**Trib3 Is Developmentally and Nutritionally Regulated in the Brain but Is Dispensable for Spatial Memory, Fear Conditioning and Sensing of Amino Acid-Imbalanced Diet**

Ttit Örd¹, Juürgen Innos², Kersti Lilleväli¹,², Triin Tekko², Silva Süt², Daima Örd³, Sulev Kõks⁴, Eero Vasar², Tõnis Örd³*

¹ Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia, 2 Department of Physiology, Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia, 3 Estonian Biocentre, Tartu, Estonia, 4 Chair of Pathological Physiology, Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia

**Abstract**

Tribbles homolog 3 (TRIB3) is a mammalian pseudokinase that is induced in neuronal cell cultures in response to cell death-inducing stresses, including neurotrophic factor deprivation. TRIB3 is an inhibitor of activating transcription factor 4 (ATF4), the central transcriptional regulator in the eukaryotic translation initiation factor 2α (eIF2α) phosphorylation pathway that is involved in the cellular stress response and behavioral processes. In this article, we study the expression of Trib3 in the mouse brain, characterize the brain morphology of mice with a genetic ablation of Trib3 and investigate whether Trib3 deficiency alters eIF2α-dependent cognitive abilities. Our data show that the consumption of a leucine-deficient diet induces Trib3 expression in the anterior piriform cortex, the brain region responsible for detecting essential amino acid intake imbalance. However, the aversive response to leucine-devoid diet does not differ in Trib3 knockout and wild type mice. Trib3 deletion also does not affect long-term spatial memory and reversal learning in the Morris water maze and auditory or contextual fear conditioning. During embryonic development, Trib3 expression increases in the brain and persists in the early postnatal stadium. Neuroanatomical characterization of mice lacking Trib3 revealed enlarged lateral ventricles. Thus, although the absence of Trib3 does not alter the eIF2α pathway-dependent cognitive functions of several areas of the brain, including the hippocampus, amygdala and anterior piriform cortex, Trib3 may serve a role in other central nervous system processes and molecular pathways.


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* E-mail: torid@ebc.ee (Tõnis Örd)

**Introduction**

TRIB3 (also known as TRB3, NIPK and SKIP3) is a kinase-like protein (pseudokinase) that was first isolated as a gene that is strongly induced during neuronal cell death caused by nerve growth factor deprivation or disruption of calcium homeostasis [1, 2]. Further studies revealed that the upregulation of Trib3 during cellular stress is mediated by the binding of activating transcription factor 4 (ATF4) to the Trib3 promoter [3, 4]. In mammalian cells, the response to diverse types of cellular stress converges on a single biochemical event—the phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) at serine 51—which inhibits general translation but selectively increases the translation rate of ATF4, leading to the activation of a multifaceted stress response gene expression program that is coordinated by ATF4 [5, 6]. Through the four known eIF2α kinases (GCN2, PERK, PKR and HRI), the eIF2α–ATF4 pathway is activated in stress situations such as amino acid or glucose deficiency, unfolded protein accumulation in the endoplasmic reticulum and oxidative damage [5], and, in accordance with the studies of the Trib3 promoter, these stresses are also characterized by the marked induction of Trib3 in different types of cells [3, 4, 7–10]. The TRIB3 protein is able to act as an inhibitor of ATF4 by directly binding to it [2, 11]. Therefore, the activation of the Trib3 promoter by ATF4 constitutes a negative feedback mechanism for regulating the activity of the eIF2α–ATF4 pathway [3, 4, 8, 12].

In the brain, the phosphorylation of eIF2α participates in several behavioral processes. Animals are unable to synthesize a subset of amino acids, termed essential amino acids (EAA), and thus need to acquire EAs from their diet. Omnivores that have a number of different food sources available need to balance their intake of different foods in order to obtain EAs in the correct proportions. When fed an otherwise complete diet lacking a single EAA, animals including mice and rats will promptly, within the
course of one meal, develop an aversive reaction towards the food, which involves substantially limiting the consumption of the food and foraging for alternative dietary sources (reviewed in [13]). This innate reaction does not depend on the gastrointestinal tract, or the senses of taste and smell, but rather on the sensing of blood amino acid levels by the anterior piriform cortex (APC) region of the brain [13]. Following consumption of an EAA-deficient meal, eIF2α is phosphorylated in the APC by GCN2, which is sensitive to intracellular levels of uncharged tRNA molecules, and mice that lack GCN2 fail to reject an EAA-imbalanced diet [14,15]. However, the mechanisms acting downstream of eIF2α to regulate feeding behavior are currently uncertain.

