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# Hypothalamic ventromedial COUP-TFII protects against hypoglycemia-associated autonomic failure

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The nuclear receptor Chicken Ovalbumin Upstream Promoter–Transcription Factor II (COUP-TFII) is an important coordinator of glucose homeostasis through its function in different organs such as the endocrine pancreas, adipose tissue, skeletal muscle, and liver. Recently we have demonstrated that COUP-TFII expression in the hypothalamus is restricted to a subpopulation of neurons expressing the steroidogenic factor 1 transcription factor, known to play a crucial role in glucose homeostasis. To understand the functional significance of COUP-TFII expression in the steroidogenic factor 1 neurons, we generated hypothalamic ventromedial nucleus-specific COUP-TFII KO mice using the cyclization recombination/locus of X-overP1 technology. The heterozygous mutant mice display insulin hypersensitivity and a leaner phenotype associated with increased energy expenditure and similar food intake. These mutant mice also present a defective counterregulation to hypoglycemia with altered glucagon secretion. Moreover, the mutant mice are more likely to develop hypoglycemia-associated autonomic failure in response to recurrent hypoglycemic or glucopenic events. Therefore, COUP-TFII expression levels in the ventromedial nucleus are keys in the ability to resist the onset of hypoglycemia-associated autonomic failure.

Hypothalamic neurons are able to detect changes in the concentration of circulating hormones and nutrients and to relay this information into adaptive signals toward peripheral organs aimed at maintaining glucose homeostasis and energy balance. Among these neurons, several studies have shown that the steroidogenic factor 1 (SF1) expressing ones, which are restricted to the hypothalamic ventromedial nucleus (VMN), are important modulators of metabolic functions through their ability to detect variations of extracellular concentrations of glucose, insulin, leptin, and orexin to coordinate insulin/glucose homeostasis (1–5). The SF1 transcription factor itself is a key actor of energy homeostasis, as SF1 knockout (KO) mice are obese (6). SF1 KO mice display an abnormal organization of the VMN, as SF1 is necessary for the terminal differentiation of the VMN neurons (7, 8). SF1 itself directs a genetic program involved in energy homeostasis and leptin action in the VMN (9). Outside these functions, SF1 neurons are also part of the neurocircuitry involved in the counterregulatory response (CRR) to hypoglycemia. To prevent hypoglycemia, organisms have developed several survival mechanisms of CRR such as decreased insulin secretion and increased glucagon secretion to quickly reestablish euglycemia (10, 11). The decreased insulin secretion is a direct effect of hypoglycemia on the pancreatic  $\beta$  cells, while the other CRRs are mainly directed by the central nervous system and in part by the SF1 neurons (12–14). Overall the SF1 neuronal population is at the interface between sensing of glucose concentrations and modulation of insulin-dependent metabolism and energy homeostasis.

Recently we have observed that Chicken Ovalbumin Upstream Promoter–Transcription Factor II (COUP-TFII) expression is restricted to a subpopulation of SF1-expressing neurons in the VMN (15). COUP-TFII is a member of the steroid/thyroid hormone receptor superfamily (16). It is expressed in multiple organs such as adipose tissue, liver, endocrine pancreas, skeletal muscle, and hypothalamus (17–20). A functional study of COUP-TFII

using transgenic mouse models has shown that a 50% of genetic inactivation of the COUP-TFII gene in all cells displayed a drastic phenotype in glucose homeostasis and energetic metabolism: these mice are hypersensitive to insulin and lean (21). To decipher COUP-TFII function in the different organs involved in energy homeostasis, we have developed genetic models of conditional COUP-TFII inactivation in specific tissues using the cyclization recombination (Cre)/locus of X-overP1 (loxP) strategy. In mice exhibiting a 50% decreased COUP-TFII expression in  $\beta$  cells, we demonstrated that COUP-TFII was necessary for pancreatic glucose-dependant insulin secretion (18, 22, 23).

Following those observations, we went on to study the hypothalamic COUP-TFII involvement in glucose homeostasis. For that purpose, we have generated a mouse model of conditional genetic inactivation of COUP-TFII in the VMN neurons. We observed that the heterozygous mutant mice displayed a leaner phenotype with improved insulin sensitivity and altered glucagon secretion in response to hypoglycemia. Moreover, these mice are more likely to develop hypoglycemia-associated autonomic failure (HAAF). Therefore, a 50% modulation of COUP-TFII levels in a neuronal subpopulation of the VMN is sufficient to induce a metabolic response and a defective CRR mechanism.

## Results

**Conditional Inactivation of COUP-TFII in SF1 Neurons.** COUP-TFII is expressed in VMN neurons but also in brain structures such as the amygdala, hippocampus, and cortical interneurons (24, 25). We were unable to detect any COUP-TFII protein expression in the VMN of the (SF1)<sup>CRE+/-</sup> COUP-TFII floxed/floxed (fl/fl) mice, while its expression was still present in the COUP-TFII fl/fl mice (Fig. S1A). COUP-TFII expression was undisturbed in the (SF1)<sup>CRE+/-</sup> COUP-TFII fl/fl mice amygdala (Fig. S1A) and in brain regions known to express COUP-TFII protein. In other SF1-expressing tissues, we did not detect COUP-TFII protein in the adrenal gland of wild-type mice (Fig. S1), and Tsai et al. have also shown that the pituitary gland does not express COUP-TFII (26).

