Perineuronal Net Formation and the Critical Period for Neuronal Maturation in the Hypothalamic Arcuate Nucleus

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47 Summary

48   In leptin-deficient *ob/ob* mice, obesity and diabetes are associated with abnormal

49   development of neurocircuits in the hypothalamic arcuate nucleus (ARC), a critical brain

50   area for energy and glucose homeostasis. As this developmental defect can be

51   remedied by systemic leptin administration, but only if given before postnatal day 28, a

52   critical period (CP) for leptin-dependent development of ARC neurocircuits has been

53   proposed. In other brain areas, CP closure coincides with the appearance of

54   perineuronal nets (PNNs), extracellular matrix specializations that restrict the plasticity

55   of neurons that they enmesh. We report that in humans as well as rodents, key subsets

56   of ARC neurons are enmeshed by PNNs. In mice, most of these neurons are both

57   GABAergic and leptin receptor-positive, including a large subset of Agrp neurons, and

58   the postnatal appearance of these PNNs coincides precisely with both closure of the CP

59   for ARC neuron development and maturation of Agrp neuron projections. Moreover,

60   postnatal PNN formation is impaired in the ARC of leptin-deficient *ob/ob* mice, and this

61   defect is rescued by leptin administration during the CP. In contrast, PNN density is

62   increased in the ARC of adult *ob/ob* mice, potentially owing to the increased activity of

63   Agrp neurons in the absence of leptin. Collectively, these findings implicate PNN

64   formation in the control of ARC neuron development and show that PNN formation in

65   this brain area is dependent on input from circulating leptin. Aberrant PNN formation in

66   the ARC of animals predisposed to obesity and diabetes offers a plausible link between

67   ARC neurocircuit dysfunction and the pathogenesis of common metabolic disorders.

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69

70   During critical periods (CPs) of early postnatal life, developing neurocircuits are

71   exquisitely sensitive to and shaped by external cues from the environment[6]. The CP for

72   ocular dominance plasticity in primary visual cortex (V1) is a well-studied example:

73   during the CP, but not before or after, visual deprivation of one eye induces a strong

74   shift of neuronal responses to the non-deprived eye[7]. The associated loss of acuity in

75   the deprived eye, clinically referred to as amblyopia, is difficult to remedy after CP

76   closure.

77   In diverse neurocircuits ranging from the mammalian visual, barrel

78   (somatosensory) and entorhinal cortices to the hippocampus and amygdala, and in

79   song nuclei in the songbird brain as well, CP closure is dependent on the formation of

80   perineuronal nets (PNNs)[8-15]. PNNs are extracellular matrix specializations composed

81   largely of hyaluronan and chondroitin sulfate proteoglycans that enmesh primarily

82   inhibitory interneurons[10,16]. Remarkably, experimentally disrupting PNNs in adult

83   animals is capable of reactivating CP plasticity in these diverse brain areas. In V1, for

84   example, PNN digestion enables restoration of vision to a previously deprived eye[8].

85   Comparable restorative effects of PNN disruption are reported across neurological and

86   psychiatric disorders as diverse as PTSD, depression, drug addiction and spinal cord

87   injury[10,16,17].

88   Recently, compelling evidence was provided in support of the existence of a CP

89   for the maturation of Agouti-related peptide (Agrp) neurons in the hypothalamic arcuate

90   nucleus (ARC)[18]. As key regulators of feeding behavior, Agrp neurons are among the

91   best-studied of all hypothalamic neurons. These GABAergic neurons co-express the

92   potent orexigen neuropeptide Y (NPY), and they project to downstream targets in

93   homeostatic circuits governing energy balance and glucose homeostasis. Maturation of

94   these projections occurs during the lactation period, concomitant with a naturally-

95   occurring leptin surge (P4-P14)[19,20], and this maturation process appears to be

96   dependent on leptin since these projections fail to develop properly in leptin-deficient

97   *ob/ob* mice (a genetic model of obesity and type 2 diabetes (T2D)). Moreover, this

98   defect in Agrp neuron development can be rescued by treatment with exogenous leptin,

99   but only if administered before P28[18,19,21]. A CP for the trophic action of leptin on Agrp

100   neuron maturation therefore exists, and its closure coincides with both the transition to

101   independent feeding and maturation of the cellular response of Agrp neurons to input

102   from leptin[22]. This CP is also a uniquely sensitive time when both over- and

103   undernutrition predispose to obesity and glucose intolerance in adulthood[23-27].

104   Here we show that PNNs enmesh key subpopulations of ARC neurons in

105   humans as well as rodents. These neurons are predominantly GABAergic and many are

106   leptin-receptor positive, including a majority of Agrp neurons. In mice, PNN formation in

107   this region coincides with both maturation of Agrp neuron projections and closure of the

108   CP for Agrp neuron development. Our finding of altered ARC PNNs in developing and

109   adult mouse models of obesity and type 2 diabetes points to a potential role for PNNs in

110   aberrant development and/or altered plasticity of ARC neurocircuits in common

111   metabolic diseases.

112

113   **Results**

114   We performed immunohistochemistry (IHC) on serial coronal sections of mouse

115   hypothalamus using *Wisteria floribunda* agglutinin, a lectin that selectively labels the *N-*

116 acetylgalactosamine residue on chondroitin sulfate (CS) chains in PNNs. Our initial

117 survey (**Supplementary Fig 1**) revealed numerous PNN-enmeshed cells localized to

118 the junction of the ARC and median eminence (ME) (**Fig 1a-d**). Compared to the rather

119 sparse distribution of PNN-enmeshed neurons in most brain areas (*e.g.,* V1), enmeshed

120 cells in the ARC-ME area are densely packed (**Fig 1e, g**). To better characterize the

121 structural features of these cells, we used high-resolution confocal microscopy

122 combined with Imaris image analysis of individually-labelled, PNN-enmeshed neurons

123 located at the periphery of the dense cluster at the ARC-ME junction. Analysis of these

124 cells (**arrow f in Fig 1c**) allowed us to better define the anatomical relationship between

125 PNNs and the neurons they enmesh without the confounding influence of labeling on an

126 adjacent, closely apposed cell. As expected, PNNs enwrap both cell soma and

127 proximal processes (**Fig 1f**), reminiscent of PNN structures described in the visual

128 cortex (**Fig 1g,h**).

129 Wholemount preparations of

130 the mediobasal hypothalamus,

131 stained with WFA and imaged

132 from either the ventricular

133 surface (**Fig 1i**) or the ventral

134 pial brain surface (**Fig 1j**),

135 revealed the presence of a

136 continuous "collar" of PNN-

137 enmeshed cells at the junction

138 of the ARC and ME.
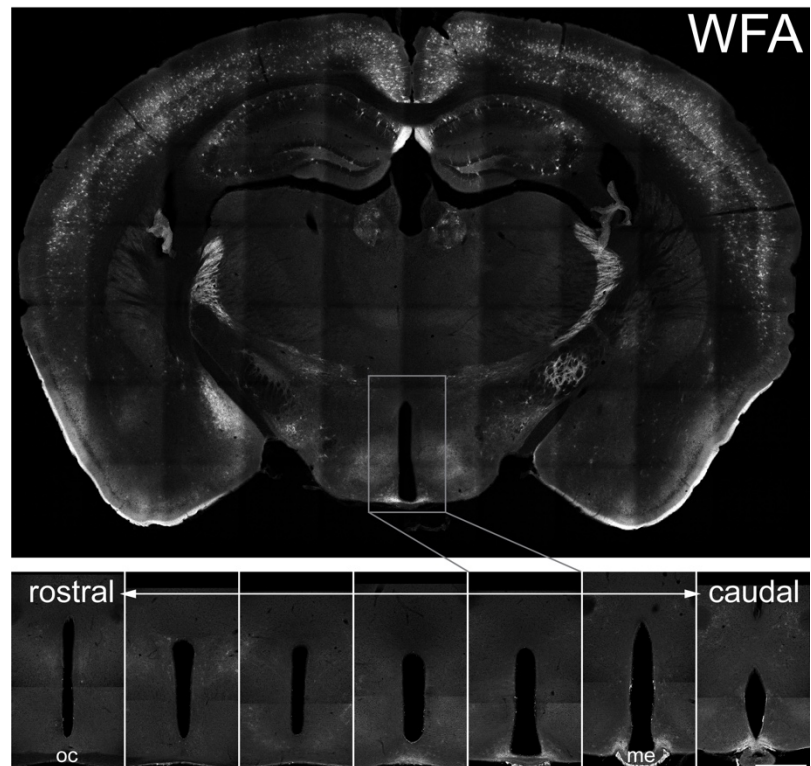
Supplementary Figure 1. Tiled panoramic confocal image of a coronal section at the level of the ARC stained with WFA (top), and corresponding serial coronal sections through the hypothalamus from the optic chiasm (oc) to the infundibular recess (bottom row), reveal a specific concentration of PNN enmeshed cells in the ventromedial ARC at its junction with the ME. Scale bar: 500 um. Images are representative of data from at least 5 animals.
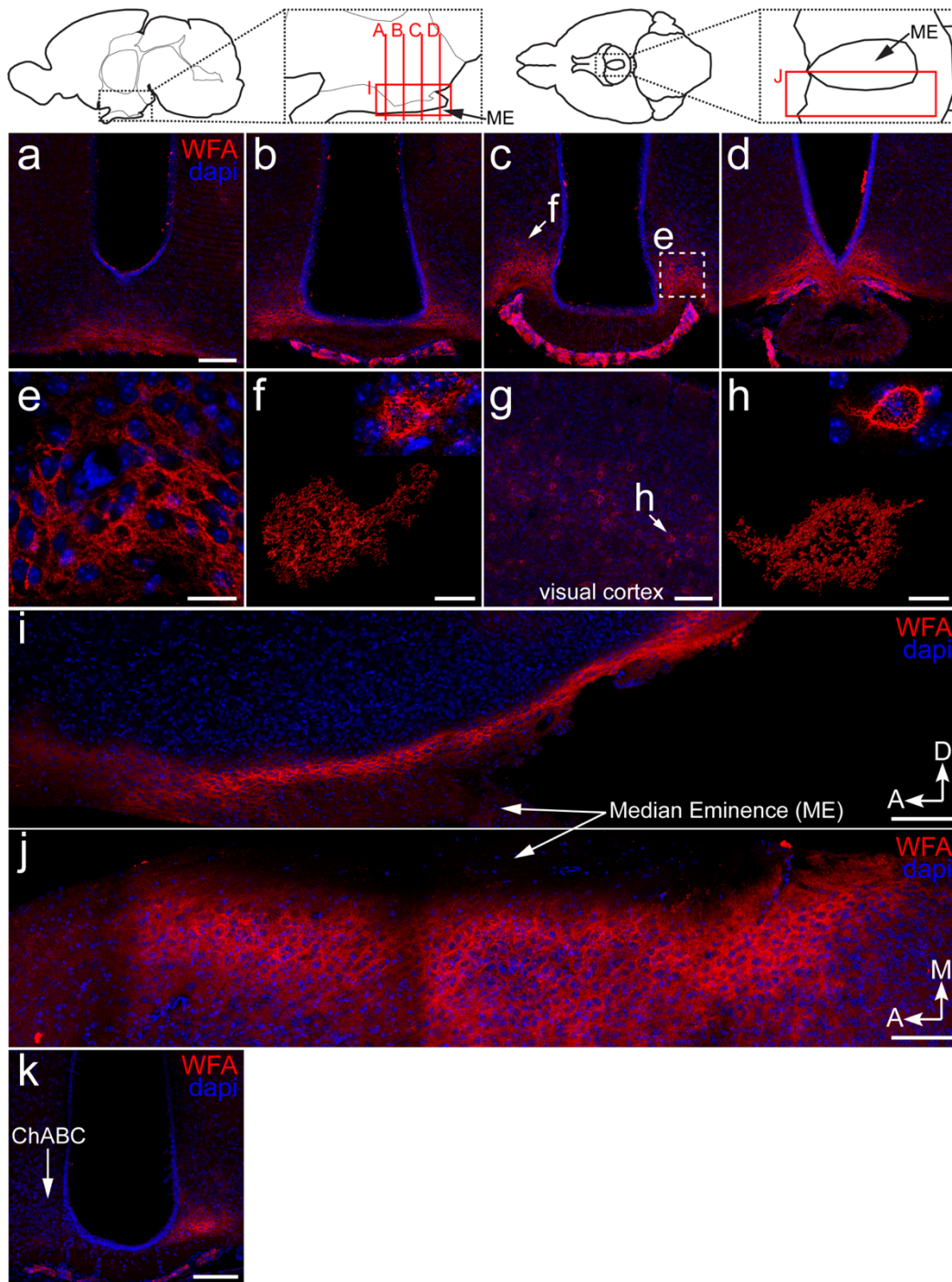
Figure 1. *Wisteria Floribunda* agglutinin (WFA)-labeling in the ventromedial ARC forms a "collar" around the ME. Diagrams at top show mid-sagittal view (left) and ventral view (right) of the mouse brain with insets showing the location and orientation of panel images. (a-d) WFA-labeled (red) coronal sections through the Arc, starting just rostral to and progressing through the ME, show a concentration of WFA-labeled cells located in the ARC at its junction with the ME. Note that the very intense staining below the ME does not correspond to labeling around neurons, but to the pia around the ME.
(e) Higher magnification image of the boxed region in (c) showing the dense cluster of WFA-labeled ARC cells.
(f) High magnification Imaris 3-dimensional rendering of an isolated WFA-labeled cell at the periphery of the dense cluster (arrow in c) reveals that WFA labels the soma and proximal processes of ARC cells. Inset shows the raw image.
(g-h) Low (g) and high (h) magnification images of PNNs labeled by WFA in the visual cortex, where they have been extensively studied, for comparison. Note similar PNN pattern between (h) and (f) wrapping the soma and proximal process.
(i-j) WFA-labeled wholemounts of the ARC viewed from the 3rd ventricle wall en-face (i) or the ventral brain surface (j) reveal the distribution of labeled ARC cells forming a "collar" around the ME, which does not contain labeling. From the ventricular surface view (i), the WFA-labeled ARC cells appear as a continuous band along the ventral margin of the ARC.
(k) WFA-labeled coronal section from a wild-type mouse sacrificed 2 days after stereotactic unilateral intra-Arc injection of Chondroitinase ABC, an enzyme that digests chondroitin sulfate carbohydrates. Scale bars: 100 um (a-d, g, i-k), 20 um (e), 10 um (f, h). Images are representative of data from at least 5 animals.

