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## **Systemic ghrelin treatment induces rapid, transient and asymmetric changes in the metabolic activity of the mouse brain**

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Short title: Brain areas responsive to ghrelin treatment.

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## **ABSTRACT**

Introduction: Ghrelin regulates a variety of functions by acting in the brain. The targets of ghrelin in the mouse brain have been mainly mapped using immunolabeling against c-Fos, a transcription factor used as a marker of cellular activation, but such analysis has several limitations. Here, we used positron emission tomography in mice to investigate the brain areas responsive to ghrelin. Methods: We analyzed in male mice the brain areas responsive to systemically-injected ghrelin using positron emission tomography imaging of  $^{18}\text{F}$ -fluoro-2-deoxyglucose ( $^{18}\text{F}$ -FDG) uptake, an indicator of metabolic rate. Additionally, we studied if systemic administration of fluorescent-ghrelin or native ghrelin display symmetric accessibility or induction of c-Fos, respectively, in the brain of male mice. Results: Ghrelin increased  $^{18}\text{F}$ -FDG uptake in few specific areas of the isocortex, striatum, pallidum, thalamus and midbrain at 0-10-min post-treatment. At the 10-20- and 20-30-min post-treatment, ghrelin induced mixed changes in  $^{18}\text{F}$ -FDG uptake in specific areas of isocortex, striatum, pallidum, thalamus and midbrain, as well as in areas of the olfactory areas, hippocampal and retrohippocampal regions, hypothalamus, pons, medulla and even the cerebellum. Ghrelin-induced changes in  $^{18}\text{F}$ -FDG uptake were transient and asymmetric. Systemically-administrated fluorescent-ghrelin labeled midline brain areas known to contain fenestrated capillaries and the hypothalamic arcuate nucleus, where a symmetric labeling was observed. Ghrelin treatment also induced a symmetric increased c-Fos labeling in the arcuate nucleus. Discussion/Conclusion: Systemically-injected ghrelin transiently and asymmetrically affects the metabolic activity of the brain of male mice in a wide range of areas, in a food intake independent manner. The neurobiological bases of such asymmetry seem to be independent of the accessibility of ghrelin into the brain.

## INTRODUCTION

Ghrelin is a stomach-derived peptide hormone [1]. Plasma ghrelin levels increase before meals, decline after meals, and then increase gradually until the next preprandial period [2]. Plasma ghrelin levels also increase in energy deficit conditions, such as fasting, malnutrition, anorexia nervosa or cachexia, among others [3–6]. Ghrelin acts via the growth hormone secretagogue receptor (GHSR) [1], which is highly expressed in some brain areas and in the pituitary [7,8]. It is widely recognized that ghrelin treatment displays a variety of neuroendocrine, autonomic, metabolic and behavioral effects in humans and rodents. Ghrelin increases plasma levels of growth hormone as well as prolactin, adrenocorticotrophic hormone and glucocorticoid [9–15]. The ghrelin-induced activation of these neuroendocrine axes together with the putative action of ghrelin on other organs, such as pancreas, promotes an increase of glycaemia [16]. Ghrelin administration also potently increases food intake and modulates rewarding aspects of appetite [17]. In addition, ghrelin modulates the activity of the autonomic nervous system and consequently increases gastric emptying and decreases blood pressure [18–20]. Preclinical studies in rodents have shown that ghrelin also influences learning and memory, but the extent to which ghrelin affects these cognitive functions in humans is currently uncertain [21,22]. Thus, ghrelin treatment displays pleiotropic central actions, some of which may have important clinical implications. However, the brain areas that sense, integrate and execute ghrelin signaling are only poorly known.

In mice and rats, GHSR is expressed in a variety of brain areas that could directly mediate the effects of ghrelin. Strikingly, however, studies using fluorescent analogs of ghrelin in mice have shown that the accessibility of plasma ghrelin is restricted to few brain areas [23–25]. In particular, systemically-injected fluorescent ghrelin diffuses into the median eminence (ME) and into the neighbor ventromedial region of the hypothalamic arcuate nucleus (ARH), which contains fenestrated capillaries of the hypophyseal portal system [23,26,27]. Systemically-injected fluorescent ghrelin also diffuses through fenestrated capillaries into the area postrema (AP) [28]. In mice, ghrelin does not seem to cross the blood-brain-barrier from the blood to the brain [29], but it can be transported towards the cerebrospinal fluid through the blood-cerebrospinal fluid barrier [30,31]. From the cerebrospinal fluid, ghrelin can target some periventricular brain areas, such as some hypothalamic nuclei [30,32]. The neuronal targets of ghrelin has been extensively mapped by assessing the induction of the immediate early gene *c-fos*, and it has been mainly reported that systemically-injected ghrelin mainly increases c-Fos levels in the ARH of mice

and rats [23,28,33–37], whereas it does not increase c-Fos levels in some areas known to express GHSR and that could mediate well-established effects of ghrelin [38]. These observations also suggest that the accessibility of plasma ghrelin to the mouse brain is limited. Nevertheless, the capability of c-Fos induction to faithfully estimate the brain areas responsive to ghrelin has important limitations. For instance, c-Fos induction may occur in neurons that are indirect targets of ghrelin. Also, *c-fos* reflects an up-regulation of some signal transduction pathways, which may be dissociated from electrical neuronal activity [39]. In addition, c-Fos does not provide information regarding neurons that are inhibited by ghrelin. Thus, the use of alternative approaches is important to complement c-Fos mapping studies and gain further insights of the central targets of ghrelin.

Here, we used positron emission tomography (PET) to assess the <sup>18</sup>F-2-deoxyglucose (FDG) uptake and estimate the changes in the metabolic activity induced by ghrelin treatment in the mouse brain. In order to maximize the chances to unmask putative effects of ghrelin on the metabolic activity of the brain, the current study was performed in male mice, which have been shown to be significantly more responsive to the orexigenic and GH secretagogue effects of ghrelin than female mice [40]. Since we found that ghrelin induces asymmetric changes in metabolic activity of the brain of male mice, we studied if systemically-administrated fluorescent-ghrelin displayed symmetric brain accessibility as well as if ghrelin treatment produced symmetric induction of c-Fos in the ARH.

## MATERIALS AND METHODS

**1. Mice.** Adult (8-12 weeks old) male C57BL/6 wild-type mice were generated in the animal facility of the IMBICE. A set of 26 mice was shipped to the animal facility of the FLENI to perform the PET studies. In both animal facilities, mice were housed in a 12-h light/dark cycle with regular chow and water available *ad libitum*. In all cases, mice were individually housed three days before the experiments, which were performed between 9 a.m. and 12 a.m. in all cases. 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 Institutes of Health, and all efforts were made to minimize suffering. All experimentation received approval from the Institutional Animal Care and Use Committees located in the Multidisciplinary Institute of Cell Biology (approval ID 17-0221).

**2. Ghrelin variants.** Ghrelin (GSS(octanoyl)FLSPEHQKAQQRKESKKPPAKLQPR) was purchased from Global Peptide (cat# PI-G-03). Fr-ghrelin [GSD(octanoyl)FLSPEHQRVQQRKESC-(DY-647P)] is a variant of ghrelin conjugated to DY-647P fluorophore through a C-terminal Cys. Fr-ghrelin was synthesized by linking DY-647P-maleimide to a 19-residues ghrelin analog that contains the initial 18 residues of the ghrelin sequence, but replacing the serine in position 3 by aspartic acid whose side-chain was amidated with octylamine, and containing a cysteine at the position 19. Fr-ghrelin was synthesized, purified by high-performance liquid chromatography and characterized by mass spectrometry, as we have done in the past [41]. Fr-ghrelin was previously validated and shown to increase food intake *in vivo* and label GHSR-expressing brain areas in wild-type mice but not in GHSR-deficient mice [31].