The allelic distribution analysis for COUP-TFII fl, wt, and (SF1)<sup>CRE</sup> alleles showed a strong lethality for the (SF1)<sup>CRE+/-</sup> COUP-TFII fl/fl mice. Out of the expected Mendelian ratio of 12.5%, only 2% of the (SF1)<sup>CRE+/-</sup> COUP-TFII fl/fl were present at birth, while we observed the expected Mendelian distribution for the (SF1)<sup>CRE+/-</sup> COUP-TFII fl/wt, (SF1)<sup>CRE+/-</sup> COUP-TFII wt/wt, COUP-TFII fl/wt, and COUP-TFII wt/wt mice with similar numbers of males and females. For the purpose of our study, we analyzed (SF1)<sup>CRE+/-</sup> COUP-TFII fl/wt male mice (SF1-COUP-

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TFII mice) phenotype and compared it to the (SF1)<sup>CRE+/-</sup> and the COUP-TFII fl/wt mice (control mice). As shown by immunoblotting, COUP-TFII protein levels were decreased by 45% in nuclear extract of SF1-COUP-TFII hypothalami compared with control mice hypothalami (Fig. S1B). These results indicate that the Cre-induced recombination of heterozygous SF1-COUP-TFII mice led to an efficient inactivation of the allele and was restricted to the VMN neurons.

**SF1-COUP-TFII Mice Have Improved Glucose Homeostasis.** SF1-COUP-TFII mice displayed similar fasting blood glucose levels than control mice (Fig. 1A), while having significantly lower blood glucose levels at the fed state than their control littermates (Fig. 1A). Despite having lower blood glucose concentrations at the fed state, the SF1-COUP-TFII mice had no significant difference in fasted or fed plasma insulin or glucagon concentrations compared with control mice (Fig. 1A). Ketone bodies concentrations remained similar, indicating comparable hepatic beta oxidation rates (Fig. S2A).

When subjected to glucose tolerance tests, 3-mo-old SF1-COUP-TFII male mice displayed improved glucose tolerance compared with their controls with higher hyperglycemia from 20 min onwards (Fig. 1B). Plasma insulin concentrations were also similar between the three mouse groups as indicated by the area under curve for insulin concentration during the test (Fig. 1B). Pancreatic insulin content was also similar to SF1-COUP-TFII (Fig. S2B), suggesting a functional pancreas.

As the SF1-COUP-TFII mice displayed enhanced glucose tolerance with no significant differences in insulin secretion, we subjected them to an i.p. insulin injection (insulin tolerance test, ITT; 0.5 U/kg) and followed blood glucose concentrations for 2 h. The insulin injection elicited a stronger drop in blood

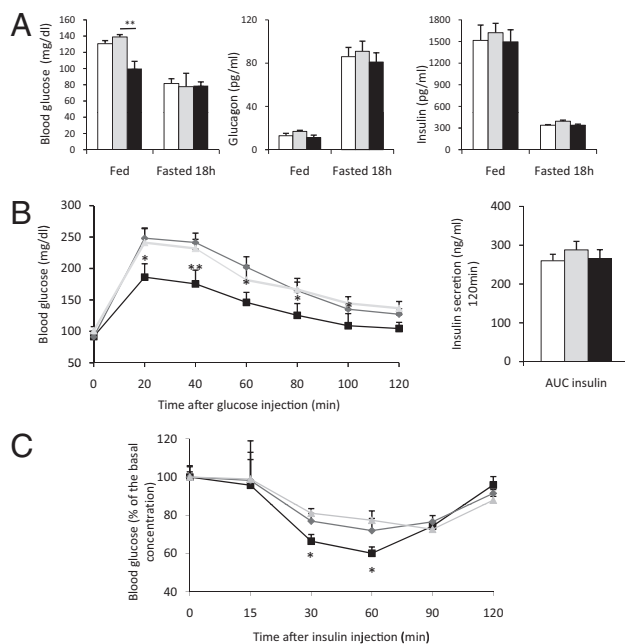
glucose concentrations of SF1-COUP-TFII mice compared with their controls (Fig. 1C). Blood glucose concentrations were similar between the three mouse groups at the end of the ITT.

When looking at insulin-dependent hepatic gene expression, such as glucokinase (GK), fatty acid synthase (FAS), or phosphoenolpyruvate carboxykinase (PEPCK), the SF1-COUP-TFII mice displayed, at the fed state, significantly greater expression of GK and FAS mRNA and lower expression of PEPCK mRNA than their control littermates (Fig. S3). The fed SF1-COUP-TFII mice showed a stronger phosphorylation of Akt protein in their liver than their controls without modification of its expression (Fig. S3). Taken together these data are indicative of overall improved insulin sensitivity in the SF1-COUP-TFII mice.

