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LEUCINE MODULATION OF THE mTOR PATHWAY FOR COGNITION MODULATION: KINETIC AND *IN VITRO* STUDIES AND MODEL DEVELOPMENT

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14. ABSTRACT
Leucine, an essential branch-chained amino acid (AA), has been shown to activate the mammalian target of rapamycin (mTOR) pathway to increase protein synthesis, and with methods for increasing brain levels, may be associated with enhanced cognitive memory formation in the brain. The current report describes studies of leucine kinetics in male Long-Evans rats, together with *in vitro* studies of the effects of leucine on the mTOR pathway and a mathematical model of the mTOR signaling pathway. We found that leucine was eliminated quickly from the blood after iv dosing (5 and 12.9 mg/kg), and levels in the brain were higher than blood, indicating active transport of leucine across the blood brain barrier (BBB). Altered brain levels of 17 other AAs suggest that the spike in leucine levels due to the iv injection tends to cause an increased brain influx of all AAs, but that competition for the L1 transporter mitigates this effect somewhat for many of the AAs that primarily make use of this system to enter the brain. *In vitro* studies using neuronal cells indicated that a single dose of leucine could not activate the mTOR pathway in a predictable manner, as indicated by increased levels of pS6K1, possibly due to incomplete translocation of the mTORC1 complex to the lysosomal membrane. Finally, in order to develop a mechanistic understanding of the impact of leucine and other amino acids on protein synthesis and neuronal plasticity, we implemented and modified a mathematical model of the mTOR signaling pathway.

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PREFACE

This research was conducted under contract FA8650-10-2-6062 with the Henry M. Jackson Foundation for the Advancement of Military Medicine (HJF). The program manager for the HJF contract was David R. Mattie, PhD (711 HPW/RHDJ).

The *in vivo* study was approved by the Wright-Patterson Air Force Base (AFB) Installation Animal Care and Use Committee (IACUC) as protocol number F-WA-2010-0121-A. The study was conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), International, in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 1996). The study was performed in compliance with DODI 3216.1.

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1.0 SUMMARY

Leucine, one of the essential branch-chained amino acids (AAs), has been shown to activate the mammalian target of rapamycin (mTOR) pathway to increase protein synthesis. It was postulated that dietary supplemental leucine administration may affect enhanced cognitive memory formation in the brain during long-term memory consolidation activities. However, limiting proper exogenous administration approaches was the paucity of disposition kinetics of leucine *in situ*. The current report describes initial sets of studies to realize leucine kinetics and regional dynamics within male Long-Evans (LE) rats, and, together with *in vitro* studies, provides answers to the effects of leucine on the mTOR pathway. Finally, a mathematical modeling framework is presented to quantitatively describe the mTOR signaling pathway.

Most published kinetic studies of leucine involve uptake of radio-labeled leucine, contained in drinking water or diet, in which leucine tissue concentrations are measured only at one time point at the conclusion of the study. For the initiation and full elaboration of mTOR pathway modulation by an exogenous substance, it was critical to determine the leucine blood kinetics and dynamics to understand the levels reaching brain tissue ultimately at regional levels. In other words, the arrival of free leucine at a tissue site and taken up by the cells would impart a signal for initiating the mTOR pathway, thus preparing the cell for enhanced protein synthesis. Initial studies were required to ensure that sufficient levels of administered leucine were getting to sites of importance in the brain. The objectives of these preliminary investigations for the *in vivo* study were: 1) to characterize leucine kinetics in LE male rats by measuring leucine tissue and blood concentrations and 2) investigate the effects of leucine administration on the blood and brain levels of all 20 AAs to ensure all AA levels were maintained during leucine administration, and the role of the blood brain barrier (BBB) in brain AA kinetics.

Leucine was dosed intravenously (iv) at 5 and 12.9 mg/kg for dose-response and time-course studies, and orally (319 mg/kg) for time-course analysis. Tissue leucine and other AA levels were measured at various time points (5 minutes to 6 hours (iv), and 30 minutes to 4 hours (oral)). The present investigation revealed that leucine was eliminated very quickly from the blood after dosing, and levels in brain were higher than blood, indicating active transport of leucine across the BBB. At 5 minutes post dosing, only 7 percent of injected leucine was detected still in blood, and at 6 hours post dosing it was down to base line levels. Similarly, of the 17 other AAs measured in brain, about two-thirds were increased, even at very early times (less than 2 minutes) after iv leucine injection. Most of those that either remained unchanged or were increased in brain are known to be transported across the BBB by the L1 large neutral AA transporter that moves leucine. Since this system is normally close to saturation, and since leucine has a high affinity for this transporter, one may speculate that the spike in leucine levels due to the iv injection tends to cause an increased brain influx of all AAs, but that competition for the L1 transporter mitigates this effect somewhat for many of the AAs that primarily make use of this system to enter the brain.

As the immediate downstream kinase to the mTOR complex, ribosomal small subunit 6 kinase 1 (S6K1) was selected as the marker of mTOR activation. Phosphorylation of S6K1 is a key step to initiating protein synthesis. Transcription activator 4E binding protein 1 (4E-BP) is further downstream of S6K1 and was also selected to determine the extent of mTOR activation. Once

phosphorylated, S6K1 then phosphorylates multiple downstream proteins, including 4E-BP and the S6 ribosomal subunit. *In vitro* studies were undertaken using mouse neuroectodermal (NE-4C) and rat hippocampal fibroblast (H19-7) cell lines, which were used as models for determining levels of activation from leucine exposure from the medium. The studies in these chosen cell lines did not indicate that a single dose of leucine could activate the mTOR pathway in a predictable manner. Although a detectable increase in phosphorylated S6K1 was seen after a single dose exposure at 5 minutes exposure to 5.25 and 52.5 µg/mL leucine, this was not replicated in subsequent experiments. To determine if the mTOR pathway could be primed to respond to a leucine stimulus, both NE-4C and H19-7 cells were incubated for 2.5 hours in a custom medium that contained no amino acids. Following the incubation period, the cells were treated with 52.5 µg/mL leucine. However, leucine alone was not able to stimulate the activity of mTOR as indicated by increased levels of phosphorylated S6K1. Interestingly, if the cells were treated with the standard complete medium after 2.5 hours in amino acid free medium, phosphorylated S6K1 levels were comparable to control levels (normal complete growth medium only) after 30 minutes. Activation of mTOR complex 1 (mTORC1) requires translocation of the complex to the lysosomal membrane where it interacts with Rag guanosine triphosphate (GTP) hydrolases (Sancak *et al.*, 2008). This recruitment to the lysosomal membrane is necessary for activation and is thought to be the process activated by growth factors, energy level increases and certain amino acids. It is possible that in the *in vitro* assays, the lack of an increase in phosphorylated S6K1was caused by incomplete translocation of the mTORC1 to the lysosomal membrane. It was also assumed that the NE-4C and H19-7 cell lines contained the required machinery to facilitate the movement of the complex through the cell; this can be verified with immunostaining techniques. Future studies should increase the incubation time to allow for translocation and activation of mTORC1. Additionally, immunofluorescence assays with antibodies to detect endogenous mTOR, RAPTOR, RagC and markers for endomembranes could be used to describe the movement of mTORC1 after amino acid (leucine) stimulation.

In order to develop a mechanistic understanding of the impact of leucine and other amino acids on protein synthesis and neuronal plasticity, we implemented and modified a mathematical model of the mTOR signaling pathway. This model was based on that developed by Jain and Bhalla (2009), which describes the pathway in a modular fashion. Our work converted the existing model from Systems Biology Markup Language (SBML) to acslX, in order to add the impact of leucine via Rag proteins as outlined further in the Section 2.0, particularly for its role in the translocation and activation of mTORC1 via Rag proteins (Sancak *et al.*, 2008).

2.0 INTRODUCTION

Leucine has been linked with cognitive enhancement in both experimental animals and humans (Hoeffer and Klann, 2009; Walker *et al.*, 2009), and it is involved in the regulation of the mammalian target of rapamycin (mTOR) signaling pathway. mTOR has two structurally and functionally distinct multi-protein complexes, mTORC1 and mTORC2 (Hall, 2008; Wang *et al.*, 2008). mTOR complexes regulate many processes such as mRNA translation, autophagy, metabolism, biogenesis, and protein synthesis in cells, including neurons. When mTOR or these pathways become deregulated, they can cause disease and cancer (Guertin and Sabatini, 2007). Protein synthesis requires both amino acids and metabolic energy, and leucine positively regulates the mTOR signaling pathway (Proud, 2002). mTOR integrates cellular nutrient (amino acid) utilization and energy-sensing pathways, and mTORC1 is reported to be promoted by inputs from branched chain amino acids, especially leucine (Lynch *et al.*, 2003; Sancak and Sabatini, 2009; Tokunaga *et al.*, 2004; Wang *et al.*, 2008). Hypothalamic mTOR signaling regulates food intake, and mTOR interacts with the protein raptor to form nutrient-sensitive complexes, signaling cell growth (Cota *et al.*, 2006; Kim *et al.*, 2003; Kimball and Jefferson, 2006; Proud, 2002; Wood *et al.*, 2008).

Stoica *et al.* (2011) reported that inhibition of mTORC1 blocks long-term synaptic plasticity and memory storage, providing direct evidence supporting the complex's active role in brain function. While short-term memory formation is associated with covalent modification of preexisting protein, long-term memory requires new protein synthesis. Neuronal protein synthesis is associated with synaptic plasticity and memory consolidation by signaling pathways that regulate mRNA translation in neurons (Buffington *et al.*, 2014; Gkogkas *et al.*, 2010; Richter and Klann, 2009; Stoica *et al.*, 2011; Tang and Schuman, 2002).

Despite its key role in cognition, the kinetics of leucine *in vivo* are not well characterized. Most published studies involve uptake of leucine-containing drinking water or diet, and tissue concentrations are only measured at terminal time points (see Fernstrom, 2005). There is a need to link *in vivo* leucine exposures, via drinking water, diet, and other routes, with brain leucine levels, in order to develop dosing regimens with the potential for cognitive enhancement in humans (dose-response).

