Neural circular RNAs are derived from synaptic genes and regulated by development and plasticity

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Circular RNAs (circRNAs) have re-emerged as an interesting RNA species. Using deep RNA profiling in different mouse tissues, we observed that circRNAs were substantially enriched in brain and a disproportionate fraction of them were derived from host genes that encode synaptic proteins. Moreover, on the basis of separate profiling of the RNAs localized in neuronal cell bodies and neuropil, circRNAs were, on average, more enriched in the neuropil than their host gene mRNA isoforms. Using high-resolution in situ hybridization, we visualized circRNA punctae in the dendrites of neurons. Consistent with the idea that circRNAs might regulate synaptic function during development, many circRNAs changed their abundance abruptly at a time corresponding to synaptogenesis. In addition, following a homeostatic downscaling of neuronal activity many circRNAs exhibited substantial up- or downregulation. Together, our data indicate that brain circRNAs are positioned to respond to and regulate synaptic function.

RESULTS

CircRNAs are enriched in brain

To systematically determine the tissue-specific expression pattern of mammalian circRNAs, we deep-sequenced rRNA-depleted total RNA samples from different mouse tissues, including brain, heart, liver, lung, and testis (Fig. 1a). From two biological replicate experiments in each tissue, we obtained a minimum of 16 × 10^6 reads and a maximum of 21 × 10^6 reads, with mappable reads ranging from 88.7 to 96.1%...
depending on the tissue (Table 1). Reads that map directly to reference genome sequences or canonical exon-exon junctions can be derived from either linear RNAs or circRNAs and were therefore used to estimate the expression of the total transcriptional output (TTO) of the corresponding gene loci. To specifically identify circRNAs, we used the remaining reads that spanned the 5' and 3' splicing sites of exon(s) of individual genes, but in reverse order (head-to-tail junction reads; Fig. 1a and Online Methods). From the five tissues, we detected a total of 13,011 unique circRNAs.

We validated the authenticity of identified circRNAs by three independent methods. First, as circRNAs usually do not possess a poly(A) tail, their representation should be depleted in a poly(A)-enriched sequencing library. Compared with rRNA-depleted total RNA sequencing, poly(A) RNA sequencing produced a much lower number of sequencing reads derived from our circRNA population (Supplementary Fig. 1a). Second, compared with linear RNAs, circRNAs are endowed with a strong resistance to the exonuclease RNase R. We therefore quantified the RNase R resistance of 20 candidate circRNAs, and all of them exhibited greater than five-fold higher resistance than the linear transcripts following RNase R treatment (Supplementary Fig. 1b). Third, we deep sequenced the cDNA products derived from 12 candidate circRNAs. For 11 of them, we observed the cDNA reads corresponding to the rolling circle RT products (Fig. 1b), which could not be detected in the cDNAs from any linear forms. This serves as direct evidence for the circular nature of the circRNA structure and, to the best of our knowledge, is the first time that the full sequences of the circRNAs have been identified. Notably, for two circRNAs (circDtnb and circEzh2), in addition to the ‘canonical’ forms that encompass all of the annotated exons between the two involved in the back-splicing, we also observed circular isoforms that consisted of the same junction sequences, but with one internal exon skipped (circDtnb) or one unannotated exon inserted (circEzh2) (Supplementary Tables 1 and 2).

Although circRNAs were observed in all of the tissues that we examined, their abundance was clearly highest in brain (Fig. 1c), where 20% of the protein-coding genes produced circRNAs (Fig. 1d and Table 1). Two factors contributed to the higher abundance of circRNAs in brain. First, many host genes that produce circRNAs were expressed exclusively in brain (Fig. 1e and Supplementary Fig. 1c). Second, on average, when a host gene was expressed in brain as well as other tissue(s), the relative contribution of circRNAs (defined as the ratio of TPM between a circRNA and the TTO of the hosting gene loci) measured in TPM, was significantly higher in brain than in all other tissues. The ratios of the relative contribution between brain and other four tissues were significantly larger than 1 (two-sided one-sample t-test, ***P < 2.2 x 10^{-15}). Tissues in c-f were obtained from two animals. Error bars represent s.d. (c,d,f).