**3. Assessment of <sup>18</sup>F-FDG uptake in the mouse brain in response to ghrelin treatment and spatial quantification.** Here, PET data was acquired two times per mouse, after ghrelin and vehicle treatments over different days, in a randomized crossover design. In the morning, mice were subcutaneously injected with ghrelin (0.06 nmol/g BW) or vehicle and intraperitoneally injected with <sup>18</sup>F-FDG (25 µCi/gr), with a variable time difference (-10 min, 0 min and +10 min) between both injections, as shown in Fig. 1. These PET studies, hereafter named E1, E2 and E3, were designed to assess <sup>18</sup>F-FDG uptake in the 0–10, 10–20 or 20–30 minutes time windows after ghrelin treatment, and had 10, 6 and 10 mice, respectively. The tested dose of ghrelin induces a transient ~50-fold increase in the plasma ghrelin levels, similar to the one observed in 5-day calorie-restricted mice [42], which return

to basal levels at ~45-min after treatment [23]. Before treatments, food pellets were removed from the home cages. After treatments, anesthesia was induced by inhalation of isoflurane (4.5% for induction and 1.5% for maintenance) supplemented with oxygen for approximately 2-3 min. PET data was acquired during 12 minutes per mouse using list-mode acquisition.

Images were acquired with a preclinical PET TriFoil Lab-PET 4, with an approximated spatial resolution of 1.2 mm full width at half maximum (FWHM) and an imaging field of view (FOV) of 3.7 cm axial and 11 cm trans-axial. Images were reconstructed using an OSEM 3D algorithm with 30 iterations to maximize the signal-to-noise ratio. If motion was detected during acquisition, a realign algorithm was applied to the temporal dynamic reconstruction using SPM12 on MATLAB. For image spatial processing, brain images were normalized to a previously generated normal C57BL/6 wild-type mice <sup>18</sup>F-FDG template, using Advanced Normalization Tools registration pipeline comprising SyN (image registration). Smoothing using an Isotropic Gaussian kernel with three times the voxel size (0.75x0.75x1.791mm) FWHM was made with SPM12 on MATLAB. <sup>18</sup>F-FDG uptake was intensity normalized to gray cerebellum mean based on the Allen Reference Atlas. A Harderian Glands mask was not applied since they do not affect registration pipeline. Statistical comparisons of the <sup>18</sup>F-FDG uptake between conditions were performed for each experiment as described in sub-section 6.

The distribution of areas with significant changes in <sup>18</sup>F-FDG uptake was assessed in regions from the Allen Reference Atlas ontology tree (at 50 μm resolution) using python scripts and the Allen Reference Space SDK [43]. The analysis was performed only on grey matter regions, excluding those with less than 2000 voxels (bilateral, equivalent to 0.25 mm<sup>3</sup>). The tree was collapsed from bottom to top until all terminal nodes met this requirement, and less than 10% of the volume was excluded in any terminal branch. The cortical areas and the superior colliculi had their layer nodes collapsed regardless of their size. This process resulted in 170 terminal nodes, from which hemilateral masks were created. Within each region, we determined the number voxels that were significantly activated or inhibited, and set a minimum threshold of 300 voxels (per side) to produce a list of responding hemilateral regions for each experiment.

**4. Visualization of brain areas accessible to systemically-injected Fr-ghrelin.** An independent set of mice was subcutaneously injected with vehicle alone (n=5) or containing Fr-ghrelin (1.2 nmol/g BW, n=6). Before treatments, food pellets were removed from the

home cages. This dose of Fr-ghrelin is the minimal dose that allows the direct visualization of Fr-ghrelin in the fixed brain (unpublished observations). Fifteen minutes after treatment, mice were anesthetized and perfused with formalin. Brains were extracted, post-fixed, immersed in 20% sucrose, and frozen. Next, a shallow longitudinal incision down the right hemisphere was made in order to track the laterality of the sections, and brains were coronally cut at 40  $\mu\text{m}$  into four equal series on a cryostat. Brain sections were sequentially mounted on glass slides, coverslipped with mounting medium containing Hoechst. Fluorescent images of the brain sections were acquired using structured illumination in a Zeiss Axio Observer D1 fluorescence microscope equipped with an Apotome.2 module and an AxioCam 506 monochrome camera. The presence of Fr-ghrelin signal was determined in 1-in-4 series from the level of the olfactory bulbs down to the cervical spinal cord. Fluorescent images were acquired with a 10x/0.45 objective, using an automated tiling strategy to image all the brain sections. Also, higher magnification images of selected regions were acquired using a 40x/0.95 objective. Tiles from each mouse were aligned for neuroanatomical analysis using the Fiji plugin TrackEM2 [44,45]. The Allen mouse brain atlas was used to identify brain sections and to describe the brain nuclei. Estimates of Fr-ghrelin signal for the different brain regions were made by considering both signal strength and number of labeled cells as compared to the signal observed in samples from vehicle-treated mice. Fr-ghrelin signal was quantified as mean fluorescent intensity and expressed in arbitrary units (A.U.)

**5. Assessment of ghrelin-induced c-Fos in the mouse brain.** Another set of mice was subcutaneously injected with vehicle alone (n=5) or containing ghrelin (0.06 nmol/g BW, n=5) between 8:00 and 10:00 a.m. Before treatments, food pellets were removed from the home cages. Two hours after treatment, mice were anesthetized and perfused with formalin. Brains of perfused mice were removed, post-fixed, immersed in 20% sucrose and frozen. As described above, a shallow longitudinal incision down the right hemisphere was made in order to track the laterality of the sections, and brains were coronally cut at 40  $\mu\text{m}$  into three equal series on a cryostat. C-Fos immunostaining was also performed as described. Briefly, sections were pretreated with 0.5%  $\text{H}_2\text{O}_2$ , treated with blocking solution (3% normal donkey serum and 0.25% TritonX in PBS) and incubated with anti-c-Fos antibody (Calbiochem/Oncogene, cat# PC38, 1:20.000) for two days at 4°C. Then, sections were treated with biotinylated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:3.000) for 1 h and with Vectastain Elite ABC kit (Vector Laboratories, cat # PK6200) for 1 h, according to manufacturer's protocols. A visible signal



was developed with 3-3'-diaminobenzidine (DAB)/Nickel solution, giving a black/purple precipitate. Sections were sequentially mounted on glass slides and coverslipped with mounting media. Bright-field images were acquired with a Nikon Eclipse 50i and a DS-Ri1 Nikon digital camera. The number of c-Fos-immunoreactive (IR) cells in the right and left ARH were estimated in coronal brain sections between bregma -1.58 and -1.94 mm. Anatomical limits of the ARH were identified using a mouse brain atlas [46]. Cells containing a distinct nuclear black/purple precipitate were quantified in one out of three complete series of coronal sections through the whole ARH. Blind quantitative analysis was performed independently by two observers and expressed as c-Fos-IR cells per coronal section per side.

**6. Statistical analysis.** Statistical analysis of the PET signal was performed using SPM12 on MATLAB. For each experiment, statistical comparisons of the  $^{18}\text{F}$ -FDG uptake between ghrelin and vehicle condition were performed using paired Student's t test. A p-value less than 0.05 was considered significant. Using a general linear model, a contrast of parameter estimates was calculated for each experiment. In order to have an accurate anatomical reference, all results of statistical differences were co-registered with the serial two photon tomography average template (50  $\mu\text{m}$  resolution version) from the Allen mouse Common Coordinate Framework (CCFv3) Reference Atlas [47].

Statistical analyses of Fr-ghrelin signal and c-Fos levels were performed using GraphPad Prism 6.0 and differences were considered significant when  $P < 0.05$ . An unpaired t-test was used to compare mean fluorescent intensity or the number of c-Fos-IR cells per section in the ARC of vehicle treated mice vs ghrelin- or Fr-ghrelin-treated mice, respectively. A paired t-test analysis was used to compare mean fluorescent intensity or the number of c-Fos-IR cells per section per side of the right and left ARH of ghrelin- and Fr-ghrelin-treated mice, respectively.

