Interplay of Dietary Fatty Acids and Cholesterol Impacts Brain Mitochondria and Insulin Action

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SUPPLEMENTARY MATERIAL

***In vivo* measurements**

Oral glucose tolerance test (GTT) was performed in week 18 of the feeding intervention after 16 hours of fasting by oral gavage of glucose (2 mg/kg body weight). Insulin tolerance (ITT) test was performed in week 19 of the feeding intervention after 4 h of fasting by intraperitoneal injection of insulin (0.25 IU/kg body weight). Blood glucose from tail vein was monitored at 0 min, 15 min, 30 min, 60 min, 90 min and 120 min using a glucose sensor (Breeze2, Bayer; Berlin, Germany).

MTT assay

To assess a non-toxic concentration of MβCD and cholesterol for CLU183 cells, MTT assay was performed. For this, cells were stimulated with various concentrations of either MβCD or cholesterol for 16 h and following incubated with 0.5 mg/ml of tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Taufkirchen, Germany) for 3 h. Sodium-dodecyl sulfate (SDS; Carl Roth, Karlsruhe, Germany) was used as positive control to induce cell death. Afterwards, dimethyl sulfoxide (DMSO; Sigma-Aldrich, Taufkirchen, Germany) was used to solubilize formazan which is the reduced form of MTT. Optical density was determined at 560 nm and 670 nm (reference wavelength) using a plate reader (TECAN, Männedorf, Switzerland).

Supplementary Table S1. Diet composition.

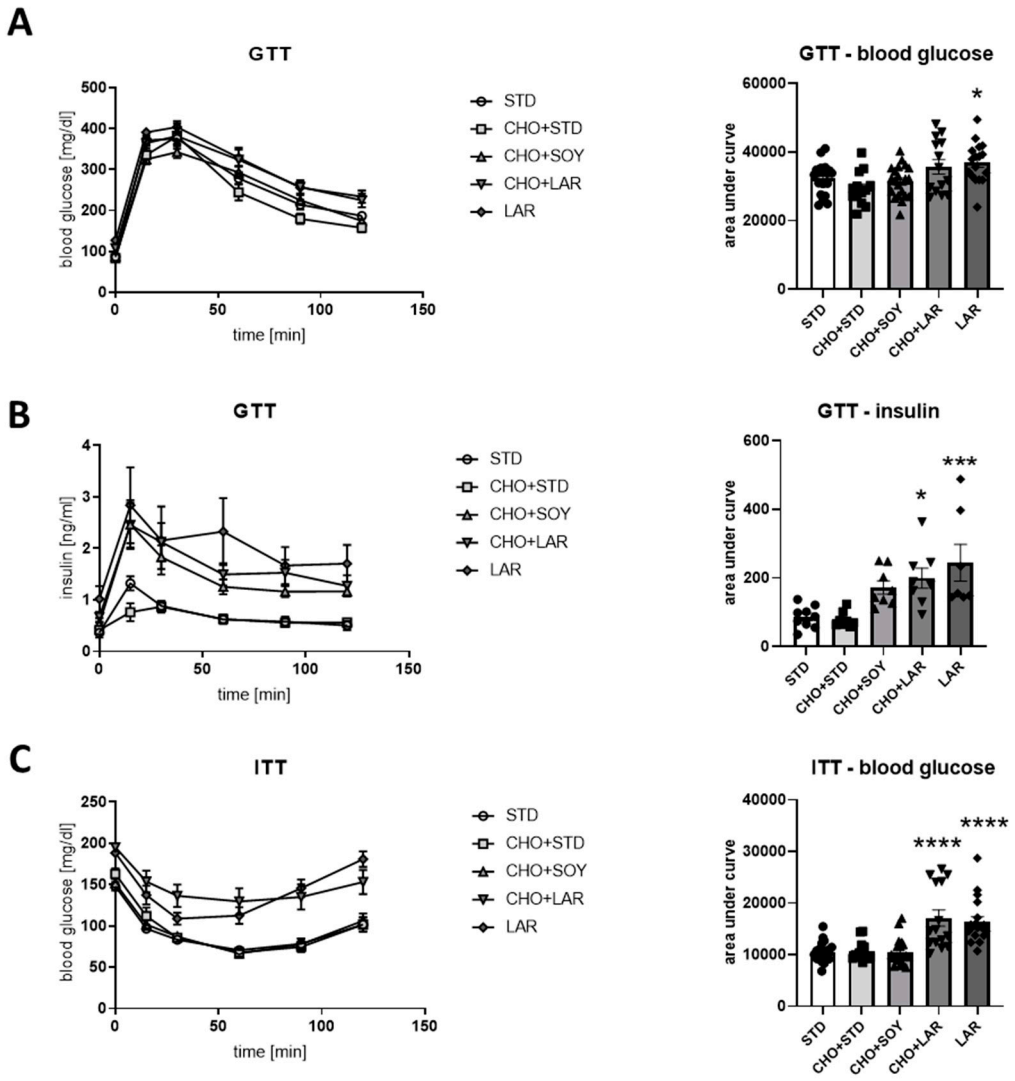
	STD	CHO+STD	CHO+SOY	LAR	CHO+LAR
Metabolizing energy (kcal/g)	3.06	3.06	4.64	4.73	4.73
Energy from carbohydrates (%)	65	65	35	35	35
Energy from protein (%)	25	25	16	20	20
Energy from fat (%)	10	10	49	45	45
Cholesterol (%)	0.00	0.75	0.75	0.00	0.75
Fatty acid composition					
Saturated fatty acids (g/100g)	0.55	0.55	4.00	7.26	7.26
Mono-unsaturated fatty acids (g/100g)	0.64	0.64	5.75	8.69	8.69
Poly-unsaturated fatty acids (g/100g)	2.01	2.01	14.50	6.36	6.36