The eIF2α–ATF4 pathway is also involved in hippocampal long-term memory formation, which is necessary for spatial learning and contextual fear conditioning. Following behavioral training, eIF2α phosphorylation in the hippocampus decreases, and, in mouse models with reduced phospho-eIF2α and ATF4 levels, the threshold for eliciting hippocampus-dependent learning is lowered [16,17]. Conversely, genetically increasing the amount of eIF2α phosphorylation in the hippocampus to a level that does not inhibit general translation, but does induce ATF4, impairs hippocampal memory [18]. In line with this, the induction of long-term potentiation, a putative cellular model of learning, is prevented by the pharmacological inhibition of eIF2α dephosphorylation in wild type hippocampal slices but is unaffected in slices from ATF4 knockout mice [17]. Thus, hippocampal long-term memory formation appears to occur via the downregulation of the phospho-eIF2α–ATF4 axis in response to behavioral training, but how this process might be influenced by endogenous modulators of ATF4 activity is unknown.

TRIB3 is a target gene and inhibitor of the eIF2α–ATF4 pathway in cell cultures, but its role and expression regulation in the brain are unclear. In the present work we characterize the pathway in cell cultures, but its role and expression regulation in the adult mouse brain and during mouse brain development, and, by utilizing a Trib3 knockout mouse line, study whether TRIB3 has an effect on the behavioral responses that are mediated by the phosphorylation of eIF2α and examine the brain morphology of mice lacking Trib3.

Materials and Methods

Animals, feeding and diets

The Trib3 knockout mouse line used in this study was generated by us by introducing a targeted deletion of the entire Trib3 protein coding region, and is based on the C57BL/6j mouse strain genetic background [19]. Mice were genotyped for knockout and wild type Trib3 alleles by PCR. The animals were maintained on a 12-hour light/12-hour dark cycle, and experimental procedures were performed during the light phase. Access to food and water was provided ad libitum, with the exception of experiments that involved an overnight period of fasting (described below).

In indicated experiments, overnight fasting was used to synchronize feeding between individual mice. During the light phase, food was available ad libitum. At the end of the light phase, the remaining food was removed from the feeder and the cage bedding was replaced to avoid the consumption of feed pellet crumbs and to minimize coprophagy. After the dark phase, ad libitum access to food was restored. Access to water was not restricted during the fasting period.

To study dietary essential amino acid limitation, synthetic diets composed of purified ingredients were used (manufactured by Research Diets, New Brunswick, NJ). The diets contained free L-amino acids as the sole source of dietary amino acids. A nutritionally complete diet containing an entire complement of amino acids was used for training and as the experimental control diet, and a corresponding diet lacking the essential amino acid leucine was used to evoke leucine deficiency. The composition of the leucine-devoid diet was adjusted with carbohydrate. The full composition of the diets used for leucine deprivation experiments is presented in Table S1. For other experiments, animals were maintained on standard commercial rodent chow.

All animal procedures were performed in accordance with the guidelines of the European Union and were approved by the Estonian National Board of Animal Experiments (resolutions number 83, 25.06.2007; 82, 25.08.2011; 8, 06.05.2013). All efforts were made to minimize suffering of animals during experimental procedures.

Total RNA isolation, RT-qPCR and RT-PCR

To study adult brain gene expression during leucine deficiency, four-month-old male wild type C57BL/6j mice were trained with synthetic nutritionally complete control diet ad libitum for one week, then fasted overnight and randomly provided either the leucine-deficient diet (Leu−) or the control diet (Leu+). Procedures were performed in an alternating order of Leu− and Leu+ group individuals. After 6 h of access to the experimental diet ad libitum, the mice were sacrificed by cervical dislocation and their brains were immediately dissected. Brain regions were identified according to the mouse brain atlas of Franklin and Paxinos [20]. The anterior piriform cortex (as defined in [20]), the hippocampus (encompassing both the dorsal and ventral hippocampus), a sample of the cerebral cortex (an approximately 3 × 3 mm region from the center of the frontal lobe, encompassing all neocortical layers) and the cerebellum (whole) were excised on ice and immediately frozen in liquid nitrogen. To quantify gene expression during mouse development, total RNA was extracted from excised embryonic or neonatal brains (excluding the cerebellum) of the indicated age using TRIzol (Invitrogen). Total RNA was isolated from the APC using the RNaseasy Micro kit and from the other adult brain regions using the RNaseasy Mini kit (both from Qiagen). Samples were homogenized with a glass-Teflon homogenizer, and on-column DNase digestion was performed according to the manufacturer’s recommendations.

