Olfactory and neuropeptide inputs to appetite neurons in the arcuate nucleus

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Abstract
The sense of smell has potent effects on appetite, but the underlying neural mechanisms are largely a mystery. The hypothalamic arcuate nucleus contains two subsets of neurons linked to appetite: AgRP (agouti-related peptide) neurons, which enhance appetite, and POMC (pro-opiomelanocortin) neurons, which suppress appetite. Here, we find that AgRP and POMC neurons receive indirect inputs from partially overlapping areas of the olfactory cortex, thus identifying their sources of odor signals. We also find neurons directly upstream of AgRP or POMC neurons in numerous other areas, identifying potential relays between the olfactory cortex and AgRP or POMC neurons. Transcriptome profiling of individual AgRP neurons reveals differential expression of receptors for multiple neuromodulators. Notably, known ligands of the receptors define subsets of neurons directly upstream of AgRP neurons in specific brain areas. Together, these findings indicate that higher olfactory areas can differentially influence AgRP and POMC appetite neurons, that subsets of AgRP neurons can be regulated by different neuromodulators, and that subsets of neurons upstream of AgRP neurons in specific brain areas use different neuromodulators, together or in distinct combinations to modulate AgRP neurons and thus appetite.

Introduction
The regulation of appetite and food intake is essential to health and survival and is evident in most or all animals. In mice and other mammals, the arcuate nucleus of the hypothalamus (ARC) has two subsets of neurons thought to play opposing roles in appetite regulation: AgRP neurons, which express agouti-related peptide, and POMC neurons, which express pro-opiomelanocortin (POMC).

AgRP neurons are activated when animals are calorically deficient[1-3]. Optogenetic or chemogenetic activation of these neurons stimulates voracious eating and various hunger-related behaviors[4-6]. Activated AgRP neurons can also induce lipogenesis, fat mass accumulation, and altered substrate utilization[7]. In contrast, POMC neurons are stimulated when energy is available[3], and optogenetic activation of POMC neurons inhibits feeding in fasted animals[8].

Interestingly, the activities of AgRP and POMC neurons can be rapidly modulated by the smell and sight of food in awake behaving mice[9-11]. Within seconds before food is consumed, the activity of AgRP neurons is inhibited while POMC neurons are stimulated. Current evidence indicates that these effects result from previously learned associations between the smell and/or sight of a particular food and its caloric content and/or nutritional value[9,12].

How might the smell of a food be conveyed to AgRP and POMC neurons to elicit these sensory effects on appetite? Previous studies have used monosynaptic rabies virus to map the locations of brain neurons presynaptic to AgRP or POMC neurons[13,14]. In the olfactory system, odor signals travel from the nasal olfactory epithelium through the olfactory bulb to the olfactory cortex, which transmits information to multiple brain areas[15]. The olfactory cortex comprises a number of anatomically distinct areas whose respective functions are not well understood. Although the rabies virus studies revealed neurons presynaptic to AgRP or POMC neurons in multiple brain areas, only one hinted at neurons upstream of AgRP neurons in an area of the olfactory cortex[14]. These results suggest that olfactory signals might be conveyed to AgRP and/or POMC neurons by the olfactory cortex but via an indirect route that cannot be detected using a monosynaptic rabies virus.

To investigate this idea, we infected AgRP or POMC neurons with PRVB177, a conditional Pseudorabies virus that travels retrogradely across multiple synapses after infecting neurons expressing Cre recombinase[16]. These experiments revealed neurons likely to be two synapses upstream of either AgRP or POMC neurons in several, partially overlapping areas of the olfactory cortex. Surprisingly, neurons directly or indirectly upstream of AgRP and POMC neurons were also seen in two amygdala areas that receive signals from the vomeronasal organ, an accessory olfactory area that appears specialized for the detection of social cues[15].

Both rabies and Pseudorabies viruses infected neurons directly upstream of AgRP and POMC neurons in numerous brain areas[13,14]. Many of these are in the hypothalamus, which regulates a large variety of physiological and behavioral functions including reproduction[17-20], appetite control[21-23] and stress[24-27]. If one could determine neuromodulators used by different upstream neurons to modulate appetite, one would have molecular/genetic tools to explore the impact of different upstream areas on AgRP or POMC neurons. These areas might convey information of different types, including sensory information, that could either boost or suppress the functions of the appetite neurons.

To this end, we first used single cell RNA sequencing to identify receptors that AgRP neurons express for neuromodulators. We then examined upstream neurons for the expression of ligands of specific receptors. These experiments revealed that AgRP neurons vary considerably in their expression of receptors for extracellular signaling molecules. They further showed that neuropeptides recognized by AgRP neuron receptors are expressed by selected subsets of neurons upstream of AgRP neurons in specific brain areas. These findings lay an important foundation for dissecting the locations of neurons that exert either positive or negative effects on appetite and those that relay olfactory sensory signals or other information to the appetite neurons. They also lay the groundwork for studies to characterize the effects of specific receptors and ligands on appetite, which could be of pharmacological importance for the development of drugs to treat appetite disorders.

Results

Appetite neurons receive input from higher olfactory areas

To explore whether appetite neurons receive information from specific higher olfactory areas, we used the polysynaptic Pseudorabies virus, PRVB177 (Fig. 1). PRVB177 has a Cre recombinase-dependent HA-thymidine kinase (TK), which allows it to replicate in Cre-expressing neurons and then travel retrogradely across sequential synapses in a time-dependent manner[16]. PRVB177 was injected into the ARC of mice expressing Cre in AgRP or POMC neurons (AgRP-Cre or POMC-Cre mice).

To examine the locations of upstream PRV-infected neurons, brain sections were immunostained for HA on subsequent days. We previously used this method in CRH-Cre mice to examine neurons upstream of hypothalamic neurons expressing CRH (corticotropin releasing hormone)[16]. Those studies confirmed that PRVB177 specifically infects only Cre-expressing neurons in the mouse brain.

We first detected PRV-infected (HA+) neurons outside the ARC on day 3 post-injection (d3pi), suggesting that these neurons are directly upstream of AgRP or POMC neurons. No infected neurons were seen in wildtype (WT) animals injected with the virus (data not shown). This is consistent with our studies using CRH-Cre mice, which showed that virus-infected neurons seen on d3pi are one synapse upstream of infected Cre-expressing neurons[16].

On d3pi, we detected PRV+ (HA+) neurons upstream of AgRP or POMC neurons in a small number of higher olfactory areas (Fig. 2, 3, and 4). Neurons upstream of POMC neurons were seen in one area off the olfactory cortex (OC), the nucleus of the lateral olfactory tract (LOT). Infected neurons upstream of both AgRP and POMC neurons were also observed in the medial amygdala (MEA). The MEA is part of the 'vomeronasal amygdala' (VA), which receives signals derived from the vomeronasal organ, an accessory olfactory area thought to be specialized for the detection of pheromones and other social cues[15]. Of all PRV+ neurons outside the ARC, the LOT contained 1.0 ± 0.6% in POMC-Cre mice, and the MEA contained 3.4 ± 1.1% in AgRP-Cre and 6.0 ± 5.3% in POMC-Cre mice (Fig. 2, 3, and 4). These results suggest that two higher olfactory areas can provide direct input to AgRP or POMC neurons.

On d4pi, PRV+ cells appeared in a number of additional olfactory areas in AgRP-Cre and POMC-Cre animals (Fig. 2, 3, and 4). In AgRP-Cre mice, we observed PRV-infected neurons in three areas of the OC: the olfactory tubercle (Tu), posterior piriform cortex (pPir), and posterior lateral cortical amygdala (PLCo). We also detected PRV+ neurons in the two areas of the vomeronasal amygdala, the posterior medial cortical amygdala (PMCo) and MEA. The percentage of PRV+ cells outside the ARC in the OC ranged from 0.4 ± 0.3% for Tu to 6.1 ± 5.2% for pPir. PRV+ neurons that first appear in a given region on d4pi are likely to be two synapses upstream of Cre-expressing neurons initially infected with PRVB177, as indicated in our previous studies of CRH-Cre mice[16].

On d4pi of POMC-Cre mice, PRV+ cells were observed in three areas of the OC: the pPir, anterior cortical nucleus (ACo), and lateral entorhinal cortex (LEnt) (Fig. 2, 3, and 4). The percentage of PRV+ neurons outside the ARC was 3.8 ± 3.7, 1.0 ± 1.0, and 0.4 ± 0.3% for these areas, respectively. As for AgRP-Cre mice, PRV infected cells were also seen in the PMCo and MEA in the VA (Fig. 2, 3, and 4).

