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AgRP neurons mediate activity-dependent development of oxytocin connectivity and autonomic regulation

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During postnatal life, leptin specifies neuronal inputs to the paraventricular nucleus of the hypothalamus (PVH) and activates agouti-related peptide (AgRP) neurons in the arcuate nucleus of the hypothalamus. Activity-dependent developmental mechanisms impact refinement of sensory circuits, but whether leptin-mediated postnatal neuronal activity specifies hypothalamic neural projections is largely unexplored. Here, we used chemogenetics to manipulate the activity of AgRP neurons during a discrete postnatal critical period and evaluated the development of AgRP inputs to the PVH and descending efferent outflow to the dorsal vagal complex (DVC). In leptin-deficient mice, targeting of AgRP neuronal outgrowth to PVH oxytocin neurons was reduced, and despite the lack of leptin receptors found on oxytocin neurons in the PVH, oxytocin-containing connections to the DVC were also impaired. Activation of AgRP neurons during early postnatal life not only normalized AgRP inputs to the PVH but also oxytocin outputs to the DVC in leptin-deficient mice. Blocking AgRP neuron activity during the same postnatal period reduced the density of AgRP inputs to the PVH of wild type mice, as well as the density of oxytocin-containing DVC fibers, and these innervation deficits were associated with dysregulated autonomic function. These findings suggest that leptin-mediated AgRP neuronal activity is required for the development of PVH connectivity and represents a unique activity-dependent mechanism for specification of neural pathways involved in the hypothalamic integration of autonomic responses.

leptin | autonomic | hypothalamus | paraventricular hypothalamic nucleus | neural development

Significance

Hypothalamic neural circuits maintain homeostasis by coordinating endocrine signals with autonomic responses to ensure that physiological responses remain in tune with environmental demands. The paraventricular nucleus of the hypothalamus (PVH) plays a central role in metabolic regulation, and the architecture of its neural inputs and axonal projections is a defining feature of how it receives and conveys neuroendocrine information. In adults, leptin regulates multiple aspects of physiology, but it also functions during development to direct formation of circuits controlling homeostatic functions. Here, we demonstrate that leptin acts to specify the input–output architecture of PVH circuits through an activity-dependent, transneuronal mechanism, which represents a unique means of neuroendocrine circuit development.

Early postnatal life is a period of dynamic brain growth and development, as young animals adapt to rapidly changing homeostatic needs and increased metabolic challenges. Accordingly, neural systems develop in response to metabolic and neuroendocrine sensory inputs that reflect the changing environment and nutritional conditions. In addition to coordinating energy intake and expenditure in response to changes in peripheral energy status, during development, the adipocyte-derived hormone leptin functions as a neurotrophic factor and directs the formation of homeostatic neural pathways (1–5). Leptin signaling is required for postnatal axon outgrowth from the arcuate nucleus of the hypothalamus (ARH) to targets in the forebrain (6), including the paraventricular hypothalamic nucleus (PVH). Leptin also specifies distinct cellular targeting patterns to functionally defined compartments of the PVH, as well as cell type-specific targeting of ascending Glucagon-like peptide-1 (GLP-1) afferents from the nucleus of the solitary tract (NTS) that carry visceral sensory information to corticotropin-releasing hormone neurons in the PVH (7, 8). These neurotrophic actions of leptin are distinct from its regulatory role in adults and appear to be restricted to a discrete postnatal critical period that ends during the fourth postnatal week (9).

In addition to secreted growth factors, axon guidance cues, and circulating molecules such as leptin, neuronal activity is critical for the organization and functional maturation of precise neural circuits during development. This phenomenon has been studied extensively in the visual system, where early patterns of neuronal activity exert significant lasting effects on ocular dominance (10); however, it is not known whether postnatal neuronal activity plays a similar role in specifying the architecture of hypothalamic circuitry. Although it is well established that development of agouti-related peptide (AgRP) inputs to preautonomic components of the PVH are dependent on environmental nutritional conditions (11, 12), the contribution of postnatal neuronal activity on development of PVH outputs to the dorsal vagal complex (DVC) in the hindbrain is not known (13, 14).

The PVH consists of multiple cell types that are organized into distinct functional compartments and integrates endocrine and visceral sensory information to coordinate autonomic responses. Within the PVH, a subpopulation of oxytocin neurons provide

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hypothalamic control of metabolism and ingestive behavior by modulating autonomic responses through projections to autonomic preganglionic neurons of the medulla and spinal cord (15–23). Oxytocin neural circuits mature relatively late in development, as mature oxytocin peptide is not detected until after birth, and axon outgrowth progressively increases during the first few weeks of postnatal life (24–26). Descending oxytocin projections that arise from caudal PVH neurons and target the DVC are negligible at postnatal day 0 (P0) but markedly increase in density postnatally, reaching adult-like levels by weaning (27). Altered oxytocin signaling has also been implicated in several neurodevelopment disorders, including Prader–Willi syndrome, Fragile X syndrome, autism spectrum disorders, and others (28–32),

Here, we assessed whether postnatal activity of AgRP neurons impacts the innervation of oxytocin neurons in the PVH, and if AgRP neuronal activity or leptin are required for the development of descending PVH oxytocin projections to the DVC. The results suggest that not only is leptin required for specifying oxytocin projections from the PVH to the DVC, but this developmental action of leptin on oxytocin neurons is receptor-independent and depends on AgRP neuronal activity, suggesting that the input–output architecture of oxytocin neurons is developmentally specified by leptin through an activity-dependent transneuronal mechanism.

Results

Activity-Dependent Development of AgRP Projections to the PVH. Previous studies have established that axonal projections from AgRP neurons reach their target destinations during the first 2 wk of postnatal life and that leptin signaling during this restricted postnatal sensitive period is required to achieve the full adult density and distribution of AgRP innervation (6, 33). Because neural activity is also known to regulate the establishment and refinement of neural circuits, we used a chemogenetic approach to inhibit AgRP neuronal activity during a critical period for postnatal development of AgRP projections to the PVH. Inhibitory DREADD (designer receptors exclusively activated by designer drugs) hM4Di receptors were selectively targeted to AgRP neurons (AgRP-Cre-hM4Di mice). Daily postnatal injections of clozapine-N-oxide (CNO; 1.0 mg/kg i.p.) were administered from P4 to P14 (Fig. 1*A*). Induction of the inhibitory hM4Di DREADD signaling pathway resulted in labeling AgRP-Cre-hM4Di neurons with the fluorescent reporter mCitrine by P10 (Fig. 1*B*). The majority of mCitrine-labeled AgRP-Cre-hM4Di-expressing neurons in the arcuate nucleus were also responsive to leptin at this age, consistent with previous reports showing that AgRP neurons are sensitive to leptin during postnatal life. Postnatal CNO administration reduced the number of leptin-induced cFos-immunoreactive nuclei in the ARH of AgRP-Cre-hM4Di mice by 67%, confirming the effectiveness of CNO-mediated neuronal inhibition of AgRP neurons in postnatal mice (Fig. 1*C*). Notably, DREADD-mediated AgRP neuronal inhibition significantly reduced the density of AgRP-immunolabeled fibers in the PVH of adult AgRP-Cre-hM4Di mice, compared with that of saline-injected control mice (Fig. 1 *D*–*F*), suggesting that the development of these projections is dependent on the activity of AgRP neurons during postnatal life.

