

Role of PTP1B in POMC neurons during chronic high fat diet: Sex differences in regulation of liver lipids and glucose tolerance

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| 1 | ROLE OF PTP1B IN POMC NEURONS DURING CHRONIC HIGH FAT DIET: SE | X |
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| 2 | DIFFERENCES IN REGULATION OF LIVER LIPIDS AND GLUCOSE TOLERANC | CE |
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29 ABSTRACT

30 Protein tyrosine phosphatase 1B (PTP1B) is a negative regulator of leptin receptor signalling and may contribute to leptin resistance in diet-induced obesity. Although PTP1B 31 inhibition has been suggested as a potential weight loss therapy, the role of specific neuronal 32 33 PTP1B signalling in cardiovascular and metabolic regulation and the importance of sex differences in this regulation are still unclear. In this study, we investigated the impact of pro-34 opiomelanocortin (POMC) neuronal PTP1B deficiency in cardiometabolic regulation in male 35 and female mice fed a high fat diet (HFD). Compared to control mice (PTP1B^{flox/flox}), male and 36 female mice deficient in POMC neuronal PTP1B (PTP1B^{flox/flox}/POMC-Cre) had attenuated body 37 weight gain (Male: -18%; Female: -16%) and fat mass (Male: -33%; Female: -29%) in response 38 to HFD. Glucose tolerance was improved by 40% and liver lipid accumulation was reduced by 39 40% in PTP1B^{flox/flox}/POMC-Cre males but not in females. Compared to control mice, deficiency 40 41 of POMC neuronal PTP1B did not alter mean arterial pressure (MAP) in male or female mice (Male: 112±1 vs. 112±1 mmHg in controls; Female: 106±3 vs. 109±3 mmHg in controls). 42 Deficiency of POMC neuronal PTP1B also did not alter MAP response to acute stress in male or 43 female compared to control mice (Male: $\Delta 32\pm 0$ vs. $\Delta 29\pm 4$ mmHg; Female: $\Delta 22\pm 2$ vs. $\Delta 27\pm 4$ 44 mmHg). These data demonstrate that POMC-specific PTP1B deficiency improved glucose 45 tolerance and attenuated diet-induced fatty liver only in male mice, attenuated weight gain in 46 males and females, but did not enhance the MAP and HR responses to a HFD or to acute stress. 47

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50 Key words: Blood pressure; obesity; leptin; glucose; liver; lipid

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52 **INTRODUCTION**

Protein tyrosine phosphatase 1B (PTP1B) is a non-transmembrane protein anchored to the cytosolic face of the endoplasmic reticulum (ER) (17). It serves as an enzyme with multiple functions including inhibition of leptin and insulin signalling (31). Obesity and pro-inflammatory proteins such as nuclear factor kB (NFkB) and increased ER stress (27) have been reported to activate PTP1B. When activated, PTP1B translocates through the cytosol to dephosphorylate plasma membrane bound janus kinase 2 (JAK2) which is attached to the leptin receptor (LR) and initiates LR signalling. This effect of PTP1B may therefore negatively influence LR signalling.

PTP1B is found in many tissues including skeletal muscle, adipose tissue, liver and the 60 61 brain (29, 31). PTP1B has been suggested as a potential weight loss and appetite suppressing target due to the reductions in body weight (BW) and food intake observed in mice with whole 62 body PTP1B deficiency (20). Some of these metabolic effects of PTP1B have been attributed to 63 central nervous system (CNS) actions (5). Although whole body and total CNS deletion of 64 PTP1B have been reported to have beneficial metabolic effects (3, 5, 20), Chiappini et al (8) 65 66 reported that ventromedial hypothalamic (VMH) deletion of Ptpn1, the gene encoding PTP1B expression, resulted in increased age-related weight gain in high fat diet (HFD) fed female mice 67 due to reductions in spontaneous motor activity and energy expenditure. These observations 68 69 suggest that PTP1B may have heterogeneous metabolic effects depending on the neuronal population in which PTP1B is expressed. 70

Because increased PTP1B attenuates LR and insulin signalling which, in turn, have been suggested to play a role in regulating sympathetic nervous system (SNS) activity and blood pressure (BP) in obesity (12, 23), there has also been interest in possible cardiovascular actions of PTP1B. Whole body PTP1B deficiency was reported to increase mean arterial pressure (MAP) in response to leptin infusion in mice fed a standard chow diet (4). However, it is not clear whether these effects on BP are due to CNS actions or to peripheral vascular effects. Thus, the potential role of neuronal-specific PTP1B in cardiovascular regulation and in modulating the chronic BP effects of CNS LR signalling are unclear, especially in conditions in which PTP1B may be activated such as in diet-induced obesity. Also, the specific neuronal populations responsible for mediating chronic cardiometabolic effects of PTP1B in obesity are unknown.

81 Pro-opiomelanocortin (POMC) neurons located within the arcuate nucleus (ARC) of the hypothalamus and in the nucleus tractus solitarius (NTS) of the brainstem are thought to be 82 important targets for leptin's effects on sympathetic activity, BP, appetite, and glucose regulation 83 84 (18, 19). We previously showed that POMC neuronal specific LR deletion abolished the chronic effects of leptin to increase BP and to reduce insulin and glucose levels (11). POMC neurons, 85 however, appear to play a lesser role in mediating leptin's effects to reduce appetite and increase 86 energy expenditure (11). Banno et al (3) showed that POMC neuronal specific PTP1B deletion 87 caused only small reductions in body weight (BW) as well as improvements in glucose 88 regulation in male mice fed a HFD. However, the role of POMC neuronal PTP1B in 89 cardiovascular regulation in dietary-induced obesity is still unclear. Importantly, there have been 90 no previous studies, to our knowledge, that have investigated potential sex differences in 91 92 cardiometabolic metabolic regulation by PTP1B in POMC neurons.