Together, these findings indicate that both AgRP and POMC appetite neurons receive information indirectly from the olfactory cortex. They suggest that one major OC area, the pPir contains neurons two synapses upstream of both AgRP and POMC neurons. They further suggest that there also neurons two synapses upstream of AgRP neurons in two additional OC areas, the Tu and the PLCo and neurons two synapses upstream of POMC neurons in two other areas of the OC, the ACo and the LEnt. Thus, while one OC area, pPir, provides indirect input to both subsets of appetite neurons, there are four additional OC areas that provide indirect input to only one or the other subset: the Tu and PLCo only to AgRP neurons and the ACo and LEnt only to POMC neurons.

Our results also suggest that both AgRP and POMC neurons receive input from the VA, which receives signals derived from the vomeronasal organ. Interestingly, both subsets receive input directly from the MEA of the VA and both also receive input indirectly from the other area of the VA, the PMCo. Given the role of the vomeronasal organ in detecting social cues, these results could indicate that such cues can affect appetite. However, since some neurons in the main olfactory bulb, which receives input derived from the nasal olfactory epithelium, project to the MEA, and there are also projections to the VA from the OC, these results could reflect information provided to the VA from the olfactory epithelium of the nose.

Appetite neurons receive input from multiple brain areas

These studies indicate that AgRP and POMC neurons also receive input from numerous other brain areas in addition to those in higher olfactory areas. Following infection of AgRP or POMC neurons, PRV-infected neurons were first detected outside the ARC on day 3 post-injection (d3pi). This is consistent with our studies using CRH-Cre mice, which showed that virus-infected neurons seen on d3pi are one synapse upstream of infected Cre-expressing neurons[16].

Three days after infection of AgRP neurons, PRV+ cells were detected in 11 non-olfactory brain areas outside the ARC (Fig. 3, 4, and 5, Extended Data Fig. 1). All but one of these areas was in the hypothalamus, which is involved in numerous endocrine controls and basic functions, including appetite, thirst, and instinctive behaviors[21-28]. The highest percentages of PRV+ cells were seen in the VMH (ventromedial hypothalamic area) and the DM (dorsal medial hypothalamic area) (Fig. 3, 4, and 5, Extended Data Fig. 1). Interestingly, some PRV+ cells were also seen in the nucleus accumbens (Acb), which is linked to reward[29]. Given that PRV-infected neurons were not detected outside the ARC until day 3, it is likely that the cells seen on day 3 after AgRP infection are directly upstream of (presynaptic to) AGRP neurons, which would be consistent with our previous studies of CRH-Cre mice[16].

Three days after infection of POMC neurons, PRV-infected neurons were also detected in multiple non-olfactory brain areas outside the ARC. Sixteen brain areas contained PRV+ cells on day 3 (Fig. 3, 4, and 5, Extended Data Fig. 1). As with those seen after AgRP infection, most of these areas were located within the hypothalamus. Interestingly, high percentages of PRV+ cells were seen in the VMH and DM, similar to what was seen for neurons upstream of AgRP neurons (Fig. 3, 4, and 5, Extended Data Fig. 1). These 16 areas are likely to contain neurons directly presynaptic to POMC neurons.

Nine non-olfactory brain areas contained PRV+ neurons on day 3 after infection of either AgRP or POMC neurons (Fig. 3 and 4), suggesting that these areas can transmit

information to both AgRP and POMC neurons. Of course, the neurons in those areas that project to AgRP and POMC neurons need not be the same. Areas with neurons upstream of AgRP but not POMC neurons were the Acb (nucleus accumbens) and the SO (supraoptic area), which are implicated in reward[29] and thirst[28], respectively. Areas with PRV-infected cells upstream of POMC but not AgRP neurons were the DlEnt, which is part of the entorhinal cortex, the MM, DTM, TuLH, RCH, ME, and PLH.

The areas with PRV+ neurons on day 3 after infection of AgRP or POMC neurons are similar to areas seen using a monosynaptic rabies virus to examine neurons presynaptic to AgRP or POMC neurons[13,14]. These are candidates for areas that could relay signals from other areas, such as higher olfactory areas, to the appetite neurons.

On day 4 after PRV-infection of AgRP or POMC neurons, PRV-infected neurons were seen in numerous additional non-olfactory brain areas (Fig. 3 and 4). PRV+ cells were seen upstream of AgRP neurons in 16 brain areas compared to 11 areas on day 3 after infection (but two seen at d3 had disappeared). And on day 4 after POMC neuron infection, PRV+ cells were seen in 34 areas versus in 16 areas on day 3 (Fig. 3 and 4). The PRV-infected neurons first seen on day 4 are likely to be two synapses upstream of AgRP or POMC neurons. Signals from these neurons are likely to be relayed to AgRP or POMC neurons by neurons presynaptic to the appetite neurons. The neurons two synapses upstream of the appetite neurons could conceivably activate or suppress neurons presynaptic to the appetite neurons, which in turn, either stimulate or suppress the appetite neurons.

AgRP neurons express numerous receptors for neuromodulators

The above experiments identified neurons directly upstream of AgRP neurons in 11 different brain areas. What are the functions of those neurons in the regulation of AgRP neurons? To gain information that could be used to dissect the roles of different upstream neurons, we sought to identify molecular markers for those neurons. For this purpose, we employed RAMUN (receptor assisted mapping of upstream neurons)[25] (Extended Data Fig. 2). The plan was to first use transcriptome profiling to define neuropeptide receptors expressed by AgRP neurons and then to determine the locations of directly upstream neurons that express the neuropeptide ligands of those receptors.

We first conducted single cell transcriptome analysis on individual AgRP neurons isolated from the ARC (Extended Data Fig. 1). We crossed AgRP-Cre mice[30] with Ai6 reporter mice[31], which have Cre-dependent expression of GFP. We dissociated cells from the ARC of these AgRP-Cre:Ai6 mice, manually isolated single GFP+ cells, and then conduced single cell RNA sequencing (scRNA-seq) on individual cells. Single-cell cDNA libraries were generated and analyzed using our previously published methods[25,32,33]. Neurons were then sequenced at an average of about 6.5 million reads per cell. Sequenced reads were mapped to the mouse genome (UCSC mm 10 assembly with GENCODE M15 release gene models). Gene expression in individual cells was determined by a cutoff of 1 FPKM (Fragments Per Kilobase of transcript per Million mapped reads).

These experiments revealed a variety of G protein-coupled receptors (GPCRs) with known ligands that are expressed by AgRP neurons (Fig. 6). These included metabotropic (GPCR) receptors for the neurotransmitters glutamate, GABA, and ADP and/or ATP. They also included a number of receptors for the biogenic amines, epinephrine/norepinephrine, histamine, or serotonin. Remarkably, they also included 23

receptors for neuropeptides and 4 for other signaling molecules. These data resemble data previously obtained by RNA sequencing of grouped AgRP neurons rather than single cells[34].

It is clear from our data that a single AgRP neuron can express multiple different GPCRs that recognize different ligands (Fig. 6). These include GPCRs for neurotransmitters, biogenic amines, and/or neuropeptides. Moreover, different neurons can express different combinations of receptors. This finding is similar to those we obtained by single cell RNA-seq of CRH (corticotropin releasing hormone) neurons in the paraventricular nucleus of the hypothalamus[25].

Another important point is that the percentage of AgRP neurons that express a particular neuropeptide receptor varies (Fig. 6). For example, Adcyapr1 was expressed in 80% of the neurons whereas, at the other extreme, Galr2 was expressed in only 5%. Given that we conducted deep sequencing of a relatively small number of AgRP neurons, the percentage expressing individual receptors could vary due to drop out effects during RNA-seq.

To compare our results with another dataset with a larger number of AgRP neurons, we also extracted data on 2,286 AgRP+ cells from a larger scale study that used single cell transcriptomics to analyze cells from the ARC and surrounding areas[35]. We found a good correspondence between the percentage of AgRP neurons expressing different neuropeptide receptors in our data and data from that study. For example, Adcyap1r1 was expressed in 80% of our AgRP neurons and 36.5% of AgRP neurons in the other dataset.

GPCRs are important cell surface molecules, which serve as drug targets for over a third of current pharmaceutical drugs[36]. The identification of GPCRs expressed by AgRP neurons lays a foundation for exploring these GPCRs as drug targets that may serve a pharmaceutical use in the control of appetite and energy metabolism.

Ligands of AgRP neuron receptors define subsets of upstream neurons

Altogether, the viral tracing experiments identified neurons directly upstream of AgRP neurons in eleven brain areas and the single cell transcriptome experiments identified AgRP neuron receptors for multiple signaling molecules, including over 20 receptors for neuropeptides (Fig. 6).

Are there subsets of upstream neurons that express certain of these neuropeptides? If so, it would provide molecular-genetic tools that could be used to dissect the functions of different identified subsets in the control of appetite.

To explore this question, we used a method we previously used to identify signaling molecules expressed by subsets of neurons directly upstream of CRH neurons[25] (Extended Data Fig. 2). This method is termed RAMUN (receptor assisted mapping of upstream neurons). To examine whether there are subsets of neurons upstream of AgRP neurons that express a specific neuropeptide ligand of an AgRP neuron receptor, we infected AgRP neurons with PRVB177, waited for 3 days for the virus to cross one synapse, and then costained brain sections for PRV and individual neuropeptides.

