C75, a Fatty Acid Synthase Inhibitor, Modulates AMP-Activated Protein Kinase to Alter Neuronal Energy Metabolism

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Running Title: Fatty Acid Synthase and AMP-Activated Protein Kinase

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Summary

C75, a synthetic inhibitor of fatty acid synthase (FAS), is hypothesized to alter the metabolism of neurons in the hypothalamus that regulate feeding behavior to contribute to the decreased food intake and profound weight loss seen with C75 treatment. In the present study, we characterize the suitability of primary cultures of cortical neurons for studies designed to investigate the consequences of C75 treatment and the alteration of fatty acid metabolism in neurons. We demonstrate that in primary cortical neurons, C75 inhibits FAS activity and stimulates carnitine palmitoyltransferase-1 (CPT-1), consistent with its effects in peripheral tissues. C75 alters neuronal ATP levels and AMP-activated protein kinase (AMPK) activity. Neuronal ATP levels are affected in a biphasic manner with C75 treatment, decreasing initially, followed by a prolonged increase above control levels. Cerulenin, a FAS inhibitor, causes a similar biphasic change in ATP levels, although levels do not exceed control. C75 and cerulenin modulate AMPK phosphorylation and activity. TOFA, an inhibitor of acetyl-CoA carboxylase, increases ATP levels, but does not affect AMPK activity. Several downstream pathways are affected by C75 treatment, including glucose metabolism and acetyl-CoA carboxylase (ACC) phosphorylation. These data demonstrate that C75 modulates the levels of energy intermediates, thus, affecting the energy sensor AMPK. Similar effects in hypothalamic neurons could form the basis for C75’s effects on feeding behavior.
Introduction

Obesity has become a worldwide health issue, affecting children and adults in developed and emerging countries (1-3). Obesity is a disorder of both energy metabolism and appetite regulation, and must be understood as a dysfunction of energy balance (3). The central nervous system (CNS) plays a critical role in the regulation of energy balance by coordinating peripheral and central signals to assess energy status and regulate feeding behavior (4-6). While it is well known that fatty acid metabolism is an important component of the peripheral regulation of energy metabolism, recent studies indicate that neurons in the hypothalamus may monitor flux through the fatty acid synthesis pathway to ascertain energy balance (7-10).

We and others have demonstrated that C75, a synthetic fatty acid synthase (FAS) inhibitor (11), decreases food intake and results in profound and reversible weight loss (8,12-14). Although inhibition of peripheral fatty acid synthesis might easily explain some of these effects, anorexia was also achieved by central (intracerebroventricular) administration of C75 (8,14). These results suggest that the FAS pathway may play a role in energy homeostasis in the brain, and that modulation of flux through this pathway in neurons or glia could alter energy levels.

FAS is a lipogenic enzyme that catalyzes the condensation of acetyl-CoA and malonyl-CoA to generate long-chain fatty acids (15). In the fed state, long-chain fatty acids are synthesized and stored as triglycerides, which are broken down for energy during periods of energy deficiency. C75 interferes with the binding of malonyl-CoA to
the β-ketoacyl synthase domain of FAS, thus inhibiting long-chain fatty acid elongation (16,17). Although it is established that FAS mRNA levels are high in lipogenic tissues such as liver, lung, and adipose (18), FAS was only recently described in brain (19,9). We have demonstrated that FAS, and other enzymes required for long-chain fatty acid synthesis, are highly expressed in neurons in many brain regions, including hypothalamic neurons that regulate feeding behavior (9), thus positioning FAS to play a role in neuronal energy metabolism.

In addition to inhibiting FAS (11), C75 stimulates carnitine palmitoyltransferase-1 (CPT-1) activity in cultured adipocytes and hepatocytes by preventing malonyl-CoA-mediated inhibition of CPT-1 (10). CPT-1 catalyzes the esterification of long-chain acyl-CoAs to L-carnitine for transport into the mitochondria for fatty acid oxidation (20). When energy reserves and fatty acid synthesis are high, elevated levels of malonyl-CoA, the endogenous inhibitor of CPT-1, prevent the oxidation of newly formed fatty acids (20,21). Conversely, during starvation, when energy levels are low, malonyl-CoA levels fall, releasing the inhibition on CPT-1, allowing fatty acids to enter the mitochondria where they are broken down for energy. While C75 can affect FAS and CPT-1 activities in peripheral tissues, its effects on neurons are unknown.

The effects of C75 on FAS and CPT-1 activities could alter energy flux through metabolic pathways in neurons. One important sensor of cellular energy balance is mammalian AMP-activated protein kinase (AMPK), a heterotrimeric protein kinase present in most mammalian tissues, composed of a catalytic subunit (α) and two
regulatory subunits (\(\beta\) and \(\gamma\)) (22,23). AMPK is most commonly activated by metabolic stresses that deplete cellular ATP and lead to an elevation of the AMP/ATP ratio (22,23), such as heat shock, exercise, ischemia/hypoxia, low glucose, and metabolic or excitotoxic insults in neurons (24).

The regulation of AMPK activity is complex, and once activated, AMPK modulates many aspects of metabolism. Acutely, AMPK phosphorylates and inactivates certain enzymes involved in biosynthetic pathways, such as HMG-CoA reductase and acetyl-CoA carboxylase, thereby preventing further ATP utilization (25,26). Additionally, AMPK stimulates catabolic processes by activating glucose uptake, glycolysis, and fatty acid oxidation in an attempt to restore cellular ATP levels (27-30). AMPK has chronic effects on these pathways via alterations in gene expression (for review see (25)). Although AMPK has been shown to be expressed the brain (24,31,32), its function in neurons is unknown. We have chosen AMPK as a candidate neuronal metabolic sensor that may be affected by C75 based on its well-established roles in energy sensing and the control of fatty acid metabolism in the periphery.

