Supplementary Data

Hypothalamic CREB regulates the expression of Pomc processing enzyme Pcsk2

Zanesco et al.

Supplementary Table S1. Macronutrient composition of the diets

Component	HFD	Standard Diet - SD
Sucrose	100	-
Dextrinized corn starch	132	-
Starch	199,5	550
Protein (casein 85%)	200	225
Soybean oil	40	45
Lard	228	-
Mineral mix	35	50
Vitamin mix	10	50
Fibers (cellulose)	50	80
L-Cystine	3	-
Choline bitartrate	2,5	-
TOTAL (g)	1000	1000

Supplementary Table S2. Key resources table

REAGENTE OR RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-pCREB	Cell Signaling	#9198
Rabbit anti-CREB	Cell Signaling	#4820
Donkey anti-rabbit IgG FITC	Abcam	#ab6798
Mouse anti-α-tubulina	Abcam	#ab7291
Lentiviral Transduction Particles		
shRNA-CREB	Sigma-Aldrich	TRCN0000301658
shRNA-CREB	Sigma-Aldrich	TRCN0000096633
shRNA-CREB	Sigma-Aldrich	TRCN0000096629
Scramble	Sigma-Aldrich	SHC002V
Chemicals, Peptides, and Recombinant Proteins		
Sodium palmiatate	Sigma-Aldrich	P9767
BSA fatty acid free	Roche Diagnostics Deutschland GmbH	10775835001
TRIzol Reagent	Thermo Fisher	Cat #15596018
High Fat Diet (HFD)	Pragsoluções Biociências	228 BAN
Chow diet	Nuvilab	CR-1
Critical commercial assays		
Rat/Mouse Neuropeptide Magnetic Bead Panel	Millipore	Cat. # RMNPMAG-83K
Taqman Gene Expression Master Mix	Applied Biosystems	Cat#4369016
Experimental Models		
Mouse: WT C57BL6 /J	Jackson Laboratory	Stock #664
POMC-Cre mouse	Jackson Laboratory	Stock #5965
B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J	Jackson Laboratory	Stock #7914
Oligonucleotides – primers gene		
Pomc	Applied	Mm00435874_m1
Cart	Applied	Mm01811203_g1
Agrp	Applied	Mm00475829_g1
Npy	Applied	Mm01410146_m1
Pcsk1	Applied	Mm01345253_m1
Pcsk2	Applied	Mm00500981_m1
Prcp	Applied	Mm00804502_m1
Cpe	Applied	Mm00516341_m1
Il1β	Applied	Mm00434228_m1
Il6	Applied	Mm00446190_m1
Il10	Applied	Mm01288386_m1
Tgfβ1	IDT	138333258
Tlr4	Applied	Mm00445273_m1
Tnf	Applied	Mm00443258_m1
Nlrp3	Applied	Mm00840904_m1

Socs3	Applied	Mm00545913_s1
Gapdh	Applied	Mm99999915_g1

Software

Excel	Microsoft	N/A
Word	Microsoft	N/A
PowerPoint	Microsoft	N/A
Prism 8.0	GraphPad Software	https://www.graphpad.com
Biorender	Biorender	N/A
Image J	National Institutes of Health	https://imagej.nih.gov/ij/

Supplementary Table S3. Quantitative analysis of western blot presented in Figure 3B.

