Original Article

MOLECULAR METABOLISM

Differential contribution of POMC and AgRP neurons to the regulation of regional autonomic nerve activity by leptin

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ABSTRACT

Objectives: The autonomic nervous system is critically involved in mediating the control by leptin of many physiological processes. Here, we examined the role of the leptin receptor (LepR) in proopiomelanocortin (POMC) and agouti-related peptide (AgRP) neurons in mediating the effects of leptin on regional sympathetic and parasympathetic nerve activity.

Methods: We analyzed how deletion of the LepR in POMC neurons ($POMC^{Cre}/LepR^{fl/fl}$ mice) or AgRP neurons ($AgRP^{Cre}/LepR^{fl/fl}$ mice) affects the ability of leptin to increase sympathetic and parasympathetic nerve activity. We also studied mice lacking the catalytic p110α or p110β subunits of phosphatidylinositol-3 kinase (PI3K) in POMC neurons.

Results: Leptin-evoked increase in sympathetic nerve activity subserving thermogenic brown adipose tissue was partially blunted in mice lacking the LepR in either POMC or AgRP neurons. On the other hand, loss of the LepR in AgRP, but not POMC, neurons interfered with leptin-induced sympathetic nerve activation to the inguinal fat depot. The increase in hepatic sympathetic traffic induced by leptin was also reduced in mice lacking the LepR in AgRP, but not POMC, neurons whereas LepR deletion in either AgRP or POMC neurons attenuated the hepatic parasympathetic nerve activation evoked by leptin. Interestingly, the renal, lumbar and splanchnic sympathetic nerve activation caused by leptin were significantly blunted in $POMC^{Cre}/LepR^{fl/fl}$ mice, but not in $AgRP^{Cre}/LepR^{fl/fl}$ mice. However, loss of the LepR in POMC or AgRP neurons did not interfere with the ability of leptin to increase sympathetic traffic to the adrenal gland. Furthermore, ablation of the p110α, but not the p110β, isoform of PI3K from POMC neurons eliminated the leptin-elicited renal sympathetic nerve activation.

Finally, we show trans-synaptic retrograde tracing of both POMC and AgRP neurons from the kidneys.

Conclusions: POMC and AgRP neurons are differentially involved in mediating the effects of leptin on autonomic nerve activity subserving various tissues and organs.

Keywords Leptin; Autonomic nervous system; Energy homeostasis; Cardiovascular regulation

1. INTRODUCTION

The adipocyte-derived hormone leptin is best known for its role in the regulation of energy homeostasis [1]. Leptin acts in the brain as part of a negative feedback mechanism to control adiposity by inhibiting food intake and increasing energy expenditure through stimulation of sympathetic nerve activity (SNA) deserving thermogenic organs such as brown adipose tissue (BAT) [1,2]. Leptin induced increases in SNA subserving white adipose tissue (WAT) may also contribute to thermogenic regulation by promoting the "browning" phenomenon [3]. Moreover, leptin-induced activation of WAT SNA has important implications on lipolysis [4–7]. More recently, we demonstrated that brain action of leptin increases parasympathetic nerve activity (PSNA) subserving the liver [8], which is consistent with the requirement of the vagal nerves for the control of hepatic function by leptin [9]. In addition, leptin increases SNA to several distinct beds that are not directly involved in the regulation of energy homeostasis. This includes the kidneys and vasculature, which are implicated in cardiovascular and hemodynamic regulation [10–12]. The arcuate nucleus of the hypothalamus (ARC) is critically involved in the regulation of several physiological processes including metabolic

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Abbreviations: α—MSH, α—melanocyte-stimulating hormone; AgRP, agouti-related peptide; ARC, arcuate nucleus of the hypothalamus; BAT, brown adipose tissue; GABA, γ-aminobutyric acid; GFP, green fluorescent protein; HFD, high fat diet; ICV, intracerebroventricular; LepR, leptin receptor; MC3/4R, melanocortin 3 or 4 receptor; PI3K, phosphatidylinositol-3 kinase; POMC, proopiomelanocortin; PRV, pseudorabies virus; PSNA, parasympathetic nerve activity; SNA, parasympathetic nerve activity; STAT3, signal transducer and activator of transcription 3; WAT, white adipose tissue

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and cardiovascular functions [5,13]. The leptin receptor (LepR) is highly expressed in the ARC and several lines of evidence point to the importance of the ARC for leptin control of both SNA and PSNA [8,11,14–18]. The ARC contains multiple unique LepR-positive neuronal populations including those expressing proopiomelanocortin (POMC) and agouti-related peptide (AgRP). Leptin increases the activity of anorexigenic POMC neurons leading to the release of POMC products such as α–melanocyte-stimulating hormone (α-MSH) that activate the postsynaptic melanocortin 3 or 4 receptor (MC3/4R) in the paraventricular nucleus and other brain nuclei [2,19]. Conversely, leptin inhibits orexigenic AgRP neurons and decreases the release of AgRP, a peptide that inhibit MC3/4R signaling. It should be noted, however, that the relationship between POMC and AgRP neurons is more complex than a simple modulation of MC3/4R in the second order neurons. This is due to the reciprocal inhibition POMC and AgRP neurons exert on each other [20,21]. In addition, these neurons co-release several biologically active molecules besides α-MSH and AgRP. For instance, the majority of POMC neurons express cocaine- and amphetamine-regulating transcript whereas a subset of these neurons produces γ-aminobutyric acid (GABA), glutamate, or acetylcholine. On the other hand, most AgRP neurons release neuropeptide Y and/or GABA [13].

Stimulation of the LepR leads to the activation of multiple intracellular pathways including the signal transducer and activator of transcription 3 (STAT3) and phosphatidylinositol-3 kinase (PI3K) [2]. Several of these pathways have been shown to be required for the tissue-specific autonomic effects of leptin. For example, inhibition of PI3K signaling prevents leptin-induced increase in sympathetic outflow to the kidney, but not to BAT [22]. However, the PI3K isoform(s) that mediate the renal sympathetic response elicited by leptin has not been determined. Class Ia PI3Ks are heterodimeric kinases consisting of a p85 regulatory subunit and a p110 catalytic subunit [23,24]. Although the two ubiquitously expressed PI3K isoforms (p110α and p110β) have high structural and enzymatic overlap they play distinct roles in cellular signaling and the regulation of physiological processes [25,26].

In the present study, we investigated the role of POMC and AgRP neurons in underlying the autonomic effects of leptin. For this, we analyzed the ability of leptin to increase SNA and PSNA subserving various beds in mice lacking the LepR in either POMC or AgRP neurons. Additionally, we assessed the role of the two PI3K catalytic subunits (p110α and p110β) in POMC neurons in mediating the renal sympathetic nerve activation evoked by leptin.

2. MATERIAL AND METHODS

2.1. Mice

We used $LepR^{fl/fl}$ mice and ROSA ($Stop^{fl/fl}$-tdTomato) reporter transgenic mice from our own colonies [18,27]. $POMC^{Cre}$ and $AgRP^{Cre}$ mice were obtained from the Jackson Laboratory (Stock# 005965 and 012899, respectively), and $p110\alpha^{fl/fl}$ mice and $p110\beta^{fl/fl}$ mice [25] were generously provided by Dr. Jean Zhao (Dana-Farber/Harvard Cancer Center). To selectively delete the LepR from POMC or AgRP neurons, female $LepR^{fl/fl}$ mice were bred with male $POMC^{Cre}$ mice or $AgRP^{Cre}$ mice, respectively. To identify Cre-positive neurons, $LepR^{fl/fl}$ mice were first crossed with ROSA ($Stop^{fl/fl}$-tdTomato) reporter animals to introduce a genetically encoded fluorescent label for visualization of Cre-expressing cells. Genotyping of mice was performed by tail snip at weaning, followed by DNA isolation and standard PCR.

Male and female, 12–16 weeks of age, $POMC^{Cre}/LepR^{fl/fl}$ mice, $AgRP^{Cre}/LepR^{fl/fl}$ mice, $POMC^{Cre}/LepR^{fl/fl}/tdTomato^{+}$ mice, $AgRP^{Cre}/LepR^{fl/fl}/tdTomato^{+}$ mice and appropriate littermate controls were used for experimentation. Animals were housed at 23 °C with a 12-hr light/dark cycle and allowed free access to tap water and standard chow or 45% high fat diet (D12451, Research Diets, Inc.). All animal protocols were approved by the University of Iowa Animal Research Committee.