We examined the expression of seven different neuropeptides in PRV+ neurons directly upstream of AgRP neurons (Fig. 7 and 8). The number of areas with a given

neuropeptide expressed in PRV+ cells varied from one to seven areas. At one extreme, Crh was detected in PRV+ cells only in the PVN. At the other, Pnoc was seen in PRV+ cells in six areas.

The percentage of PRV+ cells expressing individual neuropeptides also varied. Only 6.3 ± 1.6% of PVN PRV+ cells expressed Crh and only 7.6 ± 3.8% of VMH PRV+ neurons expressed Gal. At the other extreme, 100.0 ± 0.0% of PRV+ neurons in AH expressed Prok2. In addition, a given neuropeptide was observed in different percentages of PRV+ neurons in different areas. For example, Pnoc was seen in 10.0 ± 1.7% of PRV+ cells in PVN, but 55.9 ± 44.1% of those in MEA.

The total percentage of the PRV+ neurons expressing different neuropeptides in DM, AH, and VMH was over 100% (Fig. 7), suggesting that there are upstream neurons expressing more than one neuropeptide in these brain areas. There is no correlation between numbers of total PRV-infected neurons and percentages of neuropeptide-labeled neurons in these brain regions, indicating that higher percentages of detected neuropeptide expression in upstream AgRP neurons are not due to higher PRV infection. There is also no correlation between the total number of upstream neurons expressing a neuropeptide and the percentage of AgRP neurons expressing the receptor for that neuropeptide.

These results provide fundamental information about receptors and signaling molecules that regulate AgRP neurons. Importantly, they also provide molecular genetic tools that can now be used to probe the functions of neurons directly upstream of AgRP neurons in the control of appetite and energy metabolism.

Discussion

In these studies, we sought to gain further insight into the neural circuits that control appetite. We took a three-pronged approach (Fig. 1 and Extended Data Fig. 2). First, we used viral tracing to describe the locations of neurons directly or indirectly upstream of AgRP or POMC neurons. Second, we used scRNA-seq to identify GPCRs expressed by AgRP neurons. And third, we used a combination of viral tracing and in situ hybridization to examine the locations of neurons directly upstream of AgRP neurons that express neuropeptide ligands of AgRP GPCRs.

Inputs to AgRP and POMC neurons from higher olfactory areas

These studies indicate that both AgRP and POMC neurons receive signals from higher olfactory areas. Our results show that both AgRP and POMC neurons receive indirect input from selected areas of the olfactory cortex (Fig. 2, 3, and 4). On day 4 after infection of AgRP neurons, when the virus has likely crossed two synapses, PRV+ cells were seen in 3 areas of the OC (the Tu, pPir, and PLCo). And on day 4 after infection of POMC neurons, PRV+ cells were detected in 3 areas of the OC (the pPir, ACo, and LEnt). For both AgRP and POMC neurons, the highest percentage of PRV+ cells in the OC was observed in pPir.

Together, these results indicate that both AgRP and POMC neurons receive indirect input from the OC, but it appears that some OC areas may provide signals to AgRP versus POMC neurons. We previously found that CRH neurons receive indirect input from five areas of the OC, but those in one area, the AmPir, play a key role in stress hormone responses to volatile predator odors[16]. Interestingly, we did not detect neurons

upstream of either AgRP or POMC neurons in AmPir. What the roles are of those upstream of the appetite neurons in the OC will require future studies.

Signals from upstream OC neurons are likely to be relayed to AgRP or POMC neurons by neurons presynaptic to the appetite neurons in one or more other areas. Most or all projection neurons in the OC are glutamatergic excitatory neurons[16]. The OC neurons could, however, activate either excitatory or inhibitory neurons presynaptic to the appetite neurons and thereby either stimulate or inhibit the appetite neurons. It is thus conceivable that neurons upstream of AgRP or POMC neurons in one OC area could activate presynaptic excitatory neurons and those in another OC area activate presynaptic inhibitory neurons. In this manner, neurons upstream of AgRP or POMC neurons in different OC areas could have dramatically different effects on the activity and functions of the appetite neurons.

These studies also indicate that both AgRP and POMC neurons receive input from neurons in the vomeronasal amygdala (Fig. 2, 3 and 4), which receives sensory signals derived from the VNO[15]. PRV+ cells were seen in the MEA 3 days after infection of either subset, suggesting that the MEA contains neurons directly upstream of either AgRP or POMC neurons. PRV+ neurons were also seen 4 days after infection of either neuronal subset in another area of the vomeronasal amygdala, the PMCo. Given that the MEA and PMCo receive input derived from the vomeronasal organ, these results raise the possibility that social cues detected in the VNO could influence appetite and energy metabolism.

Our studies suggest that AgRP and POMC neurons also receive direct input from neurons in numerous other brain areas (Fig. 3, 4 and 5). On day 3 post infection, when PRV has crossed only one synapse, we observed PRV+ neurons upstream of AgRP neurons in eleven non-olfactory brain areas and PRV+ neurons upstream of POMC neurons in sixteen non-olfactory areas. For both subsets of appetite neurons, most of those areas are in the hypothalamus, which controls a variety of basic functions, including appetite and thirst, as well as numerous innate behaviors[21-28]. Many of the areas with infected cells on day 3 were the same for AgRP and POMC neurons. For both appetite neuron subsets, high percentages of PRV+ neurons were seen in the VMH and the DM. Interestingly, it has been reported that the DM (DMH) contains neurons directly upstream of AgRP neurons that affect AgRP neuron activity[37].

Receptors for extracellular signals on AgRP neurons.

Our studies clearly indicate that AgRP and POMC neurons receive direct input from neurons in multiple brain areas (Fig. 3, 4 and 5). But what are the functions of those areas in the control of appetite and energy metabolism? One way to investigate this question would involve identifying molecular markers of neurons that could be used to interrogate their functions.

The strategy we devised for this purpose was to first identify AgRP neuron receptors for neuromodulators and then use known ligands of those receptors to find the locations of upstream neurons expressing specific receptor ligands (Extended Data Fig. 2).

As a first step, we used scRNA-seq to define GPCRs expressed by AgRP neurons. GPCRs comprise the largest family of receptors in mammals and are a favored drug target due to their differential expression among cell types, including different types of neurons [36,38-40].

These experiments identified dozens of different GPCRs for extracellular signaling molecules on AgRP neurons (Fig. 6). These included GPCR receptors for neurotransmitters, such as glutamate or GABA, and receptors for biogenic amines, such as histamine and serotonin. However, the largest class of GPCRs was receptors for neuropeptides and a few other neuromodulators. We found expression of 23 different receptors for neuropeptides in AgRP neurons.

The percentage of AgRP neurons expressing different neuropeptide receptors varied widely. For example, Adcyap1r1, which recognizes PACAP and VIP, was found in 80% of AgRP neurons whereas several other receptors, such as Galr2, which recognizes galanin, was found in only 5% of AgRP neurons. As seen in our previous transcriptome studies of CRH neurons[25], different AgRP neurons expressed different combinations of receptors. It is not clear what the differential expression of GPCRs among AgRP neurons might mean. However, it does hint at a diversity of AgRP neurons that are controlled by different sets of signals to potentially guide different AgRP neuron functions.

Ligands of AgRP neuron receptors define distinct subsets of upstream neurons.
In the second step of this endeavor, we infected AgRP neurons with PRV and then costained for cells colabeled for PRV and individual neuropeptide ligands of AgRP neuron GPCRs. The goal was to determine whether neurons upstream of AgRP neurons in specific brain areas can be defined by their expression of different neuropeptides.

Our results answered this question in the affirmative. For example, the Crhr1 ligand Crh (corticotropin releasing hormone (also known as corticotropin releasing factor)) was expressed only in PRV+ neurons upstream of AgRP neurons in the PVN (Fig. 7). And the Galr2 receptor ligand Gal, was detected only in PRV+ neurons in the VMH (Fig. 7). At the other extreme, Pnoc, a ligand for the opiate receptor Oprl1, was expressed in PRV+ neurons in six different areas, including the PVN and VMH (Fig. 7 and 8). Interestingly, Pdyn, the ligand of the opiate receptor Oprk1 was expressed in PRV+ neurons in the DMH (DM) (Fig. 7 and 8). This is consistent with the report that Pdyn is expressed in DMH neurons directly upstream of AgRP neurons[37] which can inhibit AgRP neurons.

Our results show that, in certain areas, the combined percentages of PRV+ neurons expressing different neuropeptides exceed 100 percent (Fig. 7). This was the case for the VMH, AH, and DM. These findings suggest that some of these neuropeptides are expressed in some of the same upstream neurons in these areas.

