ciRS-7-miR7 regulate ischemia induced neuronal death via glutamatergic signaling

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ABSTRACT

Brain functionality resides on finely tuned regulation of gene expression by networks of non-coding RNAs (ncRNAs) such as the one composed by the circular RNA ciRS-7, the microRNA miR-7 and the long non-coding RNA Cyrano. However, very little is known on how this network regulates stress responses in neurodegeneration. Here we describe ischemia induced alterations in the ncRNA network both in vitro and in vivo and in transgenic mice lacking ciRS-7 or miR-7. Our data show that cortical neurons downregulate ciRS-7 and Cyrano and upregulate miR-7 expression upon ischemic insults. Mice lacking ciRS-7 show reduced lesion size and motor impairment, whilst the absence of miR-7 alone leads to an increase in the ischemia induced neuronal death. Moreover, miR-7 levels in pyramidal excitatory neurons regulate dendrite morphology and glutamatergic signaling suggesting a potential molecular link to the in vivo phenotype. Our data reveal a new endogenous mechanism by which ciRS-7 and miR-7 regulate the outcome of ischemic stroke and shed new light into the pathophysiological roles of intracellular networks of non-coding RNAs in the brain.
INTRODUCTION

The specialized, dynamic, and complex functionality of the brain relies on accurate regulation of gene expression, also mediated by non-coding RNAs (ncRNAs) which are particularly overrepresented in the brain and have been strongly connected with the increase of cognitive complexity in human1. MicroRNAs (miRNAs) are short (~22 nucleotides) ncRNAs known to post-transcriptionally regulate the expression of messenger RNA (mRNA) by binding short complementary sequences (seed)2 and triggering mRNA decay or inhibition of translation3. Long non-coding RNAs (lncRNAs) are molecules of regulatory RNA longer than 200 nucleotides capable of regulating gene expression by interacting with other ncRNAs such as miRNAs, or proteins through secondary structures4. Circular RNAs (circRNAs) belong to a novel class of ncRNAs that, among other functions, are able to modulate gene expression by interacting with miRNAs, hence affecting miRNAs activity on mRNA targets5. CircRNAs are generated by backsplicing of linear transcripts, a specific splicing event in which the 5’ splice site of the upstream exon binds to the 3’ splice site of the downstream exon6. Thus, in RNA-sequencing data, the reads on the backsplice junction identify the circular specific form of the transcript and the circRNA abundance is defined by the ratio of these reads to the canonical splicing reads.

These ncRNAs independently control cellular function by regulating the expression of protein coding genes, but also interact with each other. An excellent example of ncRNAs regulatory network is the brain specific network composed by two miRNAs (miR-7, miR-671), a lncRNA (Cyrano) and a circRNA (ciRS-7)5,7–9. ciRS-7 is suggested to stabilize and promote miR-7 targeting through delocalization of miR-7 in different neuronal compartments8,9. On the other end, Cyrano has a single site of almost perfect complementarity to miR-7 which induces the degradation of miR-7 through target RNA–directed miRNA degradation (TDMD)8. The regulation of miR-7 mediated by Cyrano affect ciRS-7 abundancy and localization creating a network of interactions between these molecules that at the same time indirectly affect the expression of miR-7 target genes5,8–10. Gain and loss of
function experiments and the development of knock-out animals for ciRS-7, miR-7 and Cyrano have revealed that Cyrano and ciRS-7 bind miR-7 and regulate its expression\textsuperscript{5,8,9}. Nonetheless, no information is available on how this network regulates gene expression in pathophysiological conditions.