Diet composition. Mice diets used in the feeding experiment. Standard chow diet (STD), 0.75% cholesterol in a standard diet (CHO+STD), 0.75% cholesterol in a high fat diet containing ω6-PUFA-rich soybean oil (CHO+SOY), high fat diet containing mainly lard as fat source (LAR), 0.75% cholesterol in a high fat diet containing mainly lard as fat source (CHO+LAR).

Supplementary Table S2. Primer pairs specific for each gene used in the study.

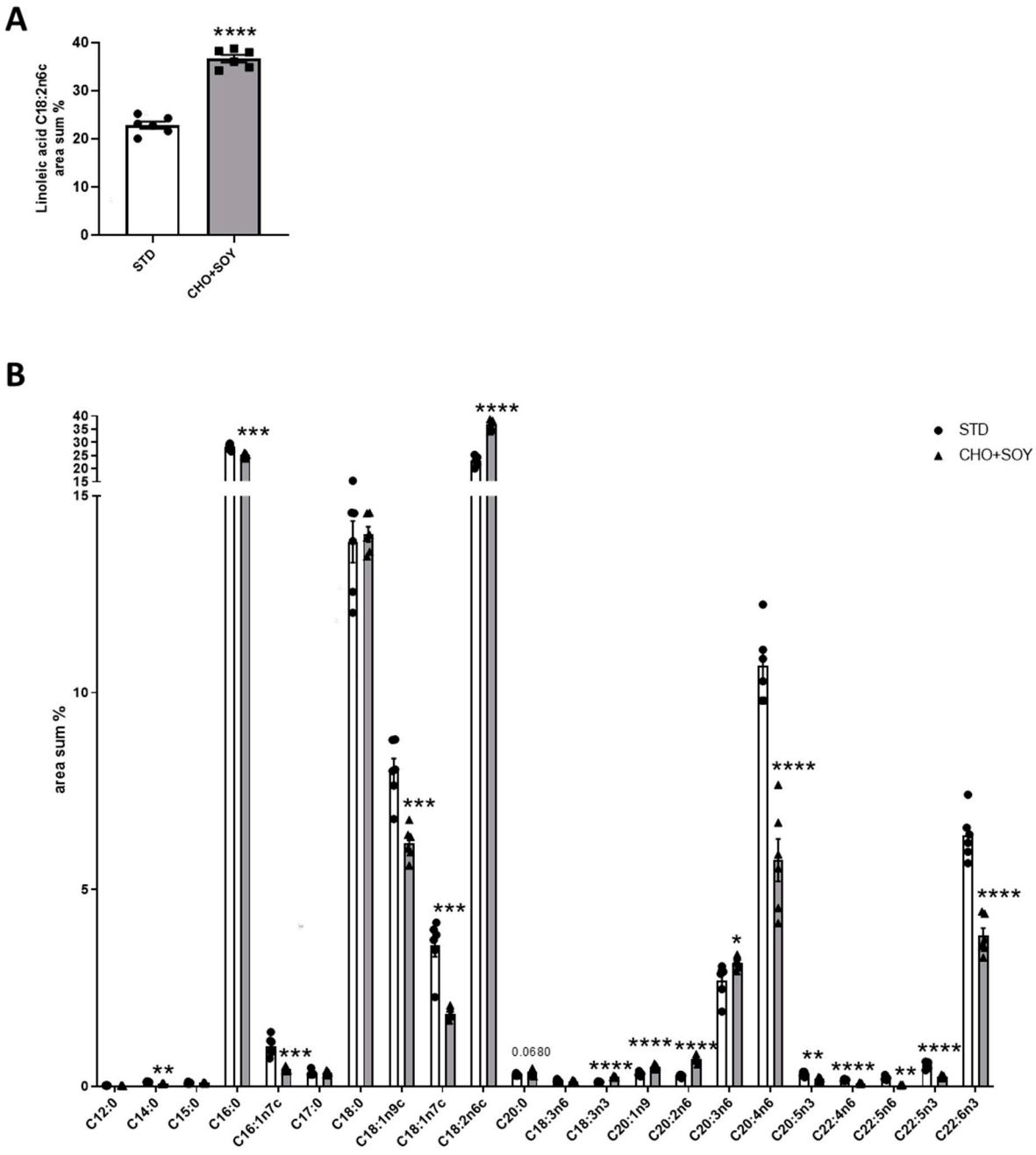
Name	Fw Primer Sequence (5´- 3´)	Rv Primer Sequence (5´- 3´)
Hmgcr	AGTACATTCTGGGTATTGCTGG	ACTCGCTCTAGAAAGGTCAATC
Srebp2	GCGTTCTGGAGACCATGGA	ACAAAGTTGCTCTGAAAACAAATCA
Fdps	CTGCAGAGTTCCTATCAGACAG	CAGGTAGAAAGAGTAGAAAGCCG
Sqle	TTGTTGCGGATGGACTCTTCTCCA	GTTGACCAGAACAAGCTCCGCAAA
Hsp60	AGTGTTCAGTCCATTGTCCC	TGACTGCCACAACCTGAAG
Sirt3	CGGCTCTATACACAGAACATCG	CATCAGCCCATATGTCTTCCC
Pgc1α	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
Mfn1	CATTGCGTTTCGGTTTTCCC	GAAGGAGCAGTAGGAGTTGAAG
Drp1	TCCCAATTCCATTATCCTCGC	CATCAGTACCCGCATCCATG
Opa1	GTGTGCTGGAAATGATTGCTC	TGGTGAGATCAAATTCCCGAG
Cox2	CCTGGTGAACTACGACTGCT	GAATAACCCTGGTCGGTTTG
Cox3	GCAGGATTCTTCTGAGCGTTCT	GTCAGCAGCCTCCTAGATCATGT
Nd1	GGATCCGAGCATCTTATCCA	GGTGGTACTCCCGCTGTAAA
Nd6	ATTAAACAACCAACAAACCCAC	TTTGGTTGGTTGTCTTGGGTT
Tbp	CTGGAATTGTACCGCAGCTT	ATGATGACTGCAGCAAATCG
β-Actin	GCCAACCGTGAAAAGATGAC	TACGACCAGAGGCATACAG