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143   As a first step to determine if these ARC WFA-labeled structures are *bone fide*

144   PNNs, we micro-injected 10 milliunits of Chondroitinase ABC (ChABC), an enzyme that

145   digests PNNs, stereotactically into the ARC of wild-type mice. Subsequent

146   histochemical analysis of

147   these animals (n=3) revealed

148   ChABC-mediated digestion

149   of ARC-ME PNNs on the

150   injected side (**Fig 1k**). Then,

151   following a previously

152   validated protocol[28], we

153   performed pre-embedding

154   WFA-DAB labeling of mouse

155   brain sections for study by

156   electron microscopy. This

157   ultrastructural analysis

158   revealed DAB electron-dense

159   deposits surrounding the

160   soma (**Fig 2b** white

161   arrowheads) and neurites

162   (**Fig 2b** white arrows) of ARC
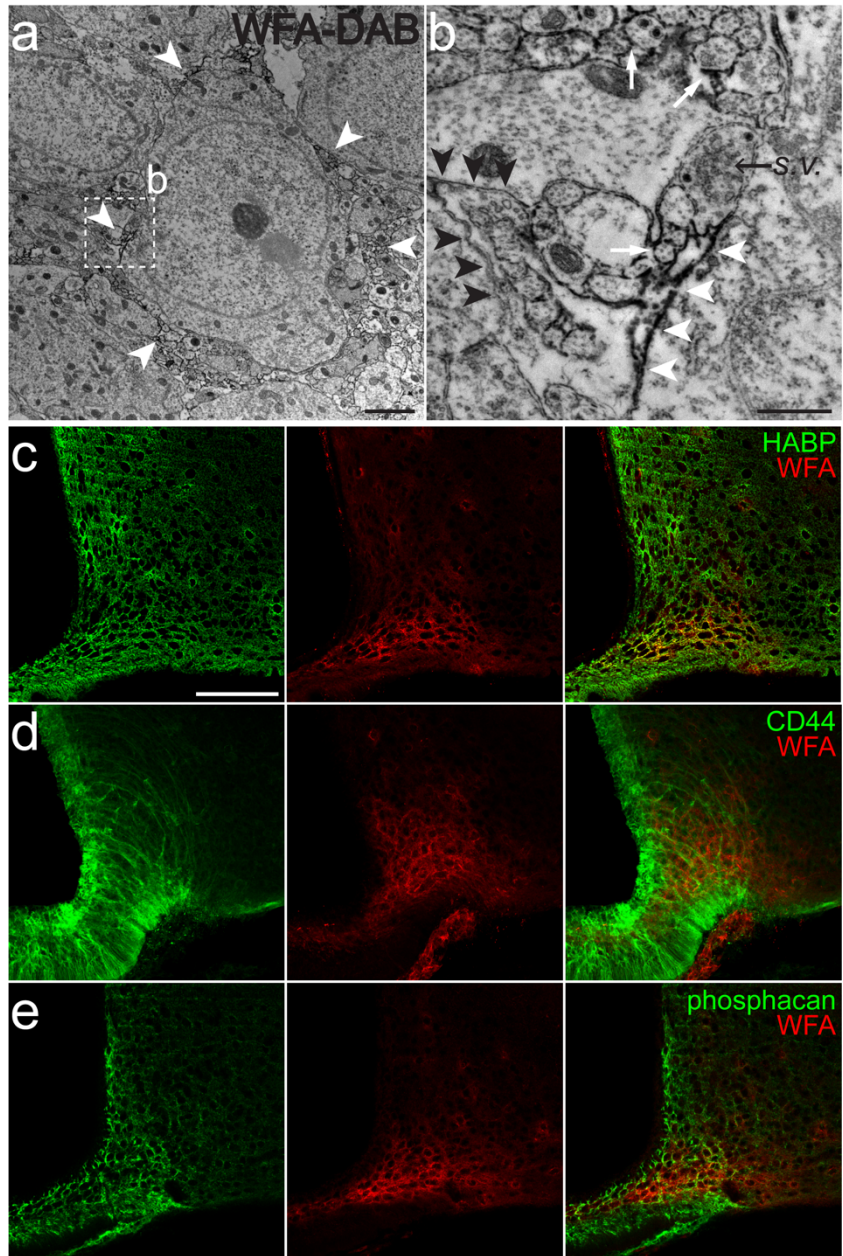
Figure 2. WFA-labelling in the ARC is observed around neuronal soma and neurites by electron microscopy and colocalizes with other major PNN components.
(a) Low-power electron micrograph of an ARC section labeled with WFA-DAB shows electron dense DAB deposits surrounding a single ARC neuron (white arrowheads).
(b) High-power electron micrograph corresponding to the boxed region in (a) shows WFA-labeling localized to the membrane around the cell soma (white arrowheads) and neurites (white arrows). Note labeling adjacent to an apparent terminal filled with synaptic vesicles (s.v.), as well as the appearance of non-labeled membranes (black arrowheads).
(c-e) Confocal images of coronal sections through the ARC stained for other PNN components, including hyaluronic acid using HABP (c, green), the cell surface receptor for hyaluronic acid, CD44 (d, green), and the chondroitin sulfate proteoglycan phosphacan (e, green), show colocalization with WFA (red) in the ARC, providing evidence that ARC WFA-labeling corresponds to PNNs.
Scale bars: 2 um (a), 500 nm (b), 100 um (c-e). Images are representative of data from at least 3 animals.

Supplementary Figure 2. Low- (a,c,e) and high-power (b,d,f correspond to boxed regions in low-power images) electron micrographs showing comparative ultrastructural distribution of WFA-DAB deposits around neurons in the cortex (a,b), hippocampus (c,d), and ARC (e,f) reveal very similar patterns of labeling localized to the soma and adjacent neurites in all 3 regions. Scale bars: 2 um (a,c,e), 500 nm (b,d,f). Images are representative of data from at least 3 animals.
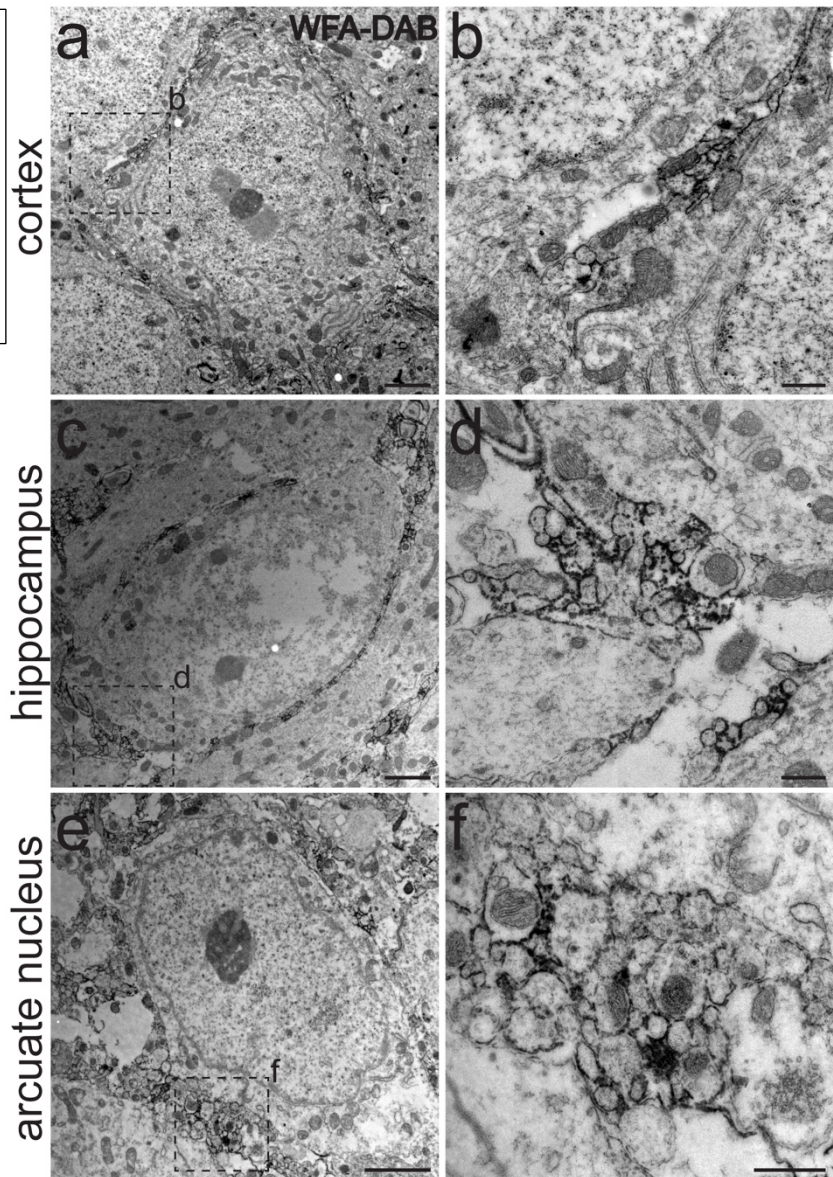
163   neurons in a distribution

164   that closely matches the

165   pattern observed in PNN-

166   enmeshed cells in the

167   cortex and hippocampus

168   (**Supplementary Fig 2**).