2.2. Intracerebroventricular (ICV) cannulation

Mice were anesthetized using either a Ketamine (91 mg/kg i.p.) and Xylazine (9.1 mg/kg i.p.) cocktail mixture or isoflurane (5% for induction; 1–2% to sustain). Once anesthetized, each mouse was placed in a stereotaxic device and implanted with a stainless-steel cannula (25 gauge, 9 mm length) into the lateral brain ventricle (0.3 mm posterior and 1.0 mm lateral to bregma and 3.0 mm below the surface of the skull) as described previously [8,18]. Mice were allowed to recover for 1 week prior to utilization of the cannula for ICV leptin or vehicle injection during sympathetic nerve recordings.

2.3. Tracing experiments

Viral transneuronal tracing experiments to characterize the projections of the ARC to the kidneys were carried out using a well-documented transneuronal isogenic recombinant pseudorabies virus (PRV-152) expressing a green fluorescent protein (GFP) [23,24]. The PRV-152 was obtained from the NIH CNNV of the University of Pittsburgh (http://www.cnnv.pitt.edu). Mice (C57BL/6J) anesthetized with Ketamine (91 mg/kg i.p.) and Xylazine (9.1 mg/kg i.p.) underwent lateral retro-peritoneal incision to gain access to each kidney. Using a Hamilton syringe, the PRV-152 (0.5 μl) was injected longitudinally into the cortex of the left and right kidney. In a subset of mice, bilateral renal denervation was performed before the injection of the PRV-152 into the kidneys. Renal denervation was performed by cutting all the nerves that subserve each kidney followed by application of 10% phenol for 10 min [28]. Sutures were used to close all the incisions. Mice were sacrificed at various time points subsequent to viral injections and perfused with PBS followed by 4% paraformaldehyde. Brains were collected post-fixed in 4% paraformaldehyde and processed for GFP immunostaining and imaging.

2.4. Immunohistochemistry

Following an overnight fast and treatment with intraperitoneal leptin (100 μg) or vehicle (10 μl of saline) for 1 h, mice were anesthetized using Ketamine/Xylazine and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were removed and stored in 4% paraformaldehyde overnight followed by sectioning using a vibratome (Leica Microsystems, Inc) into 25 μM coronal sections. For phospho-STAT3 staining, sections were incubated in 1% NaOH, 1% H_2O_2 in distilled water for 20 min at room temperature, rinsed with PBS 3 times for 10 min each, incubated with 0.3% glycine in PBS for 10 min, rinsed with PBS 3 times for 10 min each, incubated with 0.03% sodium dodecyl sulfate in PBS for 10 min. All sections were blocked in 3% normal goat serum, 0.3% Triton X-100 in PBS for 30 min at room temperature, and then incubated with primary antibody against GFP (ab290, Abcam, Inc., 1:200) or phospho-STAT3 Tyr 705 (#9131S, Cell Signaling, 1:1000) in blocking buffer for 24 h at room temperature plus an additional 48 h at 4 °C. Sections were then washed 3 times for 10 min each with PBS and 0.3% Tween20 followed by 1 h incubation with AlexaFlour488 goat anti-rabbit secondary antibody in blocking buffer and 3 additional 10 min washes in PBS and 0.3% Tween20. Sections were mounted on uncoated glass slides and coverslipped

with Vectashield antifade mounting medium with DAPI (Vector Laboratories). Images were acquired using a Zeiss LSM 710 confocal microscope.

2.5. Sympathetic and parasympathetic nerve recordings under anesthesia

Mice anesthetized with intraperitoneal administration of Ketamine (91 mg/kg body weight) and Xylazine (9.1 mg/kg body weight) underwent intubation with PE-50 to provide an unimpeded airway for the mouse to spontaneously breathe O_2 enriched room air. Next a micro-renathane tube (MRE-40, Braintree Scientific) was inserted into the right jugular vein for infusion of the sustaining anesthetic agent: α-chloralose (initial dose: 12 mg/kg, then sustaining dose of 6 mg/kg/hr). This line was also used for intravenous injection of leptin or vehicle (0.9% isotonic saline). Another MRE- 40 catheter was connected to a Powerlab via a pressure transducer and inserted into the left carotid artery for continuous measurement of arterial pressure and heart rate. Core body temperature, monitored through a rectal probe, was maintained at 37.5 °C.

Next, mice were equipped for direct multifiber SNA or PSNA recording from the nerves subserving various organs and tissues. Separate mice were used to study each nerve. The nerve fascicle that innervate the interscapular BAT was accessed through an incision in the nape of the neck. The nerve subserving the inguinal WAT was accessed through a small incision made on the right flank near the hind limb. To gain access to either the sympathetic (located by the hepatic artery; hepatic SNA) or to the parasympathetic (adjacent to the esophagus; hepatic PSNA) nerve fibers that innervate the liver, an incision was made through the abdominal wall in mice placed in the dorsal position. The pre-ganglionic nerve fibers deserving the left adrenal gland (adrenal SNA) and the post-ganglionic nerve fibers that sub-serve the left kidney (renal SNA), visceral organs (splanchnic SNA) or the hind limb region (lumbar SNA) were accessed through a retro-peritoneal incision.

Each sympathetic or parasympathetic nerve fascicle was carefully isolated from surrounding connective tissues. A bipolar platinum-iridium electrode (40-gauge, A-M Systems) was then suspended under the nerve and secured with silicone gel (Kwik-Cast, WPI). The electrode was attached to a high-impedance probe (HIP-511, Grass Instruments) and the nerve signal was amplified 10^5 times with a Grass P5 AC pre-amplifier. The amplified nerve signal was filtered at a 100- and 1000-Hz cutoff with a nerve traffic analysis system (model 706C, University of Iowa Bioengineering). The amplified and filtered nerve signal was routed to a speaker system and to an oscilloscope (model 54501A, Hewlett-Packard) to monitor the audio and visual quality of the sympathetic and parasympathetic nerve recordings and for quantification purposes. The amplified, filtered nerve signal was also directed to a MacLab analogue-digital converter (Model 8S, AD Instruments Castle Hill, New South Wales, Australia) containing the software (MacLab Chart Pro; Version 7.0) that utilizes a cursor to analyze the number of spikes/second that exceeds the background noise threshold.

Baseline SNA and PSNA were recorded over a 10 min control period before treatments with leptin (ICV: 2 μg; or intravenously: 120 μg) or insulin (ICV: 100 μU). Vehicle injections (ICV: 2 μl; intravenously: 12 μl) were used as controls. After treatments, SNA and PSNA were recorded continuously and quantified at 15 min intervals over 4 h. Any residual nerve activity that remained after death was considered background noise and thus was subtracted from the measured SNA and PSNA.

2.6. Sympathetic nerve recordings in conscious mice

In a subset of mice, renal SNA was studied in the conscious state as described in our recent report [29]. Mice were anesthetized with Isoflurane (up to 5% for induction, 1.5—2% for maintenance) and a nerve subserving the left kidney was exposed, dissected, and placed on the electrodes as described above. After an optimal positioning of the electrode (based on signal to noise ratio) was obtained, the electrodes were encased with a minimal amount (~100 μl) of silicone gel that allowed the movement of the assembly without breaking the nerve. A separate grounding wire was inserted in the nearby muscle to ground the mouse. Silk was used to stitch the recording electrodes to the spinal muscle and to secure the electrodes along the subcutaneous dorsal surface until these electrode wires were exteriorized at the nape of the neck. Mice were then gradually weaned off of isoflurane and allowed 4—5 h to regain full consciousness before the experimental protocol was started. After baseline recording of renal SNA, mice received ICV injection of leptin (2 μg) or vehicle (2 μl) and followed for 4 h. At the end of the experiment, mice were sacrificed using an overdose of Ketamine/Xylazine cocktail. As above, background noise was measured and subtracted to determine real SNA.