Figure 1 Profiling of circRNAs across tissues reveals enrichment in brain. (a) Experiment and analysis pipeline. (b) The rolling circle cDNA products from circRNAs. The gray ring represents a circRNA and the red vertical bar marks the head-to-tail junction. Two blue arcs mark the PCR primers. The red spirals on the gel image denote the 0-cycle, 1-cycle and 2-cycle RT products identified by PacBio sequencing, respectively. 11 of 12 circRNAs tested generated rolling circle products (the exception was circMyst4). (c) The percentage of circular junction reads (from Ensembl genes) from all the reads mapped on the genome is shown for different tissues, with the highest value (0.075–0.087%) in brain, followed by testis (0.028–0.029%). (d) The percentage of genes that produced circRNAs from all the expressed genes is shown across different tissues, with the highest value (20–21%) in brain, followed by testis (13%). (e) The number of circRNA host genes that are exclusively expressed in one tissue is shown across different tissues, with the highest value in brain. (f) The relative contribution of circRNA to the TTO of the same gene locus, that is, the ratio between the abundance of each circRNA and the TTO of the hosting gene loci (measured in TPM), was significantly higher in brain than in all other tissues. The ratios of the relative contribution between brain and other four tissues were significantly larger than 1 (two-sided one-sample t-test, ***P < 2.2 x 10^{-15}). Tissues in c-f were obtained from two animals. Error bars represent s.d. (c,d,f).
Table 1  Summary of RNA-seq results for five different mouse tissues

| Brain     | Heart     | Liver     | Lung      | Testis    |
|-----------|-----------|-----------|-----------|-----------|
| Total number of reads | 19,794,174 | 19,164,999 | 16,507,635 | 19,876,862 |
| Number of reads mapped on genome | 18,765,595 | 18,283,420 | 14,636,897 | 19,049,062 |
| Number of reads mapped on intronic regions | 6,884,089 | 6,971,714 | 3,028,058 | 4,629,968 |
| Number of reads mapped on intergenic regions | 2,125,517 | 1,862,307 | 2,353,147 | 3,181,043 |
| Number of reads mapped on protein coding genes | 8,290,663 | 8,085,776 | 7,775,596 | 11,156,730 |
| Number of protein coding genes expressed (TPM > 5) | 11,781 | 11,763 | 10,044 | 9,824 |
| Number of circular junction reads | 16,573 | 14,068 | 1,646 | 2,393 |
| Number of circRNA species | 6,186 | 5,664 | 989 | 1,315 |
| Number of circRNA-hosting protein coding genes | 2,569 | 2,386 | 707 | 869 |

circRNAs: synaptic gene origin and dendritic localization

Is the likelihood that a given linear transcript will be spliced to produce a circRNA related to the brain function? To address this, we conducted a Gene Ontology analysis of the genes that give rise to brain-expressed circRNAs (Online Methods). Notably, several functional groups related to synaptic function, such as synapse, synaptic part, presynaptic active zone, synapse membrane and postsynaptic density, were significantly enriched ($P < 0.001$, Fig. 2a). This enrichment was present regardless of the expression level of the host genes (Supplementary Fig. 2a).

Given the enrichment of host genes with synapse-related functions, we next examined whether the circRNAs are enriched in synaptic tissue. To address this, we prepared synaptosomes, a biochemically purified preparation that is enriched in synapses14,15, or microdissected the synaptic scaffolding molecule Homer1 transcript17, we detected circHomer1_a particles in the cell bodies and dendrites (visualized by an antibody to MAP2) of neurons (Fig. 2c). In contrast, use of an exon control probe set (using a sequence from two exons that do not form a circRNA) resulted in a markedly reduced signal with only one or two particles evident in the vicinity of the cell bodies and nuclei (Fig. 2c and Supplementary Fig. 3b). Use of additional probes developed for the detection of circRNAs derived from synapse-related genes, including circDscam, circKlh12, circElav15, circNlgn1, circGigyf2, circNbea and circRmst, resulted in a similar pattern with abundant particles present in the cell body and distributed particles observed throughout the dendritic arbor (Fig. 2c and Supplementary Fig. 3e–j). In hippocampal slices, in situ hybridization using a circHomer1_a probe revealed substantial expression of this circRNA in both somata and neuropil layers of CA1 hippocampal region (Fig. 2d). In addition to the exon control, scrambled probe and no probe control experiments (Fig. 2c and Supplementary Fig. 3c–d), we validated the specificity of our circRNA in situ hybridization by comparing the signal intensity of circRmst, circKlh12 and circGigyf2 in brain, liver and lung (Supplementary Figs. 3 and 4). Consistent with the RNA-seq data, the in situ hybridization data revealed only background levels of expression of circRmst and circKlh12 in liver and lung, whereas these circRNAs were clearly evident in hippocampal neurons. In contrast, circGigyf2 was expressed in all examined tissues as expected from RNA-seq data (Supplementary Fig. 4). To test whether the circRNA localization can mimic that of its host transcript, we performed in situ hybridization of circRmst1 and its host mRNA Rmst1 in cultured hippocampal neurons. Although signals for the circRNA and mRNA were apparent in both cell body and dendrites, they clearly did not colocalize (Supplementary Fig. 5). Given the anticipated diversity of circRNA populations, however, one must be open to counterexamples of colocalization of circRNA and mRNA when more cases are examined.

