The mRNA-Binding Protein RBM3 Regulates Activity Patterns and Local Synaptic Translation in Cultured Hippocampal Neurons

Sinem M. Sertel,1,2 Malena S. von Elling-Tammen,1 and Silvio O. Rizzoli1,2

1Institute for Neuro- and Sensory Physiology, University Medical Center Göttingen, Göttingen, 37073, Germany, and 2Cluster of Excellence “Multiscale Bioimaging: from Molecular Machines to Networks of Excitable Cells” (MBExC), University of Göttingen, Göttingen 37073, Germany

The activity and the metabolism of the brain change rhythmically during the day/night cycle. Such rhythmicity is also observed in cultured neurons from the suprachiasmatic nucleus, which is a critical center in rhythm maintenance. However, this issue has not been extensively studied in cultures from areas less involved in timekeeping, as the hippocampus. Using neurons cultured from the hippocampi of newborn rats (both male and female), we observed significant time-dependent changes in global activity, in synaptic vesicle dynamics, in synapse size, and in synaptic mRNA amounts. A transcriptome analysis of the neurons, performed at different times over 24 h, revealed significant changes only for RNA-binding motif 3 (Rbm3). RBM3 amounts changed, especially in synapses. RBM3 knockdown altered synaptic vesicle dynamics and changed the neuronal activity patterns. This procedure also altered local translation in synapses, albeit it left the global cellular translation unaffected. We conclude that hippocampal cultured neurons can exhibit strong changes in their activity levels over 24 h, in an RBM3-dependent fashion.

Key words: circadian; local translation; primary hippocampal culture; RBM3; RNA-binding protein; synaptic transmission

Significance Statement

This work is important in several ways. First, the discovery of relatively regular activity patterns in hippocampal cultures implies that future studies using this common model will need to take the time parameter into account, to avoid misinterpretation. Second, our work links these changes in activity strongly to RBM3, in a fashion that is independent of the canonical clock mechanisms, which is a very surprising observation. Third, we describe here probably the first molecule (RBM3) whose manipulation affects translation specifically in synapses, and not at the whole-cell level. This is a key finding for the rapidly growing field of local synaptic translation.

Introduction

Maintaining a synchronous pattern of day and night activity is critical for the function of all of the tissues of a mammalian organism. This is ensured by several well-established mechanisms, the first of which is the rhythmic expression of molecular clock genes in every cell throughout the day/night (Partch et al., 2014). These genes control the timing of many biological functions, such as glucose metabolism and electrical activity (Dibner et al., 2010). A second fundamental mechanism is provided by the function of the suprachiasmatic nucleus (SCN), a central pacemaker of the hypothalamus, which is in charge of the molecular clock synchronization among the cells of the animal (Welsh et al., 2010). The SCN achieves this by encoding time information in its spontaneous firing rate (low during the night, and high during the day) (Colwell, 2011), and by communicating this to other brain regions and tissues through synaptic projections and hormones (Buijs et al., 2006).
The rhythmic expression of clock genes in the SCN controls the expression and function of ion channels as the BK channels (large-conductance calcium-activated potassium channels) or L-type voltage-gated calcium channels (Colwell, 2011). The function of these proteins induces oscillations in the resting membrane potential (Pennartz et al., 2002; Kononenko et al., 2008), thereby changing the firing rates, which in turn ensures the rhythmic firing activity of the SCN, which has been demonstrated even in dispersed cultures (Green and Gillette, 1982; Herzog et al., 1998). The rhythmic firing is resistant to disturbances in the light-dark cycle (Kuhlman and McMahon, 2004; Nakamura et al., 2011), and it persists in SCN cultures that are not subjected to day/night light or temperature changes. However, clock gene expression alone is not sufficient to maintain the synchronized firing of SCN neurons in the long-term. In culture, they slowly become desynchronized, with every cell eventually assuming its own individual firing pattern that oscillates throughout the day and night cycle (Welsh et al., 1995). The desynchronization is accelerated by blocking network activity, suggesting that neuronal communication is important in maintaining the rhythm synchronicity for long time intervals (Honma et al., 2006; Yamaguchi et al., 2003).

The observation of rhythmic activity in dispersed SCN cultures prompted research also in other cell types. Fibroblast cell lines were found to exhibit molecular clock rhythmicity, albeit they lose cell synchronicity rapidly (Nagoshi et al., 2004), unless they are resynchronized by regular changes in temperature (Brown et al., 2002) or culture media (Balsalobre et al., 1998). However, many brain areas have been little investigated in relation to rhythmic activity (Paul et al., 2020). A prominent example is the hippocampus, which is involved in learning and memory, two processes that are strongly regulated by the circadian clock (Gerstner and Yin, 2010). Hippocampal activity in vivo oscillates throughout the day and night cycle (Munn and Bilkey, 2012), and its ability to respond to plasticity-inducing stimuli is also dependent on the time of day/night (Harris and Teyler, 1983). This demonstrates that the hippocampus function is governed by the 24 h cycle but leaves open the question of whether this is exclusively because of the general rhythmicity induced by the SCN, or whether this is a fundamental hallmark of the hippocampal neuron, which would persist in dissociated cultures.

To solve this question, we turned to the rat hippocampal culture. Surprisingly, we found that the culture activity exhibited significant oscillations throughout 24 h, which were accompanied by substantial changes in presynaptic activity and synapse size. In addition, we found that the abundance of RNA-binding motif 3 (RBM3), a cold-shock protein (Danno et al., 1997, 2000) that is known to promote translation (Dresios et al., 2005), also oscillates throughout 24 h, especially in synapses. Its knockdown changed the activity pattern of the neurons, as well as synapse activity and size, possibly through effects on local translation. Overall, these data suggest that hippocampal cultures exhibit endogenous changes in activity levels across 24 h, and that these patterns are under the control of RBM3.

**Materials and Methods**

**Hippocampal cultures.** Primary dissociated hippocampal cultures were prepared from newborn rats (Banker and Cowan, 1977). The hippocampi were dissected from rat brains, using animals of both sexes, with a general female to male ratio of 1:1. They were washed with Hank's balanced salt solution (Thermo Fisher Scientific). Later on, hippocampi were kept in the enzyme solution (1.6 mM cysteine, 100 mM CaCl2, 50 mM EDTA, and 25 units papain in 10 ml DMEM) for 1 h. To inactivate the enzyme solution, 5 ml DMEM (Thermo Fisher Scientific) that contains 10% FCS, 0.5% albumin, and 0.5% trypsin inhibitor was added and incubated for 15 min. Cells were further separated by mechanical disruption and were seeded on poly L-lysine (Sigma Millipore) coated circular coverslips (1.8 cm in diameter) with a density of 80,000 cells per coverslip. The neurons were kept in plating medium (3.3 mM glucose, 2 mM glutamine, and 10% horse serum in DMEM) for 1–2 h at 37°C. Afterward, the medium was exchanged to Neurobasal-A medium (with 2% supplement, 1% GlutaMax, and 0.2% penicillin/streptomycin mixture). The cultures were maintained at 37°C and 5% CO2 for ～20 d.

