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**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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Running title: POMC neuronal PTP1B and cardiometabolic regulation

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ABSTRACT

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 inhibition has been suggested as a potential weight loss therapy, the role of specific neuronal 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-opiomelanocortin (POMC) neuronal PTP1B deficiency in cardiometabolic regulation in male and female mice fed a high fat diet (HFD). Compared to control mice (PTP1B^{flox/flox}), male and female mice deficient in POMC neuronal PTP1B (PTP1B^{flox/flox}/POMC-Cre) had attenuated body weight gain (Male: -18%; Female: -16%) and fat mass (Male: -33%; Female: -29%) in response to HFD. Glucose tolerance was improved by 40% and liver lipid accumulation was reduced by 40% in PTP1B^{flox/flox}/POMC-Cre males but not in females. Compared to control mice, deficiency 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). Deficiency of POMC neuronal PTP1B also did not alter MAP response to acute stress in male or female compared to control mice (Male: Δ32±0 vs. Δ29±4 mmHg; Female: Δ22±2 vs. Δ27±4 mmHg). These data demonstrate that POMC-specific PTP1B deficiency improved glucose tolerance and attenuated diet-induced fatty liver only in male mice, attenuated weight gain in males and females, but did not enhance the MAP and HR responses to a HFD or to acute stress.

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

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 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 body PTP1B deficiency (20). Some of these metabolic effects of PTP1B have been attributed to central nervous system (CNS) actions (5). Although whole body and total CNS deletion of PTP1B have been reported to have beneficial metabolic effects (3, 5, 20), Chiappini et al (8) 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 due to reductions in spontaneous motor activity and energy expenditure. These observations suggest that PTP1B may have heterogeneous metabolic effects depending on the neuronal population in which PTP1B is expressed.

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.

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 important targets for leptin's effects on sympathetic activity, BP, appetite, and glucose regulation (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, however, appear to play a lesser role in mediating leptin's effects to reduce appetite and increase energy expenditure (11). Banno et al (3) showed that POMC neuronal specific PTP1B deletion caused only small reductions in body weight (BW) as well as improvements in glucose regulation in male mice fed a HFD. However, the role of POMC neuronal PTP1B in cardiovascular regulation in dietary-induced obesity is still unclear. Importantly, there have been no previous studies, to our knowledge, that have investigated potential sex differences in 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.

98 The main goal of the current study was to test the hypothesis that POMC neuronal
99 specific PTP1B deficiency protects against the adverse metabolic effects of a chronic HFD,
100 including weight gain, impaired glucose tolerance, and increased liver lipids, while increasing
101 BP and heart rate (HR). We also investigated potential sex differences in POMC neuronal
102 PTP1B regulation of cardiovascular and metabolic function.
103

MATERIALS AND METHODS

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

Animals

Male and female $PTP1B^{flox/flox}$ and $PTP1B^{flox/flox}/POMC-Cre$ mice were used in these studies. $PTP1B^{flox/flox}/POMC-Cre$ mice were generated by crossing $POMC-Cre$ mice that express Cre recombinase specifically in POMC neurons on a Friend Virus B (B6.FVB) background (generously provided by Dr. Joel Elmquist, University of Texas Southwestern Medical School, Dallas, TX) with $PTP1B^{flox/flox}$ mice on a mixed 129Sv/J x C57BL/6 background (generously provided by Dr. Kendra Bence, Pfizer, Cambridge, MA). The $PTP1B^{flox/flox}$ mice have LoxP sites inserted into the intronic sequence surrounding exons 6-8, which encode the PTP1B active site and surrounding parts of the catalytic domain. Therefore, crossing $POMC-Cre$ mice with $PTP1B^{flox/flox}$ mice led to the generation of mice with PTP1B deficiency only in POMC neurons. Homozygous $PTP1B^{flox/flox}$ mice from our colony were used as controls. Specificity of Cre 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 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.

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

Control $PTP1B^{flox/flox}$ (n=15) and $PTP1B^{flox/flox}/POMC-Cre$ (n=9) mice were individually 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).

Body Weight and Body Composition Analysis - High Fat Diet

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

Food Intake Response to Acute Leptin Injections

In non-fasted, non-instrumented PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre mice (20±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.

Immunohistochemistry

To provide additional confirmation of selective deletion of PTP1B in POMC neurons we used immunohistochemistry to detect expression of pSTAT3 in sections of the ARC from PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre mice. Mice were injected intraperitoneally with recombinant mouse leptin (5 mg/kg). After 45 minutes, mice were sacrificed and perfused, via a cannula inserted into the left ventricle, with phosphate-buffered saline (PBS) containing 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. Frozen brain coronal sections, 30 µm thick were cut and processed for immunofluorescence to 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% 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. After rinses (3x) with PBS, sections were incubated with biotin-conjugated anti-rabbit IgG at a dilution of 1:100 for 1 h at room temperature. After rinses with PBS, sections were incubated with Avidin conjugated DyLight 549 at dilution of 1:200 for 1 h at room temperature in a dark environment. Sections were rinsed, mounted on slides and examined in a fluorescence microscope at 556 nm wave length.

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.

Liver Composition

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

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.

Acute Air-jet Stress Test

To determine whether deleting PTP1B in POMC neurons alters MAP and HR responses to acute stress, male and female PTP1B^{flox/flox} and male and female PTP1B^{flox/flox}/POMC-Cre mice fed a HFD implanted with BP telemeters were placed in special cages used for air jet stress testing, as previously described (11). Mice were allowed to acclimate to the cages for at least 2 hours and monitored until BP was stable for at least 10 minutes. The air-jet stress was then 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 average baseline measurement from the average measurement recorded during acute stress.

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.

RESULTS

POMC Neuronal Specific PTP1B Deficiency

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

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 demonstrate that, compared with control PTP1B^{flox/flox} mice, deletion of PTP1B specifically in POMC neurons (PTP1B^{flox/flox}/POMC-Cre) had no significant effect on body weight, fat mass, or lean mass (**Figures 2A, 2B, 2C**). Glucose tolerance was not significantly altered in PTP1B^{flox/flox}/POMC-Cre mice compared to controls (**Figure 2D**). Liver lean mass was slightly reduced in PTP1B^{flox/flox}/POMC-Cre compared to controls and this was balanced by a small increase in fat mass (mg/g tissue), although the differences were not statistically significant (**Figures 2E and 2F**).

