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RESEARCH ARTICLE

Maintenance of the branched-chain amino acid transporter LAT1 counteracts myotube atrophy following chemotherapy

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Abstract

Prevention/management of cachexia remains a critical issue in muscle wasting conditions. The branched-chain amino acids (BCAA) have anabolic properties in skeletal muscle, but their use in treating cachexia has minimal benefits. This may be related to altered BCAA metabolism consequent to the use of chemotherapy, a main cancer treatment. Since this topic is minimally studied, we investigated the effect of chemotherapy on BCAA concentrations, transporter expression, and their metabolism. L6 myotubes were treated with vehicle (1.4 μL/mL DMSO) or a chemotherapy drug cocktail, FOLFIRI [CPT-11 (20 μg/mL), leucovorin (10 μg/mL), and 5-fluorouracil (50 μg/mL)] for 24–48 h. Chemotherapy reduced myotube diameter (−43%), myofibrillar protein content (−50%), and phosphorylation of the mechanistic target of rapamycin complex 1 (mTORC1) substrate S6K1^{thr389} (−80%). Drug-treated myotubes exhibited decreased BCAA concentrations (−52%) and expression of their transporter, L-type amino acid transporter 1 (LAT1; −67%). BCAA transaminase BCAT2 level was increased, but there was a reduction in PP2CM (−54%), along with increased inhibitory phosphorylation of BCKD-E1α^{ser293} (+98%), corresponding with decreased BCKD enzyme activity (−23%) in chemotherapy-treated myotubes. Decreases in BCAA concentrations were a later response, preceded by decreases in LAT1 and BCKD activity. Although supplementation with the BCAA restored myotube BCAA levels, it had minimal effects on preventing the loss of myofibrillar proteins. However, RNAi-mediated depletion of neural precursor cell-expressed developmentally downregulated gene 4 (NEdd4), the protein ligase responsible for ubiquitin-dependent degradation of LAT1, attenuated the effects of chemotherapy on BCAA concentrations, anabolic signaling, protein synthesis, and myofibrillar protein abundance. Thus, if our findings are validated in preclinical models, interventions regulating muscle amino acid transporters might represent a promising strategy to treat cachexia.

NEW & NOTEWORTHY This is the first study to attenuate chemotherapy-induced myotube atrophy by manipulating a BCAA transporter. Our findings suggest that positive regulation of amino acid transporters may be a promising strategy to treat cachexia.

branched-chain amino acids; cachexia; chemotherapy; myotubes

INTRODUCTION

Cancer cachexia is a devastating body and skeletal muscle wasting condition that contributes to poor prognosis (1), treatment outcomes (2), and reduced quality of life (3). In addition to tumor-related factors, chemotherapy is a major contributor to the loss of skeletal muscle mass in cachexia (4–8). With no available cure, the development of therapeutic strategies to mitigate the loss of skeletal muscle mass in cachexia is vital to better manage this condition.

The branched-chain amino acids (BCAA: leucine, isoleucine, and valine) regulate body weight (9), activate skeletal muscle protein synthesis (10), and have been studied in the context of muscle wasting conditions for decades (11). Although cachexia is associated with poor nutritional status, nutritional treatment with the BCAA shows some positive effects, but do not fully reverse cachexia (12–15). This may be related to altered metabolism of these amino acids.

BCAA are transported into skeletal muscle via the L-type amino acid transporter 1 (LAT1) (16) and activate skeletal muscle protein synthesis (17), a process regulated by the mammalian/mechanistic target of rapamycin complex 1 (mTORC1) (18). In addition to upstream activation by the insulin receptor substrate-1(IRS-1)/phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT) pathway (19), BCAA sensing by mTORC1 is required for full activation (20). The BCAA, mainly leucine (21), activate components of the sestrins/gator/RAG/ragulator pathway, translocating mTORC1 to the lysosomal membrane where the mTORC1 activator RHEB is localized (22). Activated mTORC1 positively regulates mRNA translation initiation (23) and ribosomal biogenesis (24), while also inhibiting protein breakdown (25, 26). In cachexia, anabolic pathways, such as mTORC1 and insulin-like growth factor1-AKT, are downregulated (6, 27), whereas catabolic pathways, mainly the autophagy/lysosomal and ubiquitin proteasome pathways (UPPs) (28, 29), are upregulated.

During BCAA catabolism, the BCAA are first transaminated by branched-chain aminotransferase (BCAT2), forming glutamate and the branched-chain α-keto acids (BCKA): 2-keto-isocaproate/4-methyl-2-oxopentanoic acid (KIC) from leucine,

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α-keto-β-methylvaleric acid/3-methyl-2-oxopentanoate (KMV) from isoleucine, and 2-keto-isovalerate/3-methyl-2-oxobutanoic acid (KIV) from valine. The BCKAs are then oxidatively decarboxylated by the branched-chain α-keto acid dehydrogenase complex (BCKD), producing their corresponding acyl CoA derivates: isovaleryl-CoA from KIC, 2-methylbutyryl-CoA from KMV, and isobutyryl-CoA from KIV, which fuel several metabolic pathways such as the TCA cycle (30).

In tumor cells, BCAT1/2 and BCKD are increased, leading to sustained BCAA catabolism and elevations of substrates that support tumor cell growth (31–33). In addition, a previous study found alterations in plasma and tissue BCAA levels in tumor- and chemotherapy-treated animals (4). However, is it not known whether the reported changes in BCAA levels in response to chemotherapy are due to altered catabolism or transport. Resolving this subject may have implications for how cachexia is treated. Here, we hypothesized that chemotherapy, a main cause of cachexia, would decrease muscle BCAA concentrations and alter BCAA catabolism, and that interventions that maintain muscle BCAA concentrations would attenuate chemotherapy-induced atrophy. We present data on the effects of chemotherapy on BCAA metabolism in skeletal muscle myotubes. We also investigated whether increasing BCAA concentrations either through supplementation with these amino acids or genetically manipulating the abundance of the BCAA transporter LAT1 would ameliorate myotube atrophy following chemotherapy.

MATERIALS AND METHODS

Antibodies

Antibodies against tropomyosin (CH1, 1:400), troponin (JLT12, 1:400), and myosin heavy chain-1 (MHC-1, 1:500) (MF-20) were obtained from Developmental Hybridoma (Iowa City, Iowa). Antibodies against phosphorylated (p) FoxO3a^{ser253} (#9466), p-4E-BP1$^{thr37/46}$ (#2855), p-AKT^{Ser473} (#4060), p-S6$^{ser235/236}$ (#4858), and its kinase, p-S6K1^{thr389} (#9234), sodium-coupled neutral amino acid transporter 1 (SNAT1) (#36057), p-BCKD-E1α^{ser293} (#40368), BCKDH-E1α (#90198), neural precursor cell-expressed developmentally downregulated gene 4 (NEdd4, #4013), and total proteins AKT (#4691), S6 (#2317), and S6K1 (#9202) were purchased from Cell Signaling Technology (Danvers, MA) and all diluted 1:1,000. Antibodies against BCAT2 (#16417-1-AP), the ubiquitin protein ligase muscle RING-finger protein-1 (MuRF1; #55456-1-AP), and protein phosphatase 1 K (PP2CM, #14573-1-AP) were purchased from Protein Tech (San Diego, CA) and diluted 1:1,000. Anti-branched-chain α-ketoacid dehydrogenase complex kinase (BDK, #PA5-31455, 1:1,000) and LAT1 (#PA5-50485, 1:500) were purchased from Invitrogen (Waltham, MA), whereas anti-γ-tubulin (#T6557, 1:10,000) and puromycin (#MABE343, 1:20,000) antibodies were obtained from Sigma Aldrich (St. Louis, MO). Anti-ubiquitin antibody (#SC-8017, 1:500) was purchased from Santa Cruz (Dallas, TX).

Myotube Growth and Treatment

L6 rat myoblasts were cultured in AMEM supplemented with 10% FBS and 1% antibiotic-antimycotic reagents at 37°C and 5% CO_2. Once confluent, myoblasts were shifted into AMEM supplemented with 1% antibiotic-antimycotic reagents and 2% horse serum [differentiation medium (DM)] to induce differentiation. DM was replenished every other day until *day 4* or *5* when myotubes were fully differentiated. Experiments began on *day 4* or *5* of differentiation depending on the batches of cells. Nevertheless, all treatment groups in a specific experiment were studied in the same batch of cells, and therefore treatments began on the same day of differentiation. Myotubes were then treated with fresh DM in combination with either the chemotherapy drug cocktail (drug: 20 μg/mL CPT11, 50 μg/mL 5-fluorouracil, and 10 μg/mL leucovorin) or vehicle [V: 1.4 μL/mL dimethyl sulfoxide (DMSO)] for 3, 6, 10, 18, 24, and/or 48 h depending on the experiment. This chemotherapy drug cocktail is used in the treatment of colorectal cancer (34) and dose was taken from a previous study in which this combination of drugs successfully induced atrophy in myotubes (7).