Leucine is an essential, branched-chain amino acid, and is moved by the large neutral amino acid (LNAA) transporter (Audus and Borchardt, 1986; Fernstrom, 2005). Leucine enters into the brain by crossing the blood-brain barrier (BBB) via the carrier-mediated transporter, system L1, which is a Na^+ independent, stereospecific, and saturable membrane protein transporter. In its movement, leucine competes with other amino acids (AAs) handled by this transporter (see Figure 1).

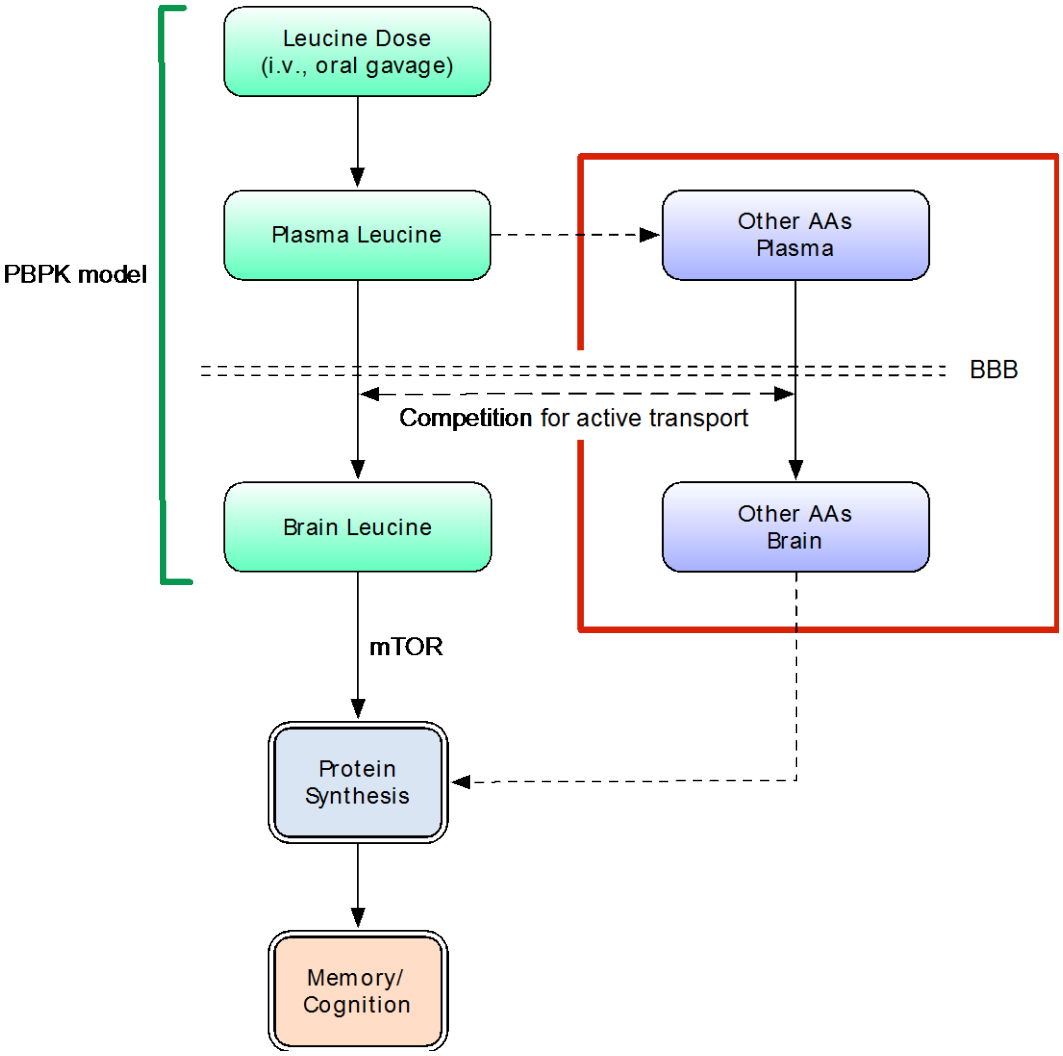


Figure 1. Schematic of Interconnected Processes for Molecular Cognition Linked with Leucine Uptake and the mTOR Signaling Pathway. Competition for active transport across the BBB occurs for those AAs that utilize the L1 large neutral AA transporter system.

The BBB is constructed by the endothelial cells of cerebral capillaries, which form very tight junctions to manage the environment of the brain and chemistry contents of the cerebral-spinal fluid. The resulting diffusion barrier controls influx of chemicals into the brain from the circulating blood so as to protect the brain from toxic insults (Ballabh *et al.*, 2004; Pardridge and Oldendorf, 1977; Smith, 2000). The BBB is surrounded by end feet of astrocytic glial cells, and pericytes are enclosed within basal lamina (Abbott, 2003; Abbott *et al.*, 2006; Engelhardt and Sorokin, 2009; Hawkins *et al.*, 2006). In addition to sodium-dependent active membrane transporters, there are also a number of facilitative AA transporters (Abbott *et al.*, 2006; Daniel *et al.*, 1977; Hawkins *et al.*, 2006; O'Kane and Hawkins, 2003; Hargreaves and Partridge, 1998; Sanchez del Pino MM *et al.* 1995; Smith *et al.*, 1984; Ueno, 2009):

Facilitative amino acid transporters

1. L1: facilitative Na^+-independent transport of large essential neutral amino acids (this system presents in both endocyte membranes (luminal and abluminal) in a 2:1 ratio)
2. y^+: facilitative transport of cationic AAs, which have affinity with cationic side chain, and are predominantly on the abluminal side
3. n: facilitative transport of glutamine system, and only present on the luminal side
4. x_G~: facilitative transport of acidic AAs, solely on the luminal border

Sodium (Na^+)-dependent transport system (found only in abluminal membrane)

1. Na^+-LNAA: Na^+-dependent transport of large neutral AAs
2. alanine (A): Na^+-dependent transport of small nonessential AAs (e.g., alanine)
3. analine-serine-cysteine (ASC): Na^+-dependent transport of some large and some neutral AAs (alanine, serine, cysteine, and glycine as well as essential AAs methionine, valine, leucine, isoleucine, and threonine)
4. nitrogen (N): Na^+-dependent transport of N rich AAs (glutamine, histidine, and asparagine)
5. excitatory acidic acid transporter (EAAT) family AAs: Na^+-dependent transport of acidic AA (aspartate and glutamate)

Table 1 shows the 20 amino acids, together with their primary associated BBB transport systems. Leucine is transported primarily by the large neutral AA facilitated transport system. Potential competitors for the L1 LNAA system include glycine, proline, threonine, cysteine, histidine, valine, isoleucine, phenylalanine, tyrosine, and tryptophan (Table 1).

Table 1. Physical Properties of Amino Acids Purchased and Analyzed in This Study

Neutral			
	Polarity:	**BBB transport:**	**MW (g/mol):**
Straight side chain			
glycine	non-polar	L1	75
alanine	non-polar	ASC	89
serine	non-polar	ASC	105
proline	non-polar	L1	115
threonine	polar	L1	119
cysteine	slightly polar	L1	121
methionine	non-polar		149
Nitrogen-rich side chain			
asparagine	non-polar		132
glutamine	polar		146
histidine	non-polar	L1	155
Branched side chain			
valine	non-polar	L1	117
leucine	non-polar	L1	131
isoleucine	non-polar	L1	131
Aromatic side chain			
phenylalanine	non-polar	L1	165
tyrosine	polar	L1	181
tryptophan	slightly polar	L1	204
Basic-positively charged			
lysine		basic Y+	146
arginine		basic Y+	174
Acidic-negatively charged			
aspartic acid		acidic X-	133
glutamic acid		acidic X-	147

Represents essential AA

Notes: AA: amino acid; ASC: alanine-serine-cysteine (transporter); basic Y+: y(+)-type cationic amino acid transporter; BBB: blood-brain barrier; L1: large neutral amino acid transporter; MW: molecular weight

Potential mechanisms for the interaction of leucine with the mTOR pathway have been suggested. In particular, studies reported with human epidermal keratinocytes (HEK) indicate that amino acids mediate the translocation of mTORC1 via Rag proteins to a compartment that also contains its activator Rheb (Sancak *et al.*, 2008). We hypothesize that leucine-driven and

Rags-mediated movement of mTORC1 also takes place in neurons, so that mTORC1 would become localized in the dendrites to facilitate local protein synthesis associated with long term memory formation (see Figure 2). Such localization, resulting from tissue/cell exposure levels to enhanced leucine concentrations, can be visualized using immunohistochemical techniques and immortalized rat hippocampal cells.

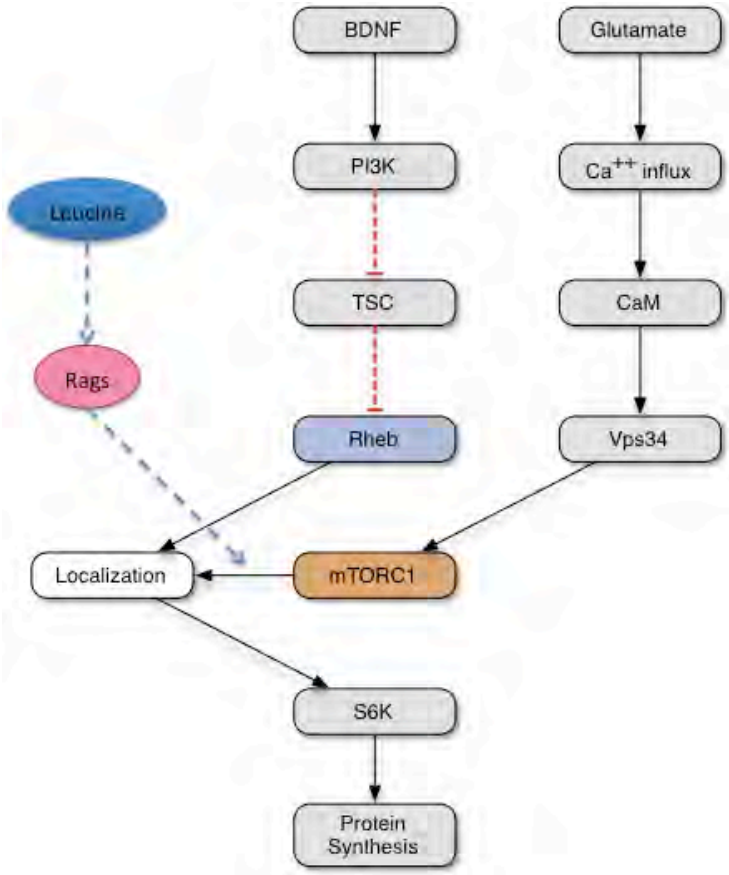


Figure 2. Schematic of the Impact of Leucine on the mTOR Protein Synthesis Pathway.
Notes: BDNF: brain-derived neurotrophic factor; CaM: calmodulin (calcium modulating protein); mTORC1: mammalian target of rapamycin complex 1; PI3K: phosphatidylinositol 3-kinase; Rags: Rag GTPases (guanosine triphosphate hydrolases); Rheb: Ras homolog enriched in brain; S6K: ribosomal small subunit 6 kinase; TSC: tuberous sclerosis complex; Vps34: Class III phosphoinositide 3-kinase