Ischemic stroke, induced by occlusion of one of the main cerebral arteries, leads to lack of oxygen and nutrients in the brain parenchyma and subsequent cell death. Lack of energy and disruption in the ion balance cause an uncontrolled release of glutamate in excitatory neurons and deficient uptake of glutamate by astrocytes induces glutamate excitotoxicity\textsuperscript{11} and subsequent oxidative stress, necrosis and apoptosis\textsuperscript{12}. Due to the complexity of the ischemic stroke pathophysiology, it is not surprising that numerous miRNAs and lncRNAs have been identified to regulate oxidative stress response and glutamate excitotoxicity contributing to the outcome of stroke\textsuperscript{13}. In fact, when the stress is sustained, miRNAs are known to be involved in adaptative sudden switches of gene expression programs\textsuperscript{14}. The efficiency of miRNA-mediated target repression depends on the availability of miRNAs to interact (expression, localization, activity) and the amount of mRNA targets that have a seed Mirna Recognition Element (MRE), which confer robustness to crucial cellular functions creating a miRNA-specific threshold\textsuperscript{15}. In this context, sudden changes in MRE containing sequences can lead to an imbalance in miRNA targeting and de-repression of specific targets. The crosstalk between miRNA and all the expressed sequences containing MRE at any given moment suggested a theory that in physiological conditions, a large number of MRE sequences compete for the binding of the miRNA\textsuperscript{16}. Moreover, the expression of specific transcript with different binding strength or MRE abundance during stress is known to be used by organisms to regulate miRNA effects in a process called target mimicry\textsuperscript{17}.
Recent studies have also highlighted a strong connection between circRNAs and the production of reactive oxygen species. However, even though single ncRNAs have been studied in the context of ischemic stroke, a comprehensive analysis of the regulatory role of ncRNAs network in stroke is still lacking. In this study, we identified the brain highly expressed circRNA, ciRS-7, as one of the seven circRNAs deregulated in ischemia-related conditions in vitro and in vivo. We expanded our analysis to other ncRNAs species and discovered additional changes in miR-7 and Cyrano which belong to the same regulatory circuitry of ciRS-7. Using ciRS-7 and miR-7 knock-out mouse models, we revealed the potential role of ciRS-7 in preventing miR-7-mediated regulation of the target mRNAs and demonstrate that ciRS-7 molecular network is involved in the regulation of stroke-induced cell death.

**RESULTS**

**Oxygen and glucose deprivation induces changes in the expression of circular RNAs**

As circRNAs were previously connected to oxidative stress response, we evaluated the potential deregulation of circRNAs in conditions mimicking ischemic stroke in vitro. We cultured murine cortical neurons isolated from embryonic day fifteen (E15) cortices, subjected them to oxygen and glucose deprivation (OGD) (Supplementary Figure S1A) and performed total RNA sequencing. To assess whether our neuronal culture is composed of cell types representative of the murine cortex, we inferred the presence of individual cell populations in our bulk RNA-seq samples through a deconvolution analysis on single-cell RNA-seq dataset of embryonic mouse brain (E14.5). The results confirmed that interneurons (Int) and pyramidal neurons (Layer V-VI) are the most abundant cell populations in our culture (Figure 1A). To validate that the culture is effectively enriched in functional Interneurons (GABAergic) and Pyramidal neurons (Glutamatergic), we stimulated our neuronal culture with GABA and glutamate and recorded the consequent induction of intracellular calcium using calcium imaging. Our data confirmed that 98% of cells in our culture responded to
glutamate and 36% to GABA, providing functional proof that our culture mostly consists of glutamatergic excitatory neurons (Figure 1B).

Ingenuity Pathway Analysis (IPA) of the differentially expressed transcripts between normoxic and OGD neurons confirmed that OGD treatment induced the activation of Hypoxia-inducible factor 1-alpha (HIF-1alpha) (Figure 1C) and downstream upregulation of glycolysis, a characteristic hallmark of OGD (Supplementary Table S1). Moreover, colorimetric cell viability assay indicated 30% decrease in neuronal viability upon the exposure (Supplementary Figure S1B), validating that our experimental setting represents a population of glutamatergic excitatory neurons that are vulnerable to ischemia-induced cell death.

CircRNAs are generated by back-splicing which produces transcripts that can be detected with back-splice junction reads (BSJ) spanning regions that are not present in regularly spliced transcripts (forward splice junction reads, FSJ) (Figure 1D). In order to identify OGD-induced alterations in circRNAs, we used CIRIquant algorithm, which considers circular specific reads (BSJ) and their ratio with linear transcript reads (FSJ), and identified 27 circRNAs with high confidence of which 7 were significantly differentially expressed (DE) between normoxic (control) and OGD conditions (Figure 1E, Supplementary Table S2). Among these DE circRNAs, ciRS-7 was the most highly expressed yielding the highest number of back-splice junctions (Figure 1F, left) and the highest circular to linear ratio (Junction ratio) (Figure 1F, right). In fact, ciRS-7 obtained a junction ratio score above 0.8, meaning that more than 80% of the transcripts originating from this locus are circular, in contrast with the other circRNAs which showed about 40% circular expression. Specifically, ciRS-7 was one of the three downregulated circRNAs upon OGD.

ciRS-7 network is altered in conditions mimicking ischemic stroke in vitro

ciRS-7 has been shown to be part of a feedback loop with miR-671 and miR-7 microRNA and indirectly, through miR-7, with the long non-coding RNA Cyrano (Figure 2A). To investigate the
relative relationship between these players in *in vitro* OGD conditions, we performed small RNA sequencing of cortical neurons exposed to OGD. Together with the previously presented dataset, we were able to capture circRNAs, mRNAs, long non-coding RNAs and small RNA transcripts (Supplementary Table S2).

