Online Appendix

Figure Legends

Fig. 1. Generation of Agrp-KI and Pomc-KI mice. *A*: Conditional knock-in of *INSR* in the β-actin 3'-untranslated region (*βac/INSR*). The *INSR* cDNA was cloned downstream of an internal ribosomal entry site (IRES) and a loxP-flanked neomycin-resistance cassette (neo), allowing Cre-mediated activation of INSR expression. We have previously shown that intercrossing mice bearing the *βac/INSR* locus with transgenic mouse lines with brain- or pancreatic β cell-specific Cre expression results in InsR reconstitution at near physiological levels in target tissues (1). *B*: Breeding strategy. *Insr*+/– mice bearing the *Ttr-INSR* transgene and the *βac/INSR* locus were intercrossed with *Insr*+/– mice bearing a construct encoding Cre recombinase under the control of either the Agrp or the Pomc promoter (2). The resulting progeny with the following genotypes, *Insr–/–*::*Ttr-INSR*::*βac/INSR*::*Agrp-(cre)* and *Insr–/–*::*Ttr-INSR*::*βac/INSR*::*Pomc-(cre)*, were denoted Agrp-knock-in (Agrp-KI) and Pomc-KI mice. WT and L1 littermates were used as controls (see experimental procedures). All mice were genotyped for recombination of the *βac/INSR* locus in tail tissues to identify and eliminate those mice with recombination and deletion of the loxP-neo-loxP cassette due to embryonic activation of Cre (3). None of the Pomc-KI mice and approximately 25% of the Agrp-KI mice were removed from the studies. >90% of all the *Insr–/–*::*Ttr-INSR*::*βac/INSR*::*Agrp-(cre)*::*Pomc-(cre)* mice (Agrp/Pomc-double KI) showed embryonic recombination at the *βac/INSR* locus and could not be studied. We analyzed exclusively male mice. The presence of *βac/INSR* alone, *Agrp-(cre)* alone, or *Pomc-(cre)* alone in *Insr–/–*::*Ttr-INSR* mice did affect body weight, glucose tolerance, or insulin tolerance (data not

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shown). *C-D*: Cre-mediated recombination visualized by GFP immunohistochemistry in *Agrp-(cre)::Rosa26-Gfp* and *Pomc-(cre)::Rosa26-Gfp* mice. 3V, third ventricle. *E-F*: Western blot analysis of liver and hindlimb muscle. Representative immunoblots of InsR β subunit and β-actin are shown.

Fig. 2 Glucose and insulin tolerance tests. *A*: Intraperitoneal glucose tolerance tests (2mg/g) in overnight-fasted 12-week-old mice (N=7-18 per group). **P*<0.05 WT *vs.* L1, Agrp-KI, and Pomc-KI. NS: no significant difference among L1, Agrp-KI and Pomc-KI. *B*: Insulin tolerance tests (0.75U/kg) in 6 hr-fasted 13-week-old mice (N=10-22 per group). Data are plotted as % of glucose values at the beginning of the test. All values are means ± SEM.

Fig. 3 Liver and muscle insulin signaling in fasted and in clamped mice. *A*: Insulin-stimulated Akt phosphorylation in liver. *B*: Akt phosphorylation in muscle of 14-to-17-week-old male mice after an overnight fast (F) or at the end of hyperinsulinemic clamps (C). Representative immunoblots are shown. Ratio of phosphorylated *vs.* total Akt was calculated and shown as means ± SEM. N=3-5. **P*<0.05 *vs.* WT, ***P*<0.01 *vs.* WT, #*P*<0.05, NS=not significant.

Fig. S4 Hepatic gene expression profile. Hepatic mRNA expression quantified by real-time RT-PCR in 14-to-17-week-old WT (white bar), L1 (blue bar), Agrp-KI (grey bar), and Pomc-KI (red bar) male mice after overnight fasting or at the end of hyperinsulinemic clamp studies. N=6-11 per group. Data are normalized against *36B4* mRNA. All values are means ± SEM. **P*<0.05 *vs.* WT, ***P*<0.01 *vs.* WT, #*P*<0.05.

