AgRP Neurons Require Carnitine Acetyltransferase to Regulate Metabolic Flexibility and Peripheral Nutrient Partitioning

**Highlights**
- Crat in AgRP neurons helps nutrient replenishment as food is available after fasting
- Crat in AgRP neurons helps maintain appropriate hepatic glucose production during fasting
- Crat in AgRP neurons facilitates peripheral substrate switching upon acute refeeding
- Crat regulates protein expression and acetylation in AgRP cells in fasting and refeeding

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**In Brief**
Reichenbach et al. demonstrate that AgRP neurons require carnitine acetyltransferase to regulate peripheral substrate switching from fatty acid to glucose utilization during fasting and the transition to refeeding. This mechanism preserves fatty acids when glucose from food becomes available and maximizes energy conservation for future periods of energy deficit.
AgRP Neurons Require Carnitine Acetyltransferase to Regulate Metabolic Flexibility and Peripheral Nutrient Partitioning

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SUMMARY

AgRP neurons control peripheral substrate utilization and nutrient partitioning during conditions of energy deficit and nutrient replenishment, although the molecular mechanism is unknown. We examined whether carnitine acetyltransferase (Crat) in AgRP neurons affects peripheral nutrient partitioning. Crat deletion in AgRP neurons reduced food intake and feeding behavior and increased glycerol supply to the liver during fasting, as a gluconeogenic substrate, which was mediated by changes to sympathetic output and peripheral fatty acid metabolism in the liver. Crat deletion in AgRP neurons increased peripheral fatty acid substrate utilization and attenuated the switch to glucose utilization after refeeding, indicating altered nutrient partitioning. Proteomic analysis in AgRP neurons shows that Crat regulates protein acetylation and metabolic processing. Collectively, our studies highlight that AgRP neurons require Crat to provide the metabolic flexibility to optimize nutrient partitioning and regulate peripheral substrate utilization, particularly during fasting and refeeding.

INTRODUCTION

The maintenance of energy and glucose homeostasis requires the ability of the brain to detect and respond to acute changes in energy state. Under conditions of energy deficit, sensing low peripheral energy availability and hypoglycemia primes the brain to initiate food intake and increase hepatic glucose production (Kleinridders et al., 2009). Agouti-related peptide (AgRP) neurons in the arcuate nucleus of the hypothalamus are a population of starvation-sensitive neurons that primarily function to increase appetite and promote energy conservation (Aponte et al., 2011; Gropp et al., 2005; Krashes et al., 2011; Luquet et al., 2005; Ruan et al., 2014). For example, adult ablation of AgRP neurons in mice causes a rapid cessation of food intake and profound body weight loss to the point of starvation (Luquet et al., 2005).

AgRP neurons primarily respond to signals of energy deficit and increase firing rates, mRNA expression, and peptide secretion (Andrews et al., 2008; Baskin et al., 1999; Briggs et al., 2011; Schwartz et al., 1998) to maintain energy homeostasis. Although AgRP neurons can respond to metabolic information in the form of hormonal feedback from ghrelin, leptin, and insulin (Andrews, 2011; Andrews et al., 2008; Ennor et al., 2007; Könner et al., 2007; van de Wall et al., 2008; Wang et al., 2013), the mechanisms underlying nutrient sensing are less clear. This is not only important during fasting but also during the transition from fasting to refeeding when there is a dynamic change in metabolic feedback information to the brain. An appropriate level of metabolic flexibility ensures that, as glucose becomes available for oxidation, such as during refeeding, high fasting fatty acid utilization and oxidation are immediately suppressed to conserve energy reserves. Indeed, the ablation of AgRP neurons increases fatty acid utilization, affects nutrient partitioning, and attenuates body weight gain in mice fed a high-fat diet (Joly-Amado et al., 2012). Thus, the ability of AgRP neurons to rapidly adjust substrate utilization promotes maximal energy conservation and is a core component of nutrient partitioning. However, the molecular mechanism responsible for this action in AgRP neurons remains unknown.

Carnitine acetyltransferase (Crat) is an important enzyme regulating metabolic flexibility in muscle and increases exercise capacity (Mutio et al., 2012; Seiler et al., 2015). Crat is a fundamental enzyme in carnitine metabolism and is one of a number of
enzymes, including carnitine palmitoyltransferase 1 (CPT1) and carnitine octanoyltransferase, involved in regulating intracellular pools of acyl-coenzyme A (CoA) esters (Ramsay and Zammit, 2004; Stark et al., 2015a). In mice with muscle-specific deletion of Crat, excessive buildup of acetyl-CoA allosterically inhibits glucose oxidation by suppressing pyruvate dehydrogenase activity and impairs the substrate switch from fatty acid to glucose oxidation during the fast/reefading transition (Muoio et al., 2012). Consequently, we hypothesized that Crat plays an important role in AgRP neurons by providing the metabolic flexibility to respond to metabolic information, particularly during fasting and the transition to refeeding, when the need to detect and respond to energy availability is most important. Our results show that Crat in AgRP neurons influences feeding behavior, hepatic substrates for gluconeogenesis and fatty acid metabolism, peripheral substrate utilization and nutrient partitioning, and the AgRP proteome during fasting and the transition to refeeding.

