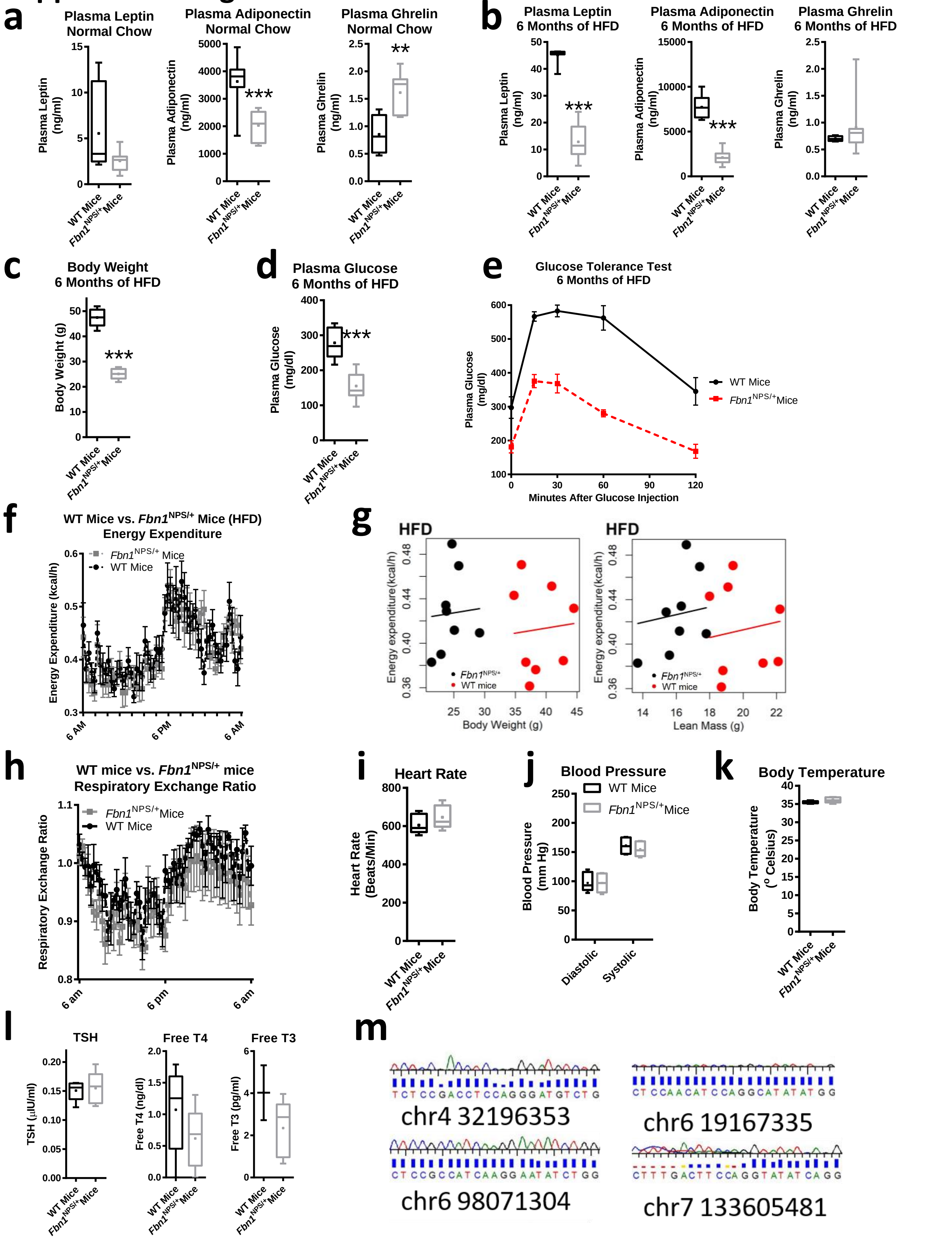
Asprosin is a centrally-acting orexigenic hormone

