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Supplemental Information.

The CNS melanocortin system directly regulates circulating cholesterol

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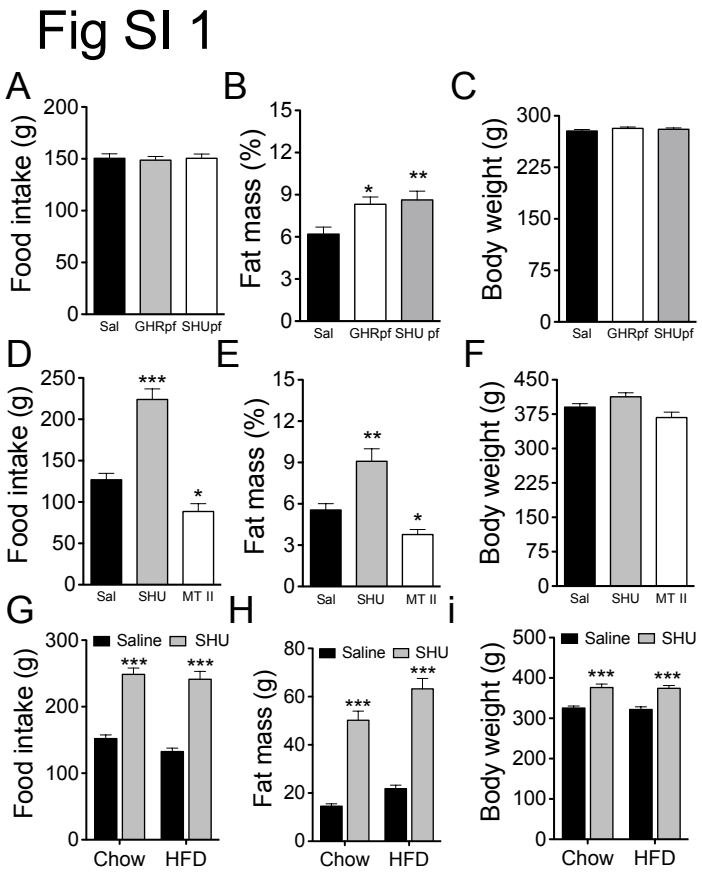

Figure S1. Food Intake, fat mass and body weight changes induced by the central infusion of ghrelin or melanocortin receptor ligands. (**A, D, G**) food intake, (**B, E, H**) fat mass and (**C, F, I**) body weight of (**A-C**) 7-day icv SHU (24 nmol/d) and MTII (1 nmol/d) infusion in rats fed *ad libitum* with chow, (**D-F**) 7-day icv ghrelin (2.5 nmol/d) and SHU (24 nmol/d) infusion in rats *pair fed* with chow; and (**G-I**) 7-day icv SHU (24 nmol/d) infusion in rats fed *ad libitum* with chow or HFD. (**A-F**) *P < 0.05; **P < 0.01; ***P < 0.001 versus saline infused control group. 1-way ANOVA, Tukey *post hoc* test. (mean ±s.e.m., n=7-8) (**G-I**) *P < 0.05; **P < 0.01; ***P < 0.001 versus controls. 2-way ANOVA, Bonferroni *post hoc* test. (mean±s.e.m., n=8)

