Hypothalamic perineuronal nets are regulated by sex and dietary interventions

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ABSTRACT

Perineuronal nets (PNNs) are widely present in the hypothalamus, and are thought to provide physical protection and ion buffering for neurons, and regulate their synaptic plasticity and intracellular signaling. Recent evidence indicates that PNNs in the mediobasal hypothalamus plays an important role in the regulation of glucose homeostasis. However, whether and how hypothalamic PNNs are regulated are not fully understood. In the present study, we examined whether PNNs in various hypothalamic regions in mice can be regulated by sex, gonadal hormones, dietary interventions, or their interactions. We demonstrated that gonadal hormones are required to maintain normal PNNs in the arcuate nucleus of hypothalamus in both male and female mice. In addition, PNNs in the terete hypothalamic nucleus display a sexual dimorphism with females higher than males, and high-fat diet feeding increases terete PNNs only in female mice but not in male mice. On the other hand, PNNs in other hypothalamic regions are not influenced by sex, gonadal hormones or dietary interventions. In summary, we demonstrated that hypothalamic PNNs are regulated in a region-specific manner and these results provide a framework to further investigate the potential functions of PNNs in regulating energy/glucose homeostasis at the interplay of sex, gonadal hormones and diets.

37 **Introduction**

38 Obesity is now recognized as a serious global health problem due to its increasing prevalence and
39 comorbidities, e.g. the metabolic syndrome. The WHO reported that over 650 million adults worldwide
40 were obese in 2016 and 40 million children under the age of 5 were overweight or obese in 2018. In U.S.,
41 the prevalence of adult obesity was 42.4% in 2017~2018 according to the Centers for Disease Control
42 and Prevention. The etiology of human obesity is not fully understood, and effective treatments for
43 obesity and associated metabolic disorders are limited. Recent studies revealed genetic and epigenetic
44 basis for variations in human body mass index (BMI) (1-3), and strikingly the majority of BMI-associated
45 genetic variants affect genes that are enriched in the brain (3,4). In particular, the brain hypothalamus
46 receives metabolic and/or hormonal signals reflecting the body's nutritional status and coordinates
47 neuroendocrine and behavioral responses to maintain body weight balance (5-7). Indeed, various
48 genetic variants associated with human obesity have been demonstrated to cause energy and/or glucose
49 dysregulations through impairing functions of neurons and/or neurocircuits within the hypothalamus
50 (8).

51

52 Perineuronal nets (PNNs) in the brain are condensed glycosaminoglycan-rich extracellular matrix
53 structures (9). PNNs typically enmesh neurons in defined circuits, and are thought to provide physical
54 protection and ion buffering for neurons, and regulate their synaptic plasticity and intracellular signaling
55 (9). PNNs abundantly exist in the forebrain regions, e.g. the cortex and the hippocampus, and the PNNs
56 levels in these regions can be regulated by animals' experience, and have been implicated in various
57 neurobiological disorders such as schizophrenia, bipolar disorder, Alzheimer's disease and addictions.
58 Recent evidence indicates that chemical disruption of PNNs in the mediobasal hypothalamus
59 significantly blunts the glucose-lowering effects of central action of fibroblast growth factor 1 (FGF1) in

60 obese Zucker diabetic fatty (ZDF) rats (10). Thus, hypothalamic PNNs may also play important roles in
61 the regulation of energy and/or glucose homeostasis. Indeed, abundant PNNs are present in multiple
62 hypothalamic regions (11,12), but whether and how these hypothalamic PNNs are regulated are not fully
63 understood.
64
65 In the present study, we sought to examine whether PNNs in various hypothalamic regions can be
66 regulated by dietary interventions in mice. Since many hypothalamic regions or neural populations are
67 sexually dimorphic and/or regulated by gonadal hormones (13,14), we also explored the potential effects
68 of sex and/or gonadal hormones on PNN levels. Our results revealed that sex, gonadal hormones and/or
69 dietary interventions can regulate PNNs in a region-specific manner and provide a framework to further
70 investigate the potential functions of PNNs in regulating energy/glucose homeostasis at the interplay of
71 sex, gonadal hormones and diets.
72

73 **METHODS**

74 **Study Animals**

75 C57BL6j male and female mice were group-housed respectively until 14 weeks of age in a temperature-
76 controlled room under a 12h:12h light-dark cycle with ad lib access to regular chow diet (5V5R-Advanced
77 Protocol PicoLab Select Rodent 50 IF/6F, PicoLab) and water. Forty nine mice are divided into 16 groups
78 by four factors (1) sex: male or female groups; (2) surgeries: receiving sham or castration
79 (CAST)/ovariectomy (OVX) surgeries at 14 weeks of age (see below); (3) dietary interventions: chronic
80 feeding with chow or a high-fat diet (HFD, D12492i Rodent Diet With 60 kcal% Fat, Research Diets, Inc.),
81 (4) nutritional status: fed or fasted at the time of perfuse (see below). Care of all animals and procedures
82 were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

83

84 **Surgery**

85 At the age of 14 weeks, either bilateral gonadectomy (CAST for males and OVX for females) or sham
86 surgeries were executed in these mice. The mice were anesthetized with inhaled 2% isoflurane. For OVX
87 in females, the dorsolateral incisions (2 cm) were made, followed by blunted separation of the
88 subcutaneous fat, and incision (1.5cm) the abdominal wall to expose the reproductive tract. The ovaries
89 were isolated and excised. The reproductive tract was returned to the peritoneum and the incisions on
90 the abdominal wall were sutured using sterile Vicryl thread (size 6); the skin incisions were sutured by
91 sterile Prolene thread. For sham surgeries in female mice, the procedures were the same, except that
92 the ovaries were kept intact. For castration in males, a central vertical incision (3 cm) was made, followed
93 by blunted separation of any subcutaneous fat to locate the vas deferens. The vas deferens were ligated
94 with Vicryl; the testes were cut and taken away from the fat pad. The skin incisions were sutured by
95 sterile Prolene thread. For sham surgeries in male mice, the procedures were the same, except that the

96 testes were kept intact.

