Perineuronal net formation during the critical period for neuronal maturation in the hypothalamic arcuate nucleus

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In leptin-deficient ob/ob mice, obesity and diabetes are associated with abnormal development of neurocircuits in the hypothalamic arcuate nucleus (ARC), a critical brain area for energy and glucose homeostasis18–22. Because this developmental defect can be remedied by systemic leptin administration, but only if given before postnatal day 28, a critical period for leptin-dependent development of ARC neurocircuits has been proposed17. In other brain areas, critical-period closure coincides with the appearance of perineuronal nets (PNNs), extracellular matrix specializations that restrict the plasticity of neurons that they enmesh8. Here we report that in humans and rodents, subsets of neurons in the mediobasal aspect of the ARC are enmeshed in PNN-like structures. In mice, these neurons are densely packed into a continuous ring that encircles the junction of the ARC and median eminence, which facilitates exposure of ARC neurons to the circulation. Most of the enmeshed neurons are both γ-aminobutyric acid-ergic and leptin-receptor positive, including a majority of Agouti-related-peptide neurons. Postnatal formation of the PNN-like structures coincides precisely with closure of the critical period for maturation of Agouti-related-peptide neurons and is dependent on input from circulating leptin, because postnatal ob/ob mice have reduced ARC PNN-like material that is restored by leptin administration during the critical period. We conclude that neurons crucial to metabolic homeostasis are enmeshed in PNN-like structures and organized into a densely packed cluster situated circumferentially at the ARC-mediated eminence junction, where metabolically relevant humoral signals are sensed.

During critical periods of early postnatal life, developing neurocircuits are exquisitely sensitive to and shaped by external cues from the environment9. The critical period for ocular dominance plasticity in the primary visual cortex is a well-studied example: during the critical period, but not before or after, visual deprivation of one eye induces a strong shift of neuronal responses to the non-deprived eye10. The associated loss of acuity in the deprived eye, clinically referred to as amblyopia, is difficult to remedy after critical-period closure. In diverse neurocircuits ranging from the mammalian visual, barrel (somatosensory) and entorhinal cortices to the hippocampus and amygdala, and in song nuclei in the songbird brain, critical-period closure is dependent on the formation of PNNs5–14. PNNs are a specialized, condensed form of extracellular matrix (ECM), which is composed largely of hyaluronic and chondroitin sulfate proteoglycans, arranged in lattice-like structures that enmesh the soma and proximal dendrites of subsets of neurons, primarily inhibitory interneurons5–14. Remarkably, the experimental disruption of PNNs in adult animals can reactivate critical-period plasticity in these diverse brain areas. In the primary visual cortex, for example, PNN digestion enables restoration of vision to a previously deprived eye15. Comparable restorative effects of PNN disruption have been reported across neurological and psychiatric disorders as diverse as post-traumatic stress disorder, depression, drug addiction and spinal cord injury16–18.

Recently, compelling evidence was provided in support of the existence of a critical period for the maturation of Agouti-related peptide (AgRP) neurons in the hypothalamic ARC19. As key regulators of feeding behaviour, AgRP neurons are among the best studied of all hypothalamic neurons. These γ-aminobutyric acid-ergic (GABAergic) neurons co-express the potent orexigen neuropeptide Y (NPY), and they project to downstream targets in homoeostatic circuits governing energy balance and glucose homeostasis. Maturation of these projections occurs during the lactation period, concomitantly with a naturally occurring leptin surge (postnatal day (P) 4–14)19,20; this maturation process appears to be dependent on leptin, because these projections fail to develop properly in leptin-deficient ob/ob mice (a genetic model of obesity and type 2 diabetes). Moreover, this defect in AgRP-neuron development can be rescued by treatment with exogenous leptin, but only if administered before P28 (refs. 14,19). Therefore, a critical period for the trophic action of leptin on AgRP-neuron maturation exists, and its closure coincides with both the transition to independent feeding and the maturation of the cellular response of AgRP neurons to input from leptin19. This critical period is also a uniquely sensitive time when both over- and undernutrition result in a predisposition to obesity and glucose intolerance in adulthood19–22.
In this study, we show that key subpopulations of ARC neurons are enmeshed in PNN-like material in humans and rodents. These neurons are predominantly GABAergic, and many are leptin-receptor positive, including most AgRP neurons. These enmeshed neurons are organized into a densely packed ring that delimits the junction of the ARC and median eminence, a circumventricular organ that provides hypothalamic neurons with access to hormones and nutrients in the circulation. Moreover, the postnatal appearance of these PNNs coincides with the closure of the critical period for AgRP-neuron development and is dependent on input from leptin.

Because PNN-enmeshed neurons in other brain areas are not tightly packed, nor are they concentrated near circumventricular organs or regulated by circulating signals, the anatomical arrangement that we report in this study appears to be unique. Nevertheless, on the basis of our findings that the condensed ECM material that enmeshes neurons at the ARC–median eminence junction is both biochemically and ultrastructurally indistinguishable from PNNs in other brain areas, we refer to them as ‘PNNs’ (rather than ‘PNN-like structures’).