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Fig SI 2

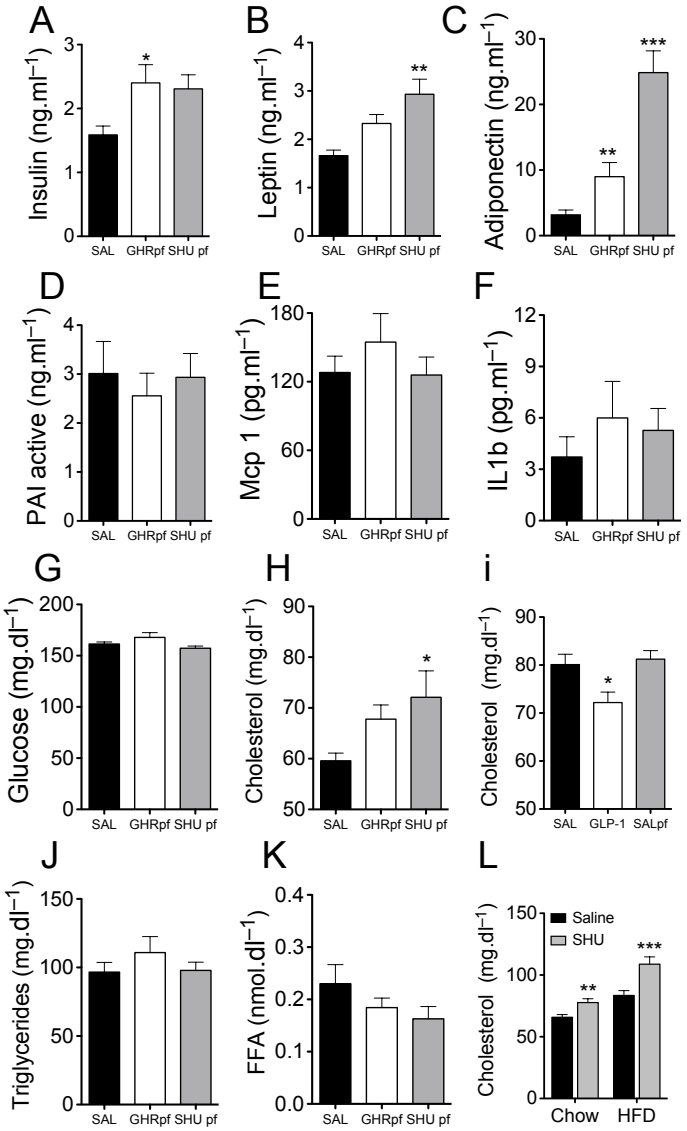

Figure S2. Plasma concentration of hormones and metabolites. (A-J) Effect of 7-day icv ghrelin (2.5 nmol/d) and SHU (24 nmol/d infusion in rats *pair fed* with chow on plasma levels of (**A**) insulin, (**B**) leptin, (**C**) adiponectin (**D**) PAI (active) (**E**) Mcp-1, (**F**) IL 1b, (**G**) glucose, (**H**) cholesterol, (**I**) triglycerides and (**J**) free fatty acids. (**K**) Effect on plasma cholesterol of 7-day icv GLP-1(2.5 nmol/d) infusion in C57 BL6 mice fed *ad libitum* with chow. (**L**) Effect on plasma cholesterol of 7-day icv SHU (24 nmol/d) infusion in rats fed *ad libitum* with chow or HFD. (**A-K**) *P < 0.05 versus saline infused control group. 1-way ANOVA. (mean±s.e.m., n=7-8) (**L**) *P < 0.05; **P < 0.01 versus controls. 2-way ANOVA, Bonferroni *post hoc* test. (mean±s.e.m., n=8)