**Calcium imaging.** Neurons were transduced with 3 μl of NeuroBurst Orange Lentivirus (Sartorius) at DIV10, and kept in the incubator for 9 additional days. For imaging, the coverslips were placed into imaging chamber and imaged with and inverted Nikon Ti eclipse epifluorescence microscope equipped with a 20× Plan Apo (Nikon) objective, an HBO-100W lamp, an IXON X3897 Andor camera, and a cage-incubator (Okolab). The temperature was set at 37°C and the atmosphere with 5% CO2 throughout the imaging session. For long-term recordings, neurons were plated in a glass-bottom 24-well plate (Cellvis) and imaged directly from the plate.

**Promoter reporter imaging.** The plasmid for the reporter promoter imaging was synthesized by GenScript, using pUC57 as a backbone. The promoter was selected as the sequence from 500 nucleotides in the upstream until 50 nucleotides in the downstream of the BMAL1 gene from *Rattus norvegicus*. The plasmid expressed EGFP under the control of this promoter. The EGFP was destabilized by adding to its C terminus the residues 422–461 of mouse ornithine decarboxylase, which provides a 2 h half-life time for the molecule (Gerstner and Yin, 2010).

**Immunostaining.** Neurons were washed with the Tyrode’s buffer (124 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 30 mM D-glucose, and 25 mM HEPES) and then fixed with 4% PFA. The immunostaining procedure for synaptotagmin1 uptake assay. In order to study the synaptic vesicle usage, we took advantage of live staining with an antibody targeting the luminal domain of Synaptotagmin1. At DIV21, coverslips with neurons were placed in a new 12-well plate (Greiner Bio-One) with 300 μl of their own Neurobasal-A medium. Neurons were incubated with 2.5 μg/ml Syt1-Atto647N antibody (105311AT1, Synaptic Systems) for 45 min. Afterward, 16.7 μM anti-mouse secondary nanobody (N2002-At542-S, Nanotag) conjugated to Atto542 was added into the medium and incubated for 15 min. Next, the neurons were washed with ice-cold Tyrode’s buffer and fixed with 4% PFA. The immunostaining procedure for synaptophysin is described in Immunostaining. In order to see the spontaneous vesicle fusion, the action potential generation was blocked by adding 5 μM TTX (Tocris Bioscience). In time-series experiments, the uptake assay was performed at different time points of the day and night. For the knocked down conditions, the uptake assay did not have secondary antibody incubation. The Syt1-Atto647N antibody was incubated for either 15 min or 1 h. An inverted Nikon Ti eclipse epifluorescence microscope (Nikon), which has a 20× Plan Apo
Puromycin assay. Puromycin (ant-pr-1, InvivoGen) is an antibiotic that interferes with mammalian translation and incorporates itself into the polypeptide chain. Coverslips were placed in a new 12-well plate with 300 μl of their own Neurobasal-A medium and were incubated with 1 μl of 0.3 mg/ml puromycin for 10 min in the incubator. Later on, they were washed twice with ice-cold Tyrode’s buffer and fixed with 4% PFA. As a control, another antibiotic called anisomycin was used. It halts the translation complex and does not allow puromycin to reach the binding site in the ribosome. Control groups were incubated with 0.13 μM anisomycin (AS562, Sigma Millipore) 10 min before puromycin treatment. Later, the immunostainings against synaptophysin, Homer1, and puromycin (MABE343, Merck Millipore) were performed as described in Immunostaining. Puromycin, anisomycin, and puromycin antibody were generous gifts from Prof. Peter Rehling (University Medical Center Göttingen, Göttingen, Germany).

Poly(A) staining. Oligo(dT) and oligo(dA) stainings were done as described previously (Chou et al., 2018). Briefly, neurons were fixed and quenched, as stated in Immunostaining. They were fixed one more time with ice-cold absolute methanol for 10 min. Cells were rehydrated first with 70% EtOH and then with 1 x Tris buffer, pH 8, for 10 min. Later on, neurons were washed with hybridization buffer [1 mg/ml yeast tRNA, 0.005% BSA, 10% dextran sulfate, 25% formamide in finalized 2× SSC (0.3 M NaCl, 30 mM trisodium citrate in water)] once and then incubated with 1000 of 1 μg/ml 30 nucleotides long either oligo(dT) or oligo (dA) conjugated to Atto647N (Sigma Millipore) for 1 h in hybridization buffer at 37°C. Samples were washed 2 times with 4× SSC and 2 times 2× SSC. The following immunostainings against synaptophysin and Homer1 were performed as specified in Immunostaining.

Transcriptomics. The mRNAseq experiments as well as the analysis were performed by Transcriptome and Genome Analysis Laboratory. Samples were sequenced with HiSeq-4000 (Illumina) with 50 bp single-end design. The alignment was performed with STAR 2.5.2a (Dobin et al., 2013), and the assignment of reads to genes was done by using featureCounts 1.5.0 (Liao et al., 2019) with R. norvegicus genome assembly r66 and gene version 91. After the count calculation of each transcript, we used the limma package to find differentially expressed transcripts (Ritchie et al., 2015). GO analysis was performed on the Webgestalt database with Ensembl gene IDs and difference folds between time points (Wang et al., 2017). The result of gene set enrichment analysis reports the pathways with p < 0.05 and false discovery rate < 0.05 (Table 1).

short-hairpin RNA (shRNA) virus preparation. The sequence for the shRNA was prepared with the help of the BLOCK-it RNAi Designer (Thermo Fisher Scientific). The shRNA sequence was synthesized by Genscript and placed in the pAAV-U6sgRNA (60958, Addgene) (Swiech et al., 2015). The plasmid of scrambled (Scr) shRNA was a generous gift of the Fornasiero laboratory (Keihan et al., 2019). Adeno-associated virus was produced in human embryonic kidney 293T (DSMZ) with three plasmids that have packaging proteins of recombinant adeno-associated virus, and were described previously (McClure et al., 2011). Human embryonic kidney cells were transfected with Lipofectamine 2000 (Thermo Fisher Scientific) using the manufacturer’s instructions. Three days later, transfected cells were harvested and centrifuged. The pellet was resuspended in 1 ml Tyrode’s buffer and was exposed to freeze/thaw cycles 3 times in 70% EtOH and dry ice mixture for lysis. After the addition of 1 μl of Nuclease (Thermo Fisher Scientific), the lysate was incubated at 37°C for 30 min and was centrifuged with 1000 x g for 5 min. The supernatant was aliquoted and stored at the –80°C freezer. The virus titration was performed by observing the GFP signal from serial dilution on transfected hippocampal cultures. The virus was used on the primary hippocampal culture at DIV15. The RBM3 shRNA sequence is as follows: CACCCGGGTC TCCCGGGCCCGAGCTGC GGCGGGGAGAGCCGGG TTTTTTT. The BMAL1 shRNA sequence is as follows: CACCGGCA AACTCAGCGAACATTTGCAGAATATGTGGCCTTGATGTTGC TTTTTTT.