**SF1-COUP-TFII Mice Have an Imbalanced Energy Homeostasis.** No significant differences in body weight and composition were found in SF1-COUP-TFII mice for the first 4 mo (Fig. 2A and B). Then, they presented a more moderate weight gain than their control littermates (Fig. 2A and B). At 6 mo, SF1-COUP-TFII mice had a significantly lower fat mass compared with their controls (Fig. 2B); the lean mass remained unaffected (Fig. 2B). As the mice displayed a difference in fat mass, food intake was measured over a 2-wk period under ad libitum feeding or during a 4 h refeeding period following an 18 h fast. Daily and induced food intakes were similar between groups (Fig. 2B and C). At similar food intake, SF1-COUP-TFII mice displayed significantly slower weight gain than their control siblings, indicating an imbalanced energy homeostasis. The SF1-COUP-TFII mice had similar circulating T4, corticosterone, growth hormone concentrations, and body temperature as their controls (Fig. S4). Uncoupling protein 1 (UCP1) mRNA levels in the brown adipose tissue were also similar (Fig. S4). Analysis of energy expenditure components, performed at thermoneutrality, did not reveal significant differences for basal metabolism, thermic feeding effect, or relative contributions of glucose and lipids to energy expenditure in the fasted or in the fed state (Fig. 2D). Basal metabolism was slightly but not significantly greater ( $P = 0.332$ ) on a per mouse base in mutant mice. Because the control mice were slightly heavier and fatter, adjustment of resting energy expenditure to body weight, lean, or fat body mass reduced the already nonsignificant differences ( $P = 0.698, 0.549,$  and  $0.719$ , respectively). Therefore, it can be confidently concluded that there is no difference in basal and resting energy expenditure between control and mutant mice. However, a significant greater cost of physical activity was observed in the mutant mice (+15%) in both fasted and fed states (Fig. 2D). This cost of activity has been measured after detailed analysis of the relationship between total energy expenditure and intensity of activity measured quantitatively by force transducers as described in ref. 27. Therefore, we went on to analyze the muscle-specific mitochondrial UCP3 expression levels (28) and observed a twofold higher expression of UCP3 in the SF1-COUP-TFII mice than their control littermates (Fig. 2E). The larger cost of physical activity observed in the SF1-COUP-TFII mice is likely due to increased uncoupling in skeletal muscles, which could contribute to increased energy expenditure.

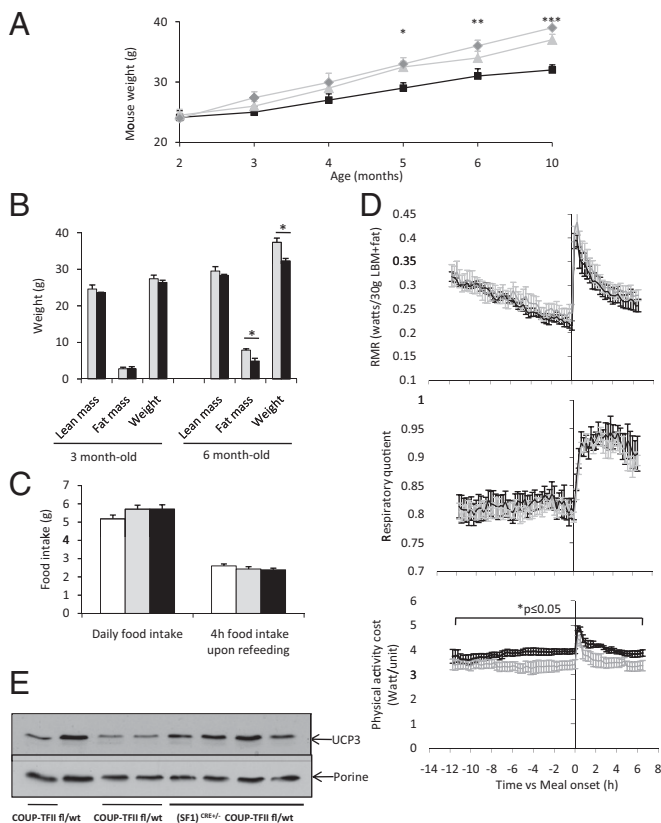
#### SF1-COUP-TFII Mice Present Hypothalamic Hypersensitivity to Insulin.

In SF1-COUP-TFII mice hypothalami, none of the described COUP-TFII target genes such as  $\gamma$ -aminobutyric acid type A receptor alpha 1 (GABAA-R  $\alpha$ 1), carnitine palmitoyltransferase I (CPT-1), or insulin-like growth factor 1 were affected (17, 29, 30) (Fig. S5). Acyl-CoA oxidase, SF1, or brain-derived neurotrophic factor genes, whose expression are known to modulate energy homeostasis, are not affected (Fig. S5). By contrast, long-term decreased COUP-TFII expression is associated with a 30% increase in pro-opiomelanocortin (POMC) gene expression (Fig. 3A). Restricted to the arcuate nucleus, POMC encodes the  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) peptide, involved in neuronal pathways controlling energy homeostasis and peripheral insulin sensitivity. This chronically elevated POMC gene expression