Altogether, our studies uncovered a rich set of molecular genetic tools that can now be used to dissect the functions of neurons upstream of AgRP neurons in specific brain areas in the control of appetite and energy metabolism.

Methods

Mice

Agrp-IRES-Cre[30] (Jax stock no: 012899), Ai6[31] (Jax stock#: 007906), Pomc-eGFP[41] (Jax stock#: 009593), and POMC-Cre[42] (Jax stock#: 005965) mice were purchased from the Jackson Laboratory. All procedures involving mouse handling were approved by the Fred Hutchinson Cancer Center Institutional Animal Care and Use Committee.

Viral vectors

PRVB177 was constructed as previously described[16]. Briefly, a CMV promoter, a flexstop-flanked sequence encoding a PRV thymidine kinase (TK) fused with a hemagglutinin (HA) epitope tag, and a SV40 polyadenylation signal, were cloned into PRV TK-BaBlu, a TK-deleted PRV Bartha strain between its gG locus sequences matching 5′ and 3′ to the lacZ sequence. Recombinant virus clones were selected and confirmed as described previously[16].

Recombinant PRVs were propagated in PK15 cells (ATCC) using a multiplicity of infection (M.O.I.) = 0.1~0.01. Three days after infection, cells were harvested by scraping. Material was frozen using liquid nitrogen and then quickly thawed in a 37°C water bath 3 times. Cell debris was then removed by centrifugation twice at 1,000xg for 5 minutes. The titer of supernatant was determined using standard plaque assays on PK15 cells, with titers expressed in plaque-forming units (PFU).

Stereotaxic injection

Stereotaxic injection was performed as previously described[16]. Virus suspension (1–2 × 10^9 PFU) was injected into the brains of mice aged 2-6 months using a 2 µl syringe at 100 nl per minute. Targeted brain areas were referenced based on a stereotaxic atlas[43] using a Stereotaxic Alignment System (David Kopf Instruments). Mice were treated with an inhalation anesthesia of 2.5% Isoflurane during injection. Animals were singly housed with regular 12 h dark/light cycles in the presence of food and water ad libitum after recovery.

Staining for neuropeptide mRNA and PRVB177 (HA)

Experiments were performed as described previously[16]. For perfusion, 4% paraformaldehyde (PFA) was used to perfuse animals transcardially. Brains were dissected out and then soaked in 4% PFA overnight. After soaking in 30% sucrose for 48 hours, the brains were frozen in OCT (Sakura) and stored at -80 °C before sectioning. For fresh frozen tissues, animals were decapitated immediately after cervical dislocation. Fresh brains were dissected out, snap frozen in isopentane mixed with dry ice, and kept at -80 °C. Brains were sectioned into 20 µm coronal sections using a cryostat.

For detection of PRV positive neurons, brain sections were incubated with biotinylated mouse anti-HA antibodies (BioLegend, #901505, 1:300) at 4°C overnight or at 37°C for an hour. Sections were then incubated with 0.5 µg/ml 4', 6-diamidino-2-phenylindole (DAPI, Sigma) and Alexa488 Streptavidin (Thermo Fisher, 1:1000) at room temperature for 1 hour followed by coverslipping with Fluoromount-G (Southern Biotech). For double staining of PRV-infected neurons expressing neuropeptides, coding regions of neuropeptide genes were amplified from mouse brain cDNA using PCR and cloned into the pCR4 TOPO vector (Thermo Fisher). Digoxigenin (DIG)-labeled cRNA probes (riboprobes) were prepared using the DIG RNA Labeling Mix (Roche). Twenty µm coronal cryostat sections of mouse brains frozen in OCT were hybridized to DIG-labeled cRNA probes at 56°C for 13–16 hours. After hybridization, brain sections were washed in 5xSSC and 0.2×SSC at 63°C for 30 minutes each consecutively. Sections were incubated with POD-conjugated anti-DIG antibodies (Roche, #11207733910, 1:2000) and biotinylated anti-HA antibodies (BioLegend, #901505, 1:300) at 4°C for overnight or at 37°C for an hour, and treated using the TSA-plus Cy3 kit (Perkin Elmer) according to manufacturer's instruction. Sections were then incubated with 0.5 µg/ml DAPI and Alexa488 Streptavidin (Thermo Fisher, 1:1000) at room temperature for 1 hour. Slides were coverslipped with

Fluoromount-G.

Dissociation of AgRP neurons and RNA-sequencing

AgRP-IRES-Cre mice[30] were crossed with Ai6[31] mice, which have Cre-dependent expression of eGFP, to generate AgRP-Cre:Ai6 mice. Arcuate nuclei of AgRP-Cre:Ai6 mice were then dissected out as previously described[33] to obtain AgRP+ cells. GFP-labelled single cells were manually isolated from AgRP-Cre:Ai6 mice using a micropipette and visualizing cells under a fluorescent microscope. Single cells were lysed, and cDNA libraries were generated similar to our previously published protocols[25,32,33]. A total of 20 cells with good quality cDNAs were analyzed to confirm the expression of AgRP and NPY. The following intron-spanning primers were used: AgRP-5', CAACTGCAGACCGAGCAGAAG, AgRP-3'-GCAGCAAGGTACCTGCTGTC, NPY-5', GGCACCCAGAGCAGAGCAC, and NPY-3', CCAGAATGCCCAAACACACG. cDNAs that confirmed expression of markers were processed for Illumina sequencing similar to previously published methods[33]. Sequenced reads were initially mapped to the mouse genome to generate reads per million (RPM) data. Later on, Fragments Per Kilobase of exon per Million mapped fragments (FPKM) data were also used with standard methods (Tophat and Cufflinks)[44,45]. The list of GPCRs was obtained from the International Union of Basic and Clinical Pharmacology (IUPHAR)/ British Pharmacological Society (BPS) website (http://www.guidetopharmacology.org).

Cell counting

Stained brain sections were imaged with AxioImager.Z1 microscope. Brain structures were identified based on a mouse brain atlas[43]. Numbers of PRV-infected cells in brain areas of every fifth section were counted. To acquire approximate total number of cells in each brain area of an animal, brain areas were judged to contain upstream neurons if they contained ≥ 2 labeled neurons in ≥ 50% of animals. All data are shown as the mean±SEM. Neuropeptide counts were confirmed with TissueFax in Fred Hutch Cancer Center Shared Resources.

Abbreviations for brain areas

Modified based on Franklin and Paxinos[43].

Acb	accumbens nucleus
ACo	anterior cortical amygdaloid area
AH	anterior hypothalamic area
AHi	amygdalohippocampal area
AmPir	amygdalo-piriform transition area
AON	anterior olfactory nucleus
aPir	piriform cortex, anterior part
ARC	arcuate hypothalamic nucleus
CA1	field CA1 of the hippocampus
CEnt	caudomedial entorhinal cortex
CxA	cortex-amygdala transition zone
DA	dorsal hypothalamic area
DG	dentate gyrus
DIEnt	dorsal intermediate entorhinal cortex
DM	dorsomedial hypothalamic nucleus
DTM	dorsal tuberomammillary nucleus
LEnt	lateral entorhinal cortex

LH	lateral hypothalamic area
LOT	nucleus of the lateral olfactory tract
LPO	lateral preoptic area
ME	median eminence
MEA	medial amygdala
MEnt	medial entorhinal cortex
MM	medial mammillary nucleus, medial part
MPA	medial preoptic area
MPO	medial preoptic nucleus
MTu	medial tuberal nucleus
OC	olfactory cortex
Pe	periventricular hypothalamic nucleus
PeF	perifornical nucleus
PH	posterior hypothalamic nucleus
PLCo	posterolateral cortical amygdaloid area
PLH	peduncular part of lateral hypothalamus
PMCo	posteromedial cortical amygdala
PMD	premammillary nucleus, dorsal part
PMV	premammillary nucleus, ventral part
pPir	piriform cortex, posterior part
PSTh	posterior subthalamic nucleus
PVN	paraventricular nucleus of the hypothalamus
RCH	retrochiasmatic area
RM	retromammillary nucleus
SCh	suprachiasmatic nucleus
SHy	septohypothalamic nucleus
SO	supraoptic nucleus
StHy	striohypothalamic nucleus
Te	terete hypothalamic nucleus
TT	tenia tecta
Tu	olfactory tubercle
TuLH	tuberal region of lateral hypothalamus
VA	vomeronasal amygdala
VEn	ventral endopiriform claustrum
VIEnt	ventral intermediate entorhinal cortex
VMH	ventromedial hypothalamic nucleus
VTM	ventral tuberomammillary nucleus

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Author contributions
D.K., N.K.H., and L.B.B. conceived the project. D.K. conducted and analyzed all of the viral tracing experiments and investigation of neuropeptides in upstream neurons and

determined ligands of AgRP neuron receptors. N.K.H. isolated single cells, prepared cDNA libraries and conducted the transcriptome analyses of single AgRP neurons and identified receptors for neuromodulators. C-Y.L and A.H. injected animals with virus, X.Y. stained brain sections, X.Y. and M.E. analyzed sections with TissueFax, and D.K., N.K.H., and L.B.B. wrote the manuscript.