## RESULTS

**Systemically injected ghrelin induces rapid, transient and asymmetric changes in the  $^{18}\text{F}$ -FDG uptake.** The effects of ghrelin on brain glucose metabolism were estimated based on the PET analysis of  $^{18}\text{F}$ -FDG uptake. The observed differences in  $^{18}\text{F}$ -FDG uptake are shown in Fig. 2-4 and outlined in Fig. 5.

In the ~0-10 min post-treatment time window, ghrelin increased  $^{18}\text{F}$ -FDG uptake in a small set of specific brain areas. In the cerebral cortex,  $^{18}\text{F}$ -FDG uptake increased in the right secondary motor area. Within the cerebral nuclei,  $^{18}\text{F}$ -FDG uptake increased in the right caudoputamen of the dorsal region of the striatum, in the right ventral part of the lateral septal nucleus, and in the right bed nuclei of the stria terminalis of the caudal pallidum. Within the thalamus,  $^{18}\text{F}$ -FDG uptake increased in the right sensory-motor cortex related areas, in the right intralaminar nuclei of the dorsal thalamus and in the left side of medial group of the dorsal thalamus. Also,  $^{18}\text{F}$ -FDG uptake increased in the right motor-related area of the superior colliculus in the midbrain.

In the ~10-20 min post-treatment time window, ghrelin treatment increased and decreased  $^{18}\text{F}$ -FDG uptake in specific brain areas. In the isocortex,  $^{18}\text{F}$ -FDG uptake decreased in the both sides of the primary and secondary motor areas, of the prelimbic and infralimbic areas and of the orbital areas (with the exception of the lateral part of the orbital area, in which  $^{18}\text{F}$ -FDG uptake decreased only in the right side). Also,  $^{18}\text{F}$ -FDG uptake decreased in the right frontal pole, the primary visual area and agranular insular area, and in the left anterior cingulate area. In primary somatosensory areas, the  $^{18}\text{F}$ -FDG uptake showed mixed responses; for instance, it increased in the left side of the mouth area whereas it decreased in the right side of the upper limb area. The  $^{18}\text{F}$ -FDG uptake decreased, with different laterality, in most the olfactory areas, with the exception of the main olfactory bulb, in which  $^{18}\text{F}$ -FDG uptake increased in the left side and decreased in the right side. The  $^{18}\text{F}$ -FDG uptake decreased in left and right CA1 and CA3 of the hippocampus, in the right dentate gyrus and in the left retrohippocampal region, endopiriform nucleus, and basolateral amygdalar nucleus. In the striatum,  $^{18}\text{F}$ -FDG uptake decreased in the right ventral striatum, which includes the nucleus accumbens and the olfactory tubercle, in the left striatum-like amygdalar nuclei and in both sides of the caudoputamen. In the pallidum,  $^{18}\text{F}$ -FDG uptake decreased in the left internal segment of the globus pallidus, in the right magnocellular nucleus and in both sides of the substantia innominata. Also,  $^{18}\text{F}$ -FDG uptake

decreased in the right sensory-motor related cortex of the thalamus, in the right external nucleus of the inferior colliculus of the midbrain, and in both sides of the lateral hypothalamic area.  $^{18}\text{F}$ -FDG uptake decreased in two regions of the pons: the right behavioral state related area, and both sides of the pontine central gray. In the medulla,  $^{18}\text{F}$ -FDG uptake showed mixed responses: increased in the right sensory-related areas and decreased in both sides of some motor-related areas (e.g., the paragigantocellular reticular nucleus, the perihypoglossal nuclei and the vestibular nuclei). In the cerebellum,  $^{18}\text{F}$ -FDG uptake decreased in both sides of two vermal regions (e.g., culmen and declive, VI) and increased in the right central lobule of the vermal regions as well as in some right hemispheric regions (e.i., simple lobule, copula pyramidis and paraflocculus) and cerebellar nuclei.

In the ~20-30 min post-treatment time window, ghrelin treatment mainly increased  $^{18}\text{F}$ -FDG uptake in specific brain areas. In the isocortex,  $^{18}\text{F}$ -FDG uptake increased in the left side of several somatosensory areas (plus the right side of the mouth area), of the auditory areas and of the temporal association areas as well as in the right side of the anteromedial visual area, of some retrosplenial areas, and of posterior parietal association areas. In the isocortex,  $^{18}\text{F}$ -FDG uptake also increased in both sides of the infralimbic area and of the medial part of the orbital area, whereas increased only in the left side of the lateral and ventrolateral parts of the orbital area. The  $^{18}\text{F}$ -FDG uptake also increased in both sides of two olfactory areas (i.e., the dorsal part of the taenia tecta, and the dorsal peduncular area) and in the right side of several areas of the hippocampal formation (including also the left side of the CA1). In the striatum,  $^{18}\text{F}$ -FDG uptake increased in both sides of the caudoputamen and the lateral septal nucleus, in the left nucleus accumbens, and in the right striatum-like amygdalar nuclei. The uptake of  $^{18}\text{F}$ -FDG increased both sides of some regions of the dorsal, ventral, medial and caudal pallidum. In the thalamus,  $^{18}\text{F}$ -FDG uptake increased in the right the sensory-motor cortex related area as well as in most of the right polymodal association cortex related areas. In the hypothalamus,  $^{18}\text{F}$ -FDG uptake increased in both sides of the periventricular zone and in the right lateral zone. In the midbrain,  $^{18}\text{F}$ -FDG uptake increased in the right ventral tegmental area, reticular nucleus, reticular part of the substantia nigra and behavioral-related areas, in the left motor-related area of the superior colliculus, as well as in both sides of the periaqueductal gray and of the pretectal regions. In the medulla,  $^{18}\text{F}$ -FDG uptake decreased in the right sensory-related areas. In the cerebellum,  $^{18}\text{F}$ -FDG uptake increased several left vermal regions [Central lobule, Culmen, Declive (VI), Pyramus (VIII), Uvula (IX), Nodulus (X)], in the left copula pyramidis and in the

left cerebellar nuclei. At the same time,  $^{18}\text{F}$ -FDG uptake decreased in two hemispheric regions: the right flocculus and both sides of the paraflocculus.

**Systemically-injected Fr-ghrelin gains access to brain areas known to contain fenestrated capillaries.** To investigate whether circulating ghrelin could differentially access the right and left sides of the brain, mice were systemically-injected with Fr-ghrelin and euthanized at 15 min after treatment. Brains were then processed, and the localization of Fr-ghrelin was imaged in the coronal brain sections using a filter set for far-red emission. Fr-ghrelin signal was observed in the epithelial cells of the choroid plexus (CP), as previously reported [30], as well as in cells of several midline structures containing fenestrated capillaries, including the subfornical organ (SFO), the subcommissural organ (SCO), the organum vasculosum of the lamina terminalis (OVLT) and the AP (Fig. 6 A-E). Fr-ghrelin signal was not detected in other brain areas protected by the blood-brain-barrier, such as the olfactory bulbs (not shown), the ventral tegmental area (not shown) or the hippocampus (Fig. 6 F). As compared to the signal found in the brain sections of vehicle-treated mice, fluorescent signal was also detected in the ARH of Fr-ghrelin-treated mice (Fig. 6 G-H). Notably, Fr-ghrelin signal in the right and the left ARH of Fr-ghrelin-treated mice were not statistically different (Fig. 6 I).

**Systemically injected ghrelin induces a similar induction of c-Fos in the left and right ARH.** To investigate whether circulating ghrelin could differentially activate the right and left ARH, mice were systemically-injected with ghrelin and euthanized at 2 h after treatment. Brains were then processed, and immuno-stained for c-Fos. As previously reported [23], we confirmed that the number of c-Fos-IR cells significantly increased only in the ARH of ghrelin-treated mice, as compared to the number of c-Fos-IR cells detected in the ARH of vehicle-treated mice (Fig. 7 A-B). Still, the number of c-Fos-IR cells in the right and the left ARH of ghrelin-treated mice were not statistically different (Fig. 7 C).