We performed immunohistochemistry (IHC) on serial coronal sections of mouse hypothalamus, using Wisteria floribunda agglutinin (WFA), a lectin that selectively labels the N-acetylgalactosamine residue on chondroitin sulfate chains in PNNs. Our initial survey (Supplementary Fig. 1) revealed numerous PNN-enmeshed cells localized to the junction of the ARC and median eminence (Fig. 1a–d). Compared with the rather sparse distribution of PNN-enmeshed neurons in most brain areas (for example, the primary visual cortex), enmeshed cells in the ARC–median eminence area were densely packed (Fig. 1e,g). To better characterize the structural features of these cells, we used high-resolution confocal microscopy combined with Imaris image analysis of individually labelled, PNN-enmeshed neurons located at the periphery of the dense cluster at the ARC–median eminence junction. Analysis of these cells (arrow f in Fig. 1c) allowed us to better define the anatomical relationship between PNNs and the neurons that they enmesh without the confounding influence of labelling on an adjacent, closely apposed cell. As expected, PNNs enwrapped both cell soma and proximal processes (Fig. 1f), in a manner reminiscent of the PNN structures described in the visual cortex (Fig. 1g,h). Whole-mount preparations of the mediobasal hypothalamus, stained with WFA and imaged from either the ventricular surface (Fig. 1i) or the ventral pial brain surface (Fig. 1j), revealed the presence of a continuous ‘collar’ of PNN-enmeshed cells at the junction of the ARC and median eminence.

As a first step to determine whether these ARC WFA-labelled structures are genuine PNNs, we micro-injected 10 mU of chondroitinase ABC (ChABC), an enzyme that digests PNNs, stereotactically into the ARC in wild-type mice. Subsequent histochemical analysis of these animals (n = 3) revealed ChABC-mediated digestion of ARC–median eminence PNNs on the injected side (Fig. 1k). Then, following a previously validated protocol, we performed pre-embedding WFA-diaminobenzidine (DAB) labelling of mouse brain sections for study with electron microscopy. This ultrastructural analysis revealed that DAB electron-dense deposits surround the cell soma (Fig. 1l,m, white arrowheads) and neurites (Fig. 1m, white arrows) of ARC neurons in a distribution that closely matches the pattern observed in PNN-enmeshed cells in the cortex and hippocampus (Supplementary Fig. 2).

In addition to the carbohydrate chains of chondroitin sulfate labelled by WFA, PNNs comprise two other major components: the chondroitin sulfate proteoglycan (CSPG) core proteins to which the chondroitin sulfate chains covalently bind, and hyaluronic acid, a long carbohydrate polymer to which CSPG core proteins non-covalently bind. Using biotinylated hyaluronic acid–binding protein (HABP)²⁴, we histochemically stained hyaluronic acid in the hypothalamus. Although HABP lightly stains the ECM throughout the brain parenchyma, we observed an increased abundance of hyaluronic acid that colocalizes with WFA at the junction of the ARC and median eminence (Fig. 1n and Supplementary Fig. 3), confirming that WFA-positive structures in this brain area contain hyaluronic acid. To identify the relevant CSPG(s) present in these PNNs, we next stained for each of the five major CSPG species found in PNNs elsewhere in the brain: aggrecan, brevican, neurocan, versican and phosphacan (PCAN). Interestingly, although each of these CSPGs was detected in various mouse brain areas (data not shown), only PCAN immunoreactivity was colocalized with WFA in the mouse ARC–median eminence (Fig. 1o and Supplementary Fig. 3).

We also immunostained for CD44, the cell-surface receptor for hyaluronic acid, and found that its expression in this brain area was largely limited to tanyocytes, with high expression localized to E3/β-tanyocytes piercing through the PNN domain and little to no expression in dorsal E2/α-tanyocytes that circumvented the PNN domain (Supplementary Fig. 4b,c). An IHC survey of CD44 expression in serial coronal sections through the hypothalamus revealed a striking colocalization with the PNN domain in the ventromedial ARC (Supplementary Fig. 4a). These findings raise the possibility that via activation of CD44, signal transduction in tanyocytes is evoked by hyaluronic acid present in PNNs.

The ARC is uniquely specialized to sense and transduce input from nutritionally relevant hormones (such as leptin and insulin) and nutrients (for example, glucose, free fatty acids and amino acids) into adaptive changes of food intake and energy metabolism. Among key neuronal subsets implicated in this homoeostatic circuitry are AgRP and pro-opiomelanocortin (POMC) neurons, the former of which is GABAergic. To identify distinct ARC neuronal subtypes among those enmeshed in PNNs (that is, the number of neurons of particular subtype/total number of PNN-enmeshed cells), we performed IHC on coronal sections from transgenic mouse lines that provided whole-cell green fluorescent protein (GFP) filling. Using glutamic acid decarboxylase 67-kDa isoform (GAD₆₇)-GFP-heterozygous knockin mice, we confirmed that most ARC neurons enmeshed in PNNs were GABAergic (81.8 ± 0.7% of ARC PNN-enmeshed cells; n = 3 GAD₆₇-GFP mice; the dots in the dot plots throughout this work represent data from independent animals; Fig. 2a–d), as is the case for PNN-enmeshed neurons in other brain areas. Three-dimensional (3D) reconstruction of GAD₆₇-GFP⁺ PNN-enmeshed cells (Supplementary Movie 1) revealed PNN enmeshing the soma and proximal dendrites of GABAergic ARC cells in a manner comparable to that of GABAergic interneurons in the primary visual cortex, with some differences in morphology, probably due to differences in cell type and process ramification (Supplementary Fig. 5 and Supplementary Movie 2).