97

98 **Food intake, body weight, glucose and body composition**

99 One week after the surgeries, mice were randomly divided into two groups to be either regular chow or
100 HFD described above ad libitum for four weeks. Food intake, body weight and fed glucose were
101 measured before the surgeries and once each week after the surgery for five weeks. The glucose was
102 measured using the glucometer via the tail vein; to avoid confounding effects from circadian clock and
103 feeding behavior on the glucose level, we always measured blood glucose in the morning of the day and
104 after a 2-hour short fasting to ensure the empty stomach. Body composition (fat mass and lean mass)
105 was examined with the Bruker minispec mq10 MRS system before the surgeries and 5 weeks afterwards.

106

107 **Perfusion and WFA staining**

108 Five weeks after the surgeries, mice were further divided into fasted and ad libitum groups before the
109 perfusion. For the fasted group, mice were fasted overnight and then perfused in the next morning; for
110 the ad libitum group, both food and water were provided ad libitum at all time before the perfusion. For
111 the perfusion, mice were anaesthetized with inhaled isoflurane and perfused with saline followed by 10%
112 formalin. The brain sections were cut at 25 µm and collected into five consecutive series. One series is
113 used for the following staining and quantification. Free-floating tissue sections were incubated overnight
114 at room temperature for 22 hours using a 1:500 dilution of biotin-labelled Wisteria floribunda agglutinin
115 (WFA) (L1516, Sigma-Aldrich) in PBS + 0.25% Triton X-100. The sections were then washed and incubated
116 for 2 hours at room temperature in 1:500 Streptavidin protein 488 (21832, Invitrogen). Images were
117 captured using a fluorescence microscope with 1s exposure time and no change of contrast for all
118 pictures to visualize various hypothalamic nuclei, including the arcuate hypothalamic nucleus (ARH), the

119 terete hypothalamic nucleus (TE) , the paraventricular hypothalamic nucleus (PVH), lateral
120 hypothalamus (LH), the anterior hypothalamus (AH), the ventromedial hypothalamic nucleus (VMH), the
121 dorsomedial hypothalamic nucleus (DMH), and the perifornical area of the anterior hypothalamus
122 (PeFAH). Fluorescence intensity of PNNs for each image was quantified using an established method by
123 the macro plugin "Perineuronal net Intensity Program for the Standardization and Quantification of ECM
124 Analysis" (PIPSQUEAK AI v5.3.9, Rewire Neuro, Inc.) (15).
125
126 **Statistics**
127 Statistical analysis was done using SPSS (SPSS 22.0.0.0, IBM SPSS Statistics) and GraphPad Prism
128 (GraphPad Prism 8.4.2, Graphpad software, LLC). Multivariate linear regression in SPSS was used to
129 investigate the influence of sex, GDX and chronic dietary intervention on various metabolic parameters
130 (cumulative food intake, body weight change, blood glucose change, fat change and lean change). If sex
131 or GDX was found to have a significant effect on any parameter, the p value for the interaction of sex
132 and GDX was tested. If any two factors were found to have significant effects, the p value for the
133 interaction of these two factors was tested.
134
135 For the PNN fluorescence intensity, in addition to the sex, GDX and chronic diet intervention, we
136 introduced one more factor (nutritional status) as mice were perfused either after an overnight fasting
137 or ad libitum. Similarly, we first used the multivariate linear regression analyses in SPSS to examine
138 effects of these 4 factors on PNNs fluorescence intensity in each hypothalamic region. If sex or GDX was
139 found to have a significant effect on any parameter, we then used the two-way ANOVA to examine the
140 interaction of sex and GDX. In case that sex had a significant effect, we used the GraphPad Prism to
141 further examine effects of dietary intervention and nutritional status in each sex separately. $P < 0.05$ was

142 considered statistically significant.

143

144 **Study approval**

145 Care of all animals and procedures were approved by the Baylor College of Medicine Institutional Animal

146 Care and Use Committee.

147

148 **Results**

149 **Distribution of WFA-labelled PNNs in the mouse hypothalamus**

150 We first surveyed the distribution of PNNs in the mouse hypothalamus using WFA immunostaining.
151 Abundant WFA-labelled PNNs were detected in multiple hypothalamic regions, including the LH, the
152 ARH, the VMH and the TE (Figure 1). In addition, we also observed modest WFA immunoreactivity in the
153 AH, the PVH and the DMH (Figure 1). Consistent with a previous report (12), WFA-labelled PNNs were
154 also observed in a newly identified hypothalamic area, namely the PeFAH (Figure 1A).

155

156 **Effects of sex, gonadal hormones and dietary interventions on metabolic parameters**

157 Given the essential roles of the hypothalamus in regulating energy homeostasis, we sought to examine
158 potential effects of dietary interventions on the level of PNNs in the hypothalamus. In order to explore
159 potential influence by sex and/or gonadal hormones, we included both male and female mice with or
160 without gonadectomy (GDX) in these analyses. Briefly, male and female C57Bl6j mice underwent sham
161 or GDX (castration for males and ovariectomy for females) at the 14 weeks of age, followed by 5-week
162 chronic feeding with either a regular chow diet or HFD. As expected, the multivariate linear regression
163 analysis revealed that compared to chow-fed groups, HFD feeding had profound effects on multiple
164 metabolic parameters, including significantly increasing calorie intake, blood glucose, body weight gain
165 and fat mass gain (Table 1). Notably, fat mass gain was significantly smaller in females than in males
166 (Table 1). We also noted that lean mass gain was significantly larger in females than in males, and that
167 GDX significantly reduced lean mass gain; in addition, there was a significant interaction between sex
168 and GDX on this parameter (Table 1). In summary, multiple metabolic parameters in mice were
169 influenced by sex, gonadal hormones, the dietary interventions, and/or their interactions, as reported
170 by others (16,17).