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Fig. S5 ICV insulin infusion induces hepatic interleukin-6 (*Il-6*) and Stat3 phosphorylation (4). *A*: Hepatic *Il-6* mRNA expression in 14-to-17-week-old WT (white bar), L1 (blue bar), Agrp-KI (grey bar), and Pomc-KI (red bar) mice after overnight fasting or at the end of hyperinsulinemic clamp studies. N=6-11 per group. Data are normalized against *36B4* mRNA. *P*=NS. *B*: Western blot analysis of Tyr705-phosphorylated and total Stat3 in liver after overnight fasting (F) or hyperinsulinemic clamps (C). Representative phospho-Tyr705 and total Stat3 immunoblots of liver lysates are shown. Ratio of phosphorylated *vs.* total Stat3 was calculated and shown as mean ± SEM. N=3-5. **P*<0.05 *vs.* WT, NS=not significant.

Fig. S6 Counterregulatory response in InsR knock-in mice. *A*: Plasma glucagon levels in 12- to 14-week-old WT (white bar), L1 (blue bar), and Pomc-KI (red bar) mice after a 6-hr fast, and 30 or 60min after insulin injection. N=6-11 per group. **P*<0.05 *vs.* WT. *B*: Normal adrenal response in Pomc-KI mice. Basal and 1 hr restraint-induced plasma corticosterone levels in 14-to-15-week-old WT (white bar), L1 (blue bar), Agrp-KI (grey bar), and Pomc-KI (red bar) mice. N=4-8 per group. ****P*<0.001 *vs.* basal.

Fig. S7 *A:* Hypothalamic expression of *Mch* and *B*: *Hcrt* in 14- to 16-week-old WT (white bar), L1 (blue bar), Agrp-KI (grey bar), and Pomc-KI (red bar) male mice after overnight fasting or hyperinsulinemic clamp. Data are normalized against *36B4* mRNA. N=6-11 per group. All values are mean ± SEM. **P*<0.05 *vs.* WT.

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Online appendix Table 1. Metabolic characteristics of experimental animals

12-week-old mice	WT	L1	Agrp-KI	Pomc-KI
Body weight (g)	31.1 ± 1.2	29.6 ± 0.9	29.1 ± 1.4	27.9 ± 1.1
Fat content (%)	9.1 ± 1.0	11.1 ± 1.3	9.9 ± 1.5	9.2 ± 0.9
Lean content (%)	85.7 ± 1.0	83.7 ± 1.3	84.5 ± 1.5	85.5 ± 0.9
Fluid content (%)	5.2 ± 0.2	5.2 ± 0.1	5.5 ± 0.4	5.4 ± 0.2
Hepatic TG (fasting, mg/g)	31.9 ± 5.5	34.1 ± 13.6	34.1 ± 11.9	43.3 ± 17.3
Hepatic TG (hyperinsulinemic clamp, mg/g)	5.2 ± 0.5	6.2 ± 1.0	5.7 ± 1.7	5.2 ± 0.3
Plasma TG (fasting, mg/ml)	0.86 ± 0.08	0.77 ± 0.12	0.62 ± 0.10	0.76 ± 0.15
Plasma TG (ad lib, mg/ml)	1.14 ± 0.10	0.69 ± 0.09**	0.50 ± 0.05**	0.54 ± 0.06**
NEFA (fasting, mg/ml)	0.89 ± 0.10	0.69 ± 0.10	ND	0.76 ± 0.06
Adiponectin (fasting, μg/ml)	6.1 ± 0.5	7.2 ± 0.5	9.7 ± 2.8	8.7 ± 2.7
Resistin (fasting, ng/ml)	0.63 ± 0.09	0.35 ± 0.05*	0.45 ± 0.12	0.56 ± 0.15
tPAI1 (fasting, ng/ml)	1.77 ± 0.42	2.30 ± 0.47	1.91 ± 0.39	1.71 ± 0.27
IL-6 (fasting, pg/ml)	33.0 ± 3.4	34.6 ± 4.0	47.2 ± 22.8	26.7 ± 0.8
TNFα (fasting, pg/ml)	55.9 ± 5.1	52.5 ± 2.4	51.4 ± 6.1	53.7 ± 5.2
15-week-old mice	**WT**	**L1**	**Agrp-KI**	**Pomc-KI**
Body weight (g)	33.1 ± 0.9	28.8 ± 1.1**	25.9 ± 1.0** †	29.7 ± 1.1*^
Length (cm)	10.4 ± 0.2	10.2 ± 0.1	9.9 ± 0.1*	10.4 ± 0.1^^
BMI (g/cm^2)	0.31 ± 0.01	0.28 ± 0.01*	0.27 ± 0.01*	0.27 ± 0.00*
IGF1 (ad lib, ng/ml)	202.9 ± 15.7	158.8 ± 5.3*	146.8 ± 4.4**	160.7 ± 7.6*

Fasting plasma parameters were measured in overnight-fasted mice. Data are means ± SEM. ND, not determined. *P<0.05 vs. WT, **P<0.01 *vs.* WT. †P<0.05 *vs.* L1. ^P<0.05 *vs.* Agrp-KI, ^^P<0.01 *vs.* Agrp-KI.