Absence of miRNA and RBP binding or translation into protein

Recent studies of two individual circRNAs suggested that they function as miRNA sponges, sequestering miRNAs4,5. Using a bioinformatics approach, we estimated the potential of the brain circRNA population to serve as miRNA sponges and concluded that, as a general class, the brain circRNAs do not exhibit a greater capacity to serve as miRNA

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RESOURCES
sponges than linear mRNAs (Supplementary Fig. 6a), consistent with recent analyses from other groups.

We also examined the possibility that brain circRNAs might function to bind or sequester RBPs. For this purpose, we predicted the binding sites of 38 RBPs based on their binding sequence motifs deposited in the database RBPD (Online Methods). CircRNAs possess a lower RBP binding density, when compared to either the coding sequence or the 3′ UTR of protein-coding genes (Supplementary Fig. 6b). This trend is consistently observed for circRNAs of different abundances (Supplementary Fig. 6b). Thus, these data indicate, based on nucleotide sequence alone, that circRNAs as a group are no more likely to bind to RBPs than linear mRNAs.

Given that we observed that neuronal circRNAs were mostly composed of protein-coding exons, we investigated their potential to be translated into peptides. Using a large mass spectrometry (MS) data set obtained from hippocampal neurons, we searched for peptides predicted by circular junctions, but were unsuccessful. The inability to detect a circRNA-derived peptide, however, could be a result of the well-known low detection sensitivity of MS-based shotgun proteomics approaches. Thus, we further studied the association of circRNAs with ribosomes. First, we performed ribosome profiling on rat brain. Similar to what was recently reported for circRNAs from a human cell line, in our rat brain samples, we did not detect a single ribosome-protected fragment (RPF) that mapped to a circRNA head-to-tail junction and could therefore serve as evidence for circRNA translation. This negative finding could be a result of the short read length of RPFs and, more importantly, the ribosome might only associate with the sequences outside the junction. To circumvent this limitation, we performed polyssome profiling on mouse brain. In contrast to mRNAs, circRNAs were enriched in the non-ribosomal RNA fraction.
Conservation of circRNA para-junctional sequences

Given that functionally important elements are often evolutionarily conserved, we examined the sequence conservation around the mouse circRNA junctions. Compared with splicing sites from the same host-exonic sequences around head-to-tail junctions were more conserved (Fig. 2e). Moreover, those sequences around common head-to-tail junctions detected in both mouse and rat were even more conserved, almost reaching the maximum PhastCons score. The observation that exonic sequences around circRNA junctions are extremely conserved evolutionarily in vertebrates is a strong indicator of their potential functional relevance.

Expression of circRNAs in brain during development

To determine whether the expression of circRNAs is developmentally regulated in brain, we profiled the circRNA population in the hippocampus over several stages: embryonic (E18), early postnatal (P1), postnatal at the beginning of synapse formation (P10) and late postnatal (P30). (d) The expression change for both circRNA and mRNA was validated using quantitative PCR for 13 circRNAs, including Homer1, Dlgap1, Romst, Myst4 and Ezh2. Error bars represent s.d. (e,f) Validation of circRNA expression changed over developmental stages using high-resolution in situ hybridization for circKlhl2 (green) at two time points, 4 (n = 26) or 21 (n = 24) d in culture. circKlhl2 expression was significantly upregulated between these developmental stages (two-sided unpaired Student’s t-test with Welch’s correction, ***P < 0.0001). The outline of the neuronal somata was identified using an antibody to MAP2 (red). Scale bar represents 10 μm.

and strongly depleted in the ribosome/polyosome-bound fractions (Supplementary Fig. 6c,d). Together, these results demonstrate that circRNAs, as a group, are unlikely to be translated into peptides.