In this study, we explored the role of FAS, CPT-1, and AMPK in neuronal energy metabolism. We utilized primary cortical neuronal cultures as a model system after demonstrating high levels of expression and activity of these key proteins, consistent with their in vivo expression patterns (9,19,24,32). The effects of C75 on ATP levels and AMPK activity were then investigated, and the consequences of altered AMPK
activity to energy production were determined. The work presented here demonstrates that C75, through both direct and indirect actions, alters neuronal energy metabolism in a biphasic fashion. These alterations are functionally important and suggest a role for AMPK in neuronal energy perception.
Experimental Procedures

**Primary Cortical Neuronal Cultures** - All experimental protocols were approved by the Johns Hopkins University Institutional Animal Care and Use Committee, and all applicable guidelines from the National Institute of Health Guide for the care and use of laboratory animals were followed. Cortices were removed from E17 Sprague-Dawley rats (Harlan, Indianapolis, IN), and were dissociated by mild trypsinization and trituration as described (33). Cells were plated on poly-D-lysine coated plastic Nunclon culture dishes at a density of 5x10^5 cells/cm^2 in Minimum Essential Media (MEM) supplemented with horse serum, fetal bovine serum, glutamine, and the antibiotics gentamycin and kanamycin. Cells were plated onto vessels as required for each type of experiment: T-25 flasks for oxidation assays; 6-well plates for Western blots, SAMS peptide assays, and HPLC analysis; 24 well plates for FAS and CPT-1 activity assays; 4 well chamber slides for immunocytochemistry; and 96 well plates for the determination of ATP levels and cell viability assays. For standard cultures cells were treated with cytosine arabinoside on day 4, and were assayed after 7-10 days in vitro. For cultures overgrown with glia, cells were not treated with cytosine arabinoside and were used for immunocytochemistry on day 6. Drug treatments were performed with vehicle or C75 (obtained from and characterized by Craig Townsend and Jill McFadden, the Department of Chemistry at the Johns Hopkins University, and from FASgen, Inc.), resuspended in RPMI; cerulenin (Sigma) resuspended in RPMI; and 5-(tetradecyloxy)-2 Furoic Acid (TOFA) (Craig Townsend and Jill McFadden) resuspended in 100% DMSO.
**Immunocytochemistry** - Cortical neurons were grown as described and harvested 7 days after plating for immunocytochemistry. Cells were fixed with 4% PFA and 20% sucrose for 20 min at 4°C, and permeated with 0.2% Triton X-100 in PBS for 10 min at 4°C. As these cultures normally contain less than 1% glial cells, cultures were also prepared in which glia were allowed to overgrow, as described, to better evaluate the expression of FAS and AMPK in glia. Cells were incubated in blocking solution (PBS containing 4% normal serum) for 1 hr at 4°C. Primary antibodies against the following antigens were diluted in blocking solution overnight at 4°C: glia fibrillary acidic protein (GFAP) (Chemicon International Temecula, CA) 1:1000; neuron-specific tubulin (NST) (Bacbo, Richmond VA) 1:1000; AMPKα(2-20) 1:500; and FAS (9) 1:1000. Cells were incubated for 1 hr at room temperature with secondary antibodies conjugated with FITC for NST and GFAP staining, or with rhodamine for FAS and AMPK staining.

**Measurement of Acetate Incorporation** - Cells were pre-treated with the indicated concentrations of vehicle or C75 for 15 min in conditioned media, and then labeled with 100 μM ³H Acetic Acid (NEN) for an additional 1.75 hours as previously described (34). Lipids were extracted with chloroform/methanol, dried under N₂ and counted using a liquid scintillation counter.
Measurement of ATP - Neurons were lysed on ice using TE buffer (100 mM Tris and 4 mM EDTA) and removed from the plate. ATP levels were then measured in the linear range using the ATP Bioluminescence Kit CLS II (Roche, Indianapolis, IN.) by following the manufacturer’s protocol, and results were read by a Perkin-Elmer Victor\textsuperscript{2} 1420.

Cell viability assay - Cortical neurons were treated for the indicated times with the indicated doses of drug, and viability was determined using the Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR). The conversion of the cell permeant non-fluorescent calcein AM dye to the intensely fluorescent calcein dye is catalyzed by intracellular esterase activity in live cells and is measured by detecting the absorbance at 485 nm/535 nm using the Perkin-Elmer Victor\textsuperscript{2} 1420.

HPLC - Adenine nucleotide levels in primary cortical neuron lysates were determined by HPLC analysis as previously described (35). Briefly, each well of a 6 well plate was washed with 2 ml of ice cold PBS, and lysed with 70 µl of ice cold 0.5 M KOH and scraped. One hundred and forty µl of H2O were added to lysates and incubated on ice for 5 min, and the pH was then adjusted to 6.5 by addition of 1 M KH\textsubscript{2}PO\textsubscript{4}. Cell lysates were spun through Microcon YM-50 centrifugal filters and stored at −80°C for subsequent HPLC analysis. The HPLC used was an Agilent 1100 LC with a variable wavelength detector. The analysis was done using Chemstation A.10.01 software.
Measurement of Fatty Acid Oxidation - Fatty acid oxidation was measured as previously described (36). Briefly, primary cortical neurons adherent to the flask were treated in triplicate with C75 at the indicated doses for the indicated times in of HAM-F10 media supplemented with 10% FBS. One-half μCi/ml (20 nmol) of [1-14C]-palmitic acid (Moravek Biochemicals, Brea, California) resuspended in β–cyclodextran (10 mg/ml in 10 mM Tris) and 2 mM carnitine was added for the last 30 min of each treatment. Flasks were fitted with serum stoppers and plastic center wells (Kontes, Vineland, New Jersey) containing glass microfiber filters (presoaked in 10 μl of 20% KOH). Following the incubation, 200 μl of 2.6 N HClO₄ was injected into the flasks and the 14CO₂ was trapped for 2 hr at 37°C. The filters were removed and quantified by liquid scintillation counting. The contents of the flasks were then hydrolyzed with 200 μl of 4 N KOH and neutralized using H₂SO₄. The water soluble products were extracted using CHCl₃/MeOH and H₂O and quantified by liquid scintillation counting. The total amount of fatty acid oxidation was obtained by addition of the 14CO₂ and water soluble products and represented as % of control or as a specific activity (nmol/hr/mg).

Measurement of Glucose Oxidation - Glucose oxidation assays were based on previously described work (37). Neurons adherent to the flask were treated in triplicate with C75 at the indicated doses for the indicated times in Krebs-Ringer bicarbonate HEPES buffer (KRBH buffer: 135 mM NaCl, 3.6 mM KCl, 0.5 mM NaH₂PO₄, 0.5 mM MgCl₂, 1.5 mM CaCl₂, 5 mM NaHO₃ and 10 mM HEPES) containing 1% BSA and 10
mM D-glucose. One-half \( \mu \text{Ci/ml} \) [U-\( ^{14} \text{C} \)]-glucose (NEN) was added for the last 30 min of each treatment and flasks were fitted as described for fatty acid oxidation assays. Reactions were stopped with the injection of 7% perchloric acid into the flask, and then 400 \( \mu \text{l} \) of benzethonium hydroxide was injected into the center well. After 2 hr at 37\(^\circ\)C, complete oxidation was quantified by measuring the amount of \( ^{14} \text{CO}_2 \) in the center well by liquid scintillation counting, and represented as % of control or as a specific activity (pmol/hr/mg).