To determine whether ARC neurons that express leptin receptors are represented among those enmeshed in PNNs, we used leptin-receptor long isoform (LepRb)-Cre/Ai14 tdTomato reporter mice to histochemically identify leptin-receptor-positive cells. These cells comprised 81.7 ± 1.5% of all PNN-enmeshed cells in the ARC (n = 3 LepRb-Cre/Ai14) (Fig. 2e–g). To identify AgRP neurons, we used NPY-GFP-transgenic mice (because NPY and AgRP are expressed in the same ARC-neuronal subset) and found these cells (which are GABAergic, and many of which express leptin receptors) also account for most ARC PNN cells (58.5 ± 1.0%; n = 3 NPY-GFP mice; Fig. 2h–j and Supplementary Movie 3). In comparison, POMC neurons composed a much smaller fraction of PNN-enmeshed cells in this brain area (13.7 ± 2.3%; n = 3 POMC-GFP mice) (Fig. 2k–m).

A separate but related question pertains to the fraction of AgRP or POMC neurons in the ARC that are enmeshed in PNNs (that is, the PNN-enmeshed neurons of particular subtype/total number of neurons of that subtype). As predicted, the fraction of AgRP neurons enmeshed in PNNs (78.3 ± 1.7% NPY-GFP neurons) is greater than the fraction of POMC neurons (43.6 ± 3.3% POMC-GFP neurons). A third peptidergic cell type found in the ARC expresses...
**Fig. 1 |** WFA labelling in the ventromedial ARC forms a ‘collar’ around the median eminence. The diagrams at the top show midsagittal (left) and ventral (right) views of the mouse brain, and the insets show the locations and orientations of the panel images. **a–d.** WFA-labelled (red) coronal sections through the ARC, starting just rostral to and progressing through the median eminence, show a concentration of WFA-labelled cells located in the ARC at its junction with the median eminence. Of note, the very intense staining below the median eminence corresponds not to the labelling around neurons but to the pia around the median eminence. **e.** Higher-magnification image of the boxed region in **c,** showing the dense cluster of WFA-labelled ARC cells. **f.** High-magnification Imaris 3D rendering of an isolated WFA-labelled cell at the periphery of the dense cluster (arrow f in **c**) reveals that WFA labels the soma and proximal processes of ARC cells. The inset shows the raw image. **g, h.** Low- (**g**) and high- (**h**) magnification images of PNNs labelled by WFA in the visual cortex, where they have been extensively studied, for comparison. A similar PNN pattern is apparent between **f** and **h** wrapping the soma and proximal process. **i, j.** WFA-labelled whole mounts of the ARC viewed from the third ventricle wall en face (**i**) or the ventral brain surface (**j**) reveal the distribution of labelled ARC cells forming a ‘collar’ around the median eminence, which does not contain labelling. From the ventricular surface view (**i**), the WFA-labelled ARC cells appear as a continuous band along the ventral margin of the ARC. **k.** WFA-labelled coronal section from a wild-type mouse killed 2 d after stereotactic, unilateral, intra-ARC injection of ChABC, an enzyme that digests chondroitin sulfate carbohydrates. **l.** Low-power electron micrograph of an ARC section labelled with WFA-DAB shows electron-dense DAB deposits surrounding a single ARC neuron (white arrowheads). **m.** High-power electron micrograph corresponding to the boxed region in **l** shows WFA labelling localized to the membrane around the cell soma (white arrowheads) and neurites (white arrows). Note the labelling adjacent to an apparent terminal filled with synaptic vesicles, as well as the appearance of non-labelled membranes (black arrowheads). **n, o.** Confocal images of coronal sections through the ARC stained for other PNN components, including hyaluronic acid, by using HABP (**o,** green) and the chondroitin sulfate proteoglycan phosphacan (**o,** green), show colocalization with WFA (red) in the ARC, providing evidence that ARC WFA labelling corresponds to PNNs. Scale bars, 100 μm (**a–d, g, i–k, n, o**); 20 μm (**e**); 10 μm (**f, h**); 2 μm (**l**); 500 nm (**m**). Images in **a–h, i, j, k, l, m** and **n, o** are representative of data from 10, 6, 5, 4 and 3 animals, respectively.
Fig. 2 | PNNs enmesh GABAergic, LepRb*, AgRP/NPY neurons in the ARC. The diagram at the top shows the midsagittal view of the mouse brain with the location and orientation of panel images. a. The dot plots show the proportion of individual neuronal subtypes enmeshed in PNNs. The dots in this and all subsequent dot plots represent data from independent animals (n = 3 animals for each neuronal subtype studied). The left plot shows the percentage of all PNN-enmeshed ARC cells that belong to a particular neuronal subtype. The right plot shows the percentage of all ARC NPY-GFP or POMC-GFP cells that are enmeshed in PNNs. b, e, h, k, n. Low-magnification images of coronal sections. c, f, i, l, o. High-magnification images of coronal sections. Images stained with WFA (red) and antibodies to GFP (green, b, h, k), dsRed (green, e), or SST (green) and AgRP (white, n) show that most PNN-enmeshed cells are GAD67-GFP* (GABAergic), LepRb* and NPY*, while few enmeshed cells express POMC or SST. d, g, j, m. High-magnification Imaris 3D surface rendering of isolated ARC PNN-enmeshed cells belonging to the various neuronal subtypes (corresponding to b, e, h, k, respectively) show PNNs wrapping the soma and proximal processes. The insets show the raw images. Supplementary Movies 1 and 3 correspond to d and j, respectively. Scale bars, 50 μm (b, e, h, k, n); 20 μm (c, f, i, l, o); 10 μm (d, g, j, m). Images in b, d, e, g, b, j, k, m and n, o are representative of data from n = 3 GAD67-GFP mice, n = 3 LepRb-Cre/Ai14 mice, n = 3 NPY-GFP mice, n = 3 POMC-GFP mice and n = 3 C57B/6 mice injected with ICV colchicine, respectively.
somatostatin (SST), which is challenging to detect histochemically because the SST peptide is rapidly exported from soma into axons. To address this issue, we administered a single intracerebroventricular (ICV) injection of colchicine to wild-type mice, which prevents the transport of SST out of the soma and thus enables somatic labelling with antibodies to SST. We report that although this approach allowed us to identify ample SST+ soma, few of these were PNN enmeshed (1.2 ± 0.6%; n = 3; Fig. 2a–o). As a confirmation of the efficacy of ICV colchicine to prevent neuropeptide export from the soma, we note that many AgRP+ PNN-enmeshed cell bodies were also observed, thus corroborating our findings in NPY-GFP mice (Fig. 2o). Together, these findings indicate that only a subset of ARC neurons—specifically, those neurons most closely linked to the control of energy balance and metabolic homeostasis—are enmeshed in PNNs.