This study examined the *in vivo* kinetics of leucine after intravenous and oral dosing in a rat model. The distribution of leucine in blood and tissues, particularly the brain as a target tissue were described. In addition, the impact that an increase in leucine had on the distribution of other amino acids was described, considering that leucine would impact AA transport across the BBB. Preliminary *in vitro* experiments were conducted to begin to assess the ability of a cellular increase in leucine to stimulate the activity of mTOR signaling. These *in vivo* and *in vitro* efforts lay the groundwork for designing the appropriate administration of a macronutrient, such as

leucine, in order to modulate mTOR signaling and, therefore, protein synthesis for improving cognitive performance. The kinetic data can be used to mathematically describe the administration, distribution, and delivery of leucine to the brain. *In silico* modeling can then simulate and predict the movement of leucine and, ultimately, extrapolate to humans.

3.0 MATERIAL AND METHODS

3.1 Chemicals

Leucine (>99 percent purity), sodium monobasic phosphate (NaH_2PO_4, 99.9 percent purity), acetonitrile, and ethanol (high performance liquid chromatography (HPLC) grade) were purchased from Sigma-Aldrich Corporation (St. Louis MO). Sodium hydroxide was obtained from Fisher Scientific (Fairlawn NJ). Deionized water with a specific resistance of 18 megaohm-cm or greater was used for the study. A standard solution in 0.1 M hydrochloric acid (HCl), containing L-isoleucine, and L-leucine, at a concentration of 250 mM was obtained from Agilent Technologies (Santa Clara CA). O-Pthalaldehyde reagent (OPA reagent) and borate buffer were purchased from Agilent Technologies (Santa Clara CA). All antibodies were purchased from Cell Signaling Technology (Beverly MA). Cell culture media and reagents were obtained from American Type Culture Collection (ATCC, Manassas VA).

3.2 *In Vitro*

The mouse neuroectodermal cell line NE-4C (CRL-2925™) and the rat hippocampal fibroblast cell line H19-7 (CRL-2526™) were purchased from ATCC (Manassas VA). The NE-4C cells were chosen as a stem cell line that could be differentiated, and the H19-7 cells were selected because they represented the hippocampal brain region, which is the target of interest for memory formation. The NE-4C cells were cultured on Primaria™ plates (Corning Inc., Corning NY), using Eagle's minimal essential medium (EMEM, ATCC) supplemented with 10 percent heat inactivated fetal bovine serum and 2 mM L-glutamine. Cells were seeded with 5×10^4 cells on 100 mm Primaria™ and allowed to grow at 37°C with 5 percent CO_2 until 85 percent confluent.

Assays were conducted in Primaria™ 6-well plates. Cells were seeded at 2×10^5/mL in a total volume of 3 mL/well and allowed to adhere and grow overnight at 37°C with 5 percent CO_2 to 90 percent confluence. H19-7 cells were cultured on poly-L-lysine coated flasks (Corning Inc.), using Dulbecco's modified Eagle medium (DMEM) supplemented with fetal bovine serum, 4.5 g/L glucose, 0.2 mg/mL G418 (gentamycin), 2 µg/mL puromycin, and 4 mM L-glutamine. Cells were seeded with 1.5×10^5 cell density and allowed to grow at 34°C (permissive temperature) with 5 percent CO_2 until about 70 to 75 percent confluent. Assays were conducted in poly-L-lysine coated 6-well plates. Cells were seeded at 2×10^5/mL in a total volume of 3 mL/well and allowed to adhere and grow overnight at 34°C with 5 percent CO_2 to 90 percent confluence.

Stock leucine was prepared in 1 M HCl at 26.25 mg/mL and diluted to 525, 52.5, 5.25, and 0.525 µg/mL dosing solutions. Temporal exposures to leucine were conducted at 5, 15, and 30 minutes. Medium in each well of a 6-well plate was aspirated, and each well washed twice with phosphate buffered saline (PBS). Fresh medium (2.94 mL) was added to each well, and then 0.06 mL of dosing solution was added and swirled to mix. The plate was incubated at 37°C for 5, 15, or 30 minutes. For amino acid starvation assays standard medium was aspirated and cells washed twice with PBS. Custom Roswell Park Memorial Institute medium (RPMI 1640 without glutamine, amino acids or glucose, supplemented to 2 mg/mL glucose (US Biological, Salem MA)) was added to the wells and incubated at 37°C for 2.5 hours. After 2.5 hours, all wells received 200 mM glutamine. The treatment groups were as follows: 1. No treatment; 2. Leucine dose (0.0525, 0.525, 5.25, or 52.5 µg/mL); 3. Removal of custom medium and add standard medium. Incubation time was 30 minutes at 37°C. Two additional controls were run with standard medium during the 2.5 hours incubation with or without a dose of 52.5 µg/mL leucine. Cells were lysed with 100 µL of NP-40 containing protease and phosphatase inhibitors (Roche cOmplete™ Mini and PhosSTOP, Sigma-Aldrich Corp.) and phenylmethylsulfonylfluoride (PMSF). The cell layer was scraped and collected in an Eppendorf tube. Protein concentration was determined using the Pierce BCA kit. Western blot analyses were performed using antibodies to phosphorylated ribosomal small subunit 6 kinase (S6K1, T389) and phosphorylated 4E binding protein 1 (4E-BP, T70).

3.3 Animals and Treatment

Male Long-Evans (LE) rats (340 ± 20 g) used throughout the experiments were obtained from Charles River Laboratories (Wilmington DE). Rats were kept in separate cages and allowed access to Purina rat chow and water *ad libitum*. A daily stock solution of leucine was dissolved in physiologic saline to prepare for intravenous (iv) dosing of 5 mg/kg and 12.9 mg/kg, via indwelling jugular vein, and oral dosing of 316 mg/kg. Volumes of iv and oral dosing were 1.5 mL/kg and 1.5 mL/100 g, respectively. Time points to collect tissue specimen for iv study were 0, 5, 15, and 30 minutes, 1, 3, and 6h post dosing, and for oral study 0.5, 1, 2, and 4 hours post dosing. Tissues collected for leucine analysis were blood, brain, kidney, liver, muscle, and brown and white fat (n=3 rats per time point). The tissues were kept frozen at -80°C prior to HPLC analysis.

3.4 Analytical Methods

3.4.1 Tissue Analysis. The frozen rat tissues were homogenized in 10 mL of 10 mM (TRIS+HCl) pH 7.4 buffer. The homogenates were centrifuged at 15350 xG for 20 minutes. Resultant supernatants were transferred, suspended in 10 percent TCA and incubated on ice for 30 minutes. The TCA-precipitated supernatants were centrifuged again at 15350 xG for 20 minutes, supernatants transferred to a fresh microfuge tube, and pellets were discarded. One µL of the supernatants were used for the HPLC amino acids analysis.

The rat plasma was centrifuged at 15350 xG for 20 minutes. The supernatant was suspended in 10 percent TCA and incubated on ice for 30 minutes. The TCA-precipitated supernatant was centrifuged at 15350 xG for 20 minutes. The resulting pellet was discarded. One µL of the supernatant was used for amino acids HPLC analysis. Rat tissues and the plasma samples were spiked with either 0 or 50 µM leucine to confirm the leucine peaks in those samples.

3.4.2 HPLC Analysis. HPLC determinations were performed with an Agilent-1100 HPLC system including a Hewlett Packard Chemstation (HP/Agilent, Wilmington DE). The HP Chemstation consisted of a quaternary gradient pump (G1312A Binary Pump) with a G1315A diode array detector, 6-mm flow cell, G1315A Fluorescence Detector, an Agilent 1313A Autosampler, a 200 µL sample loop, and HP Chemstation software. The HP Chemstation is designed to run on IBM-compatible personal computers under Microsoft Windows operating environments.

One µL of sample ultra-filtrate was injected into the HPLC system. Separation by gradient elution of amino acids was performed on a ZORBAX Eclipse analytical reverse phase column (250 x 4 mm) preceded by a ZORBAX guard column (4 x 12.5 mm, Agilent). The mobile phase consisted of a linear gradient from an initial concentration of 100 percent A (40 mM NaH_2PO_4, pH 7.8) increasing up to 100 percent B (acetonitrile:methanol:water; 45:45:10, v/v/v) in 55 minutes. The column effluent was monitored using the fluorescence detector (FLD). The FLD responses (LU-fluorescence units) corresponding to the peaks of the OPA-derivatized primary amino acids were monitored using the extinction and emission wavelengths of 250 and 410 nm, respectively. The peak areas were integrated using the HP Chemstation integration software. Amino acid concentrations were determined using standard calibration curves derived from running a pure amino acid solution.

