Hypothalamic eIF2α Signaling Regulates Food Intake

Anne-Catherine Maurin,1 Alexandre Benani,2 Anne Lorsignol,3 Xavier Brenachot,2 Laurent Parry,1 Valérie Carraro,1 Christophe Guissard,1 Julien Averous,1 Céline Jousse,1 Alain Bruhat,1 Cédric Chaveroux,1 Wafa B’chir,1 Yuki Muranishi,1 David Ron,2 Luc Pénicaud,2 and Pierre Fafournoux1,*

1UMR 1019 Nutrition Humaine, INRA, Université Clermont 1, Centre de Clermont-Ferrand-Theix, 63122 Saint Genès Champel, France
2Centre des Sciences du Goût et de l’Alimentation, UMR 6265-CNRS/1324-INRA, Université de Bourgogne, 21000 Dijon, France
3STROMAlab, UMR 5273-CNRS Université Paul Sabatier, EFS, U1031 INSERM, BP 84225, 31432 Toulouse Cedex 4, France
4University of Cambridge, Metabolic Research Laboratories and NIHR Cambridge Biomedical Research Center, Cambridge CB2 0QQ, UK
*Correspondence: pierre.fafournoux@clermont.inra.fr
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SUMMARY

The reversible phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α) is a highly conserved signal implicated in the cellular adaptation to numerous stresses such as the one caused by amino acid limitation. In response to dietary amino acid deficiency, the brain-specific activation of the eIF2α kinase GCN2 leads to food intake inhibition. We report here that GCN2 is rapidly activated in the mediobasal hypothalamus (MBH) after consumption of a leucine-deficient diet. Furthermore, knock-down of GCN2 in this particular area shows that MBH GCN2 activity controls the onset of the aversive response. Importantly, pharmacological experiments demonstrate that the sole phosphorylation of eIF2α in the MBH is sufficient to regulate food intake. eIF2α signaling being at the crossroad of stress pathways activated in several pathological states, our study indicates that hypothalamic eIF2α phosphorylation could play a critical role in the onset of anorexia associated with certain diseases.

INTRODUCTION

The regulation of food intake is one of the most essential phenomena in biology. Besides cultural and hedonic aspects, both motivation to eat and food choices largely depend on metabolic needs (Lenard and Berthoud, 2008). Part of this homeostatic regulation arises from the capacity to sense nutrient availability and to adapt food selection accordingly (Berthoud et al., 2012). The control of food intake is highly complex in the case of omnivores that have to choose among a variety of available food sources. Notably, the selection of a balanced diet is crucial to maintain the homeostasis of essential amino acids, which cannot be synthesized de novo (Harper and Peters, 1989; Morrison et al., 2012). A remarkable example of an innate mechanism governing food choice is presented by the fact that omnivorous animals will consume substantially less of an otherwise identical meal lacking a single essential amino acid (Gietzen, 1993; Harper et al., 1970). The ability to reject amino acid-imbalanced foods sources likely improves fitness by stimulating the search for healthier balanced diets (Chaveroux et al., 2010; Leung et al., 1968).

GCN2 is an ancient protein kinase that senses intracellular amino acid deficiencies (Wek et al., 1989). In response to an essential amino acid limitation, GCN2 couples the accumulation of uncharged tRNAs to the phosphorylation of eukaryotic initiation factor 2α (eIF2α) on serine 51. By this mean, GCN2 diminishes the overall protein synthesis rate, while simultaneously activating a gene expression program, mediated by the translational upregulation of the transcription factor ATF4 (Harding et al., 2000; Hinnebusch, 1993). In mammals, eIF2α is at a crossroad of a large signaling network. It can, indeed, be phosphorylated by three other protein kinases: PKR (activated by dsRNA and cytokines), PERK (activated by endoplasmic reticulum stress), or HRI (activated by heme deficiency) (Donnelly et al., 2013; Hinnebusch, 1994). Consequently, eIF2α phosphorylation is a highly conserved signal governing cell adaptation to a variety of stresses. Most importantly, the deregulation of eIF2α signaling has often been linked to various human diseases (Ron and Harding, 2007).