Postnatal Inhibition of AgRP Neuronal Activity Is Associated with Autonomic Dysfunction. Disrupted development of AgRP projections to preautonomic PVH neurons is associated with autonomic neuropathy (7). To test whether postnatal AgRP neuronal activity impacts long-term autonomic function, adult AgRP-Cre-hM4Di mice that had received postnatal injections of saline or CNO (but remained untreated after P14) were exposed to 4 °C environmental conditions for a sustained period. In response to this cold challenge, the ability to maintain core body temperature was severely impaired in adult AgRP-Cre-hM4Di mice that had received postnatal CNO treatment (Fig. 1*G*). All mice had similar baseline core temperatures before cold exposure, and after 30 min at 4 °C, the core temperature of all mice started to significantly decrease. However, by 30 min, core temperature of AgRP-Cre-hM4Di +CNO animals decreased by twice as much as all other groups, indicating their ability to maintain body temperature was quickly and severely impaired. As the duration of cold exposure increased, all mice began to lose the ability to maintain body temperature in response to the cold environment, and after 180 min, AgRP-Cre-hM4Di +CNO animals were no longer able to maintain body temperature. However, concomitant daily treatment with leptin and CNO from P4 to P14 normalized core temperature of AgRP-Cre-hM4Di mice to that of controls (Fig. 1*G*), indicating that exogenous leptin administration during postnatal life can at least partially rescue activity-dependent defects and sympathetic dysregulation.

The impact of silencing postnatal AgRP activity on gastrointestinal (GI) reflexes was tested by measuring GI transit time (GITT) in response to metabolic stimuli known to impact GI physiology. Oral gavage of Ensure led to longer GITT, but this response was blunted in AgRP-Cre-hM4Di mice treated postnatally with CNO (Fig. 1*H*). Furthermore, while acute oxytocin injection did not impact the GITT of adult mice in the fed state on a chow diet, GITT of AgRP-Cre-hM4Di mice treated with postnatal CNO significantly increased upon oxytocin administration (Fig. 1*H*). Additionally, the gut epithelial wall of CNO-treated AgRP-Cre-hM4Di mice appeared to be less permeable to macronutrient content, as evidenced by a significant decrease in the concentration of Fluorescein isothiocyanate (FITC)-labeled dextran in blood plasma after administration by oral gavage, indicating that macronutrient partitioning or nutrient absorption may be functionally impaired in adult mice that experienced reduced activity of AgRP neurons during postnatal life (Fig. 1*I*).

Leptin Is Required for Postnatal Targeting of Preautonomic PVH Oxytocin Neurons by AgRP Neurons. Leptin promotes axonal outgrowth and cellular targeting of AgRP projections to functionally and cytoarchitectonically identified compartments of the PVH (7), but whether leptin specifies AgRP inputs to other cell types is unknown. The PVH is composed of multiple cell types, including oxytocin neurons. Therefore, we quantified AgRP-immunoreactive axons in close contact with caudal PVH oxytocin neurons in *Lep*$^{ob/ob}$ mice and WT littermate controls. Consistent with previous results, AgRP inputs to the dorsal (dp), lateral (lp), and ventral medial parvocellular (mpv) compartments of the caudal PVH were significantly reduced in adult leptin-deficient animals (Fig. 2 *A*, *D*, and *G*). Moreover, by P30, the density of AgRP-immunoreactive terminals closely apposed to oxytocin neurons was significantly reduced in leptin-deficient mice compared with controls (Fig. 2 *C*, *F*, and *I*), indicating that leptin is required for AgRP axonal outgrowth to oxytocin neurons in the PVH. These changes in AgRP density are not due to leptin-induced changes in cell size because average neuron number and volume sampled were not altered by leptin deficiency (Fig. 2 *B*, *E*, and *H*).

Because AgRP innervation of PVH preautonomic neurons, including parvocellular oxytocin neurons, is especially sensitive to postnatal leptin, we used Cre-dependent, AAV-mediated axonal tracing in adult Oxytocin-Cre mice to map the projections of