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 control PTP1B^{flox/flox} mice, PTP1B^{flox/flox}/POMC-Cre mice had significant attenuations in weight gain from 14 weeks onwards ($p<0.05$), with an 18% body weight reduction in males ($p<0.05$) and a 16% reduction in females at 20 weeks of age ($p<0.01$) (**Figure 3B**). This was mainly accounted for by reduced fat mass (**Figure 3C**); in male and female PTP1B^{flox/flox}/POMC-Cre mice, fat mass (g) was reduced by 33% ($p<0.05$) and 29% ($p<0.05$), respectively, at 20 weeks of age compared to control PTP1B^{flox/flox} mice. Fat mass, as % body weight (% BW) are presented in **Figure 3D**. Total lean body mass (g) was not significantly higher in PTP1B^{flox/flox}/POMC-Cre mice compared to PTP1B^{flox/flox} mice (data not shown). Lean mass (expressed as % BW) was significantly higher in male PTP1B^{flox/flox}/POMC-Cre than in male PTP1B^{flox/flox} mice ($p<0.05$), but the increase in females was not quite statistically significant ($p=0.053$) (**Figures 3E and 3F**).

Average daily food intakes for male and female PTP1B^{flox/flox} and PTP1B^{flox/flox}/POMC-Cre mice were not significantly different from 6 to 20 weeks of age (**Figure 3G**). However, cumulative food intake over a 5 week period (weeks 10-14 inclusive) was lower in PTP1B^{flox/flox}/POMC-Cre mice compared to PTP1B^{flox/flox} ($p<0.05$) (**Figure 3H**). Fasting plasma leptin (**Figure 3I**) and insulin (**Figure 3J**) levels were also slightly lower in PTP1B^{flox/flox}/POMC-Cre mice compared to PTP1B^{flox/flox} mice at 20 weeks of age, but the differences were not statistically significant in male or female mice.

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).

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

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-Cre mice weighed significantly less than livers from PTP1B^{flox/flox} mice ($p<0.05$) (**Figure 6A**). However, when liver weight was normalized as percentage of total body weight (TBW) only male PTP1B^{flox/flox}/POMC-Cre mice livers were significantly protected from the effects of a HFD on fat accumulation compared to control PTP1B^{flox/flox} mice ($p<0.05$) (**Figure 6B**). Compared to controls, only male PTP1B^{flox/flox}/POMC-Cre mice had significantly reduced liver fat accumulation as measured by EchoMRI ($p<0.05$) (**Figure 6C**). Lean liver mass was significantly increased in male PTP1B^{flox/flox}/POMC-Cre mice compared to male ^{flox/flox} controls ($p<0.05$) (**Figure 6D**). There were no significant differences in liver lipid accumulation between female PTP1B^{flox/flox} mice and female PTP1B^{flox/flox}/POMC-Cre mice.

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**).

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

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 neuronal-specific deletion of PTP1B mice fed a HFD.

Our most important findings are that POMC neuron PTP1B deficient ($PTP1B^{flox/flox}/POMC-Cre$) mice fed a chronic HFD had attenuated weight gain and decreased 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 PTP1B deficiency on glucose tolerance and liver lipid accumulation in mice fed a HFD. Male $PTP1B^{flox/flox}/POMC-Cre$ mice fed a HFD exhibited marked improvements in glucose tolerance and reduced liver lipid accumulation compared to male control mice fed a HFD. In contrast, POMC neuron PTP1B deficiency did not protect female mice from the detrimental effects of a HFD on glucose tolerance or lipid liver accumulation. There were no significant sex differences in any other metabolic parameter analyzed in these studies.

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 dietary-induced 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, considerable effort has been focused on factors that may induce leptin resistance in the brain. As 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 hormones such as insulin (9, 21). Global and CNS specific PTP1B deficiency in mice fed a normal or HFD have been reported to reduce adiposity, increase energy expenditure, increase insulin sensitivity and improve glucose tolerance (3, 20). However, VMH specific PTP1B 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 regulation and fat metabolism depending on the neuronal population in which PTP1B is 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 the role of neuronal-specific PTP1B in regulating body weight, adiposity, glucose tolerance and 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

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 study revealed that on occasion PTP1B^{flox/flox}/POMC-Cre mice ate slightly less than PTP1B^{flox/flox} controls, which accounts for only a small part of the attenuated weight gain in mice with PTP1B deficiency in POMC neurons. Consistent with these data, multiple studies have reported that reductions in body weight of PTP1B^{flox/flox}/POMC-Cre mice fed a HFD may be due mainly to increased energy expenditure and reductions in feed efficiency rather than major reductions in food intake (3, 6, 10). Another study also noted the importance of sex differences in POMC 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 have increased motor activity compared to controls (data not shown), supporting the results of Banno et al (3).

In another study using male mice fed a normal diet, De Jonghe *et al* (10) showed that deficiency of PTP1B in POMC neurons enhanced the effects of hindbrain (4th ventricle) administration of leptin to reduce food intake and body weight compared to control mice. Whether hindbrain-mediated appetite suppression occurs with physiological levels of leptin or if this effect remains intact after chronic exposure to a HFD and obesity was not tested in these 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 from the blood to the brain, were not enhanced by POMC neuronal specific PTP1B deficiency in male or female mice fed a HFD.

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

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 triacylglycerol (TAG) in the liver. This large reduction in liver lipids was not apparent in female mice with PTP1B deficiency in POMC neurons, compared to controls. Thus, PTP1B deficiency in POMC neurons appears to have an important sex specific protective effect against liver 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 females. However, pair feeding studies would be needed to completely rule out a potential effect of the small reduction in body weight and overall adiposity as a potential cause of reduced liver lipids.

Another important finding of the present study is that there were important sex differences in the effect of POMC neuronal PTP1B deficiency on glucose regulation. A chronic HFD often causes impaired glucose regulation associated with insulin resistance in the liver as well as in other tissues such as skeletal muscle and fat (22, 30). In our study, male but not female PTP1B^{flox/flox}/POMC-Cre mice fed a chronic HFD had substantial improvements in glucose tolerance compared to control mice fed a HFD. This finding suggests an important role for 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 deletion in POMC neurons resulted in glucose intolerance and insulin insensitivity in males, but 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.

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 neurons completely abolished the rise in BP that occurred in control mice during 7 days of leptin 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 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 for female mice. Furthermore, PTP1B deficiency in POMC neurons did not enhance the HR and 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 only enhances LR signalling since leptin-mediated activation of POMC neurons has been clearly 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 factors that either inactivate POMC neurons or attenuate the effects of leptin. Another possibility 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 results of Bruder-Nascimento *et al.* (6) who reported that PTP1B deficiency in POMC neurons did not exacerbate the BP responses to chronic leptin infusion in male mice fed a normal chow

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 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 PTP1B deficiency in POMC neurons may partially offset a rise in BP despite enhanced leptin 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 POMC specific PTP1B deficiency may have effects on BP independent of changes in body weight.