171

172 **Effects of gonadal hormones on PNN in the ARH**

173 At the end of 5-week feeding, mice were then perfused either ad libitum or after an overnight fasting.
174 We then used these fixed brain samples for WFA immunostaining and then quantified the level of WFA-
175 labelled PNNs in each hypothalamic region in various conditions. In the ARH, the WFA fluorescence
176 intensity was not significantly affected by chronic HFD feeding (vs. chow), or by the acute fasting (vs. ad
177 libitum), as revealed by the multivariate linear regression analysis (Table 2). Interestingly, we noted that
178 GDX significantly reduced WFA fluorescence intensity in the ARH compared to the sham group (Table 2
179 and Figure 2A-2E). A detailed analysis at different rostral-caudal levels revealed that GDX mainly reduced
180 WFA in the rostral to medial ARH (-1.94 to -1.58 mm to Bregma) but the caudal ARH was not affected
181 (Figure 2F). Because female and male mice experienced different GDX surgeries (OVX for females and
182 CAST for males), we used a two-way ANOVA analysis to further analyze the interaction between sex and
183 GDX. We found that the inhibitory effect of GDX was independent of sex; in other words, both OVX in
184 females and CAST in males significantly reduced WFA fluorescence intensity in the ARH (Table 2 and
185 Figure 2G). We also examined effects of dietary innervations on ARH PNNs in sham or in GDX groups,
186 respectively, but did not detect any significant effect of by either chronic HFD feeding or by acute fasting
187 (data not shown). Thus, these results indicate that endogenous gonadal hormones in both males and
188 females are required to maintain normal PNNs in the ARH.

189

190 **Effects of sex and HFD feeding on PNN in the TE**

191 In the TE, female mice showed significantly higher WFA fluorescence intensity compared to males, as
192 revealed by the multivariate linear regression analysis (Table 3 and Figure 3A-3E), and this sex difference
193 existed in a rostral subdivision (-1.70 mm to Bregma) and a medial-caudal subdivision (-2.18 to -2.30 mm

194 to Bregma) of the TE (Figure 3F). Using a two-way ANOVA analysis, we further found that the significant
195 sex difference only existed in sham mice, but not in GDX mice (Figure 3G). Thus, these results indicate
196 that gonad-intact female mice have higher PNNs in the TE than gonad-intact males.
197
198 Given the clear influence by sex, we reanalyzed the effects of HFD feeding in each sex individually and
199 used a two-way ANOVA analysis followed by Sidak tests to further explore potential interactions
200 between chronic HFD feeding and acute fasting. In female mice fed ad libitum, chronic HFD feeding
201 significantly enhanced WFA fluorescence intensity in the TE compared to chow-fed females, but this
202 effect was not observed after an overnight fasting (Figure 4A-4E). This HFD-induced increases existed in
203 a rostral-medial subdivision (-1.70 to -1.94 mm to Bregma) of the TE (Figure 4F). On the other hand, in
204 male mice, neither chronic HFD feeding nor acute fasting induced any changes in WFA fluorescence
205 intensity in the TE (Figure 4G). Thus, these results indicate that chronic HFD feeding can increase PNNs
206 in the TE in female mice but not in male mice.
207
208 **Other hypothalamic regions**
209 We also analyzed effects of sex, gonadal hormones, and/or dietary interventions on WFA fluorescence
210 intensity in all other hypothalamic regions. The multivariate linear regression analysis revealed that WFA
211 fluorescence intensity in the PVH, the PeFAH, the LH, the AH, the VMH or the DMH, was not significantly
212 altered by these conditions (Table 4).
213

214 **Discussions**

215 One interesting observation of our study is that endogenous gonadal hormones are required to maintain
216 normal PNNs in the ARH in both male and female mice. The ARH in the hypothalamus has been long
217 believed to contain the first order neurons that respond to the peripheral signals, including leptin (18-
218 20), insulin (21-23), ghrelin (24), asprosin (25) and estrogens (26-28). The ARH neurons include those
219 expressing pro-opiomelanocortin (POMC) and those co-releasing agouti-related peptide (AgRP),
220 neuropeptide Y (NPY), and GABA (referred to as AgRP neurons). POMC neurons synthesize and secret an
221 anorexigenic peptide, α-melanocyte-stimulating hormone (α-MSH) which prevents overeating and body
222 weight gain (29,30). On the other hand, AgRP neurons are orexigenic (31), and activation of AgRP
223 neurons promotes eating even when mice are satiated (32,33). Recent evidence also indicates that non-
224 AgRP GABAergic neurons in the ARH provide a redundant orexigenic mechanism to drive feeding and
225 body weight gain (34). Notably, the majority of PNNs in the ARH enmeshes AgRP neurons and GABAergic
226 neurons, with only a small portion enmeshing POMC neurons (11). Interestingly, reduced PNNs in the
227 ARH is found in ob/ob mice deficient in the leptin gene and in ZDF rats with a mutated leptin receptor
228 gene (10,11), highlighting an important role of leptin signaling in maintaining the normal PNNs in the
229 ARH. Further, the central administration of FGF1 in ZDF rats can enhance PNNs in the ARH, associated
230 with a prolonged glucose-lowering benefit (10). Importantly, chemical disruption of PNNs significantly
231 shortens this glucose-lowering effect of FGF1 (10). Thus, PNNs in the ARH play an essential role in
232 regulating glucose balance.

