Cell Reports

Supplemental Information

Re-examination of Dietary Amino Acid Sensing Reveals a GCN2-Independent Mechanism

David E. Leib and Zachary A. Knight

SUPPLEMENTAL MATERIALS

SUPPLEMENTAL METHODS

Feeding experiments

Animals were singly housed and acclimated to control or leucine basal diet for at least five days prior to experiments. During experiments and for at least two days prior, food was removed from the cage at Zeitgeber time (ZT) 9:00 and replaced with fresh food at ZT 12:00 unless otherwise noted. Food pellets were weighed at ZT 12:00, 15:00, and the following day at ZT 9:00 with an Ohaus Scout Pro 400g balance to the nearest 0.1 g in order to calculate three-hour and overnight food intake. Mice were used for up to three behavioral experiments separated by at least five days on a control diet. The experimenter was not blinded to genotype or diet. Some experiments were repeated in independent cohorts of mice, including choice between K-def and control food and choice between TL-def food and control following two days of threonine/leucine deprivation.

Amino acid analysis

All amino acid analysis was performed by the UC Davis Proteomics Core. For analysis of mouse chow, a single pellet of each diet tested (control, T-def, L-def, TL-def, and K-def) was crushed with a mortar and pestle, and approximately 10 mg was transferred to a fresh tube. Next, 400 µl of 0.1-N HCl was added and vortexed. 100 µl of 10% sulfosalicylic acid was added, and this was frozen overnight. The sample was then thawed, vortexed, centrifuged, and 100 µl of supernatant was transferred to a new tube. This was then diluted 1:10 in a dilution buffer containing S-2-aminoethyl-L-cysteine as an internal control. 50 µl of this dilution was analyzed on a Hitachi L-8800 Amino Acid Analyzer. All amino acids were quantified except asparagine, aspartate, glutamic acid, and glutamine, as the Hitachi L-8800 lacked the resolution to distinguish these amino acids from each other.

For plasma amino acid analysis, group-housed wild-type mice were acclimated to control food for at least five days. On the day of plasma collection and for at least two days prior, food was removed from ZT 9:00 to ZT 12:00. Plasma was collected at ZT 12:00 (0 hours of feeding) and after one and three hours of feeding on control, T-def, L-def, or K-def food. Blood was obtained by tail snipping and collected in tubes pre-loaded with EDTA to prevent coagulation (RAM Scientific, #07-7053). The collected blood was centrifuged at 8000 g for eight minutes, then the plasma transferred to a new tube, frozen, and sent to UC Davis for analysis. To isolate the free amino acids, proteins were precipitated from the plasma by adding 1/4 volume of 10% sulfosalicylic acid, vortexing, freezing overnight, then thawing and centrifuging. Samples were then diluted 1:2.5 in buffer containing S-2-aminoethyl-L-cysteine as an internal control, and 50 µl of this dilution was analyzed on a Hitachi L-8900 Amino Acid Analyzer. All amino acids were quantified and reported in Figure 2B except cysteine, methionine, and tryptophan.

DNA sequencing of *Gcn2 -/-* locus

Genomic DNA from a *Gcn2 -/-* mouse was PCR-amplified with the following primers:

oIMR8796:	5’-TCTCCCAGCGGAATCCGCACATCG-3’
oIMR8791:	5’-TGCCACTGTCAGAATCTGAAGCAGG-3’

The PCR product was gel purified and submitted for sequencing with primer oIMR8796 (Elim Biopharmaceuticals, Inc.).

qPCR genotyping of *Gcn2 -/-* mice

Genomic DNA from wild-type and *Gcn2* -/- mice was analyzed by quantitative PCR (BioRad CFX Connect Real-Time PCR Detection System) using TaqMan Gene Expression Master Mix (Life Technologies #4369016) and the following PrimeTime primers and hydrolysis probes (IDT DNA). Probes were 5' labeled with the specified dye, and all probes contained internal Zen and 3' Iowa Black labels.