Chantemèle *et al.* (4) previously reported that whole body deficiency of PTP1B in male mice increases MAP and amplifies the BP response to leptin infusion mainly by increasing sympathetic tone. However, PTP1B deficiency did not enhance the effects of a behavioral stress (cage switching) on BP. Additional studies have shown in rats that blockade of PTP1B within the 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 effects, including increased BP and inhibition of baroreflex sensitivity, that do not appear to be mediated via POMC neurons. However, the mechanisms involved and importance of peripheral and CNS effects of PTP1B in chronic BP regulation await further investigation.

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.

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DISCLOSURES

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

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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 control diet. Body weights were measured twice weekly in PTP1B^{flox/flox} (n=15) and PTP1B^{flox/flox}/POMC-Cre (n=9) mice. EchoMRI 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:** Fat mass (% 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 PTP1B^{flox/flox}/POMC-Cre (n=6) over 120 minutes post glucose gavage. **E and F:** Whole liver lean mass (mg/g liver tissue weight) and lipid accumulation (mg/g liver tissue weight) analysis in PTP1B^{flox/flox} (n=5) and PTP1B^{flox/flox}/POMC-Cre (n=3) mice assessed with EchoMRI. Data are expressed as mean ± SEM. * *P*<0.05, **A - D:** 2-way ANOVA with post-hoc Sidak's multiple comparison; t-test following linear regression analysis; **E-F:** Unpaired Student's t-test.

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

609 weeks of age. **C:** Total fat mass (g) as measured by EchoMRI (g). **D:** Total lean mass (g) as
610 measured by EchoMRI. **E.** Total fat mass in male and female PTP1B^{flox/flox} and
611 PTP1B^{flox/flox}/POMC-Cre mice at 20 weeks of age. **F:** Average daily food intake (g). **G:**
612 Cumulative food intake from 10-14 weeks (g). **H:** Plasma leptin (ng/ml) **I:** Plasma insulin
613 (ng/ml). Data are expressed as means \pm SEM. * $P < 0.05$, **A,C,D and F:** 2-way ANOVA with
614 post-hoc Sidak's multiple comparison; t-test following linear regression analysis; **B, E, G, H and**
615 **I:** Unpaired Student's t-test.

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

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

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

Figure 8. Blood pressure and heart rate (HR) in PTP1B^{flx/flx} (n=4) and PTP1B^{flx/flx}/POMC-Cre (n=7) mice fed a HFD. Blood pressures and heart rate in PTP1B^{flx/flx} and PTP1B^{flx/flx}/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 PTP1B^{flx/flx} (n=8) and PTP1B^{flx/flx}/POMC-Cre (n=8) mice fed a HFD. **A:** MAP (mmHg) at baseline and during acute stress. **B:** Change in MAP from baseline in PTP1B^{flx/flx} (male n=3; female n=5) and PTP1B^{flx/flx}/POMC-Cre (male n=3; female n=5) during air-jet stress. **C:** HR (bpm) at baseline and during acute stress. **D:** Change in HR from baseline in PTP1B^{flx/flx} (male n=3; female n=5) and PTP1B^{flx/flx}/POMC-Cre (male n=3; female n=5) during air-jet stress. Data are expressed as mean \pm SEM. * $P < 0.05$, **A-D:** Paired Student's t-test comparing MAP and HR values during air-jet stress with pre-stress values. # $P < 0.05$, **A-D:** Unpaired Student's t-test

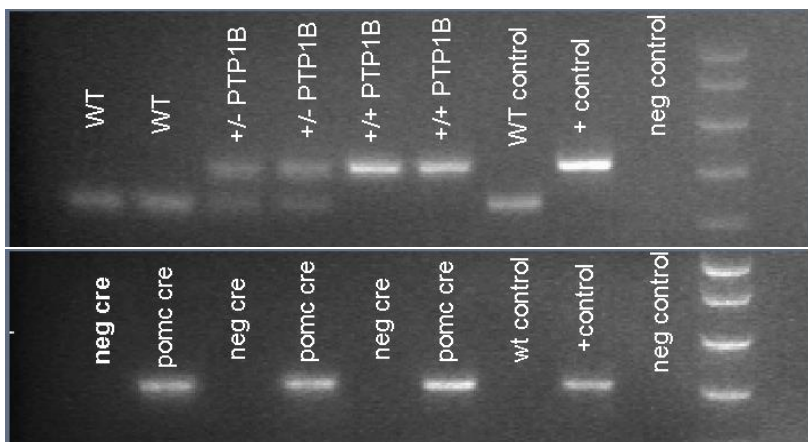
654 comparing male vs. female changes in MAP and HR during air-jet stress in PTP1B^{flox/flox} or
655 PTP1B^{flox/flox}/POMC-Cre mice.

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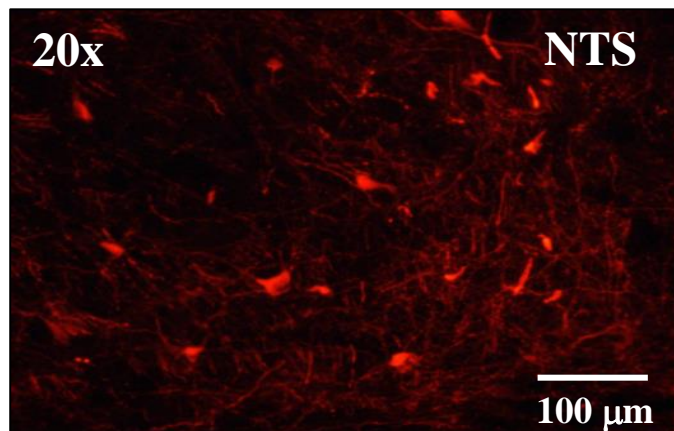
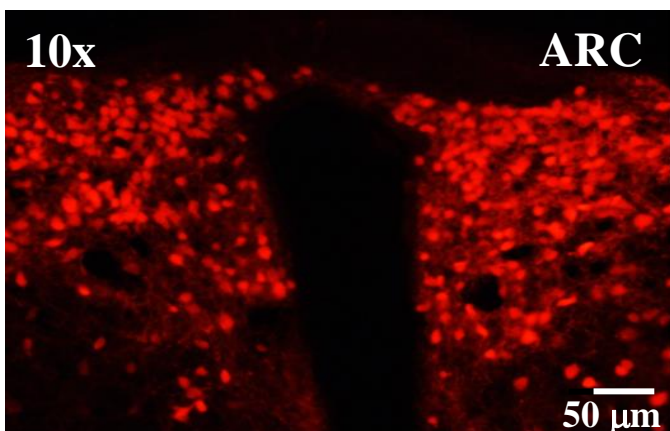
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Figure 1

