Hypothalamic WNT Signalling Is Impaired During Obesity and Reinstated by Leptin Treatment in Male Mice

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The WNT pathway has been well characterized in embryogenesis and tumorigenesis. In humans, specific polymorphisms in the T cell-specific transcription factor 7 and the WNT coreceptor, low-density lipoprotein receptor-related protein-6 (LRP-6), both prominent components of this pathway, correlate with a higher incidence of type 2 diabetes, suggesting that the WNT pathway might be involved in the control of adult glucose homeostasis. We previously demonstrated that glycogen-synthase-kinase-3β (GSK-3β), the key enzyme of the WNT pathway, is increased in the hypothalamus during obesity and exacerbates high-fat diet-induced weight gain as well as glucose intolerance. These data suggest that WNT action in the hypothalamus might be required for normal glucose homeostasis. Here we characterized whether WNT signaling in general is altered in the hypothalamus of adult obese mice relative to controls. First we identified expression of multiple components of this pathway in the murine arcuate nucleus by in situ hybridization. In this region mRNA of ligands and target genes of the WNT pathway were down-regulated in obese and glucose-intolerant leptin-deficient mice. Similarly, the number of cells immunoreactive for the phosphorylated (active) form of the WNT-coreceptor LRP-6 was also decreased in leptin-deficient mice. Leptin treatment normalized expression of the WNT-target genes Axin-2 and Cylin-D1 and increased the number of phospho-LRP-6-immunoreactive cells reaching levels of lean controls. Leptin also increased the levels of phosphorylated (inactive) GSK-3β in the arcuate nucleus, and this effect was colocalized to neuropeptide Y neurons, suggesting that inactivation of GSK-3β may contribute to the neuroendocrine control of energy homeostasis. Taken together our findings identify hypothalamic WNT signaling as an important novel pathway that integrates peripheral information of the body's energy status encoded by leptin. **(*Endocrinology* 154: 4737–4745, 2013)**

Type 2 diabetes is increasing at an alarming rate (1). During the last decade, emerging evidence suggests that the brain represents a main insulin target tissue (2, 3). However, the molecular mechanisms and the absolute impact of the brain in the pathogenesis of type 2 diabetes remain incompletely understood. Striking recent observations suggest that the WNT pathway, typically associated with its essential function in embryogenesis and tumorigenesis (4, 5), may also be involved in the pathogenesis of type 2 diabetes. Human polymorphisms in genes involved in this signaling pathway (particularly the transcription factor T cell-specific transcription factor 7, or TCF-7) were associated with an increased risk to develop type 2 diabetes (6–8). Recently, we demonstrated that glucose

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Abbreviations: aCSF, artificial cerebral spinal fluid; AKT, protein kinase B; ARC, arcuate nucleus; DKK-1, Dickkopf-1; Fz, Frizzled; GFP, green fluorescent protein; GSK, glycogen-synthase-kinase; icv, intracerebroventricular; IRS, insulin receptor substrate; LRP, LDL receptor-related protein; NPY, neuropeptide Y; PI3K, phosphoinositide 3-kinase; TCF-7, T cell-specific transcription factor 7.

activates the WNT pathway in an autocrine fashion in macrophage cell lines (9), suggesting that this pathway might function as a glucose sensory system.

The WNT pathway consists of extracellular ligands (WNTs) that bind to cell surface receptors of the Frizzled (Fz) family and associated coreceptors, the low-density lipoprotein receptor-related proteins (LRP; eg, LRP-6). In addition to multiple WNT ligands, there are also a number of endogenously secreted antagonists, including Dickkopf (DKK) proteins and secreted Fz proteins. In the absence of WNT ligands, cytoplasmic β-catenin is phosphorylated by the key enzyme glycogen-synthase-kinase-3β (GSK-3β), targeting it for proteasomal degradation. GSK-3β is held in a complex including Axin, Dishevelled, and adenomatous polyposis coli. Ligands binding to both the Fz receptors and LRP coreceptors lead to breakdown of this complex and inactivation of GSK-3β, allowing stabilization of β-catenin within the cytoplasm and translocation to the nucleus. Within the nucleus, β-catenin acts together with TCF-7 (also known as TCF-4) to activate the gene expression that is characteristic of the canonical WNT-signaling pathway (10).

The key enzyme GSK-3 is known to be involved in insulin signal transduction, as it is phosphorylated and thereby inhibited by protein kinase B (AKT) (11–13). Furthermore, inhibition of GSK-3 improves whole-body glucose homeostasis (14, 15). We recently demonstrated that hypothalamic GSK-3β activity is elevated during obesity and that neuron-specific overexpression of GSK-3β in the mediobasal hypothalamus exacerbated hyperphagia, obesity, and impaired glucose tolerance (16). In contrast, acute inhibition of this enzyme within the central nervous system improved whole-body glucose homeostasis and decreased food intake, and we demonstrated that GSK-3β facilitates the interaction between insulin- and leptin-signaling pathways in the hypothalamus (16). Inhibition of GSK-3β led to improved hypothalamic insulin signaling via activation of PI3K (phosphoinositide 3-kinase), a key intracellular mediator of both leptin and insulin action (17). The cross talk between these hormones is critical for the central regulation of glucose homeostasis, but the precise nature of the interaction remained unclear.