Total RNA concentration was determined spectrophotometrically with NanoDrop 1000 (Thermo Scientific), and 0.5 µg of total RNA was used in 10 µl first-strand CDNA synthesis reaction. The developmental brain cDNA series was synthesized with Super-Script III reverse transcriptase (Invitrogen), and the adult brain region cDNA series was synthesized with RNase H-minus M-MLV reverse transcriptase (Solis BioDyne, Estonia). Real-time PCR quantification of Trib3 mRNA and ribosomal protein L7a (Rpl7a) mRNA, which was used as the endogenous reference gene for expression normalization, was performed as described previously [19]. RT-qPCR analysis of Tribl and Trib2 expression was performed as for Trib3, using primers with the following sequences: Trib1 mRNA: 5′-GCTCGCGCTTTCAGCGAGAT-3′ (sense) and 5′-GCTCGCGCGAGCCATGTATCT-3′ (anti-sense), Trib2 mRNA: 5′-TGACCTCAAGCTGCGGAAAT-3′ (sense) and 5′-TAAGCTGCGGCTTGAATTTA-3′ (anti-sense). For end-point RT-PCR, the sequences of the primers used are as follows: Trib3 mRNA: 5′-GGCGTTATATGCTTTTTGGGAAAGA-3′ (sense) and 5′-GGCGTGCGAGGGTATGACCTT-3′ (anti-sense), Gapdh mRNA: 5′-TGTGTGCTCCTGCTGGATCTGTGA-3′ (sense) and 5′-TTGCTGTGTTAAGTGCAGAG-3′ (anti-sense). The RT-PCR products were analyzed by gel electrophoresis in agarose gel.
Aversive response to amino acid-deficient food

The protocol used for studying the effect of amino acid-deficient diet on food intake was adapted from a previously published article [15]. Four-month-old mice were used in the experiment, and for both Trib3+/+ and Trib3−/− genotypes, the experimental group contained four females and three males. The animals were arranged in a random order and the experimenter was blind to mouse genotype. Mice were trained for one week to the novelty of the synthetic control diet, single housing and food deprivation during the dark phase (overnight fasting). Single housing was used to enable the determination of food consumption by weighing the food pellets remaining in the feeder (visible food pellet crumbs found inside the cage were also included in the measurement). In the days following the training period, the animals’ food consumption was measured at set time-points throughout the light phase. On the first three days, the intake of the control diet was monitored, and on the next three days, leucine-deficient diet consumption was investigated. For every animal, the consumption of the leucine-devoid diet was compared to the amount of complete diet consumed by the same animal, which serves to control for individual variations in food intake that are independent of the dietary conditions investigated.

Morris water maze

The experimental groups consisted of three-month-old littermate Trib3+/+ and Trib3−/− mice, and both groups were sex-balanced (Trib3−/− group: 8 males and 8 females; Trib3+/+ group: 5 males and 5 females). Animal housing was not divided by genotype. To perform the experimental procedures, the animals in each cage were assigned a random order that was kept consistent over the course of the experiment. The experimenter was blind to mouse genotype. The pool diameter was 150 cm, and the water was kept at room temperature and rendered opaque by the addition of a small amount of non-toxic white putty. The platform diameter was 16 cm and its top surface was approximately 1 cm below the surface of the water. One day before the start of training, the mice were placed into the pool (with no platform) for 60 seconds to habituate them with swimming and handling. Mice were trained four times per day at intervals of approximately 45 minutes for four consecutive days. In each training trial, the animal was allowed to swim until it found the platform, but not for more than 60 seconds. If the platform was not found after 60 seconds, the mouse was guided to the platform by the experimenter. After arriving on the platform, the mouse was left there for 15 seconds before being picked up. Four different start positions, located beside the side wall of the pool at 90° intervals, were used once per day by every mouse. For each training day, the start position order was permuted for each mouse. One day after the end of the training phase, a probe trial was performed by removing the platform from the pool and allowing the mouse to search for 60 seconds. An automated video tracking system was used to monitor the swimming trajectory and time (TSE Systems GmbH, Germany). During training trials, the time required for finding the platform (escape latency) was recorded, and for probe trials, the time spent searching in each pool quadrant was recorded. Following the probe trial, reversal training was started on the same day and performed for two consecutive days. For reversal training, the platform was positioned at the quadrant opposite of the platforms’ original location and the training protocol corresponded to that of the initial training. The reversal probe trial was performed one day after the end of the reversal training phase.

