Threonine Deprivation Rapidly Activates the System A Amino Acid Transporter in Primary Cultures of Rat Neurons from the Essential Amino Acid Sensor in the Anterior Piriform Cortex1,2

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ABSTRACT Omnivores show recognition of essential (indispensable) amino acid deficiency by changing their feeding behavior within 20 min, yet the cellular mechanisms of amino acid sensation in eukaryotes are poorly understood. The anterior piriform cortex (APC) of the brain in rats or its analog in birds likely houses the in vivo amino acid chemosensor. Because amino acid transporters adapt rapidly to essential amino acid deficiency in several cell models, we hypothesized that activation of electrogenic amino acid transport in APC neurons might contribute to the function of the amino acid sensor. We evaluated transport systems in primary cultures of neurons from the APC, hippocampus and cerebellum, or glia, incubated in complete or threonine-devoid (deficient) medium. After 10 min in deficient medium, uptake of threonine or a system A-selective substrate, methyl amino-isobutyric acid, was increased 60% in APC neurons only (P < 0.05). These results demonstrated upregulation of system A, an electrogenic amino acid-sodium symporter. This depletion-induced activation required sodium, intact intracellular trafficking, and phosphorylation of signal transduction–related kinases. Efflux studies showed that other transporter types were functional in the APC; they appeared to be altered dynamically in threonine-deficient cells in response to rapid increases in system A activity. The present data provided support for the chemical sensitivity of the APC and its role as the brain area housing the indispensable amino acid chemosensor. They also showed a region-specific, phosphorylation-dependent activation of the system A transporter in the brain in response to threonine deficiency.


KEY WORDS: • deficiency • nutrient sensor • brain • amino acid • neuron culture

Maintenance of protein synthesis depends on continuous availability of essential (indispensable) amino acids (IAA).4 Thus IAA homeostasis is crucial; both whole-animal systems and cells in culture respond to IAA deprivation in ways that seek to restore the needed substrate. Omnivores uniformly reject diets containing deficiencies of IAA, showing an early hypophagic response (1–7). It is clear that neither taste nor smell can account for the detection of IAA deficiency (8–10). Also, IAA recognition in vivo is too rapid, i.e., the hypophagia is observed by 20 min (7), to be explained by mechanisms involving de novo protein synthesis.

Recognition of IAA deficiency was localized to the anterior piriform cortex (APC) by lesion studies (11) and by microinjections of nanomole amounts of the limiting IAA into the APC. The injections were selective for the limiting IAA and localized to an area of the APC (12,13) near the Area Tempes (14), now called the ventrostral APC (15). Recently, the immunoreactivity for γ-amino butyric acid (GABA)ergic terminals, cholecystokinin positive cells, and GABA transporters has been shown to be absent or very low in this area (15). As a result of this diminished inhibitory function, the APC is highly excitable. Rapid activation of the glutamatergic output cells of the APC that project to neural feeding circuits (16,17) may initiate the anorectic responses that have been observed over many years (2–13). Consistent with this idea, we have seen more phosphorylated extracellular response kinase types 1 and 2 (ERK1/2, also known as mitogen-activated


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4 Abbreviations used: AIB, 2-amino isobutyric acid; APC, anterior piriform cortex; ATA, system A amino acid transporter; BCH, 2 amino bicyclo (2,1,1) heptane-2-carboxylic acid; ERK1/2, extracellular response element kinase, also known as MAPK, mitogen-activated protein kinase; GABA, γ-amino butyric acid; GAPDH, glyceraldehyde phosphate dehydrogenase; IAA, indispensable amino acid; MeAIB, methyl amino isobutyric acid; mTOR, mammalian target of rapamy- cin; PI3K, phosphatidylinositol 3-kinase; +Thr, threonine-containing complete amino acid mix; −Thr, threonine-devoid.

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protein kinase, MAPK) in pyramidal-like cells in the APC of animals fed a threonine devoid (−Thr) than after a threonine replete (+Thr) diet for 45 min (18).

A key observation underlying the present work was that after preincubation of APC slices in a −Thr medium, the addition of either threonine or serine causes an increase in intracellular calcium in the pyramidal cell layer of the APC (19). Threonine and serine are small neutral amino acids that share the same transport system [system A(ATA), also known as SAT and GlnT] (20). In single-cell systems, both rapid and later protein synthesis-dependent mechanisms contribute to the adaptive regulation of amino acid transporters in response to complete amino acid starvation and also to deficiencies of individual IAA (21–23). System A is a Na⁺-dependent symporter and thus is electrogenic (24). Because increased transport of substrate suggests increased activity of this electronegative amino acid transport system, such an increase could excite the output of cells of the APC.