Supplemental figures

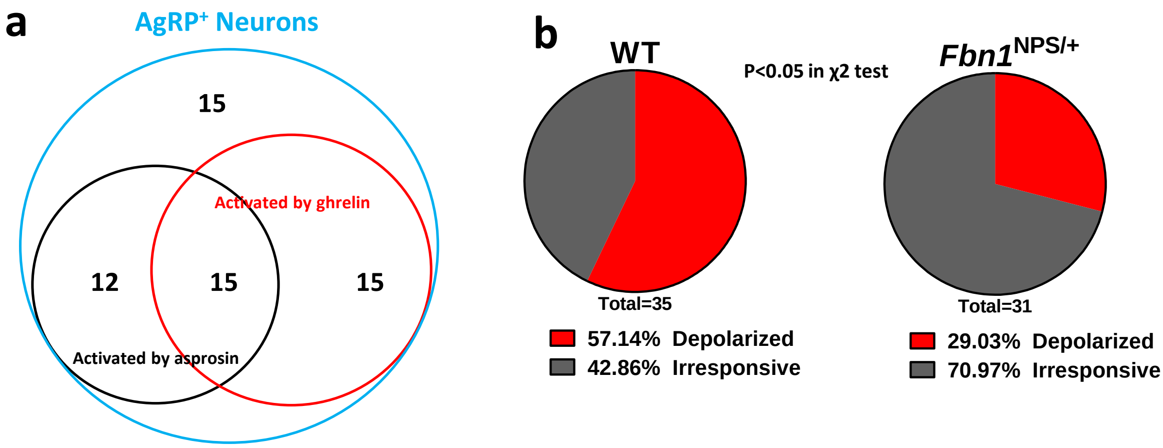
Supplemental figure legends

Supplemental tables

Supplemental Fig. 1



Supplemental Fig. 2



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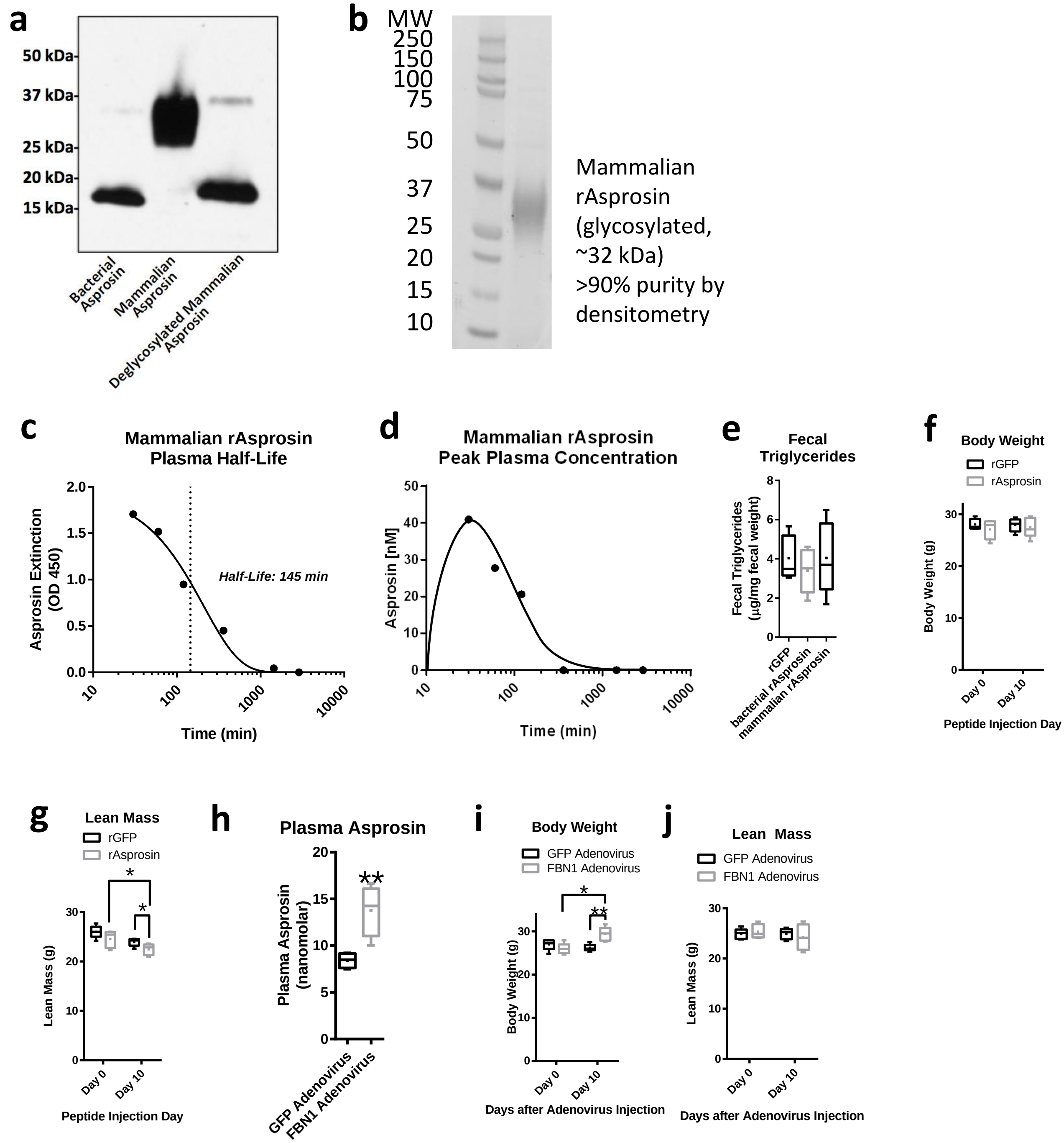

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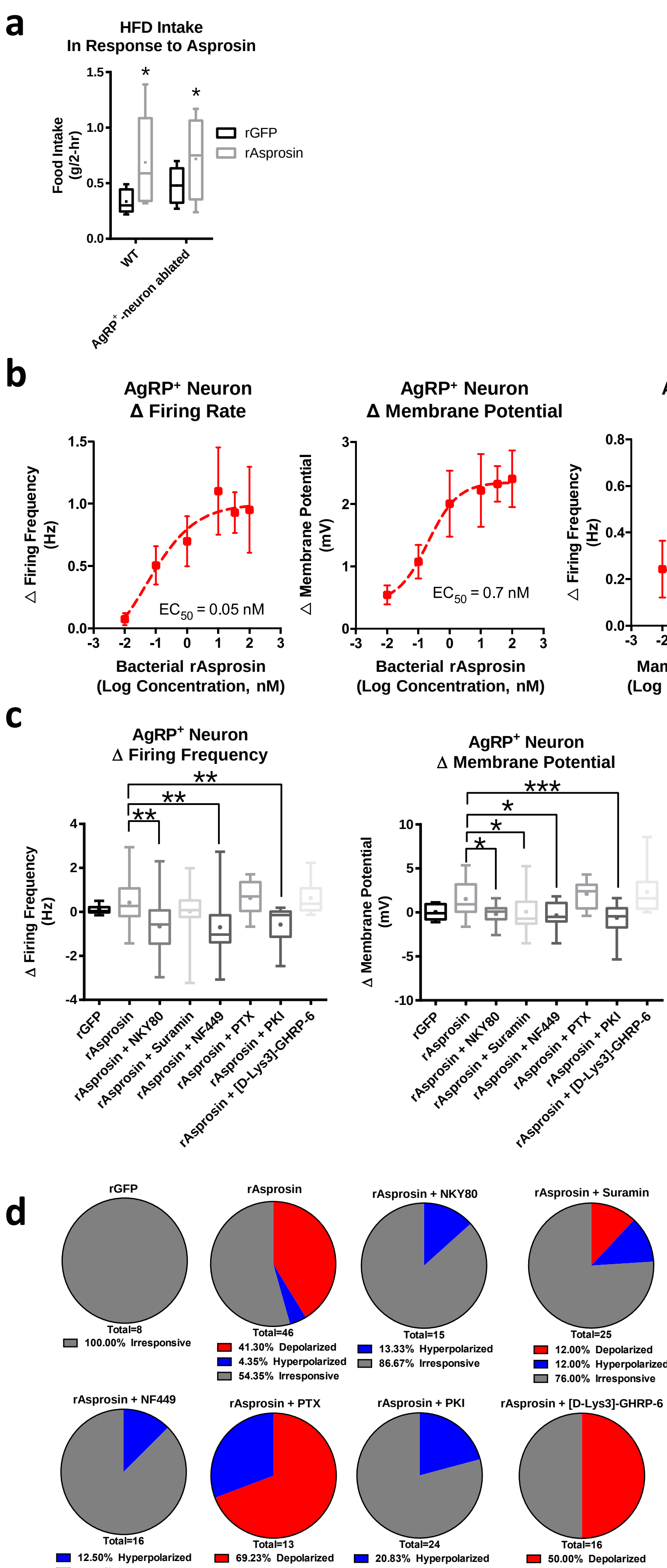

