*SI Appendix*

# Orexin-A represses satiety-inducing POMC neurons and contributes to obesity via stimulation of endocannabinoid signalling

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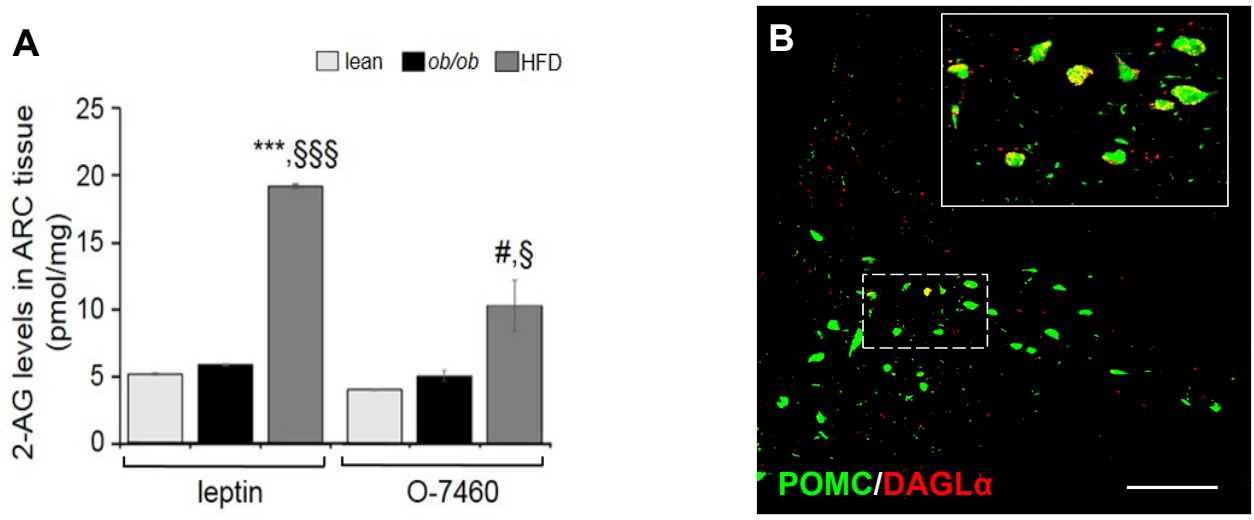
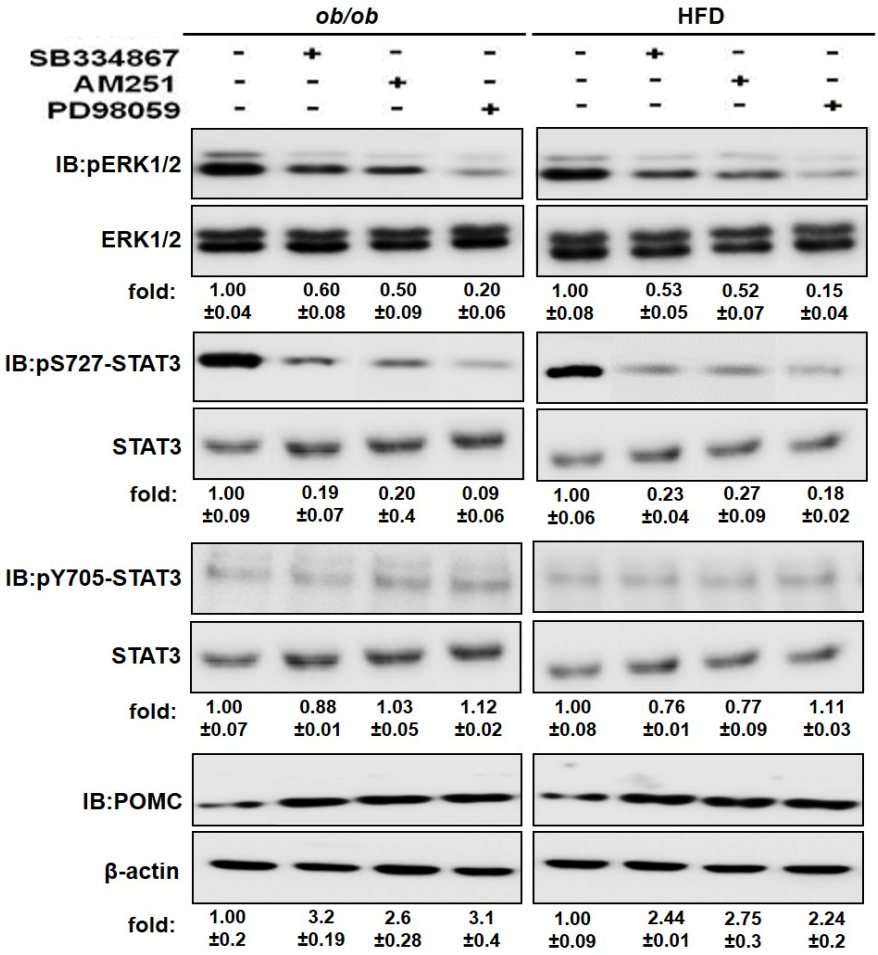
*Short title:* hypocretin1-induced 2-AG synthesis enhances appetite

| **Table 1.** 2-AG levels | | |
|---|---|---|
| **Treatments** | **Primary hypothalamic ARC neurons** | **POMC-eGFP primary neurons** |
| Untreated | 4.11±0.63 | 22.82±4.06 |
| OX-A | 65.56±7.82 ** | 55.26±6.17 # |
| OX-A+SB334867 | 3.53±0.39 (**) | 28.77±5.68 (#) |
| OX-A+O-7460 | 3.24±0.51 (**) | 19.80±5.46 (#) |
| SB334867 | 3.48±0.44 | 26.51±3.40 |
| O-7460 | 3.96±0.30 | 21.26±2.79 |