For BCAA supplementation experiments, myotubes were treated with chemotherapy drugs in DM, combined with 400 μM of each of the BCAA for 24 h. Myotubes were resupplemented with 200 μM of each BCAA for the remaining 24 h. Media were collected after 24 and 48 h.

siRNA Gene Silencing

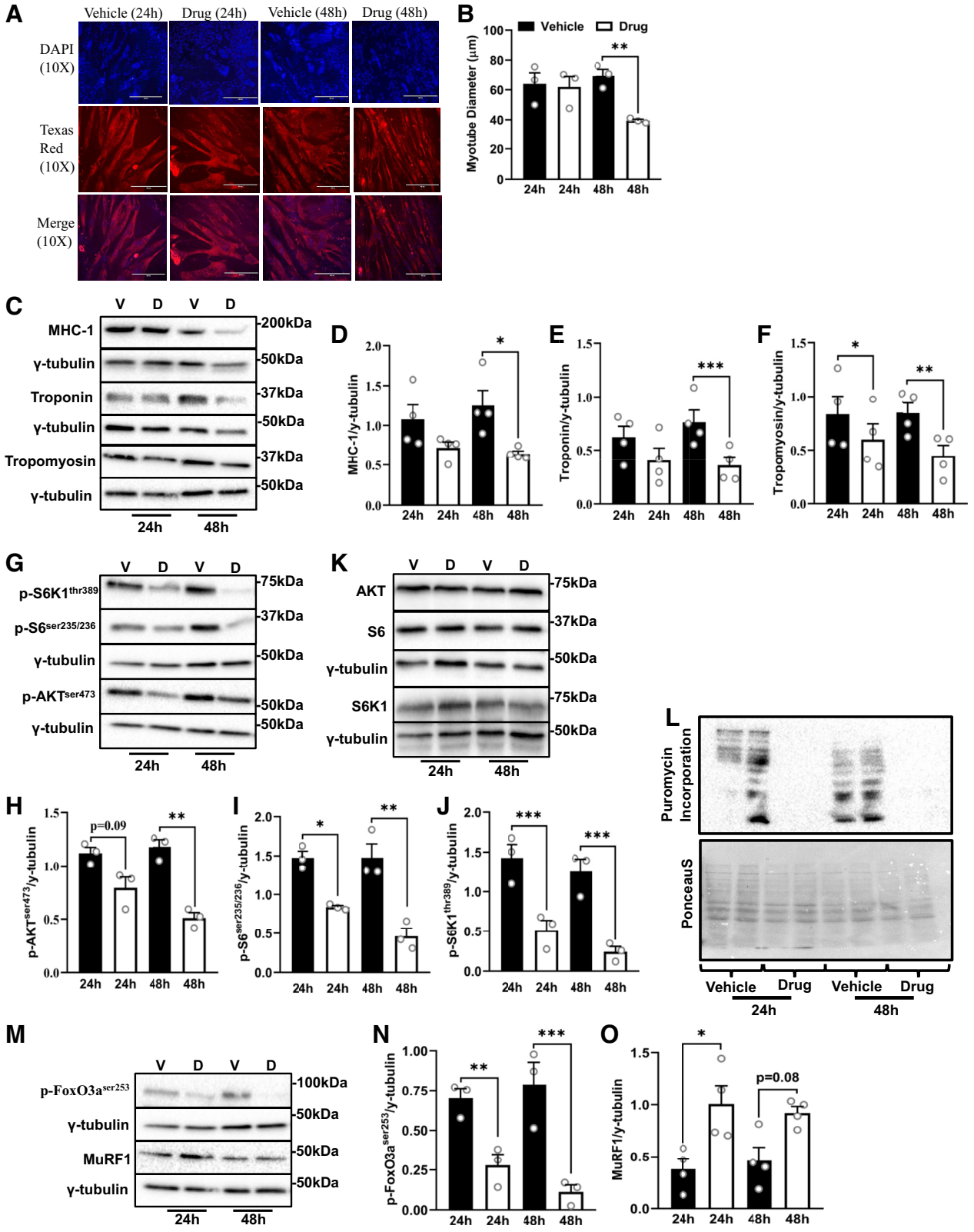
On *day 3* of differentiation, myotubes were transfected with 10 μM of NEdd4 siRNA (Sigma Aldrich, #NM_001008300): (NEdd4#1 (CCAUUUACAAGAACGGCUA[dT][dT], NEdd4#2 (UAGCCGUUCUUGUAAAUGG[dT][dT]) or 10 μM of scrambled siRNA (Sigma Aldrich, #SIC001) oligonucleotides with lipofectamine RNAiMAX (Thermo Fisher, #100014472) according to the manufacturer's instructions. At 24 h after transfection, 1 mL of DM was added to each well. Thirty hours following transfection, myotubes were treated for 48 h with chemotherapy drugs and harvested for Western blotting and HPLC analyses.

Immunofluorescence Microscopy

Vehicle- and drug-treated myotubes cultured on cover slips were fixed (4% paraformaldehyde in PBS for 10 min at room temperature), permeabilized (0.03% Triton X-100 in PBS for 5 min at room temperature), and blocked (10% horse serum in PBS) for 1 h at 37°C. Myotubes were then incubated overnight in primary antibody [2.5 μg/mL of MHC-1 in 1% bovine serum albumin (BSA) in PBS] at 4°C. Then, myotubes were washed (3 × 5 min in PBS), incubated in secondary antibody [Texas Red anti-mouse IgG secondary antibody (1:100 with 1% BSA in PBS)] for 2 h, stained for nuclei (4′,6-diamidino-2-phenylindole (DAPI)] and mounted on cover slides. Cover slips were imaged using the EVOS FL Auto microscope (Life Technologies, Waltham, MA) and the EVOS FL Auto program was used to maintain acquisition (light and brightness) settings for each slide. During imaging, researchers were blinded to treatments to avoid biasing. ImageJ was used to quantify all images by measuring the diameter of myotubes (in μm).

Western Blotting

These methods have been described previously (35). In brief, myotubes were lysed in lysis buffer [final concentration diluted in double distilled water: 1 mM ethylenediaminetetraacetic acid (EDTA), 2% sodium dodecyl sulfate (SDS), 25 mM Tris-HCl pH 7.5, 10 μL/mL protease inhibitor cocktail,



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10 μL/mL phosphatase inhibitor cocktail, and 1 mM dithiothreitol], and the bicinchoninic acid (BCA) protein assay method (Thermo Fisher) was used to determine protein concentrations. Protein extracts (~25 μg) were then electrophoresed in 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. Primary and secondary antibody incubation, imaging, and data quantification were as described (8, 35, 36).

Protein Synthesis (SUnSET Analysis)

The SUrface SEnsing of Translation (SUnSET) method has been described by others (37–39). At 48 h following the start of treatment, vehicle- and drug-treated myotubes were starved for 24 h and then treated for 30 min with 1 μM of puromycin diluted in differentiation media. Myotubes were harvested and immunoblotted against an anti-puromycin antibody and corrected to their respective Ponceau S staining.

BCKD Activity Assay

This assay was performed as previously described (40). Vehicle- and drug-treated myotubes were incubated in ^{14}C-L-valine, and BCKD activity was measured by collecting the resultant CO_2 (from BCKD's decarboxylation) on filter paper wicks soaked in 2 M NaOH. The radioactivity was counted, corrected for protein concentration, and expressed as counts per minute (CPM)/μg of protein.

Amino Acid Concentrations

Following treatment, myotubes were washed and harvested in 10% TCA. Cell lysates were then centrifuged at 2.3 *g* for 15 min, and the supernatant (containing free amino acids) was collected. The amino acids were then diluted in a ratio of 1 (sample): 2 (potassium borate buffer): 1 (0.1 N hydrochloric acid): 8 (HPLC grade water). Diluted samples were precolumn derivatized with a ratio of 1 (sample): 1 (o-phthalaldehyde, 29.28 mM). Samples were then injected into a YMC-Triart C18 column fitted onto an ultra HPLC system (Nexera X2, Shimadzu, Kyoto, Japan) connected to a fluorescence detector (Shimadzu, Kyoto, Japan; excitation: 340 nm; emission: 455 nm). A gradient solution derived from 20 mM potassium phosphate buffer (pH 6.5) (Mobile phase A) and a solution made from HPLC grade water (15%), acetonitrile (45%), and methanol (40%) at a flow rate of 0.8 mL/min were used to elute the amino acids. Amino acid standard curves were used to calculate amino acid concentrations, and all samples were normalized to total protein as previously described (41).