169   In addition to

170   chondroitin sulfate (CS)

171   carbohydrate chains labeled

172   by WFA, PNNs are

173   comprised of two other

174   major components: the CS

175   proteoglycan (CSPG) core proteins to which the CS chains covalently bind, and

176   hyaluronic acid (HA), a long carbohydrate polymer to which CSPG core proteins

177   noncovalently bind. Using biotinylated-HA-binding protein (HABP)[29], we histochemically

178   stained HA in the hypothalamus. Although HABP lightly stains the extracellular matrix

179   throughout brain parenchyma, we observed an increased abundance of HA that

180   colocalizes with WFA at the junction of the ARC and ME (**Fig 2c**), confirming that WFA

181   (+) structures in this brain area contain HA. To identify the relevant CSPG(s) present in

182   these PNNs, we next stained for each of the 5 major CSPG species found in PNNs

183   elsewhere in the brain: aggrecan, brevican, neurocan, versican and phosphacan.

184   Interestingly, although each of these CSPGs was detected in various mouse brain areas

185   (data not shown), only phosphacan immunoreactivity was co-localized with WFA in

186   mouse ARC-ME (**Fig 2e**).

187   We also immunostained for CD44, the cell surface receptor for HA, and found

188   that its expression in this brain area is largely limited to tanycytes, with high expression

189   localized to E3/β-tanycytes piercing through the PNN domain (**Fig 2d**) and little to no

190   expression in dorsal E2/α-tanycytes that circumvented the PNN domain

191   (**Supplementary Fig 3b, c**). An IHC survey of CD44 expression in serial coronal

192   sections through the hypothalamus revealed a striking colocalization with the PNN

193   domain in the ventromedial ARC (**Supplementary Fig 3a**). These findings raise the

194   possibility that via activation of CD44, signal transduction in tanycytes is evoked by HA

present in PNNs.

Supplementary Figure 3. Serial coronal sections (a) through the hypothalamus from the optic chiasm (oc) to the infundibular recess stained with WFA (red) and CD44 (green) reveal regional colocalization of the PNN constituent hyaluronan receptor CD44 and PNNs in the ventromedial Arc-ME. Low- (b) and high-magnification (c) images of a coronal section stained with WFA (red), CD44 (green), and vimentin (magenta) reveal that CD44 is expressed on β-tanycyte processes and their endfeet (arrow), but not on α-tanycyte processes or endfeet (arrowhead). Scale bar: 500 um (a), 100 um (b), 50 um (c). Images are representative of data from at least 3 animals.

196

197   The ARC is uniquely specialized to sense and transduce input from nutritionally-

198   relevant hormones (such as leptin and insulin) and nutrients (*e.g.,* glucose, free fatty

199   acids and amino acids) into adaptive changes of food intake and energy metabolism[30].

200   Among key neuronal subsets implicated in this homeostatic circuitry are Agrp and pro-

201   opiomelanocortin (Pomc) neurons, with the former being GABAergic[31]. To identify

202   distinct ARC neuronal subtypes that are among those enmeshed by PNNs (= # neurons

203   of particular subtype / total # PNN-enmeshed cells), we performed IHC on coronal

204   sections from transgenic mouse lines that provided whole cell GFP-filling. Using

205   GAD67-GFP heterozygous knock-in mice[1], we confirmed that a vast majority of ARC

206   neurons enmeshed by PNNs are GABAergic (81.8 ± 0.7% of ARC PNN-enmeshed

207   cells; n=3 GAD67-GFP mice; note that dots in dot plots throughout this work represent

208   data from independent animals) (**Fig 3a-d**), as is the case for PNN-enmeshed neurons

209   in other brain areas[10,17]. Three-dimensional reconstruction of GAD67-GFP+ PNN-

210   enmeshed cells (**Supplementary Movie 1**) revealed PNN enmeshing the soma and

211   proximal dendrites of GABAergic ARC cells in a manner comparable to that of

212   GABAergic interneurons in V1, with some differences in morphology due likely to

213   differences in cell type and process ramification (**Supplementary Fig 4** and

214   **Supplementary Movie 2**).

215   To determine if ARC neurons that express leptin receptors are represented

216   among those enmeshed by PNNs, we employed Leptin Receptor b-Cre;Ai14 td-Tomato

217   reporter mice to histochemically identify leptin-receptor positive cells. These cells

218   comprised 81.7 ± 1.5% of all PNN-enmeshed cells in the ARC (n=3 LepRb-Cre;Ai14)

219   (**Fig 3e-g**). To identify Agrp neurons, we used NPY-GFP transgenic mice (since NPY

220   and Agrp are expressed in the same ARC neuronal subset) and found that these cells

221   (which are GABAergic, and many of which express leptin receptors) also account for a

222   majority of ARC PNN cells (58.5 ± 1.0%; n=3 NPY-GFP mice) (**Fig 3h-j** and

223   **Supplementary Movie 3**). By comparison, POMC neurons comprised a much smaller

224   fraction of PNN-enmeshed cells in this brain area (13.7 ± 2.3%; n=3

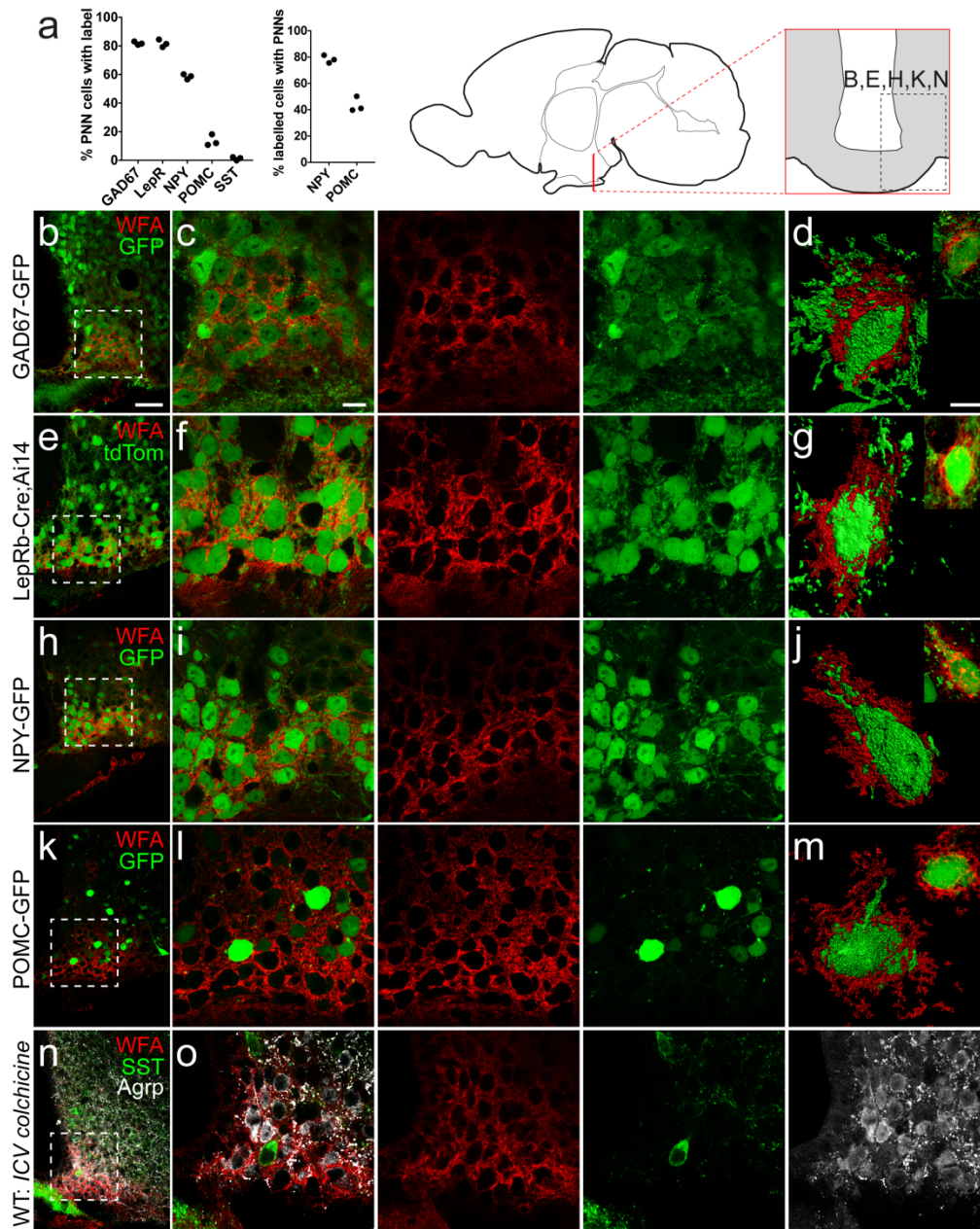225   POMC-GFP mice) (**Fig 3k-m**).

Figure 3. PNNs enmesh GABAergic, LepRb-positive, Agrp/NPY neurons in the Arc. Diagram at top shows mid-sagittal view of mouse brain with location and orientation of panel images.
(a) Dot plots show the proportion of individual neuronal subtypes enmeshed by PNNs. Dots in this and all subsequent dot plots represent data from independent animals. 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 by PNNs.
Low (b, e, h, k, n) and high (c, f, i, l, o) magnification images of coronal sections 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-positive (GABAergic), LepRb-positive, and NPY-positive, while few enmeshed cells express POMC or SST.
(d, g, j, m) High magnification Imaris 3-dimensional 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. Insets show raw images. See corresponding supplementary movies 1 and 3 for (d) and (j), respectively.
Scale bars: 50 um (b, e, h, k, n), 20 um (c, f, i, l, o), 10

226   A separate but related question pertains to the fraction of Agrp or POMC neurons

227   in the ARC that are enmeshed by PNNs (= # PNN-enmeshed neurons of particular

228   subtype / total # neurons of that subtype). As predicted, the fraction of Agrp neurons

229   enmeshed by PNNs ($78.3 \pm 1.7\%$ NPY-GFP neurons) is greater than the fraction of

230   POMC neurons ($43.6 \pm 3.3\%$ POMC-GFP neurons). A third peptidergic cell type found

231   in the ARC expresses somatostatin (SST), which is challenging to detect

232   histochemically because the SST peptide is rapidly exported from soma into axons. To

233   address this issue, we administered a single intracerebroventricular (ICV) injection of

234   colchicine to wild-type mice, which prevents transport of SST out of the soma and thus

235   enables somatic labeling with SST antibodies. We report that while this approach

236   allowed us to identify ample SST+ soma, few of these were PNN-enmeshed ($1.2 \pm$

237   0.6%; n=3) (**Fig 3n-o**). As confirmation of the efficacy of ICV colchicine to prevent

238   neuropeptide export from the soma, we note that many Agrp+ PNN-enmeshed cell

239   bodies were also observed, corroborating our findings in NPY-GFP mice (**Fig 3o**).