233

234 Here we demonstrated that PNNs in the ARH are significantly reduced by removal of the ovaries in
235 female mice, suggesting that PNNs are also regulated by ovarian hormones. Supporting this notion, co-
236 administration of both ovarian hormones (estrogen and progesterone), which simulates pregnancy, can

237 trigger PNN formation in the medial preoptic area, a hypothalamic region normally devoid of PNNs in
238 non-pregnant females (35). While the role of progesterone in energy and glucose homeostasis is not
239 clear (14), estrogen is well established to provide many metabolic benefits, including suppressing food
240 intake, increasing energy expenditure, reducing body weight gain, enhancing insulin sensitivity, and
241 lowering blood glucose (16,36-41). Both estrogen receptor α (ERα) and estrogen receptor β (ERβ) are
242 implicated in mediating these metabolic effects (42-44). Notably, abundant ERα is expressed by POMC
243 neurons in the ARH (45-47), to a less extent by AgRP neurons (48,49), while ERβ expression in the ARH
244 is minimal (50). We recently showed that intact ERα signals are required for the glucose-sensing
245 functions of ARH neurons (51). Further, estrogen can enhance glutamatergic synapses onto POMC
246 neurons (52), and increase their excitability (53,54). Importantly, we previously showed that selective
247 deletion of ERα from POMC-lineage neurons in female mice results in hyperphagia and obesity (26), and
248 attenuates estrogen-induced anorexia (55). Thus, these findings, together with our current observations
249 that PNNs are reduced by ovariectomy, suggest that PNNs in the ARH may mediate, at least a portion of,
250 estrogenic actions on energy and glucose homeostasis, and future investigations are warranted to test
251 this possibility. Interestingly, we also observed similar reductions of PNNs in the ARH of male mice
252 depleted with endogenous testosterone. Consistently, it has been reported that testosterone
253 administration can stimulate PNN formation in the forebrain of song birds (56). However, it is worth
254 noting that testosterone can be converted into estrogen by aromatase (57). Thus, it remains to be tested
255 whether the testosterone-induced PNN expression is mediated by testosterone per se or by converted
256 estrogen.
257
258 Another hypothalamic region where PNNs are regulated is the TE, where female mice showed higher
259 PNNs than males. Importantly, this sex difference was only observed in gonad-intact mice, but was

260 blunted in mice with gonads surgically removed. These results suggest that PNNs in the TE may be also
261 regulated by gonadal hormones. Supporting this possibility, estrogen has been shown to increase
262 oxytocin binding in the TE in rats (58), indicating potential actions of estrogen in this region. It has to be
263 pointed out that, unlike other hypothalamic regions (e.g. the ARH, VMH, etc.), the TE has received very
264 little attention with no more than 5 publications regarding this nucleus available in the PubMed. Early
265 neuroanatomic studies reported that cell bodies of cholecystokinin neurons and calcitonin gene-related
266 peptide (CGRP) neurons are found in the TE (59,60). In addition, the TE harbors nerve fibers that are
267 immunoreactive for NPY, α-MSH, β-endorphin, corticortropin-releasing hormone, galanin, and
268 substance P (59,60), suggesting that the TE neurons receive a wide range of innervations and inputs from
269 other neural populations. Interestingly, we found that in female mice only, PNNs in the TE was
270 upregulated by chronic HFD feeding, while such effect was not observed after an overnight fasting. It is
271 worth noting that among many hypothalamic regions expressing PNNs, the TE is the only region where
272 PNNs can be regulated by dietary interventions. Considering that the TE receives inputs from NPY and
273 α-MSH, both of which are implicated in the regulation of feeding and body weight, we suggest that TE
274 neurons and the PNNs enmeshing these neurons may play an under-appreciated role in the context of
275 energy homeostasis. This possibility warrants future investigations.
276
277 In summary, here we systemically assessed PNNs in multiple hypothalamic regions and explored
278 potential regulations by sex, gonadal hormones, dietary interventions, and their interactions. We
279 acknowledge that the limitation of the current study is its descriptive nature. However, we want to point
280 out that our results filled a gap of knowledge in the relevant field and provided a framework to develop
281 new hypotheses and design future studies to determine the physiological functions of hypothalamic
282 PNNs and to explore the regulatory mechanisms. For example, the strong regulations by gonadal

283 hormones on PNNs in the ARH suggest that gonadal hormones may act through PNNs in the ARH to
284 regulate energy and/or glucose homeostasis. Further, given that PNNs in the ARH are required to
285 mediates metabolic benefits of FGF1 (10), the efficacy of FGF1 may need to be examined in animals
286 depleted of gonadal hormones, which would provide important pre-clinical data for potential
287 applications of FGF1 or its analogs in aged men with testosterone deficiency or in post-menopausal
288 women. In addition, the dietary regulations on PNNs in the TE suggest that this understudied
289 hypothalamic nucleus may play a role in the regulation of feeding and body weight balance. Importantly,
290 since these dietary regulations only existed in female mice but not in male mice, future studies need to
291 include at least female animals, if not both. Only including male animals (a common practice in many
292 laboratories) might miss potential discoveries about PNNs in the TE or TE neurons themselves.
293

294 **Author Contributions:** NZ was involved in experimental design and most of procedures, data acquisition
295 and analyses, and writing the manuscript. ZY assisted in data analysis. HL, MY, YH, HL, CL, LT, LW, NY, JH,
296 NS, YY and CW assisted in in surgical procedures and production of study mice. LLC and TZ are involved
297 in the study design and manuscript preparation. YX is the guarantors of this work and, as such, had full
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309