Statistical Analyses

Quantified immunoblot bands were adjusted to their corresponding gamma tubulin (loading control) values. For experiments in which both Ponceau S stains and gamma tubulin data were captured, gamma tubulin blots were corrected to their respective Ponceau S data. There were no significant treatment effects on gamma tubulin levels (Fig. 1*L*, means for vehicle vs. drug at 24 h: 0.1098 ± 0.02 vs. 0.0879 ± 0.02, $P = 0.4885$; means for vehicle vs. drug at 48 h: 0.0720 ± 0.01 vs. 0.0623 ± 0.01, $P = 0.6464$; Fig. 6*R*, vehicle vs. drug vs. drug + NEdd4 siRNA: 0.0826 ± 0.01 vs. 0.1147 ± 0.01 vs. 0.1032 ± 0.01, $P = 0.6643$; Fig. 6*W*, vehicle vs. drug vs. drug + NEdd4 siRNA: 0.0691 ± 0.002 vs. 0.073 ± 0.002 vs. 0.0769 ± 0.002, $P = 0.3042$, respectively; $n = 3$ or 4). All graphs were drawn, and statistical analyses were performed using GraphPad prism 8 software. For Figs. 1–3, a two-way analysis of variance (ANOVA) with a Tukey post hoc test was used to analyze vehicle and drug treatment differences at the 24- and 48-h time points. For Figs. 4–6, a one-way ANOVA was used to analyze vehicle and drug treatment differences. Significance was determined at $P < 0.05$. All results were expressed as ±SE of at least three independent experiments (biological replicates), with each independent experiment conducted with at least three technical replicates.

RESULTS

Chemotherapy Drugs Induce Myotube Atrophy, Decrease Protein Synthesis, and Increase Measures of Protein Breakdown

Chemotherapy drug treatment reduced MHC-1 staining and myotube diameter (−43%; Fig. 1, *A* and *B*). Myotube atrophy coincided with decreases in the abundance of myofibrillar proteins at 48 h: MHC-1 (−48%), troponin (−52%), and tropomyosin (−47%) (Fig. 1, *C–F*). At the 24- and 48-h time points (percentages given are for the 48-h point), chemotherapy decreased the phosphorylation of AKT^{ser473} (−56%), S6$^{ser235/236}$ (−68%), and its kinase, S6K1^{thr389} (−80%; Fig. 1, *G–J*). There were no treatment effects on total AKT, S6, or S6K1 proteins (Fig. 1*K*). Protein synthesis measured by SUnSET analysis (Fig. 1*L*) and phosphorylation of FoxO3a^{ser253} (−85%) were also decreased, along with a treatment-induced increase in MuRF1 protein expression (+100%; Fig. 1, *M–O*).

Chemotherapy Decreases BCAA Concentrations and Their Transporter Expression

At 48 h of treatment, chemotherapy significantly reduced total BCAA levels (−52%) and tended to reduce myotube intracellular concentrations of leucine (−48%, $P = 0.08$), isoleucine (−56%, $P = 0.07$), and valine (−56%, $P = 0.06$) (Fig. 2, *A–D*). Valine concentration also tended to be lower at 24 h. No effects on alanine (Fig. 2*E*) and serine (Fig. 2*F*) were observed, but at 48 h, there were decreases in the concentrations of arginine (−65%), phenylalanine (−59%), and

Figure 1. Chemotherapy drugs induce myotube atrophy, decrease protein synthesis, and increase measures of protein breakdown. Myotubes were treated with either a chemotherapy drug cocktail [drug (D, white bars): 20 μg/mL CPT11, 50 μg/mL 5-fluorouracil, and 10 μg/mL leucovorin] or vehicle (V, black bars; 1.4 μL/mL of DMSO) for 24 and 48 h. *A*: immunofluorescence detection of MHC-1 (Bar, 400 μM). *B*: quantification of myotube diameter. Immunoblot images (*C*) and quantified data for MHC-1 (*D*), troponin (*E*), tropomyosin (*F*), as well as phosphorylated AKT^{ser473} (*G* and *H*), S6$^{ser235/236}$ (*G* and *I*), S6K1^{thr389} (*G* and *J*), and total proteins (*K*) are shown. *L*: protein synthesis was measured via SUnSET analysis. Immunoblots (*M*) and quantified data for phosphorylated p-FoxO3a^{ser253} (*N*) and MuRF1 (*O*). Data are presented as means ± SE, $n = 3$ or 4 independent experiments, with at least 3 technical replicates per experiment, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Statistical significance was determined using a two-way ANOVA followed by Tukey's post hoc test. MHC-1, myosin heavy chain-1; DAPI, 4′,6- diamidino- 2- phenylindole; V, vehicle; D, drug; h, hours; p, phosphorylated; DMSO, dimethyl sulfoxide.

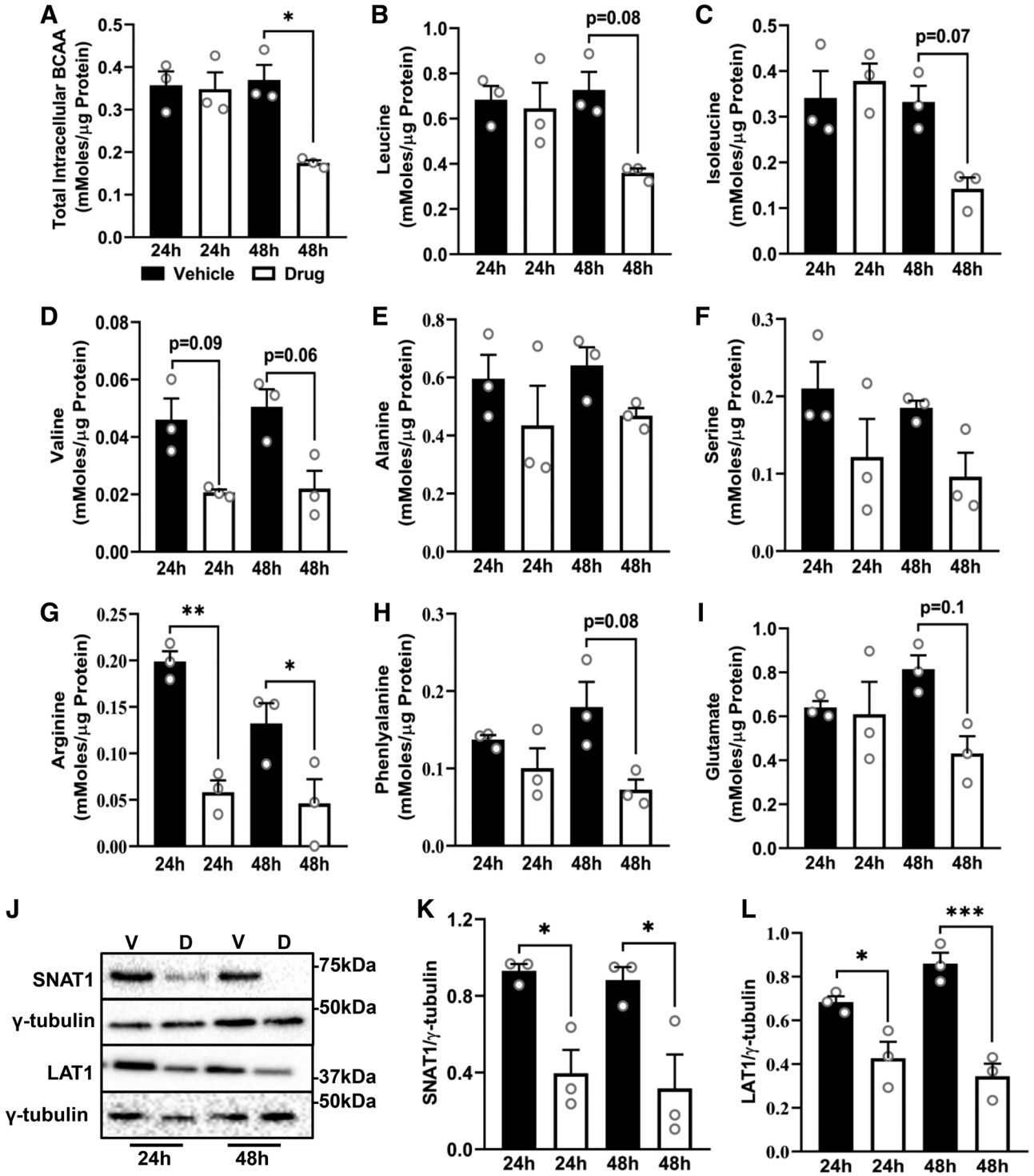

Figure 2. Chemotherapy decreases BCAA concentrations and their transporter expression. Myotubes were treated with chemotherapy drugs as described in Fig. 1. Samples were harvested and myotube intracellular concentrations of total BCAA (*A*), leucine (*B*), isoleucine (*C*), valine (*D*), alanine (*E*), serine (*F*), arginine (*G*), phenylalanine (*H*), and glutamate (*I*) were measured by HPLC. Immunoblots (*J*) and quantified data for SNAT1 (*K*) and LAT1 (*L*). Data are presented as means ± SE, $n = 3$ independent experiments, with at least 3 technical replicates per experiment, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Statistical significance was determined using a two-way ANOVA followed by Tukey's post hoc test. BCAA, branched-chain amino acid; HPLC, high-pressure liquid chromatography; LAT1, L-type amino acid transporter 1; SNAT1, sodium-coupled neutral amino acid transporter 1.

