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Short Article

# Role for Insulin Signaling in Catecholaminergic Neurons in Control of Energy Homeostasis

A. Christine Könner,[1,3,4] Simon Hess,[2] Sulay Tovar,[1,3] Andrea Mesaros,[1] Carmen Sánchez-Lasheras,[1,3] Nadine Evers,[1,3] Linda A.W. Verhagen,[1,3] Hella S. Brönneke,[5] André Kleinridders,[1] Brigitte Hampel,[1,3] Peter Kloppenburg,[2] and Jens C. Brüning[1,3,4,*]

[1]Department of Mouse Genetics and Metabolism, Institute for Genetics
[2]Biocenter, Institute for Zoology
Cologne Excellence Cluster on Cellular Stress Responses in Aging Associated Diseases, Center of Molecular Medicine Cologne, University of Cologne, D-50674 Cologne, Germany
[3]Max Planck Institute for Neurological Research, D-50931 Cologne, Germany
[4]Center for Endocrinology, Diabetes and Preventive Medicine, University Hospital Cologne, D-50924 Cologne, Germany
[5]Mouse Phenotyping Core Facility, Cologne Excellence Cluster on Cellular Stress Responses in Aging Associated Diseases, D-50674 Cologne, Germany
*Correspondence: bruening@nf-mpg.de
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## SUMMARY

**Dopaminergic midbrain neurons integrate signals on food palatability and food-associated reward into the complex control of energy homeostasis. To define the role of insulin receptor (IR) signaling in this circuitry, we inactivated IR signaling in tyrosine hydroxylase (Th)-expressing cells of mice ($IR^{\Delta Th}$). IR inactivation in Th-expressing cells of mice resulted in increased body weight, increased fat mass, and hyperphagia. While insulin acutely stimulated firing frequency in 50% of dopaminergic VTA/SN neurons, this response was abolished in $IR^{\Delta Th}$ mice. Moreover, these mice exhibited an altered response to cocaine under food-restricted conditions. Taken together, these data provide in vivo evidence for a critical role of insulin signaling in catecholaminergic neurons to control food intake and energy homeostasis.**

## INTRODUCTION

The recent identification that peripheral hormones such as insulin, leptin, and numerous others, as well as nutrients, target hypothalamic neurons to control energy homeostasis, has set the ground for detailed understanding of the neuronal circuitry underlying the control of body weight homeostasis (Belgardt et al., 2008; Konner et al., 2009; Plum et al., 2006a, 2007; Rother et al., 2008; Sánchez-Lasheras et al., 2010; Schwartz, 2006; Klöckener et al., 2011). Deletion of the IR in the brain of mice assigned IR signaling a role in regulation of fuel metabolism and reproduction in vivo (Bruning et al., 2000). Moreover, insulin signaling in the CNS not only controls body weight but also regulates peripheral glucose and fat metabolism (Koch et al., 2008; Konner et al., 2007; Obici et al., 2002).

While most studies have focused on hypothalamic insulin signaling, there is evidence that insulin has direct effects in other brain regions, such as neurons of the midbrain (ventral tegmental area [VTA] and substantia nigrar [SN]) (Elmquist et al., 1998; Figlewicz et al., 2003; Havrankova et al., 1978). Here, the IR is coexpressed with tyrosine hydroxylase (Th)—a key enzyme and marker for catecholaminergic neurons (Figlewicz et al., 2003)—and insulin administration into the VTA results in an increased formation of phosphatidylinositol 3,4,5 trisphosphate (PIP3) as a result of phosphatidylinositol 3 (PI3) kinase activation (Figlewicz et al., 2007). Therefore, insulin action in dopaminergic (DA) neurons provides a potential link between the control of food intake and the reward circuitry (Figlewicz, 2003; Figlewicz and Benoit, 2009; Palmiter, 2007).

In fact, insulin, as well as leptin and ghrelin, modulates reward seeking and drug relapse—behaviors associated with DA signaling in the mesolimbic dopamine system (Abizaid et al., 2006; Figlewicz et al., 2001, 2004, 2006, 2008; Figlewicz and Benoit, 2009; Fulton et al., 2006; Hommel et al., 2006; Jerlhag et al., 2006), and leptin signaling in the VTA directly regulates feeding behavior (Hommel et al., 2006).

To define the role of insulin signaling in catecholaminergic cells in the regulation of energy homeostasis, we have specifically altered insulin signaling in Th-expressing cells of mice and reveal an important role for insulin action in this circuitry in control of feeding and body weight.

## RESULTS

### Generation of $IR^{\Delta Th}$ Mice

To investigate the role of insulin signaling in Th-expressing cells, we inactivated the *IR* gene specifically in these cells by crossing $IR^{lox/lox}$ mice with those expressing Cre recombinase from the 3′ untranslated region of the *Th* gene ($IR^{\Delta Th}$) (Bruning et al., 2000; Lindeberg et al., 2004).

