. b, $P < 0.05$ vs. HFD *ad lib* group. c, $P < 0.05$ vs. HFD post-day 1 group. d, $P < 0.05$ vs. HFD pre-day 4 group.

brain samples failed to show significant differences in the number of TH-IR neurons positive for c-Fos in the PN [$t(5) = 0.66$, $P = 0.5376$, Fig. 5C], the PBP [$t(5) = 0.22$, $P = 0.8343$, Fig. 5D] and the IF [$t(5) = 0.44$, $P = 0.6810$, Fig. 5E] sub-regions of the VTA as well as in the number of c-Fos-IR cells in the MAcbSh [$t(5)$=0.86, $P = 0.4266$, Fig. 5F], the LAcbSh [$t(4) = 0.31$, $P = 0.7733$, Fig. 5G] and the AcbC [$t(6)$=0.74, $P = 0.4872$, Fig. 5H] sub-regions of the Acb.

3.5. Ghrelin signaling is required for the escalation of HFD consumption and full c-Fos induction in the mesolimbic pathway

In order to determine if ghrelin signaling is required for escalation of HFD consumption, GHSR-null mice were used. As previously shown, GHSR-null and wild-type displayed similar overnight food intake and body weight when fed *ad libitum* with RC (Perello et al., 2010 Zigman et al., 2005). Unlike the wild-type mice, GHSR-null mice failed to escalate their HFD consumption when 2-h daily exposed to HFD during four consecutive days [$F(3,48) = 0.02$, $P = 0.9948$] (Fig. 6A). The 22-h RC intake was unaffected through the experiment in GHSR-null 2-h exposed to HFD (Fig. 6B). In terms of the quantitative neuroanatomical analysis of the TH-IR neurons positive for c-Fos, significant interactions between genotype and diet were found in the IF [$F(1,31) = 11.94$, $P = 0.0016$, Fig. 6C], the PN [$F(1,31) = 5.38$, $P = 0.0271$, Fig. 6D] and the PBP [$F(1,31) = 7.30$, $P = 0.0111$, Fig. 6E]. In all three subdivisions of the VTA, the number of TH-IR neurons positive for c-Fos was similar between for both groups of mice when ad libitum fed with RC. The number of TH-IR neurons positive for c-Fos of HFD post-day 4 GHSR-null mice was similar as compared to values found in RC *ad lib* fed GHSR-null mice and significantly smaller as compared to the values found in HFD post-day 4 wild-type mice. Importantly, the total number of TH-IR cells in the PN, PBP and IF sub-regions of the VTA was not affected in GHSR-null mice, as compared to the wild-type mice ([$t(16) = 0.16$, $P = 0.8724$], [$t(14) = 1.21$, $P = 0.2454$], [$t(16) = 0.12$, $P = 0.9075$], respectively). In particular, 693 ± 36, 1241 ± 143 and 446 ± 18 TH-IR neurons were estimated in the PN, PBP and IF of wild-type mice, respectively, while 701 ± 21, 1430 ± 61 and 442 ± 18 TH-IR neurons were estimated in the PN, PBP and IF of the GHSR-null mice, respectively. In the Acb, significant interactions between genotype and diet were found in the number of c-Fos-IR cells in the MAcbSh [$F(1,26) = 6.28$, $P = 0.0188$, Fig. 6F], the LAcbSh [$F(1,26) = 5.84$, $P = 0.0230$, Fig. 6G] and the AcbC [$F(1,26) = 10.29$, $P = 0.0035$, Fig. 6H]. The number of c-Fos-IR cells in all sub-regions of the Acb did not significantly differ between