glutamate (−46%; Fig. 2, *G–I*). Chemotherapy drugs also reduced the expression of amino acid transporters SNAT1 (−63%) and the BCAA transporter LAT1 at 24 (−38%) and 48 h (−67%) following treatment initiation (Fig. 2, *J–L*).

Chemotherapy Alters BCAA Catabolism

Decreases in amino acid levels, especially the BCAA, made us to examine the regulation of BCAA catabolism. BCAT2

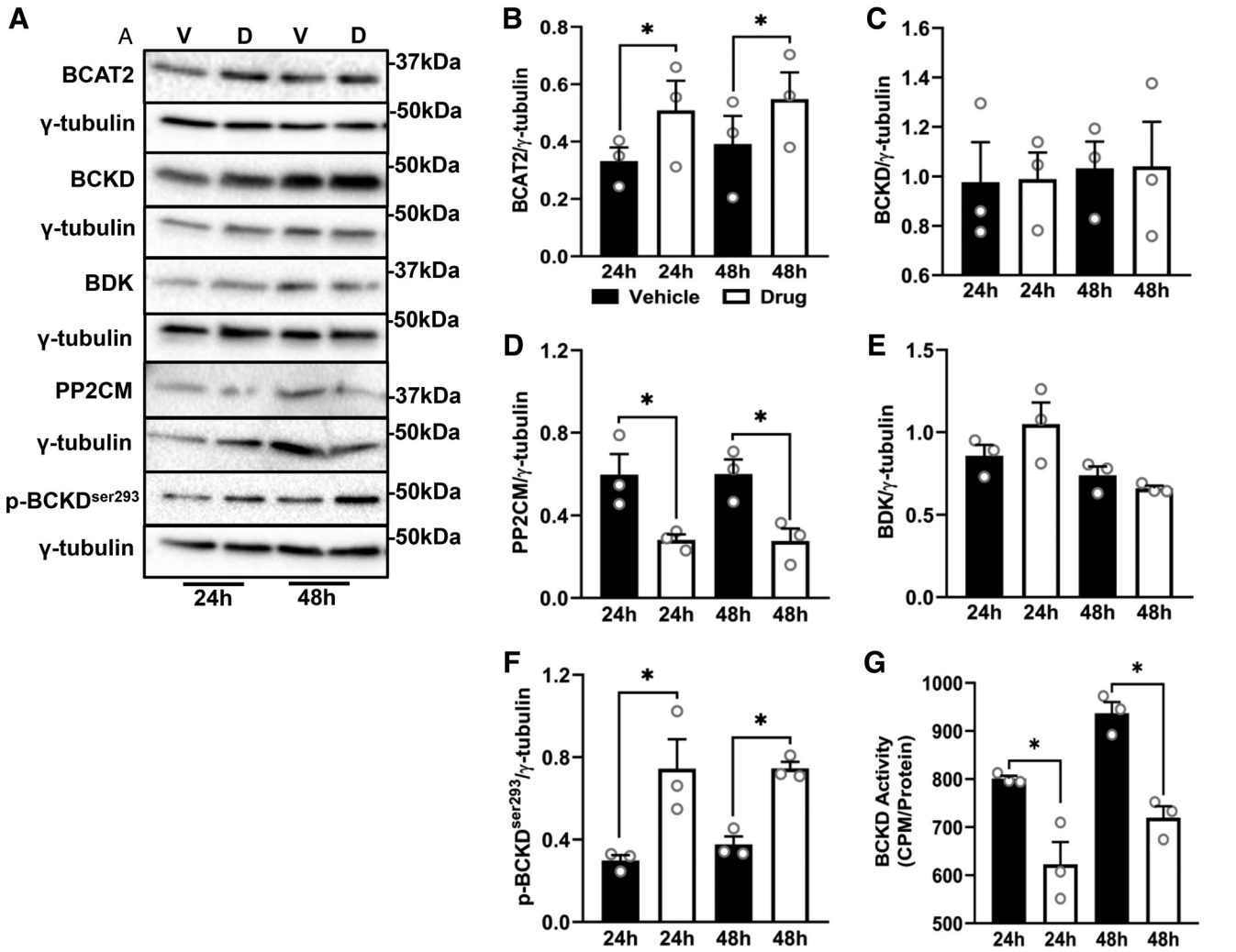

Figure 3. Chemotherapy alters BCAA catabolism. Myotubes were treated with chemotherapy drugs as described in Fig. 1. Samples were harvested and immunoblots (*A*) and quantified data for BCAT2 (*B*), BCKD-E1α (*C*), PP2CM (*D*), BDK (*E*), and p-BCKD-E1α^{ser293} (*F*) are shown. *G*: BCKD activity was measured from the release of $^{14}CO_2$ from ^{14}C-labeled valine. Data are presented as means ± SE, $n = 3$ independent experiments, with at least 3 technical replicates per experiment, $^*P < 0.05$. Statistical significance was determined using a two-way ANOVA followed by Tukey's post hoc test. BCAA, branched-chain amino acid; BCKD, branched-chain α-keto acid dehydrogenase; BDK, branched-chain α-ketoacid dehydrogenase complex kinase.

was increased at 24 (+53%) and 48 h (+40%), with no changes in BCKD protein expression following chemotherapy treatment (Fig. 3, *A*–*C*). The level of PP2CM, the BCKD phosphatase and positive regulator, was reduced as early as 24 h (−52%) after treatment began and remained decreased at 48 h (−54%, Fig. 3, *A* and *D*). The level of BDK, the BCKD kinase and negative regulator, was not different between treatments (Fig. 3, *A* and *E*). The inhibitory phosphorylation of BCKD-E1α^{ser293} was increased at 24 (+150%) and 48 h (+98%) following chemotherapy (Fig. 3, *A* and *F*). This change, along with the reduced level of PP2CM, likely contributed to the observed reduction in BCKD enzyme activity at 24 (−22%) and 48 h (−23%) (Fig. 3*G*).

Compared with the Decreases in BCAA Transporter Expression and BCKD Activity, Chemotherapy-Induced Changes in BCAA Concentrations Are a Delayed Response

Decreased LAT1 and BCAA concentrations could explain the reductions in BCAA catabolism. Therefore, we examined the time course of these changes. Chemotherapy decreased SNAT1 (−57%) and LAT1 (−76%) transporter expression starting at 18 h (Fig. 4, *A* and *B*). However, the enzyme activity of BCKD did not start to decrease (−28%) until the 24-h time point (Fig. 4*C*). Changes in the concentrations of leucine (Fig. 4*D*) and isoleucine (Fig. 4*E*) were more delayed, being noticeable only at 48 h.