**Fig. 1.** SF1-COUP-TFII mice blood parameters. (A) Blood glucose, plasma insulin, and glucagon concentrations in 5-mo-old fed or fasted male mice (18 h fast). White, (SF1)<sup>CRE+/-</sup> mice; gray, COUP-TFII fl/wt; black, (SF1)<sup>CRE+/-</sup> COUP-TFII fl/wt mice. Data are expressed as the mean  $\pm$  SEM. \* $P \leq 0.05$  ( $n = 12$ ). (B) Glucose tolerance test (GTT) and insulin secretion during GTT on 3-mo-old mice. AUC, area under curve of plasma insulin concentrations. Light gray, (SF1)<sup>CRE+/-</sup> mice; dark gray, COUP-TFII fl/wt; black, (SF1)<sup>CRE+/-</sup> COUP-TFII fl/wt mice. Data are expressed as the mean  $\pm$  SEM ( $n = 9$ ). \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ . (C) Insulin tolerance test (ITT) on 3-mo-old mice. Light gray, (SF1)<sup>CRE+/-</sup> mice; dark gray, COUP-TFII fl/wt; black, (SF1)<sup>CRE+/-</sup> COUP-TFII fl/wt mice. Data are expressed as the mean  $\pm$  SEM ( $n = 8$ ). \* $P \leq 0.05$ .





**Fig. 2.** SF1-COUP-TFII mice have imbalanced energy homeostasis. (A) Ponderal growth over a period of 10 mo. Light gray, SF1<sup>CRE+/-</sup> mice; dark gray, COUP-TFII fl/wt; black, SF1<sup>CRE+/-</sup> COUP-TFII fl/wt mice. Data are expressed as the mean  $\pm$  SEM ( $n = 32$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.005$ . (B) Body composition of 3- or 6-mo-old male mice. Gray, COUP-TFII fl/wt male mice; black, SF1<sup>CRE+/-</sup> COUP-TFII fl/wt male mice. Data are expressed as the mean  $\pm$  SEM ( $n = 5$ ). \* $P < 0.05$ ; \*\* $P < 0.01$  (C) Daily food intake and food intake during a 4 h refeeding after 18 h fast. White, SF1<sup>CRE+/-</sup> mice; gray, COUP-TFII fl/wt; black, SF1<sup>CRE+/-</sup> COUP-TFII fl/wt mice. Data are expressed as the mean  $\pm$  SEM ( $n = 8$ ). (D) Time course of resting metabolic rate (RMR), respiratory quotient (RQ), and cost of activity from 12 h before to 7 h after ingestion of a test meal ( $t = 0$ ). Gray line, COUP-TFII fl/wt mice; black line, SF1<sup>CRE+/-</sup> COUP-TFII fl/wt mice. (E) Autoradiogram of a Western blot analysis of UCP3 and porine protein expressions in mitochondria isolated from skeletal muscles.

could contribute to the observed metabolic phenotype of higher peripheral insulin sensitivity and increased energy expenditure.

When examining the signaling pathways involved in energy expenditure, such as leptin and insulin signaling, we did not observe differences in the phosphorylation status of STAT3, a transcriptional mediator of leptin (Fig. 3B). On the other hand, a greater overall phosphorylation of forkhead box protein O1 (FOXO1), a mediator of insulin transcriptional effects, was observed. In nuclear extracts from microdissected VMN, there was a significantly lower FOXO1 binding activity as demonstrated by EMSA, indicating that decreased COUP-TFII expression in the VMN lead to greater insulin sensitivity in this nucleus (Fig. 3C).

**SF1-COUP-TFII Mice Have a Defective CRR to Hypoglycemia.** As mentioned, a single dose of insulin induced a more pronounced hypoglycemia in 3-mo-old male SF1-COUP-TFII mice compared with their controls (Fig. 2C). When similar experiments were done in 5-mo-old mice, a single insulin injection elicited a more drastic drop in blood glucose concentrations in SF1-COUP-TFII mice than in their controls, which is associated with a failure to recover properly from hypoglycemia (Fig. 4A). This phenotype is reminiscent of a defective CRR to insulin-induced hypoglycemia (31). In SF1-COUP-TFII mice, the more drastic

drop in blood glucose following a single insulin injection is associated with a lower glucagon secretion 60 min after injection (Fig. 4A). At the same time, the SF1-COUP-TFII mice had higher corticosterone concentrations compared with their controls, indicating that part of the CRR is still functional and responsive to lower blood glucose concentrations (Fig. 4A). Growth hormone concentrations were similar between SF1-COUP-TFII and control mice at time 60 min (Fig. 4A).

Insulin is able to directly inhibit glucagon secretion by  $\alpha$  cells (32). To distinguish between the effect of insulin and hypoglycemia on glucagon secretion and therefore to analyze the CRR in absence of hyperinsulinemia, we went on to mimic glucopenia with an injection of 2-deoxyglucose (2-DG). An injection of 2-DG triggers a CRR including an increase in glucagon secretion independently of any insulin injection, leading to an increase in blood glucose concentrations. Upon a single injection of 2-DG, we got the expected rise in blood glucose concentrations 30 min after injection in all groups, but this elevation was blunted in the SF1-COUP-TFII mice (Fig. 4B). Glucagon concentrations were similar between the three animal groups at time 0, but SF1-COUP-TFII mice had significantly lower plasma glucagon concentrations at 30 min, indicating a defective CRR to glucopenia (Fig. 4B). Food intake, which had been previously shown to be triggered following a 2-DG injection (33, 34), remained similar between groups (Fig. 4B). The defective CRR observed in SF1-COUP-TFII mice is not associated with altered food intake.