RESULTS

Validation of Crat Deletion in AgRP Neurons

We used a cre-lox approach to delete Crat from AgRP neurons. Crat-floxed mice (Muoio et al., 2012) were crossed to AgRP-ires-cre::NPY GFP mice to generate AgRP Crat wild-type (WT) and knockout (KO) mice that expressed GFP in neuropeptide Y (NPY) neurons. To confirm cre recombination in the arcuate (ARC) nucleus, WT and KO mice were crossed to rosa(26) tdTomato reporter mice (Figures 1A–1E) and we observed >90% of NPY GFP neurons expressing the AgRP cre-dependent tdTomato reporter (Figure 1D). We used a fluorescence-activated cell sorting (FACS) approach to isolate GFP-positive NPY neurons in the ARC coupled with nested PCR to confirm the deletion of Crat in NPY (AgRP) GFP-positive neurons. NPY GFP neurons from WT (n = 5) mice showed expected RNA transcripts for Crat at ~500 bp, whereas no Crat mRNA transcripts were observed in KO mice (n = 5) (Figure 1F). A stereological analysis showed no genotype differences in total NPY GFP neuronal number and cell volume at post-natal day 14 (P14), confirming that there were no adverse developmental effects on NPY cell number and volume in the ARC (Figures 1G–1I). No differences were observed in AgRP fiber density in the paraventricular nucleus (PVN), dorsomedial hypothalamic nucleus (DMH), or lateral hypothalamus (LH) between WT and KO mice (Figures 1J–1L). Likewise, we observed no differences in adrenal weight, adrenal histology, plasma corticosterone, and adrenal Tyrosine hydroxylase and AgRP mRNA content between genotypes (Figures S1A–S1H), an important observation because AgRP is also expressed in adrenal glands (Gupta et al., 2017).

Ad Libitum and Fasting-Induced Feeding Behavior

Under conditions of ad libitum access to food, there was no difference in average 24-hr food intake (Figures 2A and 2B). Intriguingly, despite no difference in total 24-hr food intake, the microstructure of feeding behavior was altered in KO mice with significantly shorter meal duration compared with WT mice but increased meal number to compensate (Figures 2C–2E). Because AgRP neurons promote food-seeking behavior during states of negative energy balance, we examined refeeding in response to an overnight fast. KO mice exhibited significantly reduced cumulative food intake over a 24-hr period compared with WT control mice (Figures 2G and 2H), indicating an impaired feeding response after an overnight fast. Analysis of the microstructure of feeding behavior indicated that the first meal after refeeding was significantly smaller and that both average meal duration and meal size were significantly smaller compared with WT mice (Figures 2J and 2K). Collectively, these results show that deletion of Crat in AgRP neurons impairs refeeding after fasting and affects the microstructure of feeding behavior. There was no difference in body weight change or gastric emptying (Figures S2A and S2B) in WT and KO mice during fasting and refeeding.

Interestingly, there was no difference in the percentage of activated NPY neurons in WT or KO mice under fasted or refed conditions (Figure 2N) using fos as a surrogate marker for activation in NPY GFP-labeled neurons. However, an increase in fos-positive neurons in non-NPY ARC neurons during refeeding (Figure 2O) suggests a greater sensitivity to potential satiety factors associated with refeeding. Body weight gain or fat mass over a 20-week chow diet or high-fat diet (HFD) feeding period did not differ between genotypes (Figures S3A to S3B). Analysis of metabolic parameters revealed no genotype differences in heat production, oxygen consumption (VO2), or carbon dioxide production (VCO2) on a chow diet (Figures S3C–S3E). High-fat diet-fed KO mice had an increase in heat production during both the light and dark phase relative to WT controls (Figure S3F), but this was not sufficient to influence adiposity. There were no genotype differences in VO2 or VCO2 (Figures S3G and S3H). There were also no differences in body weight change or total food intake after 5 days of lepin injections (Figures S3K and S3L).