It has been established that GCN2 contributes to the aversive response to amino acid-imbalanced foods (Hao et al., 2005; Maurin et al., 2005). The genetic ablation of GCN2 throughout the brain prevents the initiation of the aforementioned food aversion (Maurin et al., 2005). However, the precise circuitry involved in the sensing and in the response to the lack of amino acid has not been fully established yet. Previous experiments have implicated the anterior piriform cortex (APC) in the sensing of postprandial blood amino acid levels and in the initiation of food aversion (Leung and Rogers, 1971). In a classical view, APC integrates olfactory information (Brunjes et al., 2005), whereas the homeostatic regulation of feeding behavior mainly involves the hypothalamus (Morton et al., 2006). This structure, particularly its mediobasal part (mediobasal hypothalamus [MBH]), is considered a major site for the integration of nutritionally relevant information originating from the periphery and mediated by circulating metabolites, hormones, and/or neural pathways (Blouet and Schwartz, 2010; Lenard and Berthoud, 2008).
Particularly, neurons located in the arcuate nucleus (ARC) of the hypothalamus are prone to sense directly peripheral nutrients through a weak blood-brain barrier of the median eminence hypothalamus are prone to sense directly peripheral nutrients. These circulating metabolites include glucose (Ciofi, 2011). These metabolites can be sensed by neurons located in the arcuate nucleus (ARC) of the hypothalamus. In particular, neurons located in the ARC are sensitive to fatty acids (Oomura et al., 1969; Pénicaud et al., 2006) and amino acids (Cota et al., 2006).

**RESULTS**

### A Leucine-Devoid Meal Activates GCN2 in the Hypothalamus

To understand the role of hypothalamic eIF2α signaling in the regulation of food intake, we first investigated whether GCN2 is expressed and activated in this area following a meal lacking one essential amino acid. We first mapped GCN2 expression in the mouse brain by in situ hybridization (ISH). In agreement with data from Costa-Mattioli et al. (2005), GCN2 mRNA was highly expressed in several areas, including piriform cortex, hippocampus, dentate gyrus, and hypothalamus (Figure S1). GCN2 mRNA was particularly found in the ARC of the MBH, a major site for nutrient sensing and food intake regulation (Figure 1A). These observations prompted us to hypothesize that food intake inhibition induced by an amino acid-imbalanced diet could result from GCN2 activation in the MBH. We then investigated whether a leucine-devoid (ΔLeu) meal could activate GCN2 in this area. Western blot analysis showed that high levels of phospho-eIF2α were detected in MBH extracts of wild-type mice fed a ΔLeu diet for 40 min (Figure 1B). No such signal was observed in extracts from GCN2 knockout (GCN2−/−) mice. More precisely, immunohistochemistry (IHC) analysis showed an important increase of phospho-eIF2α labeling in the ARC (Figure 1C). In order to check whether GCN2 activation/eIF2α phosphorylation was associated with a classical scheme of uptranslation of uORF-containing mRNA, ATF4 protein was analyzed by western blotting. Figure 1D shows that dietary leucine starvation induced a notable increase in ATF4 protein level in ARC extracts. Furthermore, GCN2 activation in the ARC was also associated with neuronal activation, as reflected by increased c-fos labeling (Figure S2A). The ARC contains at least two populations of neurons sensitive to nutrient-related signals with a major role in the regulation of food intake: orexigenic neurons expressing the neuropeptide Y (NPY), and anorexigenic neurons expressing pro-opiomelanocortin (POMC). The consumption of an amino acid-imbalanced meal promoted eIF2α phosphorylation in many cells of the ARC, including NPY and POMC neurons (Figure S2B).

### Hypothalamic GCN2 Activity Controls Food Intake

We reasoned that if GCN2 signaling in the hypothalamus plays a role to initiate food intake inhibition induced by an amino acid-imbalanced diet, then manipulations of GCN2 activity in this area should alter the anorectic response. In a first set of experiments, we assessed whether GCN2 activity in the ARC is required for the inhibition of food intake after the consumption of a ΔLeu meal.
of a ΔLeu meal. We knocked down GCN2 by lentiviral-mediated delivery of shRNA molecules bilaterally into the ARC (see Figure S3 for technical details). We had previously checked that GCN2-specific shRNA molecules led to a significant reduction of GCN2 mRNA level by 40% in ARC extracts as compared to control mice (Figure 2B). Although the injection of GCN2-shRNA lentivirus led to dispersion in both food intake response and GCN2 mRNA content, our data show that the magnitude of the aversive phenotype is correlated with GCN2 expression level in the ARC (Figure 2C).