Fear conditioning

The fear conditioning experiments were performed using the same groups of Trib3+/+ and Trib3−/− mice that were previously used in the Morris water maze, after a resting period of approximately one month. The housing arrangement and temporal order of animals in the experimental procedures was the same as in the Morris water maze. The experimenter was blind to mouse genotype. To induce leucine deficiency during the experiment, mice were first habituated to the synthetic control diet ad libitum for 12 days, and then deprived of food overnight on the night before the training day. Starting from the morning of the experimental training day, mice were fed the synthetic diet lacking leucine ad libitum. The fear conditioning training was performed five hours after the initiation of leucine deprivation. For training, a mouse was placed into the training chamber (TSE Systems GmbH), allowed to acclimatize to the context for 120 seconds, followed by two pairings of a tone (30 seconds, 75 dB, 10 kHz) with a co-terminating foot shock (2 seconds, 0.5 mA, constant current). The two pairings were separated by a 120-second pause, and mice remained in the training chamber for 15 seconds after the last pairing. Twenty-four hours after contextual fear conditioning was assayed by placing the animal into the training chamber for 5 minutes and recording its movement. Two hours later, auditory fear conditioning was assayed by placing the animal into a visually different chamber and recording its movement during a 150-second habituation (pre-tone) phase, followed by a 150-second phase of training tone presentation. The incidence of freezing (immobility except for respiration) during each testing phase was tracked with an automated system (TSE Systems GmbH). After testing, the animals were returned to a standard diet.

RNA in situ hybridization

Preparation of mouse brain sections and their analysis by in situ hybridization using digoxigenin-labeled riboprobes was performed as described previously [21]. To generate the template for the probe targeting synaptophysin (Syp) mRNA, a 463-bp cDNA fragment was PCR-amplified from mouse brain cDNA using the primers 5’-CCCAGCTTGGGGCTATCCGCCGCTT-3’ and 5’-CCGCTCGAGGTTACATCGAGCGAGCC-3’ (sense and anti-sense, with HindIII and Xhol restriction sites underlined, respectively) and cloned into the pBlueScript KS+ vector (Stratagene).

Lateral ventricle size measurement

Lateral ventricle size was studied from PFA-fixed coronal sections by measuring the area of the lateral ventricles from a microphotograph of the section, using Adobe Photoshop software, with the experimenter blind to genotype. The areas of the left and right lateral ventricle were summed for each individual. For adult mice, the section selected for the measurement was located at the level 0.1–0.22 mm posterior to bregma (figures 32 and 33 in [20]), and the anterior–posterior location of the section was determined based on the dorsal part of the third ventricle, the anterior part of the anterior commissure and the anterior part of the paraventricular thalamic nucleus. The adult study groups consisted of age-and sex-matched Trib3+/+ and Trib3−/− individuals (4 males and 3 females for each genotype) with an age of 5.5–7 months. For 9-day-old mice, the section selected for the measurement corresponded to 0.02–0.1 mm posterior to bregma (figures 31 and 32 in [20]), and the anterior–posterior location of the section was determined based on the anterior part of the anterior commissure and the shape of the dorsal part of the third ventricle. Both the adult and juvenile animals were maintained in standard laboratory conditions.
animal husbandry conditions and were not subjected to prior experimental procedures.

**Southern blot**

Ten micrograms of mouse genomic DNA was digested with NcoI (Fermentas, Lithuania) and separated by gel electrophoresis in 1% agarose gel. DNA fragments were transferred onto Hybond-N+ positively charged nylon membrane (Amersham) according to the manufacturer’s recommendations. To prepare the probe, a 1.2-kb DNA fragment corresponding to the genomic region immediately downstream of the Trib3 termination codon was purified and radiolabeled in a random-primed labeling reaction containing 50 μCi [α-32P]-dCTP (Hartmann Analytic GmbH, Germany) using the DecaLabel DNA labeling kit (Fermentas). Autoradiography of the hybridized probe was performed by storage phosphor imaging on a Typhoon Trio imager (GE Healthcare).

**Statistical analysis**

Data are expressed as the mean ± SEM, and group sizes are stated in the figure legends. To analyze the effect of leucine deficiency on gene expression in different brain regions, gene expression in leucine-devoid diet-fed and control diet-fed individuals was compared for each studied brain region with the two-tailed t test, and the resulting P-values were corrected for multiple comparisons with the Holm-Bonferroni method. Lateral ventricle size was compared all group means with correction for multiple testing using the LME. For experiments not specified above, comparisons between genotypes with the Mann–Whitney U test. For all analyses, P<0.05 was considered statistically significant.