A

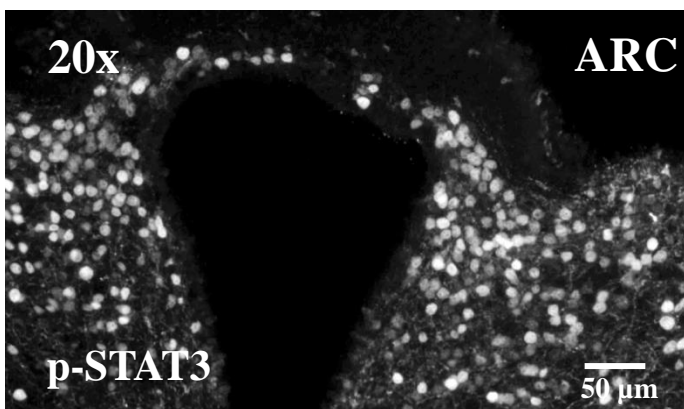


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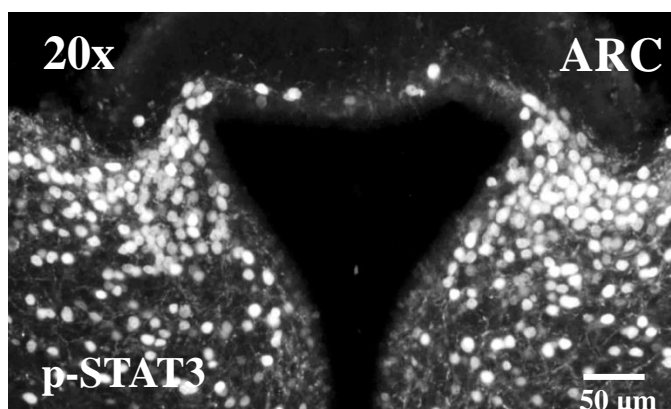


C

PTP1B^{flox/flox}



POMC/PTP1B^{flox/flox}



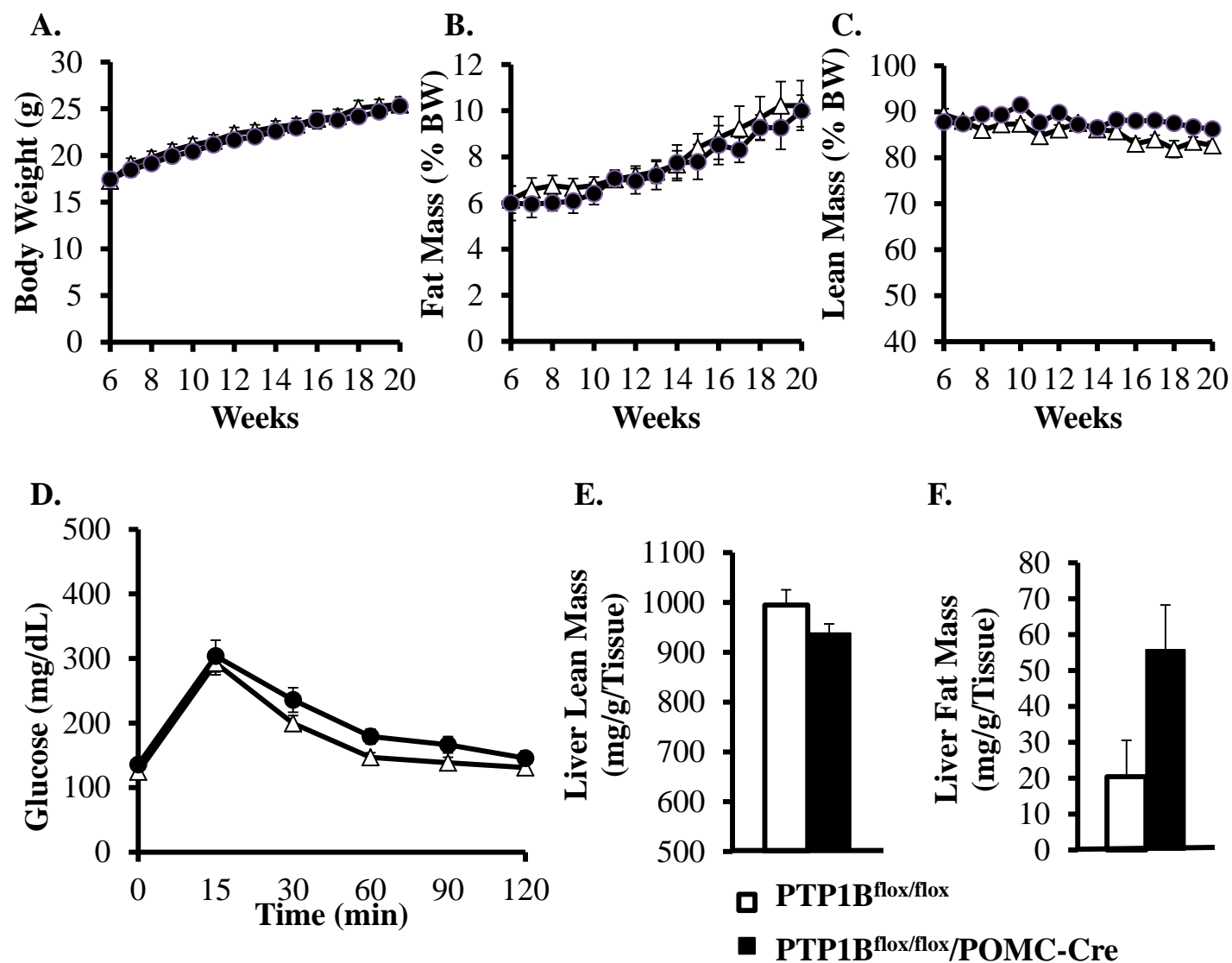
—△— PTP1B^{flx/flx}—●— PTP1B^{flx/flx}/POMC-Cre

