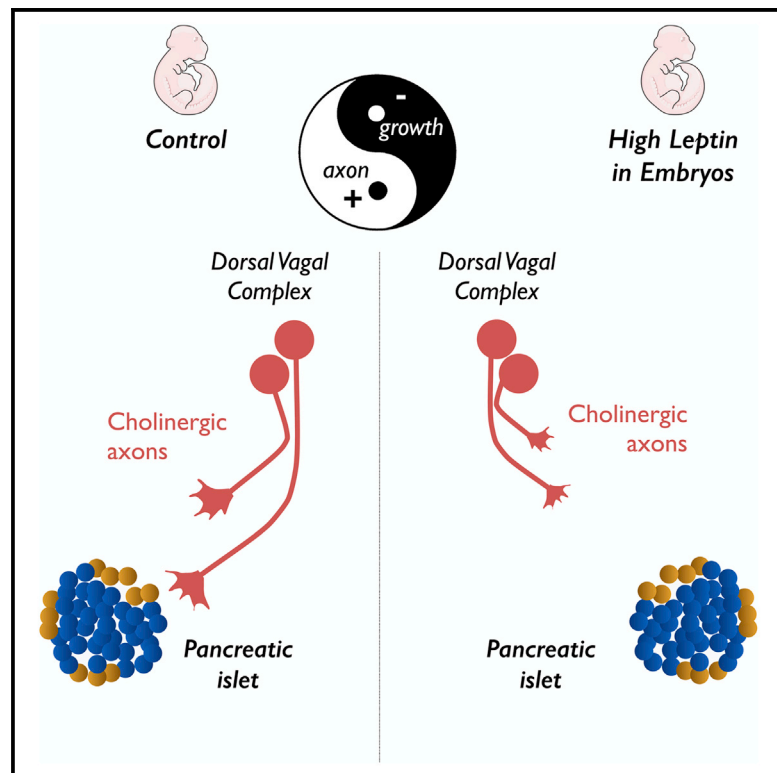


# Cell Reports

## Leptin Controls Parasympathetic Wiring of the Pancreas during Embryonic Life

### Graphical Abstract



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### In Brief

The autonomic nervous system is essential for the regulation of blood glucose levels. Croizier et al. report that prenatal leptin inhibits parasympathetic innervation of the pancreatic islets, with long-term consequences on glucose homeostasis. These findings improve our understanding of the neural mechanisms involved in the early-life origins of diabetes.

### Highlights

- Autonomic innervation of pancreatic islets develops during mid-gestation in mice
- Leptin is required for normal parasympathetic innervation of pancreatic  $\beta$  cells
- Acute leptin injections in embryos cause lifelong disturbances in glucose homeostasis
- Leptin acts directly on the hindbrain to modulate cholinergic axon growth



# Leptin Controls Parasympathetic Wiring of the Pancreas during Embryonic Life

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## SUMMARY

The autonomic nervous system plays a critical role in glucose metabolism through both its sympathetic and parasympathetic branches, but the mechanisms that underlie the development of the autonomic innervation of the pancreas remain poorly understood. Here, we report that cholinergic innervation of pancreatic islets develops during mid-gestation under the influence of leptin. Leptin-deficient mice display a greater cholinergic innervation of pancreatic islets beginning in embryonic life, and this increase persists into adulthood. Remarkably, a single intracerebroventricular injection of leptin in embryos caused a permanent reduction in parasympathetic innervation of pancreatic  $\beta$  cells and long-term impairments in glucose homeostasis. These developmental effects of leptin involve a direct inhibitory effect on the outgrowth of preganglionic axons from the hindbrain. These studies reveal an unanticipated regulatory role of leptin on the parasympathetic nervous system during embryonic development and may have important implications for our understanding of the early mechanisms that contribute to diabetes.

## INTRODUCTION

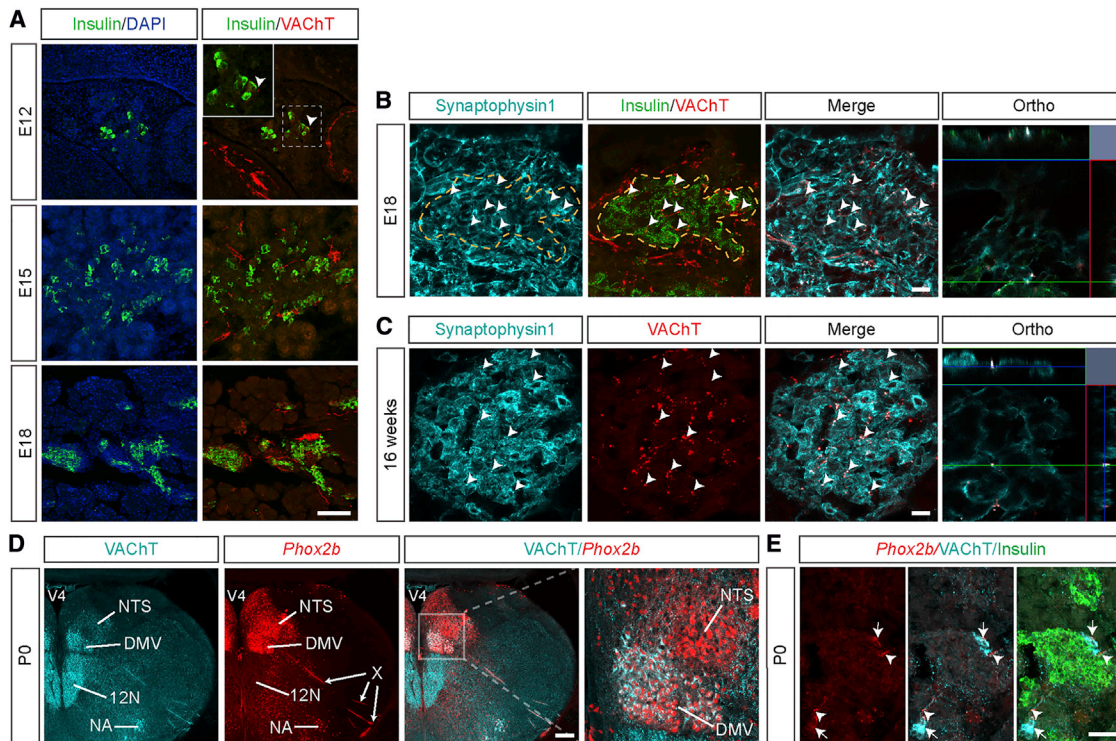
The autonomic nervous system is essential for the regulation of critical physiological functions including cardiac output, body temperature and blood glucose levels (Schmidt and Thews, 1989). Classically, the autonomic nervous system is divided into two anatomically and functionally distinct branches: the sympathetic nervous system and the parasympathetic nervous system. Consistent with a role of the autonomic nervous system in the maintenance of glucose homeostasis, sympathetic and parasympathetic neurons densely innervate the endocrine pancreas and, in particular, the islets of Langerhans (Ahrén, 2000; Thorens, 2014; Woods and Porte, 1974). Notably, the sympathetic and parasympathetic systems exert distinct actions on pancreatic islets: whereas sympathetic nerve stimulation inhibits