Figure 3. B -Original western blotting membranes

	Sample	pCreb Average Pixel	α-Tubulin Average Pixel	Ratio
Membrane 1	CT 1	9.715.782	17.208.983	0,565
	CT 2	9.759.690	12.640.083	0,772
	CT 3	12.920.196	23.643.569	0,546
	CT 4	13.846.225	19.382.033	0,714
	CT 5	4.966.426	12.104.205	0,410
	1H 1	9.665.196	17.105.861	0,565
	1H 2	19.033.602	19.182.569	0,992
	1H 3	21.980.731	20.363.740	1,079
	1H 4	6.372.497	25.018.276	0,255
	1H 5	3.357.326	17.675.719	0,190
Membrane 2	3H 1	29.070.338	20.277.861	1,434
	3H 2	22.579.844	18.709.740	1,207
	3H 3	14.382.196	24.533.447	0,586
	3H 5	10.937.004	12.139.447	0,901
	6H 1	14.232.711	21.261.690	0,669
	6H 2	15.349.409	22.665.690	0,677
	6H 3	15.310.660	22.735.740	0,673
	6H 4	10.522.447	19.163.912	0,549
	6H 5	6.631.761	27.341.347	0,243
	9H 1	23.386.024	17.075.912	1,370
	9H 2	40.116.279	22.243.225	1,804
	9H 3	22.294.610	14.404.740	1,548
	9H 4	18.357.903	28.302.640	0,649
	9H 5	4.020.276	11.566.619	0,348

pCREB/α-tubulin - Densitometry

50 uM	SD ratio	1H HFD ratio	3H HFD ratio	6H HFD ratio	9H HFD ratio
Menbrane 1	0,565	0,565	1,434	0,669	1,370
	0,772	0,992	1,207	0,677	1,804
Mean	0,67	0,78	1,32	0,67	1,59
Menbrane 2	0,546	1,079	0,586	0,673	1,548
	0,714	0,255	0,901	0,549	0,649
	0,410306	0,18994		0,242554	0,347576
Mean	0,56	0,51	0,74	0,49	0,85

pCREB/α-tubulin - Densitometry

50 uM	SD % of control	1H HFD % of control	3H HFD % of control	6H HFD % of control	9H HFD % of control
Menbrane 1	84%	85%	214%	100%	205%
	116%	148%	181%	101%	270%
Menbrane 2	98%	194%	105%	121%	278%
	128%	46%	162%	99%	116%
	74%	34%	0%	44%	62%
Mean	**100%**	**117%**	**198%**	**101%**	**237%**
Error	0,06	0,19	0,23	0,08	0,26
DP	0,20	0,61	0,75	0,26	0,85

Supplementary Table S4. Quantitative analysis of western blot presented in Figure 3D.

Figure 3. D -Original western blotting membranes

	Sample	pCreb Average Pixel	α-Tubulin Average Pixel	Ratio
Membrane 1	Crtl 1	11.484.690	15.751.033	0,729
	Crtl 2	14.428.276	17.197.154	0,839
	Crtl 3	23.742.811	31.865.397	0,745
	Crtl 4	15.095.957	37.136.480	0,406
	Crtl 5	11.737.125	25.777.024	0,455
	3DHF1	8.819.619	14.454.861	0,610
	3DHF2	16.758.326	14.186.083	1,181
	3DHF3	34.043.099	31.683.773	1,074
	1WHF1	11.103.690	15.910.033	0,698
	1WHF2	9.085.033	16.117.447	0,564
	1WHF3	37.377.714	39.384.116	0,949
	1WHF4	15.946.489	24.023.560	0,664
	2WHF1	7.744.276	16.223.033	0,477
Membrane 2	2WHF2	11.958.569	20.857.205	0,573
	2WHF3	18.612.912	17.427.740	1,068
	2WHF4	8.924.418	26.164.752	0,341
	2WHF5	15.118.388	28.441.803	0,532
	4WHF1	7.366.740	26.022.397	0,283
	4WHF2	15.238.154	22.711.569	0,671
	4WHF3	20.896.640	21.903.740	0,954
	4WHF4	17.754.803	23.366.803	0,760
	4WHF5	5.852.953	13.821.581	0,423