Although deficiency of melanocortin 4 receptors (MC4R) is known to be associated with obesity and liver steatosis, whether POMC neurons regulate liver lipids independent of effects on overall adiposity is unclear. Also, there have been no previous studies, to our knowledge, that have investigated the possible role of POMC neuronal PTP1B signalling in protecting against liver steatosis. The main goal of the current study was to test the hypothesis that POMC neuronal specific PTP1B deficiency protects against the adverse metabolic effects of a chronic HFD, including weight gain, impaired glucose tolerance, and increased liver lipids, while increasing BP and heart rate (HR). We also investigated potential sex differences in POMC neuronal PTP1B regulation of cardiovascular and metabolic function.

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104 MATERIALS AND METHODS

105 All experimental protocols and procedures were approved by the Institutional Animal 106 Care and Use Committee (IACUC) of the University of Mississippi Medical Center, Jackson, 107 Mississippi. Mice were placed in a 12-h dark (6:00 pm to 6:00 am) and light (6:00 am to 6:00 108 pm) cycle and given free access to food and water throughout the study.

109 Animals

Male and female PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre mice were used in these 110 studies. PTP1B^{flox/flox}/POMC-Cre mice were generated by crossing POMC-Cre mice that express 111 Cre recombinase specifically in POMC neurons on a Friend Virus B (B6.FVB) background 112 (generously provided by Dr. Joel Elmquist, University of Texas Southwestern Medical School, 113 Dallas, TX) with PTP1B^{flox/flox} mice on a mixed 129Sv/J x C57BL/6 background (generously 114 provided by Dr. Kendra Bence, Pfizer, Cambridge, MA). The PTP1B^{flox/flox} mice have LoxP sites 115 inserted into the intronic sequence surrounding exons 6-8, which encode the PTP1B active site 116 and surrounding parts of the catalytic domain. Therefore, crossing POMC-Cre mice with 117 PTP1B^{*flox/flox*} mice led to the generation of mice with PTP1B deficiency only in POMC neurons. 118 Homozygous PTP1B^{flox/flox} mice from our colony were used as controls. Specificity of Cre 119 120 expression in POMC neurons and selective deletion of PTP1B in POMC neurons have been reported previously (2, 5). In order to visualize Cre recombinase expression in POMC neurons 121 122 we also bred in the tomato reporter gene using B6.Cg-Gt (Rosa)26Sor/J on a C57BL/6J background purchased from Jackson Laboratories in a subset of mice. 123

124 Body Weight, Body Composition and Glucose Tolerance Analysis - Control Diet

125 Control PTP1B^{flox/flox} (n=15) and PTP1B^{flox/flox}/POMC-Cre (n=9) mice were individually
126 housed and fed a control diet (Harlan Teklad/ENVIGO, CA 170955, 4 kcal/g, 13% fat) starting

at 6 weeks of age and continuing until the experiments were completed at 29 weeks of age. Body
weights were measured twice per week from 6 - 20 weeks of age. Weekly changes in body
composition were analyzed using magnetic resonance imaging (4in1 EchoMRI-900TM, Echo
Medical System, Houston, TX). Glucose tolerance tests were completed at 20 weeks of age
(PTP1B^{*flox/flox*}, n=15 and PTP1B^{*flox/flox*}/POMC-Cre, n=6). Animals were sacrificed at 29 weeks of
age for liver lipid analysis (PTP1B^{*flox/flox*}, n=5) and PTP1B^{*flox/flox*}/POMC-Cre, n=3).

133 Body Weight and Body Composition Analysis - High Fat Diet

134 Control male (n=10) and female (n=9) PTP1B^{flox/flox} and male (n=7) and female (n=7) 135 PTP1B^{flox/flox}/POMC-Cre mice were individually housed and fed a HFD (Harlan 136 Teklad/ENVIGO, TD-0881, 4.7 kcal/g, 45% fat) starting at 6 weeks of age and continuing until 137 the experiments were completed at 29 weeks of age. Food intake and body weight were 138 measured twice per week from 6 - 20 weeks of age. Weekly changes in body composition were 139 analyzed using magnetic resonance imaging. Animals were sacrificed at 29 weeks of age for 140 liver lipid analysis.

141 Food Intake Response to Acute Leptin Injections

In non-fasted, non-instrumented PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre mice (20 \pm 2 weeks of age) fed a HFD, leptin (5 µg/g) or saline vehicle (0.2 mL) was injected intraperitoneally at 5:00 pm and food intake was measured 2, 4, 15, and 24 hours later. Food intake response to saline injection was subtracted from food intake response after leptin injection and the difference plotted as change (Δ) in food intake over 24 hours. Each animal served as its own control.

