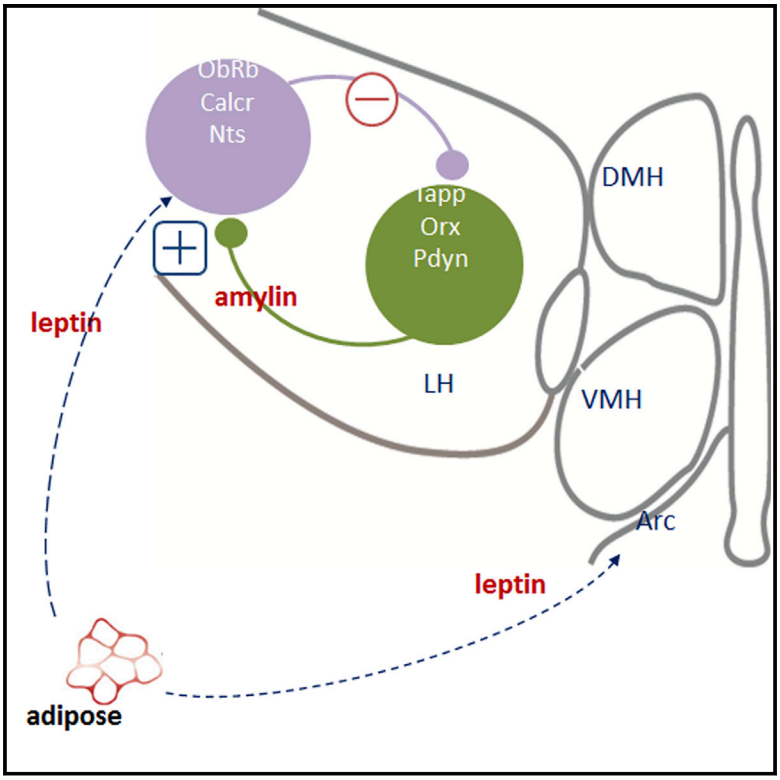
Short Article

Cell Metabolism

Hypothalamic Amylin Acts in Concert with Leptin to Regulate Food Intake

Graphical Abstract



Authors

Zhiying Li, Leah Kelly, Ibrahim Gergi, Patrik Vieweg, Myriam Heiman, Paul Greengard, Jeffrey Michael Friedman

Correspondence

friedj@rockefeller.edu

In Brief

Li et al. show that islet amyloid polypeptide (Iapp, precursor to amylin), which is cosecreted with insulin, is expressed in hypothalamic neurons and synergizes with leptin to regulate feeding. These findings provide a potential mechanism for the increased efficacy of amylin and leptin combination therapy for obesity.

Highlights

- Profiling of *Pdyn* neurons reveals hypothalamic neurons expressing *Iapp*
- Leptin regulates hypothalamic *Iapp* expression
- Amylin and leptin exerts similar electrophysiologic effects on LH *ObRb* neurons
- Amylin antagonist (i.c.v.) blunts leptin's anorexic effect in live mice acutely

Li et al., 2015, Cell Metabolism *22*, 1059–1067
December 1, 2015 ©2015 Elsevier Inc.
http://dx.doi.org/10.1016/j.cmet.2015.10.012

Cell Metabolism
Short Article

Hypothalamic Amylin Acts in Concert with Leptin to Regulate Food Intake

Zhiying Li,[1] Leah Kelly,[1] Ibrahim Gergi,[2] Patrik Vieweg,[2] Myriam Heiman,[3] Paul Greengard,[4] and Jeffrey Michael Friedman[1,5,*]
[1]Laboratory of Molecular Genetics, The Rockefeller University, New York, NY 10065, USA
[2]Linköping University, Linköping 581 83, Sweden
[3]Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02138, USA
[4]Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, NY 10065, USA
[5]Howard Hughes Medical Institute, 4000 Jones Bridge Road, Chevy Chase, MD 20815-6789, USA
*Correspondence: friedj@rockefeller.edu
http://dx.doi.org/10.1016/j.cmet.2015.10.012

SUMMARY

In this report we evaluated the functions of hypothalamic amylin in vivo and in vitro. Profiling of hypothalamic neurons revealed that islet amyloid polypeptide (*Iapp*, precursor to amylin) is expressed in neurons in the lateral hypothalamus, arcuate nucleus, medial preoptic area, and elsewhere. Hypothalamic expression of *Iapp* is markedly decreased in *ob/ob* mice and normalized by exogenous leptin. In slices, amylin and leptin had similar electrophysiologic effects on lateral hypothalamic leptin receptor *ObRb*-expressing neurons, while the amylin antagonist AC187 inhibited their activity and blunted the effect of leptin. Finally, i.c.v. infusion of AC187 acutely reduced the anorectic effects of leptin. These data show that hypothalamic amylin is transcriptionally regulated by leptin, that it can act directly on *ObRb* neurons in concert with leptin, and that it regulates feeding. These findings provide a potential mechanism for the increased efficacy of a metreleptin/pramlintide combination therapy for obesity.

INTRODUCTION

Prodynorphin (*Pdyn*), precursor of the dynorphin family of the opioid peptides, regulates diverse physiological processes including pain, stress and anxiety, learning and memory, alcohol consumption, hippocampal epilepsy, and withdrawal from narcotics (Wang et al., 2001; McLaughlin et al., 2003; Blednov et al., 2006; Loacker et al., 2007; Wittmann et al., 2009; Chefer and Shippenberg, 2006; Shin et al., 2009). Recently we found that *Pdyn* neurons in the dorsal medial hypothalamus (DMH) play a role in the feeding response to a scheduled feeding protocol that elicits food anticipatory activity (Knight et al., 2012), while deleting leptin receptor *ObRb* in *Pdyn* neurons led to increased body weight gain upon high-fat diet (HFD) treatment (Allison et al., 2015). We thus set out to study the role of *Pdyn* neurons in feeding by profiling their pattern of expression using TRAP technology. In the course of these studies, we found that a subset of lateral hypothalamic (LH) *Pdyn* neurons expresses Islet amyloid polypeptide (*Iapp*), also known as Amylin, a peptide previously shown to be expressed in pancreatic β cells. While some studies have suggested that *Iapp* is expressed in the CNS, others do not (Leffert et al., 1989; Ferrier et al., 1989). Amylin is approved for the treatment of diabetes. Importantly, amylin has been reported to synergize with leptin to reduce body weight in obese animals and humans (Roth et al., 2008), though the mechanism is not fully understood. We thus set out to confirm the expression of amylin in the CNS and evaluate its effects on neural circuits that control feeding.