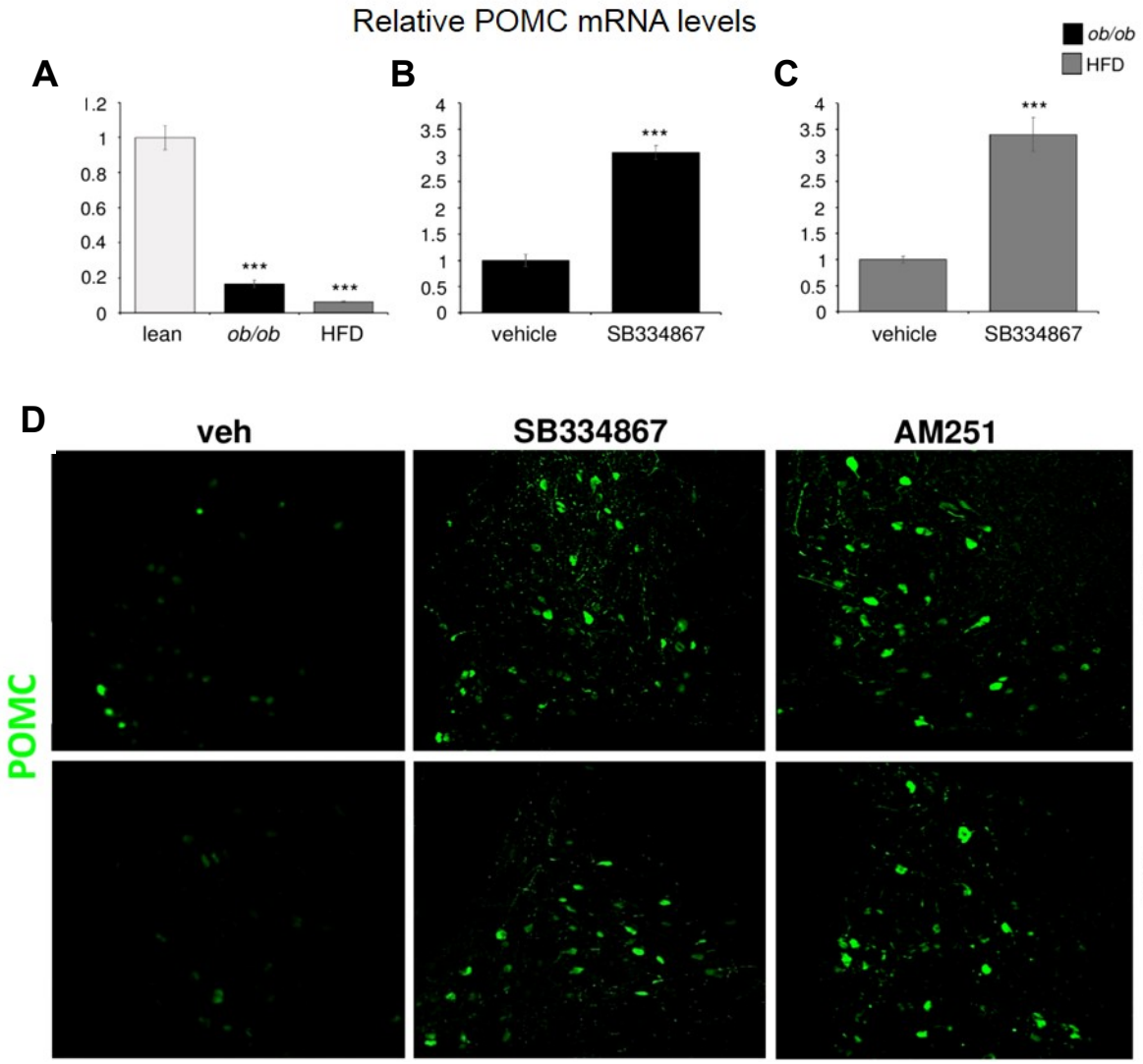

**S1 Fig. OX-A increases 2-AG synthesis in primary hypothalamic ARC neurons, POMC-eGFP neurons and in the ARC nucleus of obese mice in a manner sensitive to DAGLα inhibition**

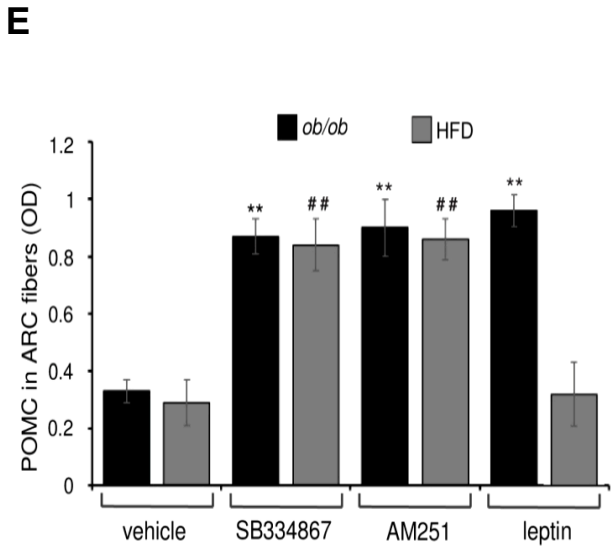
Table 1. 2-AG levels in primary hypothalamic ARC neurons or in POMC-eGFP sorted primary neurons treated with OX-A (300 nM for 30 min) in the absence or presence of SB334867 (10 μM 45 min *per se* or 15 min before OX-A exposure) or O-7460 (10 μM 45 min *per se* or 15 min before OX-A exposure). 2-AG levels were normalized *per* volume of cells (pmol/mL). Each sample contained $0.5x10^5$ cells/mL. $^{**}P<0.0005$ vs. untreated primary hypothalamic ARC neurons. $(^{**})P<0.0005$ vs. OX-A-treated primary hypothalamic ARC neurons. $^{\#}P<0.05$ vs. untreated POMC-eGFP sorted cells; $(^{\#})P<0.05$ vs. OX-A-treated POMC-eGFP sorted cells. Data are means ± SD of at least three separate experiments, each performed in triplicate. (A) 2-AG levels in the ARC of leptin- or O-7460-injected lean and obese (*ob/ob* and HFD) mice. $^{***}P<0.0005$ vs. leptin-treated lean mice; $^{§§§}P<0.0005$ vs. leptin-treated *ob/ob* mice. $^{\#}P<0.05$ vs. O-7460-treated lean mice. $^{§}P<0.05$ vs. O-7460-treated *ob/ob* mice. (B) DAGLα immunoreactivity in the ARC showing in the inset the high magnification of the boxed area. Scale bar: 50μm. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni *post-hoc* test.

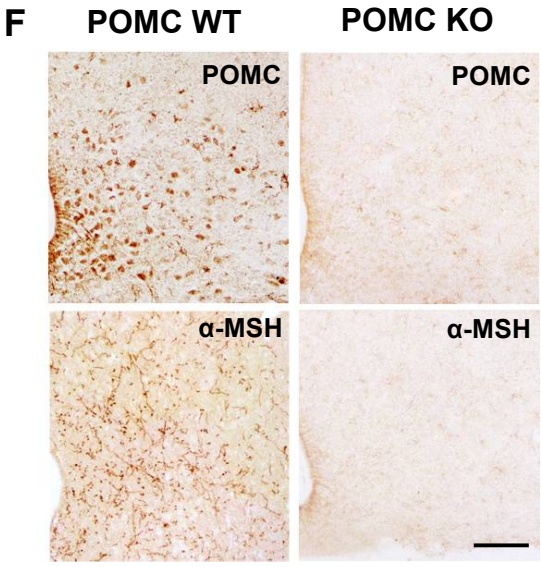



**S2 Fig. OX-A modulates POMC expression *via* ERK1/2-pSTAT3$^{Ser727}$ in obese mice**

Representative immunoblots of POMC, ERK1/2 and STAT3 expression, 6h (POMC) or 1h (ERK1/2 and STAT3) after SB334867, AM251, or PD98059 intraperitoneal injection in fed ad libitum *ob/ob* and HFD mice. Fold data represent the means ± SEM from n = 3 mice *per* group, normalized to the vehicle-treated group. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's post-hoc test.


Relative POMC mRNA levels
A
B
C
ob/ob
HFD
lean
ob/ob
HFD
vehicle
SB334867
vehicle
SB334867
***
D
veh
SB334867
AM251
POMC
ob/ob
HFD


E
ob/ob
HFD
POMC in ARC fibers (OD)
vehicle
SB334867
AM251
leptin


F
POMC WT
POMC KO
POMC
α-MSH

**S3 Fig. Effect of OX-1R or $CB_1R$ antagonists on POMC expression in obese mice**

(A-C) *Pomc* mRNA levels in the ARC of lean, *ob/ob* and HFD vehicle-injected mice (A) and in obese SB334867-injected mice (B-C). The values were normalized to the reference genes *Hprt* or *β-actin* and scaled to the expression of the respective control considered as 1. The mean of the quantitative-cycles (Cq) for the controls were: lean mice, 22.41 (SD=0.054); HFD vehicle-injected mice, 26.18 (SD=0.028); *ob/ob* vehicle-injected mice, 25.56 (SD=0.072). The Cq of the negative controls were undetectable up to 40 reaction cycles. ***$P$<0.0005 vs. lean mice (A); ***$P$<0.0005 vs. *ob/ob* vehicle-injected mice (B); ***$P$<0.0005 vs. HFD vehicle-injected mice (C). (D) Representative POMC immunohistochemical expression in the ARC of obese (*ob/ob* and HFD) vehicle- or SB334867- or AM251-injected mice [Scale bar: 100 μm]. (E) Optical density quantification of POMC immunoreactivity in neurons of obese (*ob/ob* and HFD) vehicle- or SB334867- or AM251-injected obese mice. **$P$<0.005 vs. *ob/ob* vehicle-injected mice; $^{\#\#}P$<0.005 vs. HFD vehicle-injected mice. (F) Control for specificity of POMC or α-MSH antibodies was tested by blanking the peroxidase-based immunoreactivity in POMC-KO mice (Scale bar: 100μm; see supplemental information).