insulin secretion from pancreatic  $\beta$  cells and promotes glucagon secretion from pancreatic  $\alpha$  cells, parasympathetic input stimulates the secretion of both glucagon and insulin (Bloom and Edwards, 1975). Despite the critical role of the autonomic nervous system in the regulation of islet hormone secretion, relatively little is known about the mechanisms involved in the development of the autonomic innervation of the pancreas.

The parasympathetic fibers that innervate the endocrine pancreas originate primarily from neurons in the intrapancreatic ganglia, which receive preganglionic inputs from the hindbrain via the vagus nerve (Fox and Powley, 1986). In contrast, sympathetic fibers within the pancreas originate from preganglionic cell bodies that are located in the thoracic and upper lumbar segments of the spinal cord (Furuzawa et al., 1996). Both the parasympathetic and sympathetic systems develop before birth, and autonomic nerve fibers can be observed in various peripheral organs as early as mid-gestation (Black, 1978; Burris and Hebrok, 2007; Rinaman and Levitt, 1993). Notably, the onset of autonomic innervation coincides with stages of rapid growth, differentiation, and maturation in the embryonic pancreas (Jørgensen et al., 2007).

In addition to being controlled by the autonomic nervous system, pancreatic function is also regulated by hormonal factors, including the adipocyte-derived hormone leptin. Although leptin was initially described as a regulator of energy balance and neuroendocrine function, it has subsequently been shown to influence sympathetic tone and glucose homeostasis (Enriori et al., 2011; Marino et al., 2011; Morton and Schwartz, 2011; Simonds et al., 2014). The effects of leptin are now recognized to be mediated by a distributed neural network that includes neurons located in both the hypothalamus and hindbrain (Gautron et al., 2015; Grill and Hayes, 2012; Leininger and Myers, 2008). In addition to the physiological effects it exerts during adult life, leptin provides trophic support to hypothalamic neural projections during development (Bouret et al., 2004). However, whether leptin influences the development of non-hypothalamic circuits remains unknown.

In this report, we investigated the development of the parasympathetic innervation of the endocrine pancreas. We demonstrated that exposure of the embryonic brain to leptin during a discrete developmental period results in permanent alterations in the cholinergic innervation of pancreatic  $\beta$  cells as well as long-term perturbations in glucose homeostasis. Furthermore, we showed that leptin directly inhibits cholinergic neurite



**Figure 1. Ontogeny of the Parasympathetic Innervation of Pancreatic Islets**

(A) Confocal images of VAcHT-immunopositive fibers (red fluorescence) in pancreatic islets (insulin, green fluorescence) of E12, E15, and E18 WT embryos. Arrowheads point to VAcHT fibers in close proximity to insulin-positive cells. (B and C) Representative images showing VAcHT immunoreactivity (red fluorescence) and its colocalization with synaptophysin1 labeling (turquoise fluorescence) in pancreatic  $\beta$  cells (insulin, green fluorescence) of (B) E18 WT mouse embryos and (C) 16-week-old *ob/ob* mice. Arrowheads point to double-labeled inputs (white labeling). (D) Representative images of genetically labeled cell bodies (red fluorescence) in the hindbrain of postnatal day 0 (P0) *Phox2b-Cre-tdTomato* mice. (E) Detail of *Phox2b-Cre-tdTomato* innervation of pancreatic islets (insulin, green fluorescence). Arrowheads point to td-Tomato-positive fibers that are also VAcHT-positive. Arrows point to intrapancreatic ganglia containing tdTomato. DMV, dorsomedial nucleus of the vagus nerve; NA, nucleus ambiguus; NTS, nucleus of the solitary tract; X, vagus nerve; 12N, hypoglossal nucleus; V4, fourth ventricle. Scale bars represent 100  $\mu\text{m}$  (A), 20  $\mu\text{m}$  (B and C), 200  $\mu\text{m}$  (D), and 50  $\mu\text{m}$  (E).

outgrowth from the hindbrain, suggesting that the developmental effects of leptin on parasympathetic projections involves, at least in part, a direct effect on preganglionic neurons within the hindbrain.

## RESULTS

### Ontogeny of the Parasympathetic Innervation of Pancreatic Islets

To begin to examine the development of the parasympathetic innervation of the pancreas, we first performed immunohistochemical labeling for the vesicular acetylcholine transporter (VAcHT), a well-known marker of cholinergic fibers (Weihe et al., 1996), in mouse embryonic pancreatic tissues. To visualize  $\beta$  cells, we also immunostained for insulin. VAcHT-positive fibers were found in the vicinity of pancreatic insulin-containing cells as early as embryonic day 12 (E12) (Figure 1A). However, at this age, only a few cells expressed insulin, as previously described (Jørgensen et al., 2007). The relative number of insulin-containing cells was markedly increased at E15, as was the density of

VAcHT-positive fibers surrounding  $\beta$  cells (Figure 1A). By E18, the insulin-expressing cells formed adult-like islets, and the density of cholinergic axons in  $\beta$ -cell islets had substantially increased (Figure 1A). The VAcHT-positive axons appeared to make synaptic contacts within the islets, because those axons also expressed synaptophysin1, a major synaptic vesicle membrane protein (Figures 1B and 1C). As expected, adult islets were densely innervated by VAcHT-immunopositive fibers (Figure 1C). Notably, projections containing tyrosine hydroxylase (TH), a marker of sympathetic neurons, developed in a pattern that mirrored that of the innervation of VAcHT terminals (Figure S1A).