RESULTS

Identification of Hypothalamic *Iapp* Expression

We profiled hypothalamic *Pdyn* neurons by TRAP technology using *Pdyn-eGFP-L10* transgenic mice (Heiman et al., 2008). We first confirmed that there was eutopic expression of GFP in *Pdyn-eGFP-RPL10a* BAC transgenic mice by immunohistochemistry (IHC; see Figure S1 available online). We found that *Pdyn* is expressed in hypothalamic paraventricular nucleus (PVN), LH, DMH, ventral medial hypothalamus (VMH), and Arc. The expression of GFP overlaps extensively with the published fluorescence in situ hybridization data for *Pdyn* (Allen Brain Atlas, mouse.brain-map.org) and IHC for dynorphin A (*Dyn A*). Leptin i.p. led to phosphorylation of signal transducer and activator of transcription 3 (*STAT3*) in a subset of Arc *Pdyn* neurons (11.39% ± 0.91%, n = 2; Figure S1C), indicating *Pdyn* neurons respond to leptin.

Triplicates of paired wild-type hypothalamic IP and Input RNA samples were assayed for *Pdyn*-enriched transcripts. 13822 probesets out of 45101 were present in the *Pdyn* transcriptome (Figure S2A), among which 1,729 probe sets showed a fold change (FC) > = 2. These probe sets revealed 172 GO terms (Table S1). Among *Pdyn*-enriched transcripts, 11 were known neuropeptides (Figure S2A, red dots, and Figure S2B; Table S2), and nine were verified by IHC (Figure S2C): *Pdyn*, *Orx* in the LH, arginine vasopressin (*AVP*), oxytocin (*Oxt*), galanin (*Gal*) and corticotropin releasing hormone (*Crh*) in the PVN, and pro-opiomelanocortin-alpha (*POMC*) in the Arc. However, the highest enrichment was observed for *Iapp* (35, 55, 420, and 553 folds for 4 different probe sets detecting this transcript). *Iapp* is the precursor for amylin, a peptide coexpressed with insulin in pancreatic β cells, and previous analyses on brain *Iapp* expression have yielded conflicting

results. Thus its expression or function in the CNS remains unclear. Since our data suggested that in addition to its known expression in pancreas, *Iapp* is also expressed in hypothalamic *Pdyn* neurons (Figure S3), we set out to confirm its brain expression and evaluate its function.

Profiling hypothalamic *Pdyn* neurons in *ob/ob* mice further revealed 16 transcripts that were either up or downregulated (Figure S2D, red dots, and Figure S2E; Table S3), including *Iapp*, which showed a 43- to 1,128-fold decrease (Figure 2A). No significant difference was detected between WT Input and ob Input, suggesting this downregulation was specific for *Iapp* in *Pdyn* neurons (Figure S2B). These data suggested that hypothalamic amylin, possibly coexpressed in LH *Pdyn* neurons, could play a role to regulate feeding and leptin action.

Hypothalamic Distribution of *Iapp*-Expressing Neurons

IHC using an anti-amylin antibody revealed that *Iapp* was abundantly expressed in multiple hypothalamic nuclei including cell bodies in MPO, Arc, and LH and fibers in PVN, DMH (Figure 1A). We next analyzed the coexpression of *Iapp* with other hypothalamic peptides known to regulate feeding. Consistent with the aforementioned profiling data, *Iapp* and *Pdyn* were coexpressed only in the LH and MPO. Substantial overlap was also observed between *Iapp* and *Orx* in the LH, while colocalization with *MCH*, another peptide expressed in the LH, was not observed (Figures 1B and 1C), and ~30% of LH *Pdyn* neurons did not express either *Iapp* or *Orx*. Limited colocalization was observed in the Arc *ObRb* neurons (Figure 1D), with partial coexpression in agouti-related protein (*AgRP*) but not POMC neurons (Figure 1E). Finally, while *Iapp* was not expressed in LH ObRb neurons, amylin immunopositive fibers forming *vGat* immunopositive synapse-like structures onto LH *ObRb* neurons were observed (Figure 1F), suggesting direct innervation from *Iapp* neurons to *ObRb* cells. *ObRb* neurons coexpressing neurotensin in the LH have been shown to regulate metabolism and inhibit *Orx* neurons (Leinninger et al., 2011).

Sexually Dimorphic Expression of Hypothalamic *Iapp* in Wild-Type Mice and Its Regulation by Leptin and High-Fat Diet

We next assessed whether the level of *Iapp* gene expression was altered in obese mice. In our initial BAC TRAP analyses, the RNA level for hypothalamic *Iapp* was higher in wild-type female mice than males. This was confirmed using qPCR (Figure 2B). This finding is in line with previous data showing that MPO expression of *Iapp* is seen only in postpartum female rats but not in virgin female or male rats (Dobolyi, 2009). We thus analyzed RNA levels in obese mice of both sexes.

We found that hypothalamic *Iapp* mRNA was significantly reduced in female *ob/ob* versus female WT mice (Figure 2C). The levels of RNA in WT male mice were similar to those in *ob/ob* mice of both sexes. Thus, because the levels of hypothalamic *Iapp* in WT male mice are low, a significant effect of the ob mutation to further lower the levels was not observed. In contrast, plasma amylin, which reflects the levels of pancreatic amylin, was significantly higher in both female and male *ob/ob* versus WT mice (Figure 2E).

Treatment of *ob/ob* mice with recombinant leptin for 3 months led to significant increase of hypothalamic *Iapp* mRNA in female *ob/ob* mice ($p < 0.05$, Figure 2F) and a small mRNA increase in male *ob/ob* mice which was not significant. This response was not observed after treatment for 3 or 15 days (data not shown). We also found that hypothalamic *Iapp* levels were significantly increased in both female and male *ob/ob* mice carrying a leptin transgene that leads to constitutively high levels of this hormone (Ribeiro et al., 2011; Figure 2G).

The expression of hypothalamic *Iapp* was also sexually dimorphic in diet-induced obese (DIO) mice. Hypothalamic *Iapp* expression was decreased in female DIO mice fed a HFD for 26 weeks and paradoxically significantly increased in male DIO mice (Figure 2I). A HFD also significantly increased islet *Iapp* expression in female but not in male mice, but there were not significant differences in plasma amylin concentration in either gender (Figures 2J and 2K).

Effects of Leptin, Amylin, and AC187 on LH *ObRb* Neurons

We next tested the effect of amylin on LH *ObRb* neurons in slice preparations. Patch-clamp recordings showed *ObRb* neurons were depolarized at baseline (−57.95 mV ± 0.88, n = 41), with a relatively high firing rate (8.46 Hz ± 0.88, n = 33). Bath leptin (100 nM) excited 65% and inhibited 35% of *ObRb* neurons (Figures 3A–3C). Similarly, bath amylin (100 nM) excited 62% and inhibited 38% of *ObRb* neurons (Figures 3A–3C). The effects of leptin and amylin were significantly correlated for individual neurons exposed to both treatments (Figure 3D), indicating that amylin depolarizes the same neurons that are depolarized by leptin and hyperpolarizes neurons that are hyperpolarized by leptin. This suggests that, similar to their synergistic effects on food intake and body weight, leptin and amylin could have synergistic effects on these LH neurons.