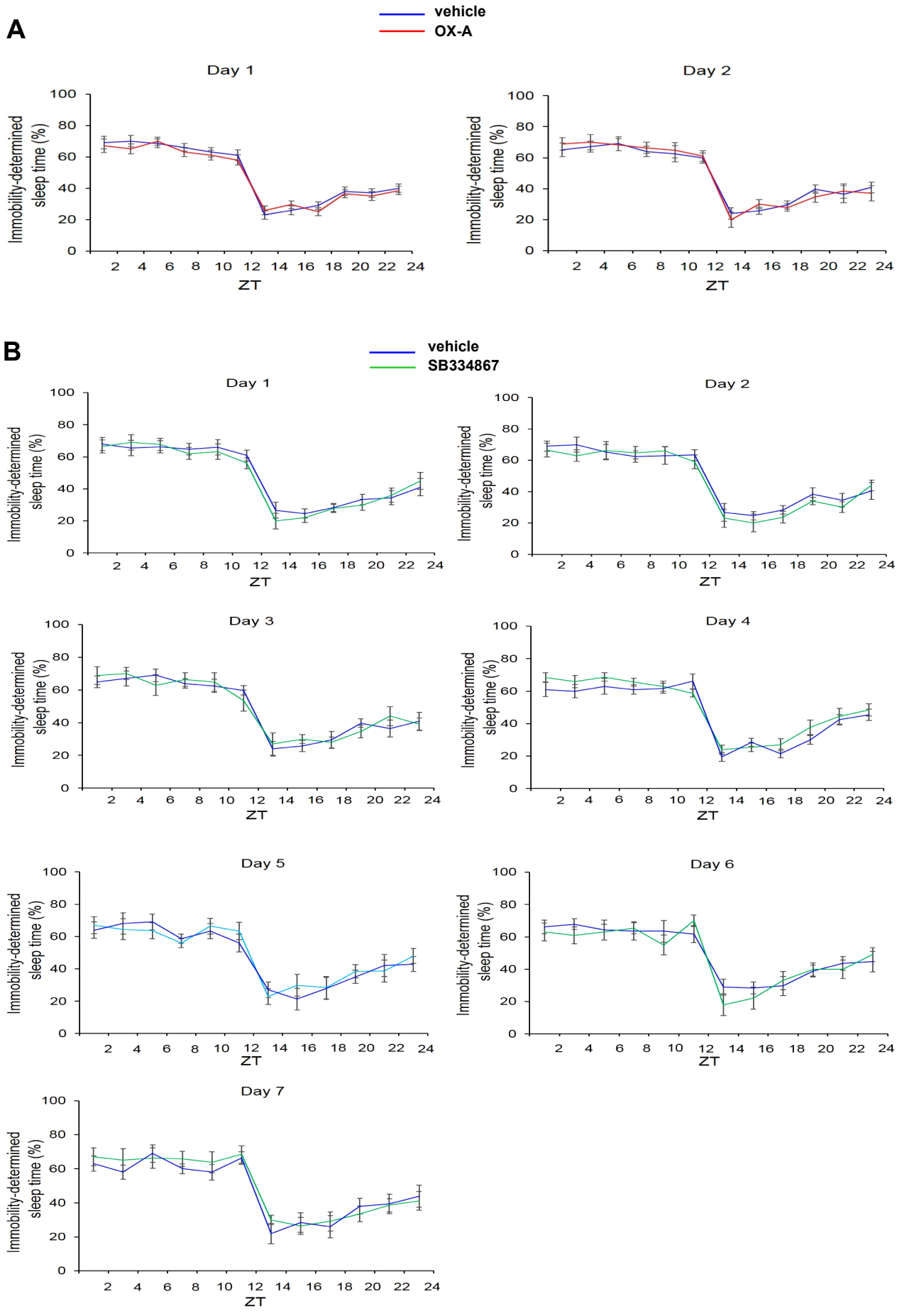
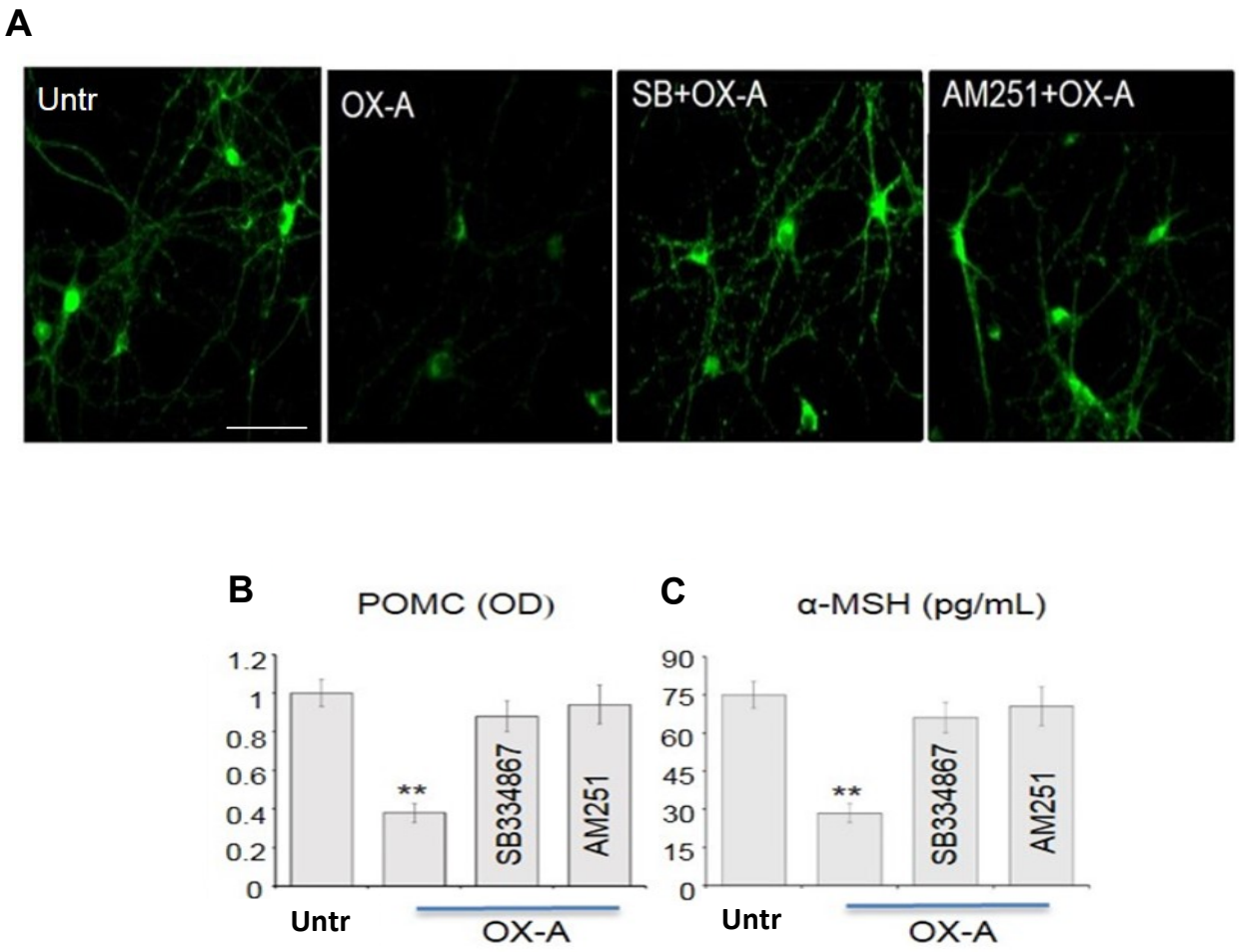

A
vehicle
OX-A
Day 1
Day 2
Immobility-determined sleep time (%)
ZT
B
vehicle
SB334867
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7

**Table 2. Descriptive statistics for selected immobility-determined sleep parameters**

| Parameter | Vehicle (mean± SEM) | OX-A (mean± SEM) | Vehicle (mean± SEM) | SB334867 (mean± SEM) | *P*-value |
|---|---|---|---|---|---|
| Total sleep time (%) | 56.2±2.6 | 54.9±3.4 | 55.3±2.2 | 57.1±5.4 | 0.421 |
| Light phase time (%) | 68.2±2.8 | 67.9±1.8 | 69.3±3.1 | 66.5±4.3 | 0.547 |
| Dark phase time (%) | 40.5±2.9 | 40.9±2.2 | 41.5±1.8 | 41.2±3.5 | 0.911 |
| Total distance travelled (m) | 106.8±11.4 | 99.8±8.3 | 117.4±12.1 | 101.4±9.2 | 0.871 |
| Light phase distance travelled (m) | 24.2±12.0 | 21.2±5.8 | 22.8±6.3 | 23.3±5.4 | 0.562 |
| Dark phase distance travelled (m) | 82.2±5.8 | 78.5±12.1 | 94.5±9.1 | 78.0±6..7 | 0.566 |