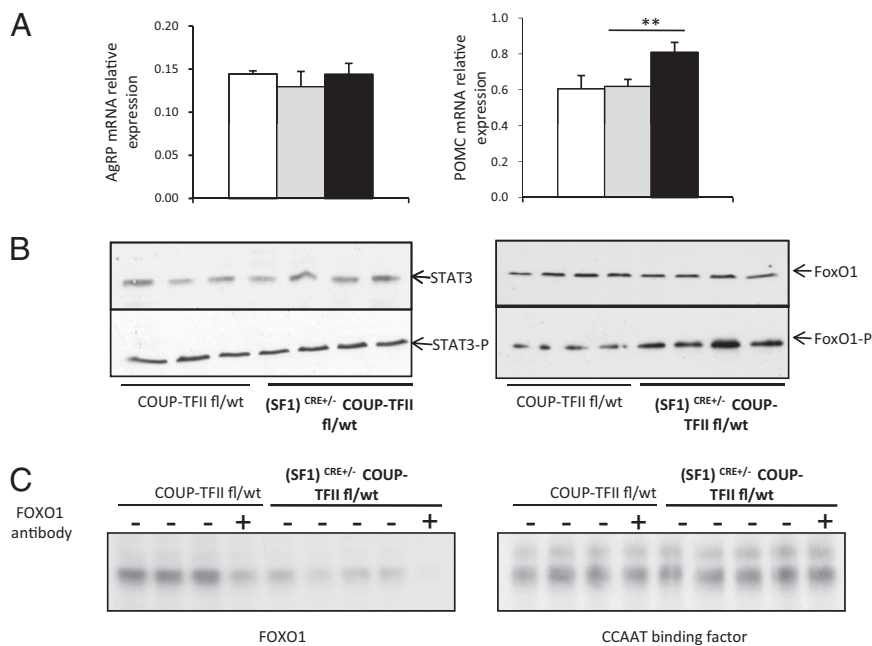
**SF1-COUP-TFII Mice Are More Prone to HAAF.** While subjected to ITTs, SF1-COUP-TFII mice displayed a stronger hypoglycemia compared with their controls (Fig. 2C). When these mice were subjected to recurrent hypoglycemia with insulin injection once a week, we observed that the blood glucose drop was aggravated and that their recovery from this drop was compromised, while the control mice remained unaffected (Fig. 5A).

This profile is reminiscent of HAAF, in which recurrent hypoglycemia leads to a degradation of the counterregulatory mechanisms. We then explored the consequences of recurrent artificial glucopenia. We subjected the mice to a single injection of 2-DG for 4 consecutive days; blood glucose concentrations were evaluated following each injection. On day 1, the SF1-COUP-TFII presented a lower increase of blood glucose concentrations compared with the control mice (Fig. 5B). Further 2-DG injections failed to induce an increase in blood glucose concentrations in SF1-COUP-TFII (Fig. 5B). Food intake over a 2 h period following the injection was also evaluated in mice receiving the daily 2-DG or saline injection. On day 3 of the daily 2-DG injection, food intake was severely impaired in the SF1-COUP-TFII mice, while it remained unaffected in the control mice (Fig. 5C), indicating that recurrent glucopenia altered the reflexive food intake. Taken together, these observations indicate that SF1-COUP-TFII mice are more prone to HAAF than control mice. When looking at the AMPK $\alpha$  subunits phosphorylation profiles, the control mice receiving 2-DG injections had normal AMPK phosphorylation, while it was blunted in SF1-COUP-TFII mice (Fig. 5D). The HAAF observed in the SF1-COUP-TFII mice is associated with an overall defective AMPK activation (35).

## Discussion

In the present work, we have shown that a 50% decrease in COUP-TFII expression in VMN neurons led to an improved insulin sensitivity. However, this phenotype is associated with defective CRR to hypoglycemia and enhanced risk of HAAF development.

The metabolic phenotype of the SF1-COUP-TFII mice showed similar postprandial thermogenesis and lipid and carbohydrate oxidation rates with greater calorie cost for physical activity. The energy expended in relation to spontaneous physical activity amounts to 10–15% of daily energy expenditure in rodents; thus, a 15% increase in physical activity cost can induce hardly more than an increase in energy expenditure of 1.5–2%. However, this can be significant when looking at the difference in fat growth over a mouse lifetime. It represents a very slow process that results from



**Fig. 3.** Decreased COUP-TFII expression in the VMN leads to an increased expression of POMC mRNA and increased insulin signaling. (A) POMC and agouti-related protein (AgRP) mRNA expression levels in the hypothalamus of fed SF1-COUP-TFII mice and their controls. White, (SF1)<sup>CRE+/-</sup> mice; gray, COUP-TFII fl/wt; black, (SF1)<sup>CRE+/-</sup> COUP-TFII fl/wt mice. Data are expressed as the mean  $\pm$  SEM ( $n = 8$ ). \* $P \leq 0.05$ . (B) Autoradiogram of a Western blot analysis of the STAT3, phosphorylated STAT3 proteins, FOXO1, and phosphorylated FOXO1 in the hypothalamus of 5-month-old male mice. (C) EMSA with nuclear extracts of SF1-COUP-TFII or COUP-TFII fl/wt mice VMN. (Left) FOXO1 binding activity in absence (-) or presence (+) of a competing anti-FOXO1 antibody. (Right) CCAAT binding activity as internal control of nuclear extracts' integrity.

