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Cell Reports
Letter



# Measuring the Ability of Mice to Sense Dietary Essential Amino Acid Deficiency: The Importance of Amino Acid Status and Timing

Dorothy W. Gietzen,[1,*] Tracy G. Anthony,[2] Pierre Fafournoux,[3] Anne-Catherine Maurin,[3] Thomas J. Koehnle,[4] and Susan Hao[1,5]
[1]University of California, Davis, Davis, CA 95616, USA
[2]Rutgers University, New Brunswick, NJ 08901, USA
[3]UMR 1019 Nutrition Humaine, INRA, Université Clermont 1, Centre de Clermont-Ferrand-Theix, 63122 Saint Genès Champanelle, France
[4]Hiram College, Hiram, OH 44234, USA
[5]Ajinomoto USA, Inc., Fort Lee, NJ 07024, USA
*Correspondence: dwgietzen@ucdavis.edu
http://dx.doi.org/10.1016/j.celrep.2016.08.021

The ability to sense and choose appropriate nutrients for survival is a topic of longstanding interest in fields ranging from molecular biology to ecology. Many have contributed to this field, particularly with regard to essential amino acids (EAAs), the precursors for protein synthesis, dating back some 100 years (reviewed in Anthony and Gietzen, 2013). A recent report (Leib and Knight, 2015) challenges previous independent but common findings on the sensing of EAA-deficient diets by rodents, including food intake and neurochemical reports over 30 years (see Koehnle et al., 2004). Specifically, Leib and Knight (2015) highlight that (1) "Mice do not reject food lacking [EAAs] as previously described," and (2) "The proposed EAA sensor, [general control nonderepressible 2 (GCN2) kinase] is not activated in the brain by EAA-deficient food." As authors of previous papers that stand in contrast to these negative findings, we collectively offer an alternate explanation for their results. Specifically, their data were obtained using experimental approaches exhibiting major differences with our previously published protocols (Anthony et al., 2004; Carraro et al., 2010; Hao et al., 2005; Koehnle et al., 2004; Maurin et al., 2005, 2014).

GCN2 is a protein kinase that has a major role in the responses to EAA deficiency in all metazoan species studied so far (the majority of the work has been done in rats and mice, given that they're comparable models for mammals, although the development of knockout technology has focused attention on the mouse). GCN2 is activated by uncharged tRNA (Hao et al., 2005; Maurin et al., 2005) when EAA levels fall below the threshold (e.g., 50 uM for leucine) (Carraro et al., 2010).

In order to study the role of GCN2 in response to EAA depletion, our experimental models consist of an overnight fast (~16–21 hr) before offering the rodents an EAA-imbalanced meal (i.e., food either devoid of or markedly low in 1 or 2 EAAs) or a control meal. This protocol ensures that the animals are sufficiently hungry to eat enough of the diet for demonstrating the response. This protocol reliably reduces circulating and/or brain concentrations of the limiting EAA within 1 hr or less (Koehnle et al., 2004; Maurin et al., 2005). For example, the concentration of leucine increases from 150–200 uM in the plasma of fasted mice to 300–500 uM following a 20–30 min meal of the control (leucine-containing) diet, whereas after a leucine-devoid meal in the same protocol, leucinemia falls to 25–50 uM (Maurin et al., 2005). Without a sufficient fasting period before offering the meal, rodents do not eat enough food to affect the concentrations of EAAs. A comparison of 3 hr and overnight fasting conducted in rats makes this clear (Koehnle et al., 2004). After a 3 hr fast, threonine levels did not decrease in brain until 2.5 hr, but by 21 min after an overnight fast, the limiting EAA (threonine or leucine) had decreased by nearly 50%.

The study by Leib and Knight (2015) presents two crucial experimental differences in comparison to our previously published studies (Carraro et al., 2010; Hao et al., 2005; Koehnle et al., 2004; Maurin et al., 2005, 2014). Leib and Knight (2015) fasted their mice for only 3 hr, and they did not make any observations before 1 hr. Thus, they failed to see the rapid drop in EAA levels, and they missed the activation period for GCN2.

In Figure 2A of their paper, Leib and Knight (2015) clearly show that their feeding protocol fails to create a rapid, imbalanced loss of EAAs because circulating concentrations of the targeted EAAs do not change until 3 hr of feeding. The kinetics of the changes in blood EAA concentrations following the EAA-deficient diets are markedly different between the two experimental protocols. Unless the dietary reduction in the limiting EAA is great enough to influence tRNA charging levels, activation of GCN2 is not to be expected. Moreover, Leib and Knight (2015) show no evidence of altered tRNA charging or direct measures of GCN2 activation in addition to their failure to reduce plasma amino acids. This, in combination with a lack of observations within the sensory period before 1 hr, leads us to conclude that this work is not a re-examination of Hao et al. (2005) and Maurin et al., (2005, 2014) but an interesting investigation unto itself.

Leib and Knight (2015) do show that both wild-type and GCN2-null mice reject EAA-devoid meals when observed over longer periods. This finding agrees with at least two previous studies (Anthony et al., 2004; Guo and Cavener, 2007). These later responses could be due to a variety of signaling systems that may or may not require GCN2 (Anthony and Gietzen, 2013; Wanders et al., 2016). After the onset of EAA deprivation, ATF4 expression is significantly induced by the

GCN2/P-eIF2α/ATF4 cascade (Carraro et al., 2010; Maurin et al., 2014). This transcription factor then controls gene-expression programs involved in the adaptation of the cell to EAA deficiency (Anthony and Gietzen, 2013; Carraro et al., 2010). Given sufficient time for transcription of these genes, a multitude of signaling events become possible at later time points including those downstream of GCN2.

In summary, the inability to effectively reduce the plasma concentration of the targeted EAA at the appropriate time can explain the differences between our studies and those of Leib and Knight (2015). As stated by Anthony and Gietzen (2013), the stimulus initiated by EAA deficiency is sensed by the brain's EAA chemosensor, the anterior piriform cortex, but "beyond this, much remains uncertain." We appreciate Leib and Knight (2015)'s efforts toward further investigation into how EAA imbalance is sensed in mammals. We suggest that differences in their findings and ours reflect significant differences in experimental design that impact the timing of EAA imbalance and thus GCN2.

## ACKNOWLEDGMENTS

The authors are grateful for the helpful comments of Drs. Ron Wek, David Ron, and Evelyn Sattlegger.

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