Feeding Behavior in Response to Intraperitoneal CCK and ICV Insulin

Given that deletion of Crat in AgRP neurons decreased meal duration under ad libitum conditions, the possibility arises that KO mice were more sensitive to satiety signals. An injection of cholecystokinin (CCK) (0.25 μg/kg, intraperitoneally [i.p.]) immediately before the dark phase (Figure 3A) highlighted that KO mice were more sensitive to i.p. CCK. KO mice exhibited reduced meal size and meal duration relative to WT mice (Figures 3B and 3C), but the meal number was significantly increased to compensate (Figure 3D). Hence, there was no overall difference in total food intake over the 24-hr period after CCK injection (Figure 3E). Central insulin suppresses food intake (Brüning et al., 2000; Niswender et al., 2003), so we also injected insulin (0.025 μU, intracerebroventricular [ICV]) into WT and KO mice and examined food intake and feeding behavior over a 24-hr period (Figure 3F). ICV insulin suppressed food intake in KO mice 2 hr after injection, an effect that was no longer evident by 6 hr or thereafter (Figures 3G and 3H). Analysis of 24-hr feeding behavior revealed that ICV insulin reduced meal size and meal duration and increased meal number in KO mice to compensate (Figures 3I–3K), resulting in no difference in 24-hr food intake between genotypes. These results are in accord with those of others (Campos et al., 2016), which highlight the ability of mice to adjust meal number to
ensure constant 24-hr food intake (under *ad libitum* conditions) despite reduced meal size and duration.

In line with the feeding response to insulin, NPY (AgRP) neurons from KO mice showed greater sensitivity to exogenous insulin (i.e. 0.75 U/kg body weight), with a significant increase in insulin-induced phosphorylated AKT (pAKT) staining in NPY (AgRP) neurons compared with saline (Figure 3L). To further examine the physiological sensitivity of NPY (AgRP) neurons to insulin, we measured pAKT in NPY neurons 30 min after refeeding and observed significantly more NPY neurons expressing pAKT in KO mice compared with WT controls (Figure 3M).

**AgRP Crat Deletion Causes a Metabolic Adaptation in Liver Function**

Analysis of plasma in fed and fasted states revealed significantly lower concentrations of insulin and triglyceride (TG) in fasted KO mice compared with WT mice (Figures 3O and 3P). Fasting significantly increased plasma corticosterone, non-esterified fatty acids (NEFA), and ketone bodies in KO mice, but we found no significant genotype effect on blood glucose (Figures 3Q–3T). Although blood glucose was not different during fasting, the increased circulating ketone bodies indicated increased fatty acid oxidation and altered hepatic liver function in KO mice.
during fasting. In the fasted state, KO mice had significantly lower liver glycogen and higher TG concentrations relative to WT mice (Figure 4A–4C). The hepatic gluconeogenic genes Pck1, Pck2, and G6pc were significantly increased in fasted KO mice relative to fasted WT mice (Figure 4D), and, consistent with increased breakdown of liver glycogen, liver glycogen phosphorylase (Pygl) gene expression was significant increased (Figure 4D).

The differences in liver glycogen and gluconeogenic genes in fasted KO mice suggest alterations in glycogenolysis and gluconeogenesis. In support of this, Glycerol kinase mRNA, the enzyme required to facilitate gluconeogenesis from glycerol, was significantly elevated in fasted KO mice (Figure 4D). Moreover, we investigated the fractional contribution of glycogenolysis and gluconeogenesis during fasting by employing a tracer-based approach to measure the sources of endogenous lipids.
glucose production (EGP), KO mice showed increased glycerol-driven gluconeogenesis after 3 hr fasting with a corresponding decrease in gluconeogenesis from phosphoenolpyruvate (Figures 4K and 4L). Moreover, blood glucose was significantly higher in KO mice compared with WT mice 15 min after an i.p. glycerol injection, indicating greater glycerol utilization for glucose production (Figure 4M). Collectively, our results suggest that the deletion of Crat from AgRP neurons affects the output of the sympathetic nervous system, which shifts liver metabolism to preferentially utilize glycerol as a substrate for gluconeogenesis.

AgRP Crat Deletion Acutely Impairs Glucose Tolerance

Initial experiments highlighted a greater increase in plasma glucose in KO compared with WT mice 15 and 30 min after oral glucose gavage (Figure 5A). Notably, blood glucose was not different between genotypes after 60 min, suggesting that AgRP Crat deletion may affect acute glycemic control rather than long-term regulation. Next we performed oral glucose tolerance tests (oGTTs) with stable isotope-labeled glucose (deuterated [6,6D2] glucose), allowing the distinction between exogenous labeled glucose and unlabeled endogenous glucose in the blood. Our results showed significantly higher levels of labeled (exogenous) glucose in the plasma of KO mice, indicating reduced glucose disposal during the oral GTT (Figures 5C and 5D). Although there was no difference in the glucose concentration from endogenous sources over the course of 120 min, the rate of change from 0–15 minutes was significantly reduced in KO mice compared to WT mice (Figures 5E and 5F), indicating a diminished ability to suppress endogenous glucose production in that time. In addition, plasma insulin levels 5 and 10 min after glucose gavage were reduced compared with WT mice (Figure 5G), underpinning the reduced glucose disposal and reduced ability to suppress endogenous glucose production observed in KO mice.