Small RNA-sequencing identified 333 upregulated and 88 downregulated miRNAs following OGD (Figure 2B). The levels of miR-671-5p remained unaltered (Figure 2B) which was confirmed independently by RT-qPCR (Supplementary Figure S2A), in contrast to previous findings of diverging miR-671-5p and ciRS-7 expression in different contexts. Instead, we detected a significant upregulation in miR-7a-5p, but not of the variant miR-7b-5p (Figure 2B, Supplementary Table S2). Similarly to human, in mouse miR-7 is redundantly encoded by three different loci (miR-7a-1, miR-7a-2, miR-7b), each is produced from different primary transcript (pri-miRNA) and precursor (pre-miRNA). Further processing, in all cases from -5p arm, give rise to two mature miR-7 sequences (miR-7a and miR-7b), differing only by a single nucleotide in the non-seed position number 10 (Figure 2C). To study the expression of these three miR-7 loci in our system, we took advantage of Global run-on sequencing (GRO-seq), which allows the capture of nuclear nascent RNA primary molecules, and compared our murine culture expression with different mouse tissues and cell lines (Supplementary Table S3). In physiological conditions, only cortical neurons and the pituitary gland actively transcribe all three independently regulated miR-7 loci (Figure 2D). The active transcription from three different loci in neuronal cells suggests a higher order of regulation of this miRNA which influences the mature forms. Under OGD conditions, only the pri-miRNAs contributing to the expression of miR-7a (miR-7a-1 and miR-7a-2) were significantly upregulated (Figure 2E), indicating that the observed upregulation occurs already at the level of transcription.

None of miR-7 passenger strands generated from the three precursors (miR-7a-1-3p, miR-7a-2-3p, and miR-7b-3p) were altered in our sequencing data (Supplementary Table S2). Moreover, we detected no changes in the host gene in which miR-7a-1 is embedded (*Hnrnpk*), and from which the...
most abundant miR-7 primary transcript is generated (Supplementary Table S2). Taken together these data show that OGD specifically regulates miR-7a-5p variant at the transcriptional and/or post-transcriptional level.

To test the possible functional relevance of the upregulation of miR-7a-5p, we acquired predicted and validated targets of miR-7a-5p using miRWalk algorithm analysis\textsuperscript{33,34} and compared their overall expression in conditions of OGD. In accordance with the upregulation of miR-7, miR-7 targets showed a significant overall downregulation (Figure 2F, Supplementary Table S4), suggesting a canonical functional repressive role of this miRNA.

The role of miR-7 interaction with ciRS-7 remains controversial, and it is thought to set the balance between a positive and negative feedback\textsuperscript{8,9} (Figure 2A). Additionally, Cyrano promotes template mediated degradation (TMD) of miR-7 through a site of almost perfect complementarity, which was shown to indirectly regulate ciRS-7 levels and localization\textsuperscript{8} (Figure 2A). In our dataset the lncRNA Cyrano was significantly downregulated upon OGD (Figure 2G), in line with the observed upregulation in miR-7a-5p (Figure 2B).

To identify a first responder to OGD in the context of this molecular circuitry we evaluate the time-dependent changes in the levels of these molecules. We subjected cortical neurons to OGD for 1, 6, 12 and 24 hours and assessed the expression level of ciRS-7, miR-7 and Cyrano. We detected a significant downregulation of ciRS-7 and Cyrano already at 6 hours after OGD, prior to the upregulation of miR-7 at 12 hours (Figure 2H). Interestingly, our data revealed a significant downregulation of miR-7 at 6h of OGD concomitantly with downregulation in ciRS-7 and Cyrano, which is in line with the previously suggested role of ciRS-7 in stabilizing miR-7\textsuperscript{9}.

\textit{ciRS-7 KO neurons exhibit differential OGD response}

The absence of ciRS-7 alters synaptic transmission in excitatory neurons and produces schizophrenia-like phenotype \textit{in vivo}\textsuperscript{9}, connecting this molecule to glutamatergic transmission. This is of particular
interest in conditions of ischemic stroke, where glutamate mediated excitotoxicity is a critical contributor to neuronal cell death\textsuperscript{12}. Our cortical neuron culture enriched in glutamatergic neurons (Figure 1B) provides a system in which these events can be studied \textit{in vitro}. Neurons cultured from ciRS-7 KO mice showed higher Ca\textsuperscript{2+}-responses to the stimulation with glutamate compared to their wild-type (WT) counterparts (Figure 3A). Consequently, ciRS-7 KO neurons also exhibited a significantly higher sensitivity to excitotoxicity upon exposure to high concentration of glutamate (Figure 3B).