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Fig SI 3

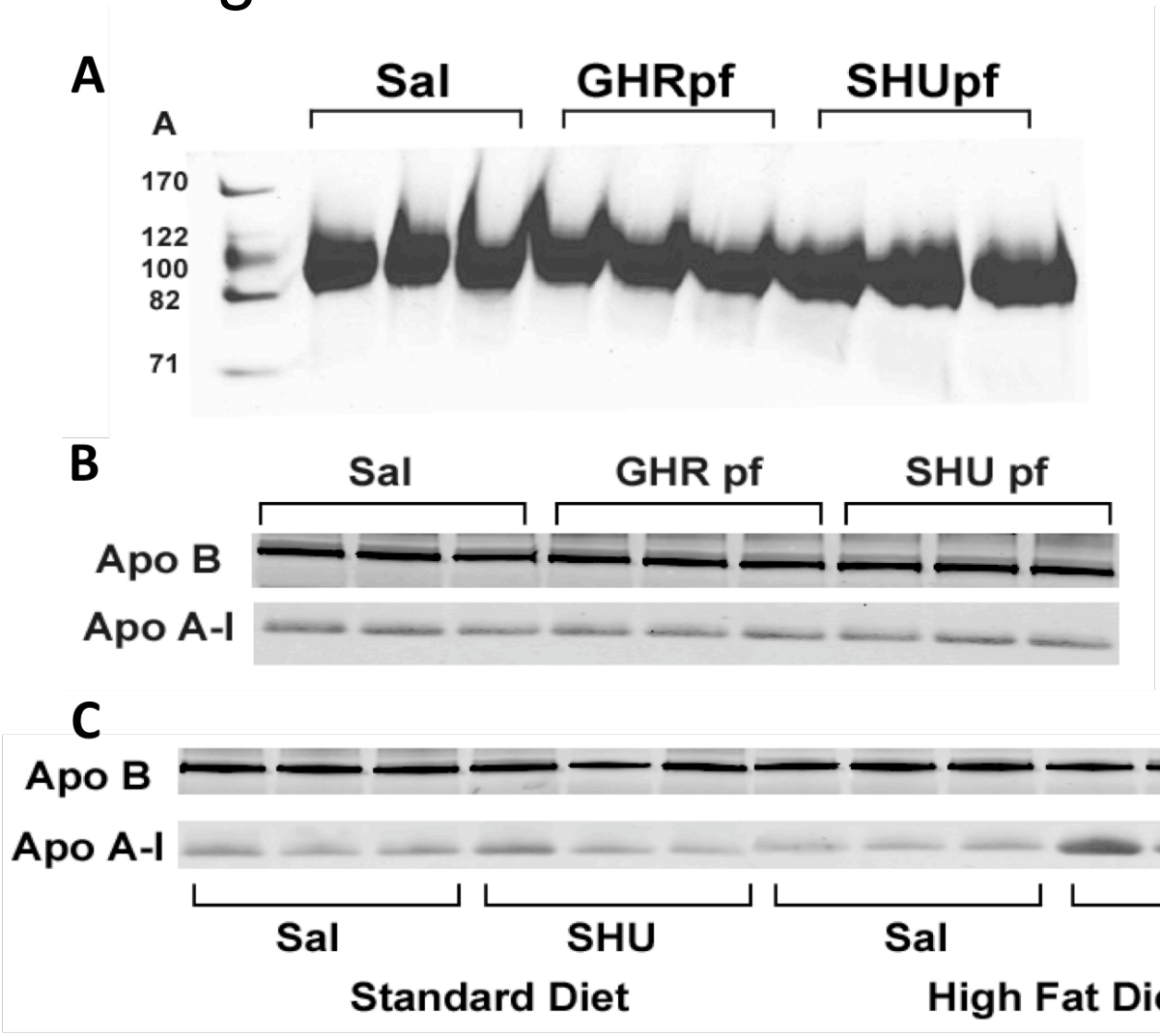

Figure S3. Study of HDL size and determination and apolipoprotein levels in plasma. (**A**) Non denaturing PAGE of HDL particles purified by ultracentrifugation obtained from pooled samples of chow *pair fed* rats infused icv for 7 days with saline, ghrelin (2.5 nmol/d) and SHU (24nmol/d). (**B-C**) (**B**) Inmunoblot for Apo B and A-I in whole plasma of chow *pair fed* rats infused icv for 7 days with saline, ghrelin (2.5nmol/d) and SHU (24nmol/d), and (**C**) 7-day icv SHU (24 nmol/d) infused rats fed *ad libitum* with chow or HFD.