2.7. Data analysis

Data are displayed as mean ± SEM. SNA and PSNA responses are expressed as percent change from baseline. Comparisons between groups were made using *t*-test, 1- or 2-way ANOVA with or without repeated measures. Appropriate post hoc tests were used when analysis of ANOVA reached significance. A value of $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Generation and validation of mice lacking LepR in POMC or AgRP neurons

In order to determine the contribution of the LepR expressed in POMC and AgRP neurons to the autonomic effects of leptin, we selectively deleted the LepR from these neurons by crossing $LepR^{fl/fl}$ mice with either $POMC^{Cre}$ mice or $AgRP^{Cre}$ mice, respectively. To visualize Cre recombination and facilitate the identification of the targeted neurons, we further crossed these mice with Cre-dependent, td-Tomato reporter mice. We verified the successful deletion of the LepR from POMC or AgRP neurons by assessing the ability of leptin to activate STAT3 in the targeted neurons. Overnight fasted control mice ($POMC^{Cre}/LepR^{wt/wt}tdTomato^{+}$ and $AgRP^{Cre}/LepR^{wt/wt}tdTomato^{+}$) as well as mice lacking the LepR in POMC neurons ($POMC^{Cre}/LepR^{fl/fl}tdTomato^{+}$) or AgRP neurons ($AgRP^{Cre}/LepR^{fl/fl}tdTomato^{+}$) were sacrificed 1 h after intraperitoneal injection of leptin (100 μg). The brains were collected and processed for phosphorylated STAT3 immunostaining.

As expected, leptin treatment increased STAT3 phosphorylation in the ARC of control mice with substantial co-localization with tdTomato-labeled POMC and AgRP neurons of $POMC^{Cre}/LepR^{wt/wt}tdTomato^{+}$ and $AgRP^{Cre}/LepR^{wt/wt}tdTomato^{+}$, respectively (Figure 1A and B). In contrast, co-localization of phospho-STAT3 with tdTomato-labeled POMC neurons was virtually eliminated in leptin-treated $POMC^{Cre}/LepR^{fl/fl}tdTomato^{+}$ mice (Figure 1C). Similarly, leptin treatment failed to increase phospho-STAT3 in tdTomato-labeled AgRP neurons of $AgRP^{Cre}/LepR^{fl/fl}tdTomato^{+}$AgRP mice (Figure 1D). Importantly, leptin-induced increase in STAT3 phosphorylation was not altered in other brain regions such as the lateral hypothalamic area (Figure S1A and B) or ventromedial hypothalamus (Figure S1C and D).

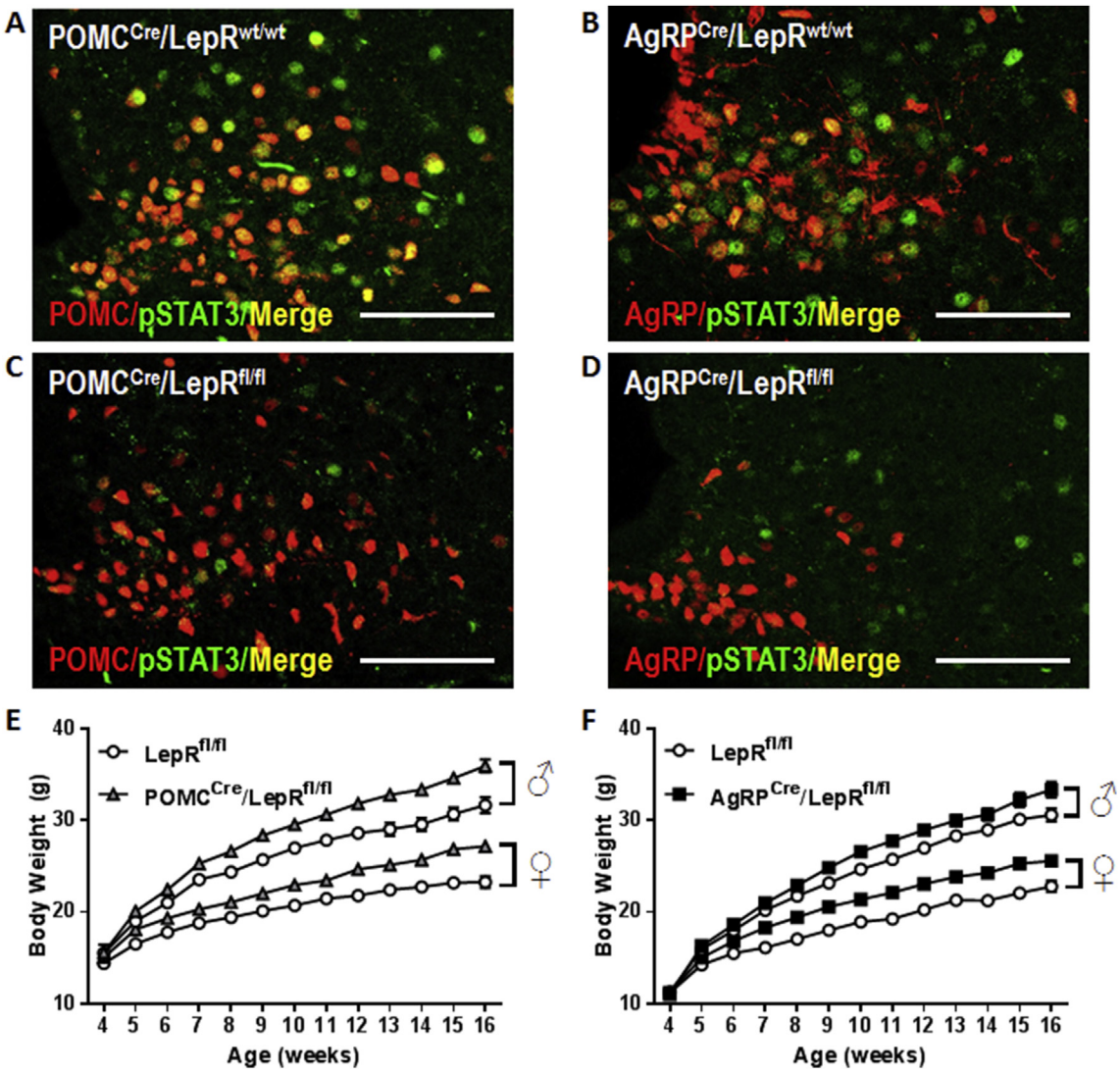

Figure 1: LepR deletion from POMC or AgRP neurons prevents leptin-induced STAT3 phosphorylation. A-B) In control mice, ICV leptin treatment increased STAT3 phosphorylation in both POMC (A) and AgRP (B) neurons. **C-D)** Loss of LepR from POMC (C) or AgRP (D) neurons abolished leptin-induced phosphorylation of STAT3 in those neurons. Images acquired at 20× magnification. Scale bar, 100 μm. **E-F)** Relative to littermate controls, male and female mice lacking the LepR in POMC (E, n = 7–19 male, 9–23 female $LepR^{fl/fl}$; n = 16–35 male, 10–27 female $POMC^{Cre}/LepR^{fl/fl}$) or AgRP (F, n = 7–9 male, 9–10 female $LepR^{fl/fl}$; n = 12–21 male, 13–17 female $AgRP^{Cre}/LepR^{fl/fl}$) neurons display increased body weight.

Consistent with previous reports [30,31], both male and female $POMC^{Cre}/LepR^{fl/fl}$ and $AgRP^{Cre}/LepR^{fl/fl}$ mice displayed a mild, but significant increase in body weight as compared to control littermates (Figure 1E and F). Furthermore, this increase in body weight was associated with increased BAT, perigonadal, and perirenal fat pad masses (Table S1) as well as elevated circulating leptin levels (e.g. males $AgRP^{Cre}/LepR^{fl/fl}$ mice: 13.2 ± 7.8 ng/ml vs controls 4.5 ± 2.8 ng/ml, $P < 0.05$). Furthermore, relative to littermate controls, $POMC^{Cre}/LepR^{fl/fl}$ mice displayed an exaggerated weight gain and adiposity when fed high fat diet (Figure S2A, B and Table S1).