3.5 Calculations and Statistics

Factory provided standards (Agilent Technologies, Santa Clara CA) of twenty primary amino acids (Table 1) were analyzed simultaneously with the HPLC method. Calibration curves, method detection limits (MDLs), sensitivities, percent recovery, and precision were determined for each amino acid. Standard curves, based on peak area, were constructed for each amino acid and were linear in their respective dynamic detection ranges. The average regression coefficient (r^2) for calibration curves was greater than 0.99 in all cases. The analytical recovery ranged from 96 to 102 percent. The precision (between-sample variability), as expressed as the coefficient of variability was excellent, ranging from 1.8 to 2.1 percent. The method detection limits for the amino acids were in the physiologically relevant range 0.2 to 1.4 µM. The analytical recovery ranged from 97.3 to 105 percent. The precision (between-sample variability), as expressed as the coefficient of variability was excellent, ranging from 1.1 to 4.5 percent. The HPLC procedure described here provides a high degree of reproducibility and reliable results. The method is capable of detecting amino acids in the range 5 to 20 µM in biological samples.

Amino acid results were analyzed with SYSTAT® 10 Statistics II statistical software (SPSS Inc., Chicago IL) using one-way analysis of variance (ANOVA) with Bonferroni multiple comparisons (Rosner, 1990). Data were analyzed as combined data per group.

3.6 Mathematical Model for mTOR Pathway

In order to develop a mechanistic understanding of the impact of leucine and other amino acids on protein synthesis and neuronal plasticity, we implemented and modified a mathematical model of the mTOR signaling pathway. This model was based on a developed and published model by Jain and Bhalla (2009). That model describes the pathway in a modular fashion (see Figure 3), and this was modified by conversion from its current form in Systems Biology Markup language (SBML) to acslX (AEgis Technologies Group, Huntsville AL). This provided a more suitable modeling platform in which to add the known regulatory impact of leucine as outlined in Section 2.0. In particular, we can incorporate its role in the translocation of the mTORC1 complex, via Rag proteins, to the lysosomal membrane, and subsequent activation of protein synthesis (Sancak *et al.*, 2008) (see Figure 2 above).

4.0 RESULTS AND DISCUSSION

4.1 *In Vivo* Results

Figure 4 depicts the time course study of leucine in blood of rats dosed with leucine intravenously. Clearance of leucine in blood was very fast after iv dosing (both 5 and 12.9 mg/kg). Even at 5 minutes post dosing, for both dose groups, only about 20 percent of the original doses were found in blood (assuming 7.5 percent blood volume). Leucine levels reached near back to blood baseline levels in low dosing group within 3 hours.

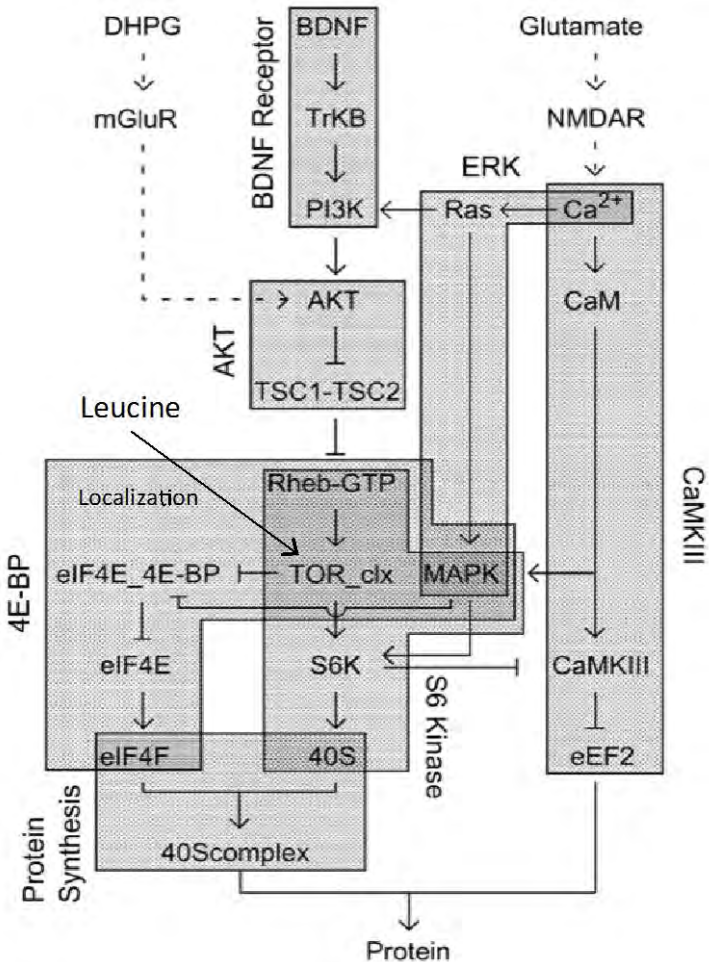


Figure 3. Schematic of the mTOR Signaling Pathway, Showing Activation of mTOR by Rheb, Following its Translocation Induced by Leucine. Adapted from Jain and Bhalla (2009). eIF4E and 40S represent control points for selective protein synthesis. Notes: AKT: protein kinase B; BDNF: brain-derived neurotropic factor; CaM: calmodulin; CaMKIII: calcium($^{2+}$)/calmodulin-dependent protein kinase; DHPG: dihydroxyphenylglycine; eEF2: eukaryotic translation elongation factor 2; eIF4E: eukaryotic translation initiation factor 4E; mGluR: metabotropic glutamate receptor; NMDAR: N-methyl-D-aspartate receptor; PI3K phosphoinositide 3-kinase; Rheb: Ras homolog enriched in brain; S6K: ribosomal S6 kinase; TrKB: tropomyosin receptor kinase B; TSC1, 2: tuberous sclerosis protein 1, 2.

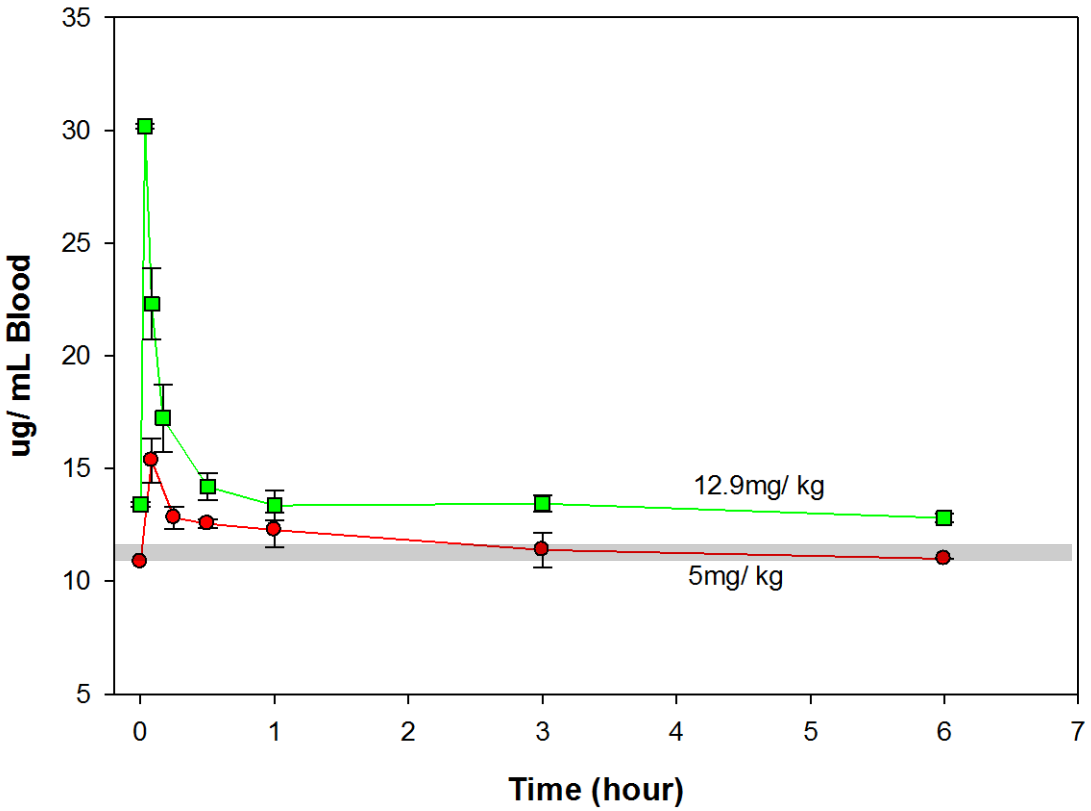


Figure 4. Leucine Concentrations in Blood of Rats Dosed with Leucine (5 and 12.9 mg/kg, iv). Rats (n=3) were injected with 5 and 12.9 mg/kg leucine via indwelling catheter, and tissue samples were collected 0 to 6 hours post dosing. The background control level of leucine was 11.02 ± 0.29 μg/ml (shaded area).

Figure 5 illustrates the time course study of leucine in brain of rats dosed with leucine intravenously. Within 5 minutes post dosing, leucine concentration in whole brain reached their highest levels, and was then slowly eliminated from brain in the low dosing group. However, the leucine level in 12.5 mg/kg group reached plateau until the end of the experiment (6 hours). Leucine concentration in brain of rats dosed with 12.9 mg/kg maintained a high concentration (approximately 29 μg/g brain) at the end of the experiment, while brain concentrations in the low dose group (5 mg/kg) reached approximately 3 μg/g on average higher over the experiment before dropping to baseline. Leucine levels in brain were higher than blood indicating active transport across the blood brain barrier. Leucine concentration in the high dose group was more than triple compared with the low dose group at 6 hours post doing. Leucine, a neutral branched side chain amino acid, is transported across the blood brain barrier by the L1 large neural amino acid transporter (Hawkins *et al.*, 2006), presumably leading to the observed high levels of leucine in brain compared with those in circulating blood.