In the periphery the majority of sympathetic neurons are catecholaminergic, and in the CNS the most abundant group of catecholaminergic cells are DA neurons of the mesencephalon (Moore and Bloom, 1979). Immunohistochemistry for endogenous Th in the brain revealed expression in the arcuate nucleus (ARC), paraventricular nucleus (PVN), VTA/SN, locus coeruleus, and rostroventrolateral medulla (see Figure S1A available online). Cre-mediated recombination was visualized by the use of a reporter mouse strain, which expresses GFP upon Ce-mediated

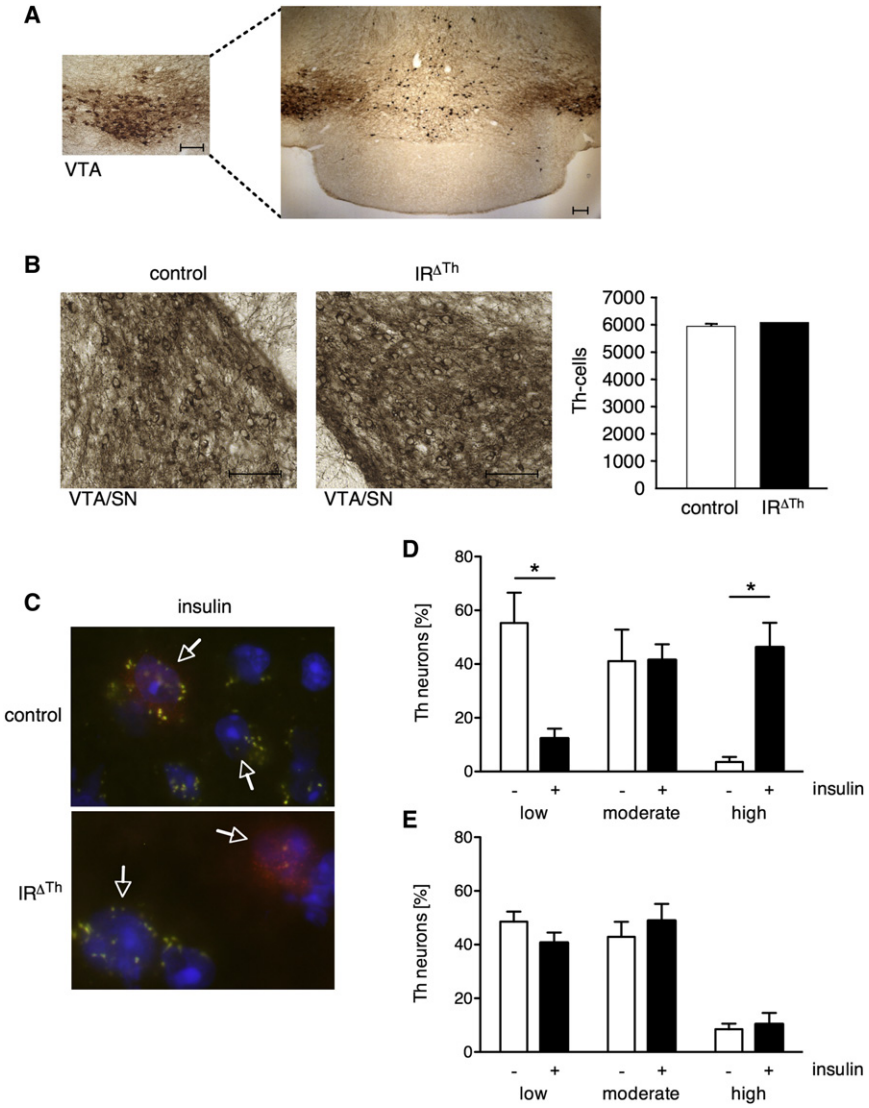


**Figure 1. Generation of Th Cell-Specific IR Knockout Mice**

(A) Cre-mediated recombination was visualized by immunohistochemistry for enhanced GFP on brain sections of double heterozygous reporter mice (*Th-IRES-Cre-Z/EG*). Cre-mediated recombination removes the loxP-flanked neomycin resistance gene; thus GFP is transcribed only in Th-expressing cells. Cre recombinase activity is present in the VTA and the substantia nigra (SN). GFP-positive cells, brown. Scale bar, 100 μm.

(B) Immunohistochemistry for Th was performed in VTA/SN sections from control and IR$^{\Delta Th}$ animals. Total number of Th-positive cells in control and IR$^{\Delta Th}$ animals is as mean ± SEM of three control and two IR$^{\Delta Th}$ animals. Th-positive cells, brown. Scale bar, 50 μm.

(C) Double immunohistochemistry of VTA/SN neurons of control and IR$^{\Delta Th}$ reporter mice was performed in overnight fasted mice, which were intravenously injected with either saline or insulin and sacrificed 10 min afterwards. Arrows indicate one Th-expressing neuron and one non-Th-expressing neuron in each panel. Blue (DAPI), DNA; red, β-gal (Th-expressing neurons); green, PIP3.

(D) Quantification of PIP3 levels in control reporter mice in the basal state (–) and after insulin (+) stimulation. Values are means ± SEM of sections obtained from three unstimulated and three insulin-stimulated control mice.

(E) Quantification of PIP3 levels in IR$^{\Delta Th}$ reporter mice in the basal state (–) and after insulin (+) stimulation. Values are means ± SEM of sections obtained from three unstimulated and four insulin-stimulated IR$^{\Delta Th}$ mice.

Displayed values are means ± S.E.M.; *$p < 0.05$.

recombination (*Z/EG-mice*) (Novak et al., 2000). These mice showed a pattern of GFP immunoreactivity in the VTA/SN and in the above described Th-positive neurons (Figure 1A).

To address whether inactivation of the IR alters development and maintenance of DA midbrain neurons, we compared the number of Th-expressing cells in control (*IR$^{lox/lox}$*) and IR$^{\Delta Th}$ mice, revealing unaltered numbers and distribution of Th-positive neurons in the VTA/SN of IR$^{\Delta Th}$ mice compared with controls (Figure 1B).

Since global brain disruption of the IR leads to hypogonadism and Th neurons in the hypothalamus can control prolactin release (Ben-Jonathan and Hnasko, 2001; Bruning et al., 2000), we assessed parameters of fertility in IR$^{\Delta Th}$ mice. However, there were no significant differences in litter-to-litter intervals, litter size, and morphology of reproductive organs between control and IR$^{\Delta Th}$ mice (Figures S1B–S1D).