Supplementation with the BCAA Increases Myotube BCAA Concentrations, but Does Not Rescue Chemotherapy-Induced Atrophy

The pattern of changes in BCAA transporter expression, BCKD activity, and intracellular BCAA concentrations suggests that maintaining BCAA concentrations might have protective effect on chemotherapy-induced atrophy. Therefore, we treated myotubes with the drugs along with coincubation with additional BCAA. Drug treatment had no effect on BCAA media concentrations, but supplementation with BCAA increased media concentrations of these amino acids

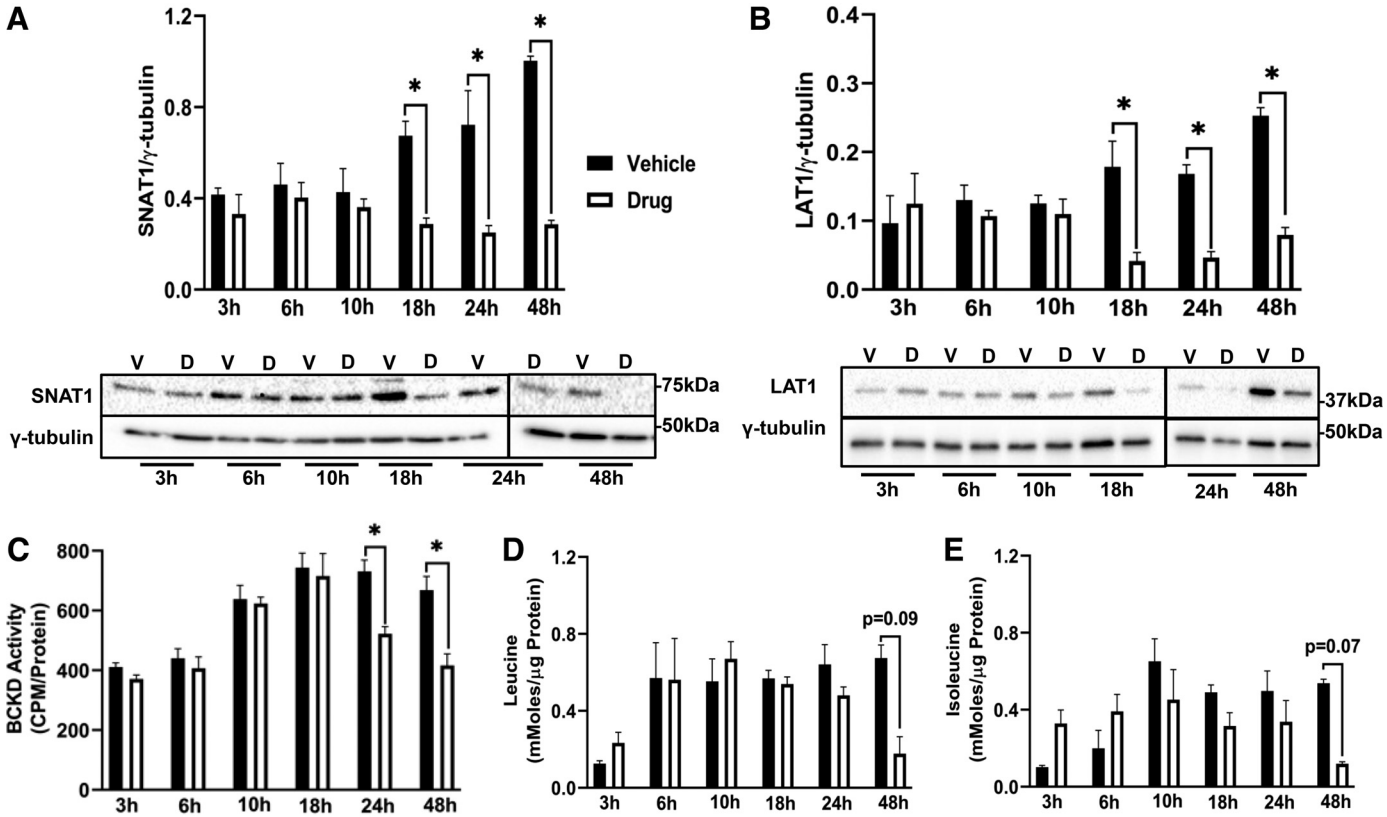

Figure 4. Compared with the decreases in BCAA transporter expression and BCKD activity, chemotherapy-induced changes in BCAA concentrations are a delayed response. Myotubes were treated as in Fig. 1, but were harvested at 3, 6, 10, 18, 24, and 48 h following the start of treatment. Immunoblots and quantified data for SNAT1 (*A*) and LAT1 (*B*). BCKD activity (*C*) and concentrations of leucine (*D*) and isoleucine (*E*) following chemotherapy. Data are presented as means ± SE, $n = 3$ independent experiments, with at least 3 technical replicates per experiment, $^*P < 0.05$. Statistical significance was determined using a one-way ANOVA followed by Tukey's post hoc test. BCAA, branched-chain amino acid; BCKD, branched-chain α-keto acid dehydrogenase; D, drug; h, hours; LAT1, L-type amino acid transporter 1; SNAT1, sodium-coupled neutral amino acid transporter 1; V, vehicle.

at 24 h (Fig. 5, *A–C*). Media supplementation with the BCAA restored myotube intracellular concentrations of these amino acids, especially for leucine and isoleucine (Fig. 5, *D–F*), but had no effect on drug-induced changes in the levels of other amino acids (Fig. 5, *G–K*) nor on the expression of their transporters (Fig. 5, *L–N*). In addition, increasing intracellular BCAA concentrations in drug-treated myotubes did not rescue mTORC1 signaling (Fig. 5, *O–Q*) or myofibrillar protein levels (Fig. 5, *R–T*).

Preventing Chemotherapy-Induced Decreases in LAT1 Level Counteracts BCAA Loss and Myotube Atrophy

NEdd4 is the ubiquitin protein ligase responsible for the ubiquitination of LAT1, targeting it for degradation (42). Therefore, we examined whether loss of NEdd4 would ameliorate the decreases in LAT1, BCAA concentrations, and myotube atrophy. We used siRNA to deplete NEdd4 (Fig. 6, *A* and *B*), which led to a reversal of chemotherapy-induced decreases in LAT1 expression (Fig. 6, *A* and *C*). In NEdd4-depleted myotubes treated with chemotherapy, intracellular BCAA (especially leucine and isoleucine) concentrations returned to their control levels (Fig. 6, *D–F*). There was also a moderation of the effects of the drugs on arginine and phenylalanine, but the effect of the drugs on glutamate and SNAT1 was not rescued following NEdd4 depletion (Fig. 6, *G–M*). Anabolic signaling (Fig. 6, *N–Q*), protein synthesis (Fig. 6*R*), and myofibrillar protein content (Fig. 6, *S–V*) were all restored, whereas ubiquitinated proteins (Fig. 6, *W* and *X*) were reduced in chemotherapy-treated myotubes depleted of NEdd4.

BCAA Levels Are Positively Correlated with Myotube Diameter, LAT1 Transporter Expression, and BCKD Activity

Intracellular BCAA concentrations showed strong and positive correlations with myotube diameter (Fig. 7*A*), BCKD activity (Fig. 7*B*), and protein expression of LAT1 (Fig. 7*C*), MHC-1 (Fig. 7*D*), and troponin (Fig. 7*E*). We also observed positive correlations between LAT1 and myotube diameter (Fig. 7*F*), and MHC-1 expression (Fig. 7*G*) and troponin expression (Fig. 7*H*). Finally, a significant positive correlation was found between BCKD activity and myotube diameter (Fig. 7*I*).

DISCUSSION

Findings from this study show that administration of anticancer drugs to skeletal muscle myotubes caused atrophy along with decreases in the concentrations of the BCAA and other amino acids, as well as reduced expression of the

