Articles in PresS. Am J Physiol Regul Integr Comp Physiol (April 20, 2011). doi:10.1152/ajpregu.00092.2011

Malonyl-CoA Mediates Leptin Hypothalamic Control of Feeding Independent of Inhibition of CPT-1a

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The authors have declared that no conflict of interest exists.

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Abstract

Hypothalamic fatty acid metabolism is involved in CNS controls of feeding and energy balance. Malonyl-CoA, an intermediate of fatty acid biosynthesis, is emerging as a significant player in these processes. Notably, hypothalamic malonyl-CoA has been implicated in leptin's feeding effect. Leptin treatment increases malonyl-CoA level in the hypothalamic arcuate nucleus (Arc), and this increase is required for leptin-induced decrease in food intake. However, the intracellular downstream mediators of malonyl-CoA's feeding effect have not been identified. A primary biochemical action of malonyl-CoA is the inhibition of the acyltransferase activity of carnitine palmitoyltransferase-1 (CPT-1). In the hypothalamus, the predominant isoform of CPT-1 that possesses the acyltransferase activity is CPT-1 liver type (CPT-1a). To address the role of CPT-1a in malonyl-CoA's anorectic action, we used a recombinant adenovirus expressing a mutant CPT-1a that is insensitive to malonyl-CoA inhibition. We show that arcuate nucleus overexpression of the mutant CPT-1a blocked the malonyl-CoA-mediated inhibition of CPT-1 activity. However, the overexpression of this mutant did not affect the anorectic actions of leptin or central cerulenin for which an increase in Arc malonyl-CoA level is also required. Thus, CPT-1a does not appear to be involved in the malonyl-CoA's anorectic actions induced by leptin. Furthermore, long-chain fatty acyl-CoA's, substrates of CPT-1a, dissociate from malonyl-CoA's actions in the Arc under different feeding states. Together, our results suggest that Arc intracellular mechanisms of malonyl-CoA's anorectic actions induced by leptin are independent of CPT-1a. The data suggest that target(s) rather than CPT-1a mediates malonyl-CoA action on feeding.

Introduction

Obesity is a major health problem and a major cause of insulin-resistance and diabetes. An imbalance between energy intake and energy expenditure can lead to overweight and contribute to the development of obesity and the metabolic syndrome. The hypothalamus plays a critical role in the CNS control of feeding and energy balance (21, 28). A large body of evidence now shows that fatty acid metabolism participates in this action of the hypothalamus (1, 3, 5-7, 9, 11, 12, 15, 17, 18, 20, 23, 24). In this regard, malonyl-CoA, an intermediate in fatty acid biosynthesis, is emerging as a significant player in the hypothalamic control of feeding and body energy balance (5-7, 9, 11, 12, 15, 18), Recent data have linked malonyl-CoA signaling action to the hypothalamic intracellular pathways of leptin in the central regulation of energy balance (6, 9). In the hypothalamus, leptin treatment increases malonyl-CoA level via inhibiting AMP-activated protein kinase (AMPK) and activating acetyl-CoA carboxylase (ACC) (2, 6). Notably, the increase of hypothalamic malonyl-CoA induced by leptin takes place specifically in the arcuate nucleus (Arc) (6), a critical site in mediating leptin's central actions on feeding and energy balance (28). Despite these findings, the intracellular mechanisms by which malonyl-CoA impacts feeding remain unclear. It is known that malonyl-CoA inhibits the acyltransferase activity of carnitine palmitoyltransferase-1 (CPT-1) that converts long-chain fatty acyl-CoA (LCFA-CoA) to long chain acylcarnitine (9, 23). CPT-1 has liver- and muscle-isoforms, with the hypothalamus mainly expressing the liver-isoform (CPT-1a) (23). Pharmacological studies have demonstrated that ICV administration of compound ST-1326, a specific CPT-1a inhibitor, inhibits Arc CPT-1a activity, increases cellular LCFA-CoA's levels, and reduces food intake (23). Accumulation of hypothalamic LCFA-CoA's has been suggested to have anorectic effects as ICV oleic acid (which can form LCFA-CoA's in cells) was shown to reduce food intake (24). Given that malonyl-CoA is a physiological inhibitor of CPT-1, inhibition of Arc CPT-1a and the ensuing increase of LCFA-CoA's have been proposed to mediate malonyl-CoA's anorectic signaling actions. However, a growing body of evidence now strongly challenges this hypothesis (16). For example, we previously demonstrated that exogenous leptin upregulates malonyl-CoA level without affecting the level of LCFA-CoA's in the Arc (6). The result thus casts doubt on the role of CPT-1a as a mediator of malonyl-CoA's action in leptin feeding pathways. To clarify the role of CPT-1a

in the Arc, we used a recombinant adenovirus expressing a mutant CPT-1a that is insensitive to malonyl-CoA inhibition (10). Using this mutant, we examined the feeding responses of the animals with a disruption of malonyl-CoA-mediated inhibition on CPT-1a acyltransferase activity.

Methods

Animal preparations for feeding experiments: Animal experiments were performed in accordance with the guidelines of the Canadian Council for Animal Care, and were approved by the University of Alberta Animal Policy and Welfare Committee. Male Sprague–Dawley rats (225–300 g) were purchased from Charles River Laboratories. The rats were housed in a controlled (12h light/12h dark) environment, and were allowed *ad. lib.* access to standard laboratory chow and water unless otherwise noted. Before the feeding experiments were started, the rats were handled daily and subjected to mock injections to minimize the stress from the experimental manipulation. In the leptin study, leptin or vehicle was administered at 1h before the dark onset. Food intakes at 3h and 23h (overnight) after the dark onset were monitored. Overnight body weight changes (24h after the injection) were also monitored. In the cerulenin study, at 5h before the dark onset, food was removed. Then, cerulenin or vehicle was administered and food was not provided until the dark onset. Food intakes were monitored at 3h and 19h (overnight) after dark onset. Overnight body weight changes (24h after the injection) were also monitored.

Brain sample preparations: At the designated time points, rats were euthanized by decapitation. Brains were rapidly dissected (within 1 min) and coronal brain sections were prepared using a cryostat or a brain matrix (Roboz Surgical Instrument, Maryland). Individual hypothalamic nuclei including arcuate nucleus (Arc), ventromedial nucleus (VMN), lateral hypothalamic area (LHA) and paraventricular nucleus (PVN) were dissected according to the established protocol (6). The accuracy of the dissection was verified by comparing the characteristic neuropeptide mRNA levels as detailed previously (6).

Recombinant adenoviruses: The adenoviruses were delivered into the Arc (1×10^7 p.f.u. / μl; 0.4μl per side) by bilateral stereotaxic injection (6). The coordinates were: anterior–posterior: -2.8 mm; dorsal–ventral: -9.5 mm; and medial–lateral: ±0.4 mm. The accuracy of the injections was confirmed by histological analysis as described previously (6). The adenovirus encoding MCD contains the full length human MCD (hMCD) coding sequence (27). The adenovirus encoding the wild type CPT-1a contains the nucleotide sequence (58-2700) including the entire coding region of rat CPT1a and the same sequence

was used to generate the mutant CPT-1a with the 593-methionine residual mutated to serine residual (22). The feeding experiments with leptin and cerulenin were conducted in the second week following the initial delivery of the adenoviruses. Significantly high levels of protein expressions or enzyme activities were reliably detected after one week and these high levels last until at least fourteen days (two weeks) following the delivery of the viruses.

Cannulation surgery and intracerebroventricular injection: Cannulas were implanted into either the lateral or 3rd ventricle based on the established protocol (6). The accuracy of placement was confirmed by angiotensin-2 drinking test or histological analysis as previously described (6). After surgery, daily food intake and body weight were monitored. After body weights returned to the levels before surgery and the rats were fully habituated to the experimental manipulations, bolus injections of the chemicals (leptin and cerulenin) were administered ICV.