Figure 3

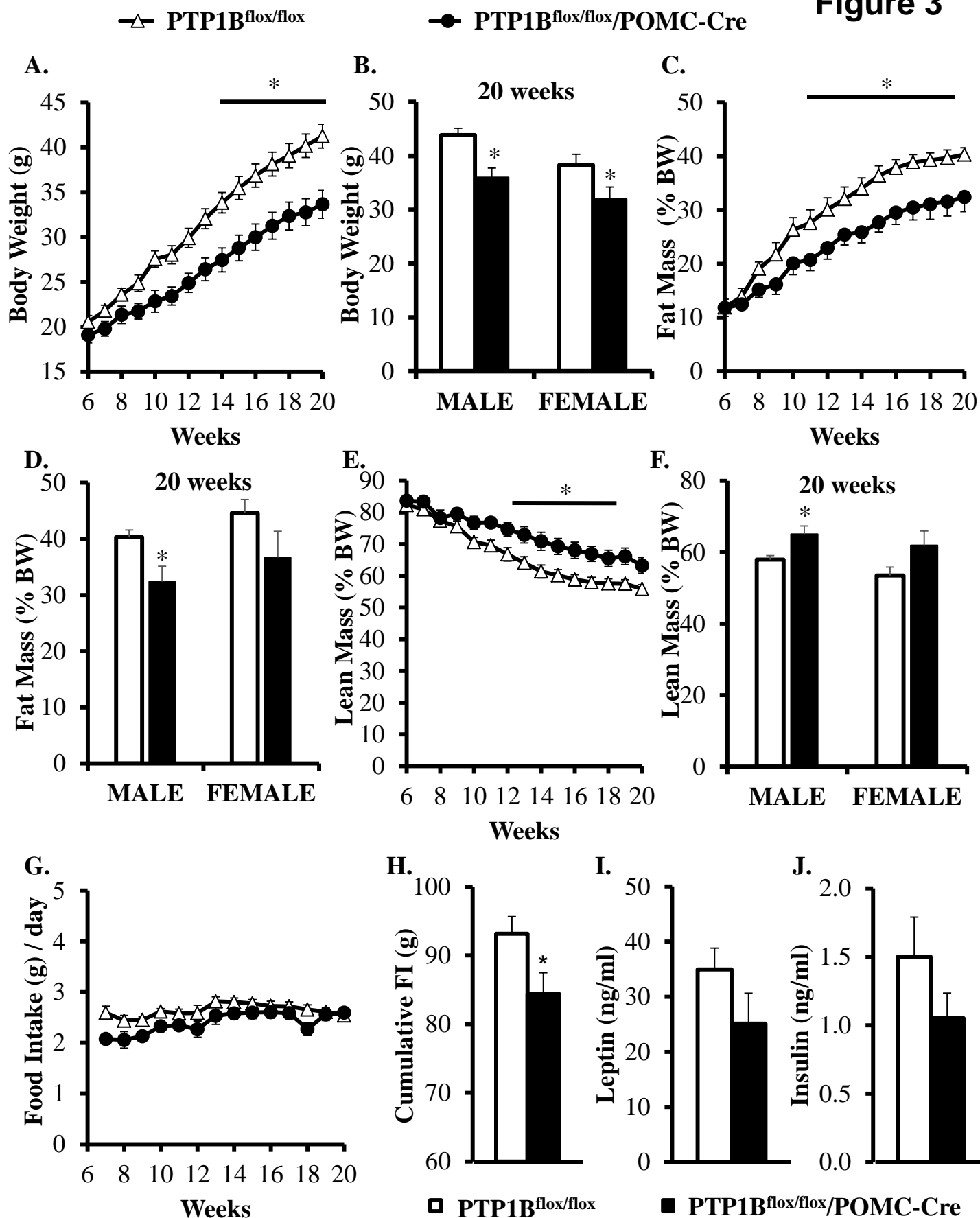


Figure 4

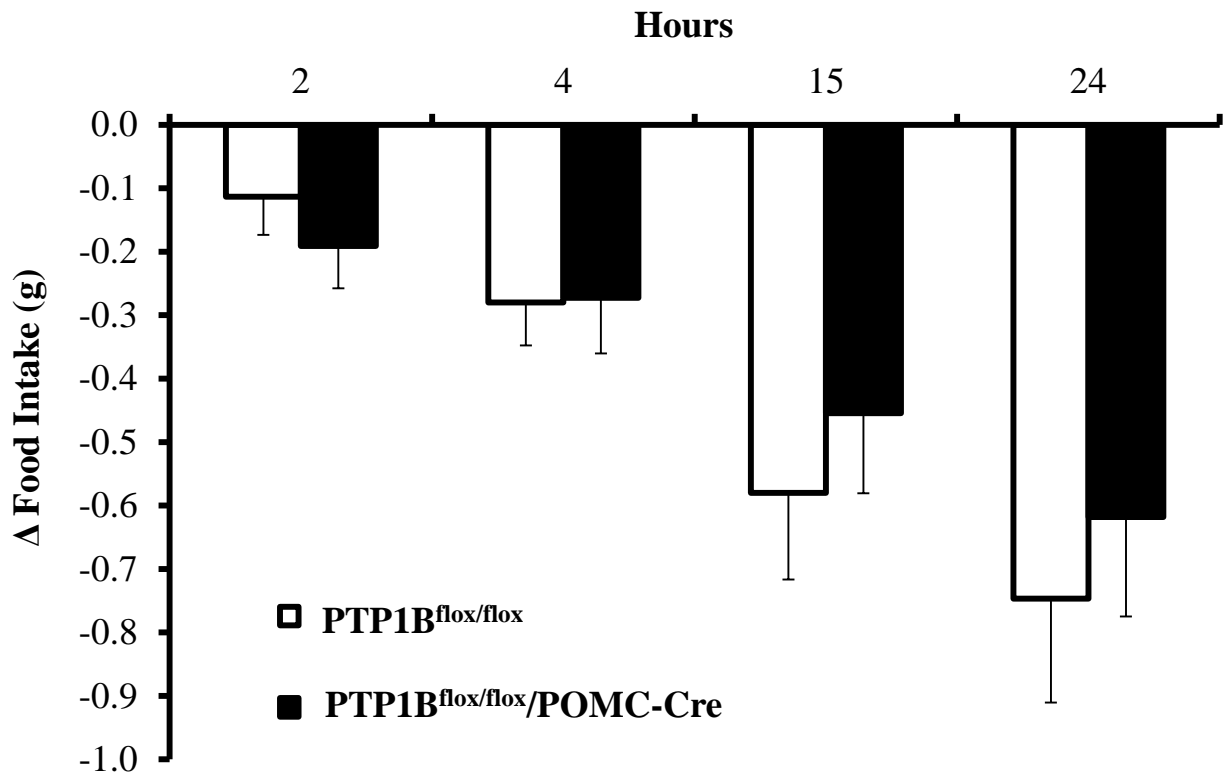


Figure 5

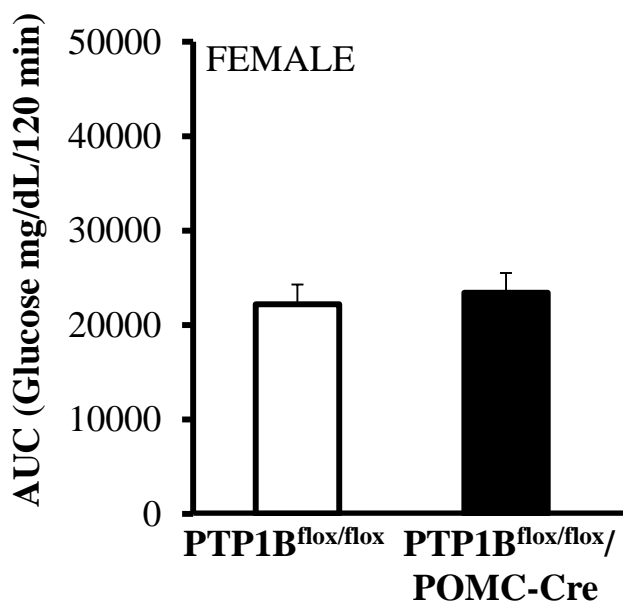