Based on the association of the WNT pathway with type 2 diabetes, and our observation of a central role of the key WNT-responsive enzyme GSK-3β in mediation of central actions of leptin and insulin, in the current study we tested the hypothesis that WNT signaling is active in the hypothalamus of adult mice and is activated by the key body weight-regulatory hormone leptin. Therefore, we initially characterized the gene expression pattern of most components of this pathway in the hypothalamus of wild-type mice and then investigated whether key genes are differentially expressed between wild-type and leptin-deficient obese mice in the arcuate nucleus (ARC), the key region involved in neuronal control of metabolism. We furthermore analyzed whether leptin is involved in expression of mRNA for major WNT target genes and whether leptin regulates the WNT-coreceptor LRP6 on a post-translational level. To assess the potential importance of WNT signaling in regulating energy homeostasis via acting specifically in the hypothalamus, we additionally tested whether leptin selectively alters WNT activity in leptin-responsive neurons of the hypothalamus, expressing neuropeptide Y (NPY).

Materials and Methods

Animals

All procedures involving animals were licensed under German animal ethics legislation and received approval by the federal public authority for animal ethics. Experiments that were performed in Australia were conducted in accordance with the Monash University Animal Ethics Committee guidelines. All experiments used male mice that were purchased from Janvier. All mice (wild-type and $Lep^{ob/ob}$ mice) were on the C57BL/6JRj genetic background. Additionally, we used NPY-green fluorescent protein (GFP) mice (B6.FVB-Tg [Npy-hrGFP]1Lowl/J) which were drawn from the breeding colonies of the Monash University (Melbourne, Australia). Animals were 2 months old and housed individually under standard conditions with a light-dark cycle of 12 hours. The ambient temperature for mice was 23°C. Apart from the dark phase before the experiments, all animals had access to standard chow diet. For central administration of drugs, cannulas were stereotaxically implanted into the left lateral ventricle as described previously (17).

Expression of genes that are involved or targets of WNT signaling in the brain detected by in situ hybridization

To determine the central expression pattern of genes that are involved in or are targets of the WNT-pathway, we performed in situ hybridization on brains from $Lep^{+/+}$ mice. For differential expression of mice with and without impaired glucose tolerance, we performed a separate experiment employing one group of $Lep^{+/+}$ and one group of $Lep^{ob/ob}$ mice. Riboprobes complementary to the genes were generated from cloned cDNA from the hypothalamus of *Mus musculus*. cDNA synthesis was performed by using a cDNA synthesis kit (Invitrogen), according to the manufacturer's instructions. Primers used for amplification of the fragments were designed using Primer Select (Supplemental Table 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org; Lasergene; DNA-Star Software). DNA fragments were ligated into pGEM-T-Easy (Promega), transformed into DH5-α *Escherichia coli*, and sequenced. For cRNA synthesis of antisense riboprobes by in vitro transcription, SP6-polymerase or T7-polymerase (Invitrogen) was used. To generate the sense control for all riboprobes, cRNA synthesis was performed by the converse polymerase. As previ-

ously described (18), forebrain sections (16 μm) were collected throughout the extent of the ARC onto a set of 12 slides, with 12 sections mounted on each slide. Accordingly, slides spanned the hypothalamic region approximating from −2.7 to −0.4 mm relative to Bregma according to the atlas of the mouse brain (19). In situ hybridization and analysis were performed as described previously (18). A detailed description of primer selection for the genes that were analyzed and their sequences are provided in Supplemental Table 1.

To test whether leptin activates expression of WNT genes, we performed in situ hybridization for mRNA of these genes on coronal brain cryosections of mice that were injected with leptin. Therefore, leptin-deficient $Lep^{ob/ob}$ mice and $Lep^{+/+}$ mice received either a leptin (2 mg/kg) or a vehicle (PBS) ip injection 2 hours before decapitation, and in situ hybridization was performed as described above (n = 6/group).

Immunohistochemistry

We analyzed whether leptin acts on the Fz coreceptor LRP-6 in the hypothalamic ARC. Therefore, leptin-deficient $Lep^{ob/ob}$ mice and $Lep^{+/+}$ mice received either a leptin (2 mg/kg) or a vehicle (PBS) ip injection 15 minutes before transcardial perfusion and immunohistochemistry were performed (n = 5–6/group), using an anti-phospho-LRP-6 Ser1490 antibody (catalog no. 2568; Cell Signaling Technology). To analyze whether DKK-1 antagonizes the WNT pathway at the level of phospho-LRP-6, wild-type mice received an intracerebroventricular (icv) injection of either DKK-1 (1 μg in 1 μL artificial cerebral spinal fluid [aCSF]) or vehicle (aCSF), 15 minutes before transcardial perfusion and immunohistochemistry were performed using the same antibody as described above (n = 9/group).

To further establish whether leptin interacts with the WNT pathway in neurons, we investigated the effect of leptin on phosphorylation of GSK-3β (Ser9) in NPY-GFP mice. Phosphorylation of GSK-3β at Ser9 is critical to inactivate the enzyme (11–13). Therefore, 8-week-old mice (n = 5/group) were fasted for 16 hours and received a single icv injection of either leptin (2 μg/ in 1 μL aCSF) or aCSF 45 minutes before transcardial perfusion and immunohistochemistry were performed using an anti-phospho-GSK-3β (Ser9, catalog no. 9323; Cell Signaling Technology) antibody as described elsewhere (17, 20). For visualization, the second antibody Alexa Fluor 594 (Invitrogen) was used and analyzed by fluorescence light microscopy (Axio Imager.M2; Zeiss).