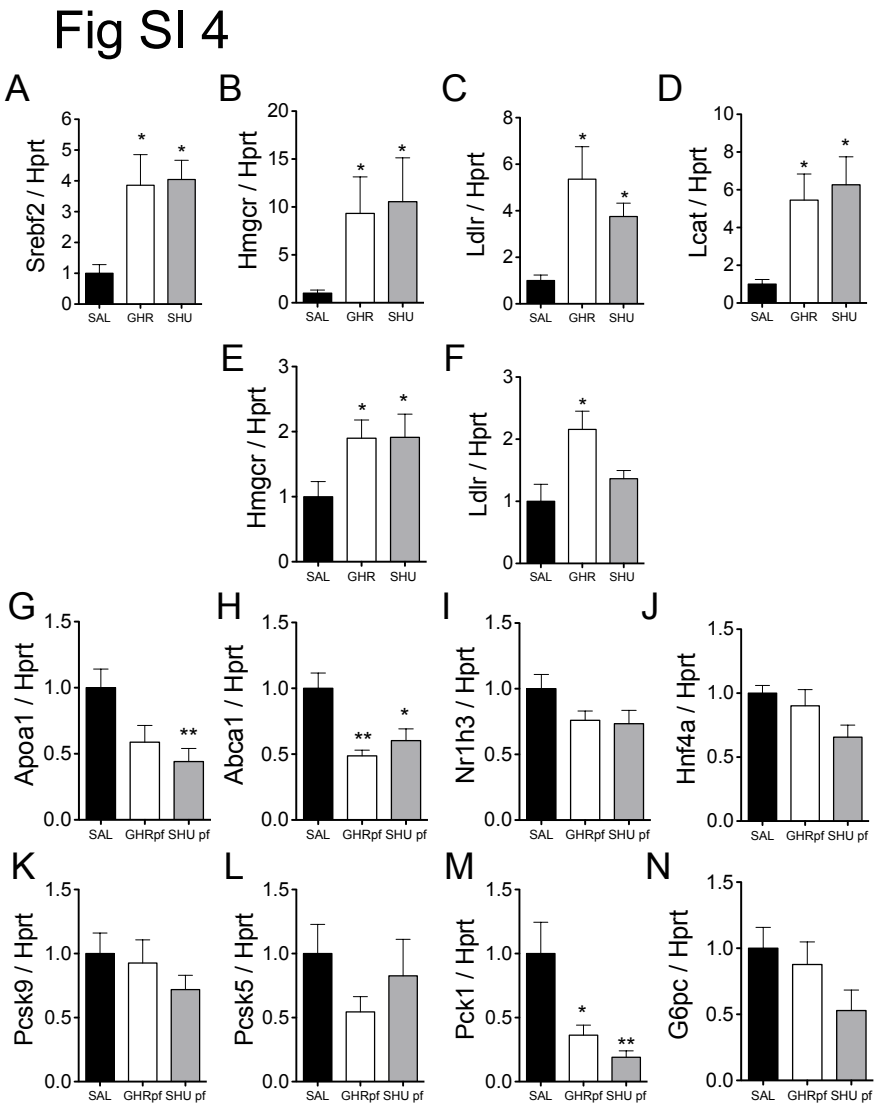

Figure S4. Analysis of hepatic gene expression changes induced by central infusion of ghrelin or SHU . (**A-D**) Effect of 7-day icv ghrelin (2.5 nmol/d) and SHU (24 nmol/d) infusion in *ad libitum* chow fed rats on hepatic mRNA expression of (**A**) Srebf2, (**B**) Hmgcr, (**C**) Ldlr, (**D**) Lcat. (**E-F**) Effect of acute injection (4-hours) of ghrelin (10 µg/2.5 µL) or SHU (25 µg/2.5 µL) in *ad libitum* chow-fed rats on hepatic mRNA expression of (**E**) Hmgcr and (**F**) Ldlr. (**G-N**) Effect of 7-day icv ghrelin (2.5 nmol/d) and SHU (24 nmol/d) infusion in *pair* fed chow rats on hepatic mRNA expression of (**G**) Apoa1, (**H**) Abca1, (**I**) Nr1h3 (LXR), (**J**) Hnf4a, (**K**) Pcsk9, (**L**) Pcsk 5, (**M**) Pck1 and (**N**) G6pc.. *P < 0.05; **P < 0.01; ***P < 0.001 versus saline infused control group, 1-way ANOVA. (mean±s.e.m., n=7-8)

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Fig SI 5

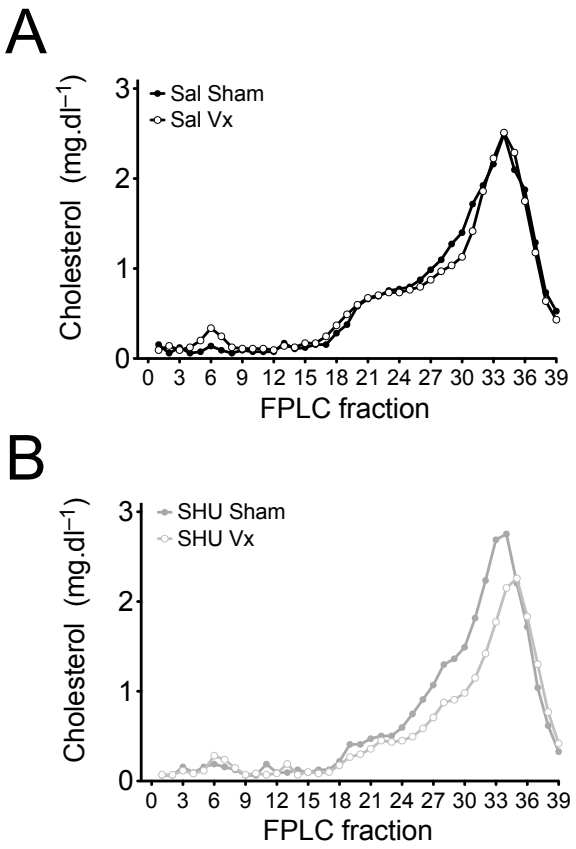

Figure S5. Effect of hepatic vagotomy in the changes induced by central infusion of SHU in HDL-C. (**A-B**) Effect of hepatic vagotomy (Vx) in pair fed rats infused for 7 days with saline (**A**) or SHU (24 nmol/d) (**B**). Plasma samples were obtained 3 hours after the onset of the light phase, and were pooled (7-8 individuals per group) before separation by FPLC.