We then performed a glucose uptake assay in various tissues to determine the capacity of the endogenous response to oral glucose to regulate glucose uptake. 15 min after glucose load, glucose uptake was lower in white adipose tissue (WAT) and skeletal muscle, with a paradoxical increase in the hypothalamus (Figure 5H). By 30 min after glucose load, all tissues showed greater glucose uptake in KO compared with WT mice (Figure 5I). Fos coexpression within ARC NPY GFP neurons was examined 10 min after an oral glucose load, which should reduce Fos coexpression in NPY neurons because their activity is reduced. The results showed more Fos-expressing NPY (AgRP) neurons in KO compared to WT mice (Figures 5J–5L), consistent with lack of suppression of Fos activity in response to a glucose load. Collectively, these results suggest that the deletion of Crat from AgRP neurons attenuates their ability to acutely sense glucose and engage the appropriate mechanisms to clear plasma glucose within a 30-min time frame.

AgRP Crat Deletion Impairs Peripheral Nutrient Partitioning

KO mice showed a shift toward increased fatty acid utilization with a lower respiratory exchange ratio (RER) during both light and dark phases of the day (Figures 6A and 6B) when fed an ad libitum chow diet. In addition, heatmap analysis of RER values from individual mice clearly shows reduced RER values during fasting and refeeding (Figures 6C and 6D). Importantly, the change in RER in response to refeeding was delayed in KO mice, suggesting that the deletion of Crat from AgRP neurons influences peripheral nutrient partitioning to incoming nutrient availability. In line with greater fatty acid utilization, KO mice showed a greater clearance of lipids from the plasma following an olive oil gavage (Figure 6G). Moreover, we observed an adipose tissue (inguinal white fat) gene expression profile that would predict increased lipolysis in a fasted state (Figure 6H), which is consistent with increased plasma NEFA levels during fasting in KO mice (Figure 3S). Specifically, we observed an increase in KO mice in Atgl and lipoprotein lipase (Lpl), which hydrolyze TGs in intracellular lipid droplets and lipoproteins, respectively. Similarly, protein kinase A (Pka), an enzyme induced by adrenergic stimulation that increases lipolysis and phosphoglycolate phosphatase (Pgp), an enzyme required to liberate glycerol by dephosphorylating glycerol-3-phosphate (Mugabo et al., 2016), were both increased in KO mice. We also performed a lipoprotein lipase inhibition assay using tyloxapol (300 mg/kg body weight i.p. after a 5 hr fast) (Schoetz et al., 1957) and observed a significant increase in plasma TG in KO mice (Figure 6I), suggesting greater efflux from the liver for utilization in peripheral tissues such as muscle. Indeed, the increased muscle TG concentration in the fed state shown in Figure 6J and increased plasma TG concentration in KO mice 3 hr after refeeding (Figure 5E) support this idea as a mechanism to restock muscle lipid content. We also observed that fasting increased Pgp mRNA expression, muscle glycogen phosphorylase (Pygm), and Glut4, whereas there was no increase in Cpt1b (Figure 6K). Norepinephrine turnover was reduced in fasted KO mice but significantly increased in refed KO mice compared with WT controls (Figures 6L and 6M) underlying altered communication between the central nervous system and muscle in KO mice. Collectively, these studies show that Crat is required in AgRP neurons to regulate nutrient partitioning by regulating fatty acid utilization, gene expression, and metabolism.

Figure 3. Sensitivity to Insulin and CCK

(A–K) CCK (0.25 µg/kg, i.p.) (A) or insulin (0.025 µL, IV) (F) produced smaller meal size (B and I), shorter meal duration (C and J) with a compensatory increase in meal number (D and K) in KO mice compared with WT controls. As a result, there were no differences in overall food intake 24 hr after CCK injections (E) and no differences 6 hr after insulin injections (G), although KO mice had significantly lower food intake 2 hr after ICV insulin administration (H).

(L–N) Analysis of pAKT staining in AgRP/NPY neurons 10 min after exogenous insulin injection (0.75 U/kg, i.p.) (L) or 30 min after refeeding (M) showed that KO mice have a higher sensitivity to circulating insulin. Representative micrographs of NPY/pAKT coexpression ARC sections during fasting and 30 min after refeeding (the white bar represents 100 µm) (N).