The cholinergic fibers that innervate the pancreas mainly originate from the dorsomedial nucleus of the vagus nerve (DMV). Most of these fibers run within the vagus nerve and terminate on the pancreatic ganglia, where they form synapses with interneurons (Fox and Powley, 1986). To assess the proportion of cholinergic preganglionic fibers that directly innervate the embryonic pancreatic ganglia and islets, we used a transgenic mouse in which the fluorescent protein tdTomato is selectively expressed in cell bodies and neuronal processes of a subset of

nucleus of the solitary tract (NTS)/DMV neurons (*Phox2b-Cre*; tdTomato mice; Figure 1D; Scott et al., 2011). We observed that a significant proportion of the VACHT-immunoreactive fibers in the pancreatic islets also contained the red fluorescent signal of tdTomato. In addition, a number of cholinergic intrapancreatic ganglia also contain tdTomato (Figure 1E). These data suggest that the cholinergic fibers that are found in pancreatic islets might originate from hindbrain neurons as well as intrapancreatic ganglia (Figures 1D and 1E).

### Lack of Leptin Affects the Pattern of Cholinergic Innervation of the Pancreas

Leptin is known to regulate the activity of the autonomic nervous system during adulthood (Arteaga-Solis et al., 2013; Simonds et al., 2014), but whether leptin is involved in the development of the autonomic nervous system remains unknown. Accordingly, we next examined the density of cholinergic fibers in the islets of leptin-deficient (*ob/ob*) embryos. Although the overall timing of development and the distribution of VACHT fibers were relatively similar between *ob/ob* and wild-type (WT) embryos (Figures 1A and 2A), we observed clear differences in the density of fibers. There were 1.3-fold more labeled fibers in the islets of E18 *ob/ob* mice than in those of E18 WT mice (Figure 2B).

To determine whether the changes in the cholinergic innervation of islets that were observed in the *ob/ob* embryos were permanent, we studied pancreatic islet innervation in adult mice. The density of VACHT fibers that was observed in the islets of adult *ob/ob* mice was 3-fold higher than that observed in those of WT mice (Figure 2C). We also examined the density of fibers that contained vasoactive intestinal polypeptide (VIP), another marker of parasympathetic innervation, and found a 3-fold increase in the density of VIP-labeled fibers in the islets of adult *ob/ob* mice (Figure 2G).

### A Single Prenatal Leptin Injection Has Long-Term Structural Effects on Islet Innervation

If leptin is a critical regulator of the cholinergic innervation of the pancreas, exposing embryos to leptin should change VACHT fiber density. We therefore injected leptin directly in the ventricular cavity of WT and *ob/ob* embryos at E12. The density of VACHT-positive fibers was reduced by 1.9-fold and 2.1-fold in the leptin-treated WT and *ob/ob* embryos, respectively, compared to their control littermates (Figure 2B). The effects of prenatal leptin on parasympathetic innervation appear to be permanent, because the density of VACHT fibers was also reduced by 1.4-fold and 1.3-fold in adult WT and *ob/ob* animals, respectively, that were prenatally treated with leptin (Figures 2D and 2E). Similarly, a single prenatal leptin injection reduced the density of VIP fibers in pancreatic islets by 2-fold (Figure 2F). In contrast, prenatal leptin did not appear to influence the sympathetic innervation of the pancreas, as the density of TH-labeled fibers was similar between leptin- and vehicle-treated *ob/ob* mice (Figure S1B). In addition, prenatal leptin treatment did not influence islet size (Figure 2E) or  $\beta$  cell area (Figure 2H) in *ob/ob* mice but did reduce islet size by 1.4-fold in WT mice (Figure 2D). Because the parasympathetic system is also involved in glucagon secretion (Patel, 1984; Thorens, 2014), we also examined the parasympathetic

innervation of glucagon-producing  $\alpha$  cells. The density of VACHT fibers innervating pancreatic  $\alpha$  cells was similar between leptin- and vehicle-treated mice (Figures S2A–S2C). Together, these data indicate that prenatal leptin exposure has long-term effects on the parasympathetic innervation of pancreatic  $\beta$  cells but does not lead to a widespread disruption of the autonomic inputs to the pancreas.

