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Title: Melanin-Concentrating Hormone acts through hypothalamic kappa opioid system and p70S6K to stimulate acute food intake

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Abstract: Melanin-Concentrating Hormone (MCH) is one of the most relevant orexigenic factors specifically located in the lateral hypothalamic area (LHA), with its physiological relevance demonstrated in studies using several genetically manipulated mice models. However, the central mechanisms controlling MCH-induced hyperphagia remain largely uncharacterized. Here, we show that central injection of MCH in mice deficient for kappa opioid receptor (k-OR) failed to stimulate feeding. To determine the hypothalamic area responsible for this MCH/k-OR interaction, we performed virogenetic studies and found that downregulation of k-OR by adeno-associated viruses (shOprkl-AAV) in LHA, but not in other hypothalamic nuclei, was sufficient to block MCH-induced food intake. Next, we sought to investigate the molecular signaling pathway within the LHA that mediates acute central MCH stimulation of food intake. We found that MCH activates k-OR and that increased levels of phosphorylated extracellular signal regulated kinase (ERK) are associated with downregulation of phospho-S6 Ribosomal Protein. This effect was prevented when a pharmacological inhibitor of k-OR was co-administered with MCH. Finally, the specific activation of the direct upstream regulator of S6 (p70S6K) in the LHA attenuated MCH-stimulated food consumption. Our results reveal that lateral hypothalamic k-OR system modulates the orexigenic action of MCH via the p70S6K/S6 pathway.



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Dear Editor,

We will be grateful if the enclosed Ms entitled “**Melanin-Concentrating Hormone acts through hypothalamic *kappa* opioid system and p70S6K to stimulate acute food intake**” could be considered for publication as an Original Article at *Neuropharmacology*.

We confirm that this work is original and has not been published elsewhere nor is it currently under consideration for publication elsewhere.

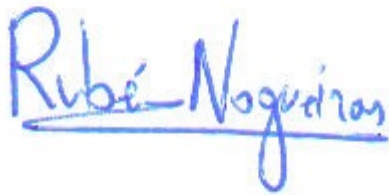
All authors listed have contributed sufficiently to the project to be included as authors. To the best of our knowledge, no conflict of interest exists. We have included funding sources after the discussion.

MCH is an orexigenic neuropeptide specifically located in the lateral hypothalamus that increases food intake and adiposity, having a strong impact in energy homeostasis. In the present study, by a combination of genetic and pharmacological approaches we uncover that its acute orexigenic effect is mediated via the *k*-OR system. In addition, we carry out some detailed mechanistic studies showing that the p70S6K/S6 signaling pathway in the lateral hypothalamic area (LHA) mediates MCH-induced feeding. More specifically, the highlights of our manuscript are:

- Central MCH fails to stimulate food intake in *k*-OR-deficient mice.
- Only *k*-OR specifically located in the LHA modulates MCH-induced feeding.
- Central MCH activates *k*-OR in the LHA, and reduces phosphorylated levels of S6 ribosomal protein. This effect is blunted in rats pre-treated with a specific *k*-OR antagonist.
- The genetic activation of p70S6K in the LHA reduces MCH-induced feeding.

These findings are quite significant because despite the fact that the orexigenic effect of MCH has been reported almost two decades ago; the involved hypothalamic molecular pathways remain largely unknown.

Sincerely yours,

A handwritten signature in blue ink that reads "Rubén Nogueiras". The signature is written in a cursive style with a horizontal line underlining the name.

Ruben Nogueiras, PhD

Reviewer 1:

As acknowledged by the authors, the reduced hyperphagic effect of MCH led to by the knockdown of k-OR in the LH was less than that produced for the i.c.v. injection of k-OR antagonists. They attributed the reduced effect to the possibility that a "lower number of neurons are affected by the virogenetic approach". This could be a valid explanation. However, the authors cannot exclude the possibility that the MCH action through the k-OR occur in regions other than the LH. Indeed, the nucleus Accumbens (NAc) appears as good target as the LH for the k-OR-mediated effects of MCH. MCHR1 is strongly expressed in the NAc and k-OR agonists are known to act in the NAc.

REPLY:

We completely agree with the reviewer. We cannot rule out the possibility of extra-hypothalamic MCH actions through the kappa opioid receptor system. Indeed, the possibility of this interaction in the NAc is quite feasible since the MCHR1 is highly expressed in this area. In fact, we plan to address this issue and its functional significance in the future but feel that is out of the scope of the current manuscript. In the current study, we have focused on hypothalamic MCH/k-OR interaction to simplify the complex central mechanisms involved on food intake regulation considering that MCHR and *kappa* opioid receptor are widely expressed within the central nervous system.

We do consider the comment from the reviewer very appropriate, and will highlight this issue by adding the following sentence:

"Moreover, we cannot rule out a potential extra-hypothalamic MCH action through k-OR system. Indeed, a good candidate is the NAc where MCHR1 is strongly expressed. Further studies to address this issue are clearly merited".

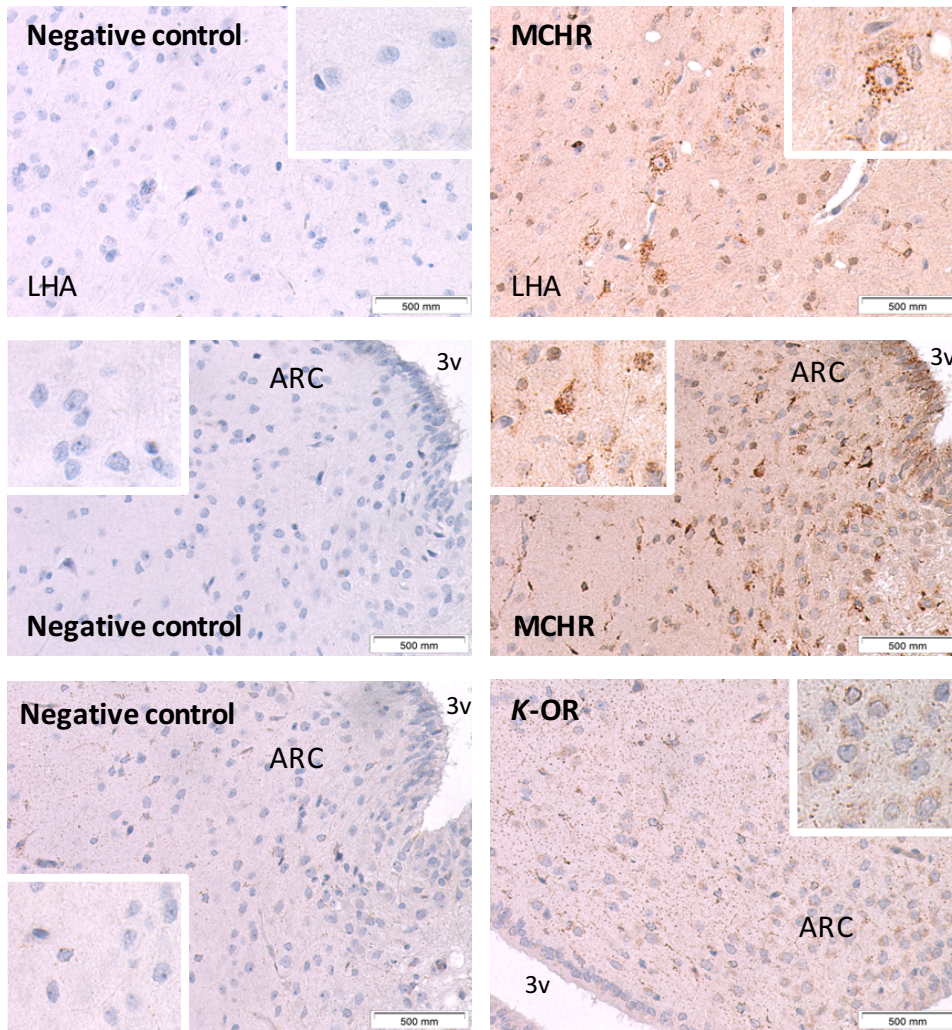
Reviewer 2:

I have a concern regarding the colocalization MCHR/KOR. This was done with antibodies which specificity needs to be proven. To have worked with commercial antibodies directed at GPCRs I can attest that the majority are not selective. I know of researchers who wanted to raise antisera to the MCHR and never found specificity. The authors need to test these antibodies and not only accept the data of the companies which established them.