Table 1. The biological process pathways determined by analyzing difference among the transcriptomes measured at different time points

| Description | Normalized enrichment score |
|-------------|-----------------------------|
| 0800 vs 1400 |                             |
| Ribonucleoprotein complex subunit organization | –1.92 |
| RNA splicing | –2 |
| Ribonucleoprotein complex biogenesis | –2.32 |
| 0800 vs 2000 |                             |
| Ribonucleoprotein complex biogenesis | –2.23 |
| Axon development | 1.94 |
| Cell morphogenesis involved in differentiation | 1.95 |
| Dendrite development | 1.93 |
| Synapse organization | 2 |
| Synaplin-plexin signaling pathway | 1.9 |
| Cell part morphogenesis | 1.91 |
| Synaptic transmission, glutamatergic | 1.97 |
| Regulation of neuron projection development | 1.88 |
| Neuron projection organization | 1.86 |
| 0800 vs 0200 |                             |
| Vesicle-mediated transport in synapse | 1.96 |
| Synaptic vesicle cycle | 1.97 |
| Glutamate receptor signaling pathway | 1.97 |
| Response to ammonium ion | 2 |
| Serotonin receptor signaling pathway | 1.93 |
| Amine transport | 2.02 |
| Regulation of trans-synaptic signaling | 1.92 |
| Response to anesthetic | 1.92 |
| Synaptic transmission, GABAergic | 2.03 |
| Regulation of postsynaptic membrane | 1.9 |
| Neurotransmitter receptor levels |                             |
| 2000 vs 1400 |                             |
| Defense response to other organism | 1.99 |
| Cytokine-mediated signaling pathway | 1.98 |
| Endothelium development | 1.97 |
| Integrin-mediated signaling pathway | 1.96 |
| Leukocyte migration | 1.91 |
| Humoral immune response | 1.85 |
| 0200 vs 1400 |                             |
| Cell adhesion mediated by integrin | 2.25 |
| Cilium organization | 2.16 |
| Smoothed signaling pathway | 2.06 |
| Extracellular structure organization | 2.05 |
| Integrin-mediated signaling pathway | 1.96 |
| Embryonic morphogenesis | 1.87 |
| Skeletal system development | 1.87 |
| Connective tissue development | 1.84 |
| Cardiovascular system development | 1.82 |
| Skin development | 1.8 |
| 0200 vs 2000 |                             |
| Cilium organization | 2.38 |
| Smoothed signaling pathway | 2.29 |
| Amine transport | –2.23 |
| Synaptic transmission, GABAergic | –2.42 |
| GABA signaling pathway | –2.16 |
| Synaptic vesicle cycle | –2.03 |
| Neurotransmitter transport | –2.03 |
| Regulation of neurotransmitter levels | –1.99 |
| Acid secretion | –1.96 |
| Response to amine | –2 |

We performed a gene set enrichment analysis by comparing the transcriptomes obtained at different time points, using the Webgestalt database (Wang et al., 2017). The table shows up to 10 nonredundant biological process pathways whose p value was < 0.05 and false discovery rate was < 0.05.

Experimental design and statistical tests. To find correlations between two groups, Pearson’s correlation was used (see Figs. 1E, 2B, 6F). For long-term calcium imaging where individual neurons were traced, the Friedman test followed by Dunn’s multiple comparisons test was used (see Figs. 4B, 5E, 8B,D, 9H,I, 10F). For parametric time-series data, the one-way ANOVA test followed by Dunnett’s multiple comparisons test was used (see Figs. 3D,G,H,J,K, 9G, 10H,J). For analysis between groups, we used (Nikon) objective, an HBO-100W lamp, and an IXON X3897 Andor camera, was used for imaging, and the images were analyzed using MATLAB (The MathWorks).

We observed the GFP signal from serial dilution on transfected hippocampal cultures. The virus was used on the primary hippocampal culture at DIV15. The RBM3 shRNA sequence is as follows: CACCCGGGTC TCCCGGGCCCGAGCTGC GGCGGGGAGAGCCGGG TTTTTTT. The BMAL1 shRNA sequence is as follows: CACCGGCA AACTCAGCGAACATTTGCAGAATATGTGGCCTTGATGTTGC TTTTTTT.
Figure 1. The average neuronal activity in dissociated hippocampal cultures oscillates during the day/night. **A**, To determine the firing pattern of dissociated hippocampal neurons, we transfected cultured hippocampal neurons with the genetically encoded \( \text{Ca}^{2+} \) indicator NeuroBurst at DIV10. Starting at DIV18, we imaged neurons every 4 h, relying on a continuous 45 s recording protocol. This provided a sample of activity at the particular time points, while being sufficiently mild to avoid phototoxicity. **B**, To visualize the overall activity at every time point, we...
Mann–Whitney test (see Figs. 6B, 7B,D,F,H1J,K, 10A-G). For multiple comparisons, we used two-way ANOVA test followed by Tukey’s multiple comparisons test (see Fig. 3CF). Tests were performed by GraphPad Prism software version 8.3.0 (GraphPad Software).

**Results**

**Neuronal activity changes throughout 24 h in dissociated hippocampal cultures**

Primary hippocampal cultures are widely used, as they are relatively simple to prepare and maintain most of the important functional features of the *in vivo* neurons (Dotti et al., 1988). We used them here, relying on a classical protocol (Banker and Cowan, 1977) that dissociates the hippocampi of newborn rats and results in mixed glia and neuron cultures. Most of the neurons are glutamatergic (>90%) (Benson et al., 1994) and have a mature morphology and synapse development already at ~12 DIV.