(O–T) Analysis of plasma insulin (O), triglycerides (P), ketone bodies (Q), glucose (R), NEFA (S), and corticosterone (T) from fed and fasted AgRP Crat mouse. All data are expressed as mean ± SEM; n = 7; a, significant at p < 0.05.
Crat Deletion Changes the AgRP Proteomic Response to Fasting and Refeeding

To examine how Crat deletion in AgRP neurons affects intracellular protein expression during fasting and refeeding, we used a FACS approach to collect NPY GFP neurons from WT and KO mice under fed, fasted, and refed conditions and analyzed protein abundance using mass spectrometry (Figure 7A). A comparison of WT versus KO mice under fed conditions revealed 32 proteins with a significant difference in abundance. Of these 32, 7 were more than 2-fold higher in NPY neurons, and 3 were more than 2-fold lower in NPY neurons from KO versus WT mice (Figure 7B; Table S1). Of the 32 proteins, 10 were grouped into metabolic pathways (KEGG pathways ID 1100) (Figure 7C), and 3 were grouped to glycolysis (KEGG pathway ID 10). Two proteins within the metabolic KEGG pathway that were more than 2-fold higher in KO were NADH dehydrogenase 1 alpha subcomplex subunit 2 (Ndufa2) and methylenetetrahydrofolate dehydrogenase (Mthfd1), an oxidoreductase that can use nicotinamide adenine dinucleotide (NAD+) or nicotinamide adenine dinucleotide phosphate (NADP+) as acceptors.

In the fasted state, we identified 131 significantly different proteins in KO compared with WT NPY neurons. Of these 131, 84 were more than 2-fold higher, and 17 were more than 2-fold decreased (Table S2). KEGG identified 79 enriched pathways atgl, adipose triglyceride lipase; Cpt1a, carnitine palmitoyltransferase 1a; Fasn, fatty acid synthase; G6p, glucose-6-phosphatase, catalytic; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Gk, glycerol kinase, transcript variant 1; Gsk3b, glycogen synthase kinase 3 beta; Il-6, interleukin 6; Lpl, lipoprotein lipase; Pck1, phosphoenolpyruvate carboxykinase 1 (cytosol); Pck2, phosphoenolpyruvate carboxykinase 2 (mitochondrial); Pygl, glycogen phosphorylase, liver; Scd1, stearoyl-coenzyme A desaturase 1. All data are expressed as mean ± SEM; n = 7–10. Two-way (repeated measures where appropriate) ANOVA with Tukey’s post hoc analysis; a, significant at p < 0.05.
with metabolic pathways (32 of 131) the most enriched (Figure 7D). The two most significantly increased proteins in KO versus WT NPY neurons in the fasted state were mitochondrial cytochrome b–c1 complex subunit 6 (Uqcrh) and ATP-dependent 6-phosphofructokinase, platelet type (Pfkp), which catalyzes the third step of glycolysis. The 2 most significantly decreased proteins in NPY neurons from KO versus WT mice were NADH dehydrogenase (ubiquinone) Fe-S protein 6 (Ndufs6) and ATP synthase, H+ transporting, mitochondrial F1F0 complex, subunit e (Atp5K). For a complete list, see Table S2.

During refeeding, we observed 190 significantly different proteins in NPY neurons from KO versus WT mice. Of these 190, 39 were more than 2-fold increased and 47 were more than 2-fold decreased in NPY neurons from KO versus WT mice. KEGG identified 59 enriched pathways, many of which were similar to those reported in the fasted group, including the enrichment of the metabolic pathway (50 of 190) (Figure 7E; Table S3). The 2 most significantly increased metabolic pathway proteins in NPY neurons from KO versus WT mice were NADH dehydrogenase (ubiquinone) 1 subunit C2 (Ndufc2) and dolichyl-diphosphooligosaccharide protein (Rpn1). The 3 most significantly decreased proteins in NPY neurons from KO versus WT mice were ATP synthase protein 8 (Mtatp8), arginase 1 (Arg-1), and NADH dehydrogenase (ubiquinone) Fe-S protein 6 (Ndufs6).

Because acetyl CoA is the sole donor of acetyl groups for acetylation (Pietrocola et al., 2015), Crat may play an important indirect role in intracellular acetylation of proteins. Therefore, we examined whether Crat deletion affected the protein acetylation signature within NPY neurons from KO versus WT mice. Our results indicated that, under fed conditions, 14.15% versus...
Figure 6. AgRP Crat Deletion Impairs Peripheral Nutrient Partitioning

(A–F) KO mice have a lower respiratory quotient (RER) under ad libitum conditions (A and B) and during fasting and refeeding (C–F). Each column of the heatmap represents one animal, and each row represents the average RER per hour; black boxes on the left side represent the dark phase, with black dotted lines indicating the change in light conditions. The white line represents the time point of refeeding. KO mice have a delayed switch from fatty acid to glucose utilization.