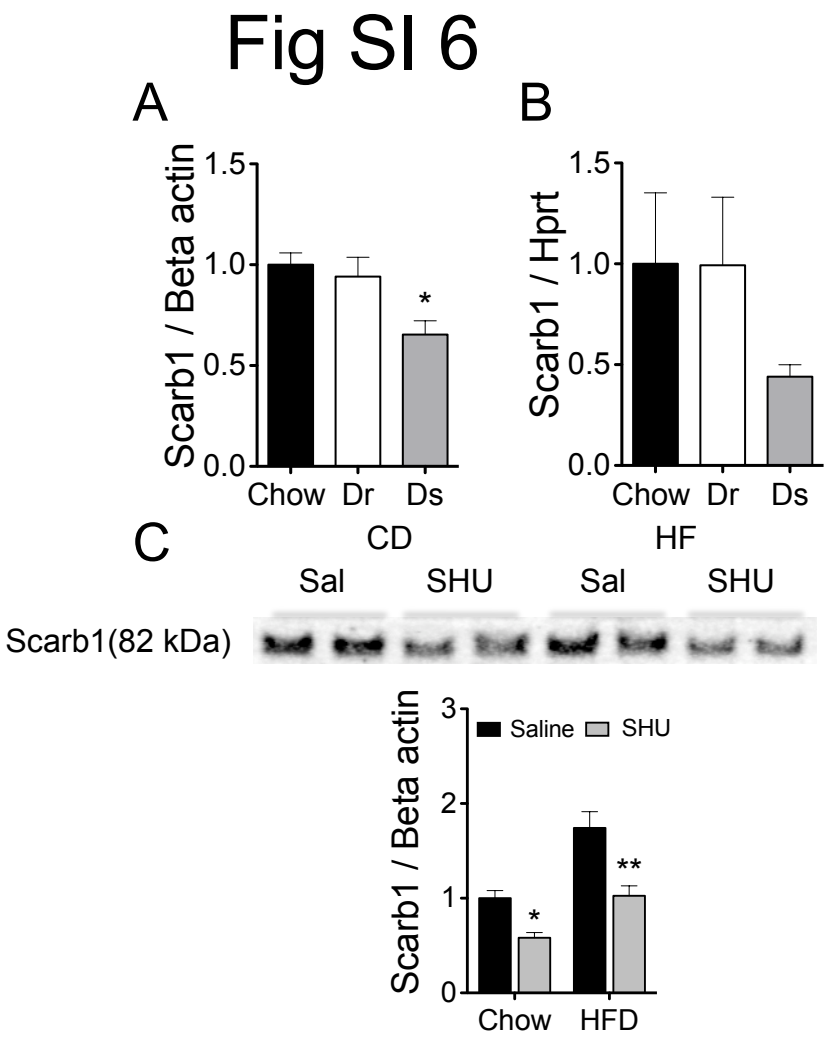

Figure 6S. Hepatic SR-BI levels in Diet Induced Obese rats and chronically icv SHU infused rats acutely exposed to High fat Diet. Protein (**A**) and (**B**) gene expression levels of Scarb1 (SR-BI) in chow fed rats, Dr and Ds rats. (**C**) Hepatic SR-BI protein expression in 7-day icv SHU (24 nmol/d) infused rats fed *ad libitum* with chow or HFD. (**A, B**). *P < 0.05 versus Standard chow and DIO resistant groups. 1-way ANOVA. (mean±s.e.m., n=6-7) (**C**) *P < 0.05; **P < 0.01 versus controls. 2-way ANOVA, Bonferroni *post hoc* test. (mean±s.e.m., n=6)

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Supplemental Table 1. Primers used for real time PCR.

Gene	Specie	Accession number	Forward sequence (5'-3')	Reverse sequence (5'-3')	product (bp)
Abca1 (ABCA 1)	rat	NM_178095	**GGTTTGGGGAGGAAATTGAT**	**AACCATCCACAGCAACCTTC**	162
Apoa1 (APO A I)	rat	NM_012738	**CCTGGATGAATTCCAGGAGA**	**TCGCTGTAGAGCCCAAACTT**	192
G6pc (Gluc 6Pase)	rat	NM_013098	**ACCCTGGTAGCCCTGTCTTT**	**GGGCTTTCTCTTCTGTGTCG**	150
Hmgcr (HMGcAR)	rat	NM_013134	**TGCTTGGTTTCTGGCTCTTT**	**TTAACCCATTGGAGGTGAGC**	230
Hnf4a (HNF4α)	rat	NM_022180	**AAATGTGCAGGTGTTGACCA**	**CACGCTCCTCCTGAAGAATC**	178
hprt (HPRT)	rat	NM_012583	**CAGTCCCAGCGTCGTGATTA**	**AGCAAGTCTTTCAGTCCTGTC**	139
Lcat (LCAT)	rat	NM_017024	**CTGGCTTTGGCAAGACCTAC**	**TACCAGTCCTGCCAGCTTCT**	191
Ldlr (LDLr)	rat	NM_175762	**CAGTGCGGCGTAGGATTG**	**GGATCACAGACCCGAAATGT**	120
Nr1h3 (LXRα)	rat	NM_031627	**TGATGCTGAATTTGCTCTGC**	**GGCTCACCAGCTTCATTAGC**	185
Nr1h4 (FXR-1)	rat	NM 021745	**CAGCCACAGATCTCCTCCTC**	**TCTTTGTCACAGGCATCTCG**	160
Nr5a2 (LRH-1)	rat	NM_021742	**TGAAAGCTGCAAGGGTTTCT**	**CTTGTACATTGGCCCGAACT**	211
Pck1 (PEPCK)	rat	NM_198780	**CCCAGGAGTCACCATCACTT**	**TTCGTAGACAAGGGGGACA**	205
Pcsk5 (PCSK 5)	rat	XM_001078022	**AGGTGGAGTGGATCCAACAG**	**CCGTGTAGCCTCTCTTCCAG**	189
Pcsk9 (PCSK 9)	rat	NM_199253	**GAGTGGAGGAGCAACAGAGG**	**TTTCAGGCAGTCACATCAGC**	205
Scarb1 (SR B1)	rat	NM_031541	**CAAGAAGCCAAGCTGTAGGG**	**CCCAACAGGCTCTACTCAGC**	230
Srebf2 (SREBP 2)	rat	NM_001033694	**GACAGGATGAAGCCACCATT**	**GGCAAGAGACCTGAGTCCTG**	214
Mrpl32 (L32)	mouse	NM 029271	**GCCAGGAGACGACAAAAAT**	**AATCCTCTTGCCCTGATCC**	131
Nr1h4 (FXR-1)	mouse	NM_009108	**GGCCTCTGGGTACCACTACA**	**ACATCCCCATCTCTCTGCAC**	179
Nr5a2 (LRH-1)	mouse	NM_030676	**CCCAAACGTGAGGAACAACT**	**TATCGCCACACACAGGACAT**	241
Scarb1 (SR B1)	mouse	NM_016741	**CAGGCTGTGGGAACTCTAGC**	**GAAAAAGCGCCAGATACAGC**	248