Gcn2 -/- specific qPCR:
Forward: 5'-GTGTTTGGCCAGCATTGTC-3'
Reverse: 5'-GGATCCACTAGCCATATGTAACTT-3'
Probe (FAM-labeled): 5'-TACATATGCTGTGCCTGTGGAGGC-3'

Wild-type-specific qPCR:
Forward: 5'-CATATGGCTAGCAGTCATGGAG-3'
Reverse: 5'-GTGGATAATGGAGCTCGTTCTTA-3'
Probe (FAM-labeled): 5'-AGAGAGGGCTCAGTGGTTGAGAGA-3'

Control qPCR:
Forward: 5'-GTGAAGGCTGATTCTTTGTGG-3'
Reverse: 5'-AGAAATGTCTCTGTCGCTCAAG-3'
Probe (HEX-labeled): 5'-AAAAGACCCCTACGCCCCAACTC-3'

As a DNA loading control, control qPCR was multiplexed in the same reaction as wild-type- or *Gcn2* -/- specific qPCR reactions. The amount of wild-type and *Gcn2* - DNA was quantified relative to control by ΔCt and expressed in Figure S1 as the fold average for wild-type or *Gcn2* -/- mice, respectively.

Western blots
For western analysis after one hour of feeding, mice were fasted overnight, then re-fed either control, T-def, or K-def food for one hour. For western analysis after twelve hours of feeding, food was removed from the cage from ZT 9:00 to 12:00, then mice were fed either control or T-def food until ZT 24:00. After feeding, mice were sacrificed by cervical dislocation, their brains rapidly dissected, and the APC, MBH, and liver collected. A cytoplasmic protein extract was then prepared by a modification of the Dignam and Roeder protocol (Dignam et al., 1983). Dissected tissue was incubated in hypotonic lysis buffer (10 mM HEPES pH 7.9, 60 mM KCl, 2 mM MgCl2, 0.6% NP-40, and 10% glycerol) on ice for ten minutes, then disrupted with a pestle and centrifuged at 1600 g at 4ºC for five minutes. The supernatant was collected and total protein concentration determined by BCA assay (Pierce #23225). 30 mg of total protein was separated on a tris-glycine gradient gel (Bio-Rad #456-9036) according to manufacturer's protocol, then bands for p-EIF2A, total EIF2A, or GCN2 visualized by standard western blotting techniques using the following antibodies: p-EIF2A (Cell Signaling #9921 or #3398, 1:1000 in 2% BSA in TBS-T), total EIF2A (Cell Signaling #9722, 1:1000 in 3% milk in TBS-T), or GCN2 (Abcam #137543, 1:1000 in 3% milk in TBS-T). HRP activity was killed with 1% sodium azide in TBS-T for one hour, then blots were re-probed for beta-actin (Cell Signaling #7074, 1:1000 in 3% milk in TBS-T). Band densitometry was performed in ImageJ. p-EIF2A / total EIF2A ratios were calculated by first normalizing p-EIF2A and EIF2A band intensities to beta-actin loading controls, then dividing normalized p-EIF2A values by normalized total EIF2A values. Data are presented as fold p-EIF2A / EIF2A relative to the average for wild-type mice fed control food.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Apparent rapid sensing of EAA deficiency may reflect neophobia. Related to Figure 1.
(A) Feeding paradigm used in (B-E). (B) Wild-type (n=6) and *Gcn2* -/- (n=3) mice did not consume a significantly different amount of T-def food compared to control in the first three hours of feeding, with no significant effects of genotype or interaction between diet and genotype . Overnight, mice consumed less T-def food than control ($p<0.0001$), but genotype and interaction between diet and genotype had no significant effect. (C) Wild-type mice (n=11) did not consume a significantly different amount of L-def food compared to control in the first three hours of feeding. Overnight, the mice consumed less L-def than control ($p<0.0001$). (D) Wild-type (n=16) and *Gcn2* -/- (n=8) mice consumed significantly less TL-def food than control in the first three hours of feeding ($p=0.0049$) and overnight ($p<0.0001$), with a significant effect for genotype in the first three hours of feeding ($p=0.0135$) but not overnight, and no significant interaction between genotype and diet at either time point. (E) Wild-type (n=31) and *Gcn2* -/- (n=8) mice consumed significantly less novel control food than familiar control food in the first three hours of feeding ($p=0.021$), and *Gcn2* -/- mice consumed less than wild-type mice ($p=0.0349$), but there was no significant interaction between diet and genotype. Overnight, there were no significant effects of diet, genotype, or interaction between diet and genotype.