Supplementary Figure S1: High-fat diets decrease insulin sensitivity.



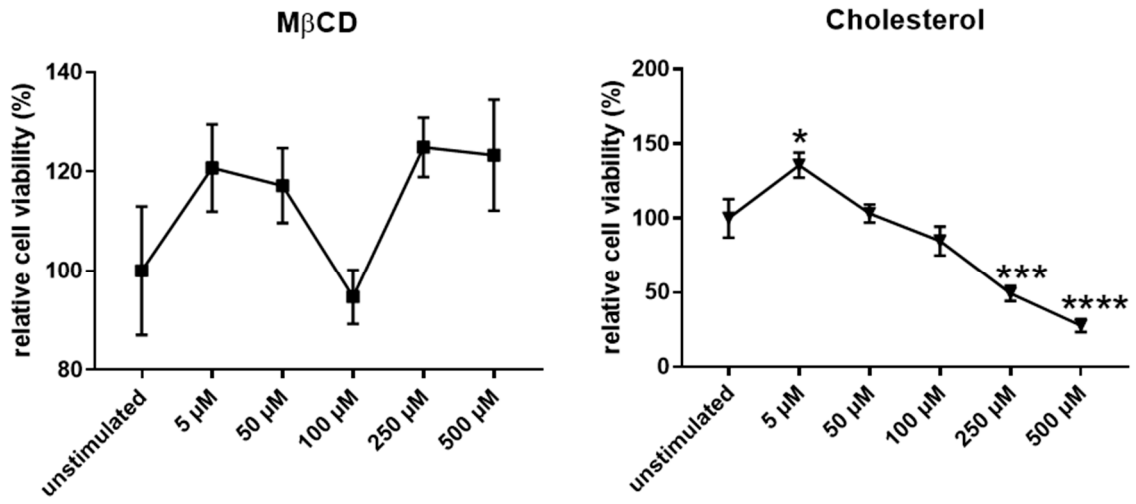
Supplementary Figure S1: High-fat diets decrease insulin sensitivity. (**A**,**B**) Glucose tolerance test (GTT) and (**C**) insulin tolerance test (ITT) after 18 and 19 weeks of feeding intervention. All values are displayed as median ± SEM with a total n of 13-21 per group. Statistics: One-way-ANOVA with Dunnett post hoc test for multiple comparisons. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. *: vs STD

Supplementary Figure S2: Changes in relative abundance of fatty acids in serum due to dietary intervention.