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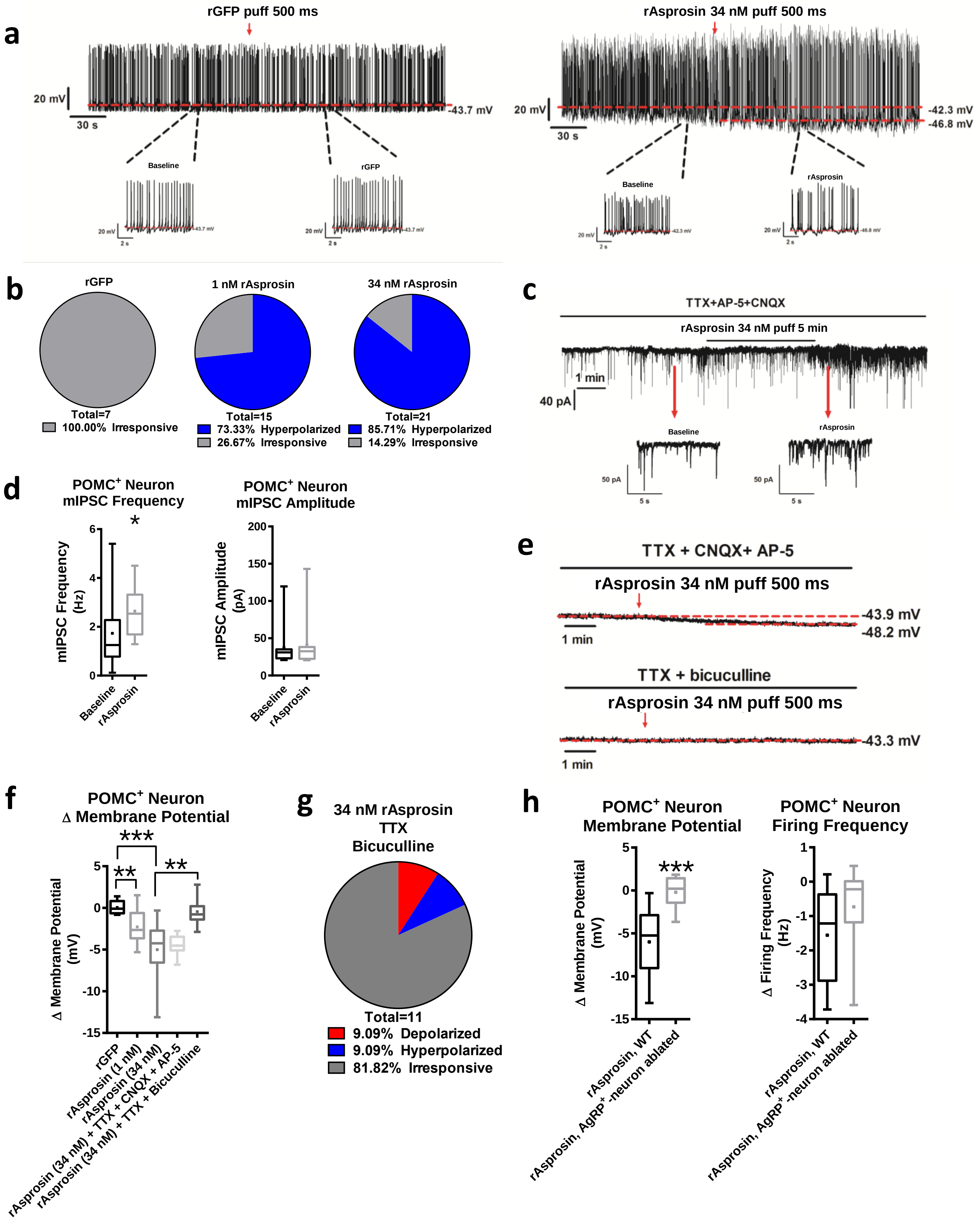

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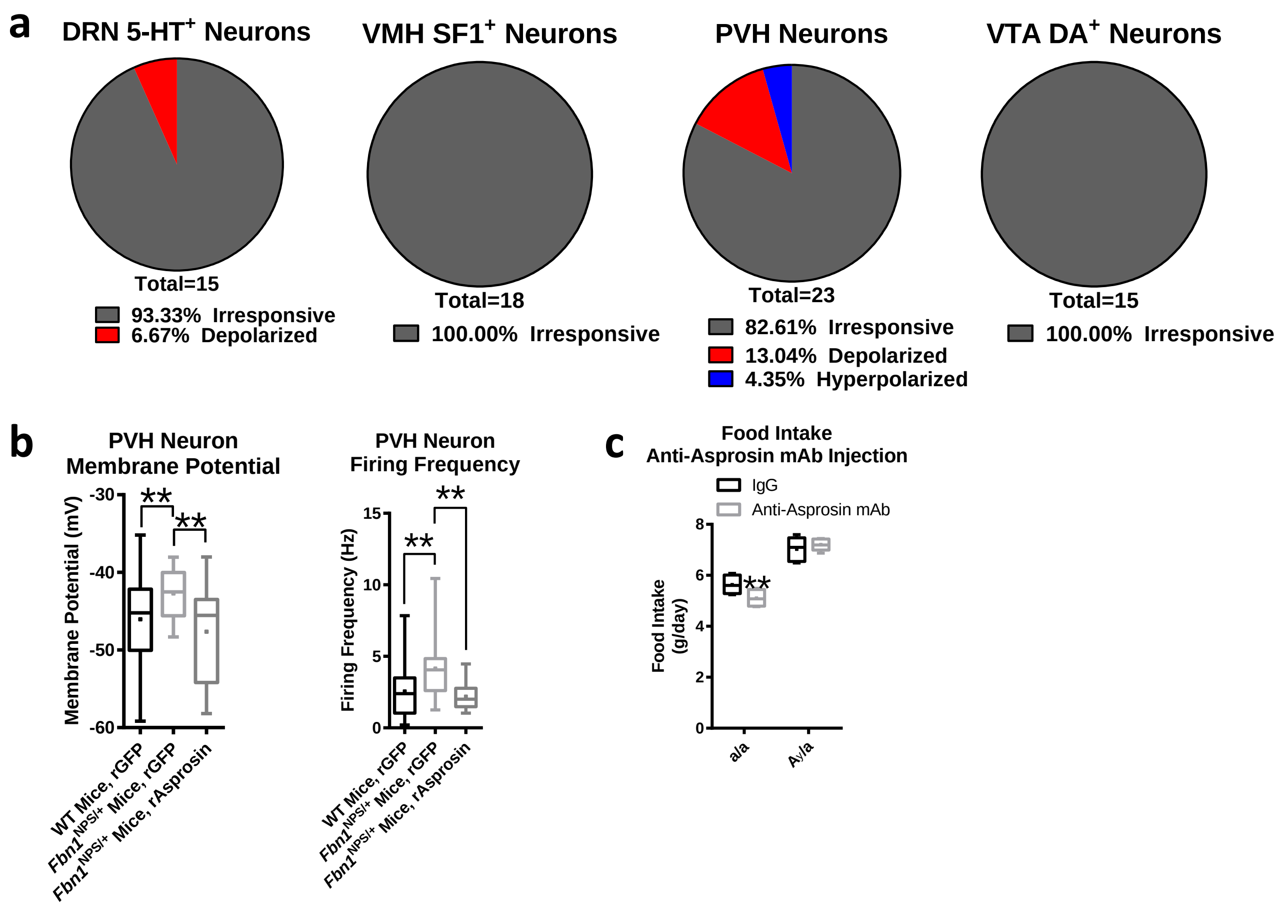