## DISCUSSION

We show here that ghrelin treatment induces rapid, transient and asymmetric changes in the metabolic activity of the brain of male mice. In contrast, treatment with fluorescent ghrelin results in a non-asymmetric labeling of most brain areas with fenestrated capillaries, and ghrelin treatment induces a symmetric elevation of c-Fos expression at the ARH. Thus, systemically-injected ghrelin recruits most central neuronal networks in an asymmetric fashion in male mice, and the neurobiological basis of such asymmetry seems to be independent of the accessibility of ghrelin into the brain or the rapid action of ghrelin at the ARH level.

To our knowledge, the current study provides the first assessment of food intake-independent ghrelin-induced changes of the  $^{18}\text{F}$ -FDG uptake in the mouse brain. Preclinical-PET is a powerful non-invasive and quantitative technology that generates a three-dimensional nuclear imaging data set and allows the assessment of functional processes in specific brain areas of live animals [48]. Importantly, preclinical-PET displays low spatial and temporal resolutions that must be considered when interpreting results. Indeed, such low resolution of preclinical-PET studies together with the fact that we found that ghrelin affected glucose metabolism in many brain areas that are highly interconnected undermined our capability to delineate a precise and sequential neuronal circuit recruited by ghrelin. Given the limits in spatial resolution, current studies may have also failed to reveal changes in the  $^{18}\text{F}$ -FDG uptake when different cells displayed simultaneous increase and decrease of glucose metabolism within the same brain area. Finally, results presented here should be interpreted as average responses due to the group-wise statistical analysis performed. Despite all these considerations, the current study resulted in a valuable strategy that provided an alternative analysis of the central targets of circulating ghrelin.

In line with the notion that ghrelin treatment induces neuroendocrine effects, we found that ghrelin also affects glucose metabolism in the hypothalamus. Specifically, ghrelin induced a symmetric reduction of the glucose metabolism in both sides of the hypothalamic lateral zone in the 10-to-20 min posttreatment period whereas increased the glucose metabolism in periventricular zone of the hypothalamus, which includes the paraventricular nucleus and the ARH, in the 20-to-30 min posttreatment period. Also, ghrelin treatment affected the glucose metabolism in several brain areas that are known to control the autonomic function, including specific areas, such as the agranular insular area, the anterior

cingulate area and the basolateral amygdala, or larger brain regions, such as the hypothalamus, nuclei of the pons or the medulla, in which the specific area responsive to ghrelin could not be precisely identified due to the low spatial resolution of the preclinical-PET [49,50]. Ghrelin increased the glucose metabolism in both sides of the periaqueductal gray that regulates not only autonomic function, but also motivated behaviors.

Ghrelin affects complex functions such as the ability to process information of the pleasurable (hedonic) value of rewarding stimuli or the actions to seek them [17,51]. Here, we found that ghrelin affected brain metabolism in structures of the limbic system, such as the nucleus accumbens, in the right ventral tegmental area and in the ventral pallidum (both sides of substantia innominate and the right magnocellular nucleus). The nucleus accumbens is involved in cognitive processing of motivation, reward and reinforcement learning. The nucleus accumbens receives innervations from the prefrontal cortex (the prelimbic and infralimbic cortex), the ventral hippocampus, the midline thalamic nuclei, the intralaminar nuclei of the thalamus and the ventral tegmental area, and strongly innervates the ventral pallidum. The ventral pallidum controls the processing and execution of motivated behaviors [52]: it receives inputs from the nucleus accumbens, as well as from the ventral tegmental area, and projects to the medial dorsal nucleus of the dorsal thalamus, which in turn projects to the prefrontal cortex and the ventral striatum [53,54]. Ghrelin treatment also increased glucose metabolism in other brain areas related to the mesolimbic system such as the dorsal thalamus as well as the prelimbic, limbic, frontal pole, orbital and cortical amygdalar areas of the cerebral cortex. Also, ghrelin rapidly reduced glucose metabolism in the olfactory tubercle, which is located in the ventral striatum and is a processing center that translates sensory and rewarding information into subsequent goal-directed behaviors [55]. The olfactory tubercle receives primary innervations from the olfactory bulbs and the ventral tegmental area and it is highly interconnected with the nucleus accumbens, the ventral pallidum and other centers of the basal ganglia. The olfactory tubercle also recruits the striatum-like amygdalar nuclei [55], that showed decreased glucose metabolism in response to ghrelin. Of note, ghrelin decreases glucose metabolism in the hypothalamic lateral zone, which receives numerous innervations and projects to different center of the limbic system, including the ventral pallidum, the ventral tegmental area and the nucleus accumbens [56]. In line with the reports indicating that ghrelin affects memory and learning, we found that ghrelin also affected the glucose metabolism in the Ammon's horns and in the dentate gyrus of the hippocampus. The hippocampus plays a major role in the formation of spatial representations and episodic

memory, and its crosstalk with the mesolimbic pathway helps to control reward associative processes important for memory-guided decision-making [57]. The hippocampus receives innervations from the ventral tegmental area, and indirectly innervates the ventral tegmental area via the nucleus accumbens and the ventral pallidum.

We found that ghrelin treatment rapidly affected the glucose metabolism in brain areas that are part of the basal ganglia, which are a collection of interconnected subcortical brain nuclei integrated to cortico-basal ganglia-thalamo-cortical loops that modulate not only voluntary motor actions but also learning, working memory, decision making and reward processing [58,59]. These functional loops include motor, associative and limbic loops that converge, integrate and exchange information, at different levels, in order to control complex goal-directed behaviors [59]. The larger center of the basal ganglia is the striatum, which is considered an input area [60]. Here, ghrelin increased glucose metabolism not only in the ventral (nucleus accumbens and olfactory tubercle) striatum, as discussed above, but also in the dorsal (caudoputamen) striatum. The dorsal striatum receives innervations from the cerebral cortex (corticostriatal projections), the thalamus (thalamostriatal projections), the SNC (nigrostriatal projections) and the ventral tegmental area (mesostriatal projections), among others [60]. Interestingly, ghrelin rapidly increases glucose metabolism in cortical motor areas, which originate corticostriatal projections, and in the intralaminar nuclei of the dorsal thalamus, which originate most thalamostriatal projections [61]. The striatum projects to other basal ganglia: the reticular part of the substantia nigra and the external and internal globus pallidus of the dorsal pallidum. The external globus pallidus projects to the subthalamic nucleus that, in turn, project to the internal globus pallidus and the reticular part of the substantia nigra, which are the major output nuclei of the motor loop via their projections to thalamic nuclei [60]. We found that ghrelin decreases glucose metabolism in the internal globus pallidus whereas increases the glucose metabolism in the reticular part of the substantia nigra, in the external globus pallidus, and in some nuclei of the dorsal thalamus. These thalamic nuclei act as relay stations between the basal ganglia and the cerebral cortex in order to close motor and associative loops. In the 10-to-20 min period, ghrelin decreased glucose metabolism in primary and secondary motor areas, suggesting that these areas are controlled in a dynamic fashion.

Ghrelin treatment also affected glucose metabolism in brainstem areas that belong to the reticular formation, which is an interconnected network of brain nuclei that serve as a major integration and relay system to coordinate functions essential for life. The reticular

formation projects to both the cerebral cortex, via the ascending reticular activating system in order to sort sensory stimuli and to maintain behavioral arousal and consciousness, as well as to the spinal cord, via the reticulospinal tracts in order to play premotor functions on the control of movements and to control of respiratory and cardiovascular functions. Among the areas that are part of the reticular formation, ghrelin changed the glucose metabolism in the midbrain reticular nucleus, the perihypoglossal nuclei, the gigantocellular reticular nucleus as well as in another less defined behavioral state-, motor- and sensory-related areas of the brain stem. Also, ghrelin affects the glucose metabolism in some layers the inferior and superior colliculus, which project to the paramedian pontine reticular formation, and in vermal and hemispheric regions of the cerebellum, which are reciprocally connected to the reticular formation via the inferior cerebellar peduncles and help integrate visual, auditory, and vestibular stimuli in motor coordination.