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Supplemental Information.

Supplemental Material and Methods:

Animals:

Surgical procedures:

Acute intracerebroventricular (icv) treatments: Male Wistar rats were anesthetized with ketamine (86 mg/kg i.p.)/xylazine (12.9 mg/kg,i.p.) and a 22-gauge stainless steel cannula (Plastics One, Roanoke, VA) was placed in the 3rd-cerebral ventricle, and fixed to the skull with dental cement and two stainless steel screws. The stereotaxic coordinates used were: anteroposterior: -0.80 mm; lateral: 1.4mm; dorsoventral: -3.50mm, from bregma. Following surgery, the animals received a single dose of buprenorphin (0.28 mg/kg, s.c.; Buprenex; Reckitt Benckiser Healthcare). After a week of recovery, correct placement of the cannula was verified by icv administration of angiotensin II (5 ng/μL in 2.5 μL of 0.9% saline solution). Rats that failed to drink a minimum of 5 ml of water within 60 min after the administration of angiotensin were removed from the studies.

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Chronic intracerebroventricular infusion: Male Wistar rats (250-350 g) or C57Bl6 (12-15 week old) were anasthetized with ketamine (86 mg/kg i.p.)/xylazine (12.9 mg/kg,i.p.). A stainless steel cannula (Brain infusion kit, Alzet Durect, CA) was placed in the right lateral cerebral ventricle. The stereotaxic coordinates used in rats were: anteroposterior: -0.80 mm; lateral: 1.4 mm; dorsoventral: -3.50 mm, from bregma. The stereotaxic coordinates used in mice were: anteroposterior: -0.70 mm; lateral: 1.2 mm; dorsoventral: -2.50 mm, from bregma. The cannula was fixed to the skull with dental cement and two stainless steel screws. The icv cannula was connected through a polyvinyl tube to an osmotic minipump filled with the treatment or vehicle (0.9% saline solution), for 7-14 days of infusion. Osmotic minipumps (model 2001-2002 for rats and 1003D or 1007D for mice; Alzet Durect, CA) were implanted subcutaneously in the interscapular space. The skin was sutured and the animals received a single dose of buprenorphin (0.28 mg/kg, s.c.; Buprenex; Reckitt Benckiser Healthcare).

Hepatic vagotomy: Male Wistar rats (250-275 g) were anasthetized with ketamine (86 mg/kg i.p.)/xylazine (5 mg/kg,i.p.). A laparotomy was performed in the midline. Hepatic ligaments were severed and the stomach was carefully pulled caudally and the right and central lobes of the liver were reflected rostrally to expose fascia containing the hepatic branch of the left vagal trunk. Under a binocular microscope, the hepatic vagal branch was identified and transected. Special care was taken to remove all connective tissue between the stomach, liver and esophagus, without

damaging the ventral trunk of the vagus. In the sham-operated animals the same procedure was followed except the scission of the nerve branch. Saline solution was applied to the abdominal cavity to avoid dryness of the viscera. The abdomen was sutured and the animals received a single dose of buprenorphin (0.28 mg/kg, s.c., Buprenex; Reckitt Benckiser Healthcare). The animals were allowed to recover for 7 days prior to undergo chronic intracerebroventricular infusion.

Gene expression quantification: Total RNA was extracted from frozen tissue samples using RNeasy mini columns (Qiagen, Valencia, CA), following manufacturer's instructions and concentration was determined by spectrophotometry. After DNAse I treatment (Invitrogen), 2 μg of total RNA were used to generate cDNA templates for RT-PCR, using random hexamers, dNTPs, RNAse inhibitor, and retrotranscriptase Superscript III (all reagents from Invitrogen, Carlsbad, CA). qPCR was performed using SYBR Green I DNA master mixture (BioRad, Hercules CA) according to the standard protocol using approximately 70 ng template cDNA. All primers were used at a final concentration of 0.5 μM. A standard curve was used to obtain the relative concentration of each experimental gene; values were normalized to the concentration of Hypoxanthine-guanine phosphoribosyltransferase (Hprt) in rat samples; or ribosomal protein 32 (Mrpl32) in mouse samples.