In the VTA, insulin has previously been shown to activate the PI3 kinase cascade, which catalyzes the generation of PIP3 from phosphatidylinositol 4,5 diphosphate (PIP2) (Figlewicz et al., 2007). Therefore, the effect of Th neuron-restricted IR deficiency on insulin's ability to activate the PI3 kinase pathway was determined. In the basal state, little immunoreactive PIP3 was detectable in Th-expressing neurons of control and IR$^{\Delta Th}$ reporter mice (Figures 1C–1E). In control mice, insulin treatment resulted in activation of PIP3 formation in Th-expressing and non-Th-expressing neurons of the VTA/SN (Figures 1C and 1D). In contrast, in IR$^{\Delta Th}$ mice, insulin stimulation resulted in PIP3 formation in non-Th-expressing cells, but it failed to activate PIP3 formation in Th-expressing neurons (Figures 1C and 1E). These data indicate efficient and specific IR inactivation in Th-expressing cells in the VTA/SN of IR$^{\Delta Th}$ mice.

## Insulin Increases Firing of VTA/SN Dopaminergic Neurons

To determine differences in the insulin responsiveness between control mesencephalic VTA/SN DA neurons and DA neurons lacking the IR, we performed perforated patch clamp recordings (Figures 2A–2F). The spontaneous spike frequency was not significantly different between control and IR$^{\Delta Th}$ DA neurons (controls, 2.1 ± 0.2 Hz, n = 25; IR$^{\Delta Th}$, 1.8 ± 0.2 Hz, n = 27; $p > 0.05$; Figure 2D). In 50% (8 out of 16) of control mesencephalic

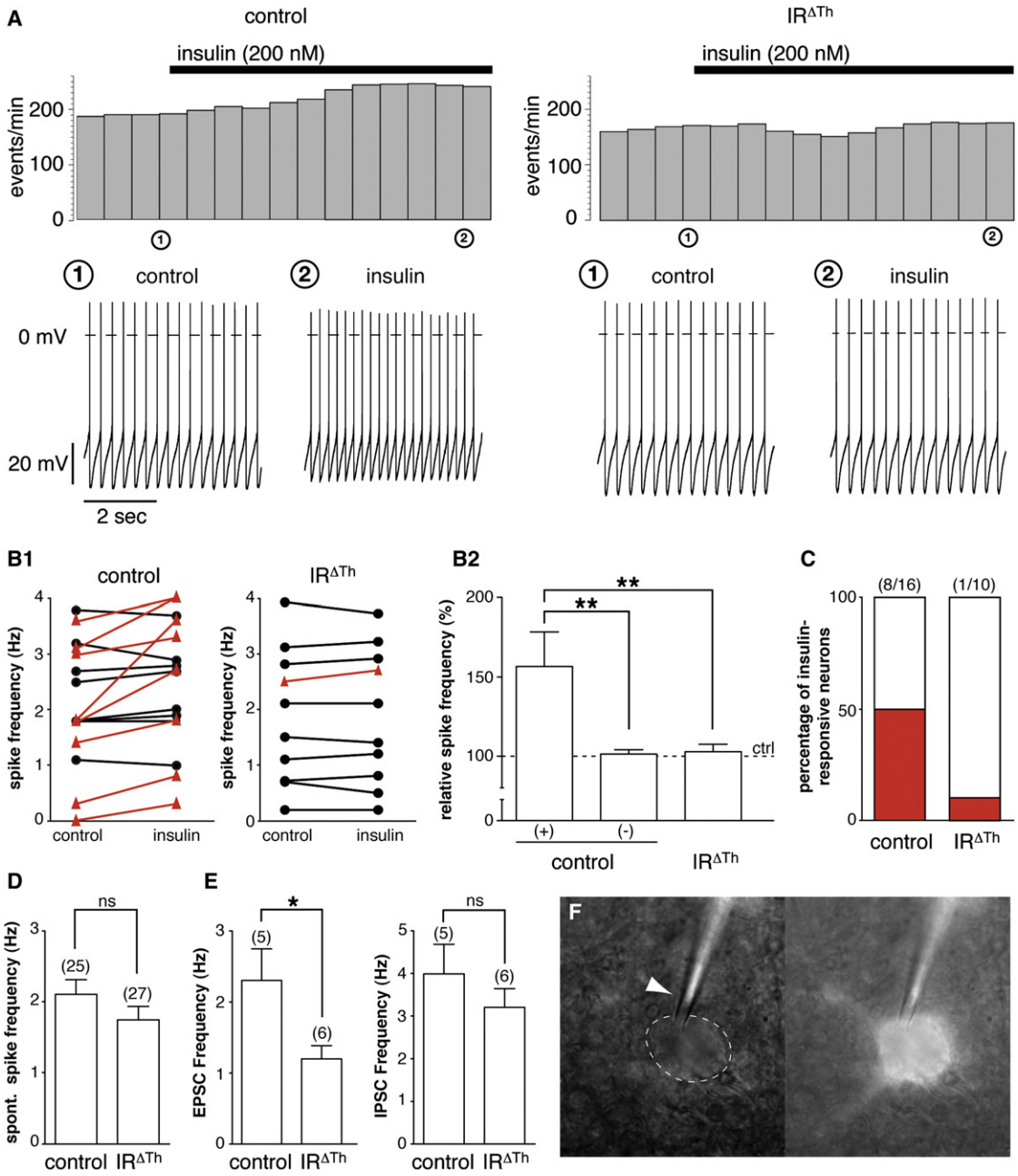


**Figure 2. Insulin Increases the Frequency of Action Potentials in Mesencephalic Dopaminergic Neurons**

(A) (Top) Peristimulus time histogram (bin width, 60 s) of recordings from a control (left) and an $IR^{\Delta Th}$ neuron (right) during insulin (200 nM) application. (Bottom) Original traces from the recordings shown above at two different time points (indicated by numbers).