240   Together, these findings indicate that only a subset of ARC neurons – specifically, those

241   neurons most closely linked to control of energy balance and metabolic homeostasis –

242   are enmeshed by PNNs.
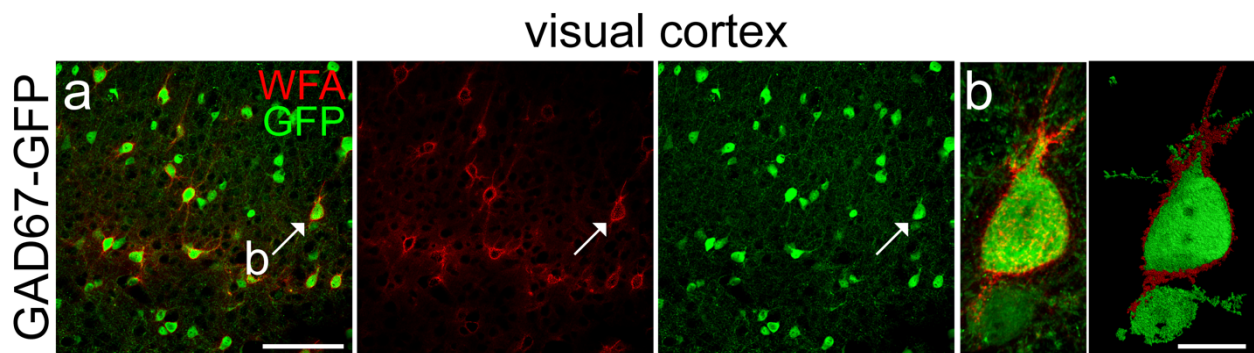
Supplementary Figure 4. PNNs enmesh GABAergic interneurons in the visual cortex.
(a) Confocal image of a coronal section through the primary visual cortex of a GAD67-GFP mouse stained with WFA (red) and GFP antibody (green) shows many PNN enmeshed GABAergic interneurons in layer II-III. Arrow indicates cell shown in (b).
(b) High magnification confocal image (left) and Imaris 3-dimensional surface rendering (right) of an isolated PNN-enmeshed GABAergic interneuron shows PNNs wrapping the soma and proximal processes. Scale bars: 100 um (a), 20 um (b). Images are representative of data from at least 5 animals.

243   A key question raised by these observations is whether PNNs contribute to

244 closure of the CP for ARC neurocircuit development, as is true of V1 and other brain

245 areas. As a first step to address this question, we performed a developmental time-

246 series analysis of PNN formation over the lactation and periweaning period from

247 postnatal day 10 (P10) to P30 in wildtype mice (**Fig 4**). This approach was based on

248 evidence that leptin-dependent maturation of ARC-ME neurocircuits occurs during a CP

249 that closes ~P28[18,19,21], so PNNs must appear during this period if they are to contribute

250 to closure of this CP. WFA labeling revealed only a very faint signal at P10 (10.0 ± 0.8

251 intensity units; n=4 C57B/6 mice) that lacked the typical PNN honeycomb configuration

252 (**Fig 4a**). By P21, WFA labeling intensity had increased by more than 2-fold (27.4 ± 1.7

253 intensity units; n=5 mice) and structural features of PNNs were evident (**Fig 4b**). WFA

254 intensity was further increased at P30 (46.2 ± 2.6 intensity units; n=3 mice), by which

255 time PNN structures appeared fully formed (**Fig 4c**).

256   The time course of ARC PNN formation during postnatal development in wildtype

257 mice was closely paralleled by the maturation of Agrp neuron projections, quantified as

258 an increase in Agrp fiber density in the ARC (0.23 ± 0.07% at P10; 0.87 ± 0.11% at P21;

259 1.48 ± 0.14% at P30) (**Fig 4d**). We note that at CP closure (~P28), both PNNs and

260 Agrp fiber density in the ARC were transiently increased over values characteristic of

261 adult mice (P90: WFA 34.1 ± 2.5 intensity units; Agrp fiber density 1.05 ± 0.09%).

262 During postnatal development, the domain of CD44 expressing tanycytes also

263 expanded from medial (where it covered only the ME β-tanycytes at P21) to lateral and

264 dorsal along the ventricular lining, paralleling the progressive appearance of PNNs (**Fig

265 4e,f**; compare yellow and white arrows and arrowheads).

Figure 4. PNN formation in the ARC occurs during the lactation and periweaning period, corresponding with the maturation of Agrp neurons. (a-c) Confocal images of coronal sections stained with WFA (red), Agrp (green), and dapi (blue) from postnatal wild-type mice at age P10 (a), P21 (b), and P30 (c). PNN staining intensity and ARC Agrp fiber density increase in parallel over this time period. (d) Dot plot shows correlated increase in WFA intensity and Agrp fiber density in the ARC from P10 to P30, as well at P90. Dots (WFA intensity in red, Agrp density in black) represent values from individual animals and horizontal bars represent the mean. WFA intensity is represented by the average over all voxels in the ARC region of interest, with range 0-255. Agrp fiber density is measured as the volume of Agrp+ voxels divided by the total volume of the ARC region of interest. (e, f) High magnification confocal images of coronal sections stained with WFA (red) and CD44 (green) show that CD44 expression in tanycyte processes and endfeet extends from (e) more medial ME β-tanycytes at P21 (yellow arrowhead indicates cell bodies and yellow arrow indicates endfeet) to (f) more laterally located β-tanycytes with processes penetrating the ARC at P30 (white arrowhead and arrow), concomitant with the increase in PNN intensity over the same period. Scale bars: 100 um (a-c), 50 um (e, f). Images are representative of data from at least 3 animals.
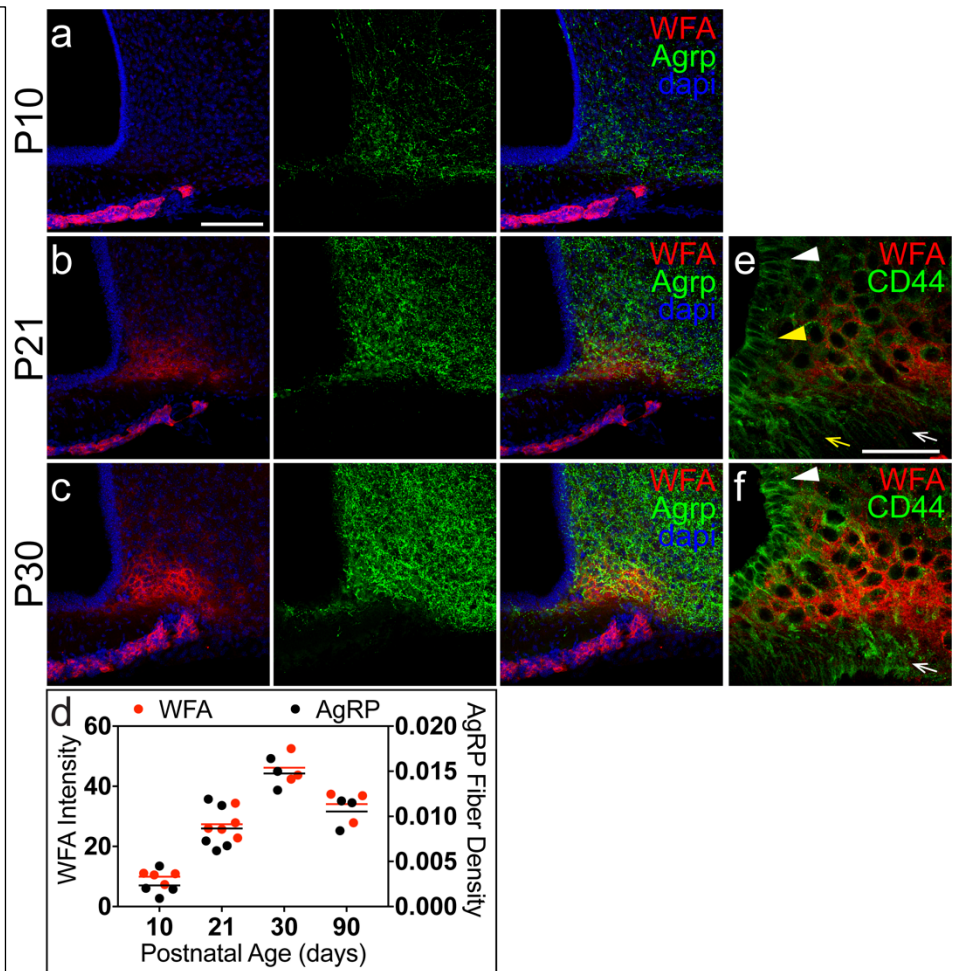
The tight temporal association between ARC PNN

267   formation and maturation of Agrp neuron projections

268   coincides closely with closure of the CP for Agrp neuron development (~P28)[18]. Given

269   the key role played by leptin in the latter process, we next sought to investigate whether

270   input from leptin influences ARC PNN formation. To this end, we performed a

271   developmental time-series analysis of ARC WFA labeling in *ob/ob* pups and *ob/+*

272   control littermates at P15, P21, and P30. At each of these ages, we found that in *ob/ob*

273   mice, ARC WFA intensity (normalized to the mean value of control littermates) was

274   significantly below that detected in controls (*P15*: 0.92 ± 0.01, n=3 *ob/ob* vs. 1.00 ±

275   0.00, n=2 *ob/+*, p=0.002; *P21*: 0.89 ± 0.01, n=5 *ob/ob* vs. 1.00 ± 0.02, n=5 *ob/+*,

15

276   p=0.0005; *P30*: 0.87 ± 0.01, n=5 *ob/ob* vs. 1.00 ± 0.00, n=5 *ob/+*, p<0.0001) (**Fig 5a-d,**

277   **g**).

Figure 5. 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), show reduced WFA labeling and apparent disruption of PNN architecture in the ARC.
(e-f) Confocal images of ARC sections from *ob/ob* pups that received daily i.p. injections of leptin (f) or vehicle (e) from P10 to P30 before being euthanized for analysis with WFA (red) and Agrp (green). Leptin administration during this critical period appeared to restore WFA labeling intensity and PNN architecture. Insets in (c-f) show higher magnification of the ventromedial ARC region indicated by the arrowhead, revealing an increase in Agrp expression within neuronal soma in leptin deficiency.
(g-h) 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 onward with daily i.p. leptin (red open circle) or vehicle (black open circle) injection (h). Values were normalized to the mean WFA intensity of the control groups (*ob/+* or *ob/ob*-veh). Dots represent values from individual animals. Horizontal bars represent the mean with error bars showing SEM. *two-tailed t-test p=0.002, P15; p=0.0005, P21; p<0.0001, P30; p=0.02, P30 rescue. Scale bars: 100 um (a-f), 20 um (insets in c-f). Images are representative of data from each group.

