Loss of hypothalamic Furin affects POMC to proACTH cleavage and feeding behavior in high-fat diet-fed mice

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ABSTRACT

Objective: The hypothalamus regulates feeding and glucose homeostasis through the balanced action of different neuropeptides, which are controlled and activated by the proprotein convertases PC1/3 and PC2. However, the recent association of polymorphisms in the proprotein convertase FURIN with type 2 diabetes, metabolic syndrome, and obesity, prompted us to investigate the role of FURIN in hypothalamic neurons controlling glucose and feeding.

Methods: POMC-Cre+/− mice were bred with Furin−/− mice to generate conditional knockout mice with Furin-deletion in neurons expressing proopiomelanocortin (POMC FurKO), and Furin+/− mice were used as controls. POMC FurKO and controls were periodically monitored on both normal chow diet and high fat diet (HFD) for body weight and glucose tolerance by established in-vivo procedures. Food intake was measured in HFD-fed FurKO and controls. Hypothalamic POMC mRNA was measured by RT-qPCR. ELISAs quantified POMC protein and resulting peptides in the hypothalamic extracts of POMC FurKO mice and controls. The in-vitro processing of POMC was studied by biochemical techniques in HEK293T and CHO cell lines lacking Furin.

Results: In control mice, Furin mRNA levels were significantly upregulated on HFD feeding, suggesting an increased demand for FURIN activity in obeseogenic conditions. Under these conditions, the POMC FurKO mice were hyperphagic and had increased body weight compared to Furin+/− mice. Moreover, protein levels of POMC were elevated and ACTH concentrations markedly reduced. Also, the ratio of α-MSH/POMC was decreased in POMC FurKO mice compared to controls. This indicates that POMC processing was significantly reduced in the hypothalami of POMC FurKO mice, highlighting for the first time the involvement of FURIN in the cleavage of POMC. Importantly, we found that in vitro, the first stage in processing where POMC is cleaved into proACTH was achieved by FURIN but not by PC1/3 or the other proprotein convertases in cell lines lacking a regulated secretory pathway.

Conclusions: These results suggest that FURIN processes POMC into proACTH before sorting into the regulated secretory pathway, challenging the dogma that PC1/3 and PC2 are the only convertases responsible for POMC cleavage. Furthermore, its deletion affects feeding behaviors under obesogenic conditions.

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Endothalamic hormones and neuropeptides convey signals between the hypothalamus and other metabolic organs inducing either satiety or appetite. These ligands, as well as many of their cell surface receptors, are synthesized as inactive precursor proteins and require the proteolytic activity of specific endopeptidases as a first step towards activation [6]. After these cleavage steps by endopeptidases, further trimming and amidation are often needed for full activation [7,8]. Most of the cleavages are performed by proprotein convertases (PCs), a seven-member family of subtilisin-like serine proteases with selectivity for basic amino acid substrates involved in energy and glucose homeostasis including the pancreatic a-cells [20]. While PC1/3 and PC2 have been extensively studied in the context of obesity and metabolic dysfunction, very little is known about the role of hypothenal FURIN in vivo by deleting Furin in POMC neurons.

2. MATERIALS AND METHODS

2.1. Cell culture and transfection
The human embryonic kidney (HEK) 293 T cell line was cultured in DMEM/F-12 without phenol red (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. 24 h after seeding, cells (= 60% confluency) were transfected with plasmids encoding human c-myc-tagged-POMC plasmid (within pcDNA3 backbone) and human Furin, or PCSK1, mouse Pcsk5a, Pcsk5b, Pcsk6 or human PCSK7 using X-tremEGENE™ 9 (Roche) according to the manufacturer's protocol. As negative control, cells were transfected with pcDNA3 plasmid, indicated in Figure 4A—B and Figure 1 as mock.

2.2. Western blot
Cells were lysed in 1 × lysis buffer (20 mM Tris—HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycero phosphate, 1 mM Na3VO4, 1 µg/ml leupeptin (Cell Signaling Technology)). The conditioned media were collected for methanol precipitation after overnight incubation of the cells in 1 mL of serum free medium (DMEM/F-12 without phenol red). 12.5 µg of BSA were added as control of methanol precipitation. The precipitated proteins were dissolved in 1 × sample buffer (Tris base 0.0625 M, SDS 0.07 M (2%), glycerol 10%, bromophenol blue) and prepared for western blot. Western blot was performed according to the standard procedures using NuPAGE 10% Bis-Tris minigels and NuPAGE MES SDS running buffer 1× (Thermo Fisher Scientific). The primary antibodies used were mouse anti-myc (homemade), and anti-ACTH (clone A2A3, RRID: 2905636) directed against the C-terminus of ACTH so can recognize proACTH. Mouse anti-FURIN (MON152 [34]), and rabbit anti-PC1/3, anti-PC5/6 A, anti-PC5/6 B, anti-PACE4, and anti-PC7 (homemade) were used to detect each PC in either cell lysates or conditioned media. The secondary antibodies were anti-mouse or anti-rabbit labeled with horseradish peroxidase (HRP) (Dako).

