Systems/Circuits

Coordinated Regulation of Hepatic Energy Stores by Leptin and Hypothalamic Agouti-Related Protein

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Like obesity, prolonged food deprivation induces severe hepatic steatosis; however, the functional significance of this phenomenon is not well understood. In this study, we show that the fall in plasma leptin concentration during fasting is required for the development of hepatic steatosis in mice. Removal of leptin receptors from AGRP neurons diminishes fasting-induced hepatic steatosis. Furthermore, the suppressive effects of leptin on fasting-induced hepatic steatosis are absent in mice lacking the gene encoding agouti-related protein (*Agrp*), suggesting that this function of leptin is mediated by AGRP. Prolonged fasting lead to suppression of hepatic sympathetic activity, increased expression of acyl CoA:diacylglycerol acyltransferase-2 in the liver, and elevation of hepatic triglyceride content and all of these effects are blunted in the absence of AGRP. AGRP deficiency, despite having no effects on feeding or body adiposity in the free-fed state, impairs triglyceride and ketone body release from the liver during prolonged fasting. Furthermore, reducing CNS *Agrp* expression in wild-type mice by RNAi protected against the development of hepatic steatosis not only during starvation, but also in response to consumption of a high-fat diet. These findings identify the leptin-AGRP circuit as a critical modulator of hepatic triglyceride stores in starvation and suggest a vital role for this circuit in sustaining the supply of energy from the liver to extrahepatic tissues during periods of prolonged food deprivation.

Introduction

Hepatic steatosis develops with obesity and also with starvation. Accumulation of triglycerides in the liver is determined by the balance of input processes (nonesterified free fatty acid [FFA] influx, *de novo* lipogenesis, and triglyceride synthesis) and output processes (β-oxidation, very-low-density lipoprotein [VLDL] secretion). In obesity, hepatic steatosis is generally thought to be caused by increased influx of FFA and insulin-stimulated *de novo* lipogenesis (Brown and Goldstein, 2008; Postic and Girard, 2008). We have shown that impairment of leptin signaling in the brain, a classical feature of obesity, is also a causal factor in the development of hepatic steatosis in free-fed mice (Warne et al., 2011). During fasting, plasma insulin concentrations and *de novo* lipogenesis are reduced (Hellerstein et al., 1991; Timlin and Parks, 2005), β-oxidation and FFA influx are increased, and VLDL secretion is not detectably altered (Haude and Völcker, 1991; LeBoeuf et al., 1994). Accordingly, it is commonly assumed that fasting-induced hepatic steatosis arises from increased influx of FFA to the liver. However, the extent to which the brain participates in the development of fasting-induced hepatic steatosis is not known.

One major function of the hormone leptin is to convey the abundance of peripheral energy stores to the brain. Plasma leptin concentrations decrease precipitously with fasting, which signals the brain to trigger adaptive responses to lower energy expenditure and to increase appetite (Farooqi and O'Rahilly, 2009). During fasting, there is marked upregulation of several hypothalamic orexigenic genes, including agouti-related protein (*Agrp*), neuropeptide Y (*Npy*), melanin-concentrating hormone (*Mch*), and *Orexin*. In contrast to the lateral hypothalamic MCH- and orexin-expressing neurons (Leinninger et al., 2009), AGRP-expressing neurons possess functional leptin receptors and are direct leptin targets (Kaelin et al., 2006; van de Wall et al., 2008; Olofsson et al., 2013). Accordingly, the fasting-induced increase in *Agrp* expression can be prevented by leptin treatment (Ebihara et al., 1999; Mizuno and Mobbs, 1999; Wilson et al., 1999; Korner et al., 2001). AGRP is a potent orexigen and transgenic overexpression of *Agrp* causes obesity (Ollmann et al., 1997; Schwartz et al., 2000). However, mice with genetic deletion of *Agrp* (Qian et al., 2002) or progressive degeneration of AGRP neurons (Xu et al., 2005b) show normal regulation of energy balance. The surprising absence of a body weight phenotype in these mice suggests that the function of AGRP in body weight regulation can be readily compensated by other mechanisms. Indeed, other constituents of AGRP neurons, such as the classical neurotransmitter GABA and NPY, have been shown to be required for feeding (Wu

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et al., 2008; Wu et al., 2009; Atasoy et al., 2012; Wu et al., 2012). Therefore, the absolute requirement of AGRP, the namesake of the AGRP neurons, in metabolic regulation remains unsettled.

In this study, we reveal a critical role for the leptin-AGRP axis in the regulation of hepatic energy storage during periods of prolonged food deprivation. This regulation may serve as an adaptive mechanism to ensure sustained energy supply from the liver to extrahepatic tissues.

Materials and Methods

Mouse genetics and maintenance. Male C57bl/6J mice (originally from The Jackson Laboratory and subsequently bred in-house) were used for most studies. Male *Lep*$^{ob/ob}$ mice were purchased from The Jackson Laboratory. *Agrp*$^{-/-}$ mice were generated by Deltagen. Briefly, an IRES-*lacZ-NeoR* cassette was targeted into ES cells derived from the 129/OlaHsd mouse substrain. The targeting event replaced a 255 bp genomic region, chr8:108,091,292-108,091,546 (MGSCv37), in and around the first coding exon of *Agrp*, including the translational start site. Targeting was confirmed in the embryonic stem cell line by Southern hybridization with a radiolabeled probe hybridizing outside of and adjacent to the arm of the targeting construct. F1 mice were generated by breeding *Agrp*$^{+/-}$ chimeras with C57bl/6 females; progeny were then intercrossed and the line was subsequently maintained on a mixed genetic background. Genotyping was performed using allele-specific PCR with primer sets flanking the targeting site (for wild-type allele detection) or spanning the distal targeted breakpoint (for mutant allele detection) and the forward primer located within the IRES-*lacZ-NeoR* cassette. Mice lacking one copy of the *Agrp* gene (*Agrp*$^{+/-}$) were used as littermate controls. *Tg.AgrpCre/+* mice (Xu et al., 2005a) and *Lepr*$^{fl/fl}$ mice (van de Wall et al., 2008) were bred to generate *Tg.AgrpCre/+*;*Lepr*$^{fl/+}$ mice that subsequently were bred with *Lepr*$^{fl/fl}$ mice to generate *Tg.AgrpCre-Lepr*$^{fl/fl}$ (hereafter referred to as *Agrp-LeprKO*) mice. The control littermates used in this study were all *Lepr*$^{fl/fl}$. *Tg.AgrpCre* mice have been used and validated in a number of independent studies (Gropp et al., 2005; Xu et al., 2005b; Kitamura et al., 2006; Claret et al., 2007; Könner et al., 2007; Zhang et al., 2008; van de Wall et al., 2008; Al-Qassab et al., 2009).

All mice were group housed in a pathogen-free, temperature- (22°C), humidity-, and light (0700 h-1900 h lights on)–controlled environment with *ad libitum* regular chow (mouse diet 5008; Purina), a low fat diet (LFD; 10 kcal% fat, D12450B; Research Diets), or a high-fat diet (HFD; 60 kcal% fat, D12492; Research Diets) and free water access. For fasting procedures, mice were placed into a new, clean cage with no food but free access to water at the start of the fast. All procedures were approved by the University of California–San Francisco Institutional Animal Care and Use Committee.

Body composition and food intake. Whole-body lean and fat mass were determined by EchoMRI at the University of California–San Francisco Diabetes and Endocrinology Research Center Metabolic Core Facility. Fat pad weights were determined after postmortem dissection and removal of connective tissue. Food intake was measured using a Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments). Mice were singly housed for 4 d before being housed in the CLAMS and were allowed 24 h to acclimatize to the new CLAMS cages before taking measurements.

Intracerebroventricular injection. Anesthetized (100 mg/kg ketamine, 5 mg/kg xylazine, i.p.) mice were mounted onto a stereotaxic apparatus (model 1900; David Kopf Instruments) and implanted with a guide cannula (Plastics One) into the right lateral ventricle (anteroposterior −0.3 mm, lateral −1.0 mm relative to bregma; −2.7 mm below the skull; Warne et al., 2011). Buprenorphine (0.1 mg/kg i.p.) was provided for analgesia immediately after surgery and as required. Correct placement was verified by a robust drinking response to angiotensin II (0.1 mg/ml i.c.v. [Sigma] at 10 nl/s via a 2.9 mm injector [Plastics One]) and by postmortem examination.

Leptin treatment. For intraperitoneal treatment, mice received injections of leptin (3 mg/kg in PBS; National Institute of Diabetes and Digestive and Kidney Diseases); controls received injections of vehicle (PBS, pH 7.8, 10 ml/kg). For intracerebroventricular treatment, mice were injected with 2 μg of leptin (in aCSF); controls received injections of vehicle (aCSF, 1 μl). For the majority of studies, mice received 3 injections intraperitoneally or intracerebroventricularly over the 30 h fasting period, one at the onset of the fast, a second 10 h later, and a third 20 h after the initiation of the fast; tissues were collected 30 h after the food was removed. For studies examining *in vitro* release of FFA from adipose explants, mice were fasted for 25 h and received intraperitoneal injections at the onset of the fast and after 12 and 24 h of fasting. For studies examining hepatic VLDL secretion after fasting, mice were fasted for 36 h and received intraperitoneal injections at the onset and after 12, 24, and 35 h of fasting.

Norepinephrine turnover assay. Norepinephrine turnover was calculated from the disappearance of liver norepinephrine concentration with time after norepinephrine biosynthesis blockade using α-methyl-*p*-tyrosine (α-MPT). Mice were injected with α-MPT (250 mg/kg in saline i.p.), a subset of mice was immediately killed by cervical dislocation, and tissues were collected for norepinephrine turnover rate calculations; the remaining mice received a second intraperitoneal injection of α-MPT (125 mg/kg) 3 h later; tissues were collected 6 h after the first intraperitoneal injection. For comparison of norepinephrine turnover in control and *Agrp*$^{-/-}$ mice under fasting conditions, α-MPT treatment was started 24 h after the initiation of a fast and no food was present throughout (totaling a 30 h fast). For examination of the effects of AgRP administration, fed mice were deprived of food and received an intracerebroventricular injection of aCSF (1 μl) or mAGRP$_{82\text{-}131}$ (1 nM; Phoenix) with 5 min of the first intraperitoneal α-MPT injection.

RNAi. For fasting studies, 10-week-old C57bl/6J male mice were injected with either 5 μg of negative control dicer-substrate RNA (DsiRNA; NC1; Integrated DNA Technologies) or 5 μg of a DsiRNA directed against *Agrp* (MMC.RNAI.N007427.12.1; Integrated DNA Technologies). Mice then either remained *ad libitium* fed for 30 h or were deprived of food for 30 h, after which time tissues were collected. Treatment of the fed and fasted groups was performed and tissues were collected at the same times of day. In the *ad libitum* fed group, food intake was monitored for 3 d before the treatment (at 24 h intervals) and was measured 24 h after DsiRNA treatment. For LFD/HFD studies, on day 0, 8-week-old male C57bl/6J mice were provided with either HFD or LFD *ad libitum*, which they remained on until the end of the study. On days 2 and 4, mice from each dietary group were injected with either 5 μg of the negative control DsiRNA (NC1) or 5 μg of a DsiRNA against *Agrp*, as outlined above. All tissues were collected on day 6 at the same time of day.