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600 # Tables

601 **Table 1. Effects of sex, GDX and diet on various metabolic parameters.**

	B	p value	95.0% Confidence Interval	
			Lower Bound	Upper Bound
Cumulative intake (calorie)				
Female vs Male#	-13.234	0.298	-38.542	12.075
GDX vs Sham##	7.461	0.554	-17.798	32.721
High fat diet vs Regular chow###	56.660	p < 0.001***	31.306	82.013
Body weight change (g)				
Female vs Male#	-0.448	0.620	-2.255	1.359
GDX vs Sham##	0.532	0.556	-1.275	2.339
High fat diet vs Regular chow###	5.389	p < 0.001***	3.582	7.196
Blood glucose change (mg/dL)				
Female vs Male#	13.141	0.128	-3.915	30.198
GDX vs Sham##	1.143	0.893	-15.913	18.200
High fat diet vs Regular chow###	37.838	p < 0.001***	20.782	54.894
Fat change (g)				
Female vs Male#	-2.052	0.012*	-3.633	-0.471
GDX vs Sham##	1.495	0.063	-0.085	3.076
P for the interaction of Sex and GDX		0.451		
High fat diet vs Regular Chow###	5.539	p < 0.001***	3.958	7.120
P for the interaction of Sex and Diet		0.226		
Lean change (g)				
Female vs Male#	1.405	p < 0.001***	0.828	1.983
GDX vs Sham##	-0.805	0.007**	-1.383	-0.228
P for the interaction of Sex and GDX		p < 0.001***		
High fat diet vs Regular chow###	-0.107	0.710	-0.684	0.470

602

603 # Here we use male mice as reference. ## Here we use sham mice as reference. ### Here we use

604 regular chow as reference.

605

606

607 **Table 2. Effects of sex, GDX, diet and fasting on PNNs in the ARH.**

	B	p value	95.0% Confidence Interval	
			Lower Bound	Upper Bound
Female vs Male#	3216.040	0.710	-14091.082	20523.162
GDX vs Sham##	-28525.814	0.002**	-45832.935	-11218.692
High fat diet vs Regular chow###	5478.986	0.527	-11858.261	22816.234
Fast vs Ab libitum####	1070.635	0.902	-16266.612	18407.883

608 # Here we use male mice as reference. ## Here we use sham mice as reference. ### Here we use

609 regular chow as reference. ####Here we use fed mice as reference.

610

611

612

613 **Table 3. Effects of sex, GDX, diet and nutritional status on PNNs in the TE.**

	B	p value	95.0% Confidence Interval	
			Lower Bound	Upper Bound
Female vs Male#	37265.520	0.004**	12278.584	62252.456
GDX vs Sham##	8878.365	0.478	-16108.572	33865.301
High fat diet vs Regular chow###	3443.149	0.783	-21587.281	28473.579
Fast vs Ab libitum####	-2328.269	0.852	-27358.698	22702.161

614 # Here we use male mice as reference. ## Here we use sham mice as reference. ### Here we use regular

615 chow as reference. ####Here we use fed mice as reference.

616

617

618 **Table 4. Effects of sex, GDX, diet and nutritional status on PNNs in the PVH, PeFAH, LH, AH, VMH and**

619 **DMH.**

	B	p value	95.0% Confidence Interval	
			Lower Bound	Upper Bound
PVH				
Female vs Male[#]	1093.487	0.785	-6950.901	9137.874
GDX vs Sham[##]	1801.150	0.649	-6123.285	9725.586
High fat diet vs Regular chow[###]	7111.050	0.082	-948.109	15170.209
Fast vs Ab libitum[####]	1437.498	0.721	-6621.661	9496.657
PeFAH				
Female vs Male[#]	10127.575	0.447	-16501.677	36756.827
GDX vs Sham[##]	12003.295	0.361	-14228.883	38235.473
High fat diet vs Regular chow[###]	12968.496	0.332	-13709.655	39646.646
Fast vs Ab libitum[####]	3879.818	0.771	-22798.333	30557.969
LH				
Female vs Male[#]	48723.960	0.118	-12839.004	110286.924
GDX vs Sham[##]	25737.089	0.397	-34907.897	86382.074
High fat diet vs Regular chow[###]	-22043.519	0.475	-83719.530	39632.491
Fast vs Ab libitum[####]	-17950.520	0.560	-79626.531	43725.490
AH				
Female vs Male[#]	9830.509	0.347	-11017.746	30678.764
GDX vs Sham[##]	6991.586	0.496	-13545.796	27528.969
High fat diet vs Regular chow[###]	6079.521	0.560	-14807.017	26966.059
Fast vs Ab libitum[####]	599.040	0.954	-20287.498	21485.578
VMH				
Female vs Male[#]	8833.935	0.639	-28921.823	46589.693
GDX vs Sham[##]	6705.915	0.718	-30486.859	43898.688

High fat diet vs Regular chow###	-14866.204	0.432	-52691.292	22958.884
Fast vs Ab libitum####	-19095.426	0.314	-56920.514	18729.662
DMH				
Female vs Male#	-2075.195	0.135	-4826.375	675.985
GDX vs Sham##	267.179	0.843	-2442.978	2977.336
High fat diet vs Regular chow###	1587.169	0.252	-1169.064	4343.401
Fast vs Ab libitum####	-487.298	0.723	-3243.531	2268.934

Here we use male mice as reference. ## Here we use sham mice as reference. ### Here we use regular chow as reference. ####Here we use fed mice as reference.

624 ## Figure Legends

625 **Figure 1. Distribution of WFA-labelled PNNs in mouse hypothalamus.** Representative fluorescent
626 microscopic images showing WFA-labelled PNNs (green) and DAPI counterstaining (purple) in a series of
627 coronal brain sections at the level of -0.82 mm (A), -1.34 mm (B), -1.58 mm (C) and -1.82 mm (D) relative
628 to the Bregma. Scale bars = 200 µm. AH, the anterior hypothalamus; ARH, the arcuate hypothalamic
629 nucleus; DMH, the dorsomedial hypothalamic nucleus; LH, the lateral hypothalamus; PeFAH, the
630 perifornical area of the anterior hypothalamus (PeFAH); PVH, the paraventricular hypothalamic nucleus;
631 TE, the terete hypothalamic nucleus; VMH, the ventromedial hypothalamic nucleus.