A key question raised by these observations is whether PNNs contribute to closure of the critical period for ARC-neuromodulatory development, as is true of the primary visual cortex and other brain areas. As a first step to address this question, we performed a developmental time-series analysis of PNN formation over the lactation and perinatal period from P10–30 in wild-type mice (Fig. 3).

This approach was based on evidence that leptin-dependent maturation of ARC–median-eminence neurcircuitry occurs during a critical period that closes ~P28 (refs. 1,4), so PNNs must appear during this period if they are to contribute to closure of this critical period. WFA labelling revealed only a very faint signal at P10 (10.0 ± 0.8 intensity units; n = 4 C57B/6 mice) that lacked the typical PNN honeycomb configuration (Fig. 3a). By P21, WFA labelling intensity had increased by more than twofold (27.4 ± 1.7 intensity units; n = 5 mice), and the structural features of PNNs were evident (Fig. 3b). WFA intensity was further increased at P30 (46.2 ± 2.6 intensity units; n = 3 mice), by which time the PNN structures appeared fully formed (Fig. 3c).

The time course of ARC PNN formation during postnatal development in wild-type mice was closely paralleled by the maturation of AgRP-neuron projections, quantified as an increase in AgRP-fibre density in the ARC (0.23 ± 0.07% at P10; 0.87 ± 0.11% at P21; 1.48 ± 0.14% at P30) (Fig. 3d). We note that at critical-period closure (~P28), both PNNs and AgRP-fibre density in the ARC transiently increased over values characteristic of adult mice (P90: WFA 34.1 ± 2.5 intensity units; AgRP-fibre density 1.05 ± 0.09%). During postnatal development, the domain of CD44-expressing tanyocytes also expanded from medial (where it covered only the median-eminence β-tanyocytes at P21) to lateral and dorsal along the ventricular lining, paralleling the progressive appearance of PNNs (Supplementary Fig. 4d,e; comparison of yellow and white arrows and arrowheads).

The tight temporal association between ARC PNN formation and maturation of AgRP-neuron projections coincides closely with the closure of the critical period for AgRP-neuron maturation (~P28). Given the key role played by leptin in the latter process, we next sought to investigate whether input from leptin might influence ARC PNN formation. To this end, we performed a developmental time-series analysis of ARC WFA labelling in ob/ob pups and ob/+ control littersmates at P15, P21 and P30. At each of these ages, we found that in ob/ob mice, ARC WFA intensity (normalized to the mean value of control littersmates) was significantly below that detected in controls (P15: 0.92 ± 0.01, n = 3 ob/ob versus 1.00 ± 0.00, n = 2 ob/+; P21: 0.89 ± 0.01, n = 5 ob/ob versus 1.00 ± 0.02, n = 5 ob/+; P = 0.0005; P30: 0.87 ± 0.01, n = 5 ob/ob versus 1.00 ± 0.01, n = 5 ob/+; P = 0.0001) (Fig. 4a–d).