Chemicals: Leptin was obtained from A. F. Parlow, National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases (USA). For ICV injection, a dose of 15μg of leptin dissolved in PBS was chosen based on our previous studies (6). Cerulenin was obtained from Sigma (St. Louis, MO) and 125μg in 25% DMSO/75% PBS (vehicle) was used in ICV injection as previously described (1).

Quantifications of malonyl-CoA, long-chain fatty acyl-CoA's and long-chain acylcarnitines: The CoA recycling assay was performed to measure the malonyl-CoA level and high performance liquid chromatography (HPLC) was used to measure the levels of long-chain acyl-CoA's (consisting of palmitoyl-CoA, oleoyl-CoA and stearoyl-CoA) as detailed elsewhere (6). To measure the levels of the long-chain acylcarnitines, brain tissues were extracted with acetonitrile and 2-propanol (6). The extracts were dried under streams of nitrogen and were reconstituted in 300 μl acetonitrile and n-butanol (1:1). The samples were then filtered and loaded into HPLC coupled with mass spectrometer (13). The levels of long-chain acylcarnitines (consisting of palmitoylcarnitine, oleoylcarnitine and stearoylcarnitine) were quantitated as detailed before (13).

Malonyl-CoA decarboxylase (MCD) activity assay: Adenovirus encoding human MCD or GFP (null) was stereotaxically delivered into the Arc. Individual hypothalamic nuclei (Arc, VMN, LHA and PVN) were dissected from coronal brain sections. The MCD activity assay was performed based on established protocol (6, 27).

Carnitine palmitoyltransferase-1 (CPT-1) activity assay: The brain was removed from the skull within 40 seconds and was immediately sectioned using the brain matrix. The mediobasal hypothalamic area (MBH), LHA and PVN were quickly dissected on ice and the tissues were immediately homogenized in the cold lysis buffer (0.25M sucrose, 5mM Tris-HCl and 1mM EGTA, pH 7.4). The crude homogenate was centrifuged at 800g for 10 minutes at 4°C. The resulting pellet was washed by resuspension in 2 volumes of the lysis buffer and was then centrifuged at 800g. This step was repeated twice to maximize the yield of the mitochondrial fraction. The combined supernatant was centrifuged at 6000g for 15 minutes at 4°C. The resulting pellet (crude mitochondrial fraction) was gently resuspended in the lysis buffer and was used in the activity assay using a radiometric method (20).

Antibodies and Western blotting: The CPT-1a antibody (Ab) was generated as described elsewhere (29). Actin (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the loading control in the Western blots. The procedures of protein electrophoresis, transfer and Ab detection were performed based on standard Western protocol (Invitrogen, California). Densitometry was performed using Scion Image software (Scion, Frederick, MD).

Statistical analysis: Data are reported as means with SE. Data consisting of two groups were analyzed by Student t-test. Data consisting of three groups were analyzed by One-way ANOVA. These One-way ANOVAs, when they yielded significant overall effects, were further analyzed by Newman-Keuls Multiple Comparison Test for group comparisons. Data consisting of four or six groups were analyzed by Two-way ANOVA. These Two-way ANOVAs, when they yielded significant overall effects, were further analyzed by Bonferroni posttests for group comparisons. For all tests, $P<0.05$ indicated significance.

Results

The M593S CPT-1a mutant is insensitive to malonyl-CoA inhibition. In this study, we used a M593S CPT-1a mutant to address the role of CPT-1a in malonyl-CoA-mediated anorectic actions. The M593S mutation results in an impaired interaction between malonyl-CoA and the malonyl-CoA binding site in CPT-1a (22). We first verified malonyl-CoA insensitivity of this mutant using yeast cells that do not possess endogenous CPT-1 acyltransferase activity (22). We transfected yeast cells with the vector that expresses this mutant (CPT-1a mt) or the wild type CPT-1a (CPT-1a wt), and then measured CPT-1 acyltransferase activity using the extracts from these cells. We found that malonyl-CoA inhibitory effect on the mutant was nearly abolished (Fig. 1A). Next, we evaluated CPT-1 acyltransferase activities in the hypothalamus. The adenovirus encoding the mutant CPT-1a (CPT-1a mt), the wild type CPT-1a (CPT-1a wt), or the null virus was delivered bilaterally into the Arc. Two weeks following delivering the viruses, rats were euthanized and CPT-1a protein levels were quantified in individual hypothalamic nuclei (Arc, VMN, LHA and PVN). Compared to the rats injected with the null adenovirus, CPT-1a (wt or mt) adenoviral infections induced increases in CPT-1a protein levels in the Arc while the CPT-1a protein levels were not altered in the VMN, LHA or PVN (Fig. 1B). Concomitant with the increased protein levels, CPT-1 acyltransferase activities were also increased selectively in the mediobasal hypothalamic area (MBH) encompassing the Arc (Fig. 1C). Together, these data demonstrate Arc-specific overexpressions of CPT-1a as well as activations of CPT-1 following the stereotaxic Arc delivery of the viruses. We then evaluated the response of CPT-1a to exogenous malonyl-CoA. We prepared crude mitochondrial extract from the mediobasal hypothalamic (MBH) region of the animals with Arc overexpressing the wild type CPT-1a or the mutant CPT-1a. Exogenous malonyl-CoA (50 μM) (20) was then added to the extracts and CPT-1 activity assay was conducted. As expected, we observed that the mutant CPT-1a was resistant to the inhibitory effect of malonyl-CoA (Fig. 1D).

Increase in Arc malonyl-CoA is required for leptin's anorectic actions. Before addressing the role of CPT-1a in leptin's malonyl-CoA signaling pathway, we confirmed the importance of malonyl-CoA in the central control of feeding and in leptin's anorectic actions. The adenovirus encoding malonyl-CoA

decarboxylase (Ade-MCD) (27), which lowers malonyl-CoA level (9), was administered into the Arc of rats. Consistent with the previous finding (9), Arc delivery of the Ade-MCD increased daily food intakes and body weight gains, compared to the rats treated with the null virus (Fig. 2A). Delivery of the Ade-MCD induced an increase in MCD activity in the Arc (Fig. 2B), while the MCD activity was not altered in the VMN (Fig. 2B) or in the LHA and PVN (LHA: MCD, 87 ± 4.7 % vs. null, 100 ± 13 %; PVN: MCD, 105 ± 1 % vs. null, 100 ± 10 %). In addition, following the delivery of the Ade-MCD, the malonyl-CoA level in the Arc was reduced (Fig. 2C). We then administered (ICV) leptin to the rats with Arc-specific activation of MCD. As we demonstrated in the previous study (6), leptin treatment increased malonyl-CoA level in the Arc (leptin: 220%, PBS: 100%; $P<0.05$). We further showed that the MCD overexpression attenuated the level of the increase in malonyl-CoA and antagonized the anorectic actions by leptin treatment (Fig. 2D). These results confirm that the increase in Arc malonyl-CoA level is a significant contributor to leptin's anorectic effects.