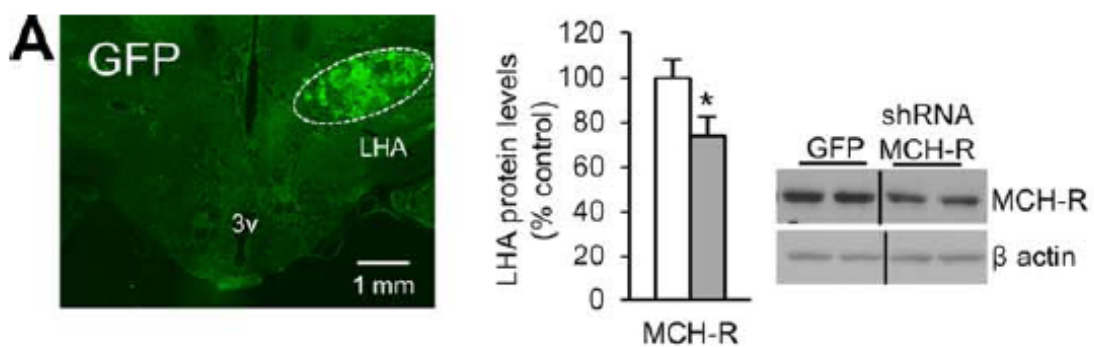
REPLY:

We share the reviewer's concerns regarding antibody specificity and it is something we critically appraise in our papers. For the ones used in this manuscript we have carried out different validation procedures to test their reliability. Some of the information related to this issue was already reported in previous papers (e.g. see Imbernon et al., *Gastroenterology*, 2013, and Imbernon et al., *Hepatology*, 2016), and we did not find it appropriate to redo the experiments here. Nevertheless, please allow us to recapitulate some of this information about the rabbit-MCHR (Abnova PAB16225) and goat anti-k-OR (Sigma SAB2501442) antibodies.

Regarding MCHR we first performed standard validation methods (with/without primary antibodies) that exhibited specific hypothalamic staining as shown below:

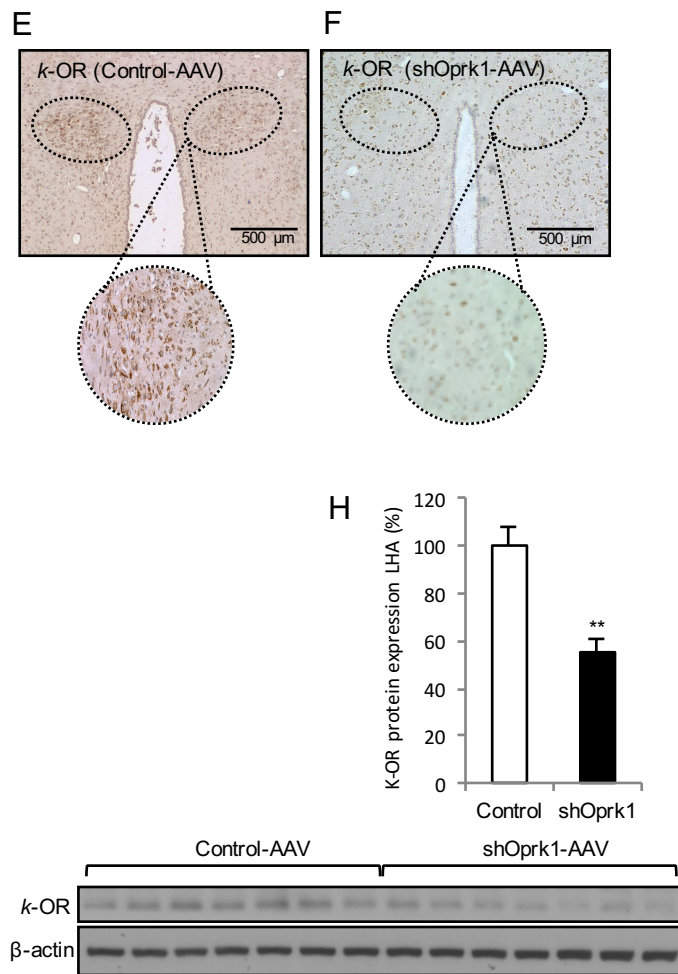


This specificity was further validated using a combination of genetic knock-down of the receptor and WB. When we knock-down MCHR by shRNA-viral particles specifically in the lateral Hypothalamic area (LHA), the protein expression of MCHR analyzed by western blot is significantly reduced as shown in the figure below (Figure 2A from Imbernon et al., Hepatology, 2016):

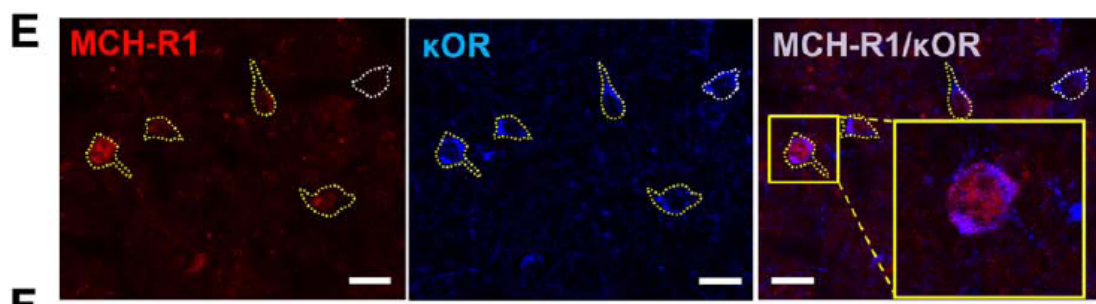


Similarly, in the present study, the genetic down-regulation of *k*-OR in specific hypothalamic areas (paraventricular nucleus, PVH and LHA) using a shOprk1-AAV clearly resulted to a significant decrease in *k*-OR protein expression tested by different

techniques such as immunohistochemistry (for PVH) and western blot (for LHA) (Figures 2E-F, H):



In addition, using the goat anti-*k*-OR (Sigma SAB2501442) antibody it has been demonstrated a positive and negative *k*-OR immunoreactivity for tdTomato cells expressing MCHR1 (Figure 3E, Imbernon et al., Hepatology, 2016):



Some of these assays were carried out in the laboratory of Dr. Zsolt Liposits, who is one of the most reputed scientists in the field of brain immunohistochemistry.

Another concern is that KOR is significantly expressed on MCH expressing neurons. The authors need to analyze their data with this caveat in mind.

REPLY:

We agree that the biological significance of *k*-OR expression on MCH neurons is yet unclear. We feel that our data showing that exogenous pharmacological blockade with *k*-OR-antagonists block exogenous MCH-induced food intake indicates that the *k*-OR is exerting its effect downstream of the MCHR. Having said that, we share the reviewer's view that the functional interaction of the *k*-OR- and MCH-systems is likely to go further than the effect here described. The presence of *k*-OR in MCH neurons indicates that the activity of MCH neurons is also regulated by endogenous opioid-neurons. In fact, it is known that dynorphin neurons are co-localized with MCHR1 neurons (Chee et al 2013). Therefore, these findings provide the anatomical and biochemical substrate of a strong functional relationships among both systems. Whether this interaction is related to energy balance remains to be established.

We have now added the following text to the discussion: "Nevertheless, it is becoming clear that the functional interaction of the *k*-OR- and MCH-systems is likely to go further than the effect described here. The presence of *k*-OR in MCH neurons indicates that the activity of MCH neurons is also regulated by endogenous opioid-neurons. In fact it is known that dynorphin neurons are co-localized with MCHR1 neurons (Chee et al 2013) and that *k*-OR is co-localized with MCH neurons (Parks et al., 2014). These anatomical and biochemical findings imply strong functional relationships between the two systems. Further studies assessing the role of the *k*-OR as a potential mediator of MCH in other brain systems like dopaminergic reward areas such as the NAc and VTA are warranted. Similarly, the functional significance of the *k*-OR in MCH neurons and putative regulation by endogenous opioid peptides needs to be uncovered".

Finally, a new reference (Parks et al., 2014) has been added to the references list in the manuscript (highlight).

The highlights of our manuscript are:

- Central MCH fails to stimulate food intake in *k*-OR-deficient mice.
- *k*-OR, in the lateral hypothalamic area (LHA), modulates acute MCH-induced feeding.
- Central MCH activates *k*-OR in the LHA, and reduces phosphorylated levels of S6 ribosomal protein.
- The genetic activation of p70S6K in the LHA reduces MCH-induced feeding.