We then tested whether the activation of the GCN2/eIF2α pathway alone in the MBH is sufficient to elicit an anorexic response. For that purpose, we pharmacologically activated the GCN2/eIF2α pathway in the MBH independently of the initial stimulus (one essential amino acid-devoid diet). We injected L-leucinol or vehicle (NaCl) into the third cerebral ventricle of adult mice and recorded their food intake. L-leucinol is known to increase the intracellular level of uncharged Leu-tRNA by inhibiting Leucyl-tRNA synthetase and to consecutively activate GCN2 (Ashe et al., 2001). Figure 3A shows that intracerebroventricular (i.c.v.) administration of L-leucinol caused a rapid decrease in food intake of wild-type mice (between −30% and −40% in the first 3 hr following injection), whereas it had no effect on GCN2 knockout (GCN2−/−) mice. In a parallel experiment, we evaluated GCN2 activation in response to L-leucinol administration into the third ventricle, by assessing phospho-eIF2α immunoreactivity on brain slices (Figure 3B). L-leucinol induced the phosphorylation of eIF2α in the MBH of wild-type mice, whereas it had no effect in GCN2 knockout mice. No phospho-eIF2α labeling was found in extrahypothalamic areas, demonstrating that GCN2 activation occurred specifically in the vicinity of the injection site. Particularly, no phospho-eIF2α signal was observed in the APC (Figure 3B). Furthermore, the administration of L-leucinol into the third ventricle also resulted in the accumulation of ATF4 protein in the ARC (Figure 3C). Overall, these results show that pharmacological activation of GCN2 in the MBH is sufficient to induce an anorexic response.

**Figure 2. GCN2 Knockdown in the ARC Markedly Blunts the Aversive Response to a ΔLeu Meal**

Lentivectors encoding GCN2-specific shRNA or scramble sequence were delivered bilaterally into the ARC of wild-type male mice (see Figure S3 for technical details). (A) GCN2-shRNA delivery in the ARC resulted in a loss of the aversive response to a ΔLeu meal (*p < 0.05, unpaired Student’s t test; six to seven males per group). The relative consumption of a ΔLeu versus Ctr diet during a 1 hr meal was expressed as the ratio of consumption (Δ% ± SEM) of the ΔLeu diet compared to the consumption of the Ctr diet by the same animal. We verified that GCN2 knockdown did not affect the intake of Ctr diet (see Table S1). At the end of the experiment, mice were sacrificed, then the ARC was dissected to extract total RNA. (B) GCN2-shRNA lentiviral delivery in the ARC resulted in a 40% decrease of the mean level of GCN2 mRNA expression in the ARC. Results are given as mean ± SEM (**p < 0.01, unpaired Student’s t test; six to seven males per group). (C) The strength of association between levels of food intake inhibition and GCN2 expression in the ARC was analyzed by the Pearson correlation test. See also Figure S3 and Table S1.

**elf2α Signaling in the Hypothalamus Regulates Food Intake**

Our data have demonstrated that GCN2 activity in the hypothalamus controls food intake. We next investigated whether elf2α, the substrate for GCN2, is involved in the signaling process leading to food intake inhibition. In order to raise hypothalamic phospho-elf2α levels while bypassing GCN2 activation, we used salubrinal, a drug that has been shown to prevent phospho-elf2α dephosphorylation by inhibiting the phosphatase complexes (Boyce et al., 2005).

I.c.v. administration of salubrinal into the third ventricle induced a marked decrease in food intake as measured on a balanced diet, from −40% to −50% 1 hr and 2 and 3 hr, respectively, after the beginning of the meal, as compared to vehicle injection (Figure 4A). We then confirmed that salubrinal injection into the third ventricle resulted in an increased phosphorylation of elf2α in the hypothalamus, but not in the APC (Figure 4B).
Western blot analysis showed increased ATF4 protein expression in the ARC following L-leucinol administration into the third ventricle. After overnight starvation, mice were treated for 2 hr with L-leucinol, and ARC tissues were harvested. Both cytoplasmic and nuclear protein extracts were prepared from pooled ARC tissues of four mice per group. See also Table S1.

Again, the increased level of phospho-eIF2α in the ARC was associated with ATF4 protein overexpression (Figure 4C). These results demonstrated that increasing phospho-eIF2α level in the MBH leads to food intake inhibition. We can thus conclude that eIF2α phosphorylation in the MBH appears to be a key event in the control of food intake.