3.2. LepR ablation from POMC and AgRP neurons interferes with leptin control of SNA subserving BAT and WAT

We began our assessment of the contribution of POMC neurons and AgRP neurons to the sympathetic effects of leptin by examining the consequence of deleting the LepR in POMC or AgRP neurons on the BAT sympathoexcitatory effects of leptin. While control mice exhibited a robust increase in BAT SNA in response to ICV leptin (2 μg), this effect was partially blunted in both $POMC^{Cre}/LepR^{fl/fl}$ mice and $AgRP^{Cre}/LepR^{fl/fl}$ mice (Figure 2A and B). The partial attenuation of the BAT sympathoexcitatory effects of leptin in mice lacking the LepR in POMC or AgRP neurons is consistent with the mild obesity displayed by these animals.

Interestingly, whereas POMC and AgRP neurons were both involved in mediating leptin-evoked sympathetic outflow to BAT, leptin-induced sympathetic activation to the inguinal WAT was completely ablated in $AgRP^{Cre}/LepR^{fl/fl}$ mice but remained normal in $POMC^{Cre}/LepR^{fl/fl}$ mice (Figure 2C and D). Of note, ICV vehicle treatment, which was used as control, did not change SNA to BAT, WAT, or to any other tissue tested in our studies.

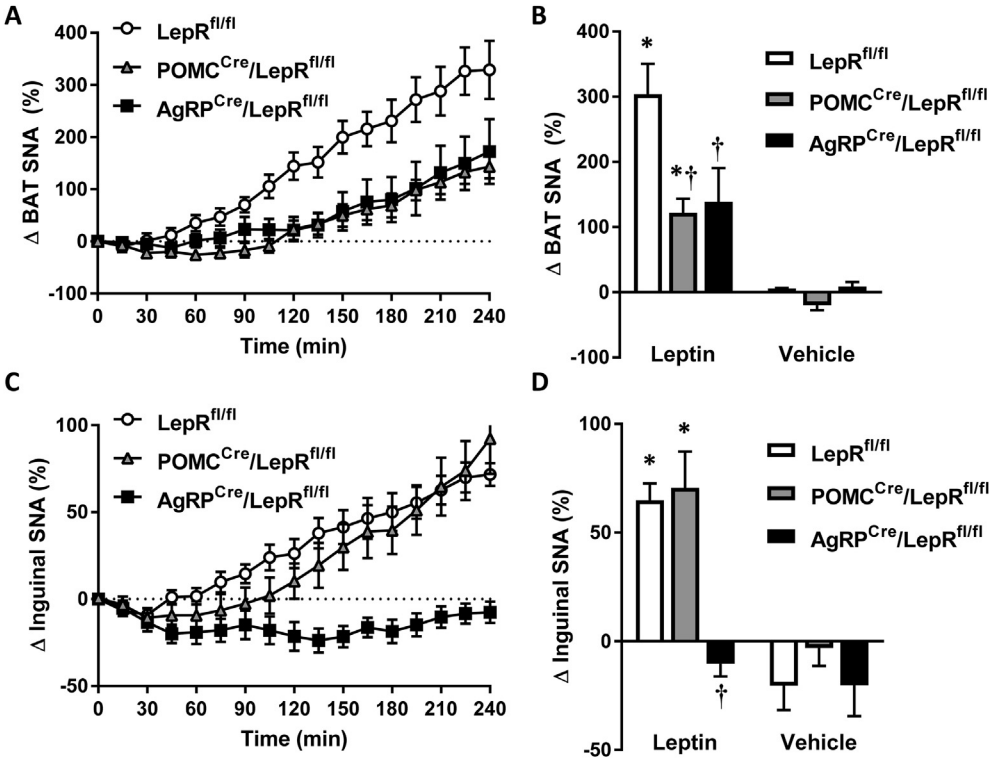

Figure 2: Contrasting effects of LepR ablation in POMC and AgRP neurons on leptin control of BAT and WAT SNA. A—B) Leptin-induced increase in BAT SNA is partially attenuated in both POMC^{Cre}/LepR$^{fl/fl}$ and AgRP^{Cre}/LepR$^{fl/fl}$ mice (n = 11 leptin treated LepR$^{fl/fl}$ mice; n = 9 leptin treated POMC^{Cre}/LepR$^{fl/fl}$ mice; n = 9 leptin treated AgRP^{Cre}/LepR$^{fl/fl}$ mice; n = 9 vehicle treated LepR$^{fl/fl}$ mice; n = 7 vehicle treated POMC^{Cre}/LepR$^{fl/fl}$ mice; n = 5 vehicle treated AgRP^{Cre}/LepR$^{fl/fl}$ mice). **C—D)** Sympathetic nerve activation to inguinal WAT elicited by ICV leptin is impaired in AgRP^{Cre}/LepR$^{fl/fl}$ mice, but not POMC^{Cre}/LepR$^{fl/fl}$ mice (n = 15 leptin treated LepR$^{fl/fl}$ mice; n = 13 leptin treated POMC^{Cre}/LepR$^{fl/fl}$ mice; n = 17 leptin treated AgRP^{Cre}/LepR$^{fl/fl}$ mice; n = 5 vehicle treated LepR$^{fl/fl}$ mice; n = 6 vehicle treated POMC^{Cre}/LepR$^{fl/fl}$ mice; n = 6 vehicle treated AgRP^{Cre}/LepR$^{fl/fl}$ mice). Bar graphs represent the averages of the last hour of SNA recordings. Comparisons were made using 2-way ANOVA with or without repeated measures, *p < 0.05 vs genotype matched vehicle, †p < 0.05 vs leptin-LepR$^{fl/fl}$ controls.

3.3. Leptin's effect on hepatic autonomic activity is differentially altered by LepR deletion from POMC and AgRP neurons

We next assessed the effects of deleting the LepR in POMC or AgRP neurons on the control of hepatic autonomic nerve activity by leptin. In line with our previous report [8], in control mice ICV leptin increased both SNA and PSNA subserving the liver. The hepatic SNA response to leptin was not significantly different in POMC^{Cre}/LepR$^{fl/fl}$ mice relative to controls, but there was a trend towards a reduced response. On the other hand, AgRP^{Cre}/LepR$^{fl/fl}$ mice displayed a significantly attenuated increase in hepatic SNA in response to ICV leptin (Figure 3A and B), indicating that leptin evoked hepatic sympathetic nerve activation is in part dependent on neuronal populations other than those expressing POMC and AgRP. Interestingly, the hepatic PSNA response to ICV leptin was completely abolished in both POMC^{Cre}/LepR$^{fl/fl}$ and AgRP^{Cre}/LepR$^{fl/fl}$ mice (Figure 3C and D), suggesting a co-requirement of leptin action on multiple neuronal populations to elicit the hepatic parasympathetic response.

3.4. LepR in POMC neurons is necessary for the lumbar, splanchnic, and renal but not adrenal, sympathetic effects of leptin

In order to more broadly assess the importance of POMC neurons and AgRP neurons in mediating the regional sympathoexcitatory effects of leptin, we investigated the lumbar, splanchnic, renal, and adrenal SNA responses evoked by leptin. ICV leptin increased lumbar and splanchnic (Figure 4A—D) as well as renal SNA (Figure 5A and B) in control mice, but not in POMC^{Cre}/LepR$^{fl/fl}$ mice. In contrast, the ICV leptin-induced increase in lumbar, splanchnic, and renal SNA was normal in AgRP^{Cre}/LepR$^{fl/fl}$ mice relative to controls. Of these, lumbar and renal SNA responses were completely ablated in POMC^{Cre}/LepR$^{fl/fl}$ mice while these mice retained a minor but greatly attenuated splanchnic SNA response to leptin. This was mirrored by a trend towards an attenuation of the splanchnic SNA response to leptin in AgRP^{Cre}/LepR$^{fl/fl}$ mice accompanied by near-complete preservation of the lumbar and renal SNA responses. Interestingly, ICV leptin treatment increased adrenal SNA to a similar extent in POMC^{Cre}/LepR$^{fl/fl}$, AgRP^{Cre}/LepR$^{fl/fl}$ mice and littermate controls (Figure 4E and F). These data indicate that leptin control of adrenal SNA involve neuronal populations other than POMC and AgRP neurons. These findings further support the specificity of the disruption of the SNA responses after ablation of the LepR in POMC or AgRP neurons.