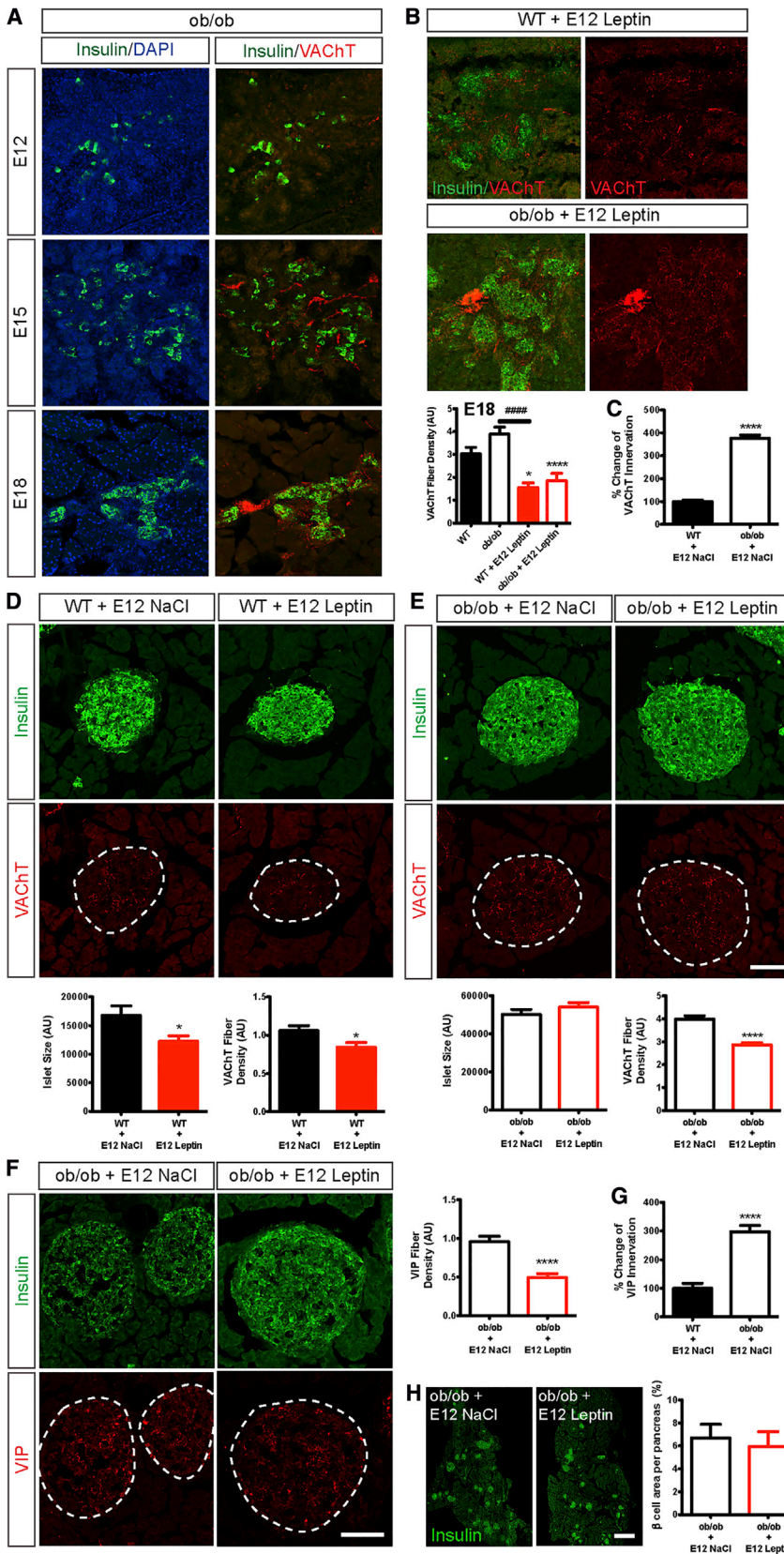
### Injection of Leptin during Embryonic Life Causes Long-Term Disturbances in Glucose Homeostasis

Because prenatal leptin exerted an enduring effect on  $\beta$  cell innervation, we next examined whether it was associated with disturbances in glucose homeostasis. The pre- and post-weaning growth curves (body weights) of prenatally injected WT and *ob/ob* mice were undistinguishable from those of vehicle-injected mice (Figures 3A–3D). Consistent with these data, daily food intake and body composition were similar between leptin-treated and control mice (Figures S3A–S3C). However, clear differences were observed in several indices of glucose homeostasis. Compared with vehicle-treated *ob/ob* mice, *ob/ob* mice that were prenatally treated with leptin displayed elevated levels of glucose 60–120 min following a glucose challenge (Figure 3E). However, glucose tolerance test results of the leptin-treated WT mice were similar to those of the controls (Figure 3I). Nevertheless, WT mice that were prenatally exposed to leptin displayed impaired insulin tolerance compared to control mice (Figure 3J). These differences were not observed in leptin-treated *ob/ob* mice (Figure 3F). Moreover, leptin-treated WT and *ob/ob* mice displayed normal fed, fasting, and re-fed glucose levels (Figures 3G and 3K). In addition, serum insulin (Figures 3H and 3L), glucagon (Figure S2D), and C-peptide levels (Figure S3D) were similar between leptin-treated and control *ob/ob* mice. However, the glucose-stimulated insulin secretion in leptin-treated *ob/ob* mice was decreased compared to that in control mice when measured 45 min after the glucose injection (Figure 3M). In contrast, this defect in insulin secretion in leptin-injected embryos was not observed upon L-arginine stimulation (Figure 3N). Furthermore, hepatic gluconeogenesis appeared normal, because injection of pyruvate resulted in similar glucose responses in leptin-treated and control mice (Figure 3O). Other markers of liver function, such as hepatic triglyceride content and free fatty acid levels, also appeared normal in prenatally treated mice (Figures 3P and 3Q).

### Leptin Acts Directly on Cholinergic Neurons in the Hindbrain to Blunt Axon Growth

The parasympathetic nerve fibers that innervate the pancreas mainly originate from hindbrain neurons located in the DMV (Fox and Powley, 1986). Most of these fibers travel within the vagus nerve and terminate on the pancreatic ganglia (Fox and Powley, 1986). To identify leptin's potential site of action, we first examined where leptin was taken up by intracerebroventricularly injecting fluorescently labeled bioactive leptin (16 kDa) into embryos (Balland et al., 2014; Vauthier et al., 2013). As early as 5 min after injection, fluorescent leptin was observed in cells in the wall of the fourth ventricle (Figure 4A). By 45 min after injection, a significant amount of fluorescently labeled leptin was detected in the hindbrain





## Figure 2. Prenatal Leptin Exposure Impairs Parasympathetic Innervation of Pancreatic Islets

(A) Confocal images of VACHT-positive fibers (red fluorescence) in close proximity to insulin-positive cells (green fluorescence) in E12, E15, and E18 *ob/ob* embryos.

(B) Confocal images and quantification of the density of VACHT fibers (red fluorescence) innervating pancreatic  $\beta$  cells (insulin, green fluorescence) in E18 WT and *ob/ob* embryos that were injected with vehicle or leptin at E12 (1  $\mu$ g per embryo) (n = 4 per group).

(C) Percentage change in the innervation of pancreatic islets by VACHT fibers between adult WT and *ob/ob* mice.