DISCUSSION

Our present data highlight an important role of eIF2α signaling in the hypothalamus in the control of feeding behavior in mammals. Most particularly, the results described herein show a crucial role of the hypothalamic eIF2α kinase GCN2 in mediating anorexia toward foods with an imbalanced amino acid composition. Such a nutritional stress can be frequent for wild omnivorous animals. For instance, rodents are often confronted to partial essential amino acid deficiency when the sole protein source is a plant, often lacking some essential amino acids (Chaveroux et al., 2010). This pathway is exploited by omnivores to recognize depressions in blood amino acid levels, resulting in a behavioral response that limits consumption of imbalanced foods (Hao et al., 2005; Maurin et al., 2005) and favors, by default, a balanced diet.

Previous results have involved the APC in the detection of amino acid imbalances leading to food aversion. These conclusions have been first established from cell ablation experiments and localized injections of the limiting amino acid (Leung and Rogers, 1971). More recently, it was found that GCN2 activity was induced in the APC following the consumption of an amino acid-imbalanced meal (Hao et al., 2005; Maurin et al., 2005). Our present data demonstrate that hypothalamic GCN2 activity is sufficient to initiate food intake inhibition. This conclusion is strengthened by a previous work of Blevins et al. showing that threonine injection in the lateral hypothalamus partially blunts food aversion resulting from a threonine imbalanced diet (Blevins et al., 2003). Current available data do not allow the determination of the relative contribution of GCN2 activity in APC and hypothalamus in the response to an amino acid-imbalanced diet. Our data show that a partial GCN2 knockout in the ARC inhibits the response to a leucine-deficient meal. This result indicates that the hypothalamus is a crucial sensing area involved in the early detection of imbalanced diets. Nevertheless, this conclusion does not exclude the possibility that the APC could play a role in the regulation of food aversion. Indeed, the APC is a well-recognized site for the integration of olfactory informations (Brunjes et al., 2005), particularly in the conditioned avoidance of foods (Choi et al., 2011). It could be hypothesized that dietary amino acid imbalances could be sensed by GCN2, on the one hand, in the ARC leading to an immediate decrease in food intake and, on the other hand, in the APC for eliciting a learned avoidance behavior.

Besides sensing amino acid limitation, the hypothalamus also detects increases in blood amino acid concentration. Cota et al. showed that leucine injection into the third ventricle has...
Signal intensity was quantified using ImageJ software. Results are given as percentage of vehicle control ± SEM. The molecular events downstream of eIF2α phosphorylation leading to changes in neuronal activity and food intake inhibition remain to be identified. The phosphorylation of eIF2α promotes a decrease in the overall protein synthesis and an increase in the translation of several mRNAs including ATF4, thereby altering gene expression at the transcriptional level. Therefore, eIF2α signaling in the hypothalamus may promote the aversive response through effects on both translation and transcription. However, two observations suggest that an interaction between ATF4 and GABA receptors could be involved in this process. First, a robust, specific, and reversible interaction between these two proteins has been shown (Nehring et al., 2000; White et al., 2000). GABAB receptor (GABAB-R) subunit and ATF4 are colocalized at the membrane surface of neurons, and their interactions may affect their activities (Nehring et al., 2000). Second, GABAergic signaling has been described to modulate feeding (Itó et al., 2013; Wu et al., 2009) and, particularly, the responses to amino acid deprivation (Truong et al., 2002). Further works will be required to investigate the role of an interaction between ATF4 and GABAB-R in the control of food intake.

Considering that eIF2α phosphorylation is at the crossroad of several stress pathways activated in several pathological states (Donnelly et al., 2013), our results may have implications in the context of disease-associated anorexia. Several diseases (i.e., cancer, infectious diseases) are very often associated with an inflammatory state, which may result in a wasting disorder consisting of a combination of both increased metabolic rate and anorexia (Tisdale, 2009). The mechanisms involved in the establishment of anorexia are hardly identified. However, cytokines resulting from inflammatory states have been proposed to be one component acting on the CNS (Buchanan and Johnson, 2007; Laviano et al., 2012). We could hypothesize that peripheral inflammation could lead to eIF2α phosphorylation in the hypothalamus and could be one of the signaling processes leading to anorexia. Preliminary data show that intraperitoneal lipopolysaccharide (LPS) administration, a commonly used model to experimentally induce the full spectrum of symptoms associated with inflammation (André et al., 2008), dramatically reduces food intake in association with an increase of eIF2α phosphorylation in the hypothalamus (data not shown). Taken together, our data highlight that hypothalamic eIF2α signaling could contribute to the establishment of disease-associated anorexia. The knowledge of the signaling pathways controlling appetite will help to understand the pathophysiology of food intake disorders and is a prerequisite for their successful treatment.