2.3. Generation of POMC/Furin KO mice
POMC-Cre+/− (B6-FVB-Tg (Pomc-cre)1Low/l, JAX stock #010714) mice were bought from Jackson Laboratories (https://www.jax.org). Furin+/− mice were described before [35]. Mice were backcrossed at least 5 times to a C57Bl6J background. All the mice were housed in standard cages on a 12-hour day/night cycle and fed a standard rodent chow (10 KJ% fat, 13 KJ% protein, 77 KJ% carbohydrates) or high fat diet (HFD) (45 KJ% fat, 20 KJ% protein, 35 KJ% carbohydrates) in a conventional facility of the KU Leuven. Food and water were provided ad libitum. All experiments were approved by the KU Leuven Animal Welfare Committee, following the guidelines provided in the Declaration of Helsinki (KU Leuven project number 034/2020). For food intake experiments, POMC-Cre mice were individually housed in single grid cages with 5 days of acclimatization and 3 days of food intake measurements.
2.4. Intraperitoneal glucose tolerance test (IPGTT)
Mice were fasted overnight or 4 h and intraperitoneally injected with 1.5–2 mg/g body weight of d-glucose in PBS. Blood glucose levels were monitored at indicated time-points using a Contour Glucometer (Roche).

2.5. Real time quantitative PCR (RT-qPCR)
RNA from snap frozen mouse hypothalamus was isolated with the Nucleospin RNA II kit according to the manufacturer’s protocol. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Primers were designed using the Primer 3 plus software. RT-qPCR was performed with a CFX Connect Real-Time PCR Detection System from Bio-Rad using SYBR Green supermix (Bio-Rad). Data is represented as $2^{-\Delta \Delta C_{T}}$. Primers for mouse genes and the Cre recombinase gene from bacteriophage P1 are listed in Table S1.

2.6. Peptide isolation from hypothalamus
Hypothalamic extracts were generated based on the technique in [36]. Extractions were optimized for each protein/peptide using known amounts of the protein/peptide spiked into the hypothalamus and recovery/interference assessed. Each frozen hypothalamus was disrupted in cold 0.1 M HCl using a Qiagen TissueRuptor. The resulting homogenates were centrifuged at 5000 g at 4 °C and supernatants transferred to a low-protein retention tube. A 50 μL aliquot was removed to a separate tube for total protein measurement using a BCA protein assay (see below), then a stabilizing buffer of 0.1 M tris/0.1% BSA was added to the extracts before freezing at −80 °C. The hypothalamic extracts were diluted 1:3 with 1/C14 homogenates were centrifuged at 5000 g at 4 °C for 20 min and supernatants transferred to a low-protein protein tube. A 50 μL aliquot was removed to a separate tube for total protein measurement using a BCA protein assay (see below), then a stabilizing buffer of 0.1 M tris/0.1% BSA was added to the extracts before freezing at −80 °C. The hypothalamic extracts were diluted 1:3 with 1/C14

2.7. POMC ELISA
The total POMC in each hypothalamus was detected by using a 2-site immunometric assay as described previously [11,37,38]. Briefly, monoclonal antibody A1A12 (RRID: 2756529) was added, followed by HRP-labeled avidin as well as the enzyme substrate for the final detection. This assay has a sensitivity of 10 pmol/L and is specific for POMC and proACTH, and does not detect ACTH or α-MSH [39].

2.8. ACTH ELISA
The ACTH detection and quantification in mouse hypothalamic samples was performed by using a 2-site immunometric assay as described [11,37]. Briefly, The ACTH ELISA plates were coated with monoclonal antibody A1A12 (RRID: 2756529). The detection antibody was monoclonal antibody A2A3 (RRID: 2905636), directed against the C-terminus of ACTH, and labeled directly with HRP. The standards were prepared from human pituitary ACTH, provided by the National Institute of Biological Standards and Control, London, UK. This assay has a sensitivity of 1.1 pmol/L and has previously been shown to have <0.1% cross reactivity with POMC and does not detect α-MSH, ACTH 18–39 or ACTH 1–24.