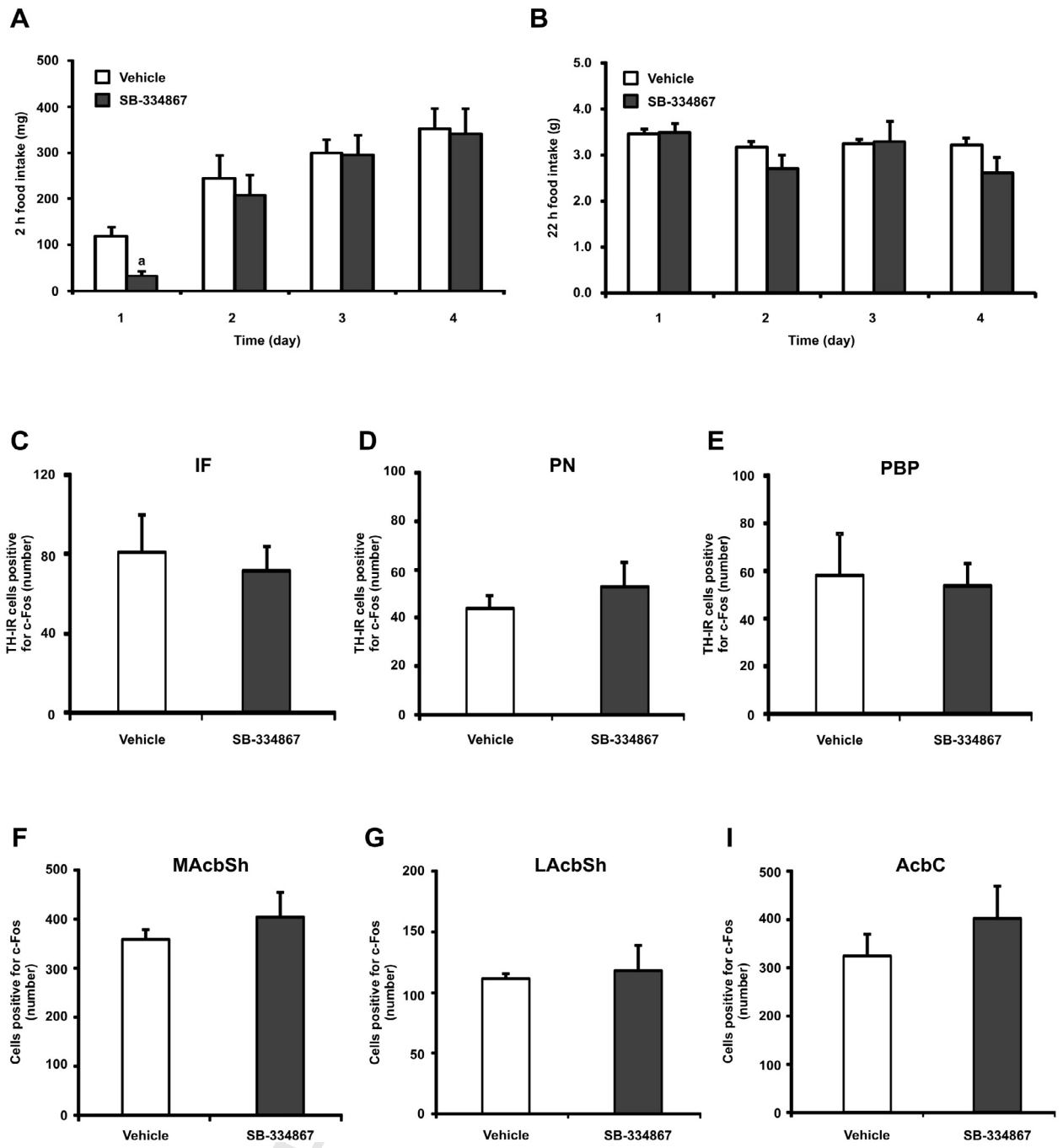

Fig. 5. Pharmacologic orexin signaling blockage fails to affect both escalation of HFD consumption induced by daily and time-limited access to HFD and c-Fos induction in the mesolimbic pathway.
Panel A and B show 2 h HFD consumption and daily 22 h RC consumption, respectively, in vehicle- and SB-334867-treated mice with daily and time-limited access to HFD for 4 days. Panels C to E show the total number of TH-IR cells positive for c-Fos in the IF (C), PN (D) and PBP (E) while panels F to H show the number of c-Fos-IR cells in the MAcbSh (F), LAcbSh (G) and AcbC (H) of vehicle- and SB-334867-treated mice. Values are the mean ± SEM and were compared by one-way ANOVA (A-B, $n = 10$ vehicle- and $n = 8$ SB-334867-treated mice) or t-Test (C-H, $n = 3$ vehicle- and $n = 5$ SB-334867-treated mice). a, $P < 0.05$ vs. vehicle-treated mice under the same experimental condition.