We examined the adaptive responses of cultured brain cells to IAA deficiency by measuring the uptake of labeled threonine, or System A substrates, after preincubation in either a +Thr or −Thr medium. Neurons cultured from the APC, hippocampus, and cerebellum or glia were used. Transporter subtypes were determined by incubation with and without selective inhibitors of the various amino acid transporters. To examine the signaling pathways involved, we also tested the effects of kinase inhibitors on the uptake of ATA substrates by the cells.

**MATERIALS AND METHODS**

Tissue for the cell culture studies was obtained from weanling albino male rats (Wistar, Harlan-France, Gannat, France). They were maintained and used in accordance with the guidelines of the French Committee for Animal Care. For 1–2 d after arrival in the vivarium and before their use for preparation of cell culture, they had free access to standard stock diet pellets containing at least 25% protein and water.

**Cell culture.** The methods for neuron culture were adapted from the protocol used by Brewer (25) for the primary culture of adult rat hippocampal neurons. Rats were killed by rapid decapitation and their brains quickly removed and placed in PBS (4°C) for sterile dissection. The APC was removed as diagrammed in Gietzen et al. (26). The remaining parts of the slice from which the APC was taken were used for the primary culture of glial cells. The whole hippocampus was removed and a sagittal 2-mm slice was taken for the culture of hippocampal neurons. A slice of cerebellar tissue from the lateral cerebellar hemisphere was taken for the culture of cerebellar neurons. Tissues from 4 rats, taken separately according to tissue type, were pooled and cell cultures were prepared (25) using Neurobasal/B-27, a serum-free, bicarbonate-buffered medium optimized for the survival of adult rat hippocampal neurons (25,27). For glial culture, the cells were resuspended in DMEM (Sigma-Aldrich, L'Isle d'Abeau Chesnes, France) supplemented with 10% fetal calf serum. Cultures were maintained in an incubator at 37°C under a 5% CO₂/95% air atmosphere.

**Confirmation of cell types.** Adult neurons from many brain areas cultured in Neurobasal/B-27 elaborate neurites after a few days in culture, and maintain their morphological, neurochemical and electrophysiologic phenotypes (25,28,29). To verify cell types present in the cultures, immunocytochemical labeling for neurons and glia was performed by incubation for 45 min at 37°C with a mixture of rabbit polyclonal anti-neurofilament 200 (Sigma) and mouse monoclonal anti-glia fibrillary acidic protein (Sigma) antibodies, respectively. Secondary antibodies were rhodamine-conjugated donkey anti-rabbit Ig, and fluorescein-conjugated donkey anti-mouse Ig (Santa Cruz Biotechnology, Santa Cruz, CA). Neurons and glial cells were counted in randomly selected cultures by several observers. Under these conditions, we found ~10% glial cells among the neurons, and no visible neurons in the glial cultures.

**Transport.** For uptake we used either radiolabeled L-[3-3H]-threonine or α-[1-14C]-methylaminoisobutyric acid (MeAIB). For efflux we used 2 amino [L-[3-14C]}-isobutyric acid (AIB, each: Sp act. 1.85 GBq/μmol, Amersham, Bucks, UK)). Uptake was measured in cells attached to the bottoms of 48- or 24-well plates as described previously (30). Cell cultures were washed free of growth medium and then incubated for 0–180 min in either a complete balanced neurobasal medium prepared in our laboratory containing all the amino acids (+Thr) or the same medium devoid of threonine (−Thr). At the assigned time, the incubation medium was removed and transport was initiated by adding transport medium containing 100 μmol/L of the substrate and 74 Bq of either labeled threonine or MeAIB for uptake, or AIB for efflux. The transport medium contained (in mmol/L): 120 NaCl, 26 NaHCO₃, 5 KCl, 2.5 CaCl₂, 1.2 Na₂HPO₄, 1.2 KH₂PO₄, 1 MgSO₄, 10 glucose and 10 HEPES, pH 7.2. To achieve a sodium-free transport medium for determination of sodium dependency, sodium salts were replaced with appropriate comparable salts and sodium was replaced isometrically by N-methyl-d-glucamine. See Calculations below for further description of the determinations of transport types. MeAIB was used to determine transport via system A. Similarly, 2-amino bicyclo (2,2,1) heptane-2-carboxylic acid (BCH) was used for system L [reviewed in (31)]. Cells were incubated for 10 to 120 min in −Thr medium and then rinsed before application of transport medium containing labeled substrate for the measurement of uptake. The reaction was stopped by rinsing with cold (4°C) substrate-free transport medium. Labeled substrate taken up from the transport medium by the cells was measured as a function of time using the 2-, 5-, 10- and 15-min time points, and transport rates were calculated. The uptake of threonine by other transport systems, ASC, L and passive diffusion, was calculated by difference using results from uptake in the presence of selective analogs for the various systems and with or without Na⁺-free media (see Calculations).