Hepatic triglyceride secretion. Mice fasted for 36 h were injected intraperitoneally with Triton WR-1339 (500 mg/kg; Sigma) and blood was collected before and 2, 4, and 6 h after injection. No food was provided during this treatment period.

In vitro explant preparation. Liver slices (1 mm thick, 50 mg) were preincubated for 2 h in Krebs-Ringer bicarbonate buffer (pH 7.4, 37°C) in a humidified atmosphere saturated with 95% O_2-5% CO_2; buffer was replaced after 1 and 1.5 h. Slices were then incubated in fresh buffer containing norepinephrine-HCl (Sigma) or no drug for 2 h, after which time tissue was processed for RT-PCR analyses. Epididymal adipose tissue was removed postmortem, cut into explants (50 mg), and preincubated in Krebs-Ringer bicarbonate buffer (pH 7.4, 37°C) supplemented with 5% FFA-free BSA (Sigma) for 2 h; buffer was replaced after 1 and 1.5 h. Adipose explants were then incubated in fresh buffer containing 0.1 μM isoprenaline-HCl (Sigma) or no drug. Buffer was collected 1, 2, and 4 h later for analysis of FFA concentrations.

Biochemical assays. Liver triglycerides were extracted using the Folch method and the concentration was determined using a colorimetric assay (Sigma). Liver norepinephrine was extracted in 0.01 N HCl containing 1 mM EDTA and 4 mM sodium metabisulfite. Leptin, insulin, and norepinephrine concentrations were measured using ELISA kits (Crystal Chem or Alpco). Blood glucose concentrations were measured using a Freestyle glucometer (Abbott Diabetes Care). FFA, β-hydroxybutyrate, and acetoacetate concentrations were measured using colorimetric kits (Wako Chemicals). Tissue mRNA expression was determined by quantitative RT-PCR; RNA was extracted, reverse transcribed, and then PCR amplified (7900HT Fast Real-Time PCR System) using specific TaqMan gene

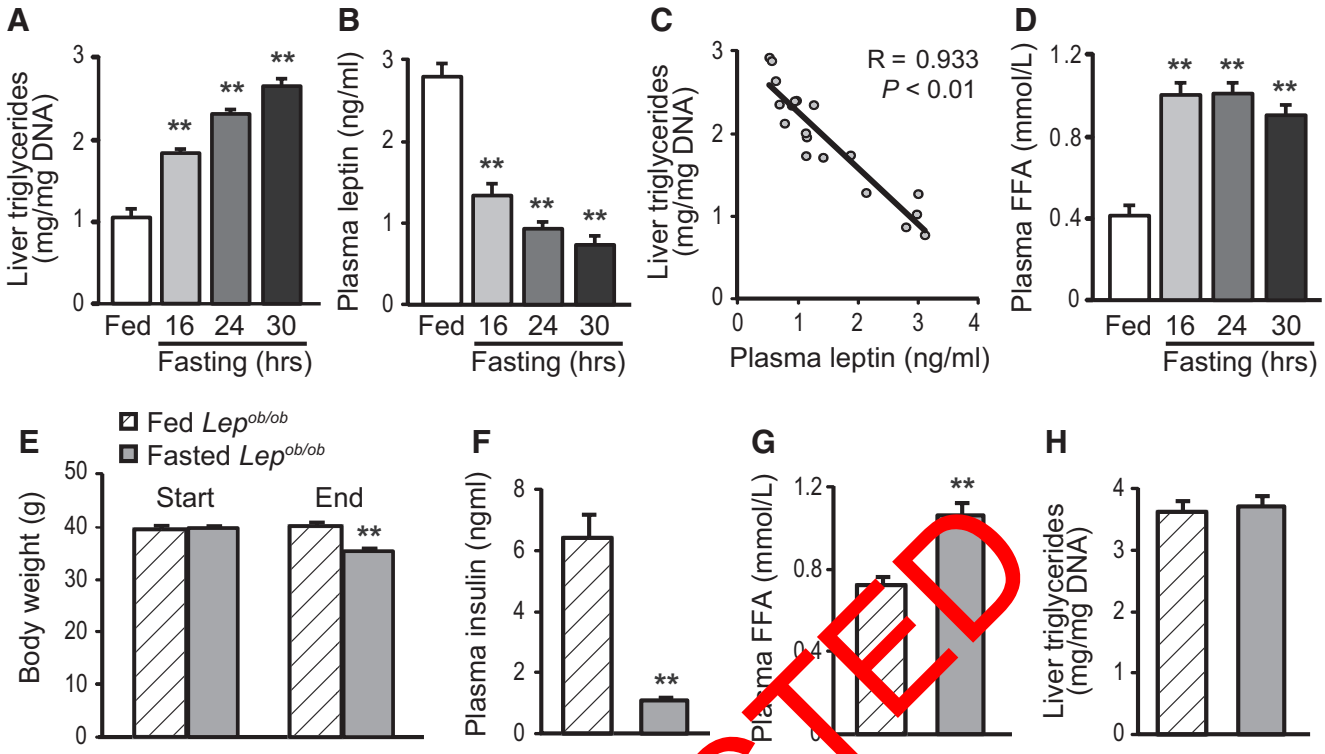

Figure 1. Leptin concentrations during starvation correlate with liver triglyceride content in wild-type mice, but leptin-deficient *Lep*$^{ob/ob}$ mice fail to develop hepatic steatosis with starvation. ***A–C***, Increasing the duration of fasting ($n = 5$/time point) results in a progressive increase in liver triglyceride content (***A***) and reduction in plasma leptin concentrations (***B***) that are negatively correlated (***C***). ***D***, In contrast, the plasma FFA concentrations are elevated with 16 h fasting but do not increase in plasma concentration further with greater fasting duration. ***E–H***, Comparison of 7-week-old *ad libitum*-fed or 36-h-fasted *Lep*$^{ob/ob}$ male mice ($n = 5$/group) on body weight change between start and end of the experiment (***E***), the terminal plasma concentrations of insulin (***F***) and FFA (***G***) and liver triglyceride (***H***) content. Data are shown as means ± SEM. ** < 0.01 compared with fed (for ***A***, ***B***, ***D***, one-way ANOVA followed by Tukey's *post hoc* tests; for ***C***, Pearson's correlation; for ***E***, repeated-measures ANOVA and Tukey's *post hoc* tests, and for ***F–H***, unpaired Student's *t* tests).

expression assays and Universal PCR Master Mix (Applied Biosystems). β-Actin was used to normalize expression.

Statistics. All data are normally distributed (Shapiro Wilk test). Data from two groups were compared using two-tailed unpaired Student's *t* tests. Data from three or more groups were analyzed using one- or two-way ANOVAs, as appropriate, followed by *post hoc* tests. Data with within-subjects measurements were analyzed using repeated-measures ANOVA. For linear regression, Pearson's correlation was used. All data are expressed as the mean ± SEM. Significance was defined at $p < 0.05$.

Results

Decline of plasma leptin levels correlates closely with the increase of hepatic triglyceride content in fasted wild-type mice

Liver triglyceride levels progressively increased along with the duration of fasting (16, 24 or 30 h; Fig. 1*A*; $F_{(3,16)} = 63.0$, $p < 0.0001$) in wild-type mice, whereas plasma leptin concentrations progressively decreased (Fig. 1*B*; $F_{(3,16)} = 48.9$, $p < 0.0001$). These two parameters exhibited a significant inverse correlation (Fig. 1*C*; $r = 0.93$, $p < 0.01$). Plasma FFA concentrations increased with 16 h of food deprivation, but showed no further increase in concentration with greater fasting duration (Fig. 1*D*; $F_{(3,16)} = 26.0$, $p < 0.0001$). Therefore, the fall in plasma leptin concentration correlates closely with the progressive development of hepatic steatosis in food-deprived wild-type animals.

Leptin-deficient mice fail to increase hepatic triglyceride stores with prolonged fasting

To investigate whether the ability to regulate leptin levels is required to increase hepatic triglyceride content during fasting, we first examined whether leptin-deficient *Lep*$^{ob/ob}$ mice were able to elevate liver triglyceride levels with prolonged fasting, as wild-type mice do markedly. Depriving food from 7-week-old *Lep*$^{ob/ob}$ mice for 36 h resulted in body weight loss (Fig. 1*E*), lower plasma insulin concentrations (Fig. 1*F*), and greater plasma FFA concentrations (Fig. 1*G*). However, in stark contrast to wild-type mice, there was no significant increase in liver triglyceride content in *Lep*$^{ob/ob}$ mice with fasting (Fig. 1*H*). This lack of increase in liver triglyceride content with fasting was unlikely due to a lack of capacity of the *Lep*$^{ob/ob}$ liver to accumulate more fat, because *Lep*$^{ob/ob}$ mice doubled the triglyceride content in their liver as they aged from 7 to 14 weeks under free-fed conditions (7 weeks: 3.6 ± 0.2 mg/mg DNA, 14 weeks: 7.3 ± 0.2 mg/mg DNA, $p < 0.001$). Therefore, the lack of fasting-induced hepatic steatosis in *Lep*$^{ob/ob}$ mice could be due to the complete absence of leptin, a fundamental regulator of the phenomenon.