16

279   Based on these results, we hypothesized that like Agrp neuron maturation,

280   formation of PNNs in the ARC is dependent on input from circulating leptin. To test this

281   hypothesis, we administered either leptin or vehicle to *ob/ob* pups according to a

282   schedule reported to mimic the postnatal leptin surge that was used to define the CP for

283   Agrp neuron maturation[18]. Specifically, *ob/ob* pups received a daily intraperitoneal

284   injection of either leptin (10 mg/kg) or vehicle from P10 to P30, and were euthanized

285   one day later for IHC analysis. As predicted, ARC WFA intensity was significantly

286   increased in leptin-compared to vehicle-treated *ob/ob* pups (*P30 rescue*: 1.15 ± 0.02,

287   n=3 *ob/ob-leptin* vs. 1.00 ± 0.01, n=2 *ob/ob-vehicle*, p=0.02) (**Fig 5e, f, h**). There was

288   no significant difference in weight gain between the leptin and vehicle-treated groups.

289   Interestingly, the presence of Agrp+ soma in the ARC of vehicle-treated pups, but not

290   leptin-treated pups (insets in **Fig 5e, f**) appears to offer confirmatory evidence of leptin

291   action, which is known to reduce Agrp expression[32]. A similar pattern was also

292   observed when Agrp staining between *ob/+* and *ob/ob* pups was compared at P30

293   (insets in **Fig 5c, d**).

294   To determine how ARC PNNs are affected by persistent leptin deficiency in

295   adulthood, we performed WFA labeling of 12 week-old *ob/ob* mice and age-matched

296   C57B/6 mice followed by high-resolution confocal microscopy and Imaris 3-dimensional

297   image analysis (**Fig 6a, b**). Paradoxically, in the ARC-ME area, WFA intensity in *ob/ob*

298   mice (normalized to the mean value of age-matched controls) was significantly higher

299   than in controls (1.24±0.05, n=5 *ob/ob* vs. 1.00±0.03, n=5 wt; p=0.004)(**Fig 6e**),

300   whereas no such difference in intensity was detected in primary visual cortex of the

301   same animals (1.00±0.01, n=5 *ob/ob* vs. 0.99±0.01, n=5 wt; p=0.2) (**Supplementary**

Fig 5). To investigate whether this increase of ARC PNN density 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 age 12 weeks) or were fed standard chow and sacrificed at the same age. We report that whereas no difference in ARC WFA intensity was detected between HFD and chow-fed cohorts (1.00±0.00, n=5 hfd vs. 1.00±0.00, n=3 chow), tanycytic CD44 expression was increased in the former group (1.11±0.03, n=5 hfd vs. 1.00±0.01, n=5 chow;
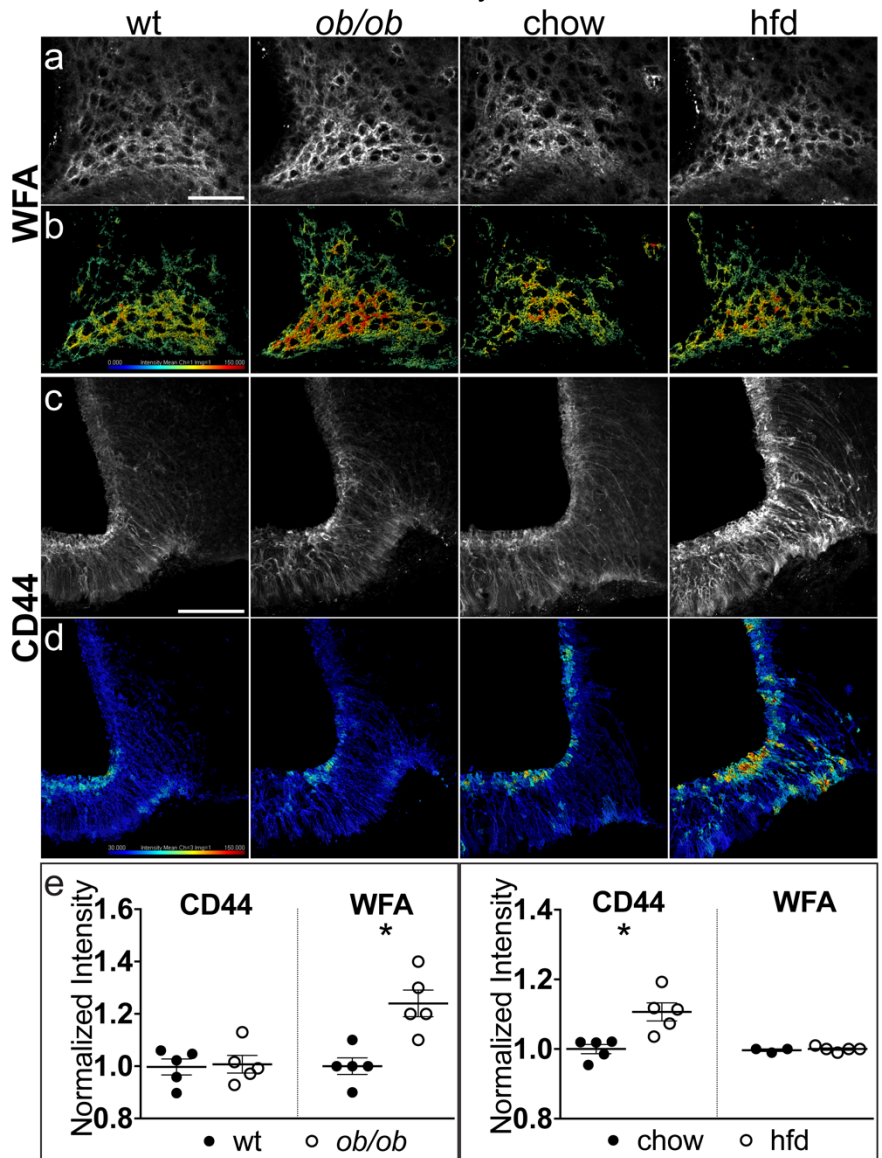

Figure 6. Leptin-deficient *ob/ob* mice and DIO mice fed a high fat diet, two rodent models of obesity and glucose intolerance, exhibit altered ARC PNNs or PNN constituents. (a, b) High magnification confocal images (a) of coronal sections stained with WFA and corresponding Imaris spectral images (b) showing voxel intensity from C57B/6 wt, *obob*, chow-fed, or high-fat diet (HFD) -fed mice. Adult *obob* mice exhibited increased ARC PNN intensity compared to wt, but there was no difference between mice fed chow and HFD. (c, d) High magnification confocal images (c) of coronal sections stained with CD44 and corresponding Imaris spectral images (d) showing voxel intensity from C57B/6 wt, *obob*, chow fed, or HFD fed mice. DIO mice fed HFD exhibited increased tanycyte CD44 expression compared with chow fed mice, with no difference observed between wt and *obob* mice. Note that wt and *obob* mice are age-matched 3 mo old mice fed standard chow diet. Chow and HFD mice were C57B/6 wt mice that were fed either chow or hfd for 12 weeks (beginning at age 3 mo. and sacrificed at age 6 mo.). Spectral reference line shown in (b) with intensity range 0 to 150 and (d) with range 30 to 150. (e) Dot plots show normalized intensity values for CD44 and WFA in the ARC of wt and *obob* mice (left) and chow and HFD mice (right). Values were normalized to the mean WFA or CD44 intensity of the control groups (wt or chow). Dots represent values from individual animals. Horizontal bars represent the mean with error bars showing SEM. *two-tailed t-test p=0.004 for WFA *obob* v. wt, p=0.007 for CD44 HFD v. chow. Scale bars: 50 um (a), 100 um (c). Images are representative of data from each group.

18

325 p=0.007)(**Fig 6c-e**). In contrast, no difference in tanycytic CD44 expression was

326 observed between *ob/ob* and chow-fed control mice (1.01±0.03, n=5 *ob/ob* vs.

327 1.00±0.03, n=5 wt; p=0.8). Together, these data suggest that increased PNN density in

328 the ARC of adult *ob/ob* mice is secondary to leptin deficiency rather than to obesity *per*

329 *se*, whereas the reverse is true for the increase of tanycytic CD44 expression in HFD-

330 fed (but not *ob/ob)* mice.
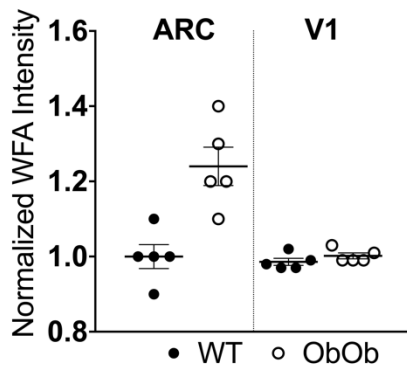
Supplementary Figure 5. Dot plot shows normalized intensity values for WFA in the ARC and V1 of wt and *obob* mice. Increased WFA intensity in *obob* mice was seen in the Arc, but not in V1. Values were normalized to the mean WFA intensity of wt ARC or V1. Dots represent values from individual animals. Horizontal bars represent the mean with error bars showing SEM.

To determine if PNNs are present in the ARC of other mammalian species, we performed WFA staining in the hypothalamus of both rats and humans. We report that PNNs are present in the ARC-ME of all 3 species (**Supplementary Fig 6**). As in the mouse, a majority of cells enmeshed by PNNs in the human ARC are NPY/Agrp neurons, with both the soma and proximal dendrites of these neurons wrapped by WFA+ material (**Fig 7** and **Supplementary Movie 4**). Interestingly,

340 PNN-enmeshed cells in humans are more sparsely distributed than those in mice, which

341 enhances the ability to see how the mesh structure associates with the neuronal

342 contours.