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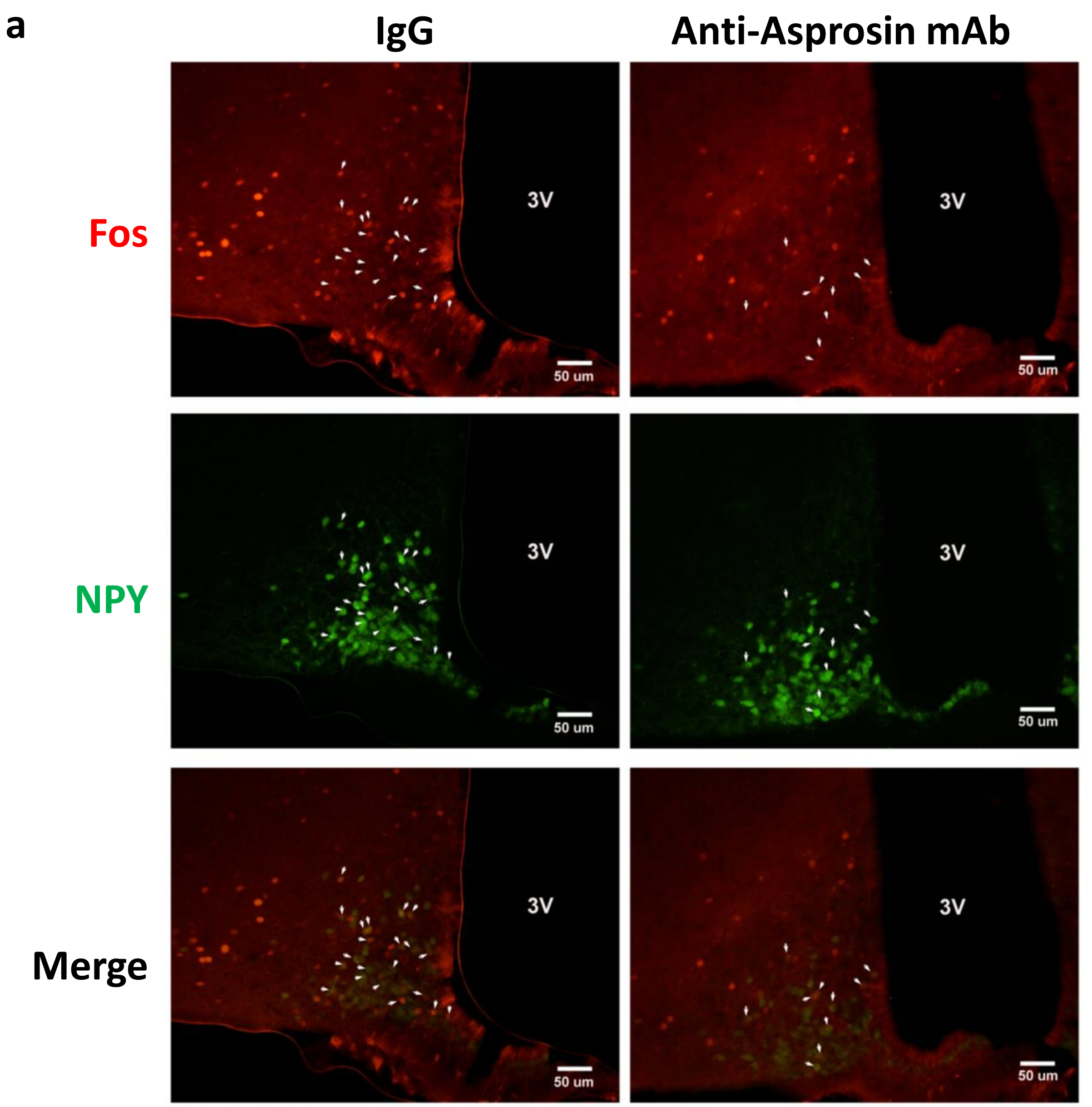
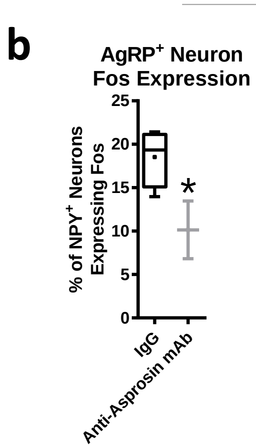
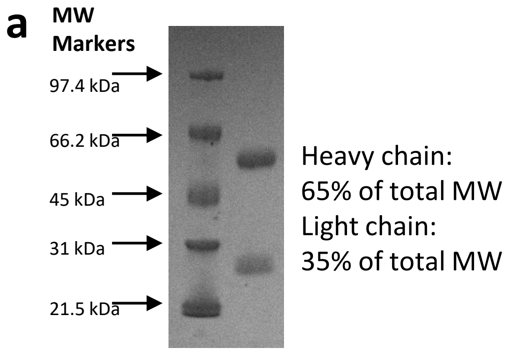