Blocking malonyl-CoA inhibition of CPT-1 acyltransferase activity does not affect leptin's anorectic actions. We injected the adenovirus encoding wild type CPT-1a (Ade-CPT-1a wt), the mutant CPT-1a (Ade-CPT-1a mt) or the null (Ade-null) into the Arc of rats. Three to four days following the virus infections, daily food intakes and body weights returned to the pre-injection levels. At least through the eighth day following the initial injection of the viruses, no significant body weight or feeding differences were found among these rats (data not shown). We then assessed the role of malonyl-CoA inhibition of CPT-1 in leptin's anorectic actions. During the second week following the initial delivery of the viruses (around the eleventh day), leptin was injected to the rats. We found that Arc overexpression of CPT-1a mt (malonyl-CoA insensitive) did not affect leptin-induced feeding inhibition or weight loss (Fig. 3A). During this period (the second week following the injection of the viruses), the adenoviral infections did not affect the daily (24h) food intakes as compared to the basal pre-injection levels, and as in the first week described above, no significant differences of 24h food intakes were found among all treated groups (basal level: 100 ± 3.2 %, Ade-null: 103 ± 2.0 %, Ade-CPT-1a wt: 97 ± 2.1 %, Ade- CPT-1a mt: 95 ± 5.8 %). Concomitant with producing similar anorectic effects, leptin induced similar increases in Arc malonyl-CoA levels in all treated groups (Fig. 3B). Since malonyl-CoA inhibition of CPT-1 acyltransferase activity

is reversible (22), the CPT-1 activity assay using tissue extract is not informative in evaluating the *in vivo* effect of malonyl-CoA on CPT-1 activity. CPT-1 acyltransferase activity converts long-chain fatty acyl-CoA's (LCFA-CoA's) to long-chain acylcarnitines (LC-AC's) (13). We therefore assessed CPT-1a activity by measuring the levels of LC-AC's. As expected, activation of Arc CPT-1a increased LC-AC's levels in the Arc (Fig. 3C). Leptin reduced LC-AC's levels in the Arc that ectopically expresses the wild type CPT-1a (Fig. 3C), indicating an inhibition of CPT-1 activity by leptin in these animals. In contrast, leptin did not affect LC-AC's levels in the Arc overexpressing the mutant CPT-1a (Fig. 3C), suggesting that leptin-induced accumulation of malonyl-CoA does not inhibit the CPT-1 activities in these animals. In parallel with the changes in the levels of LC-AC's, levels of total LCFA-CoA's (substrates for CPT-1a) were reduced by CPT-1a activation (Fig. 3D). Leptin induced the increase in LCFA-CoA's levels in the Arc ectopically expressing CPT-1a wt, while it did not affect LCFA-CoA's levels in the Arc overexpressing the CPT-1a mt (Fig. 3D). It should be noted that these biochemical assays were performed at the time when the feeding experiment with leptin was conducted (i.e. in the second week following viral injections). We also demonstrated (Fig. 1) that the effect of overexpressing the mutant CPT-1a on antaganizing malonyl-CoA-mediated inhibition remained significant at a later time point (i.e. two weeks following the viral injections). Thus, we demonstrated that during the period when the leptin-induced inhibition of CPT-1a were blocked, the leptin-induced feeding inhibition was not affected. Taken together, these data demonstrate that blocking malonyl-CoA inhibition of CPT-1a and the resulting increase in LCFA-CoA's level does not affect leptin's anorectic actions.

Blocking malonyl-CoA inhibition of CPT-1 acyltransferase activity does not affect the anorectic action of central cerulenin. To assess the role of CPT-1a in the specific context of malonyl-CoA signaling actions, we examined the feeding response to central cerulenin that is an inhibitor of fatty acid synthase (FAS). FAS uses malonyl-CoA as a substrate and blocking FAS activity increases malonyl-CoA level (15). We first show that overexpressing MCD attenuated the level of cerulenin-induced increase in Arc malonyl-CoA, and blocked the feeding inhibition induced by cerulenin treatment (Fig. 4A). These data demonstrate that an increase in Arc malonyl-CoA level is required for cerulenin's anorectic effects. As expected, Arc overexpression of CPT-1a mt did not affect the anorectic effects by cerulenin, and

cerulenin treatment increased Arc malonyl-CoA to a similar level in all treated groups (Fig. 4B). It should be noted that the inhibition of FAS reduces the synthesis of long-chain fatty acids/acyl-CoA's (14), which would contribute to lowering the LC-AC's levels via mass action. As a result, we did not monitor the CPT-1 activity following cerulenin treatment. However, we found a similar level of increase (1.5-2 fold) in Arc malonyl-CoA by cerulenin compared to that seen following leptin treatment (compare Fig. 4B with Fig. 3B). We thus assume that cerulenin treatment produced the same effect on CPT-1a activity as leptin treatment did. Together, our data indicate that malonyl-CoA inhibition of Arc CPT-1a is not required for the anorectic effects of either leptin or cerulenin.

Changes in long-chain acyl-CoA's level in the Arc are dissociated from those in malonyl-CoA levels under fasting and refeeding conditions. Under different nutritional states such as fasting and refeeding, circulating leptin level changes in tight association with that of hypothalamic malonyl-CoA level (9, 14). If LCFA-CoA's act as downstream mediator of malonyl-CoA action in leptin's intracellular signaling pathways, the levels of LCFA-CoA's should also change in association with the level of malonyl-CoA. To assess this hypothesis, we examined the Arc levels of LCFA-CoA's and malonyl-CoA under fasting and refeeding conditions. Unexpectedly, we found that Arc LCFA-CoA's levels were increased by fasting even though the malonyl-CoA level decreased (Fig. 5A), and these changes were reversed upon re-feeding (Fig. 5A). Thus, in the Arc, the changes in LCFA-CoA's levels appear to dissociate from those in malonyl-CoA level under different feeding states. It is known that CPT-1-mediated fatty acid β-oxidation activity in the brain is trivial as compared to the periphery (16). In determining the size of brain LCFA-CoA's pool, other actions/pathways such as exchange with the circulation, the action of acyl-CoA synthetase (ACS) and brain acyl-CoA hydrolase (BACH) play more prominent roles (Fig. 5B and ref. (16). Here, we showed that fasting elevated the fatty acid levels in the circulation while re-feeding brought down the elevated levels (Fig. 5C). Furthermore, the BACH activity was lowered by fasting and elevated to pre-fasting level following re-feeding (Fig. 5D). Although the physiological relevance of these changes is unclear, these metabolic events provide an interpretation for the observed dissociation of LCFA-CoA's from malonyl-CoA.

Discussion

CPT-1a, a key enzyme in regulating mitochondrial fatty acid β-oxidation, has been proposed to be a candidate for mediating hypothalamic malonyl-CoA's anorectic actions. In the CNS, as fatty acid β-oxidation activity is trivial, malonyl-CoA-mediated regulation of CPT-1 acyltransferase activity is not as significant in the brain as it is in the periphery (16). Indeed, in our current and previous studies (6), we found no change of the Arc levels of either LCFA-CoA's (substrates of CPT-1a), or LC-AC's (products of CPT-1a), upon leptin administration under normal conditions. These results suggest that leptin treatment does not affect CPT-1 acyltransferase activity in the Arc. Thus, Arc CPT-1a may not be implicated in leptin's central actions on feeding under normal conditions. In particular, our data indicate that CPT-1a is not a critical component of malonyl-CoA signaling mechanisms in leptin's anorectic actions. Some potential mechanisms underlie this conclusion. First, due to the inherent heterogeneity of CNS cells (28), malonyl-CoA metabolism and CPT-1a expression might not take place in the same cells (31). Thus, the change of malonyl-CoA level in response to leptin may occur in a population of cells that does not express CPT-1a. Secondly, malonyl-CoA produced by the two isoforms of ACC (ACC-1 and ACC-2) has different effects on CPT-1a activity. Compared to ACC2, the ACC-1-associated malonyl-CoA does not significantly affect CPT-1a-mediated fatty acid β-oxidation (19). It should be noted that we have demonstrated leptin specifically activates ACC1 to increase malonyl-CoA level (6). It follows that malonyl-CoA may not inhibit Arc CPT-1a in leptin's anorectic actions. Finally, due to the inherent nature of low activity, the CPT-1a in the Arc may not be subject to the regulation by malonyl-CoA particularly when malonyl-CoA is increased. Under physiological conditions, the already low activity of CPT-1a in the Arc may be resistant to a further inhibition by malonyl-CoA. Under artificial conditions such as when wild type CPT-1a is ectopically overexpressed, leptin treatment does inhibit CPT-1a activity and reduce LCFA-CoA's levels. However, the blockades of these changes fail to affect leptin's anorectic actions. Therefore, in the Arc, malonyl-CoA inhibition of CPT-1a seems unlikely to play a key role in mediating leptin's feeding actions. Our data also show that leptin's effect on body weight is independent of malonyl-CoA-mediated CPT-1a inhibition. Together with the results of food intake, we speculate that leptin's action on body energy expenditure would also be independent of malonyl-CoA's inhibitory effect on CPT-1a.