To determine whether primary hippocampal cultures show differences in their electrical activity in dependence on the time of day/night, we performed long-term calcium imaging, using a genetically encoded calcium indicator, NeuroBurst (Sartorius). To sample culture activity regularly, we imaged the neurons (starting at DIV18) every 4 h, for 45 s (Fig. 1A). This enabled us to obtain a fluorescence-based measure of the activity of the individual neurons at the particular time points (Fig. 1B), which we termed “activity score.” Individual neurons exhibited changes in the activity score throughout the day and night, with the examples shown in Figure 1B having strong peaks at 6:00 and 14:00. Such changes in activity were observed for all neurons investigated (see a selection in Fig. 1C). To test whether the activity of the different neurons was synchronized, we performed this experiment with four different culture preparations, in which we tracked 156 different neurons. Their average activity score showed significant changes throughout the measurement (Fig. 1D), with activity being high at 14:00, dropping until ~22:00, and rising again after 02:00, before dropping again for several hours, and finally rising one more time before the end of our measurements. Randomizing the timing of the individual neuronal measurements eliminates all significant changes (Fig. 1G,H), which suggests that the results obtained here are unlikely to be because of chance, but are rather because of synchronous culture activity. The pattern observed does not conform to a precise 24 h pattern (Fig. 1D). Two possible interpretations could be made. First, the cultures exhibit their own pattern of activity, which does not relate to a 24 h rhythm. Second, the activity of the individual neurons does conform to a 24 h rhythm, but they are partially desynchronized, so that a 24 h rhythm is no longer observed at the whole culture level, especially when averaging results across different cultures, as in Figure 1D. The second interpretation appeared probable, since 24 h patterns are difficult to maintain with precision even in SCN cultures (Honma et al., 1998). Interestingly, the first 32 h of the recordings overlapped well with the next 32 h (Fig. 1E). Similar observations were made for younger cultures (DIV10; Fig. 1F). Again, the pattern did not seem to conform to a simple 24 h cycle.
Figure 3. Synaptic vesicle recycling measurements confirm the existence of activity oscillations throughout 24 h. A, To measure the presynaptic activity, we performed a Syt1 uptake assay (Matteoli et al., 1992; Kraszewski et al., 1995) at DIV18, at different times of day and night. To label the recycling vesicles, an Atto647N-conjugated Syt1 antibody was added to the cell culture medium for 45 min (1). The antibody recognizes a luminal (intravesicular) epitope and is taken up during synaptic vesicle recycling. The 45 min incubation is sufficient to saturate all of the recycling vesicles (2), and thereby provides an estimate for the total recycling pool. The nonrecycling (reserve) pool of vesicles, which is larger than the recycling pool (Rizzoli and Betz, 2005), is not depicted here. To then obtain an estimate for the overall activity of the neurons at the particular time points, we applied Atto532-conjugated secondary nanobodies (NB) that target the Syt1 antibody, for 15 min (3). The nanobodies label only a subset of the vesicles, in proportion to the activity levels (4). The neurons were subsequently fixed, and were immunostained for synapticophysin (Syph) to label presynaptic compartments. To determine whether the assay indeed functioned, we blocked network activity with TTX, which only allows the Syt1 antibodies to bind to surface epitopes, or to spontaneously recycling vesicles (Truckenbrodt et al., 2018). B, Exemplary images of neurons tested at 08:00 or 20:00, along with a TTX treatment example. Scale bar, 10 μm. C, F, The Syt1 and NB intensities were measured, with and without TTX treatment. Each symbol represents the average intensity of synapses in one image. N = 4 independent
To test this in more detail, we analyzed whether the activity patterns of individual neurons correlated significantly to the 24 h pattern of a bona fide molecular clock gene. We expressed in our cultures a destabilized GFP molecule, relying on the promoter of the clock gene BMAL1. The fluctuations in the GFP amounts, which report the BMAL1 promoter activity, conformed to a 24 h cycle, with peaks at night and lower values during the day (Fig. 2). In parallel, we analyzed the activity patterns of the GFP-expressing neurons (Fig. 2). We found that their activity patterns correlated significantly (albeit negatively) to the BMAL1 promoter activity. This implies that the activity of individual neurons in these cultures can be seen as exhibiting a 24 h rhythm, albeit this is difficult to observe when averaging many neurons and independent cultures, because of a partial desynchronization.

The dynamics of the synaptic vesicles also change throughout 24 h

Oscillations in neuronal activity should also be reflected at the synaptic level, especially in the synaptic vesicle dynamics. The vesicle behavior can be analyzed with precision by using antibodies that detect the luminal (intravesicular) domain of the vesicular calcium sensor synaptotagmin 1 (Syt1) (Matteoli et al., 1992; Kraszewski et al., 1995). The antibodies are taken up by synaptic vesicles during their recycling, since they expose the luminal epitopes during exocytosis, and thus enable the antibody to penetrate into the vesicles, and to be endocytosed (Fig. 3A). We incubated the cultures every 6 h with fluorescently conjugated Syt1 antibodies for 45 min. This time interval is sufficient to label (saturate) all active synaptic vesicles, and therefore to provide a measure of the active vesicle pool size (Truckenbrodt et al., 2018). We then applied to the cultures fluorescently conjugated nanobodies that recognize the Syt1 antibodies, for 15 min. The nanobodies bind Syt1 antibodies that are exposed to the surface during vesicle activity (Fig. 3A). This short incubation interval does not saturate all binding sites (Truckenbrodt et al., 2018), and therefore provides a measure of synaptic activity at the respective time point, rather than a measure of the vesicle pool size. To confirm the validity of this assay, we compared normal neurons with neurons in which network activity was blocked using the Na+ channel inhibitor TTX. TTX blocked active vesicle recycling, and therefore reduced both the antibody and nanobody stainings (Fig. 3B,C,F), as expected.

These measurements suggested that the size of the actively recycling vesicle pool is relatively constant throughout 24 h (Fig. 3D), but the synaptic activity, as measured by the nanobody intensity, exhibits significant differences (Fig. 3G). None of these measurements showed any changes in cultures maintained constantly in TTX, as expected (Fig. 3E,H).

Overall, these experiments confirm the idea that, at the synapse level, neurons show changes in their activity patterns throughout 24 h. As these measurements only targeted the active vesicles, which make up only ~50% of all vesicles (Rizzoli and Betz, 2005), we sought to also obtain a measurement of the entire vesicle pool, by immunostaining the synapses at different time points, relying on the vesicle marker synaptophysin (Takamori et al., 2006). This again showed changes during 24 h, with a substantial increase at 02:00 (Fig. 3I,K). We observed similar behavior for a marker of the postsynaptic density, Homer1 (Fig. 3K). No changes could be detected in the number of synapses (Fig. 3J). These results suggest that not only neuronal and synapse activity, but also synapse size, depends on the time of day/night.