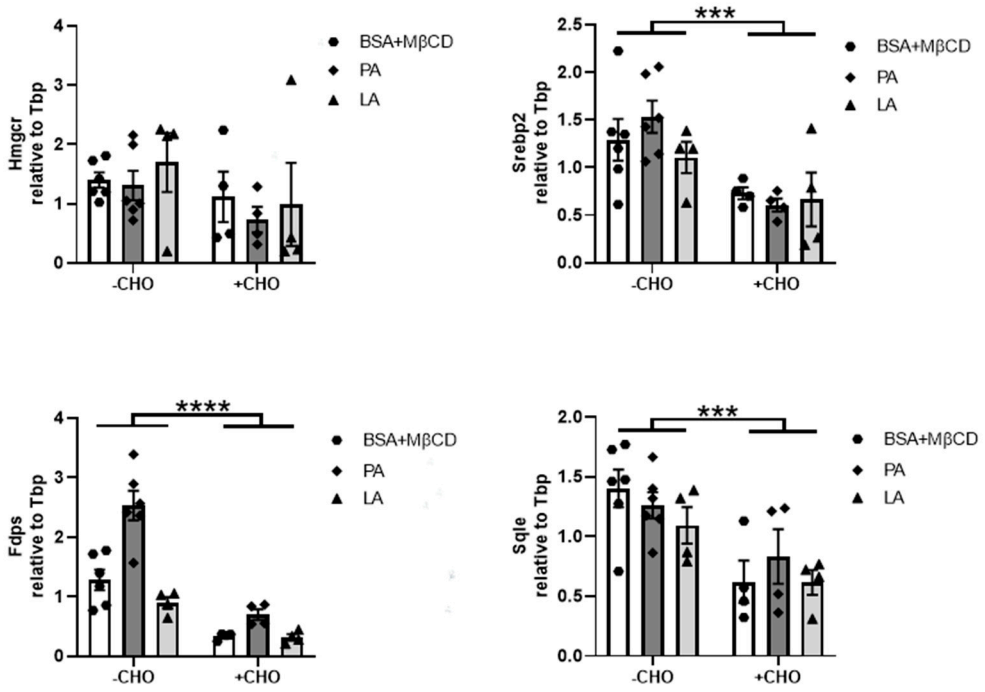
Supplementary Figure S2. Changes in relative abundance of fatty acids in serum due to dietary intervention. (**A**) Relative abundance of linoleic acid in serum. (**B**) Relative fatty acid composition in serum**.** All values are displayed as median ± SEM with a total n of 6 mice per group. Statistics: Multiple Student's t-test for unpaired samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs STD group.

Supplementary Figure S3: Determination of concentration for MβCD and cholesterol in hypothalamic neurons.