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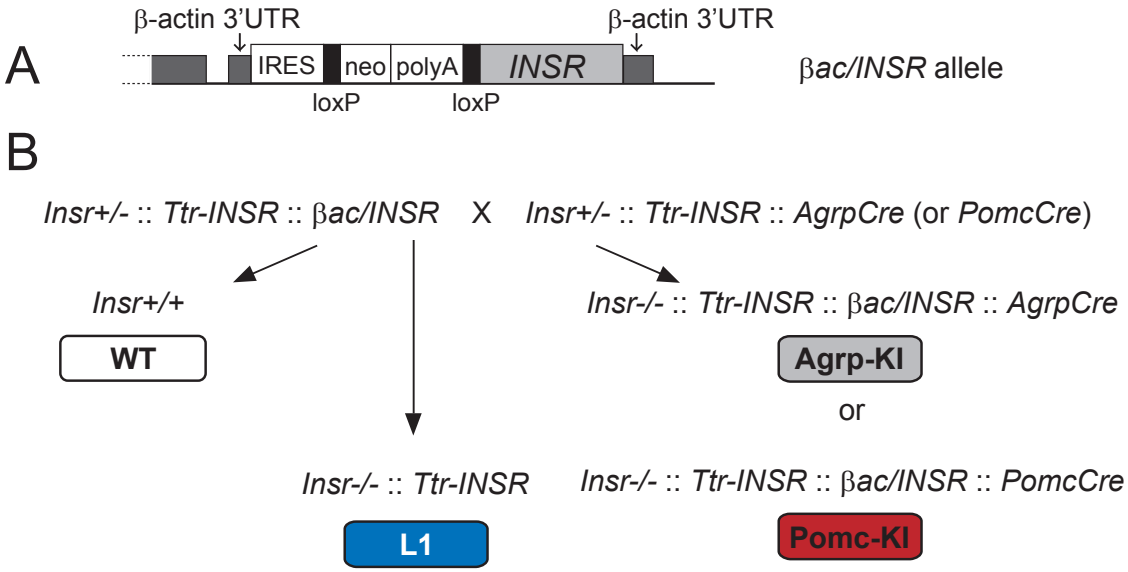
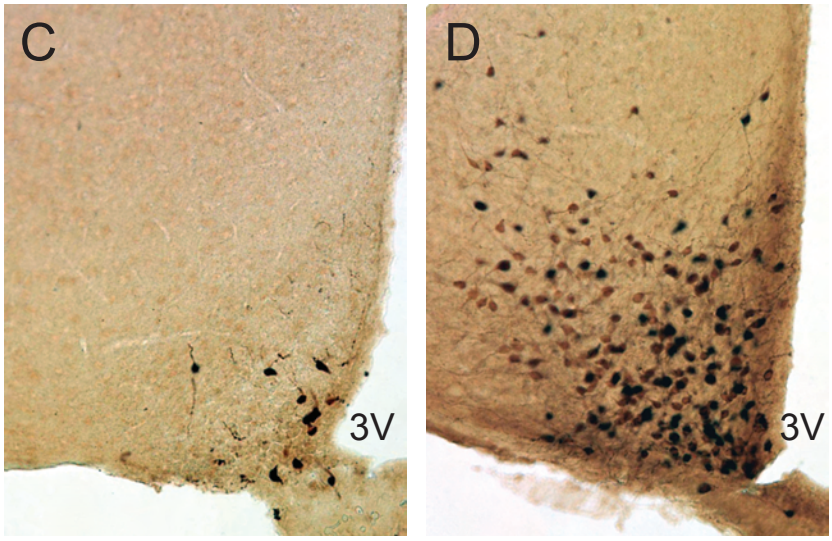
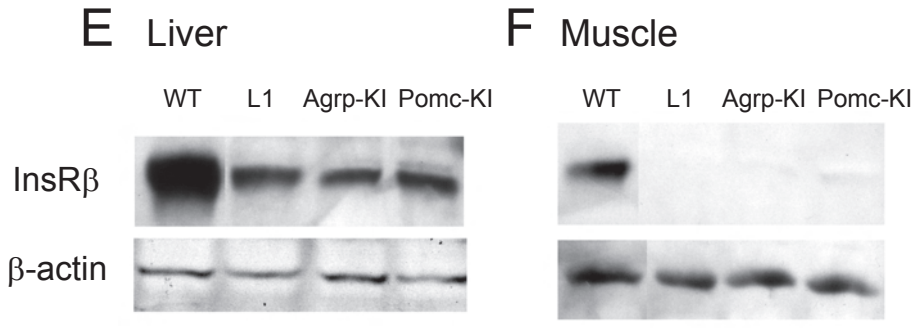
Lin et al, Figure S1