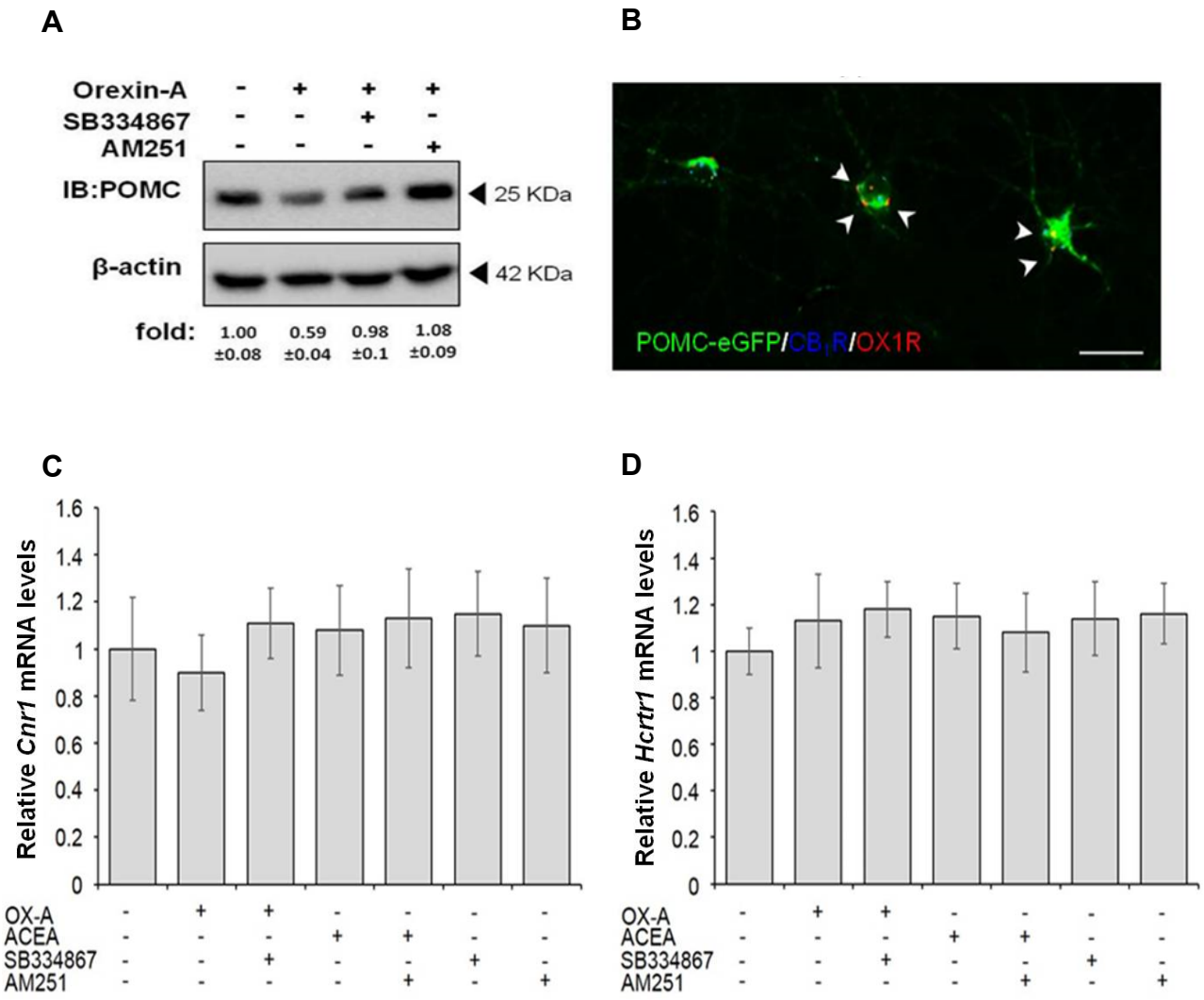
## S4 Fig. Effects of OX-A or SB334867 on the immobility-determined sleep time

(A-B) Graphs showing no effect of OX-A or SB334867 on the average immobility-determined sleep time in mice during 2 days of OX-A or SB334867 treatment (A) or 7 days of SB334867 treatment (B). All plots are based on 24h of data, presented in 2h time bins. Dara are means ± SEM. n=6 per group. (Table 2). Statistical analysis was derived from all the days of recording under a 12h : 12h LD cycle at 100 lux.



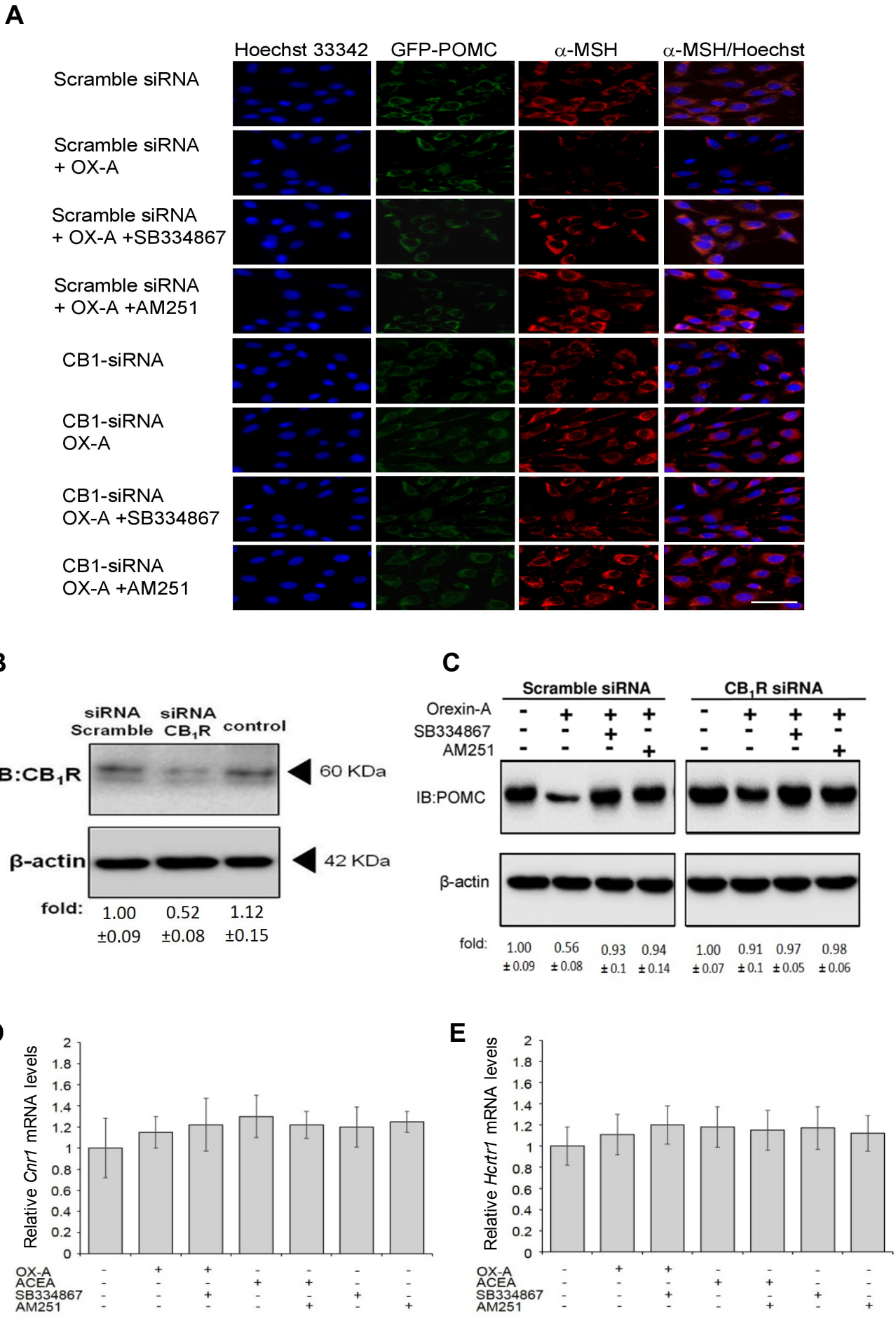

**S5 Fig. OX-A-mediated reduction of POMC expression and α-MSH levels in primary cultures of hypothalamic ARC neurons**

(A) Representative POMC immunocytochemical expression in primary hypothalamic ARC neurons from wild-type mice after treatment with OX-A (300 nM for 30 min) in the absence or presence of SB334867 (10 μM 15 min before OX-A exposure) or AM251 (0.5 μM 15 min before OX-A exposure). [Scale bar: 50μm]. (B) Optical density quantification of POMC immunoreactivity in primary hypothalamic ARC neurons treated as described in (A). (C) α-MSH levels (pg/mL) in primary hypothalamic ARC neurons treated as indicated in (A). Results represent the mean ± SEM of three separate experiments, each performed in duplicate. ***P*<0.005 vs. untreated cells (two-way ANOVA followed by Bonferroni's *post-hoc* test).