Supplemental Fig. 8



b

STNET[...]TYSLQISSTPLYKKKELNQLEDKYDKDYLSGELGDNLKMKIQVLLH – COOH		
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	KELNQLED	922.5
	LNQLEDKY	63.4
	QLEDKYDK	95.2
	EDKYDKDY	4.5
	KYDKDYLS	44.2
	DKDYLSGE	929.6
	DYLSGELG	14,094.4
	LSGELGDN	111.9
	GELGDNLK	39.4
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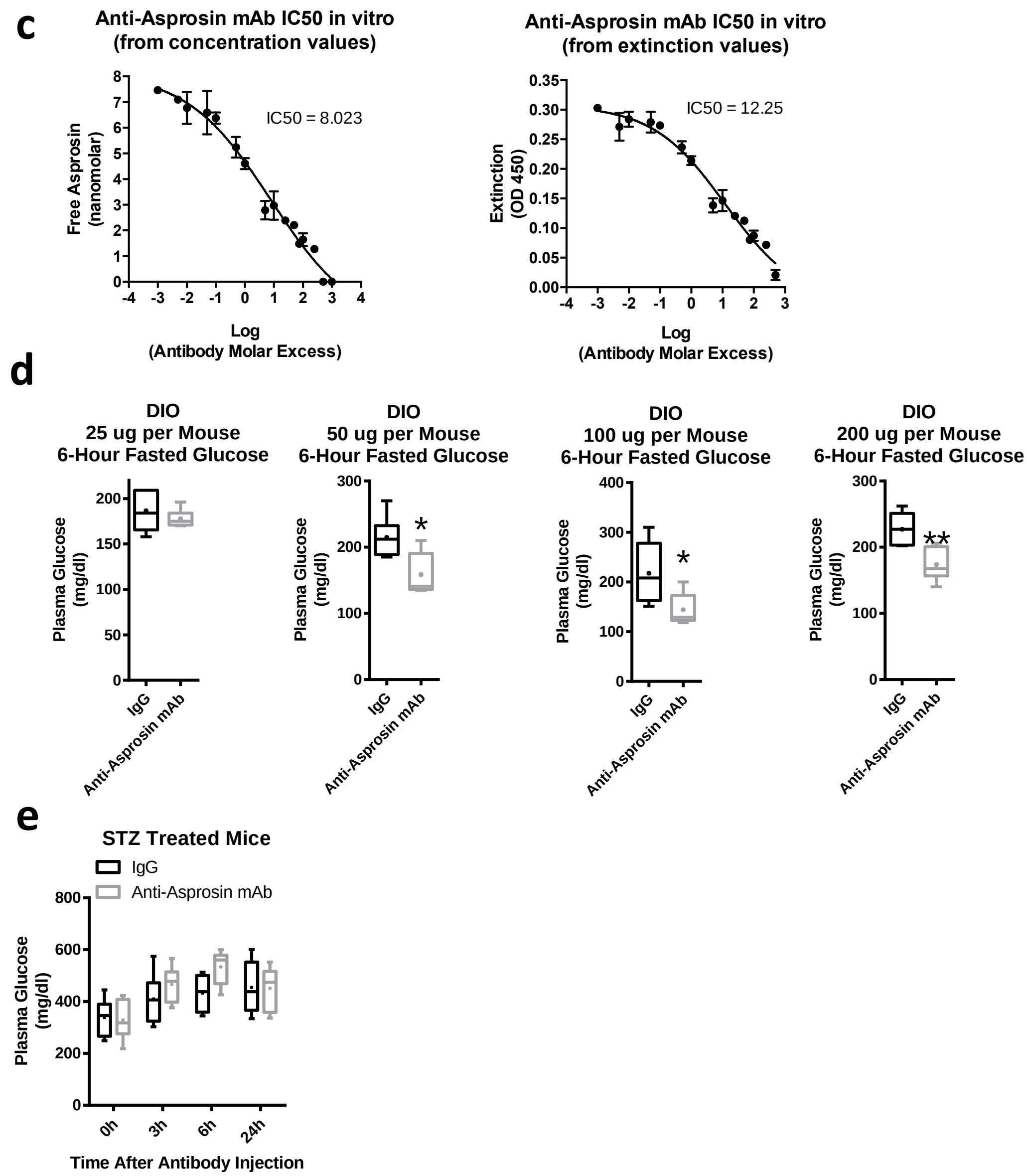
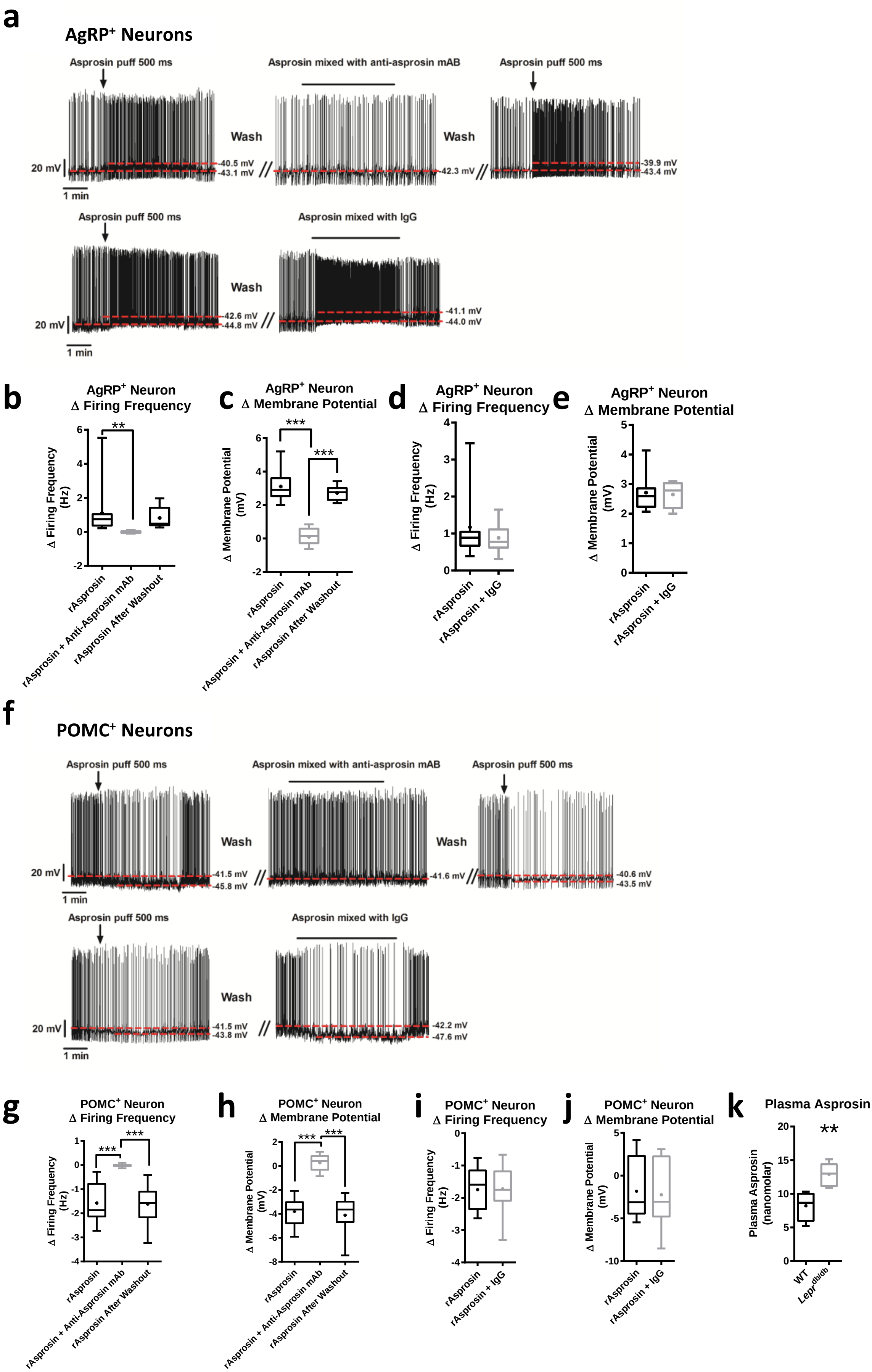


Supplemental Fig. 9



Supplemental figure legends

Supplemental Fig. 1 *Fbn1*$^{NPS/+}$ mice mimic human NPS and are protected from diet-induced obesity and diabetes (**a**) Plasma ELISA for leptin, adiponectin, and ghrelin in WT and *Fbn1*$^{NPS/+}$ mice on normal chow (n = 6, P = 0.06). (**b**) Plasma ELISA for leptin, adiponectin, and ghrelin in WT and *Fbn1*$^{NPS/+}$ mice after 6 months on high-fat diet (n = 6). (**c**) Body weight of WT and *Fbn1*$^{NPS/+}$ mice after 6 months on high-fat diet (n = 6). (**d**) Plasma glucose in WT and *Fbn1*$^{NPS/+}$ mice after 6 months on high-fat diet (n = 6). (**e**) Glucose tolerance test in WT and *Fbn1*$^{NPS/+}$ mice after 6 months of high-fat diet (n = 6, P = 0.0001). (**f**) Energy expenditure over 24 hr in WT and *Fbn1*$^{NPS/+}$ mice on HFD for 3 months (P = 0.74). (**g**) Analysis of energy expenditure of *Fbn1*$^{NPS/+}$ mice and WT littermates on HFD for 3 months by ANCOVA (n = 5 per group, body weight P = 0.633 and lean mass P = 0.437). (**h**) Respiratory Exchange Ratio over 24 hr in WT and *Fbn1*$^{NPS/+}$ mice (n = 5 per group, P = 0.39). (**i**) Heart rate, blood pressure (**j**) and body temperature (**k**) of WT and *Fbn1*$^{NPS/+}$ mice (n = 5 for NPS, n = 7 for WT). (**l**) Plasma ELISA for TSH, free T4, and free T3 in WT and *Fbn1*$^{NPS/+}$ mice (n = 5 per group). (**m**) Representative sequence traces of *Fbn1*$^{NPS/+}$ mice. **P < 0.01, and ***P < 0.001. Statistical tests used: two-tailed *t*-test (**a-d,i-m**) and two-way ANOVA (**e,f,h**).