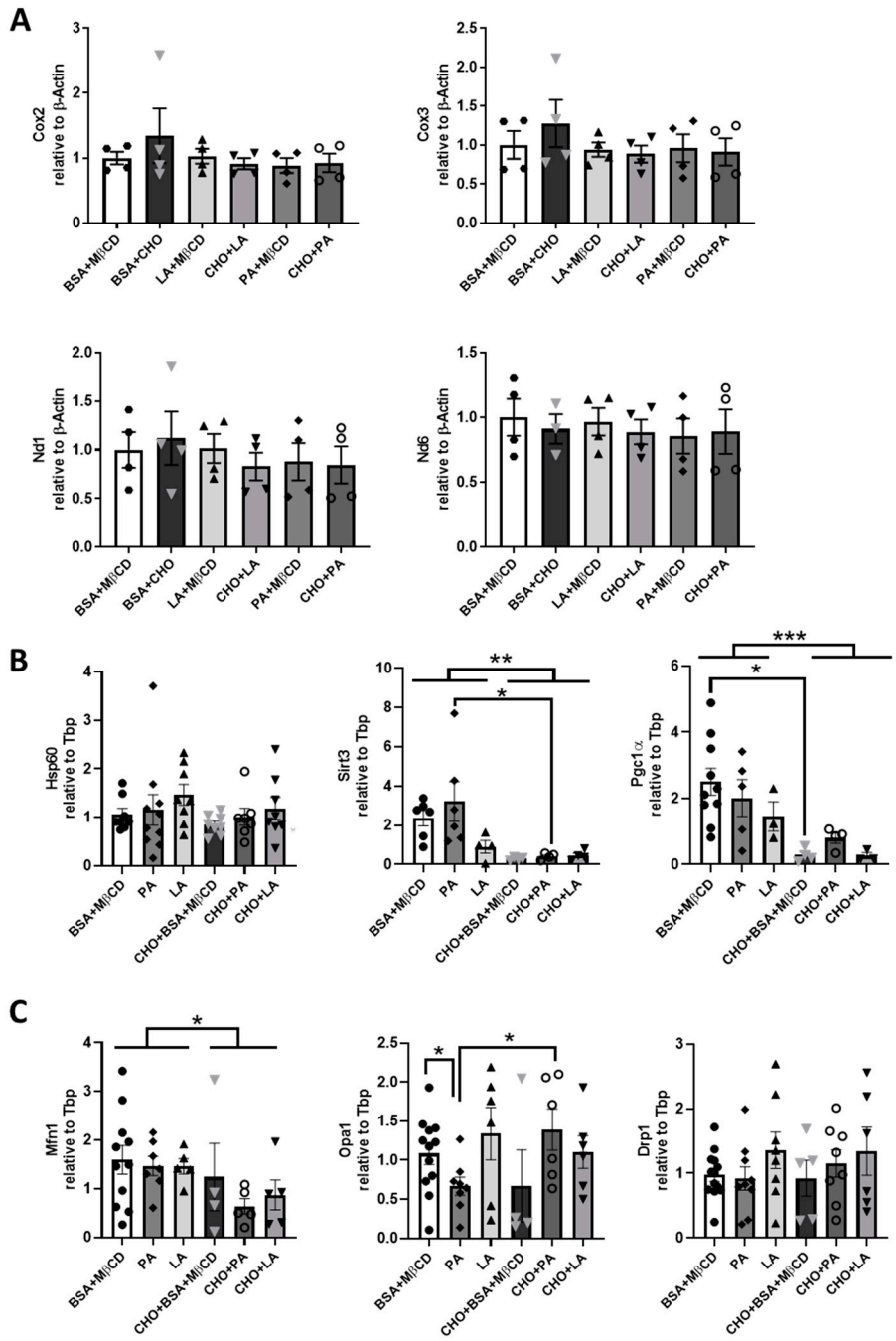
Supplementary Figure S3. Determination of concentration for MβCD and cholesterol in hypothalamic neurons. Cell viability in % relative to unstimulated CLU183 cells using MTT assay with a total n of 8 per group. Statistics: One-way-ANOVA with Dunnett's multiple comparisons test. All the data are presented as mean ± SEM. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ vs unstimulated control.

Supplementary Figure S4: Cholesterol regulates endogenous cholesterol biosynthesis in hypothalamic neurons.



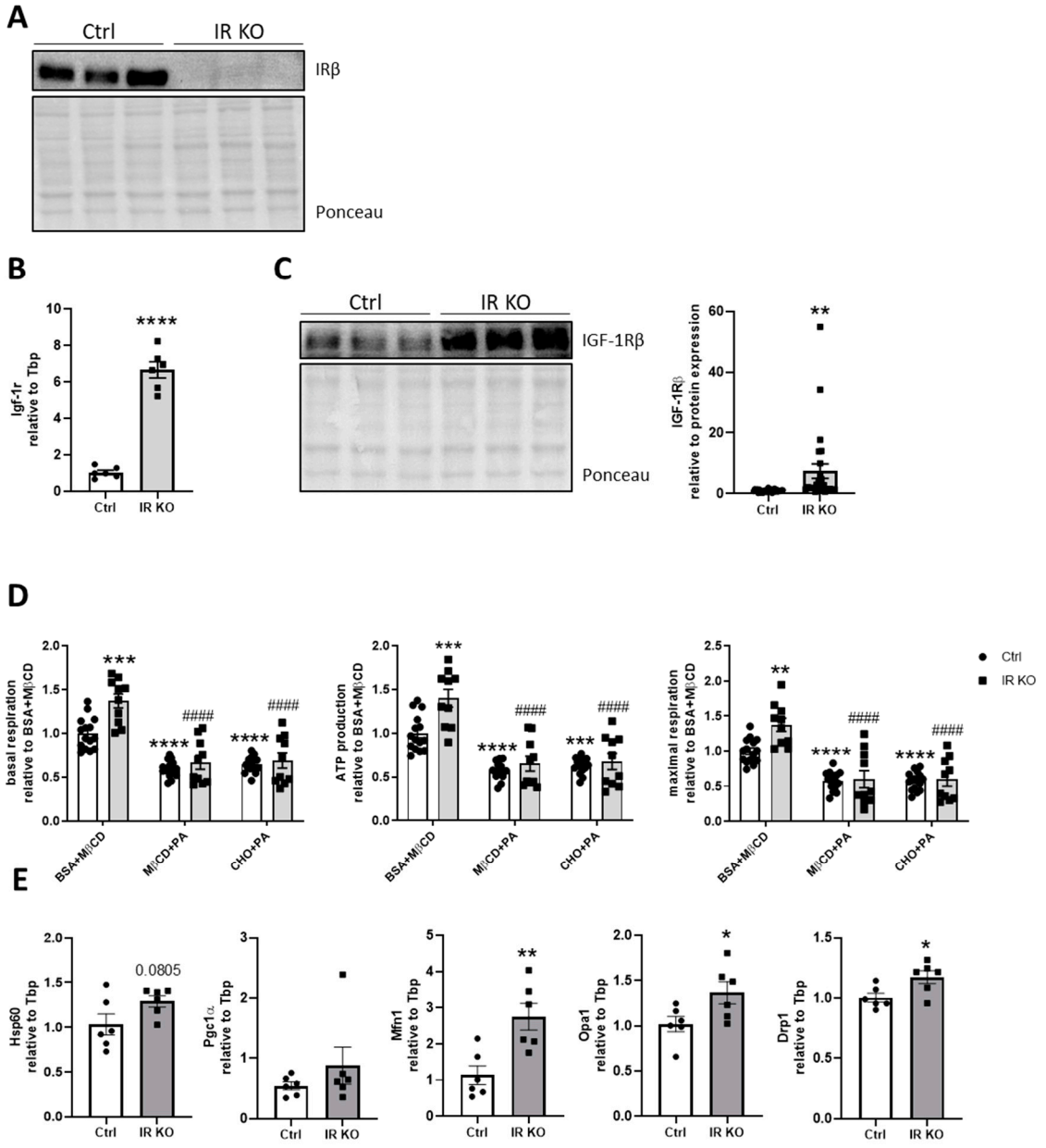
Supplementary Figure S4: Cholesterol regulates endogenous cholesterol biosynthesis in hypothalamic neurons. Relative mRNA expression of *Hmgcr*, *Srebp2*, *Fdps*, and *Sqle*. Data of three independent experiments with a total n= 4-6. All the data are presented as mean ± SEM. *** $p < 0.001$, **** $p < 0.0001$.