The effects of leptin were next tested in the presence of the amylin antagonist AC187. AC187 (100 nM) significantly inhibited the effects of leptin on both leptin-depolarizing and hyperpolarizing neurons. In addition, AC187 alone also inhibited leptin-depolarized *ObRb* neurons and activated leptin-hyperpolarizing ObRb neurons (Figures 3E and 3F), suggesting that endogenous amylin tonically regulates the activity of these leptin regulated neurons. Voltage-clamp recordings confirmed that *ObRb* neurons receive spontaneous excitatory inputs (Figure 3G). However, bath leptin or amylin did not significantly change sEPSC amplitudes in these neurons (bottom right panels), suggesting that both leptin and amylin exert their effects postsynaptically. In aggregate, these data predict that inhibition of amylin action should blunt the response to leptin in vivo.

AC187 Antagonizes Leptin's Anorexic Effect in Mice

We measured food intake in ad lib fed wild-type mice treated with i.c.v. AC187 or PBS in the lateral ventricle prior to treatment with i.p. leptin or PBS. In the absence of AC187, leptin significantly suppressed food intake (Figure 4A). Pretreatment with AC187 significantly blunted the anorectic effect of i.p. leptin with intakes similar to i.p. PBS control mice. Consistent with the in vitro effect of AC187 alone to alter neural activity, AC187 also increased food intake in mice treated with i.p. PBS by ~1.5-fold during the first 2 hr (Figures 4A and 4B). In both cases, however, the effect of the antagonist was transient. This is likely a result of leptin action on other brain regions such as Arc and/or the clearance of AC187.

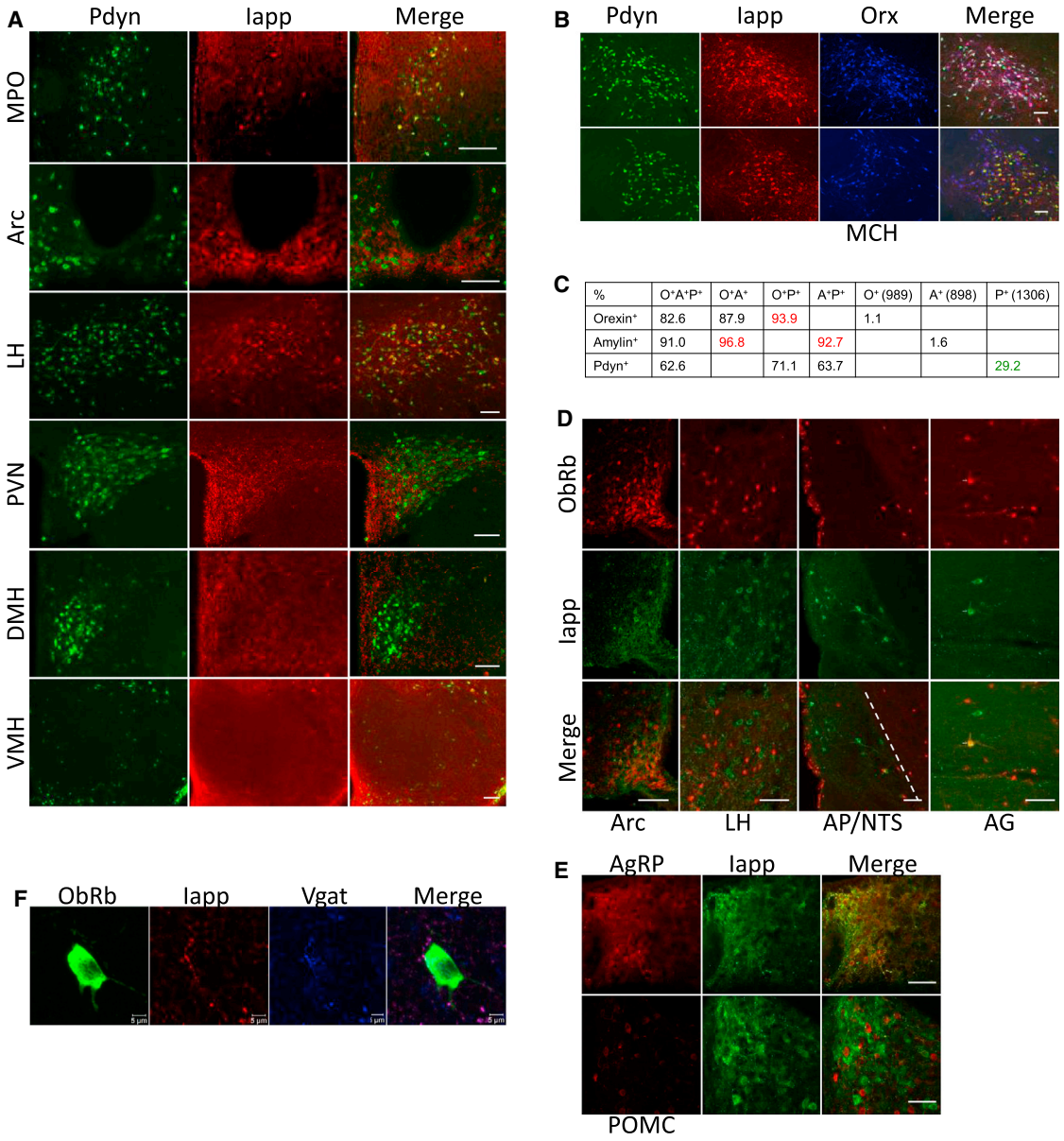


Figure 1. IHC Characterization of Hypothalamic *Iapp* Expression

(A) Hypothalamic slices from adult *Pdyn* BAC TRAP mice were stained for *GFP* (green) and *Iapp* (red). Scale bars, 100 μm.

(B) IHC for *GFP* (green), *Iapp* (red), or *Orx* or *MCH* (blue, top or lower row) in the LH of *Pdyn* BAC TRAP mice. Scale bar, 100 μm.

(C) Double and triple colocalization between *Orx*, *Iapp*, and *Pdyn* were counted from images in (B). A total of 91% *Iapp*-expressing (Amylin$^+$) neurons were triple-positive for *Orx*, amylin, and *Pdyn* ($O^+A^+P^+$), while 96.8% were double-positive for *Orx* and amylin (O^+A^+) and 92.7% were double-positive for *Pdyn* and amylin (A^+P^+).