Supplemental Fig. 2 Response of feeding-related neuron populations to asprosin (**a**) VENN-diagram of a set of AgRP^{+} neurons tested and response to asprosin and ghrelin (rAsprosin: 34 nM; Ghrelin: 300 nM). (**b**) Ghrelin-mediated activation of AgRP^{+} neurons from *Fbn1*$^{NPS/+}$ mice and WT mice (rAsprosin: 34 nM; Ghrelin: 300 nM, $p < 0.05$ in χ^2test).

Supplemental Fig. 3 Mammalian asprosin is glycosylated and has a plasma half-life of approximately 145 minutes. (**a**) Immunoblot for mammalian-expressed rAsprosin (lane 2). The same sample was enzymatically deglycosylated and loaded in lane 3. Bacterially expressed rAsprosin control in lane 1. (**b**) Coomassie Blue stained SDS PAGE gel of mammalian rAsprosin (glycosylated, ~32 kDa). (**c**) Plasma half-life of mammalian rAsprosin as determined by ELISA after subcutaneous injection of 60 µg mammalian rAsprosin. (**d**) Peak plasma asprosin concentration as determined by ELISA after subcutaneous injection of 60 µg of mammalian rAsprosin. (**e**) Fecal triglyceride content in mice during 24 hr following injection with GFP, bacterial recombinant asprosin, or mammalian recombinant asprosin (60 µg/mouse rGFP, 30 µg/mouse bacterial rAsprosin, and 60 µg/mouse mammalian rAsprosin, $n = 5$ per group). (**f**) Body weight of WT mice before and after 10 days of daily subcutaneous injection with 30 µg rGFP or bacterial rAsprosin ($n = 5$). (**g**) Lean mass of WT mice before and after 10 days of daily subcutaneous injection with 30 µg rGFP or bacterial rAsprosin, as determined by MRI ($n = 5$). (**h**) Plasma ELISA for Asprosin in mice with adenovirus-mediated overexpression of GFP or *FBN1* in the liver ($n = 5$). (**i**) Body weight of WT mice before and 10 days after injection of a GFP or *FBN1* adenovirus ($n = 5$). (**j**) Lean mass of WT mice before and 10 days after injection of a GFP or *FBN1* adenovirus, as determined by MRI ($n = 5$). *$P < 0.05$, and **$P < 0.01$. Statistical tests used: two-tailed *t*-test (**h**), one-way ANOVA (**e**), and two-way ANOVA (**f,g,i,j**).

Supplemental Fig. 4 Asprosin employs the Gα_s-cAMP-PKA pathway to activate AgRP$^+$ neurons in a dose-responsive manner (**a**) Cumulative food intake on HFD during 2 hr after IP injection of GFP or bacterial recombinant asprosin in WT control or AgRP$^+$ ablated mice (30 μg/mouse, *n* = 6 per group, *P* = 0.02). (**b**) AgRP$^+$ neuron firing frequency and membrane potential changes in response to increasing concentrations of recombinant asprosin produced bacterially or in mammalian cells (Firing frequency: bacterial rAsprosin 0.01 nM *n* = 9, 0.1 nM *n* = 11, 1 nM *n* = 8, 10 nM *n* = 12, 34 nM *n* = 24, 100 nM *n* = 14; mammalian rAsprosin: 0.01 nM *n* = 12, 0.1 nM *n* = 15, 1 nM *n* = 15, 10 nM *n* = 14, 34 nM *n* = 15, 100 nM *n* = 15. Membrane potential: bacterial rAsprosin 0.01 nM *n* = 13, 0.1 nM *n* = 11, 1 nM *n* = 13, 10 nM *n* = 10, 34 nM *n* = 33, 100 nM *n* = 15; mammalian rAsprosin: 0.01 nM *n* = 13, 0.1 nM *n* = 16, 1 nM *n* = 15, 10 nM *n* = 15, 34 nM *n* = 16, 100 nM *n* = 15). (**c**) Changes of AP firing frequency and membrane potential in AgRP$^+$ neurons after treatment with rGFP, bacterial rAsprosin, and in the presence of different inhibitors (Firing frequency: rGFP *n* = 8, rAsprosin *n* = 39, rAsprosin + 100 μM NKY80 *n* = 15, rAsprosin + 50 μM suramin *n* = 16, rAsprosin + 20 μM NF449 *n* = 16, rAsprosin + 50 μM PTX *n* = 12, rAsprosin + 1 μM PKI *n* = 23, rAsprosin + 100 μM [D-Lys3]-GHRP-6 *n* = 13. Membrane potential: rGFP *n* = 8, rAsprosin *n* = 44, rAsprosin + 100μM NKY80 *n* = 15, rAsprosin + 50 μM suramin *n* = 25, rAsprosin + 20 μM NF449 *n* = 16, rAsprosin + 50 μM PTX *n* = 13, rAsprosin + 1 μM PKI *n* = 24, rAsprosin + 100 μM [D-Lys3]-GHRP-6 *n* = 16). (**d**) Response ratio of AgRP$^+$ neurons after treatment with rGFP, bacterial rAsprosin, and in the presence of different inhibitors (rGFP *n* = 8, rAsprosin *n* = 46, rAsprosin + 100μM NKY80 *n* = 15, rAsprosin + 50 μM suramin *n* = 25, rAsprosin + 20 μM NF449 *n* = 16, rAsprosin + 50 μM PTX *n* = 13, rAsprosin + 1 μM PKI *n* = 24, rAsprosin + 100 μM [D-Lys3]-GHRP-6 *n* = 16). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Statistical tests used: one-way ANOVA (**c**), and two-way ANOVA (**a**).