Peripheral or central replacement of leptin during fasting mitigates fasting-induced hepatic steatosis

In focusing on how leptin mediates hepatic steatosis during prolonged fasting, we sought to determine the extent to which increased liver triglycerides specifically result from the fall in leptin levels that occurs during fasting. Wild-type mice were injected intraperitoneally with leptin three times during a 30 h fast; fasted controls received three intraperitoneal injections of vehicle. Exogenous leptin treatment prevented plasma leptin concentrations from falling with fasting (Fig. 2*A*; $F_{(2,12)} = 19.0$, $p = 0.0002$) and significantly reduced the fasting-induced accumulation of liver triglycerides (Fig. 2*B*; $F_{(2,12)} = 43.98$, $p < 0.0001$). Exoge-

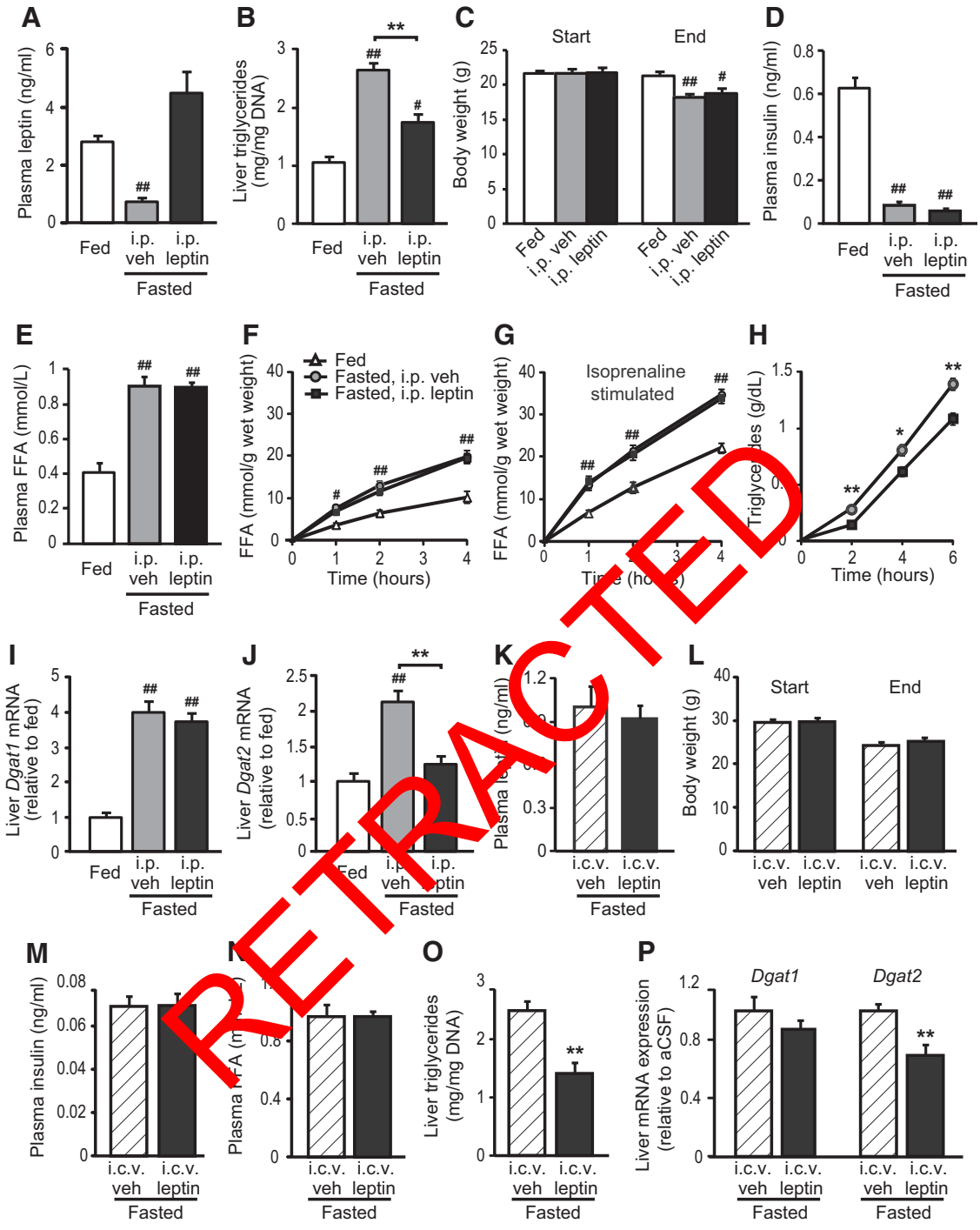

Figure 2. Preventing the decline in leptin during starvation attenuates hepatic steatosis development. ***A–E***, Comparison of mice that were *ad libitum* fed or injected intraperitoneally with vehicle (veh) or leptin ($n = 5$/group) during a 30 h fast on plasma leptin concentrations at the end of the study (***A***), terminal liver triglyceride levels (***B***), experimental start and end body weights (***C***), and the terminal plasma concentrations of insulin (***D***) and FFA (***E***). ***F***, ***G***, *In vitro* basal (***F***) and isoprenaline (0.1 μM)-stimulated (***G***) release of FFA from epididymal adipose tissue biopsies with time ($n =$ 4 mice/group) from mice treated *in vivo* as described for ***A–E***. ***H***, *In vivo* release of triglycerides in 36-h-fasted mice treated with four intraperitoneal injections of vehicle or leptin ($n = 6$/group). ***I***, ***J***, Terminal liver mRNA expression of *Dgat1* (***I***) and *Dgat2* (***J***) for the mice described in ***A–E***. ***K–P***, Comparison of mice that received three intracerebroventricular injections of vehicle or leptin ($n =$ 6/group) during a 30 h fast on terminal plasma leptin concentrations (***K***), experimental start and end body weights (***L***), terminal plasma concentrations of insulin (***M***) and FFA (***N***), liver triglyceride content (***O***) and liver mRNA expression of *Dgat1* and *Dgat2* (***P***). Data are shown as means ± SEM. #$p < 0.05$, ##$p < 0.01$ compared with fed, *$p < 0.05$, **$p < 0.01$ compared with fasted vehicle treated or as indicated (for ***A***, ***B***, ***D***, ***E***, ***I***, ***J***, two-way ANOVA; for ***C***, ***F***, ***G***, ***L***, repeated-measures ANOVA; for ***K***, ***M–P***, unpaired Student's *t* tests).

nous leptin treatment did not affect body weight loss (Fig. 2*C*; significant effects of the experimental groups [$F_{(2,16)} = 5.1$, $p = 0.02$], time [start vs end, $F_{(1,8)} = 13.4$, $p = 0.006$], and an interaction between factors [$F_{(2,16)} = 5.7$, $p = 0.01$]) or the changes in plasma insulin concentrations (Fig. 2*D*; $F_{(2,12)} = 105.3$, $p < 0.0001$) or plasma FFA concentrations (Fig. 2*E*; $F_{(2,12)} = 35.4$, $p < 0.0001$) with fasting. In accordance with the plasma measures, *in vitro* FFA release from white adipose tissue explants taken from fasted mice was greater than that of explants taken from fed mice and was further increased by isoprenaline treatment (Fig. 2*F*,*G*).

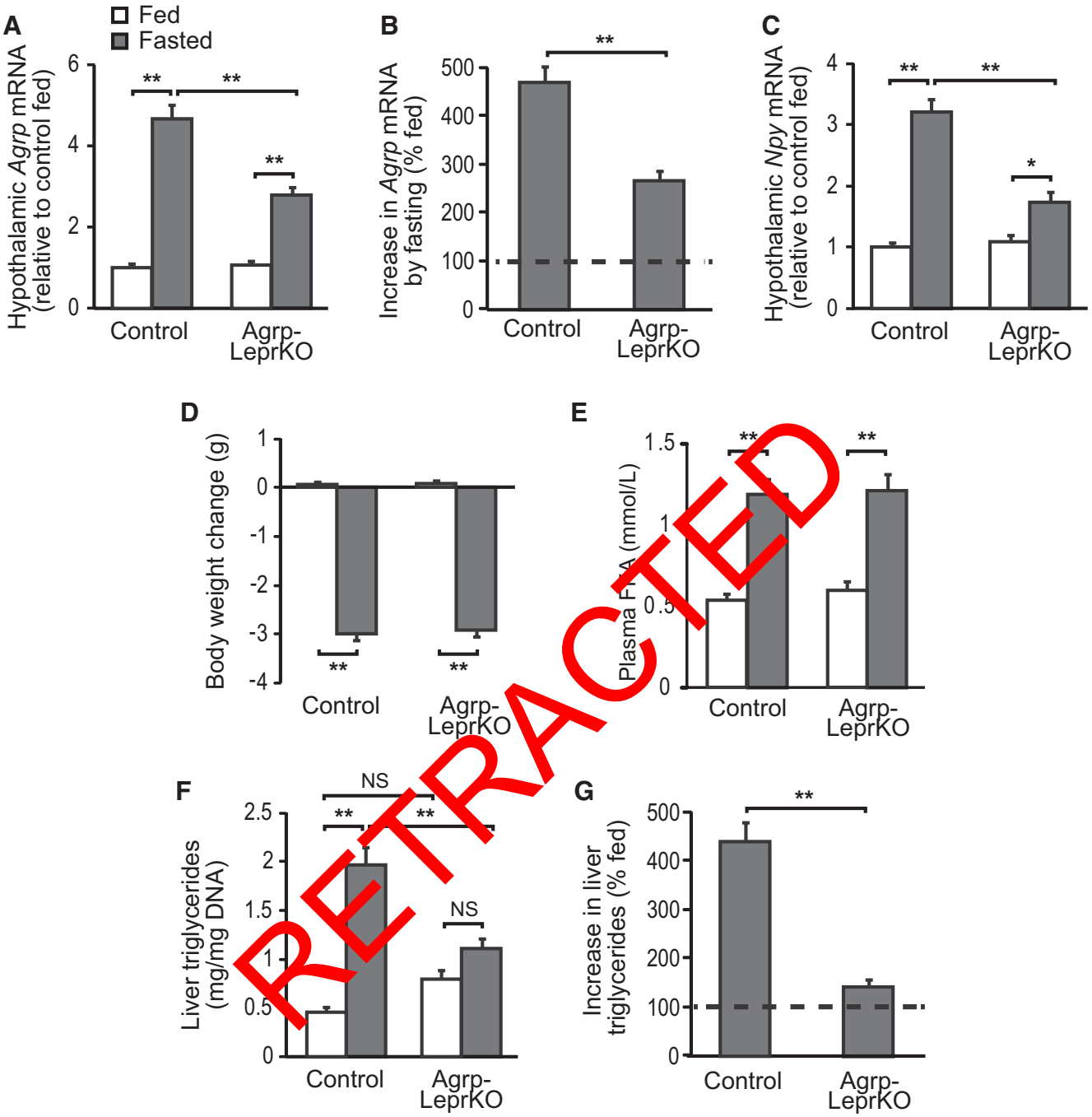

Figure 3. Mice lacking leptin receptors in AGRP neurons do not demonstrate fasting-induced hepatic steatosis. Five-week old weight-matched control and *Agrp-LeprKO* mutant mice either remained *ad libitum* fed or were fasted for 30 h ($n = 5$/group). ***A***, ***B***, Hypothalamic *Agrp* mRNA expression, expressed all relative to the control, fed group (***A***) or with the fasted group expressed as a percentage of the fed group of that respective genotype (***B***). ***C***, Hypothalamic mRNA expression of *Npy*. ***D***, Body weight change over the 30 h treatment period. ***E***, Terminal plasma FFA concentrations. ***F***, ***G***, Liver triglyceride content expressed as the total tissue measurement (***F***) or with the fasted group expressed as a percentage of the fed group of that respective genotype (***G***). Data are shown as means ± SEM. *$p < 0.05$, **$p < 0.01$ as indicated (two-way ANOVA and Tukey's *post hoc* tests except ***B*** and ***G***, which used unpaired Student's *t* tests).

Using this assay, no differences in *in vitro* FFA release were detected from white adipose tissue explants obtained from fasted mice treated *in vivo* with either vehicle or leptin (Fig. 2*F*,*G*; *in vitro* basal release: *in vivo* treatment $F_{(2,9)} = 4.2$, $p = 0.05$; time $F_{(3,27)} = 125.2$, $p < 0.0001$; interaction $F_{(6,27)} = 3.8$, $p < 0.007$; *in vitro* isoprenaline treatment: *in vivo* treatment $F_{(2,9)} = 10.6$, $p = 0.0043$; time $F_{(3,27)} = 342.9$, $p < 0.0001$; interaction $F_{(6,27)} = 5.7$, $p = 0.0006$). These data indicate that the leptin-dependent changes in liver triglyceride accumulation during fasting are not the secondary result of differences in the rate of lipolysis in white adipocytes.