Since preclinical-PET studies showed that ghrelin induces asymmetric changes on the metabolic mouse brain activity, we evaluated the accessibility of systemically injected Fr-ghrelin into the entire mouse brain, and found that Fr-ghrelin labels the CP, the ARH, the AP, the SFO and the OVLT, all of which contain fenestrated capillaries and express GHSR [31,62,63]. Among the brain labeled areas labeled in our experimental conditions, the ARH is only a bilateral structure, located each side of the third ventricle, and we found that Fr-ghrelin labeled with similar intensity both of its sides. Of note, systemically-injected radiolabeled ghrelin was also shown to label the olfactory bulb in the mouse brain [21,64], in line with the notion that the OB contains a permeable blood-brain-barrier [65,66]. Despite the OB, as well as other odor-processing brain regions, seem to contain GHSR-expressing cells [7,67,68], we failed to detect Fr-ghrelin labeling in the OB nor in any other brain area lacking fenestrated capillaries. Thus, circulating Fr-ghrelin seems to quickly gain access to a limited number of brain regions, as we and others have observed before using different fluorescent ghrelin analogs and additional strategies that include enzymatic amplification steps aimed to enhance the signal of the tracers [23,25,26]. Of note, the size of the brain areas that were labeled with systemically-injected Fr-ghrelin is below the spatial resolution of the preclinical-PET strategy, and, consequently, the extent to which ghrelin affects its <sup>18</sup>F-FDG uptake could not be determined with the current methodology. As an alternative strategy to test the putative asymmetry in the central effects of ghrelin, we assessed if systemically-injected ghrelin induces an asymmetric increase of the marker of neuronal activation c-Fos in the ARH, the key hypothalamic region mediating the orexigenic effect of



ghrelin [42], and found no evidence of laterality. Thus, systemically-injected ghrelin does not seem to display an asymmetric accessibility or induction of c-Fos in the ARH.

Notably, ghrelin treatment in humans also induces asymmetric effects on blood-oxygen-level dependent signal in brain areas associated to motor and limbic systems [69–74]. Thus, the mechanisms underlying the region-specific functional lateralization of the human brain in response to ghrelin may be clarified using mice as an experimental model. For instance, it is well established that systemically-injected ghrelin in mice mainly targets the ARH, which could, consequently, recruit other areas with some degree of lateralization. Within the ARH, ghrelin mainly acts on neurons producing agouti-related protein (AgRP), which are required and sufficient to mediate the orexigenic effects of ghrelin [24,26,75]. AgRP neurons innervate many brain areas in which we detected changes of the glucose metabolism, including the reticular part of the substantia nigra, the olfactory areas, the medial amygdalar nucleus, the ventral pallidum, the lateral hypothalamic area and the ventral tegmental area [76–80]. Thus, AgRP neurons of the ARH could be responsible of some of the observed effects of ghrelin on brain glucose metabolism; however, it remains to be determined if the projections from the ARH display some degree of lateralization. Importantly, the asymmetric effects of ghrelin on the metabolic brain activity may result from the inherent lateralization of some brain functions [81]. For instance, mesolimbic system in rats show inherent functional lateralization as indicated by the observations that the right cortex and nucleus accumbens contain higher basal levels of DA, as compared to the left structures, and that the right nucleus accumbens receives greater dopaminergic neurotransmission, as compared to the left nucleus accumbens, in response to cerebellar stimulation [82,83]. Also, some limbic centers in rats display unilateral <sup>18</sup>F-FDG uptake in response to the systemic administration of an opioid receptor agonist [84]. Similarly, the anterior cingulate shows asymmetric metabolism of the endogenous opioid peptides in humans [85]. In humans and rodents, the hypothalamic control of some neuroendocrine systems and food intake was also shown to display some degree a functional asymmetry [86,87]. Alternatively, the asymmetric effects of ghrelin on the brain glucose metabolism might be due to the action of the hormone on the peripheral fibers of the autonomic nervous system (e.g. vagus nerve [88]), which show anatomical asymmetry due to the differential innervation of the organs located in the left and right sides of the thoracic and abdominal cavities [89]. Further studies in mice would likely help to better understand the neurobiological basis of the asymmetric effects of ghrelin on the brain metabolism.

In conclusion, our study provides an alternative systematic analysis of the brain areas responsive to ghrelin treatment in male mice. The study unmasks several central targets of ghrelin in the mouse brain that had not been described before, and provides the first indication that the central actions of ghrelin display asymmetric effects, in a food intake-independent manner. We hope the current detailed map of the ghrelin-responsive brain areas will be a valuable tool for the future delineation of the neuronal circuits that mediate the different neuroendocrine, autonomic and behavioral actions of ghrelin. Future studies will be required to investigate the extent to which current observations regarding the actions of ghrelin in male mice apply to female mice.

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## FIGURE LEGENDS

**Fig. 1. Overall experimental design.** The figure summarizes the experimental design used in the current study. The pink region represents the expected time-course of plasma ghrelin after subcutaneous injection, indicated by the blue arrow, common to the three experimental conditions (E1, E2, E3). Plasma ghrelin is expected to remain low at basal levels of fed mice in the case of vehicle injection. The green regions represent the expected time course of  $^{18}\text{F}$ -FDG uptake after its intraperitoneal injection, marked by a red solid line and arrow for each experimental condition, followed by a red dashed line and a yellow box representing the onset and duration of the inhalatory anesthesia, and a gray box to indicate the acquisition period.

**Fig. 2. Differential  $^{18}\text{F}$ -FDG uptake during the 0–10 minutes time windows after ghrelin treatment (E1).** Panel **A** shows  $^{18}\text{F}$ -FDG PET coronal t-static images for the comparison of ghrelin vs. vehicle treated mice, co-registered and overlaid with a serial two photon tomography average mouse template. The maps are colored using a dual black-to-orange and black-to-blue color lookup Fig. 5 (shown on the left) to indicate increased and decreased  $^{18}\text{F}$ -FDG uptake, respectively, starting from the significance threshold of  $p < 0.05$ . Panel **B** show three volumetric views of the regions with significant changes in  $^{18}\text{F}$ -FDG uptake. From left to right, a front-top-left, front-top, and front-top-right views are shown. A saturated orange or blue color indicate increased or decreased uptake, respectively. These views were generated using Fiji [44] and the Scalable Brain Atlas [90] Composer. Scale bar: 4 mm divided into 1 mm segments.

**Fig. 3. Differential  $^{18}\text{F}$ -FDG uptake during the 10–20 minutes time windows after ghrelin treatment (E1).** Panel **A** shows  $^{18}\text{F}$ -FDG PET coronal t-statistic images for the comparison of ghrelin vs. vehicle treated mice, co-registered and overlaid with a serial two photon tomography average mouse template. The maps are colored using a dual black-to-orange and black-to-blue color lookup Fig. 5 (shown on the left) to indicate increased and decreased  $^{18}\text{F}$ -FDG uptake, respectively, starting from the significance threshold of  $p < 0.05$ . Panel **B** show three volumetric views of the regions with significant changes in  $^{18}\text{F}$ -FDG