Melanin-Concentrating Hormone acts through hypothalamic *kappa* opioid system and p70S6K to stimulate acute food intake

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ABSTRACT

Melanin-Concentrating Hormone (MCH) is one of the most relevant orexigenic factors specifically located in the lateral hypothalamic area (LHA), with its physiological relevance demonstrated in studies using several genetically manipulated mice models. However, the central mechanisms controlling MCH-induced hyperphagia remain largely uncharacterized. Here, we show that central injection of MCH in mice deficient for kappa opioid receptor (*k*-OR) failed to stimulate feeding. To determine the hypothalamic area responsible for this MCH/*k*-OR interaction, we performed virogenetic studies and found that downregulation of *k*-OR by adeno-associated viruses (shOprk1-AAV) in LHA, but not in other hypothalamic nuclei, was sufficient to block MCH-induced food intake. Next, we sought to investigate the molecular signaling pathway within the LHA that mediates acute central MCH stimulation of food intake. We found that MCH activates *k*-OR and that increased levels of phosphorylated extracellular signal regulated kinase (ERK) are associated with downregulation of phospho-S6 Ribosomal Protein. This effect was prevented when a pharmacological inhibitor of *k*-OR was co-administered with MCH. Finally, the specific activation of the direct upstream regulator of S6 (p70S6K) in the LHA attenuated MCH-stimulated food consumption. Our results reveal that lateral hypothalamic *k*-OR system modulates the orexigenic action of MCH via the p70S6K/S6 pathway.

Keywords:

Hypothalamus, Melanin-Concentrating Hormone, *kappa*-opioid receptor, food intake.

Chemical compounds studied in this article:

Melanin-Concentrating Hormone (MCH) (PubChem CID: 24868207)

Naloxone Hydrochloride (PubChem CID: 5464092)

Norbinaltorphimine dyhydrochloride (norBNI) (PubChem CID: 5480230)

1. Introduction

In mammals, MCH is a cyclic 19-amino acid neuropeptide synthesized in the LHA and zona incerta (ZI). This neuropeptide is a critical hypothalamic regulator of energy homeostasis, having effects on both feeding behavior and energy expenditure. Central administration (Qu et al., 1996), or transgenic overexpression (Ludwig et al., 2001) of MCH increases food intake, whereas deletion of MCH (Alon and Friedman, 2006; Shimada et al., 1998) or its receptor (Chen et al., 2002) reduces body weight. In addition, MCH can modulate glucose metabolism (Kong et al., 2010; Ludwig et al., 2001) and peripheral lipid metabolism in the white adipose tissue (Imbernon et al., 2013) and liver (Imbernon et al., 2016; Pissios et al., 2006). Although it is well recognized that MCH acts in a way to preserve energy, the molecular mechanisms underlying MCH action are poorly understood. Previous studies indicate that MCH acts in concert with other neuropeptides, such as NPY or POMC in the arcuate nucleus (ARC) (Griffond and Risold, 2009), and orexin in LHA (Guan et al., 2002). However, there is a general lack of information regarding the interaction with other hypothalamic systems. Opioid receptors are widely expressed within the hypothalamus (Mansour et al., 1994), and we have previously demonstrated that the *k*-OR system in the ARC is able to modulate the orexigenic effect of ghrelin (Romero-Picó et al., 2013). A potential interaction among MCH and the opioid system to regulate food intake and food addiction was postulated previously (Lopez et al., 2011), but the mechanistic aspects and the specific brain areas where this interaction occurs remain unexplored. Since LHA seems to be a link between homeostatic and hedonic centers controlling feeding behavior, and *k*-OR co-localizes with MCH receptor in the LHA (Imbernon et al., 2016), we investigated whether MCH may act via the opioid system within this hypothalamic area to drive acute food consumption.

Here we use multiple pharmacological and genetic approaches to provide evidence that MCH-induced food intake is mediated through the activation of *k*-OR signaling specifically within the LHA. This MCH/*k*-OR interaction involves reduced phospho-S6, and the functional relevance of this mechanism was demonstrated by the

fact that genetic constitutive activation of p70S6K in the LHA blocked MCH-induced feeding. These results confirm the relevant role of the hypothalamic *kappa* opioid system as a modulator of MCH orexigenic effect; positioning *k*-OR as a common neuronal signaling pathway integrating central orexigenic signals.

2. Material and methods

2.1. Animal Procedures and Chemicals

Male Sprague-Dawley rats (200-250 g) were housed individually and maintained on 12:12-h light-dark cycle at constant temperature ($21\pm 1^\circ\text{C}$) and humidity (40-50%). They were allowed *ad libitum* access to water and standard chow from Scientific Animal (proteins 16%, carbohydrates 60%, and fat 3%). In all procedures implying surgery, rats were anesthetized by an intraperitoneal injection of ketamine-xylazine (ketamine 100mg/Kg BW + xylazine 15 mg/Kg BW).

Adult (8-10 weeks old) wild type (WT) C57BL6 mice, mutant mice (*Oprk1*^{-/-}) that lack functional *k*-OR subtype 1 (B6.129S2-*Oprk1*^{tm1kff}/J, The Jackson Laboratory) (Imbernon et al., 2016; Romero-Picó et al., 2013), were maintained on an *ad libitum* chow diet in Specific Pathogen Free (SPF) conditions and housed with a 12:12-h light-dark cycle. For surgery, mice were anesthetized by an intraperitoneal injection of 138 mg ketamine/Kg BW + 7 mg xylazine/Kg BW.

Intracerebroventricular (i.c.v.) central injections were performed between 9:00-10:00 am. All animal procedures were conducted in accordance with the standards approved by the Faculty Animal Committee at the University of Santiago de Compostela, and experiments were performed in agreement with the rules of Laboratory Animal Care and International Law on Animal Experimentation. Opioid receptors antagonists: naloxone hydrochloride (non-selective) and norBNI (nor-Binaltorphimine dihydrochloride), a selective *k*-OR antagonist were purchased by Tocris (St Lois, MO, USA). Melanin-Concentrating Hormone (MCH, H-1482) was provided by Bachem (Bubendorf, Switzerland).

2.2. Pharmacological studies

Two different approaches were addressed in this study. First, to identify MCH targets we used two groups: 1) control rats (i.c.v. vehicle) and 2) MCH treated rats (i.c.v. MCH). Second, to study the effect of opioid receptor antagonists on acute

MCH-induced food intake, rats were fed *ad libitum* and organized in three groups (n=10/per group) receiving double i.c.v. injection: 1) vehicle + vehicle; 2) vehicle + MCH; 3) opioid receptor antagonist + MCH. I.c.v. cannulae aimed at the lateral ventricle were implanted as describe previously (Romero-Picó et al., 2013). After 5 days of recovery, opioid receptor antagonist was administered i.c.v. 20 min prior MCH injection. The doses employed to inhibit total opioid receptors (naloxone) and *k*-OR (norBNI) was 75 nmol and 40 nmol, respectively (Romero-Picó et al., 2013). The dose of MCH utilized to stimulate acute food intake at 2h was 8.4 nmol/rat in 5µl. WT and *Oprk*^{-/-} mice (n=8 *per* group) received a single injection of vehicle or MCH (4.2 nmol/mouse in 2 µl) as previously described (Imbernon et al., 2016). We measured food intake over 2h and then animals were sacrificed and brains frozen at -80 °C. For molecular studies in rats, we sacrificed the animals at 15, 30, 90 or 120 min and froze the brains at -80°C until nuclei isolation.

2.3. Genetic approaches

We used adeno-associated viruses (AAV) encoding scramble or short hairpin *kappa* opioid receptor subtype 1 mRNA (shOprk1) as previously described (Imbernon et al., 2016; Romero-Picó et al., 2013). Stereotaxic injections of AAV into specific brain areas was carried out using previously established coordinates (Imbernon et al., 2016; Romero-Picó et al., 2013): PVH (\pm 0.5 mm from the midline, 1.9 mm posterior to bregma, and 8 mm ventral from the surface of the skull); ARC (\pm 0.3 mm lateral, - 2.8 mm antero-posterior, and 10.2 mm dorso-ventral); LHA (\pm 2 mm lateral, - 2.85 mm antero-posterior, and 8.1 mm dorso-ventral). For each nucleus studied we set up four groups (n=10 rats /per group): 1) scramble-AAV + vehicle i.c.v.; 2) scramble-AAV + MCH i.c.v.; 3) shOprk-AAV + vehicle i.c.v.; 4) shOprk-AAV + MCH i.c.v. 15 days after the stereotaxic surgery, an i.c.v. cannula was implanted in the lateral ventricle and 5 days later, we administered vehicle (saline) or MCH i.c.v. and measured food consumption at 2h. Then, rats were sacrificed and brains were frozen at -80°C.

We utilized null adenoviruses (Control-Ad) or adenoviruses encoding a constitutively active p70S6Kinase form (CAS6K-Ad) (Blouet et al., 2008) to study the molecular cascade involved in MCH action. Plasmid pRK7-HA-S6K1-F5A-E389-

R3A was courteously gift by Dr. Clémence Blouet. Cloning and package in Ad5-CMV-GFP adenoviruses was performed at Viral Vector Production Unit (Universitat Autònoma de Barcelona). Four groups (n=10 rats *per* group) were subjected to stereotaxic surgery: 1) Control-Ad + vehicle i.c.v.; 2) Control-Ad + MCH i.c.v.; 3) CAS6K-Ad + vehicle i.c.v.; 4) CAS6K-Ad + MCH i.c.v. Adenoviruses were bilaterally injected in the LHA (\pm 2 mm lateral, - 2.85 mm antero-posterior, and 8.1 mm dorso-ventral). One week after surgery, an i.c.v. cannula was implanted and 5 days later we i.c.v. injected vehicle or MCH. At 2h, we measured food intake and rats were sacrificed and brains were stored at -80°C until analysis.