We next investigated whether intrahepatic alterations in lipid handling could account for the differences in triglyceride content. Secretion of VLDL from the liver, which was determined by the plasma accumulation of triglycerides after injection of the lipoprotein lipase inhibitor Triton WR-1339 (Tyloxapol), was lower in mice that were treated with leptin during fasting compared with concurrently fasted but vehicle-injected mice (Fig.

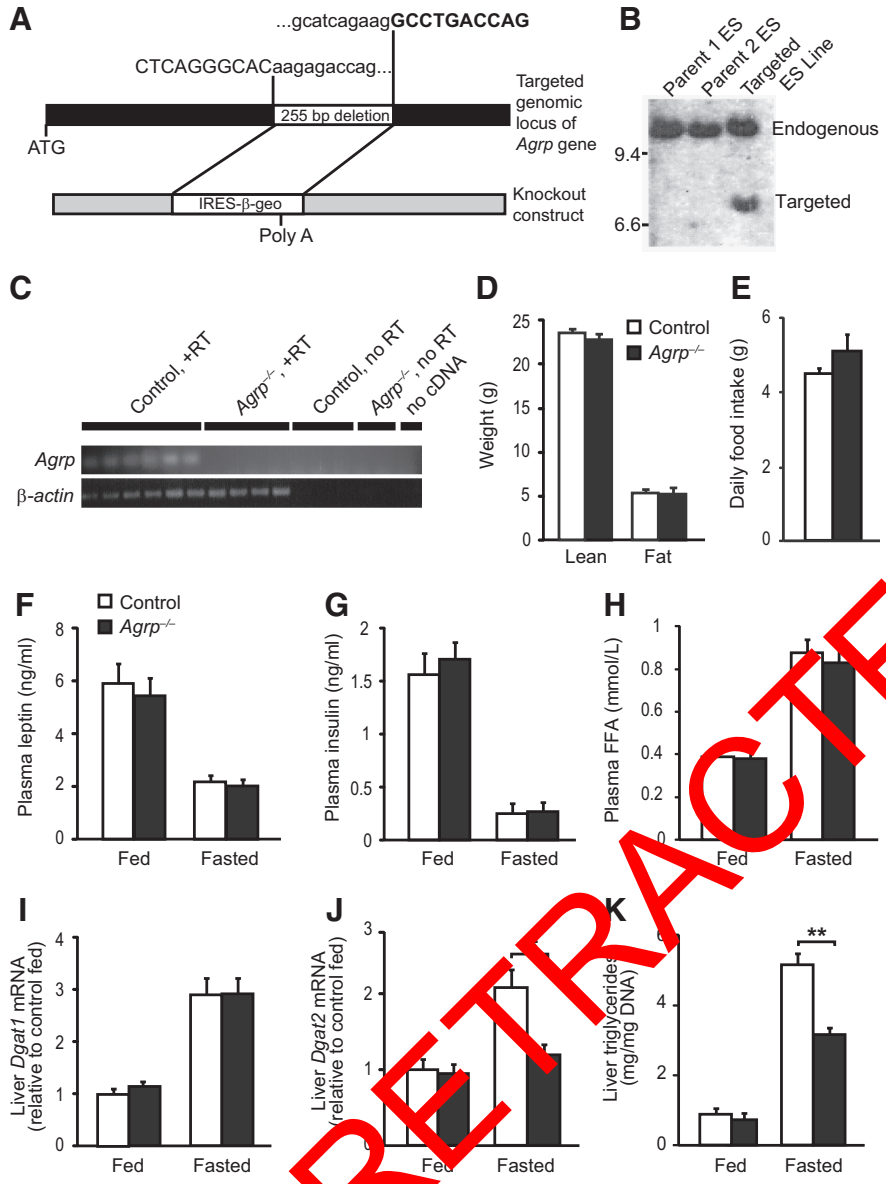

Figure 4. AGRP is required for the development of hepatic steatosis during starvation. ***A***, Diagrammatic representation of the genomic sequence of *Agrp* and the knock-out construct. ***B***, Confirmation of the targeting event from the homologous recombination event; Southern blot hybridization was performed on genomic DNA from ES cell lines that were digested by BamHI and probed with a radiolabeled DNA fragment that hybridizes outside of and adjacent to the knock-out construct arm. ***C***, Semiquantitative real-time PCR analyses of *Agrp* and β-actin mRNA in the hypotalami of control ($Agrp^{+/-}$, $n = 6$) and $Agrp^{-/-}$ ($n = 4$) mice; '+RT' indicates the presence of reverse transcriptase (RT) in the PCR; "no RT" indicates the lack of RT in the PCR. ***D***, Whole-body lean and fat mass of 12 week-old $Agrp^{-/-}$ mice and littermate controls ($n = 6–9$/group). ***E***, Twenty-four-hour *ad libitum* food intake of 12-week-old mice ($n = 5–8$/group). ***F–K***, Control and $Agrp^{-/-}$ mice were either *ad libitum* fed or fasted for 30 h ($n = 5–6$/group). Shown are plasma concentrations of leptin (***F***), insulin (***G***), and FFA (***H***) and liver *Dgat1* mRNA expression (***I***), *Dgat2* mRNA expression (***J***), and triglyceride levels (***K***) at the end of the 30 h experiment. Data are shown as means ± SEM. $^{**}p < 0.01$ as indicated (for ***D***, ***E***, unpaired Student's *t* tests; for ***F–K***, two-way ANOVA followed by Tukey's *post hoc* tests).

2*H*; significant effects of intraperitoneal treatment $F_{(1,8)} = 70.2$, $p < 0.0001$; time $F_{(3,24)} = 420.1$, $p < 0.0001$; interaction $F_{(3,24)} = 5.8$, $p = 0.004$). This suggests that the reduction in liver triglyceride content with leptin replacement was not the result of increased VLDL output. We then examined hepatic triglyceride synthesis in response to fasting. The terminal and only committed step in this process is catalyzed by two acyl CoA:diacylglycerol acyltransferase enzymes, DGAT1 and DGAT2 (Cases et al., 1998; Cases et al., 2001). The mRNA expression of both *Dgat1* (Fig. 2*I*; $F_{(2,12)} = 47.9$, $p < 0.0001$) and *Dgat2* (Fig. 2*J*; $F_{(2,12)} = 18.0$, $p = 0.0002$) was increased in the liver after 30 h of fasting. Interestingly, leptin treatment did not affect the increase in hepatic *Dgat1* mRNA expression in response to fasting, but completely prevented the increase in *Dgat2* mRNA expression (Fig. 2*I*,*J*).

To determine whether the effects of changing leptin levels on liver triglyceride content are centrally mediated, we injected leptin or vehicle (aCSF) into the lateral ventricle of the brains of wild-type mice three times during a 30 h fast. Mice that received intracerebroventricular leptin during fasting did not show any differences in plasma leptin, insulin, or FFA concentrations, prefasting or postfasting body weight, or liver *Dgat1* mRNA expression compared with vehicle-injected mice, but exhibited significantly lower liver triglyceride content and *Dgat2* mRNA expression (Fig. 2*K–P*), phenocopying the effects of peripheral leptin administration. These findings demonstrate that preventing plasma leptin concentrations from falling during fasting, either by administration of exogenous leptin to wild-type mice or on examining mice lacking leptin altogether ($Lep^{ob/ob}$), significantly attenuates the development of fasting-induced hepatic steatosis. These results suggest that the capacity to downregulate central leptin action is required for the full development of hepatic steatosis with prolonged fasting.

Removal of leptin receptors from AGRP neurons diminishes fasting-induced hepatic steatosis

Expression of AGRP is dramatically increased upon fasting, an effect that can be prevented by exogenous leptin treatment (Baskin et al., 1999b; Ebihara et al., 1999; Mizuno and Mobbs, 1999; Wilson et al., 1999; Korner et al., 2001; Takahashi and Cone, 2005). To determine whether direct leptin action on AGRP neurons is important for the development of fasting-induced hepatic steatosis, we generated mice in which leptin receptors were specifically removed from AGRP neurons (*Agrp-LeprKO* mice). Because such deletion of leptin receptors from AGRP neurons results in obesity and alterations in fat metabolism (van de Wall et al., 2008), which could secondarily affect hepatic lipid accumulation, we chose to study 5-week-old preobese *Agrp-LeprKO* mutant mice that were weight matched to their littermate controls (control mice: 26.6 ± 0.4 g, mutant mice: 26.7 ± 0.2 g). Although hypothalamic *Agrp* mRNA expression was not different between free-fed control and mutant mice, fasting-induced *Agrp* upregulation was significantly attenuated in the mutant mice (Fig. 3*A*,*B*; significant effects of genotype [$F_{(1,16)} = 18.4$, $p = 0.0006$], fasting [$F_{(1,16)} = 160.5$, $p < 0.0001$], and a

genotype-fasting interaction [$F_{(1,16)} = 20.5$, $p = 0.0003$]), suggesting that reduced leptin signaling in AGRP neurons during fasting is important for the upregulation in *Agrp* mRNA expression. Similar patterns were observed for hypothalamic *Npy* mRNA expression (Fig. 3*C*; significant effects of genotype [$F_{(1,16)} = 19.8$, $p = 0.0004$], fasting [$F_{(1,16)} = 84.7$, $p < 0.0001$], and a genotype-fasting interaction [$F_{(1,16)} = 25.1$, $p = 0.0001$]). Control and *Agrp-LeprKO* mutant mice showed similar fasting-induced changes in body weight (Fig. 3*D*; significant effect of fasting [$F_{(1,16)} = 743.4$, $p < 0.0001$]) and plasma FFA concentrations (Fig. 3*E*; significant effect of fasting [$F_{(1,16)} = 68.2$, $p < 0.0001$]); however, the mutant mice exhibited a blunted ability to increase liver triglyceride content with fasting (Fig. 3*F*,*G*, significant effects of genotype [$F_{(1,16)} = 5.4$, $p = 0.03$], fasting [$F_{(1,16)} = 64.5$, $p < 0.0001$], and a genotype-fasting interaction [$F_{(1,16)} = 28.2$, $p < 0.0001$]). These results suggest that direct leptin action on AGRP neurons is important for the development of fasting-induced hepatic steatosis.