**Efflux.** To determine whether transport-stimulated amino acid transport systems, such as systems L and ASC, are present on the APC cells used in these experiments, efflux of the amino acid analog, 2-amino isobutyric acid (AIB) was studied after allowing 1-14C-AIB to equilibrate in the cells overnight. After loading with labeled AIB and before the efflux studies, cells were incubated with or without threonine for 15 min as described above. Then efflux of the label into the medium (with and without Na⁺) was measured at various time points up to 20 min in response to the addition of lysine, glutamine or threonine to the medium (50 mmol/L, final concentration).

**Kinase inhibitors.** Because Franchi-Gazzola and colleagues (32) showed that the rapid adaptive increase of system A activity with amino acid deprivation depends on ERK1/2 activation, we conducted transport studies in the presence of PD98059, an inhibitor of these kinases. Cells, prepared as above, were incubated in +Thr or −Thr medium, as usual; PD 98059 (50 mmol/L; Calbiochem) was included in the uptake medium. Uptake of [14H]-Thr was measured as described above. Also several studies have shown effects of amino acid depletion and repletion on activation of the kinase p70s6k, a factor in the mammalian target of rapamycin (mTOR) pathway, and phosphatidylinositol 3-kinase (PI3K) in various cell types [reviewed in (33,34)]. Because these kinases also affect system A transport activity, we used their inhibitors, rapamycin for the mTOR pathway and wortmannin for PI3K (both from Calbiochem, Darmstadt, Germany), each at 100 mmol/L. To see an inhibitory effect of either rapamycin or wortmannin, it was necessary to add these inhibitors 90 min before initiation of the transport studies; each was also present during the incubation in +Thr or −Thr medium. Finally, to determine whether the transporter activation seen here was due to recruitment of transporters to the membrane, as shown for glucose uptake by its transporters, types 1 and 4, by Molero et al. (35), we also used nocardazole (2 or 33 mmol/L; Calbiochem) in the medium. After treatment with nocardazole, rapamycin, wortmannin or vehicle, uptake of MeAIB was measured as described above at 5, 10 and 15 min.

**Amino acid determination.** Cells were maintained in a complete medium and rinsed before being used. Amino acid analysis was done after 0, 15, 30, 60, 90 or 120 min incubation in either a +Thr or −Thr medium. After incubation in +Thr or −Thr medium and a
final rinse, the cells were harvested in ethanol/acetic acid (5%). Concentrations of intracellular amino acid were determined using postcolumn ninhydrin detection on an automatic amino acid analyzer (BIO-TEK Kontron Instruments, St. Quentin en Yvelines, France). The pellet containing cell proteins was lyophilized and resuspended in NaOH (0.2mol/L) for protein determinations using the method of Bradford (36).

**RT-PCR.** Total RNA was extracted from cells grown in 25-mL plastic flasks using RNeasy mini kits (Qiagen, Hilden, Germany). After RNA integrity had been confirmed by ethidium bromide staining according to kit directions, RT-PCR was done using a OneStep RT-PCR Kit (Qiagen). To estimate the relative abundance of transcripts for two major isoforms of system A (ATA1 and ATA2) in cells incubated in +Thr or −Thr medium, semiquantitative RT-PCR was done using glyceraldehyde phosphate dehydrogenase (GAPDH) and β-actin as controls. Oligonucleotide primers designed for the reverse transcriptase products and internal controls were included in all samples and amplified at the same time. PCR products were separated by electrophoresis through a 1% agarose gel, stained with ethidium bromide and quantified using a digital imaging system (Alpha Innotech, San Leandro, CA).