Glucose tolerance test

In this experiment, we tested whether the prominent glucose-lowering effects of leptin are mediated via the WNT pathway. Therefore we inhibited WNT signaling, using the WNT antagonist DKK-1 prior to the assessment of leptin's effect on glucose tolerance. $Lep^{ob/ob}$ mice were fasted for 16 hours and divided into 3 groups. The first group was icv injected with the WNT antagonist DKK-1 (1 μg in 1 μL aCSF; R&D Systems; 5897-DK/CF), followed by ip leptin 30 minutes later (2 mg/kg body weight in PBS). The second group was injected with vehicle icv (aCSF) followed by leptin, whereas the third group received 2 vehicle injections ([icv: aCSF; ip: PBS; n = 7 each group]). The glucose tolerance test (1 mg glucose/kg body weight) was performed 30 minutes after the second injection, and glucose levels were measured in blood collected from the vena facialis as described previously (17).

Statistics

The data were analyzed by one-way ANOVA followed by a Holm-Sidak comparison test, as appropriate, using SigmaStat statistical software (Jandel). When the data failed equal variance or normality tests, they were analyzed by one-way ANOVA on ranks followed by Dunn's multiple-comparison test. The results are presented as means ± SEM, and differences were considered significant if $P < .05$.

Results

Genes involved in the WNT pathway are expressed in the mediobasal hypothalamus

To morphologically characterize the WNT pathway in the brain, we first analyzed the mRNA expression pattern of genes that encode selected ligands (WNT-4, WNT-7a), receptor and coreceptor (Fz-5, LRP-6), other downstream components of the pathway (Axin-1, Dishevelled, GSK-3β, β-catenin, and TCF-7), or target genes (Axin-2 and Cyclin-D1). An initial screen was carried out on wild-type $Lep^{+/+}$ mice. The hybridization signal for almost all investigated genes was not confined to the hypothalamus and could be observed in extrahypothalamic regions with the highest density in the hippocampus, cortex, and thalamus. However, most genes were strongly expressed in the mediobasal hypothalamus, and many showed particular prominence in the ARC (Figure 1). The control sense probes generated low-intensity nonspecific signals (two examples, Axin-1 and GSK-3β, are shown in Figure 1).

In our recent study, we demonstrated that the antagonist Dickkopf-1 was increased in $Lep^{ob/ob}$ mice compared with wild-type mice, suggesting that WNT signaling is down-regulated in these mice (16). Therefore, we further characterized the expression of selected ligands and target genes in the ARC. A comparison of gene expression between $Lep^{+/+}$ mice and leptin-deficient $Lep^{ob/ob}$ mice revealed that expression of the ligands WNT-7a and WNT-4 was significantly down-regulated by about 60% and 70% in the ARC of $Lep^{ob/ob}$ mice compared with wild types (Figure 2, a and b; $P = .004$; $P = .038$). Moreover, target gene expression of Axin-2 and Cyclin-D1-mRNA was down-regulated by about 60% and 25%, respectively, in the ARC of $Lep^{ob/ob}$ mice in comparison with the controls (Figure 2, c and d; $P = .002$; $P = .043$).

To test whether the lack of leptin is responsible for the differential down-regulation of WNT target genes in $Lep^{ob/ob}$ mice, we investigated the effect of leptin replacement on the expression of these genes in the ARC. Leptin treatment completely restored Axin-2 and Cyclin-D1