Figure S2. *Gcn2* -/- mice are homozygous for a GCN2 loss-of-function allele. Related to Figure 1.
Gcn2 -/- mice are reportedly homozygous for a targeted mutation in which a single loxP site replaces exon 12 of *Gcn2*, a necessary part of GCN2's ATPase domain. (A) To confirm their genotype, the genomic region flanking the loxP insertion was PCR-amplified and sequenced. The sequencing chromatogram clearly shows the junctions of the genomic DNA sequence with the loxP insertion. The position of the *Gcn2* -/- specific forward genotyping qPCR primer used in (B) is also depicted. (B) Two qPCR primer sets were designed to distinguish the wild-type and *Gcn2* -/- alleles. The probe and reverse primer in the wild-type-specific probe and primer set bind within exon 12, whereas the forward primer of the *Gcn2* -/- specific probe and primer set spans the junction between the loxP insertion and genomic DNA in the *Gcn2* -/- mouse. Genomic DNA from six wild-type and six *Gcn2* -/- mice was analyzed using these primers. Wild-type-specific qPCR detected less than 10,000-fold less DNA in *Gcn2* -/- mice compared to wild-type controls, far less than the two-fold difference expected if the *Gcn2* -/- mice were only heterozygous for the mutation (dotted line). Similarly, *Gcn2* -/- specific qPCR detected close to 10,000-fold less DNA in wild-type mice than *Gcn2* -/- mice.

Figure S3. Additional controls for western blots. Related to Figure 2.
(A) Wild-type mice were given ad libitum access to food, fasted overnight, or fasted and then re-fed for one hour. Protein extracts made from APC, MBH, and liver from these mice were analyzed by western blot for p-EIF2A (Ser-51), total EIF2A, and beta-actin loading control. (B) Fasting had no significant effect on EIF2A phosphorylation in the APC, MBH, or liver. Re-fed mice had significantly reduced EIF2A phosphorylation in the APC compared to ad libitum fed mice ($p=0.0268$), but not in the MBH or liver. (C) Western blots for GCN2 from wild-type and *Gcn2* -/- APC, MBH, and liver extracts demonstrate that GCN2 is expressed in each of these tissues.

Figure S4. Consumption of lysine-deficient food does not activate GCN2 in the brain. Related to Figure 3.
(A) Wild-type and *Gcn2* -/- mice were fed control or K-def food for one hour or 12 hours. Food

intake in grams by each mouse is listed above the top panel. Protein extracts made from APC, MBH, and liver from these mice were analyzed by western blot for p-EIF2A (Ser-51), total EIF2A, and beta-actin loading control. (B) After one hour of feeding, basal level p-EIF2A / EIF2A in the APC was significantly lower in *Gcn2* -/- mice than wild-type controls ($p<0.0001$), with no effect of diet or interaction between diet and genotype. There were no significant effects of diet or genotype in the MBH or liver. (C) After twelve hours of feeding, 2-way ANOVA revealed a significant interaction between diet and genotype ($p=0.0130$), where lysine seemed to reduce EIF2A phosphorylation in wild-type but increase EIF2A phosphorylation in *Gcn2* -/- mice. In the MBH and liver, *Gcn2* -/- mice had significantly lower phosphorylation of EIF2A than wild-type mice ($p=0.0119$ and 0.0023).