148 Immunohistochemistry

To provide additional confirmation of selective deletion of PTP1B in POMC neurons we used 149 immunohistochemistry to detect expression of pSTAT3 in sections of the ARC from 150 PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre mice. Mice were injected intraperitoneally with 151 recombinant mouse leptin (5 mg/kg). After 45 minutes, mice were sacrificed and perfused, via a 152 cannula inserted into the left ventricle, with phosphate-buffered saline (PBS) containing 153 154 phosphatase inhibitor (Roche Inc., USA); tissues were collected and kept overnight in formalin, after which the solution was switched to 30% sucrose and tissues were kept overnight at 4°C. 155 Frozen brain coronal sections, 30 µm thick were cut and processed for immunofluorescence to 156 157 verify the presence of p-STAT3 immunoreactivity. Sections were rinsed in PBS and then incubated in blocking solution (PBS, 0.3% Triton X-100) for 24 hrs and pre-incubated with 5% 158 159 normal horse serum in PBS for 1 h at room temperature. After rinses with PBS, sections were incubated with rabbit anti-p-STAT3 (Cell Signaling, MA) at a dilution 1:100 for 48 hrs at 4°C. 160 After rinses (3x) with PBS, sections were incubated with biotin-conjugated anti-rabbit IgG at a 161 dilution of 1:100 for 1 h at room temperature. After rinses with PBS, sections were incubated 162 with Avidin conjugated DyLight 549 at dilution of 1:200 for 1 h at room temperature in a dark 163 environment. Sections were rinsed, mounted on slides and examined in a fluorescence 164 microscope at 556 nm wave length. 165

166 Oral Glucose Tolerance Test

D-glucose (3 mg/kg of lean body mass plus 1 mg/kg of fat mass) was administered by gavage after a 5-h fast in 20±2 week-old male and female PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre mice fed a HFD. Blood samples were collected by tail snip, and blood glucose was measured using glucose strips (ReliOn) at baseline, 15, 30, 60, 90, and 120 minutes after glucose administration.

172 Liver Composition

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Whole livers from male and female PTP1B^{*flox/flox*} mice and PTP1B^{*flox/flox*}/POMC-Cre fed a HFD were harvested and analyzed for fat and lean mass composition using EchoMRI.

We also performed Oil Red-O staining in frozen liver sections from $PTP1B^{flox/flox}$ and PTP1B^{flox/flox}/POMC-Cre mice to assess liver lipids. Sections (10 µm thick) were fixed in 10% buffered formalin for 5 minutes and stained for 10 minutes with 0.5% Oil Red-O in 60% isopropyl alcohol. The slides were washed several times in water and counterstained in Mayer's hematoxylin for 30 s and mounted in aqueous mounting media.

Liver triglyceride was analyzed from male and female PTP1B^{flox/flox} and male and female 180 PTP1B^{flox/flox}/POMC-Cre mice using a colorimetric assay (BioVision K622, Milpitas, CA 95035 181 USA) according to the manufacturer's instructions. Briefly, 100 mg liver samples were 182 homogenized in a Douncer homogenizer in 5% NP-40. Samples were centrifuged and 183 supernatant was isolated and diluted (1:1000). Samples were incubated with lipase at room 184 temperature for 20 min to convert triglyceride to glycerol. Reaction mix was then added and the 185 samples were incubated for a further 60 min at room temperature, protected from light. The 186 samples were read at absorbance 540 nm in a microplate reader. 187

188 Measurement of Blood Pressure and Heart Rate

At 20±2 weeks old, male and female PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre mice fed a HFD were anesthetized with 2% isoflurane and, under sterile conditions, a telemetry probe (TA11PA-C10, Data Science, MN) was implanted in the left carotid artery and advanced into the aorta. Seven to ten days after recovery from surgery, MAP and HR were measured by telemetry, 24 hours/day for 4 consecutive days using computerized methods for data collection as previously described (11, 14). Daily MAP and HR were obtained from the average of 12:12 light:dark recording using a sampling rate of 500 Hz with a duration of 10 seconds every 10–minute period.

197 Acute Air-jet Stress Test

To determine whether deleting PTP1B in POMC neurons alters MAP and HR responses 198 to acute stress, male and female PTP1B^{flox/flox} and male and female PTP1B^{flox/flox/}POMC-Cre mice 199 fed a HFD implanted with BP telemeters were placed in special cages used for air jet stress 200 testing, as previously described (11). Mice were allowed to acclimate to the cages for at least 2 201 hours and monitored until BP was stable for at least 10 minutes. The air-jet stress was then 202 203 administered in 5-second pulses every 10 seconds for 5 minutes while BP and HR were measured continuously. Changes in BP and HR to acute stress were measured by subtracting the 204 average baseline measurement from the average measurement recorded during acute stress. 205

206 Statistical Analyses

Data are expressed as mean \pm SEM. Significant differences between two groups were determined by Student's *t*-test. Significant differences between two groups over time were determined by two-way ANOVA where possible followed by the Sidak's multiple comparisons test. Differences between groups over time were determined by t-test following linear regression analysis. A *p* value of <0.05 indicates a significant difference.

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215 **RESULTS**

216 POMC Neuronal Specific PTP1B Deficiency

PCR data demonstrated that PTP1B^{flox/flox}/POMC-Cre animals were homozygous for 217 PTP1B^{flox/flox} and expressed Cre recombination (Figure 1A). We also confirmed positive 218 expression of Cre-recombinase within POMC neurons of homozygous PTP1B^{flox/flox}/POMC-Cre 219 mice. Whole brain sections from a subset of PTP1B^{flox/flox}/POMC-Cre mice inbred for the tomato 220 red reporter gene showed tomato red fluorescence as an indicator of Cre-recombinase expression 221 in the arcuate nucleus (ARC) (-2.18 mm from bregma) of the hypothalamus and the nucleus 222 tractus solitarius (NTS) (-6.72 mm from bregma) where POMC neurons are known to be located 223 (Figure 1B). Furthermore, we used immunohistochemistry to detect expression of pSTAT3, a 224 225 major leptin signalling protein, in sections of the arcuate nucleus (-2.18 mm from bregma) in PTP1B^{*flox/flox*}/POMC-Cre and PTP1B^{*flox/flox*} mice following acute IP leptin injection (**Figure 1C**). 226 Deletion of PTP1B specifically in POMC neurons resulted in a markedly greater pSTAT3 227 staining compared to PTP1B^{*flox/flox*} mice. 228

Body Weight, Body Composition and Glucose Tolerance Test of PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre Mice Fed a Control Diet.