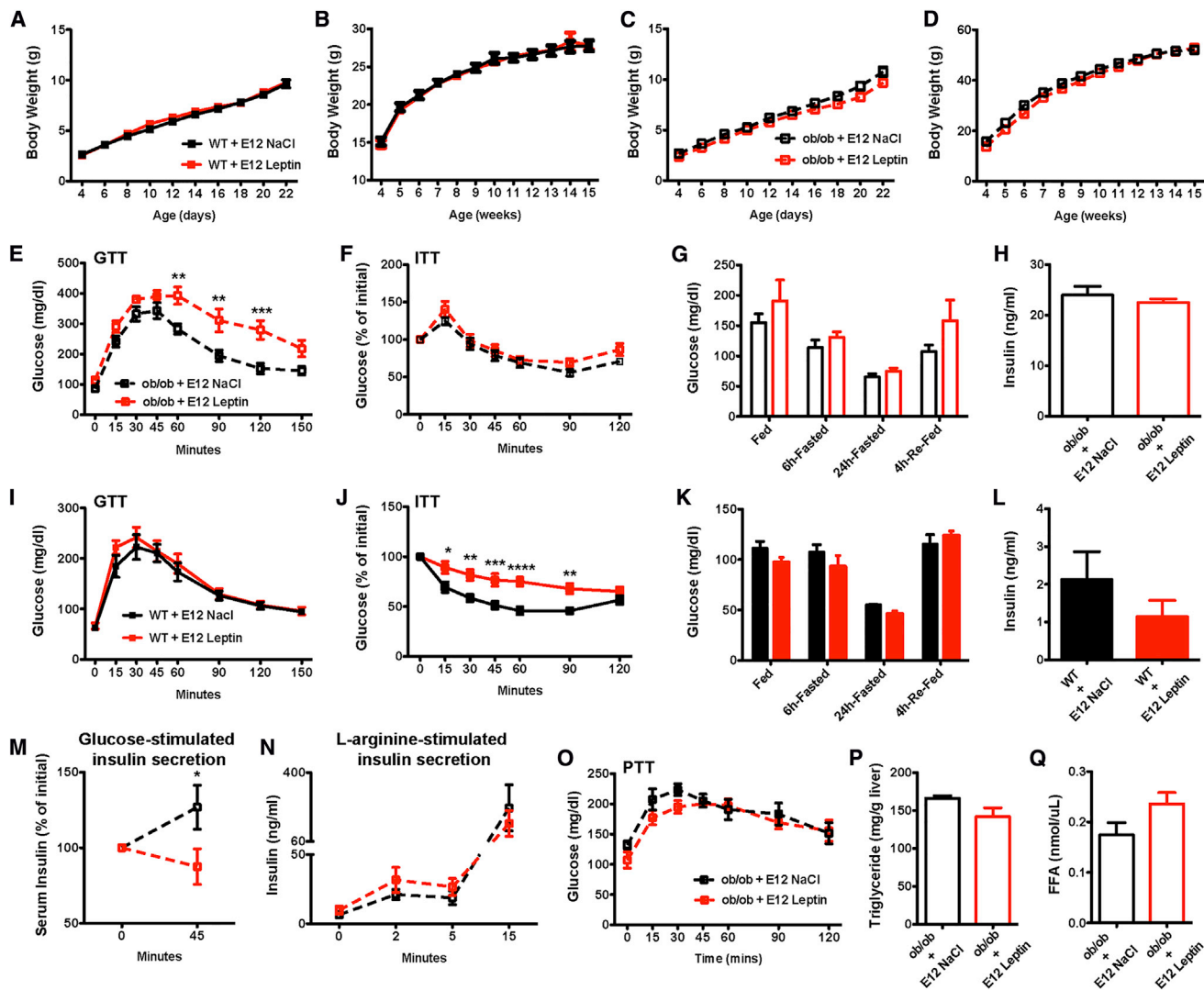
(D and E) Confocal images and quantification of pancreatic islet size (insulin, green fluorescence) and of the density of VACHT fibers (red fluorescence) innervating islets in 16-week-old (D) WT and (E) *ob/ob* mice that were injected with leptin or vehicle at E12 (n = 3–6 per group).

(F) Confocal images and quantification of the density of VIP-positive fibers (red fluorescence) innervating pancreatic  $\beta$  cells (insulin, green fluorescence) in 16-week-old WT and *ob/ob* mice that were injected with vehicle or leptin at E12 (1  $\mu$ g per embryo) (n = 4 per group).

(G) Percentage change in the innervation of pancreatic islets by VIP fibers between adult WT and *ob/ob* mice.

(H) Photomicrographs and quantification of the  $\beta$  cell area in pancreases of 16-week-old *ob/ob* mice that were prenatally injected with leptin or vehicle (n = 4 per group).

Scale bars represent 100  $\mu$ m (A–F) and 1 mm (G). \*p < 0.05 versus WT (B) and versus WT + NaCl at E12 (D); \*\*\*\*p < 0.0001 versus *ob/ob* (B), versus WT + NaCl at E12 (C), and versus *ob/ob* + NaCl at E12 (E and F); #####p < 0.0001 between the indicated groups (B). Values are shown as the mean  $\pm$  SEM.



### Figure 3. Prenatal Leptin Exposure Causes Lifelong Dysregulation of Glucose Homeostasis

(A–D) Pre- (A and C) and post-weaning (B and D) growth curves (body weights) of WT (A and B) and *ob/ob* mice (C and D) that were injected with leptin or vehicle at E12 ( $n \geq 10$  per group).

(E–L) Glucose (E and I) and insulin (F and J) tolerance tests of 10- to 12-week-old *ob/ob* (E and F) and WT mice (I and J) that were injected with leptin or vehicle at E12 ( $n = 5–8$  per group for the glucose tolerance test [GTT] and  $n = 7–8$  per group for the insulin tolerance test [ITT]). Blood glucose levels in 13-week-old *ob/ob* (G) and WT mice (K) that were prenatally injected with leptin or vehicle ( $n = 3–7$  per group). Plasma insulin levels in 16-week-old *ob/ob* (H) and WT (L) mice that were injected with leptin or vehicle at E12 ( $n = 5–7$  per group).