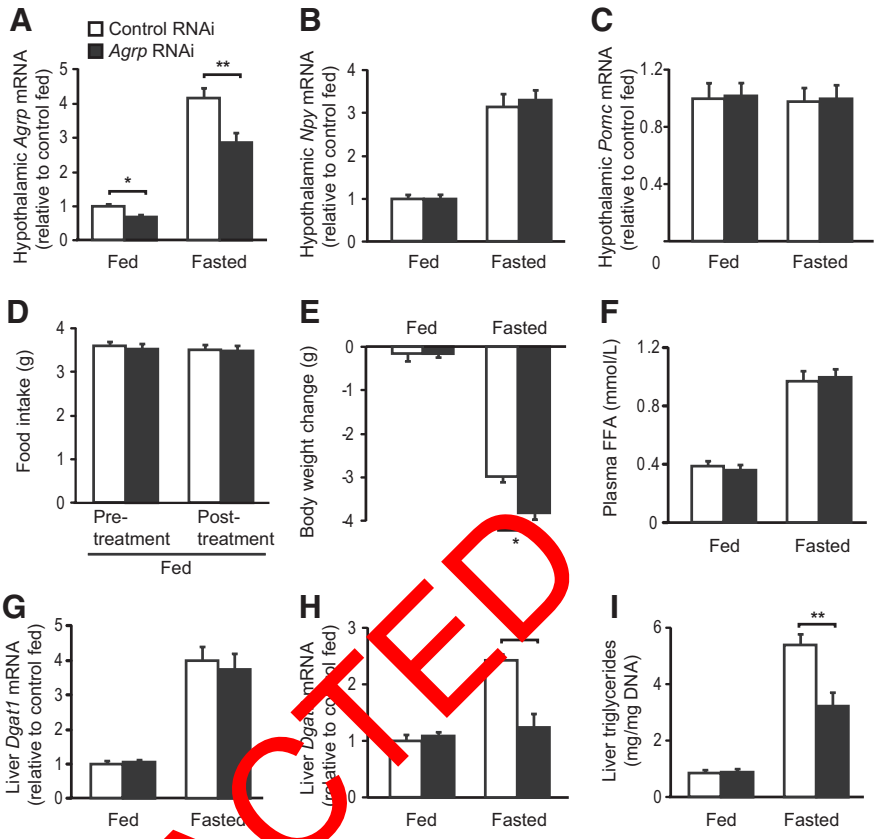

Figure 5. Knock-down of *Agrp* mRNA expression using RNAi attenuates fasting-induced hepatic steatosis. Assessment of the effects of an intraperitoneal injection of a negative control DsiRNA (NC1) or a DsiRNA directed against *Agrp* in 10-week-old male mice that remained *ad libitum* fed for 30 h or were immediately fasted for 30 h ($n = 5$/group). The four groups of mice were weight matched before treatment and dietary manipulation. Hypothalamic mRNA expression of *Agrp* (***A***), *Npy* (***B***), and *Pomc* (***C***) at the end of the 30 h experiment confirmed that the DsiRNA treatment against *Agrp* only reduced *Agrp* mRNA expression. ***D***, Twenty-four-hour food intake of the *ad libitum* fed groups before and after DsiRNA treatment. ***E***, Body weight change over the 30 h experiment. ***F–I***, Terminal plasma FFA concentrations (***F***) and liver *Dgat1* mRNA expression (***G***), *Dgat2* mRNA expression (***H***), and triglyceride levels (***I***) at the end of the 30 h experiment. Data are shown as means ± SEM. *$p < 0.05$, **$p < 0.01$ as indicated (two-way ANOVA and Tukey's *post hoc* tests).

Agrp$^{-/-}$ mice have normal energy balance in the fed state but cannot increase hepatic fat content during prolonged fasting

Removal of leptin receptors from AGRP neurons could affect the function of all components of AGRP neurons, including AGRP, NPY, and GABA. To determine whether the AGRP neuropeptide is required for the development of fasting-induced hepatic steatosis, we generated mice that lack the *Agrp* gene (*Agrp*$^{-/-}$) by gene targeting (Fig. 4*C*). Because *Agrp*$^{-/-}$ mice were maintained on a mixed genetic background and differences in genetic background could cause significant phenotypic variations, it was important to compare mutants with their littermate controls to minimize the impact of genetic background variation. Therefore, we set up breeding between *Agrp*$^{-/-}$ and *Agrp*$^{+/-}$ mice so that 50% of the progeny were *Agrp*$^{-/-}$ mutants and the remaining littermates could serve as controls (*Agrp*$^{+/-}$). Consistent with a previous study using independently generated mice (Qian et al., 2002), the *Agrp*$^{-/-}$ mice generated for this study had a body composition and food intake that were similar to control littermates (Fig. 4*D*,*E*). Fasting for 30 h produced similar changes in plasma leptin, insulin, and FFA concentrations and liver *Dgat1* mRNA expression in control and *Agrp*$^{-/-}$ mice (Fig. 4*F–I*; significant effects of fasting [$F_{(1,17)} = 47.9$, $p < 0.0001$, $F_{(1,17)} = 94.5$, $p < 0.0001$, $F_{(1,17)} = 71.0$, $p < 0.0001$, $F_{(1,17)} = 59.9$, $p < 0.0001$, respectively], no significant effects of genotype or genotype-fasting interactions). However, the increase in liver *Dgat2* mRNA with prolonged fasting evident in control mice was not observed in *Agrp*$^{-/-}$ mice (Fig. 4*J*; significant effects of genotype [$F_{(1,17)} = 8.6$, $p = 0.009$], fasting [$F_{(1,17)} = 17.1$, $p = 0.0007$], and a genotype-fasting interaction [$F_{(1,17)} = 6.7$, $p = 0.02$]). Consistent with altered hepatic *Dgat2* expression in *Agrp*$^{-/-}$ mice, liver triglyceride levels with fasting were also significantly lower in *Agrp*$^{-/-}$ mice compared with control mice (Fig. 4*K*; significant effects of genotype [$F_{(1,17)} = 23.9$, $p = 0.0001$], fasting [$F_{(1,17)} = 225.8$, $p < 0.0001$], and a genotype-fasting interaction [$F_{(1,17)} = 17.4$, $p = 0.0006$]). These findings demonstrate that AGRP is required for the full development of starvation-induced hepatic steatosis.

Knock-down of *Agrp* mRNA expression by RNAi impairs fasting-induced hepatic steatosis in wild-type mice

To further confirm the importance of AGRP in regulating fasting-induced hepatic steatosis, we investigated whether knock-down of *Agrp* expression within the CNS by RNAi would reduce liver triglyceride levels in adult wild-type mice that had undergone a prolonged fast. RNAi using siRNA is an effective tool to suppress the expression of genes (Whitehead et al., 2009). Traditionally, siRNAs are chemically synthesized 21-mers with a central 19 bp duplex region and symmetric 2 base 3′ overhangs on each end. In contrast, dicer-substrate RNAs (DsiRNAs) are chemically synthesized 27-mer RNA duplexes that have increased potency compared with traditional siRNAs (Kim et al., 2005). We showed recently that the majority of AGRP neurons within the hypothalamus are in direct contact with the systemic circulation and can readily take up blood-borne substances (Olofsson et al., 2013), raising the unique possibility that a DsiRNA directed against *Agrp* could be administered by peripheral injection. Wild-

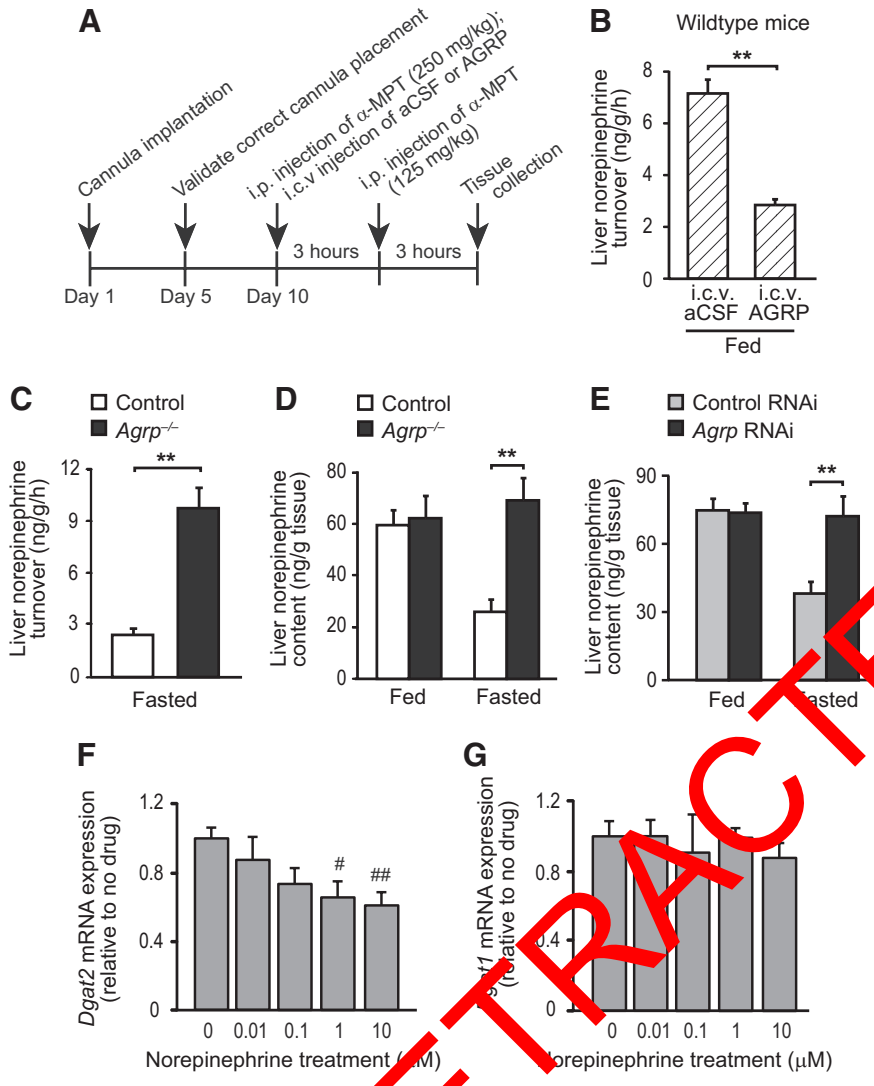

Figure 6. AGRP inhibits hepatic sympathetic tone and is required for suppression of hepatic sympathetic activity during starvation. ***A***, Experimental timeline of the data presented in ***B***. ***B***, Liver norepinephrine turnover rate in 10 week-old male wild-type mice treated intracerebroventricularly with either aCSF or AGRP ($n = 5$/group). ***C***, Liver norepinephrine turnover rate of 30-h-fasted control and *Agrp*$^{-/-}$ mice ($n = 5$/group). ***D***, Liver norepinephrine content of *ad libitum* fed or 30-h-fasted control and *Agrp*$^{-/-}$ mice ($n = 5$/group). ***E***, Liver norepinephrine content of wild-type mice treated with a negative control (NC1) or *Agrp*-specific DsiRNA and subsequently remaining *ad libitum* fed or fasted for 30 h ($n = 5$/group). ***F***, ***G***, *In vitro* liver explant mRNA expression of *Dgat2* (***F***) and *Dgat1* (***G***) after exposure for 2 h to no drug or to increasing concentrations of norepinephrine ($n = 4$/group). Data are shown as means ± SEM. $^{**}p < 0.01$ as indicated, $\#p < 0.05$, $\#\#p < 0.01$ compared with no drug (for ***B***, ***C***, unpaired Student's *t* tests; for ***D***, ***E***, two-way ANOVA and Tukey's *post hoc* tests; for ***F***, ***G***, one-way ANOVA and Tukey's *post hoc* tests).