**Calculations and statistics.** Each reported observation is based on measurements made in at least three separate trials done at different times; each trial was carried out on separate cultures; each culture was prepared from tissues pooled from 4 rats. Uptake of labeled threonine or MeAIB by cells is expressed as mmol/g protein. Efflux of AIB is expressed as mmol/L of medium. Unidirectional influx was estimated by linear regression using substrate accumulation for threonine over a period of 0–15 min or for MeAIB over 0–20 min. Definitions for the components of total threonine uptake were as follows:

**Na+**-independent uptake = measured without **Na+** in the medium

**Na+**-dependent uptake = total uptake − **Na+**-independent uptake

System A = total uptake − uptake in the presence of MeAIB

System ASC = Na+ dependent uptake − system A

System L = **Na+**-independent uptake − measured without **Na+** in the presence of BCH

Passive diffusion = uptake measured without **Na+** in the presence of BCH.

After finding overall significance using ANOVA, comparisons between different cell types and treatments were performed by post-hoc testing using preplanned comparisons. The least significant differences test was used for differences among means and Dunnett’s test for uptake and efflux when comparing with values at 0 time. When only two groups were compared, a two-tailed Student’s t test was used. Values for t were calculated by linear regression. Analyses were done using the SAS Statistical package (SAS/STAT version 6.12 for Windows, 95, SAS Institute, Cary, NC). Differences were considered significant at P < 0.05.

**RESULTS**

**Amino acid concentrations in neurons and glia after incubation in a −Thr medium.** After incubation in the control (+Thr) medium, the intracellular concentrations of threonine in APC, hippocampal and cerebellar neurons, and glia were 1033 ± 161, 590 ± 187, 986 ± 240 and 951 ± 211 nmol/g protein, respectively. These values did not change over time in the +Thr medium. After transfer to −Thr medium, the intracellular concentrations of threonine fell rapidly in all cell types (Table 2). By 15 min, threonine had decreased to ~30% of the initial value in all cell types and brain areas (all P < 0.0001). In contrast to the glia and neurons from the other brain areas, the APC neurons had significant increases in the concentrations of five other amino acids (Table 2). There were significant (P < 0.05) increases compared with 0 time, for serine, glycine, valine, isoleucine and phenylalanine after 15 min of incubation in −Thr medium in the APC. These increases persisted to 120 min (Table 2). As noted above, all 20 amino acids that are used for protein synthesis were measured in all cell types and brain areas. Except for increases in amino acids in the APC neurons and the general decrease in the limiting amino acid, threonine, the only other significant change was an increase in the nonessential, non-limiting amino acid, serine, in hippocampus at 60 min (P < 0.05, not shown). In cerebellar neurons and glia, there were no changes in any amino acids except the decreased limiting amino acid, threonine, already mentioned.

**Effect of incubation in −Thr medium on [3H]-Thr uptake in neurons and glia.** The rate of labeled threonine uptake by APC neurons was significantly increased (60%; P < 0.05) after 10 min of incubation in the −Thr medium and remained at that high level for each of the longer incubation periods by comparison with 0 time and with cells incubated in the control (+Thr) medium (Fig. 1). For cells preincubated in +Thr medium, there were no changes from the control rate (0 time) during the 60-min trial (values for later times were unchanged from the rate at 0 time indicated by the bar on the left of Fig. 1). In the presence of the specific system A substrate, MeAIB, the upregulation of labeled threonine transport after threonine deprivation was blocked. This indicates the involvement of the amino acid transport system A (ATA) in the upregulation (black bars in Fig. 1). Similarly, there were no effects of threonine deprivation on the **Na+**-dependent ASC system, although this system did contribute to the total threonine uptake. In addition, there was no effect of threonine deprivation on the **Na+**-independent system L or the nonspecific, passive diffusion-mediated influx of label (Table 3).

The increase (P < 0.05) in [3H]-Thr accumulation into APC neurons after incubation in −Thr medium was **Na+** dependent. The **Na+**-dependent uptake systems studied here were ATA and ASC (Fig. 1, Table 3). There were no changes in [3H]-Thr uptake in the cells from the other brain areas nor in glia with threonine deprivation (Table 3), although glia showed more **Na+**-dependent threonine transporters under all conditions (P < 0.0001). Because effects due to the presence or absence of threonine in the medium in hippocampal, cerebellar or glial cells were not present, the increases in [3H]-Thr uptake appeared to be specific for APC neurons. Therefore, we focused the remainder of our studies on neurons cultured from the APC, using glia as a control tissue.