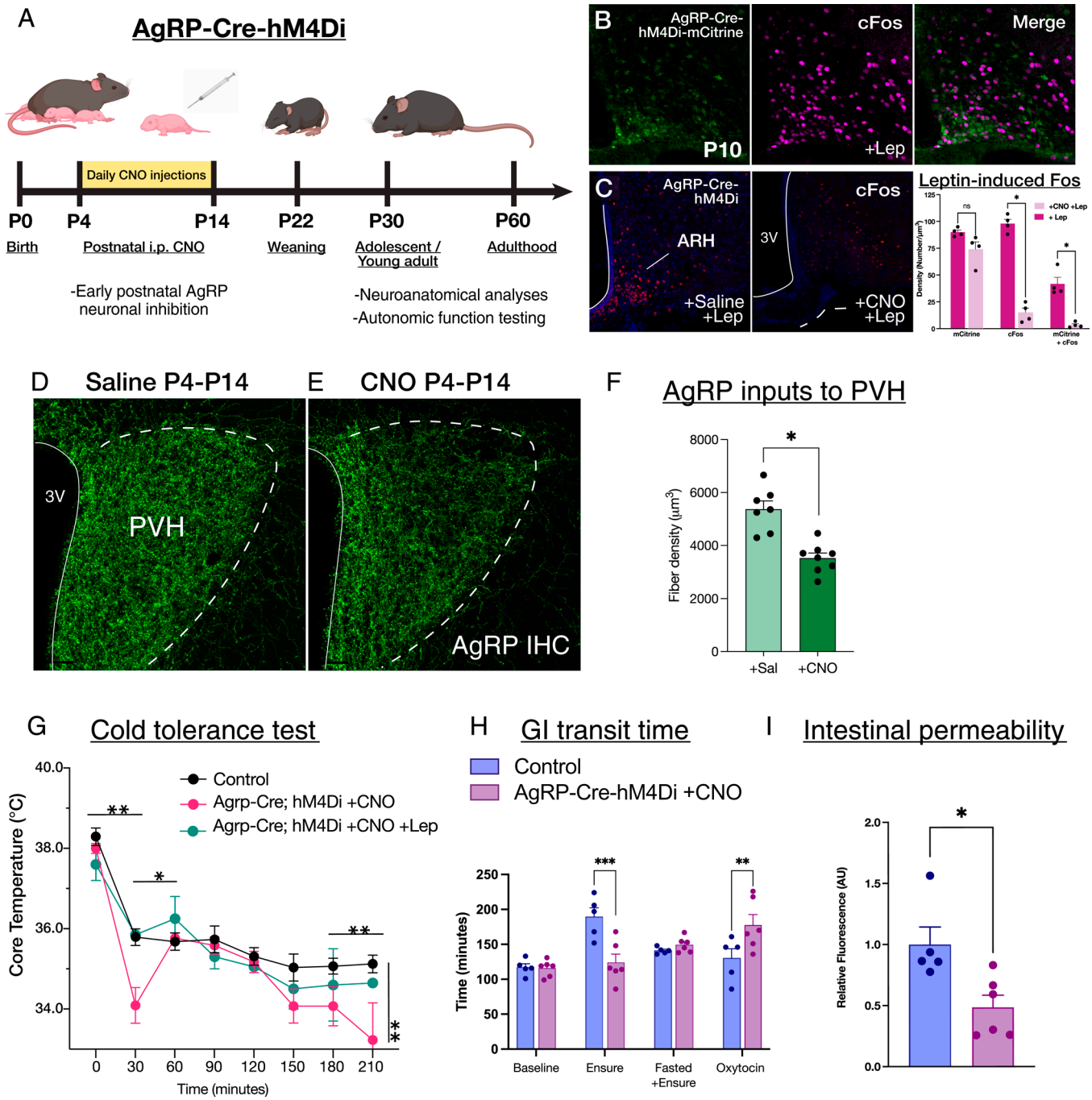

Fig. 1. Postnatal activity of AgRP neurons specifies axon outgrowth and regulates autonomic output. Schematic of experimental paradigm. Mice received daily CNO (1.0 mg/kg i.p.) injections from P4 to P14, and neuroanatomical experiments and tests of autonomic function were conducted in adolescence and adulthood unless denoted otherwise (*A*). Confocal images illustrating ARH AgRP-Cre-hM4Di mCitrine expression and localization of leptin-induced cFos at P10 (*B*). Confocal images illustrating ARH leptin-induced cFos-immunoreactive nuclei in mCitrine-labeled neurons in AgRP-Cre-hM4Di mice that had received i.p. injection of saline or CNO 1 h prior to leptin injection (*C*). Postnatal DREADD-mediated AgRP neuronal inhibition results in significantly decreased AgRP fiber density in the PVH at P30, compared with control AgRP-Cre-hM4Di mice that received postnatal i.p. saline injections (*D–F*). Early postnatal AgRP neuron inhibition resulted in impaired thermoregulatory responses when exposed to a cold environment in adulthood (*G*), as well as dysregulated GI transit time in response to multiple GI metabolic stimuli (*H*) and altered intestinal permeability (*I*). Two-way repeated-measures ANOVA with multiple comparisons was used to compare groups in the cold challenge assay, unpaired *t* tests were used to compare data between two groups. Differences between groups were considered statistically significant at $P < 0.05$. Asterisk denotes statistically significant comparisons.

these neurons in the dorsomedial medulla (Fig. 3*A*). Viral transduction and location of AAV injection was confirmed by colocalization of tdTomato fluorescence and oxytocin immunolabeling (Fig. 3 *B–D*) at the injection site. In these mice, we observed tdTomato-labeled axons in the DVC (Fig. 3 *E–G*), which is composed of the NTS, the dorsal motor nucleus of the vagus nerve (DMX), and the area postrema (AP). The tdTomato-labeled Oxytocin-Cre axonal inputs were not uniformly distributed within the DVC and appeared to target specific regional domains and subnuclei of the DVC. The medial, gelatinous, and commissural subnuclei of the NTS at the level of the AP contained the highest density of tdTomato-labeled oxytocin axons (Fig. 3 *F* and *G*). These regions are known to receive input from vagal afferents and are associated with viscerosensory transmission, especially gastric functions (27). At the level of the hindbrain with an open 4th ventricle, just anterior to the most rostral appearance of the AP,

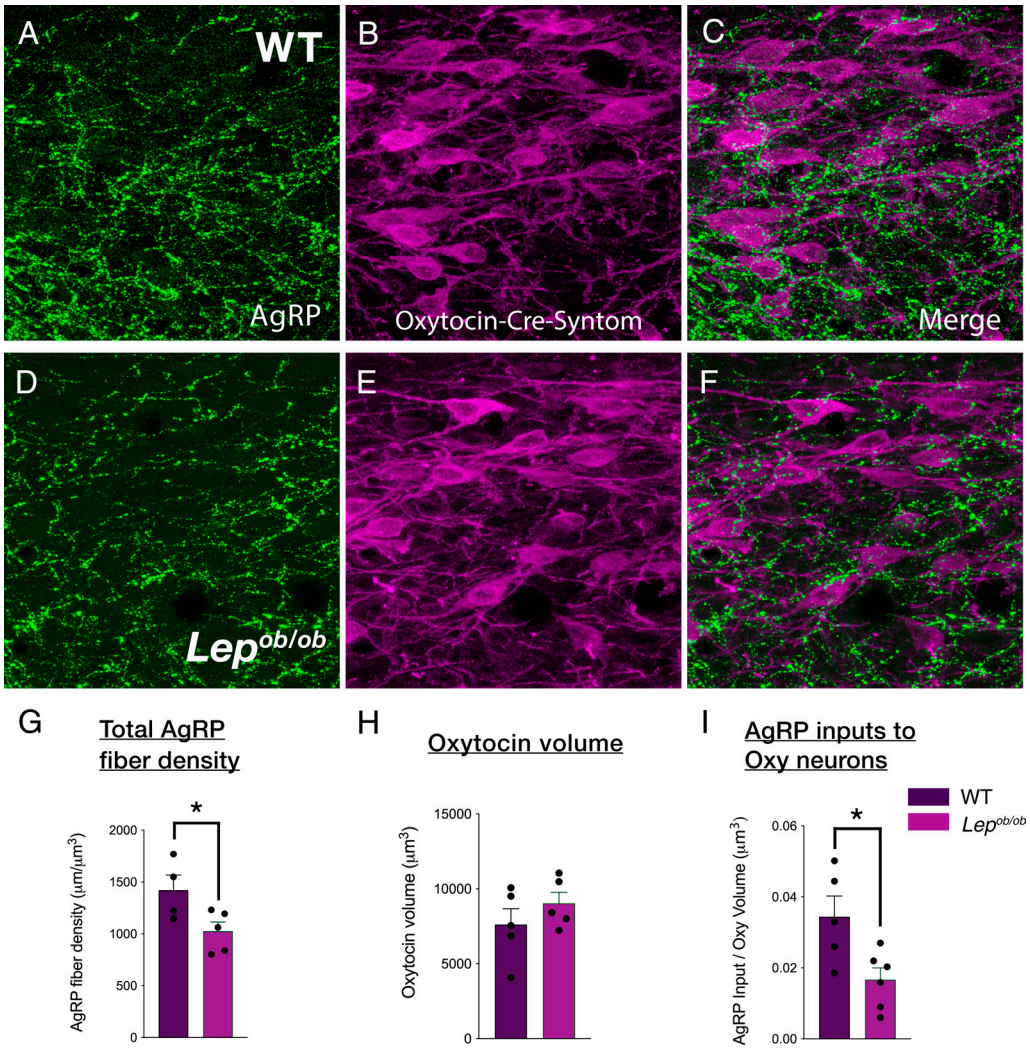

Fig. 2. Leptin specifies targeting of AgRP projections to PVH oxytocin neurons. Confocal images depicting AgRP-immunoreactive axons (green) and Oxytocin-Cre-SynTom fluorescence (magenta) in Oxy-Cre-SynTom-WT (*A–C*) and leptin-deficient Oxy-Cre-SynTom-OB mice (*D–F*) in the PVH at P30. Density of total AgRP-immunoreactive axons within the PVH was significantly decreased in leptin-deficient mice (*A*, *D*, and *G*). No differences were observed in oxytocin volume (*B*, *E*, and *H*). AgRP-immunoreactive axons targeting Oxytocin-Cre neurons were also significantly decreased in leptin-deficient mice compared with WT controls (*C*, *F*, and *I*).