2.9. α-MSH ELISA
Detection and quantification of α-MSH was performed by using a newly developed competitive ELISA based on a single polyclonal antibody (RRID: 2756515) produced by Prof. Sharon Wardlaw, Columbia University [40,41]. This antibody is specific for C-terminal amidated α-MSH, and shows no cross reactivity with POMC, ACTH, or the free acid form of α-MSH that has not been amidated. The antibody (1 mg/L) was added to an ELISA plate previously coated with 10 mg/L rabbit anti-IgG (Sigma Aldrich, UK). Biotin labelled α-MSH (created by combining biotin and α-MSH at a 50:1 M ratio) was added to the wells at 100 pmol/L, followed by α-MSH standards (Abcam) in the range of 15–1000 pmol/L or samples. The unlabeled standards or samples competed with the labelled α-MSH to bind the polyclonal anti-α-MSH

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**Figure 1:** Furin and Pomc mRNA levels are increased in a chronic high-fat-diet in Fur<sup>Cre<sup> control mice. Relative mRNA expression in hypothalami of 23-week-old male Fur<sup>Cre<sup> mice fed either a NCD or a HFD for 15 weeks. The mice were in a fed state at the moment of the euthanasia (A) Furin, (B) Pomc, (C) Agrp, (D) Npy. n = 3–6 mice/group.

**A** Relative expression (to Gapdh) in Hypothalamus

**B** Relative expression (to Gapdh) in Hypothalamus

**C** Relative expression (to Gapdh) in Hypothalamus

**D** Relative expression (to Gapdh) in Hypothalamus

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**Figure 1 Caption:** Furin and Pomc mRNA levels are increased by a chronic high-fat-diet in Fur<sup>Cre<sup> control mice. Relative mRNA expression in hypothalami of 23-week-old male Fur<sup>Cre<sup> mice fed either a NCD or a HFD for 15 weeks. The mice were in a fed state at the moment of the euthanasia (A) Furin, (B) Pomc, (C) Agrp, (D) Npy. n = 3–6 mice/group.

**Notes:**

- **NCD:** normal chow diet; **HFD:** high fat diet.
- **IPGTT:** intraperitoneal glucose tolerance test
- **RT-qPCR:** real-time quantitative PCR
- **ELISA:** enzyme-linked immunosorbent assay
- **ACTH:** adrenocorticotropic hormone
- **α-MSH:** α-melanocyte-stimulating hormone
- **SEM:** standard error of the mean
antibody. Then, avidin-labelled HRP and the enzyme substrate were added to generate the signal measured at OD 450 nm. This assay has a sensitivity of 20 pmol/L.

2.10. Bicinchoninic acid (BCA) protein assay
Hypothalamus extracts were diluted 1:9 with 1× PBS and total protein quantified using the Pierce RapidGold BCA protein assay kit (ThermoFisher Scientific). The assay was performed according to the manufacturers’ instructions.

2.11. Metabolic labeling experiment
Transfected cells were metabolically labelled as described before [42]. After 40 min pulse labelling with 1 mCi 35S-methionine the cells were lysed and the lysates immune-precipitated with A2A3 and myc antibodies bound to protein G sepharose (Pharmacia Biotech).

2.12. Statistical analysis
Results are expressed as means ± SEM. Statistical analysis was performed by unpaired Student’s t test or one-way ANOVA with Sidak’s multiple comparisons test for grouped analysis, or repeated measure two-way ANOVA for pairwise time-specific differences between genotypes. A value of p < 0.05 was considered significant. *p < 0.05, **p < 0.01, ***p < 0.001.

3. RESULTS
We found that hypothalamic Furin mRNA levels were increased twofold in the group of control (Furfl/fl) mice on HFD compared to control animals on a normal chow diet (NCD), suggesting that FURIN activity might be important in regulating energy homeostasis (Figure 1A). POMC expression was also strongly increased (Figure 1B), while Agrp