Competing interests

The authors declare that they have no competing interests. L.B.B was on the Board of Directors of International Flavors & Fragrances during most of this work.

Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper. Additional data and materials related to this paper may be requested from the authors.

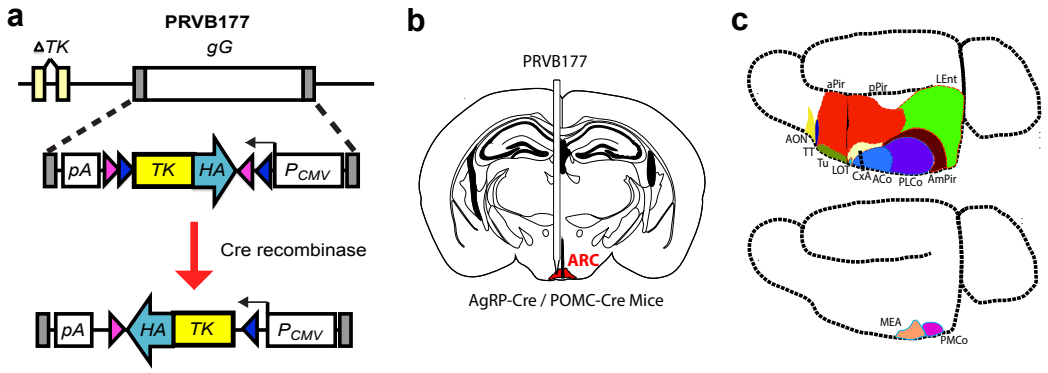


Fig. 1 | Strategy to assess possible input to ARC AgRP or POMC neurons from higher olfactory areas. a, Cre recombinase causes irreversible expression of TK-HA from PRVB177, allowing it to replicate and cross synapses. *gG*, *gG* locus; *PCMV*, cytomegalovirus promoter; *pA*, polyadenylation signal. **b**, PRVB177 was injected into the ARC (red) of AgRP-Cre or POMC-Cre mice. **c,** Brain sections were then immunostained for PRV (HA) to identify PRV-infected neurons in areas of the olfactory cortex (above) or vomeronasal amygdala (below).

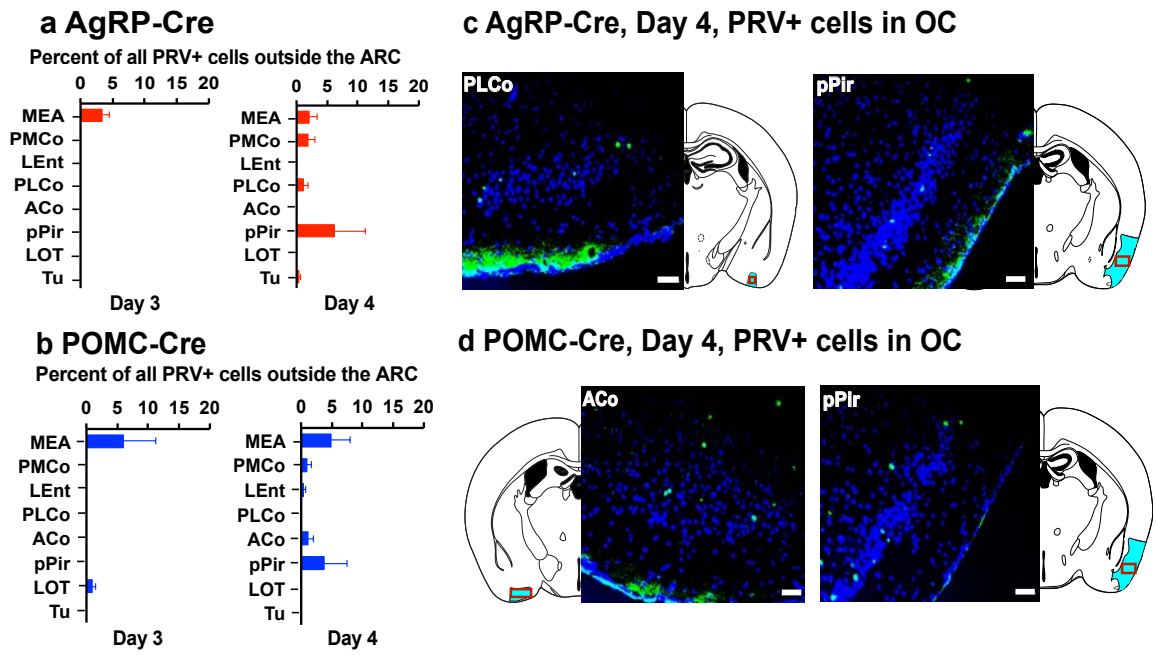


Fig. 2 | AgRP and POMC neurons receive input from higher olfactory areas. a, **b**, Mean percentages of PRV+ neurons outside the ARC in olfactory areas 3 or 4 days after injection of AgRP-Cre **(a)** or POMC-Cre **(b)** mice with PRVB177. Post-injection day 3: AgRP-Cre (n = 16); POMC-Cre (n = 4). Post-injection day 4: AgRP-Cre (n = 6); POMC-*Cre* (n = 4). Error bars indicate s.e.m. See Methods for full names of abbreviated brain areas. **c**, **d**, Photographs and diagrams of olfactory area sections with neurons immunostained for PRV on day 4 post-injection of AgRP-Cre **(c)** or POMC-Cre **(d)** mice. PRV+(HA+), green; DAPI counterstain, blue. Scale bars, 100 μm. Corresponding areas on diagrams are labeled with red rectangles in OC areas indicated in cyan.