**Results**

Consumption of essential amino acid-deficient diet induces Trib3 mRNA expression in the anterior piriform cortex

When mice are fed a diet lacking an EAA, the concentration of that EAA is reduced in the blood and GCN2-dependent phosphorylation of eIF2α ensues in the APC, the region containing the brains’ chemosensor for EAA deprivation [14,15]. To determine whether Trib3 is upregulated in the brain in response to EAA deficiency, adult mice were habituated to a nutritionally complete synthetic diet for one week, which was followed by overnight fasting to synchronize feeding before being provided either a synthetic diet lacking the EAA leucine (Leu−) or the corresponding leucine-containing complete diet (control; Leu+). After 6 h, the mice were sacrificed, and RT-qPCR was used to quantify Trib3 mRNA expression in various brain regions. In the complete diet-fed mice, the Trib3 expression level is very similar in the frontal cerebral cortex, the APC and the hippocampus, while in the cerebellum it is approximately 5-fold higher (Figure 1). In leucine-deprived mice, Trib3 mRNA abundance is increased 3-fold in the APC, compared to Leu+ mice, and it is also slightly but statistically significantly elevated in the cerebral cortex, while no significant change in Trib3 expression is detectable in the hippocampus or cerebellum (Figure 1).

In addition to Trib3, there are two other Drosophila Tribbles homologs in mammals, Trib1 and Trib2, RT-qPCR analysis of Trib1 and Trib2 expression in the adult mouse brain was performed in the same brain regions and dietary conditions as for Trib3. The results show that in complete diet-fed mice, Trib1 expression level does not vary between the APC, the cerebral cortex and the cerebellum, and is approximately 50% lower in the hippocampus compared to the other studied regions (Figure S1A), while the level of Trib2 expression is uniform in all of the four studied brain regions (Figure S1B). Neither Trib1 nor Trib2 demonstrate a significant change in expression level in any of the studied brain regions in response to the consumption of leucine-deficient diet (Figure S1A and B). Thus, in comparison with the other Tribbles homologs, Trib3 displays a unique expression pattern in the adult mouse brain, with elevated basal expression in the cerebellum compared with the cerebrum, and Trib3 is the only gene in the Tribbles homolog family that is induced in the APC by amino acid deficiency.

Deletion of Trib3 does not influence the rejection of amino acid-imbalanced diet

Because the aversive response to an amino acid-deficient diet is dependent on the modulation of eIF2α phosphorylation [14,15], and because Trib3 was revealed to be induced by EAA depletion in the EAA-sensitive APC region of the brain (Figure 1) and is known to regulate eIF2α-ATF4 pathway activity during amino acid limitation in cell cultures [12], we sought to explore the importance of Trib3 for the sensing of amino acid-imbalanced diet in mice. We have recently generated a Trib3-deficient mouse line by introducing a targeted deletion of the protein coding region of the Trib3 gene, as detailed in Figure S2A and B. As expected, RT-PCR analysis of Trib3 expression in mouse brain readily detects Trib3 mRNA in Trib3−/− and Trib3+/− individuals, while Trib3 mRNA is undetectable in Trib3−/− littermates (Figure S2C).
To study the aversion to amino acid-deficient diet, Trib3+/+ and Trib3−/− mice were fasted 12 h overnight and presented with either Leu+ or Leu− diets during the day, and food intake was measured at 0.5 h to 12 h time-points. As depicted in Figure 2A, both Trib3+/+ and Trib3−/− mice demonstrate a similar and substantial rejection of Leu− food starting from 0.5 h, and, at the later time-points (4 h and 12 h), where the intra-group variation is lower, the consumption of Leu− diet is decreased by approximately 30% compared to Leu+ for both genotypes. The proportion of food intake repression for EAA-deficient diet is comparable to results that other researchers have obtained for wild type mice [15]. In both males and females, the body weight of littermate wild type and Trib3-deficient mice habituated to the synthetic complete diet (Leu+) is similar (Figure 2B), as is the amount of weight lost due to overnight fasting (Figure 2C). Thus, the loss of Trib3 does not alter the self-restriction behavior of mice in response to amino acid-insufficient food.

Trib3 is dispensable for spatial and reversal learning in the Morris water maze

Excessive hippocampal ATF4 activity resulting from eIF2α phosphorylation has been associated with impaired spatial memory and learning [17,18], and the hippocampus also expresses Trib3 (Figure 1). To examine the role of Trib3 in long-term spatial memory, we studied the performance of Trib3+/+ and Trib3−/− mice in the Morris water maze, a hippocampus-dependent task in which mice learn to escape from opaque water onto a submerged platform by following spatial cues [22]. As shown in Figure 3A, Trib3-deficient mice and their wild type counterparts demonstrate similar and consistent improvement in the time required to find the hidden platform (escape latency) over the first three training days, and no further improvement is displayed by either genotype on training day four. One day after the last training day, a probe test was performed by removing the platform and allowing the mice to search for one minute. Both genotypes exhibited a strong preference for the quadrant of the pool that previously contained the platform (target quadrant), spending nearly 50% of the time there, indicating that no significant differences exist between the genotypes in spatial learning ability (Figure 3B).