type mice were injected intraperitoneally with a DsiRNA directed against either *Agrp* (5 μg per mouse) or directed against a negative control sequence and then immediately fasted for 30 h. A separate cohort of mice, matched for age, sex, and body weight, were similarly treated with the RNAi reagents but allowed to feed *ad libitum*. Hypothalamic mRNA expression of *Agrp* (Fig. 5*A*; significant effects of intraperitoneal treatment [$F_{(1,16)} = 16.7$, $p = 0.0009$] and fasting [$F_{(1,16)} = 169.2$, $p < 0.0001$]), but not *Npy* (Fig. 5*B*; only a significant effect of fasting [$F_{(1,16)} = 109.2$, $p = 0.0001$]) or proopiomelanocortin (*Pomc*; Fig. 5*C*), was reduced by this DsiRNA treatment. The extent to which this treatment knocked down *Agrp* expression may have been underestimated because the DsiRNA against *Agrp* was injected at the beginning of the 30 h experiment whereas *Agrp* mRNA expression was measured at the end. Treatment with the DsiRNA against *Agrp* did not affect food intake or body weight in mice that were free-fed (Fig. 5*D*,*E*), but did cause greater weight loss in mice that were fasted (Fig. 5*E*; significant effects of DsiRNA treatment [$F_{(1,16)} = 13.1$, $p = 0.002$], fasting [$F_{(1,16)} = 930.9$, $p < 0.0001$], and a treatment-fasting interaction [$F_{(1,16)} = 19.2$, $p = 0.0005$]). Treatment with a DsiRNA against *Agrp* did not alter plasma FFA concentrations or liver *Dgat1* mRNA expression (Fig. 5*F*,*G*; significant effects only of fasting [$F_{(1,16)} = 84.2$, $p < 0.0001$, $F_{(1,16)} = 127.9$, $p < 0.0001$, respectively]), but did result in significantly lower liver *Dgat2* mRNA expression and triglyceride content in mice that were fasted, but interestingly not in those that were *ad libitum* fed (Fig. 5*H*,*I*; significant effects of DsiRNA treatment [$F_{(1,16)} = 13.3$, $p = 0.002$, $F_{(1,16)} = 11.1$, $p = 0.004$, respectively], fasting [$F_{(1,16)} = 28.2$, $p < 0.0001$, $F_{(1,16)} = 16.6$, $p < 0.0001$, respectively], and a treatment-fasting interaction [$F_{(1,16)} = 17.$, $p = 0.0007$, $F_{(1,16)} = 11.8$, $p = 0.003$, respectively]). These results further strengthen the notion that upregulation of AGRP during fasting is required for the development of hepatic steatosis.

AGRP inhibits hepatic sympathetic activity and is required for fasting-induced suppression of sympathetic activity in the liver

Prolonged fasting leads to suppression of sympathetic activity in a number of peripheral tissues, including the liver, which is independent of adrenal medullary secretion (Young and Landsberg, 1977, 1979; Young et al., 1984; Landsberg, 2006). This finding has also been observed in human subjects (O'Dea et al., 1982; Young et al., 1984). Because AGRP is increased during fasting, we investigated whether increased central AGRP action leads to suppression of liver sympathetic activity. Hepatic sympathetic activity was determined by measuring the norepinephrine turnover (NETO) rate, which is calculated from the time-dependent decline in norepinephrine levels after administration of a tyrosine hydroxylase inhibitor (α-MPT) to prevent norepinephrine synthesis (Spector et al., 1965; Brodie et al., 1966). Wild-type mice were weight-matched before the injections. A single intracerebroventricular (i.c.v.) injection of AGRP resulted in a significantly lower hepatic NETO rate when compared with the vehicle injected group (Fig. 6*A*,*B*). Consistent with this result, NETO rates were significantly higher in the livers of fasted *Agrp*$^{-/-}$ mice than in fasted controls (Fig. 6*C*). These findings indicate that AGRP inhibits hepatic sympathetic activity and, accordingly, that AGRP is required for the suppression of hepatic sympathetic activity in starvation.

Norepinephrine inhibits *Dgat2* mRNA expression in liver explants

Liver norepinephrine content was lower after 30 h of fasting in control mice compared with that of *ad libitum* fed mice; however,

this difference was completely absent in *Agrp*$^{-/-}$ mice, resulting in abnormally high norepinephrine content in the livers of fasted *Agrp*$^{-/-}$ mice (Fig. 6*D*; significant effects of genotype [$F_{(1,17)} = 9.3$, $p = 0.007$] and a genotype-fasting interaction [$F_{(1,17)} = 7.3$, $p = 0.02$]). Similarly, compared with control DsiRNA treatment, knock-down of *Agrp* using DsiRNA did not alter hepatic norepinephrine content in free-fed mice, but did result in significantly greater hepatic norepinephrine content in fasted mice such that no fall in norepinephrine content was evident with fasting (Fig. 6*E*; significant effects of the DsiRNA treatment [$F_{(1,16)} = 6.9$, $p = 0.02$], fasting [$F_{(1,16)} = 9.2$, $p = 0.008$], and a treatment-fasting interaction [$F_{(1,16)} = 7.6$, $p = 0.01$]). To determine whether norepinephrine affects lipid synthesis in the liver directly, liver explants from wild-type mice were treated with increasing doses of norepinephrine (0–10 μM). Norepinephrine treatment caused a dose-dependent decrease in *Dgat2* ($F_{(4,15)} = 3.7$, $p = 0.03$), but not *Dgat1*, mRNA expression (Fig. 6*F*,*G*), suggesting that the decrease in liver sympathetic tone with fasting may stimulate liver *Dgat2* mRNA expression and, consequently, triglyceride synthesis.

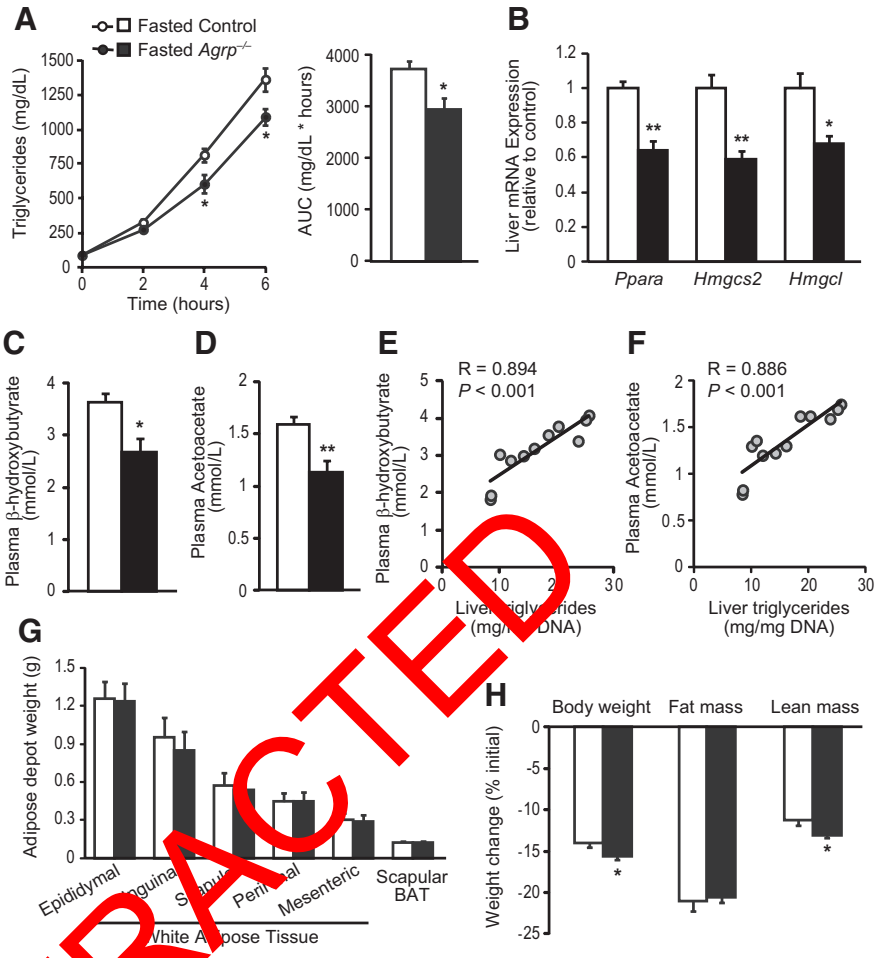

Figure 7. AGRP is required for the proper production of ketone bodies and release of VLDL from the liver during starvation. Control and *Agrp*$^{-/-}$ mice were starved for 36 h, after which time measurements were made ($n = 5$–6/group). ***A***, *In vivo* triglyceride release from the liver, presented as the plasma triglyceride concentrations every 2 h after injection of Tyloxapol and a histogram of the area under the curve (AUC) for the total 6 h experiment. ***B***–***D***, Liver mRNA expression of genes regulating (*Ppara*) and involved in (*Hmgcs2* and *Hmgcl*) ketogenesis and plasma concentrations of the ketone bodies (***B***), β-hydroxybutyrate (***C***), and acetoacetate (***D***) at the end of the 36 h fast. ***E***, ***F***, Positive correlation of plasma β-hydroxybutyrate (***E***) and acetoacetate (***F***) concentrations with 36-h-fasted liver triglyceride content. ***G***, Adipose depot weights at the end of the 36 h fast (BAT, brown adipose tissue). ***H***, Fasting-induced body weight, fat, and lean mass changes (expressed as a percentage of the initial values for each mouse). Data are shown as means ± SEM. $^*p < 0.05$, $^{**}p < 0.01$ as indicated (for ***A***, line graph, repeated-measures ANOVA; for ***A***, histogram, and ***B***–***D***, ***G***, ***H***, unpaired Student's *t* tests; for ***E***, ***F***, Pearson's correlations).