(M and N) Glucose- (M) and L-arginine-stimulated (N) insulin secretion in adult *ob/ob* mice that were injected with leptin or vehicle at E12 ( $n = 3–5$  per group).

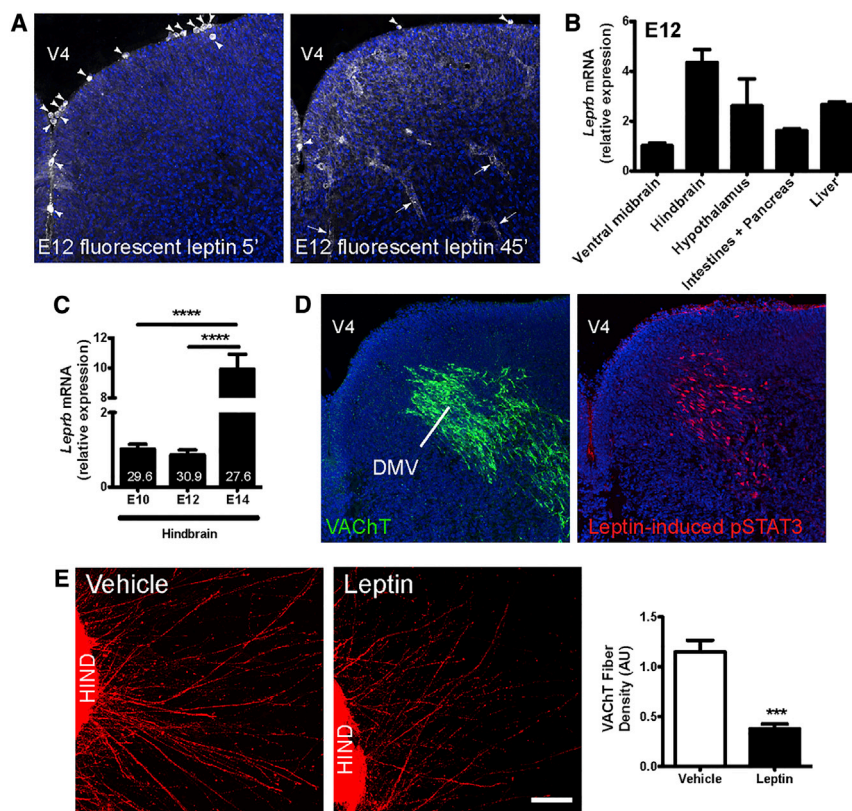
(O) Pyruvate tolerance test of 8-week-old *ob/ob* mice that were prenatally injected with leptin or vehicle ( $n = 6$  per group).

(P and Q) Plasma triglyceride (P) and free fatty acids (FFA) (Q) levels in 16-week-old *ob/ob* mice that were injected with leptin or vehicle at E12 ( $n = 4–7$  per group).

\* $p < 0.05$  versus *ob/ob* + NaCl at E12 (M); \*\* $p < 0.01$  versus *ob/ob* + NaCl at E12 (E); \*\*\* $p < 0.001$  versus *ob/ob* + NaCl at E12 (E) and versus WT + NaCl at E12 (J); and \*\*\*\* $p < 0.0001$  versus WT + NaCl at E12 (J). Values are shown as the mean  $\pm$  SEM.

parenchyma and vessels (Figure 4A). Fluorescent leptin was also observed in the parenchyma and vessels of the spinal cord near the cholinergic neurons of the ventral motor column (Figure S4A). Less fluorescent leptin was observed in other brain regions, including the hypothalamus (Figure S4B). Fluorescently labeled leptin was also observed in peripheral tissue, including the liver and pancreas, 45 min after injection (Figures S4C and S4D).

Consistent with a primary action of central leptin on hindbrain neurons, relatively high levels of leptin receptor (*Leprb*) mRNA were found in the embryonic hindbrain. At E12, *Leprb* mRNA levels were two to four times higher in the hindbrain than in other brain regions such as the ventral midbrain or hypothalamus (Figure 4B). Moreover, *Leprb* mRNA levels in the hindbrain in E14 embryos were 5- to 10-fold greater than those in E10–E12 embryos (Figure 4C). During embryonic life, *Leprb* mRNA is also



**Figure 4. Leptin Blocks Cholinergic Axonal Growth from the Embryonic Hindbrain**

(A) Representative photomicrographs of the hind-brain showing cell bodies (arrowheads) and vessels (arrows) labeled with fluorescent leptin (1 $\mu$ g per embryo, white labeling) 5 and 45 min after it was intracerebroventricularly injected into E12 embryos. (B) Relative expression of *Leprb* mRNA in various brain areas and peripheral organs of E12 WT mouse embryos (n = 3 per group).

(C) Relative expression of *Leprb* mRNA in the hind-brain of E10, E12, and E14 WT mouse embryos (n = 3–5 per age). Numbers in bar graphs indicate Ct values. (D) Confocal images of VACHT-immunoreactive cells (green fluorescence) and leptin-induced pSTAT3-immunopositive cells (red fluorescence) in the DMV of E12 WT embryos.

(E) Confocal images and quantification of the overall density of VACHT-positive fibers in organotypic cultures of isolated E12 hindbrains. The cultures were incubated with leptin (100 ng/ml) or vehicle for 48 hr (n = 5–6 explants per group).

DMV, dorsomedial nucleus of the vagus nerve; HIND, hindbrain; V4, fourth ventricle. Scale bar, 100  $\mu$ m. \*\*\*p < 0.001 versus vehicle (E); and \*\*\*\*p < 0.0001 between indicated groups (D). Values are shown as the mean  $\pm$  SEM.

expressed in several peripheral organs that are known to be innervated by the vagus nerve, including the intestines, pancreas, liver, and stomach (Figure 4B).