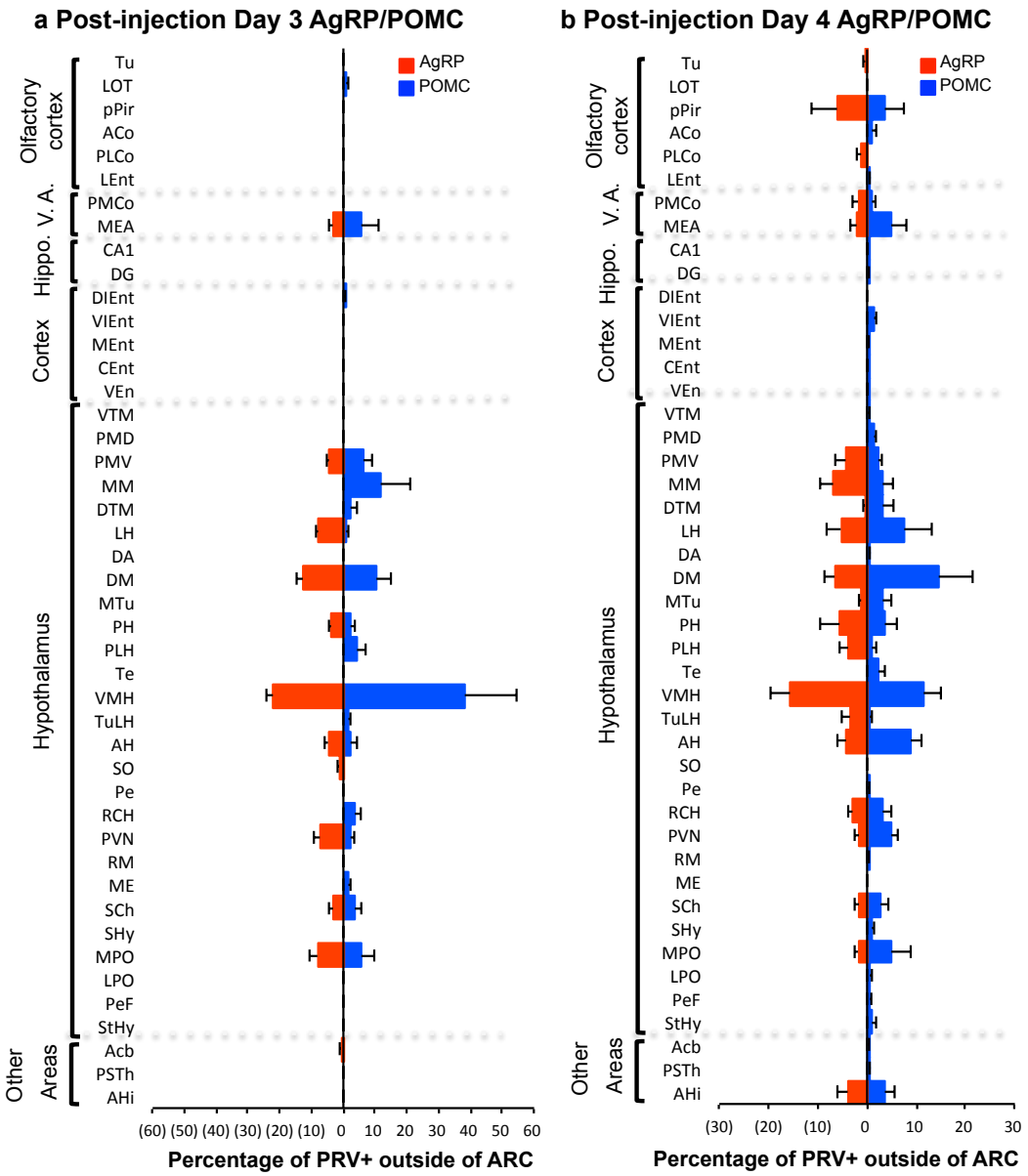


Fig. 3 | Brain areas with PRV+ neurons after infection of AgRP or POMC neurons. Bar graphs show the percentages of all non-ARC PRV+ cells in individual brain areas three **(a)** and four **(b)** days after infection of ARC AgRP (red) or POMC (blue) neurons. Post-injection day 3: AgRP-Cre (*n* = 16); POMC-Cre (n=4). Post-injection day 4: AgRP-Cre (*n* = 6); POMC-Cre (n=4). Error bars indicate s.e.m. V.A., vomeronasal amygdala; Hippo., hippocampus. See Methods for full names of abbreviated brain areas.

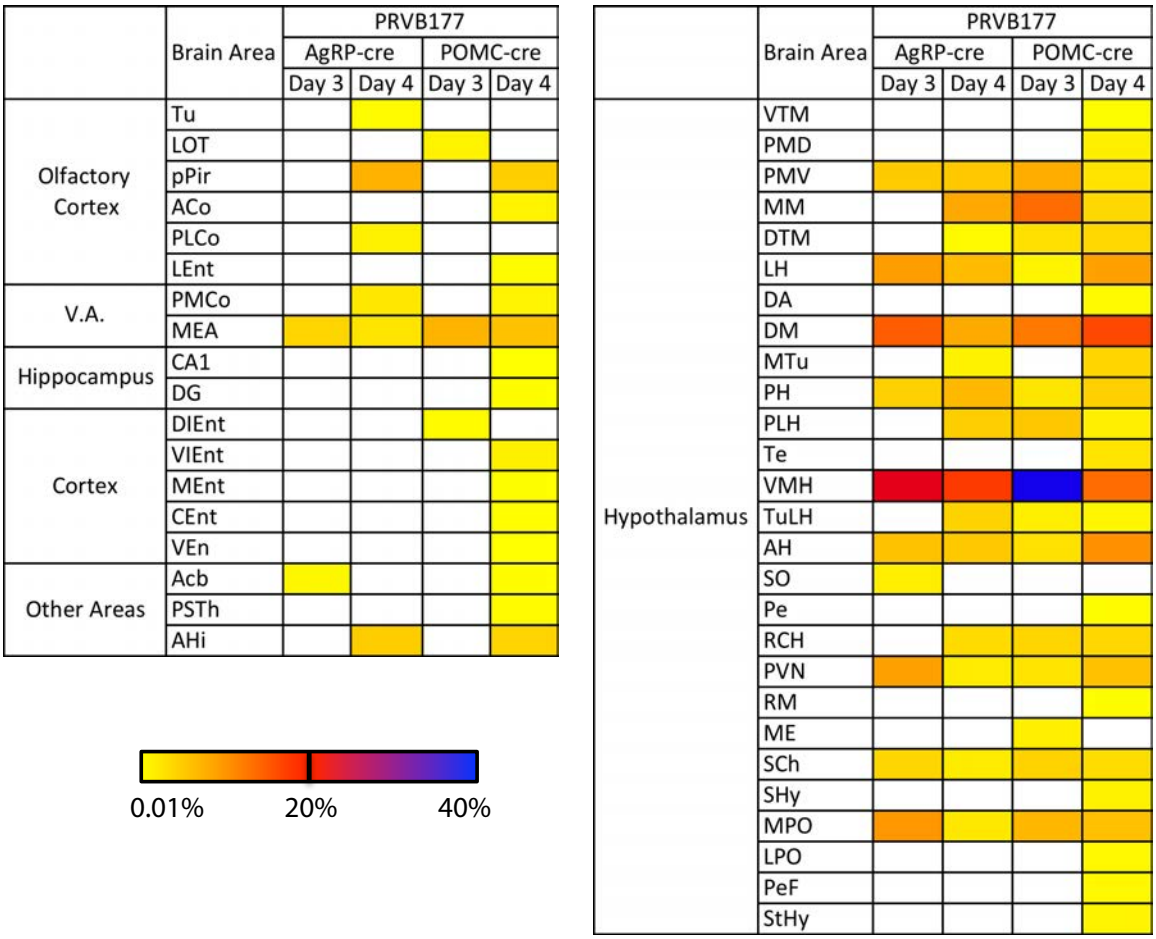


Fig. 4 | Heat map of brain areas with neurons upstream of AgRP and POMC neurons. Colored boxes indicate approximate percentages of all non-ARC PRV+ neurons seen in individual brain areas (white indicates none) on day 3 or 4 after ARC injection of AgRP-Cre or POMC-Cre mice with PRVB177. Post-injection day 3: AgRP-Cre (*n* = 16); POMC-Cre (n=4). Post-injection day 4: AgRP-Cre (*n* = 6); POMC-Cre (n=4). Areas with PRV+ neurons on day 3 are directly upstream of AgRP or POMC neurons. Areas with PRV+ neurons on day 4 but not day 3 are indirectly upstream of AgRP or POMC neurons. V.A., vomeronasal amygdala. See Methods for full names of abbreviated brain areas.

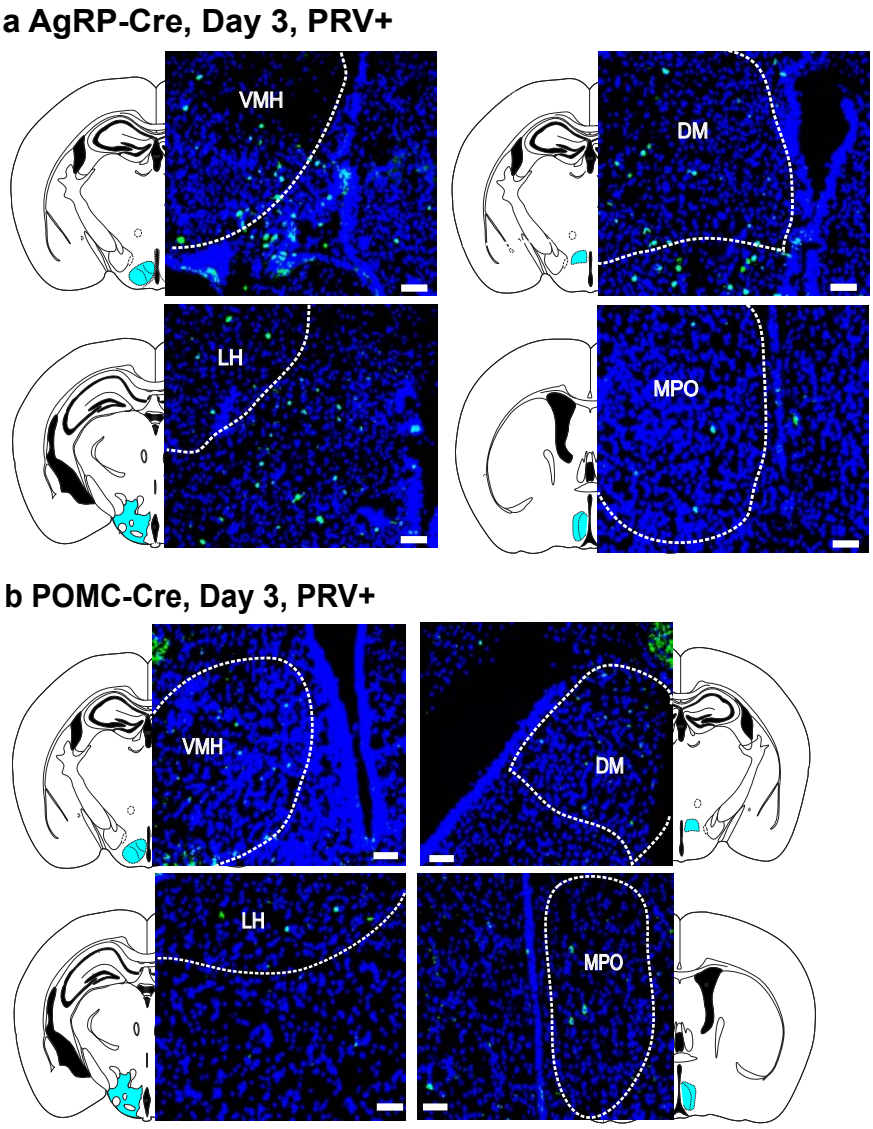


Fig. 5 | Non-olfactory areas with PRV+ neurons directly upstream of AgRP or POMC neurons. Photographs show cells immunostained for PRVB177 (green) in different areas on day 3 after injection of AgRP-Cre **(a)** or POMC-Cre **(b)** mice. DAPI counterstain, blue. Corresponding areas on diagrams are labeled with cyan. Scale bars, 100 μm. Dotted lines indicate locations of brain areas.