**Measurement of CPT-1 Activity** - CPT-1 activity was measured using digitonin permeabilization (38). Drugs and vehicle controls were added as indicated for each experiment. After 2 hr, the medium was removed, cells were washed with PBS, and incubated with 700 \( \mu \text{l} \) of assay medium consisting of: 50 mM imidazole, 70 mM KCl, 80 mM sucrose, 1 mM EGTA, 2 mM MgCl\(_2\), 1 mM DTT, 1 mM KCN, 1 mM ATP, 0.1% fatty acid free bovine serum albumin, 70 \( \mu \text{M} \) palmitoyl-CoA, 0.25 \( \mu \text{Ci} \) [methyl-\( ^{14} \text{C} \)]L-carnitine (Amersham Pharmacia Biotech, Piscataway, NJ), 40 \( \mu \text{g} \) digitonin, with or without 100 \( \mu \text{M} \) malonyl-CoA. After incubation for 6 min at 37\(^\circ\)C, the reaction was stopped by the addition of 500 \( \mu \text{l} \) of ice-cold 4 M perchloric acid. Cells were then harvested and centrifuged at 13,000 x g for 5 min. The pellet was washed with 500 \( \mu \text{l} \) ice-cold 2 mM perchloric acid and centrifuged again. The resulting pellet was resuspended in 800 \( \mu \text{l} \) dH\(_2\)O and extracted with 400 \( \mu \text{l} \) of butanol. The butanol phase, representing the acylcarnitine derivative, was measured by liquid scintillation counting.
**Western Blot Analysis** - Cells were lysed using an identical method and buffer as described for the AMPK activity assay. Samples were boiled and run on 10% polyacrylamide gels, and transferred to PVDF membrane. Blots were successively probed, stripped, and reprobed with the following antibodies: anti-pAMPKα, AMPKα (Cell Signalling, Beverly, MA), and sheep anti-FAS (39). Samples for the ACC/pACC westerns were run on separate 5% polyacrylamide gels and probed with either anti-ACC or anti-phospho-Ser79 ACC antibodies (40).

**Measurement of AMP-Activated Protein Kinase Activity** - AMPK activity was determined by performing SAMS peptide assays as previously described (41). Neurons plated on 6 well culture dishes were lysed using 350 μl per well of Triton X-100 lysis buffer: 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1% Triton X-100, 250 mM sucrose, 50 mM NaF, 5 mM NaPP1, 1 mM dithiothreitol, 50 μg/ml Leupeptin, 0.1 mM Benzamidine, and 50 μg/ml trypsin inhibitor. Three wells were pooled per condition, and AMPKα was immunoprecipitated in the presence anti-AMPKα(2-20) antibody coupled to Protein A/G beads (Santa Cruz, CA). Immunoprecipitates were then washed and resuspended in 4X assay buffer and kinase activity was assessed by measurement (for 20 min at 30°C) of the incorporation of 32P into the synthetic SAMS peptide substrate, HMRSAMSGLLHLVKRR, (Princeton Biomolecules). Samples were spotted on P81 phosphocellulose paper, washed extensively, and quantitated by Cerenkov counting.
Each sample was corrected for protein concentration and reported either as % of control or as pmol/min/mg.

**Electrophysiology and mEPSC analysis** - Whole cell patch clamp recordings were performed from cortical cultures at 14-21 days in vitro. To isolate AMPA-mediated mEPSCs, neurons were continuously perfused with artificial cerebral spinal fluid (aCSF) at a flow rate of <1 ml/min. The composition of aCSF was as follows (in mM): 150 NaCl, 3.1 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, 0.1 DL-APV, 0.005 strychnine, 0.1 picrotoxin, and 0.001 tetrodotoxin (TTX). The osmolarity of the aCSF was adjusted to 305–310, pH was 7.3-7.4. Intracellular saline consisted of (in mM): 135 CsMeSO₄, 10 CsCl, 10 HEPES, 5 EGTA, 2 MgCl₂, 4 Na-ATP, and 0.1 Na-GTP. This saline was adjusted to 290-295 mOsm, pH 7.2.

Once the whole-cell recording configuration was achieved, neurons were voltage clamped and passive properties were monitored throughout. In the event of a change in $R_s$ or $R_i$ greater than 15% during the course of a recording the data were excluded from the set. mEPSCs were acquired through an Axopatch 200B amplifier (Axon Instruments, Union City, CA), filtered at 2 kHz and digitized at 5 kHz. Sweeps (20 seconds) with zero latency were acquired until a sufficient number of events were recorded (minimum of 5 minutes). Data was continuously recorded only after a period of 1-2 minutes where the cell was allowed to stabilize. mEPSCs were manually detected with MiniAnalysis (Synaptosoft Inc, Decatur, GA) by setting the amplitude threshold to RMS * 3 (usually 4 pA). Once a minimum of 100 events was collected
from a neuron, the amplitude, frequency, rise time (time to peak), decay time (10%-90%), and passive properties were measured. In all electrophysiological experiments, a similar amount of data (n) was acquired from each experimental group (i.e. DMSO, Drug). Data from each group was then averaged and statistical significance determined by the student T test. Data were never reused or transferred from one experimental group to another (DMSO controls were exclusive).

**Statistical analysis** - Data are presented as means ± standard error of the mean from multiple determinations (n>4). Unless otherwise noted, data were analyzed by One-way ANOVA with Dunnett post test to compare treated samples with controls. Differences from post tests were considered statistically significant at *, P < 0.05; **, P<0.01; ***, P<0.001. For the analysis of AMPK activity results (Fig. 5C and D) each time point was compared with control samples by performing unpaired one-tailed t-tests.
Results

**FAS and AMPK are expressed in neurons in primary culture** - To establish whether primary cortical neuronal cultures could serve as a model for biochemical studies designed to evaluate the effects of FAS inhibition/CPT-1 stimulation on neuronal metabolism, immunocytochemistry was performed to identify the cell type(s) that express FAS and AMPK in vitro, and to determine if these patterns of expression recapitulate in vivo expression (9,19) (Fig. 1). Double-labeling immunocytochemistry was performed for NST and either FAS (Fig. 1A) or AMPK (Fig. 1B) to determine localization in neurons, and for glial fibrillary acidic protein (GFAP) and either FAS (Fig. 1C) or AMPK (Fig. 1D) to determine localization in glia. FAS immunoreactivity was uniformly seen in NST-positive cells, indicating neuronal expression of FAS, consistent with the in vivo pattern of expression (9,19). FAS localization was most prominent in cell bodies, where it co-localized with NST (Fig. 1A). Less FAS-immunoreactivity was visualized in NST-positive processes. The catalytic subunits of AMPK (α1 and α2) have been localized to neurons throughout the developing and adult rat brain (24,32). Neuronal expression of AMPKα was confirmed in the cortical cultures by double-immunolabeling (Fig. 1B).