(B) Effect of insulin on the spike frequency of mesencephalic DA neurons from control and $IR^{\Delta Th}$ mice. (B1) The dots and triangles indicate the change in firing frequency of the individual neurons (control, n = 16; $IR^{\Delta Th}$, n = 10). Red triangles show insulin-responsive cells according to the three times SD criterion (see the Supplemental Experimental Procedures), and black dots represent nonresponsive cells. (B2) Relative change in firing frequency during application of insulin (200 nM) in responsive (+) and nonresponsive (−) control and $IR^{\Delta Th}$ neurons. Neurons that were silent under control conditions (n = 1) were not included.

(C) Percentage of control (8/16) and $IR^{\Delta Th}$ (1/10) mesencephalic DA neurons that responded to bath application of 200 nM insulin with a significant increase in firing frequency.

(D) Spontaneous frequency in DA neurons of control (n = 25) and $IR^{\Delta Th}$ (n = 27) mice. Neurons that were silent under control conditions (n = 1) were not included.

(E) Frequency of excitatory postsynaptic currents (EPSCs, left) and inhibitory postsynaptic currents (IPSCs, right) of DA neurons of control (n = 5) and $IR^{\Delta Th}$ (n = 6) mice.

(F) Fluorescence image of a mesencephalic DA neuron during a perforated-patch clamp recording (left) and after conversion to the whole-cell configuration (right). To test the integrity of the perforated-patch clamp recording, the pipette was backfilled with 0.02% tetraethylrhodamine-dextran.

Displayed values are means ± S.E.M.; *p < 0.05; **p < 0.01.

DA neurons, insulin significantly increased the spike frequency on average by 57% ± 22% (Figures 2A–2C). In contrast, only one $IR^{\Delta Th}$ DA neuron out of ten responded to insulin (Figures 2A–2C). This insulin effect in control cells was also observed in conditions where synaptic input was blocked (data not shown), indicating cell-autonomous insulin action to increase DA neuron firing. In addition, analysis of synaptic input revealed a decreased excitatory postsynaptic current (EPSC) frequency in $IR^{\Delta Th}$ DA neurons as compared to control DA neurons (controls, 2.3 ± 0.4 Hz, n = 5; $IR^{\Delta Th}$, 1.2 ± 0.2 Hz, n = 6; $p < 0.05$; Figure 2E) while the frequency of the inhibitory input (IPSCs) remained unchanged (controls, 4.0 ± 0.7 Hz, n = 5; $IR^{\Delta Th}$, 3.2 ± 0.4 Hz, n = 6; $p > 0.05$; Figure 2E). Thus, insulin modulates the intrinsic firing properties in a substantial subset of mesencephalic DA neurons and promotes the establishment or maintenance of excitatory synaptic connections of these cells.

## Increased Adiposity in $IR^{\Delta Th}$ Mice

To investigate the impact of IR inactivation in Th-expressing cells on the regulation of energy homeostasis, body weight of control and $IR^{\Delta Th}$ mice was monitored. While body weight of *Th-IRES-Cre* mice is comparable to control littermates (Figure S1E), both female and male $IR^{\Delta Th}$ mice exhibited an increased body weight from approximately 7 weeks of age on as compared to controls (Figures 3A and 3B). Consistent with the increased body weight, $IR^{\Delta Th}$ mice displayed an increased epigonadal fat pad mass at the age of 20 weeks compared to controls (Figures 3C and 3D). Also brown adipose tissue mass was increased in $IR^{\Delta Th}$ mice at the age of 20 weeks (Figures 3E and 3F). In vivo magnetic resonance spectrometry confirmed the presence of adiposity, as body fat content was increased in $IR^{\Delta Th}$ mice as compared to controls (Figures 3G and 3H). Increased adiposity in $IR^{\Delta Th}$ mice was also reflected in an augmented adipocyte size (Figure 3I) and in increased plasma leptin concentrations (Figures 3J and 3K). Taken together, these results indicate that inactivation of the *IR* gene in Th-expressing cells results in increased body weight and adiposity.

## Hyperinsulinemia in $IR^{\Delta Th}$ Mice

We next assessed glucose metabolism in control and $IR^{\Delta Th}$ mice. However, there were no alterations in glucose tolerance, insulin sensitivity, or plasma insulin concentrations in young $IR^{\Delta Th}$ mice compared to controls (Figures S2A–S2F). However, serum insulin concentrations of $IR^{\Delta Th}$ mice were significantly increased at an age of 20 weeks, when obesity persisted longer and became more prominent (Figures S2G and S2H). Nevertheless, fasted and fed blood glucose levels remained unaltered in $IR^{\Delta Th}$ mice also at this age (Figure S2I). Collectively, these results indicate that mild insulin resistance develops in $IR^{\Delta Th}$ mice likely secondarily to obesity.