**MeAIB uptake in APC neurons.** Uptake of [1-14C] MeAIB, a system A selective substrate, was measured in APC neurons after 10, 30, 60 or 120 min incubation in +Thr or −Thr medium. MeAIB uptake in the −Thr neurons was increased maximally at 10 min (Fig. 2) and maintained this increased rate of uptake for the 120-min experimental period (not shown). Rates for MeAIB uptake into APC neurons were consistent after incubation times of 10, 30, 60 or 120 min, showing a pattern similar to the results using [3H]-Thr as the

<table>
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<th>Gene</th>
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<td>ATAI</td>
<td>5’-GAGAAAAAGGAGATTGCCAG-3’</td>
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<tr>
<td>ATAI</td>
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<td>5’-TTAACGGCGATCAAGTAGTCCG-3’</td>
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</tbody>
</table>

1 Abbreviations: ATA, system A amino acid transporter; GAPDH, glyceraldehyde phosphate dehydrogenase.
substrate (Fig. 1). After +Thr incubation, MeAIB uptake rates were 0.22 ± 0.01 [mean ± SD for μmol/(g protein ⋅ min)] after 10 min and 0.20 ± 0.01 after 120 min. After −Thr incubation, MeAIB uptake rates were increased (P < 0.05) to 157–167% of +Thr values, and remained similarly elevated over the measured times, 0.35 ± 0.01 after 10 min and 0.34 ± 0.02 after 120 min in −Thr medium. MeAIB uptake was sodium dependent (Fig. 2). These results confirmed the activation of system A in Thr-deficient neurons.

Efflux of amino [1-14C]-isobutyric acid (AIB). Amino isobutyric acid is a substrate for several transport systems, many of which (not including system A) can be trans-stimulated. Therefore, we expected increased levels of label in the medium after incubation with appropriate substrates, indicating that the substrates in the extracellular medium had trans-stimulated the outward flux of AIB from the previously loaded cells. Following AIB loading, the cells were incubated in +Thr or −Thr medium for 15 min. After preincubation with +Thr medium, efflux of labeled AIB from the cells was unchanged from control levels by added threonine (Fig. 3A). Efflux was stimulated at 12.5 and 20 min by the addition of lysine or glutamine to the media (Fig. 3A). These increases in efflux were independent of sodium in the medium (not shown). In contrast, there were dynamic changes in the −Thr cells because glutamine-stimulated efflux of AIB (measured at 5, 10, 12.5 and 20 min) was increased to 119% of the 5-min value by 10 min and markedly decreased at 20 min (t² = 0.802, Fig. 3B). Glutamine itself is transported by any of several possible transport systems; at least 31 transport systems for glutamine, 14 for glutamate, and 14 for aspartate, have been reviewed recently by Bode (37). Similarly, lysine evoked an increase of AIB efflux to 117% of control at 10 min in the −Thr cells and caused a similar decrease after 12.5 min (t² = 0.987, Fig. 3B). Lysine is not a substrate for system A (38), but is carried by the Na⁺ independent system L and others (39). Therefore, in these cells, both glutamine and lysine affected AIB efflux when added to the extracellular medium. These studies show not only the presence of trans-stimulation in these cells and the competence of amino acid transporters other than ATA in the APC cells, but also that the −Thr condition affected other transport types besides system A. The differences after only 15 min in the −Thr medium suggest secondary responses to the upregulation of the ATA system in the −Thr condition (22). System A should not have been activated in these efflux studies in which Na⁺ was not essential in the medium because System A is Na⁺ dependent. Also, these efflux data show that the data for significant upregulation

![Image](https://academic.oup.com/jn/article-abstract/133/7/2156/4688352/23-November-2018)
only of system ATA in the uptake studies were not due to the absence of the other transporters in the +Thr or −Thr cells. These data are consistent with the increases of valine, isoleucine and phenylalanine in APC neurons (Table 2) after 15 min in −Thr medium because neither isoleucine nor phenylalanine is a substrate for system A.