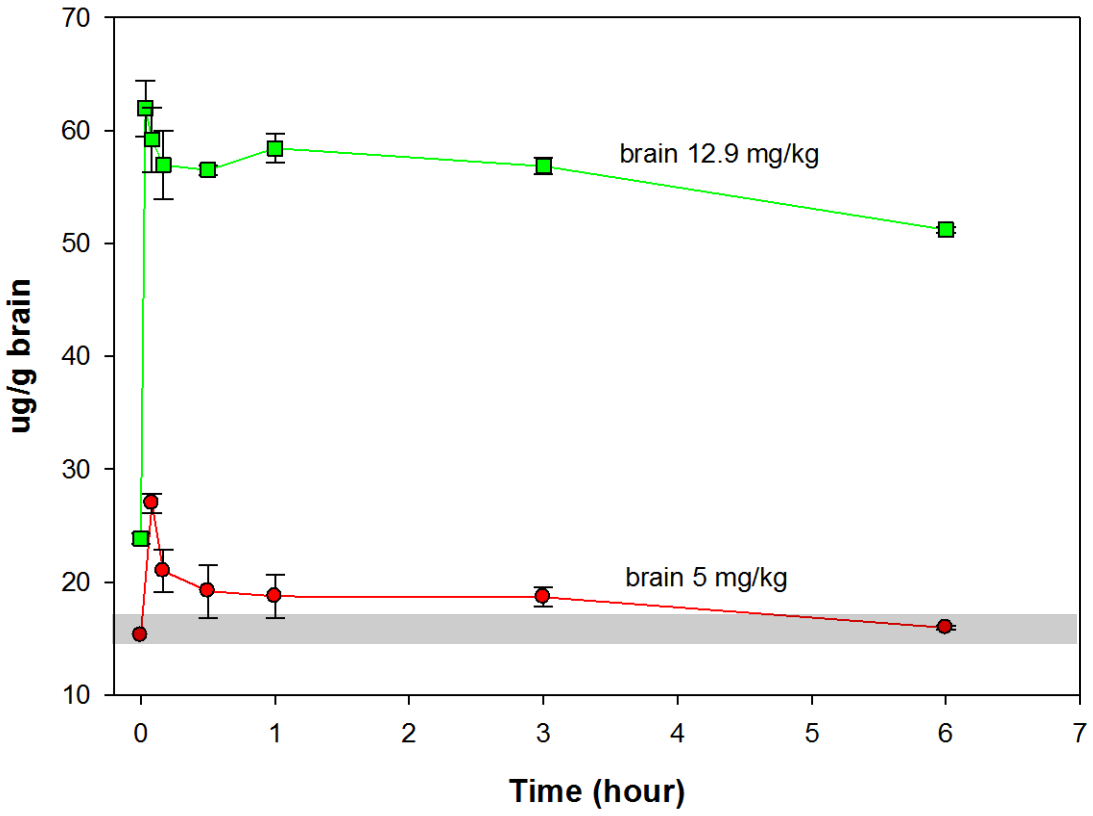


Figure 5. Leucine Concentration in Rat Brain following iv Dosing. Rats (n=3) were injected with 5 and 12.9 mg/kg leucine via indwelling catheter, and brain samples were collected 0 to 6 hours post dosing. The background control level of leucine was 15.61 ± 1.22 μg/g brain (shaded area).

Figure 6 depicts the time course of leucine in blood and brain of rats dosed with leucine orally (316.4 mg/kg). Leucine absorbed through gastrointestinal tract reached its highest level in blood within 30 minutes, and almost reached to the control levels at the end of experiment at 4 hours. Leucine levels in brain became higher than blood at approximately 1.5 hours post dosing, and stayed higher than blood, again indicating active transport at the BBB. Leucine levels almost reached the control levels at 4 hours post dosing.

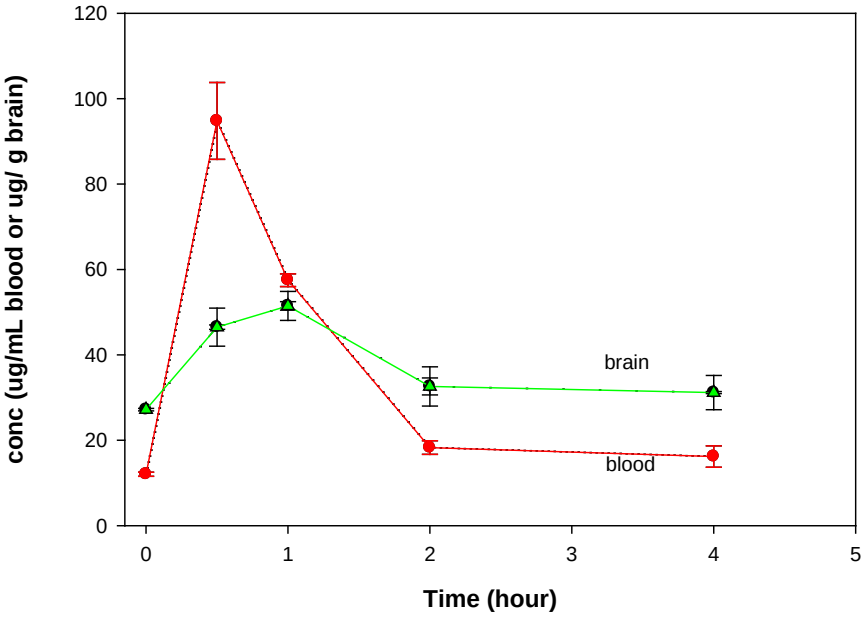


Figure 6. Time Course Study of Leucine Levels in Blood and Brain of Rats Following Oral Dosing with Leucine. Rats (n=3) were orally dosed with leucine (316.4 mg/kg), and blood and brain samples were collected 0 to 4 hours post dosing.

The time course of the concentration of leucine in tissues after iv injection is presented in Figures 7 and 8. The data reveal that overall kinetic behaviors are similar at both doses (5 and 12.9 mg/kg). At early time points in the 5 mg/kg group, the highest concentration is detected in kidney, muscle, liver, and brown fat. White fat, brain, and blood had the lowest concentrations in this group. The relative order of tissue concentrations is unchanged except for liver. At 30 minutes post dosing, liver concentration is higher than muscle, but returned to below the muscle level 1 hour post dosing. In the 12.9 mg/kg group the highest concentration was observed in muscle, brown fat, kidney, and liver. Brain, white fat, and blood had the lowest concentration. In both dosing groups, blood had the lowest levels of leucine throughout the experimental period.

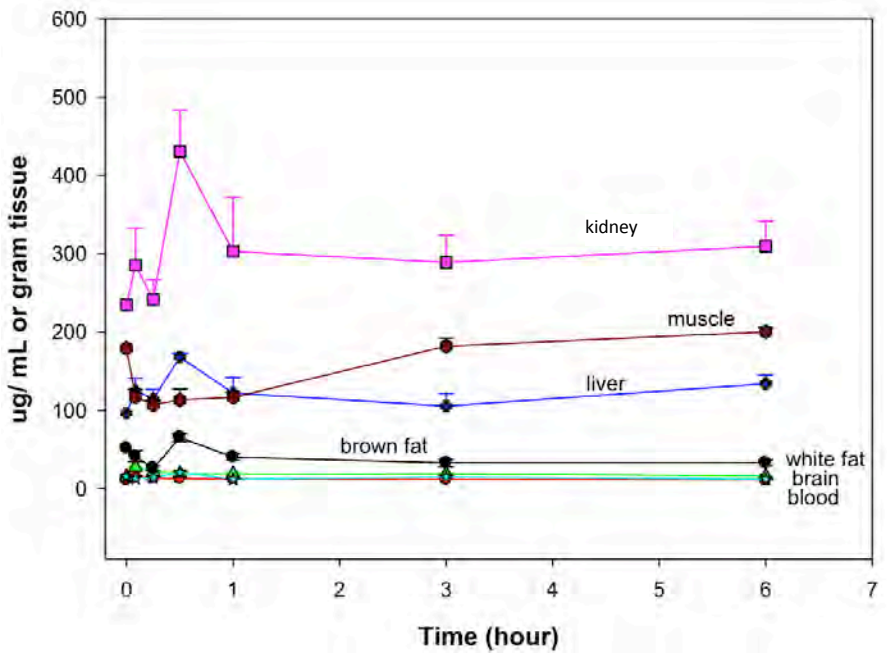


Figure 7. Leucine Concentrations in Tissues of Rats Dosed with 5 mg/kg Leucine. Rats (n=3) were injected with leucine and tissues were collected 0 to 6 hours post dosing.

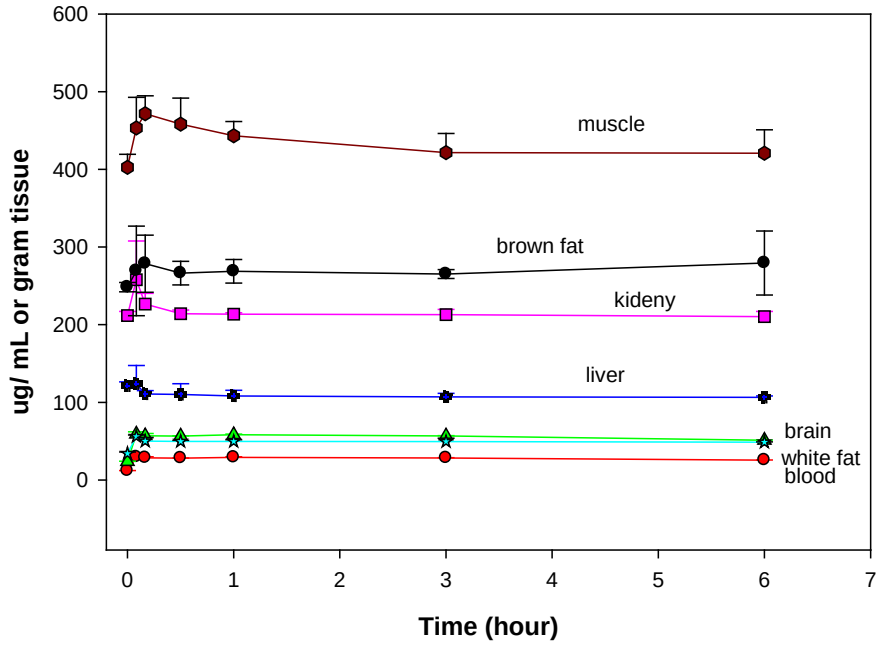
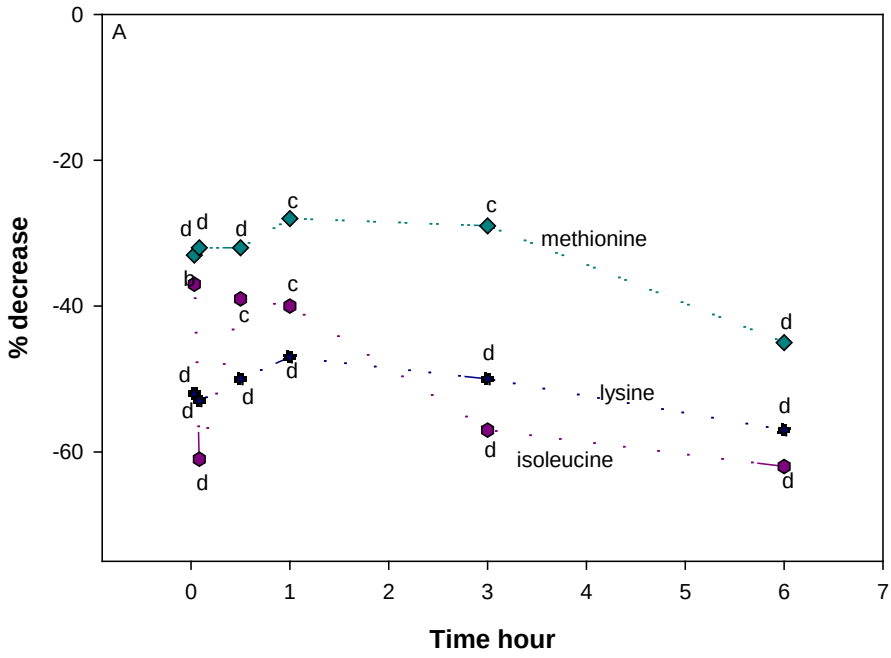