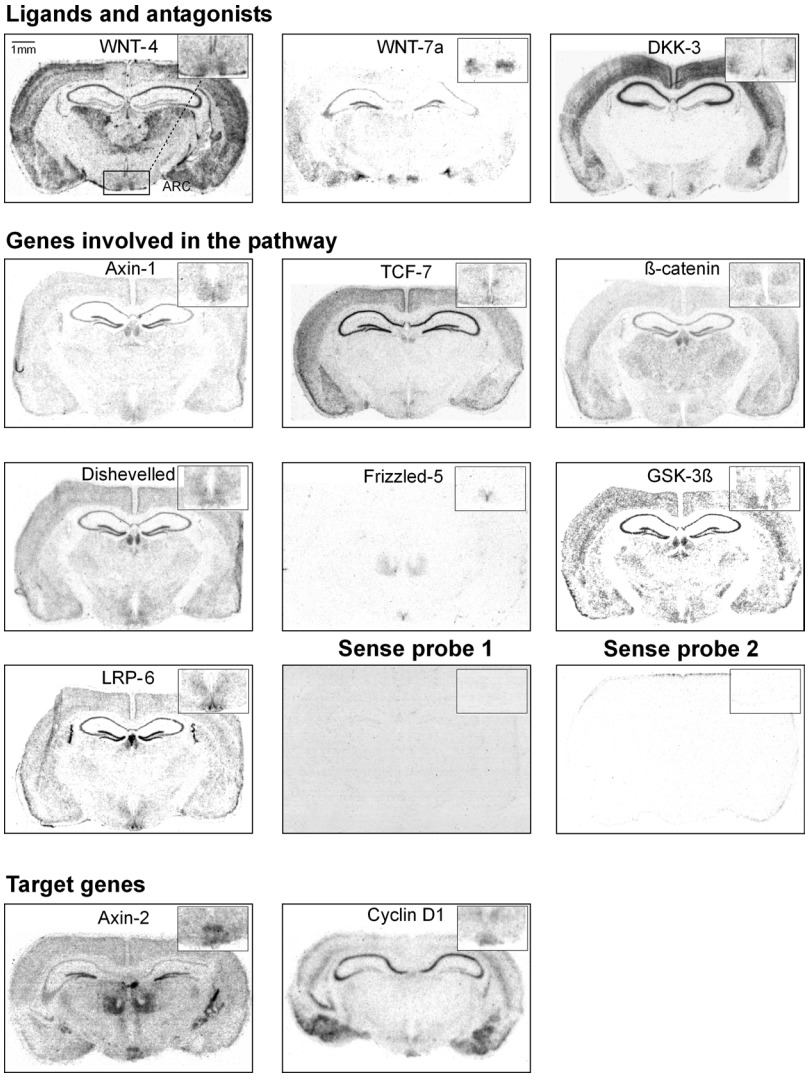

Figure 1. Genes encoding members of the WNT pathway are expressed in the hypothalamus Autoradiographs of wild-type mouse brain sections after in situ hybridization to an antisense ^{35}S-labeled riboprobe binding to genes that are involved in WNT signaling. Most genes whether encoding a ligand (WNT-4, WNT-7a), an antagonist (DKK-3) to the Fz receptor, are part of the pathway (Axin-1, Dishevelled, GSK-3β, β-catenin, Fz-5, LRP-6 and TCF-7), or are target genes (Axin-2 and Cyclin-D1) are expressed in the ARC of the hypothalamus. Outside of the hypothalamus for some genes hybridization occurred in the hippocampus, cortex, and thalamus. Representative for all respective sense riboprobes 2 images are shown (sense probe 1 for Axin 1 and 2 for GSK-3β). Inserts depict binding of the riboprobes to the ARC.

mRNA levels, such that no difference relative to wild-type mice was detected (Figure 2, c and d; n = 5–6/group).

Effects of leptin on the activity of the WNT coreceptor LRP-6

Having established that components of the WNT pathway are expressed in the ARC, and that leptin activates WNT target gene expression, as a possible mechanism we tested whether leptin activates the coreceptor LRP-6, which is an essential modulator of WNT activity at the receptor level. LRP-6 is activated by phosphorylation at Ser1490 (21) and therefore, we used immunohistochemistry to investigate whether leptin increases the number of phospho-LRP-6 (Ser1490)-immunoreactive cells in the ARC. Leptin-deficient Lep$^{ob/ob}$ mice exhibited a significant 50% reduction of phospho-LRP-6 (Ser1490)-immunoreactive cells in the ARC compared with lean controls (Figure 3a; $P = .009$). Leptin fully restored phospho-LRP-6 (Ser 1490) immunoreactivity to the wild-type controls (Figure 3a; vehicle Lep$^{ob/ob}$ mice vs ip leptin Lep$^{ob/ob}$ mice; $P = .013$). In line with these findings, antagonizing WNT signaling via icv injection of DKK-1 reduced the number of phospho-LRP-6 (Ser1490)-immunoreactive cells by about 25% in the ARC of lean wild-type relative to vehicle-injected mice (Figure 3b; $P = .019$; n = 10 animals/group). Because we had previously identified a potent impairment of glucose homeostasis followed by icv DKK-1 injection, we tested whether this ligand also antagonized the ability of leptin to decrease blood glucose (16). Whereas leptin robustly improved glucose homeostasis in Lep$^{ob/ob}$ mice ($P = .047$) after vehicle pretreatment, this effect was totally abolished in mice that were icv injected with DKK-1 prior to the leptin challenge (Figure 3c; n = 7/group).

Leptin inhibits GSK-3β in NPY neurons

Hypothalamic leptin and insulin signaling converge in individual neurons, controlling transcription of important neuropeptides that regulate energy homeostasis, such as NPY (22). The catabolic action of leptin involves inhibition of orexigenic NPY neurons (23), which is required to suppress hepatic glucose production (24). Having established that leptin increases WNT target gene expression and acts on the Fz coreceptor LRP-6 in the ARC, we further investigated whether leptin specifically inhibits GSK-3β in NPY neurons. Therefore, we first identified the proportion of NPY neurons that contain phospho-GSK-3β and are responsive to leptin treatment. Leptin treatment did not affect NPY staining, as shown in Figure 4a. However, leptin treatment enhanced the number of phospho-GSK-3β (Ser9)-positive