Supplemental Fig. 5 Asprosin inhibits anorexigenic $POMC^{+}$ neurons indirectly through a GABAergic mechanism (**a**) Representative AP firing traces of $POMC^{+}$ neurons after GFP and bacterial recombinant asprosin treatment. (**b**) Response ratio of $POMC^{+}$ neurons after GFP, 1nM and 34nM bacterial recombinant asprosin treatment (rGFP *n* = 7, 1 nM rAsprosin *n* = 15, 34 nM rAsprosin *n* = 21). (**c**) Representative miniature inhibitory postsynaptic current (mIPSC) trace of $POMC^{+}$ neurons before and after bacterial recombinant asprosin treatment in the presence of inhibitors (AP-5: 30 μM, CNQX: 30 μM and TTX: 1 μM). (**d**) mIPSC frequency and amplitude in $POMC^{+}$ neurons before and after bacterial recombinant asprosin treatment (*n* = 10 per group). (**e**) Representative AP firing traces of $POMC^{+}$ neurons before and after bacterial recombinant asprosin in the presence of various inhibitors. (**f**) Amplitude changes of $POMC^{+}$ membrane potential afterGFP or bacterial recombinant asprosin treatment only, and in the presence of different inhibitors (AP-5: 30 μM, CNQX: 30 μM and TTX: 1 μM; TTX: 1 μM and bicuculline: 50 μM; rGFP *n* = 7, 1 nM rAsprosin *n* = 15, 34 nM *n* = 21, 34 nM + TTX + CNQX + AP5 *n* = 12, 34 nM + TTX + bicuculline *n* = 10). (**g**) Response ratio of $POMC^{+}$ neurons after 34 nM bacterial recombinant asprosin treatment in the presence of TTX + bicuculline. (**h**) Amplitude changes of $POMC^{+}$ neuron membrane potential and firing frequency in WT and $AgRP^{+}$ neuron ablated mice after treatment with bacterial recombinant asprosin (rAsprosin: 34 nM; firing frequency: rAsprosin, WT *n* = 8;

rAsprosin, AgRP$^+$-ablated *n* = 17. Membrane potential: rAsprosin, WT *n* = 6; rAsprosin, AgRP$^+$-ablated *n* = 23). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Statistical tests used: two-tailed *t*-test (**d,h**) and one-way ANOVA (**f**).

Supplemental Fig. 6 Asprosin acts via AgRP$^+$ neurons and requires intact melanocortin signaling (**a**) Response rates of 5-HT$^+$ neurons in the dorsal raphe nuclei (DRN), SF1$^+$ neurons in the ventromedial hypothalamic nucleus (VMH), neurons in the paraventricular nucleus of the hypothalamus (PVH), and dopamine (DA$^+$) neurons in the ventral tegmental area (VTA; rAsprosin: 34 nM). (**b**) Membrane potential and firing frequency of PVH neurons from *Fbn1*$^{NPS/+}$ mice after ICV injection with 0.5 μg GFP or Asprosin. WT mice with GFP injection served as controls (rAsprosin: 0.5 μg; rGFP: 0.5 μg). (**c**) Cumulative food intake during 24 hr after IP injection of 250 μg IgG or anti-asprosin mAb in WT control (a/a) or Agouti yellow (A^y/a) mice (KK background, *n* = 5 per group). ***P* < 0.01. Statistical tests used: one-way ANOVA (**b**), and two-way ANOVA (**c**).

Supplemental Fig. 7 Asprosin immunologic neutralization reduces Fos expression in AgRP$^+$ neurons (**a**) Immunofluorescence for Fos (top), NPY-GFP (center), and a merged composite (bottom) in the arcuate nucleus of mice receiving either IgG control, or anti-asprosin antibody followed by an overnight fast (100 μg/250 μl/mouse; scale bar indicates 50 μm). (**b**) Count of double positive neurons from **a** (IgG *n* = 4 total images, anti-asprosin mAb *n* = 2 total images). **P* < 0.05. Statistical tests used: two-tailed *t*-test (**b**).

Supplemental Fig. 8 Characterization of the anti-asprosin neutralizing antibody (**a**) SDS Page gel of the anti-asprosin mAb showing heavy chain (top band) and light chain (bottom band). Percentage of contribution to total molecular weight by heavy chain and light chain, respectively, was calculated by densitometry. (**b**) Epitope mapping for the asprosin epitope detected by the anti-asprosin mAb. The binding epitope is highlighted in red. (**c**) Elisa against recombinant asprosin pre-incubated with various concentrations of anti-asprosin mAb. 50% inhibitory concentration (IC_{50}) based on concentration values was 8.023 nM, based on absolute extinction values 12.25 nM. (**d**) Plasma glucose in mice with diet-induced obesity after a single injection of various concentrations of anti-asprosin mAb (n = 6 per group). (**e**) Plasma glucose in mice with chemical ablation of pancreatic β-cells by (Streptozotocin – STZ – treatment) in response to IgG or anti-asprosin mAb (n = 6). *$P < 0.05$, and **$P < 0.01$. Statistical tests used: two-tailed *t*-test (**d**), and two-way ANOVA (**e**).

Supplemental Fig. 9 The anti-asprosin neutralizing antibody reversibly inhibits asprosin's effect on $AgRP^+$ and $POMC^+$ neurons (**a**) Representative traces of $AgRP^+$ neurons in response to a bacterial recombinant asprosin puff, followed by asprosin preincubated with 100-fold excess anti-asprosin mAb, followed by asprosin, or the same preincubated with IgG control antibody (rAsprosin: 34 nM). (**b**) Firing frequency response of $AgRP^+$ neurons to bacterial recombinant asprosin, asprosin preincubated with anti-asprosin mAb, and response to asprosin after washout (rAsprosin n = 14, rAsprosin + mAb n = 14, washout n = 8; rAsprosin: 34 nM). (**c**) Membrane potential response of

AgRP$^+$ neurons to bacterial recombinant asprosin, asprosin preincubated with anti asprosin mAb, and response to asprosin after washout (rAsprosin n = 14, rAsprosin + mAb n = 14, washout n = 8; rAsprosin: 34 nM). (**d**) Firing frequency response of AgRP$^+$ neurons to bacterial recombinant asprosin and IgG control antibody (rAsprosin n = 7, rAsprosin + IgG n = 7; rAsprosin: 34 nM). (**e**) Membrane potential response of AgRP$^+$ neurons to bacterial recombinant asprosin and IgG control antibody (rAsprosin n = 7, rAsprosin + IgG n = 7; rAsprosin: 34 nM). (**f**) Representative traces of POMC$^+$ neurons in response to a bacterial recombinant asprosin puff, followed by asprosin preincubated with 100-fold excess anti-asprosin mAb, followed by asprosin, or the same preincubated with IgG control antibody (rAsprosin: 34 nM). (**g**) Firing frequency response of POMC$^+$ neurons to bacterial recombinant asprosin, asprosin preincubated with anti-asprosin mAb, and response to asprosin after washout (rAsprosin n = 24, rAsprosin + mAb n = 24, washout n = 24; rAsprosin: 34 nM). (**h**) Membrane potential response of POMC$^+$ neurons to bacterial recombinant asprosin, asprosin preincubated with anti-asprosin mAb, and response to asprosin after washout (rAsprosin n = 20, rAsprosin + mAb n = 20, washout n = 20; rAsprosin: 34 nM). (**i**) Firing frequency response of POMC$^+$ neurons to bacterial recombinant asprosin and IgG control antibody (rAsprosin n = 15, rAsprosin + IgG n = 15; rAsprosin: 34 nM; IgG: 500 μg/mL). (**j**) Membrane potential response of POMC$^+$ neurons to bacterial recombinant asprosin and IgG control antibody (rAsprosin n = 31, rAsprosin + IgG n = 31; rAsprosin: 34 nM; IgG: 500 μg/mL). (**k**) Elisa for plasma asprosin in 8-week-old male WT and *Lepr*$^{db/db}$ mice (WT n = 5; *Lepr*$^{db/db}$ n = 6). $^{**}P < 0.01$, and $^{***}P < 0.001$. Statistical tests used: two-tailed t-test (**d,e,i-k**), and one-way ANOVA (**b,c,g,h**).