Table S1. Diet compositions. Related to Figure 2.
The exact formulation of each diet, expressed in grams per kilogram (bold font). The results of independent amino acid analysis are included to the right of the formula amount, where applicable (regular font). All diets are isocaloric and contain the same salt content.

Supplemental References
Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res *11*, 1475-1489.

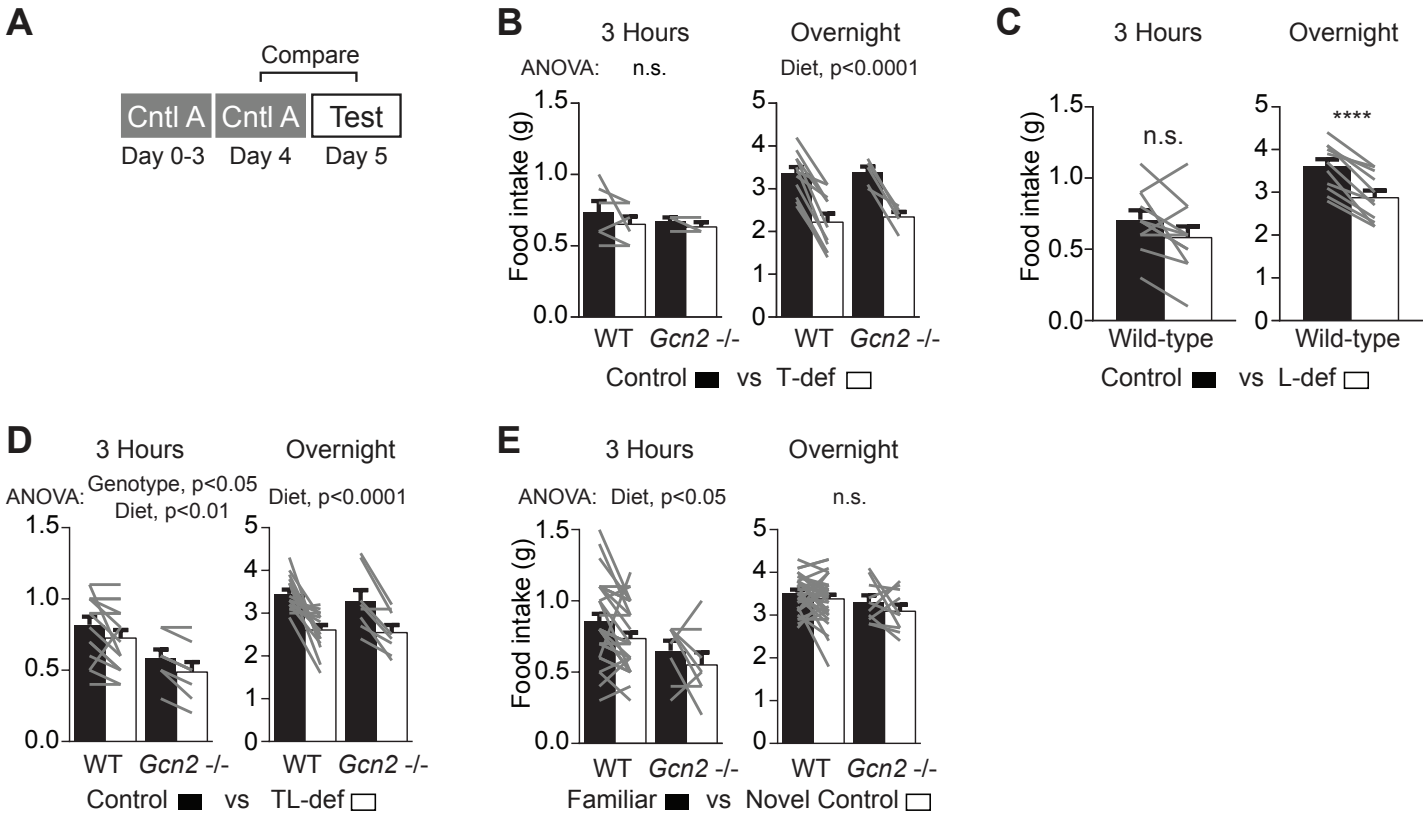

Figure S1. Apparent rapid sensing of EAA deficiency may reflect neophobia

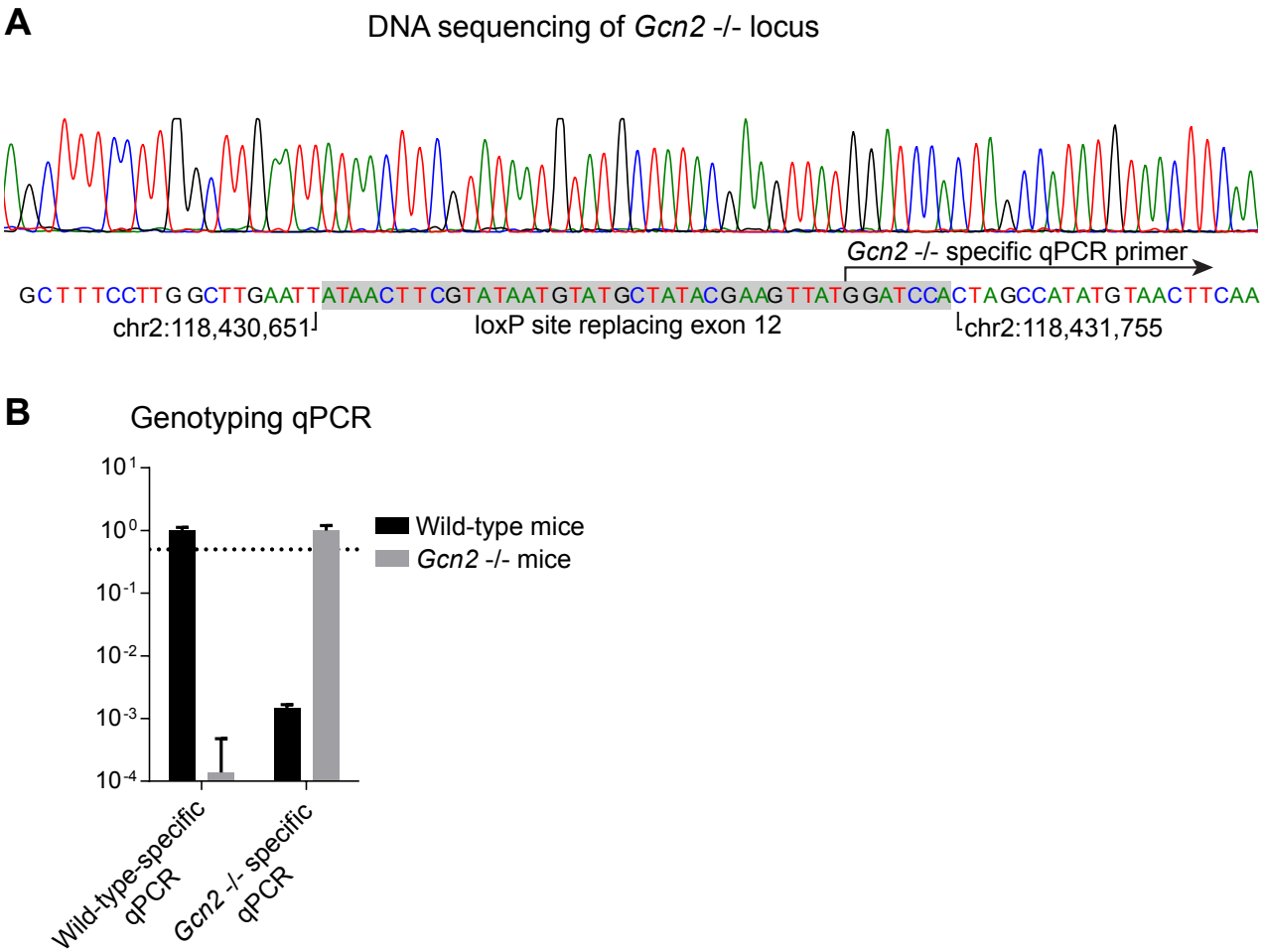

Figure S2. Confirmation of genotype of GCN2 knockout mice by sequencing and qPCR