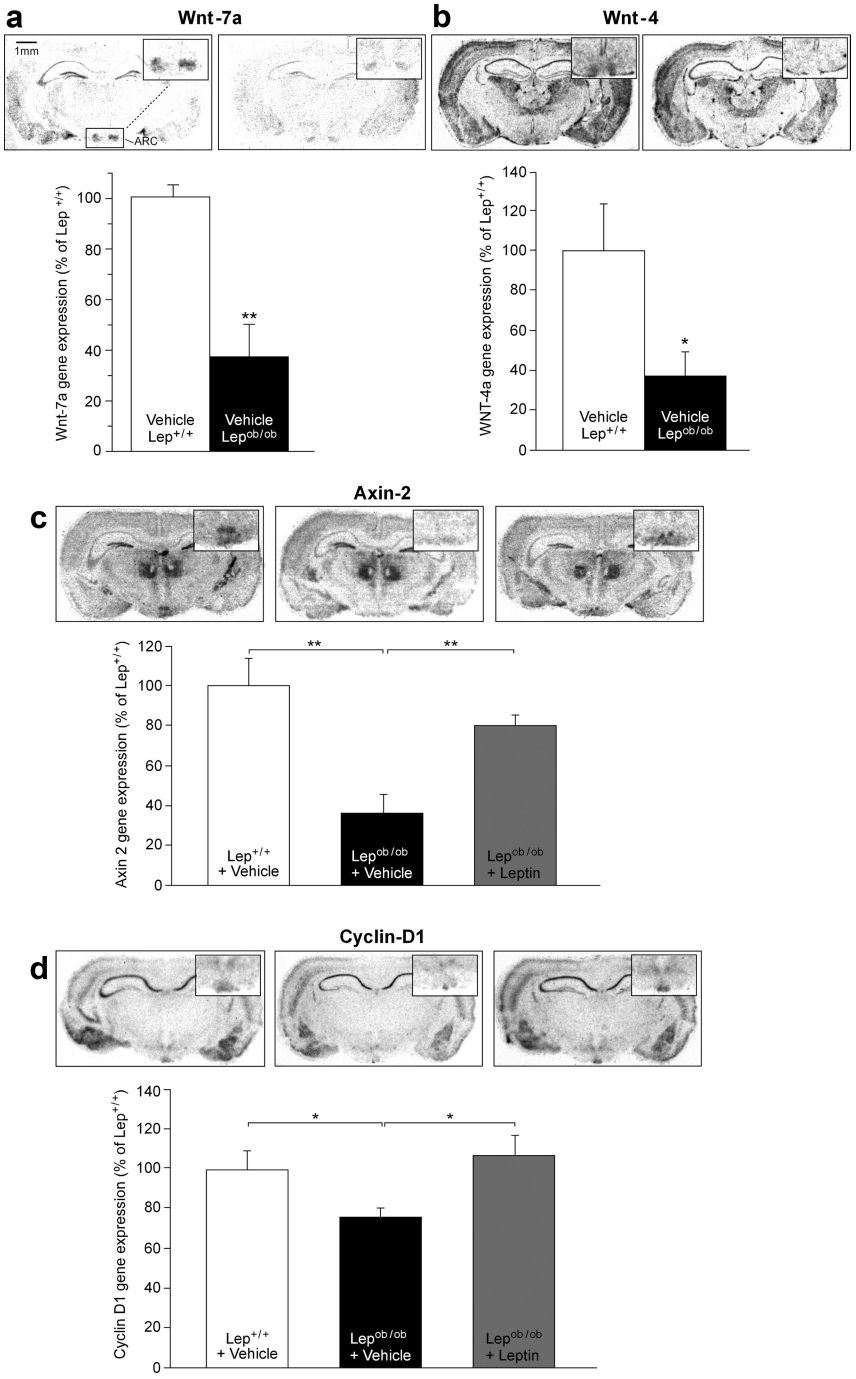

Figure 2. Differential mRNA regulation of ligands and target genes in the hypothalamic ARC of Lep $^{+/+-}$ and Lep$^{ob/ob}$ mice. For some genes, 2 examples of a WNT-receptor agonist (WNT-7a [panel a], WNT-4 [panel b]) and for 2 target genes of the WNT-pathway (Axin-2 [panel c], Cyclin-D [panel d]), differential gene expression between Lep$^{+/+-}$ and Lep$^{ob/ob}$ mice was analyzed. Both, mRNA expression of target genes and agonist were down-regulated in the Lep$^{ob/ob}$ mice compared with the controls. An ip leptin injection (2 mg/kg body weight) 2 hours before decapitation restored the decreased target gene expression of Axin-2 (c) and Cyclin-D1 (d) in Lep$^{ob/ob}$ mice. The upper panels depict autoradiographs of the respective genes, whereas in the lower panels a bar graph generated from quantification of the signal in the ARC (5–6 animals in each group) of representative brain sections is shown. Inserts in the upper panels depict binding of the riboprobes to the ARC. Lep$^{ob/ob}$ mice, leptin-deficient mice; Means ± SEM, *, $P \leq .05$; **, $P \leq .01$.

cells in the ARC by 30% (Figure 4b; $P < .01$), and increased the number of immunoreactive NPY-GFP neurons coexpressing phospho-GSK-3β (Ser9) by 50% in these mice (Figure 4c; $P < .01$).

Discussion

The WNT-pathway plays a well-established role in embryogenesis and tumorigenesis. Recently, however, it was demonstrated that glucose activated the WNT-pathway in an autocrine fashion in vitro (9). Furthermore, polymorphisms within the gene encoding TCF-7 contribute to an increased risk of developing type 2 diabetes in humans (6–8). These findings, together with our recent observation that the key enzyme of the WNT pathway, GSK-3β, plays an essential role in the neuronal control of food intake and glucose metabolism (16), strongly suggested that hypothalamic WNT signaling may have a key role in the neuroendocrine control of metabolism.

The surprising finding that all investigated genes involved in the WNT pathway are expressed in the ARC of the hypothalamus of the adult mouse brain indicates that this pathway might be associated with the hypothalamic control of body weight and glucose metabolism. The expression of some components (eg, DKK-3 and TCF-7), however, appears to be heterogeneous, indicating differential processing of WNT components in different neuron subpopulations in the ARC. Whether this differential mRNA expression is reflected on the protein level remains to be investigated. The ligands WNT-7a (25) and WNT-4 (26), which activate the WNT-pathway, were down-regulated in the ARC of Lep$^{ob/ob}$ mice. This is consistent with our previous finding that the known receptor antagonist (DKK-1) was in-