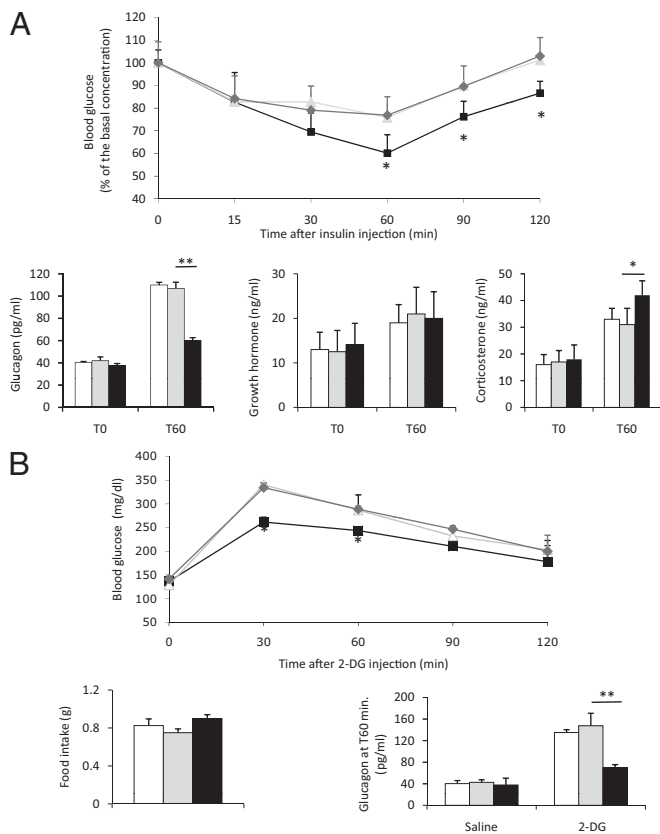
very small, quite undetectable differences in daily energy balance (<2%). Now, considering the hypothalamic transduction pathways whose expression has been altered by a decrease in COUP-TFII expression in the SF1 neurons, POMC expression was increased. POMC neurons receive strong excitatory outputs from the VMN (36). One of the consequences of COUP-TFII decreased expression in the VMN neurons could be an increase in POMC expression in the arcuate nucleus. This could contribute to the overall hypersensitivity to insulin and the leaner phenotype observed in SF1-COUP-TFII mice (37), as activation of central melanocortin pathways enhances insulin sensitivity (38) and energy expenditure (37). Moreover, the SF1-COUP-TFII mice phenotype is quite similar to the one observed in mice invalidated for the FOXO1 protein in the SF1 neurons (SF1-FOXO1 mice) (39), a mouse model with hypothalamic hypersensitivity to insulin. This could be due to the fact that in SF1-COUP-TFII mice, FOXO1 binding activity on its target sequences is decreased in the VMN, mimicking part of the SF1-FOXO1 mice phenotype. Therefore, some of the transcriptional pathways altered in the neurons of the SF1-FOXO1 mice could be shared by the COUP-TFII transcriptional program. A few years ago, our team had shown that COUP-TFII expression itself is directly repressed by FOXO1 (22). Therefore, part of the SF1-FOXO1 mice phenotype could also be due to a decreased COUP-TFII expression in the SF1 neurons.

The leaner phenotype associated with an insulin hypersensitivity is also similar to the one displayed by mice with a global COUP-TFII genetic inactivation at the heterozygous state (COUP-TFII<sup>+/-</sup> mice) (21). Now we could ponder the part played by the decreased expression of COUP-TFII in the VMN of the COUP-TFII<sup>+/-</sup> mice global phenotype. Even though part of it is clearly due to the defective development of the adipose tissue, COUP-TFII hypothalamic decreased expression could be part of the overall improved glucose homeostasis. Moreover, the SF1-COUP-TFII have a hypersensitivity to insulin preceding the difference in adiposity (Fig. 2C), while in the COUP-TFII<sup>+/-</sup> mice, this hypersensitivity was solely attributed to the lack of adipose tissue. Our recently published research of polymorphisms within the human COUP-TFII gene has shown a correlation between the presence of the SNP RS3743462 on the COUP-TFII human gene regulatory region and improved insulin sensitivity in nonobese patient cohort (40). Taken all together, these observations lead us to speculate that the maintenance of COUP-TFII expression in

metabolic tissues including VMN neurons is one of the actors involved in global insulin sensitivity.