343

344
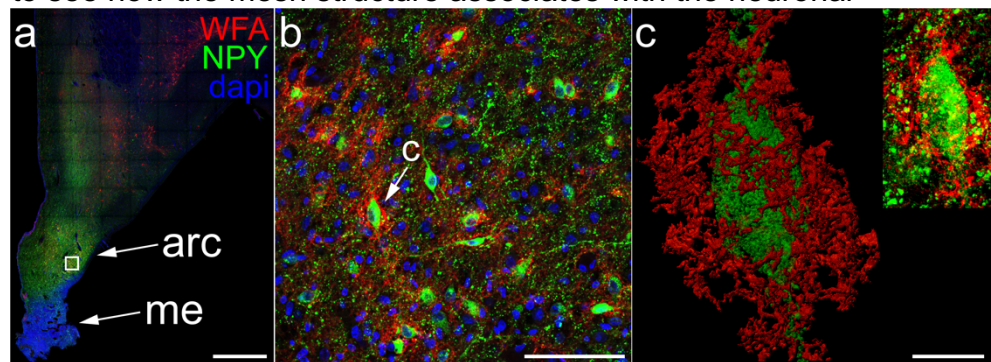
Figure 7. PNNs enmesh NPY+ neurons in the human Arc. (a) Multi-tile confocal image of a coronal section through the human mediobasal hypothalamus stained with WFA (red), NPY (green), and dapi (blue). Boxed region shows location of image in (b). (b) High magnification image shows many PNN-enmeshed NPY+ cells in the human Arc. Arrow indicates cell shown in (c). (c) High magnification Imaris 3-dimensional surface rendering of human ARC PNN-enmeshed NPY+ cell shows PNNs wrapping the soma and proximal processes, as in the mouse ARC and PNN-enmeshed cells in other brain areas. Inset shows raw image with WFA (red) and NPY (green). See corresponding supplementary movie 4. Scale bars: 2 mm (a), 100 um (b), 10 um (c). Images are representative of data from 2 human specimens.
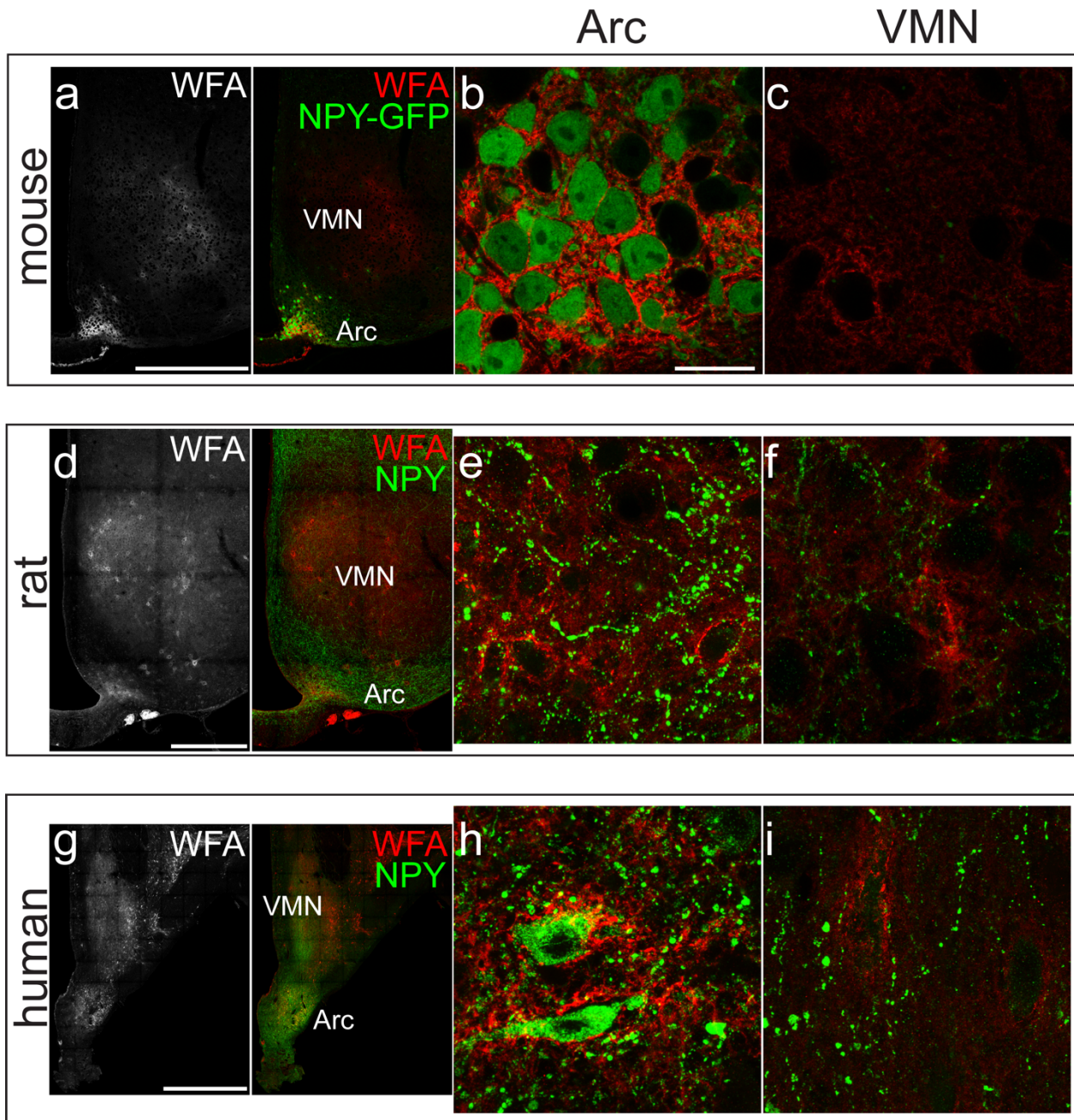
Supplementary Figure 6. PNNs are conserved across the mouse, rat, and human Arc.
(a, d, g) Low magnification confocal images of coronal sections from mouse (a), rat (d), and human (g), stained with WFA and GFP (Npy-GFP mice in (a)) or NPY (d, g). Across species, the ARC displays more intense WFA-labeling than the nearby VMN, where WFA labeling is diffuse. This is confirmed in higher magnification confocal images of the ARC (b, e, h) and VMN (c, f, i). Scale bars: 500 um (a, d), 20 um (b, c, e, f, h, i), 4 mm (g).

Discussion

346   We report that PNNs enmesh Agrp and other leptin receptor-expressing neurons

347 in the ARC (most of which are GABAergic, similar to neurons enmeshed by PNNs in

348   visual cortex and other brain areas), and that their appearance during postnatal

349   development coincides closely with both maturation of Agrp neuron projections and

350   closure of the CP for leptin-mediated regulation of Agrp neuron development[18].

351   Situated at the junction of the ARC and ME, these PNNs are detected in humans as

352   well as rodents, and their formation appears to be sensitive to input from leptin, being

353   deficient in the ARC of postnatal *ob/ob* mice, and restored by leptin administration to

354   *ob/ob* mice during the CP. Together, these findings support a model in which PNNs

355   contribute to closure of the CP for the development of neurocircuits crucial to control of

356   energy balance and glucose metabolism in adulthood*,* and suggest that leptin regulation

357   of ARC neuron development during the CP involves an action on PNN formation. To

358   our knowledge, these findings also offer the first demonstration of PNN formation that is

359   responsive to input from a circulating hormone.

360   In sharp contrast to the deficiency of PNNs we observed in the ARC during

361   postnatal development, adult *ob/ob* mice exhibit an overabundance of ARC PNNs. To

362   explain this paradoxical finding, we draw upon both *in vivo*[9,33,34] and *in vitro*[35] evidence

363   that PNN formation is driven by activity of the enmeshed neuron. Thus, leptin induces

364   depolarization and increases the excitability of Agrp neurons during the CP (prior to

365   ~p21-23)[22], so leptin action on these neurons may constitute a stimulus to PNN

366   formation during this time. During subsequent development, however, a progressive

367   increase in the expression of ATP-sensitive potassium channels by Agrp neurons leads

368   to a "phenotype switch", whereby leptin now exerts the hyperpolarizing effect

369   characteristically observed in adult Agrp neurons[22]. Consequently, Agrp neuron activity

370   is predicted to be reduced in leptin-deficient mice during the CP, but increased in

371   adulthood, and PNN formation parallels these changes.

372   Available evidence suggests both that ARC neurocircuits are highly plastic during

373   development and that this plasticity is markedly reduced upon CP closure. Thus,

374   whereas Agrp neuron ablation induces life-threatening starvation when it occurs in adult

375   mice, it has little or no phenotypic impact when induced shortly after birth[36]. Similarly,

376   CRISPR-mediated deletion of leptin receptors selectively from Agrp neurons in adults

377   recapitulates most of the phenotype of whole body leptin deficiency (hyperphagia,

378   obesity and diabetes), whereas the same deletion has little detectable effect when

379   induced during development[37]. Combined with evidence that over the course of the CP,

380   when Agrp neurons innervate their downstream targets[18,19,21], their response to leptin

381   switches from excitatory to inhibitory[22], these data collectively point to the existence of a

382   mechanism whereby Agrp-linked circuit plasticity during postnatal development is

383   sharply constrained in adulthood. PNNs are likely candidates to mediate this effect, as

384   they can limit plasticity both through direct signaling effects of CSPGs on the enmeshed

385   neuron and by providing a scaffold for binding regulatory molecules such as Otx2[14]

386   (which suppresses plasticity) and the chemorepulsive molecule Semaphorin 3a[38] (which

387   promotes growth of projections away from the enmeshed neuron)[8-15,38].

388   Such a role for PNNs has important implications for understanding how

389   nutritional excess during postnatal development affects ARC neurocircuits in ways that

390   can predispose to obesity and T2D in adulthood[30,39,40]. Specifically, over-nutrition

391   during lactation, due either to maternal HFD consumption[23,25-27] or to culled litter size[24],

392   *1)* reduces numbers of ARC neurons expressing leptin receptors. *2)* decreases leptin

393   responsiveness of these neurons[23,24], 3) impairs formation of ARC projections within

394   hypothalamic feeding circuits[23,25], and 4) predisposes to excess body adiposity and

395   metabolic dysregulation in adulthood[25-27]. Similarly, epidemiological evidence suggests

396   that in humans, late gestation and early childhood are particularly sensitive periods

397   when environmental exposures can shape a predisposition to metabolic disease in

398   adulthood[41]. Interestingly, early gestational undernutrition is also reliably associated

399   with an increased risk of adult obesity[42-44], and this effect can be partially reversed by

400   leptin treatment during the lactation period[45]. Since PNNs sharply limit the plasticity of

401   neurons that they enmesh, and since elsewhere in the brain, the developmental

402   appearance of PNNs heralds CP closure[17], we interpret our discovery that ARC neurons

403   become enmeshed by PNNs at a time corresponding to CP closure as being of potential

404   relevance to experience-dependent plasticity in neurocircuits for energy balance and

405   glucose homeostasis. It is possible, for example, that experimental re-activation of ARC

406   plasticity in adults will ameliorate metabolic dysfunction, as has been observed following

407   re-induction of plasticity in the visual cortex in models of amblyopia[8].

408   In addition to limiting plasticity during development, PNNs can be altered in

409   adulthood in ways that impact the function of the neurons they enmesh, and the variable

410   effect of different CSPGs, hyaluronan and other PNN components to influence plasticity

411   is well-documented[17]. PNN composition can also change in response to injury,

412   inflammation, or neurodegeneration in ways that further restrict the plasticity of

413   enmeshed neurons[10]. Thus, either a deficiency[46-48] or an overabundance[10] of PNNs or

414   individual PNN components can have deleterious effects on circuit behavior and

415   neurological function. As one example, functional recovery after spinal cord injury is

416   limited by "strengthening" of PNNs owing to inflammation and reactive gliosis, such that

417   recovery is improved by PNN disruption[17].

418   In this context, it is notable that diet-induced obesity (DIO) induced by HFD

419   feeding is associated with reactive gliosis involving activation of both microglia and

420   astrocytes in the same ARC area where PNNs are found[49], and recent work suggests

421   that these glial responses are both necessary and sufficient for obesity in this

422   setting[50,51]. In this context, our finding of increased tanycyte expression of the

423   hyaluronan receptor CD44 in mice with DIO is of interest because it raises the

424   possibility that environmental exposures in adulthood (*e.g.,* consuming a HFD) can

425   influence signaling between PNN constituents (*e.g.,* hyaluronan) and adjacent cells

426   (*e.g.,* tanycytes) in the ARC-ME. Reports that tanycytes transport circulating leptin into

427   the mediobasal hypothalamus[52] heighten the potential importance of such interactions.