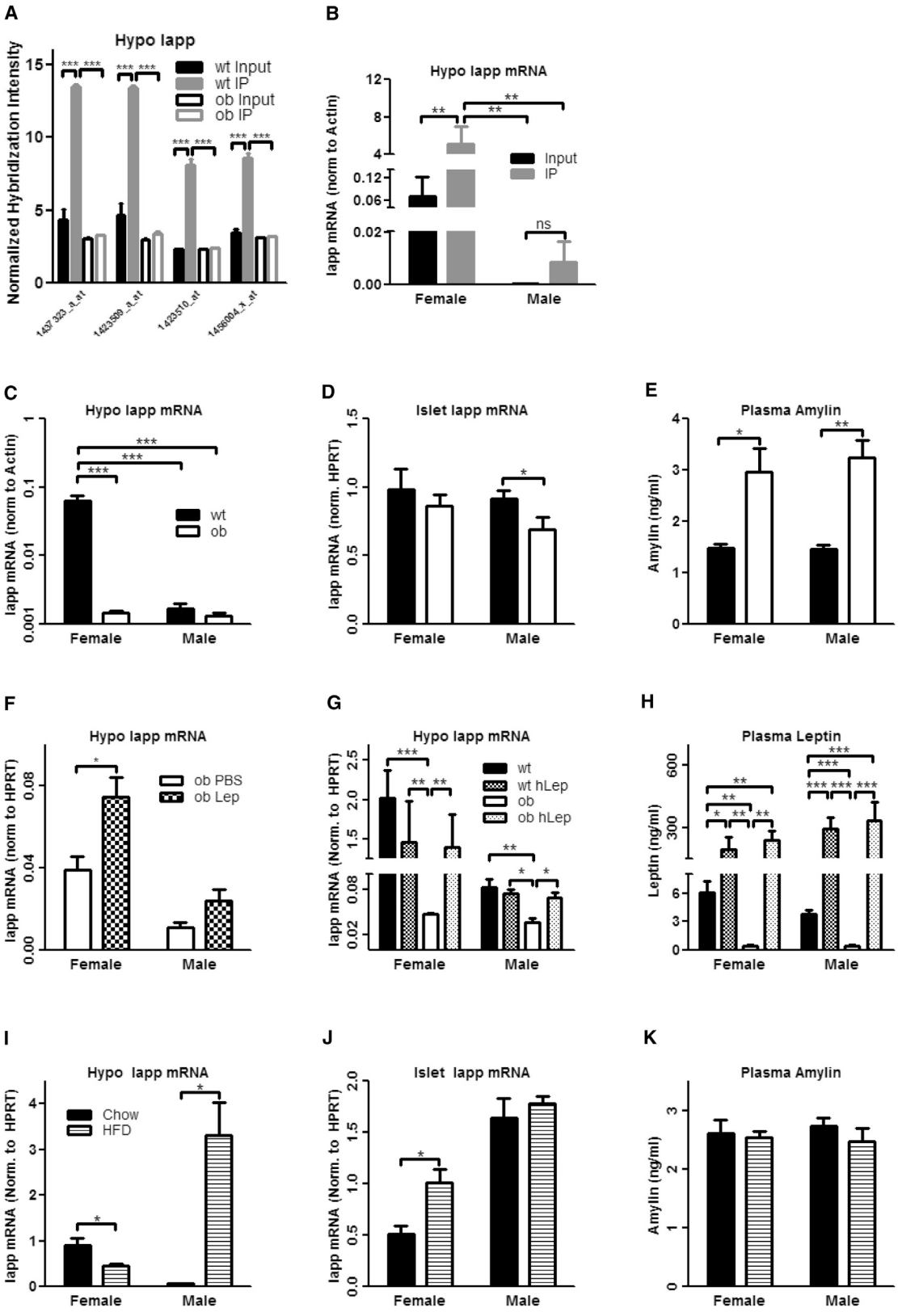
(D) IHC for *tdTomato* (red) and *Iapp* (green) in the Arc, LH, AP/NTS, and AG from adult *ObRb-IRES-Cre* and *Rosa26-CAG-LSL-tdTomato* double-positive mice. Scale bars, 100 μm.

(E) IHC for *Iapp* (green) and *AgRP* or *POMC* (red, top or lower row) in the Arc from wild-type mice. Scale bars, 50 μm.

(F) IHC for *GFP* (green), Iapp (red), and Vgat (blue) in the LH of adult *ObRb-IRES-Cre* and *Eef1a1-LSL-eGFP-RPL10a* double-positive mice. Scale bar, 5 μm.

DISCUSSION

In order to identify transcripts in neurons that control feeding, we molecularly profiled hypothalamic *Pdyn* neurons using BAC TRAP (Heiman et al., 2008). We found that *Pdyn* neurons respond to leptin and coexpress many other neuropeptides: *POMC/Cart* (Arc), *Orx* (LH), and *Avp*, *Oxt*, *Gal*, and *Crh* (PVN). Unexpectedly, we also identified hypothalamic neurons that express *Iapp*, the precursor to amylin. We found that hypothalamic *Iapp* expression is positively regulated by leptin and that its expression is sexually dimorphic in WT mice fed ad lib with regular chow or HFD. We also found that amylin modulates the activity of LH *ObRb* neurons in a similar manner to leptin and that CNS amylin acts in concert with leptin to regulate feeding in vivo.



(legend on next page)

Overall, these data establish hypothalamic amylin as a neuropeptide that is leptin regulated. Finally, the ability of an amylin antagonist delivered i.c.v. to blunt the response to leptin further suggests that CNS amylin can modulate leptin's effects in vivo.

Iapp was originally identified as a pancreatic polypeptide cosecreted with insulin from β cells (Leffert et al., 1989; Ferrier et al., 1989). Its anorexic effect has been extensively studied (Chance et al., 1993, 1991; Lutz et al., 1994; Rushing et al., 2001), and it has also been shown to potentiate the weight-reducing effect of leptin and reverse leptin resistance in obese animals (Roth et al., 2008). Studies on CNS amylin expression have yielded conflicting results (Leffert et al., 1989; Ferrier et al., 1989). Consequently, a peripheral mechanism accounting for amylin's anorexic effect has been proposed (Riediger et al., 2004, Potes et al., 2010). In this model, area postrema (AP) is the primary target of pancreatic amylin and projects to hypothalamic nuclei (Arc, LH, and VMH) that regulate feeding via the nucleus of the solitary tract, lateral parabrachial nucleus, central amygdala, and lateral subdivisions of the bed nucleus of the stria terminalis. Other studies, however, have suggested AP-independent pathways (Chance et al., 1991; Lutz et al., 2001). In line with this, our data confirmed that *Iapp*-expressing neurons reside in the hypothalamus (somas in MPO, Arc, and LH; fibers in PVN and DMH) and other brain regions (Figure S4). The failure to detect *Iapp* expression in previous studies likely resulted from insufficient sensitivity. The *Iapp* transcript in whole hypothalamus is orders of magnitude lower than many other hypothalamic neuropeptides, but is as abundant as these transcripts when assayed after polysome precipitation from *Pdyn* neurons (Figures S2A and S2B). These data indicate that while *Iapp* expression in the hypothalamus is sparse, *Iapp* is expressed at fairly high levels in neurons that express it.