pCREB/α-tubulin - Densitometry

50 uM	SD ratio	3D HFD ratio	1W HFD ratio	2W HFD ratio	4W HFD ratio
Membrane 1	0,729139	0,610149	0,697905	0,477363	0,283092
	0,838992	1,181322	0,563677	0,573354	0,670942
	0,745097			1,068005	0,954022
Mean	0,771076	0,895735	0,630791	0,706241	0,636019
Menbrane 2	0,406	1,074	0,949	0,341	0,760
	0,455		0,664	0,532	0,423
Mean	0,43	1,07	0,81	0,44	0,59

pCREB/α-tubulin - Densitometry

50 uM	SD % of control	3D HFD % of control	1W HFD % of control	2W HFD % of control	4W HFD % of control
Menbrane 1	95%	79%	91%	62%	37%
	109%	153%	73%	74%	87%
	97%			139%	124%
Menbrane 2	94%	249%	220%	79%	176%
	106%		154%	123%	98%
Mean	**100%**	**96%**	**90%**	**80%**	**104%**
Error	0,11	0,30	0,24	0,14	0,14
DP	0,38	0,95	0,79	0,45	0,46

Supplementary Table S5. Quantitative analysis of western blot presented in Figure 4B.

Figure 4. B -Original western blotting membranes

Sample	pCreb Average Pixel	α-Tubulin Average Pixel	Ratio
SD1	12.538.054	11.535.205	1,08694
SD2	7.420.811	28.224.083	0,26292
SD3	8.602.154	24.351.276	0,35325
SD4	15.229.326	23.071.690	0,66009
SD5	22.502.276	23.965.154	0,93896
Média			0,66043
9H HFD1	4.054.497	17.488.790	0,231834
9H HFD2	9.317.397	21.090.397	0,441784
9H HFD3	11.247.811	21.333.619	0,527234
9H HFD4	4.121.276	20.242.912	0,203591
9H HFD5	4.243.740	16.696.397	0,254171

pCREB/α-tubulin - Densitometry

50 uM	SD	9H HFD
Membrane 1	165%	35%
	40%	67%
	53%	80%
	100%	31%
	142%	38%
Mean	100%	50%

Supplementary Table S6. Quantitative analysis of western blot presented in Figure 4G.

Figure 4. G -Original western blotting membranes						
	pCreb	α-Tubulin		pCREB/α-tubulin - Densitometry		
Sample	Average Pixel	Average Pixel	Ratio	50 uM	SD	4W HFD
SD1	5.789.447	11.668.276	0,49617		108%	143%
SD2	8.344.740	15.123.205	0,55178		120%	273%
SD3	9.259.447	20.320.983	0,45566	Membrane 1	99%	78%
SD4	12.089.811	18.486.912	0,65397		142%	135%
SD5	2.547.740	17.563.205	0,14506		31%	53%
Mean			0,46053	Mean	100%	137%
4W HFD1	9.713.276	14.775.569	0,657388			
4W HFD2	18.058.983	14.352.790	1,258221			
4W HFD3	6.888.740	19.155.497	0,359622			
4W HFD4	10.929.033	17.515.619	0,623959			
4W HFD5	6.626.861	26.917.397	0,246192			