Supplemental tables

Supplemental Table 1

a Anthropometric measurements

	Body Weight	Height	BMI	Age
Unit	*kg*	*cm*	*kg/m2*	*years*
NPS1	24.8	158.4	9.9	25
NPS2	41	177.6	13	18.7
reference value	*73.4±0.85**	*162.9±0.30**	*18.5-24.9†*	

b Body composition

	Total Body Potassium (TBK)				Dual-energy X-ray absorptiometry (DXA)				Air displacement plethysmography (BodPod)		Lohman-4C		
	TBK	FFM	FM	%FM	BMC	FFM	FM	%FM	Body Volume	O18 dilutlion TBW	FFM	FM	%FM
Unit	*g*	*kg*	*kg*	*%*	*kg*	*kg*	*kg*	*%*	*l*	*kg*	*kg*	*kg*	*%*
NPS1	52.1	21.6	3.12	12.6	1.558	19.1	5.75	32.2	22.773	12.74	19.72	5.04	20.3
NPS2	74.8	30.6	10.21	24.9	1.98	31.59	9.33	22.8	38.573	20.02	29.72	11.05	27.1

FFM: Fat Free Mass, FM: Fat Mass, BMC: Bone Mineral Content, TBW: Total Body Water

c Total body water

	TBW-2H	TBW-18O	Turnover rate kH	Turnover rate kO	lean body mass	body fat	body fat	VCO2
Unit	*%WT*	*%WT*	*d-1*	*d-1*	*kg*	*kg*	*%*	*mol/d*
NPS1	53.8	51.9	-0.12	-0.16	17.27	7.53	30.4	10.7
NPS2	50.92	49.32	-0.1	-0.13	27.7	13.3	32.44	13.55

d Energy expenditure by doubly labeled water method

Unit	*kcal/kg BW*	*kcal/kg FFM*	*BMR (kcal/d)*	*PAL (TEE/BMR)*
NPS1	54.8	68.9	942	1.44
NPS2	42	57.9	1212	1.42

PAL: Physical Activity Level, TEE: Total Energy Expenditure, BMR: Basal Metabolic Rate

e Energy expenditure by indirect calorimetry

	BMR measured	Predicted BMR by height/weight (Schofield)	Measured BMR vs predicted (Schofield)		Predicted BMR by weight only (Schofield)	Measured BMR vs predicted (Schofield)		PAL (TEE/BMR)
Unit	*kcal/d*	*kcal/d*	*kcal/d*		*kcal/d*	*kcal/d*		
NPS1	942	884	57	6.5%	854	88	9.3%	1.16
NPS2	1212	1159	53	4.6%	1094	118	9.7%	1.45

TEE: Total Energy Expenditure, BMR: Basal Metabolic Rate, PAL: Physical Activity Level

f

	24 hr EE	24 hr EE	24 hr RQ	24 hr HR	24 hr activity	Sleep EE	Sleep EE	Sleep RQ	Sleep HR	Sleep activity	BMR	BMR	BMR RQ	BMR HR	BMR activity
Unit	*kcal/min*	*kcal/kg*		*BPM*	*CPM*	*kcal/min*	*kcal/kg*		*BPM*	*CPM*	*kcal/min*	*kcal/kg*		*BPM*	*CPM*
NPS1	0.76	46.9	0.85	101	66	0.56	32.3	0.88	86	86	0.65	38	0.8	89	1
NPS2	1.22	42.9	0.87	105	102	0.91	31.9	0.82	99	99	0.84	29.6	0.74	96	17

EE: Energy Expenditure, RQ: Respiratory Quotient, CPM: Counts per Minute, BMR: Basal Metabolic Rate

g

	Walk 2.5 mph	Walk 2.5 mph	Walk 2.5 mph RQ	Walk 2.5 mph HR	Walk 2.5 mph activity	Walk 3 mph	Walk 3 mph	Walk 3 mph RQ	Walk 3 mph HR	Walk 3 mph activity	Free Time	Free Time	Free Time RQ	Free Time HR	Free Time activity
Unit	*kcal/min*	*kcal/kg*		*BPM*	*CPM*	*kcal/min*	*kcal/kg*		*BPM*	*CPM*	*kcal/ min*	*kcal/ kg*		*BPM*	*CPM*
NPS1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.86	50.1	0.83	112	111
NPS2	3.63	127.4	0.92	144	1168	4.39	154.3	0.95	167	1270	1.29	45.4	0.89	108	120

EE: Energy Expenditure, RQ: Respiratory Quotient, CPM: Counts per Minute, BMR: Basal Metabolic Rate

Supplemental Table 1 Anthropometric measurements, body composition and energy expenditure of two individuals with NPS

(**a**) Anthropometric measurements: Body weight, height, and computed BMI.*[30]; †Normal values calculated for sedentary and active females age 24 and 18 years, respectively, according to[14] . (**b**) Body composition obtained using the total body potassium (TBK) method, dual-energy X-ray absorptiometry (DXA), and BioPod air displacement plethysmography and computed body composition using the Lohman-4C model. (**c**) Total body obtained using the doubly-labeled water method and derived parameters. (**d**) Energy expenditure as derived from TBW measurements. (**e**) Energy expenditure by indirect calorimetry. (**f**) Energy expenditure during 24 hr and sleep phase by indirect calorimetry. (**g**) Energy expenditure during walking at various paces and unallocated free time by indirect calorimetry.

Supplemental Table 2

a Vitals

	BP			HR			Respirations	Temp
Unit	mm Hg	mm Hg	mm Hg	BPM	BPM	BPM	RPM	°F
NPS1	132/73	145/74	128/79	94	97	97	18	98.7
NPS2	142/95	139/97	129/82	98	103		20	97.6
reference value	133±20/79±11*			68±11*			12-20	97.6-99.6

b Hormones

	Leptin	Ghrelin	Adiponectin
Unit	ng/ml	pg/ml	µg/ml
NPS1	5.7	891	8.1
NPS2	11.1	522	8.5
reference value	8.0 – 38.9	520 - 700	5 – 37

Supplemental Table 2 Vitals and select hormone concentrations of two individuals with NPS

(**a**) Blood pressure (measured in triplicate), heart rate (measured in triplicate/duplicate), respirations, and body temperature of two individuals with NPS. * Reference values from[31]. (**b**) Plasma Leptin, Ghrelin, and Adiponectin in two individuals with NPS.

Additional References

32. National Center for Health Statistics. Anthropometric Reference Data for Children and Adults: United States, 2011–2014. 1–46 (2016).

33. Herbert, A., Cruickshank, J. K., Laurent, S., Boutouyrie, P. Reference Values for Arterial Measurements Collaboration. Establishing reference values for central blood pressure and its amplification in a general healthy population and according to cardiovascular risk factors. *Eur. Heart J.* **35,** 3122–3133 (2014).