2.4. RNA isolation and TaqMan

Total RNA isolation from PVH, ARC, and LHA was performed following TRIZOL Reagent manufacture's protocol. Reverse transcriptase (RT) and real-time PCR (TaqMan) was done as previously described (Lopez *et al*, 2008). Primers used in this study are depicted in Table I (Supplementary information).

2.5. Western Blot

Total protein extract was obtained from LHA using lysis buffer (50 mM Tris-HCl pH=7.5, 1 mM EGTA, 1 mM EDTA, 1% Triton-X100, 1 mM Sodium Orthovanadate, 50 mM Sodium Fluoride, 5 mM Sodium Pyrophosphate, 0.27 M sucrose). We charged 20 μ g of total protein in 8-10% SDS-PAGE acrylamide gels. Proteins were transferred to Immuno-Blot PVDF membranes (BIORAD) with 0.2 or 0.45 μ m pore size. The membranes were subsequently blocked with 3-5% BSA in TBS 0.1% tween (TBS-T) for 1h. Membranes were incubated overnight with primary antibodies in blocking solution at 1:1000 dilution: goat anti-k-OR (Sigma SAB2501442), rabbit anti-phospho-ERK (Cell Signaling #4370), rabbit anti-p70S6Kinase (Cell Signaling #9202), rabbit anti-phospho-S6 ribosomal protein (Ser235/236) (Cell Signaling #2211), mouse anti-S6 ribosomal protein (Cell Signaling #2317) and mouse anti- β -Actin (Sigma A5316). Afterwards, membranes were washed three times 10 minutes with TBS-T, incubated 1h with secondary antibodies (DAKO) at 1:5000 dilutions, and again washed before protein detection using ECL chemiluminescent western blot substrate (Thermo Scientific). Images were quantified by ImageJ software.

2.6. Immunohistochemistry and GFP visualization

Brains were fixed in 4% formaldehyde calcium. Paraffin-embedded coronal brain sections (5 μ m) were dried overnight at 55-60 °C, de-paraffined with xylene and then rehydrated. Antigenic recuperation was performed incubating 20 min at 97°C in a 10 mM Tris-EDTA buffer. For immunohistochemistry (IHC), sections were incubated overnight at 4°C with goat anti-*k*-OR receptor (Sigma SAB2501442) diluted 1:3000, rabbit anti-phospho-S6 (Cell Signaling #2211) (1:200), or rabbit anti-p70S6Kinase (Cell Signaling #9202) (1:100) in EnVision Flex Antibody diluent (DAKO). After three washes, sections were incubated with LSAB-DAKO secondary for 30 min. Images were captured in a conventional microscopy (Olympus XC50). Quantification of phospho-S6 immuno-positive signal was performed through Frida software.

Immunofluorescence was destined to test specific nuclei injection and co-localization studies. Sections were incubated with rabbit antibody against green fluorescent protein (GFP) (Abcam ab290) (1:1000), goat anti-*k*-OR receptor (Sigma SAB2501442), or rabbit anti-MCHR (Abnova PAB16225). To visualize green positive signal, we used a goat anti-rabbit Alexa 488 (for GFP) or donkey anti-goat Cy2 (for *k*-OR) as secondary antibodies (1:500). To visualize red positive signal, we used donkey anti-rabbit Cy3 (for MCHR) (Jackson ImmunoResearch laboratories, UK). Images were captured in a confocal microscopy (Leica TCS-SP2).

2.7. Statistical Analysis and Data Presentation

Results are expressed as mean \pm SEM. GraphPad Prism (version 4.0) was used for the data analysis. Comparison between two groups was performed using an unpaired *t*-test. One-way ANOVA followed by Bonferroni's multiple comparison test was used to compare three treatments (vehicle, MCH, and norBI+MCH). Two-way ANOVA was used to examine interactions between variables (*k*-kOR silencing x MCH or S6K activation x MCH effect) followed by a Bonferroni's post-hoc test. Sample size and statistical values are defined in each figure legend. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Pharmacological or genetic inhibition of the *k*-OR System blunts MCH-orexigenic effect

Central MCH administration in rats significantly increased food intake after 2h (vehicle vs MCH, $p < 0.01$) (Figures 1A and 1B). The orexigenic effect of MCH was completely blocked by the general opioid receptor antagonist naloxone (75 nmol) (MCH vs MCH+naloxone, $p < 0.001$) (Figure 1A). This effect was likely mediated by the *k*-OR receptor since the specific opioid receptor antagonist norBNI (40 nmol) significantly blocked the hyperphagic action induced by MCH at 2h to similar extent (MCH vs norBNI+MCH, $p < 0.05$) (Figure 1B).

Dose of antagonists used were chosen based on a dose- and time-response experiment. We found that 30 $\mu\text{g}/\text{rat}$ (equivalent to 75 nmol naloxone and 40 nmol norBNI) (i.c.v.) is the minimal amount required to significantly reduce food intake over 1-2h in fasted rats (Supplementary Figure S1A and S1B).

In our experimental model, we did not include a treatment group with naloxone or norBNI alone, without co-injection of MCH, since it was previously demonstrated by our group that under the same experimental conditions, the non-selective inhibition of opioid system with naloxone, or the pharmacological inhibition of *k*-OR using norBNI have not a *per se* effect on food intake during the acute measurement of food consumption (2h) (Romero-Picó et al., 2013).

Naloxone and norBNI were delivered 20 min prior to MCH administration. It has been shown, that at 20 min, i.c.v. norBNI produces transient effects at both mu- and delta- receptors (Horan et al., 1992). Since norBNI is a long-lasting antagonist (up to 3 weeks *in vivo*) (Bruchas et al., 2007), we hypothesized that if the effects of norBNI mediating MCH-induced food intake were specific, the injection of norBNI 24h prior to MCH administration would recapitulate its response. We found that identically to 20 min prior to MCH injection, norBNI injected 24h prior to MCH blunted its orexigenic action (Supplementary Figure S2). Moreover, this additional experiment reinforces the fact that norBNI *per se* has not an anorectic effect 2h after i.c.v.

In order to confirm the physiological relevance of the *k*-OR system on acute

MCH-induced feeding, we i.c.v. administered MCH in mice lacking functional *k*-OR. We found that contrary to the orexigenic effect of i.c.v. MCH in wild type mice, MCH was not able to stimulate food intake in *Oprk*^{-/-} mice (Figure 1C). These results demonstrate that MCH needs a functional central *k*-OR system to stimulate food consumption.

3.2. The functional interaction between MCH and *k*-OR occurs in the LHA

Recent data have shown that MCHR/*k*-OR receptors are co-expressed in LHA (Imbernon et al., 2016). To investigate if this coincidence was restricted to the LHA or it could be found in other hypothalamic sites, we performed double IHC of MCHR and *k*-OR. Our data showed strong co-localization of both receptors in the ARC (Figure 1D-F) and the PVH (Figure 1G-I). Besides this morphological evidence, to evaluate the specific hypothalamic nucleus responsible of the MCH-*k*-OR interaction, we stereotaxically delivered AAV encoding a shRNA against *k*-OR (shOprk1) in those places (Figure 2A). Infection efficiency was assessed by expression of GFP (Figure 2B-D) and decreased *k*-OR levels in the PVH (Figure 2E and 2F), LHA (Figure 2G and 2H), and ARC (Figure 2I). To exclude any shRNA-induced saturation of endogenous neuronal microRNAs pathway potentially masking our results, we analyzed mRNA expression of mir124 and mir138 in LHA, two markers of toxicity (van Gestel *et al*, 2014). No changes were observed in the expression of mir124 and mir138 after the knockdown of *Oprk1* in the LHA compared to AAV encoding scramble shRNA, indicating that the use of shOprk1-AAV is viable for mRNA *Oprk1*-silencing (Figure 2J). Of note, we have previously demonstrated that this shRNA for *k*-OR does not affect expression of the other opioid receptors, supporting the specificity of this knockdown (Romero-Picó et al., 2013).