**Effect of kinase inhibition on the rapid activation of system A.** The activation of system A, as shown by increased uptake of labeled threonine in APC neurons previously incubated in −Thr medium, was inhibited in the presence of the ERK1/2 inhibitor, PD98059. This suggests that ERK1/2 are involved in the activation of system A in APC neurons (Table 4), as was shown by Franchi-Gazzola et al. (32) in cultured human fibroblasts. Also, the addition of nocodazole at 33 μmol/L, but not at 2 μmol/L, blocked the rapid increase of system A activity, as measured by the uptake of the selective system A substrate, MeAIB, in a dose-related effect. Upregulation of system A in APC neurons was also inhibited after a 90 min preincubation with rapamycin, an inhibitor of mTOR, and wortmannin, which inhibits PI3K (Fig. 4). Again in these studies, transport was Na⁺ dependent.

**Expression of ATA1 and ATA2 in APC neurons and glia after incubation in −Thr media.** The relative abundance of transcripts for two isoforms of system A (ATA1 and ATA2) and for control transcripts, GAPDH and β-actin, was determined in the APC neurons and glia previously incubated with or without threonine for 2 (not shown), 3 or 5 h by semiquantitative RT-PCR (Fig. 5). Both GAPDH and β-actin were affected by incubation in −Thr medium as demonstrated by the increased density of the bands with increased duration of the incubation in −Thr medium. Because GAPDH responses to the −Thr medium appeared more pronounced, the relative abundance of ATA1 and ATA2 was determined by comparison using β-actin as the control. The problem of the appropriate control for RT-PCR gels is beyond the scope of this paper, but has been addressed (40). Inspection of the gel (Fig. 5) reveals that ATA2 could actually be the best control because it was unchanged throughout in both cell types. In contrast, message for ATA1 was increased (P < 0.05 vs. +Thr, both relative to the β-actin bands) in APC neurons after 5 h. The expression of ATA1 was lower in glia than in neurons (P < 0.05).

**FIGURE 2** Uptake of [14C]-methyl amino isobutyric acid (MeAIB) into anterior piriform cortex (APC) neurons from rats after 10 min incubation in media: +Thr, −Thr (both containing Na⁺) or without Na⁺. There was little MeAIB uptake and no effect of −Thr in the cells incubated without Na⁺. Data for 30, 60 and 120 min did not differ within an incubation type but are not included in the figure for simplicity because they overlapped the lines given. Values are means ± SEM, n = 3 separate experiments. *Significantly different from MeAIB uptake ([μmol/g cell protein]) in the −Thr cells at 15 and 20 min, P < 0.05.

### DISCUSSION

Removal of threonine from the incubation medium of neurons cultured from APC resulted in a rapid and selective increase in the transport activity of the system A transporter, ATA. These are the first observations to implicate selective activation of any electrogenic amino acid transport system by IAA depletion in neurons from the APC, the putative IAA chemosensor. The ATA1 is alternatively termed SAT1, GlnT

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**TABLE 3**

<table>
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<tr>
<th>Component²</th>
<th>APC</th>
<th>Cerebellum</th>
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<tr>
<td></td>
<td>+Thr</td>
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<tr>
<td>Total</td>
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1 **Values are means ± SD for at least 3 separate cultures each prepared from tissue from 4 rats.** *Different from +Thr cells for the component indicated.**

2 **Components of uptake were calculated as described in Methods.**

3 **Total Thr uptake in controls (+Thr) differed by cell type (overall ANOVA, P < 0.0001); differences were due to differences between glia and neurons.**
or SA2, and is a member of the vesicular GABA transporter family (37). This rapid response to the –Thr medium was seen only in APC neurons, not in neurons from hippocampus or cerebellum, and not in glia. These findings are consistent with mediation of the rapid recognition of IAA deficiency in vivo by the APC and the easy excitability of these neurons as described above. There are numerous reports of cells showing upregulation of ATA2 by amino acid limitation including glial cells (41), human hepatoma cells (42) and cultured fibroblasts (43). Here, rapid upregulation of ATA occurred in neurons. Because ATA1 is the “neuron-specific” system A transporter, in contrast to ATA2 (44), we suggest that the isoform responsive to –Thr media in our neurons is ATA1.

**Amino acid concentrations.** Because threonine concentrations were reduced in all cell types, whereas the increases of other amino acids were greatest in APC, far less in hippocampus and not present in either cerebellum or in glia, it appears that the unique feature of amino acid concentrations in APC is the increase in several of the nonlimiting amino acids, rather than the decreased threonine. The increases in the intracellular concentrations of serine and glycine in the APC neurons at 15 min are consistent with a rapid increase in system A activity because they are the preferred substrates for this transporter system (20).