428   That this obesity-associated increase of tanycyte CD44 content is not observed in *ob/ob*

429   mice despite their severe obesity phenotype is consistent with evidence that

430   hypothalamic gliosis is also not observed in these animals[53], presumably reflecting the

431   requirement for an intact leptin signal in this response. These considerations collectively

432   highlight the need for future investigation into the extent to which both PNN composition

433   and ARC neuronal plasticity are altered in models of obesity, diabetes and related

434   metabolic disorders.

435

436   **Methods**

437   *Animals*

438 GAD67-GFP knock-in[1], LepRb-Cre;Ai14 reporter[2,3], Npy-GFP[4], POMC-GFP[5], and wild-

439 type C57B/6 mice (Jackson Labs), age P60 to P120, were used to characterize

440 neuronal and glial subtypes associated with ARC PNNs. Wild-type C57B/6 mice, age

441 P0 to P90 were used for developmental time-series studies to characterize PNN

442 formation and Agrp neuron maturation. To study the effects of leptin deficiency on PNN

443 formation, we used *ob/ob* mice (Jackson Labs). To characterize PNNs in rats, we used

444 the Wistar strain (Harlan). Both sexes were used for all studies. Mice were on 12h:12h

445 light-dark cycle in 5/cage group housing. Animals were perfusion-fixed with saline and

446 4% paraformaldehyde (PFA), the brains were extracted, post-fixed overnight at 4°C,

447 then sectioned using a vibratome (50 um) or cryostat (12 um). The Institutional Animal

448 Care and Use Committees at St. Joseph's Hospital and Medical Center and the

449 University of Washington approved all animal procedures.

450

451 *Human Specimens*

452 Three brains (ages 23, 64, and 71 years with postmortem intervals 8, 9, and 12 hours,

453 respectively) were collected at autopsy. Multiple wholemount blocks were dissected

454 from the hypothalamus along the 3rd ventricle from each brain, their positions along the

455 3rd ventricle wall were documented, and the tissue was immersion-fixed in 4% PFA at

456 4°C for 24 hours. All specimens were collected with informed consent and in

457 accordance with the St. Joseph's Hospital and Medical Center Committee on Human

458 Research (IRB no. 10BN159).

459

460 *Stereotactic Intracranial Injections*

461   Animals were head-fixed with ear bars in a custom digital stereotactic rig used to

462   perform unilateral intra-ARC injections of Chondroitinase ABC (10 milliunits in 100

463   nanoliters sterile PBS) at the coordinates (0.3 mm lat, 1.1 mm posterior, 5.6 mm depth)

464   relative to bregma, and sacrificed 48h later. For ICV colchicine administration, 1 ul of

465   colchicine (10 ug/ul in sterile PBS) was injected at (1.3, 0, 1.5) relative to bregma and

466   animals were sacrificed 24h later.

467

468   *Postnatal leptin administration*

469   We crossed ob/+ mice to generate litters containing *ob/ob* pups. Litters were genotyped

470   at P1-2 and litter sizes were adjusted to 6-8 pups to standardize nutrition during

471   lactation period. Leptin (10 mg/kg i.p.; made available via Dr. A.F. Parlow; National

472   Hormone & Peptide Program, CA) or vehicle was administered on a daily basis at noon

473   from P10-P30 and the animals were euthanized 24 hours after the last injection.

474

475   *Wholemount Dissection*

476   After cervical dislocation, the mouse brain was removed from the skull and 3V

477   wholemounts were freshly dissected using principles similar to those described for LV

478   wholemounts[54]. Briefly, 3V wholemounts were dissected by performing a

479   ventriculotomy of the 3V from ventral to dorsal and rostral to caudal. The exposed

480   ventricle walls were immersion-fixed in 4% PFA at 4°C overnight prior to

481   immunostaining.

482

483   *Immunohistochemistry and Microscopy*

484   For *Wisteria Floribunda* agglutinin (1:500, Sigma L1516) or HA-binding protein (1:50,

485   AMSbio AMS.HKD-BC41) staining, sections were incubated in WFA or HABP in PBS

486   with 0.5% TX followed by streptavidin-Alexa 488 or 561 (1:1000, Invitrogen Molecular

487   Probes) in PBS/0.5% TX, each for 24h at 4°C. For immunostaining, sections were

488   incubated in primary and secondary antibodies in PBS/0.5% TX and 5% normal goat

489   serum for 24-48h at 4°C. Primary antibodies: chicken anti-GFP (1:500, Aves Labs GFP-

490   1020), rabbit anti-dsRed (1:1000, Clontech 632496), rabbit anti-Agrp (1:200, Phoenix

491   Pharmaceuticals H-003-57), rabbit anti-Npy (1:1000, Abcam ab30914), rat anti-

492   somatostatin (1:500, EMD Millipore MAB354), chicken anti-vimentin (1:500, EMD

493   Millipore AB5733), rabbit anti-CD44 (1:1000, Abcam ab157107), and rat anti-

494   phosphacan DSD-1 (1:500, EMD Millipore MAB5790-I). Secondary antibodies:

495   conjugated to Alexa Fluor dyes (goat or donkey polyclonal, 1:500, Invitrogen). Confocal

496   images were taken on a Leica SPE.

497

498   *Electron Microscopy*

499   Adult C57B/6 mice (Jackson Labs) were transcardially perfused with 4% PFA and 0.5%

500   glutaraldehyde (EMS) in 100 mM phosphate buffer (PB). Brains were post-fixed at 4°C

501   overnight, and 50 μm coronal sections were cut on a Leica VT1000 S vibratome. Pre-

502   embedding IHC was performed using WFA (Sigma L1516), amplified with Vectastain

503   Elite ABC kit (Vector Laboratories), and developed with DAB as described previously[28].

504   Sections were postfixed in 1% osmium tetroxide for 30 min and then embedded in

505   Durcupan ACM epoxy resin (Fluka, Sigma-Aldrich)[55]. For reconstruction of WFA-

506   labeled neurons, we cut ~200 serial semithin (1.5 μm) sections on an Ultracut UC-6

507   ultramictrotome. Selected semithin sections were glued to resin blocks and detached

508   from glass slides by repeated freeze-thaw. Ultrathin sections (60-80 nm) were then cut

509   and placed on Formvar-coated single-slot grids, stained with lead citrate, and examined

510   at 80 kV on a FEI Tecnai G2 Spirit transmission electron microscope equipped with a

511   Morada CCD digital camera (Olympus).

512

513   *Image Analysis and Quantification*

514   To quantify the proportion of various ARC neuronal subtypes enmeshed by PNNs, we

515   used high resolution confocal z-stacks to reconstruct the ventromedial arcuate region

516   containing PNN structures in two coronal sections containing the median eminence

517   (Bregma -1.7 and -2.0). Only reporter-labelled cells that were completely enmeshed

518   (360°) by WFA-labelled PNN matrix were counted as positive. To analyze the intensity

519   of WFA or CD44 labeling, high resolution confocal z-stacks were volumetrically

520   analyzed in Imaris image analysis software (Bitplane) and voxel intensities throughout

521   the region of interest (e.g. Arc) were determined (intensity unit range, 0-255) and

522   compared across study groups. Raw images were then rendered as 3-dimensional

523   spectral images corresponding to individual voxel intensities. For Agrp fiber density

524   measurements, we used Imaris software to determine the volume of Agrp-labeled

525   positive voxels, above a set intensity threshold, as a fraction of the total volume in the

526   measured region of interest.

527

528   *Statistical Analysis*

529   Descriptive statistics and two-tailed t-tests were computed in GraphPad Prism 7. P-

530   value less than 0.05 was considered statistically significant. Unless otherwise stated,

531   dot plots show dots representing data from individual animals with mean and standard

532   error of the mean shown as bars.

533

534   *Data Availability*

535   All raw images and data analysis presented here are available upon request.

536

537   **Figure Legends**

538

539   **Figure 1.** *Wisteria Floribunda* agglutinin (WFA)-labeling in the ventromedial ARC forms

540   a "collar" around the ME. Diagrams at top show mid-sagittal view (left) and ventral view

541   (right) of the mouse brain with insets showing the location and orientation of panel

542   images.

543   (a-d) WFA-labeled (red) coronal sections through the Arc, starting just rostral to and

544   progressing through the ME, show a concentration of WFA-labeled cells located in the

545   ARC at its junction with the ME. Note that the very intense staining below the ME does

546   not correspond to labeling around neurons, but to the pia around the ME.

547   (e) Higher magnification image of the boxed region in (c) showing the dense cluster of

548   WFA-labeled ARC cells.

549   (f) High magnification Imaris 3-dimensional rendering of an isolated WFA-labeled cell at

550   the periphery of the dense cluster (arrow in c) reveals that WFA labels the soma and

551   proximal processes of ARC cells. Inset shows the raw image.

552   (g-h) Low (g) and high (h) magnification images of PNNs labeled by WFA in the visual

553   cortex, where they have been extensively studied, for comparison. Note similar PNN

554   pattern between (h) and (f) wrapping the soma and proximal process.

555   (i-j) WFA-labeled wholemounts of the ARC viewed from the 3rd ventricle wall en-face (i)

556   or the ventral brain surface (j) reveal the distribution of labeled ARC cells forming a

557   "collar" around the ME, which does not contain labeling. From the ventricular surface

558   view (i), the WFA-labeled ARC cells appear as a continuous band along the ventral

559   margin of the ARC.

560   (k) WFA-labeled coronal section from a wild-type mouse sacrificed 2 days after

561   stereotactic unilateral intra-Arc injection of Chondroitinase ABC, an enzyme that digests

562   chondroitin sulfate carbohydrates.

563   Scale bars: 100 um (a-d, g, i-k), 20 um (e), 10 um (f, h). Images are representative of

564   data from at least 5 animals.

565

566   **Figure 2.** WFA-labelling in the ARC is observed around neuronal soma and neurites by

567   electron microscopy and colocalizes with other major PNN components.

568   (a) Low-power electron micrograph of an ARC section labeled with WFA-DAB shows

569   electron dense DAB deposits surrounding a single ARC neuron (white arrowheads).

570   (b) High-power electron micrograph corresponding to the boxed region in (a) shows

571   WFA-labeling localized to the membrane around the cell soma (white arrowheads) and

572   neurites (white arrows). Note labeling adjacent to an apparent terminal filled with

573   synaptic vesicles (s.v.), as well as the appearance of non-labeled membranes (black

574   arrowheads).

575 (c-e) Confocal images of coronal sections through the ARC stained for other PNN

576 components, including hyaluronic acid using HABP (c, green), the cell surface receptor

577 for hyaluronic acid, CD44 (d, green), and the chondroitin sulfate proteoglycan

578 phosphacan (e, green), show colocalization with WFA (red) in the ARC, providing

579 evidence that ARC WFA-labeling corresponds to PNNs.

580 Scale bars: 2 um (a), 500 nm (b), 100 um (c-e). Images are representative of data from

581 at least 3 animals.

582

583 **Figure 3.** PNNs enmesh GABAergic, LepRb-positive, Agrp/NPY neurons in the Arc.

584 Diagram at top shows mid-sagittal view of mouse brain with location and orientation of

585 panel images.

586 (a) Dot plots show the proportion of individual neuronal subtypes enmeshed by PNNs.