Then, we analyzed the acute effect of MCH i.c.v. on food intake, and expectedly we found that cumulative food intake was significantly increased in Control-AAV animals. The orexigenic action of MCH i.c.v. was maintained when *k*-OR was down-regulated in the ARC or PVH (Figure 2K). However, when *k*-OR was down-regulated in the LHA, we observed a significant interaction between *k*-OR silencing and MCH orexigenic response ($P_{\text{interaction}}=0.0121$, $F=7.106$, $DF=1$). While control rats treated with MCH showed a marked hyperphagia during the 2h period post-injection ($P_{\text{vh vs MCH}}<0.0001$), MCH-induced food intake was significantly decreased when we knocked down the *k*-OR specifically in LHA ($P_{\text{MCH vs}}$

shOprk/MCH=0.0087) (Figure 2K). These results suggest that *k*-OR signaling in the LHA is essential for the effect of MCH on feeding.

3.3. MCH increases phosphorylated levels of ERK in the LHA

Once we had established that *k*-OR located in the LHA was contributing to MCH-induced hyperphagia, we investigated the downstream molecular mechanisms involved in the interaction between MCH and *k*-OR within the LHA. Since the inhibition of *k*-OR affects the orexigenic MCH response, we postulated that the *kappa* opioid system might act downstream MCH signaling.

First, we corroborated the accuracy of nuclei isolation by assessing the mRNA expression of specific markers, namely pro-opiomelanocortin (POMC) in the ARC, corticotropin-releasing hormone (CRH) in the PVH, and proOrexin in the LHA (Supplementary figure S3).

k-OR activation is known to activate extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK), and p38 MAPK *in vitro* and *in vivo* (Bruchas and Chavkin, 2010). Since the best characterized opioid-induced Mitogen-Activated Protein Kinases (MAPK) network to date is ERK1/2, we analyzed the activity of ERK under central MCH stimulation. We observed that MCH induced the phosphorylation of ERK in the LHA at 15 min ($p < 0.05$) (Figure 3A). This molecular effect rapidly disappeared, with the molecular changes not detected at 30 or 120 min. Thus, the rapid phosphorylation of ERK in the LHA after 15 min i.c.v. MCH injection denotes an activation of *k*-OR system by MCH. The increased levels of phospho-ERK were accompanied by reduced ribosomal S6 activity in LHA (Figure 3B), since rats treated with MCH showed a significant reduction of phosphorylated-S6 form in the LHA, an effect absent after 90 min (Figure 3C-I).

3.4. Activation of p70S6Kinase in LHA impairs MCH orexigenic effect

If the hypothesis that MCH acts in the LHA by attenuating the S6 activity is correct, the activation of its upstream direct regulator would be expected to block the orexigenic effect of MCH. In order to test this idea, we injected an adenovirus encoding a constitutive active form of p70S6K (CAS6K-Ad) in the LHA (Figure 1A). We confirmed the efficiency of the viral vector by detecting increased p70S6K protein levels in the LHA 12 days after administration of CAS6K-Ad compared to control rats (Figure 4B-D). Although the highest expression of p70S6K was detected

at day 12, activation of p70S6K was significant from day 4 (Figure 4E). Next, we i.c.v. administered vehicle or MCH (8.4 nmol) in rats previously injected with either Control-Ad or CAS6K-Ad in LHA, and we found that the activation of p70S6K in the LHA attenuated MCH-induced food intake ($P_{\text{MCH and CAS6K interaction}}=0.009$, $F=7.646$, $DF=1$) (Figure 4F). A post-hoc test indicated that CAS6K-Ad rats stimulated with MCH ate significantly less than control-MCH animals ($p<0.05$).

At the molecular level, we confirmed the up-regulation of p70S6K form in the LHA under CAS6K-Ad activation ($p<0.001$) (Figure 4G). On the other hand, to further validate the overexpression model we checked the direct downstream target of p70S6K. The ratio phospho-S6/S6 increased when p70S6K was constitutively activated under MCH i.c.v. stimulation ($p<0.05$) (Figure 4H).

4. Discussion

Molecular/synaptic mechanisms of how MCH controls feeding have previously been detailed by the DiLeone group (Georgescu et al., 2005; Sears et al., 2010). They demonstrated that MCH regulates glutamate receptor activity in the nucleus accumbens (NAc), where it acts to modulate feeding behavior. However, within the hypothalamus the molecular mechanisms responsible of MCH-induced orexigenic response are not resolved. Here, in keeping with previous findings (Lopez et al., 2011), we observe that central non-selective pharmacological inhibition of the opioid system with naloxone impairs acute MCH-induced appetite. Furthermore, our results also document that the *kappa* opioid system located in LHA plays an important role in modulating the MCH-mediated orexigenic response in rats. Certainly, the degree of blockade observed for virogenetic-induced knockdown of *k*-OR in the LHA was lower than for the i.c.v. pharmacological blockade. This observation was somewhat expected since it is well known that a lower number of neurons are affected by the virogenetic approach. Moreover, we cannot rule out a potential extra-hypothalamic MCH action through *k*-OR system. Indeed, a good candidate is the NAc where MCHR1 is strongly expressed. Further studies to address this issue are clearly merited. Whatever the explanation, our data conclusively show that within the LHA the mechanism of action of MCH involves the hypothalamic *k*-OR system and led us to hypothesize that that *k*-OR system may play an important role modulating the acute MCH orexigenic response similarly to what occurs with ghrelin in ARC. Noteworthy, we previously demonstrated that the blockade of *k*-OR signaling in ARC

is sufficient to interfere with the orexigenic ghrelin response mediated by NPY in an AMPK-independent manner (Romero-Picó et al., 2013).

The role of the opioid system on feeding regulation is well established (Bodnar, 2015; Gosnell et al., 1986; Nogueiras et al., 2012). Within the mesolimbic dopamine reward system, opioids promote appetite for palatable foods through the ventral tegmental area (VTA) and the NAc network (Noel and Wise, 1995). Besides the hedonic aspects of feeding regulation by the opioid system, opioid receptors are widely expressed in the hypothalamus (Mansour et al., 1994) and interact with homeostatic signals to control food intake. Indeed, the cross-talk between the opioid system and ARC neuropeptides, such as POMC (Pennock and Hentges, 2011), AgRP (Hagan et al., 2001), and NPY (Kotz et al., 1993; Schick et al., 1991) has been previously described. MCH receptors and *k*-OR are co-localized in different hypothalamic areas including ARC, PVN, and LHA, however we found that MCH-induced hyperphagia was prevented by genetic inhibition of *Oprk1* only in LHA, indicating that this hypothalamic area is the most relevant for MCH/*k*-OR interaction [in terms of food intake](#). Nevertheless, it is becoming clear that the functional interaction of the *k*-OR- and MCH-systems is likely to go further than the effect described here. The presence of *k*-OR in MCH neurons indicates that the activity of MCH neurons is also regulated by endogenous opioid-neurons. In fact it is known that dynorphin neurons are co-localized with MCHR1 neurons (Chee et al 2013) and that *k*-OR is co-localized with MCH neurons (Parks et al., 2014). These anatomical and biochemical findings imply strong functional relationships between the two systems. Further studies assessing the role of the *k*-OR as a potential mediator of MCH in other brain systems like dopaminergic reward areas such as the NAc and VTA are warranted. Similarly, the functional significance of the *k*-OR in MCH neurons and putative regulation by endogenous opioid peptides needs to be uncovered.

The LHA is historically considered as a feeding center that connects the homeostatic circuitries to the hedonic ones (Castro et al., 2015). While MCH is mainly produced in the LHA, MCH neurons project to numerous hypothalamic and extra-hypothalamic areas, where MCHR is expressed at the post-synaptic level. Thus, MCH does seem to have a role in the LHA through its interaction with *k*-OR system, despite the low levels of MCH receptors detected in this area (Chee et al., 2013). Although we found a clear crosstalk between MCH and *k*-OR in the LHA, we cannot rule out that this

interaction may also cause molecular changes on extra-hypothalamic nuclei to where MCH neurons project, such as VTA or NAc. Further experiments are necessary to answer these questions and to elucidate the whole MCH neuronal network.