Immunoblot analysis: For apolipoprotein determination in FPLC fractions, identical volume (15μL) of the eluted samples were separated by SDS-PAGE,

transferred to polyvinylidene fluoride (PVDF) paper, and blotted with antibodies against ApoA-I (Novus Biologicals, 1:5000) and Apo B (Milipore, 1:5000). For plasma apolipoprotein determination, whole plasma was initially diluted ten times with ice-cold RIPA buffer (100 mM Tris HCl; 300 mM NaCl; 0.2% SDS; 1% deoxycholate; 2% NP-40; complete EDTA-free, 1 tablet/50 ml, pH 7.4). Protein concentrations were determined by BCA method and 25µg per sample were separated by SDS-PAGE. Proteins were transferred to PVDF paper, and blotted with antibodies against ApoA-I and Apo B as described above. For Scarb1 (SRBI) protein quantification, liver samples (100mg aprox.) were homogenized in ice cold RIPA buffer using a Tissuelyzer (Quiagen), the protein concentration was assessed by BCA method and 30µg per sample were separated by SDS-PAGE. Proteins were transferred to PVDF paper, and blotted with antibodies against SRBI (Novus, Biologicals, 1:5000) and beta actin (Novus Biologicals, 1:10000). Immunoreactive proteins were detected by incubating the blots with fluorescently labeled species-specific secondary antibodies (Rockland Inmnologicals, Molecular Probes; Invitrogen) and visualized by the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Lipoprotein separation by fast liquid performance chromatography (FPLC): Trunk blood from rats was collected in EDTA+ aprotinin-containing tubes. Blood from mice was collected from tail veins using EDTA-coated Microvette® tubes (Sarstedt, Nuremberg, Germany). Cholesterol distribution among the various classes of lipoproteins was analyzed by subjecting 200 µl of pooled plasma (6-8 individuals

per group) by fast phase liquid chromatography gel filtration on two Superose 6 HR columns in tandem. Each fraction (0.5 ml) was collected for cholesterol determination by colorimetric assay (Thermo Electron, Pittsburgh, Pennsylvania, USA).

Clearance analysis of HDL Cholesterol: HDL (*d* = 1.063-1.21) was prepared from human plasma by sequential centrifugation and was labeled with [cholesterol-1,2-^{3}H]cholesteryl oleoyl ether (American Radiolabeled Chemicals, Inc.) by exchange from donor liposomal particles using partially purified human plasma cholesterol ester transfer protein [41]. After exchange, donor particles were removed by floatation at *d* 1.08 and labeled HDL particles were re-isolated by floatation at *d* 1.21. The resulting labeled HDL was exhaustively dialyzed against phosphate-buffered saline and the specific radioactivity was estimated to be 640,000 dpm/μg HDL cholesterol. *Ad libitum* chow fed rats infused chronically icv with SHU 9119 (10nmol/day, 4 days) or saline (12 per group) were lightly anesthetized with isoflurane and injected with labeled HDL in 0.5 ml of phosphate-buffered saline via the tail vein. Blood was collected and plasma was immediately separated and the radioactivity was determined with a β-counter. The radioactivity of the 0.5 min post-injection was defined as 100% of the injected radioactivity and representative of the initial plasma cholesterol pool. Rats with highest radioactivity value at 0.5min post-injection were included in the analysis. Clearance curves were averaged per treatment group and fitted with a non linear regression for two phase decay with a least square fit. Both curves were compared with extra sum of squares F test.

Analysis of HDL size by non denaturing electrophoresis:

Two milliliters of plasma were pooled from 2 animals of same treated group. KBr was added to raise the density to 1.063g/mL and samples were spun at 50000rpm at 10ºC for 12 hours in a Beckman Optima XL ultracentrifge. The supernatant (1mL) was carefully removed; the density of the remaining volume was raised by adding KBr up to 1.21g/mL. Samples were spun at 50000rpm at 10ºC fro 48 hours. The supernatant (200µL) was carefully collected and the protein content was determined by the bicinchoninic acid (BCA) method using a commercial kit (Pierce). The samples were diluted in non denaturing running buffer (25mM Tris base, 192mM Glycine) and 12µg of protein per well were loaded in a 4-15% Tris glycine poliacrilamide gel. Electrophoresis was performed at 80V for 16 hours at 4ºC. The protein content of the gel was revealed using Comassie brilliant blue staining. The size of HDL was compared with a High Molecular Weight marker (GE healthcare).

Quantification of blood parameters:

Lincoplex assays (Linco Research, St. Charles, Missouri, USA) were used for quantification of plasma insulin, leptin, adiponectin, Plasminogen activator inhibitor (PAI)-1 active antigen, Monocyte chemotactic protein (MCP)-1 and interleukin (IL) 1b levels. Plasma glucose and non-esterified fatty acid levels (NEFA) were measured using commercially available enzymatic assay kits from Wako (Autokit Glucose, and NEFA C, Wako, Neuss, Germany). Total plasma cholesterol and triglyceride levels were determined using Infinity Cholesterol reagent and Infinity Triglyceride reagent

(Thermo Electron, Pittsburgh, Pennsylvania, USA) unless otherwise stated. All assays were performed according to the manufacturer's instructions.