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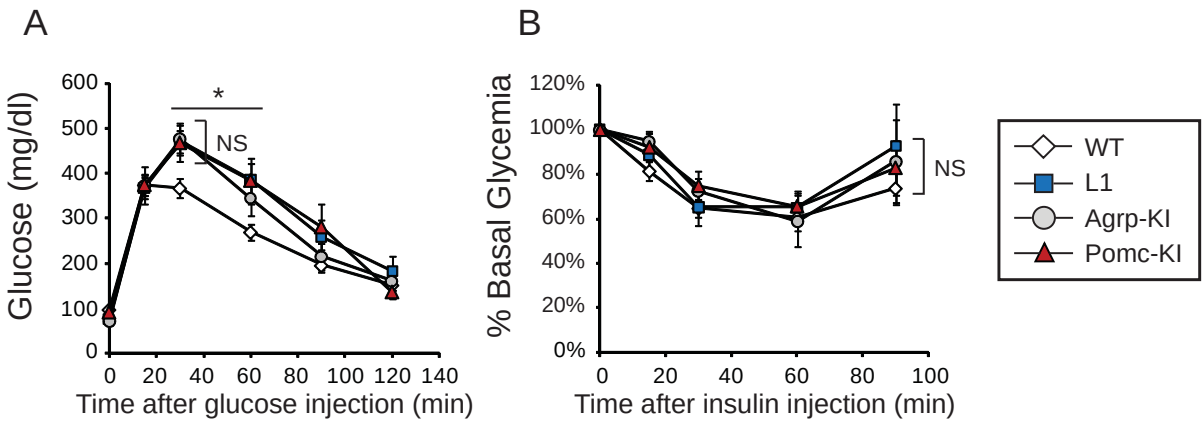