We next examined the distribution of labeling for pSTAT3, a commonly used surrogate marker of leptin receptor activation, in embryos that received a central injection of leptin. After 45 min, robust pSTAT3 immunoreactivity was observed in the DMV, and the distribution of pSTAT3-positive cells overlapped with that of VACHT neurons (Figure 4D). In addition, central leptin increased the number of pSTAT3-labeled cells in the ventral motor column of the spinal cord, where cholinergic neurons are located (Figure S4E). In contrast, pSTAT3 immunoreactivity was not observed in other tissues that took up fluorescent leptin, such as the hypothalamus, liver, and pancreas (Figures S4F–S4H). However, it remains possible that leptin might activate other canonical LepRb signaling pathways, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt, in regions and tissues that do not exhibit STAT3 phosphorylation after leptin administration. Nevertheless, these results suggest that central leptin might influence the parasympathetic innervation of the pancreas by primarily acting, at least in part, on hindbrain neurons.

To determine if leptin acts directly on preganglionic cholinergic neurons of the hindbrain to influence axon growth, we next performed a series of in vitro experiments in which hindbrain explants were microdissected, placed in a collagen matrix, and exposed to leptin or vehicle. After 48 hr, the density of cholinergic axons extending from hindbrain explants in which leptin was

added to the culture medium was 3.3-fold less than that in control explants (Figure 4E). Together, these data provide direct evidence that leptin acts on hindbrain neurons to inhibit cholinergic axon growth.

## DISCUSSION

Both the sympathetic and parasympathetic branches of the autonomic nervous system are critical regulators of glucose homeostasis (Ahrn, 2000; Thorens, 2014; Woods and Porte, 1974). However, the mechanisms that underlie the development of the autonomic nervous system in general, and of the parasympathetic innervation of the endocrine pancreas in particular, remain elusive. In the present study, we report that cholinergic innervation of pancreatic islets develops during mid-gestation under the influence of leptin. Our results indicate that prenatal leptin decreases parasympathetic innervation of the endocrine pancreas, which alters glucose homeostasis without affecting body weight or body composition. These data are in good agreement with a direct role of leptin in glucose regulation. Mice with a genetic leptin deficiency (*ob/ob* mice) or leptin receptor deficiency (*db/db* mice) display hyperphagia and obesity as well as insulin resistance and diabetes. While increased food intake and body adiposity clearly contribute to the impaired glucose metabolism that is observed in these mouse models, several observations suggest that leptin regulates glucose metabolism independently of its effects on energy balance. Caloric restriction has only a moderate beneficial effect on insulin sensitivity and hyperglycemia in *ob/ob* and *db/db* mice (Morton et al., 2005; Wyse and Dulin, 1970), and leptin administration to



leptin-deficient mice ameliorates hyperglycemia and hyperinsulinemia (Farooqi et al., 1999; Pellemounter et al., 1995), even when differences in food intake are controlled for pair-feeding (Hedbacker et al., 2010; Schwartz et al., 1996). The exact mechanisms underlying the insulin resistance observed in leptin-injected embryos remain to be investigated, but it is possible that prenatal leptin alters glucose production in the liver and/or glucose uptake by muscle. Our data are also in good agreement with other studies that have reported an inhibitory action of leptin on the parasympathetic system. For example, central leptin administration in adult mice has been shown to reduce parasympathetic outflow, causing changes in airway diameter and lung function (Arteaga-Solis et al., 2013).

Leptin's regulation of the parasympathetic innervation of the pancreas likely involves direct effects on hindbrain neurons rather than direct effects on the embryonic pancreas. Central leptin injection induced STAT3 phosphorylation in DMV neurons, but not in the pancreas. In addition, the hindbrain contains the highest density of leptin receptors of any brain region, and central injection of fluorescently labeled leptin resulted in rapid accumulation of fluorescence in the hindbrain. Furthermore, direct exposure of hindbrain explants to leptin inhibited cholinergic axon growth. Notably, the developmental effects of prenatal leptin appear to be restricted to the innervation of pancreatic  $\beta$  cells, because the cholinergic innervation of pancreatic  $\alpha$  cells was unaffected by prenatal leptin. The parasympathetic nerve fibers that innervate the pancreas originate mainly from the DMV, and the majority of these fibers terminate on the pancreatic ganglia, which, in turn, innervate pancreatic  $\beta$  cells (Fox and Powley, 1986). Therefore, it is likely that the reduction in the cholinergic innervation of pancreatic  $\beta$  cells results from decreased innervation of the pancreatic ganglia by preganglionic autonomic fibers that originate in the hindbrain and that distinct pancreatic ganglia are responsible for the innervation of  $\alpha$  and  $\beta$  cells. More generally, our data suggest that normal preganglionic innervation of pancreatic ganglia is important for normal cholinergic innervation of islets by postganglionic neurons. In addition, we cannot rule out the possibility that some of the perturbations in glucose homeostasis that were observed after prenatal leptin administration resulted from changes in the autonomic innervation of the liver, although liver function appeared normal in leptin-injected embryos.

Leptin is one of the first major metabolic hormones to appear during development. White adipose tissue (the main source of leptin production in adult animals) is minimal at early ages, yet mouse fetuses do contain significant leptin levels in their blood as early as E12 (Udagawa et al., 2006; Y. Ishii and S.G.B., unpublished data). Various tissues produce leptin during embryonic development. On E13, high levels of leptin gene expression are found in the fetal liver and cartilage/bone structures, followed by cardiac expression between E16 and E18 (Hoggard et al., 1997, 2000). In addition to being produced by the embryo itself, dams also contain high levels of leptin during pregnancy, but whether maternal leptin crosses the placenta and reaches the embryo during early to mid-gestation (i.e., when brain development is initiated) remains unclear. Circulating leptin levels increase markedly during the postnatal period and exhibit a "surge" (Ahima et al., 1998). The inhibitory effect of leptin