From these results, we hypothesized that, like AgRP-neuron maturation, formation of PNNs in the ARC is dependent on input from circulating leptin. To test this hypothesis, we administered either leptin or vehicle to ob/ob pups according to a schedule reported to mimic the postnatal leptin surge that was used to define the critical period for AgRP-neuron maturation1. Specifically, ob/ob pups received a daily intraperitoneal injection of either leptin (10 mg kg–1) or vehicle from P10 to P30, and were killed 1 d later for IHC analysis. As predicted, ARC WFA intensity was significantly higher in leptin-treated than vehicle-treated ob/ob pups (P30 rescue: 1.15 ± 0.02, n = 3 ob/ob-leptin versus 1.00 ± 0.01, n = 2 ob/ob-vehicle) (Fig. 4e,f,h). There was no significant difference in weight gain between the leptin- and vehicle-treated groups. Interestingly, the presence of AgRP+ soma in the ARC in vehicle-treated pups but not leptin-treated pups (insets in Fig. 4e,f) appears to offer confirmatory evidence of leptin action, which is known to decrease AgRP expression26. A similar pattern was also observed when AgRP staining between ob/+ and ob/ob pups was compared at P30 (insets in Fig. 4c,d).
Fig. 4 | Leptin-deficient ob/ob mice have impaired PNN formation during postnatal development that can be rescued by leptin administration during the critical period. a–d, Confocal images of ARC sections from ob/ob (b, d) and ob/+ (a, c) control littermates at P15 (a, b) and P30 (c, d), stained with WFA (red) and AgRP (green). Arrowheads indicate the ARC region where the earliest PNN formation is seen at P15 in ob/+ mice, but not in ob/ob littermates. Images in a–d are representative of data from 2, 3, 5 and 5 animals, respectively. e, f, Confocal images of ARC sections from ob/ob pups that received daily intraperitoneal injections of leptin (f) or vehicle (e) from P10 to P30 before being killed for analysis with WFA (red) and AgRP (green). Leptin administration during this critical period appeared to restore WFA labelling intensity and PNN architecture. The insets in c–f show higher magnification of the ventromedial ARC region indicated by the arrowheads, revealing an increase in AgRP expression within neuronal soma in leptin deficiency. The images in e and f are representative of data from 2 and 3 animals, respectively. g, h, The dot plots show normalized intensity values for WFA in the ARC of P15, P21 and P30 ob/+ (filled circles) and ob/ob (open circles) mice (g), or P30 ob/ob mice treated from P10 onwards with daily intraperitoneal leptin (red open circle) or vehicle (black open circle) injection (h). Values are normalized to the mean WFA intensity of the control groups (ob/+ or ob/ob-veh). The dots represent values from independent animals (n = 2 ob/+ and n = 3 ob/ob (P15); n = 5 ob/+ and n = 5 ob/ob (P21); n = 5 ob/+ and n = 5 ob/ob (P30); n = 2 ob/ob-veh and n = 3 ob/ob-lep (P30 rescue)). The horizontal bars represent the mean. There was a consistent decrease in ARC PNN intensity across multiple postnatal ages in ob/ob mice compared with their ob/+ littermates, which appeared to be restored in ob/ob mice at P30 by leptin administration during the critical period (*P21 ob/ob 0.89 ± 0.01 versus ob/+ 1.00 ± 0.02, two-tailed t-test P = 0.0005, t = 5.545, d.f. = 8, 95% confidence interval (CI) of the difference: −0.151 to −0.062; P30 ob/ob 0.87 ± 0.01 versus ob/+ 1.00 ± 0.01, two-tailed t-test P = 0.0001, t = 8.975, d.f. = 8, 95% CI of the difference: −0.168 to −0.099). Scale bars, 100 μm (a–f); 20 μm (insets in e–f).
To determine how ARC PNNs are affected by persistent leptin deficiency in adulthood, we performed WFA labelling of 12-week-old ob/ob mice and age-matched C57B/6 mice and subsequent high-resolution confocal microscopy and Imaris 3D image analysis (Supplementary Fig. 6a,b). Paradoxically, in the ARC–median eminence area, the WFA intensity in ob/ob mice (normalized to the mean value for age-matched controls) was significantly higher than that in controls (1.24 ± 0.05, n = 5 ob/ob versus 1.00 ± 0.03, n = 5 wild-type; P = 0.004) (Supplementary Fig. 6e), whereas no such difference in intensity was detected in the primary visual cortex of the same animals (1.00 ± 0.01, n = 5 ob/ob versus 0.99 ± 0.01, n = 5 wild-type; P = 0.2) (Supplementary Fig. 7; of note, the ARC WFA intensity data shown in Supplementary Fig. 7 are replicated from the left panel in Supplementary Fig. 6e to make two different points—comparing ARC WFA to ARC CD44 intensity in Supplementary Fig. 6e and comparing ARC WFA to primary visual cortex WFA intensity in Supplementary Fig. 7). To investigate whether this increase in ARC PNN intensity in ob/ob mice was the result of leptin deficiency or was instead secondary to obesity, we compared WFA-staining intensity between cohorts of wild-type C57B/6 mice that were either made obese through high-fat diet (HFD) feeding for 12 weeks (beginning at the age of 12 weeks) or were fed standard food and killed at the same age. We report that, whereas no difference in ARC WFA intensity was detected between HFD and standard-fed cohorts (1.00 ± 0.00, n = 5 HFD versus 1.00 ± 0.00, n = 3 standard feed), tanyctye CD44 expression was increased in the former group (1.11 ± 0.03, n = 5 HFD versus 1.00 ± 0.01, n = 5 standard feed; P = 0.007; Supplementary Fig. 6c–e). In contrast, no difference in tanyctye CD44 expression was observed between ob/ob and wild-type control mice (1.01 ± 0.03, n = 5 ob/ob versus 1.00 ± 0.03, n = 5 wild-type; P = 0.8). Together, these data suggest that increased PNN intensity in the ARC of adult ob/ob mice is secondary to leptin deficiency rather than to obesity per se, whereas the reverse is true for the increase of tanyctye CD44 expression in HFD-fed (but not ob/ob) mice.

To determine whether PNNs are present in the ARC of other mammalian species, we performed WFA staining in the hypothalamus in both rats and humans. We report that PNNs are present in the ARC–median eminence of all three species (Supplementary Figs. 8 and 9). As with the results in mice, most cells enmeshed in PNNs in the human ARC were NPY/AgRP neurons, and both the soma and proximal dendrites of these neurons were wrapped by WFA+ material (Supplementary Fig. 8 and Supplementary Movie 4). Interestingly, PNN-enmeshed cells in humans were more sparsely distributed than those in mice, thus enhancing the ability to observe how the mesh structure associates with the neuronal contours.