To test if this increased sensitivity to glutamate is affecting gene expression changes in OGD, we subjected the ciRS-7 KO cortical cultures to OGD following the previous experimental design (Supplementary Figure S1A) and performed total RNA-seq. OGD induced deregulation in total of 5767 genes in ciRS-7 KO neurons compared to normoxic conditions, of which the 85\% were shared with WT neurons (Figure 3C). In response to OGD, 871 genes were differentially expressed only in ciRS-7 KO neurons, while the expression 1260 genes was exclusively altered in WT neurons subjected to OGD. ciRS-7 WT specific genes altered in OGD were functionally enriched in mitochondrial processes, DNA metabolism, regulation of cell-cycle and oxidative stress-induced apoptosis (Figure 3D). The 871 KO-specific genes were instead involved in developmental and pro-regenerative processes (\textit{progenitor migration to the cortex, DNA replication, transcription, cerebellar cortex development}) (Figure 3E). Thus, even though the genetic predisposition to glutamate sensitivity of ciRS-7 KO would suggest a deleterious outcome in the OGD response, ciRS-7 KO neurons in OGD regulate pathways of resilience. In support of this, ciRS-7 KO neurons did not show increased cell death compared to WT in response of OGD treatment (Supplementary Figure S2B). Interestingly, the lack of ciRS-7 did not affect OGD induced changes in the expression of miR-7 and Cyrano (Figure 3F). Similar to WT neurons, Cyrano lncRNA was downregulated in ciRS-7 KO neurons responding to OGD (Figure 3F, Supplementary Table S5). Moreover, even though ciRS-7
KO neurons stably express lower levels of miR-7a-5p\textsuperscript{9} (Figure 3F, Supplementary Table S5), small-
RNA sequencing revealed a significant upregulation of miR-7a-5p in response to OGD also in these
neurons (Figure 3F, Supplementary Table S5). In line with our previous findings, we detected no
changes in miR-671-5p, miR-7b-5p and miR-7 passenger strands expression (Supplementary Table
S5). Nonetheless, whilst the expression in miR-7a-5p in OGD remained lower in ciRS-7 KO neurons
compared to WT neurons (Figure 3F), the targets of miR-7a-5p, both predicted and validated, showed
a significant global repression in these conditions (Figure 3G).

Alteration of ciRS-7 network affects ischemic stroke outcome in vivo

To evaluate the involvement of ciRS-7 network in ischemic stroke \textit{in vivo}, \textit{BALB/c} mice were
subjected to permanent middle cerebral artery occlusion (pMCAo) and levels of ciRS-7, Cyrano and
miR-7a-5p were evaluated by qPCR at six hours, one day and five days post-ischemia. In line with
our \textit{in vitro} results, we detected a significant downregulation of ciRS-7 and Cyrano and upregulation
of miR-7a-5p in the peri-ischemic cortex (PI) at one day post-ischemia (dpi) compared to contralateral
cortex (CL) (Figure 4A). Moreover, similar to OGD, we failed to detect alterations in miR-671-5p
levels (Supplementary Figure S2C).

To evaluate the functional relevance of the ambiguous behavior of ciRS-7 KO neurons which showed
 glutamate sensitivity but lack of increased cell death in OGD, we assessed whether ciRS-7 KO mice
subjected to transient ischemic stroke would show altered vulnerability to ischemic damage and
associated sensorimotor impairments. The size of the ischemic lesion was measured using Magnetic
Resonance Imaging (MRI) and behavioral deficits were assessed using Neurological Severity Scores
(NSS) at acute (1dpi), subacute (3dpi) and chronic (7dpi) timepoints after transient middle cerebral
artery occlusion (tMCAo) (Figure 4B). At 1dpi, ciRS-7 KO mice showed a significant reduction in
the ischemic lesion volume compared to their WT controls (Figure 4C) and significantly ameliorated
motor deficits (Figure 4D). Interestingly, ciRS-7 KO animals exhibited a reduced lesion size only transiently at the acute timepoint (1dpi), showing no delay in the progression of the damage (Supplementary Figure S3A,B).

To identify the molecular changes associated with the acute reduction in the lesion size and motor deficits of the ciRS-7 KO mice, mice were sacrificed at 1dpi and peri-ischemic and the contralateral cortex was used for mRNA sequencing. IPA analysis of the differentially expressed genes revealed that ciRS-7 KO animals exhibited substantial inhibition in the pathways related to neuronal and tissue damage, cytokine and interleukin release and calcium influx, in their peri-ischemic cortex compared to WT controls (Figure 4E, Supplementary Table S6). Moreover, the differentially expressed genes were mostly contributing to pathways of necrosis (205 genes) and quantity of cytokine (41 genes), suggesting that the decreased lesion size in the ciRS-7 KO mice may be due to inhibition of necrotic pathways and reduction in cytokine release.