a Neurotransmitter GPCRs

Ligand	Gene	Single AgRP Neurons																		
Glutamate	*Grm5*															■	■			
	Grm7		■				■	■		■	■	■							■	■	
GABA	*Gabbr1*	■		■			■	■			■	■	■	■	■	■	■	■	■	■	■
	Gabbr2	■		■												■			■		
ADP/ATP	*P2rx4*	■											■	■					■	■	■
	P2rx6			■																	
	P2ry1													■	■						
	P2ry14		■	■																	

b Biogenic amine GPCRs

Ligand	Gene	Single AgRP Neurons																		
Epinephrine, Norepinephrine	*Adra1a*			■											■			■		
	Adra2a	■																		
Histamine	*Hrh3*	■						■		■	■			■	■		■	■	■		■
Serotonin	*Htr1a*	■												■			■	■			■
	Htr1b									■											
	Htr6													■							

c Neuropeptide/other GPCRs

Ligand	Gene	Single AgRP Neurons																		
Pacap, Vip	*Adcyap1r1*			■	■	■	■	■		■	■	■	■	■		■	■	■	■	■	■
Melanin-concentrating hormone	*Mchr1*						■										■				■
Neuropeptide FF	*Npffr1*							■													
	Npffr2									■											
Opiates	*Oprl1*	■						■			■	■	■	■			■	■	■	■	■
	Oprk1																				■
Cortistatin, Somatostatin	*Sstr1*	■								■					■						■
	Sstr4																		■		
Prokineticin	*Prokr2*						■							■				■			■
Calcitonin, Cgrp, Amylin, Adrenomedullin	*Calcr*	■		■			■				■	■	■	■	■		■	■	■	■	■
Ghrelin	*Ghsr*	■					■	■		■			■								
Cannabinoid	*Cnr1*											■									■
Corticotropin-releasing factor	*Crhr1*		■	■																	
Galanin	*Galr2*																		■		
Orexin	*Hcrtr1*		■				■														
Melonocortin	*Mc3r*	■		■										■		■	■		■	■	
Neuropeptide Y	*Npy1r*	■					■							■							
	Npy2r	■					■	■		■	■	■	■	■		■	■	■	■	■	■
	Npy5r	■																			
Neurotensin	*Ntsr2*															■					
Bradykinin	*Bdkrb2*										■										
Chemokine	*Cxcr6*		■										■								
Neuronostatin	*Gpr107*				■					■						■			■	■	■
Lysophosphatidic acid	*Lpar1*	■																			
Prostanoid	*Ptger1*													■							
Lysophospholipid	*S1pr3*	■																			
Thrombin	*F2r*																	■		■	

Fig. 6 | AgRP neurons differentially express numerous GPCRs. Transciptome data from individual AgRP neurons indicate that single AgRP neurons can express multiple GPCR genes and that those genes are expressed in different combinations in different neurons. Shown here are GPCRs with known ligands indicated on the left. Colored boxes indicate expression in individual AgRP neurons. GPCRs expressed in AgRP neurons included GPCRs for neurotransmitters (**a**), biogenic amines **(b)**, and neuropeptides and other ligands **(c)**.

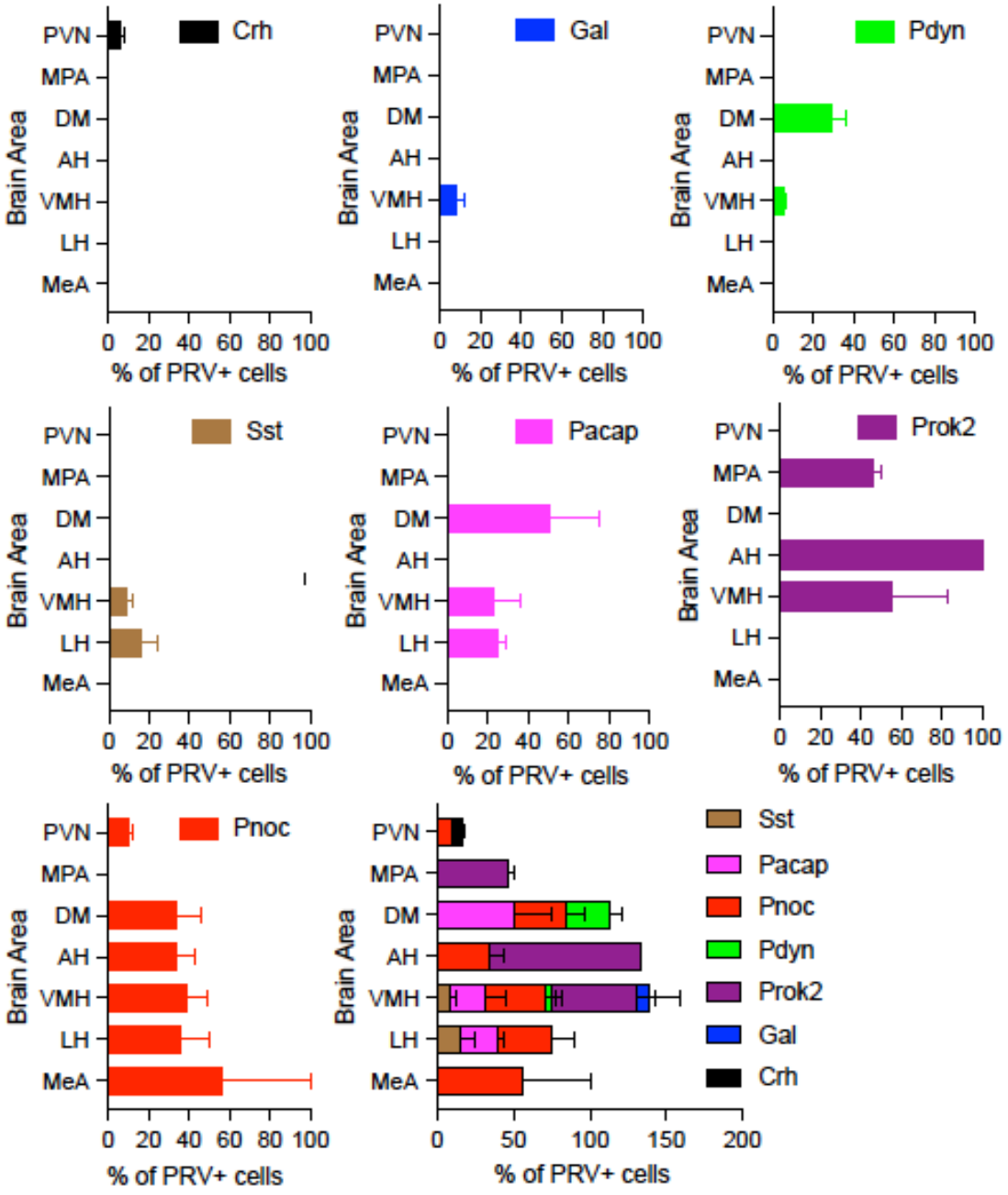


Fig. 7 | Expression of specific neuropeptides in PRV+ cells directly upstream of AgRP neurons in different areas. Graphs show the percentages of all non-ARC PRV+ neurons colabeled for individual neuropeptides in different areas on day 3 after injecting AgRP-Cre mice with PRVB177 (n = 3-4). Error bars indicate s.e.m. See Methods for full names of abbreviated brain areas.