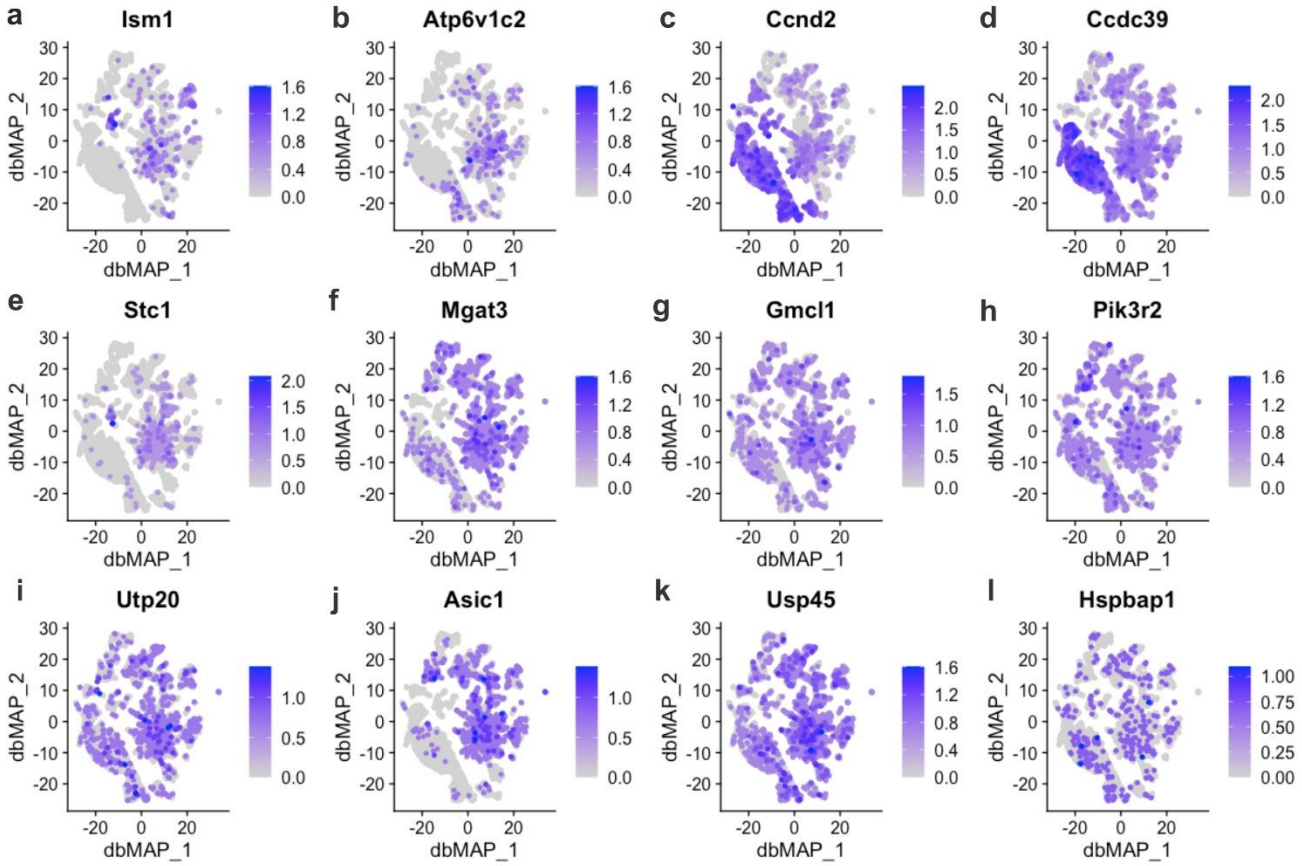
Supplementary Table S7. Quantitative analysis of western blot presented in Figure 5B.

Figure 5. B -Original western blotting membranes							
	pCreb	α-Tubulin		pCREB/α-tubulin - Densitometry			
Sample	Average Pixel	Average Pixel	Ratio	50 uM	Saline	SP 2h	SP 4h
Saline 1	10.323.983	17.085.154	0,604266		91%	108%	89%
Saline 2	7.904.740	10.067.497	0,785174		119%	117%	77%
Saline 3	7.664.983	14.193.154	0,540048	Membrane 1	82%	136%	103%
Saline 4	5.151.468	7.148.033	0,720683		109%	164%	108%
Mean			0,662543			139%	115%
SP 2h 1	11.688.933	16.308.569	0,716736	Mean	100%	133%	99%
SP 2h 2	12.963.397	16.685.154	0,776942				
SP 2h 3	15.475.154	17.118.861	0,903983				
SP 2h 4	15.538.317	14.273.740	1,088595				
SP 2h 5	24.649.731	26.698.154	0,923275				
SP 4h 1	5.501.740	9.281.033	0,592794				
SP 4h 2	8.377.740	16.385.326	0,511295				
SP 4h 3	6.827.175	9.969.326	0,684818				
SP 4h 4	15.253.125	21.294.740	0,716286				
SP 4h 5	22.178.539	29.193.447	0,759709				