However, a definitive conclusion requires direct measurement of energy expenditure in the experimental paradigm.

There have been increasing concerns (16) with the hypothesis that inhibiting Arc CPT-1a with the ensuing increases of LCFA-CoA's can produce anorectic effects (23, 24). Our results provide further evidence to support the notion that these biochemical events are not implicated in leptin's anorectic actions. In addition, particularly under acute experimental conditions (24), intracellular LCFA-CoA's levels may not go up following the treatment with long-chain fatty acids (16, 24). Moreover, a growing body of evidence now shows that accumulation of hypothalamic LCFA-CoA's is indeed associated with increase in food intake, and increases in adiposity and body weight (3, 4, 25). Notably, ghrelin, an orexigenic factor that potently stimulates feeding, increases hypothalamic LCFA-CoA's levels while inhibits hypothalamic ACC, which reduces malonyl-CoA level (3). The observed increases in LCFA-CoA's levels suggest that the action of LCFA-CoA's, at least in ghrelin's hypothalamic pathways, can dissociate from that of malonyl-CoA. In line with this prediction, we demonstrate a dissociation of the levels of LCFA-CoA's from that of malonyl-CoA under fasting and re-feeding conditions. It is also worth pointing out that Arc overexpression of acyl-CoA synthetase (ACS) raising LCFA-CoA's levels does not induce the expected anorexigenic actions (personal communication with Dr. Jason Dyck, University of Alberta). Together, these data strongly challenge the proposed anorectic role of Arc LCFA-CoA's, and they also argue against the view that LCFA-CoA's can act as effectors of Arc malonyl-CoA-mediated anorectic actions.

Further challenge to a role of CPT-1a and LCFA-CoA's as mediators of malonyl-CoA feeding action comes from the study using compound C89b, a CPT-1 activator (1). It was expected that C89b treatment, by activating CPT-1a and thus reducing LCFA-CoA's levels, would stimulate food intake. Surprisingly, the study demonstrated that C89b induced the same feeding response, i.e. an inhibition, as the increase in Arc malonyl-CoA level (1). These C89b data also directly contradict the previous finding that CPT-1a inhibition (with ST1326) reduces feeding. Because opposing actions on the same targets produce similar feeding effects, these data further suggest that CPT-1a and LCFA-CoA's are not implicated in the hypothalamic control of feeding. In support of this prediction, our results are also unfavorable for a

significant role of Arc CPT-1a *per se* in the CNS control of feeding and body weight. In our studies, we were unable to detect significant changes of either food intake or body weight gain following Arc-specific activation of CPT-1a. Furthermore, we demonstrated that CPT-1a activity (by measuring the levels of long-chain acylcarnitines) in the Arc was not significantly altered under either fasting or re-feeding condition (*ad. lib.* fed: 100 ± 17 %, fasted: 104 ± 14 %, re-fed: 112 ± 10 %). Taking these findings together, Arc CPT-1a is unlikely to play a direct and key role in the central controls of feeding and energy balance.

The recent discovery of the brain-specific CPT-1 isoform, CPT-1c (26), may provide some insights into differential roles of CPT-1 isoforms in the central control of feeding and body energy balance. CPT-1c is structurally similar to CPT-1a and CPT-1b, but does not have an appreciable CPT acyltransferase activity (26, 30). Notably, CPT-1c has been implicated in the hypothalamic control of energy balance (30). Given that CPT-1c exhibits a high amino acid sequence similarity to the other CPT-1 members, those CPT-1a regulators (i.e. ST1326 and C89b) may have affected CPT-1c with the same pharmacological action, thus resulting in the same feeding effect. Furthermore, we anticipate that CPT-1c is an alternative downstream mediator in malonyl-CoA's anorectic signaling action. Indeed, our studies have provided evidence that CPT-1c is a downstream mediator of the malonyl-CoA action in leptin Arc anorectic signaling pathways (addressed in another manuscript).

Perspectives and Significance

Our data strongly suggest that the intracellular downstream pathways mediating Arc malonyl-CoA's anorectic effects induced by leptin are independent of CPT-1a. Our study thus leaves open the possibility of other target(s) as mediators of Arc malonyl-CoA anorectic signaling actions.

Acknowledgements: The studies are funded by a grant from the Canadian Diabetes Association and a fellowship from Heart and Stroke Foundation of Canada (awarded to S.G.). G.D.L. is a scientist of the Alberta Heritage Foundation for Medical Research (AHFMR). We thank Ms. Amy Barr (University of Alberta) for the preparation (amplification) of the recombinant adenoviruses. We thank Mr. Ken Strynadka and Mr. Thomas Panakkezhum (University of Alberta) for the HPLC analysis of long-chain fatty acyl-CoA's. F.G.H., D.S., N.C. and P.C. wish to acknowledge the grants from Ministry of Education and Science, Spain (Grant SAF2007-61926 to F.G.H.), and from Instituto de Salud Carlos III (Grant CIBERobn CB06/03/0026 to F.G.H. and research contract to P.C.).

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Figure Legends

Figure 1: **M593S mutant of CPT-1a is insensitive to malonyl-CoA inhibition.**

Yeast extract (10μg of protein) of wild type CPT-1a or M593S mutant CPT-1a was incubated with increasing concentrations of malonyl-CoA, and the CPT-1 activities in the extract were measured (n=4-5). (B) The adenovirus (Ade) expressing the control (null, n=6), wild type CPT-1a (CPT-1a wt, n=6) or M593S mutant CPT-1a (CPT-1a mt, n=6) was administered into the arcuate nucleus (Arc). Two weeks following the administration of the viruses, the rats were euthanized. The individual hypothalamic nuclei tissues (Arc, VMN, LHA and PVN) were dissected from the brain sections and the CPT-1a protein levels were examined by Western blotting. Two representative blots from the Arc of each group are shown and the ratios of the band intensity of CPT-1a to that of β-actin were quantitated (n=6). * and **, vs. null, $P<0.05$. (C) Two weeks following Arc administration of the adenoviruses encoding the null, CPT-1a wt and CPT-1 mt (n=6), the CPT acyltransferase activities in the MBH encompassing the entire Arc and some VMN, LHA and the PVN were measured. The CPT-1a activities of individual VMN tissues were not measured. * and **, vs. null, $P<0.05$. (D) Two weeks following the delivery of the viruses (encoding CPT-1a wt and CPT-1a mt), the rats were euthanized. The mediobasal hypothalamus (MBH) area containing the Arc was dissected and the crude mitochondrial fraction was prepared. Exogenous malonyl-CoA (50μM) was added to the mitochondrial preparation and CPT-1 activity assay was conducted (n=8). The percentages of the activity inhibition by malonyl-CoA (compared to the assay without the addition of exogenous malonyl-CoA) are presented. *, vs. CPT-1a wt, $P<0.05$.

Figure 2: The increase of Arc malonyl-CoA is required in leptin's anorectic actions.

(A) The adenovirus encoding the malonyl-CoA decarboxylase (MCD, n=5) or eGFP (null, n=5) was administered into the Arc on day 0. Daily food intakes and daily body weights were monitored. The daily food intake before the virus injection was used as the baseline level. The differences between the daily food intake from day 1 through day 7 and the basal level are presented. The daily body weight from day 1 through day 7 was compared to day 0 and the percentages of the body weight change are presented. Food intakes: * and **, vs. null, $P<0.05$; ***, vs. null, $P=0.05$. Body weights: *, **, *** and ****, vs. null, $P<0.05$. (B) After one week following Arc delivery of the adenoviruses (MCD, n=5; null, n=5), the rats were euthanized. The MCD activities in individual hypothalamic nuclei (Arc, VMN, LHA and PVN) were measured. The MCD activities from the Arc (n=5) and the VMN (n=5) are shown. *, vs. null, $P<0.05$. (C) The rats were subject to the similar procedures as described in (B). The malonyl-CoA levels (n=3-4) in the Arc were measured. *, vs. null, $P<0.05$. (D) After one week following the virus delivery, a bolus injection of leptin (15 μg, in PBS) was given by ICV before the dark cycle. Then, the food intakes at 3h after the dark onset were monitored (n=6-9). *, vs. null/PBS, $P<0.05$; **, vs. MCD/PBS, $P<0.05$; †, vs. null/leptin, $P<0.05$. Malonyl-CoA levels in the Arc were measured and the percentages of the increase of malonyl-CoA level by leptin (as compared to PBS) are shown (n=6-9). *, $P<0.05$, vs. null.