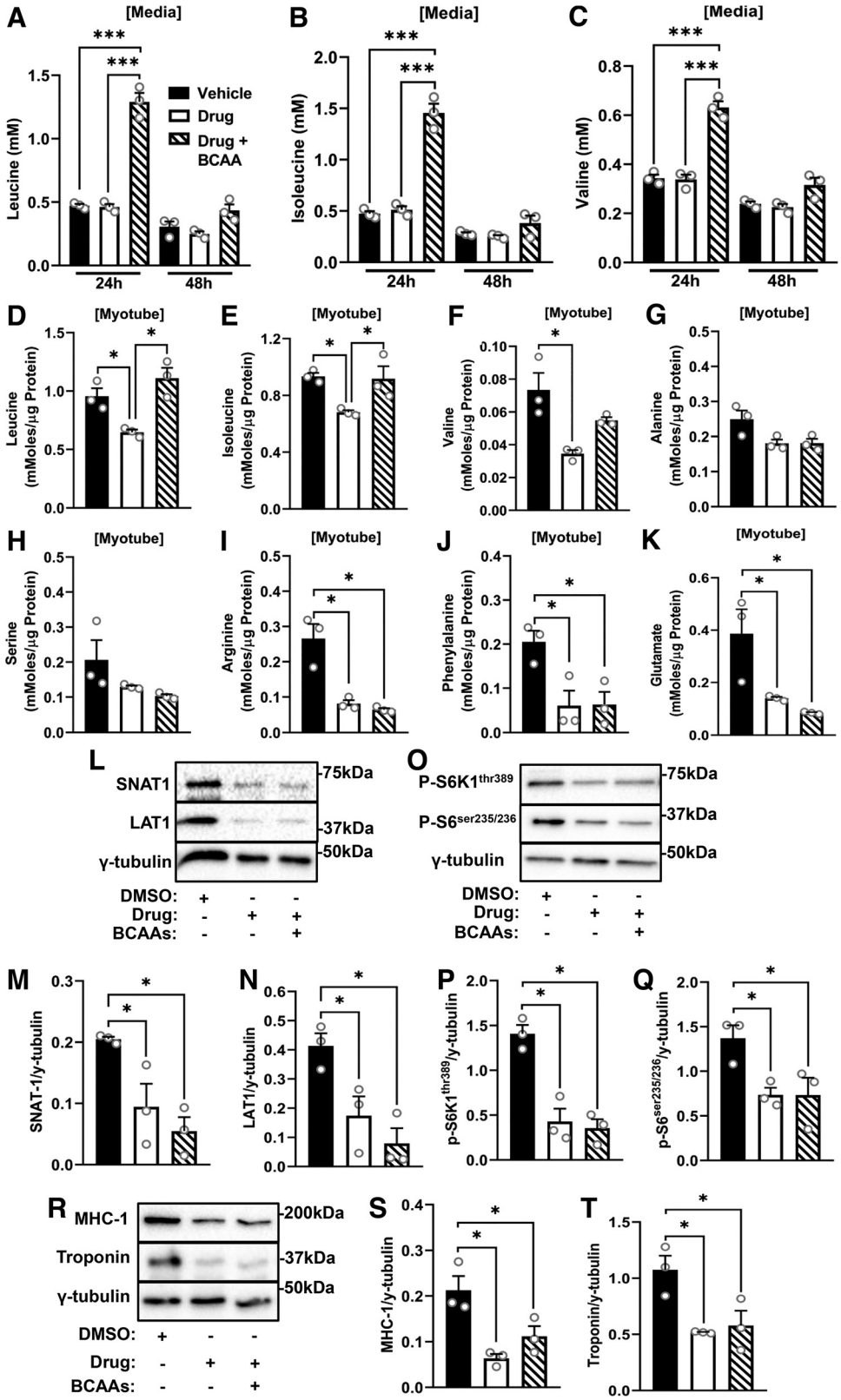

Figure 5. Supplementation with BCAA increases myotube BCAA concentrations but does not rescue chemotherapy-induced atrophy. Myotubes were treated as in Fig. 1, but a separate group of myotubes was treated with chemotherapy and supplemented with 400 μM BCAA for 24 h, followed by treatment with 200 μM BCAA for the remaining 24 h. Media concentrations of leucine (*A*), isoleucine (*B*), and valine (*C*), as well myotube intracellular levels of leucine (*D*), isoleucine (*E*), valine (*F*), alanine (*G*), serine (*H*), arginine (*I*), phenylalanine (*J*), and glutamate (*K*) were measured by HPLC. Immunoblots (*L*, *O*, and *R*) and quantified data for SNAT1 (*M*), LAT1 (*N*), p-S6K1^{thr389} (*P*), p-S6$^{ser235/236}$ (*Q*), MHC-1 (*S*), and troponin (*T*) are shown. Data are presented as means ± SE, $n = 3$ independent experiments, with at least 3 technical replicates per experiment, $^{*}P < 0.05$; $^{***}P < 0.001$. Statistical significance was determined using a one-way ANOVA followed by a Tukey's post hoc test. BCAA, branched-chain amino acid; HPLC, high-pressure liquid chromatography; LAT1, L-type amino acid transporter 1; MHC-1, myosin heavy chain-1; SNAT1, sodium-coupled neutral amino acid transporter 1.

amino acid transporters LAT1 and SNAT1. These changes occurred in parallel to altered expression of some of the key enzymes involved in the proximal steps of BCAA catabolism, leading to decreased BCKD activity, the rate-limiting enzyme in BCAA catabolism. Importantly, we showed that maintaining LAT1 expression was sufficient in attenuating the loss of anabolic signaling, protein synthesis, and myofibrillar protein abundance in chemotherapy drug-treated myotubes.

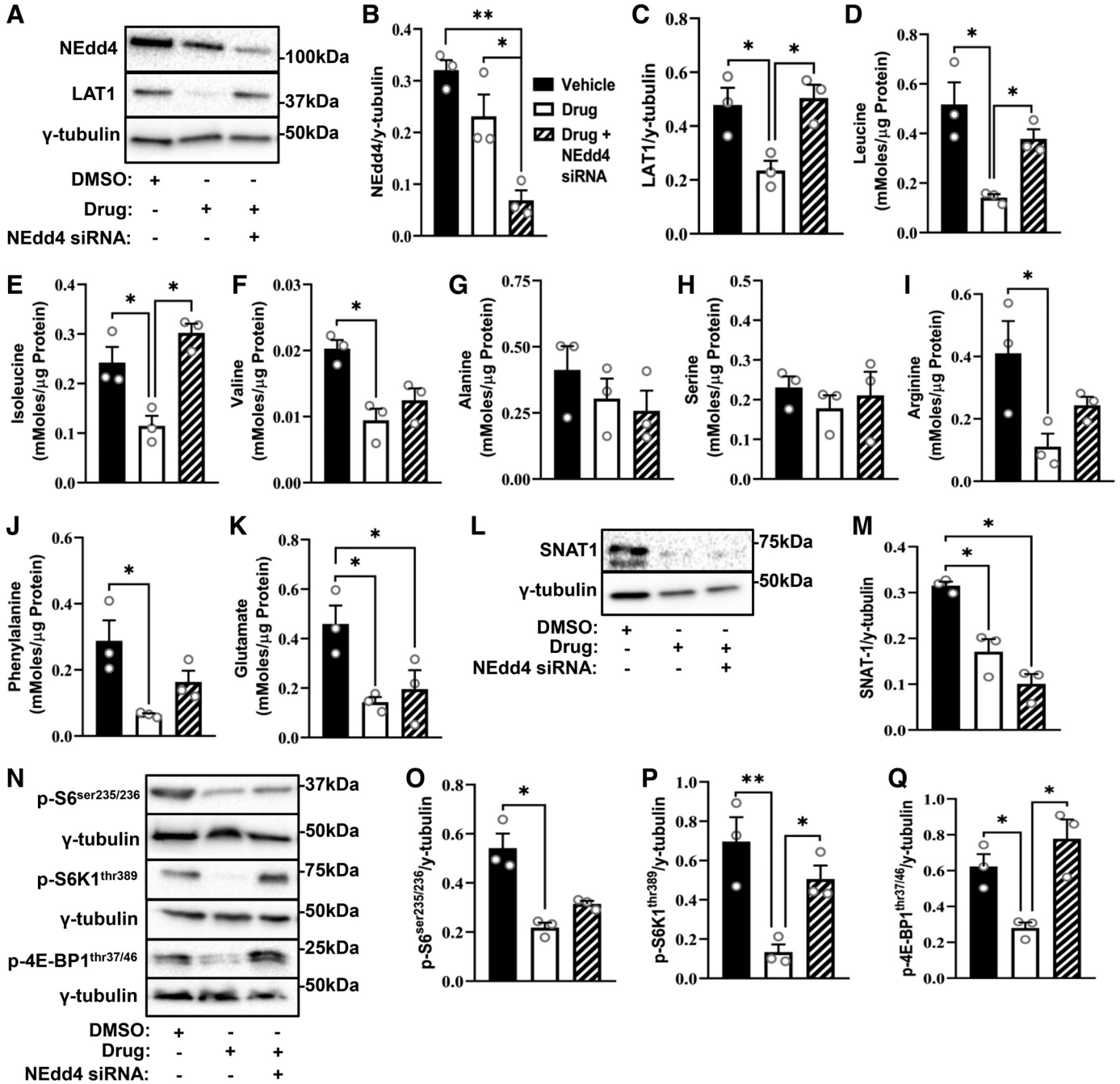

Figure 6. Preventing chemotherapy-induced decreases in LAT1 level counteracts BCAA loss and myotube atrophy. L6 myotubes were treated with control or NEdd4 siRNA oligonucleotides for 24 h, followed by treatment with the chemotherapy drug cocktail for an additional 48 h. Immunoblots (*A*) and quantified data for NEdd4 (*B*) and LAT1 (*C*) in myotubes from the different treatment groups. Concentrations of leucine (*D*), isoleucine (*E*), valine (*F*), alanine (*G*), serine (*H*), arginine (*I*), phenylalanine (*J*), and glutamate (*K*) were measured via HPLC. Immunoblots (*L* and *N*) and quantified data for SNAT1 (*M*), p-S6$^{ser235/236}$ (*O*), p-S6K1^{thr389} (*P*), and p-4E-BP1$^{thr37/46}$ (*Q*). *R*: protein synthesis was measured via SUnSET analysis. Immunoblots (*S* and *W*) and quantified data for MHC-1 (*T*), troponin (*U*), tropomyosin (*V*), and ubiquitinated proteins (*X*). Data are presented as means ± SE, $n = 3$ independent experiments, with at least 3 technical replicates per experiment, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Statistical significance was determined using a one-way ANOVA followed by a Tukey's post hoc test. BCAA, branched-chain amino acid; h, hours; HPLC, high-pressure liquid chromatography; LAT1, L-type amino acid transporter 1; NEdd4, neural precursor cell-expressed developmentally downregulated gene 4; SNAT1, sodium-coupled neutral amino acid transporter 1.