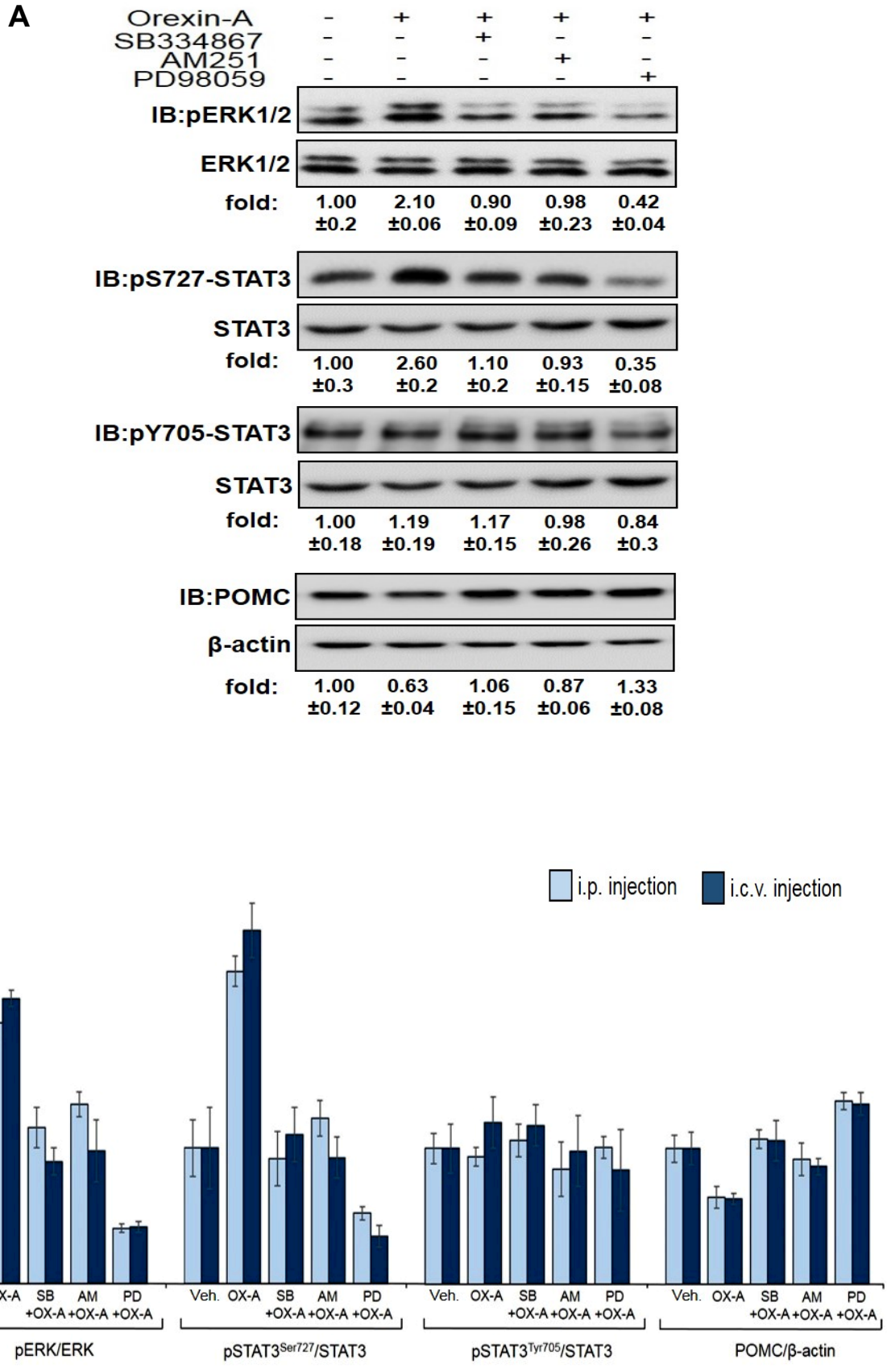

**S6 Fig. OX-A reduces POMC expression in POMC-eGFP sorted primary neurons**

(A) Representative immunoblots of POMC expression in POMC-eGFP sorted primary neurons treated with OX-A (300 nM for 30 min) in the absence or presence of SB334867 (10 μM 15 min before OX-A exposure) or AM251 (0.5 μM 15 min before OX-A exposure). Results represent the means ± SEM of three separate experiments, each performed in duplicate. Fold data was normalized to the untreated cells. (B) Confocal microscopy image showing $CB_1R$/OX-1R immunocoexpression (arrowheads) at the plasma membrane of POMC-eGFP sorted primary neurons [Scale bar: 20μm]. (C,D) *Cnr1* (C) or *Hcrtr1* (D) mRNA levels in POMC-eGFP sorted primary neurons treated with $CB_1R$ or OX-1R agonists (ACEA, 0.5 μM or OX-A, 300nM for 30 min, respectively) in the absence or presence of the respective antagonist (SB334867, 10μM 45 min *per se* or 15 min before OX-A exposure; AM251, 0.5 μM 45 min *per se* or 15 min before ACEA exposure). The values were normalized to the reference genes *Hprt* or *β-actin* and scaled to the expression of the respective control considered as 1. The means of the quantitative-cycles (Cq) for the untreated cells were 23.51 (SD=0.065) for *Cnr1,* and 22.34 (SD=0.071) for *Hcrtr1*. The Cq of the negative controls were undetectable up to 40 reaction cycles.


A
Hoechst 33342
GFP-POMC
α-MSH
α-MSH/Hoechst
Scramble siRNA
Scramble siRNA + OX-A
Scramble siRNA + OX-A +SB334867
Scramble siRNA + OX-A +AM251
CB1-siRNA
CB1-siRNA OX-A
CB1-siRNA OX-A +SB334867
CB1-siRNA OX-A +AM251
B
siRNA Scramble
siRNA CB1R
control
IB:CB1R
60 KDa
β-actin
42 KDa
fold:
1.00 ±0.09
0.52 ±0.08
1.12 ±0.15
C
Scramble siRNA
CB1R siRNA
Orexin-A
SB334867
AM251
IB:POMC
β-actin
fold:
1.00 ± 0.09
0.56 ± 0.08
0.93 ± 0.1
0.94 ± 0.14
1.00 ± 0.07
0.91 ± 0.1
0.97 ± 0.05
0.98 ± 0.06
D
Relative Cnr1 mRNA levels
OX-A
ACEA
SB334867
AM251
E
Relative Hcrtr1 mRNA levels
OX-A
ACEA
SB334867
AM251

**S7 Fig. POMC and α-MSH expression in mHypoA-POMC/GFP cells transfected with $CB_1R$ siRNA**

(A) Representative α-MSH immunofluorescence of mHypoA-POMC/GFP cells transfected with scrambled or $CB_1R$ siRNA sequences and treated with OX-A (300 nM for 30 min) in the absence or presence of SB334867 (10 μM 15 min before OX-A exposure) or AM251 (0.5 μM 15 min before OX-A exposure). [Scale bar: 20μm]. (B) Representative immunoblots of $CB_1R$ expression in mHypoA-POMC/GFP cells transfected with scrambled or $CB_1R$ siRNA sequences. The $CB_1R$ expression in non-transfected cells is also shown. Results represent the means ± SEM of three separate experiments, each performed in duplicate. Fold data were normalized to the scrambled siRNA-treated cells. (C) Representative immunoblots of POMC expression in mHypoA-POMC/GFP cells treated as described in (A). Results represent the means ± SEM of three separate experiments, each performed in duplicate. Fold data were normalized to the scrambled or CB1R siRNA-treated cells. (D,E) *Cnr1* (D) or *Hcrtr1* (E) mRNA levels in mHypoA-POMC/GFP cells treated with CB1R or OX-1R agonists (ACEA, 0.5 μM or OX-A, 300 nM, respectively, for 30 min) in the absence or presence of the respective antagonist (SB334867, 10 μM 45 min *per se* or 15 min before OX-A exposure; AM251, 0.5 μM 45 min *per se* or 15 min before ACEA exposure). The values were normalized to the reference genes *Hprt* or β-actin and scaled to the expression of the respective control considered as 1. The mean of the quantitative-cycles (Cq) for the untreated cells were 21.78 (SD=0.061) for *Cnr1* and 21.59 (SD=0.052) for *Hcrtr1*. The Cq of the negative controls were undetectable up to 40 reaction cycles.