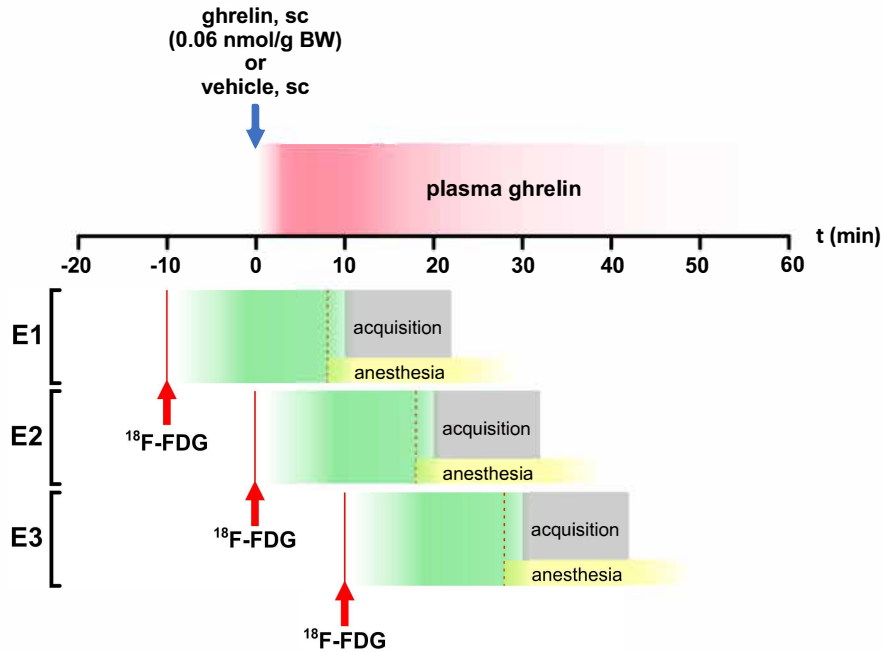
uptake. From left to right, a front-top-left, front-top, and front-top-right views are shown. A saturated orange or blue color indicate increased or decreased uptake, respectively. These views were generated using Fiji [44] and the Scalable Brain Atlas [90] Composer. Scale bar: 4 mm divided into 1 mm segments.

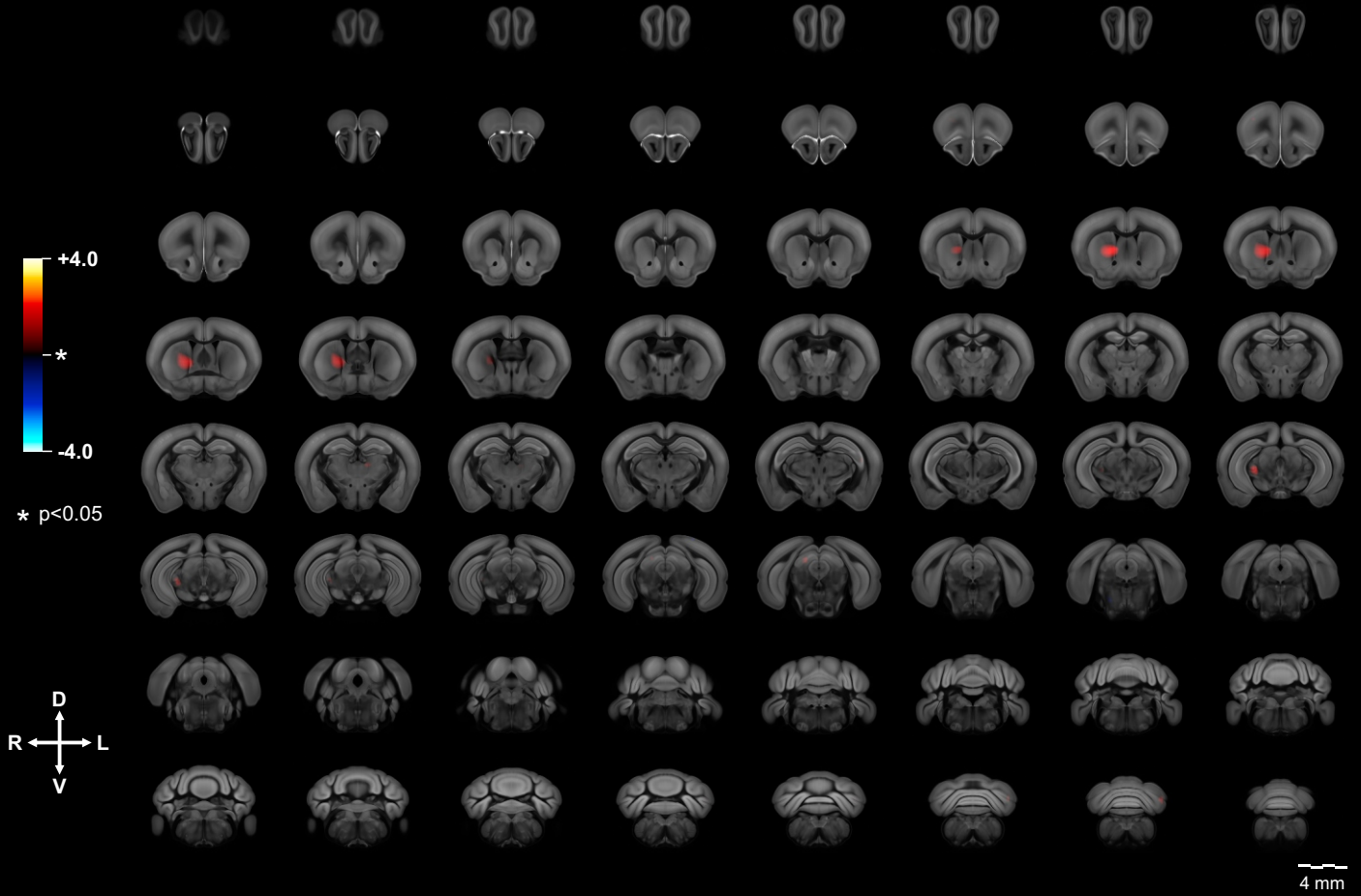
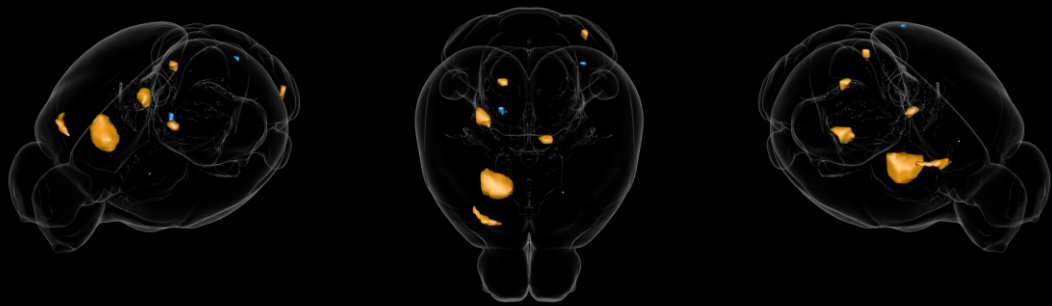
**Fig. 4.** Differential  $^{18}\text{F}$ -FDG uptake during the 20–30 minutes time windows after ghrelin treatment (E3). Panel **A** shows  $^{18}\text{F}$ -FDG PET coronal t-static images for the comparison of ghrelin vs. vehicle treated mice, co-registered and overlaid with a serial two photon tomography average mouse template. The maps are colored using a dual black-to-orange and black-to-blue color lookup Fig. 5 (shown on the left) to indicate increased and decreased  $^{18}\text{F}$ -FDG uptake, respectively, starting from the significance threshold of  $p < 0.05$ . Panel **B** show three volumetric views of the regions with significant changes in  $^{18}\text{F}$ -FDG uptake. From left to right, a front-top-left, front-top, and front-top-right views are shown. A saturated orange or blue color indicate increased or decreased uptake, respectively. These views were generated using Fiji [44] and the Scalable Brain Atlas [90] Composer. Scale bar: 4 mm divided into 1 mm segments.