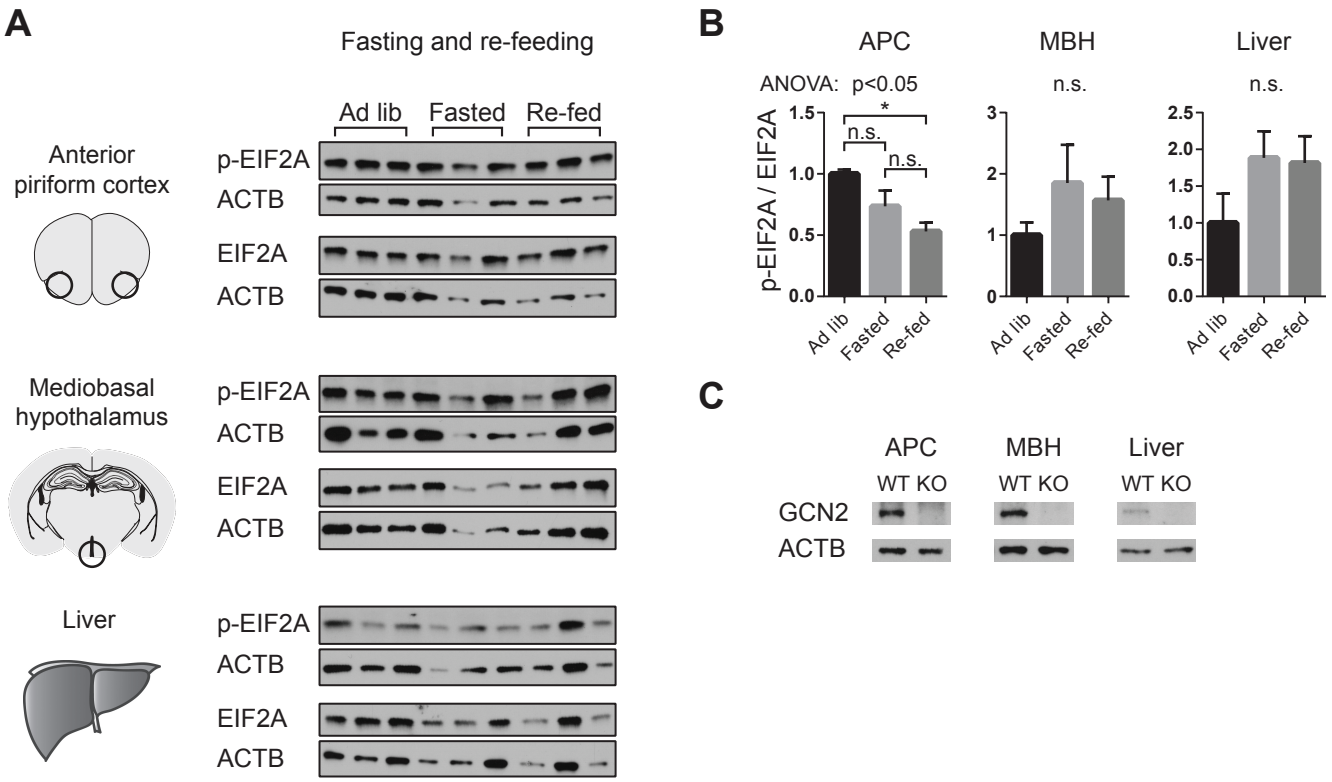

Figure S3. Additional controls for western blots

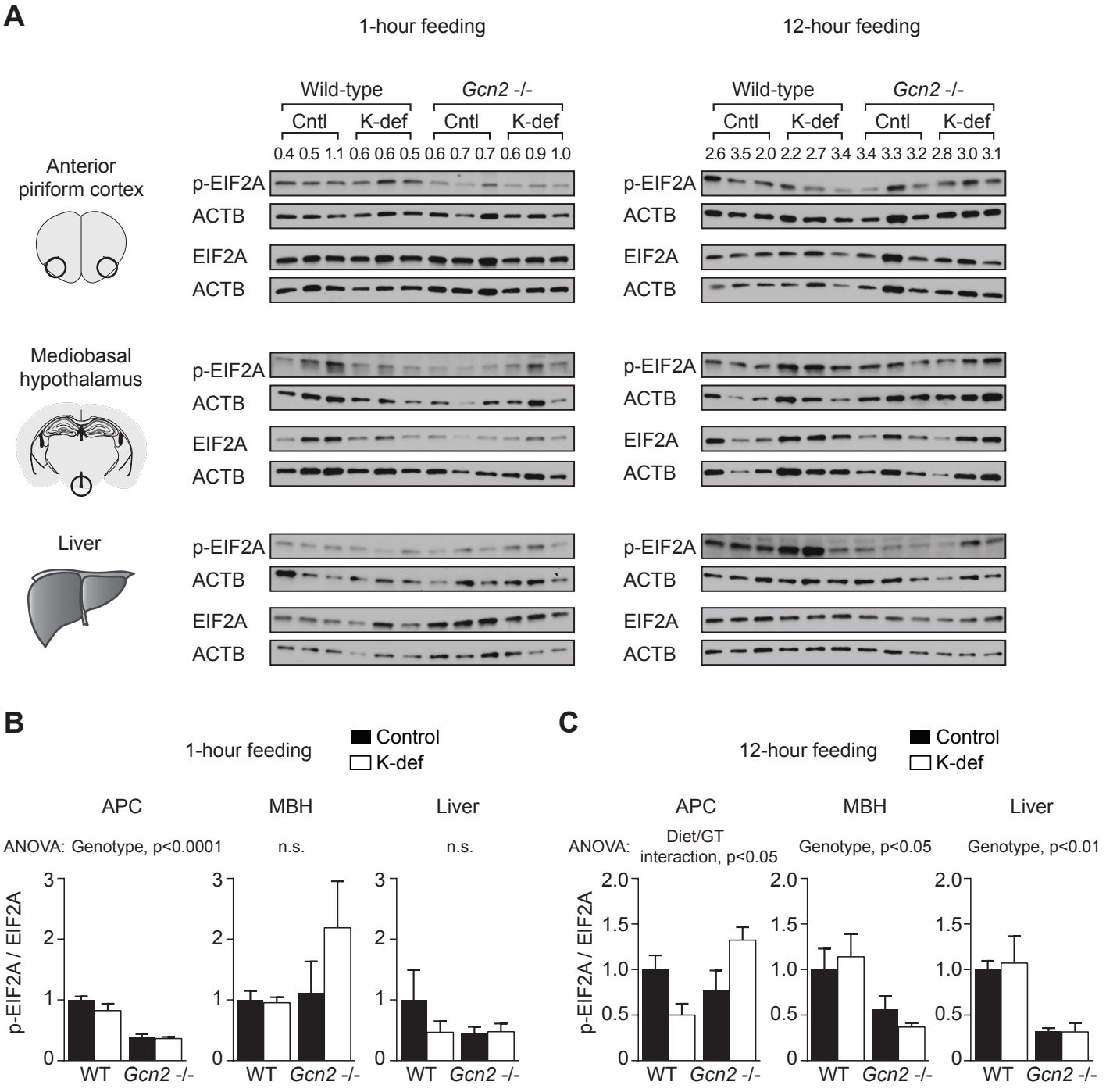

Figure S4. Consumption of lysine-deficient food does not activate GCN2 in the brain