632

633 **Figure 2. GDX reduces PNNs in the ARH.** (A-D) Representative fluorescence microscopic images showing
634 WFA-labelled PNNs (green) in the ARH of female sham (A), female OVX (B), male sham (C) and male CAST
635 mice (D). Scale bars = 200 µm. ARH, the arcuate hypothalamic nucleus. (E) Quantification of the total
636 WFA fluorescence intensity in the ARH from sham vs. GDX mice. N = 24 or 25 mice per group. Data are
637 presented with mean ± SEM with individual data points. **, P<0.01 in unpaired two-tailed Student's t-
638 tests. (F) Quantification of the WFA fluorescence intensity at various rostral-caudal levels of the ARH
639 from sham vs. GDX mice. N = 24 or 25 mice per group. Data are presented with mean ± SEM. *, P<0.05
640 and **, P<0.01 in unpaired two-tailed Student's t-tests at each level. (G) Quantification of the total WFA
641 fluorescence intensity in the ARH from female or male mice with sham or GDX surgeries. N = 12 or 13
642 mice per group. Data are presented with mean ± SEM with individual data points. *, P<0.05 in two-way
643 ANOVA analysis followed by Holm-Sidak post hoc tests.

644

645 **Figure 3. PNNs in the TE display a sexual dimorphism.** (A-D) Representative fluorescence microscopic
646 images showing WFA-labelled PNNs (green) in the TE of female sham (A), male sham (B), female OVX
647 (C), and male CAST mice (D). Scale bars = 200 µm. TE, the terete hypothalamic nucleus. (E) Quantification
648 of the total WFA fluorescence intensity in the TE from female vs. male mice. N = 24 or 25 mice per group.
649 Data are presented with mean ± SEM with individual data points. **, P<0.01 in unpaired two-tailed
650 Student's t-tests. (F) Quantification of the WFA fluorescence intensity at various rostral-caudal levels of
651 the TE from female vs. male mice. N = 24 or 25 mice per group. Data are presented with mean ± SEM. *,
652 P<0.05 and **, P<0.01 in unpaired two-tailed Student's t-tests at each level. (G) Quantification of the
653 total WFA fluorescence intensity in the TE from female or male mice with sham or GDX surgeries. N = 12
654 or 13 mice per group. Data are presented with mean ± SEM with individual data points. **, P<0.01 in
655 two way ANOVA analysis followed by Sidak post hoc tests.
656
657 **Figure 4. HFD feeding increases TE PNNs in female mice.** (A-D) Representative fluorescence microscopic
658 images showing WFA-labelled PNNs (green) in the TE of female mice that were fed chow ad libitum (A),
659 HFD ad libitum sham (B), chow after an overnight fasting (C), and HFD after an overnight fasting (D).
660 Scale bars = 200 µm. TE, the terete hypothalamic nucleus. (E) Quantification of the total WFA
661 fluorescence intensity in the TE from female mice with various dietary interventions. N = 6 mice per
662 group. Data are presented with mean ± SEM with individual data points. *, P<0.05 in two-way ANOVA
663 analysis followed by Sidak post hoc tests. (F) Quantification of the WFA fluorescence intensity at various
664 rostral-caudal levels of the TE from female mice fed with chow or HFD ad libitum. N = 6 mice per group.
665 Data are presented with mean ± SEM. *, P<0.05 and **, P<0.01 in unpaired two-tailed Student's t-tests
666 at each level. (G) Quantification of the total WFA fluorescence intensity in the TE from male mice with

667 various dietary interventions. N = 6 or 7 mice per group. Data are presented with mean ± SEM with

668 individual data points. No significance was detected by in two way ANOVA analysis.