Combined male and female data in mice fed a normal diet from 6 weeks of age 231 demonstrate that, compared with control PTP1B^{flox/flox} mice, deletion of PTP1B specifically in 232 POMC neurons (PTP1B^{flox/flox}/POMC-Cre) had no significant effect on body weight, fat mass, or 233 lean mass (Figures 2A, 2B, 2C). Glucose tolerance was not significantly altered in 234 PTP1B^{flox/flox}/POMC-Cre mice compared to controls (Figure 2D). Liver lean mass was slightly 235 reduced in PTP1B^{flox/flox}/POMC-Cre compared to controls and this was balanced by a small 236 increase in fat mass (mg/g tissue), although the differences were not statistically significant 237 (Figures 2E and 2F). 238

Body Weight and Body Composition of PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre Mice Fed a HFD

Combined male and female data shown in Figure 3A demonstrate that compared with 241 control PTP1B^{flox/flox} mice, PTP1B^{flox/flox}/POMC-Cre mice had significant attenuations in weight 242 gain from 14 weeks onwards (p < 0.05), with an 18% body weight reduction in males (p < 0.05) 243 and a 16% reduction in females at 20 weeks of age (p < 0.01) (Figure 3B). This was mainly 244 accounted for by reduced fat mass (Figure 3C); in male and female PTP1B^{flox/flox}/POMC-Cre 245 mice, fat mass (g) was reduced by 33% (p<0.05) and 29% (p<0.05), respectively, at 20 weeks of 246 age compared to control PTP1B^{*flox/flox*} mice. Fat mass, as % body weight (% BW) are presented in 247 Figure 3D. Total lean body mass (g) was not significantly higher in PTP1B^{flox/flox}/POMC-Cre 248 mice compared to PTP1B^{flox/flox} mice (data not shown). Lean mass (expressed a % BW) was 249 significantly higher in male PTP1B^{flox/flox}/POMC-Cre than in male PTP1B^{flox/flox} mice (p<0.05), 250 but the increase in females was not quite statistically significant (p=0.053) (Figures 3E and 3F). 251 Average daily food intakes for male and female PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-252 Cre mice were not significantly different from 6 to 20 weeks of age (Figure 3G). However, 253 cumulative food intake over a 5 week period (weeks 10-14 inclusive) was lower in 254 PTP1B^{flox/flox}/POMC-Cre mice compared to PTP1B^{flox/flox} (p<0.05) (Figure 3H). Fasting plasma 255 leptin (Figure 3I) and insulin (Figure 3J) levels were also slightly lower in 256 PTP1B^{flox/flox}/POMC-Cre mice compared to PTP1B^{flox/flox} mice at 20 weeks of age, but the 257 258 differences were not statistically significant in male or female mice.

259 Impact of POMC Neuronal Specific PTP1B Deficiency on Food Intake Responses to Leptin

Using saline IP injection as a baseline control, leptin injection resulted in similar 24 hour reductions in food intake in $PTP1B^{flox/flox}$ and $PTP1B^{flox/flox}/POMC$ -Cre male and female mice fed a HFD (Figure 4). There were no significant sex differences in the anorexic effect of acute leptin
injections (data not shown).

264 Impact of POMC Neuronal Specific PTP1B Deficiency on Glucose Tolerance

Sex differences in the responses to a glucose tolerance test (GTT) were noted at 20 weeks 265 of age and data for male and female mice were therefore analyzed separately. Male 266 PTP1B^{flox/flox}/POMC-Cre mice had significantly improved glucose tolerance compared to male 267 PTP1B^{*flox/flox*} mice (p < 0.05) as evidenced by a 40% reduction in area under the curve (AUC) 268 (p < 0.05) (Figures 5A and 5B). Female PTP1B^{flox/flox} control mice had substantially better 269 glucose tolerance and lower AUC compared to male PTP1B^{flox/flox} mice. However, female 270 PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre mice had similar glucose tolerances and there were no 271 significant differences in AUC (Figure 5C and 5D). 272

273 Impact of POMC Neuronal Specific PTP1B Deficiency on Liver Lipid Accumulation

Mice were sacrificed at 29±1 week of age and livers harvested from PTP1B^{flox/flox}/POMC-274 Cre mice weighed significantly less than livers from PTP1B^{*flox/flox*} mice (p < 0.05) (Figure 6A). 275 However, when liver weight was normalized as percentage of total body weight (TBW) only 276 male PTP1B^{flox/flox/}POMC-Cre mice livers were significantly protected from the effects of a HFD 277 on fat accumulation compared to control PTP1B^{flox/flox} mice (p < 0.05) (Figure 6B). Compared to 278 controls, only male PTP1B^{flox/flox}/POMC-Cre mice had significantly reduced liver fat 279 accumulation as measured by EchoMRI (p < 0.05) (Figure 6C). Lean liver mass was significantly 280 increased in male PTP1B^{flox/flox}/POMC-Cre mice compared to male ^{flox/flox} controls (p<0.05) 281 (Figure 6D). There were no significant differences in liver lipid accumulation between female 282 PTP1B^{*flox/flox*} mice and female PTP1B^{*flox/flox*}/POMC-Cre mice. 283

Liver sections from male PTP1B^{*flox/flox*}/POMC-Cre mice had reduced lipid content compared to PTP1B^{*flox/flox*} mice as shown by a reduction in Oil Red O staining (representative images) **Figure 7A**. Significant reductions in liver triacylglycerol were also observed only in male PTP1B^{*flox/flox*}/POMC-Cre mice compared to PTP1B^{*flox/flox*} mice (p<0.05) (**Figures 7B**).