## Increased Food Intake in $IR^{\Delta Th}$ Mice

To further analyze the mechanism underlying the increase in body weight and adiposity of $IR^{\Delta Th}$ mice, energy intake and energy expenditure were assessed in these mice. While food intake of *Th-IRES-Cre* mice was comparable to C57BL/6 control littermates (Figure S1F), $IR^{\Delta Th}$ mice displayed an increased food intake both at 6 and 13 weeks of age as compared to controls (Figures 4A–4D). Since *Th-IRES-Cre*-mediated IR inactivation is not only restricted to DA midbrain neurons, we next aimed to specifically address the role of IR signaling in the VTA in control of feeding behavior. Thus, we stereotactically injected either an adeno-associated virus (AAV) expressing GFP (AAV-GFP) or an AAV expressing Cre (AAV-Cre) bilaterally in the VTA of $IR^{lox/lox}$ mice. Analysis of food intake revealed that 14 days after injection of the AAV-vectors—concomitant with the predescribed maximum of AAV-driven transgene expression—animals correctly targeted with AAV-Cre in the VTA exhibited a higher food intake than those injected with AAV-GFP and those where injection of AAV-Cre had failed to correctly target the VTA (Figures S2J and S2K). Collectively, these data indicate that IR inactivation in Th neurons and likely those in the VTA causes hyperphagia.

In contrast, indirect calorimetry revealed unaltered oxygen consumption in male $IR^{\Delta Th}$ mice as compared to control mice, and female $IR^{\Delta Th}$ mice only exhibited decreased energy expenditure during daytime (Figures 4E and 4F). Basal locomotor activity in both female and male $IR^{\Delta Th}$ mice was also indistinguishable to control animals (Figures 4G and 4H).

## $IR^{\Delta Th}$ Mice Exhibit Altered Cocaine-Induced Locomotor Activity

The role of the DA system in the behavioral effects of cocaine is well-established (Di Chiara and Imperato, 1988). Moreover, food deprivation increases the reinforcing efficacy of cocaine self-administration (Bell et al., 1997), and cocaine-induced locomotor activity is increased in food-deprived rats (Bell et al., 1997). Thus, to address whether insulin signaling in Th-expressing cells alters cocaine's motor-activating effects, we compared cocaine-induced locomotor activity in control and $IR^{\Delta Th}$ mice that were restricted to 80% of their normal daily food intake. Cocaine treatment significantly increased locomotor activity in control mice, but the ability of cocaine to induce locomotor activity was largely attenuated in $IR^{\Delta Th}$ mice (Figure 4I). However, cocaine's ability to enhance locomotor activity was comparable between ad libitum-fed control and $IR^{\Delta Th}$ mice (Figure S3A). Collectively, these experiments reveal a role for insulin action in the DA system to modulate the response to cocaine dependent on metabolic state.

Food deprivation also affects appetitive behaviors, including increased drinking of sweet solutions (Sheffield and Roby, 1950; Smith and Duffy, 1975), while insulin decreases acute sucrose intake as well as sucrose self-administration in rats (Figlewicz et al., 2008). Thus, we aimed to investigate whether lack of insulin signaling in Th-expressing cells has an effect on preference for a sucrose solution. Animals were given a choice between two bottles: one bottle contained water and the other bottle a sucrose solution (1%, 2%, 4%, or 8% sucrose, w/v). Mice lacking the IR in Th-expressing cells exhibited a trend for increased sensitivity to a sucrose solution of 1% and 2% (w/v) (Figure 4 J). However, sensitivity to sucrose solutions of higher concentrations (i.e., 4% and 8%, w/v) was comparable between control and $IR^{\Delta Th}$ mice (Figure 4J). These experiments indicate that insulin action in Th neurons may also contribute to the regulation of food-reward-related behavior, although this aspect clearly needs further detailed investigation in different behavioral paradigms.