**EXPERIMENTAL PROCEDURES**

**Animals, Experimental Diets, and Food Intake Measurements**

The generation of C57BL/6J GCN2 null mice has been described in detail elsewhere (Harding et al., 2000; Maurin et al., 2005). Maintenance of the mice and all experiments were approved by our institutional-animal care and use committee (CEMEA18-13), in conformance with French and European Union laws (details about permissions are given in Supplemental Experimental Procedures). Experimental diets were manufactured in our institute facilities (INRA, Unité de Préparation des Aliments Expérimentaux, Jouy-en-Josas, France).
Tissue Preparation
For tissue sampling (western blots, RT-qPCR), brains were rapidly removed in order to either collect and freeze MBH in liquid nitrogen or freeze whole brain for 2 min in isopentane cooled with dry ice. Samples were stored at −80°C until processing. For ARC dissection, brain sections 500 μm thick were prepared by using a cryostat, and ARCs were punched out using a stainless steel needle (starting approximately at −1.46 mm relative to bregma and using anatomical landmarks from a mouse brain stereotaxic atlas). For ATF4 western blot analysis, nuclear proteins were extracted from fresh ARC tissue using an adult mouse brain matrix (ASI Instruments). Nuclear protein extraction, western blot, and RT-PCR analysis are described in Supplemental Information. In order to perform IHC and ISH experiments (details in Supplemental Experimental Procedures), brains were previously fixed before freezing by transcardially perfusing mice with ice-cold 4% paraformaldehyde. Coronal sections 20 μm thick were cut with a cryostat and collected.

Lentiviral shRNA Delivery into the ARC
Details about lentiviral GCN2-shRNA strategy are given in Figure S3 and in the Supplemental Experimental Procedures. Lentiviral delivery of GCN2-specific shRNA or scramble sequence was performed bilaterally into the ARC of 12 wild-type C57BL/6 male mice (Javvier). Stereotaxic coordinates were taken relative to the bregma (anteroposterior [AP], −1.7 mm; lateral [L], ±0.3 mm) and to the skull (dorsosventral [DV], −5.8 mm). The lentiviral prepation (106 U/ml; 1 μl/side) was injected at a rate of 0.2 μl/min.

L-Leucinol Administration into the Third Ventricle
A total of 10 GCN2−/− mice and 12 GCN2+/− mice underwent stereotaxic surgery to implant a chronic stainless steel cannula (Brain Infusion Kit 2; ALZET) in the third cerebral ventricle using the following coordinates from bregma: AP, −0.825 mm; L, 0 mm; and DV, −5 mm. The cannula was fixed to the skull using screws and dental cement. Injections of L-Leucinol (Sigma-Aldrich; 10 nmol in 1 μl NaCl) or vehicle (NaCl 0.9%) into the third cerebral ventricle were performed over 1.5 min in conscious mice.

Salubrinal Administration into the Third Ventricle
Cannulas were implanted as described for the L-leucinol experiment. Injections into the third cerebral ventricle were performed in conscious mice. After 6 hr starvation, half of the mice received an i.c.v. injection of vehicle, i.e., 2.5 μl of 0.5% DMSO diluted in artificial cerebrospinal fluid (aCSF; Tocris), whereas the other half received an i.c.v. injection of 2.5 μl of 100 μM salubrinal (Sigma-Aldrich) diluted in aCSF as previously described by Methippara et al. (2009). Injections were performed over 1.5 min.

Statistical Analysis
All data, presented as mean ± SEM, have been analyzed using XLSTAT. We performed either a two-way ANOVA (Fisher’s post hoc) or paired or unpaired t-test into the third cerebral ventricle were performed in conscious mice. After 6 hr starvation, half of the mice received an i.c.v. injection of vehicle, i.e., 2.5 μl of 0.5% DMSO diluted in artificial cerebrospinal fluid (aCSF; Tocris), whereas the other half received an i.c.v. injection of 2.5 μl of 100 μM salubrinal (Sigma-Aldrich) diluted in aCSF as previously described by Methippara et al. (2009). Injections were performed over 1.5 min.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.01.006.

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