Ingredient (g/kg)	Cntl A	Cntl B		T-dev		L-dev		TL-dev		K-dev		AA-dev	L-basal
L-Arginine	**10**	**10**	12	**10**	12	**10**	12	**10**	12	**10**	12	**0**	**5**
L-Histidine-HCl-H_2O	**6**	**6**	5	**6**	18	**6**	6	**6**	5	**6**	6	**0**	**3**
L-Isoleucine	**8**	**8**	9	**8**	8	**8**	8	**8**	9	**8**	11	**0**	**4**
L-Leucine	**12**	**12**	15	**12**	15	**0**	0.3	**0**	0.2	**12**	15	**0**	**4.8**
L-Lysine-HCl	**14**	**14**	16	**14**	20	**14**	17	**14**	18	**0**	0	**0**	**7**
L-Methionine	**6**	**6**	9	**6**	7	**6**	7	**6**	9	**6**	7	**0**	**3**
L-Phenylalanine	**8**	**8**	9	**8**	10	**8**	10	**8**	10	**8**	9	**0**	**4**
L-Threonine	**8**	**8**	9	**0**	0	**8**	9	**0**	0	**8**	9	**0**	**4**
L-Tryptophan	**2**	**2**	3	**2**	3	**2**	3	**2**	3	**2**	3	**0**	**1**
L-Valine	**8**	**8**	10	**8**	10	**8**	10	**8**	9	**8**	9	**0**	**4**
L-Alanine	**10**	**10**	28	**10**	14	**10**	14	**10**	11	**10**	11	**0**	**10**
L-Asparagine-H_2O	**5**	**5**		**5**		**5**		**5**		**5**		**0**	**5**
L-Aspartate	**10**	**10**		**10**		**10**		**10**		**10**		**0**	**10**
L-Cystine	**4**	**4**	2	**4**	2	**4**	3	**4**	2	**4**	2	**0**	**4**
L-Glutamic Acid	**30**	**30**		**30**		**30**		**30**		**30**		**0**	**30**
L-Glutamine	**5**	**5**		**5**		**5**		**5**		**5**		**0**	**5**
Glycine	**10**	**10**	12	**10**	12	**10**	12	**10**	13	**10**	13	**0**	**10**
L-Proline	**5**	**5**	6	**5**	6	**5**	6	**5**	6	**5**	6	**0**	**5**
L-Serine	**5**	**5**	10	**5**	9	**5**	10	**5**	10	**5**	10	**0**	**5**
L-Tyrosine	**4**	**4**	5	**4**	5	**4**	5	**4**	5	**4**	5	**0**	**4**
Total Amino Acids	**170**	**170**		**162**		**158**		**150**		**156**		**0**	**127.8**
Corn starch	**550.5**	**550.5**		**558.5**		**562.5**		**570.5**		**564.5**		**645.5**	**592.7**
Maltodextrin 10	**125**	**125**		**125**		**125**		**125**		**125**		**200**	**125**
Cellulose	**50**	**50**		**50**		**50**		**50**		**50**		**50**	**50**
Corn oil	**50**	**50**		**50**		**50**		**50**		**50**		**50**	**50**
Mineral mix S10001	**35**	**35**		**35**		**35**		**35**		**35**		**35**	**35**
Sodium bicarbonate	**7.5**	**7.5**		**7.5**		**7.5**		**7.5**		**7.5**		**7.5**	**7.5**
Vitamin mix V10001	**10**	**10**		**10**		**10**		**10**		**10**		**10**	**10**
Choline bitrartrate	**2**	**2**		**2**		**2**		**2**		**2**		**2**	**2**
Red dye, FD&C#40	**0**	**0**		**0.025**		**0.025**		**0**		**0**		**0**	**0.05**
Blue dye, FD&C#1	**0.05**	**0.025**		**0.025**		**0**		**0.025**		**0.025**		**0.025**	**0**
Yellow dye, FD&C#5	**0**	**0.025**		**0**		**0.025**		**0.025**		**0.025**		**0.025**	**0**

Table S1. Diet compositions