the dorsomedial NTS also received substantial oxytocin innervation (Fig. 3*E*). The NTS was generally more densely innervated by PVH oxytocin axons than the DMX, while the AP and underlying subpostremal area exhibited minimal axonal labeling (Fig. 3 *F* and *G*). Within the DMX, a considerable number of inputs were found along the dorsolateral edges of the medial gastric columns and the lateral celiac columns (Fig. 3*E*). At mid-AP levels of the caudal DVC, (Fig. 3 *F* and *G*), within the most lateral part of the medial NTS, along the dorsolateral edge of the underlying DMX, a very dense oxytocin terminal field was observed, presumably where the rostral A2 noradrenergic neurons and caudal C3 adrenergic cell groups are known to be located. A few scattered axons were seen in NTS subnuclei lateral to the solitary tract (ts).

Leptin and Postnatal Development of Descending PVH Oxytocin Projections to the DVC. To determine whether PVH oxytocin neurons are sensitive to the postnatal actions of leptin, mice received acute injection of either saline or leptin (10 mg/kg i.p.) at P16 and subsequently perfused for immunohistochemical localization of cFos. Leptin treatment resulted in a moderate number of cFos-immunoreactive nuclei in multiple parts of the PVH, primarily the dorsal, ventral, and medial parvocellular regions (Fig. 3 *H* and *I*). These functionally defined subcompartments are located in the caudal PVH and project to the DVC, where they are known to regulate GI functions (34–37). However, although leptin-induced cFos was observed in the PVH at P16, it was virtually absent within oxytocin neurons (Fig. 3 *H* and *I*). The density of oxytocin-immunolabeled neurons that coexpress LepRb-targeted tdTomato was also low at P16 (Fig. 3 *J–L*), suggesting that leptin receptors are not broadly expressed on PVH oxytocin neurons during postnatal life. However, PVH oxytocin neurons appear to receive dense inputs by axons containing LepRb-Cre-tdTomato fluorescence, presumably derived from other neuronal populations that provide direct inputs to PVH oxytocin neurons (Fig. 3 *J–L*). Therefore, although PVH oxytocin neurons do not appear to express LepRb and are not directly stimulated by postnatal leptin, development of descending oxytocin projections may take place through an alternative mechanism, perhaps through target-dependent signaling at its receptor. To explore the possibility that oxytocin receptor-expressing neurons in the DVC are activated by leptin during postnatal development, mice with Oxytocin Receptor-Venus fluorescence were assessed for leptin-induced phospho-STAT3 (pSTAT3)-immunoreactivity. Robust induction of pSTAT3-immunoreactivity was primarily localized to the medial and commissural NTS subnuclei, as well as the adjacent gracile nucleus, while pSTAT3 nuclear labeling was absent in