Additional methodological details for specific experiments:

Effect of ghrelin on cholesterol levels in mice: Wild-type 129SV wild type mice were injected with ghrelin as previously described [42] for 7 days. Body composition was assessed, the animals were sacrificed and trunk blood was collected. For the assessment of cholesterol levels in *Ghr*$^{-/-}$, *Ghsr*$^{-/-}$ and dKO mice, 12-15 month old male mice fed standard chow were fasted overnight and 150 µL of blood was collected in EDTA-coated tubes. Total plasma cholesterol was measured in plasma, and pooled group samples were prepared for lipoprotein separation by FPLC and cholesterol content was determined in the obtained fractions. In addition hepatic gene expression was assessed in 6-8 month old male dWT and *Ghsr-/-* mice fed with standard chow after a 24-hour fast. Liver was collected and immediately frozen and stored at -80º C.

Effect of the lack of MC3 / 4-R on cholesterol levels in mice: Cholesterol levels were assessed in 6-month old male *Mc4r-/-* mice that had been fed for 3 months with low (10% kJ/fat; LFD), moderate (45% kJ/fat; HFD) or very high (60% kJ/fat; VHFD) fat and were fasted overnight. Total plasma cholesterol was measured using a Beckman Synchron CX7.

Male *Mc4r-/-*mice, and wild type littermates (7-8 weeks old) as well as body fat matched C57Bl6 mice maintained at all times on standard chow (8-9 months old),

were simultaneously fasted for 6 hours, and tail-bled for quantification of total cholesterol in plasma. Body composition was assessed by QMR.

Acute intracerebroventricular (icv) administration of ghrelin or melanocortin receptor ligands: To investigate the acute effect of the blockade of central melanocortin system activity on the hepatic synthesis of cholesterol, ghrelin (10 μg/2.5 μL) or SHU (25 μg/2.5 μL) was injected into the 3rd ventricle of male Wistar rats (300-325 g) fed *ad libitum* after the onset of the light phase. Prior to the injection the food was removed from the cages and the animals were sacrificed 4 hours after the treatment.

To investigate the acute effect of the activation of the central melanocortin system on plasma cholesterol levels, male Wistar rats maintained on standard chow (525-625 g) or on the high-fat diet for 11 months (750-900 g) were fasted for 24 h. Fasting began 3 hours before the onset of the dark phase. MT II (3 nmol/2.5 μL) or control 0.9% saline solution was injected twice over 18 h (1 h before dark phase and 4 h before sacrifice) into the 3rd ventricle.

Chronic icv infusion of ghrelin or melanocortin receptor ligands: Male Wistar rats maintained on *ad libitum* chow were chronically infused with ghrelin (2 nmol/d icv) for 14 days. An additional *pair*-fed group was identically infused with ghrelin, but access to food was restricted to the amount consumed by the saline-infused control group each day. In separate parallel experiments rats received SHU (24 nmol/d) or MTII (1 nmol/d) for 7 days, or else ghrelin (2 nmol/d) plus SHU (24

nmol/d) in *pair*-fed rats. Food intake, body weight and body composition were monitored. At the end of the infusion, trunk blood and liver were collected and and stored at -80º C. For HDL-cholesterol clearance test, rats were infused chronically icv with SHU 9119 (10nmol/day) or saline for 4 days using implanted osmotic minipumps. Rats were fed *ad libitum* except during the dark phase prior the experiment, in which all rats received the food eaten the previous day (25g) by control group rats. On the day of the experiment, labeled HDL in 0.5 ml of phosphate-buffered saline was injected via the tail veins between 9am and11am. Blood samples were collected up to 6 hours, the plasma was immediately separated and radioactivity was determined using a β-counter.

Chronic icv infusion of GLP-1: Male C57Bl6 mice maintained on *ad libitum* chow were chronically infused with GLP-1 (7-37) amide (2.5 nmol/d icv) for 7 days. An additional *pair*-fed group was identically infused saline, but access to food was restricted to the amount consumed by the GLP-1-infused control group each day, prior to sacrifice following the treatment period.

Study of feeding response in rats resistant (DR) or sensitive (DIO) to high fat diet: The *ad libitum* food intake in DIO– resistant (Dr) and DIO-sensitive (Ds) rats fed with high fat diet, and in a group of chow-fed rats was assessed during 24 hours starting the measurement just before the onset of the dark phase. Then, the food was removed from the cages for the next 24 hours and returned before the onset of

the dark phase, and the food intake was measured during the first 24 hours of refeeding.