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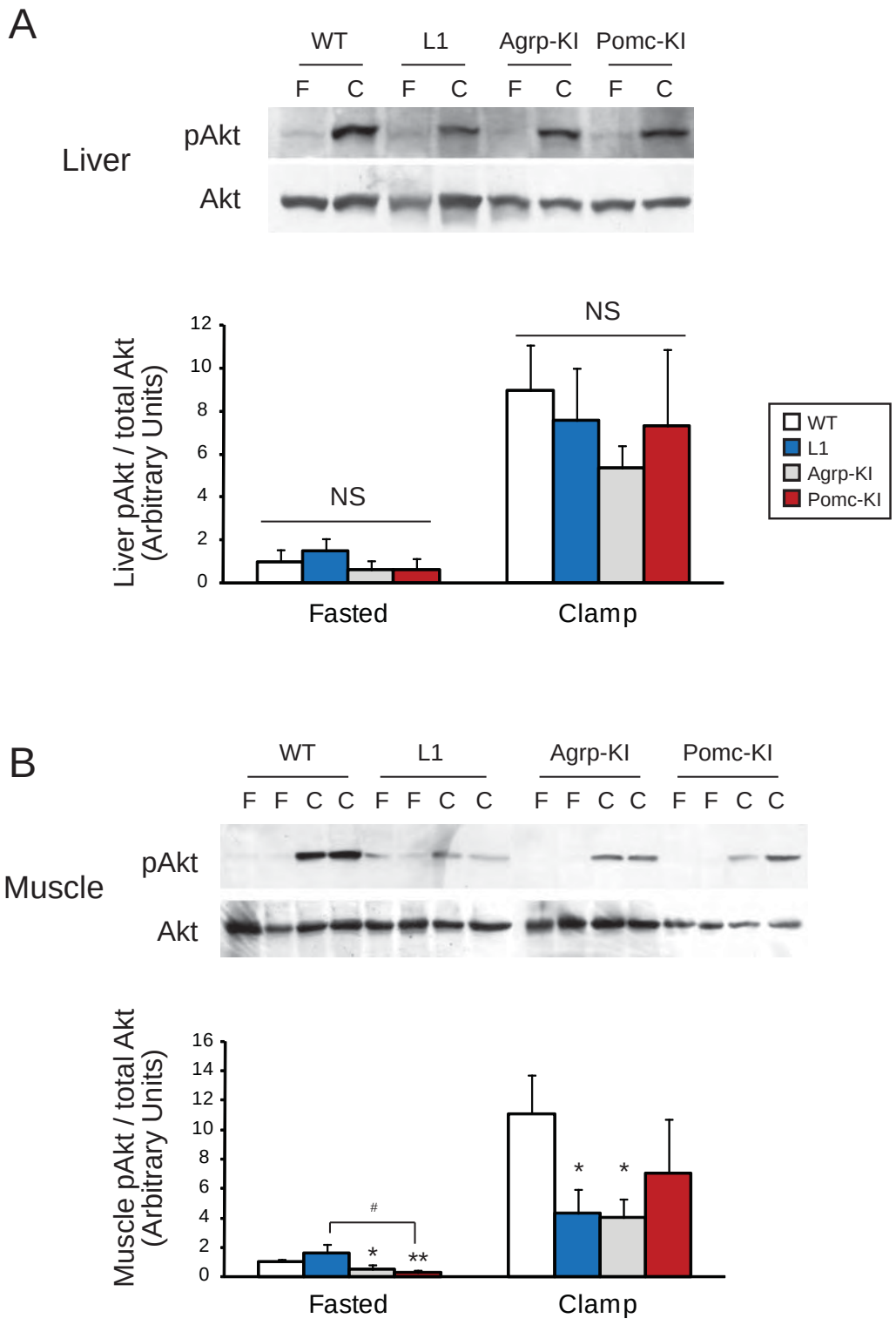

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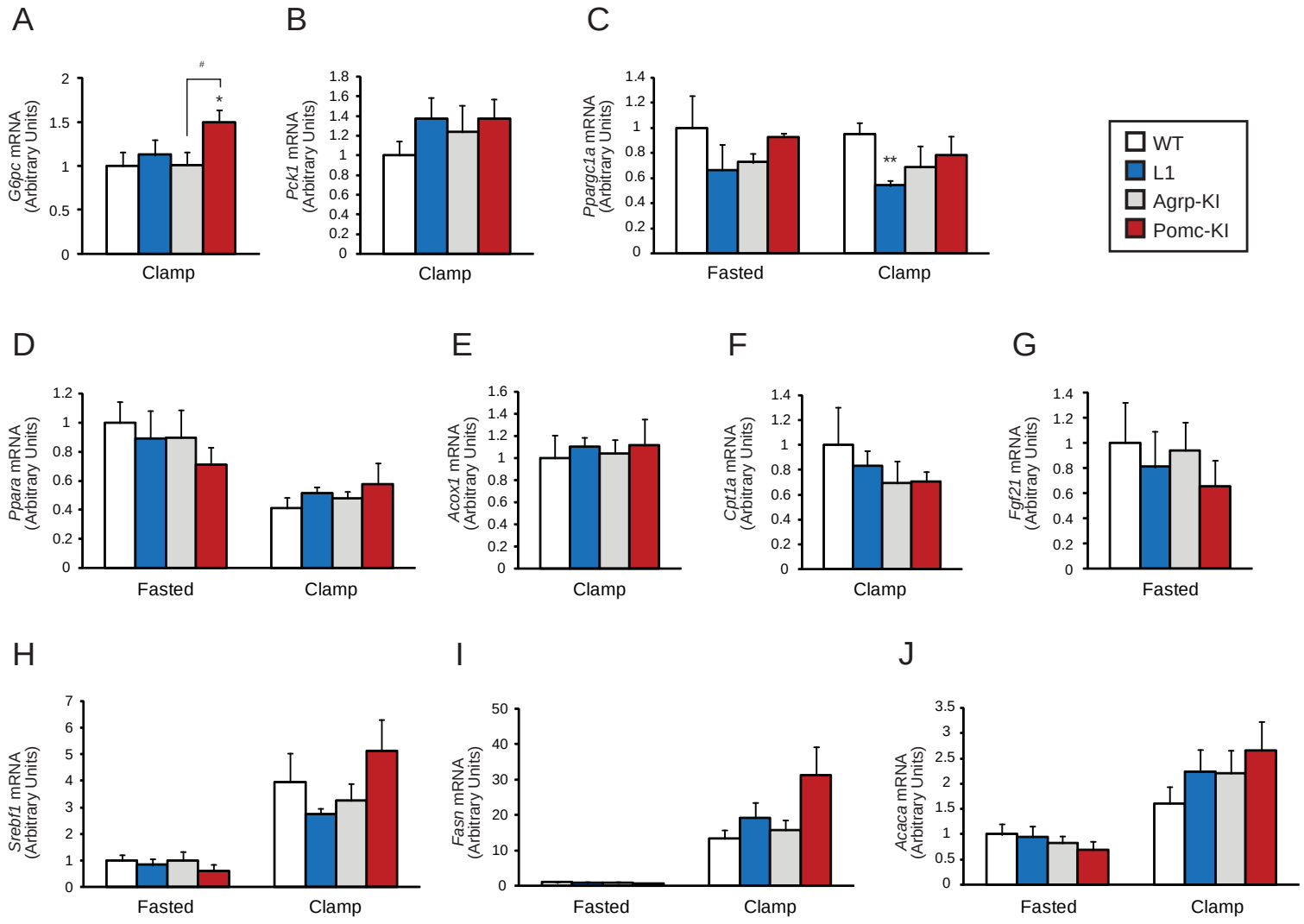