Similar to the ICV treatment, intravenous administration of leptin (120 μg) increased renal SNA in control and AgRP^{Cre}/LepR$^{fl/fl}$ mice, but not in POMC^{Cre}/LepR$^{fl/fl}$ mice (Figure 5C) confirming the importance of POMC neurons for the renal sympathoexcitatory effects of leptin. The non-responsiveness of mice lacking the LepR in POMC neurons appears specific to leptin as indicated by the comparable increase in renal SNA elicited by ICV insulin (100 μU) in POMC^{Cre}/LepR$^{fl/fl}$ mice relative to control littermates (Figure 5D). Thus, deletion of the LepR from POMC neurons does not result in an overall impairment of neuronal function.

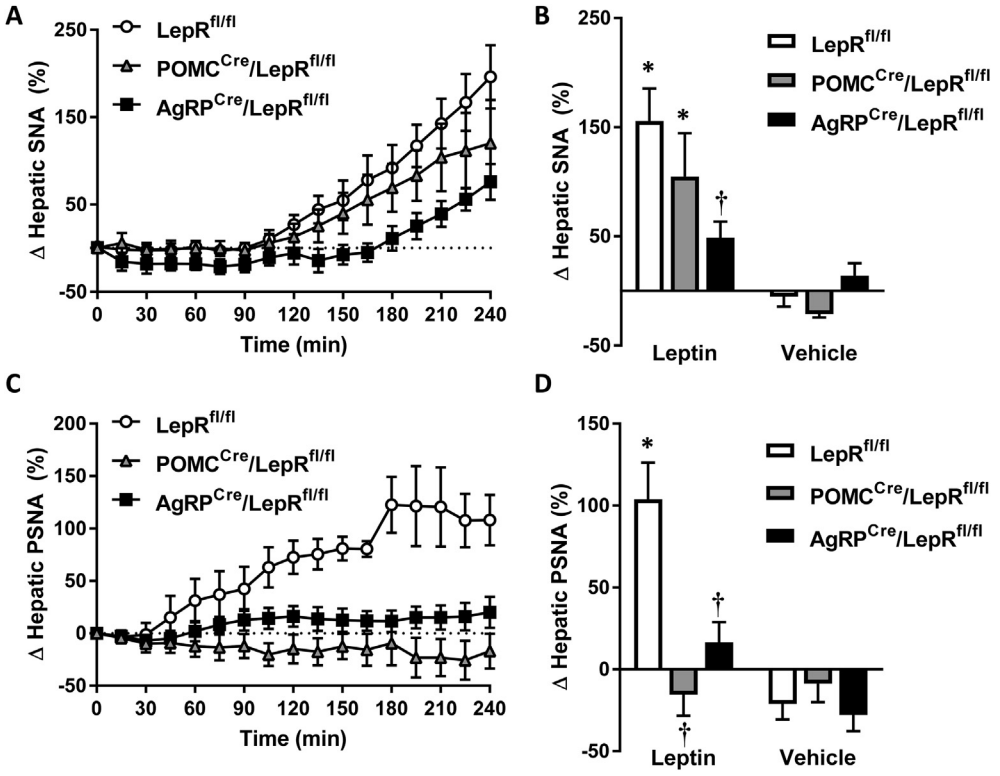

Figure 3: Loss of LepR in POMC and AgRP neurons affects differentially the effects of leptin on hepatic autonomic nerve activity. A—B) Leptin stimulation of hepatic SNA was partially blunted in $AgRP^{Cre}/LepR^{fl/fl}$ mice, but not $POMC^{Cre}/LepR^{fl/fl}$ mice (n = 12 leptin treated $LepR^{fl/fl}$ mice; n = 9 leptin treated $POMC^{Cre}/LepR^{fl/fl}$ mice; n = 6 leptin treated $AgRP^{Cre}/LepR^{fl/fl}$ mice; n = 3 vehicle treated $LepR^{fl/fl}$ mice; n = 3 vehicle treated $POMC^{Cre}/LepR^{fl/fl}$ mice; n = 9 vehicle treated $AgRP^{Cre}/LepR^{fl/fl}$ mice). **C—D)** The increase in hepatic PSNA evoked by leptin was abolished in both $POMC^{Cre}/LepR^{fl/fl}$ and $AgRP^{Cre}/LepR^{fl/fl}$ mice (n = 15 leptin treated $LepR^{fl/fl}$ mice; n = 13 leptin treated $POMC^{Cre}/LepR^{fl/fl}$ mice; n = 17 leptin treated $AgRP^{Cre}/LepR^{fl/fl}$ mice; n = 5 vehicle treated $LepR^{fl/fl}$ mice; n = 6 vehicle treated $POMC^{Cre}/LepR^{fl/fl}$ mice; n = 6 vehicle treated $AgRP^{Cre}/LepR^{fl/fl}$ mice). Bar graphs represent the averages of the last hour of SNA and PNSA recordings. Comparisons were made using 2-way ANOVA with or without repeated measures, *$p < 0.05$ vs genotype matched vehicle, †$p < 0.05$ vs leptin-$LepR^{fl/fl}$ controls.

3.5. Predominant role of the PI3K p110α over p110β in POMC neurons in leptin control of renal sympathetic nerve traffic

To further assess the importance of POMC neurons in mediating the sympathetic effects of leptin, we tested the consequence of disrupting in POMC neurons of a key LepR signaling pathway, PI3K signaling. Thus, we generated mice lacking either the catalytic p110α or p110β subunit of PI3K specifically in POMC neurons ($POMC^{Cre}/p110\alpha^{fl/fl}$ and $POMC^{Cre}/p110\beta^{fl/fl}$). Body weight of $POMC^{Cre}/p110\alpha^{fl/fl}$ mice and $POMC^{Cre}/p110\beta^{fl/fl}$ mice was not different from that of wild type littermates when the mice were fed either normal chow or high fat diet (Figure 6A and B).

Previous studies have implicated PI3K signaling in underlying the sympathoexcitatory effects of leptin to the kidney but not to BAT, hind limb, or adrenal gland [22,32]. This evidence combined with the findings above implicating POMC neurons in mediating leptin control of renal SNA led us to focus our attention on the effects of deleting the PI3K subunits in POMC neurons on leptin-induced renal sympathetic activation. Interestingly, the renal SNA response to ICV leptin was substantially reduced in $POMC^{Cre}$ mice/$p110\alpha^{fl/fl}$ mice (43 ± 32%) relative to controls (284 ± 50%, $p < 0.05$). In contrast, deletion of p110β in POMC neurons did not alter the ICV leptin-induced increase in renal SNA (279 ± 36%) indicating that specifically the p110α subunit of PI3K in POMC neurons is necessary for leptin to increase renal SNA. To ensure that anesthesia did not contribute to the blunted renal SNA response to leptin in $POMC^{Cre}/p110\alpha^{fl/fl}$ mice, we measured the renal sympathetic effects of leptin in the conscious state. Consistent with the data obtained in the anesthetized state, we found that the leptin-evoked increase in renal SNA was blunted in conscious $POMC^{Cre}/p110\alpha^{fl/fl}$ mice, but not in $POMC^{Cre}/p110\beta^{fl/fl}$ mice (Figure 6C and D). Together, these findings point to the importance of leptin signaling in POMC neurons, and specifically through p110α, to control sympathetic traffic to the kidney.

3.6. POMC and AgRP neurons innervate the kidneys

The evidence above implicating POMC neurons in leptin control of renal SNA prompted us to investigate the anatomical links between these neurons and the kidneys using pseudorabies tracing experiments. Consistent with previous reports [33,34], injection of GFP-expressing pseudorabies virus (PRV-152) into the kidneys labels the ARC with the earliest infection in the ARC appearing 4—5 days after PRV-152 inoculation (Figure S3A and B). GFP staining in the ARC became maximal at the latest time point examined (8 days after inoculation, Figure S3C). As expected, renal denervation abolished PRV-152-induced GFP labeling in the ARC (Figure S3D and E) confirming that PRV-152 transport to the ARC is mediated by the renal nerves.