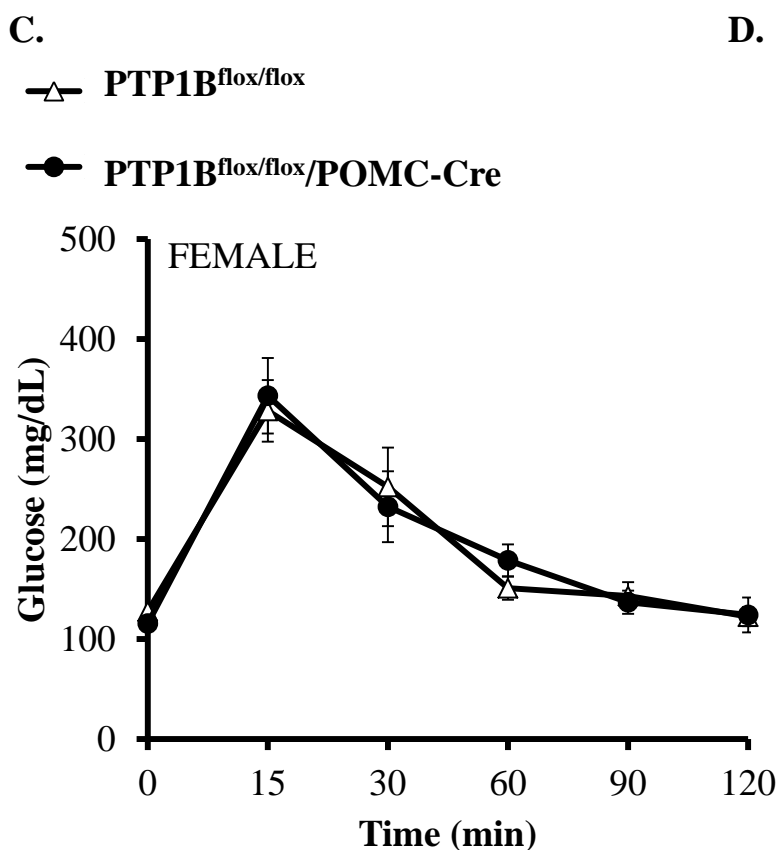
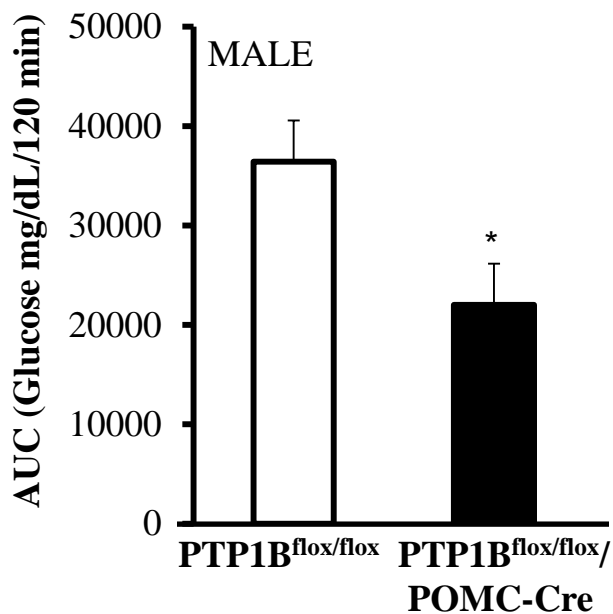
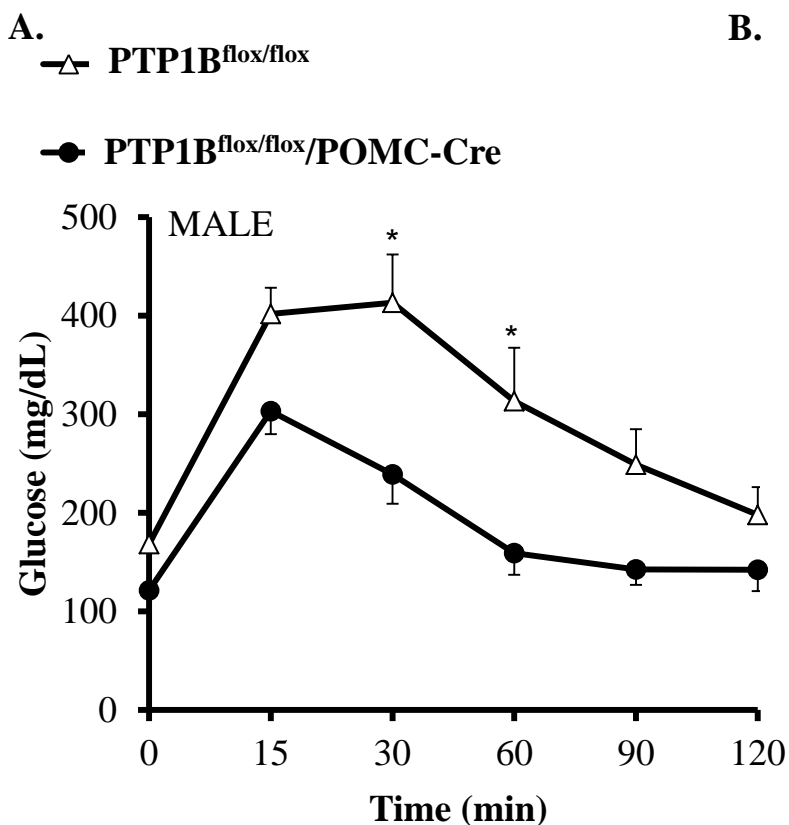