Agrp deficiency leads to impairment of energy release from the liver during prolonged fasting

Hepatic triglycerides are assembled into VLDL and secreted from the liver, and these lipoproteins are transported to extrahepatic tissues such as muscle for use as energy (Olpin, 2004). During starvation, ketone bodies are generated in the liver predominantly from the β-oxidation of fatty acids and serve as a vital energy source to many organs, including the brain. Liver triglycerides can also be hydrolyzed to fatty acids (Reid et al., 2008; Romeo et al., 2008; He et al., 2010; Wu et al., 2011), and stimulation of hepatic hydrolase activity leads to a marked increase in β-hydroxybutyrate, a vital ketone body (Reid et al., 2008). Our results show that AGRP is required for the increase in hepatic triglyceride stores in response to starvation. To gain insight into the physiological importance of this AGRP-dependent effect, we examined the capacity of *Agrp*$^{-/-}$ mice to produce and secrete energy substrates that can be used by other tissues during starvation. The release of VLDL from the liver was lower in fasted *Agrp*$^{-/-}$ mice compared with fasted controls (Fig. 7*A*; significant effects of genotype [$F_{(1,9)} = 8.5$, $p = 0.02$], time [$F_{(3,27)} = 257.6$, $p < 0.0001$], and a genotype-time interaction [$F_{(3,27)} = 4.5$, $p = 0.01$]). The mRNA expression of peroxisome proliferator-activated receptor α (*Ppara*), 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*Hmgcs2*), and 3-hydroxymethyl-3-methylglutaryl-CoA lyase (*Hmgc1*), genes involved in ketogenesis, was significantly lower in the livers of fasted *Agrp*$^{-/-}$ mice (Fig. 7*B*). Accordingly, plasma concentrations of the ketone bodies β-hydroxybutyrate and acetoacetate were significantly lower in fasted *Agrp*$^{-/-}$ mice and were closely correlated with the lower liver triglyceride content of these mice (Fig. 7*C*–*F*). In contrast, fasting blood glucose concentrations were not significantly different between genotypes (control mice, 43.2 ± 1.4 mg/dL; *Agrp*$^{-/-}$ mice, 41.8 ± 2.4 mg/dL, $p = 0.64$). Although no significant differences in total fasting-induced fat mass loss or individual white and brown fat pad weights were observed between fasted control and *Agrp*$^{-/-}$ mice, a greater loss of lean mass and body weight was observed in *Agrp*$^{-/-}$ mice with fasting (Fig. 7*G*,*H*). These results suggest that AGRP-dependent development of hepatic steatosis in starvation is important to sustain the levels of liver-derived energy substrates.

AGRP mediates the effects of leptin on hepatic lipid metabolism during starvation

We next sought to determine whether AGRP acts coordinately, downstream of leptin action, to control hepatic lipid metabolism during prolonged fasting. *Agrp*$^{-/-}$ and control mice were fasted for 30 h, during which time they were administered leptin or vehicle intraperitoneally. Leptin treatment increased plasma leptin concen-

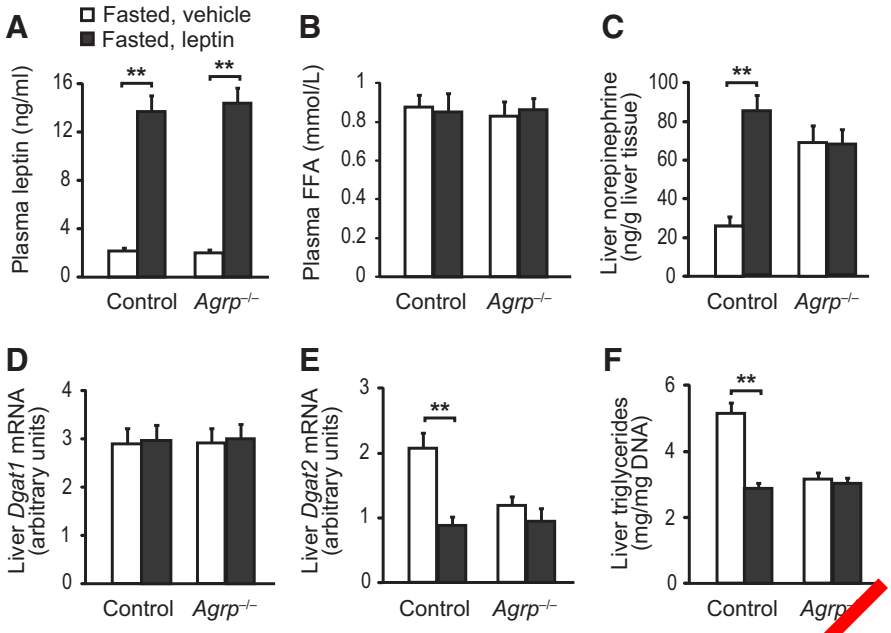

Figure 8. The effects of leptin on hepatic lipid metabolism during starvation are mediated by AGRP. Comparison of 12-week-old male control and *Agrp* $^{-/-}$ mice after a 30 h fast during which time mice received three intraperitoneal injections of either vehicle or leptin ($n = 5$/group). Measurements were made from tissues at the end of the 30 h fast. ***A***, ***B***, Plasma concentrations of leptin (***A***) and FFA (***B***). ***C–F***, Liver norepinephrine content (***C***), *Dgat1* mRNA expression (***D***), *Dgat2* mRNA (***E***), and triglyceride content (***F***). Data are shown as means ± SEM. $^*p < 0.05$, $^{**}p < 0.01$ as indicated (two-way ANOVA and Tukey's *post hoc* tests).

trations to similar degrees in control and *Agrp* $^{-/-}$ mice (Fig. 8*A*; significant effect of intraperitoneal treatment [$F_{(1,16)} = 193.5$, $p <$ 0.0001]) but did not affect plasma FFA concentrations in either genotype (Fig. 8*B*). Although leptin treatment increased liver norepinephrine levels (significant effects of intraperitoneal treatment [$F_{(1,16)} = 13.9$, $p = 0.002$] and a treatment-genotype interaction [$F_{(1,16)} = 15.34$, $p = 0.001$]) and reduced hepatic *Dgat2* (significant effects of intraperitoneal treatment [$F_{(1,16)} = 16.4$, $p = 0.0009$], genotype [$F_{(1,16)} = 5.4$, $p = 0.03$], and a treatment-genotype interaction [$F_{(1,16)} = 7.04$, $p = 0.02$]), but not *Dgat1* mRNA expression, or triglyceride levels (significant effects of intraperitoneal treatment [$F_{(1,16)} = 26.4$, $p < 0.0001$], genotype [$F_{(1,16)} = 15.4$, $p = 0.001$], and a treatment-genotype interaction [$F_{(1,16)} = 21.1$, $p = 0.0003$]) in fasted control mice, it was ineffective in producing these effects in fasted *Agrp* $^{-/-}$ mice (Fig. 8*C–F*). These results suggest that during prolonged fasting, leptin exerts its effects on hepatic lipid metabolism by modulating AGRP expression.

Knock-down of *Agrp* expression mitigates HFD-induced hepatic steatosis

It has been shown that hepatic steatosis is rapidly induced within 3 d of high-fat feeding (Samuel et al., 2004). The substantial reduction in fasting-induced hepatic steatosis seen in AGRP deficiency raised the possibility that this pathway could be manipulated to alleviate hepatic steatosis associated with diet-induced obesity. We therefore investigated whether acute knock-down of *Agrp* by RNAi could prevent the early development of HFD-induced hepatic steatosis in wild-type mice. C57BL/6J mice were placed on an *ad libitum* HFD or LFD and were subsequently injected with a DsiRNA directed against *Agrp* or a negative control sequence on the second and fourth days. Mice were killed after 6 d of dietary treatment. Regardless of diet, treatment with DsiRNA against *Agrp* resulted in significantly lower hypothalamic *Agrp* mRNA expression (Fig. 9*A*; effect of treatment [$F_{(1,16)} = 18.0$, $p = 0.0007$], no significant effect of diet or diet-treatment interaction) without affecting hypothalamic *Npy* or *Pomc* expression (Fig. 9*B*,*C*). Mice treated with control or *Agrp*-specific RNAi exhibited similar increases in body weight (Fig. 9*D*; effect of diet $F_{(1,16)} = 103.4$, $p < 0.0001$), fat pad weight (Fig. 9*E*; effect of diet $F_{(1,16)} = 187.83$, $p < 0.0001$), and plasma insulin concentrations (Fig. 9*F*; effect of diet $F_{(1,16)} = 15.9$, $p = 0.001$) after HFD feeding, but showed no significant changes in plasma FFA concentrations (Fig. 9*G*). Treatment with *Agrp*-specific DsiRNA resulted in higher hepatic norepinephrine content (Fig. 9*H*; effects of DsiRNA treatment $F_{(1,16)} = 7.1$, $p = 0.02$ and a treatment-diet interaction $F_{(1,16)} = 4.9$, $p = 0.04$), unaltered *Dgat1* mRNA expression (Fig. 9*I*; only a significant effect of diet $F_{(1,16)} = 56.4$, $p < 0.0001$), lower hepatic *Dgat2* mRNA expression (Fig. 9*J*; significant effects of DsiRNA treatment [$F_{(1,16)} = 4.110$, $p = 0.061$], diet [$F_{(1,16)} = 14.47$, $p = 0.0002$], and a treatment-diet interaction [$F_{(1,16)} = 4.038$, $p = 0.0628$]) and liver triglyceride content (Fig. 9*K*; significant effects of treatment [$F_{(1,16)} = 6.191$, $p = 0.025$], diet [$F_{(1,16)} = 19.88$, $p = 0.0005$], and a treatment-diet interaction [$F_{(1,16)} = 4.038$, $p = 0.0628$]) in the HFD-fed, but not LFD-fed, mice. This result opens the possibility that suppression of *Agrp* expression using RNAi could be used to alleviate obesity-associated hepatic steatosis.

Discussion

In this study, we explored the physiological role and therapeutic potential of the leptin-AGRP axis in the regulation of hepatic triglyceride storage. Our results highlight several key points. First, we present evidence that, in addition to the increased influx of FFA to the liver, the decline in plasma leptin concentrations and the consequent increase in AGRP expression in the brain is an important determinant in the development of starvation-induced hepatic steatosis. Genetic deletion of *Agrp* leads to attenuation of hepatic steatosis induced by prolonged fasting and insufficient release of energy substrates from the liver. These data suggest that the development of hepatic steatosis in starvation is not simply the result of the passive accumulation of excess circulating FFA. Rather, the increase in hepatic triglyceride stores during starvation is an important adaptive response under CNS control and is necessary to sustain the supply of energy from the liver to extrahepatic tissues to meet the energy demands of these tissues. Our data suggest that the reduced energy supply from the liver during starvation in *Agrp* $^{-/-}$ mice may lead to increased muscle wasting, an adverse effect on health and survival if food deprivation were to persist. Intrahepatic lipid content increases in healthy, nonobese human male subjects during fasting and is positively correlated with the concentration of plasma β-hydroxybutyrate (Moller et al., 2008), similar to what was observed in this study. However, it is difficult to compare the magnitude of hepatic steatosis directly in mice and humans fasting for similar durations. A typical 8-week-old male mouse loses 10–13% of its body weight after an overnight (16 h) fast. However, in humans, 4–6 d of total starvation are needed to achieve a 5%

weight loss (Hoggard et al., 2004). Furthermore, responses to fasting are altered by preexisting obesity in humans (Hoggard et al., 2004).