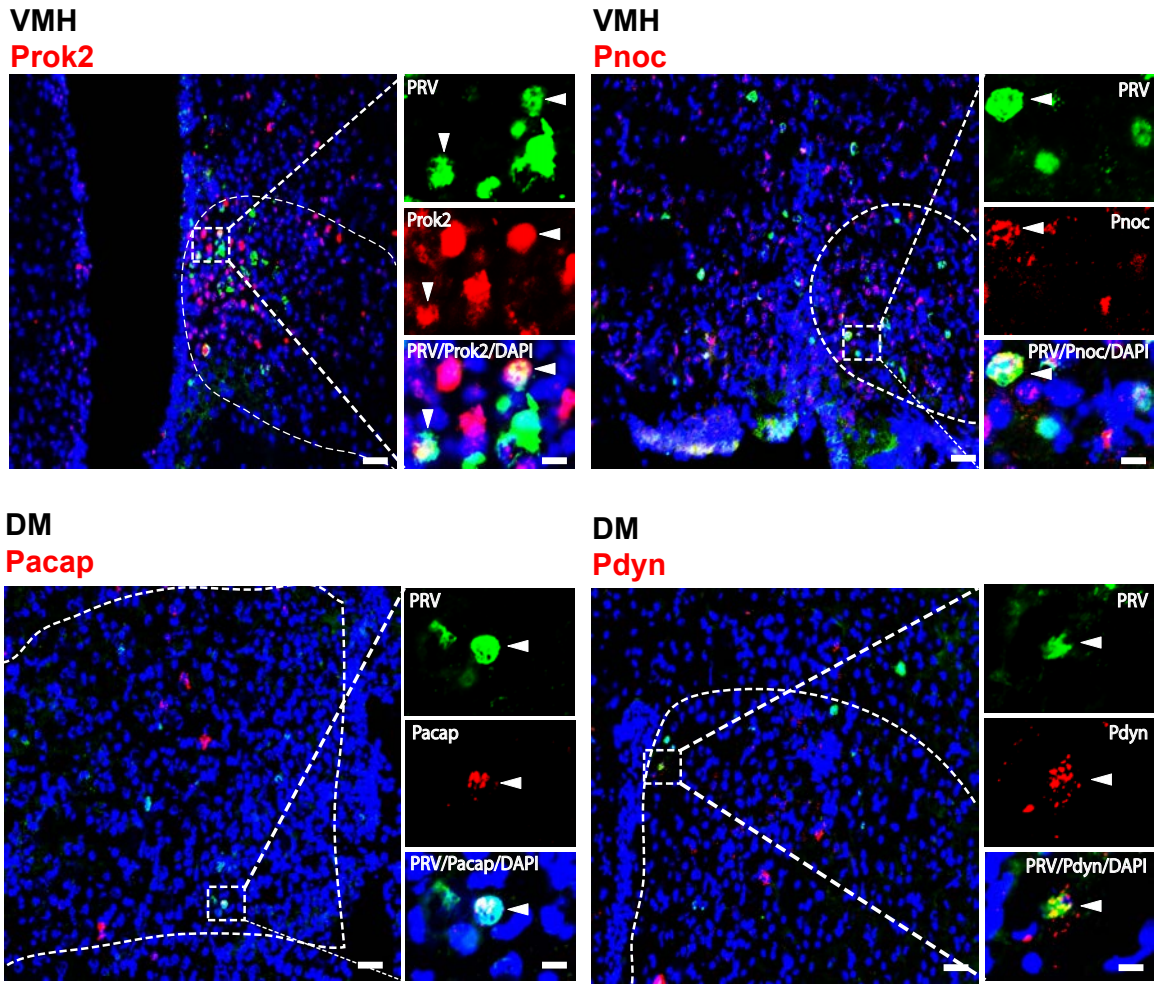
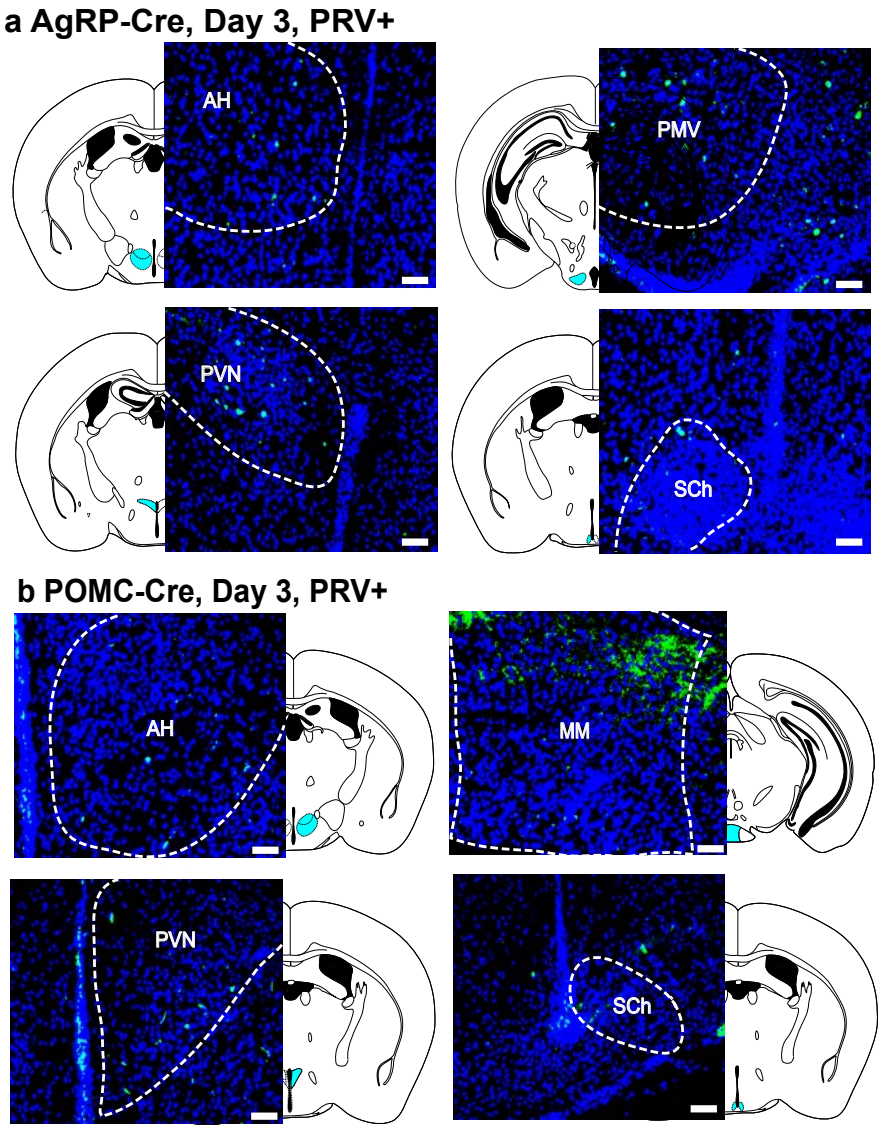
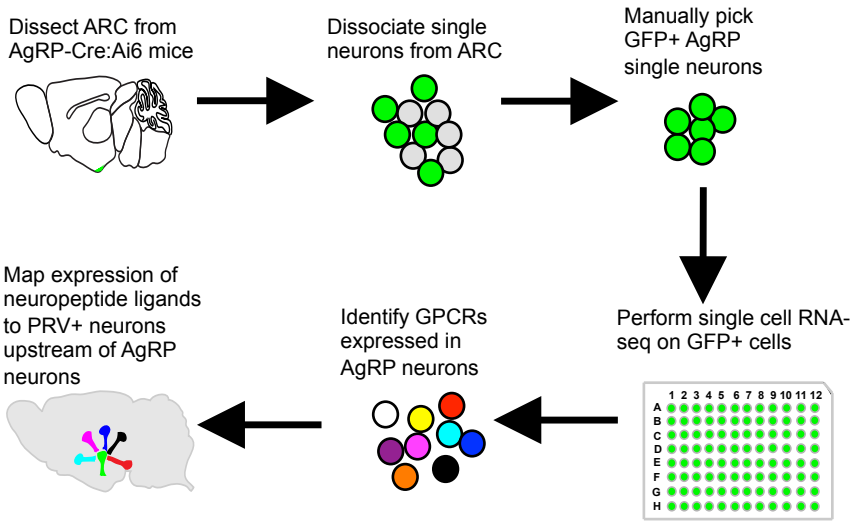


Fig. 8 | Expression of individual neuropeptides in single PRV+ cells. Photographs show expression of individual neuropeptides (Prok2, Pnoc, Pacap, or Pdyn) in single PRV+ neurons in VMH or DM. PRV+, green; neuropeptide+, red; DAPI+, blue. Brain areas are indicated by dotted lines. Boxed areas are shown at higher magnification at right. Scale bars, 100 μm (left) and 20 μm (right).



Extended Data Fig. 1 | Additional non-olfactory areas with PRV+ neurons directly upstream of AgRP or POMC neurons. Photographs show cells immunostained for PRVB177 (green) in different areas on day 3 after injection of AgRP-Cre **(a)** or POMC-Cre **(b)** mice. DAPI, blue. Corresponding areas on diagrams are labeled with cyan. Scale bars, 100 μm. Brain areas are indicated by dotted lines.



Extended Data Fig. 2 | Identification of GPCRs expressed in AgRP neurons and upstream neurons expressing their ligands. The ARC was dissected from AgRP-Cre:Ai6 mice and dissociated into single cells. Single GFP+ AgRP neurons were manually isolated and subjected to single cell RNA-seq. Transcriptome data was used to identify GPCRs expressed in single AgRP neurons and those with known ligands determined. The expression of individual neuropeptide ligands in neurons directly upstream of AgRP neurons was determined by infecting AgRP neurons with PRVB177 and costaining brain sections for PRV and different neuropeptides.

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