Figure 6

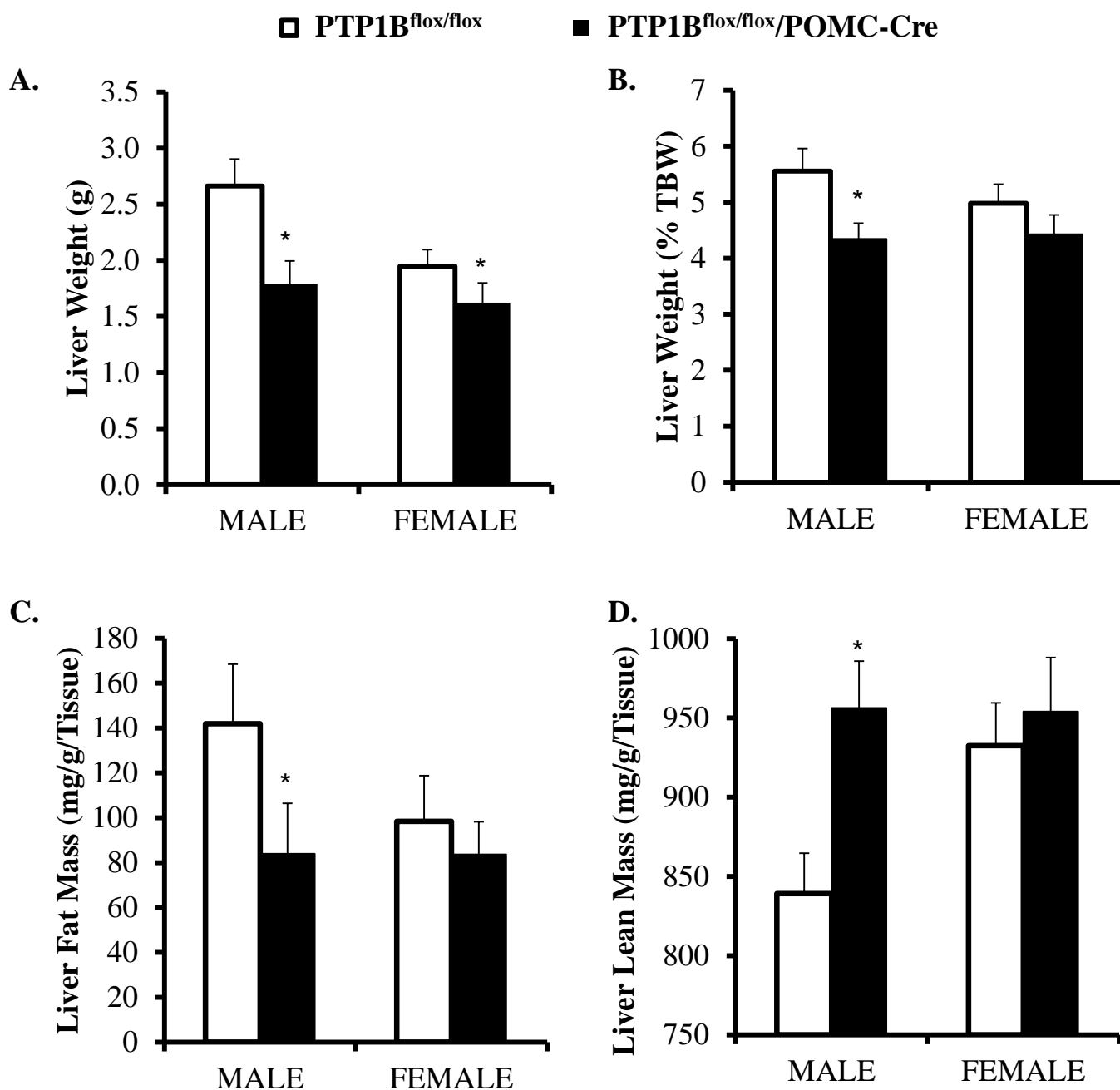
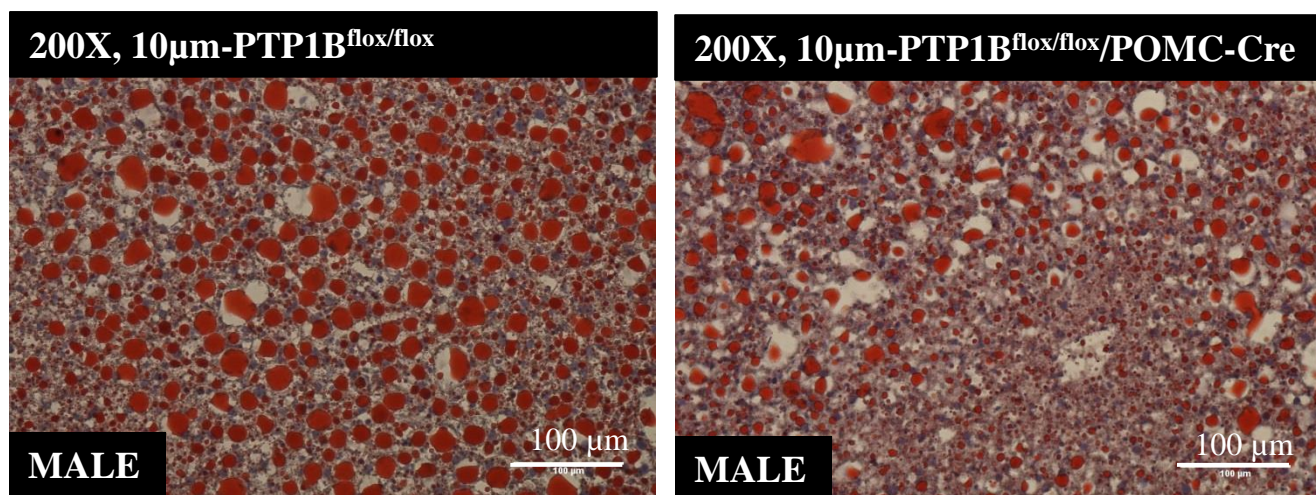


Figure 7

A.