The Morris water maze can also be used to study spatial reversal learning by repositioning the platform and challenging the mice to learn the new platform location. Recently, it has been published that mice with a forebrain-specific postdevelopmental disruption of the eIF2α kinase PERK have unaltered learning in the Morris water maze but are impaired in reversal learning, implicating the eIF2α phosphorylation pathway in behavioral flexibility [23]. Therefore, we also studied reversal learning ability in Trib3 knockout mice. For this experiment, the hidden platform was relocated to the opposite quadrant. As indicated in Figure 3C, Trib3+/+ and Trib3−/− mice display similar escape latency of nearly 30 seconds on the first day of reversal training, and, for both genotypes, the escape latency improves drastically on the second reversal training day, with Trib3-deficient as well as wild type individuals swimming to the hidden platform in approximately 10 seconds on average. In accordance with this result, no significant differences are observable between Trib3+/+ and Trib3−/− mice in

Figure 2. Rejection of amino acid-imbalanced diet in mice is not influenced by the deletion of Trib3. (A) Consumption of a diet lacking the essential amino acid leucine (Leu−), compared to the consumption of a corresponding nutritionally complete control diet (Leu+), was measured for each animal at the indicated time-points by weighing the remaining food. For each animal, the intake of Leu− diet was compared to that of the Leu+ diet, and the average difference in the consumption of the Leu− diet relative to the Leu+ diet is expressed in percent ± SEM for each genotype. (B) Body weight and (C) body weight loss due to overnight food deprivation do not differ between Trib3-deficient and wild type adult mice. For B and C, four-month-old group-housed Trib3+/+ (n = 5 for both males and females) and Trib3−/− (n = 8 for both males and females) mice were maintained on the Leu+ diet ad libitum for two weeks to determine their diet-habituated body weight, followed by a single iteration of overnight fasting. The data in B and C are presented as the group means ± SEM. doi:10.1371/journal.pone.0094691.g002
the quadrant occupancy pattern of a probe trial performed after reversal training (Figure 3D). The swimming speeds exhibited in 60-second sessions at different phases of the experiment are similar for both genotypes (Figure 3E), indicating that locomotor ability in the Morris water maze is not compromised by the lack of Trib3.

Trib3-deficient mice display normal contextual and auditory fear conditioning

In a fear conditioning experiment, animals learn to associate an aversive stimulus with a neutral stimulus or context [24], and long-term fear memory is known to involve eIF2α but the genes acting downstream of eIF2α are uncertain [16,17,25]. We trained Trib3 knockout and wild type mice with two pairings of tone with co-terminating foot shock, and to induce Trib3, the animals were fed a leucine-deficient diet ad libitum from the morning of the training day until the end of the measurements (performed one day after training). Auditory fear conditioning, which requires the amygdala but not the hippocampus [24], was tested by presenting the training tone in a chamber different from the training chamber and measuring the amount of time spent freezing, an indicator of fear. Trib3+/+ and Trib3−/− mice demonstrated a similar robust increase in freezing time during the tone period compared to the pre-tone period (Figure 4A). Contextual fear conditioning, which
requires both the amygdala and the hippocampus [24], was tested by placing the animal into the training chamber and tracking its movement over the course of 5 minutes. The amount of time spent freezing did not differ significantly between mice lacking Trib3 and their wild type counterparts, with both genotypes freezing for more than 50% of the time spent in the chamber (Figure 4B). Thus Trib3 does not affect long-term fear memory.

Trib3 expression increases during embryonic mouse brain development

In Drosophila, the Tribbles protein is expressed in the embryo and participates in early embryonic development [26–28]. Therefore, we sought to examine the expression of Tribbles homologs in the developing mammalian brain. RNA was extracted from mouse brains ranging from embryonic day (E) 14 to postnatal day (P) 4, and the abundance of Trib1, Trib2 and Trib3 mRNA was determined by RT-qPCR. The results show that Trib3 expression increases steadily from E14 to E18, by approximately 4-fold in total (Figure 5A). After birth (P0), the level of Trib3 mRNA is decreased by approximately 50% compared to E18, however, this decrease appears to be transient, as a resumption of elevated Trib3 expression is evident at over following days, with a peak at P2 reaching nearly 6-fold higher than E14 (Figure 5A). Unlike Trib3, the changes in Trib1 and Trib2 expression over the course of mouse brain development are very mild, with both genes demonstrating expression variations of less than 2-fold from E14 to P4 (Figure S3A and B). To study whether the loss of Trib3 expression affects the mRNA levels of the other Tribbles homologs in the developing brain, we quantified the expression levels of Trib1 and Trib2 in littermate Trib3+/+, Trib3−/− and Trib3−/− mouse brains at P3. The results, presented in Figure 5B, show a lack of compensatory regulation of Trib1 or Trib2 expression in the developing brain in response to Trib3 deletion. These results indicate a possible role for Trib3 in the pathways regulating brain development.