Figure 3: Blocking malonyl-CoA inhibition of CPT-1 acyltransferase activity does not affect the anorectic action of leptin.

(A) During the second week following the Arc administration of Ade-CPT-1a wt, Ade-CPT-1a mt or Ade-null, a bolus injection of leptin (15 μg in PBS) was given by ICV to the rats before the dark cycle. Overnight food intake and body weight were monitored (n=6). The values from the rat having a targeted Arc overexpression of CPT-1a and an increase of CPT acyltransferase activity were included in the data analysis. *, vs. null/PBS, $P<0.05$; **, vs. CPT-1a wt/PBS, $P<0.05$; ***, vs. CPT-1a mt/PBS, $P<0.05$. (B,C and D) After one week following the Arc delivery of the adenoviruses (CPT-1a wt, CPT-1a mt and null), a bolus injection of leptin (15 μg in PBS) was given by ICV to the rats. The Arc levels (at 3h after ICV injection) of the malonyl-CoA (B, n=5-7), long-chain acylcarnitines (C, n=5-6) and the long-chain acyl-CoA's (D, n=5-8) were measured. NS: the differences between CPT-1a mt/PBS and CPT-1a mt/leptin are not significant. (B) *, vs. null/PBS, $P<0.05$; **, vs. CPT-1a wt/PBS, $P<0.05$; ***, vs. CPT-1a mt/PBS, $P<0.05$. (C) *, CPT-1a wt/PBS vs. null/PBS, $P<0.05$; **, CPT-1a mt/PBS vs. null/PBS, $P<0.05$; †, CPT-1a wt/leptin vs. CPT-1a wt/PBS, $P<0.05$; NS, CPT-1a mt/leptin vs. CPT-1a mt/PBS, not significant. (D) *, CPT-1a wt/PBS vs. null/PBS, $P<0.05$; **, CPT-1a mt/PBS vs. null/PBS, $P<0.05$; †, CPT-1a wt/leptin vs. CPT-1a wt/PBS, $P<0.05$; NS, CPT-1a mt/leptin vs. CPT-1a mt/PBS, not significant.

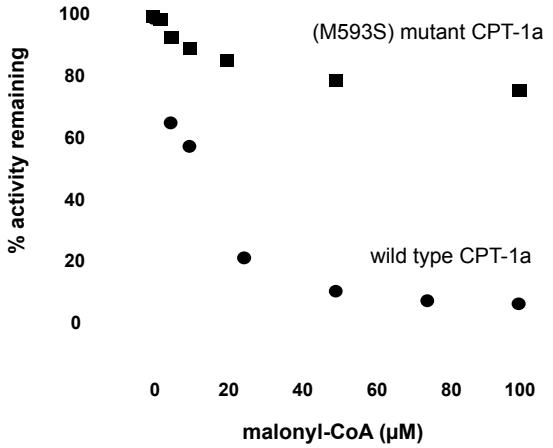
Figure 4: Blocking malonyl-CoA inhibition of CPT-1 acyltransferase activity does not affect the anorectic action of cerulenin.

(A) Ade-MCD or Ade-null was delivered into the Arc of rats. After one week following Arc delivery of the adenoviruses, a bolus injection of cerulenin (125μg, in 25% DMSO) was given by ICV before the dark cycle. Then the malonyl-CoA levels in the Arc were measured. The percentages of the increase of the malonyl-CoA level by cerulenin (as compared to 25% DMSO) are shown (n=6-7). Following ICV injection of cerulenin, the food intakes at 3h after the dark onset and the overnight (24h) body weight changes were also monitored (n=6-7). *, $P<0.05$, vs. null. (B) Ade-CPT-1a wt, Ade-CPT-1a mt or Ade-null was delivered into the Arc of rats. After one week following Arc delivery of the adenoviruses, a bolus injection of cerulenin (125μg, in 25% DMSO) was given by ICV before the dark cycle and overnight food intake and overnight body weight were monitored (n=8-10). Malonyl-CoA levels in the Arc were also measured and the percentages of the increase of the malonyl-CoA level by cerulenin (as compared to 25% DMSO) are shown (n=8-10). *, vs. null/vehicle, $P<0.05$; **, vs. CPT-1a wt/vehicle, $P<0.05$; ***, vs. CPT-1a mt/vehicle, $P<0.05$.

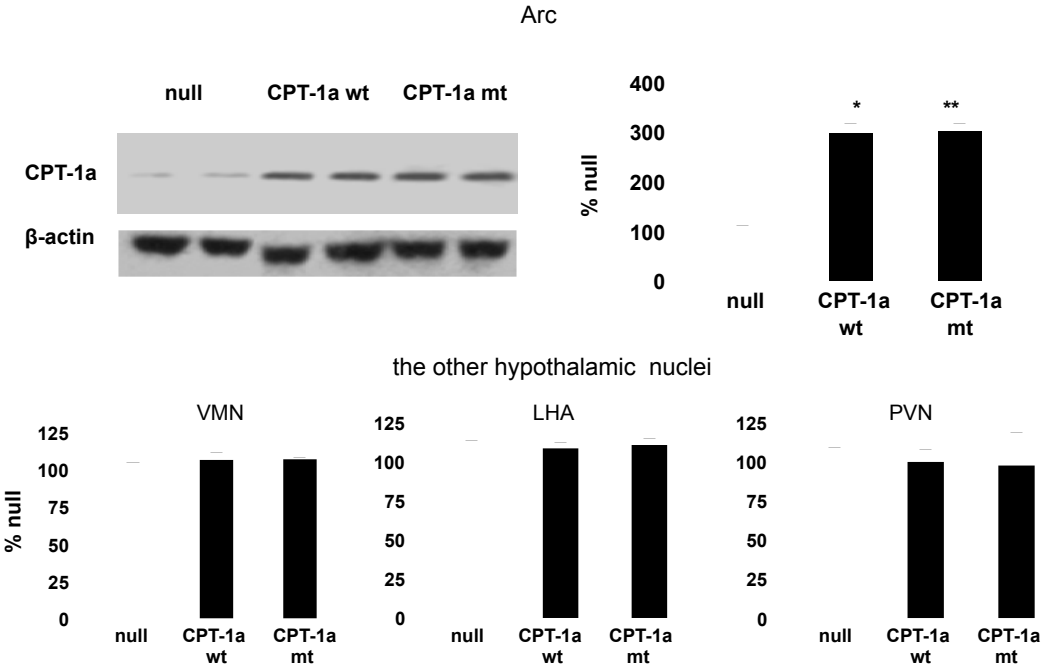
Figure 5: The changes of long-chain acyl-CoA's levels in the Arc are dissociated from the changes of malonyl-CoA levels under fasting and re-feeding conditions.