Figure 3 Regulated expression of brain circRNAs during development. (a) Heatmap of circRNA expression across four different developmental stages showing the regulation of several circRNA clusters between P0 and P10, the time at which synapses typically form. The abundance of circRNAs across four developmental stages is depicted on a scale from red (low) to yellow (high). A developmentally upregulated cluster consisting of 43 circRNAs exhibiting an early peak at E18 or P1 and then declined over subsequent developmental time points. A developmentally downregulated cluster consisting of 26 circRNAs, as a group, are unlikely to be translated into peptides.

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hippocampus following the establishment of mature neural circuits (P30) (Supplementary Fig. 7). There was a clear shift in the circRNA expression pattern associated with the onset of synaptogenesis at P10 (Fig. 3a). Notably, the circRNAs that were consistently upregulated during hippocampal development were produced from the gene loci that also code for proteins enriched with synapse-related functions (Fig. 3b). In contrast, no enrichment of any functional categories could be observed for the gene loci showing the opposite (downregulated) circRNA dynamic expression pattern.

We next examined the relationship between the expression of a circRNA and its linear host comparing the earliest (E18) and latest (P30) developmental stages. We found that many circRNAs change their expression independent of their host transcripts during synaptogenesis (Fig. 3c). We validated 13 circRNA and mRNA pairs with different expression patterns using quantitative PCR (Fig. 3d). Dlgap1, whose protein product is a core component of postsynaptic density (PSD), showed a >20-fold increase in circRNA expression at P30 when compared with E18, whereas the mRNA expression increased by less than four fold. Genes such as Myst4, Klhl2 and Aagab markedly increased their circRNA expression over the course of development, whereas their mRNA expression markedly decreased. In contrast, Caenl1c showed mRNA remained unchanged. Using high-resolution in situ hybridization in cultured hippocampal neurons, we further validated the developmental regulation of circKlhl2 that exhibited strong upregulation during development (Fig. 3e). Analysis of the average fluorescence intensity at an early and late developmental stage (neurons cultured beginning at P1, days in vitro = 4 or 21) revealed a significant enhancement of the circKlhl2 expression levels (P < 0.0001; Fig. 3f). Taken together, our data from high-throughput sequencing, quantitative PCR and in situ hybridization indicate that the expression of circRNAs is developmentally regulated in neurons and that many circRNAs change their expression independent of their host linear transcripts.

**Neuronal plasticity changes circRNA expression**

If circRNAs regulate synaptic function, then their expression levels might be modulated by alterations in neuronal activity and plasticity. We induced homeostatic synaptic plasticity in cultured hippocampal neurons by manipulating neuronal activity using bicuculline, an antagonist to the GABA A receptor. Treatment with bicuculline enhanced excitatory neuronal network activity, leading to a homeostatic decrease in the mini-excitatory postsynaptic current (mEPSC) amplitude, without a change in mEPSC frequency (Fig. 4a)²⁰. Following substantial decreases in circRNA expression along developmental stages, whereas the
induction of homeostatic plasticity, the circRNA population exhibited dynamic behavior: the expression of 37 circRNAs was enhanced (Fig. 4b), whereas that of 5 circRNAs was reduced. In contrast, most of their linear host transcripts showed no substantial change in expression level (Fig. 4b). We validated the plasticity-induced changes in four circRNA candidates using quantitative PCR (Fig. 4c). We also visualized directly the circRNA expression changes after homeostatic plasticity for additional candidates using in situ hybridization. CircHomer1_a was significantly upregulated in primary hippocampal neurons (P < 0.0005 for somata and P < 0.0028 for dendrites) (Fig. 4d,e) and hippocampal slices (Fig. 4f). Taken together, these data indicate that circRNA expression levels are regulated by neural plasticity, suggesting that they are important for regulating synaptic transmission and/or local translation.

**DISCUSSION**

Eukaryotic circRNAs are a class of low-abundance, but biochemically stable, cellular RNAs that possess neither a 5′ nor a 3′ end. The property of circularity has contributed to their relative anonymity (until recently), as most of the transcriptome-wide studies begin with the purification of a poly(A)+ RNA fraction. Similar to other recent studies, we sequenced and analyzed rRNA-depleted samples that allow one to analyze circRNAs and their linear host transcripts in a quantitative manner. Whereas circRNA identification relied on available genome annotation in previous studies, we set up a computational pipeline that does not rely on exon annotations or assume canonical splice sites, and can therefore identify circRNAs derived from previously unannotated exons and transcripts. This allowed us to identify the circRNAs in rat, which, to date, has a relatively incomplete transcriptome annotation.