In contrast, minimal FAS immunoreactivity was visualized in GFAP-positive cells, indicating that there was relatively little FAS expressed in glia (Fig. 1C). Co-localization was detected only in occasional glial processes, where FAS may serve a role in membrane synthesis. AMPK immunoreactivity did not co-localize with GFAP, indicating
that AMPK was not highly expressed in glia, confirming the expression pattern for AMPK seen in the cortex in vivo (32). For these experiments, cortical cultures were allowed to overgrow with glia so that a larger population of glia could be examined, as these cultures normally contain less than 1% glia. These results indicate that FAS and AMPK expression is predominantly neuronal, and is consistent with in vivo expression patterns. FAS and AMPK were predominantly expressed in neuronal cell bodies, also consistent with in vivo studies. These data demonstrate the robust expression of FAS and AMPK in neurons in primary cultures and support the use of these cultures for the current studies.

**C75 inhibits FAS and stimulates CPT-1 and fatty acid oxidation in neuronal cultures** - C75 effectively inhibits the activity of purified FAS (11), and the activity of FAS in adipose tissue (8). The IC$_{50}$ of C75 for FAS activity in cortical cultures was determined. Primary cultures of cortical neurons were prepared as described in Methods, and assayed for FAS activity by measuring the incorporation of [$^3$H]-acetic-acid into lipid (Fig. 2A). C75 treatment caused a dose-dependent decrease in acetate incorporation, with an approximate IC$_{50}$ of 40 µg/ml. These results indicate that C75 can inhibit FAS activity in primary cortical neurons.

In addition to FAS inhibition, C75 has been shown to stimulate carnitine palmitoyltransferase-1 (CPT-1) activity, increasing fatty acid oxidation and ATP levels in 3T3-L1 adipocytes (10). CPT-1 is an integral mitochondrial membrane protein that
catalyzes the esterification of long-chain acyl-CoAs to L-carnitine, which is the rate-limiting step in the transport of these acyl moieties from the cytosol into the mitochondria, where they undergo fatty acid oxidation (20). CPT-1 is sensitive to inhibition by malonyl-CoA, which is important for signaling the availability of fuels (42), (43). In mammals, CPT-1 exists as two isoforms, liver and muscle (L-CPT-1 and M-CPT-1, respectively), that are similar in sequence but differ kinetically with respect to their malonyl-CoA sensitivities and $K_m$'s for carnitine (44),(45). C75 has been shown to compete with malonyl-CoA at its binding site on CPT-1 in several cell types tested (10).

Based on RT-PCR results, the primary cortical neurons used here contain both the liver and muscle CPT-1 isoforms (data not shown). To determine the effect of C75 on neuronal CPT-1 activity, CPT-1 activity assays were performed by measuring acylcarnitine formation. C75 significantly increased CPT-1 activity (217% of control), whereas malonyl-CoA inhibited activity to approximately 30% of control as expected (Fig. 2B). Although previous studies indicated that minimal fatty acid oxidation occurs in neurons as compared with other tissues (46), experiments were performed to determine whether C75 could alter the rate of fatty acid oxidation in cortical neurons, as it did in peripheral tissues (10). The level of total fatty acid oxidation was determined by combining the $^{14}$C labeled acid soluble and CO$_2$ products obtained after the addition of [1-$^{14}$C]-palmitate. Though the average basal rate of fatty acid oxidation is low in these neurons, (0.316 nmol/h/mg), C75 does significantly increase the total fatty acid oxidation to 146% of control after 2 hr (Fig. 2C). The increase seen with C75 in both
CPT-1 activity and fatty acid oxidation suggests that C75, through modulation of these metabolic pathways, may influence cellular energy balance.

**C75 increases ATP levels in primary cortical neuronal cultures** - FAS utilizes acetyl-CoA, malonyl-CoA (synthesized through the action of acetyl-CoA carboxylase (ACC) in an ATP-dependent step), and NADPH to generate palmitate. We hypothesized that C75-induced alterations in fatty acid metabolism might affect neuronal energy balance, which could signal a change in energy status. To investigate this, the effect of C75 on ATP levels was determined (Fig. 3). Treatment of cortical neuronal cultures with C75 for 2 hr showed a significant dose-dependent increase in cellular ATP levels, up to 267.7% of control (Fig. 3A), consistent with C75’s effects on other cell types, including 3T3-L1 adipocytes (10). ATP levels then decreased with higher doses of C75, possibly through compensatory mechanisms. Neurons can be very sensitive to sudden changes in cellular ATP, and sudden changes in ATP levels may be a signal of cell stress prior to cell death (47). Therefore, viability assays were performed to demonstrate that even at extremely high doses of C75, minimum loss of neuronal viability is observed (Fig. 3B). The effect of 40 µg/ml of C75 on neuronal ATP levels was sustained, maintaining ATP levels above control values for at least 15 hr after treatment (Fig. 3C), with no significant loss of viability (Fig. 3D), suggesting that, although this alteration in neuronal energy status is significant and prolonged, it does not negatively impact neuronal viability. For the remainder of these studies, 40 µg/ml
C75 was used. These results demonstrate that a single dose of C75 significantly affects neuronal ATP levels for a prolonged time course.

C75 has a biphasic effect on ATP levels - We next examined the effect of C75 on ATP levels over short time intervals (Fig. 4). Elucidation of the effects of C75 on ATP levels at earlier time intervals was considered important for two reasons. The time course of C75’s action on ATP levels could clarify the mechanism of this effect on ATP levels. Additionally, cellular energy sensors such as AMPK may monitor the relative changes in cellular energy, not only the absolute amount of ATP. When sampled at short time intervals, C75 treatment caused a decrease in ATP levels, which dropped to approximately 65% of control at 2.5 min (Fig. 4A). By 30 min, ATP levels began to recover, returning to control levels by 1 hr, after which ATP levels increased (Fig. 3C). Though short lived, this transient decrease in ATP that occurs prior to the prolonged increase in ATP may play an important role in the downstream effects of C75.

