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Chair: Stefan Bröer

Amino acid signaling to mRNA translation: Central role of mTOR

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The increase in muscle protein synthesis that occurs in response to consumption of a complete meal by a fasted individual is a result not only of increased availability of the substrates used for synthesizing protein, *i.e.* amino acids, but also to nutrient- and hormone-induced activation of intracellular signaling pathways that regulate mRNA translation. Arguably, one of the most important nutrient-regulated signaling pathways in muscle involves a complex of proteins that includes the protein kinase referred to as the mammalian target of rapamycin (mTOR), the regulatory associated protein of mTOR (raptor), the Ras homolog enriched in brain (Rheb), the proline-rich Akt substrate (PRAS)40, LST8 (also known as GβL), and possibly other as yet unidentified proteins. Together, these proteins form the TOR complex 1 (TORC1). Nutrients such as the branched-chain amino acid leucine alter the conformation and/or composition of the TORC1 complex, resulting in increased mTOR protein kinase activity. For example, the amino acid-induced activation of TORC1 correlates with decreased amounts of raptor and PRAS40 present in mTOR immunoprecipitates, suggesting that amino acids alter the interaction of mTOR with both proteins. Amino acids also increase the proportion of Rheb present in the GTP bound form, an event that is critical for maximal TORC1 activation. However, the mechanism(s) through which amino acids act to alter either TORC1 conformation or Rheb association with GTP is incompletely defined. Upregulated nutrient signaling through TORC1 leads to increased phosphorylation of several proteins that play important roles in regulating the mRNA binding step in translation initiation including the eukaryotic initiation factor (eIF)4E binding protein (4E-BP)1 and the ribosomal protein S6 kinase S6K1. S6K1 subsequently phosphorylates eIF4B and eukaryotic elongation factor (eEF)2 kinase. Phosphorylation of 4E-BP1 by mTOR results in its release from the inactive 4E-BP1•eIF4E complex allowing the mRNA cap binding protein eIF4E to associate with eIF4G and eIF4A to form the active eIF4F complex. Phosphorylation of eIF4B, an activator of the RNA helicase activity of eIF4A, promotes its association with the eIF4F complex. Depending on the cell type, amino acid-induced assembly of the eIF4F complex can lead to increased global rates of protein synthesis and also to changes in the selection of mRNAs for translation, thereby altering the pattern of gene expression at the protein level. For example, activation of TORC1 preferentially increases the translation of mRNAs encoding proteins such as eEF1A, eEF2, and many ribosomal proteins, thereby increasing the capacity of the cell to synthesize protein. Our recent studies have identified a new target for TORC1 signaling, the catalytic ε-subunit of the guanine nucleotide exchange factor, eIF2B. In these studies, we found that leucine addition to leucine-deprived cells causes a redistribution of the eIF2Bε mRNA from an untranslated, non-polysomal fraction into polysomes, leading to increased incorporation of [³⁵S]methionine into eIF2Bε protein. The shift of eIF2Bε mRNA into polysomes and increased synthesis of eIF2Bε protein are both prevented by pre-treatment with the specific mTOR inhibitor rapamycin. Because eIF2B is an important regulator of global rates of protein synthesis, an increase in its expression is likely an important component in the increased capacity for mRNA translation associated with mTOR activation.

In summary, TORC1 represents a nexus through which nutrients and hormones act to acutely regulate mRNA translation. Moreover, by specifically increasing the translation of mRNAs encoding proteins involved in mRNA translation, activation of TORC1 upregulates the capacity to synthesize protein.

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Class 3 GPCRs as broad-spectrum L-amino acid sensors

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Class 3 G-protein-coupled receptors (GPCRs) are typified by a large extracellular domain that includes a nutrient sensing Venus Fly Trap domain (VFT) and a Cys-rich domain that couples nutrient binding in the VFT to the activation of G-proteins on the internal face of the 7-transmembrane domain motif. Class 3 GPCRs include receptors that are selective for the amino acid L-glutamate (mGlu₅) and the glutamate metabolite, γ -amino-butyrate; the so-called GABA(B) receptors. Recent work demonstrates that one subgroup of the class 3 receptors that includes the extracellular Ca²⁺-sensing receptor (CaR), the T1R1/T1R3 taste receptor and the recently cloned ortholog of the fish 5.24 receptor, GPRC6A, are all broad-spectrum L-amino acid-sensing receptors that are coupled to phosphoinositide turnover, intracellular Ca²⁺ mobilization and, perhaps, other intracellular signalling pathways. CaR homodimers are selective for aromatic and aliphatic L-amino acids, GPRC6A homodimers are selective for basic and aliphatic L-amino acids and T1R1/T1R3 heterodimers are selective for aliphatic and polar L-amino acids. Although the amino acid residues required for ligating the α -amino and α -carboxylate functional groups are tightly conserved in many class 3 GPCRs, the side chain binding cleft of the broad-spectrum amino acid sensing receptors is predicted to be substantially larger and recent work suggests that short peptides may be developed as selective receptor activators. The physiological significance of amino acid sensing is known to include the regulation of growth promoting and metabolism-regulating signalling pathways and in appetite control. Expression of the CaR in endocrine tissue, the gut, kidney and hypothalamus of the brain, provides potential molecular explanations for various aspects of L-amino acid sensing.

Closing the GATs. Metabolic consequences of blocking GABA uptake

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Reuptake of the inhibitory neurotransmitter GABA is largely accomplished via high affinity GABA uptake systems (GATs). The majority of uptake in the brain is *via* GAT1, with some contribution from GAT3. These transporters are located on both neurons and glia, although it is likely that GAT1 is found on neurons and glia, while GAT3 is mostly found on glial processes.