288 Impact of POMC Neuronal Specific PTP1B Deficiency on Blood Pressure and Heart Rate

Compared to control mice, deficiency of PTP1B specifically in POMC neurons did not significantly alter MAP in male or female HFD fed mice. Therefore, the BP data for males and females were combined in **Figure 8A**. There were also no significant differences observed in systolic or diastolic pressures in mice with POMC specific PTP1B deficiency compared to control mice fed a chronic HFD (**Figure 8B and 8C**). HR was similar in PTP1B^{flox/flox}/POMC-Cre mice compared to PTP1B^{flox/flox} mice (**Figure 8D**) fed a HFD.

Impact of POMC Neuronal Specific PTP1B Deficiency on Blood Pressure and Heart Rate Responses to Acute Stress

Pre-stress resting measurements of MAP were not significantly different in PTP1B^{flox/flox} 297 compared to PTP1B^{flox/flox}/POMC-Cre mice (Figure 9A). In response to acute stress, MAP of 298 PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre mice increased by 29±4 and 32±0 mmHg in males and 299 27±4 and 22±2 in females, respectively (Figures 9B). At baseline, HR was not significantly 300 different in PTP1B^{flox/flox} compared to PTP1B^{flox/flox}/POMC-Cre mice (Figure 9C). Acute stress 301 raised HR equally in PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre mice (Figure 9D). Female 302 PTP1B^{flox/flox}/POMC-Cre mice had an attenuated MAP response to acute stress compared to male 303 PTP1B^{flox/flox}/POMC-Cre as shown in Figure 9B. No other sex differences were observed. 304

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307 **DISCUSSION**

An important goal of this study was to test the hypothesis that POMC neuronal specific PTP1B deficiency protects against the adverse metabolic effects of dietary-induced obesity, including glucose intolerance and liver steatosis, while exacerbating increases in BP and HR. We also tested whether there were sex differences in the cardiometabolic effects of POMC neuronalspecific deletion of PTP1B mice fed a HFD.

important findings POMC PTP1B deficient 313 Our most are that neuron (PTP1B^{flox/flox}/POMC-Cre) mice fed a chronic HFD had attenuated weight gain and decreased 314 315 whole body fat accumulation without measurable decreases in daily food intake compared to control mice fed a HFD. We also found important sex differences in the effect of POMC neuron 316 PTP1B deficiency on glucose tolerance and liver lipid accumulation in mice fed a HFD. Male 317 PTP1B^{flox/flox}/POMC-Cre mice fed a HFD exhibited marked improvements in glucose tolerance 318 and reduced liver lipid accumulation compared to male control mice fed a HFD. In contrast, 319 POMC neuron PTP1B deficiency did not protect female mice from the detrimental effects of a 320 HFD on glucose tolerance or lipid liver accumulation. There were no significant sex differences 321 in any other metabolic parameter analyzed in these studies. 322

Another important, albeit surprising, finding of our study was that PTP1B deficiency in POMC neurons did not significantly enhance BP and HR responses to a HFD or to acute stress in male or female mice, compared to control mice fed a HFD. These findings suggest that blockade of the actions of PTP1B in POMC neurons may offer beneficial metabolic effects in dietaryinduced obesity, especially in male mice, without significantly raising BP.

Diet induced obesity is associated with resistance to many of leptin's metabolic effects, including its ability to suppress appetite, enhance glucose tolerance and to protect against lipid accumulation in various tissues such as the liver (25). However, leptin's effect to enhance sympathetic nervous system (SNS) activity and therefore to increase BP and HR appears to be preserved, resulting in "selective" leptin resistance in obese subjects (24). Multiple mechanisms have been proposed to explain leptin resistance (16, 24, 26) but the factors contributing to selectivity of leptin's effects on SNS activity, BP, food intake, glucose regulation and liver lipid accumulation in obesity remain unclear.

Since many of the cardiometabolic responses to LR activation are initiated in the CNS, 336 considerable effort has been focused on factors that may induce leptin resistance in the brain. As 337 338 a negative regulator of LR signalling, PTP1B has been considered as a potential contributor to diet-induced leptin resistance as well as a modulator of the metabolic responses to other 339 hormones such as insulin (9, 21). Global and CNS specific PTP1B deficiency in mice fed a 340 normal or HFD have been reported to reduce adiposity, increase energy expenditure, increase 341 insulin sensitivity and improve glucose tolerance (3, 20). However, VMH specific PTP1B 342 343 deficiency appears to increase rather than decrease weight gain and adiposity in female mice (8). These observations suggest that PTP1B may have different modes of action on body weight 344 regulation and fat metabolism depending on the neuronal population in which PTP1B is 345 346 expressed, although sex differences could also be a potential contributing factor (8). To our knowledge there have been no previous studies that have explored potential sex differences in 347 348 the role of neuronal-specific PTP1B in regulating body weight, adiposity, glucose tolerance and 349 liver lipids.