Our data are consistent with a previously reported sexual dimorphism of *Iapp* expression in the MPO of maternally behaving female rats (Dobolyi, 2009), though we also found significantly different levels of hypothalamic *Iapp* expression between female and male mice. In addition, leptin deficiency markedly reduced hypothalamic *Iapp* expression in female mice but not in male which already express low *Iapp* levels at baseline. This downregulation was not observed in islet *Iapp*, suggesting a tissue-specific mechanism for amylin regulation. Exogenous leptin significantly elevated hypothalamic *Iapp* in both genders, suggesting that leptin regulation is not gender specific, but the precise mechanism remains unknown. HFD treatment also led to sexually dimorphic and tissue-specific modulation of hypothalamic *Iapp* expression. We speculate that HFD-induced leptin resistance underlies this modulation. Previous studies of the effects of a combination of leptin plus amylin have only been carried out in male DIO rats, although this combination reduces body weight in both male and female human patients (Roth et al., 2008; http://www.takeda.com/news/2010/20100223_3746.html).

We found that amylin modulates the electrophysiologic activity of LH *ObRb* neurons in a manner similar to leptin. These neurons are a heterogeneous population, with ~65% being excited and 35% being inhibited by both leptin and amylin. This correlation is consistent with the synergy between leptin and amylin in vivo. Furthermore, we found that inhibition of amylin signaling with AC187 blunted leptin's effects on both leptin-activated and leptin-inhibited neurons, suggesting a constitutive activation and inhibition of LH *ObRb* neurons by CNS amylin. Together with our IHC data showing putative synaptic structures between amylin immunopositive fibers and *ObRb* immunopositive cell bodies, it is likely that amylin-expressing neurons innervate LH *ObRb* neurons directly. Supporting this hypothesis are our unpublished data showing that calcitonin receptor (*Calcr*), the core component of amylin receptor, is enriched in *ObRb* neurons (FC = 3.06, corrected p = 3.5e-06). Previous data (Leinninger et al., 2011) showed that LH neurotensin-expressing *ObRb* neurons innervate *Orx* neurons directly and inhibited them through direct and indirect pathways. In aggregate, these data suggest a reciprocal circuit between LH *ObRb* and LH *Orx*/amylin neurons. However, our data do not exclude the possibility that some amylin immunopositive fibers came from Arc or brain regions outside the hypothalamus.

Previous studies suggested that amylin-induced pSTAT3 activation in the VMH, which may involve IL-6, underlies amylin's additive or synergistic effect with leptin (Roth et al., 2008; Le Foll et al., 2015; Moon et al., 2011). Consistent with this, we showed that i.c.v. AC187 blunt leptin's anorexic effect in vivo. We also found that treatment of mice with i.c.v. AC187 increased food intake in ad lib-fed PBS control mice. This is consistent with our electrophysiological data showing that AC187 inhibited endogenous activities of LH *ObRb* neurons in the absence of leptin or amylin. This confirms a functional role of CNS amylin in exerting a tonic effect to modulate leptin signaling and suppress food intake. Taken together, our data established a role of hypothalamic amylin in modulating the neuronal activity of *ObRb*-expressing neurons, providing insight into the mechanistic interaction between leptin and amylin that may underlie the synergistic effect of a leptin/amylin combination for the treatment of obesity. Future studies of mice with a conditional loss of *Iapp* in

Figure 2. Characterization of Hypothalamic *Iapp* Expression

(A) Normalized hybridization intensity of *Iapp* probe sets from triplicates of hypothalamic WT Input, WT IP, ob Input, and ob IP samples obtained from *Pdyn* BAC TRAP mice (n = 6–8, ***p < 0.001).

(B) *Iapp* gene expression from quadruplicates of female and male hypothalamic WT Input and WT IP samples (n = 6–8, **p < 0.01).

(C) *Iapp* gene expression from hypothalamic total RNA extracts of individual WT and *ob*/*ob*, female, and male mice (n = 4, ***p < 0.001).

(D) Islet *Iapp* expression from WT and *ob*/*ob* female and male mice (n = 3 or 7, *p < 0.05).

(E) EIA of plasma amylin from WT and *ob*/*ob* female and male mice (n = 4–7, *p < 0.05, **p < 0.01).

(F) Hypothalamic *Iapp* expression from leptin or PBS-treated female and male *ob*/*ob* mice (n = 3 or 4, *p < 0.05).

(G) Hypothalamic *Iapp* expression from WT and *ob*/*ob*, double- and nontransgenic Lap-tTA and Tre-hLeptin mice (n = 4, *p < 0.05, **p < 0.01, ***p < 0.001).

(H) EIA of plasma amylin from the mice in G (human leptin in double-transgenic mice and mouse leptin in nontransgenic mice, *p < 0.05, **p < 0.01, ***p < 0.001).

(I) Hypothalamic *Iapp* expression from HFD-treated female and male mice (n = 3 or 4, *p < 0.05).

(J) Islet *Iapp* expression from the mice in (I).

(K) EIA of plasma amylin from the mice in (I).