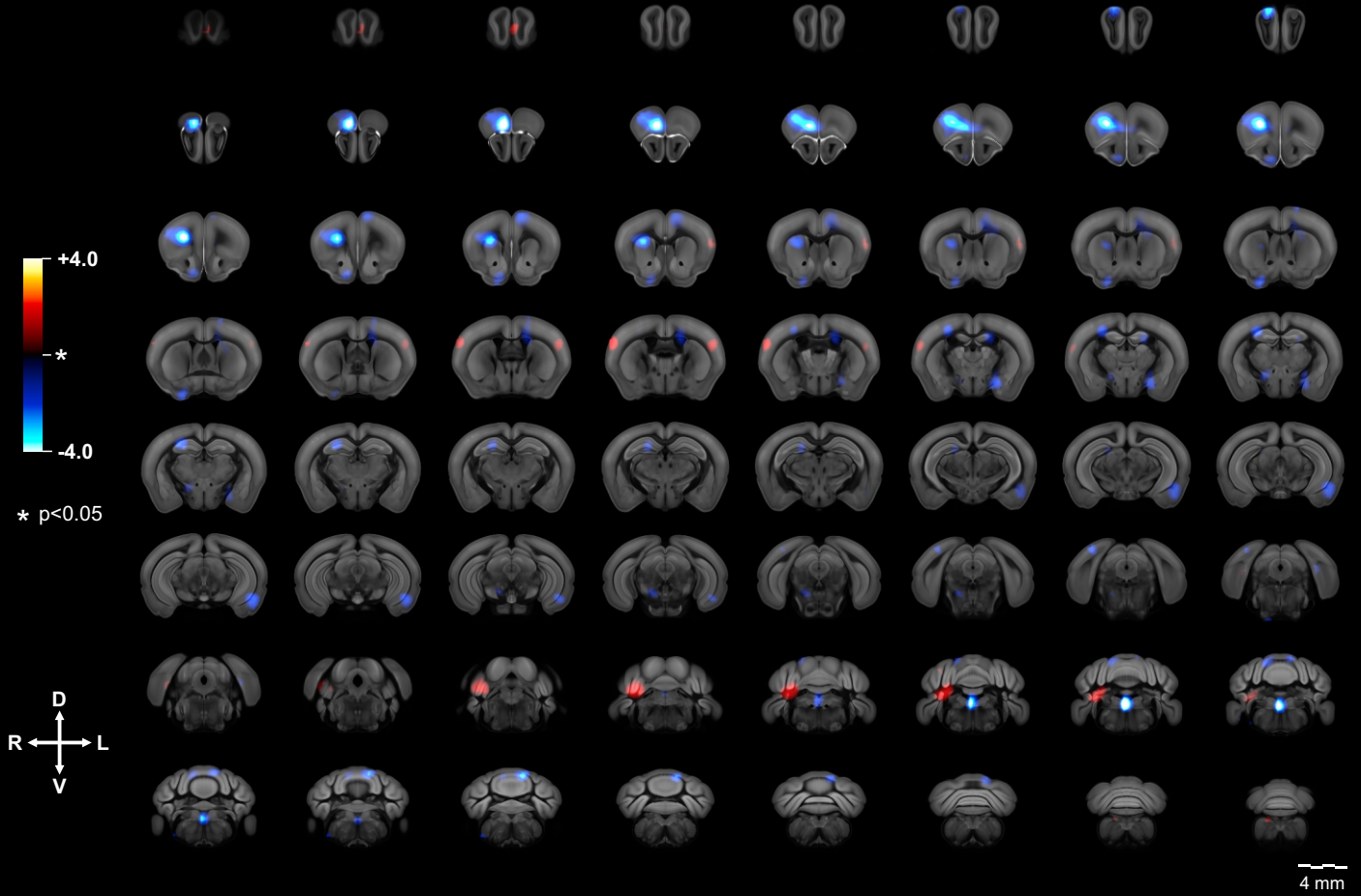
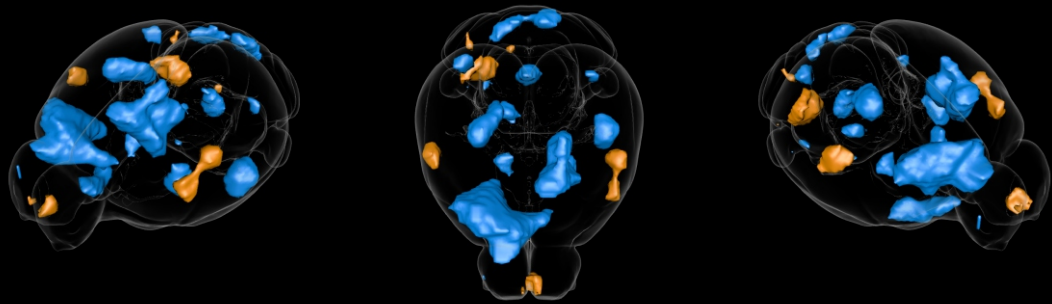
**Fig. 5.** Summary of brain areas with significant change of  $^{18}\text{F}$ -FDG uptake in each experimental condition. The figure summarizes the brain areas where a significant change of  $^{18}\text{F}$ -FDG uptake was observed for each experimental condition (E1, E2, E3). The references for the colors and side of the signals are shown as an independent diagram at the bottom right. Specifically, orange indicates increased  $^{18}\text{F}$ -FDG uptake, blue indicates decreased  $^{18}\text{F}$ -FDG uptake, and gray indicates unchanged  $^{18}\text{F}$ -FDG uptake.

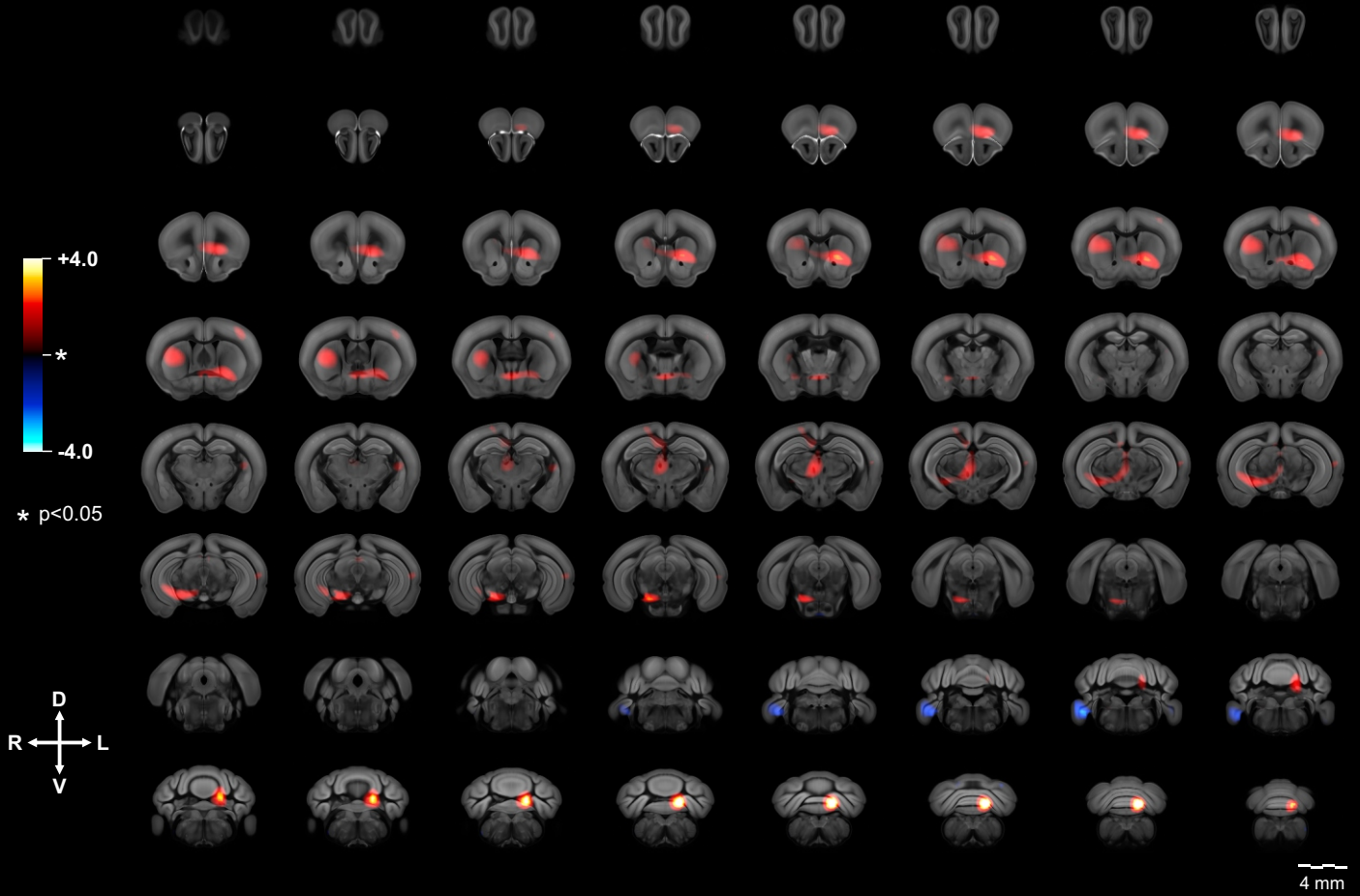
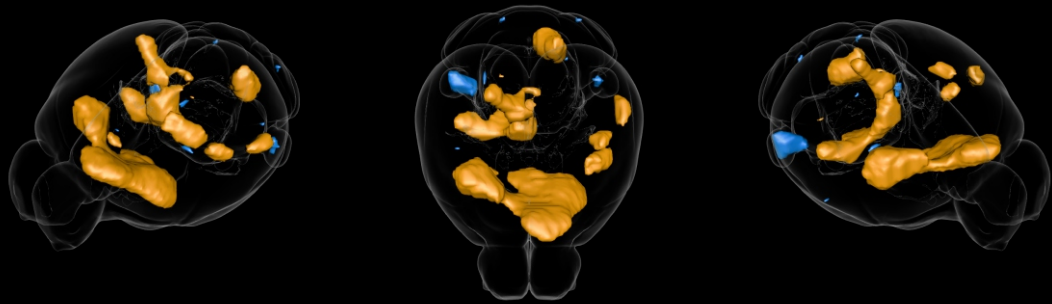
**Fig. 6.** Localization of fluorescent signal in mice systemically-injected with Fr-ghrelin. Panels **A-F** show representative microphotographs of the Fr-ghrelin signal observed in the CP (**A**), SFO (**B**), SCO (**C**), OVLT (**D**), AP (**E**) and hippocampus (**F**) of mice that were SC-injected with Fr-ghrelin. Panels **G-H** show and the right and left ARH of vehicle (**G**) and Fr-ghrelin (**H**) treated mice, respectively. Each panel shows a high magnification image of the areas marked in the low magnification image. Cell nuclei were labeled with Hoechst. Arrows point at Fr-ghrelin signal. Panel **I** shows the quantification of fluorescent signal in the right and left ARH of mice that were SC-injected with Fr-ghrelin. pc: posterior commissure; CA1, CA2, CA3: hippocampal fields 1, 2 and 3; DG: dentate gyrus. Scale bars, 100  $\mu\text{m}$  (low magnification, 10x objective) and 10  $\mu\text{m}$  (high magnification, 40x objective).