In order to identify the major cellular responders mediating the observed alterations in gene expression profiles, we carried out deconvolution analysis by using adult mouse brain scRNA-seq data\(^3\) to define the contribution of different cell populations to our bulk mRNA sequencing dataset. This analysis identified neurons and astrocytes as major cellular responders contributing to the differences between the WT and ciRS-7 KO gene expression profiles (Figure 4F). ciRS-7 KO mice showed increased neuronal involvement in the peri-infarct cortex compared to the WT mice in line with the significantly decreased tissue death in ciRS-7 KO mice, as measured by MRI (Figure 4C). At the same time, ciRS-7 KO animals exhibited a decrease in the contribution of astrocytes in the peri-ischemic cortex.

We then collected brain slices of ischemic ciRS-7 KO mice at 7dpi, timepoint of maximum immune response\(^3\), for staining with the astrocytic glial fibrillary acidic protein (GFAP). As expected, ischemic stroke induced the expression of GFAP in the peri-infarct cortex in both WT and ciRS-7
KO mice. However, ciRS-7 KO mice showed significantly reduced astrogliosis in this area compared to WT animals (Figure 4G).

At molecular level, tMCAo induced a significant downregulation of ciRS-7 in WT mice at 1dpi in the peri-ischemic cortex, whereas the levels of miR-7-5p and Cyrano remained unaltered (Figure 4H, Supplementary Figure S3C). However, ciRS-7 KO mice showed a significant upregulation of miR-7-5p and unaltered levels of Cyrano (Figure 4H). These data suggest a correlation between the absence of ciRS-7 and the upregulation of miR-7 in response to transient ischemic stroke.

cIRS-7 prevents miR-7 effects on glutamatergic signaling in excitatory neurons

Previous studies have identified the involvement of miR-7 in response to oxidative stress, in particular to low glucose. To establish the possible functional link between the upregulation of miR-7 in ciRS-7 KO mice in conditions of ischemic stroke, we subjected cre-loxP inducible miR-7 KO mice to tMCAo (Figure 5A, Supplementary Figure S4A,B). We failed to detect changes in the lesion volume as measured by MRI between miR-7 KO and WT mice at acute timepoint (1dpi) (Supplementary Figure S4C). In line with this data, miR-7 KO mice did not show any differences in motor deficits compared to their WT controls (Supplementary Figure S4D). However, we detected an increase in the lesion volume in miR-7 KO mice (Figure 5B) compared to their WT controls at later timepoint (7 dpi), which was not associated with changes in Neuroscore (Supplementary Figure S4E), suggesting a potential role of miR-7 in preventing the ischemia induced cell death.

To understand the functional role of miR-7 in ischemic stroke, we first validated that miR-7 has functional target sites in our system through the analysis of miR-7 target binding sites using Ago2 HITS-CLIP sequencing data in pyramidal excitatory neurons (Figure 5C). We identified RISC-associated binding sites on expressed transcripts in mature projection neurons and the analysis of minimal seed site hexamer of miR-7 revealed 645 potential targets in the 3’ UTRs (Figure 5D). As expected, ciRS-7 obtained the highest number of functional binding sites (n=139) and accounted for
more than 11% of the total reads (Supplementary Table S7). The overlap between Ago2 HITS-CLIP physical targets and the downregulated genes in the peri-ischemic cortex of ciRS-7 KO mice, where miR-7 is upregulated, highlighted 276 common genes (Figure 5E). These genes were functionally enriched in pathways related to glutamatergic synaptic transmission and morphological changes of neuronal projections (Figure 5F, Supplementary Figure S5).

Given the central role of glutamatergic signaling in ischemia, these miR-7 potential targets in excitatory neurons might relate the improved ischemic outcome and the increased levels of miR-7 in ciRS-7 KO mice. To test this connection, we transduced ciRS-7 WT and KO neurons with lentivirus overexpressing GFP and miR-7a or GFP only, under the neuronal specific human Synapsin 1 (hSyn) promoter. Efficiency of infection was assessed through fluorescence microscopy (Supplementary Figure S6A) and we validated with RT-qPCR that miR-7a-5p levels resembled the endogenous levels in conditions of OGD (Supplementary Figure S6B).