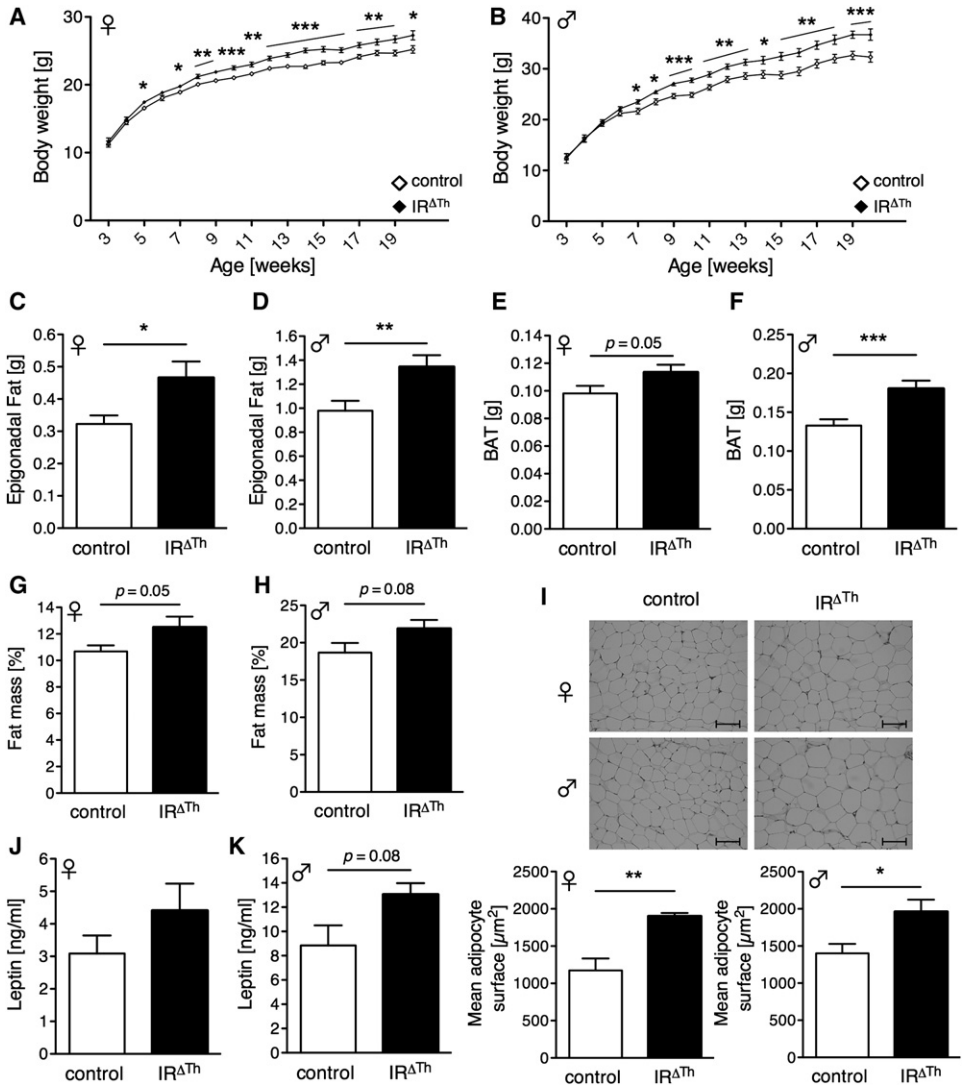


**Figure 3. Increased Body Weight and Adiposity in IR$^{\Delta Th}$ Mice**

(A) Average body weight of female control (◇) and IR$^{\Delta Th}$ (◆) mice (n = 27–30).
(B) Average body weight of male control (◇) and IR$^{\Delta Th}$ (◆) mice (n = 13–22).
(C) Parametrial fat pad weight of female control and IR$^{\Delta Th}$ mice (n = 29) at the age of 20 weeks.
(D) Epididymal fat pad weight of male control and IR$^{\Delta Th}$ mice (n = 15–17) at the age of 20 weeks.
(E) Brown adipose tissue (BAT) weight of female control and IR$^{\Delta Th}$ mice (n = 29) at the age of 20 weeks.
(F) Brown adipose tissue (BAT) weight of male control and IR$^{\Delta Th}$ mice (n = 15–17) at the age of 20 weeks.
(G) Average body fat content of female control and IR$^{\Delta Th}$ mice (n = 29) at the age of 20 weeks measured by NMR.
(H) Average body fat content of male control and IR$^{\Delta Th}$ mice (n = 15–17) at the age of 20 weeks measured by NMR.
(I) H&E stain of epididymal/parametrial adipose tissue of female and male control and IR$^{\Delta Th}$ mice at the age of 20 weeks. Scale bar, 50 μm. Quantification of mean adipocyte surface in epididymal/parametrial adipose tissue of female (n = 4) and male (n = 4–5) control and IR$^{\Delta Th}$ mice at the age of 20 weeks.
(J) Serum leptin concentrations of female control and IR$^{\Delta Th}$ mice (n = 17–20) at the age of 20 weeks.
(K) Serum leptin concentrations of male control and IR$^{\Delta Th}$ mice (n = 7–9) at the age of 20 weeks.
Displayed values are means ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001.

## Decreased Tyrosine Hydroxylase and Dopamine Receptor 2 Expression in IR$^{\Delta Th}$ Mice

Next, expression of different genes critically involved in dopamine signaling in the mesolimbic and nigrostriatal circuitry was determined in control and IR$^{\Delta Th}$ mice. This revealed decreased mRNA expression of Th and dopamine receptor 2 (D2R) in the VTA of IR$^{\Delta Th}$ mice as compared to controls (Figure S3B). On the other hand, expression of monoamine oxidase B (MAOB), catechol-O-methyl transferase (COMT), aromatic L-amino acid decarboxylase (AAAD), and the dopamine transporter (DAT) was unaltered in the VTA of IR$^{\Delta Th}$ mice as compared to controls (Figure S3B). Moreover, expression of MAOB, COMT, D1R, D2R, and D3R were unaltered in the NAc and CPu of these mice (Figures S3C and S3D). However, in the absence of functional