Enlarged lateral ventricles in Trib3−/− mice

To study the effect of germine Trib3 gene inactivation on brain morphology, sections from adult littermate Trib3+/+ and Trib3−/− mouse brains were visualized by RNA in situ hybridization of Syn mRNA, which encodes the synaptic marker synaptophysin [29]. The results show that the expression pattern of Syn is similar in Trib3+/+ and Trib3−/− individuals, and that the gross morphology of many prominent brain structures is unaltered by Trib3 deficiency (Figure 6A). However, the lateral ventricles are noticeably enlarged in mice lacking Trib3 compared to the corresponding wild type mice (Figure 6B). To study this further, lateral ventricle area was measured from coronal brain sections of a group of Trib3+/+ and Trib3−/− mice at 5.5–7 months of age. As depicted in Figure 6C, the size of the lateral ventricles is significantly increased, by 47% on average, in adult Trib3-deficient mice compared to wild type individuals. Similarly, in juvenile (P9) mice, lateral ventricle size is approximately 2-fold greater in Trib3−/− mice than in corresponding Trib3+/+ individuals (Figure 6D). Thus, Trib3 may serve a function associated with the ventricular system in the brain.

Discussion

TRIB3 is known to be upregulated in neuronal cell cultures subjected to cell death-inducing stresses [1,2,30], but its role and...
regulation in the brain under physiological conditions have not been investigated previously. In this article, we studied the expression of Trib3 in the mouse brain during adulthood and development, the brain morphology of Trib3-deficient mice, and characterized the behavioral phenotype of Trib3 knockout mice, including their long-term spatial memory, fear memory and response to amino acid-insufficient food.

In the adult mouse brain, our experiments revealed that Trib3 is markedly induced in the APC region by dietary EAA insufficiency within 6 hours. Previous knowledge of transcriptional changes in the APC in response to amino acid deficiency is relatively scarce. Consumption of an EAA-incomplete meal leads to the depletion of the limiting amino acid in blood plasma, and, in the brain, the concentration of the limiting amino acid is decreased in the APC, leading to eIF2α phosphorylation [15,31]. The phosphorylation of eIF2α is coupled to the upregulation of ATF4 [5], which acts as a master transcriptional activator of C/EBP-ATF composite sites [6], a type of stress-sensitive regulatory element. In various continuous cell lines, examination of the Trib3 promoter has revealed that the upregulation of Trib3 in response to chemical inducers of endoplasmic reticulum stress and oxidative stress is mediated by the binding of ATF4 to a C/EBP-ATF site [3,4]. Therefore, it is likely that the mechanism of Trib3 regulation by nutrients in vivo in the brain also proceeds via the C/EBP-ATF composite site in the Trib3 promoter.

Mice carrying a targeted deletion of Trib3, generated recently by us [19], have no apparent physical defects, allowing for behavioral testing to be performed. Our experiments uncovered that a lack of Trib3 does not affect access to EAA-imbalanced diet, which is dependent on the APC, long-term spatial memory, which is dependent on the hippocampus, or fear conditioning, which is dependent on the amygdala. These behavioral paradigms require the modulation of eIF2α phosphorylation in the brain, and for long-term memory consolidation, the control of ATF4 levels appears to be the crucial function of phospho-eIF2α [14,15,17,18]. In light of cell culture-based data which demonstrates that Trib3 provides negative feedback inhibition of ATF4 activity [3,4,6,12], the behavioral test results obtained for Trib3-deficient mice are unexpected. It is possible that Trib3 does not significantly inhibit ATF4 activity in the brain during normal physiology, or that the inhibitory effect of Trib3 does not extend to the particular aspect of ATF4 function that is necessary for memory formation, which is proposed to be the antagonism of CREB [16]. Alternatively, possible ATF4-dependent or -independent effects of Trib3 in the brain are masked in Trib3+/- mice by slight alterations in nervous system development or the activation of intracellular signaling mechanisms that are able to compensate for the absence of Trib3.

The importance of Trib3 expression in the brain may also be revealed in behavioral responses that are currently unexplored in Trib3-deficient mice. In mammals, two additional Tribbles homologs, Trib1 and Trib2, are present along with Trib3. However, the molecular and physiological functions of the three Tribbles homologs have diverged [32], and the ability to interact with ATF4 has only been shown in the case of Trib3. This reduces the likelihood that Trib1 and Trib2 could compensate for the deletion of Trib3 in the context of ATF4 activity regulation, and we did not detect altered expression of Trib1 or Trib2 in the brain of neonatal Trib3 knockout mice. Further, our data demonstrate that the expression pattern of Trib3 in the brain is distinct from that of the other members of the Tribbles homolog family. Notably, only Trib3 was induced in the APC by EAA deprivation and only Trib3 exhibited prominent upregulation during embryonic brain development.