Increased threonine uptake in –Thr hippocampal neurons

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Increased threonine uptake in –Thr hippocampal neurons

or SA2, and is a member of the vesicular GABA transporter family (37). This rapid response to the –Thr medium was seen only in APC neurons, not in neurons from hippocampus or cerebellum, and not in glia. These findings are consistent with mediation of the rapid recognition of IAA deficiency in vivo by the APC and the easy excitability of these neurons as described above. There are numerous reports of cells showing upregulation of ATA2 by amino acid limitation including glial cells (41), human hepatoma cells (42) and cultured fibroblasts (43). Here, rapid upregulation of ATA occurred in neurons. Because ATA1 is the “neuron-specific” system A transporter, in contrast to ATA2 (44), we suggest that the isoform responsive to –Thr media in our neurons is ATA1.

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Increased threonine uptake in –Thr hippocampal neurons

<table>
<thead>
<tr>
<th></th>
<th>APC</th>
<th>Glia</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>µmol/(g protein · min)</td>
<td></td>
</tr>
<tr>
<td>+Thr Vehicle</td>
<td>0.44 ± 0.02a</td>
<td>0.77 ± 0.09</td>
</tr>
<tr>
<td>PD98059</td>
<td>0.45 ± 0.03a</td>
<td>0.78 ± 0.09</td>
</tr>
<tr>
<td>−Thr Vehicle</td>
<td>0.69 ± 0.05d</td>
<td>0.74 ± 0.08</td>
</tr>
<tr>
<td>PD98059</td>
<td>0.44 ± 0.03a</td>
<td>0.88 ± 0.08</td>
</tr>
</tbody>
</table>

1 Values are means ± SD for at least 3 experiments, each done on separate cultures using pooled tissue from 4 weanling rats. Means in a column with different letters differ, P < 0.05.

2 Abbreviation: ERK1/2, extracellular response element kinase.
did not occur at the early times studied here. This is in contrast to the results of Kilberg et al. (45) who used human hippocampal system ASC transporter expressed in oocytes. The only suggestion that system A activity was active in our hippocampal neurons was an increase in the concentration of serine at 60 min. There was no effect of -Thr incubation in hippocampal or cerebellar neurons at times relating to the in vivo anorectic response, before 30 min (7). Moreover, these brain areas have not been implicated in amino acid chemosensation in vivo, as reviewed in Leung and Rogers (46).

**Derepression of ATA in APC neurons after 10 min of threonine deficiency.** The -Thr medium produced a rapid 60% increase in Na\(^+\)-dependent uptake of both labeled threonine and MeAIB, indicating that this rapid adaptation was due to system A activity. System A is characterized by stereoselectivity, trans-inhibition, dependence on a transmembrane Na\(^+\) gradient and can be regulated by a wide variety of intracellular and extracellular signals (23,47). System A is a widely expressed amino acid transporter in mammalian cells and mediates the Na\(^+\)-coupled uptake of small aliphatic amino acids. Alanine, serine, glycine and glutamine are particularly good substrates of system A, but other amino acids including threonine and proline are also transported (22,38,39). Also, threonine induces electrogenic current in oocytes transfected with the rat neuronal ATA1 gene (38), showing that threonine is indeed a competent substrate for neuronal ATA1, and its transport is electrogenic.

Early system A activation in an amino acid-free medium invokes a decrease in trans-inhibition. When the substrate concentrations are high, the transporter is thought to be trans-inhibited by its substrate amino acids and/or Na\(^+\) binding to the carrier, or to an associated protein (48), keeping the transporter in an inactivated form (21,23). A reduction in the intracellular pool of neutral amino acid substrates would release the transporter from inhibition. Our observations of increased threonine uptake by system A after incubation in -Thr medium are consistent with the derepression of system A that occurs consistently in several cell types (47). According to Varoqui et al. (49), ATA1 in rat brain is associated mainly with the plasma membranes of glutamatergic neurons. Thus, we speculate that the activation of electrogenic transport via ATA1 in APC neurons may be a clue to activation of the principal output neurons of the APC, which are glutamatergic (50) and are activated by a -Thr diet (18,51,52).