We validated our findings with several independent approaches. Notably, using PacBio deep sequencing of the cDNA products derived from candidate circRNAs, we observed reads that corresponded to the rolling circle RT products, which serves as direct evidence for the circular nature of the RNA and provides the full-length sequences of the circRNAs. On the basis of the sequences, we identified, to the best of our knowledge for the first time, circRNA isoforms with the same head-to-tail junctions, but different internal exon composition. As observed in this study and other previous reports, multiple circRNAs with different junctions could form from the same gene loci. The identification of circRNA isoforms with the same junction, but different internal exons, adds another layer to circRNA diversity. The fact that the internal exon composition cannot be simply predicted using junction exons necessitates the experimental determination of full-length sequence of a circRNA before any further functional investigation.

We found that circRNAs were most abundant in brain, consistent with a recent report analyzing circRNAs in fly heads. Furthermore, the brain-expressed circRNAs were derived from gene loci that also code for proteins enriched for synapse-related functions. To examine whether circRNAs themselves might be associated with the function of synapses, we studied both synaptosomes and the microdissected neuropil from hippocampal slices and found in these samples a relative enrichment of circRNAs compared with their host linear transcripts. In addition, to the best of our knowledge for the first time, we visualized individual circRNA species directly both in vitro and in vivo (in hippocampal slices). We observed the localization of circRNAs in both the cell body and the dendrites of neurons, similar to what has been observed for both miRNAs and other regulatory RNAs such as mRNAs.

These findings are interesting in light of recent debate in the field concerning the question of circRNA function. Thus far, clear functions have been established for two circRNAs. A recent study suggested that the majority of circRNAs are mere side products of pre-mRNA splicing. Other studies have suggested that circRNAs may have biological functions based on the observation that even lowly expressed circRNAs are regulated. As conservation in evolution often implies functionality, we analyzed the conservation of mouse circRNA sequences across vertebrates. Compared with splicing sites not involved in circRNA biogenesis, the exonic sequences around the circRNA head-to-tail junctions showed higher conservation. Moreover, we analyzed the overlap of circRNAs detected in rat and mouse, and found that 23.6% of the circRNAs identified in mouse neuropil were also expressed in rat neuropil (Supplementary Fig. 2e). This observation is consistent with a recent study in which 20% of mouse circRNAs were detected in human cell lines, but higher than in another study in which only 4% of the mouse circRNAs were identified in human samples. The difference might be explained by different sampling depths, as most identified circRNAs were expressed at low levels and might therefore ‘stochastically’ escape detection. Indeed, the circRNAs detected in both mouse and rat samples were clearly of much higher abundance than those detected in only one sample (Supplementary Fig. 2f).

Here we present evidence for the developmental regulation of circRNAs in neurons. There were many circRNAs whose abundance changed independent of the host linear transcript, suggesting a circRNA-specific regulation of biogenesis and/or turnover. The development of the CNS and brain involves neuronal maturation, neurite outgrowth and synaptogenesis. Non-coding RNAs such as miRNAs and IncRNAs have emerged as important components for regulating these developmental processes.

Recently, for example, the lncRNA RMST was identified as a factor that is important for neuronal differentiation as well as a co-regulator of SOX2, a mediator of neuronal stem cell fate. We identified a set of circRNAs that were differentially expressed in the mouse hippocampus at different developmental stages (E18 to P30). A circRNA that was markedly downregulated at later stages arose from the host gene coding for Rmst, thereby supporting a potential function of circRNAs in brain development. In contrast, the expression of circRkh1@ was increased at P30 (P21) compared with E18 (P4), indicating a putative role of this circRNA during synaptogenesis or when mature synapses have formed. In summary, we found a shift in the expression pattern for a large set of circRNAs associated with the onset of synaptogenesis, indicating a role of circRNAs in hippocampal development.