(G) KO mice showed greater TG clearance after olive oil load.

(H) KO iWAT mRNA levels as log2 fold expression relative to WT controls.

(I) KO mice exhibit greater plasma TG levels after inhibiting LPL with tyloxapol (300 mg/kg body weight).

(J) Muscle triglycerides are significantly increased in fed KO mice compared with WT mice.

(K) KO muscle mRNA levels as log2 fold expression relative to WT controls.

(L and M) Noradrenaline turnover in fasted and refed muscle.

CD36, CD36 antigen, transcript variant 1; Cpt1b, carnitine palmitoyltransferase 1b; Cs, citrate synthase; Glut4, glucose transporter 4; InsR, insulin receptor; Pgp, phosphoglycolate phosphatase; Pka, protein kinase A; Pygm, glycogen phosphorylase, muscle. All data are expressed as mean ± SEM; n = 7–10. Two-way (repeated measures where appropriate) ANOVA with Tukey’s post hoc analysis; a, significant at p < 0.05.
Figure 7. Crat Deletion Affects Protein Regulation in AgRP Neurons
(A) Hypothalami of mice under fed, fasted, and refed conditions were dissociated, and NPY GFP-positive cells were sorted by FACS for proteomic analysis. (B) Significantly changed proteins were subjected to enrichment analysis using string database 10.0. (C–E) The KEGG metabolic pathway had the highest enrichment under fed (C), fasted (D), and refed conditions (E). (F) Enrichment analysis of acetylated proteins using FunRich 3.0 revealed that fewer proteins in KO are acetylated in the fasted and refed state.

Abat, 4-aminobutyrate aminotransferase; Acdi6, acyl-CoA synthetase long-chain family member 6; Aldh2, aldehyde dehydrogenase 2, mitochondrial; Aldh5a1, aldehyde dehydrogenase family 5, subfamily A1; Aldoc, aldolase C, fructose-bisphosphatase; Arg1, arginase, liver; Atp5a1, ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1; Atp5j2, ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F2; Atp5k, ATP synthase, H+ transporting, mitochondrial F0 complex, subunit e; Atp6v0a1, ATP synthase, H+ transporting, mitochondrial F1F0 complex, subunit e; Atp6v1a, ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit; Atp6v1b2, ATPase,
13.80% of proteins were acetylated in WT and KO NPY neurons, respectively (Figure 7F). However, under conditions of fasting, 15.93% of proteins in WT NPY neurons were acetylated, whereas this was significantly lower at 13.88% in KO NPY neurons (p < 0.05). Under refed conditions, 15.06% and 13.61% of proteins were acetylated from WT and KO NPY neurons (p < 0.05), respectively (Figure 7F).

DISCUSSION

AgRP activation engages neural circuits to prevent and/or adapt to states of hunger by promoting energy conservation and euglycemia. However, the ability of AgRP neurons to acutely respond to signals of incoming energy availability is also important to conserve energy, as illustrated by increased fatty acid utilization and attenuated body weight gain on a HFD in AgRP neonatally ablated mice (Joly-Amado et al., 2012). This nutrient partitioning involves effectively and rapidly switching off peripheral fatty acid oxidation in favor of storage, characteristic of the transition from a fasted to a refed state. Our current studies suggest that Crat affects AgRP neuronal function predominantly during fasting and the transition to refeeding. This is based on observations in which the deletion of Crat in AgRP neurons changes food intake and feeding behavior after fasting; hepatic function during fasting, which includes a shift to glycerol as a preferred gluconeogenic substrate; peripheral glucose clearance in response to an acute glucose load; peripheral fatty acid oxidation during fasting and in response to refeeding; and the intracellular protein abundance and acetylation status within AgRP neurons, particularly during fasting and refeeding. Thus, the influence of Crat on AgRP neuronal function is most relevant during fasting and in the transition to refeeding. This occurs at a time when metabolic processing in AgRP neurons is most relevant to control nutrient partitioning to promote maximal energy conservation.