Importantly, we found that POMC neurons can be labeled from the kidneys (Figure 7A). Of note, AgRP neurons were also labeled after PRV-152 injection into the kidneys demonstrating that these neurons also project to the kidneys (Figure 7B). The relatively small number of POMC and AgRP neurons labeled from the kidneys is consistent with the low transfection of the ARC at this time point (Figure S3B).

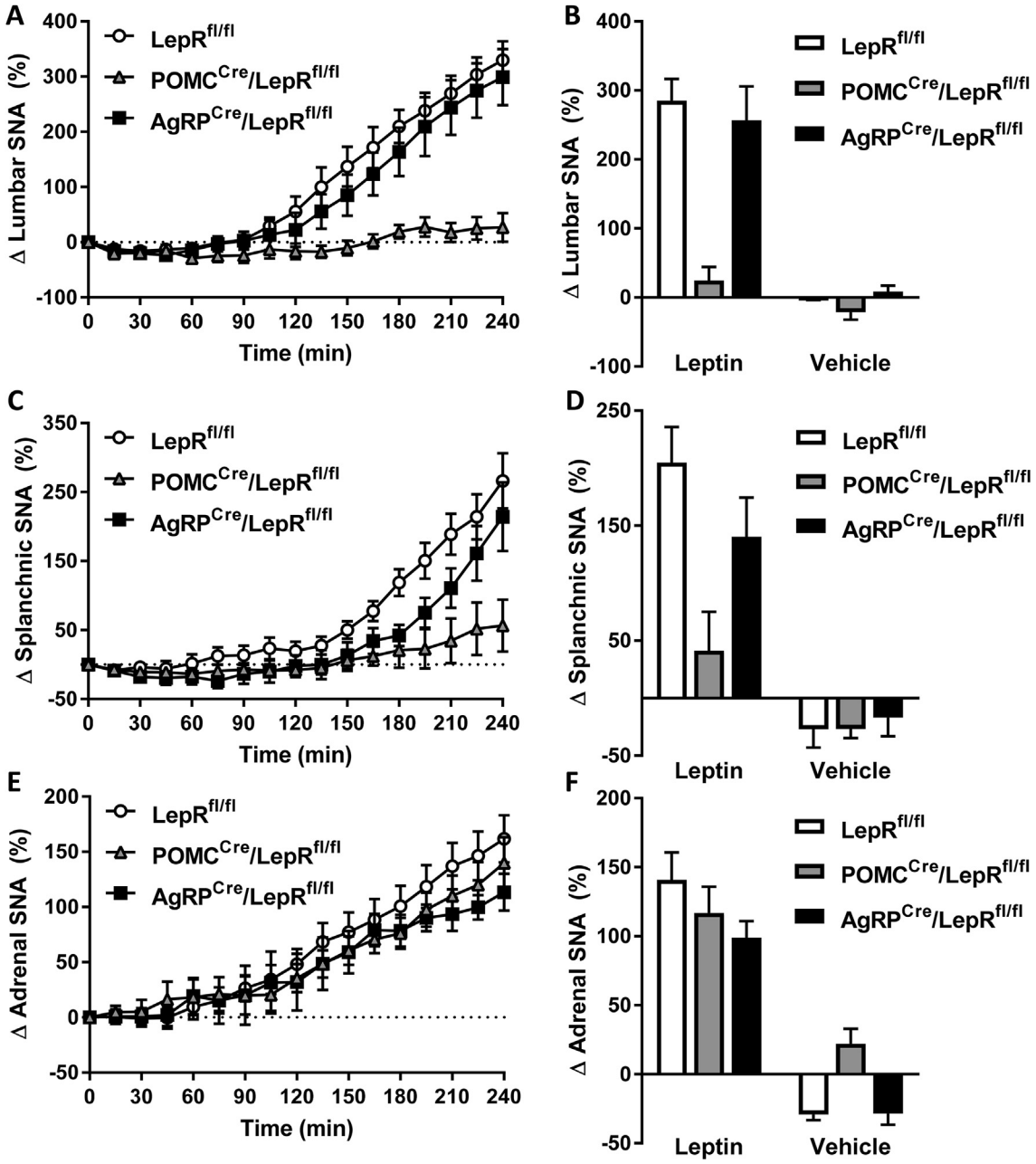

Figure 4: The LepR on POMC, but not AgRP, neurons is required for leptin activation of lumbar and splanchnic SNA, but not adrenal SNA. A–D) ICV leptin (2 μg) increased lumbar (A-B, n = 13 leptin treated $LepR^{fl/fl}$ mice; n = 8 leptin treated $POMC^{Cre}/LepR^{fl/fl}$ mice; n = 8 leptin treated $AgRP^{Cre}/LepR^{fl/fl}$ mice; n = 5 vehicle treated $LepR^{fl/fl}$ mice; n = 6 vehicle treated $POMC^{Cre}/LepR^{fl/fl}$ mice; n = 5 vehicle treated $AgRP^{Cre}/LepR^{fl/fl}$ mice), and splanchnic (C-D, n = 12 leptin treated $LepR^{fl/fl}$ mice; n = 9 leptin treated $POMC^{Cre}/LepR^{fl/fl}$ mice; n = 6 leptin treated $AgRP^{Cre}/LepR^{fl/fl}$ mice; n = 3 vehicle treated $LepR^{fl/fl}$ mice; n = 4 vehicle treated $POMC^{Cre}/LepR^{fl/fl}$ mice; n = 5 vehicle treated $AgRP^{Cre}/LepR^{fl/fl}$ mice) SNA in control and $AgRP^{Cre}/LepR^{fl/fl}$ mice; but not in $POMC^{Cre}/LepR^{fl/fl}$ mice. **E–F)** The leptin-evoked increase in SNA subserving the adrenal gland was not affected in $POMC^{Cre}/LepR^{fl/fl}$ and $AgRP^{Cre}/LepR^{fl/fl}$ mice (n = 14 leptin treated $LepR^{fl/fl}$ mice; n = 9 leptin treated $POMC^{Cre}/LepR^{fl/fl}$ mice; n = 7 leptin treated $AgRP^{Cre}/LepR^{fl/fl}$ mice; n = 3 vehicle treated $LepR^{fl/fl}$ mice; n = 3 vehicle treated $POMC^{Cre}/LepR^{fl/fl}$ mice; n = 3 vehicle treated $AgRP^{Cre}/LepR^{fl/fl}$ mice). Bar graphs represent the averages of the last hour of SNA recordings. Comparisons were made using 2-way ANOVA with repeated or without measures, *$p < 0.05$ vs genotype matched vehicle, †$p < 0.05$ vs leptin-$LepR^{fl/fl}$ controls.

4. DISCUSSION

Our data demonstrate an important functional distinction between POMC and AgRP neurons in leptin control of regional autonomic nerve activity, supporting the idea that these neuronal populations mediate divergent aspects of leptin actions. We found that: 1) the leptin-evoked increase in BAT SNA was partially blunted in mice lacking the LepR in either POMC or AgRP neurons; 2) LepR on AgRP, but not POMC, neurons is required for leptin control of SNA subserving the inguinal WAT and liver; 3) hepatic PSNA response to leptin requires both POMC and AgRP neurons; 4) POMC, but not AgRP, neurons mediate the lumbar, splanchnic, and renal sympathetic nerve activation evoked by leptin; 5) the ability of leptin to increase sympathetic traffic to the adrenal gland is independent of POMC and AgRP neurons; and 6) the p110α, but not p110β, subunit of PI3K in POMC neurons is required for the effect of leptin on renal SNA. Together these findings demonstrate