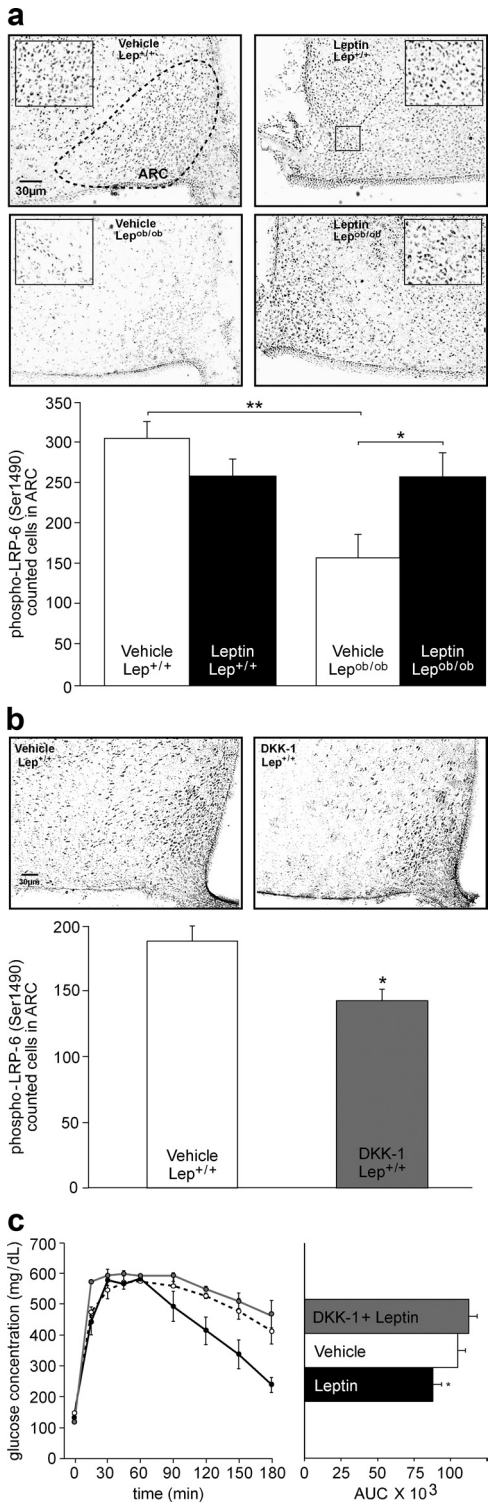

Figure 3. Leptin interacts with the WNT pathway at the level of the coreceptor LRP-6 a, Immunoreactivity of phospho-LRP-6-positive cells is decreased in the ARC of leptin-deficient mice compared with lean wild-type mice. Leptin reversed the reduced number of phospho-LRP-6 (Ser1490)-immunoreactive cells in the ARC of $Lep^{ob/ob}$ mice. Mice received either a leptin (2 mg/kg) or a vehicle (PBS) ip injection 15 minutes before transcardial perfusion. Inserts depict representative images of phospho-LRP-6 immunoreactivity in the ARC of $Lep^{ob/ob}$ and control mice. A bar graph shows counted cells immunoreactive for phospho-LRP-6 in the ARC of representative sections of each animal

creased in this area (16), suggesting impaired receptor activation of this pathway in the ARC of $Lep^{ob/ob}$ mice. The finding that gene expression of the WNT targets Axin-2 (27) and Cyclin-D1 (28) was also regulated in the same manner strongly suggests that the activity of the WNT pathway is functionally impaired in the hypothalamus of obese mice. This novel concept is further corroborated by reduced phospho-LRP-6 immunoreactivity in the ARC of $Lep^{ob/ob}$ mice, which suggests reduced endogenous WNT receptor activity. The mRNA of the transcription factor TCF-7 was not regulated by leptin treatment or genotype (Supplemental Figure 1), suggesting that leptin does not regulate this factor on a transcriptional level.

The adipokine leptin can improve glucose metabolism, independent of its effects on energy homeostasis (17, 29, 30). We have recently established that these effects of leptin occur very rapidly, suggesting that they involve post-translational modifications in hypothalamic signaling pathways (17). Leptin sensitized insulin action in the hypothalamus upstream of PI3K via altering phosphorylation of insulin receptor substrate (IRS)-1 at Ser307 and Ser612 (17). Recently, we found parallel affects by pharmacologic inhibition of GSK-3β (16). Because leptin inactivates GSK-3β in vitro (31, 32), a direct action of this hormone on canonical WNT signaling in the adult hypothalamus appears likely. This idea is supported by the finding that leptin treatment normalizes reduced gene expression of the WNT target genes (Axin-2 and Cyclin D1) in the ARC of leptin-deficient mice and inactivates GSK-3β, as indicated by increased phospho-Ser9 GSK-3β immunoreactivity in the ARC. Because leptin was capable of normalizing the reduced phospho-LRP-6 immunoreactivity seen in the ARC in $Lep^{ob/ob}$ mice, it is plausible that leptin selectively activates the WNT pathway via modulation of WNT receptor activity. Phosphorylation at

Legend to Figure Continued. . .
(n = 5–6/group). b, Immunoreactivity of phospho-LRP-6-positive cells decreased after DKK-1 administration. Wild-type mice received icv either DKK-1 (1 μg in aCSF) or vehicle (aCSF), 15 minutes before transcardial perfusion, and immunohistochemistry was performed using anti-phospho-LRP-6 (n = 9/group). Phospho-LRP-6 (Ser1490)-immunoreactive cells in the ARC decreased after DKK-1 injection (P = .019). Inserts depict representative images of phospho-LRP-6 immunoreactivity in the ARC. c, icv injection of DKK-1 blocks the glucose-lowering effect of leptin. ip glucose tolerance test (1g glucose/kg) after icv injection of DKK-1 (1 μg in 1 μL aCSF) and/or ip injection of leptin (2 mg/kg in PBS, n = 7 each group). Thirty minutes after the icv injection of DKK-1, leptin was injected, 30 minutes before glucose and blood glucose levels were measured. DKK-1 blocked the leptin effect and $Lep^{ob/ob}$ mice remained glucose intolerant, whereas leptin alone improved glucose tolerance in $Lep^{ob/ob}$ mice compared with the control group (n = 7/group; P = .047). Vehicle/leptin (black circles); DKK-1/ leptin (gray circles); Vehicle/vehicle (white circles). Data show means ± SEM. *, $P \leq .05$; **, $P \leq .01$. AUC, area under the curve.