In summary, we report that PNNs enmesh AgRP- and other leptin-receptor-expressing neurons in the ARC (most of which are GABAAergic, similarly to neurons enmeshed by PNNs in the visual cortex and other brain areas), and that their appearance during postnatal development coincides closely with both the maturation of AgRP-neuron projections and the closure of the critical period for leptin-mediated regulation of AgRP-neuron development. Situated at the junction of the ARC and median eminence, these PNNs are present in humans as well as rodents, and their formation appears to be sensitive to input from leptin, being deficient in the ARC of postnatal ob/ob mice and restored by leptin administration to ob/ob mice during the critical period. Unlike PNN-enmeshed neurons in other brain areas, which tend to be sparsely distributed, these neurons form a densely packed ring that circumscribes the median eminence—a circumventricular organ that facilitates processing of metabolically relevant humoral input—at its junction with the ARC. Together, these findings describe an apparently unique brain structure implicated in the closure of the critical period for the development of neurocircuits crucial to controlling energy balance and glucose metabolism in adulthood. Our findings also suggest that leptin regulation of ARC neuron development during the critical period involves an action on PNN formation.

In sharp contrast to the deficiency of PNNs that we observed in the ARC during postnatal development, we observed an overabundance of ARC PNNs in adult ob/ob mice. To explain this paradoxical finding, we draw on both in vivo 27,28 and in vitro 29 evidence that PNN formation is driven by the activity of enmeshed neurons. Thus, leptin induces depolarization and increases the excitability of AgRP neurons during the critical period (before ~P21–23)30; consequently, leptin action on these neurons may constitute a stimulus to PNN formation during this time. However, during subsequent development, a progressive increase in the expression of ATP-sensitive potassium channels by AgRP neurons leads to a ‘phenotype switch’, whereby leptin exerts the hyperpolarizing effect characteristically observed in adult AgRP neurons31. Consequently, AgRP-neuron activity is predicted to be decreased in leptin-deficient mice during the critical period but increased in adulthood, and PNN formation parallels these changes.

Available evidence suggests that ARC neurocircuits are highly plastic during development and that this plasticity is markedly decreased after critical-period closure. Thus, whereas AgRP-neuron ablation induces life-threatening starvation when it occurs in adult mice, it has little or no phenotypic effect when induced shortly after birth32. Similarly, clustered regularly interspaced short palindromic repeat–mediated deletion of leptin receptors selectively from AgRP neurons in adults recapitulates most of the phenotype of whole-body leptin deficiency (hyperphagia, obesity and diabetes), whereas the same deletion has little detectable effect when induced during development33. Combined with evidence that, over the course of the critical period, when AgRP neurons inner-vate their downstream targets44, their response to leptin switches from excitatory to inhibitory35, these data collectively point to the existence of a mechanism whereby AgRP-linked circuit plasticity during postnatal development is sharply constrained in adulthood. PNNs are probable candidate mediators of this effect, because they can limit plasticity both through direct signalling effects of CSPGs on the enmeshed neuron and by providing a scaffold for binding regulatory molecules, such as homeobox protein Otx236, which suppresses plasticity, and the chemoattractive molecule semaphorin-3A37, which promotes growth of projections away from the enmeshed neuron38-42.

Such a role for PNNs has important implications for understanding how nutritional excess during postnatal development affects ARC neurocircuits in ways that can result in predisposion to obesity and type 2 diabetes in adulthood. Specifically, overnutrition during lactation, due to either maternal HFD consumption18,20-22, or culled litter size19, (1) decreases numbers of ARC neurons expressing leptin receptors, (2) decreases the leptin responsiveness of these neurons13,19, (3) impairs the formation of ARC projections within hypothalamic feeding circuits4,30 and (4) results in predisposition to excess body adiposity and metabolic dysregulation in adulthood36-38. Similarly, epidemiological evidence suggests that in humans, late gestation and early childhood are particularly sensitive periods when environmental exposures can shape a predisposition to metabolic disease in adulthood44. Interestingly, early gestational undernutrition is also reliably associated with an increased risk of adult obesity45,46, and this effect can be partially reversed by leptin treatment during the lactation period46. Because PNNs sharply limit the plasticity of neurons they enmesh, and because elsewhere in the brain, the developmental appearance of PNNs heralds critical-period closure37, we interpret our discovery that ARC neurons become enmeshed in PNNs at a time corresponding to critical-period closure as being of potential relevance to experience-dependent plasticity in neurocircuits for energy balance and glucose homeostasis. It is possible, for example, that experimental reactivation of ARC plasticity in adults may ameliorate metabolic...
In this context, it is notable that diet-induced obesity through HFD feeding is associated with reactive gliosis involving the activation of both microglia and astrocytes in the same ARC area where PNNs are found\(^4\). Recent work suggests that these glial responses are both necessary and sufficient for obesity in this setting\(^5\). The finding of increased tanyocyte expression of the hyaluronan receptor CD44 in mice with diet-induced obesity is of interest because it raises the possibility that environmental exposures in adulthood (for example, consuming a HFD) can influence signalling between PNN constituents (for example, hyaluronan) and adjacent cells (for example, tanyocytes) in the ARC–median eminence. Reports that tanyocytes transport circulating leptin into the mediobasal hypothalamus\(^6\) highlight the potential importance of such interactions. That this obesity-associated increase of tanyocyte CD44 content is not observed in ob/ob mice despite their severe obesity phenotype is consistent with evidence that hypothalamic gliosis is also not observed in these animals\(^5\), thus presumably reflecting the requirement for an intact leptin signal in this response.