The brain is the most plastic organ and its circuits undergo tight regulation and modification throughout the entire lifespan of animals. Both the stability and flexibility of neuronal networks is central to all behavior, including learning and memory. Experience-dependent alterations in the connectivity of neural networks can result in plasticity of intrinsic excitability and synaptic strength. We induced homeostatic plasticity by treating cultured hippocampal neurons with the GABA_A receptor antagonist bicuculline and observed a dynamic change in circRNA expression. Notably, a circRNA (circHomer1_a) derived from the Homer1 linear transcript was the most significantly upregulated circRNA after plasticity. The Homer1 protein has a major role in the organization of the postsynaptic density. It is known that neuronal activity causes an increase in expression of an immediate early gene variant of Homer1, Homer1a, whereas the expression of Homer1b/c is relatively unchanged. The unbalanced regulation of different Homer1 isoforms contributes to homeostatic downscaling such that the truncated protein encoded by Homer1a interferes with the
native interaction between mGluR and Homer 1b/c-encoded functional scaffold protein. The head-to-tail junction formed in circHomer1_a uses the splicing donor of intron 5, which is only required to splice the Homer 1b/c transcripts, but not the Homer 1a transcript. Thus, the biogenesis of circHomer1_a could compete with that of Homer 1b/c mRNA. Upregulation of circHomer1_a could then prevent the potential over-expression of Homer 1b/c, which would otherwise be detrimental to homeostatic synaptic downscaling. It is therefore conceivable that transcriptional upregulation, the predominant usage of an upstream polyadenylation signal, and circRNA biogenesis work together to achieve the same goal, that is, to reduce the interaction of surface mGluR and scaffold Homer protein. However, it should be noted that only a few circRNAs showed co-regulation with their host genes; more common was the observation that circRNAs exhibited changes independent of the cognate mRNA following plasticity.

Finally, as a heterogeneous group of transcripts, it is very likely that circRNAs affect cellular and neuronal function via a diverse set of mechanisms. The different data sets accumulated in this study should serve as a rich resource for future functional research, where genetic perturbation of specific circRNAs followed by careful phenotypic examination in different in vitro and/or in vivo neuronal systems will be needed to shed more light on circRNA function in the nervous system and specifically to address their role in learning and memory.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. The sequencing data have been deposited at Gene Expression Omnibus (GEO) under accession number GSE61991.

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AUTHOR CONTRIBUTIONS

X.Y. and G.T. designed and performed all of the bioinformatic analysis and edited the manuscript. I.V., A.B., T.W., I.E. and G.A. designed, conducted and analyzed the experiments and edited the manuscript, with the help of M.W., C.G., C.Q., T.C., X.W., J.H., H.L. and W.S. S.S. performed and analyzed the electrophysiology experiments. W.C. and E.M.S. conceived and supervised the project, and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Tissue collection and hippocampal microdissections. Wild-type C57B6 or C57BL/6j-Etv1-ARRAY TSS88 (male and female) mice and Sprague Dawley (male) rats were housed in standard cages and fed standard lab chow and water ad libitum. All animal work was performed following regulations of German animal welfare law. For the developmental studies, hippocampi were dissected from mice at the age of E18, P1, P10 and P30. For tissue profiling, two wild-type C57B6 male mice at the age of 20 weeks were used to dissect the brain, heart, liver, lung and testes. To profile distinct sub-neuronal compartments (soma and neuropil), C57BL/6j-Etv1-ARRAY TSS88 male and female mouse and rat male hippocampal slices (500 μm) were prepared from 4–5-week-old animals and microdissected as described previously for rat5. All tissues were collected in RNA later (Ambion) and subsequently lysed in Trizol (Invitrogen) to extract RNA following the manufacturer's instructions.

Preparation of synaptosomes. Synaptosomes were purified from 20 hippocampi of adult mice (4 weeks old) as previously described15. The hippocampi were homogenized in ice-cold sucrose buffer (320 mM sucrose, 5 mM HEPS, pH 7.4) with a 15-ml Teflon-glass tissue grinder and homogenized with eight even strokes. The homogenate was subjected to three differential centrifugations (1,000 g for 10 min, supernatant was further subjected to centrifugation at 12,000 g for 10 min followed by 13,000 g for 10 min) before applying one gradient centrifugation (3%, 10%, 15% and 23% PercollPlus (GE Healthcare) in sucrose buffer at 31,000 g for 30 min). The latter resulted in separation into five different fractions. The fraction at the interface of the 15% and 23% Percoll contained the most pure synaptosomes, as verified by western blot (enrichment of AMPA receptor subunit, GluR4, substantial depletion of glial fibrous marker protein GFAP), and was therefore used for all experiments performed in this study.