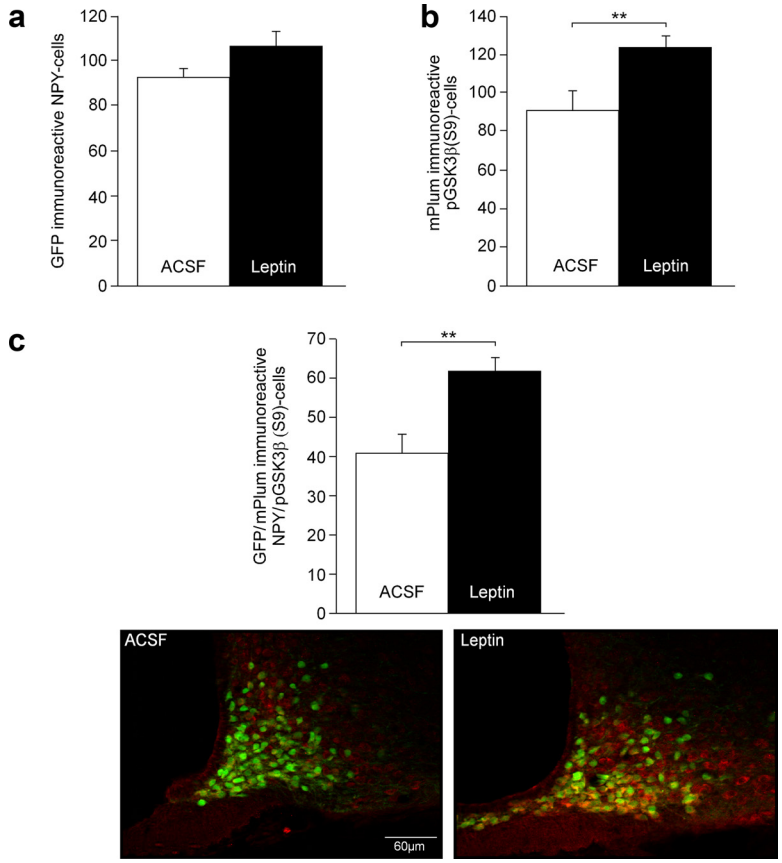

Figure 4. Leptin activates WNT signaling via GSK-3β inhibition in NPY neurons Immunoreactivity of phospho-GSK-3β (Ser9)-positive cells increases after icv leptin administration. NPY-GFP mice received either leptin (2 μg in 1 μL aCSF) or vehicle (aCSF) 45 minutes before transcardial perfusion, and immunohistochemistry was performed using anti-phospho-GSK-3β (n = 5/group). a, The bar graph shows the counted cells positive for NPY-GFP cells. Leptin administration did not affect NPY staining. b, Phosphorylation of GSK-3β (Ser9) increased after leptin administration. c, The ratio of NPY and GSK-3β (Ser9)-positive cells increased after leptin administration. Images show immunoreactive staining of cells, positive for GSK-3β (Ser9, red) in NPY neurons (green) after icv treatment with either vehicle or leptin. Data show means ± SEM. **, $P \leq .01$. IR, insulin receptor; JAK, Janus kinase.

Ser1490 of the coreceptor LRP-is required for Fz receptor activation (21) and therefore for intact WNT signaling. Whether leptin directly activates the WNT coreceptor, eg, via intrinsic janus-kinase 2 activity, or whether this effect occurs via yet unknown indirect mechanisms remains to be identified in future studies.

The hypothesis that the WNT pathway is functional in the adult mouse hypothalamus is supported by the finding that the WNT antagonist DKK-1, contrary to leptin, reduces the number of phospho-LRP-6-immunoreactive cells in the ARC of wild-type mice. Intriguingly, DKK-1 icv injection, 30 minutes before leptin, totally abolished the glucose-lowering properties of the hormone. This suggests that the glucose-lowering effects of leptin largely depend on neuronal signaling via the WNT pathway and, in particular, on the level of phosho-LRP-6. It is plausible that the catabolic action of leptin is also transduced by hypothalamic WNT signaling, because we previously observed that GSK-3β overexpresssion in the ARC increased food intake and body weight and was associated with leptin resistance (16). Consistent with increased food intake and body weight, phospho-GSK-3β (Ser9) immunoreactivity was abundant in NPY neurons of the mediobasal hypothalamus, suggesting that this key WNT-signaling kinase is involved in regulating activity of these prominent orexigenic neurons. Interestingly, more than 70% of leptin-induced phospho-GSK-3β (Ser9) cells were identified as NPY neurons. The catabolic action of leptin involves inhibition of orexigenic NPY neurons (23); hence it seems possible that leptin inhibits the NPY neurons via involvement of the WNT pathway, particularly GSK-3β. Whether the remaining 30% of leptin-induced phospho-GSK-3β (Ser9) cells in the medial basal hypothalamus are pro-opiomelanocortin neurons remains to be identified in future studies.