both groups of mice when ad libitum fed with RC. The number of c-Fos-IR cells in all Acb sub-regions of HFD post-day 4 GHSR-null mice was similar as compared to values found in RC *ad lib* fed GHSR-null mice and significantly smaller as compared to the values found in HFD post-day 4 wild-type mice. In the LHA, a significant interaction between genotype and diet was found in the number of orexin-IR cells positive for c-Fos [$F(1,20) = 5.38$, $P = 0.0311$]. No significant differences were detected between RC *ad lib* fed GHSR-null and wild-type mice (21 ± 8 and 13 ± 11 orexin-IR cells positive for c-Fos, respectively). In contrast, a significant increase in the number of orexin-IR cells positive for c-Fos was found in both GHSR-null and wild-type mice on HFD post-day 4 (76 ± 9 and 272 ± 39, respectively, $P < 0.001$ vs. same group on RC); still, the magnitude of this increase was significantly smaller in GHSR-null mice as compared to wild-type mice. Importantly, the number of orexin-IR cells in the LHA was not affected in GHSR-null mice as compared to the wild-type mice (1827 ± 136 vs. 1668 ± 77, respectively, $t(15) = 1.11$, $P = 0.2838$).

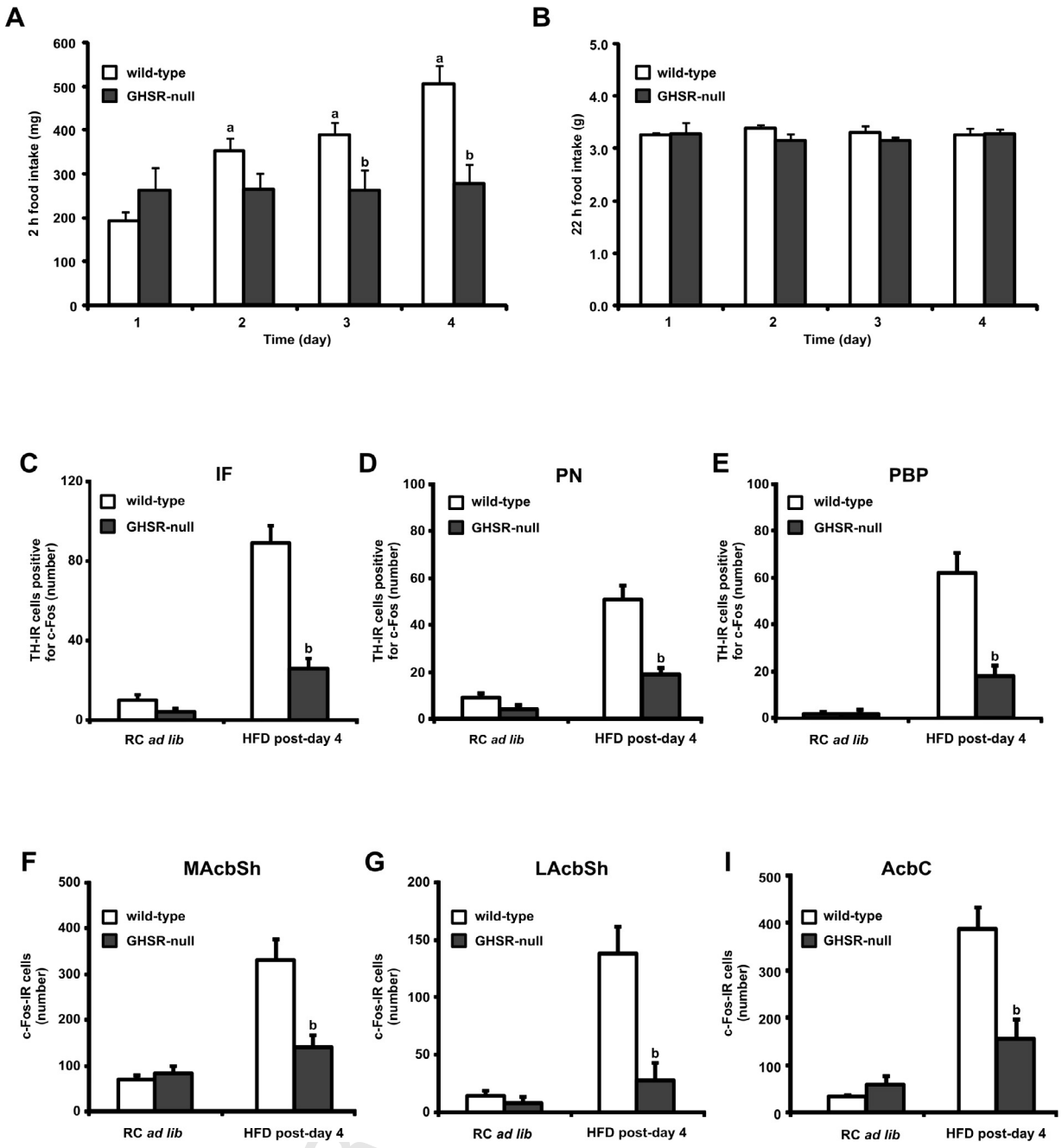

Fig. 6. GHSR signaling is required for both escalation of HFD consumption and full c-Fos induction in the mesolimbic pathway induced by daily and time-limited access to HFD.
Panel A and B show 2 h HFD consumption and daily 22 h RC consumption, respectively, in wild-type and GHSR-null mice with daily and time-limited access to HFD for 4 days. Panels C to E show the total number of TH-IR cells positive for c-Fos in the IF (C), PN (D) and PBP (E) while panels F to H show the number of c-Fos-IR cells in the MAcbSh (F), LAcbSh (G) and AcbC (H) of wild type and GHSR-null mice. Values are the mean ± SEM, and were compared by a two-way ANOVA. $n = 24$ and 16, wild-type and GHSR-null, respectively. a, $P < 0.05$ vs. same group at day 1. b, $P < 0.05$ vs. wild-type mice same day.

4. Discussion

Here, we used a simple rodent model of binge eating in which animals fed ad libitum with RC have a time-limited daily exposure to HFD. Using similar models, previous studies have shown an escalation in food intake among the initial binge eating events until the magnitude of the palatable food consumption finally stabilizes (Bake et al., 2014; Berner et al., 2008; Davis et al., 2007; Sindelar et al., 2005; Wojnicki et al., 2008); however, the molecular substrates involved in this escalation profile have not been studied. Intake escalation is a relevant feature of these binge eating models because it is associated to the feeling of loss of control over eating, which is a typical characteristic of human binge eating episodes (Goeders et al., 2009; Perello et al., 2014). Of course, human binge eating episodes are a much more complex behavior affecting people for long periods of time (Wolfe et al., 2009). Still, the study of rodent models in a short term fashion can be useful to get insights into some of the neuronal circuits recruited under particular conditions, as it has been previously done in the drugs of abuse research field (Goeders et al., 2009; Steketee and Kalivas, 2011). Some of the