The transient decrease in ATP levels prior to the increase caused by C75 treatment was compared to the effects on ATP levels produced by other reagents that inhibit FAS or that inhibit other steps in the fatty acid synthesis pathway (Fig. 4B). One possible explanation for the initial decrease in ATP is that in the setting of FAS inhibition, there may be a period of futile cycling of ACC, the rate-limiting enzyme in long-chain fatty acid synthesis. Following FAS inhibition, ACC may continue to synthesize malonyl-CoA, thus consuming ATP, while malonyl-CoA decarboxylase would
continue to degrade malonyl-CoA. If so, other reagents that inhibit FAS should cause a similar transient decrease in ATP levels, while those that inhibit ACC should not.

To test this hypothesis, ATP levels were determined following treatment with TOFA and cerulenin, inhibitors of ACC and FAS, respectively (48,49). Cerulenin is a natural product FAS inhibitor, and does not directly affect CPT-1 activity (50). Concentrations of inhibitors were tested for their abilities to inhibit the incorporation of \[^{3}H\]acetic acid into lipid to a similar extent as 40 \(\mu\)g/ml C75 (data not shown). The cerulenin and TOFA concentrations that were chosen corresponded to or exceeded the IC\(_{50}\) for FAS inhibition compared to the dose of C75 employed. These concentrations were found to have no significant effect on neuronal viability for the treatment duration used in these studies (data not shown).

Treatment of neuronal cultures for 2 hr with increasing concentrations of cerulenin caused no significant increase in ATP levels above control (Fig. 4C); however, a decrease in ATP (to approximately 70% of control), comparable to that seen with C75, was observed at early time points (5 min-1 hr) (Fig. 4D). Similar to C75, full recovery of ATP levels to control values was seen by 2 hr. In contrast, inhibition of ACC with increasing concentrations of TOFA increased ATP levels significantly to approximately 240% of control (Fig. 4E). However, unlike the inhibition of FAS with C75 or cerulenin, no decrease in ATP was observed from 5 min to 1 hr with 20 \(\mu\)g/ml TOFA (Fig. 4F). These data demonstrated that FAS inhibition by either C75 or cerulenin transiently, but significantly, reduced ATP levels. In contrast, TOFA, which acts proximally in the
pathway prior to ATP consumption, did not cause a decrease in ATP levels. Collectively, these data support a futile cycling hypothesis and indicate that manipulation of the fatty acid synthesis pathway can affect energy levels in neurons.

To confirm the changes in ATP levels with C75 treatment, and to determine AMP levels, HPLC was performed on lysates prepared from primary cortical neuronal cultures. In most cases, it is the combined rise in AMP and the fall in ATP levels that activates AMPK (25). AMP and ATP levels were found to change reciprocally in response to C75 treatment (Fig. 4G). At 5 min following C75 treatment, AMP was increased significantly above control. The converse was seen after 2 hr of treatment, a time at which ATP levels are just beginning to increase. Since AMP and ATP levels display a reciprocal relationship with C75 treatment, it is likely that ATP was generated through catabolic reactions, not through the action of adenylate kinase (25,51).

**C75 treatment modulates AMPK phosphorylation and activity** - The long term increase in ATP levels seen with C75 might alter the activity of proteins that function to monitor energy levels. To determine whether the alteration in neuronal energy levels observed with C75 treatment was physiologically important, the effect of C75 on a potential downstream effector, AMPK, was examined. The sensitivity of neurons to changes in energy status and the well-established role of AMPK as an energy sensor that activates catabolic pathways when energy states are low made AMPK an attractive target for the consequences of C75’s effects. To be fully activated AMPK must be phosphorylated once bound to AMP (25). To assess the phosphorylation status of
AMPK, western blot analysis was performed on extracts from C75- and vehicle-treated cultures. AMPK phosphorylation initially increased compared to control (at 5 and 30 min), after which phosphorylation decreased (Fig. 5A). No changes in the overall level of AMPK or FAS protein were observed with C75 treatment. As anticipated, the level of AMPK phosphorylation was inversely related to the level of ATP in cells in culture (Fig 5B).

The altered phosphorylation status suggested that C75 affected the activity of AMPK. To directly measure AMPK activity, SAMS peptide activity assays were performed (Fig. 5C). In control cultures, AMPK activity was 0.3446 pmol/min/mg. AMPK activity was altered by C75 treatment in agreement with AMPK phosphorylation. Thus, at 5 min, when ATP levels were decreased, AMPK activity was increased to approximately 125% above control (Fig. 5C). As ATP levels began to increase after 30 min of C75 treatment, AMPK activity decreased, and was significantly reduced to 25% of control levels at 2 hr. AMPK activity remained decreased for at least 4 hr.

All three reagents, C75, cerulenin, and TOFA, affected ATP levels to some degree, albeit with different profiles (Fig. 4). Thus, a biphasic effect on ATP levels (an initial reduction followed by an increase) occurred with C75 and cerulenin, but not with TOFA. TOFA caused an increase in ATP levels, but did not cause an initial decrease. While both cerulenin and C75 caused transient decreases in ATP levels, only C75 treatment resulted in an increase in ATP above basal levels. We wished to determine whether all reagents also affected AMPK activity, or whether only certain profiles of ATP
level fluctuations correlated with changes in AMPK activity. This is a relevant point; while C75 and cerulenin influence food intake, TOFA treatment alone does not, but TOFA reverses the anorexic effect of C75 in mice (8). SAMS peptide assay was performed on cortical cultures treated with cerulenin or TOFA (Fig. 5D). Although cerulenin treatment significantly decreased AMPK activity, this effect was not as great as that of C75. Surprisingly, TOFA, which had caused a significant increase in ATP levels, did not affect AMPK activity. These results suggested that it was not the absolute level of ATP that influences AMPK activity, but a fluctuation (a decrease followed by an increase) in ATP levels that affected AMPK activity.