Supplementary Figure S5: Cholesterol regulates mitochondrial function and dynamics in hypothalamic neurons.



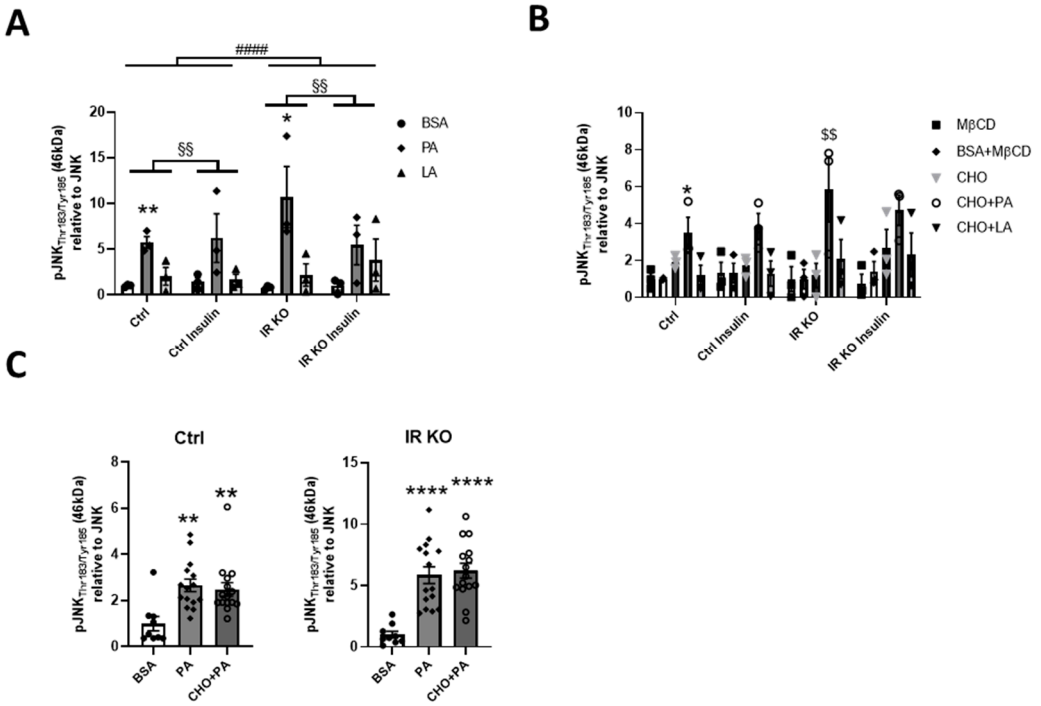
Supplementary Figure S5. Cholesterol regulates mitochondrial function and dynamics in hypothalamic neurons. (**A**) Relative mRNA expression of *Cox2*, *Cox3*, *Nd1*, and *Nd6* in stimulated neurons. Data of four independent experiments with a total n= 4. Relative mRNA expression of (**B**) *Hsp60*, *Sirt3*, *Pgc1α*, (**C**) *Mfn1*, *Opa1*, and *Drp1* in stimulated neurons. Data of three independent experiments with a total n= 4-12. All the data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Supplementary Figure S6: Characterization of insulin receptor-deficient hypothalamic neurons.