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proposed mechanisms mediating intake escalation during the successive exposures to drugs of abuse include tolerance, behavioral sensitization or habit formation, and the mesolimbic dopamine pathway have been suggested as a potential target underlying these behavioral changes (Berridge, 2007; Steketee and Kalivas, 2011). Importantly, the current study was performed using satiated mice that were exposed to HFD at a time of the day when spontaneous food intake is minimal and while they remained with free access to RC. Thus, this type of HFD consumption appears to be mainly due to the palatable nature of the stimulus and involves rewarding, rather than homeostatic, aspects of eating. In order to characterize the activation of the mesolimbic pathway under this experimental condition, we evaluated c-Fos induction in VTA dopamine neurons – identified as TH-IR cells – and in Acb neurons – the main VTA dopamine target. The use of c-Fos as a surrogate marker for neuronal activation is a powerful tool since it is highly expressed when cells become activated and strongly affects target gene expression (Hoffman and Lyo, 2002). Indeed, c-Fos has been used by others and by us to map the neuroanatomical profiles of activation of the reward-related pathways (Konkle and Bielajew, 2004; Valdivia et al., 2014). Importantly, though, the absence of c-Fos expression is not proof of the lack of involvement on a given neuronal population in a particular neuronal circuitry.

The role of the VTA dopamine neurons in the rewarding aspect of food intake is a matter of debate (Salamone and Correa, 2012). The VTA dopamine neurons are responsive to acute intake of palatable foods and also able to regulate food consumption (Schultz, 2010; Valdivia et al., 2014). In response to the successive exposures to a rewarding stimulus, the role of the VTA dopamine system is rather sophisticated: the Acb dopamine signaling increases after the first exposure and then habituates with the repeated exposures and becomes a predictive signal of the stimulus (Salamone and Correa, 2012; Schultz, 2010). On top of this functional complexity, the VTA dopamine neurons are located in diverse sub-regions within this nucleus that, in turn, are part of different neuronal circuits for which their independent physiological role is virtually unknown (Ferreira et al., 2008; Ikemoto, 2007 Lammel et al., 2011). Here, we found that IF and PBP dopamine neurons are activated in mice exposed to either one or four HFD consumption events; however, the level of activation was higher in mice exposed to four HFD consumption events. Conversely, the level of activation of the PN dopamine neurons did not differ in mice exposed to either one or four HFD consumption events. Thus, the IF and PBP dopamine neurons seem to display a differential response through the escalation process while the PN dopamine neurons activation's seems to be independent of the number of HFD consumption events. Notably, the IF dopamine neurons were the only VTA dopamine neuronal subpopulation activated in anticipation to the fourth HFD consumption event. Thus, current data support the notion that dopamine neurons located in specific sub-divisions of the VTA are differentially involved in diverse aspects of binge eating behaviors.