587 Dots in this and all subsequent dot plots represent data from independent animals. The

588 left plot shows the percentage of all PNN-enmeshed ARC cells that belong to a

589 particular neuronal subtype. The right plot shows the percentage of all ARC Npy-GFP

590 or POMC-GFP cells that are enmeshed by PNNs.

591 Low (b, e, h, k, n) and high (c, f, i, l, o) magnification images of coronal sections stained

592 with WFA (red) and antibodies to GFP (green) (b, h, k), dsRed (green) (e), or SST

593 (green) and Agrp (white) (n) show that most PNN-enmeshed cells are GAD67-GFP-

594 positive (GABAergic), LepRb-positive, and NPY-positive, while few enmeshed cells

595 express POMC or SST.

596 (d, g, j, m) High magnification Imaris 3-dimensional surface rendering of isolated ARC

597 PNN-enmeshed cells belonging to the various neuronal subtypes (corresponding to b,

598   e, h, k, respectively) show PNNs wrapping the soma and proximal processes. Insets

599   show raw images. See corresponding supplementary movies 1 and 3 for (d) and (j),

600   respectively.

601   Scale bars: 50 um (b, e, h, k, n), 20 um (c, f, i, l, o), 10 um (d, g, j, m). Images are

602   representative of data from at least 3 animals.

603

604   **Figure 4.** PNN formation in the ARC occurs during the lactation and periweaning

605   period, corresponding with the maturation of Agrp neurons.

606   (a-c) Confocal images of coronal sections stained with WFA (red), Agrp (green), and

607   dapi (blue) from postnatal wild-type mice at age P10 (a), P21 (b), and P30 (c). PNN

608   staining intensity and ARC Agrp fiber density increase in parallel over this time period.

609   (d) Dot plot shows correlated increase in WFA intensity and Agrp fiber density in the

610   ARC from P10 to P30, as well at P90. Dots (WFA intensity in red, Agrp density in black)

611   represent values from individual animals and horizontal bars represent the mean. WFA

612   intensity is represented by the average over all voxels in the ARC region of interest, with

613   range 0-255. Agrp fiber density is measured as the volume of Agrp+ voxels divided by

614   the total volume of the ARC region of interest.

615   (e, f) High magnification confocal images of coronal sections stained with WFA (red)

616   and CD44 (green) show that CD44 expression in tanycyte processes and endfeet

617   extends from (e) more medial ME β-tanycytes at P21 (yellow arrowhead indicates cell

618   bodies and yellow arrow indicates endfeet) to (f) more laterally located β-tanycytes with

619   processes penetrating the ARC at P30 (white arrowhead and arrow), concomitant with

620   the increase in PNN intensity over the same period.

32

621   Scale bars: 100 um (a-c), 50 um (e, f). Images are representative of data from at least 3

622   animals.

623

624   **Figure 5.** Leptin-deficient *ob/ob* mice have impaired PNN formation during postnatal

625   development that can be rescued by leptin administration during the critical period.

626   (a-d) Confocal images of ARC sections from *ob/ob* (b,d) and *ob/+* (a,c) control

627   littermates at P15 (a,b) and P30 (c,d), stained with WFA (red) and Agrp (green), show

628   reduced WFA labeling and apparent disruption of PNN architecture in the ARC.

629   (e-f) Confocal images of ARC sections from *ob/ob* pups that received daily i.p. injections

630   of leptin (f) or vehicle (e) from P10 to P30 before being euthanized for analysis with

631   WFA (red) and Agrp (green). Leptin administration during this critical period appeared

632   to restore WFA labeling intensity and PNN architecture. Insets in (c-f) show higher

633   magnification of the ventromedial ARC region indicated by the arrowhead, revealing an

634   increase in Agrp expression within neuronal soma in leptin deficiency.

635   (g-h) Dot plots show normalized intensity values for WFA in the ARC of P15, P21, and

636   P30 *ob/+* (filled circles) and *ob/ob* (open circles) mice (g), or P30 *ob/ob* mice treated

637   from P10 onward with daily i.p. leptin (red open circle) or vehicle (black open circle)

638   injection (h). Values were normalized to the mean WFA intensity of the control groups

639   (*ob/+* or *ob/ob*-veh). Dots represent values from individual animals. Horizontal bars

640   represent the mean with error bars showing SEM. *two-tailed t-test p=0.002, P15;

641   p=0.0005, P21; p<0.0001, P30; p=0.02, P30 rescue.

642   Scale bars: 100 um (a-f), 20 um (insets in c-f). Images are representative of data from

643   each group.

644

645

646 **Figure 6.** Leptin-deficient *ob/ob* mice and DIO mice fed a high fat diet, two rodent

647 models of obesity and glucose intolerance, exhibit altered ARC PNNs or PNN

648 constituents.

649 (a, b) High magnification confocal images (a) of coronal sections stained with WFA and

650 corresponding Imaris spectral images (b) showing voxel intensity from C57B/6 wt, *obob*,

651 chow-fed, or high-fat diet (HFD) -fed mice. Adult *obob* mice exhibited increased ARC

652 PNN intensity compared to wt, but there was no difference between mice fed chow and

653 HFD.

654 (c, d) High magnification confocal images (c) of coronal sections stained with CD44 and

655 corresponding Imaris spectral images (d) showing voxel intensity from C57B/6 wt, *obob*,

656 chow fed, or HFD fed mice. DIO mice fed HFD exhibited increased tanycyte CD44

657 expression compared with chow fed mice, with no difference observed between wt and

658 *obob* mice. Note that wt and *obob* mice are age-matched 3 mo old mice fed standard

659 chow diet. Chow and HFD mice were C57B/6 wt mice that were fed either chow or hfd

660 for 12 weeks (beginning at age 3 mo. and sacrificed at age 6 mo.). Spectral reference

661 line shown in (b) with intensity range 0 to 150 and (d) with range 30 to 150.

662 (e) Dot plots show normalized intensity values for CD44 and WFA in the ARC of wt and

663 *obob* mice (left) and chow and HFD mice (right). Values were normalized to the mean

664 WFA or CD44 intensity of the control groups (wt or chow). Dots represent values from

665 individual animals. Horizontal bars represent the mean with error bars showing SEM.

666 *two-tailed t-test p=0.004 for WFA *obob* v. wt, p=0.007 for CD44 HFD v. chow.

667   Scale bars: 50 um (a), 100 um (c). Images are representative of data from each group.

668

669   **Figure 7.** PNNs enmesh NPY+ neurons in the human Arc.

670   (a) Multi-tile confocal image of a coronal section through the human mediobasal

671   hypothalamus stained with WFA (red), NPY (green), and dapi (blue). Boxed region

672   shows location of image in (b).

673   (b) High magnification image shows many PNN-enmeshed NPY+ cells in the human

674   Arc. Arrow indicates cell shown in (c).

675   (c) High magnification Imaris 3-dimensional surface rendering of human ARC PNN-

676   enmeshed NPY+ cell shows PNNs wrapping the soma and proximal processes, as in

677   the mouse ARC and PNN-enmeshed cells in other brain areas. Inset shows raw image

678   with WFA (red) and NPY (green). See corresponding supplementary movie 4.

679   Scale bars: 2 mm (a), 100 um (b), 10 um (c). Images of representative of data from 2

680   human specimens.

681

682   **Supplementary Figure 1.** Tiled panoramic confocal image of a coronal section at the

683   level of the ARC stained with WFA (top), and corresponding serial coronal sections

684   through the hypothalamus from the optic chiasm (oc) to the infundibular recess (bottom

685   row), reveal a specific concentration of PNN enmeshed cells in the ventromedial ARC at

686   its junction with the ME. Scale bar: 500 um. Images are representative of data from at

687   least 5 animals.

688

689 **Supplementary Figure 2.** Low- (a,c,e) and high-power (b,d,f correspond to boxed

690 regions in low-power images) electron micrographs showing comparative ultrastructural

691 distribution of WFA-DAB deposits around neurons in the cortex (a,b), hippocampus

692 (c,d), and ARC (e,f) reveal very similar patterns of labeling localized to the soma and

693 adjacent neurites in all 3 regions. Scale bars: 2 um (a,c,e), 500 nm (b,d,f). Images are

694 representative of data from at least 3 animals.

695

696 **Supplementary Figure 3.** Serial coronal sections (a) through the hypothalamus from

697 the optic chiasm (oc) to the infundibular recess stained with WFA (red) and CD44

698 (green) reveal regional colocalization of the PNN constituent hyaluronan receptor CD44

699 and PNNs in the ventromedial Arc-ME. Low- (b) and high-magnification (c) images of a

700 coronal section stained with WFA (red), CD44 (green), and vimentin (magenta) reveal

701 that CD44 is expressed on β-tanycyte processes and their endfeet (arrow), but not on α-

702 tanycyte processes or endfeet (arrowhead).

703 Scale bar: 500 um (a), 100 um (b), 50 um (c). Images are representative of data from at

704 least 3 animals.

705

706 **Supplementary Figure 4.** PNNs enmesh GABAergic interneurons in the visual cortex.

707 (a) Confocal image of a coronal section through the primary visual cortex of a GAD67-

708 GFP mouse stained with WFA (red) and GFP antibody (green) shows many PNN

709 enmeshed GABAergic interneurons in layer II-III. Arrow indicates cell shown in (b).

710 (b) High magnification confocal image (left) and Imaris 3-dimensional surface rendering

711 (right) of an isolated PNN-enmeshed GABAergic interneuron shows PNNs wrapping the

712   soma and proximal processes. Scale bars: 100 um (a), 20 um (b). Images are

713   representative of data from at least 5 animals.

714

715   **Supplementary Figure 5.** Dot plot shows normalized intensity values for WFA in the

716   ARC and V1 of wt and *obob* mice. Increased WFA intensity in *obob* mice was seen in

717   the Arc, but not in V1. Values were normalized to the mean WFA intensity of wt ARC or

718   V1. Dots represent values from individual animals. Horizontal bars represent the mean

719   with error bars showing SEM.

720

721   **Supplementary Figure 6.** PNNs are conserved across the mouse, rat, and human Arc.

722   (a, d, g) Low magnification confocal images of coronal sections from mouse (a), rat (d),

723   and human (g), stained with WFA and GFP (Npy-GFP mice in (a)) or NPY (d, g).

724   Across species, the ARC displays more intense WFA-labeling than the nearby VMN,

725   where WFA labeling is diffuse. This is confirmed in higher magnification confocal

726   images of the ARC (b, e, h) and VMN (c, f, i). Scale bars: 500 um (a, d), 20 um (b, c, e,

727   f, h, i), 4 mm (g).

728

729   **Supplementary Movie 1.** Imaris 3-dimensional surface rendering of an isolated PNN-

730   enmeshed (red) GAD67-GFP+ ARC neuron (green).

731   **Supplementary Movie 2.** Imaris 3-dimensional surface rendering of an isolated PNN-

732   enmeshed (red) GAD67-GFP+ V1 interneuron (green).

733   **Supplementary Movie 3.** Imaris 3-dimensional surface rendering of an isolated PNN-

734   enmeshed (red) Npy-GFP+ ARC neuron (green).

735   **Supplementary Movie 4.** Imaris 3-dimensional surface rendering of an isolated PNN-

736   enmeshed (red) NPY+ human ARC neuron (green).

737

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739

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