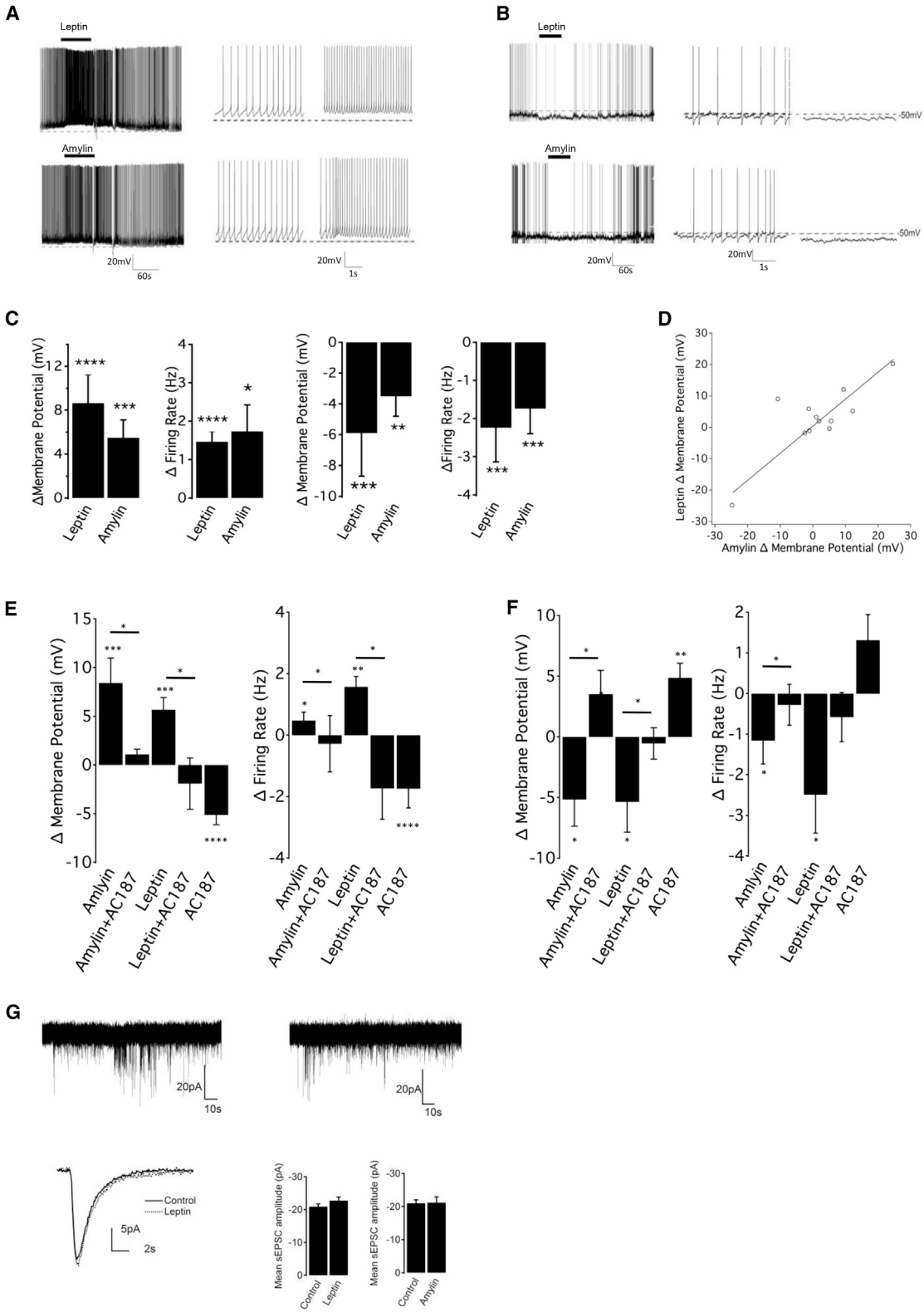


Figure 3. Effects of Leptin and Amylin on LH *ObRb* Neurons

(A) Five minutes (left) and 5 s (right) current-clamp recording of a leptin-depolarizing neuron in the presence of 100 nM leptin or amylin (top or bottom). Black bars, leptin or amylin.

(legend continued on next page)

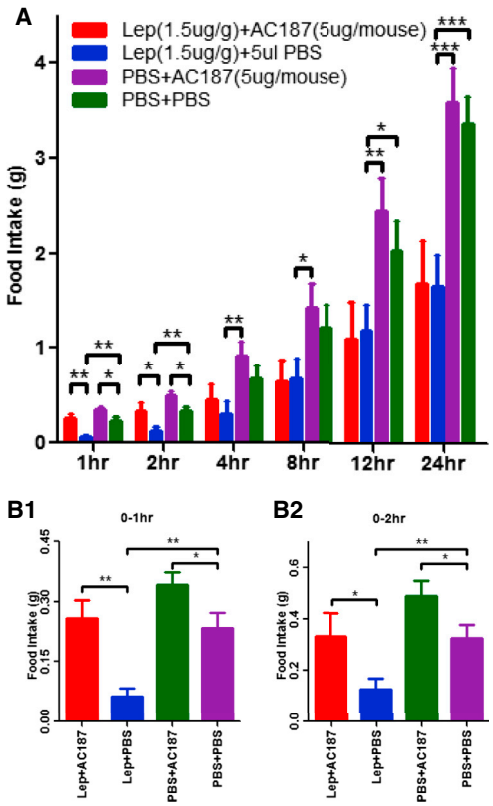


Figure 4. Acute i.c.v. Application of AC187 Temporarily Abolishes Leptin's Anorexic Effect

(A) Twenty-four hour accumulated food intake at 1, 2, 4, 8, 12, and 24 hr time points for the four experimental groups: (1) i.p. leptin+i.c.v. AC187 (red, n = 12), (2) i.p. leptin+i.c.v. PBS (blue, n = 11), (3) i.p. PBS+i.c.v. AC187 (purple, n = 12), and (4) i.p. PBS+i.c.v. PBS (green, n = 15). *p < 0.05, **p < 0.01, and ***p < 0.001.

(B1 and B2) Data at 1 and 2 hr time points were plotted with appropriate scales.

(C) Detailed food consumption (mean and SEM) at each time point for all groups.

C Food consumption at each time points for the four experimental groups in A.

	i.p. leptin+i.c.v. AC187, n=12		i.p. leptin+i.c.v. PBS, n=11		i.p. PBS+i.c.v. AC187, n=12		i.p. PBS+i.c.v. PBS, n=15	
Time (hr)	mean (g)	s.e.m. (g)	mean (g)	s.e.m. (g)	mean (g)	s.e.m. (g)	mean (g)	s.e.m. (g)
1	0.26	0.05	0.06	0.02	0.34	0.03	0.23	0.04
2	0.33	0.09	0.13	0.04	0.49	0.06	0.33	0.05
4	0.46	0.15	0.31	0.12	0.92	0.14	0.67	0.14
8	0.64	0.22	0.68	0.21	1.43	0.25	1.22	0.24
12	1.10	0.38	1.18	0.28	2.45	0.34	2.03	0.30
24	1.68	0.44	1.65	0.34	3.58	0.36	3.37	0.28

orexin neurons will be needed to establish the molecular mechanisms by which amylin receptor signaling influences leptin signaling in LH *ObRb* neurons. A fuller understanding of the cellular mechanisms could provide new approaches to improve leptin signaling and sensitivity. In summary, we identified a population of LH *lapp*-expressing neurons and established an important role for central amylin to modulate the activity of LH *ObRb* neurons. We further find that amylin blockade decreases the effects of leptin on these neurons in vivo and in vitro, suggesting hypothalamic amylin acts in concert with leptin. This provides insights into the cellular mechanism that could underlie the synergistic or additive effect of leptin and amylin in feeding. Further genetic studies ablating amylin production only in the CNS or LH will be necessary to confirm this.

EXPERIMENTAL PROCEDURES

Mice

All animal experiments were conducted under the guideline of The Rockefeller University Center of Comparative Biosciences and NIH. All procedures were approved in protocol 14697 and 13606. Mice were housed at 68°F–72°F on 12 hr light-dark cycles.

Pdyn BAC TRAP mice were generated as described before (Heiman et al., 2008) and bred to C57BL/6J (Jackson Laboratories, stock number 000664) for five generations before use. Transgenic mice were bred to heterozygous

(B) Example of 5 min (left) and 5 s (right) current-clamp recording of a leptin-hyperpolarizing neuron in the presence of 100 nM leptin or amylin (top or bottom). Black bars, leptin or amylin.