(A) Some rats were fasted for 48h (fasted, n=9), and the other rats were re-fed for 3h after being fasted for 48h (re-fed, n=6). Arc levels of long-chain fatty acyl-CoA's and malonyl-CoA were measured. *Ad. lib.* fed (n=6) was used as the control. *, fasted vs. *ad. lib.* fed, $P<0.05$; **, re-fed vs. fasted, $P<0.05$. (B) A schematic diagram of the metabolism of brain cellular long-chain fatty acyl-CoA's (LCFA-CoA's) is shown. Intracellular long-chain fatty acid (LCFA) either synthesized *de novo* or transported from the circulation is esterified by acyl-CoA synthetase (ACS) to form LCFA-CoA's. Hydrolysis by acyl-CoA hydrolase and the CPT-1a-mediated mitochondrial β-oxidation are two pathways that lower the cellular LCFA-CoA's levels. In the brain, acyl-CoA hydrolase (BACH) action plays a major role, while the mitochondrial β-oxidation is a minor pathway, in controlling the cellular LCFA-CoA's levels. (C) The rats were subjected to the fasting and re-feeding procedure as described in (A). The plasma levels of free fatty acid were measured (n=5). *Ad. lib.* fed was used as the control. *, fasted vs. *ad. lib.* fed, $P<0.05$; **, re-fed vs. fasted, $P<0.05$. (D) Some rats were fasted for one overnight (fasted, n=5) and the other rats were re-fed for 3h after being fasted for two overnights (re-fed, n=3). Arc acyl-CoA hydrolase activities were measured. Note: the acyl-CoA hydrolase activity levels between 24h-fasting and 48h-fasting are comparable. *, vs. *ad. lib.* fed, $P<0.05$.

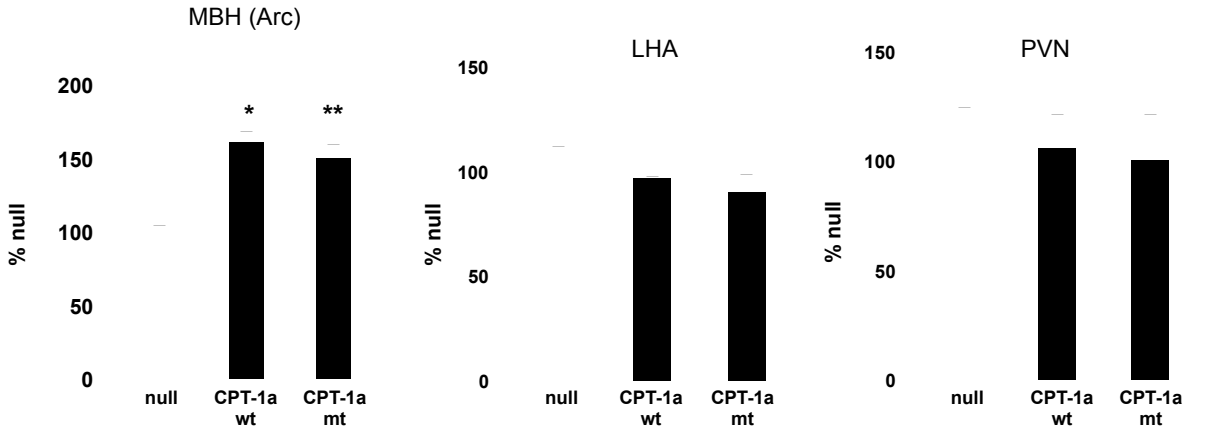
A **Malonyl-CoA sensitivity of M593S mutant of CPT-1a expressed in yeast cell**



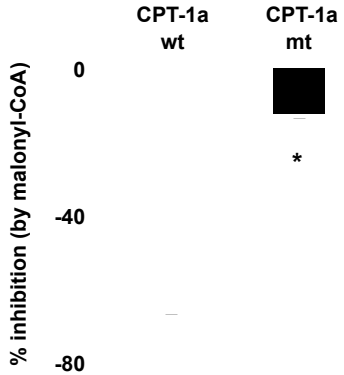
B **CPT-1a protein levels following arcuate nucleus (Arc) administration of the adenoviral vector encoding CPT-1a**



C **CPT-1 activities following arcuate nucleus (Arc) administration of the adenoviral vector encoding CPT-1a**

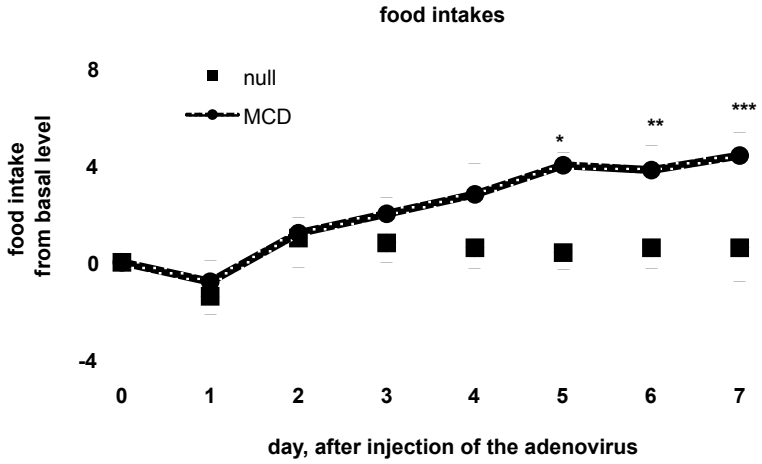


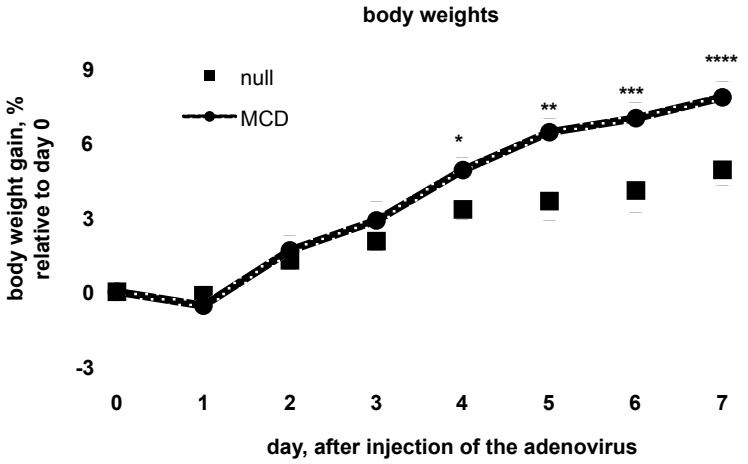
D **Malonyl-CoA sensitivity of the mutant CPT-1a in the Arc**



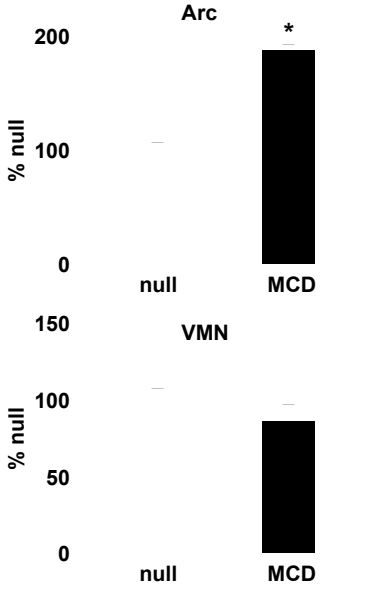
(Figure 1)

A Daily food intake and body weight following arcuate nucleus (Arc) administration of the adenoviral vector encoding malonyl-CoA decarboxylase (MCD)

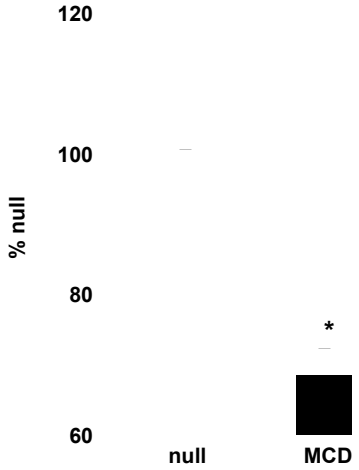




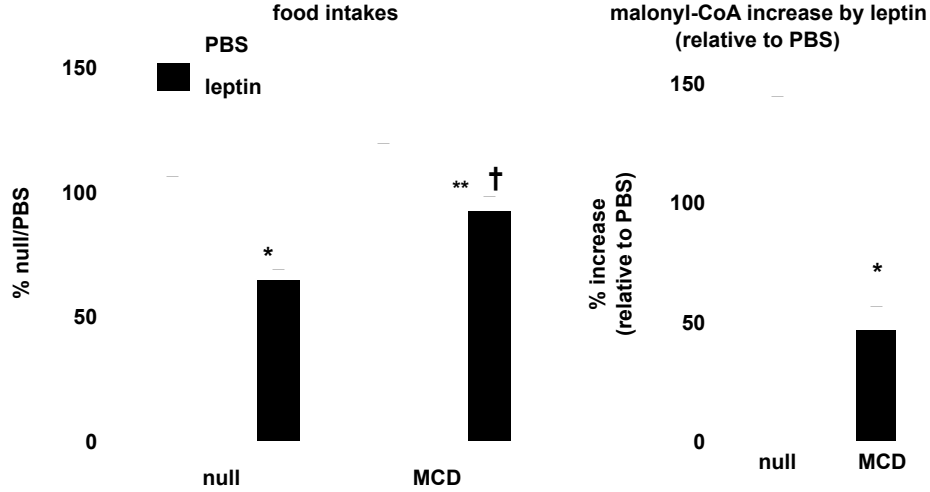
B MCD activities following Arc delivery of the Ade-MCD