B.

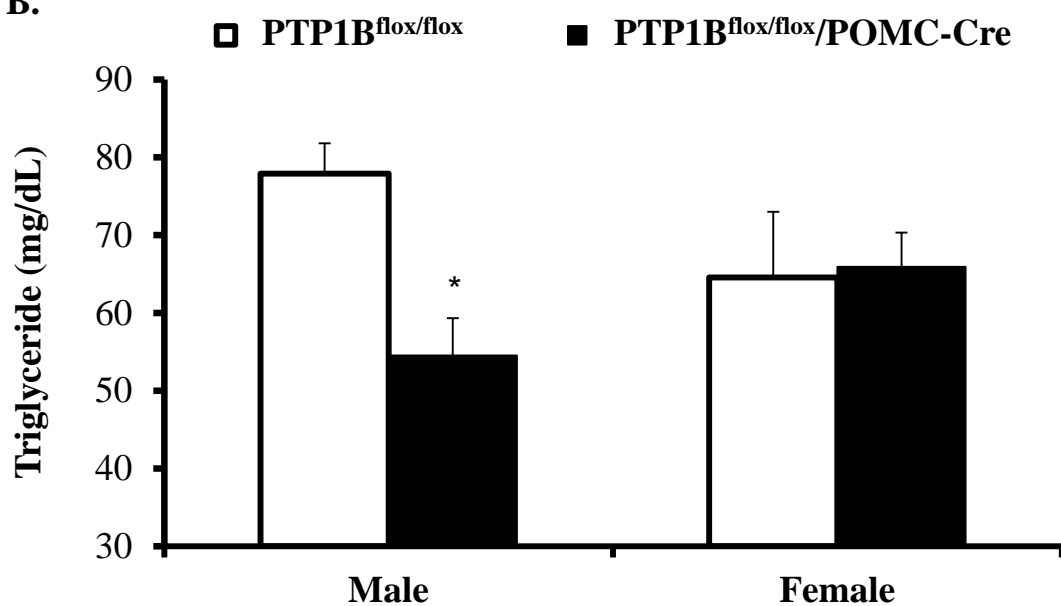


Figure 8

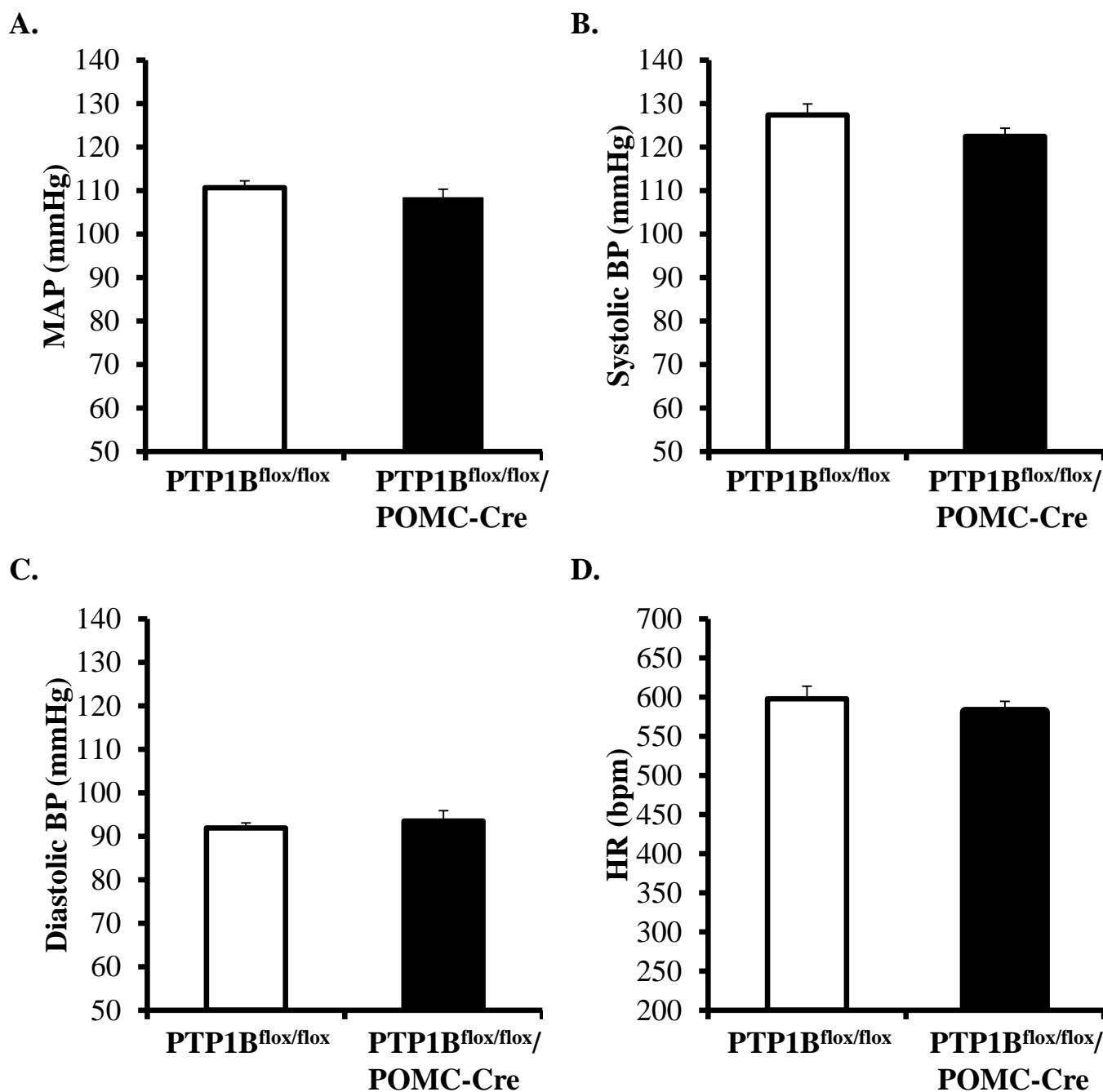


Figure 9

□ PTP1B^{flx/flx}

■ PTP1B^{flx/flx}/POMC-Cre