Figure 8. Leucine Concentrations in Tissues of Rats Dosed with 12.9 mg/kg Leucine. Rats (n=3) were injected with leucine and tissues were collected 0 to 6 hours post dosing.

Figure 9 illustrates percent changes of other AAs by leucine iv injection (5 mg/kg) in plasma and brain. At 5 mg/kg iv-dosing level, AAs in plasma (Figure 9A) revealed that methionine, lysine, and isoleucine were decreased at all time points. At 15 minutes post dosing, isoleucine decreased the most, and no plasma AAs were increased at this dose. AAs in brain (Figure 9B) showed lysine, proline, and valine increased while aspartic acid and isoleucine decreased. Isoleucine in brain decreased the most (by 54 percent). This result was also the case for the12.9 mg/kg iv dose. As with oral dosing, isoleucine decreased in both plasma and brain.

At the 12.9 mg/kg iv-dosing level, plasma (Figure 10A) showed tryptophan levels were increased. At 5 minutes post dosing, isoleucine increased by 84 percent, then decreased to near control levels by 1 hour post doing. Tryptophan increased from the control value by 56 percent at 1 hour, 55 percent at 3 hours, and 66 percent at the end of the study. Brain tissue (Figure 10B) revealed that lysine, serine, tryptophan, and proline were increased, while methionine and threonine decreased. At 5 minutes post dosing, proline was elevated by 95 percent, and then by 40 percent at the end of study. Lysine increased to the highest levels after 30 minutes and was statistically significantly increased at all time points.

At the 316 mg/kg oral dosing level, glycine, histidine, and alanine AAs in plasma (Figure 11A) had increased and only cysteine level decreased of the 20 amino acid detected. At 1 hour post dosing glycine increased by 120 percent, then by 78 percent at 2 hours, and then to the control level by 4 hours. In brain tissue (Figure 11B), proline, isoleucine, lysine, histidine, and tryptophan were elevated and no AAs were decreased. Proline was increased statistically significantly at all time points. At 2 hours post dosing, proline increased by 132 percent; by the end of the study, proline increased by 171 percent. Proline in brain tissue increased the most (by 171 percent) at the end of study. High levels of proline in the brain were also observed in the iv-dosing studies (5 and 12.9 mg/kg, Figures 9B and 10B).



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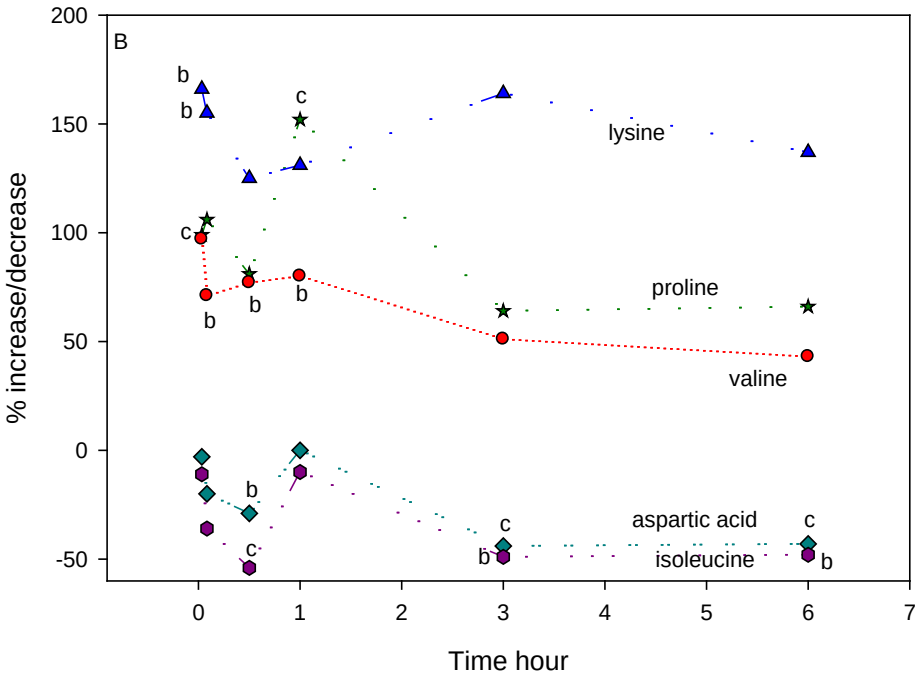
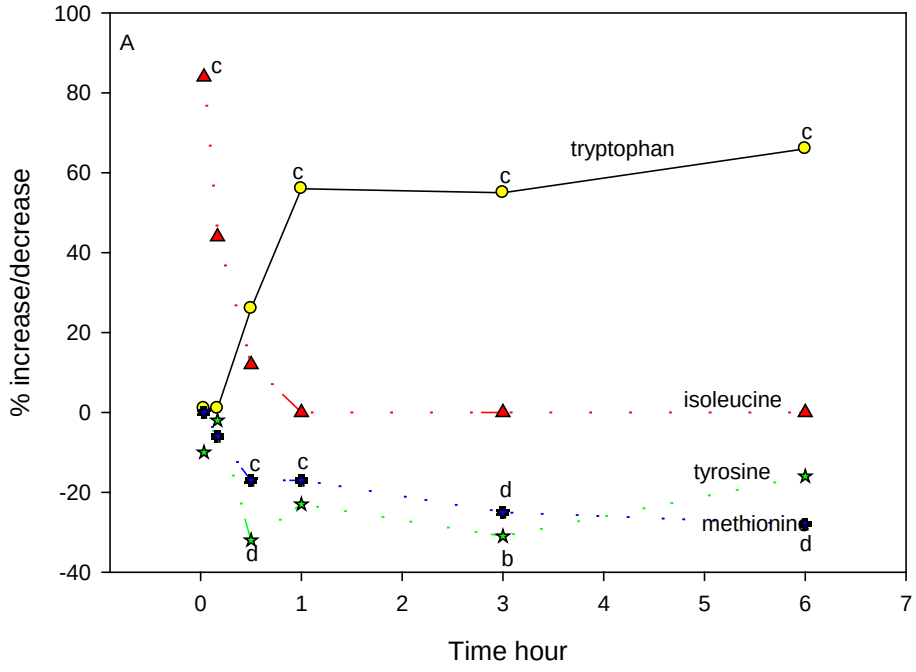


B

Figure 9. Percent Increase/Decrease of AAs by Leucine Injection (5 mg/kg) in (A) Plasma and (B) Brain. Rats (n=3) were injected with 5 mg/kg leucine via indwelling catheter, and samples were collected 0 to 6 hours post dosing. [b]Significantly different from control at $p<0.05$. [c]Significantly different from control at $p<0.01$. [d]Significantly different from control at $p<0.001$.