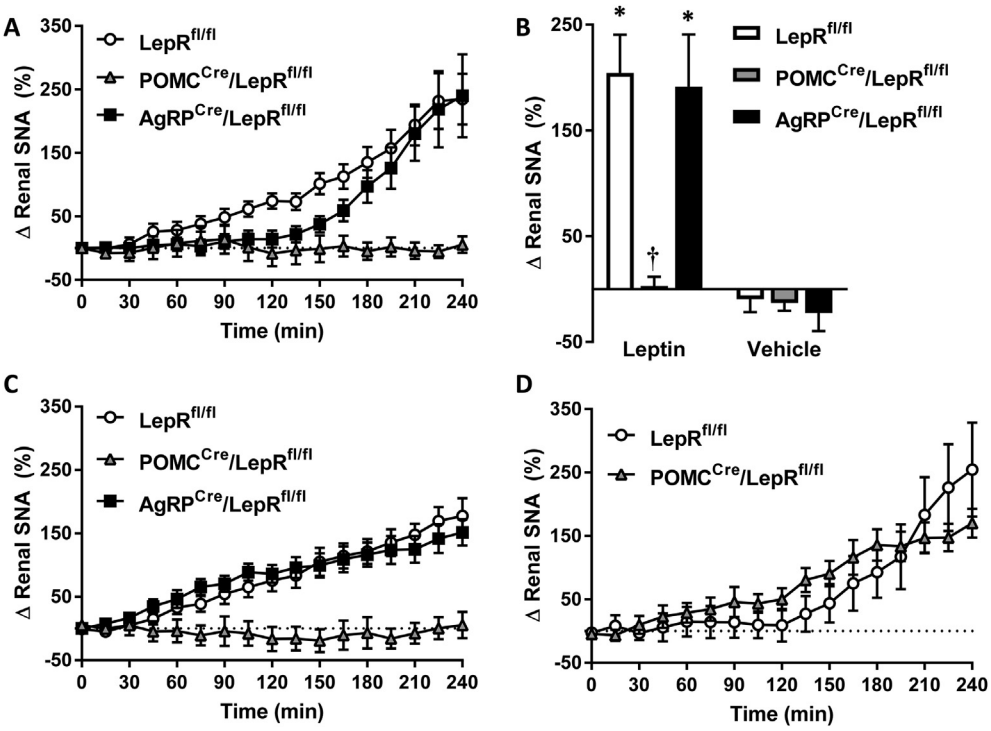

Figure 5: LepR ablation from POMC, but not AgRP, neurons effects selectively leptin control of renal SNA. **A–C)** Leptin administered ICV (2 μg, A-B, n = 13 leptin treated $LepR^{fl/fl}$ mice; n = 8 leptin treated $POMC^{Cre}/LepR^{fl/fl}$ mice; n = 8 leptin treated $AgRP^{Cre}/LepR^{fl/fl}$ mice; n = 5 vehicle treated $LepR^{fl/fl}$ mice; n = 6 vehicle treated $POMC^{Cre}/LepR^{fl/fl}$ mice; n = 5 vehicle treated $AgRP^{Cre}/LepR^{fl/fl}$ mice) or intravenously (120 μg, C, n = 16 $LepR^{fl/fl}$ mice; n = 8 $POMC^{Cre}/LepR^{fl/fl}$ mice; n = 8 $AgRP^{Cre}/LepR^{fl/fl}$ mice) increased renal SNA in control ($LepR^{fl/fl}$) and $AgRP^{Cre}/LepR^{fl/fl}$ mice, but not in $POMC^{Cre}/LepR^{fl/fl}$ mice. **D)** $POMC^{Cre}/LepR^{fl/fl}$ mice displayed a normal increase in renal SNA in response to ICV insulin (n = 6 $LepR^{fl/fl}$ mice; n = 8 $POMC^{Cre}/LepR^{fl/fl}$ mice). The bar graph represents the averages of the last hour of SNA recordings. Comparisons were made using 2-way ANOVA with or without repeated measures, *$p < 0.05$ vs genotype matched vehicle, †$p < 0.05$ vs leptin-$LepR^{fl/fl}$ controls.

that POMC and AgRP neurons are differentially involved in mediating the regional autonomic effects of leptin.

We previously demonstrated the importance of the ARC in mediating the BAT sympathoexcitatory effect of leptin [15,18]. This was indicated by the increased BAT SNA when leptin was administered directly into the ARC [15] whereas ARC-specific LepR deletion abolished the BAT sympathetic response evoked by leptin [18]. Here, we show that both POMC and AgRP neurons contribute to leptin-induced BAT sympathetic nerve activation although neither is fully required. The partial loss of BAT SNA response to leptin is consistent with the mild obesity phenotype observed in mice lacking the LepR in POMC or AgRP neurons (Figure 1 and [30,31]). These findings are in line with the hypothesis that the net ability of leptin to increase BAT sympathetic outflow and regulate body weight is the result of an additive effect separately originating from each neuronal population, such that the contributions of POMC and AgRP neurons to BAT SNA are not redundant but rather are functionally independent. This is supported by the demonstration that mice lacking the LepR in both POMC and AgRP neurons present with a more severe obesity phenotype relative to mice bearing LepR deletion from POMC and AgRP neurons individually [31]. It will be interesting to determine how deletion of the LepR from both POMC and AgRP neurons affects the BAT SNA response to leptin.

In contrast to the partial contributions of POMC and AgRP neurons in mediating leptin's effect on BAT SNA, the ability of leptin to increase sympathetic outflow to the inguinal WAT required LepR on AgRP, but not POMC, neurons. Leptin has been involved in the regulation of lipolytic processes in the inguinal WAT through the sympathetic nerves [4–7]. Of note, sympathetic transmission has also been implicated as an important mechanism underlying the development of beige adipocytes in the inguinal WAT depot [35]. For instance, "beiging" of the inguinal fat pad as a metabolic adaptation during cold exposure requires β3-adrenoreceptors [36]. Further studies into a potential role of leptin in this process are warranted to determine a potential contribution for AgRP neurons in the control of metabolic function via regulation of inguinal WAT SNA, in particular in the context of obesity and during cold exposure.

The autonomic nervous system plays a key role in the control of various hepatic functions including lipid metabolism and glucose production [8,37,38]. Moreover, leptin action in the ARC has been implicated in the regulation of liver functions [39]. For instance, virally-induced expression of the LepR in the ARC of LepR deficient animals improves peripheral insulin sensitivity via enhanced suppression of glucose production [9]. We previously demonstrated that leptin action in the ARC increases both SNA and PSNA subserving the liver [8]. Here, we extend these findings by demonstrating that POMC and AgRP neurons play distinct roles in mediating the effect of leptin on hepatic SNA as well as PSNA. In keeping with the notion that leptin's metabolic actions are for the most part distributed across distinct neuronal populations, the hepatic sympathetic response to leptin was partially dependent on AgRP neurons. However, this response occurred in a manner completely independent from POMC neurons. Thus, a significant portion of the leptin-induced increase in hepatic SNA is unaccounted for by POMC or AgRP neurons.

Strikingly, the hepatic PSNA response to leptin required LepR on both POMC and AgRP neurons. This phenotype was unique among the nerves tested in this study, as it was the only autonomic response