Although a large amount of literature has focused on the important role of AgRP neurons to increase energy-conserving mechanisms during fasting, little research has focused on the mechanisms that enable AgRP neurons to respond to acute refeeding. In the muscle, Crat regulates metabolic flexibility by facilitating the substrate switch during the transition from fasting to refeeding (Muolio et al., 2012; Seiler et al., 2015). These actions attenuate the development of metabolic diseases such as obesity and type 2 diabetes. Our results show that Crat in AgRP neurons is required to maintain metabolic flexibility during the transition from fasting to refeeding and suggest a distinct function of Crat in substrate switching, irrespective of tissue type. KO mice not only showed increased fatty acid utilization during ad libitum and fasted conditions, as indicated by the lower RER, they also showed a blunted switch from fatty acid to glucose utilization during the fasting-to-refeeding transition. Moreover, the deletion of Crat in AgRP neurons improves whole-body glycerol and TG handling and the response to lipoprotein lipase inhibition in the same manner as AgRP ablation (Joly-Amado et al., 2012). Although this did not influence body fat loss during a single bout of fasting, we predict that this would affect energy homeostasis during a model of chronic energy deficit, such as calorie restriction or feeding entrainment, when AgRP neurons are most important to restore energy homeostasis (Andermann and Lowell, 2017).

The importance of Crat in AgRP neurons to maintain metabolic flexibility and nutrient partitioning was also observed after glucose administration because blood glucose was significantly higher in KO mice 15 and 30 min after gavage, primarily because of impaired glucose clearance, but returned to levels in WT mice at 60 and 120 min. Together with RER changes, these results show that Crat blunts, but does not prevent, the responsiveness of AgRP neurons. This observation is supported by measures of glucose-stimulated glucose uptake, which shows deficits in glucose uptake in inguinal white adipose tissue (iWAT) and muscle at 15 min but not at 30 min. Interestingly, it is after this 30-min time point that glucose clearance is no longer different in WT and KO mice during an oral GTT.

Cort deletion affected the acetylation profile of proteins within AgRP neurons during fasting and refeeding but not in the fed state, which further highlights the important role of Crat in AgRP neurons during fasting-to-refeeding transition. This is
consistent with others who showed that Crat plays an important role in intracellular acetylation of proteins (Davies et al., 2016; Madiraju et al., 2009) by regulating acetyl group availability for acetylation (Pietrocola et al., 2015). Further analysis of proteomic data highlighted that Crat regulates metabolic processing primarily during fasting and refeeding. Differentially regulated proteins included those involved in sirtuin (SIRT) activity, NADH metabolism, uncoupling activity, glycolysis, and mitochondrial dynamics. We also noted a number of differences related to proteins involved in synaptic function and endocytosis. This supports the concept that increased synaptic activity requires greater energetic demands via mitochondrial transport (Li et al., 2004), dynamics (Dietrich et al., 2013), and appropriate mitochondrial processing. Although a number of proteins regulating mitochondrial function are known to influence AgRP function and energy homeostasis, including mitofusins (Dietrich et al., 2013), AMP-activated protein kinase (AMPK) (Claret et al., 2007), SIRT (Dietrich et al., 2010), PGC1a (Gill et al., 2016), and uncoupling protein-2 (Andrews et al., 2008), none of these studies have illustrated the importance of AgRP metabolic pathways during fasting and the refeeding transition.

Because AgRP neurons play a fundamental role in food intake, we examined the effects of Crat deletion on food intake and feeding behavior. Under ad libitum conditions, meal duration was significantly reduced; however, animals increased the meal number so that overall food intake was not different between genotypes. These results are consistent with Campos et al. (2016), in which inactivation of a subpopulation of AgRP to parabrachial calcitonin gene-related peptide (CGRP) neurons (AgRP → CGRP Bel) reduced meal duration but mice were able to increase the meal number to maintain appropriate food intake. Moreover, this study showed that the inability to engage the AgRP → CGRP Bel pathway heightened sensitivity to satiety cues (Campos et al., 2016), consistent with our observations that KO mice are more sensitive to feedback signals of satiety (i.p. CCK and ICV insulin). AgRP neurons projecting to the lateral hypothalamus, anterior bed nucleus of the stria terminalis, PVN, and paraventricular thalamus also increase the drive to eat (Betley et al., 2013) independent from the AgRP

EXPERIMENTAL PROCEDURES

Animals

All experiments were conducted in compliance with the Monash University Animal Ethics Committee guidelines. Male mice were kept under standard laboratory conditions with free access to food (chow diet, catalog no. 8720610, Barastoc Stockfeeds, Victoria, Australia) and water at 23 °C in a 12-hr light/dark cycle and were group-housed to prevent isolation stress unless otherwise stated. All male mice were aged 8 weeks or older for experiments unless otherwise stated. Additional information about animals can be found in the Supplemental Experimental Procedures.

Food Intake Experiments

Mice were single-housed in specialized feeding cages (BioDAQ, Research Diets, NJ, USA), and recordings were analyzed after a 3-day acclimation period. Feeding behavior after fasting and short-term fasting (3 hr) combined with i.p. CCK (0.25 μg/kg) or ICV insulin (0.025 μU in 1.5 μL) administered at the onset of the active period were analyzed.
Metabolic Studies
For labeled or unlabeled oral GTTs (1.5 g/kg) and glycerol tolerance test (GlyTT, 1 g/kg i.p.), mice were fasted from 8 a.m., and tests were performed 5 hr later, at 1 p.m. Labeled glucose enrichment in plasma samples was measured via gas chromatography-mass spectrometry (GC-MS) (for details, see the Supplemental Experimental Procedures).