C Malonyl-CoA levels following Arc delivery of the Ade-MCD

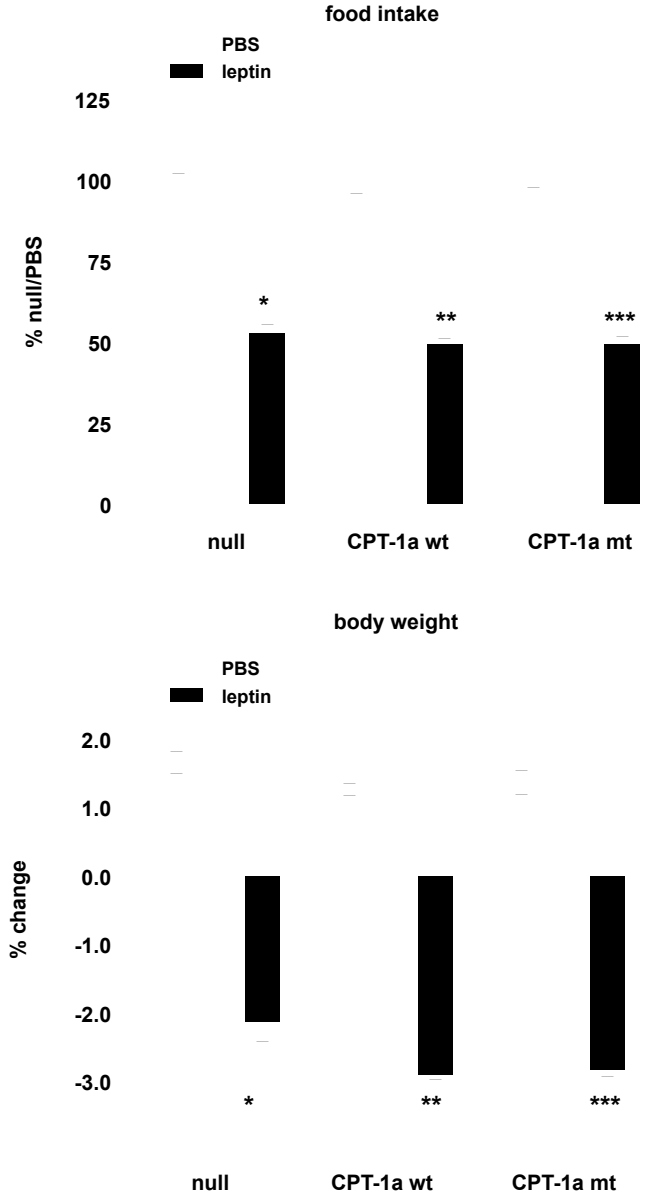


D Leptin's effects on food intakes and malonyl-CoA levels with Arc overexpression of MCD

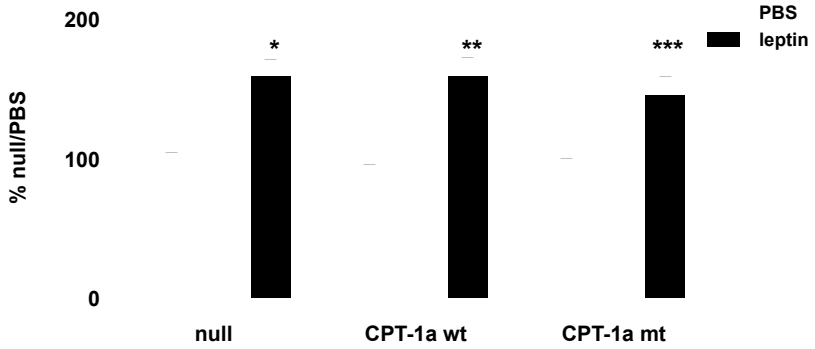


(Figure 2)

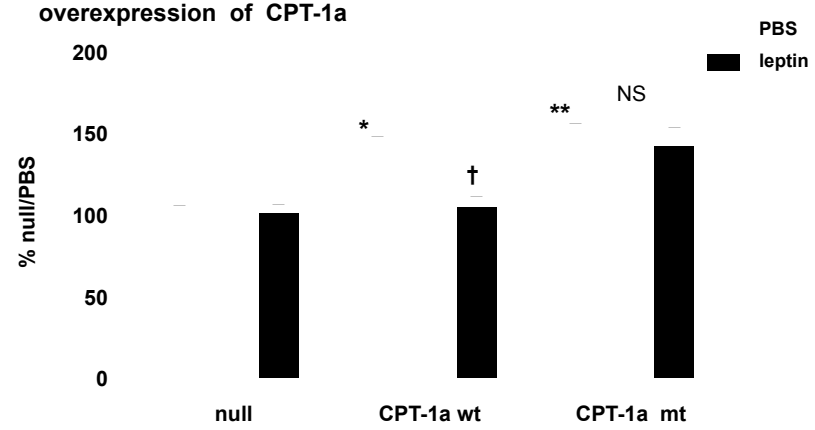
A Leptin's effects on food intake and body weight with Arc overexpression of CPT-1a



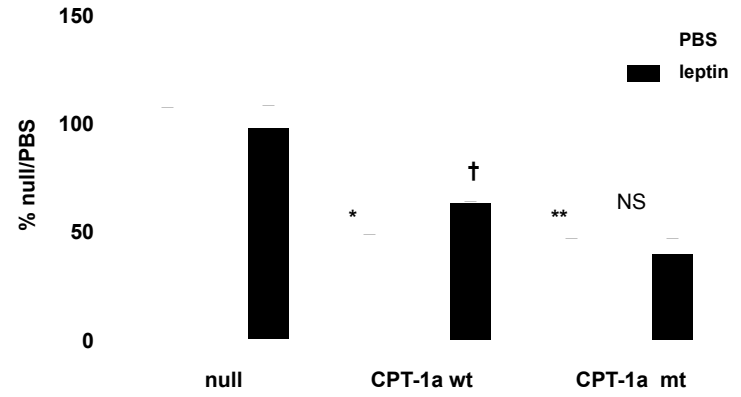
B Leptin's effects on Arc malonyl-CoA levels with Arc overexpression of CPT-1a



C Leptin's effects on long-chain acylcarnitine levels with Arc overexpression of CPT-1a

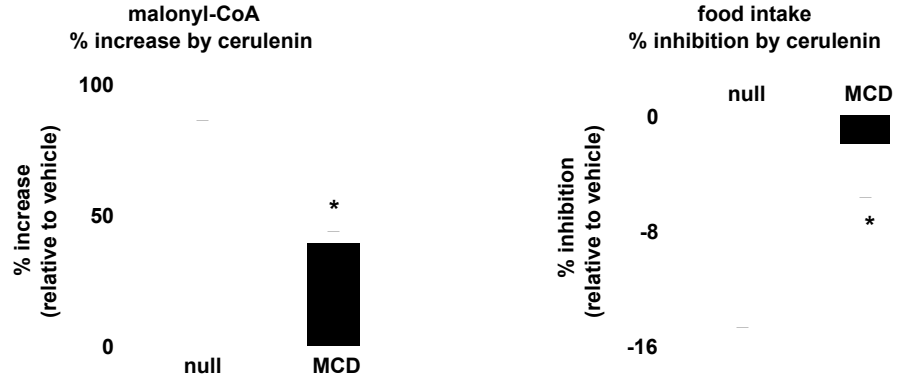


D Leptin's effects on long-chain acyl-CoA's levels with Arc overexpression of CPT-1a