Many of the previous studies on nutritional management/prevention of cachexia have focused on the anabolic roles of the BCAA. Although, quantitatively speaking, nonoxidative disposal (protein synthesis) is the main use of BCAA and other amino acids (17), BCAA can also be metabolized in muscles to yield ketoacids (30), beta-hydroxy-beta-methylbutyrate (HMB, from leucine) (43), and glutamine (44), all of which serve vital functions, including anabolism (30). BCAA is also important for muscle ammonia disposal (45), a metabolite that has been

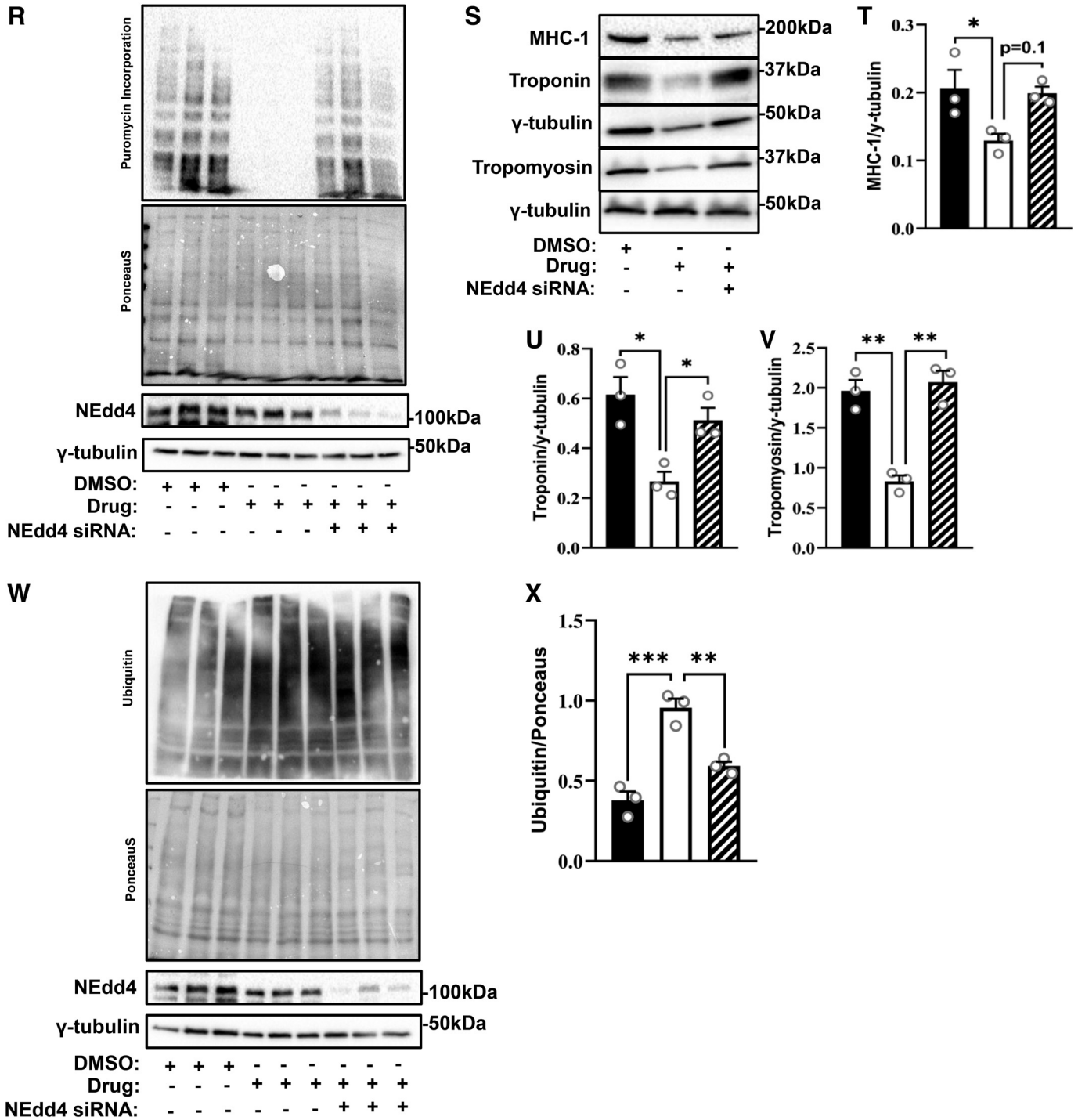

Figure 6. *Continued*

implicated in muscle atrophy (46, 47). Therefore, the less than stellar results of interventions that use BCAA (or protein) supplements to fully reverse cachexia (12) might result from altered metabolism of the BCAA, a subject that has received little attention.

The reductions in myotube BCAA concentrations might result from increased protein synthesis, increased oxidation, and or reduced transport. The data presented here and in our previous study (8), as well as reports by others (5, 39), clearly rule out the first option. Our observation of reduced BCKD activity leaves reduced transport as the possible explanation. Indeed, our time course analyses suggest that reduced expression of the amino acid transporters was the earliest response, followed by reduced oxidation. This implies that the latter observation was a response to the reduced transporter expression so as to prevent the fall in muscle amino acid levels, which did not appear until the 48-h time point.