Our analysis of Trib3 expression during mouse brain development revealed that the abundance of Trib3 mRNA in the brain increases from E14 to E18 and remains high in the neonatal brain. During vertebrate brain development, a substantial proportion of newly generated neurons undergo programmed cell death, which is caused in part due to the limited availability of neurotrophic factors [33]. In the mouse forebrain, programmed cell death is prevalent during the period from E12 to E18, encompassing both proliferative and postmitotic neurons [34]. Therefore, it is possible that the induction of Trib3 during embryonic brain development is related to neurotrophic factor deficiency or neuronal cell death. Consistent with this assumption, Trib3 is upregulated in neurally differentiated PC12 cells and superior cervical ganglion neurons in response to nerve growth factor deprivation [1,35,36]. In addition to neuronal cells, BV-2 microglial cells also upregulate Trib3 expression under certain conditions [37,38]. Thus, the origin of
Trib3 expression during central nervous system development warrants evaluation in future studies.

Examination of the brain morphology of adult and juvenile Trib3-deficient mice revealed increased lateral ventricle size compared to mice with wild type Trib3. In humans, lateral ventricle size increases with aging and enlarged ventricles are associated with age-related brain disorders, including Alzheimer’s disease [39], however, lateral ventricle volume also displays a relatively large amount of variability in healthy, non-elderly humans [40]. Further experiments are needed to study the dynamics of Trib3 deficiency-related ventricular expansion during mouse brain aging, as well as the neurological, neuroanatomical and behavioural importance of this effect, and to elucidate the mechanism by which the lack of Trib3 results in enlarged lateral ventricles.

In conclusion, we establish that Trib3 expression increases in the mouse brain during the progression of embryonic brain development, and, in the adult brain, Trib3 is induced by dietary essential amino acid deprivation. Nevertheless, mice homozygous for a germline deletion of Trib3 are normal regarding several aspects of cognitive functioning, including spatial learning and re-learning, fear memory, and the self-restriction of amino acid-deficient diet intake.

Supporting Information

Figure S1 Trib1 (A) and Trib2 (B) expression levels in various regions of the adult mouse brain. Wild type mice consumed either a diet lacking leucine (Leu−; n = 5) or a corresponding control diet containing leucine (Leu+; n = 5), and, after 6 h of feeding, gene expression in the indicated brain regions was quantified by RT-qPCR. The results are presented as the mean ± SEM, and expressed relative to the level in the anterior piriform cortex of the control diet (Leu+) group. (TIF)

Figure S2 Targeted disruption of the mouse Trib3 gene. (A) Schematic representation of the gene targeting strategy used to generate the Trib3-deficient allele. Filled and unboxed figures indicate exonic regions containing the Trib3 protein coding sequence and mRNA untranslated regions, respectively. The 5′ and 3′ homology arms (3.1 and 1.9 kb, respectively) for the homologous recombination event were selected to flank the genomic region corresponding to the Trib3 protein coding sequence. The homology regions were PCR-amplified and cloned into a targeting vector that contained a phosphoglycerate kinase (pgk) promoter-driven neomycin resistance cassette (pgk-NeoR) for positive selection and a thymidine kinase promoter-driven diphtheria toxin A expression cassette (tk-DTA) for negative selection. The NoI restriction sites that generate the genomic DNA fragments detected in panel B are indicated by unfilled vertical arrowheads. (B) Verification of the targeted disruption by Southern hybridization. NoI-digested genomic DNA was transferred onto membrane and probed with a radiolabeled 1.2-kb genomic fragment corresponding to the region immediately downstream of the Trib3 stop codon. The expected size of the target fragment is 2.8 and 1.9 kb for the wild type and mutant alleles, respectively. (C) RT-PCR analysis of Trib3 gene expression in P3 brain samples from wild type, heterozygous mutant and homozygous mutant littermate mice (n = 2 per genotype). Gapdh was amplified from the same samples as a positive control gene. The results from negative control reactions, which contained either total RNA that had not been subjected to reverse transcription (No RT) or water instead of template solution (No template), are also shown. (TIF)

Table S1 Composition of the diets used to study leucine deficiency. (PDF)

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Author Contributions

Conceived and designed the experiments: Tiit Oord TT KL Tönis Oord JS DO. Analyzed the data: Tiit Oord KL TT Tönis Oord EV SK. Contributed reagents/materials/analysis tools: Tiit Oord Tönis Oord SS KL TT DO JI SK EV. Wrote the paper: Tiit Oord. Coordinated the project: Tönis Oord.

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