Synaptic mRNA amounts are subject to change over 24 h

Along with brain activity, brain metabolism also changes throughout the day and night cycle, including aspects as transcription and translation, which have been shown to exhibit strong circadian rhythmicity (Noya et al., 2019). We therefore proceeded to test whether such changes could also be observed in cultured hippocampal neurons. We analyzed the mRNA levels in the cultures, relying on FISH, performed with fluorescently conjugated oligonucleotides containing multiple thymidine (dT) moieties. These label specifically the polyadenylated tails of mRNAs, and showed measurable signals throughout the cells, including synaptic areas (Fig. 4A). We analyzed the FISH signals and found that they changed throughout the 24 h (Fig. 4B). Nevertheless, these results demonstrate that dynamic changes of the mRNA levels take place over time in dissociated hippocampal cultures.

The abundance of RBM3 changes over 24 h, especially at synapses

To determine the molecular mechanisms responsible for the changes in neuronal activity, synapse morphology, and mRNA amounts throughout 24 h, we analyzed the transcriptome of the cultures at different times of day/night, using mRNA sequencing (mRNAseq) (Fig. 5A, N = 6 independent experiments). Although several overall changes could be seen among the different sets of genes, relating to processes, such as synaptic transmission and neuronal morphogenesis (Table 1), only one transcript showed a significant differential expression when the results from six different culture preparations were combined (Fig. 5B): RNA-binding motif 3 (Rbm3). This molecule showed the same general pattern of expression as bona fide clock genes, such as like Bmal1 and Per2 (Fig. 5C), but its variation among different cultures was small enough to result in significant differences between the time points, unlike Bmal1 or Per2. We assume that the desynchronization between different cell cultures is strong enough to mask the rhythmicity of Bmal1 or Per2, albeit at least the former is
RBM3 controls the firing pattern in primary hippocampal cultures

To determine whether RBM3 affects neuronal activity, we resorted to long-term calcium imaging (as in Fig. 1), in cultures subjected to RBM3 knockdown, or to the transfection of a scrambled oligonucleotide control. The RBM3 knockdown reduced the amounts of the protein significantly (Fig. 6A,B), albeit not completely. When analyzing in parallel the calcium signals from the knockdown cultures and their controls, it became obvious that activity could still be detected in both conditions (Fig. 6C,D), but that the patterns were different. The RBM3 knockdowns had peaks of activity at precisely the time points when the control cultures had their minimal activity (Fig. 6E,F). This relation was significant (Fig. 6F), implying that RBM3 has an important role in regulating the pattern of neuronal activity.

To test whether these effects also translated to synaptic vesicle activity, we relied on the Syt1 uptake assay used in Figure 3. We chose the time point that exhibited the highest difference in activity in calcium imaging (18:00; Fig. 6), and incubated the cultures with Syt1 antibodies for 15 min, to determine the activity levels (Fig. 7A–D), or for 60 min, in different coverslips, to measure the total size of the actively recycling vesicle pool (Fig. 7E-H). As cultures undergoing the knockdown treatment may be more fragile than unmodified cultures, we avoided the assay relying on mixtures of nanobodies and antibodies (from Fig. 3A), which involves multiple buffer changes that may harm the cultures, and we simply relied on separate 15 or 60 min incubations. Both measurements showed significant differences, with RBM3 knockdown enhancing synaptic function (Fig. 7B,F), in agreement with the change in activity observed at 18:00 in calcium imaging (Fig. 6). Performing these measurements in the presence of TTX, which blocks network activity, resulted in no significant differences between the knockdowns and the controls (Fig. 7D,H).

Finally, immunostainings for synaptophysin or Homer1, performed to determine the synapse size (as in Fig. 3I–K), suggested that RBM3 knockdown significantly influences the postsynapse size (Fig. 7I–K). Overall, these results demonstrate that RBM3 is strongly involved in the changes of synaptic and neuronal function throughout 24 h.

RBM3 controls local translation at the postsynapse

As mRNA amounts varied throughout 24 h (Fig. 4), and as RBM3 is an mRNA binding protein, we next sought to determine whether the RBM3 knockdown influences the mRNA levels in synapses, where the highest RBM3 oscillations were observed (Fig. 5). We repeated the FISH experiments performed in Figure 4, either in RBM3 knockdown neurons or in controls (Fig. 8). No significant changes could be observed: neither when relying on synaptophysin as a synaptic marker (Fig. 8A,B), nor when relying on the postsynaptic marker Homer1 (Fig. 8C,D). This suggests that RBM3 does not influence the synaptic mRNA levels, at least not sufficiently for detection with this FISH assay.
As RBM3 has been strongly linked to translation (Dresios et al., 2005; Smart et al., 2007), we next analyzed its potential influence on this process. We relied on an assay that reports the translation sites, the so-called puromycin assay (Hafner et al., 2019). Puromycin is an antibiotic that binds to the P site of the ribosome and incorporates itself into the polypeptide chain. This results in the polypeptide chain being released from the ribosome prematurely (Fig. 9A), thereby stopping the translation process. A subsequent immunostaining with a specific puromycin antibody reports all of the stopped translation sites, thereby providing an accurate estimate of ongoing translation in the particular cellular area. We combined this assay with the RBM3 knockdown (Fig. 9B) and found that this treatment significantly reduced local translation in postsynapses (Fig. 9G).
translation levels remaining in RBM3 knockdowns were close to the background levels, measured by pretreating the cultures with anisomycin, an antibiotic that halts the ribosomal complex and prevents the incorporation of puromycin (Fig. 9D,E,G). A similar trend was also observed in presynapses (Fig. 9C,F), albeit the local translation levels were too low for a clear differentiation between RBM3 knockdowns and controls (Fig. 9H). Importantly, when we analyzed the effects of the knockdown at the level of the whole cells, no significant difference could be measured (Fig. 9I). This suggests that RBM3 controls local translation in postsynapses, and possibly also in presynapses, but its effects do not extend, under these conditions, to the organization of translation in the entire cell.

Importantly, to study the influence of molecular clock on the primary hippocampal culture, we repeated several of the assays presented above with a knockdown for a core clock gene, Brain, and muscle ARNT like 1 (BMAL1). The significant reduction in BMAL1 gene expression on BMAL1 shRNA was verified using qPCR. Surprisingly, we have not observed any substantial changes in the BMAL1 knocked down neurons (Fig. 10). This confirms that the observations
Figure 7. Synaptic activity and morphology are modified on RBM3 shRNA treatment. A, E, To measure synaptic vesicle recycling, we relied on the Syt1 uptake assay, applying the antibodies for 15 or 60 min, in different experiments. The assays were performed on DIV18 neurons, at 18:00. GFP is used as a reporter for the expression of shRNA or a scrambled control sequence. Typical images are shown. Scale bar, 20 μm. B, F, The Syt1 intensity in 15 and 60 min incubations were measured. Symbols represent the mean of each image. Error bar indicates mean ± SEM.
we made above are specific for RBM3, which again underlines the importance of this protein for neuronal activity in these cultures.