![Figure 2: The absence of Furin in POMC-neurons leads to hyperphagia and increased body weight after a short HFD period.](image-url)
and Npy were decreased (Figure 1C–D), consistent with previous studies with HFD-diet fed mice [43–45]. To establish the possible role of hypothalamic FURIN in controlling energy and glucose homeostasis, we generated a conditional knockout mouse model in which Furin was deleted in POMC neurons. Pomc-Cre+/− mice were bred with Fur−/− mice to study the potential role of Furin specifically in anorexigenic POMC neurons (Figure 2A). We confirmed the Cre-mediated Furin recombination by RT-qPCR analysis of both Furin (lacking exon 2) and Cre mRNA in the hypothalamus of POMCFurKO and Fur−/− mice (Fig. S1). When fed on NCD, body weight of POMCFurKO mice was similar to Fur−/− control mice (Figure 2B). However, on HFD POMC FurKO mice became significantly heavier than Fur−/− mice from 2 weeks of HFD onwards (Figure 2C). Consistent with the increase in body weight, food intake of POMC FurKO and Fur−/− mice (Fig. S2). Glucose tolerance was normal in Pomc−/− mice after 3 weeks of HFD (Figure 2D). The ACTH and α-MSH content was decreased, indicating defective POMC processing (Figure 3E–F). The ACTH relative to the α-MSH content was decreased, albeit not significantly (Figure 3G).

In order to determine whether POMC processing was directly dependent on FURIN activity, we analyzed the in-vitro processing of the POMC precursor in ΔFurHEK293T cells, lacking FURIN, co-transfected with Pomc and Furin, PC1/3, PACE4, PC5/6 A, PC5/6 B, or PC7. (Figure 4). PC4 was not included because it is not active in cells without a regulated secretory pathway. The POMC detected in the cell lysates of ΔFur-HEK293T cells was equally present and each PC was efficiently expressed in each experimental condition (Figure 4A–B). The resulting POMC cleavage products were detected in the conditioned media and in the cell lysate of ΔFurHEK293T cells (Figure 4C–D). Interestingly, FURIN was the only PC able to efficiently cleave POMC at the cleavage site KR164, resulting mainly in 23 KDa proACTH. A minute amount of glycosylated and mature ACTH forms (gACTH and mACTH, respectively) could be detected after prolonged exposure. PC7 was able to cleave the same consensus site, albeit much less efficiently (Figure 4C). Under these conditions, in the absence of a regulated secretory pathway, PC1/3 was unable to cleave POMC. These results are consistent with previous studies showing that POMC expression levels in POMCFurKO mice were significantly higher than in the controls, while the mRNA expression levels of AgRP and Npy were not changed (Figure 3A). At protein level, the absolute amount of POMC was increased in hypothalamic lysates of HFD-fed POMCFurKO mice (Figure 3B), whereas ACTH was significantly decreased (Figure 3C), and α-MSH slightly reduced, although not significantly (Figure 3D). The ACTH and α-MSH amounts relative to POMC were strongly decreased, indicating defective POMC processing (Figure 3E–F).

Figure 3: POMC expression and processing is altered in HFD-fed POMCFurKO mice. (A) Relative mRNA expression of Npy, Agrp, and Pomp in the hypothalami of 10-week-old male POMCFurKO (pink bars) and Fur−/− (blue bars) mice on HFD for 2 weeks (n = 4–5 mice/group). **P < 0.01 determined by unpaired t-test. All data are represented as mean ± SEM. The total content of POMC (B), ACTH (C), αMSH (D), and the ratio ACTH/POMC (E), αMSH/POMC (F), and ACTH/αMSH (G) in the hypothalami of 11-week-old male POMCFurKO and Fur−/− mice fed on HFD for 3 weeks (n = 4–6 mice/group). ***P < 0.001 determined by unpaired t-test. For RT-qPCR analyses, the values were normalized to Gapdh expression levels; all data are represented as mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
Figure 4: POMC is selectively cleaved by FURIN at the C-terminal site of ACTH (KR164) into proACTH and β-lipotropin (LPH) in ΔFurHEK293T cells. Western blot analysis of POMC and each co-transfected PCs in cell lysate (A–B) and proACTH and ACTH in medium (C) of Furin-deficient HEK293T cells co-transfected with POMC and different PCs. The blot in A (upper panel) is labeled with an anti-myc antibody. The lower panel shows a ponceau S-staining of the cell lysates as loading reference. (B) Western blot analysis of each transfected PC detected either in the medium or in the cell lysate. As negative control was used medium or cell lysate from ΔFurHEK293T transfected with a different PC. The blot in C is labeled with the A2A3 antibody directed against the free carboxy-terminus of ACTH. One minute-exposure of the entire blot (upper panel), and 5 min exposure of the lower part of the blot (lower panel) to show the less abundant low MW ACTH forms. Mouse pituitary protein extract (25 μg) was used as positive control (first lane). (D) Metabolic labeling of Furin-deficient HEK293T cells transfected with POMC alone or together with FURIN (40 min pulse). (E) New model for POMC processing in hypothalamus. The antibody used to detect POMC-myc is depicted in blue, and in orange the antibody used to detect proACTH and ACTH (glycosylated: gACTH, and mature: mACTH). In ΔFurHEK293T cells lacking the secretory pathway, FURIN preferentially cleaves the first POMC cleavage site, most likely in the TGN. The same cleavages are mediated by PC1/3 in cells with a regulated secretory pathway probably requiring the pH conditions of the ISGs. Cleavage of proACTH to ACTH is performed by PC1/3, the enzymes providing redundancy are probably FURIN and/or PC2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
prorenin cannot be cleaved by PC1/3 in cells lacking a regulated secretory pathway but is cleaved in neuroendocrine cells [47,48]. It was suggested that the carboxyterminal tail of PC1/3, which is cleaved off in secretory granules, has an autocrine/paracrine function in the TGN. Moreover, POMC was cleaved to proACTH by FURIN inside the ΔFurHEK293T cells after a short 40 min pulse labeling, which allows newly synthesized protein to reach the TGN, but is too short for secretion (Figure 4D). A schematic of POMC cleavage mediated by FURIN, together with the specific antibodies used to detect POMC, proACTH and ACTH, is represented in Figure 4E. To corroborate these unexpected results that challenge the dogma that only PC1/3 and PC2 are involved in the processing of POMC into ACTH/α-MSH, we repeated these experiments in FURIN deficient CHO cells (RPE.40 cells [49]) with similar results (Fig. S3).