A
Orexin-A
SB334867
AM251
PD98059
IB:pERK1/2
ERK1/2
fold: 1.00 ±0.2 2.10 ±0.06 0.90 ±0.09 0.98 ±0.23 0.42 ±0.04
IB:pS727-STAT3
STAT3
fold: 1.00 ±0.3 2.60 ±0.2 1.10 ±0.2 0.93 ±0.15 0.35 ±0.08
IB:pY705-STAT3
STAT3
fold: 1.00 ±0.18 1.19 ±0.19 1.17 ±0.15 0.98 ±0.26 0.84 ±0.3
IB:POMC
β-actin
fold: 1.00 ±0.12 0.63 ±0.04 1.06 ±0.15 0.87 ±0.06 1.33 ±0.08
B
i.p. injection
i.c.v. injection
OD integration
Veh. OX-A SB +OX-A AM +OX-A PD +OX-A
pERK/ERK
pSTAT3Ser727/STAT3
pSTAT3Tyr705/STAT3
POMC/β-actin

**S8 Fig. Effect of the intraperitoneal or intracerebroventricular injection of OX-A on the ERK1/2, STAT3 or POMC expression**

(A) Representative immunoblots of POMC, ERK1/2 and STAT3 expression after OX-A intracerebroventricular (i.c.v.) injection in lean mice (fed *ad libitum*) pretreated with SB334867, AM251, PD98059 (6h, POMC; 1h, ERK1/2 and STAT3). (B) Bargraphs showing the optical density of immunoblots for POMC, ERK1/2 and STAT3 expression in lean mice (fed ad libitum) after OX-A, SB334867+OX-A, AM251+OX-A, or PD98059+OX-A-intraperitoneal (i.p., blue light; see Fig.2A) or i.c.v. (blue) injection. Fold data represent the means ± SEM from n = 3 mice per group, normalized to the vehicle-treated group. No difference was found between the values obtained after i.p. and i.c.v. drugs injection. Statistical analysis was run using two-way ANOVA followed by the Bonferroni's post-hoc test.

## MATERIALS AND METHODS

**Animals and drugs.** Experiments were performed under institutional approval and according to the guidelines of the institutional ethical code and the Italian (decree law n. 116/92) and European (Official Journal of European Community L358/1 12/18/1986) regulations for the care and use of laboratory animals. Since orexin levels exhibit a robust diurnal fluctuation (levels slowly increase during the dark period or active phase (i.e. ZT13-24) and decrease during the light period or rest phase [1]), all the mice were housed under 12h light : 12h dark cycle, light on at 8:00 PM, i.e. ZT0, for at least 4 weeks before killing at ZT20-22. The mice, housed in standard cages in a temperature and humidity-controlled vivarium, received ad libitum chow and water. Adult male C57BL/6j mice were obtained from Charles River Laboratories (Sulzfeld, Germany); male mice with spontaneous nonsense mutation of the *ob* gene for leptin (*ob/ob*, JAX mouse strain) B6.V-Lepob/J and WT *ob* gene expressing homozygous siblings of different ages were obtained from breeding *ob* gene heterozygotes, and genotyped with PCR. Nine-week-old male mice were made obese by being fed 7 weeks of high-fat diet (HFD) (diet HFD TD97366 providing 4.7 Kcal/g as 49% fat, 18% protein and 33% carbohydrate; Harlan Laboratories). The POMC-eGFP transgenic mice (C57BL/6J-Tg [Pomc-EGFP]1Low/J) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA).
Mice were injected intraperitoneally with vehicle (saline), OX-A (Tocris; 40μg/Kg 2h prior to perfusion or killing; n =6 mice per group), OX-1R antagonist, SB334867 (Tocris; 30mg/Kg 3h *per se* or 1h before OX-A injection), $CB_1R$ antagonist AM251 (Tocris; 10mg/Kg 3h *per se* or 1h before OX-A injection), leptin (Sigma Aldrich; 5 mg/kg, 2h before perfusion or killing), the ERK inhibitor PD98059 (Sigma-Aldrich; 10mg/Kg 3h *per se* or 1 h before OX-A injection) or the DAGLα inhibitor O-7460 (12mg/kg 3h before killing). In some experiments mice were treated with intracerebroventricular (i.c.v.) injection of OX-A (10 nmol for 1h), in the absence or presence of SB334867 (5 nmol 30 min before OX-A injection) or AM251 (20 nmol 30 min before OX-A injection). For the i.c.v administration of drugs a polyethylene guide cannula (SP-10 polyethylene tube, Natsume Seisakusho, Tokyo, Japan) was implanted into the left lateral ventricle of animals under sodium pentobarbital anesthesia [50 mg/kg body weight, intraperitoneal (i.p.) injection] as described previously [2].

**Immunohistochemistry.** Under deep pentobarbital anesthesia (60 mg/kg, i.p.), mice were perfused transcardially with 4% (wt/vol) paraformaldehyde/0.1M phosphate buffer, pH7.4 (PB). The brains were cut with a Leica CM3050S cryostat into 10μm-thick serial sections in the coronal plane, collected in alternate series and processed for immunofluorescence or immunoperoxidase with specific primary antibodies. The following primary antibodies were used: goat anti-OX-1R antibody (Santa Cruz; specificity for immunoreactivity tested by Heydendael et al. [3]); rabbit anti-$CB_1R$ antibody (anti C terminus 461-472, Abcam; specificity for immunoreactivity tested by von Rüden et al. [4]); goat anti-OX-A (Santa Cruz; specificity for immunoreactivity tested by Blanco-Centurion et al. [5]); guinea pig anti-DAGLα (kindly provided by Prof. Ken Mackie; specificity for immunoreactivity verified by Cristino et al. [6]); rabbit anti-α-MSH (Abcam) and goat anti-POMC (Santa Cruz) for which

the specificity of immunoreactivity was tested in the present study by using POMC-KO mice (B6.129X1-Pomctm2Ute/J, obtained from the Jackson Laboratory. Bar Harbor, ME, USA) through peroxidase-based immunohistochemistry. Control for the specificity of the secondary antisera was performed by omitting the primary antiserum before the addition of the secondary antisera. All the primary antibodies were diluted 1:100–1:300 in donkey serum. Immunofluorescence was revealed by specific Alexa-488, -546 or -350 secondary donkey anti-IgGs (Invitrogen LifeTechnology) and analyzed with a Leica DMI6000 fluorescence microscope equipped with a confocal laser scanning Microscopy (Leica TCS SP8). Quantitative analysis was performed by Leica DFC320 cooled digital CCD camera (Leica Microsystems). Images were digitally acquired at the same magnification and processed for fluorescence determination at the single cell level with Metamorph Imaging Software (Leica MetaMorph © AF). For each section, the optical density zero value was assigned to the background (i.e., a tissue portion devoid of stained cell bodies or fibers).

**Preembedding Immunogold Silver-Enhanced Electron Microscopy**. Under deep pentobarbital anesthesia, mice were perfused transcardially with 3% (vol/vol) paraformaldehyde/0.5–1% glutaraldehyde in PB. Double preembedding immunogold labeling was performed on the ARC sections (50-µm-thick) of lean mice. The sections were incubated free-floating overnight at 4°C with the primary antibodies (rabbit anti-$CB_1R$ antibody, anti C terminus 461-472, and goat anti-OX-1R), all diluted 1:100 in donkey serum blocking solution with 0.02% saponin. Subsequently, the sections were incubated in a mixture of 10nm (for $CB_1R$) and 6nm (for OX-1R) gold-conjugated secondary antibodies (Aurion), diluted 1:30 in donkey serum blocking solution with 0.02% saponin. Sections were treated with 0.5% OsO4 in PB for 10 min at 4 °C, dehydrated in an ascending series of ethanol and propylene oxide and embedded in TAAB 812 resin (TAAB). During dehydration, sections were treated with 1% uranyl acetate in 70% ethanol (vol/vol) for 15 min at 4 °C. Ultrathin (60nm thickness) sections were cut by vibratome (Leica), collected on Formovar-coated, single- or multiple-slot (50-mesh) grids and stained with 0.65% lead citrate. Electron micrographs were taken with the TEM microscope (FEI Tecnai G2 Spirit TWIN). The TEM observation was limited to the series sectioned up to 0.6–0.8 µm depth from the external surface of preembedded immunolabeled tissue. Additional sections were processed in parallel as controls of reaction by omitting both or one of the primary antibodies from the mixture. No labeling was detected in the control samples.