The Acb plays a key role processing rewarding stimuli and acting as a limbic-motor interface, in which learned associations of motivational significance are converted into goal-directed behaviors (Ikemoto, 2007; Kelley, 2004). The Acb can be divided into distinct regions that display different but overlapping functions: the MAcbSh, which is more involved in the control of reward processing itself, and the LAcbSh and, particularly, the AcbC more involved in the cognitive processing of motor functions related to reward and reinforcement (Ikemoto, 2007; Kelley, 2004). Notably, the different Acb sub-regions display different patterns of mesolimbic connectivity: the MAcbSh is innervated by dopamine neurons located in the IF, PN and medial PBP while the LAcbSh and AcbC are more selectively innervated by lateral PBP dopamine neurons (Ikemoto, 2007). Here, we found that the AcbC neurons are activated in mice exposed to either one or four HFD consumption events; however, the level of activation was higher after four HFD consumption events. Thus, the activation of the AcbC seems to depend on the number of HFD consumption events. LAcbSh neurons seem to be exclusively activated after four HFD consumption events, while MAcbSh neurons are similarly activated after one or four HFD consumption events. The Acb activation presumably occurs as a consequence of the VTA dopamine neurons activation, since food rewards trigger phasic Acb dopamine release that, in turn, increases c-Fos (Day et al., 2007; Mendoza et al., 2005; Park and Carr, 1998). Current results suggest that consummatory rather than anticipatory aspects of feeding are associated with the activation of the mesolimbic pathway. This possibility agrees with previous microdialysis studies showing that Acb dopamine release mainly occurs during the consummatory phase of feeding and not during the anticipatory phase (Sahr et al., 2008; Wilson et al., 1995). Notably, an activation of the MAcbSh neurons was detected in anticipation to the fourth HFD consumption event, similarly as found for the IF dopamine neurons. The fact that the IF dopamine neurons selectively innervate the MAcbSh suggests that these two simultaneous phenomena are linked (Ikemoto, 2007). MAcbSh activation is associated to increases in locomotor activity (Zahm, 2000); however, no changes in the food anticipatory locomotor activity were detected in mice daily and time-limited exposed to HFD in our experimental conditions (although, we confirmed that anticipatory locomotor activity to HFD is increased in calorie restricted mice). Notably, other studies have also failed to detect anticipatory locomotor activity after repeated exposure to a palatable food in ad libitum fed mice depending on both the diet used and the time of day at which the experiment is performed (Bake et al., 2014; Merrer and Stephens, 2006).

Sensitization is a form of neuronal plasticity in which repeated exposure to a stimulus leads to an enhanced response to that stimulus together with a long-lasting increase in behavioral activation and Acb dopamine release (Steketee and Kalivas, 2011). Previous reports have shown that VTA dopamine neurons excitability is enhanced by a history of repeated exposure to amphetamine, cocaine or ethanol; and this augmented dopamine signaling is associated with behavioral sensitization to these stimuli (Steketee and Kalivas, 2011). Current data show that daily and time-limited HFD consumption is associated not only to an intake escalation over successive exposures but also to an enhanced activation of specific VTA dopamine neurons sub-populations. Thus, escalation in high fat intake during repeated binge eating episodes can be considered not only a form of sensitization but also a potential manner to conceptualize this eating behavior. Intermittent bingeing on other palatable foods, including sugar solutions, vegetable fat or sweet-fat mixtures, has been shown to affect Acb dopamine turnover and D1 and/or D2 signaling (Avena et al., 2009). However, future studies will be required in order to elucidate the molecular mechanisms mediating escalation in high fat intake. Interestingly, HFD *ad lib* group failed to activate the mesolimbic pathway after the fourth 2-h HFD consumption event. Thus, intermittency in the HFD consumption is required for the persistent activation of the mesolimbic system. Notably, Acb dopamine release in response to sucrose is detected only when rats are exposed to an intermittent regime but not when rats have ad libitum access to it (Rada et al., 2005). Similarly, chronic exposure to nicotine desensitizes the mesolimbic system to a further acute nicotine treatment (Benwell et al., 1995). Therefore, it could be hypothesized that the mesolimbic dopamine activity is also desensitized when a palatable food is constantly eaten.