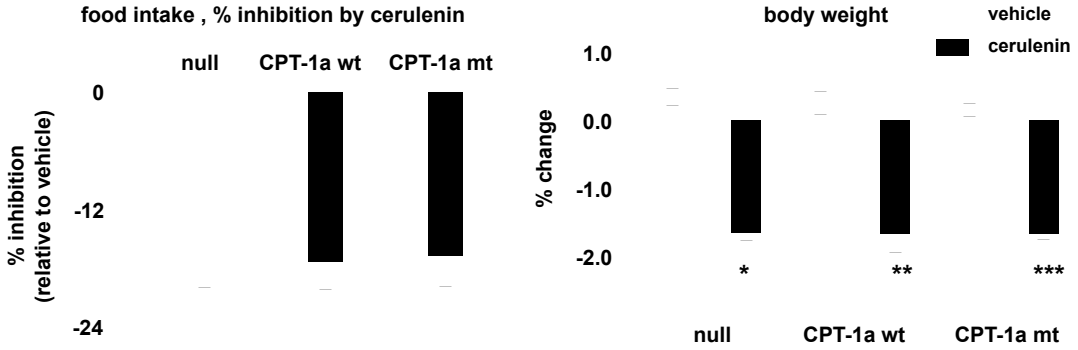


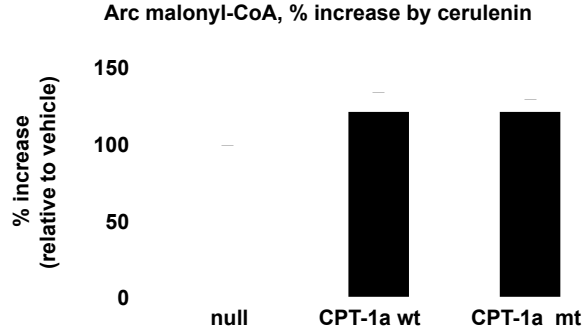
(Figure 3)

A Cerulenin's effects on food intake and body weight with Arc overexpression of MCD



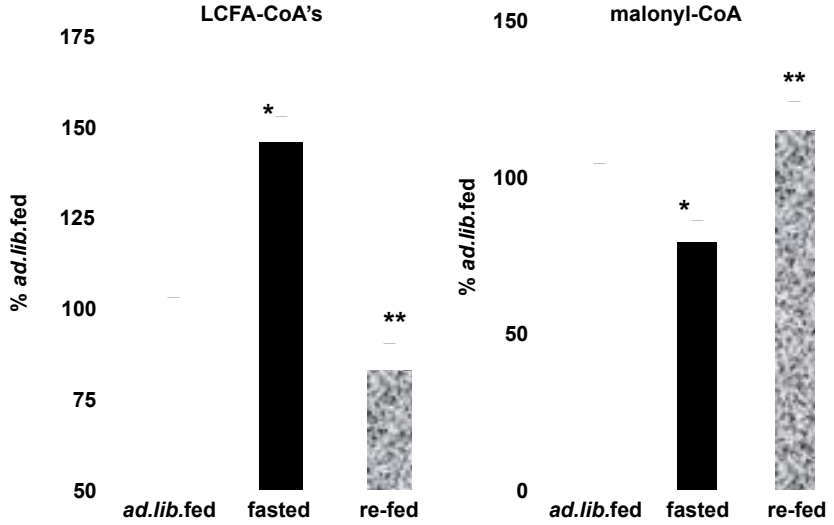
B Cerulenin's effects on food intake, body weight and Arc malonyl-CoA levels with Arc overexpression of CPT-1a



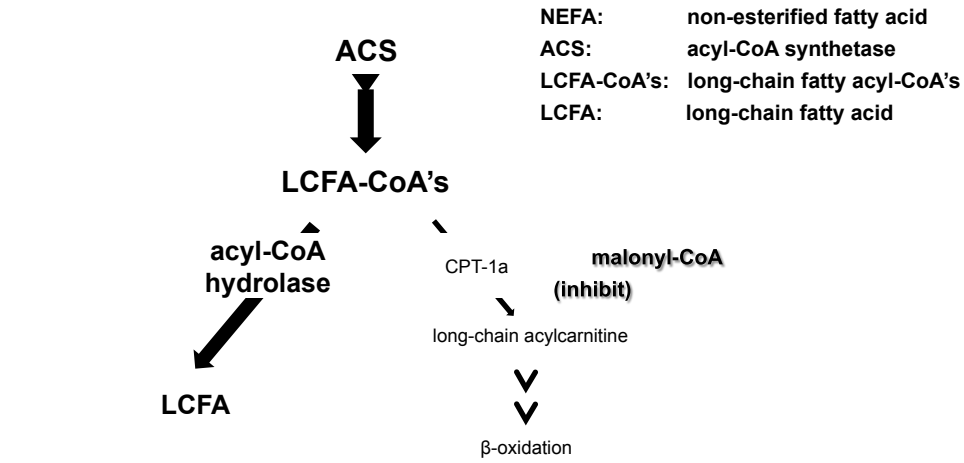


(Figure 4)

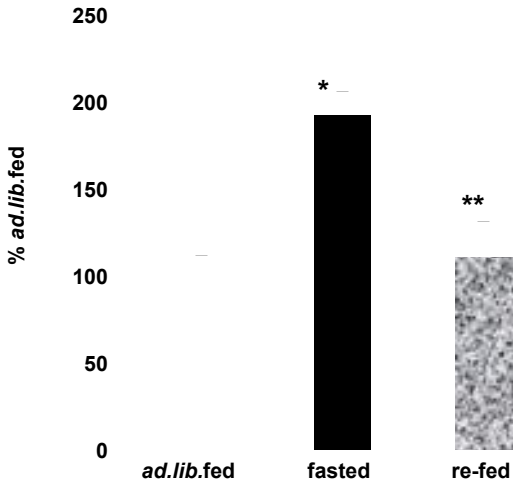
A Arc levels of long-chain fatty acyl-CoA's and malonyl-CoA under fasting and re-feeding states



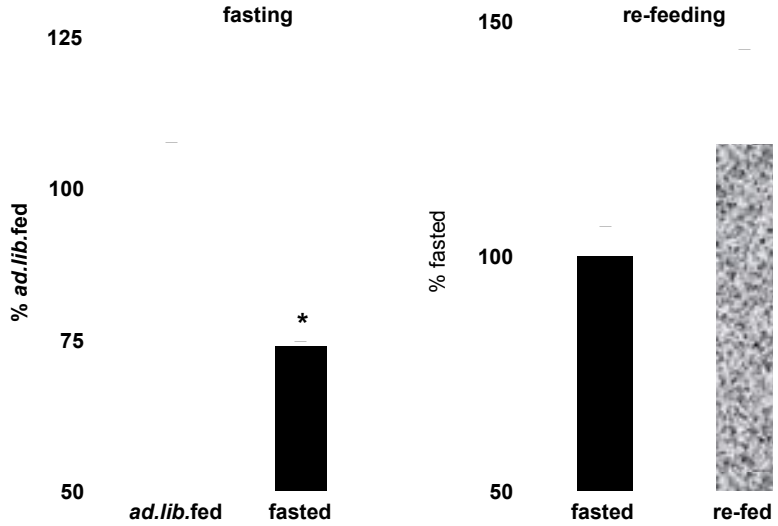
B Diagram of brain cellular long-chain fatty acyl-CoA metabolisms



C Plasma non-esterified fatty acid levels under different feeding states



D Arc acyl-CoA hydrolase activities under different feeding states



(Figure 5)