(C) Bath application of leptin and amylin (100 nM each) led to significant increases in membrane potential and firing rate in leptin-depolarizing neurons (n = 22, p < 0.0001 for leptin; n = 14 or 16, p < 0.001 or 0.05 for amylin), and significant decreases in membrane potential and firing rate in leptin-hyperpolarizing neurons (n = 12, p < 0.001 for leptin; n = 10, p < 0.01 or 0.001 for amylin). Data for leptin and amylin are not always from the same neuron.

(D) Correlation between the effects of amylin and leptin on the membrane potential of *ObRb* neurons. Data are from neurons sequentially treated with leptin and amylin separate by a washout (n = 13, Spearman Rank correlation $r^2 = 0.8132$, p < 0.01).

(E) 100 nM AC187 significantly blocks the effects of leptin and amylin on membrane potential (p < 0.05 or p < 0.01, n = 9) and firing rate (p < 0.05, n = 6) of leptin-depolarizing neurons. AC187 alone significantly decreased membrane potential firing rate (p < 0.0001, n = 13–14). Data for leptin and amylin are not from the same neuron.

(F) AC187 significantly reduces the effects of amylin and leptin on the membrane potential (p < 0.05 or p < 0.01, n = 7–8) and firing rate (p < 0.05 or p = 0.1094, n = 7–8) of leptin-hyperpolarizing neurons. AC187 alone significantly increased membrane potential (p < 0.001, n = 17), but not firing rate (p = 0.2188, n = 6). Data for leptin and amylin are not from the same neuron.

(G) Voltage-clamp traces showing spontaneous EPSCs in the absence or presence of 100 nM leptin (top left or right). Bottom trace shows mean sEPSC in both conditions. Right panel shows mean sEPSC amplitude in the presence or absence of leptin and amylin (p = 0.0625 and p > 0.999, n = 7 or 6).

ob/+ mice (Jackson Laboratories, stock number 000632) to generate transgenic *ob*/*ob* mice. *ObRb-IRES-Cre* and *Rosa26-CAG-LSL-tdTomato* mice (Jackson Laboratories, stock number 008320 and 007914) were used to generate mice in which *tdTomato* was specifically expressed in *ObRb*-expressing cells. An *Eef1a1-LSL-eGFP-RPL10a* mouse (Stanley et al., 2013) was also used to generate mice in which *eGFP-RPL10a* was specifically expressed in *ObRb*-expressing cells. *Lap-tTA* (Jackson Laboratories, stock number 003563), and *Tre-hLeptin* (Ribeiro et al., 2011) mice were used to generate double transgenic strains in which constitutive expression of a leptin transgene led to high levels of plasma leptin in the absence of doxycyclin.

IHC and Fluorescence Imaging

Antibodies against *GFP*, *DynA*, *Orx*, *AVP*, *Oxt*, *Gal*, *Crh*, *AgRP*, *POMC*, *MCH*, *Th*, *Vgat*, *vGlut2*, *Iapp*, and *tdTomato* were used (see details in Supplemental Experimental Procedures). Images were acquired using an inverted Zeiss LSM 780 laser scanning confocal microscope. Settings were kept constant for all images that underwent cell counting by Imaris.

Quantitative Real-Time PCR, Isolation of Pancreatic Islets, Leptin Infusion, Retro-Orbital Bleeding, and Plasma Hormone Measurements

Procedures were carried out as previously described (see Supplemental Experimental Procedures for details).

Lateral Ventricle Cannulation

Single-injection cannula (Plastics 1, C313GS-4/SP, 2.5 mm projection) was inserted into the brain (1.2 mm lateral and 0.4 mm back from the bregma) of isofluorane/O_2-anesthetized and stereotaxic-anchored (KOPF 942) mice. Wound healing and home-cage behavior were monitored. Body weight recovery to no less than 90% of presurgery amount within 7 days was the inclusion criteria.

Acute Feeding Procedure

Cannulated mice were housed on reverse light cycle (10 a.m.–10 p.m. dark). Food intake and body weight were measured daily to evaluate acclimation. On the day of experiment, food intake and body weight were measured and dirty bedding was replaced 2 hr before dark onset. Mice showing normal fluctuations were included. One hour later, mice were given i.c.v. AC187 (5 ug/5ul, Abcam, ab141150) or PBS through matched cannula internal (Plastics 1, C313IS-4/SP, 3.5 mm projection). Forty minutes later, mice were given i.p. recombinant mouse leptin (1.5 ug/10 ul/g of body weight, Amylin Pharmaceuticals) or PBS in the same order as during i.c.v. injection. Food intake was measured at 1, 2, 4, 8, 12, and 24 hr after dark onset under red light.

Slice Preparation and Electrophysiology

ObRb-IRES-Cre and *Rosa26-CAG-LSL-tdTomato* double-positive mice (3–8 weeks old) were used to patch LH *ObRb* neurons as described before. See details in Supplemental Experimental Procedures.

Statistics

Results were expressed as mean ± SEM. Student t test, one-way ANOVA, or two-way ANOVA was used to assess the significance of difference of the mean or the comparative analyses. For electrophysiological recordings, when data were distributed normally (Shapiro-Wilk test), statistical differences were determined using a two-tailed paired or unpaired Student's t test. Alternatively, Wilcoxon tests were used. Differences were considered significant at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article at http://dx.doi.org/10.1016/j.cmet.2015.10.012.

AUTHOR CONTRIBUTIONS

M.H. and P.G. made the *Pdyn* BAC TRAP mice. Z.L. researched the data and wrote the manuscript. L.K. researched data for Figure 3 and edited the manuscript. I.G. and P.V. provided technical support on researching data for Figure 4. J.M.F. advised the project and edited the manuscript.

ACKNOWLEDGMENTS

We thank Nathaniel Heintz, Yotam Sagi, Marc Schneeberger Pane, and Susan Korres (The Rockefeller University); Joseph P. Doyle (Takeda Pharmaceutical); Joseph D. Dougherty (Washington University); Giovanni Ceccarini (University Hospital of Pisa); and The Rockefeller University Bio-Imaging Center, Comparative Bioscience Center, Genomic Resource Center, and Antibody and Bioresource Core Facility for their contribution and support. This work is supported by funding from The JPB Foundation.

Received: September 29, 2014
Revised: August 5, 2015
Accepted: October 22, 2015
Published: November 19, 2015

REFERENCES

Allison, M.B., Patterson, C.M., Krashes, M.J., Lowell, B.B., Myers, M.G., Jr., and Olson, D.P. (2015). TRAP-seq defines markers for novel populations of hypothalamic and brainstem LepRb neurons. Mol. Metab. *4*, 299–309, http://dx.doi.org/10.1016/j.molmet.2015.01.012.