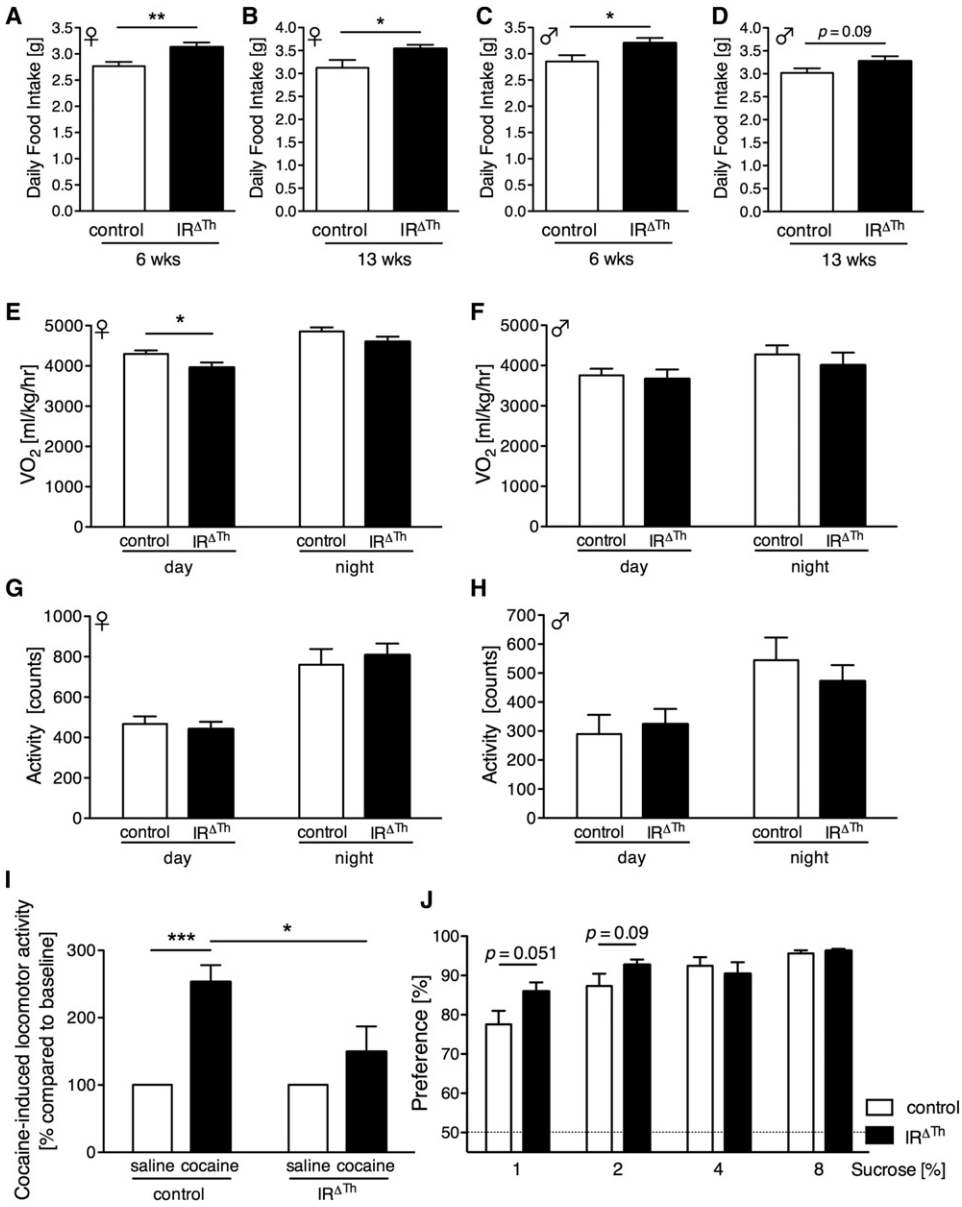


**Figure 4. Increased Food Intake and Altered Response to Cocaine in $IR^{\Delta Th}$ Mice**

(A) Daily food intake of female control and $IR^{\Delta Th}$ mice at the age of 6 weeks (n = 11–12).

(B) Daily food intake of female control and $IR^{\Delta Th}$ mice at the age of 13 weeks (n = 12–13).

(C) Daily food intake of male control and $IR^{\Delta Th}$ mice at the age of 6 weeks (n = 10–13).

(D) Daily food intake of male control and $IR^{\Delta Th}$ mice at the age of 13 weeks (n = 11).

(E) Mean oxygen consumption ($VO_2$) corrected for lean body mass of female control (n = 12) and $IR^{\Delta Th}$ (n = 7) mice measured by indirect calorimetry at the age of 19 weeks.

(F) Mean oxygen consumption ($VO_2$) corrected for lean body mass of male control (n = 6) and $IR^{\Delta Th}$ (n = 5) mice measured by indirect calorimetry at the age of 19 weeks.

(G) Basal locomotor activity of female control (n = 12) and $IR^{\Delta Th}$ (n = 7) mice at the age of 19 weeks.

(H) Basal locomotor activity of male control (n = 6) and $IR^{\Delta Th}$ (n = 5) mice at the age of 19 weeks.

(I) Food-restricted (maintained at 80% of normal average daily food intake) control (n = 11) and $IR^{\Delta Th}$ (n = 9) mice were injected intraperitoneally with either vehicle (saline) or cocaine (20 mg/kg BW). Cocaine-induced locomotor activity is expressed as percentage compared to total distance moved under baseline conditions (saline).

(J) Sucrose preference expressed as percentage of total intake of a sucrose solution for food-restricted (maintained at 80% of normal average daily food intake) control (n = 10) and $IR^{\Delta Th}$ (n = 15) mice in a two-bottle choice paradigm.

Displayed values are means ± S.E.M.; *p < 0.05; **p < 0.01; ***p < 0.001.

IR signaling, decreased Th expression in the VTA/SN does not alter dopamine content of the VTA/SN, the NAc, or the CPu (Figure S3E).

## DISCUSSION

In the last several years substantial evidence has emerged, showing that peripheral hormonal signals such as insulin, leptin, and ghrelin are able to modulate the reward circuitry. Both insulin and leptin administration into the VTA result in an increased formation of PIP3, the lipid product of activated PI3 kinase (Figlewicz et al., 2007). In our model, insulin's ability to activate the PI3 kinase pathway was blunted specifically in VTA Th neurons of $IR^{\Delta Th}$ mice, indicating successful inactivation of the IR in these cells and that insulin stimulates PI3 kinase in these cells in a cell-autonomous manner and not indirectly via synaptic transmission.

It has previously been demonstrated that intraventricular insulin administration prevents the expression of a place preference for high-fat food, decreases lick rates for sucrose solutions in a lickometer task, and decreases sucrose self-administration in rats that are not food deprived (Figlewicz et al., 2004; Figlewicz et al., 2006). Moreover, direct injection of insulin into the VTA blocks VTA-initiated feeding of sucrose pellets by administration of a μ-opioid agonist, while VTA insulin injection did not change baseline sucrose pellet intake, indicating that insulin's effect on food reward may only be relevant when there is adequate stimulation or drive within the VTA (Figlewicz et al., 2007). Our results in mice with targeted inactivation of insulin signaling in Th cells and their obese, hyperphagic phenotype reveals a critical role for insulin signaling in this circuitry to control feeding and fat mass long-term. Moreover, the results of our study support a direct role for insulin signaling in the brain reward system, as $IR^{\Delta Th}$ mice exhibited altered cocaine-evoked locomotor activity and indications of altered sensitivity to a sucrose solution.