Whilst overexpression of miR-7 in WT neurons did not affect the overall neuronal glutamate excitability, increased levels of miR-7 in ciRS-7 KO neurons significantly reduced their response to glutamate (Figure 5G), thus reverting the genotype of glutamate sensibility of the ciRS-7 KO neurons. This appeared to be a glutamate specific effect, as the overexpression of miR-7 did not affect GABAergic response (Supplementary Figure S6C).

Finally, to evaluate the functional impact of the potential targets of miR-7 involved in morphological changes of neuronal projections (Figure 5F), we performed live imaging analysis of neurite outgrowth of ciRS-7 KO and WT neurons infected with miR-7 lentivirus. In agreement with the calcium imaging experiment, we were able to detect a significant increase in the neurite length when over expressing miR-7 in ciRS-7 KO cells, but not in WT neurons (Figure 5H). This result is in line with a previous study demonstrating a direct effect of miR-7 overexpression in controlling neurite length in a neuroblastoma cell line. However, circRNAs are known to be poorly expressed in cell lines due of...
their high proliferation rate\textsuperscript{42}. So, we wondered whether this miR-7 direct effect on neurite length was due to lack of ciRS-7 interference. A study identifying circRNAs in neuronal cell lines and tissue discovered that human neuroblastoma cell line expresses an exiguous amount of ciRS-7\textsuperscript{42}. In addition, we validated that murine neuroblastoma cell line N2A also express negligible levels of ciRS-7 even when differentiated using retinoid acid, albeit the levels of Cyrano and miR-7 are more abundant (Supplementary Figure S7A,B,C).

**DISCUSSION**

Here we identify how the ncRNAs network composed of ciRS-7 – miR-7 – Cyrano regulates cellular responses to OGD \textit{in vitro} and ischemic stroke \textit{in vivo}. In OGD \textit{in vitro} model, we identified a new endogenous relationship between ciRS-7 and miR-7 than previously shown as ciRS-7 and Cyrano downregulation occurred ahead of miR-7 upregulation which was not dependent on ciRS-7 presence. In \textit{in vivo} conditions, the absence of ciRS-7 alleviated cellular death and subsequent sensorimotor deficits in response to ischemic stroke whereas the lack of miR-7 led to a more extensive tissue damage. These effects were partially executed via regulation of glutamatergic signaling by miR-7. Together these data highlight an endogenous regulatory role for the ciRS-7 network in mediating cellular stress responses under pathophysiological conditions.

We hypothesize that our results on the ncRNAs network composed by ciRS-7, miR-7 and Cyrano reflects a stress response mediated by miR-7 that is modulated by ciRS-7 and Cyrano expression. In mouse, ciRS-7 harbors 130 MREs (binding sites) for miR-7\textsuperscript{9} and, because of its extremely high expression in neuron\textsuperscript{5,9}, the low levels of miR-7 in physiological conditions\textsuperscript{5} are likely to be bound to ciRS-7, as suggested by our pyramidal excitatory neurons CLIP-seq data and others\textsuperscript{9,10}. Moreover, Cyrano, which is also very abundant in neurons, harbors a single almost complementary site for miR-7 which mediates miR-7 degradation\textsuperscript{8}. Our data show that in physiological conditions strong and high
abundant miR-7 binding competitors (ciRS-7/Cyrano) interact with limited regulator (miR-7) whereas in conditions of ischemic stroke, both \textit{in vitro} and \textit{in vivo}, there is a concomitant removal of ciRS-7 and Cyrano and subsequent upregulation of miR-7. Our data showing a downregulation in Cyrano prior to the upregulation of miR-7 is in line with previously known dynamics between these two molecules as Cyrano triggers miR-7 degradation through TDMD\textsuperscript{8}. On the other hand, here we show for the first time the induction of miR-7 in the absence of ciRS-7 suggesting that expression level of miR-7 is not dependent on ciRS-7 in conditions of ischemic stroke. This data, together with the lack of involvement of miR-7 levels in ciRS-7 downregulation identified new endogenous relationship induced by stress between miR-7 and ciRS-7 in respect of what it has been previously shown in physiological conditions\textsuperscript{8,9}.