Polysome profiling. Mouse brains were collected, snap frozen in liquid nitrogen and stored at –80 °C. Frozen mouse whole brain was pulverized under liquid nitrogen and the powder lysed in 1 ml of lysis buffer (10 mM HEPES pH 7.4, 150 mM KCl, 10 mM MgCl2, 0.5% NP40 (vol/vol), 0.5 mM DTT, 100 μM nitrogen) with 200 μl proteinase K in 1% SDS (pH 4.5), followed by ethanol precipitation, then converted to double stranded DNA using Trizol (Invitrogen). The DNA was then treated with Trizol (Invitrogen) followed by RNA isolation. Reverse transcription was performed using random hexamers and reverse transcriptase (SSIII, Invitrogen). Quantitative PCR was done using SYBR green master mix (Roche). For RNA transcripts, one primer was designed to anneal at the circular junction whereas the other was within the circRNA transcript. For linear transcripts, both primers were designed to amplify segments within the gene (top1k, top2k, top3k, top5k, top10k), and then tested for the enrichment of GO terms for the circRNA-hosting genes within the same gene set. All quantitative PCR primers are listed in Supplementary Table 4.

Processing of PacBio sequencing data. Circular consensus reads (CCSreads) obtained from PacBio sequencing were aligned to custom database (consisting of different scaffolds from both linear mRNAs and circRNAs) using Blast (parameters: -evalue 1E-10 -word_size 9). We reported the alignments with identity higher than 95% for both linear and rolling-circle products using an in-house perl script.

Conservation analyses. The positions of rat circRNAs were converted to mouse (mm9) genome coordinates using the UCSC LiftOver tool, then were intersected with mouse circRNA using BEDTools. To examine the evolutionary conservation of the sequences around mouse circRNA junctions, we downloaded PhastCons scores for alignment of 29 vertebrate genomes with mouse (mm9) from (http://hgdownload.soe.ucsc.edu/goldenPath/mm9/phastCons30way/vertebrate/). To rule out possible biases, we compared the sequences around the splicing sites involved against those not involved in the formation of head-to-tail junction from the same gene locus.

Gene Ontology enrichment analysis. We performed Gene Ontology enrichment analysis using DAVID (http://david.abcc.ncifcrf.gov). The background gene set consists of all expressed genes (TTO > 0.01) and the test gene set consists of all expressed circRNA-hosting genes. In Supplementary Figure 2a, we chose a background gene set consisting of the 1,000, 2,000, 3,000, 5,000, 10,000 most highly expressed genes (top1k, top2k, top3k, top5k, top10k), and then tested for the enrichment of GO terms for the circRNA-hosting genes within the same gene set, respectively.
High-resolution in situ hybridization in primary cells and slices. Dissociated rat hippocampal neurons were prepared and maintained as described previously.47, 48 4-week-old rats were perfused with 1× PBS and 4% paraformaldehyde solution (vol/vol), the lung and liver were dissected, sliced to 1 mm, and fixed for 3 h. Lung and liver cells were dissociated and plated to poly-l-lysine–coated dishes as described.67 We performed in situ hybridization using the QuantGene ViewRNA miRNA ISH Cell Assay for Fluorescence miRNA and RNA In situ Hybridization (RNA FISH) with custom-made probes targeting the circRNA exon junctions or the cognate mRNA (see Supplementary Table 5 for circRNA probe sequences). Cultured neurons (DIV 26–28 or DIV 4/21 for developmental studies) were fixed for 1 h at 21–23 °C using a 4% paraformaldehyde solution (4% paraformaldehyde, 5.4% glucose (wt/vol), 0.01 M sodium metaperiodate in lysisine-phosphate buffer). The in situ hybridization was performed following the manufacturer’s protocol omitting the dehydration/rehybridization step as well as the protease treatment. Dendrites were stained using an anti-Map2 antibody (Millipore AB5622, 1:1,000 dilution). Liver cells were stained with anti-Albumin antibody (Abcam ab106582, 1:50 dilution) and lung cells with anti-Heme Oxygenase 1 antibody (Abcam ab13243, 1:200 dilution). For in situ hybridization in hippocampal slices, slices were dissected and fixed overnight in 4% PFA in PBS solution at 4 °C, gently shaking. Hippocampal slices were embedded in 4% low melt agarose and 30-μm sections were prepared using a vibratome (Leica VT 1200s). The in situ hybridization was performed as described above with a few modifications from the manufacturer’s protocol: the slices were post-fixed for 10–15 min at 21–23 °C, washed with PBS and incubated for 15 min with the detergent solution. After completion of the in situ hybridization the slices were blocked for 1 h at 21–23 °C in 1% BSA/1% Triton X-100 in PBS (wt/vol for BSA and vol/vol for Triton X-100). Slices were incubated with the primary antibody anti-Map2 (Millipore AB5622, 1:1,000 dilution) overnight at 4 °C to stain dendrites, washed three times with 1× PBS and incubated with the secondary antibody (Invitrogen, A11008, Alexa 488-goat anti-rabbit; 1:1,000 dilution) stain dendrites, washed three times with 1× PBS and incubated with the secondary antibody (Invitrogen, A11008, Alexa 488-goat anti-rabbit; 1:1,000 dilution) and DAPI (1:1,000 dilution) to visualize nuclei for 2 h at 21–23 °C.