Figure 1

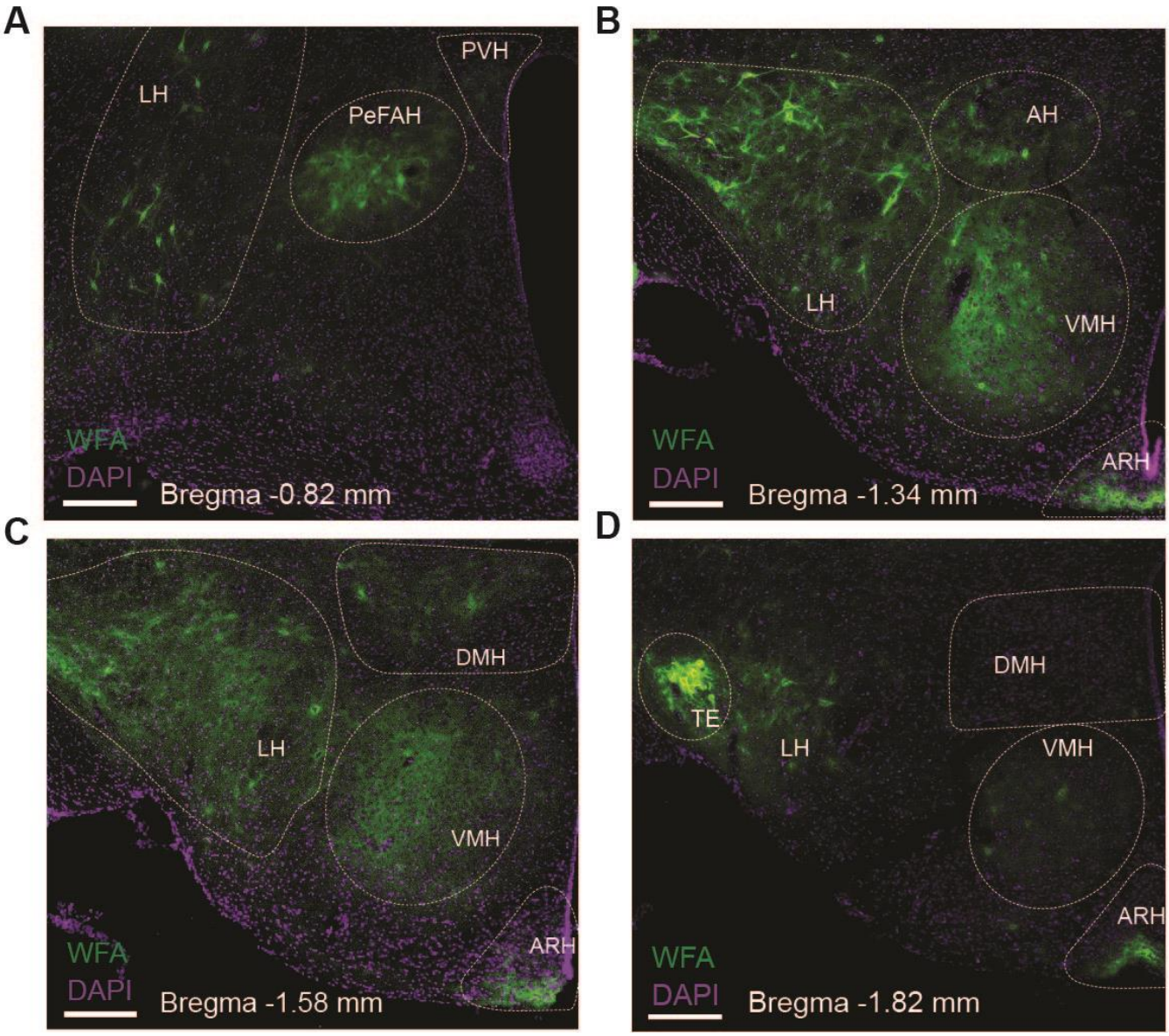

A
LH
PeFAH
PVH
WFA
DAPI
Bregma -0.82 mm
B
AH
LH
VMH
ARH
WFA
DAPI
Bregma -1.34 mm
C
DMH
LH
VMH
ARH
WFA
DAPI
Bregma -1.58 mm
D
DMH
TE
LH
VMH
ARH
WFA
DAPI
Bregma -1.82 mm

Figure 2

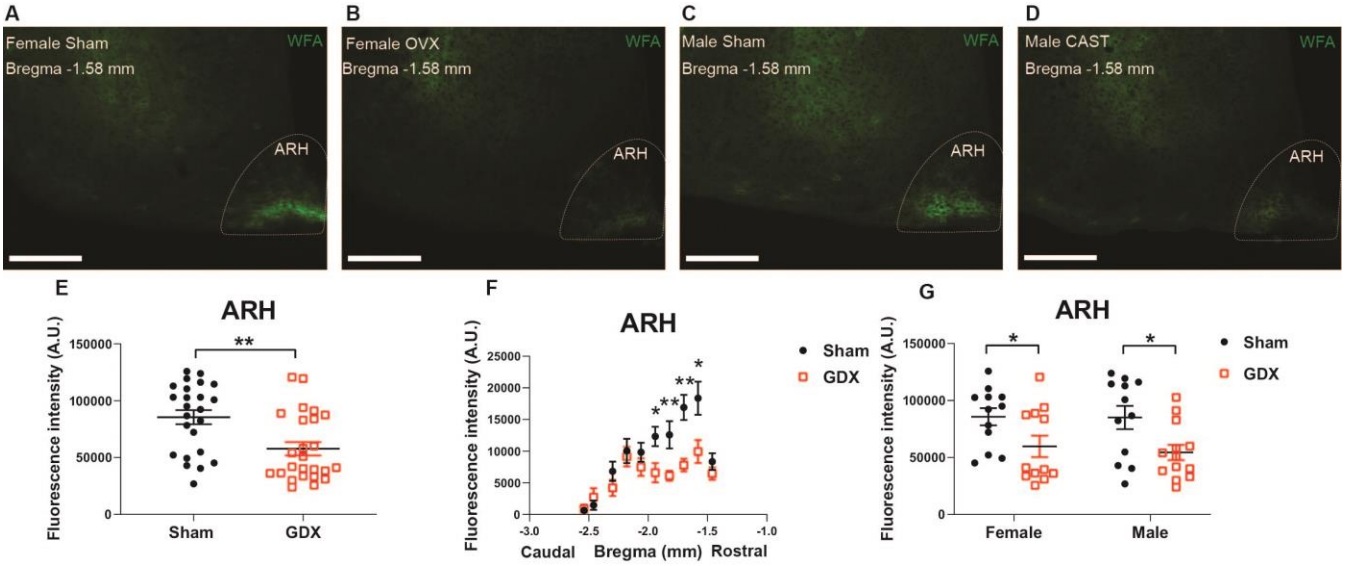

A
Female Sham
Bregma -1.58 mm
WFA
ARH
B
Female OVX
Bregma -1.58 mm
WFA
ARH
C
Male Sham
Bregma -1.58 mm
WFA
ARH
D
Male CAST
Bregma -1.58 mm
WFA
ARH
E
ARH
Fluorescence intensity (A.U.)
150000
100000
50000
0
**
Sham
GDX
F
ARH
Fluorescence intensity (A.U.)
25000
20000
15000
10000
5000
0
-3.0
-2.5
-2.0
-1.5
-1.0
Caudal
Bregma (mm)
Rostral
Sham
GDX
G
ARH
Fluorescence intensity (A.U.)
150000
100000
50000
0
Female
Male
Sham
GDX