In our previous work, we have observed that COUP-TFII is expressed in ~60–65% of the SF1 neurons (15). We can speculate that its expression pattern is defining a unique subpopulation of SF1-expressing neurons and that its expression in this subpopulation is key for the organism to protect itself against recurrent hypoglycemic episodes. Indeed, one of the most striking features of the SF1-COUP-TFII mice is that recurrent insulin injections revealed that the CRR to hypoglycemia is deteriorating faster than in control mice, leading to HAAF. Therefore, decreased COUP-TFII expression in the hypothalamus could enhance this desensitization to recurrent hypoglycemia and the onset of HAAF. Under normal situations, hypoglycemia triggers a CRR in which the fall in glucose concentrations leads to neuroendocrine and behavioral responses designed to restore euglycemia as fast as possible. One of these behavioral defenses against a fall in glycemia is a sugar-craving sensation and induced food intake (41). In the SF1-COUP-TFII mice, upon a single bout of glucopenia, the food intake reflex was not yet altered. We observed a significantly higher concentration of corticosterone related to the stronger hypoglycemia. There has been evidence indicating that greater concentrations of corticosterone could reduce the autonomic symptom response to recurrent hypoglycemia, leading to hypoglycemia unawareness, and contribute to the onset of HAAF (42, 43). In the SF1-COUP-TFII mice, the lack of a proper glucagon secretion in the context of a higher corticosterone secretion will therefore exacerbate the onset of HAAF. Moreover, as the glucopenic events are repeated, the food intake reflex is altered and could contribute to decrease blood glucose concentrations and therefore further degradation of the CRR. In a mouse model of insulinopenic diabetes, we observed that hypothalamic COUP-TFII expression was at its lowest (15). It is now well documented that in insulinopenic diabetic rodents, the CRR to hypoglycemia is altered (44). As a decreased COUP-TFII expression in the VMN neurons is involved in the onset of HAAF, it is therefore possible that in type I diabetes, this desensitization could be enhanced by the intrinsic decrease of COUP-TFII expression in the VMN neurons.

One of the ways the central nervous system is able to initiate a proper and in-range CRR is through the glucose-sensitive neurons' (GSNs) ability to detect minute variations in glucose concentrations (12, 13, 45, 46). We cannot directly argue on the

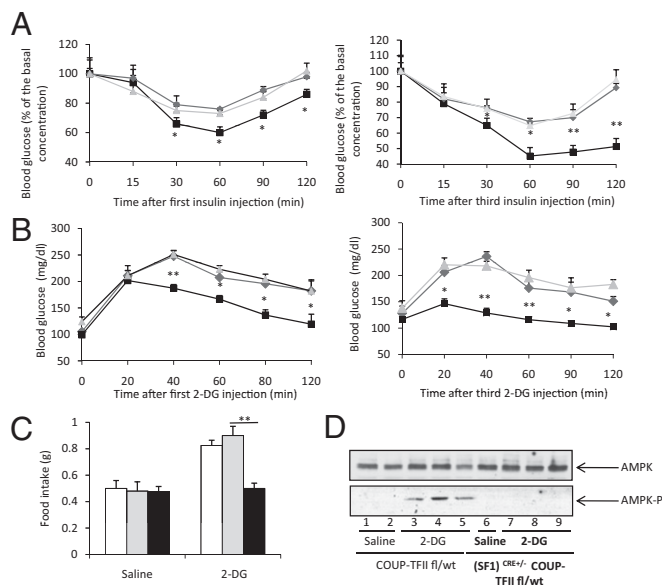


**Fig. 4.** SF1-COUP-TFII mice have a defective CRR to hypoglycemia. (A, Upper) ITT in 5-mo-old male mice (Left). Light gray, (SF1)<sup>CRE+/+</sup> mice; dark gray, COUP-TFII fl/wt; black, (SF1)<sup>CRE+/+</sup> COUP-TFII fl/wt mice. Data are expressed as the mean ± SEM (n = 8). \*P ≤ 0.05. (Lower) Glucagon, corticosterone, and growth hormone concentrations at time 0 and 60 min. White box, (SF1)<sup>CRE+/+</sup> mice; gray box, COUP-TFII fl/wt; black box, (SF1)<sup>CRE+/+</sup> COUP-TFII fl/wt mice. Data are expressed as the mean ± SEM (n = 8). (B, Upper) Blood glucose concentrations following a 2-DG injection (Left). Light gray, (SF1)<sup>CRE+/+</sup> mice; dark gray, COUP-TFII fl/wt; black, (SF1)<sup>CRE+/+</sup> COUP-TFII fl/wt mice. Data are expressed as the mean ± SEM (n = 8). (Lower) Food intake over a 2 h period following a single 2-DG injection and glucagon secretion at time 60 min of the 2-DG injection. White box, (SF1)<sup>CRE+/+</sup> mice; gray box, COUP-TFII fl/wt; black box, (SF1)<sup>CRE+/+</sup> COUP-TFII fl/wt mice. Data are expressed as the mean ± SEM (n = 6). \*P ≤ 0.05; \*\*P ≤ 0.01.