The role of miR-7 in ischemic stroke has been studied \textit{in vivo} with controversial results\textsuperscript{43,44}. Our large cohort of animals revealed a high biological variability in expression levels of miR-7 in WT animals subjected to transient ischemic stroke. This can be explained by the presence of feedback loops between ciRS-7, Cyrano and miR-7 and suggests the idea that ciRS-7 and Cyrano may behave as a buffer to regulate miR-7 expression. In line with this idea, ciRS-7 KO mice subjected to tMCAo significantly overexpress miR-7 one day post-ischemia and show a reduced stroke lesion size and motor deficits. Furthermore, at 7 dpi, miR-7 inducible KO mice showed an exaggerated ischemic lesion size compared to WT animals, reinforcing the idea of a role of miR-7 in regulating ischemic damage. Moreover, our data is in line with literature showing that the levels of miR-7 are downregulated at early timepoints in ischemic stroke and that upregulation in miR-7 is necessary for stroke recovery\textsuperscript{45}. These data together propose a model in which ciRS-7 and Cyrano regulate miR-7 and possibly influence its upregulation acting as a “buffering” system.
Due to the lack of extensive changes in miR-7 targets in both ciRS-7, Cyrano and miR-7 KO animals in physiological conditions\(^8,9\), the exact role of ciRS-7 as an influencer of miR-7 target gene expression has remained controversial. Our data show a significant shift in the overall expression of miR-7 targets in conditions of OGD (Figure 2F), where a concerted downregulation of two strong miR-7 regulators, ciRS-7 and Cyrano, occurs. Our data suggests that the inducible nature of this network may explain the reported lack of extreme effects on miR-7 targets in physiological conditions. This is supported by studies showing that single miRNA mutants exhibit a phenotype solely under stress conditions\(^46,47\). Moreover, removal of a single competing target for endogenously low expressed miRNAs, such as miR-7, is not expected to induce a relevant change in gene expression in physiological conditions\(^15\). Finally, particularly for non-coding RNAs, cells are prone to adaptation upon knock-out, generating an imbalance in output between knock-out and knock-down experiments, as previously experienced\(^9\).

We used the analysis of Ago2 CLIP-seq dataset to discover physiological interactions of miR-7 with the downregulated genes in ciRS-7 KO tMCAo animals in which miR-7 is upregulated. Our data suggest a role of miR-7 in regulating glutamatergic signaling and neuronal outgrowth. The central role of glutamate in ischemic stroke pathophysiology is well established\(^12\), contributing to the neuronal loss upon exaggerated release and impaired clearance of glutamate in the synaptic cleft\(^11\). Overexpression of miR-7 failed to induce changes in glutamatergic signaling and neuronal outgrowth in wild-type cortical neurons. However, in the absence of ciRS-7, miR-7 overexpression dampened the neuronal glutamatergic response which likely contributes to the reduced cellular damage in conditions of ischemic stroke.

Our hypothesis of ciRS-7 acting as a buffer on miR-7 mediated effects is strengthened further by our data showing that miR-7 overexpression promotes neurite outgrowth only in ciRS-7 KO neurons,
which lack the ciRS-7 buffering effect. In line with our results, previously published data indicate
that miR-7 overexpression regulates neurite length in a neuroblastoma cell line\textsuperscript{41}. Of note is that
murine neuroblastoma cell lines express negligible amounts of ciRS-7 even when differentiated with
retinoic acid, suggesting a more direct effect of miR-7 in the absence of its regulator. A recent study
has highlighted a potential role of miR-7 in controlling energy homeostasis in hypothalamic
neurons\textsuperscript{39}, linking this miRNA to conditions in which the energetic balance is strongly altered, such
as in ischemic stroke. However, alteration in the levels of miR-7 in hypothalamic neurons were not
accompanied by changes in the expression of ciRS-7 or Cyrano\textsuperscript{39}. Together with our data showing
how the type of ischemic insult activates ciRS-7 network with different dynamics, this suggests that
the network responds in a stress type, strength, and duration dependent manner.

In summary, here we identify a role of ciRS-7 as a regulator of cellular stress response under OGD
\textit{in vitro} and ischemic stroke \textit{in vivo}. Upregulation of miR-7 following cerebral ischemia is critical for
the control of ischemic stroke induced damage and post-ischemia plasticity and depends on the levels
of ciRS-7. Our data support the role for ciRS-7 as a buffer of miR-7 effects against unwanted changes,
thus behaving as a “safe-guide” system. Finally, we propose a regulatory role for ciRS-7-miR-7
network in glutamatergic signaling through miR-7 target genes, hence providing a novel mechanism
which controls post-ischemia neuronal damage. More broadly, this study suggests a role of
intracellular network of non-coding RNAs in regulating pathophysiological processes in the brain.
MATERIAL AND METHODS