In conclusion, we report that the ARC–median eminence junction, a brain area that specializes in processing humoral input relevant to fuel homeostasis, is marked by a cluster of tightly packed, PNN-enmeshed neurons, including most AGRP neurons. The formation of these PNNs coincides with the closure of the critical period for ARC-neuron development and, postnatally maturated AGRP neurons, is dependent on input from the adipocytokine hormone leptin. Future investigation is needed to clarify the extent of ARC-neuron development and, like postnatal maturation, a brain area that specializes in processing humoral input relevant to fuel homeostasis.

**Methods**

**Animals.** GAD\(^{-}\)-GFP-knockin\(^7\), Lepr\(^{-}\)/Cre/Ai14-reporter\(^8\), NPY-GFP\(^9\), POMC-GFP\(^10\) and wild-type C57B/6 mice (The Jackson Laboratory), age P60–120, were used to characterize the neuronal and glial subtypes associated with ARC PNNs. Wild-type C57B/6 mice, age P0–90 were used for developmental time-series studies to characterize PNN formation and AGRP neuron maturation. To study the effects of leptin deficiency on PNN formation, we used ob/ob mice and their ob/ob littermate controls (The Jackson Laboratory). To characterize PNNs in rats, we used the Wistar strain (Harlan). Both sexes were used for all studies. Mice were on a 12:12 h light–dark cycle and housed in groups of five per cage. Animals were perfusion-fixed with saline and 4% paraformaldehyde (PFA), the brains were extracted and post-fixed overnight at 4 °C, then sectioned with a vibratome (Leica VT1200S; 50 μm) or cryostat (Thermo Fisher Scientific; Cryostat X56; 12 μm). The Institutional Animal Care and Use Committees at St. Joseph’s Hospital and Medical Center and the University of Washington approved all animal procedures. All animal studies were conducted in compliance with the relevant ethical regulations.

**Human specimens.** Three brains (ages 23, 64 and 71, with post-mortem intervals 8, 9 and 12 h, respectively) were collected at autopsy. Multiple whole-mob blocks were dissected from the hypothalamus along the third ventricle from each brain, their positions along the third ventricle wall were documented, and the tissue was immersion-fixed in 4% PFA at 4 °C for 24 h. All specimens were collected with informed consent and in ethical compliance with the St. Joseph’s Hospital and Medical Center Committee on Human Research (Institutional Review Board approval no. 10BN159).

**Electron microscopy.** Adult C57B/6 mice were transcardially perfused with 4% PFA and 0.5% glutaraldehyde (ElectroN Microscopy Sciences) in 100 mM phosphate buffer. Brains were post-fixed for 4 °C overnight, and 50 μm coronal sections were cut on a Leica VT1000 S Vibrating Blade Microtome (Leica Biosystems). Pre-embedding IHC was performed by using WFA, amplified with an ELITE ABC Kit (Vector Laboratories) and developed with DAB\(^1\). Sections were post-fixed in 1% osmium tetroxide for 30 min and then embedded in Durcupan ACM epoxy resin (Sigma-Aldrich).\(^1\) To reconstruct WFA-labelled neurons, we cut ~20 serial semi-thin (1.5 μm) sections on an Ultracut UC6 ultra-microtome (Leica Microsystems). Selected semi-thin sections were then cut into ultrathin sections (60–80 nm) and placed on Formvar-coated single-slot grids, stained with lead citrate and examined at 80 kV on a Tecnai G\(^2\) Spirit (FEI Company) transmission electron microscope equipped with a Morada CCD digital camera (Olympus).

**Immunohistochemistry and microscopy.** For WFA (1:500, L1516; Sigma-Aldrich) or biotinylated HABP (1:50, AMS.HKD-BC41; AMS Biotechnology) staining, primary antibodies were incubated in WFA or HABP in PBS with 0.5% Triton X-100 followed by streptavidin–Alexa Fluor 488 or 561 (1:1,000; Invitrogen Molecular Probes) in PBS/0.5% Triton X-100, each for 24 h at 4 °C. For immunostaining, sections were incubated in primary and secondary antibodies in PBS/0.5% Triton X-100 and 5% normal goat serum for 24 h at 4 °C. Primary antibodies were: chicken anti-GFP (1:500, GFP-1020; Aves Labs); rabbit anti-DsRed (1:1,000, 632496; Takara Bio Clontech); rabbit anti-AGRP (1:200, H-0037–53; Phoenix Pharmaceuticals); rabbit anti-NPY (1:1,000, ab39014; Abcam); rat anti-SST (1:500, MAB354; EMD Millipore); chicken anti-vimentin (1:500, AB5733; EMD Millipore); rabbit anti-CD44 (1:1,000, ab157107; Abcam); and rat anti-DSG-1 (1:500, MAB5790-I; EMD Millipore). Secondary antibodies were conjugated to Alexa Fluor dyes (goat or donkey polyclonal, 1:500; Thermo Fisher Scientific). Confocal images were taken on a Leica SPE confocal microscope (Leica Microsystems).

**Statistical analysis.** Descriptive statistics and two-tailed \(t\) tests were calculated in Prism version 7 (GraphPad). \(P < 0.05\) was considered statistically significant. Unless otherwise stated, dot plots show dots representing data from independent animals, with the mean and s.e.m. shown as bars.
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Author contributions
Z.M., K.M.A. and M.W.S. conceived and designed the study; Z.M., K.M.A., E.C., V.H.P., J.M.S., J.M.B., R.H., M.E.M., H.T.N., J.M.G.V., L.M.Z. and M.W.S. acquired, analysed and interpreted the data; Z.M. and M.W.S. drafted and revised the manuscript. All authors approved the final version of the manuscript.