Our results indicate that PTP1B deficiency specifically in POMC neurons only modestly attenuated weight gain in male and female mice fed a high fat diet and that this was accounted for predominantly by reductions in fat mass. There was a slight but significant effect of POMC 353 neuron PTP1B deficiency to reduce cumulative food intake but not on the acute effect of leptin injections to reduce food intake. Careful analysis of weekly food intake over the duration of the 354 study revealed that on occasion PTP1B^{flox/flox}/POMC-Cre mice ate slightly less than PTP1B^{flox/flox} 355 controls, which accounts for only a small part of the attenuated weight gain in mice with PTP1B 356 deficiency in POMC neurons. Consistent with these data, multiple studies have reported that 357 reductions in body weight of PTP1B^{flox/flox/}POMC-Cre mice fed a HFD may be due mainly to 358 increased energy expenditure and reductions in feed efficiency rather than major reductions in 359 food intake (3, 6, 10). Another study also noted the importance of sex differences in POMC 360 361 neuronal regulation of body weight, energy expenditure and obesity (7). Metabolic phenotyping data collected on the animals examined in this study suggest that PTP1B^{flox/flox}/POMC-Cre may 362 have increased motor activity compared to controls (data not shown), supporting the results of 363 Banno et al (3). 364

In another study using male mice fed a normal diet, De Jonghe et al (10) showed that 365 deficiency of PTP1B in POMC neurons enhanced the effects of hindbrain (4th ventricle) 366 administration of leptin to reduce food intake and body weight compared to control mice. 367 Whether hindbrain-mediated appetite suppression occurs with physiological levels of leptin or if 368 369 this effect remains intact after chronic exposure to a HFD and obesity was not tested in these 370 studies. Our current study demonstrated that the appetite suppressing effects of physiological levels of systemically administered leptin, which better mimics the normal route of leptin access 371 372 from the blood to the brain, were not enhanced by POMC neuronal specific PTP1B deficiency in male or female mice fed a HFD. 373

Although male and female mice with POMC neuron PTP1B deficiency had reductions in
body weight compared to controls, we found a sex difference in liver lipid accumulation. PTP1B

376 deficiency in POMC neurons reduced liver lipids by 40% in male mice as assessed by three different methods: oil red-O staining, Echo-MRI, and biochemical measurement of 377 triacylglycerol (TAG) in the liver. This large reduction in liver lipids was not apparent in female 378 mice with PTP1B deficiency in POMC neurons, compared to controls. Thus, PTP1B deficiency 379 in POMC neurons appears to have an important sex specific protective effect against liver 380 381 steatosis in dietary-induced obesity. This finding suggests that PTP1B may play a major role in contributing to development of fatty liver in obesity in males but may be of lesser importance in 382 females. However, pair feeding studies would be needed to completely rule out a potential effect 383 384 of the small reduction in body weight and overall adiposity as a potential cause of reduced liver lipids. 385

Another important finding of the present study is that there were important sex 386 differences in the effect of POMC neuronal PTP1B deficiency on glucose regulation. A chronic 387 HFD often causes impaired glucose regulation associated with insulin resistance in the liver as 388 well as in other tissues such as skeletal muscle and fat (22, 30). In our study, male but not female 389 PTP1B^{flox/flox}/POMC-Cre mice fed a chronic HFD had substantial improvements in glucose 390 tolerance compared to control mice fed a HFD. This finding suggests an important role for 391 392 POMC neuron PTP1B in development of HFD-induced glucose intolerance in males but not in females. These results complement those presented by Shi et al (28) who demonstrated that LR 393 394 deletion in POMC neurons resulted in glucose intolerance and insulin insensitivity in males, but 395 not in females, compared to controls.

Fatty liver is a well-recognized cause of insulin resistance and impaired glucose regulation. Sex differences in glucose tolerance caused by POMC neuronal-specific PTP1B deficiency may therefore be related, in part, to differences in liver lipids since only males with PTP1B deficiency in POMC neurons were protected against liver steatosis. However, the mechanisms responsible for these sex differences in glucose regulation caused by POMC neuronal PTP1B deficiency are still unclear and warrant further investigation. Also, it is important to note that control female mice fed a HFD had considerably better glucose tolerance than males fed a HFD, warranting further investigation of these sex differences.

404 Our previous studies demonstrated that POMC neurons mediate most of the chronic effects of leptin to raise BP (11, 13, 18, 19). For example, LR deficiency specifically on POMC 405 neurons completely abolished the rise in BP that occurred in control mice during 7 days of leptin 406 407 infusion (13). Because PTP1B is a negative regulator of LR activation, we hypothesized that selective deficiency of PTP1B in POMC neurons would increase BP in obese mice fed a HFD. In 408 409 contrast to our hypothesis, we did not observe any major differences in MAP or HR in mice with POMC-specific PTP1B deficiency compared to control mice. This was true for male as well as 410 for female mice. Furthermore, PTP1B deficiency in POMC neurons did not enhance the HR and 411 412 BP responses to an acute air jet stress in male or female mice.