Discussion

Our results suggest that hippocampal neurons in dissociated cultures maintain synchronized activity patterns, which are reproducible between independent coverslips and preparations, as demonstrated by both calcium imaging and measurements of synaptic vesicle dynamics. At the same time, their transcriptomes also show a tendency to synchronize, albeit only one protein, RBM3, showed significant differences between different time points, when multiple cultures were considered. RBM3 manipulations resulted in profound changes in the neuronal activity patterns. A potential mechanism for the RBM3 function may be through its modulation of local translation in synapses (Fig. 7), which it appears to affect in a specific fashion, with less influence on global translation.

An important issue is why peaks of activity appear at similar time points across different cultures. We speculate that the time of making the cultures is relevant, and we kept this parameter constant throughout all experiments, with the rats being delivered at 10:00 A.M. to 11:00 A.M., and the resulting cultures placed in the incubator between 4:00 P.M. and 5:00 P.M. The density of the cultures may also influence their synchronization in terms of activity, as mentioned in the Introduction, and as detailed in the following paragraphs.

Activity patterns in dissociated hippocampal neurons

Primary hippocampal cultures are prepared from mechanically and enzymatically dissociated hippocampi. The loss of the third dimension is a dramatic change for the network dynamics. At the same time, not having hormonal and temporal input from other regions makes it more difficult to synchronize the neurons in a culture. This is already known from SCN cultures, where the neurons demonstrate individual rhythmicity (Welsh et al., 1995), and can maintain 24 h rhythmicity when plated at high densities (Honma et al., 1998), but lose rhythmicity when the network communication is perturbed (Yamaguchi et al., 2003). Together, these findings suggest that network communication is essential for rhythmicity and synchronization in cultures, and that low-density cultures will lose synchronization relatively rapidly.

In view of these arguments, it was unclear whether hippocampal neurons would be able to synchronize over long periods in the culture, as the SCN neurons do (Watanabe et al., 1993). Interestingly, our findings are consistent with the observations...
Figure 9. RBM3 knockdown decreases translation at the postsynapse. A, To measure local translation at the synapse (1), we used the puromycin assay. Puromycin binds to the P site in the ribosome (2). It incorporates itself into the polypeptide chain and releases the polypeptide chain prematurely (3). A subsequent immunostaining for puromycin (4) enables an estimation of the amount of local translation. As a negative control, we treated the cultures with anisomycin (see D), which prevents puromycin binding. B, C, Puromycin immunostainings are shown, along with Homer1 or Syph stainings, to indicate postsynaptic and presynaptic sites. Scale bars, 2.5 μm. D, To measure the puromycin effect after protein synthesis inhibition, (1) we used anisomycin as a negative control before puromycin treatment. Anisomycin blocks the amino acid transfer to the polypeptide chain (2). Therefore, puromycin cannot be incorporated into the polypeptide chain (3). A subsequent immunostaining for puromycin (4) enables an estimation of the amount of puromycin incorporation, which have overcome the anisomycin effect. E, F, As a negative control, we performed the puromycin assay together with anisomycin as in D. Typical images for the negative control of the puromycin assay are shown together with Homer1 (postsynaptic marker) or Syph (presynaptic marker) staining. Scale bar, 2.5 μm. G, Puromycin staining intensities for the Homer1 areas are shown. Each dot represents the mean of an image. Error bar indicates mean ± SEM. N = 4 independent experiments; n = 20 images. The RBM3 knockdowns show significantly less translation at postsynapses (one-way ANOVA, followed by Dunnett’s multiple comparisons test). p = 0.0029. H, Puromycin staining intensities for the Syph areas are shown. Each dot represents the mean of an image. Error bar indicates mean ± SEM. N = 4 independent experiments; n = 20 images. The RBM3 knockdowns appear to lower translation, but the overall levels in presynapses are too close to the negative controls (anisomycin) for this difference to be determined with precision (the difference was not significant when tested by a Kruskal–Wallis test, followed by Dunn’s multiple comparisons test). p = 0.0536. I, An analysis of the global puromycin levels in RBM3 knockdowns and controls. Each dot represents the mean of an image. Error bar indicates mean ± SEM. N = 4 independent experiments. No significant difference was observed by a Kruskal–Wallis test, followed by Dunn’s multiple comparisons test, p = 0.9719. ns, not significant.
on SCN. Dissociated hippocampal neurons exhibit high or low activity at specific times of day/night, albeit a clear 24 h rhythmicity cannot be observed when averaging results across different cultures.

Neuronal activity was not the only factor that presented such a behavior. Presynaptic activity, synapse size, and mRNA amounts at the synapse also were changing throughout 24 h. These observations suggest that one of the most commonly used models for synaptic research, the primary hippocampal culture, has a time-dependent behavior. This makes it extremely important to acknowledge the timing of experiments performed with these cultures.
RBMs3 connects molecular clock genes to neuronal function

It has been repeatedly demonstrated that the molecular clock regulates genes that control neuronal activity, as discussed in the Introduction. This makes them excellent candidates for the regulation of rhythmic activity in cultured neurons. Surprisingly, we did not find any of the core clock genes to have a very clear transcription pattern in these cultures, which implies that they may not be very well synchronized among different neurons and different cultures, unlike RBM3. This molecule has been found in many time-series transcriptomics datasets as a daily (24 h) rhythmic gene (Yan et al., 2008; Zhang et al., 2014; Pembroke et al., 2015; Renaud et al., 2015; Terajima et al., 2017; Noya et al., 2019; Ray et al., 2020), and its oscillations in expression may be independent of at least some components of the molecular clock, as they still persist in BMAL1 KO cells (Ray et al., 2020). Overall, our work cannot state whether the RBM3 oscillations are controlled by the central molecular clock machinery in hippocampal cultures. However, its stronger synchronization (across cultures) than that of the canonical clock genes implies that Rbm3 expression may be independent from them.

Other than being a rhythmically expressed gene in the literature, RBM3 is a cold-shock protein, whose expression is induced in hypothermia conditions. For example, keeping a culture at 32°C instead of 37°C for 24 h induces Rbm3 expression (Chappell et al., 2001; Yang et al., 2019). Such temperature changes are not possible in the wells of a closed plate in the incubator, which eliminates the possibility that Rbm3 expression was synchronized by temperature changes in our experiments.