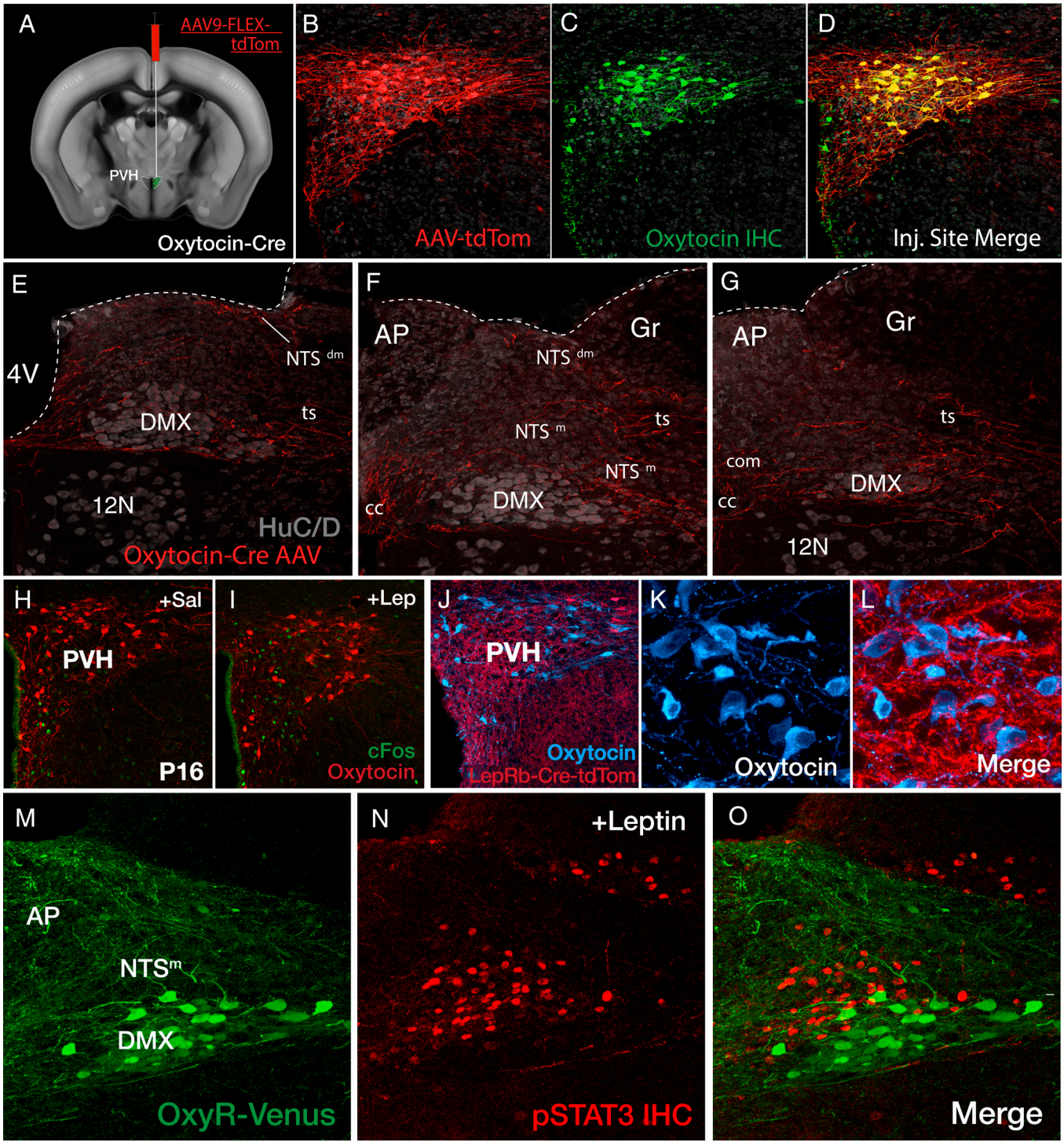

Fig. 3. Leptin signaling in oxytocin and oxytocin receptor-expressing neurons in the PVH and DVC. Stereotaxic injection of Cre-dependent AAV-tdTomato virus into the PVH of adult Oxytocin-Cre mice labels projections of neurons expressing Oxytocin-Cre (*A*). Confocal images depicting representative injection site of AAV-tdTomato virus (red) into Oxytocin-Cre-expressing neurons and oxytocin-immunolabeled neurons (green) in the caudal PVH (*B–D*). TdTomato-labeled Oxytocin-Cre axonal inputs (red) are shown at three levels of the dorsomedial medulla after AAV-mediated recombination. Neurons (gray) are immunolabeled with the pan-neuronal marker HuC/D (*E–G*). Confocal images of oxytocin (red) and cFos (green) immunolabeling in the PVH of mice injected with i.p. saline or leptin at P16 (*H* and *I*). Representative confocal images of oxytocin-immunolabeled neurons (blue) and LepRb-Cre-tdTom fluorescence (red) in caudal regions of the PVH at P18 taken with 20× (*J*) and high-magnification 63× objectives, respectively (*K* and *L*). Confocal image of OxyR-expressing neurons in the DVC at P30, visualized by OxyR-Venus fluorescence (*M*). Representative image of pSTAT3-immunoreactivity (red) in the DVC of OxyR-Venus mice injected i.p. with leptin (*N*). Merged image showing pSTAT3-immunoreactive nuclei in response to i.p. leptin injection and DVC OxyR-Venus labeled neurons (*O*). Abbreviations: PVH, paraventricular nucleus of the hypothalamus; DVC, dorsal vagal complex; AP, area postrema; DMX, dorsal motor nucleus of the vagus nerve; Gr, Gracile nucleus; NTSm, medial subnucleus of the solitary tract; NTSdm, dorsomedial subnucleus of the solitary tract; 3V, third ventricle; 4V, fourth ventricle; cc, central canal; ts, solitary tract.

oxytocin receptor neurons, suggesting that, like PVH oxytocin neurons, oxytocin receptor-expressing DVC neurons are likewise not directly responsive to leptin (Fig. 3 *M–O*).

Activity-Dependent Development of Oxytocin Inputs to the DVC. To compare the ontogeny of oxytocin projections to the DVC in WT and *Lep*$^{ob/ob}$ mice, oxytocin axons were visualized

by genetically targeting the synaptophysin-tdTomato (SynTom) axonal reporter to Oxytocin-Cre expressing neurons of WT (Oxytocin-Cre-SynTom-WT) and leptin-deficient (Oxytocin-Cre-SynTom-OB) mice at P8, P16, P30, and P60 d of age (Fig. 4 *A*–*H*, all ages quantified in Fig. 4 *I*–*L*). Oxytocin-Cre-SynTom labeled projections to the DVC were markedly reduced in *Lep*$^{ob/ob}$ mice by P16 (Fig. 4 *B*, *F*, and *J*) and remained low into adult life at P30 (Fig. 4 *C*, *G*, and *K*) and P60 (Fig. 4 *D*, *H*, and *L*). No changes were detected in the number of Oxytocin-Cre-SynTom labeled neurons in the PVH at P16 or P30 (Fig. 4 *M*–*Q*). The leptin-dependent innervation of PVH oxytocin neurons by AgRP neurons may provide an activation signal that subsequently impacts the development of oxytocin projections to downstream targets. To test this, we blocked postnatal AgRP neuron activity using DREADD-mediated inhibition and assessed oxytocin innervation of the DVC. Postnatal CNO treatment did not impact the number or density of PVH oxytocin neurons in AgRP-Cre-hM4Di mice (Fig. 5 *A* and *B*, quantified in Fig. 5*G*). However, after early postnatal AgRP neuronal activity was inhibited, the density of oxytocin projections found in the DVC was significantly reduced. Furthermore, early postnatal activity of AgRP neurons has long-term effects, as evidenced by sustained deficits in DVC oxytocin innervation found in peripubertal mice at P30 (Fig. 5 *C*, *D*, and *H*), as well as in adult animals at P60 and older (Fig. 5 *E*,