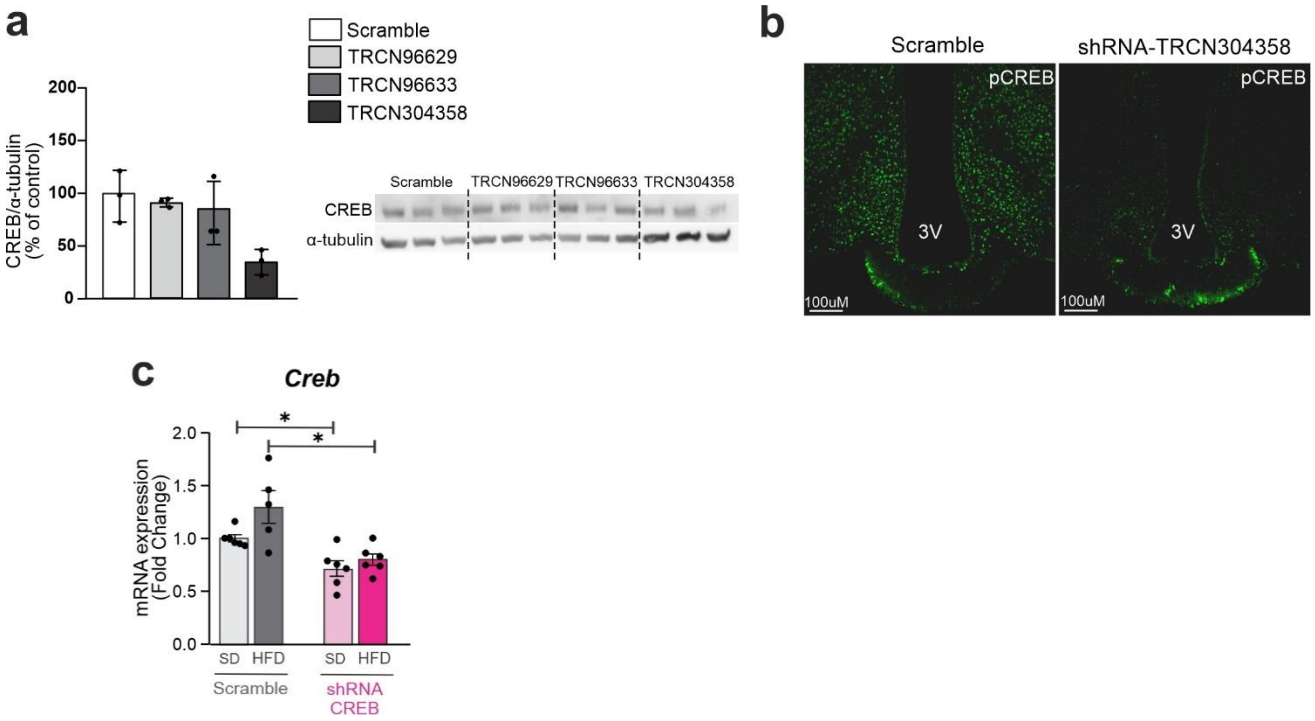
Supplementary Table S8. Quantitative analysis of western blot presented in Supplementary Figure S2a.