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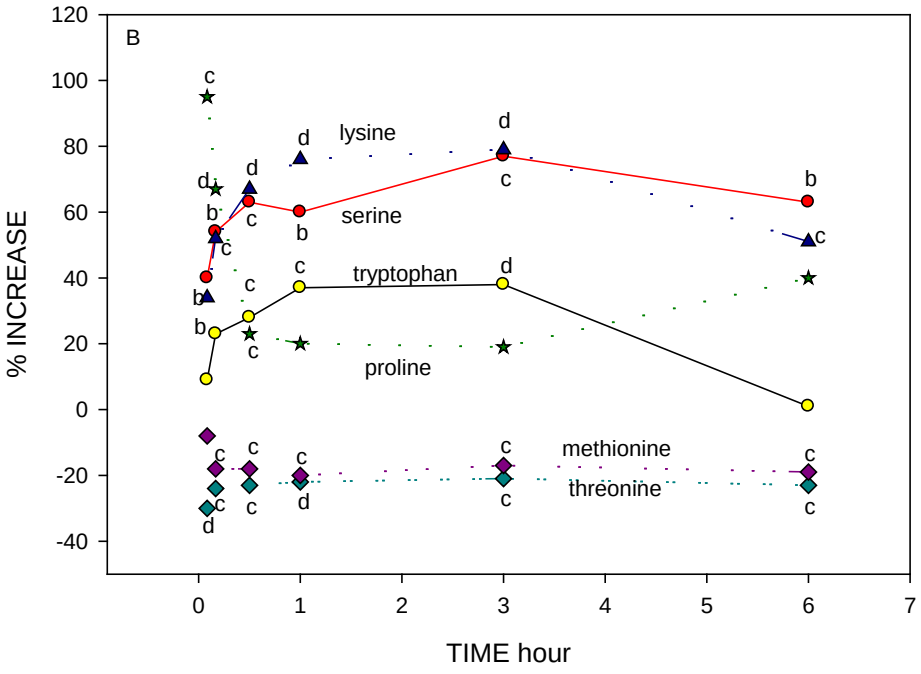
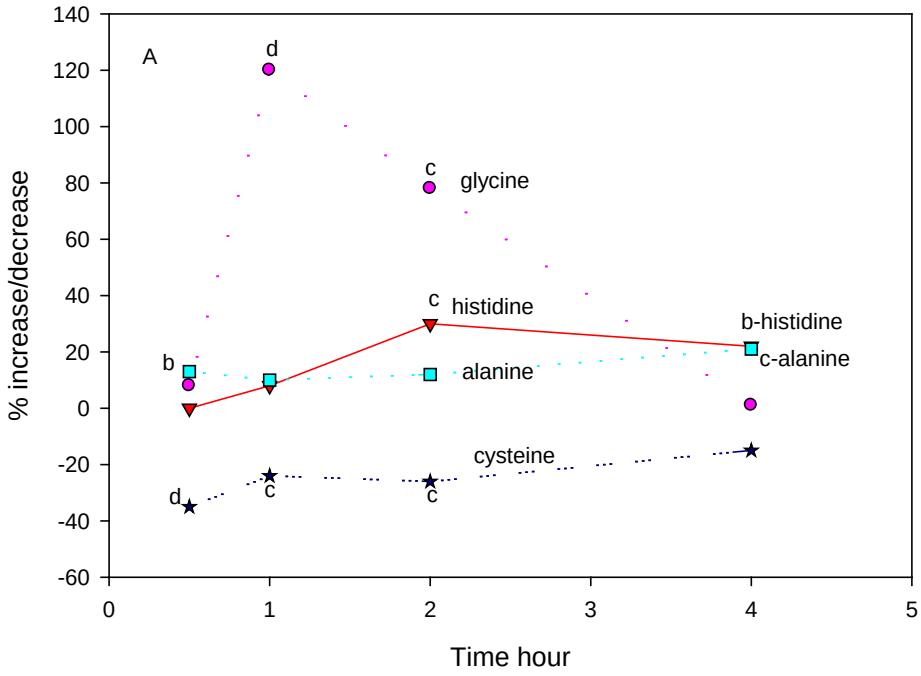


B

Figure 10. Percent Increase/Decrease of AAs by Leucine Injection (12.9 mg/kg) in (A) Plasma and (B) Brain. Rats (n=3) were injected with 12.9 mg/kg leucine via indwelling catheter, and samples were collected 0 to 6 hours post dosing. [b]Significantly different from control at $p<0.05$. [c]Significantly different from control at $p<0.01$. [d]Significantly different from control at $p<0.001$.



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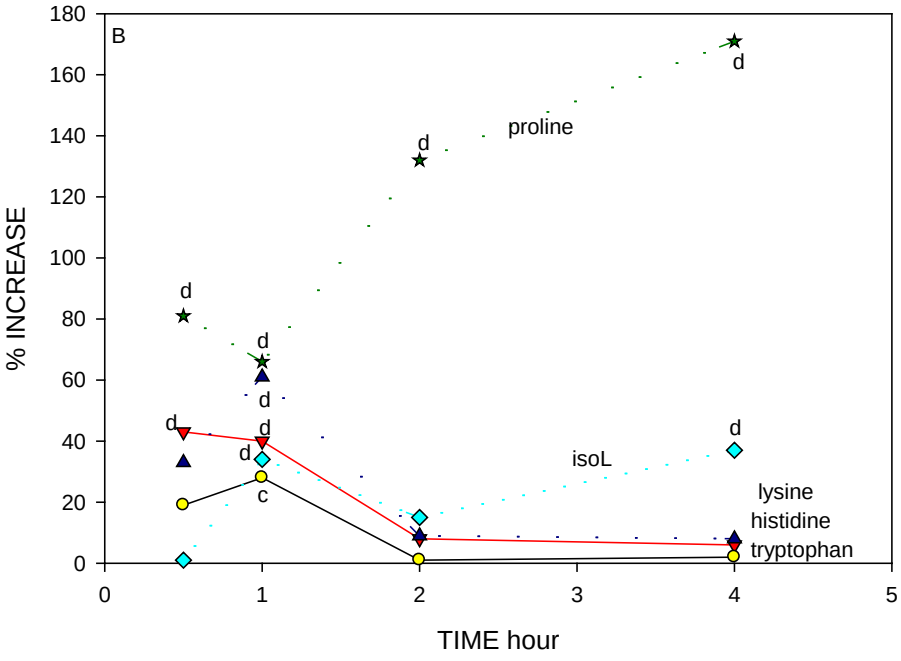

B

Figure 11. Percent Increase/Decrease of AAs by Leucine Oral Dosing (316 mg/kg) in (A) Plasma and (B) Brain. Rats (n=3) were orally dosed with leucine (316 mg/kg), and samples were collected 0 to 4 hours post dosing. [b]Significantly different from control at $p<0.05$. [c]Significantly different from control at $p<0.01$. [d]Significantly different from control at $p<0.001$.

4.2 *In Vitro* Results

4.2.1. Temporal Exposures. Based on the current understanding of mTOR pathway response kinetics, it was hypothesized that a dose of leucine could activate mTOR, and that this activation would be detectable by increases in phosphorylated S6K1 (at the threonine AA at position 389 of S6K1, or T389) and phosphorylated 4E-BP (T70) *in vitro*. Figure 12 shows the relative levels of phosphorylated S6K1 and 4E-BP, when detectable, after 5, 15, and 30 minutes exposures to 0.0525 to 525 µg/mL leucine. The pH of the 525 µg/mL solution used for the 15 minutes exposure was low enough to cause a pH change in the medium. The diminished intensity of the phosphorylated S6K1 band in Figure 12B is likely a result of the decreased pH and not an actual change in mTOR activity. This concentration was not used for any other exposures. Fifteen and 30 minute exposures resulted in no observable changes in levels of phosphorylated S6K1, and phosphorylated 4E-BP was not detectable (Figure 12B and C). Levels of phosphorylated S6K1 appeared to increase after a 5 minutes exposure to 52.5 and 5.25 µg/mL leucine but not 0.525 or 0.0525 µg/mL leucine (Figure 12A). However, this effect was not replicated in subsequent exposures (data not shown). The levels of phosphorylated 4E-BP did not change relative to control after a 5 minutes exposure.

4.2.2 Amino Acid Starvation. In order to determine if the mTOR pathway could be manipulated to activate in response to a dose of leucine, NE-4C and H19-7 cells were grown in amino acid free medium for 2.5 hours before exposure to 52.5 µg/mL leucine. Figure 13 A and B show the response of NE-4C cells to leucine after a period of amino acid starvation. The first lanes show the basal levels of phosphorylated S6K1 when cells are grown in standard, complete medium. The addition of leucine to cells grown in complete medium (Figure 13B, lane 2), shows that levels of phosphorylated S6K1 did not change from control. When cells were grown in medium without amino acids, the addition of leucine did not cause an increase in the level of phosphorylated S6K1 (Figure 13 A and B). This result was replicated in H19-7 cells, as well (Figure 13C). NE-4C cells that were grown in amino acid free medium and then switched to complete medium showed levels of phosphorylated S6K1 that were comparable to control (Figure 13 B, lane 5).

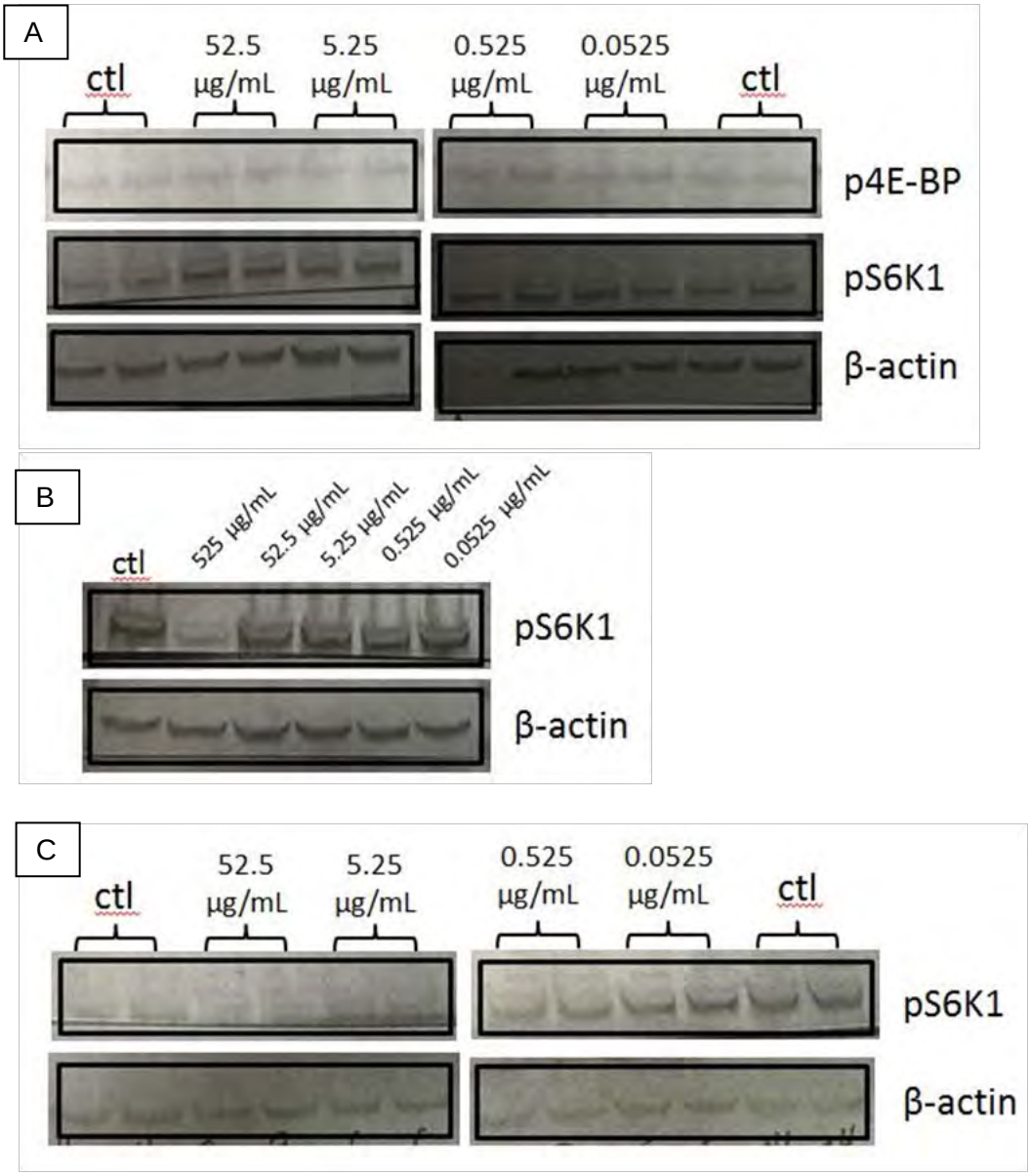

Figure 12. Estimation of Activation of mTOR Signaling in NE-4C Cells. Representative Western blots from NE-4C cells exposed to leucine for (A) 5 minutes, (B) 15 minutes, and (C) 30 minutes. β-actin was the loading control. Assays were run in duplicate. Note: ctl: control