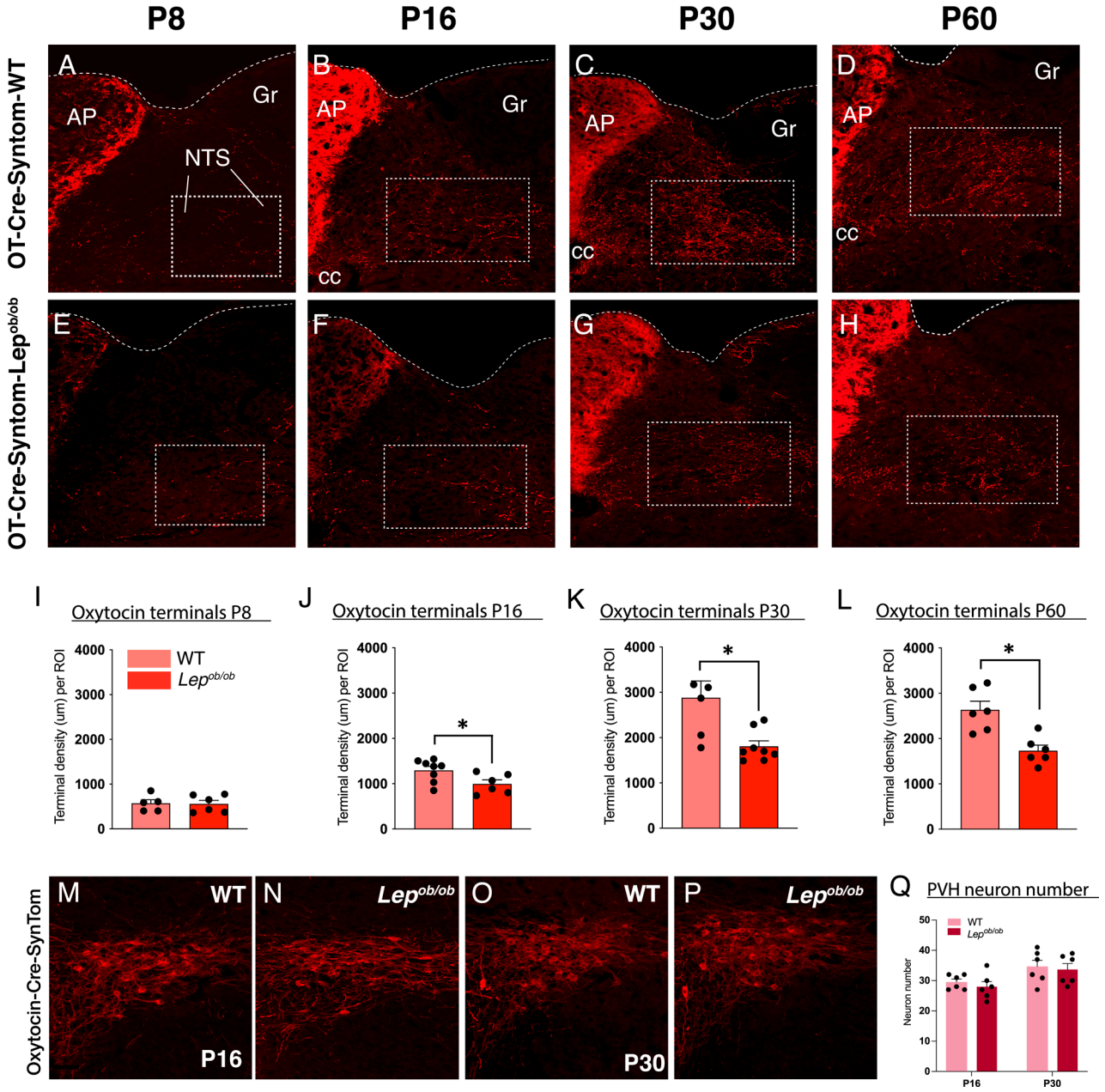

Fig. 4. Postnatal ontogeny of DVC oxytocin terminals is impaired in leptin-deficient mice. Density and distribution of DVC oxytocin terminals at postnatal ages P8, P16, P30, and P60 visualized by genetic targeting of fluorescent synaptophysin-tdTomato fusion protein (red) to neurons expressing Oxytocin-Cre (*A*–*H*). In WT mice (Oxy-Cre-SynTom-WT), Oxy-Cre-SynTom axons are observed in the caudal DVC as early as P8 (*A*), significantly increase in density by P16 (*B*), and reach adult-like levels by P30 (*C*), which are maintained at P60 (*D*). In leptin-deficient mice (Oxy-Cre-SynTom-Lep$^{ob/ob}$), a significant decrease in Oxy-Cre-SynTom labeled terminals emerged in the DVC at P16 (*B*, *F*, and *J*) and was maintained at P30 (*C*, *G*, and *K*) and P60 d of age (*D*, *H*, and *L*). No changes were detected in the number of Oxytocin-Cre-SynTom labeled neurons in the PVH at P16 or P30 (*M*–*Q*). Boxes indicate location of Region of interest (ROIs) used for quantitative analysis. Unpaired *t*-tests were used to test for significant differences between genotypes at each age examined. Asterisk denotes *P*-values < 0.05. Error bars indicate mean ± SEM; circles represent individual values. Images are maximum intensity projections from confocal image stacks collected through 30um-thick sections. Abbreviations: AP, area postrema; Gr, Gracile nucleus; NTS, nucleus of the solitary tract; cc, central canal.

F, and *I*). Thus, in contrast to the direct organizational actions of postnatal leptin on AgRP axonal targeting, these findings suggest that the specification of descending PVH oxytocin projections to the DVC requires activity-dependent AgRP neuronal outgrowth.

Because leptin activates AgRP neurons during postnatal life (38), we hypothesized that early postnatal activation of AgRP neurons in *Lep*$^{ob/ob}$mice could effectively function to restore the impaired PVH AgRP projections of *Lep*$^{ob/ob}$ mice, and potentially other circuits downstream, including oxytocin projections to the DVC. Leptin-deficient AgRP-Cre-hM3Dq-*Lep*$^{ob/ob}$ mice that received daily early postnatal CNO administration showed significantly higher densities of AgRP-immunoreactive terminals contacting oxytocin neurons in the PVH of adult mice, compared with those observed in *Lep*$^{ob/ob}$ mice that did not receive DREADD-mediated early postnatal AgRP neuronal activation (Fig. 6 *A*–*E*). Moreover, the density of oxytocin-immunoreactive fibers and terminals in the DVC was also greater in *Lep*$^{ob/ob}$ mice that were treated postnatally with CNO (Fig. 6 *F*–*I*). Together with the inhibitory DREADD data, these results suggest that leptin activates AgRP neurons during the postnatal critical period to promote AgRP axonal outgrowth to oxytocin neurons in the PVH and that this leptin-induced activity is required for oxytocin connectivity and autonomic regulation (Fig. 6*J*).

Discussion

As a central regulator of energy balance, the PVH integrates multiple sources of hormonal, viscerosensory, and extrasensory information to effectively coordinate metabolic state with feeding behavior and autonomic regulation. Key among these signals is leptin, which informs the brain about the status of energy stores in peripheral tissues (1, 39). Subpopulations of PVH neurons impact multiple aspects of autonomic regulation and feeding behavior through direct projections to regions such as the NTS, parabrachial nucleus, and lateral hypothalamus (34, 35, 40–43). Thus, changes in the input–output architecture of the PVH will likely alter how it functions to integrate these diverse signals and regulate downstream effectors of neuroendocrine and autonomic function.

Targeting of AgRP inputs to preautonomic PVH neurons, but not to neuroendocrine neurons in the PVH, can be sustainably rescued by treatment of *Lep*$^{ob/ob}$ mice with exogenous leptin during the first 2 wk of postnatal life (6, 7). During this same period, targeting of GLP-1 afferents from the NTS to specific subpopulations of PVH neurons are likely mediated by cell-autonomous leptin receptor signaling (8, 44). For example, in the present study, AgRP inputs to oxytocin neurons in the PVH are reduced in adult *Lep*$^{ob/ob}$ mice; and although ascending viscerosensory inputs from NTS Preproglucagon (PPG) neurons to PVH neurons are dependent on leptin, GLP-1 inputs to oxytocin neurons are unchanged (8), suggesting that the developmental actions of leptin can permanently specify the balance in sensory information normally conveyed to oxytocin neurons by at least these two afferent pathways.