Figure Suppl 2. A -Original western blotting membranes								
	pCreb	α-Tubulin			Densitometria (%)			
Sample	Average Pixel	Average Pixel	Ratio		Scramble	shTRCN96629	shTRCN96633	shTRCN304358
Scramble 1	26079861,00	18071861,00	1,44		75%	94%	120%	36%
Scramble 2	26603104,00	13617497,00	1,95		101%	92%	68%	46%
Scramble 3	27338569,00	11379669,00	2,40		124%	86%	68%	22%
Mean			**1,93**	**Mean**	**100%**	**91%**	**85%**	**34%**
shTRCN96629	25775033,00	14217912,00	1,81					
shTRCN96629	20176740,00	11373426,00	1,77					
shTRCN96629	**17621326,00**	10547033,00	1,67					
shTRCN96633	25186154,00	10882912,00	2,31					
shTRCN96633	16094619,00	12295376,00	1,31					
shTRCN96633	21521861,00	16414255,00	1,31					
shTRCN304358	17215447,00	24807225,00	0,69					
shTRCN304358	22145518,00	25029690,00	0,88					
shTRCN304358	9652447,00	22946154,00	0,42					

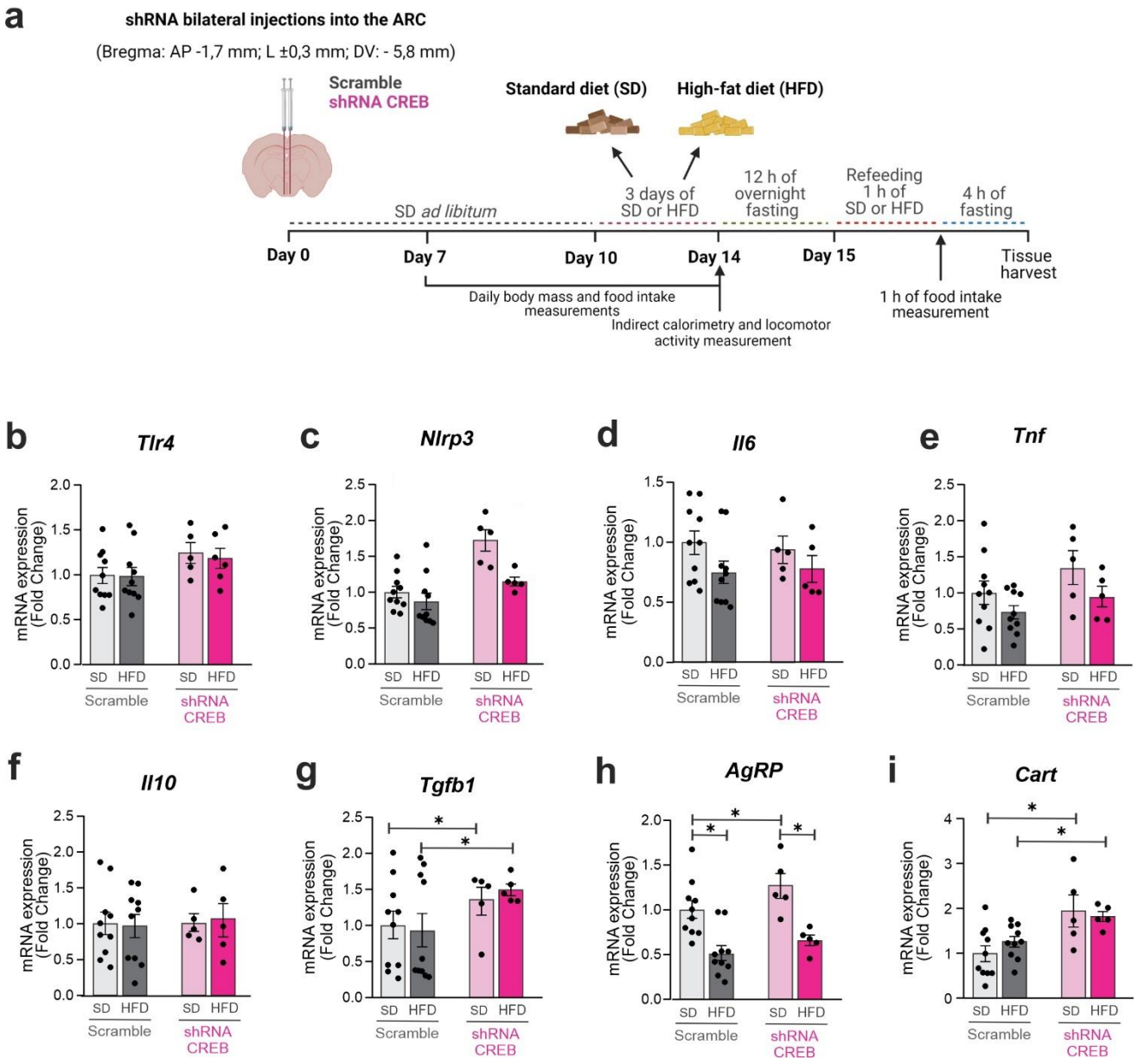
1 Supplementary Figures



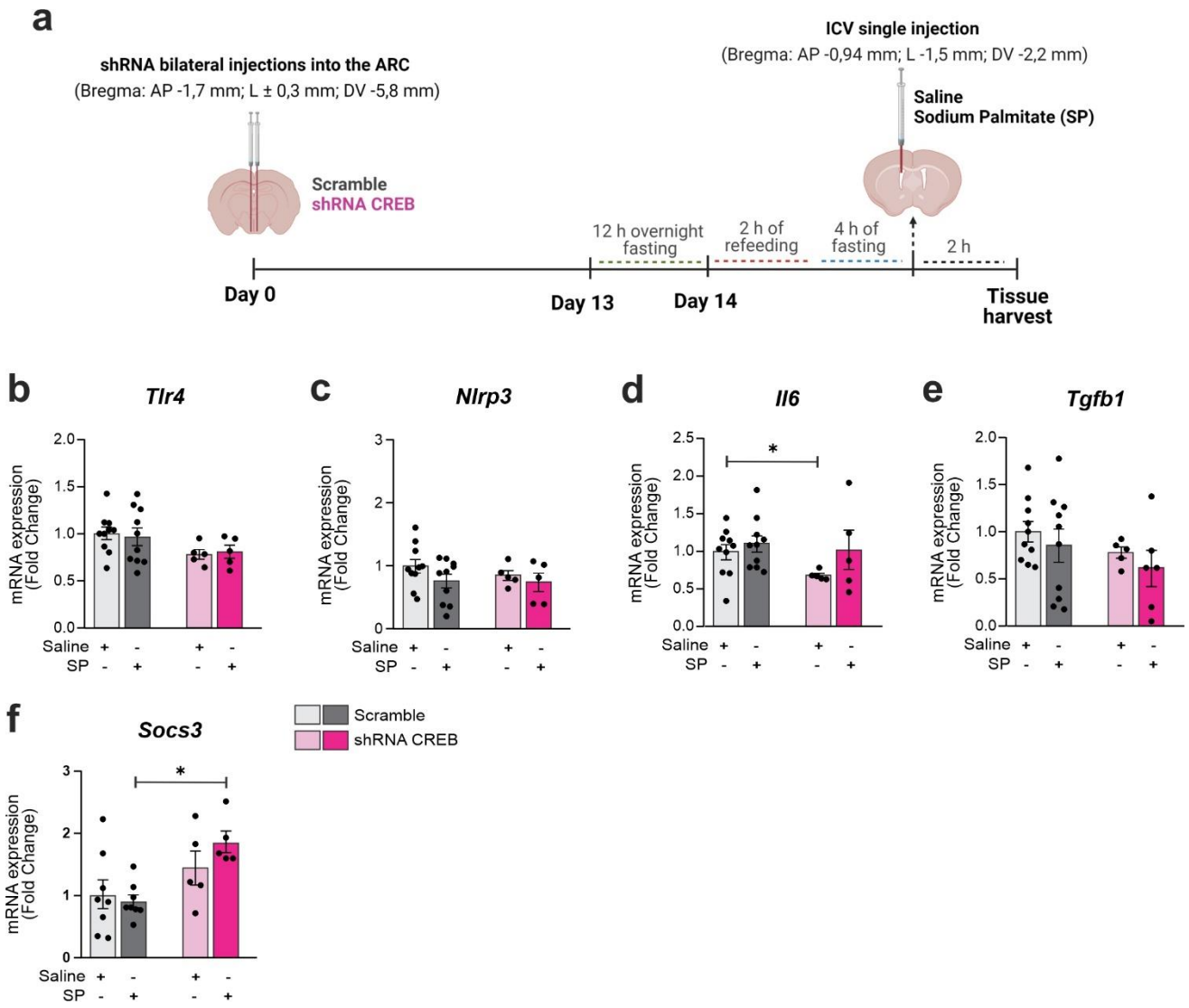
Supplementary Figure S1. Major Creb1 transcriptional targets in the Arc-ME. Gene expression of **(a)** *Ism1*; **(b)** *Atp6v1c2*; **(c)** *Ccnd2*; **(d)** *Ccdc39*; **(e)** *Stc1*; **(f)** *Mgat3*; **(g)** *Gmcl1*; **(h)** *Pik3r2*; **(i)** *Utp20*; **(j)** *Asic1*; **(k)** *Usp45* and **(l)** *Hspbap1*, the 12 major Creb1 transcriptional targets with higher relative importance (excluding unclassified/predicted genes).