_C75 affects both metabolic pathways downstream of AMPK and neuronal activity._ To determine whether the changes seen with C75 regarding ATP levels and AMPK activity were physiologically relevant to neurons, the activities of pathways that are regulated by AMPK were examined after C75 treatment. It is known that when activated, AMPK inactivates ACC via phosphorylation (30,52). No change in ACC phosphorylation status could be detected at early time points, when AMPK was activated at 5 min (data not shown); however, at 2 hr of C75 treatment, when AMPK is unphosphorylated and inactive, the level of phosphorylated ACC present was drastically reduced, suggesting that the status of AMPK is affecting downstream ACC activity (Fig. 6A).
AMPK has been shown to enhance glycolytic flux thru the phosphorylation and activation of the bi-functional enzyme, phosphofructokinase/fructose 2,6-bisphosphatase (PFK-FBPase-2) (27,28,53). Therefore, to determine whether the change in AMPK activity with C75 treatment affected glucose utilization, glucose oxidation assays were performed (Fig. 6B). The average basal glucose oxidation rate for untreated neurons was 0.3140 pmol/hr/mg. C75 stimulated glucose oxidation above this basal rate at 30 min and 1 hr (140 and 120% of control, respectively, Fig. 6B), suggesting that increased glucose oxidation may represent a source of the increased ATP observed with C75 treatment, likely through the modulation of AMPK activity.

We next questioned whether C75 would have an effect on neuronal activity based on the fact that it appeared to alter neuronal energy metabolism, and hypothalamic c-Fos expression (54). To investigate potential effects of FAS inhibitors on synaptic transmission, we applied either C75 or cerulenin to cortical neurons in culture and measured AMPA-mediated miniature excitatory postsynaptic currents (mEPSCs). Applying cerulenin over a 30-minute period resulted in little change in frequency or amplitude (n=4) of mEPSCs (Fig. 6C). However, application of C75 over the same time period resulted in a dramatic increase in mEPSC frequency, while having no effect on mEPSC amplitude (n=4) (Fig. 6C and D). In addition to acute application of FAS inhibitors, cerulenin and C75 were also applied chronically (2 hrs), and the AMPA-mediated mEPSCs relative to DMSO control neurons determined. Neurons treated with C75 exhibited a 15-fold increase in mEPSC frequency, while neurons treated with cerulenin showed a slight, but not significant increase in frequency (~2-fold)(Fig. 6E). In
contrast, application of either C75 or cerulenin to cultured neurons did not affect mEPSC amplitude.

For each drug, we also measured various passive parameters of AMPA miniature events. For instance, both C75 (DMSO = 2.21 ± 0.02 ms; C75 = 2.01 ± 0.02 ms) and cerulenin (DMSO = 2.10 ± 0.01 ms; C75 = 1.91 ± 0.05 ms) significantly reduced the decay time of mEPSCs, while each also showed a trend toward a reduction in mEPSC rise time (data not shown). Further, passive properties from each group were also measured. We found no change in access resistance from either C75 or cerulenin groups, though we did find that C75 caused a significant increase in input resistance when compared to DMSO control neurons (DMSO = 427 ± 47 mOhms; C75 = 610 ± 70 mOhms), while cerulenin treatment resulted in a non-significant effect on this parameter (DMSO = 476 ± 61 mOhms; cerulenin = 571 ± 59 mOhms). Together, these results demonstrate that the cellular changes induced by C75 affect many downstream processes, including neuronal firing and multiple downstream targets of AMPK, and are thus physiologically relevant.
Discussion

In the present study, we demonstrate that alteration of fatty acid metabolism, specifically through the inhibition of FAS, had significant effects on cellular energy levels that in turn modulates metabolism to increase neuronal energy balance. For these studies, a previously well-established primary cortical neuron model system was utilized (33). This model permitted biochemical analysis of the metabolic effects of C75 in neurons, and allowed for the identification of four consequences of C75 treatment: 1) the inhibition of FAS leads to a rapid, transient decrease in ATP; 2) there is rapid activation of AMPK, accompanied by the activation of downstream energy producing/conserving pathways that increase ATP levels; 3) activation of CPT-1 and fatty acid oxidation may contribute to increased ATP levels; and 4) neuronal activity is increased (Fig. 7). The reciprocal change in AMP and ATP levels following C75 treatment supports the model that ATP is generated through catabolic reactions, and not through the alteration of adenylate kinase activity, which is further supported by the increases in both glucose and fatty acid oxidation with C75 treatment.

C75 has the same dual action in neurons, inhibiting FAS and activating CPT-1, as it does in previously examined peripheral tissues. The effect of C75 on CPT-1 is not of the same magnitude in neurons as in other tissues; this can be explained by the limiting enzymes that control fatty acid oxidation in neurons (55). We propose that this dual action of C75 contributes to the biphasic change in ATP levels. The initial decrease in ATP may be due to the direct inhibition of FAS and the temporary energy
consuming futile cycling of ACC that follows. The decrease in ATP seen with the inhibition of FAS by C75 and cerulenin, and the lack thereof when ACC is inhibited by TOFA, support this model. The increase in ATP at later time points following the inhibition of ACC with TOFA could be explained by the conservation of ATP that is no longer utilized by ACC and/or a decrease in malonyl-CoA levels with a subsequent increase in CPT-1 activity. Following the initial decrease, the prolonged increase in ATP may occur through multiple mechanisms, one of which is the direct activation of CPT-1, while another is indirect through the actions of downstream effectors such as AMPK that respond to the initial decrease in ATP.

We propose that it is the initial decrease in ATP following C75 treatment with the corresponding increase in AMP that activates AMPK. A similar decrease in ATP, without a transient activation of AMPK, is seen with cerulenin; however, both compounds cause a significant decrease in AMPK activity. This is consistent with the similar but less dramatic effect on weight loss and anorexia seen with cerulenin when compared to C75 (8,14,12). Of the fatty acid synthesis inhibitors tested, C75 is the most effective at weight loss in vivo and at modulating AMPK activity in vitro. These results suggest that it may be the fluctuations in the changes in cellular energy levels, not only the absolute values they attain, that are important for C75’s action. There is precedent for such a mechanism in the regulation of feeding. It is the transient decrease and then rise of plasma glucose, as opposed to simply a change in absolute plasma glucose concentration, that is detected by the brain and important for triggering meal initiation (for review, see (56)).
While TOFA, which inhibits ACC, increased ATP levels in this study, it did not affect AMPK activity. Similarly, TOFA had no effect on feeding behavior in vivo, whereas pretreatment with TOFA actually reversed the anorexic effect of C75 (8). A somewhat different result was obtained by Wakil and collaborators (57), who found that Acc2−/− mutant mice had an increase in food intake compared with wild-type mice. These effects may be the result of compensatory responses observed in mice with chronic alterations in ACC activity.