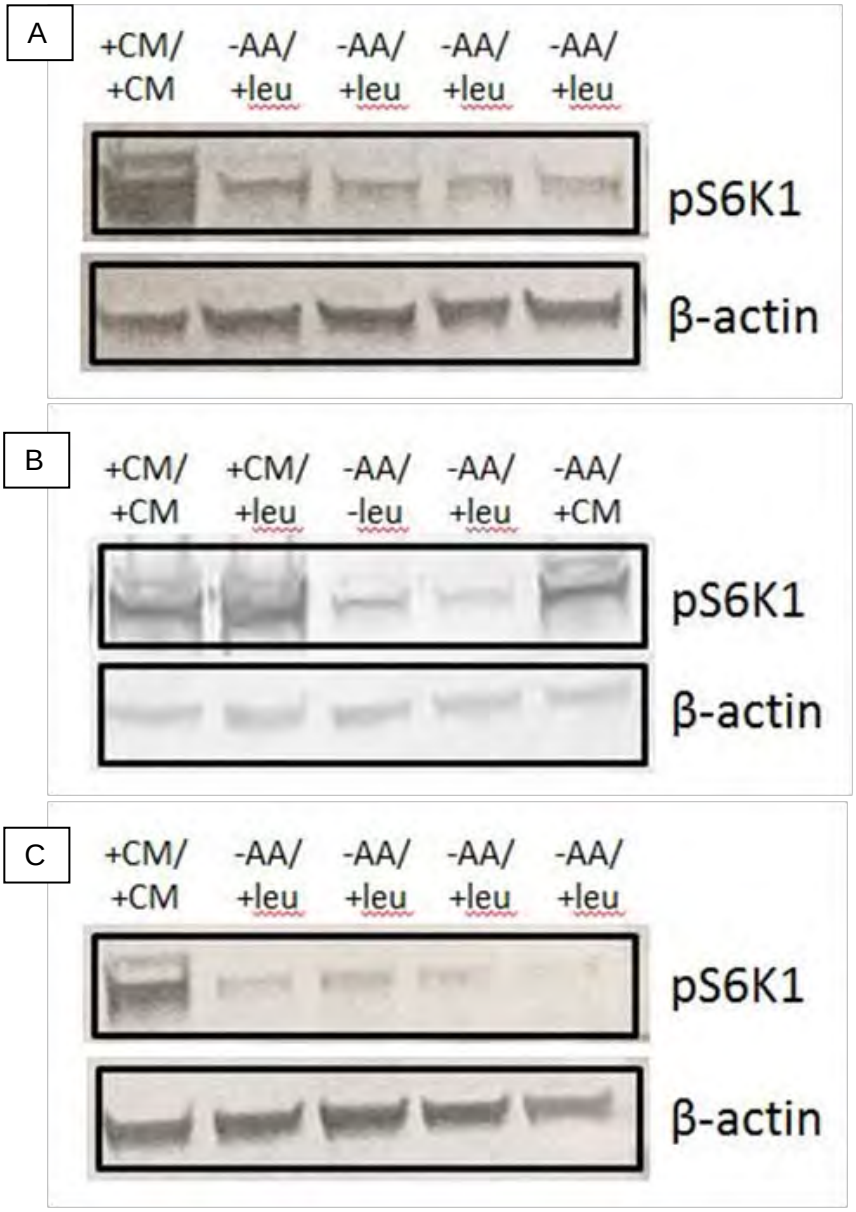

Figure 13. Evaluation of the Ability of Leucine to Activate mTOR Signaling after Amino Acid Starvation. Representative Western blots from (A, B) NE-4C and (C) H19-7 cells exposed to 52.5 µg/mL leucine after amino acid starvation. Notes: AA: amino acid; CM: complete medium; leu: leucine

4.3 Mathematical Modeling Results

The mathematical model developed by Jain and Bhalla (2009, in the supplementary material) was converted from SBML to acslX, in preparation for adding a module that describes the impact of leucine on the mTOR signaling pathway, although no model simulations were run in the new format.

5.0 CONCLUSIONS

Blood and brain leucine levels were predictably and significantly increased in both blood and brain as a result of both iv and oral gavage administration in rat. The gavage studies in particular support the feasibility of enhancing brain leucine levels by the oral route as a precondition for possible leucine-driven cognitive enhancement. Leucine administration initiated highly complex time- and dose-dependent responses in both plasma and brain levels of many other AAs. No obvious general chemical or biological AA properties were determined from the limited studies of brain levels (e.g., no sub-brain regions studied) and uptake behavior (e.g., radiotracer or inhibitor studies) observed in these studies. Nevertheless, knowing that most AAs have been reported to use the L1 large neutral AA transport, and that this system is normally close to saturation, and knowing leucine has one of the highest affinities for this transporter, one may speculate that the spike in leucine levels due to the iv injection tends to cause an increased brain influx of all AAs. However, the competition for the L1 transporter mitigates this effect somewhat for many of the AAs that primarily make use of this system to enter the brain.

As the immediate downstream kinase to the mTOR complex, S6K1 was selected as the marker of mTOR activation. Phosphorylation of S6K1 is a key step to initiating protein synthesis. 4E-BP is further downstream of S6K1 and was also selected to determine the extent of activation. Once phosphorylated, S6K1 then phosphorylates downstream proteins including 4E-BP and the S6 ribosomal subunit. *In vitro* studies using neuronal cells did not indicate that a single dose of leucine could activate the mTOR pathway in a predictable manner. Although a detectable increase in phosphorylated S6K1 was seen after a 5 minute exposure to 5.25 and 52.5 µg/mL leucine, this was not replicated in subsequent experiments. To determine if the mTOR pathway could be primed to respond to a leucine stimulus, both NE-4C and H19-7 cells were incubated for 2.5 hours in a custom medium that contained no amino acids. Following the incubation period, the cells were treated with 52.5 µg/mL leucine. Leucine alone was not able to stimulate the activity of mTOR as indicated by increased levels of phosphorylated S6K1. Interestingly, if the cells were treated with the standard complete medium after 2.5 hours in amino acid free medium, phosphorylated S6K1 levels were comparable to control levels (normal complete growth medium only). Activation of mTORC1 requires translocation of the complex to the lysosomal membrane where it interacts with Rag guanosine triphosphate (GTP) hydrolases (Sancak *et al.*, 2008). This recruitment to the lysosomal membrane is necessary for activation and is thought to be signaled by growth factors, energy levels, and amino acids. It is possible that in the *in vitro* assays the lack of an increase in phosphorylated S6K1was caused by incomplete translocation of the mTORC1 complex to the lysosomal membrane, which would require all amino acids for its movement. It was also assumed that the NE-4C and H19-7 cell lines contain all the required machinery to facilitate the movement of the complex through the cell. This was not verified in these current studies, but could be verified with immunostaining techniques in the future.

Future study of the required substances needed and the timing of the events for eliciting MTor responses as seen in the literature are recommended. Future studies should also consider pulsed increases of chemical mixtures (such as in dietary events that include main diet chemical changes with enhanced leucine levels), and increase in incubation time to allow for translocation and activation of mTORC1. Additionally, immunofluorescence assays with antibodies to endogenous mTOR, RAPTOR, RagC, and markers for endomembranes could be used to

describe the movement of mTORC1 after amino acid (leucine) stimulation. These data would provide the basis for the extension of the mathematical model of the mTOR pathway to include the impact of leucine. The kinetic data for leucine could in turn be used for the development of a pharmacokinetic model that would be able to describe, and predict leucine levels in the brain as a result of specific leucine dosing scenarios. Ultimately, integration of these PK and PD models, suitably validated, could lead to the development of a multi-scale model with predictive capacities, and in particular, the ability to optimize leucine administration for its impact on end-points associated with cognitive function.

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LIST OF ACRONYMS

4E-BP	4E binding protein 1
AA	amino acid
AAALAC	Association for the Assessment and Accreditation of Laboratory Animal Care
ANOVA	analysis of variance
ASC	analine-serine-cysteine
ATCC	American Type Culture Collection
BBB	blood brain barrier
DMEM	Dulbecco's modified Eagle medium
DTIC	Defense Technical Information Center
EAAT	excitatory amino acid transporter
EMEM	Eagle's minimal essential medium
FLD	fluorescence detector
GTP	guanosine triphosphate
H19-7	rat hippocampal fibroblast cell line 19-7
HCl	hydrochloric acid
HEK	human epidermal keratinocytes
HJF	Henry M. Jackson Foundation for the Advancement of Military Medicine
HPLC	high performance liquid chromatography
iv	intravenous
LE	Long-Evans
LNAA	large neutral amino acid
MDL	method detection limit
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
NE-4C	mouse neuroectodermal cell line 4C
OPA	O-pthalaldehyde
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonylfluoride
RPMI	Roswell Park Memorial Institute
S6K1	ribosomal small subunit 6 kinase
SBML	systems biology markup language