**Lipid Extraction and 2-AG measurement.** After treatments, the lean and obese mice were killed by cervical dislocation, the brains removed and the ARC rapidly dissected. Tissue samples were pooled and homogenized in 5 vol chloroform/methanol/ Tris·HCl 50mM (2:1:1 by volume) containing 50pmol of d5-2-arachidonoylglycerol (d5-2-AG) as internal standards. Homogenates were centrifuged at 13,000 × g for 16min (4°C), the aqueous phase plus debris were collected and four times extracted with 1vol chloroform. The lipid-containing organic phases were dried and pre-purified by open-bed chromatography on silica columns eluted with increasing concentrations of methanol in chloroform. Fractions for 2-AG measurement were obtained by eluting the columns with 9:1 (by volume) chloroform/methanol and then analyzed by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS). LC-APCI-

MS analyses were carried out in the selected ion monitoring mode, using m/z values of 384.35 and 379.35 (molecular ions +1 for deuterated and undeuterated 2-AG). Values are expressed as pmol per mg of wet tissue extracted. For 2-AG levels in the cells, the primary hypothalamic ARC neurons or POMC-eGFP sorted primary neurons were treated with OX-A (300nM for 30min) in the absence or presence of SB334867 (10 μM for 45min *per se* or 15min before OX-A exposure) or AM251 (0.5 μM for 45min *per se* or 15min before OX-A exposure). After treatment, cells and supernatant were collected, homogenized and analyzed as indicated for tissue sample. 2-AG levels were normalized per mL of cell + medium. Each sample contained $0.5x10^5$ cells/mL.

**Quantification of PI(4,5)$P_2$**

PI(4,5)$P_2$ was extracted from untreated or OX-A-treated POMC-eGFP sorted primary neurons (300 nM, 30min) in the absence or presence of SB334867 (10 mM, 45min *per se* or 15min before OX-A treatment). PI(4,5)$P_2$ was quantified using the PI(4,5)$P_2$ mass ELISA kit (Echelon Biosciences, Salt Lake City, UT, USA) following the manufacturer's instructions. Briefly, extracted lipids were re-suspended in PBS 0.25% Protein Stabilizer, incubated with a PI(4,5)$P_2$ detector protein for 1h at room temperature and then added to a 96-well PI(4,5)$P_2$-coated microplate. A peroxidase-linked secondary detection reagent and a colorimetric substrate were added to detect the PI(4,5)$P_2$ detector protein binding to the plate. The colorimetric signals were measured at 450nm using a Tecan Genios Pro instrument. The data represent PI(4,5)$P_2$ levels in pmol/10000 cells calculated from the standard curve generated according to the manufacturer's instructions.

**α-MSH and OX-A measurements in mice.** After treatment, blood samples (50μL each) from lean and obese mouse were collected, centrifuged and plasma and serum fractions frozen at -80 °C until assay. The α-MSH serum levels were determined using a commercial ELISA Kit (MyBioSource, San Diego, CA, USA) according to the manufacturer's instructions. The minimum detectable concentration of α-MSH was 3.12 pg/mL. The intra- and inter-assay precision were ‹8% and ‹10%, respectively.
For OX-A determinations, the cerebrospinal fluid (CSF), collected from the cisterna magna as described [7] was immediately frozen on dry ice and stored at -80°C until assay. The CSF OX-A levels were measured with commercially ELISA KIT (Phoenix Pharmaceuticals Inc, Burlingame, CA,USA) according to the manufacturer's instruction. A maximum of 15μL of CSF could be acquired from each mouse. Therefore, CSF samples from two mice were pooled to yield the 25μL required for the assay. Triplicate samples were assayed and levels determined against a known standard.

**Western blot analysis.** Lean and obese mice, treated as described elsewhere, were killed by cervical dislocation, the brains removed and the ARC rapidly dissected. Tissue samples were homogenized in 1xTNE buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA) containing 10% Triton X-100, protease and phosphatase inhibitor mixtures (Sigma Aldrich). Protein concentrations were analyzed using Lowry protein assay (Bio-Rad Laboratories) to allow the loading of the same amount of proteins (20μg) on SDS/PAGE.

Proteins transferred onto PVDF membranes were blocked with 5% non-fat dry powdered milk dissolved in 1xTBST (20mM Tris, 137mM NaCl, 0.1% Tween-20) for 2h at room temperature. After blocking, the membranes were incubated with primary antibodies dissolved in TBST containing 5% non-fat powdered milk. The used primary antibodies were: ERK1/2 (1:1000; Cell Signaling); pERK1/2Thr202/Tyr204 (1:1000; Cell Signaling); STAT3 (1:2000; Abcam); pSTAT3Ser727 (1:1000; Cell Signaling); pSTAT3Tyr705 (1:1000; Cell Signaling) and POMC (1:800; Abcam). The membranes, rinsed with TBST, were incubated for 1h with HRP-conjugated goat anti-rabbit secondary antibody (1:3000; Biorad) and goat anti-mouse only for STAT3 (1:4000; Biorad) at RT. A monoclonal β-actin antibody (1:4000; Sigma-Aldrich) was used to check for equal protein loading. Reactive bands were detected by chemiluminescence (Clarity ECL; Biorad). Images were analyzed on a ChemiDoc station with ImageJ software. For $CB_1R$ and POMC expression in cell cultures, after treatment, cell homogenates were subjected to electrophoresis in 10% polyacrylamide gel and transferred to PVDF membranes. Membranes were blocked with non-fat dry powdered milk for 2h and incubated over-night at 4 °C with a rabbit polyclonal anti-$CB_1R$ (1:500; Calbiochem) and anti-POMC antibody (1:800; Abcam) whereas incubation were for 1h at RT with HRP-conjugated goat anti-rabbit secondary antibody (1:1500 for CB1R and 1:2500 for POMC; Biorad). The monoclonal anti-β-actin (1:4000; Sigma-Aldrich) was used as the reference protein expression. Detection was performed using chemiluminescence (Clarity ECL; Biorad). Images were analyzed on a ChemiDoc station with ImageJ software.

***Pomc*, *Cnr1* and *Hcrtr1* mRNA quantification by RT-PCR analysis.** *Pomc*, *Cnr1* and *Hcrtr1* mRNA were quantified by RT-PCR analysis using the primers designed by Allele-Id software version 7.0 (Biosoft International) and synthesized (HPLC-purification grade) by MWG-Biotech. For *Pomc* mRNA quantification, tissue samples were dissected from n=6 animals per group, collected in RNA Later (Invitrogen Life Technology) and homogenized in 1.0 mL of trilzol (Invitrogen) following the manufacturer's instructions. For *Cnr1* and *Hcrtr1* expression, mHypoA-POMC/GFP or POMC-eGFP sorted cells was collected in 1.0 mL of trizol (Invitrogen) following the manufacturer's instructions. Total RNA was extracted according to manufacturer recommendations, dissolved in RNase-free water, and further purified by spin cartridge using the PureLink-micro RNA purification system (Invitrogen). Total RNA was dissolved in RNA storage solution (Ambion Life Technology) and UV-quantified by a Bio-Photometer (Eppendorf). Concentration and integrity of RNA samples were also evaluated by the RNA-6000-Nano lab-on-chip assay using a 2100 Bioanalyzer equipped with a 2100 Expert Software (Agilent Technology) following the manufacturer's instruction. For all of the tested samples, the RNA integrity number was greater than 7.5 relative to a 0–10 scale. One microgram of total RNA, as evaluated by the 2100 Bioanalyzer, was reverse-transcribed in cDNA. Optimized primers for SYBR green analysis and optimum annealing temperatures were designed by Allele-Id software version 7.0 (Biosoft International) and were synthesized (HPLC-purification grade) by MWG-Biotech (POMC: F-primer AACGCCATCATCAAGAAC, R-primer TTCCTAACACAGGTAACTCTA; Cnr1: F-primer GGGCACCTTCACGGTTCTG, R-primer GTGGAAGTCAACAAAGCTGTAGA; Hcrtr1: F-primer GTTATCTGCCCATCAGTGTCCTC,

R-primer: GGTGAAGCAGGCGTAGACG; Hprt: F-primer TTGACACTGGTAAAACAATGC, R-primer GCCTGTATCCAACACTTCG; Actb: F-primer CCAGGCATTGCTGACAGG, R-primer TGGAAGGTGGACAGTGAGG). For each target, all mRNA sequences at http://www.ncbi.nlm.nih.gov/gene/ were aligned and common primers were designed. Relative gene-expression calculation, corrected for PCR efficiency and normalized with respect to the reference genes *Hprt* and β-actin was performed by the IQ5 software. Significance analysis (limit p≤0,05) was performed by the REST® software, according to Pfaffl [8].