Importantly, our data show that the crosstalk MCH/*k*-OR reaches the physiological target of the growth factor-activated p70S6K, namely the 40S subunit of the S6 ribosomal protein that play a key role in modulating translational efficiency. The phosphorylation of S6 ribosomal protein is a valuable hallmark of neuronal activity and its biological role in the brain is one of the major challenges nowadays. p70S6K possesses autoinhibitory and catalytic domains. Activation of p70S6K occurs through a complex series of phosphorylation events on eight or more serine or threonine residues. As reviewed by Dufner and Thomas (Dufner and Thomas, 1999), these phosphorylation sites are S404, S411, S418, S424 and T421 on the C-terminal autoinhibitory domain and T229, S371 and T389, which are critical for catalytic activity. Autoinhibitory sites, on the other hand, are thought to be phosphorylated by members of the mitogen-activated protein kinase (MAPK) family, p38 and ERK (Mukhopadhyay et al., 1992) and elicit a conformational change to facilitate phosphorylations in the catalytic domain. Previous studies linked ERK with the regulation of S6K (Lee et al., 2016). Although these studies were undertaken in a different context, during dopaminergic neuronal differentiation of human neuronal stem cells, the authors observed that the increase in phospho-ERK was accompanied by a reduction in phospho-p70S6K. Conversely, inhibition of ERK significantly increased the levels of phospho-p70S6K without activation of Akt and mTOR, indicating that ERK might directly inhibit p70S6K. Due to the complexity of p70S6K activity, we decided to focus on its direct downstream target in order to identify if *k*-OR activation by MCH and increased phosphorylated ERK (phospho-ERK) levels would imply changes in the active (phosphorylated) form of S6. Our results show that *in vivo* the increased level of phospho-ERK induced by i.c.v. MCH administration was accompanied by a significant reduction of phospho-S6 protein levels at 15 min. This observation also suggests that p70S6K is regulated by ERK signaling pathway during the acute control of food intake *in vivo* studies. This rapid response mediated by the activation of *k*-OR might trigger other molecular cascades to regulate food consumption since the highest orexigenic physiological response induced by MCH is observed at 2h where molecular changes in phospho-ERK and phospho-S6 are absent.

Nevertheless, this is the first report of the link between hypothalamic *k*-OR activation and the regulation of p70S6K pathway through MCH in the central nervous system (CNS), demonstrating the crosstalk between ERK and S6 during acute food intake regulation mediated by MCH.

Finally, it has been previously reported that depression of p70S6K pathways in the mediobasal hypothalamus (MBH) during fasting promotes cellular mechanisms to stimulate appetite whereas the specific activation of p70S6K reduces food intake and protects against the adverse effects of a high fat diet (Blouet et al., 2008). Our findings indicate that the activation of p70S6K in LHA decreases the food intake promoted by MCH, thus providing additional evidence of the relevance of this kinase on the control of appetite.

5. Conclusion

In summary, we describe a novel interaction by which the ERK and p70S6K/S6 cascades are modulated by MCH through *k*-OR activation during the acute MCH-induced orexigenic response. We found that (a) administration of MCH rapidly activates *k*-OR in the LHA and increases phospho-ERK protein levels; (b) elevated phospho-ERK levels reduce S6 activity in this area; and (c) at the functional level, constitutive activation of S6 upstream kinase (p70S6K) in LHA significantly decrease the acute food intake induced by MCH. Thus, our findings indicate that *k*-OR integrates central MCH signaling with the p70S6K/S6 pathway to regulate the action of MCH on acute food intake regulation (Figure 5).

Taking into account that MCH plays a major role in the control of energy homeostasis, and that MCH receptor antagonists are in the pipeline of several pharmaceutical companies, our data support the relevance of the hypothalamic *kappa* opioid system as a potential drug target. In keeping with this, the recent approval of non-selective opioid receptor antagonists for treatment of obese patients adds translational value to our findings.

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Supplementary data

Supplementary information related to this article is available at the *Neuropharmacology* website.

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Figure Legends

Figure 1. Central blockade of *kappa* opioid receptor (*k*-OR) disrupts MCH orexigenic response in rats and mice animal models. (A-B) MCH-stimulated food intake upon 2 hours after intracerebroventricular (i.c.v.) pre-administration of either vehicle or (A) 75 nmol naloxone or (B) 40 nmol norBNI in rats (n=10 *per* group). (C) MCH-induced food consumption 2 hours after i.c.v. injection of either vehicle or 4.2 nmol MCH in wild type (WT) mice (n=12-13 mice/group), and mutant mice for functional *k*-OR (*Oprk1*^{-/-}) (n=10-11 mice/group). (D-I) Representative images of two-color immunofluorescence showing a subpopulation of ARC neurons that exhibits immunoreactivity for (D) MCHR (in red), (E) *k*-OR (in green) and (F) a merged image of the two. Corresponding immunofluorescent labeling was also carried out in the PVH: singly-labeled neurons appear either (G) red or (H) green, whereas (I) co-expression (arrowhead) is indicated by yellow. Scale bars: 75 μ m. Bar graphs on the right indicate the degree of co-expression. Values are represented as means \pm SEM. Acute food intake in (A-B) rats was analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons, while food consumption in (C) mice was evaluated by a student t-test. * represents differences compared to vehicle and # indicates differences compared to MCH-treated animals.

Figure 2. Blockade of *k*-OR in the LHA using an AAV-particles lead to a reduction of food intake elicited by MCH. (A) Illustration of the specific rat hypothalamic nuclei (PVH, ARC, and LHA) where shOprk1-AAV were injected to knockdown *k*-OR signaling. (B-D) Representative GPF visualization of targeted hypothalamic nuclei: (B) PVH, (C) ARC, (D) LHA. Scale bars: 500 μ m. (E-F) Demonstration of *k*-OR silencing by immunohistochemistry after 2 weeks of either (E) Control-AAV or (F) shOprk-AAV injection in PVH. Scale bars: 500 μ m. (G-H)) mRNA *Oprk1* or protein expression of *k*-OR after shOprk-AAV injections in LHA measured by (G) TaqMan and (H) western blot (n=8 *per group*). (I) mRNA *Oprk1* levels in ARC following injection of either Control-AAV or shOprk1-AAV into this nucleus (n=8 *per group*). (J) mir124 and mir138 mRNA expression in LHA to test the viability of shOprk-AAV once targeted into the LHA (n=8 *per group*). (K) Food intake stimulated by MCH (8.4 nmol) in rats at 2 hours under specific silencing of *k*-OR into the PVH (n=10 *per group*), ARC (n=10 *per group*), or LHA (n=20 *per group*). Values are expressed as means \pm SEM. Student t-test was performed to (E-I) confirm significant reduction of *k*-OR receptor, or (J) assay mir124 and mir138 expression. Two-way ANOVA followed by Bonferroni's multiple comparison was used to evaluate the interaction MCH/*k*-OR system on food consumption. a=MCH orexigenic effect (p<0.001), b=shOprk-AAV knockdown effect, and c=significant MCH/shOprk-AAV knockdown interaction (p<0.05) in (K) rats stereotaxically treated with either Control-AAV or shOprk-AAV in LHA.

Figure 3. Rapid activation of *k*-OR mediated by MCH lead to a reduction of phosphor-S6 in LHA. (A) Western Blot for phosphorylated levels of ERK performed in LHA rat samples, and normalized against β -actin after 15, 30, and 120min i.c.v. MCH (8.4 nmol) administration (n=8 *per group*). Lines reflect the cropped sites of the western blot images. (B-I) Immunohistochemistry detection of (C-I) phospho-S6 (pS6) expression, and (B) localization in LHA in rats treated with (C, G) vehicle, (D, H) MCH (8.4 nmol), and (E, I) norBNI (40 nmol)/MCH (8.4 nmol) at (C-E) 15 min, and (G-I) 90 min (C) (n=6-10 *per group*). Scale bars: (B) 2mm; (C-I) 500 μ m. Bar graphs on the right indicate the protein levels expressed as percentage normalized to vehicle group. Values are represented as means \pm SEM. For all pairwise comparisons a (A) student t-test was used, whereas in cases with three groups, a (C-I) one-way

ANOVA followed Bonferroni's multiple comparisons were used. * indicates differences compared to vehicle and # indicates differences compared to MCH-treated animals.

Figure 4. Constitutive activation of p70S6K (CAS6K) in LHA decreases the food intake in rats centrally treated with MCH. (A) Injection of Ad vectors encoding for a green fluorescent protein (GFP) to test a specific site of expression in LHA. Scale bar: 1mm. (B-D) Immunohistochemistry protein detection of p70S6K in either (B) Control-Ad or (C) CAS6K-Ad rats ($n=4$ per group), and (D) negative control. Scale bars: 500 μ m. (E) Western blot for p70S6K performed in LHA samples and normalized against β -actin from day 0 (control) to day 12 ($n=4$ per group). (F) Cumulative food intake stimulated by i.c.v. MCH (8.4 nmol) at 2 h under the activation of p70S6K using CAS6K-Ad particles ($n=10$ per group). (G) Protein levels of p70S6K measured by western blot and corrected by β -actin in either Control-Ad or CAS6K-Ad vehicle rats. (H) Ratio of phosphor-S6/S6 analyzed by western blotting and normalized against β -actin to validate the constitutively activation of p70S6K ($n=10$ per group). Values of bar graphs are represented as means \pm SEM. Two-way ANOVA followed by a Bonferroni's Multiple Comparison was used to (F) evaluate the interaction between CAS6K activation and MCH orexigenic effect. a=MCH significant effect, and c=significant interaction. Student's t-test analysis was used to evaluate (E) the expression of p70S6K over 12 days in rats treated with CAS6K-Ad compared to control rats, and (G, H) the activation of p70S6K/S6 pathway in CAS6K-Ad rats compared to Control-Ad animals. $P<0.05$ was considered statistically significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) compared to Control-Ad group.