In rodents, orexin signaling modulates HFD consumption, without effects on RC intake (Choi et al., 2010; Thompson and Borgland, 2011; Valdivia et al., 2014). Here, we confirmed that spontaneous HFD consumption in mice is reduced by an orexin 1 receptor

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antagonist the first day of exposure. We also found that LHA orexin neurons were activated by one or four HFD consumption events. In addition, we found that pharmacological blockage of the orexin 1 receptor signaling failed to affect the escalation of HFD consumption and the activation of the mesolimbic pathway. Thus, current data suggest that the initial HFD consumption in ad libitum fed mice requires orexin 1 receptor signaling; however, the neuronal mechanisms that take place in the escalation effect are independent of the orexin signaling. Interestingly, orexin 1 receptor blockade attenuates the development, but not the expression, of cocaine sensitization (Thompson and Borgland, 2011). In contrast, wild-type mice given an orexin 1 receptor antagonist and orexin knock-out mice display normal sensitization and locomotor response to morphine (Sharf et al., 2010). Also, orexin 1 receptor blockade fails to affect cocaine potentiation of brain stimulation-induced reward in mice (Riday et al., 2012).

It is well established that ghrelin signaling affects rewarding aspects of eating in both rodent and human beings (Perelló and Zigman, 2012). For instance, ghrelin signaling in rodents enhances the performance in the HFD conditioned place preference test and increases the motivation for HFD pellets in progressive ratio paradigms (Perello et al., 2010). Here, we found GHSR signaling is required for the escalation of HFD consumption and for the full activation of the mesolimbic pathways under this experimental condition. These observations point out a role for the ghrelin signaling on HFD sensitization. Interestingly, GHSR-deficient mice also exhibit diminished behavioral activation and reinforcement responses to cocaine and nicotine (Abizaid et al., 2011; Jerlhag and Engel, 2011), and it has been proposed that ghrelin signaling could induce a form of central sensitization in which animals are more reactive to rewarding stimuli (Wellman et al., 2013). Indeed, it has been shown that GHSR-deficient mice display a reduced anticipatory locomotor response to scheduled meals (Blum et al., 2009). To our knowledge, current results are the first report of the requirement of the ghrelin signaling for HFD escalation in rodents. Although the brain targets mediating ghrelin's effects on binge eating are currently unknown, it possible to hypothesize that they involve ghrelin signaling on the mesolimbic pathway. The VTA dopamine neurons express GHSR and respond to ghrelin by increasing the action potential frequency and the Acb dopamine release (Abizaid et al., 2006; Jerlhag et al., 2007). Also, rewarding foods-induced Acb dopamine release is reduced in GHSR knock-out mice (Egecioglu et al., 2010), and selective GHSR expression in catecholaminergic cells, including the VTA dopamine neurons, is sufficient to mediate ghrelin-induced conditioned place preference for HFD (Chuang et al., 2011). The possibility that ghrelin signaling could involve a form of central sensitization that affects the intake of palatable foods may be clinically relevant and deserves future studies as alterations in ghrelin signaling seem to contribute to the magnitude of binge eating episodes in some, but not all, patients with binge eating disorder (Geliebter et al., 2008).

In summary, the current model suggests that the VTA dopamine neurons are differentially recruited, depending of the number of HFD consumption events, in mice with daily and time-limited access to this diet. Additionally, our data show that the ghrelin signaling is required for the escalation of HFD consumption under these experimental conditions.

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Author contributions

Study conception and design: Valdivia, Cornejo, De Francesco, Reynaldo, Perello.Acquisition of data: Valdivia, Cornejo, De Francesco, Reynaldo.Analysis and interpretation of data: Valdivia, Cornejo, De Francesco, Reynaldo, Perello.Drafting of manuscript: Valdivia, De Francesco, Perello.Critical revision: Valdivia, Cornejo, De Francesco, Reynaldo, Perello.

Uncited references

Q6

Pankevich et al. (2010).

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