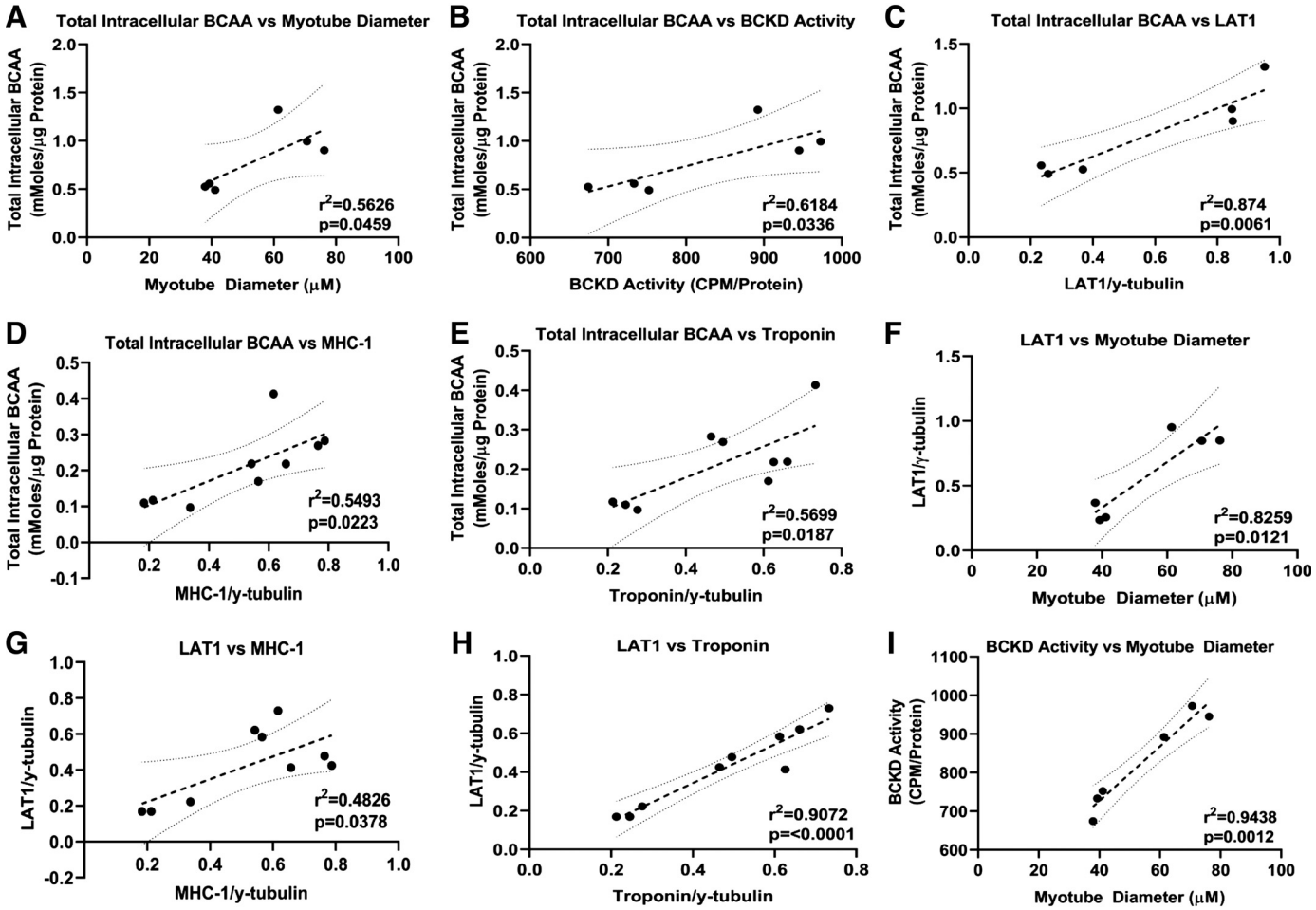

Figure 7. BCAA levels are positively correlated with myotube diameter, LAT1 transporter expression, and BCKD activity. Correlations between total intracellular BCAA and myotube diameter (*A*), BCKD activity (*B*), protein expression of LAT1 (*C*), MHC-1 (*D*), and troponin (*E*). Correlations between LAT1 and myotube diameter (*F*), MHC-1 (*G*), and troponin (*H*). *I*: BCKD activity and myotube diameter correlation. Data were analyzed using linear regression, and 95% confidence intervals are denoted. These charts are drawn from the data in Figs. 1–3. BCAA, branched-chain amino acid; BCKD, branched-chain α-keto acid dehydrogenase; LAT1, L-type amino acid transporter 1; MHC-1, myosin heavy chain-1

Although it is possible that the decrease in LAT1 might result from altered rates of synthesis or degradation, our data in the NEdd4 depletion experiments suggest that altered degradation is responsible at least in part for the chemotherapy-induced decrease in LAT1 expression. Interestingly, NEdd4 level at 48 h of treatment was lower in chemotherapy drug-treated control (drug treatment, but without siRNA). This likely results from the myotubes attempting to prevent further declines in LAT1 by reducing NEdd4 level. We cannot comment on the mechanism regulating NEdd4 or whether factors other than NEdd4 are implicated in the regulation of LAT1 in response to chemotherapy drugs.

A major finding of this work is that simply increasing BCAA concentrations in the incubation medium, which restored intracellular BCAA levels, did not prevent myotube atrophy or restore mTORC1 signaling. This might be related to impaired signaling from the BCAA to mTORC1, a question that should be addressed in future studies. Our initial plan to use CRISPR/CAS9 system to increase LAT1 expression was not successful due to technical issues. However, we believe our indirect approach of using RNA interference to deplete NEdd4 is advantageous for two reasons. First, an indirect approach to maintain LAT1 level by using RNAi to deplete NEdd4, the ubiquitin protein ligase that targets LAT1 for proteasomal degradation (42), represents a more physiological titration of LAT1 level. Second, a previous study with overexpression of LAT1 was unable to increase intracellular AA concentrations (48), likely because the overexpressed LAT1 triggers an increase in NEdd4 level, leading to the degradation of LAT1. Our indirect approach with NEdd4 depletion both increased LAT1 expression and restored myofibrillar protein levels. The fact that our NEdd4 knockdown approach (which increased LAT1) was more effective in reversing chemotherapy-induced myotube atrophy compared with the mere addition of the BCAA may be explained by the fact that NEdd4 depletion partially restored the levels of arginine [an mTORC1 signaling activator (33)] and phenylalanine (Fig. 6, *I* and *J*). However, we think it is unlikely that this would totally explain the beneficial effects of maintaining LAT1 level. This is because supplementation with BCAA alone, which restored intracellular BCAA levels [the main anabolic amino acids for skeletal muscle (17)] did not rescue myofibrillar protein abundance, and NEdd4 depletion did not rescue the levels of other

amino acids. Thus, LAT1 may play roles in myotube anabolism by mechanisms additional to facilitating the transport of its cognate amino acids (49, 50). Interestingly, some amino acid transporters are needed for full mTORC1 activation (51). Whether LAT1 is involved in mTORC1 activation and whether or not LAT1 regulates proteolysis represent an area of future inquiry.

We observed higher BCAT2 expression along with reduced glutamate concentrations in drug-treated myotubes. These changes suggest that BCAT2 action is geared toward BCAA production from the BCKA, perhaps as a way to preserve BCAA levels. This would be consistent with the observed reduction in BCKD activity. Glutamate can also be metabolized into glutamine in the skeletal muscle (52), an amino acid reported to increase antioxidant capacity (53). The observed reduction in glutamate levels might lead to reduced capacity of the myotubes to handle chemotherapy-induced increases in reactive oxygen species levels (54). In addition, as reported in our previous study, chemotherapy reduced substrate (glucose) availability in myotubes (8). Since arginine (whose level was reduced by chemotherapy drugs) can be converted to glucose (55, 56), the reduction in arginine level in chemotherapy drug-treated myotubes would worsen glucose homeostasis in these myotubes. It is also worth mentioning that not only the level of LAT1 but also SNAT1 level was reduced. However, the fact that maintaining LAT1, which transports the BCAA and a few other amino acids [phenylalanine, tyrosine, tryptophan, methionine, and histidine (16)], suffices to reverse the loss of myofibrillar proteins in drug-treated myotubes underlines the significance of the BCAA and especially LAT1 in myotube anabolism. In further support of this point, we showed strong and significant positive correlations between LAT1, myotube diameter, and BCAA concentrations.

Our observation of a strong positive correlation between BCKD activity and myotube size is intriguing. Because the BCAA are anabolic for muscle, including serving as substrates for protein synthesis, one would envisage competition between BCAA oxidation and these same amino acids being used for protein synthesis. The observed strong positive correlation between BCKD activity and myotube size points to an anabolic effect of BCAA oxidation (at least at the level of BCKD). Although this link requires further study, the relationship between the two is somewhat analogous to the positive effect of autophagy on skeletal muscle health (57).

A limitation of this study is that in our siRNA experiments, NEdd4 may regulate the levels of substrates other than LAT1. Therefore, although NEdd4 depletion increased LAT1 content with a concomitant increase in intracellular BCAA levels, we cannot rule out the contributions of other substrates of NEdd4 to the observed benefits of NEdd4 depletion. Also, because this is an in vitro model, we are unable to investigate the contributions of other tissues, including adipose tissue and liver [where >80% of BCKD's oxidative capacity resides (58)], to the altered BCAA catabolism. Therefore, in vivo studies investigating the effects of cancer and chemotherapy on similar measures as in our study are warranted. Nonetheless, by using a clinically relevant chemotherapy cocktail (59), this in vitro model allows us to explore the direct mechanistic effects of chemotherapy on skeletal muscle cell atrophy and amino acid metabolism.

In sum, we demonstrated profound negative effects of chemotherapy drugs on the concentrations, metabolism, and transporter expression of the BCAA in myotubes. These perturbations were not corrected by simple supplementation with additional BCAA in the incubation medium, but using RNA interference against NEdd4 to prevent the decline in LAT1 expression rescued the atrophy phenotypes, including improvements in anabolic signaling, protein synthesis, and myofibrillar protein abundance. Although these findings suggest that interventions that preserve LAT1 have therapeutic potential in cancer cachexia, our findings would need to be replicated in preclinical animal models. Finally, using LAT1 manipulation to rescue muscle atrophy in cancer cachexia would need to be done in a tissue-specific (skeletal muscle) manner because whole body increases in LAT1 expression might have undesirable consequences in other tissues, especially in cancer cells (16).

DATA AVAILABILITY

Data will be made available upon reasonable request.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.M. and O.A.J.A. conceived and designed research; S.M. performed experiments; S.M. analyzed data; S.M. and O.A.J.A. interpreted results of experiments; S.M. prepared figures; S.M. drafted manuscript; S.M. and O.A.J.A. edited and revised manuscript; S.M. and O.A.J.A. approved final version of manuscript.

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