As a cold shock protein, RBM3 activates the translation machinery. Several studies have demonstrated that RBM3 enhances polysome formation, by phosphorylation of translation initiation factors and by changing the miRNA level (Chappell et al., 2001; Dresios et al., 2005). RBM3 has been described to enhance the translation of specific genes in hypothermia, thereby protecting synaptogenesis (Yan et al., 2019; Zhu et al., 2019). Moreover, although RBM3 is primarily located at the nucleus, one isoform has been found in dendrites, where it colocalizes with a ribosomal protein (Smart et al., 2007). In summary, these observations suggest that RBM3 is important for synaptic function, probably because of its role in translation, and possibly in local synaptic translation. Our findings suggest that RBM3 does not change the overall mRNA availability in synapses, but that it specifically changes local translation in synapses, without affecting the global translation. This effect may result in strong changes in synaptic activity, as explained below.

RBM3 may regulate synaptic function through local translation

Local translation appears to be an essential resource for neurons, since they need to strengthen or prune their connections in response to changes in synaptic activity. This implies that new proteins, as synaptic receptors, need to be incorporated dynamically in synapses. As neurons have extremely long neurites (Ishizuka et al., 1995), transport from the cell body would probably fail to satisfy the protein turnover needs of the synapses. To cope with this logistics challenge, neurons would need to place the translation machinery in synapses.

For a long time, electron microscopy images of synapse have demonstrated the presence of polyribosomes in the dendritic shaft and in the postsynapse (Steward and Levy, 1982; Ostroff et al., 2018). Later studies have shown that other components of the translational machinery, such as tRNAs, translation initiation factors, and elongation factors are present in synapses (Steward and Levy, 1982; Tiedge and Brosius, 1996; Sutton and Schuman, 2006). Despite these observations, direct evidence for translation in all synaptic compartments, and especially in the presynapse, has been difficult to obtain until recent assays demonstrated this thoroughly for both synaptic boutons and dendritic spines (Hafner et al., 2019).

Functional data have also offered strong support to the idea that local translation is an important feature of the synapses. Synaptic plasticity has been shown to depend on local translation (Miller et al., 2002). This process has also been linked to memory formation (Jones et al., 2018). Furthermore, electrical activity can be affected by the local translation as well, as in the case of the calyx of Held (Scarnati et al., 2018), where the inhibition of protein synthesis enhances spontaneous activity.

Overall, these observations suggest that local translation has important effects on synaptic transmission, and hence on plasticity, which last for hours. It is therefore evident that disturbing local translation would affect synaptic transmission, which in turn would influence the general network activity, as we observed in RBM3 KD experiments.

To our knowledge, this is the first time that RBM3 has been linked to changes in local translation, or to long-term neuronal activity changes. At the same time, our work demonstrates that broad changes take place in neuronal activity depending on the time of day/night, even in a simple model like dissociated hippocampal neurons in culture. This suggests that these cultures, which are far more common than SCN cultures, could become a useful model for circadian rhythm studies. Finally, the link between RBM3 and local translation may provide substantial further insight in the future, especially as the local translation field is now rapidly progressing through numerous innovative tools and concepts (Holt et al., 2019).

References

Balsalobre A, Damiola F, Schibler U, Gene C (1998) A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell 95:929–937.
Banker GA, Cowan WM (1977) Rat hippocampal neurons in dispersed cell culture. Brain Res 126:397–342.
Benson DL, Watkins FH, Steward O, Banker G (1994) Characterization of GABAergic neurons in hippocampal cell cultures. J Neurocytol 23:279–295.
Brown SA, Zumbrunn G, Fleury-Olela F, Pretner N, Schibler U (2002) Rhythms of mammalian body temperature can sustain peripheral circadian clocks. Curr Biol 12:1574–1583.
Bujis RM, Scheer FA, Kreier F, Yi C, Bos N, Goncharuk VD, Kalsbeek A (2006) Organization of circadian functions: interaction with the body. Prog Brain Res 153:341–360.
Chappell SA, Owens GC, Mauro VP (2001) A 5′ leader of Rbm3, a cold stress-induced mRNA, mediates internal initiation of translation with increased efficiency under conditions of mild hypothermia. J Biol Chem 276:36917–36922.
Chou CC, Zhang Y, Umoh ME, Vaughan SW, Lorenzini I, Liu F, Sayegh M, Donlin-Asp PG, Chen YH, Duong DM, Seyfried NT, Powers MA, Kukar T, Hales CM, Gearing M, Cairns NJ, Boylan KB, Dickson DW, Bademakers R, Zhang YJ, et al. (2018) TDP–43 pathology disrupts nuclear pore complexes and nucleocytoplasmic transport in ALS/FTD. Nat Neurosci 21:228–239.
Colwell CS (2011) Linking neural activity and molecular oscillations in the SCN. Nat Rev Neurosci 12:553–569.
Danno S, Nishiyama H, Higashitsuji H, Yokoi H, Xue JH, Itoh K, Matsuda T, Fujita J (1995) Decreased expression of mouse Rbm3, a cold-shock protein, in Sertoli cells of cryptorchid testis. Am J Pathol 146:1685–1692.
Dibner C, Schibler U, Albrecht U (2010) The mammalian circadian timing system: organization and coordination of central and peripheral clocks. Annu Rev Physiol 72:517–549.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR (2013) STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29:15–21.

Dotti CG, Sullivan CA, Banker GA (1998) The establishment of polarity by hippocampal neurons in culture. J Neurosci 18:1454–1468.

Dresios J, Aschrafi A, Owens GC, Vanderklish PW, Edelman GM, Mauro VP (2005) Cold stress-induced protein Rbm3 binds 60S ribosomal subunits, alter microRNA levels, and enhances global protein synthesis. Proc Natl Acad Sci USA 102:1865–1870.

Gerster JR, Yin JC (2010) Circadian rhythms and memory formation. Nat Rev Neurosci 11:577–588.

Green DJ, Gillette R (1982) Circadian rhythm of firing rate recorded from single cells in the rat suprachiasmatic brain slice. Brain Res 245:198–200.

Hafner AS, Donlin-Asp PG, Leitch B, Herzog E, Schuman EM (1992) Exo-endocytosis of environmental input. Eur J Neurosci 20:1113.

Harris KM, Teyler TJ (1983) Age differences in a circadian influence on hippocampal LTP. Brain Res 261:69–73.

Herzog ED, Takahashi JS, Block GD (1998) Clock controls circadian period in isolated suprachiasmatic nucleus neurons. Nat Neurosci 1:708–713.

Holt CE, Martin KC, Schuman EM (2019) Local translation in neurons: visualization and function. Nat Struct Mol Biol 26:557–566.

Honma S, Shirakawa T, Kamiya M, Honma KI (1998) Circadian periods of single suprachiasmatic neurons in rats. Neurosci Lett 250:157–160.

Honma S, Shirakawa T, Nakamura W, Honma KI (2000) Synaptic communication of cellular oscillations in the rat suprachiasmatic neurons. Neurosci Lett 294:113–116.