AMPK, known for its role in peripheral tissues as an energy sensor, is predominantly expressed in neurons of the CNS, and may mediate at least some of the downstream changes in energy production that occur as a consequence of C75 treatment. The presence of AMPK in neurons and its relatively low level of expression or absence in glia as indicated by this study is consistent with published work demonstrating that AMPK is present in most peripheral tissues and neurons but is absent from unactivated glia (32). The absence of glial AMPK expression may be indicative of the differences in energy flux or monitoring between the two cell types. It has been estimated that 95% of brain energy usage is accounted for by neurons and is mainly due to excitatory signaling (58,59), and that the glutamate released by neurons and its uptake by astrocytes during excitation serves to couple neuronal synaptic activity with astrocytic glucose usage and lactate production. The production of lactate by the glia generates 2 ATP, whereas the oxidation of 2 lactate molecules by neurons would provide 36 ATP. Therefore, the lesser energy flux through glia as compared with
neurons may translate into a reduced need for energy sensing molecules such as AMPK.

This activation of AMPK triggers a cascade of potential downstream effects in an attempt to restore cellular energy levels, thus leading to an increase in ATP. To determine the physiological relevance of altering AMPK activity, two downstream pathways were examined. It has been shown that when energy stores are low, when fatty acid breakdown is favored over synthesis, ACC is phosphorylated and inactivated by AMPK (52). When AMPK is inactivated following C75 treatment, ACC phosphorylation is dramatically reduced, suggesting that ACC activity is affected by the change in AMPK activity. Additionally, AMPK is known to affect glucose metabolism, (60,61), and therefore the effect of C75 on glucose oxidation was examined, and found to increase at early time points (30 min). C75’s stimulation of glucose oxidation is consistent with the correction of hyperglycemia observed in ob/ob mice (8) and with a recent report that C75 increases glucose metabolism (60). We propose that it is the increase in both fatty acid and glucose oxidation that contributes to an energy rich state, and that once this energy rich state is obtained, compensatory mechanisms inhibit AMPK and return glucose oxidation to control levels.

In addition to altering multiple aspects of cellular metabolism, C75 treatment appears to alter neuronal activity. We have shown that C75 has a robust effect on the frequency of mEPSCs, while having no discernable effect on mEPSC amplitude. This effect appears to be specific to C75. In regards to the mechanism by which C75 may
affect mEPSC frequency, it likely occurs via a change at the level of the presynaptic terminal. It is well accepted that changes in mEPSC frequency, especially robust changes over relatively short time periods, are due to an increase in release probability at the presynaptic terminal (62). However, there are many possibilities that could change terminal release probability. It is beyond the scope of this study to identify the precise molecular mechanism involved in C75 enhancement of synaptic transmission.

The ability of C75 to affect these parameters, while cerulenin does not, may reflect the larger effect of C75 on ATP levels compared to that achieved with cerulenin. Recently published work demonstrates that C75 increases neuronal activity in several neuronal types tested in a slice preparation, leading the authors to conclude that C75 is a non-specific neuronal activator (63). This study is inconsistent with a previous paper, in which c-Fos expression was utilized to assess the effect of C75 on neuronal activity; investigators demonstrated that C75 induced c-Fos expression in specific regions of the hypothalamus and brainstem, not global neuronal activation (54).

Being inhibited by ATP and activated by long-chain acyl-CoAs, ATP-sensitive K$^+$ channels ($K_{\text{ATP}}$) may also serve as metabolic sensors (64-66). It has been suggested that $K_{\text{ATP}}$ channels in the hypothalamus are involved in the regulation of feeding, and that activation of $K_{\text{ATP}}$ channels leads to an increase in feeding (67). The increase in neuronal activity described in this study could result from the inhibitory effect of increased ATP on $K_{\text{ATP}}$ channels, leading to depolarization and increased firing, potentially affecting feeding behavior. The metabolic state of the cell is coupled to electrical activity via $K_{\text{ATP}}$ channels in many tissues (68).
The regulation of brain FAS expression is different from that of peripheral tissues (9) and it is becoming apparent that, even though the brain does not use fats as fuel, as fatty acid oxidation is limiting (46,55), neuronal energy balance may be regulated by modulating the breakdown of fatty acids. This is a model supported by the present study and by recent work described by Obici S. et al., in which the inhibition of CPT-1 decreases food intake (7). We, however, propose that it is the breakdown rather than the build up of fatty acids through C75’s stimulation of CPT-1 that leads to an energy positive state. This energy surplus could signal the neuron that it is in the fed state, and assuming the same mechanisms are in play in hypothalamic neurons, inhibit feeding. The apposing results seen by our group and those of Obici et al. suggest that perhaps it is the fluctuations in activities in lipid synthetic and degradative pathways that are important in regulating feeding behavior, not just the activities of these pathways.

Collectively, the results presented here demonstrate that C75 treatment has several effects on neuronal energy metabolism and on neuronal activity. By altering glucose and fatty acid metabolism, and thus neuronal energy levels, C75 may influence energy perception, at least partially through the modulation of AMPK activity. We believe that the effect of C75 on neuronal energy is physiologically relevant due to the robust effect on neuronal activity and on downstream energy sensing molecules such as AMPK, followed by alterations in pathways that are regulated by AMPK (ACC phosphorylation and glucose metabolism). This shift in the energy state of the neuron could signal the CNS, via altering neuronal activity, to inhibit feeding. Understanding
the effect of modulating fatty acid metabolism on neuronal energy perception could lead to a better understanding of the mechanisms underlying feeding behavior and could potentially lead to new therapeutic targets for weight loss.

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Figure Legends

Fig. 1. **FAS and AMPK are abundant in cortical neurons, with little to no expression in glia.** Localization of FAS (A) and AMPK (B) in primary rat cortical neurons was demonstrated by double-labeling immunocytochemistry for neuron-specific tubulin, represented in red and FAS or AMPK represented in green. Double-labeling immunocytochemistry for GFAP represented in red and FAS (C) or AMPK (D) represented in green using primary rat cortical neurons allowed to overgrow with glia.