However, since Th is the first, rate-limiting enzyme in catecholamine synthesis and neurons of both the peripheral and central nervous system use catecholamines as neurotransmitters, and thus IR inactivation in our model occurred in different Th-positive brain regions besides the VTA, the observed phenotype cannot directly be accounted for by impaired insulin action in DA VTA neurons. For example, the IR is also expressed in noradrenergic neurons in the NTS, which are involved in the satiation process (Blevins and Baskin, 2010; Werther et al., 1987). Moreover, inheritant to the technical approach of this study, the presented findings could also have been influenced by neuroadaptive changes due to lack of IR signaling in Th neurons during development. Nevertheless, the fact that obesity in $IR^{\Delta Th}$ mice develops as a consequence of hyperphagia in the absence of detectable major alterations in energy expenditure or circulating catecholamine concentrations (data not shown), and the clear trend for hyperphagia upon acute AAV-Cre-mediated IR deletion in the VTA, provides further support for the notion that insulin resistance in DA midbrain neurons might indeed contribute significantly to the phenotype observed in these animals.

Mechanistically, we demonstrate that insulin has a significant excitatory effect in a major subpopulation of DA VTA/SN neurons. Moreover, Th neuron-specific deletion of the IR results in a reduction of excitatory input on DA VTA/SN neurons and abolished the aforementioned cell-autonomous effect on these cells. A potential mediator of insulin's effects on firing frequency is the PI3 kinase pathway. We and others have previously demonstrated that insulin in a PI3 kinase-dependent activation of $K_{ATP}$-channels can lead to cell-autonomous hyperpolarization of neurons (Konner et al., 2007; Plum et al., 2006b; Spanswick et al., 2000). Since insulin leads to an increase in spontaneous firing in DA VTA/SN neurons, it is unlikely that $K_{ATP}$-channels are the downstream target of PI3 kinase signaling in these cells. However, insulin can modulate activity of the DAT at different levels in a PI3 kinase-dependent manner (Carvelli et al., 2002; Figlewicz et al., 1994). Thus, increased insulin-mediated DAT activity might lead to a higher dopamine clearance, resulting in the disinhibition of DA VTA/SN cells in a D2R-dependent mechanism (Uchida et al., 2000). However, future studies will have to further address the detailed mechanism(s) of how insulin controls VTA/SN neuronal excitability.

Importantly, research over the last several years suggested a role for the DA system in the development of obesity, as body mass index is negatively correlated with D2R density in striatal regions (Volkow et al., 2008; Wang et al., 2001). Moreover, in rats, diet-induced obesity is linked to deficits in mesolimbic dopamine neurotransmission, and obesity-prone rats exhibit reduced D2R expression levels (Geiger et al., 2008, 2009; Johnson and Kenny, 2010). Along this line, reduced D2R expression in $IR^{\Delta Th}$ mice may point toward an additional effect of insulin in the regulation of dopamine controlled feeding.

Collectively, our study reveals a critical role for insulin action in catecholaminergic neurons in long-term control of feeding. The further elucidation of the exact neuronal subpopulation(s) and cellular mechanisms responsible for this effect may thus define potential targets for the treatment of obesity.

## EXPERIMENTAL PROCEDURES

### Animal Care

All animal procedures and euthanasia were reviewed by the animal care committee of the University of Cologne, were approved by local government authorities (Bezirksregierung Köln), and were in accordance with National Institutes of Health guidelines. Mouse husbandry was performed as previously described (Konner et al., 2007).

### Generation of $IR^{\Delta Th}$ Mice

*Th-IRES-Cre* mice (Lindeberg et al., 2004) were mated with $IR^{lox/lox}$ mice, and breeding colonies were maintained by mating $IR^{lox/lox}$ with *Th-IRES-Cre-*$IR^{lox/lox}$ ($IR^{\Delta Th}$). Only animals from the same mixed background strain generation were compared to each other.

### Food Intake and Indirect Calorimetry

Food intake was measured over a 10 day period, during which mice were housed individually in regular cages using food racks. Food racks were weighed every day and daily food intake was calculated as the average daily intake of chow within the time stated. Indirect calorimetry was measured in a Calorimetry Module (CLAMS, Oximax Windows 4.00, Columbus Instruments, Columbus, OH, USA) as previously described (Mesaros et al., 2008).

### Analysis of Body Composition

Nuclear magnetic resonance (NMR) was employed to determine whole-body composition of live animals using the NMR Analyzer minispec mq7.5 (Bruker Optik, Ettlingen, Germany).

### Cocaine-Induced Locomotor Activity

$IR^{lox/lox}$ (control) and $IR^{\Delta Th}$ mice that were either ad libitum fed or restricted to 80% of their average daily food intake (starting 3 days prior to the experiment)

were injected intraperitoneally with either vehicle (saline) or cocaine (20 mg/kg BW), and the distance moved by each mouse over a period of 45 min was measured using an automated video-based system in an open field (50 × 50 cm, VideoMot 2, TSE Systems, Bad Homburg, Germany).

### Statistical Methods

Data sets were analyzed for statistical significance using either a two-tailed unpaired Student's t test or, if applicable (i.e., comparison of locomotor activity under basal conditions and after cocaine treatment), a two-tailed paired Student's t test. In one case, i.e., the comparison of average daily food intake of AAV-Cre VTA hit mice with either AAV-GFP or AAV-Cre VTA missed mice, a one-tailed unpaired Student's t test was performed. All p values below 0.05 were considered significant. All displayed values are means ± SEM; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus controls.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.cmet.2011.03.021.

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