on parasympathetic development during embryonic life differs from its effect on hypothalamic neural projections during postnatal life. During postnatal life, leptin acts as a powerful neurotrophic agent that promotes the formation of neural circuits that originate in the arcuate nucleus (Bouret et al., 2012, 2004). The mechanisms underlying the opposing effects of leptin on hypothalamic and hindbrain axon growth remain to be investigated. However, recent data have suggested that the transition from stimulatory to inhibitory effects of leptin on neuronal activity occurs in parallel with the acquisition of functional ATP-sensitive potassium channels (Baquero et al., 2014), and these data support the hypothesis that leptin activates distinct membrane-linked and intracellular pathways in hindbrain and hypothalamic neurons during development. Prenatal leptin exposure could also result in changes in insulin levels that could then, in turn, indirectly affect the autonomic innervation of the pancreas. Supporting this idea, central leptin administration in adult rats decreases glucose-stimulated insulin secretion (Muzumdar et al., 2003). In addition, recent studies have demonstrated that the effects of a perinatal high-fat diet on pancreatic parasympathetic innervation and glucose-dependent insulin secretion depended on hypothalamic insulin signaling (Vogt et al., 2014). However, our *in vitro* experiments, which showed that exposing isolated hindbrain explants to leptin blunted axon growth, support a direct role of leptin in cholinergic axon growth.

In conclusion, our data show the importance of an optimal hormone balance during prenatal life for lifelong glucose regulation and correct organization of parasympathetic inputs to pancreatic islets. These findings might be important for physiopathological conditions that are associated with abnormally elevated leptin levels during critical periods of growth and development. For example, maternal obesity is associated with elevated levels of leptin in both the maternal and fetal circulation (Luo et al., 2013), and epidemiological studies have demonstrated a strong association between maternal obesity and the offspring's predisposition to diabetes (Boney et al., 2005). Better understanding of the relationship between prenatal leptin and the programming of metabolic diseases will be crucial as we seek to develop interventional studies to ameliorate and hopefully reverse such metabolic malprogramming.

## EXPERIMENTAL PROCEDURES

### Animals

*ob/ob* mice and their WT littermates were produced in our breeding colony by pairing heterozygous male and female mice (JAX mice, stock 000632). The day of conception (sperm-positive vaginal smear) was designated as E0. For all experiments, on day one after birth, litter size was adjusted to six or seven pups to ensure adequate and standardized nutrition until weaning. Only male mice were studied. All animal procedures were conducted in compliance with and approved by the Institutional Animal Care and Use Committee of the Saban Research Institute of the Children's Hospital of Los Angeles. For detailed information regarding the analytical time points, determination of body weight and composition, serum analyses, and assessment of glucose homeostasis, see [Supplemental Experimental Procedures](#).

### VACHT, Synaptophysin, Tyrosine Hydroxylase, HNF4 $\alpha$ , Insulin, and Glucagon Immunohistochemistry and Image Analyses

For immunostaining of embryonic tissue, pregnant females were anesthetized and the embryos collected at E12, E15, and E18. The embryos



were then fixed in a solution of 4% paraformaldehyde. For immunostaining of adult tissue, mice were transcardially perfused with 4% paraformaldehyde. Tissue sections were processed for immunofluorescence using standard procedures. The primary antibodies used for immunohistochemistry were as follows: rabbit anti-VACHT (1:500; Synaptic Systems), mouse anti-synaptophysin1 (1:500; Synaptic Systems), rabbit anti-TH (1:2,000; Immunostar), goat anti-HNF4 $\alpha$  (1:500; Santa Cruz), and guinea-pig anti-insulin (1:500; Abcam). For detailed information regarding image acquisition and quantification, see [Supplemental Experimental Procedures](#).

### Isolated Hindbrain Explant Cultures

Brains were collected from E12 embryos. The hindbrain (consisting of the DMV and NTS) was then carefully dissected from the rest of the brain under a stereomicroscope. Explants (n = 5–6 cultures per group) were cultured onto a rat tail collagen matrix (BD Biosciences) using standard procedures (Bouret et al., 2004). After a 48-hr incubation with leptin (100 ng/ml; Peprotech), or with vehicle, the explants were fixed in paraformaldehyde, and cholinergic neurites extending from the explants were stained with rabbit anti-VACHT (1:1,000; Synaptic Systems). See [Supplemental Experimental Procedures](#) for additional details regarding image analysis.

### Intra-embryonic Leptin Injections

Timed-pregnant mice carrying E12 *ob/ob* and WT embryos were anesthetized, and the uterine horns were gently placed outside the abdominal cavity. Using a Nanofil syringe with a 35G needle attachment (World Precision Instruments), 1  $\mu$ g murine leptin (1 mg/ml; Peprotech) or vehicle (NaCl 0.9%) was then delivered into each embryo intracerebroventricularly. Each experiment included offspring from at least 11–14 litters.

### Fluorescent Leptin Assays

Using a Nanofil syringe with a 35G needle attachment (World Precision Instruments), 1  $\mu$ g fluorescently labeled bioactive leptin (Cisbio Bioassays) (Balland et al., 2014; Vauthier et al., 2013) was intracerebroventricularly injected into E12 embryos. Embryos were sacrificed 5 and 45 min later to assess tissue leptin uptake by confocal microscopy.

### pSTAT3 Immunohistochemistry

Leptin (1  $\mu$ g; Peprotech) was intracerebroventricularly injected into E12 embryos as described above. Controls received equivolume injections of vehicle (0.9% NaCl). The embryos were collected 45 min later and fixed with a solution of 2% paraformaldehyde. Frozen coronal (head) or sagittal (body) sections were cut at 30  $\mu$ m and processed for pSTAT3 immunostaining as previously described (Bouret et al., 2012).

### Statistical Analysis

All values are presented as the mean  $\pm$  SEM. Statistical analyses were conducted using GraphPad Prism (version 5.0a). For each experiment, slides were numerically coded to obscure the treatment group. Statistical significance was determined using unpaired two-tailed Student's t test and two-way ANOVA followed by the Bonferroni post hoc test when appropriate.  $p \leq 0.05$  was considered statistically significant.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.02.088>.

### AUTHOR CONTRIBUTIONS

S.C. and S.G.B. designed the study. S.C. and S.G.B. interpreted the results. S.C. performed experiments and analyzed the data. S.C. contributed to all figures. V.P. contributed to Figures 4 and S4. S.C. and S.G.B. wrote the paper, with input from the other authors.

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