Fig. 2. **Inhibition of FAS/stimulation of CPT-1 activity and fatty acid oxidation by C75.** (A) Primary cortical neurons were treated for 2 hr with increasing concentrations of C75 (10-60 μg/ml), and the level of FAS activity was assayed by measuring the amount of [³H]-acetic acid incorporated into extractable lipids compared to untreated samples. (B) After a 2 hr treatment with 30 μg/ml C75 or 100 μM malonyl-CoA CPT-1 activity was determined using digitonin permeabilization and isolation of the ¹⁴C-labeled acylcarnitine derivative. (C) The effect of 40 μg/ml of C75 over time on the total oxidation of [1-¹⁴C]-palmitate was calculated as the sum of ¹⁴CO₂ and ¹⁴C labeled acid soluble products generated during a 30 min incubation with [1-¹⁴C]-palmitate. ***, P<0.001.

Fig. 3. **C75 changes neuronal ATP levels.** (A) Primary cortical neurons were treated for 2 hr with increasing concentrations of C75 (20-180 μg/ml). Changes in ATP levels were evaluated by luminescence and are represented as a % of untreated controls. **(B)**
Calcein dye uptake was used to evaluate neuronal viability as a percent of untreated controls after a 2 hr treatment with increasing concentrations of C75 (20-180 μg/ml). ATP levels (C) and neuronal viability (D) were assessed as described above over time (2-15 hr) with 40 μg/ml C75. ATP levels in (C) are significantly different from control at all reported time points. *, P<0.05; ***, P<0.001.

Fig. 4. The effect of fatty acid synthesis inhibitors on neuronal nucleotide pools. (A) ATP levels were assessed by luminescence over a short time course, between 2.5 and 1 hr, using 40 μg/ml of C75. Values at 2.5, 5, 10 and 15 min are significantly different from control, ***, P<0.001. (B) Fatty acid synthesis pathway: acetyl-CoA carboxylase (ACC) catalyzes the ATP dependent conversion of acetyl-CoA to malonyl-CoA. Fatty acid synthase (FAS) catalyzes the condensation reaction of acetyl-CoA and malonyl-CoA to form palmitate (C₁₆). Malonyl-CoA decarboxylase (MCD) converts malonyl-CoA back to acetyl-CoA. Both C75 and cerulenin inhibit FAS, whereas TOFA inhibits ACC activity. (C) The response of ATP levels to increasing concentrations of cerulenin (5-20 μg/ml) at 2 hr as compared to untreated control samples and 40 μg/ml C75. (D) The change in ATP over time (5-120 min) using 15 μg/ml of cerulenin compared to untreated control. Values at 5, 15, and 30 min are significantly different from control, ***, P<0.001. (E) The response of ATP levels to increasing concentrations of TOFA (10-40 μg/ml) as compared to untreated controls, 40 μg/ml C75, and DMSO concentrations comparable to 20 and 40 μg/ml TOFA. (F) The change in ATP over time (5–60 min) using 20 μg/ml of TOFA compared to untreated controls. Values at 60 min are significantly different from control, ***, P<0.001. (G) ATP and AMP levels were
determined by HPLC after treatment for 5 min and 2 hr with 40 μg/ml C75. *, P<0.05; **, P<0.01; ***, P<0.001.

Fig. 5. **The effect of C75 on AMPK.** (A) Western blot analysis of cortical lysates from control samples (C₁ and C₂) and those treated with 40 μg/ml of C75 over time (5 min–4 hr) were performed utilizing antibodies detecting both phosphorylated AMPK catalytic isoforms, α₁ and α₂, (top), unphosphorylated AMPKα (middle), and FAS (bottom) were utilized. (B) A synopsis of the effect of 40 μg/ml C75 on ATP levels over time compared to (C) the effect of 40 μg/ml C75 over the same time course (5 min-4 hr) on AMPK activity, as determined by performing SAMS peptide assays on cortical neuron lysates. (D) AMPK activity determined after 5 min and 2 hr treatments with cerulenin and TOFA (15 μg/ml and 20 μg/ml, respectively). *, P<0.05; **, P<0.01, ***, P<0.001.

Fig. 6. **Downstream effects of C75.** (A) Western blot analysis utilizing antibodies recognizing the phosphorylated (top) and unphosphorylated (bottom) forms of ACC was performed using control lysates and those treated for 2 hr with 40 μg/ml C75. (B) The effect of 40 μg/ml C75 over time on the complete oxidation of [1-14C] glucose, as measured as a function of 14CO₂ product generation. (C) C75 enhances synaptic transmission by altering mEPSC frequency. Two neurons, (17 days in vitro), were patched clamped and mEPSCs baseline (t=0) were recorded. Either cerulenin (left traces; 15 μg/ml) or C75 (right traces; 40 μg/ml) was perfused during the recording and mEPSCs were acquired at the time indicated. Calibration = 50 pA, 500 ms. (D) Summary data from experiments described in A. At each time point, both mEPSC
frequency (top) and mEPSC amplitude (bottom) were normalized to values recorded at baseline (T=0) and then plotted vs. time. (E) Summary histograms from chronic applications of either C75 or cerulenin to neurons (14-21 days in vitro). Data were acquired after 2 hr of incubation in neuronal growth media with DMSO, cerulenin, or C75. Normalized values were obtained by dividing drug averages by DMSO averages. C75 and cerulenin were normalized to individual DMSO treated neuronal populations. *, P<0.05; **, P<0.01.

Fig. 7. **Model for C75 action on energy metabolism.** The inhibition of FAS and stimulation of CPT-1 by C75 alters flux thru both fatty acid synthesis and breakdown pathways. This altered flux leads to altered neuronal metabolism through the stimulation of fatty acid oxidation, and the alteration of glucose metabolism that is modulated by a change in AMPK activity. Acetyl-CoA carboxylase (ACC); fatty acid synthase (FAS); carnitine palmitoyltransferase-1 (CPT-1); phosphorylated AMP-activated protein kinase (p-AMPK); tricarboxylic acid cycle (TCA); electron transport chain (ETC).
Figure 2
Figure 3
Figure 4
Figure 5

A

| C1 | 5' | 30' | 2hr | 4hr | C2 |
|----|----|-----|-----|-----|----|
| pAMPKα | ![Image of pAMPKα](image1) |
| AMPKα | ![Image of AMPKα](image2) |
| FAS | ![Image of FAS](image3) |

B

[ATP] (% of control)

C

AMPK Activity (% of control)

D

AMPK Activity (% of control)

Cerulenin

| 5' | 2hr |
|----|-----|
| ![Image of Cerulenin](image4) |

TOFA

| 5' | 2hr |
|----|-----|
| ![Image of TOFA](image5) |
Figure 6
Figure 7
C75, a fatty acid synthase inhibitor, modulates AMP-activated protein kinase to alter neuronal energy metabolism

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