**Body weight and food intake measurements.** For body weight, obese and lean mice were injected daily with vehicle or SB334867 (Tocris, 30 mg/kg, i.p., n=6 mice per group) for 7 days. Body weight was measured both prior and after 7 days of treatment. In other experiment, lean mice were injected daily with vehicle or OX-A (Tocris; 40 μg/kg, i.p., n=6 mice per group) and the body weight or food intake was measured prior to OX-A injection and after 24-h or 48-h OX-A treatment. Food intake was controlled by supplying to each mouse a pre-weighed, fixed amount (50 g) of pellets in a bowl. To determine the amount of consumed food, all the remaining pellets was weighed at the specific time points.

**Study population and hormone measurements.** We studied 50 unrelated white young male adults (mean age ± SD= 43.11 ± 1.18 years; mean BMI [95% confidence interval (CI)]= 48.11± 0.98kg/m$^2$) who had suffered from obesity for at least 5 years. The population was recruited at the Obesity Outpatient Clinic of the Department of Clinical and Experimental medicine of Federico II University Hospital of Naples, Italy. All the data were compared to that obtained from 50 male healthy, normal weight, volunteers (mean age ± SD= 40.11 ± 2.48 years; mean BMI [95% confidence interval (CI)]= 21.38± 1.71kg/m$^2$).
Secondary causes of obesity were excluded, and no patient was an alcohol abuser or under pharmacological treatment for any disease. The physical and biochemical characteristics of the population, evaluated in all subjects, were measured by routine laboratory methods. A venous blood sample was collected from each patient at 8.00 AM after overnight fasting. The research was approved by the Ethics Committee of the Faculty of Medicine, University of Naples Federico II (authorization n° 193/06, 25/10/2006; amendment n° 193/06/ESES1, 1/10/2014), and was carried out according to the Helsinki II Declaration.
After centrifugation, plasma and serum fractions were frozen at -80°C until assay for the hormone measurements. Circulating levels of α-MSH were measured in serum fractions using a commercial radioimmunoassay (RIA) kit (Eurodiagnostica, Malmo, Sweden). The minimum detectable concentration of α-MSH was 3pmol/L and the intra-and inter-assay coefficients of variation of the assay were 11.8 and 13.0%, respectively.
Serum OX-A levels were measured by using the ultra-sensitive Fluorescent EIA Kit (Phoenix Pharmaceuticals Inc, Burlingame, CA, USA) following the manufacturer's instructions. Before measurement, serum OX-A was extracted using Sep-Pak C18 columns (Waters, Milford, MA) and eluated slowly with 80% acetonitrile. The samples were evaporated and the dry residue, dissolved in water and used for Fluorescent EIA Kit. The minimal detectable concentration was less than 40 pg/mL.

**Cell cultures and hormone analysis**. mHypoA-POMC/GFP were cultured in Dulbecco's modified Eagle's medium (Life Tecnologies) supplemented with 10% fetal bovine serum (Life Tecnologies), penicillin (50 units/mL) and streptomycin (50 µg/mL), at 37°C in 100 mm culture dishes (Life Tecnologies) gassed with an atmosphere of 95% air-5% $CO_2$.
Primary cultures of hypothalamic ARC neurons, derived from neonatal 0- or 1-day-old C57BL/6 (Charles River) or POMC-eGFP transgenic mice, were obtained as described [9]. Briefly, the ARC was quickly dissected and mechanically dispersed in $Ca^{2+}$- and $Mg^{2+}$-free buffered Hanks'balanced salt solution. Tissues were dissociated enzymatically (0.125% trypsin solution, 37°C for 20 min) and mechanically. Cells were plated at a density of $2x10^4$ cells/$cm^2$ on polylysine-coated coverslips and grown in Neurobasal medium supplemented with 2% B27, 0.5 mM L-glutamine, penicillin (50U/mL) and streptomycin (50 µg/mL). Cells were used between 6 and 8d in vitro. More than 80% of primary cultured cells were positive for neuronal marker NeuN antibodies as determined by immunocytochemistry.
POMC expression and cellular α-MSH content were analyzed by immunocytochemical technique and a commercial ELISA Kit, respectively. Briefly, for POMC immunocytochemical study, the primary ARC neurons were treated with OX-A (300nM for 30 min) in the absence or presence of SB334867 (10µM for 45 min *per se* or 15 min before OX-A exposure) or AM251 (0.5µM for 45 min *per se* or 15 min before OX-A exposure). After treatments, the cells were washed three time with PBS and, finally, fixed for 20 min with paraformaldehyde (4% vol/vol). These preparations were finally rinsed with PBS and blocked in PBS-containing BSA (2% wt/vol). Goat anti-POMC (Santa Cruz) antibody was used as a primary antibody. After 18h at 4°C, the cells were washed and exposed to Alexa-488 secondary donkey anti-IgGs (Invitrogen LifeTechnology) for 2h in the dark. Immunocytochemical study was performed by Leica DMI6000 fluorescence microscope equipped with a Leica DFC320 cooled digital CCD camera (Leica Microsystems). For α-MSH measurement, primary hypothalamic ARC neurons were treated for 4h as described above. After treatments, α-MSH levels were determined in the supernatants using a commercial ELISA Kit (MyBioSource, San Diego, CA, USA) according to the manufacture's instructions. The minimum detectable concentration of α-MSH was 3.12 pg/mL. The intra- and inter-assay precision were ‹8% and ‹10%, respectively.

***Cnr1* gene silencing.** *Cnr1* silencing was obtained by transfecting mHypoA-POMC/GFP cells with endoribonuclease-prepared siRNA sequences (EMU088771; Sigma-Aldrich) using Lipofectamine 2000 (Life Tecnologies) and following the manufacturer's instructions. The siRNA silencing efficiency was determined 24h after the initial transfection by measuring mRNA levels. Western Blot technique was used to detect the expression of $CB_1R$ and POMC in $CB_1R$ silenced mHypoA-POMC/GFP cells. Proteins were subjected to electrophoresis in 10% polyacrylamide gel and transferred to PVDF membranes. Membranes were blocked with non-fat dry powdered milk for 2h and incubated over-night at 4 °C with a rabbit polyclonal anti-$CB_1R$ (1:500; Calbiochem) and anti-POMC antibody (1:800; Abcam) whereas incubation were for 1h at RT with HRP-conjugated Goat α-Rabbit secondary antibody (1:1500 for $CB_1R$ and 1:2500 for POMC; Biorad). The monoclonal

anti-β-actin (1:4000; Sigma-Aldrich) was used as the reference protein expression. Detection was performed using chemiluminescence (Clarity ECL; Biorad). Images were analyzed on a ChemiDoc station with ImageJ software.

**Fluorescence scan cell sorter (FSCS) purification.** Cells isolated by FSCS were prepared from ARC of 0 or 1 day old POMC-eGFP mice as described [10]. The cells were sorted using the BD FSCS Aria III system (Becton Dickinson) to give two cell populations, GFP-positive and -negative cells. The sample was kept cold at all times to minimize RNA degradation and cell death during sorting. Viable cells, gated by their forward and side scatter characteristics, were seeded on polylysine-coated coverslips. After 18 h, POMC-eGFP neurons were treated and analysed as described above.

**Video-tracking (sleep) analyses.** Video-tracking analyses were based on an established protocol [11]. Mice were entrained to a 100 lux 12h : 12h LD cycle for 2 days during the OX-A treatment or 7 days for SB334867 treatment and then transferred to identically sized cages in new light-tight chambers. The single 12h : 12h LD cycle was recorded with a near-infrared CCTV camera (Maplin Electronics), at 3 frames/s, in the AVI file format. Recording began within 48h of the transfer to the new cage, and an acrylic block was placed under the food hopper to keep the mouse in the recording field at all times. Video files were stored on a digital hard drive recorder prior to analysis. Two parameters were extracted from the video footage: immobility-determined sleep time and distance travelled. Sleep time is expressed as a percentage of recording time. Immobility sensitivity was set at 95% to prevent the detection of movements caused by breathing during sleep.

**Statistical analysis.** Data are expressed as mean ± SEM unless otherwise indicated and were analyzed by GraphPad Prism 6 software, version 6.05 (GraphPad, Inc.). Statistical differences among groups were determined by either Student's t-test or two-way ANOVA followed by post hoc Bonferroni tests for comparison among means. A level of confidence of $P<0.05$ was employed for statistical significance. For the study on obese patients, the comparison between the means of variables measured in obese and non-obese men was analyzed by the Mann-Whitney *U*-test. Correlations were analyzed by Pearson Test. $P<0.05$ was considered as statistically significant.

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