It is well established that overexpression of AGRP stimulates food intake (Ollmann et al., 1997; Rossi et al., 1998; Ebihara et al., 1999) and that acute activation of AGRP neurons evokes feeding (Aponte et al., 2011; Krashes et al., 2011). However, recent studies suggest that AGRP itself may not be necessary for the evoked feeding responses elicited by acute activation of AGRP neurons (Aponte et al., 2011). Instead, NPY and GABA, both components of AGRP neurons (Broberger et al., 1998; Cowley et al., 2001), are likely required for evoked feeding responses (Atasoy et al., 2012). Similarly, the anorectic response caused by acute ablation of AGRP neurons has been attributed to a melanocortin-independent, GABA-dependent mechanism (Wu et al., 2008; Wu et al., 2009; Wu et al., 2012). Consistent with this notion, transgenic mice with chronic defects in GABA release by AGRP neurons show altered energy balance (Tong et al., 2008), whereas *Agrp*$^{-/-}$ mice have normal feeding, body adiposity, plasma leptin, insulin, and FFA levels (this study) and respond normally to the anorectic effects of leptin and refeed normally after a fast (Qian et al., 2002). Therefore, the effects of AGRP on body weight can be readily compensated by other mechanisms. The existence of compensatory regulation is illustrated by the findings that acute killing of the AGRP neurons in adulthood results in severe weight loss (Luquet et al., 2005), whereas killing of these neurons in neonates, a critical period in hypothalamic development, causes obesity and hyperinsulinemia (Joly-Amado et al., 2012). In this study, we show that *Agrp*$^{-/-}$ mice exhibit impaired hepatic steatosis development and are completely unresponsive to the effects of leptin on hepatic lipid metabolism during prolonged food deprivation. Although life-long absence of the AGRP could promote compensatory regulation of liver lipid metabolism, acute knock-down of *Agrp* with RNAi in fasted wild-type mice recapitulates the phenotypes seen in *Agrp*$^{-/-}$ mice, arguing against this possibility. Therefore, our results indicate that the AGRP neuropeptide plays an indispensable role in hepatic lipid metabolism during starvation. Future experiments using cellular lipidomics, stable isotope tracer methods, and mass spectrometry will help in our understanding of how cellular and tissue lipid partitioning and hepatic energy substrate fluxes are affected in *Agrp*$^{-/-}$ mice.

AGRP neurons are uniquely located in the mediobasal hypothalamus adjacent to a circumventricular organ, the median eminence, where fenestrated blood vessels are present (Ciofi et al., 2009; Mullier et al., 2010; Morita and Miyata, 2012). This property enables AGRP neurons to be more responsive to blood-borne metabolic signals such as leptin (Olofsson et al., 2013). AGRP neurons project to multiple regions of the brain, including the arcuate nucleus (notably onto POMC neurons), paraventricular nucleus, dorsomedial hypothalamus, lateral hypothalamic area, and the parabrachial nucleus of the hindbrain (Cowley et al., 1999; Elias et al., 1999; Wilson et al., 1999; Cowley et al., 2001; Wu et al., 2009). Therefore, modulation of AGRP function could affect many of its target neurons, and the functional integrities of these higher order neuronal circuits are likely to be important for proper regulation of AGRP neurons by leptin. Although AGRP and α-MSH act on the same receptors, AGRP may play a more dominant role during starvation because *Agrp* mRNA expression increases markedly, whereas *Pomc* mRNA expression is only moderately reduced (Baskin et al., 1999a; Mizuno et al., 1999; Wilson et al., 1999). It is intriguing that *Agrp*$^{-/-}$ mice have an impaired ability to modulate hepatic sympathetic activity, *Dgat2* expression, and triglyceride levels in the fasted

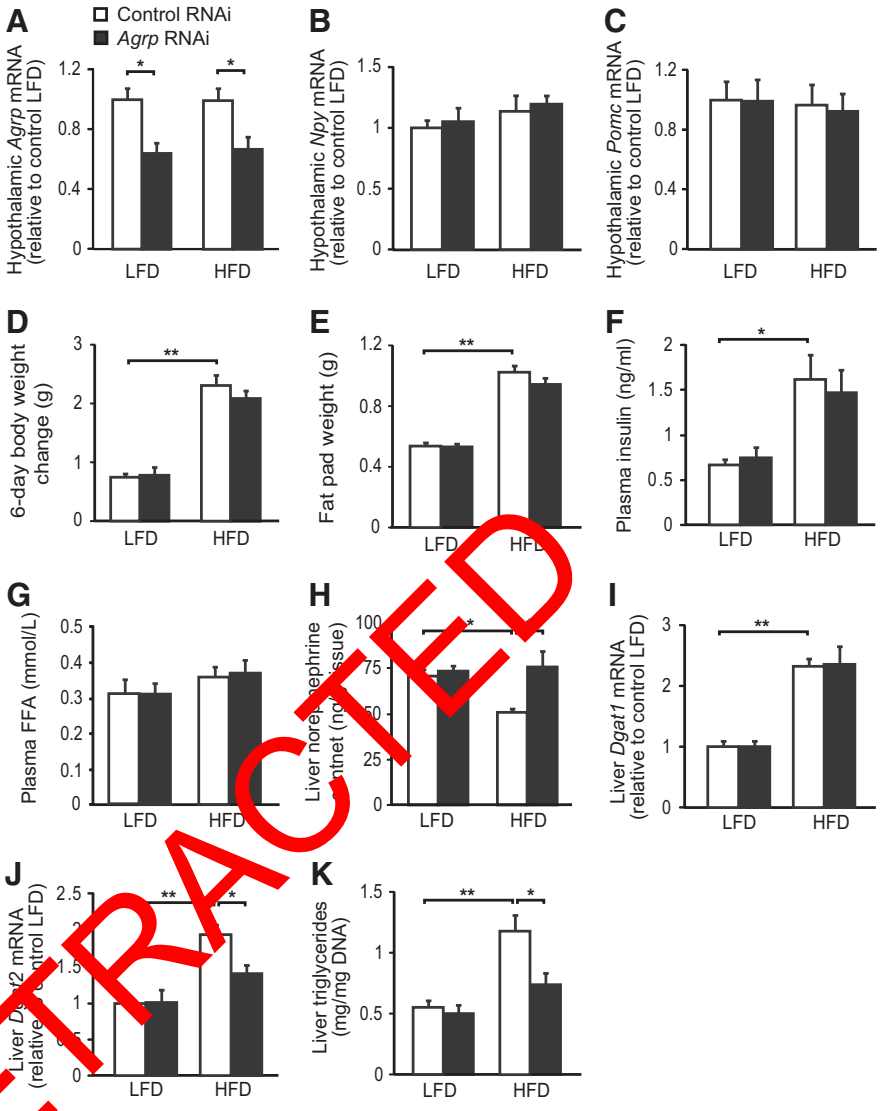

Figure 9. Knock-down of *Agrp* expression using RNAi attenuates HFD-induced hepatic steatosis. Comparison of the effects of 2 injections spaced 2 d apart of a control DsiRNA or a DsiRNA directed against *Agrp* in 8-week-old male mice ($n = 5$/group) in mice previously free-fed with a HFD or LFD for 2 d and subsequently maintained on the HFD or LFD with unrestricted access for a further 4 d. All groups were weight matched before diet change. ***A–C***, Hypothalamic mRNA expression of *Agrp* (***A***), *Npy* (***B***), and *Pomc* (***C***) at the end of the experiment. ***D***, Body weight change over the 6 d experiment. ***E–G***, Measurements of total white adipose tissue depot weight (***E***) and plasma concentrations of insulin (***F***) and FFA (***G***) at the end of the 6 d experiment. ***H–K***, Liver norepinephrine content (***H***), *Dgat1* expression (***I***), *Dgat2* mRNA expression (***J***), and triglyceride content (***K***) at the end of the experiment. Data are presented as the means ± SEM. *$p < 0.05$, **$p < 0.01$ as indicated (two-way ANOVA and Tukey's *post hoc* tests).

state, but these parameters appear largely normal in the fed state. *Agrp* expression is low in the fed state, but increases dramatically upon fasting, concomitant with and dependent on the decline in plasma leptin levels (Baskin et al., 1999b; Mizuno and Mobbs, 1999; Wilson et al., 1999; Takahashi and Cone, 2005). These observations suggest that AGRP function is more important during starvation, when body's energy reserves are diminishing, and that one key function of this pathway is to increase hepatic triglyceride content to provide energy substrate for ketone body and VLDL formation.

It should be noted that intracerebroventricular administration of AGRP or melanocortin receptor antagonists suppresses sympathetic nerve activity in white adipose tissue (Egawa et al., 1991; Yasuda et al., 2004; Brito et al., 2007; Nogueiras et al., 2007; Chao et al., 2011), similar to what we have observed with AGRP action on hepatic sympathetic activity. However, body fat is mobilized normally upon food deprivation in animals with significant reduction in AGRP (Leitner and Bartness, 2008). Furthermore, starvation-induced increases in lipid mobilization still proceed normally in chronically decerebrate rats in which all forebrain structures are disconnected from the brainstem, thereby blocking forebrain descending sympathetic outflow to white adipose tissue (Harris et al., 2006). Consistent with these results, we did not detect any differences in circulating FFA concentrations in $Agrp^{-/-}$ mice compared with controls under either fed or fasting conditions, nor in fasted wild-type mice treated with *Agrp*-specific or control DsiRNA sequences. These results suggest that defects in hepatic lipid metabolism in $Agrp^{-/-}$ mice are not due to defects in FFA release from white adipose tissue.

A hallmark feature of common obesity, including diet-induced obesity, is leptin resistance. We have shown that impairment of brain leptin signaling is a causal factor for the development of hepatic steatosis in free-fed mice independent of hyperphagia or obesity (Warne et al., 2011). However, the parallels between prolonged fasting and obesity are complicated by the fact that *Agrp* mRNA expression is dramatically upregulated with fasting but not with HFD feeding. Nevertheless, increased AGRP neuronal firing rate (Diano et al., 2011), AGRP innervation onto target neurons in the hypothalamus (Newton et al., 2013), and diminished ability of leptin to inhibit AGRP release (Li et al., 2000; Breen et al., 2005; Enriori et al., 2007) have each been documented in diet-induced obese mice. Consistent with these findings, AGRP immunoreactivity shows a U-shaped correlation with BMI in humans, with the highest AGRP expression in the lowest and the highest BMI quartiles (Alkemade et al., 2012). Therefore, obesity may be associated with an increase in AGRP function that is not reflected by changes in *Agrp* mRNA expression.

It is generally thought that the development of hepatic steatosis is an early sign of liver damage, which can progress to nonalcoholic steatohepatitis, cirrhosis, and end-stage liver diseases (Browning and Horton, 2004). There is considerable debate over the direct association between hepatic steatosis and insulin resistance (Monetti et al., 2007; Jornayvaz et al., 2011). Despite this, leptin treatment improves insulin sensitivity in congenital lipodystrophy, a condition associated with severe hepatic steatosis, and is regulated via mechanisms that are independent of food intake (Shimomura et al., 1999). Recent evidence shows that leptin improves hepatic lipid metabolism in mouse model of lipodystrophy through modulation of the sympathetic nervous system (Miyamoto et al., 2012), supporting the notion that targeting the CNS–liver pathway could have beneficial health effects. Nonalcoholic fatty liver disease affects >30% of the Western population (Browning et al., 2004). Our data show that knock-down of *Agrp* expression by peripheral administration of RNAi in wild-type mice is effective at reducing hepatic steatosis induced by starvation or HFD consumption, raising the possibility that AGRP is an accessible brain target for therapeutic intervention for the treatment of nonalcoholic fatty liver disease.

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