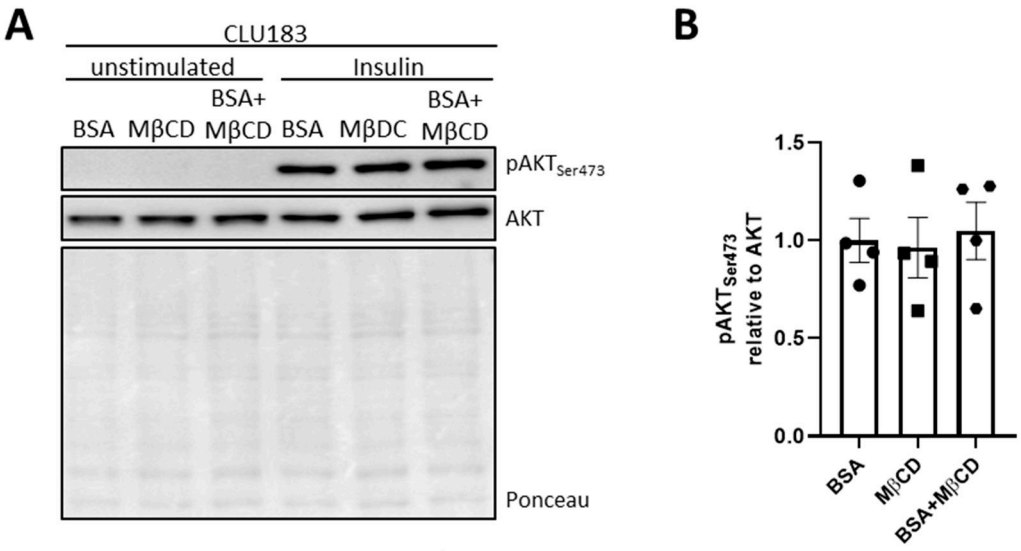
Supplementary Figure S6. Characterization of insulin receptor-deficient hypothalamic neurons. (**A**) Protein expression of IR in control and IR KO CLU183 cells. (**B**) Relative mRNA expression of Igf-1r. (**C**) Protein expression of IGF-1R in control and IR KO CLU183 cells and densitometric analysis. (**D**) Relative oxygen consumption rate (basal respiration, ATP production, and maximal respiration) in stimulated control and IR KO cells. (**E**) Relative mRNA expression of *Hsp60*, *Pgc1α*, *Mfn1*, *Opa1*, and *Drp1* in control and IR KO cells. (B) Dense intensity of IGF-1R was normalized to Ponceau, which was verified on the same Western blot membrane as a loading control and calculated relative to the control (Ctrl) group. A representative blot is shown. (B-E) Data of three independent experiments with a total n= 6-26. All the data are presented as mean ± SEM. Statistics: Multiple Student's t-test for unpaired samples and one-way-ANOVA with Tukey's post hoc test for multiple comparisons., * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. * vs Ctrl: BSA+MβCD, #### vs IR KO: BSA+MβCD.

Supplementary Figure S7: Palmitate but not cholesterol induces JNK activation.