Competing interests
The authors declare no competing interests.

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- **n/a**
- **Confirmed**
  - The exact sample size \((n)\) for each experimental group/condition, given as a discrete number and unit of measurement
  - An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
  - The statistical test(s) used AND whether they are one- or two-sided
    - *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
  - A description of all covariates tested
  - A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
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  - For null hypothesis testing, the test statistic (e.g. \(F\), \(t\), \(r\)) with confidence intervals, effect sizes, degrees of freedom and \(P\) value noted
    - Give \(P\) values as exact values whenever suitable.
  - For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
  - For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
  - Estimates of effect sizes (e.g. Cohen’s \(d\), Pearson’s \(r\)), indicating how they were calculated
  - Clearly defined error bars
    - State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on [statistics for biologists](#) may be useful.

Software and code

Policy information about availability of computer code

| Data collection                  | Leica Application Suite confocal image capture software |
|---------------------------------|--------------------------------------------------------|
| Data analysis                   | GraphPad Prism7 statistical analysis software and Imaris image analysis software (Bitplane) version 9.1 |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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### Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | sample sizes were calculated based on prior work quantifying confocal image intensity and immunostaining, with pilot data using the antibodies/staining to be quantified to provide an estimate of inter-subject variability |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | none |
| Replication | immunostaining for various markers presented was confirmed across multiple samples, including different mouse strains (C57B/6 and CD1) and in diverse species (mouse, rat, human) |
| Randomization | allocation to experimental groups receiving different treatment (HFD vs chow feeding) was random |
| Blinding | images were de-identified with the investigator performing quantification blinded to the study groups. |

### Reporting for specific materials, systems and methods

#### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| [x] Unique biological materials |
| [x] Antibodies |
| [x] Eukaryotic cell lines |
| [x] Palaeontology |
| [x] Animals and other organisms |
| [x] Human research participants |

#### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| [ ] ChIP-seq |
| [ ] Flow cytometry |
| [ ] MRI-based neuroimaging |

#### Unique biological materials

Policy information about availability of materials

Obtaining unique materials transgenic mice presented are commercially available from Jackson Laboratories.

#### Antibodies

| Antibodies used | chicken anti-GFP (1:500, Aves Labs GFP-1020), rabbit anti-dsRed (1:1000, Clontech 632496), rabbit anti-Agrp (1:200, Phoenix Pharmaceuticals H-003-57), rabbit anti-Npy (1:1000, Abcam ab30914), rat anti-somatostatin (1:500, EMD Millipore MAB8354), chicken anti-vimentin (1:500, EMD Millipore ABS733), rabbit anti-CD44 (1:1000, Abcam ab157107), and rat anti-phosphacan DSD-1 (1:500, EMD Millipore MAB5790-I). |

| Validation | chicken anti-GFP: 175 citations on labome.com (https://www.labome.com/product/Aves-Labs/GFP-1020.html), 86 published figures in IHC on benchsci.com (https://app.benchsci.com/product/Aves%20Labs/GFP-1020/figures); rabbit anti-dsRed: 143 citations on labome.com (https://www.labome.com/product/Takara-Bio-Clontech/632496.html); rabbit anti-Agrp: validated for IHC in Shin et al., J Comp Neurol 2008, also 9 citations with use in IHC on manufacturer’s website (https://www.phoenixpeptide.com/products/view/Antibodies/H-003-57); rabbit anti-Npy: 24 citations on manufacturer’s website (https://www.abcam.com/neuropeptide-y-antibody-ab30914.html?productWallTab=ShowAll#top-358), 7 citations on labome.com with 4 including use for IHC in mouse (https://www.labome.com/product/Abcam/ab30914.html), 18 published figures on benchsci.com with use in immunostaining/immunofluorescence (https://app.benchsci.com/product/Abcam/AB30914/figures), rat anti-somatostatin: 90 citations on labome.com including 32 with use in IHC in mouse (https://www.labome.com/product/EMD-Millipore/MAB8354.html); chicken anti-vimentin: 36 citations on labome.com including 8 with use in IHC in mouse (https://www.labome.com/product/EMD-Millipore/ABS733.html); rabbit anti-CD44: 34 citations on manufacturer’s website
Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals
- GAD67-GFP knock-in, LepRb-Cre-Ai14 reporter, Npy-GFP, POMC-GFP, and wild-type C57B/6 mice (Jackson Labs), age P60 to P120, were used to characterize neuronal and glial subtypes associated with ARC PNNs. Wild-type C57B/6 mice, age P0 to P90 were used for developmental time-series studies to characterize PNN formation and Agrp neuron maturation. To study the effects of leptin deficiency on PNN formation, we used ob/ob mice and their ob/+ littermate controls (Jackson Labs). To characterize PNNs in rats, we used the Wistar strain (Harlan). Both sexes were used for all studies. Mice were on 12h:12h light-dark cycle in 5/cage group housing.

Wild animals
- the study did not involve use of wild animals

Field-collected samples
- the study did not involve use of field-collected samples

Human research participants

Policy information about studies involving human research participants

Population characteristics
- Brain specimens were collected from 3 patients: (1) 23 year old woman with cervical spinal cord glioma, (2) 64 year old man with occipital lobe glioblastoma, and (3) 71 year old man with temporal lobe hemorrhagic stroke.

Recruitment
- Human brain specimens were collected from deceased patients undergoing autopsy, with no inclusion/exclusion criteria except the requirement of being notified in time to collect the specimen with a post-mortem interval less than 24 hours.