Figure 5. Scheme summarizing the mechanism of action used by MCH in the LHA to regulate the acute food intake. (A) i.c.v. MCH administration rapidly activates k -OR by enhancing phospho-ERK, and the activation of k -OR has an inhibitory effect on p70S6K/S6 pathway leading to an increase of food intake over 2 h. The reduction of appetite caused by (B) pharmacological inhibition of k -OR (norBNI) or (C) CAS6K-Ad activation in LHA demonstrate a crosstalk between the physiological orexigenic response of MCH and these two systems.

Figure 1

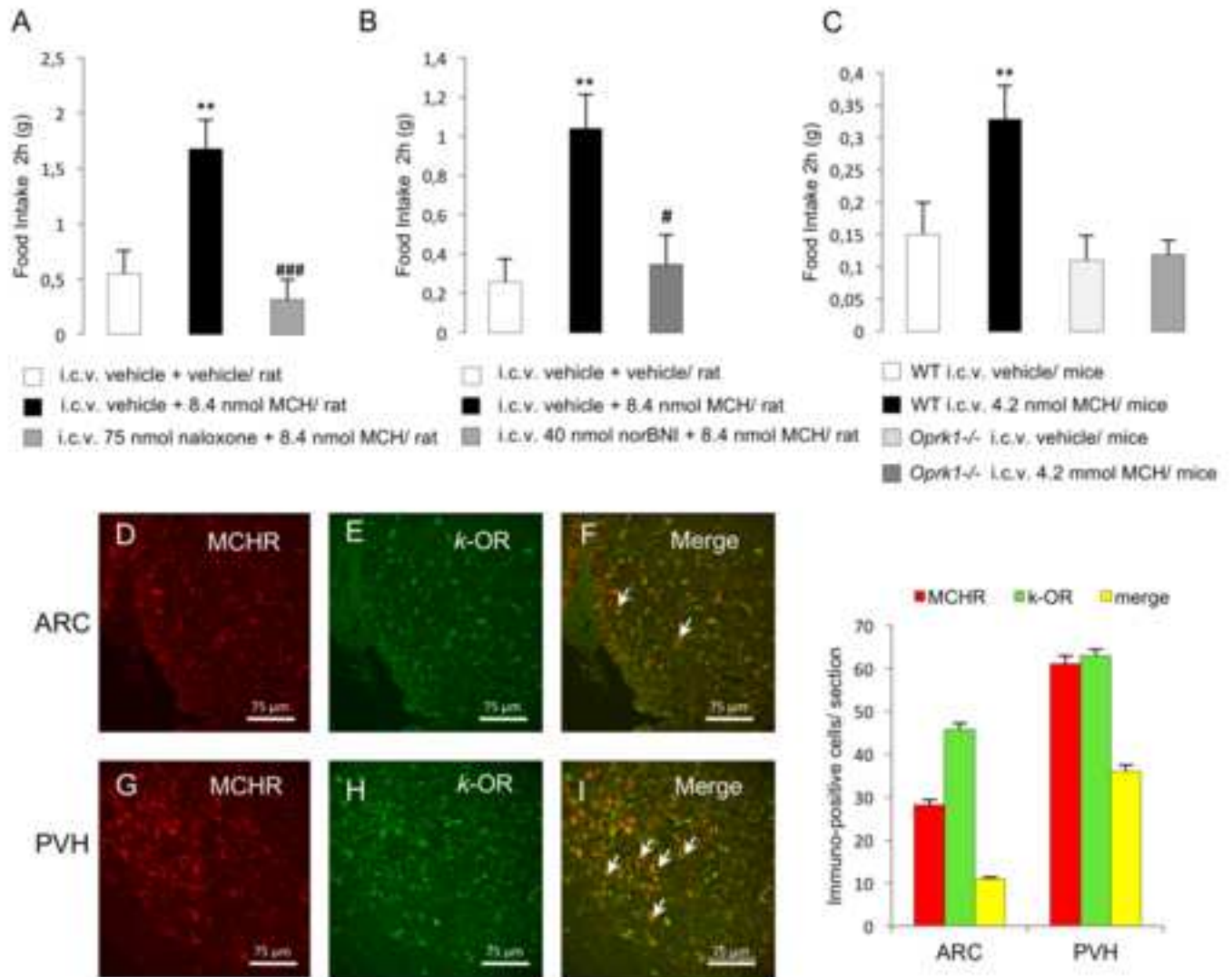


Figure 2

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Figure 2

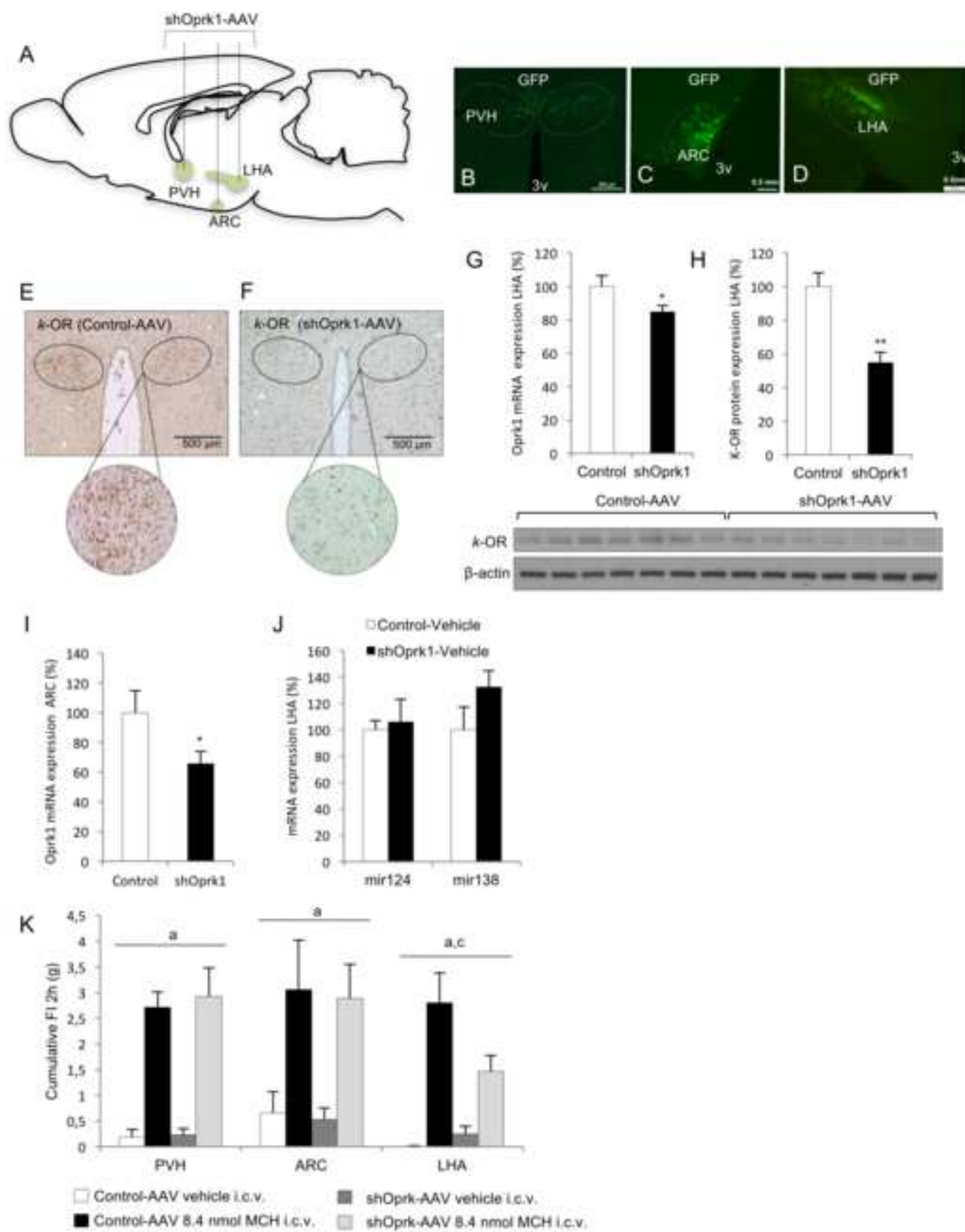


Figure 3

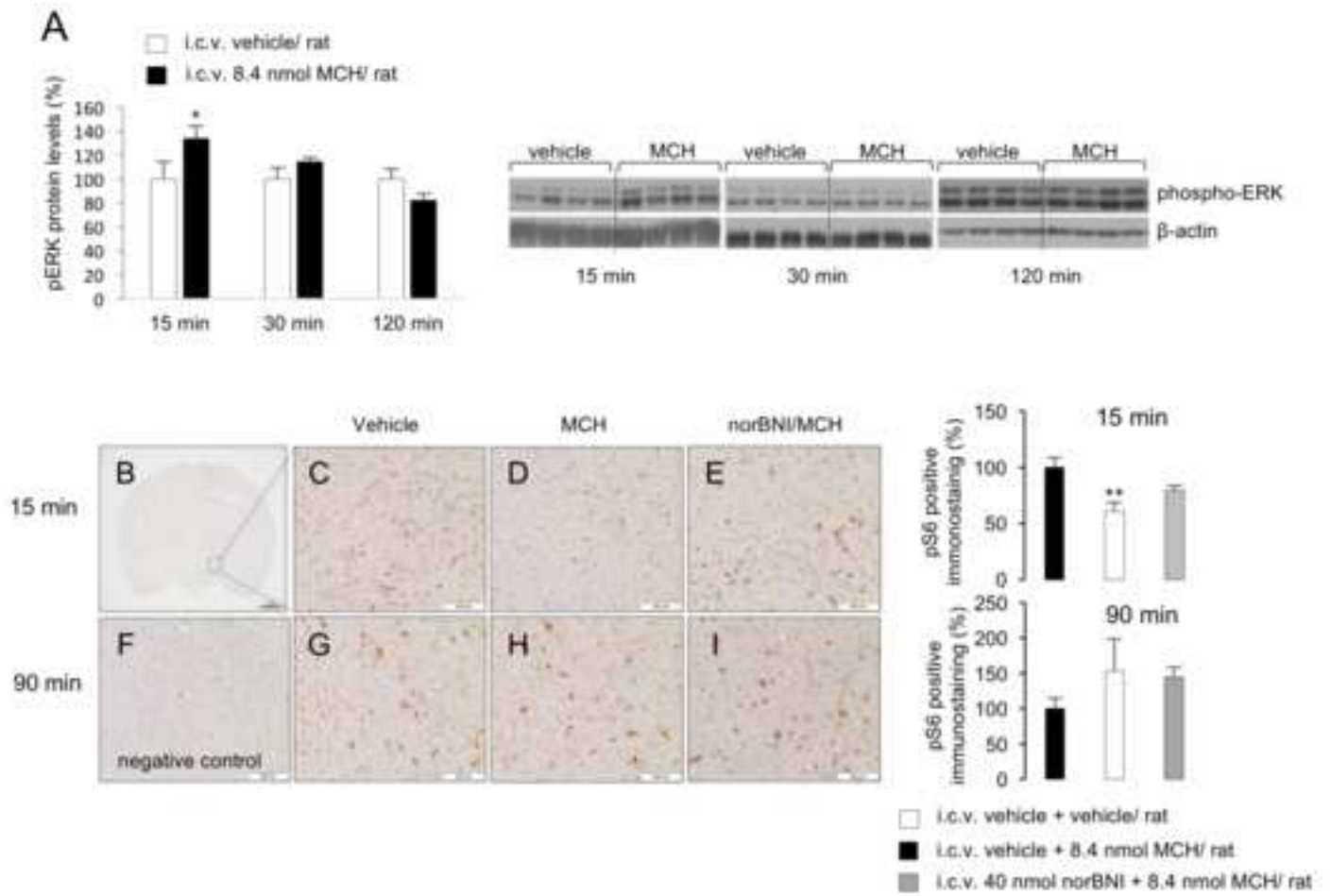


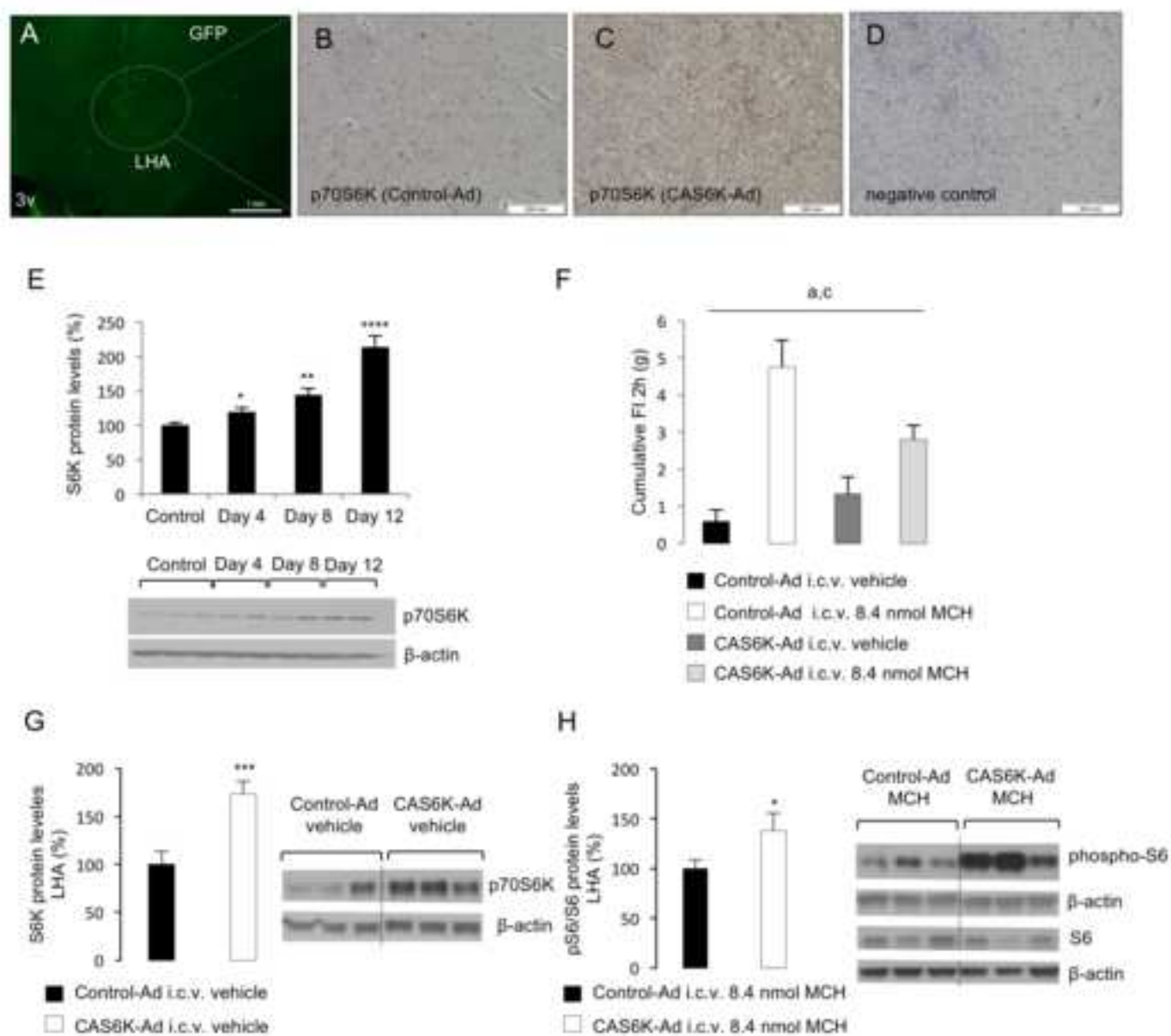
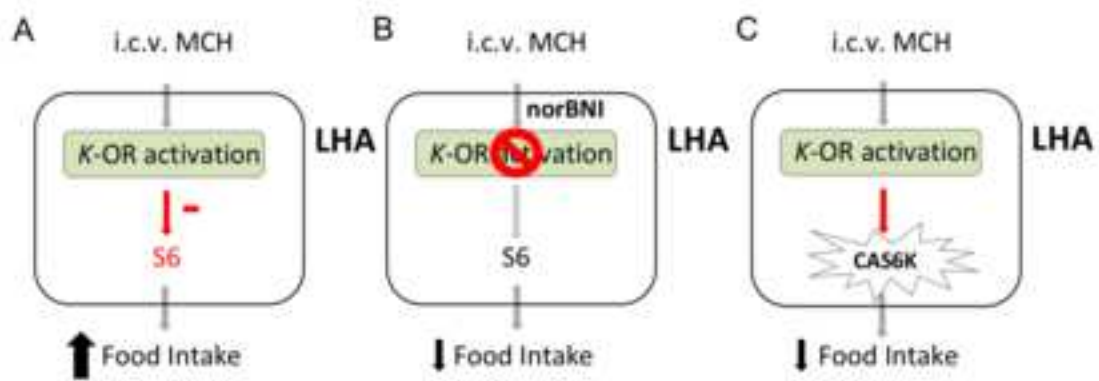
Figure 4[Click here to download high resolution image](#)**Figure 4**

Figure 5



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