The contribution of GABA uptake to energy metabolism has been described as “minimal” and hence it has been speculated that GABA uptake is unlikely to make any meaningful contribution to metabolism. In order to discover what the metabolic consequences of blockade of GABA uptake actually were, we employed a Guinea pig cortical slice preparation, coupled with targeted neuropharmacology, ¹³C NMR spectroscopy and multivariate statistics to determine the effect of inhibition of GATs.

In brief, slices (350 μM) were prepared from the Guinea pig cortex (excised from newly sacrificed animal) in the para-sagittal plane using a McIlwain tissue chopper. Slices were incubated in a modified, gassed (95% O₂, 5% CO₂) Krebs-Henseleit buffer with 10 mM glucose to allow metabolic recovery for 1 h, then washed and immediately incubated with [3-¹³C]sodium pyruvate (control) and two different concentrations of the GAT inhibitor of choice. We used the broad spectrum inhibitor guvacine, the GAT1-specific inhibitors SKF899776a, tiagabine, NNC711 and CI-966, as well as the GAT3 specific inhibitor (*S*)-SNAP 5114. These were typically used at concentrations around the K_i, as well as 10 x this concentration (to allow for non-specific effects). Following 1h of incubation the experiment was stopped by rapid vacuum filtration and the slices extracted using chloroform/methanol, lyophilized and resuspended in ²H₂O for ¹H/¹³C NMR analysis at 14.1 T. The net flux into isotopomers of Glu, Gln, GABA, Asp, Ala and lactate was determined as well as the total metabolite pool size of lactate, Glu, GABA, Asp, Ala and Gln.

The broad spectrum inhibitor guvacine produced a metabolic profile that resembled that of the GABA-B agonist Baclofen, suggesting that the increased GABA concentration on GAT1/GAT3 inhibition is active at GABA-B receptors. The centrally active GAT1 inhibitor tiagabine produced a profile that was most similar to that of SKF899776a, although the two ligands could still be distinguished from one another using principal components analysis (PCA). CI-966 produced a unique metabolic profile, the nearest match to which from our library was that of the type II mGluR antagonist APICA, suggesting that CI-966 may act more on astrocytic GATs and/or interact with astrocytic mGluR. The GAT3 inhibitor SNAP 5114 was distinguishable from the other GAT ligands using PCA. The metabolic profiles generated by the inhibitors did not closely resemble that of any GABA-C agonist or that of any GABA-A agonist that we have studied to date. The response is the opposite of that seen at GABA-C suggesting that GATs and GABA-C receptors are not closely localized. Our ability to interpret the response to GABA-A ligands is hampered by lack of specific agonists for this receptor.

Taken together, these data suggest that GABA which builds up in the synapse is most likely to act upon GABA-B receptors, that the individual GAT inhibitors may well be acting on different subpopulations of GATs, that GAT inhibition produces a negative (decreased) impact on brain metabolism and that it is possible to distinguish between activity of the various ligands using this approach.

Neutral amino acid transporters of the kidney and intestine

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The epithelial cells of the renal proximal tubule and of the intestine are important sites of amino acid transport. A defect in this amino acid transport leads to compromised amino acid absorption, which manifests as an excess of amino acids in the urine and/or faeces. Several such disorders have been identified, including Hartnup disorder and iminoglycinuria.

Hartnup disorder is an autosomal recessive inherited disorder, which is characterised by the excretion of large amounts of neutral amino acids in the urine. Clinical symptoms are variable, and can include a photosensitive skin rash, episodes of cerebellar ataxia and other neurological symptoms. Recently, we cloned and characterised a novel sodium-dependent neutral amino acid transporter called B⁰AT1. We and others have since identified mutations in B⁰AT1 that cause Hartnup disorder by inactivating the transporter (Seow *et al.*, 2004; Kleta *et al.*, 2004). However, causative mutations in B⁰AT1 were not found in all affected individuals. Together with the variability of clinical symptoms, this suggests that Hartnup disorder may be genetically heterogeneous.

To investigate this further, we studied several newly acquired families and reevaluated those families where causative mutations were not previously detected. Genomic sequencing of B⁰AT1 revealed disease-associated mutations in each of these Hartnup disorder families, including six novel missense mutations and one nonsense mutation. Functional analysis of these mutations by expression of B⁰AT1 cRNA in *Xenopus laevis* oocytes*, demonstrated that the transporter is inactivated by all novel mutations detected in these families. This suggests that B⁰AT1 is the major gene involved in Hartnup disorder.

Iminoglycinuria is an autosomal recessive disorder characterized by increased excretion of imino acids (proline and hydroxyproline) and glycine in the urine. While the renal transport of imino acids and glycine is always defective in iminoglycinuria, intestinal transport is not always affected. Moreover, some obligate heterozygotes show hyperglycinuria, while others do not. This variability suggests the involvement of multiple genes and/or alleles (Chesney, 2001).

Among the known renal and intestinal amino acid transporters, several candidates exist that are potentially involved in iminoglycinuria – these include IMINO, PAT1, PAT2 and XT2. IMINO is a sodium-dependent proline transporter that we have cloned and characterised. PAT1 and PAT2 are both proton-dependent proline and glycine transporters, while XT2 is a putative glycine transporter. We are currently investigating the role of these candidates in iminoglycinuria.

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**Xenopus laevis* oocytes were harvested by surgery of anaesthetized frogs (MS-222, 1.5g/l). The procedure was approved by the Animal Experimentation Ethics Committee of the Australian National University.