Blednov, Y.A., Walker, D., Martinez, M., and Harris, R.A. (2006). Reduced alcohol consumption in mice lacking preprodynorphin. Alcohol *40*, 73–86.

Chance, W.T., Balasubramaniam, A., Zhang, F.S., Wimalawansa, S.J., and Fischer, J.E. (1991). Anorexia following the intrahypothalamic administration of amylin. Brain Res. *539*, 352–354.

Chance, W.T., Balasubramaniam, A., Stallion, A., and Fischer, J.E. (1993). Anorexia following the systemic injection of amylin. Brain Res. *607*, 185–188.

Chefer, V.I., and Shippenberg, T.S. (2006). Paradoxical effects of prodynorphin gene deletion on basal and cocaine-evoked dopaminergic neurotransmission in the nucleus accumbens. Eur. J. Neurosci. *23*, 229–238.

Dobolyi, A. (2009). Central amylin expression and its induction in rat dams. J. Neurochem. *111*, 1490–1500.

Ferrier, G.J., Pierson, A.M., Jones, P.M., Bloom, S.R., Girgis, S.I., and Legon, S. (1989). Expression of the rat amylin (IAPP/DAP) gene. J. Mol. Endocrinol. *3*, R1–R4.

Heiman, M., Schaefer, A., Gong, S., Peterson, J.D., Day, M., Ramsey, K.E., Suárez-Fariñas, M., Schwarz, C., Stephan, D.A., Surmeier, D.J., et al. (2008). A translational profiling approach for the molecular characterization of CNS cell types. Cell *135*, 738–748.

Knight, Z.A., Tan, K., Birsoy, K., Schmidt, S., Garrison, J.L., Wysocki, R.W., Emiliano, A., Ekstrand, M.I., and Friedman, J.M. (2012). Molecular profiling of activated neurons by phosphorylated ribosome capture. Cell *151*, 1126–1137.

Le Foll, C., Johnson, M.D., Dunn-Meynell, A.A., Boyle, C.N., Lutz, T.A., and Levin, B.E. (2015). Amylin-induced central IL-6 production enhances ventromedial hypothalamic leptin signaling. Diabetes *64*, 1621–1631, http://dx.doi.org/10.2337/db14-0645.

Leffert, J.D., Newgard, C.B., Okamoto, H., Milburn, J.L., and Luskey, K.L. (1989). Rat amylin: cloning and tissue-specific expression in pancreatic islets. Proc. Natl. Acad. Sci. USA *86*, 3127–3130.

Leinninger, G.M., Opland, D.M., Jo, Y.H., Faouzi, M., Christensen, L., Cappellucci, L.A., Rhodes, C.J., Gnegy, M.E., Becker, J.B., Pothos, E.N., et al. (2011). Leptin action via neurotensin neurons controls orexin, the mesolimbic dopamine system and energy balance. Cell Metab. *14*, 313–323.

Loacker, S., Sayyah, M., Wittmann, W., Herzog, H., and Schwarzer, C. (2007). Endogenous dynorphin in epileptogenesis and epilepsy: anticonvulsant net effect via kappa opioid receptors. Brain *130*, 1017–1028.

Lutz, T.A., Del Prete, E., and Scharrer, E. (1994). Reduction of food intake in rats by intraperitoneal injection of low doses of amylin. Physiol. Behav. *55*, 891–895.

Lutz, T.A., Mollet, A., Rushing, P.A., Riediger, T., and Scharrer, E. (2001). The anorectic effect of a chronic peripheral infusion of amylin is abolished in area postrema/nucleus of the solitary tract (AP/NTS) lesioned rats. Int. J. Obes. Relat. Metab. Disord. *25*, 1005–1011.

McLaughlin, J.P., Marton-Popovici, M., and Chavkin, C. (2003). Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. J. Neurosci. *23*, 5674–5683.

Moon, H.S., Chamberland, J.P., Diakopoulos, K.N., Fiorenza, C.G., Ziemke, F., Schneider, B., and Mantzoros, C.S. (2011). Leptin and amylin act in an additive manner to activate overlapping signaling pathways in peripheral tissues: in vitro and ex vivo studies in humans. Diabetes Care *34*, 132–138.

Potes, C.S., Lutz, T.A., and Riediger, T. (2010). Identification of central projections from amylin-activated neurons to the lateral hypothalamus. Brain Res. *1334*, 31–44.

Ribeiro, A.C., Ceccarini, G., Dupré, C., Friedman, J.M., Pfaff, D.W., and Mark, A.L. (2011). Contrasting effects of leptin on food anticipatory and total locomotor activity. PLoS ONE *6*, e23364, http://dx.doi.org/10.1371/journal.pone.0023364.

Riediger, T., Zuend, D., Becskei, C., and Lutz, T.A. (2004). The anorectic hormone amylin contributes to feeding-related changes of neuronal activity in key structures of the gut-brain axis. Am. J. Physiol. Regul. Integr. Comp. Physiol. *286*, R114–R122.

Roth, J.D., Roland, B.L., Cole, R.L., Trevaskis, J.L., Weyer, C., Koda, J.E., Anderson, C.M., Parkes, D.G., and Baron, A.D. (2008). Leptin responsiveness restored by amylin agonism in diet-induced obesity: evidence from nonclinical and clinical studies. Proc. Natl. Acad. Sci. USA *105*, 7257–7262.

Rushing, P.A., Hagan, M.M., Seeley, R.J., Lutz, T.A., D'Alessio, D.A., Air, E.L., and Woods, S.C. (2001). Inhibition of central amylin signaling increases food intake and body adiposity in rats. Endocrinology *142*, 5035.

Shin, E.J., Jang, C.G., Bing, G., Park, D.H., Oh, C.H., Koo, K.H., Oh, K.W., Yamada, K., Nabeshima, T., and Kim, H.C. (2009). Prodynorphin gene deficiency potentiates nalbuphine-induced behavioral sensitization and withdrawal syndrome in mice. Drug Alcohol Depend. *104*, 175–184.

Stanley, S., Domingos, A.I., Kelly, L., Garfield, A., Damanpour, S., Heisler, L., and Friedman, J.M. (2013). Profiling of glucose-sensing neurons reveals that GHRH neurons are activated by hypoglycemia. Cell Metab. *18*, 596–607.

Wang, Z., Gardell, L.R., Ossipov, M.H., Vanderah, T.W., Brennan, M.B., Hochgeschwender, U., Hruby, V.J., Malan, T.P., Jr., Lai, J., and Porreca, F. (2001). Pronociceptive actions of dynorphin maintain chronic neuropathic pain. J. Neurosci. *21*, 1779–1786.

Wittmann, W., Schunk, E., Rosskothen, I., Gaburro, S., Singewald, N., Herzog, H., and Schwarzer, C. (2009). Prodynorphin-derived peptides are critical modulators of anxiety and regulate neurochemistry and corticosterone. Neuropsychopharmacology *34*, 775–785.