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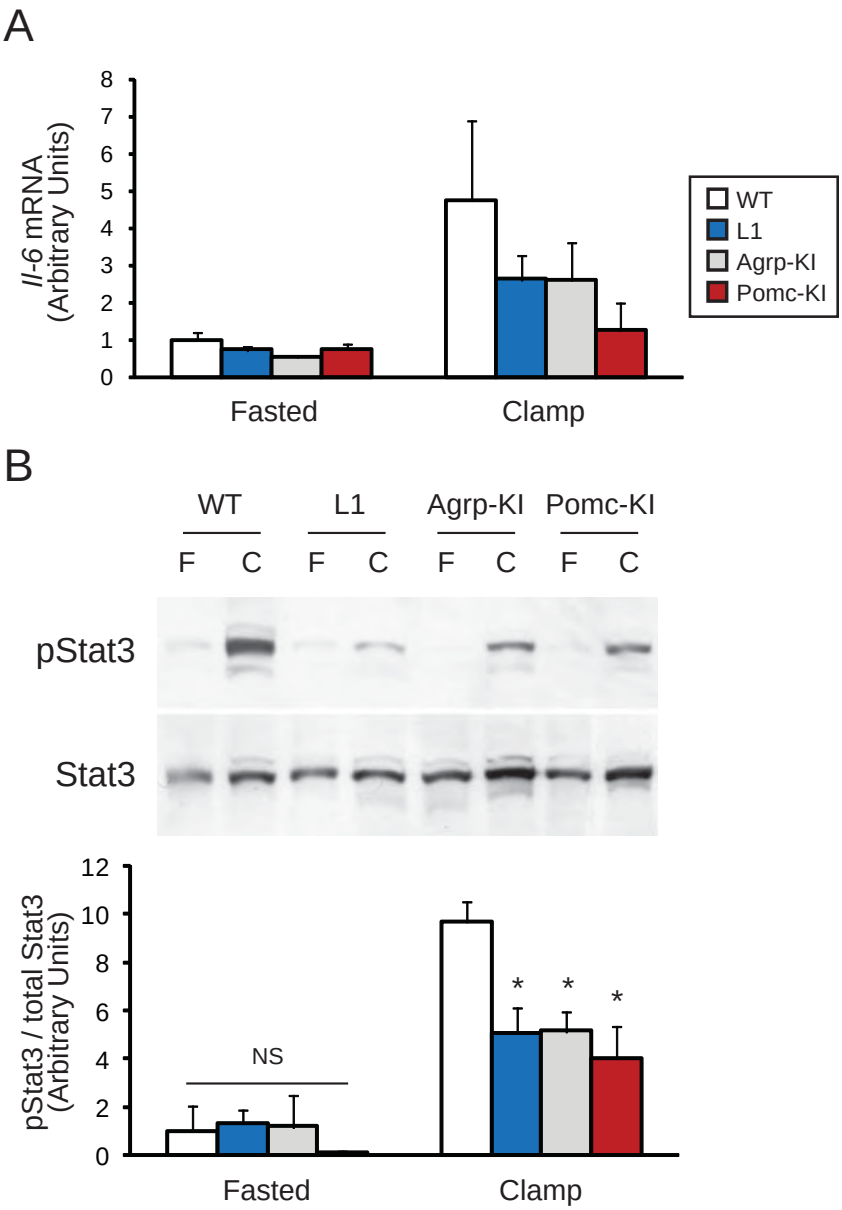