These surprising results are difficult to explain if one assumes that PTP1B inactivation 413 only enhances LR signalling since leptin-mediated activation of POMC neurons has been clearly 414 415 demonstrated to stimulate SNS activity and raise BP (11, 13, 15, 18). A possible explanation for these findings is that PTP1B signalling in POMC neurons may modulate the effects of additional 416 417 factors that either inactivate POMC neurons or attenuate the effects of leptin. Another possibility 418 is that PTP1B does not substantially reduce activity of the downstream pathways associated with chronic SNS and BP effects of leptin in POMC neurons. Consistent with this possibility are the 419 420 results of Bruder-Nascimento et al. (6) who reported that PTP1B deficiency in POMC neurons 421 did not exacerbate the BP responses to chronic leptin infusion in male mice fed a normal chow

422 diet. Although their results are not strictly comparable to our findings since we investigated the impact of PTP1B deficiency in obese mice fed a HFD, it is clear that PTP1B deficiency in 423 424 POMC neurons does not raise BP in male or female mice on a normal or HFD. A potential limitation of these findings, however, is the possibility that reduced body weight associated with 425 PTP1B deficiency in POMC neurons may partially offset a rise in BP despite enhanced leptin 426 427 signalling, although we did not find any differences in day or night BP or BP responses to stress. Further experiments utilizing weight-matched mice may be useful in determining whether 428 429 POMC specific PTP1B deficiency may have effects on BP independent of changes in body 430 weight.

Chantemèle et al. (4) previously reported that whole body deficiency of PTP1B in male 431 mice increases MAP and amplifies the BP response to leptin infusion mainly by increasing 432 sympathetic tone. However, PTP1B deficiency did not enhance the effects of a behavioral stress 433 (cage switching) on BP. Additional studies have shown in rats that blockade of PTP1B within the 434 435 NTS may be important for maintaining normal baroreflex sensitivity (1). Taken together, these results suggest that global deficiency of PTP1B may have multiple adverse cardiovascular 436 effects, including increased BP and inhibition of baroreflex sensitivity, that do not appear to be 437 438 mediated via POMC neurons. However, the mechanisms involved and importance of peripheral and CNS effects of PTP1B in chronic BP regulation await further investigation. 439

440 Summary and Perspectives

These data indicate that PTP1B deficiency in POMC neurons attenuates weight gain, adiposity, liver lipid accumulation and improves glucose tolerance without significantly altering BP or HR in male mice fed a HFD. PTP1B deficiency in POMC neurons also attenuated weight gain and adiposity in female mice fed a HFD but did not protect against liver lipid accumulation or glucose intolerance. Our findings therefore suggest that PTP1B in POMC neurons may exacerbate the adverse metabolic effects of obesity induced by a chronic HFD in male mice to a greater extent than females although the mechanisms for these sex differences remain unknown. Taken together these data indicate important sex differences in the regulation of glucose and liver lipid accumulation by PTP1B in POMC neurons.

Although our observations indicate that deficiency of PTP1B in POMC neurons does not increase BP, global PTP1B deficiency appears to cause hypertension via mechanisms that remain to be elucidated. The potential adverse cardiovascular effects of PTP1B blockade may limit development of this therapeutic approach for obesity and associated metabolic abnormalities such as liver steatosis, insulin resistance and diabetes mellitus. Further studies are needed to determine whether novel therapeutic strategies can be developed to avoid deleterious cardiovascular effects while retaining beneficial metabolic actions of PTP1B blockade.

457

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463 **DISCLOSURES**

464 No conflicts of interest, financial or otherwise are declared by the authors.

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586 FIGURE LEGENDS

Figure 1. Genotype confirmation using PCR and immunofluorescence analysis. A: PTP1B
(+/+), heterozygotes (+/-) and POMC-Cre positive mice. Positive (+) and negative (-) DNA
samples. B: Tomato reporter gene expression in POMC-Cre positive neurons of a homozygous
PTP1B^{flox/flox}/POMC-Cre mouse in arcuate nucleus (ARC) and nucleus tractus solitarius (NTS).
C: Immunohistochemistry of pSTAT3 signalling in the ARC of a PTP1B^{flox/flox} and
PTP1B^{flox/flox}/POMC-Cre mouse injected IP with leptin.

Figure 2. Body composition, glucose tolerance and liver lipid analysis of mice fed a normal 593 control diet. Body weights were measured twice weekly in PTP1B^{flox/flox} (n=15) and 594 PTP1B^{flox/flox}/POMC-Cre (n=9) mice. EchoMRI for lean and fat mass were conducted once per 595 596 week for the duration of the study. A: Body weight (g) from 6 to 20 weeks of age. B: Fat mass 597 (% Body Weight) from 6 to 20 weeks of age. C: Lean mass (% Body Weight) from 6 to 20 weeks of age. **D**: After a 5 hour fast, glucose tolerance was measured in PTP1B^{flox/flox} (n=15) and 598 PTP1B^{flox/flox}/POMC-Cre (n=6) over 120 minutes post glucose gavage. E and F: Whole liver 599 lean mass (mg/g liver tissue weight) and lipid accumulation (mg/g liver tissue weight) analysis in 600 PTP1B^{flox/flox} (n=5) and PTP1B^{flox/flox}/POMC-Cre (n=3) mice assessed with EchoMRI. Data are 601 expressed as mean ± SEM. * P<0.05, A - D: 2-way ANOVA with post-hoc Sidak's multiple 602 comparison; t-test following linear regression analysis; E-F: Unpaired Student's t-test. 603