Increased influx of the BCAA and phenylalanine in APC neurons, which are not preferred substrates for system A, could have occurred via a trans-stimulated increase in the activity of other transporters, such as system L, as reviewed in (53). Both uptake and efflux data show that systems L and ASC were present in the neurons. We also observed increased efflux of the nonmethylated AIB after incubation with glutamine and lysine in these cells, showing the competence of trans-stimulated systems in our APC neurons. This increase was converted into a decrease at 20 min in threonine-deficient neurons, showing dynamic responses among the other transporters as expected (23). The increase in labeled threonine uptake seen after -Thr was Na\(^+\) dependent. This suggests the involvement of systems such as A or ASC. Tews et al. (54) previously implicated system ASC in the blood brain barrier in the imbalanced amino acid model. Yet, the inhibition of threonine uptake by MeAIB and the upregulation of MeAIB uptake were taken to rule out system ASC in our APC neurons.

**Involvement of phosphorylation and kinase activity.** At least two kinase pathways have been described that respond to differences in intracellular amino acid concentrations. These include the MAP kinase pathways, resulting in ERK and c-Jun N-terminal kinase (32), and the mTOR pathway, along with its downstream signaling control of the activity of p70S6K and 4E-BP1 phosphorylation (55,56). Both p70S6K and PI3K are activated similarly in muscle cells (34). In a T-lymphoblastoid cell model, amino acid deprivation induces a rapid decrease in the activity of p70S6K (33). Franchi-Gazzola et al. (32) reported that rapamycin has no effect on the activation of system A activity, indicating that the mTOR pathway is not necessary in their system. In contrast, in our APC neurons, not only PD 98059, but also both rapamycin and wortmannin, which inhibit p70S6K and PI3K, respectively, inhibited the adaptive increase of system A activity induced by threonine deprivation.

Yet another mechanism is suggested by results for intracellular trafficking of biogenic amine transporters, including the GABAergic transporter, reviewed in Blakely and Bauman (57), by reports of movement of ATA2 to the plasma membrane (41), and by the present results. Nocodazole, at the higher dose used here, inhibited adaptive transporter upregulation in threonine-deficient APC neurons. This is similar to the blockade of glucose transport in adipocytes by the same concentrations of nocodazole (35). In adipocytes, the activity of nocodazole prevented movement of the transporters to the cell membrane (35). Thus, movement of ATA to the plasma membrane may be involved in its activation in APC neurons as well. In sum, inhibitors of several phosphorylation-dependent processes prevented the -Thr upregulation of ATA in APC neurons. Thus, phosphorylation of existing proteins,
rather than protein synthesis per se, i.e., early rather than later mechanisms must be involved.

System A isofoms. Longer incubation times in a 3-Thr medium are characterized by additional protein synthesis–related cellular responses, such as upregulation of genes for transporters and synthetase enzymes in several systems (22,53,58). In addition, amino acids have roles in the regulation of protein synthesis via mechanisms other than those of growth factors, peptides and hormones in their effects on the translation of mRNA (59–62). We studied the expression of ATA1 and ATA2 by RT-PCR, and determined that only neuronal ATA1 was increased after 5 h in a-Thr (Fig. 5). Upregulation of message for ATA1 in the neurons would be consistent with a role for the ATA1 isoform in the earlier responses noted above. Yet, the rapid activation of system ATA1 that occurred in neurons from the highly chemically sensitive APC shows similar timing to the in vivo anorectic response induced by IAA-deficient diets in rats (7), too early for the adaptation to be due to increase in message for the transporter.

Many of the signaling pathways suggested by these results, which accompany the rapid upregulation of ATA1 activity, such as ERK1/2 Pi3K and p70S6K are phosphorylation dependent and could contribute to the activation of the glutamatergic output cells of the APC that are involved in the anorectic (behavioral) response. Alternatively, activation of electrogentic ATA1 could increase the intracellular sodium concentration, which would cause firing of these cells directly, and thus play a role in IAA signaling in omnivores.

Future studies are warranted to determine the signaling pathways extending from the decreased limiting amino acid in the APC, through the activation of ATA1 and increased excitability of the output cells, to activation of the anorectic response to IAA deficiency via action on neural feeding circuits (13,17,63). All of this must occur before the changes in feeding behavior, which occur in <20 min (7). Nonetheless, we suggest that the present results provide an important step in these pathways and a new piece of this fascinating puzzle.

LITERATURE CITED