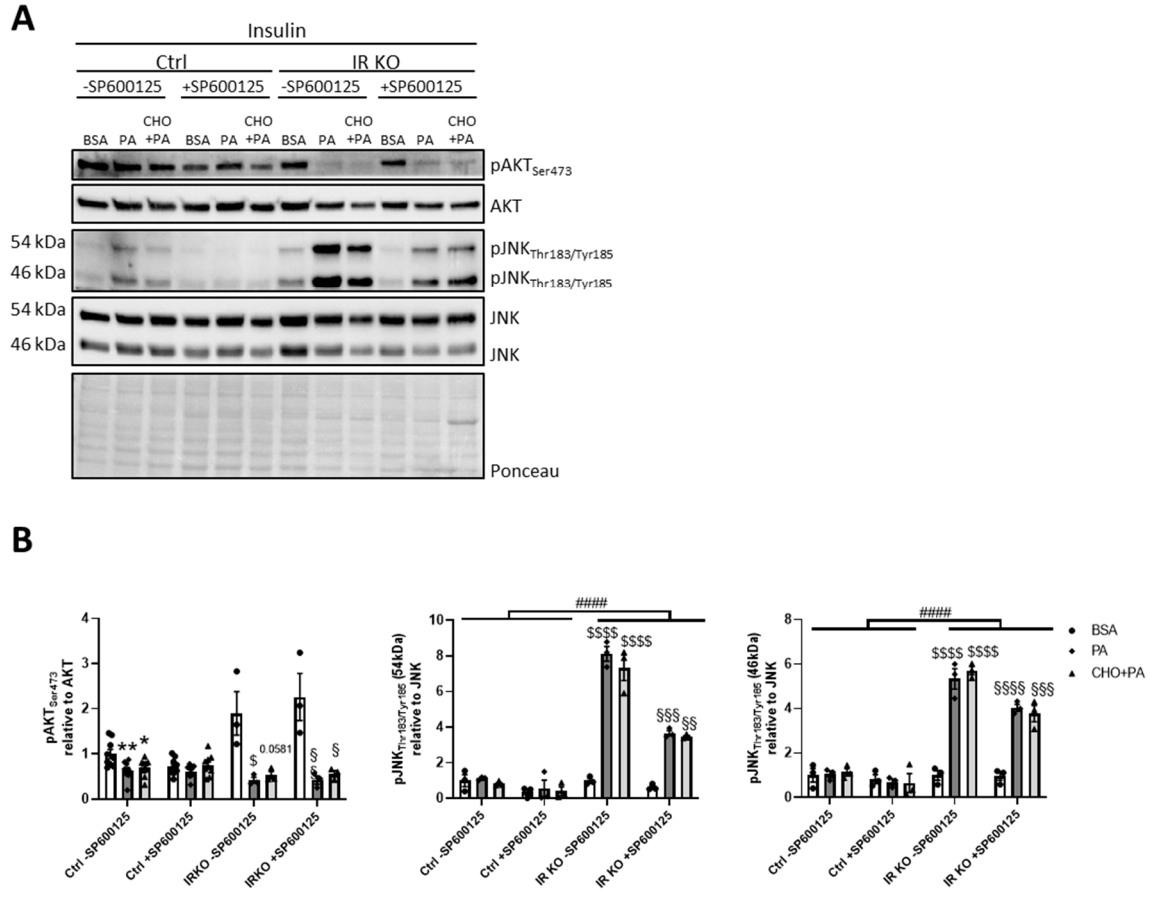
Supplementary Figure S7. Palmitate but not cholesterol induces JNK activation. (**A-C**) Densitometric analysis of the protein expression of pJNK Thr183/Tyr185 and JNK in stimulated control or IR KO CLU183 cells. All values are displayed as median ± SEM. Data of three independent experiments with a total n= 3-15. Dense intensity of pJNK Thr183/Tyr185 to total JNK protein, which was verified on the same Western blot membrane as a loading control and calculated relative to the respective control (BSA or BSA+MβCD) group in each gel. Statistics: Two-way-ANOVA with Tukey's post hoc test for multiple comparisons of Ctrl vs IR KO and one-way-ANOVA with Tukey's post hoc test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. *: vs BSA(Ctrl) or BSA+MβCD(Ctrl), $: vs BSA(IRKO) or BSA+MβCD(IRKO), §§: unstimulated vs Insulin stimulated, ####: Ctrl vs IR KO.

Supplementary Figure S8: Investigating different control stimulations on insulin sensitivity in hypothalamic neurons.



Supplementary Figure S8. Investigating different control stimulations on insulin sensitivity in hypothalamic neurons. (**A**) Protein expression of pAKT Ser473, AKT and (**B**) densitometric analysis in stimulated neurons. Dense intensity of pAKT Ser473 was normalized total AKT, which was verified on the same Western blot membrane as a loading control and calculated relative to the control (BSA) group. A representative blot is shown. All values are displayed as median ± SEM. Data of four independent experiments with a total n = 4. Statistics: One-way-ANOVA with Tukey's post hoc test for multiple comparisons.

Supplementary Figure S9: Palmitate- and palmitate/cholesterol-induced insulin resistance is independent of JNK activation.



Supplementary Figure S9. Palmitate- and palmitate/cholesterol-induced insulin resistance is independent of JNK activation. (**A**) Protein expression in stimulated control or IR KO CLU183 cells of pAKT Ser473, AKT, pJNK Thr183/Tyr185, JNK and (**B**) densitometric analysis. Dense intensity of pAKT Ser473 was normalized total AKT protein and for pJNK Thr183/Tyr185 to total JNK protein, which was verified on the same Western blot membrane as a loading control and calculated relative to the control (BSA) group in each gel. A representative blot is shown. All values are displayed as median ± SEM. Data of three independent experiments with a total n = 3-9. Statistics Two-way-ANOVA with Tukey's post hoc test for multiple comparisons of Ctrl vs IR KO and one-way-ANOVA with Tukey's post hoc test for multiple comparisons * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. *: vs Ctrl -SP600125 BSA, $, $$$$: vs IR KO -SP600125 BSA, §, §§, §§§, §§§§: vs IR KO +SP600125 BSA, #: Ctrl vs IR KO, 0.0581 vs IR KO -SP600125.