Data from our AAV tracing experiments confirm that descending projections from PVH oxytocin neurons provide direct inputs to distinct regions of the DVC, where they influence autonomic function and several aspects of homeostasis by modulating the impact of incoming visceral sensory information (16–18, 21, 27, 36, 41, 42, 45–52). These descending oxytocin projections appear to be established during the postnatal period (27), when animals begin to explore solid food in preparation for independent ingestion (53), and also corresponds to when the postnatal leptin surge functions to impact the organization of afferents to the PVH (54). Our analysis of oxytocin projections to the DVC in *Lep*$^{ob/ob}$ mice suggests that leptin is required for postnatal specification of these projections, without affecting the number of PVH oxytocin neurons. However, in contrast to the direct receptor-mediated developmental actions of leptin on AgRP and GLP-1 afferents to the PVH, postnatal development of descending oxytocin projections to the DVC appears to be independent of direct LepRb-mediated signaling, because these cells do not display LepRb expression or STAT3 activation in response to postnatal leptin treatment. This observation suggests an indirect mechanism of leptin action on neural development, perhaps through activity-dependent activation of axon targeting. Thus, leptin signaling appears to function indirectly during postnatal life to alter the development of PVH

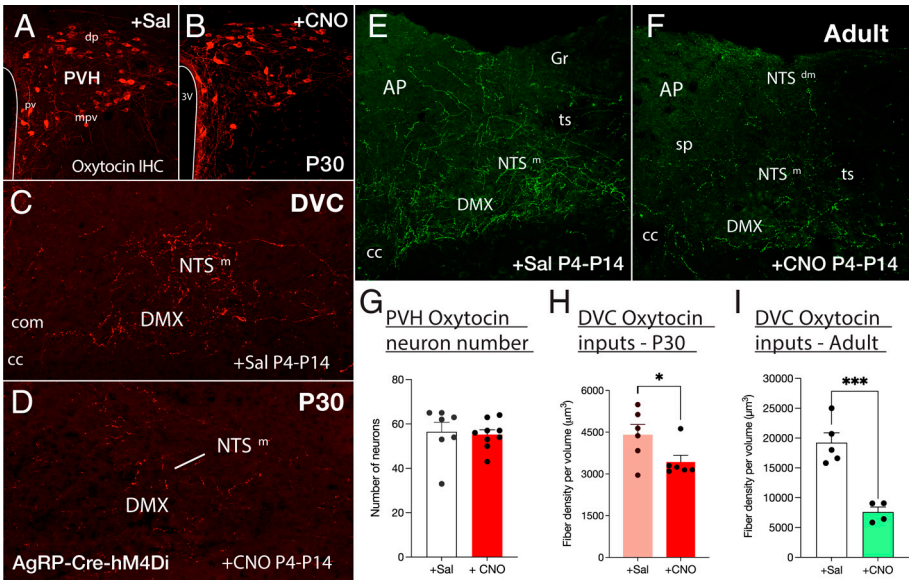

Fig. 5. Early postnatal AgRP neuronal activity specifies descending oxytocin projections to the DVC. Maximum intensity projection confocal images of oxytocin-immunolabeled neurons (red) in caudal PVH regions known to contain preautonomic neurons in AgRP-Cre-hM4Di mice at P30 that received daily postnatal injection of either saline or CNO i.p. from P4 to P14 (*A*, *B*, and *G*). Early postnatal DREADD-mediated AgRP neuronal inhibition led to a significant reduction in the density of oxytocin-immunolabeled inputs to the DVC at P30 (*C*, *D*, and *H*) and adult animals at P60 or older (*E, F,* and *I*).

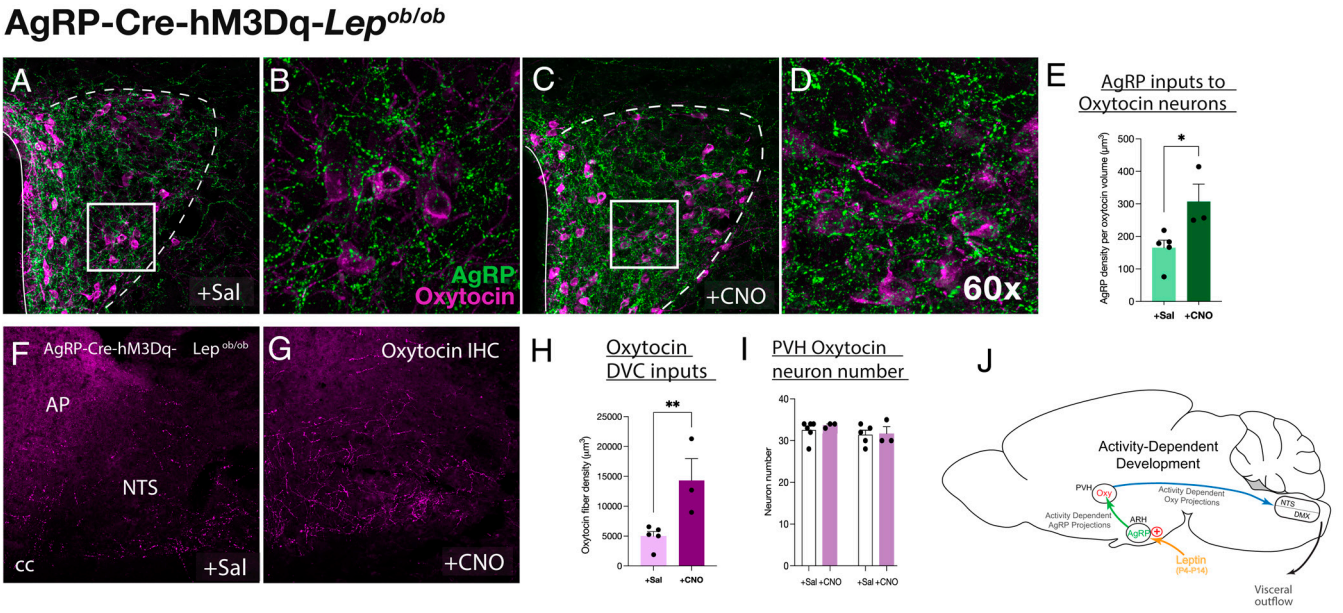

Fig. 6. Early postnatal activation of AgRP neurons in leptin-deficient AgRP-Cre-hM3Dq-Lep$^{ob/ob}$ mice restores impaired AgRP and oxytocin projections in Lep$^{ob/ob}$ mice. Representative images of AgRP (green) and oxytocin (magenta) immunolabeling in the PVH of AgRP-Cre-hM3Dq-Lep$^{ob/ob}$ mice that received saline (*A* and *B*) or CNO (*C* and *D*) daily from P4 to P14. DREADD-mediated early postnatal AgRP neuronal activation in AgRP-Cre-hM3Dq-Lep$^{ob/ob}$ mice that received daily CNO showed significantly elevated densities of AgRP-immunoreactive terminals closely apposed to oxytocin neurons in the PVH in adulthood, compared with those observed in Lep$^{ob/ob}$ mice that received saline (*E*). Density of oxytocin-immunoreactive fibers and terminals in the DVC was significantly higher in AgRP-Cre-hM3Dq-Lep$^{ob/ob}$ mice that were treated postnatally with CNO (*F*–*H*). No change was observed in PVH oxytocin neuron number in any of the groups tested (*I*). Schematic of proposed activity-dependent mechanism of action: During postnatal days (P4 to P14 leptin levels surge in neonatal mice and activate (depolarize; red plus symbol) AgRP neurons in the ARH. This early postnatal AgRP neuronal activation is required for normal development of AgRP axonal projections to the PVH where they innervate oxytocin neurons. Development of neural projections from oxytocin neurons to the DVC is also dependent on AgRP neuronal activity through a transneuronal mechanism of activity-dependent neural development, with downstream effects on autonomic function (*J*). Boxes on 20× images correspond to adjacent higher magnification images, and denote location of ROIs used for analysis. Confocal images taken with 20× (*A*, *C*, *F*, and *G*) and high- magnification (*B* and *D*) objectives, respectively. Abbreviations: Agi, AgRP-Cre-hM4Di; AgQO, AgRP-Cre-hM3Dq; PVH, paraventricular nucleus of the hypothalamus; ARH, arcuate nucleus of the hypothalamus; DVC, dorsal vagal complex; AP, area postrema; DMX, dorsal motor nucleus of the vagus nerve; NTS, nucleus of the solitary tract; 3V, third ventricle; cc, central canal.

oxytocin neurons, in contrast to its regulatory actions documented in adult rodents (16, 22, 55–57).