Central insulin and leptin signaling plays a very important role in regulating peripheral glucose metabolism (3, 17, 33–36). We previously showed that the central glucose-lowering properties of leptin appear to be indirect. Rather than a direct action on the IRS/PI3K pathway, leptin sensitizes insulin action through this pathway (17). We demonstrated that GSK-3β has an essential control in the neuronal control of glucose homeostasis via sensitization of this pathway (16). This, together with the current study suggests that hypothalamic GSK-3β signaling through the WNT pathway not only has a pivotal role in the neuroendocrine control of glucose homeostasis, it also represents a critical signaling pathway utilized by leptin, and this may be a key mechanism providing convergence in the actions of leptin and insulin. The fact that leptin increased phospho-LRP-6 immunoreactivity and inhibited GSK-3β in NPY neurons suggests that the sensitization effect of leptin on insulin signaling is mediated via the WNT pathway. This hypothesis is supported by the data published in Reference 16, which revealed that inhibition of GSK-3β sensitizes insulin signaling via the IRS/PI3K pathway. Furthermore phospho-AKT has been robustly shown to phosphorylate and inactivate GSK-3β (11–13), which

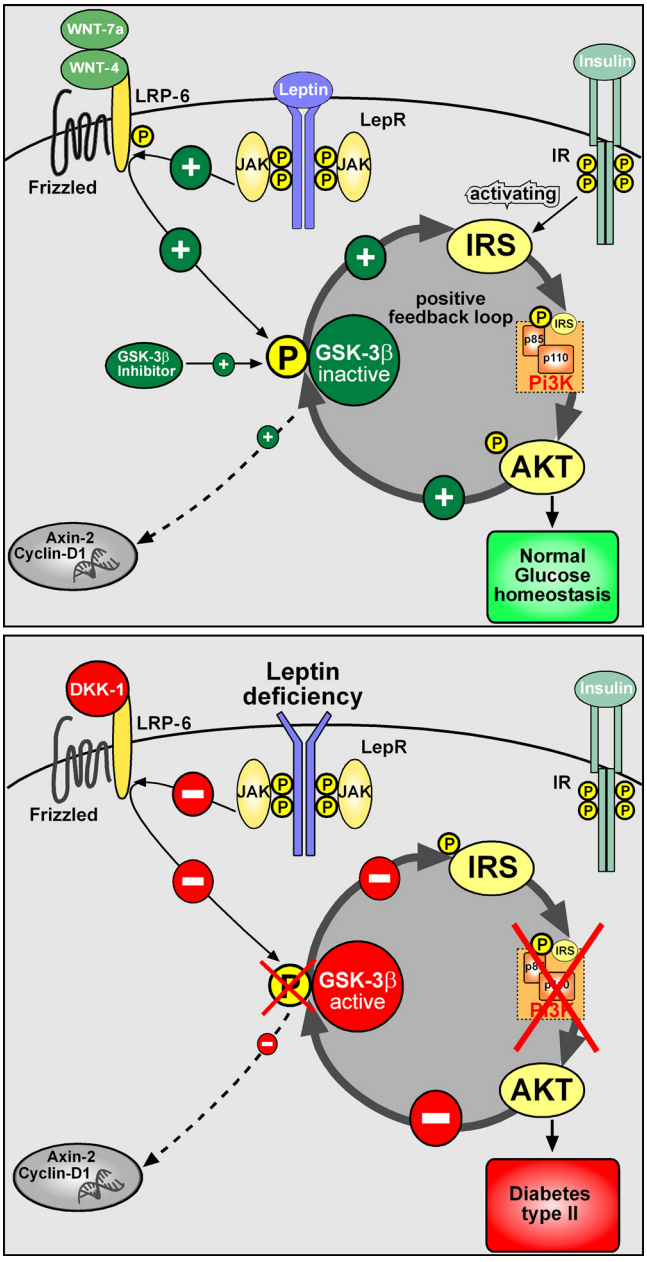

Figure 5. The WNT pathway in the hypothalamus: model proposing potential interactions of leptin, insulin and WNT signaling in the hypothalamus. a, In nondiabetic, leptin-sensitive animals leptin activates LRP-6, resulting in inactivation of GSK-3β (This inactivation can also be induced artificially by administration of a GSK-3β inhibitor). Consequently a positive feedback loop might be triggered in which the phosphorylation of inhibitory phosphorylation sites on IRS-1 by GSK-3β is reduced. This modification on IRS-1 might result in activation of the IRS-PI3K pathway by insulin, resulting in increased phospho-AKT. Phospho-AKT, in turn, might enhance this mechanism by further inactivating GSK-3β. b, In leptin-deficient mice leptin no longer activates LRP-6. Enhanced GSK-3β activity will inhibit IRS-1 through phosphorylation on inhibitory sites. This, in turn, will lead to hypothalamic insulin resistance and subsequently to the development of type-2 diabetes. LepR, leptin receptor; IR, insulin receptor; JAK, Janus kinase.

suggests a possible feedback loop in hypothalamic neurons by which glucose homeostasis is maintained. A model summarizing the potential interaction of leptin and insulin signaling with the WNT pathway is presented in Figure 5.

Taken together, our data strongly support a central role of WNT signaling in the neuronal control of metabolism. This is supported by the fact that WNT signaling is differentially regulated by endogenous leptin deficiency and leptin-replacement therapy on various hierarchical levels. This includes the ligands WNT-4 and WNT-7a, the coreceptor LRP-6, the key enzyme GSK-3β (16), and the target genes Axin-2 and Cyclin-D1. Aberrant WNT signaling has also been associated with the pathogenesis of diabetic retinopathy (37), coronary artery disease (38), osteoporosis (38), cancer, and Alzheimer's disease (39). The proposed role of leptin in the brain in regulating WNT signaling might unravel an important link between type 2 diabetes and the pathogenesis of these other severe diseases.

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