Figure 3

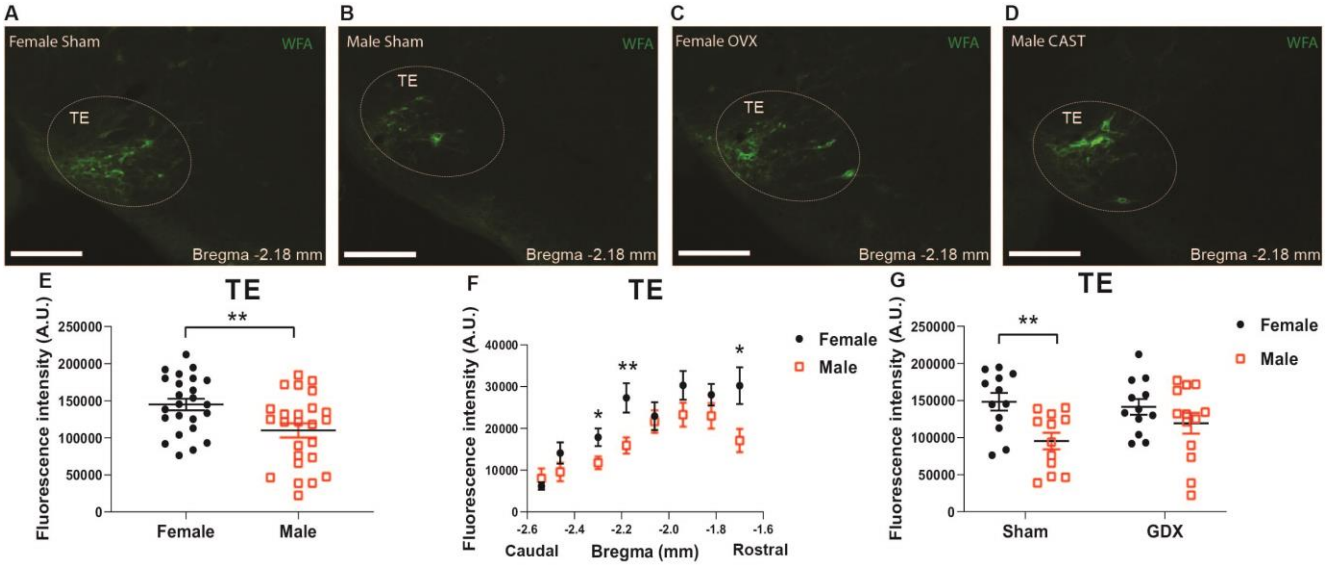
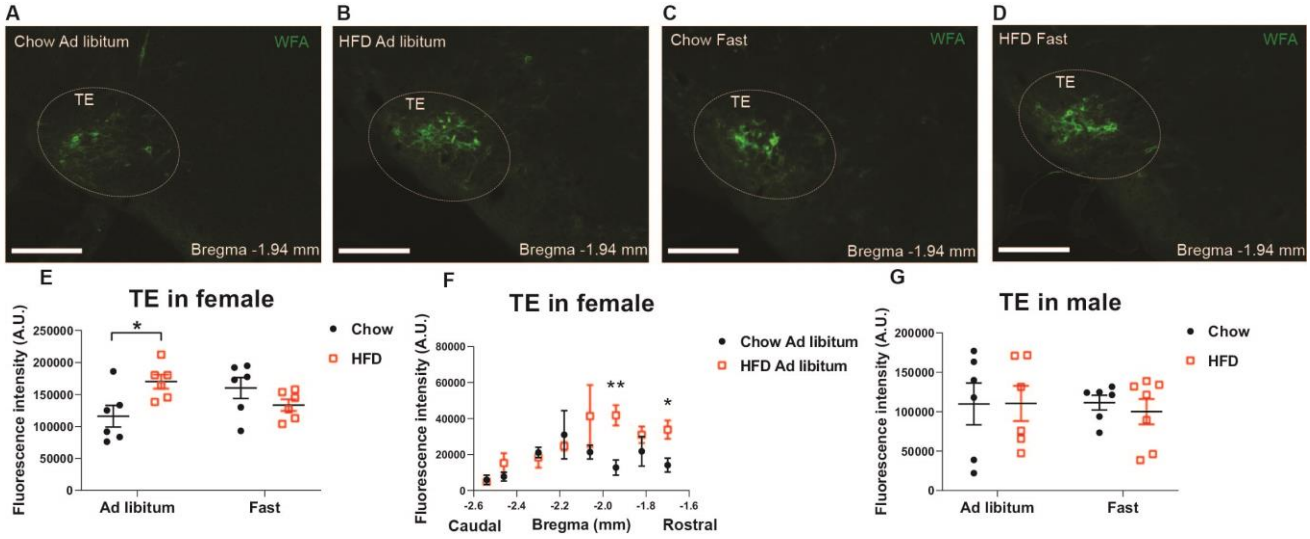

A
Female Sham
WFA
TE
Bregma -2.18 mm
B
Male Sham
WFA
TE
Bregma -2.18 mm
C
Female OVX
WFA
TE
Bregma -2.18 mm
D
Male CAST
WFA
TE
Bregma -2.18 mm
E
TE
Fluorescence intensity (A.U.)
250000
200000
150000
100000
50000
0
**
Female
Male
F
TE
Fluorescence intensity (A.U.)
40000
30000
20000
10000
0
*
**
*
-2.6
-2.4
-2.2
-2.0
-1.8
-1.6
Caudal
Bregma (mm)
Rostral
Female
Male
G
TE
Fluorescence intensity (A.U.)
250000
200000
150000
100000
50000
0
**
Sham
GDX
Female
Male

Figure 4


A
Chow Ad libitum
WFA
TE
Bregma -1.94 mm
B
HFD Ad libitum
TE
Bregma -1.94 mm
C
Chow Fast
WFA
TE
Bregma -1.94 mm
D
HFD Fast
WFA
TE
Bregma -1.94 mm
E
TE in female
Fluorescence intensity (A.U.)
250000
200000
150000
100000
50000
0
*
Ad libitum
Fast
Chow
HFD
F
TE in female
Fluorescence intensity (A.U.)
80000
60000
40000
20000
0
**
*
-2.6
-2.4
-2.2
-2.0
-1.8
-1.6
Caudal
Bregma (mm)
Rostral
Chow Ad libitum
HFD Ad libitum
G
TE in male
Fluorescence intensity (A.U.)
200000
150000
100000
50000
0
Ad libitum
Fast
Chow
HFD