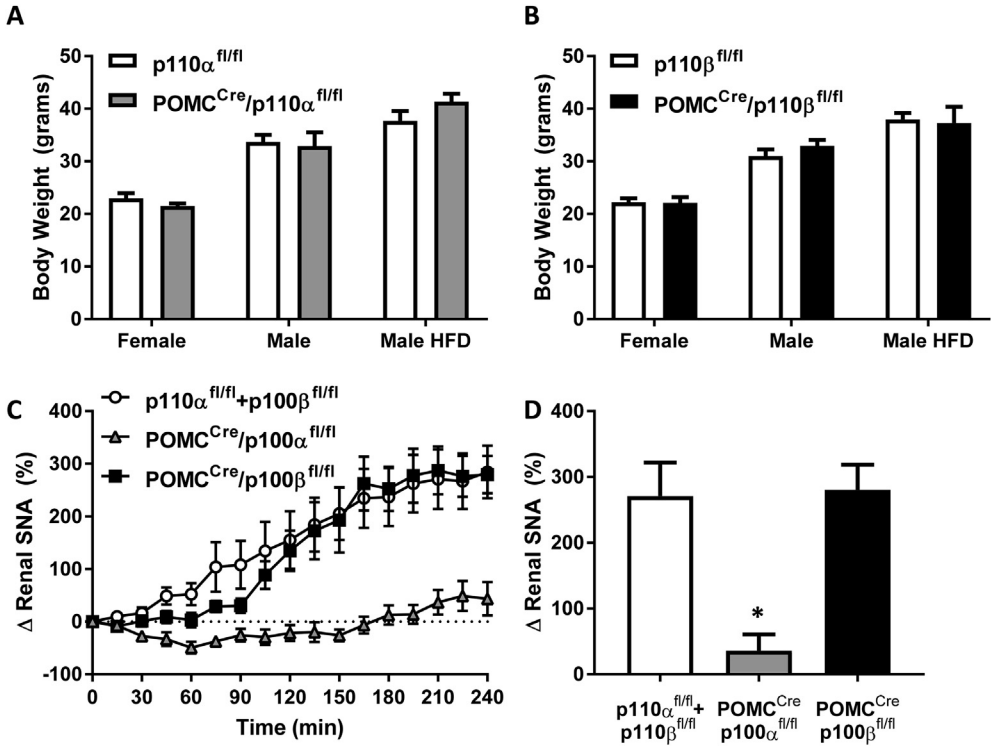

Figure 6: Effects of POMC neurons-selective deletion of p110α or p110β catalytic subunits of PI3K. A–B) Deletion of the p110α (A) or p110β (B) had no effect on body weight in male or female mice fed normal chow or male mice fed a high fat diet (HFD). **C–D)** Leptin-induced increase in renal SNA, measured in the conscious state, was abolished in mice lacking the p110α, but not p110β, PI3K in POMC neurons (n = 9 p110α$^{fl/fl}$ + p110 β$^{fl/fl}$ mice; n = 6 POMC^{Cre}/p110α$^{fl/fl}$ mice; n = 6 p110 β$^{fl/fl}$ mice). The bar graph represents the averages of the last hour of SNA recordings. Comparisons were made using *t*-test, 1- or 2-way ANOVA with or without repeated measures, *p < 0.05 vs p110α$^{fl/fl}$ + p110 β$^{fl/fl}$ mice.

evoked by leptin that could be completely ablated by loss of the LepR from either POMC or AgRP neurons. Thus, our data indicate that leptin promotes sympathetic and parasympathetic activation to the liver through distinct neuronal populations, providing novel insights into the central mechanisms underlying leptin-mediated regulation of hepatic functions.

Interestingly, we observed a consistent requirement for leptin action on POMC, but not AgRP, neurons to increase lumbar, splanchnic, and renal sympathetic outflow. SNA to these beds is essential for the regulation of various physiological processes including blood pressure. The importance of POMC neurons in mediating the renal sympathetic activation to leptin is further confirmed by our demonstration that POMC neurons innervate the kidneys and the inability of leptin to increase renal SNA in mice lacking the p110α catalytic subunit of PI3K in these neurons. Of note, leptin failed to increase blood pressure in mice carrying POMC neurons-specific ablation of the LepR [40] or Insulin Receptor Substrate 2, a key mediator of PI3K activation by the LepR [41]. Renal SNA has also emerged as a key contributor to glucose homeostasis through the control of glucose excretion in the urine. For instance, reduced renal SNA appears to account for the elevated glycosuria and subsequent improved glucose tolerance in mice bearing hypothalamic-restricted loss of the *pomc* gene [42]. However, the contribution of LepR on POMC neurons to the regulation of urinary glucose excretion is not known, but is worth investigating. The relevance of the kidney-projecting AgRP neurons that we identified to the control of renal SNA by stimuli other than leptin also merits examination.

LepR deletion from POMC or AgRP neurons did not interfere with the ability of leptin to increase sympathetic activity of nerves subserving the adrenal gland suggesting the involvement of other neuronal populations. Consistent with this possibility, we recently implicated neurons in the parabrachial nucleus in the control of adrenal SNA [43]. Moreover, we showed that LepR ablation from neurons in the parabrachial nucleus augments glucose mobilization in response to noxious stimuli [43]. It will be interesting to directly examine the requirement of the LepR in neurons within the parabrachial nucleus for leptin-induced sympathetic nerve activation to the adrenal gland.

The involvement of POMC and AgRP neurons in the control of autonomic nerve activity subserving various beds is consistent with the pleiotropic actions of these neurons. The divergent signaling capacities of POMC and AgRP neurons afforded by the presence of multiple neurotransmitters [13] may underlie the regional SNA and PSNA responses evoked by leptin action in these neurons. In support of such possibility leptin control of lumbar SNA involve both MC3/4R and glutamate receptors in the second order neurons [11] whereas the renal sympathoexcitatory effect of leptin is mediated by the MC4R [44]. Neuroanatomical studies have revealed the variety of axonal projections emanating from POMC and AgRP neurons, which innervate various brain nuclei [45,46]. These observations combined with the heterogenicity and functional diversity of POMC and AgRP neurons [21,47,48] raise the possibility that different subsets of these neurons may control specific subcircuits to influence autonomic nerve activity to distinct tissues.

In conclusion, our data show that POMC and AgRP neurons contribute to leptin control of regional activity of the autonomic nervous system. These findings support the notion that leptin engages different

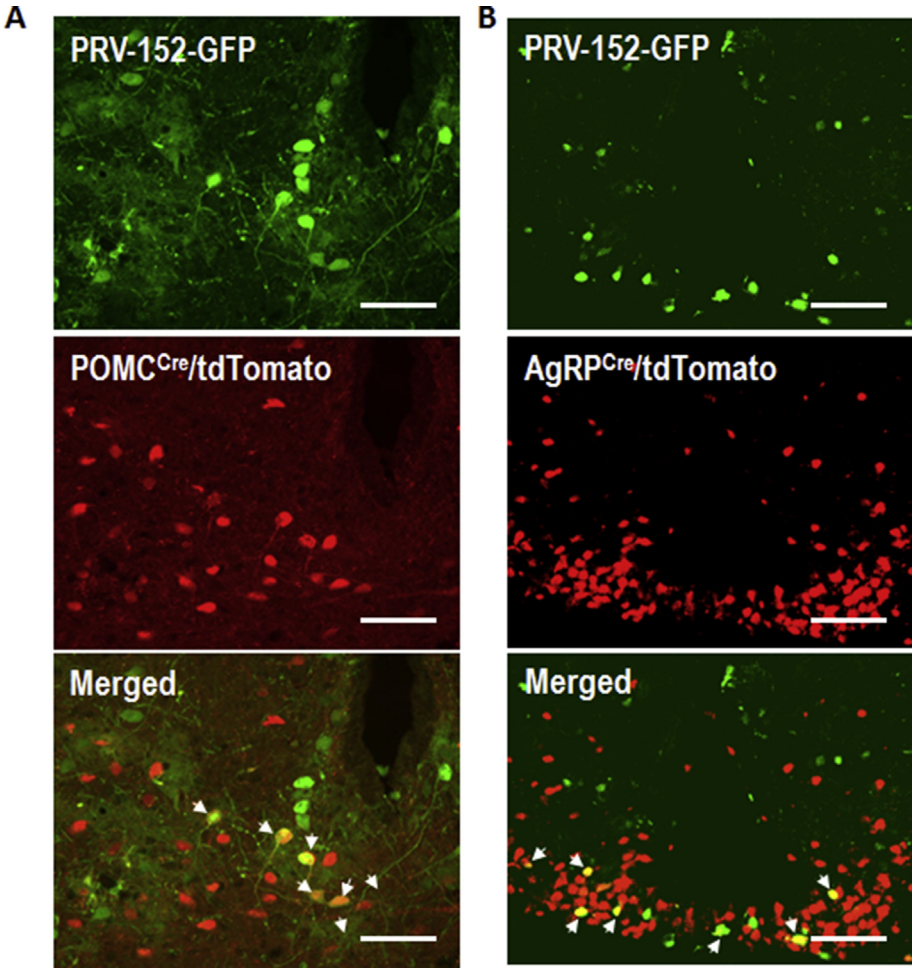

Figure 7: Trans-synaptic retrograde tracing of POMC and AgRP neurons from the kidneys. A–B) GFP-expressing PRV-152 pseudorabies virus injected into the left and right kidneys trans-synaptically labels, after 5 days, the ARC POMC (A) and AgRP neurons (B) in POMC^{Cre}/tdTomato mice and AgRP^{Cre}/tdTomato mice, respectively. Images acquired at 20× magnification. Scale bar, 100 μm.

neuronal populations in a selective manner to control the activity of the sympathetic and parasympathetic nervous systems.

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CONFLICT OF INTEREST

The authors have no conflict of interest relevant to this study.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.molmet.2017.12.006.

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