Supplementary Figure S2. Characterization of hypothalamic CREB knockdown. Adult male C57BL/6J mice were submitted to bilateral injection of lentivirus particles (Scramble and shRNA CREB) into the arcuate nucleus (ARC). Three distinct lentiviruses were tested for their efficiency to inhibit the expression of hypothalamic CREB. **(a)** Western blot of hypothalamic samples obtained 14 days after CREB down-regulation with different lentiviral-shRNA sequences; **(b)** Coronal brain section of mediobasal hypothalamus 14 days after the bilateral injection of shRNA CREB (TRCN304358) and Scramble particles into the ARC were immunostained with pCREB; the confocal microscope acquisition settings were kept the same for both groups. Hypothalamic mRNA expression of Creb in different conditions; **(c)** Adult male C57BL/6J mice were submitted a bilateral injection of lentivirus particles (Scramble or shRNA CREB) into the arcuate nucleus (ARC) and, after 10 days, were fed on SD or HFD for 3 days. Data are presented as means ± SEM. Two-way ANOVA followed by Sidak's post hoc test was used for statistical analyses. *p≤0,05 in comparison with groups indicated in the figure.



Supplementary Figure S3. Hypothalamic CREB down-regulation slightly modulates inflammatory markers and neuropeptides. Adult male C57BL/6J mice were submitted to bilateral injection of lentivirus particles (Scramble and shRNA CREB) into the arcuate nucleus (ARC) and, after 10 days, were fed on SD or HFD for 3 days. **(a)** Schematic representation of experimental protocol; mRNA gene expression of inflammatory **(b-g)** and neuropeptides **(h-i)**. Data are presented as means ± SEM. N= 5 - 15 mice in two different cohorts. Two-way ANOVA followed by Sidak's post hoc test was used for statistical analyses. *p≤0,05 in comparison with groups indicated in the figure.



Supplementary Figure S4: Hypothalamic CREB knockdown does not modulate inflammatory markers upon palmitic acid stimulus. Adult male C57BL/6J mice were submitted a bilateral injection of lentivirus particles (Scramble or shRNA CREB) into the arcuate nucleus (ARC) and, after 14 days, were ICV injected with saline or sodium palmitate (SP) 30 µM. **(a)** Schematic representation of experimental protocol, hypothalami were harvested 2 hours after injection. **(b-f)** mRNA inflammatory markers. Data are presented as means ± SEM. N= 5-15 mice in two different cohorts. Two-way ANOVA followed by Sidak's post hoc test was used for statistical analyses. *p≤0,05 in comparison with groups indicated in the figure.