fact of COUP-TFII being expressed in such neurons, but as GSNs to hypoglycemia are quite exclusively located in the VMN and greatly involved in the CRR (47, 48), as COUP-TFII is expressed in 65% of the VMN neurons, and as these neurons are known to be involved in the CRR, it is therefore reasonable to propose that GSNs might express COUP-TFII. If they are not colocalized, presynaptic inputs could exist between these two populations, allowing reciprocal regulation. Therefore, COUP-TFII expression could input directly or indirectly on the central detection of glycemic variations by the GSNs and therefore be part of the CRR. One interesting feature of the SF1-COUP-TFII mice is that glucagon secretion is not affected in a significant manner at the fasted state. Recently, it has been shown that the CRR initiation depends on the speed at which blood glucose concentrations fall: with a slow fall, as it occurs during a fasting period, hepatportal sensors are responsible for the sympatho-adrenal response to lowering glucose concentrations, while with a rapid drop such as insulin-induced hypoglycemia, the brain glucose sensors located in the VMN take over by triggering glucagon secretion (49). In regards to our observations, COUP-TFII expression levels in the VMN SF1 neurons will only contribute to the latter.

Overall, the phenotype of the SF1-COUP-TFII mice is very progressive, and we could ponder if the continuous hypersensitivity to insulin could participate to the onset of a defective CRR. It is interesting to note that in younger mice, the only visible metabolic feature is the hypersensitivity to insulin, which precedes the difference in adiposity. Then, as mice age, if they are not subjected to ITT tests, the first insulin injection in 4–5-mo-old mice reveals a phenotype of defective CRR associated to the hypersensitivity. Experiments indicate that the insulin's inhibitory effect on glucagon release in a CRR is also in part mediated at the level of the VMN (50) and blockade of the sympathetic and parasympathetic inputs to α cells prevents the hypoglycemia-induced glucagon release (51, 52). Therefore, insulin can directly affect the way the VMN is able to trigger properly a CRR. We suggest that a continuous central hypersensitivity to insulin, as indicated by the increased FOXO1 phosphorylation, could contribute to the onset of a defective CRR. As the hypoglycemic events are more frequent, the HAAF is set and associated to an overall defective activation of the energy sensor, AMPK.

In type 1 diabetic patients, iatrogenic hypoglycemia is one of the most recurrent complication and remains one of the limiting factors in maintaining a lifetime of glycemic control (53). One of the challenges is to understand the mechanisms by which the organism is able to detect a fall in glucose concentrations and to initiate a proper and in-range CRR without, in the long term, altering the central nervous system glucose detection threshold. With the SF1-COUP-TFII mouse model and later the identification of the genetic pathways altered, we expect to provide further insights in the molecular mechanisms involved in the etiology of the HAAF. The fact that COUP-TFII is a nuclear receptor whose activity is modulated by a ligand (54) could open the search



**Fig. 5.** SF1-COUP-TFII mice are more prone to HAAF. (A) Recurrent ITT, 5-mo-old male mice were subjected to a weekly insulin tolerance test. (Left) First insulin injection; (Right) third insulin injection. Light gray, (SF1)<sup>CRE+/+</sup> mice; dark gray, COUP-TFII fl/wt; black, (SF1)<sup>CRE+/+</sup> COUP-TFII fl/wt mice. Data are expressed as the mean ± SEM (n = 10). \*P ≤ 0.05; \*\*P ≤ 0.01. (B) Blood glucose concentrations following recurrent 2-DG injections on 5-mo-old male mice. (Left) First injection; (Right) third 2-DG injection. Light gray, (SF1)<sup>CRE+/+</sup> mice; dark gray, COUP-TFII fl/wt; black, (SF1)<sup>CRE+/+</sup> COUP-TFII fl/wt mice. Data are expressed as the mean ± SEM (n = 6). \*P ≤ 0.05. (C) 2 h food intake following three 2-DG injection. White box, (SF1)<sup>CRE+/+</sup> mice; gray box, COUP-TFII fl/wt; black box, (SF1)<sup>CRE+/+</sup> COUP-TFII fl/wt mice. Data are expressed as the mean ± SEM (n = 8). (D) Autoradiogram of a Western blot analysis of the AMPKα and phosphorylated AMPKα in the hypothalamus of 5-mo-old male mice after four recurrent 2-DG injections.



for therapeutic molecules involved in protection against the deleterious effects of recurrent hypoglycemia. Therefore, COUP-TFII appears to be a good candidate as a potential target for therapeutic intervention in pathologies associated with a defective sensing of hypoglycemia and in the onset of HAAF.

## Materials and Methods

The transgenic mouse line (SF1)<sup>CRE+/-</sup> COUP-TFII fl/wt was generated by crossing the COUP-TFII fl/wt mice previously described (18), with transgenic mice expressing the Cre recombinase under the control of SF1 promoter (1). In this study the phenotype of transgenic male (SF1)<sup>CRE+/-</sup> COUP-TFII fl/wt mice were compared with control COUP-TFII fl/wt and (SF1)<sup>CRE+/-</sup> male mice. Mice were housed in colony cages with a 12-h light/12-h dark cycle in a temperature-controlled environment (20°C) and fed ad libitum with

a standard laboratory chow diet: composition 65% carbohydrate, 11% lipids, and 24% proteins (SAFE). All procedures were performed in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals. Animal experimentation permit number A-75-1845 was obtained from the French Ministère de L'enseignement Supérieur et de la Recherche. Supplemental details regarding methods can be found in *SI Materials and Methods*.

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