**Fig. 7.** Induction of c-Fos in mice systemically-injected with ghrelin. Panel **A-B** show a representative microphotograph of c-Fos immunolabeling in the right and left ARH of mice that were SC-injected with vehicle (**A**) or ghrelin (**B**). Panel **C** shows the quantification of the number of c-Fos-IR cells in the right and left ARH of mice that were SC-injected with ghrelin. Scale bars, 100  $\mu\text{m}$  (low magnification, 10x objective).



**A****B**

**A****B**

**A****B**



Region	Structure name	E1	E2	E3	
<b>Isocortex</b>	Frontal pole, cerebral cortex	●	●	●	
	Primary motor area	●	●	●	
	Secondary motor area	●	●	●	
	Primary somatosensory area, nose	●	●	●	
	Primary somatosensory area, lower limb	●	●	●	
	Primary somatosensory area, mouth	●	●	●	
	Primary somatosensory area, upper limb	●	●	●	
	Primary somatosensory area, unassigned	●	●	●	
	Supplemental somatosensory area	●	●	●	
	Ventral auditory area	●	●	●	
	Anteromedial visual area	●	●	●	
	Primary visual area	●	●	●	
	Anterior cingulate area, dorsal part	●	●	●	
	Anterior cingulate area, ventral part	●	●	●	
	Prelimbic area	●	●	●	
	Infralimbic area	●	●	●	
	Orbital area, lateral part	●	●	●	
	Orbital area, medial part	●	●	●	
	Orbital area, ventrolateral part	●	●	●	
	Agranular insular area, dorsal part	●	●	●	
	Retrosplenial area, lateral agranular part	●	●	●	
	Retrosplenial area, ventral part	●	●	●	
	Anterior area	●	●	●	
	Temporal association areas	●	●	●	
	<b>Olfactory areas</b>	Main olfactory bulb	●	●	●
		Accessory olfactory bulb	●	●	●
		Anterior olfactory nucleus	●	●	●
		Taenia tecta, dorsal part	●	●	●
		Taenia tecta, ventral part	●	●	●
		Dorsal peduncular area	●	●	●
Piriform area		●	●	●	
Postpiriform transition area		●	●	●	
<b>Hippocampal region</b>	Field CA1	●	●	●	
	Field CA3	●	●	●	
	Dentate gyrus, molecular layer	●	●	●	
	Dentate gyrus, granule cell layer	●	●	●	
<b>Retrohippocampal region</b>	Entorhinal area, lateral part, layer 3	●	●	●	
	Entorhinal area, lateral part, layer 5	●	●	●	
	Entorhinal area, lateral part, layer 6a	●	●	●	
	Parasubiculum	●	●	●	
	Subiculum	●	●	●	
<b>Cortical subplate</b>	Endopiriform nucleus, dorsal part	●	●	●	
	Endopiriform nucleus, ventral part	●	●	●	
	Basolateral amygdalar nucleus, post. part	●	●	●	
<b>Striatum</b>	Caudoputamen	●	●	●	
	Nucleus accumbens	●	●	●	
	Olfactory tubercle	●	●	●	
	Lateral septal nucleus, rostral part	●	●	●	
	Lateral septal nucleus, ventral part	●	●	●	
	Striatum-like amygdalar nuclei	●	●	●	
<b>Pallidum</b>	Globus pallidus, external segment	●	●	●	
	Globus pallidus, internal segment	●	●	●	
	Substantia innominata	●	●	●	
	Magnocellular nucleus	●	●	●	
	Medial septal nucleus	●	●	●	
	Bed nuclei of the stria terminalis	●	●	●	

Region	Structure name	E1	E2	E3
<b>Thalamus</b>	Thalamus, sensory-motor cortex related	●	●	●
	Medial group of the dorsal thalamus	●	●	●
	Midline group of the dorsal thalamus	●	●	●
	Intralaminar nuclei of the dorsal thalamus	●	●	●
	Medial habenula	●	●	●
<b>Hypothalamus</b>	Lateral habenula	●	●	●
	Periventricular zone	●	●	●
<b>Midbrain</b>	Hypothalamic lateral zone	●	●	●
	Inferior colliculus, external nucleus	●	●	●
	Substantia nigra, reticular part	●	●	●
	Ventral tegmental area	●	●	●
	Midbrain reticular nucleus	●	●	●
	Superior colliculus, motor related	●	●	●
	Periaqueductal gray	●	●	●
	Pretectal region	●	●	●
	Midbrain, behavioral state related	●	●	●
	<b>Pons</b>	Pontine central gray	●	●
Pons, behavioral state related		●	●	●
<b>Medulla</b>	Medulla, sensory related	●	●	●
	Paragigantocellular reticular nucleus	●	●	●
	Perihypoglossal nuclei	●	●	●
	Medial vestibular nucleus	●	●	●
<b>Cerebellum</b>	Lobule III	●	●	●
	Lobules IV-V	●	●	●
	Declive (VI)	●	●	●
	Pyramus (VIII)	●	●	●
	Uvula (IX)	●	●	●
	Nodulus (X)	●	●	●
	Simple lobule	●	●	●
	Copula pyramidis	●	●	●
	Paraflocculus	●	●	●
	Flocculus	●	●	●
Cerebellar nuclei	●	●	●	

	left decrease	left n/s	left increase
right decrease	●	●	●
right n/s	●	●	●
right increase	●	●	●