Endogenous Glucose Production
Mice were injected with deuterated water (D2O) (0.9% NaCl in D2O, 25 mL/kg bodyweight, i.p.) and 10% D2O mixed into drinking water. Five hours after injection, mice were subjected to a fast, and plasma samples (20 μL) were taken after 3 and 18 hr of fasting. Enrichment was measured via GC-MS (Kowalski et al., 2015; Supplemental Experimental Procedures).

Glucose-Stimulated Glucose Uptake
To localize glucose uptake in tissue, we performed oral GTT (1.5g/kg) combined with intraperitoneal injection of tritiated 2-deoxy-D-glucose (2-[3H]-DG) tracer (10 μCi in 200 μL saline; see the Supplemental Experimental Procedures for details).

CLAMS
To assess the energy expenditure of AgRP Crat mice during ad libitum chow feeding, fasting, and refeeding and HFD conditions, single-housed mice were placed in a comprehensive lab animal monitoring system (Oxymax-CLAMS), and recordings were analyzed after a 1-day acclimation period.

Analysis of Blood Chemistry
Detailed protocols can be found in the Supplemental Experimental Procedures.

PCR
Total RNA was extracted from tissue with Qiazol (Qiazol Sciences) according to the instruction manual (see the Supplemental Experimental Procedures for a detailed description of the procedure). cDNA synthesis and real-time qPCR were performed as described previously (Stark et al., 2015b). For primer details, see the Supplemental Experimental Procedures.

Ex Vivo Fat Oxidation Assay
Animals were anesthetized with isoflurane. Tissues were collected and incubated in Krebs buffer containing 14C-oleate (1 μCi/mL), and 14CO2 was captured in 1 M NaOH for a period of 2 hr and measured as described previously (Meex et al., 2015).

Norepinephrine Turnover
Norepinephrine was measured by high performance liquid chromatography (HPLC), and turnover rates were calculated after log transformation by log(saline) – log(alpha-methyl-p-tyrosine [aMPT]) / 0.434, where 0.434 is an estimate for the kinetic rate (Brodie et al., 1966; for details; see the Supplemental Experimental Procedures).

Proteomics of AgRP Neurons
Peptides were analyzed using nano liquid chromatography-mass spectrometry (for details, see the Supplemental Experimental Procedures). Acquired .raw files were analyzed with MaxQuant (v1.5.5.1) to globally identify and quantify proteins across the different conditions. Data visualization and statistical analyses were performed in Perseus (v1.5.5.3). After removing the reversed and known contaminating proteins, the label-free quantification (LFQ) values were log2-transformed, and the reproducibility across the biological replicates was evaluated by Pearson’s correlation analysis. The replicates were grouped into WT fed versus KO fed, WT fasted versus KO fasted, and WT refed versus KO refed. A two-sample t test was performed to obtain the significantly expressed proteins, with p < 0.05. Significantly changed proteins were further analyzed to investigate association with KEGG pathways and gene ontology (GO) terms using the String database. Enrichment analysis for compartmentalization of acetylated proteins was performed against the Uniprot database (rodents, taxon ID 9989) with FunRich 3.0.

Immunohistochemistry
Immunohistochemistry was performed as reported previously (Wu et al., 2014) using the following primary antibodies: c-Fos rabbit polyclonal immunoglobulin G (IgG) (sc-52, Santa Cruz Biotechnology) and phospho-AKT (Ser473) rabbit monoclonal IgG (9271s, Cell Signaling Technology). The secondary antibody for both was Alexa Fluor goat anti-rabbit 594 antibody (A-11037, Invitrogen). Fiber density, cell number, and volume were measured with a design-based unbiased stereology method (Stereoinvestigator, MicroBrightField, VT, USA) as described previously (Lemus et al., 2010).

Statistical Analysis
Statistical analyses were performed using GraphPad Prism for MacOS X. Data are represented as mean ± SEM. Two-way ANOVAs with post hoc tests were used to determine statistical significance. A two-tailed Student’s unpaired t test was used when comparing genotype only. p < 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, three figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.01.067.

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AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES
UCP2 mediates ghrelin’s action on NPY/AgRP neurons by lowering free radicals. Nature 454, 846–851.


Carnitine acetyltransferase mitigates metabolic inertia and muscle fatigue during exercise. Cell Metab. 22, 65–76.