4. DISCUSSION

In this study, we have demonstrated that hypothalamic Furin in mice is essential for the regulation of energy homeostasis under obeseogenic conditions. Our results revealed that the absence of Furin in POMC neurons caused an increase in body weight and food intake on HFD. In addition, we demonstrated that FURIN-dependent processing of neuropeptides in feeding-controlling neurons can be linked to the metabolic state of the animals. This is consistent with the 2-fold increased expression of Furin during HFD compared to the NCD in control mice. Most remarkable is our observation that FURIN is most likely a POMC to proACTH convertase in the hypothalamus.

The hyperphagia and increased body weight observed in POMC FurKO mice suggest the melanocortin system as a potential source of FURIN substrates linked to the phenotype. PC1/3 and PC2 have generally been considered the only PC processing enzymes of POMC [8]. Our in vitro findings put FURIN forward as a new enzymatic player of the melanocortin system in hypothalamic neurons. Furin and PC2, in hypothalamic neurons, the cleavage step of ACTH into α-MSH is exclusively performed by PC2 and PC2 null mice therefore have undetectable levels of α-MSH in hypothalamus [10].

Our results provide evidence for the model presented in Figure 4D. Furin appears to be the only PC able to cleave POMC in the TGN, while PC1/3 can cleave it in ISGs. The subsequent PC cleavage at the aminoterminus of proACTH necessary to generate ACTH can be performed by PC1/3 but possibly also by FURIN based on the near-normal levels of ACTH in PCSK1 null patients [13]. This newly identified role of Furin in the processing of POMC warrants reassessment of other cleavages of peptide hormones and neuropeptides. For instance, PCSK1 null patients have reduced but detectable amounts of GLP-1 in serum [13,52].

Besides the direct effect that the impaired processing of POMC might have on the phenotype of POMC FurKO mice, loss of FURIN might also affect other substrates which might indirectly affect the melanocortin pathway and therefore the observed phenotype. These substrates include for instance the V-ATPase subunit ATP6AP1/Ac45, shown to be cleaved by Furin in β cells [26,27], and involved in the acidification and hence secretion of granules [53–55]. However, the activity of PC2, which has an acidic pH optimum, is not severely affected based on the (near) normal amounts of α-MSH.

Furthermore, other molecules, such as semaphorins, crucial for development of the melanocortin system in hypothalamic neurons [56,57] and brain-derived neurotrophic factor (BDNF), which has a crucial role in regulating energy homeostasis [58,59], are potential FURIN substrates and therefore might be possible contributors to the obese-like phenotype.

In conclusion, in this work we have unveiled the importance of FURIN activity in the regulation of energy balance in hypothalamic neurons during a high metabolic challenge. In particular, our results strongly indicate that FURIN activity becomes critical in POMC neurons during a HFD regimen, likely through the proteolytic cleavage of POMC which is required to overcome the augmented metabolic demand.

DATA AVAILABILITY

No data was used for the research described in the article.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

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