Although the findings reported here provide evidence for a unique role for leptin and activity-dependent AgRP neuronal outgrowth in the hypothalamus, activity-dependent axonal targeting has been well documented in other sensory neural systems. Studies in the visual system of multiple species have demonstrated that neural activity, either spontaneous or patterned, is required for normal development of central projections (10, 58, 59). Similarly, patterns of innervation in cerebellum and cerebral cortex are also dependent on neural activity (60–62). Based on our observations, the development of AgRP axonal outgrowth to the PVH and downstream targeting of oxytocin projections to the DVC appear to depend on the postnatal activity of AgRP neurons. Chemogenetic silencing of AgRP neuronal activity during the previously defined postnatal period for innervation of PVH neurons caused a lasting reduction in the density of AgRP inputs in adult mice. Moreover, we observed that chemogenetic activation of AgRP neurons during the same postnatal period normalizes the density of oxytocin projections to the DVC in *Lep$^{ob/ob}$* mice, suggesting that modulation of AgRP neuronal activity is sufficient to exert a lasting effect on the density of oxytocin projections to the DVC. However, the developmental effects of AgRP neuronal activity on oxytocin projections may not be limited to the first 2 wk of life, as is the case for the effects of leptin on the development of AgRP inputs to the PVH (9). For example, peripubertal silencing of AgRP neuronal activity using chemogenetics impacts the structure and function of cortical neurons, as well as food intake, indicating that these circuits may retain their plasticity longer (63). Together with previous results in *Lep$^{ob/ob}$* mice (6), our findings suggest that perturbations in leptin, or other signals that alter firing of AgRP neurons during postnatal development, may lead to permanent changes in PVH connectivity.

The anatomical changes in descending projections from PVH oxytocin neurons to DVC targets correspond to disruptions in cold tolerance and GI motility, thereby raising the possibility that postnatal AgRP activity may exert a lasting effect on autonomic function. Consistent with this, PVH oxytocin neurons have been implicated in control of adaptive thermogenesis and energy expenditure, both of which are impacted by leptin signaling (64–67). Moreover, neurons in the PVH, including oxytocin neurons, provide direct projections to regions containing preganglionic autonomic neurons that provide motor control of visceral functions (34, 68–71). Consistent with a modulatory role for oxytocin in gastric function (71), our data suggest that a decrease in oxytocin projections to the DVC is associated with decreased GITT and that administration of circulating oxytocin increased GITT in CNO-treated AgRP-Cre-hM4Di mice, compared with controls (Fig. 1*H*). In contrast to a reduction in GITT typically induced by oxytocin in adult animals, our data suggest that environmental conditions during postnatal development permanently impair multiple neural pathways involved in the hypothalamic integration of autonomic responses. Alternatively, it is plausible that the developmental manipulations of AgRP activity impact projections from other AgRP targets that normally innervate the DVC and regulate metabolic function. For example, feeding dams a high-fat diet during both prenatal life and lactation caused opposing changes in numbers of oxytocin and CRF neurons in the PVH, with corresponding changes in their inputs to the DVC (23). Nevertheless, the

structural dependency between AgRP activity and PVH oxytocin neurons suggests a hypothalamic-to-brainstem pathway by which PVH oxytocin may function as a downstream mediator of hypothalamic leptin to regulate meal-related satiety signals and other GI functions (16, 17, 72).

Previous ex vivo electrophysiological studies determined that leptin excites AgRP neurons during postnatal life, in contrast to its hyperpolarizing action in mature mice after they transition to independent feeding (38). Thus, the ability of leptin to activate AgRP neurons during postnatal development, when their projections are extending into the PVH, may function as a temporally restrained developmental signal analogous to retinal activity or experience during the critical period for development of the visual system (10, 58, 60). Moreover, this activity-dependent developmental action of leptin may extend to downstream targets involved in autonomic regulation. Impairment of oxytocin projections to the DVC caused by silencing postnatal AgRP neuron activity suggests that activity-dependent AgRP neuronal outgrowth to the PVH may provide a neural substrate for hypothalamic modulation of ingestive behavior and metabolism. Integration of neuroendocrine and visceral information through longitudinal oxytocin connections appears to regulate autonomic responses independent of cell autonomous LepRb signaling. Thus, in addition to direct receptor-mediated actions of leptin on development of AgRP projections to the PVH, regulatory factors that alter the activity of AgRP neurons during key postnatal developmental periods may also exert a lasting impact on targeting of AgRP axons in the PVH, as well as the downstream projections of cells innervated by AgRP neurons. Although determining how leptin calibrates topographic maps of AgRP innervation to functionally distinct components of the PVH requires additional studies, the findings reported here support the notion that activity-dependent patterning of AgRP inputs to the PVH, and subsequent development of oxytocin projections from the PVH to the DVC, represents the first demonstration of an activity-dependent mechanism for hormonal specification of hypothalamic circuitry.

Materials and Methods

Please see *SI Appendix* for detailed methodological information.

Animals. Mice were housed at 22 °C on a 12 h light–dark cycle and provided ad libitum access to a standard chow diet (PicoLab Rodent Diet 20 #5053) and water. Mice were weaned at P22 and maintained in their home cages with mixed genotype littermates until they were used for experiments. All animal care and experimental procedures were approved by the Institutional Care and Use Committee of Vanderbilt University and the Saban Research Institute at Children's Hospital Los Angeles. All procedures were performed in accordance with the guidelines of the NIH.

Experimental Paradigms and Analyses. Data are presented as group mean values ± SEM, as well as individual data points. Statistical analyses were performed using GraphPad Prism software (Version 9.5). Two-way repeated measures ANOVA with multiple comparisons was used to compare groups in the cold challenge assay. One-way ANOVA followed by a pairwise post hoc test was used to test for comparisons between three groups. Student's *t* test was used to compare data within two groups, using paired and unpaired tests where appropriate. Differences between groups were considered statistically significant at $P < 0.05$.

Data, Materials, and Software Availability. Study data are included in the article and/or *SI Appendix*. Image data have been deposited in Open Science Framework (https://osf.io/ytk7s/) (73).

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