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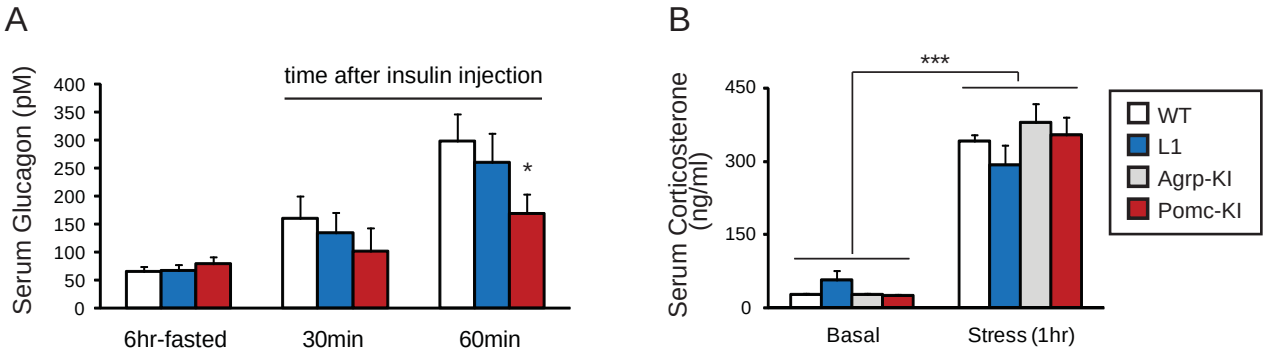

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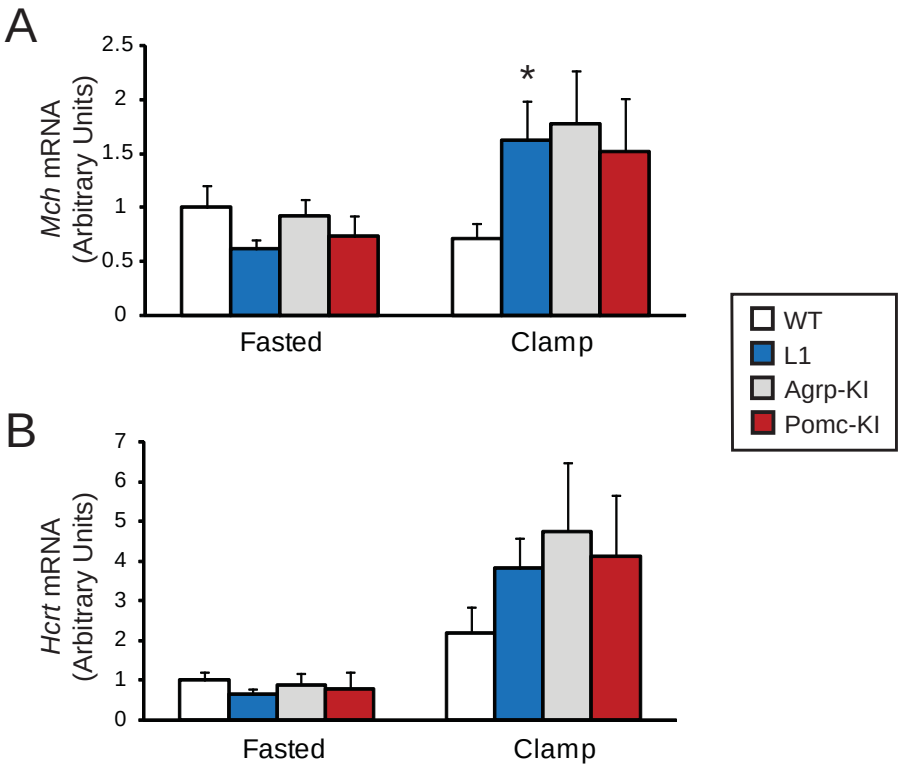

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