Image acquisition and processing. Confocal microscopy was performed using a Zeiss LSM780 confocal laser fluorescence microscope system. Image analysis was done from z stacks of confocal image series of 10–30 confocal planes taken at 35–50-μm intervals using a 40× oil immersion objective. For analysis, conducted in a non-blind manner, cell bodies were circumscribed and dendrites straightened using the software ImageJ. To quantify the in situ signal in cell bodies, the average fluorescence intensity was measured per z stack and normalized to the area of the circumscribed cell body. In dendrites the particle abundance was determined using a custom MATLAB script, then the number of particles was normalized to the area of the straightened dendrite. The statistical significance of the in situ data was tested using either an unpaired t-test or Mann-Whitney U test according the normality of the distribution pre-tested using Lilliefors test.

Electrophysiology. Whole-cell patch-clamp recordings were made with an Axopatch 200B amplifier from cultured hippocampal neurons (DIV 28–29) bathed in HBs containing 11 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 30 mM glucose, 10 mM HEPES [pH 7.4; ~310 mOsm] plus 1 μM TTX and 20 μM bicuculline. Whole-cell pipette internal solution contained 120 mM potassium gluconate, 20 mM KCl, 0.1 mM EGTA, 2 mM MgCl2, 2 mM ATP, 0.4 mM glucuronate triphosphate, 10 mM HEPES (pH 7.2; ~300 mOsm) and the pipette resistances ranged from 4–6 MΩ. Bicuculline was added in conditioned media for 12 h and the media was replaced with the HBS 15 min before recording. Neurons were voltage clamped at ~70 mV while the series resistance was left uncompensated during the recordings. mEPSCs were analyzed offline using Stimfit software by employing a template-matching algorithm. Recordings were started 5 min after patching and the recording duration usually ranged from 5–10 min. Statistical differences between experimental conditions were determined by Mann-Whitney U test.

Potential miRNA binding sites. To quantify miRNA binding sites, exonic sequences within each circRNA were concatenated using Ensembl annotation, and the number of predicted miRNA binding sites (7mer-m8)49 for all miRNA (deposited in miRBase version 19)10 was counted. As a control, the same procedure was performed on CDS and 3′ UTR of the protein-coding genes.

Potential RBP binding sites. We predicted the RBP binding sites based on their sequencing motifs deposited in RBDB2. Predicted RBP binding sites on circRNAs were compared to those on CDS and 3′ UTR of protein-coding genes.

Potential translatability of circRNAs. To estimate the translational capacity of circRNAs, we studied their association with ribosome complexes. We performed polysome profiling on mouse brain and ribosome footprinting on rat brain. Sequencing reads from four fractions of mouse brain (free, 60S, 80S and polysome) and RPs of rat brain were aligned to circRNAs using BWA, and reads spanning the circular junctions were counted and converted to TPM as described above.

To investigate potential peptides arising from circRNA candidates, a liquid chromatography mass spectrometry sequencing was conducted on total lysate from 21-d-old primary neurons without any pharmacological or electrophysiological treatment. The genomic representation of circRNA candidates was translated in six potential frames (three frames per strand) and the position of the circRNA junction was recorded. This custom database was merged together with the rat protein RefSeq database and was used as a template for peptide matching with Mascot. A custom Perl script was used to identify peptides crossing the circular junction position—such peptides could only arise from circRNA translation.

Statistical analysis. For statistical analysis, two-sided one-sample t-test (Fig. 1f), two-sided unpaired t-test with Welch’s correction (Figs. 2b and 3c) and two-sided Mann–Whitney U test (Fig. 4a.e) were performed. In Figures 1f, 2b, and 3c, whiskers show extreme data points no more than 1.5 times the interquartile range; and in Figures 3f and 4a.e whiskers show minimum to maximum. The statistical significance of the in situ data was tested using either an unpaired t-test or Mann–Whitney U test since the normality of the distribution was pretested using Lilliefors test. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those generally employed in the field. Data collection and analysis were not performed blind to the conditions of the experiments and no randomization of data was performed.

A Supplementary Methods Checklist is available.

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