Figure 3. Body composition and plasma analysis of male (n=8) and female (n=7) $PTP1B^{flox/flox}$ and male (n=5) and female (n=7) $PTP1B^{flox/flox}/POMC$ -Cre mice during HFD feeding. Body weight and food intake were measured twice weekly. EchoMRIs for lean and fat mass were conducted once per week for the duration of the study. **A:** Body weight (g) from 6 to 20 weeks of age. **B**. Body weight in male and female $PTP1B^{flox/flox}$ and $PTP1B^{flox/flox}/POMC$ -Cre mice at 20 weeks of age. **C**: Total fat mass (g) as measured by EchoMRI (g). **D**: Total lean mass (g) as measured by EchoMRI. **E**. Total fat mass in male and female PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre mice at 20 weeks of age. **F**: Average daily food intake (g). **G**: Cumulative food intake from 10-14 weeks (g). **H**: Plasma leptin (ng/ml) **I**: Plasma insulin (ng/ml). Data are expressed as means \pm SEM. * *P*<0.05, **A**,**C**,**D** and **F**: 2-way ANOVA with post-hoc Sidak's multiple comparison; t-test following linear regression analysis; **B**, **E**, **G**, **H** and **I**: Unpaired Student's t-test.

Figure 4. Food intake response to acute leptin administration. The (Δ) change in 24 hr food intake after a saline injection subtracted from food intake response after leptin injection (5mg/kg, IP) in PTP1B^{flox/flox} (n=15) and PTP1B^{flox/flox}/POMC-Cre (n=11) mice. Data are expressed as means ± SEM. **P*<0.05, 2-way ANOVA with post-hoc Sidak's multiple comparison.

5. Glucose tolerance measured over 120 minutes in PTP1B^{flox/flox} 620 Figure and PTP1B^{flox/flox}/POMC-Cre mice fed a HFD. Glucose tolerance in male (n=10) and female (n=7) 621 PTP1B^{*flox/flox*} and male (n=7) and female (n=7) PTP1B^{*flox/flox*}/POMC-Cre mice measured after a 5 622 hour fast. A: Male PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre blood glucose measured over 120 623 minutes post glucose gavage. B: Blood glucose AUC for male PTP1B^{flox/flox} and 624 PTP1B^{flox/flox}/POMC-Cre. C: Female PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre blood glucose 625 measured over 120 minutes post glucose gavage. D: Blood glucose AUC for female PTP1B^{flox/flox} 626 and PTP1B^{flox/flox}/POMC-Cre. Data are expressed as mean ± SEM. * P<0.05, A & C: 2-way 627 628 ANOVA with post-hoc Sidak's multiple comparison, n=7-10/group; **B & D**: Unpaired Student's 629 t-test.

Figure 6. Whole liver lipid accumulation analysis in male (n=10) and female (n=9) $PTP1B^{flox/flox}$

631 and male (n=6) and female (n=7) PTP1B^{*flox/flox*}/POMC-Cre mice fed a HFD. Liver fat and lean

mass were assessed using EchoMRI. A: Whole liver weight (g). B: Liver weight as percentage
of total body weight (TBW). C: Fat mass (mg/g liver weight) D: Lean mass (mg/g liver weight).
Data are expressed as mean ± SEM. * *P*<0.05, A-D: Unpaired Student's t-test.

Figure 7. Liver triacylglycerol content in male (n=6) and female (n=4) PTP1B^{flox/flox} and male (n=4) and female (n=6) PTP1B^{flox/flox}/POMC-Cre mice fed a HFD. **A:** PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre liver sections stained for Oil Red O and visualized at 200X magnification. The nucleus was counterstained with Mayer's hematoxylin. **B:** PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre liver triacylglycerol content (mg/dL). Data are expressed as mean ± SEM. * P<0.05, **B:** Unpaired Student's t-test.

Figure 8. Blood pressure and heart rate (HR) in PTP1B^{*flox/flox*} (n=4) and PTP1B^{*flox/flox*}/POMC-Cre (n=7) mice fed a HFD. Blood pressures and heart rate in PTP1B^{*flox/flox*} and PTP1B^{*flox/flox*}/POMC-Cre mice were measured for 12/12 hrs day/night for 4 consecutive days. **A:** Mean Arterial Pressure (MAP). **B:** Systolic blood pressure (BP). **C:** Diastolic blood pressure (BP). **D:** Heart Rate (HR). Data are expressed as mean \pm SEM. * *P*<0.05, **A-D:** Unpaired Student's t-test.

Figure 9. Mean arterial pressure (MAP) and heart rate (HR) responses to acute air-jet stress in 646 PTP1B^{flox/flox} (n=8) and PTP1B^{flox/flox}/POMC-Cre (n=8) mice fed a HFD. A: MAP (mmHg) at 647 baseline and during acute stress. **B:** Change in MAP from baseline in PTP1B^{flox/flox} (male n=3; 648 female n=5) and PTP1B^{flox/flox}/POMC-Cre (male n=3; female n=5) during air-jet stress. C: HR 649 (bpm) at baseline and during acute stress. D: Change in HR from baseline in PTP1B^{flox/flox} (male 650 n=3; female n=5) and PTP1B^{flox/flox}/POMC-Cre (male n=3; female n=5) during air-jet stress. Data 651 are expressed as mean \pm SEM. * P<0.05, A-D: Paired Student's t-test comparing MAP and HR 652 values during air-jet stress with pre-stress values. # P < 0.05, A-D: Unpaired Student's t-test 653

654 comparing male vs. female changes in MAP and HR during air-jet stress in PTP1B^{flox/flox} or
 655 PTP1B^{flox/flox}/POMC-Cre mice.

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C PTP1B^{flox/flox}



POMC/PTP1B^{flox/flox}



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Control Diet

Figure 2





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