Ishinaka N, Cowan WM, Aron DR (1995) A quantitative analysis of the dendritic organization of pyramidal cells in the rat hippocampus. J Comp Neurol 362:17–45.

Jones KJ, Templet S, Zenoura K, Kuzniakowska B, Pena FX, Hwang H, Lei DJ, Honma S, Shirakawa T, Kawasaki T, Nakamura W, Yamazaki S, Kudo T, Cutler T, Colwell CS, Block GD (2011) Age-related decline in circadian output. J Neurosci 31:10201–10205.

Noy A, Colaneri D, Brünig F, Spiller A, Mirscof D, Opitz L, Mann M, Tyagarajan SK, Robles MS, Brown SA (2019) The forebrain suprachiasmatic transcriptome is organized by clocks but its proteome is driven by sleep. Science 366:eaaa2642.

Ostroff LE, Watson DJ, Cao G, Parker PH, Smith H, Harris KM (2018) Shifting patterns of polyribosome accumulation at synapses over the course of hippocampal long-term potentiation. Hippocampus 28:416–430.

Partch CI, Green CB, Takahashi JS (2014) Molecular architecture of the mammalian circadian clock. Trends Cell Biol 24:90–99.

Paul JR, Davis JA, Goode AK, Becker BK, Fussiler A, Meador-Woodruff A, Gamble KL (2020) Circadian regulation of membrane physiology in neural oscillators throughout the brain. Eur J Neurosci 51:109–130.

Pembroke WG, Babbs A, Davies KE, Ponting CP, Oliver PL (2015) Temporal transcriptomics suggest that twin-peaking genes reset the clock. Elife 4: e10518.

Pennartz CM, De Jeur MT, Bos NP, Schap J, Geurtsen AM (2002) Diurnal modulation of pacemaker potentials and calcium current in the mammalian circadian clock. Nature 416:286–290.

Peretti D, Bastide A, Radford H, Vertly N, Molloy C, Martin MG, Moreno JA, Steinert JR, Smith T, Dinsdale D, Willis AE, Mallucci GR (2015) RBM3 mediates structural plasticity and protective effects of cooling in neurodegeneration. Nature 518:236–239.

Ray S, Vakula KU, Stangherlin A, Howell SA, Snijders AP, Damodaran G, Reddy AB (2020) Circadian rhythms in the absence of the clock gene Bmal1. Science 367:800–806.

Renaud J, Duman F, Kheelouai M, Foisset SR, Letourneur F, Bienvenu T, Khwaja O, Doreseuil O, Billuart P (2015) Identification of intellectual disability genes showing circadian clock-dependent expression in the mouse hippocampus. Neuroscience 308:11–50.

Ritchie ME, Shipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 43:e47.

Rizzolo SO, Betz WJ (2005) Synchrony vesicle pools. Nat Rev Neurosci 6:57–69.

Scarnati MS, Kataria R, Biswas M, Paradiso KG (2018) Active presynaptic release in mammalian brain, and altered transmitter release in the mammalian clock, and altered transmitter synthesis after protein synthesis inhibition. Elife 7:e36697.

Smart F, Aschrafi A, Atkins A, Owens GC, Pilotte J, Cunningham BA, Vanderklish PW (2007) Two isoforms of the cold-inducible mRNA-binding protein RBM3 localize to dendrites and promote translation. J Neurochem 101:1367–1379.

Steward O, Levy WB (1982) Preferential localization of polyribosomes under the base of dendritic spines in granule cells of the dentate gyrus. J Neurosci 2:284–291.

Sureban SM, Ramalingam S, Natarajan G, May R, Subramaniam D, Nimura J, Takamori S, Holt M, Stenius K, Lemke EA, Grønborg M, Riedel D, Urlaub H, Schuman EM, Banker GA (1988) The establishment of polarity by interacting with the neurodegeneration-associated protein TDP-43. J. Neurosci., February 10, 2021

Munn RG, Bilkey DK (2012) The firing rate of hippocampal CA1 place cells is modulated with a circadian period. Hippocampus 22:1325–1337.

Nagoshi E, Saini C, Bauer C, Laroche T, Naef F, Schibler U (2004) Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillator activity passaged to daughter cells. Cell 119:693–705.

Nakamura TJ, Nakamura W, Yamazaki S, Kudo T, Cutler T, Colwell CS, Block GD (2011) Age-related decline in circadian output. J Neurosci 31:10201–10205.

Noy I, Colanero D, Brünig F, Spiller A, Mirscof D, Opitz L, Mann M, Tyagarajan SK, Robles MS, Brown SA (2019) The forebrain suprachiasmatic transcriptome is organized by clocks but its proteome is driven by sleep. Science 366:eaaa2642.
Truckenbrodt S, Viplav A, Jähne S, Vogts A, Denker A, Wildhagen H, Fornasiero EF, Rizzoli SO (2018) Newly produced synaptic vesicle proteins are preferentially used in synaptic transmission. EMBO J 37:e98044.

Wang J, Vasaikar S, Shi Z, Greer M, Zhang B (2017) WebGestalt 2017: a more comprehensive, powerful, flexible and interactive gene set enrichment analysis toolkit. Nucleic Acids Res 45:W130–W137.

Watanabe K, Koibuchi N, Ohtake H, Yamaoka S (1993) Circadian rhythms of vasopressin release in primary cultures of rat suprachiasmatic nucleus. Brain Res 624:115–120.

Welsh DK, Logothetis DE, Meister M, Reppert SM (1995) Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. Neuron 14:697–706.

Welsh DK, Takahashi JS, Kay SA (2010) Suprachiasmatic nucleus: cell autonomy and network properties. Annu Rev Physiol 72:551–577.

Wu G, Anafi RC, Hughes ME, Kornacker K, Hogenesch JB (2016) MetaCycle: an integrated R package to evaluate periodicity in large scale data. Bioinformatics 32:3351–3353.

Wang J, Vasaikar S, Shi Z, Greer M, Zhang B (2017) WebGestalt 2017: a more comprehensive, powerful, flexible and interactive gene set enrichment analysis toolkit. Nucleic Acids Res 45:W130–W137.

Welsh DK, Logothetis DE, Meister M, Reppert SM (1995) Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. Neuron 14:697–706.

Zhang R, Lahens NF, Ballance HI, Hughes ME, Hogenesch JB (2014) A circadian gene expression atlas in mammals: implications for biology and medicine. Proc Natl Acad Sci USA 111:16219–16224.

Zhu X, Yan J, Bregere C, Zelmer A, Goerne T, Kaphammer JP, Guzman R, Wellmann S (2019) RBM3 promotes neurogenesis in a niche-dependent manner via IMP2-IGF2 signaling pathway after hypoxic-ischemic brain injury. Nat Commun 10:14.