Primary cortical neuron culture preparation and OGD treatment

Primary cortical neurons were prepared from C57BL/6J and C57BL/6N-Cdr1asem1Nikr (Cdr1as KO and their WT counterpart) embryonic day 15 embryonal cortices. After dissection and removal of the meninges, cortices were incubated 15 minutes at 37 °C in a solution of Krebs buffer (0.126 M NaCl, 2.5 mM KCl, 25 mM NaHCO3, 1.2 mM NaH2PO4, 1.2 mM MgCl2, 2.5 mM CaCl2, supplemented with 45mM BSA, 0.8% of 3.85% MgSO4 and 1% Pen/Strep, pH 7.4) and 0.025% (w/v) trypsin (Sigma-Aldrich, T 9201). Tissue was then treated with 0.008% w/v DNaseI (Sigma-Aldrich, DN25) and 0.026% w/v trypsin inhibitor (Sigma-Aldrich, T9003) and centrifuged at 300 x g for 3 minutes. Cell pellet was resuspended in 3ml of DNaseI/Trypsin solution and then diluted in 7ml of Krebs. After centrifugation at 300 x g for 3 minutes the pellet containing embryonic neurons was resuspended in cortical neurons growth media: Neurobasal (Gibco 21103049), B27 Supplement (Gibco, 17504044), 0.2 mM L-glutamine (Lonza, BE17-605E), 0.01 mg/ml Penicillin/Streptomycin (Gibco, 15140122). Cells were plated on Poly-D lysine (Sigma-Aldrich, P6407) freshly precoated plates (50 μg/ml in sterile water plates for 1h at 37 °C and washed in sterile water prior use). Different density was used for 6-well plates (1.8 million cells per well), 48-well plates (125.000 cells per well), 13mm plastic coverslips (30.000 cells per coverslip). After 5 days in culture half of the in cortical neurons growth media was changed to fresh. Experiments were performed 7 days after the isolation day. Cells were maintained in the incubator 37 °C, 5% CO2. For OGD treatment experiments, before hypoxia induction the media was changed to Normoxic (cortical neurons growth media with Neurobasal changed to Gibco A2477501 supplemented with D-Glucose 25mM and Sodium Pyruvate 0.2mM) or OGD (cortical neurons growth media with Neurobasal changed to Gibco A2477501). Normoxic cells were then put back in the incubator, OGD cells were incubated in hypoxic chamber 37 °C, 5%CO2, 1%O2 (SCI-tive N, Ruskinn Technology). After the OGD timepoint cells were harvested for RNA extraction or subjected to MTT colorimetric test.
MTT test

Cell viability was measured from 48-well plates treating the cells with 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide), MTT reagent (Sigma-Aldrich, TOX1) diluted with culture media at a final concentration of 120 μM. Triton-X 100 1% v/v (Sigma-Aldrich, X100) treated wells were used as a positive control for this assay. Plates were incubated for 3-5h at +37°C. After, medium was discarded, formazan crystals were dissolved with dimethyl sulfoxide (DMSO) (Sigma-Aldrich, D2650) for 30min at RT in the dark. Absorbance was read at 585nm using Wallac 1420 Victor2 microplate reader (Perkin Elmer). Wells without cells were used as background and subtracted from the absorbance data, all the six technical replicates were plotted for each of the three biological replicate and the data was normalized on normoxic WT neurons or WT neurons infected with GFP only.

Neurite length measurement

Neurons were seeded at a density of 125,000 cells/well in 48-well plates. After OGD exposure or lentiviral infection, neurons were imaged at day 7 for 48 hours with IncuCyte® S3 Live Cell Analysis System (Essen BioScience Ltd.) in bright field and green channel live cell images (two 10x magnification images per well). Acquired data were analysed with the Incucyte® Neurotrack Analysis Software Module (Sartorius) considering the average value of the two images taken for each of the nine technical replicates for all the three biological replicates.

RNA isolation and qRT-PCR

Total RNA was extracted from primary cells and ipsilateral/contralateral animal cortex using TRIzol™ Reagent (Invitrogen) following the manufacturer’s instructions. 1μl of GlycoBlue™ Coprecipitant (Ambion) was added at the isopropanol step in each sample. RNA was quantified using a Nanodrop 2000 spectrophotometer. 1 μg of total RNA was used for reverse-transcription
ofmRNAs, circRNA, IncRNAs and pri-miRNA species with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer’s protocol. SYBR Green qPCR Master Mix (High ROX) (Bimake) and custom designed oligos were used to quantify mRNAs, circRNAs, and IncRNAs following the manufacturer’s indications. Mature microRNAs were reverse-transcribed with TaqMan™ MicroRNA Reverse Transcription Kit (Invitrogen) using miRNA-specific primers supplied in the TaqMan® probe kit for qPCR following the manufacturer’s protocol. Mature microRNAs and pri-microRNA PCRs were performed using Maxima Probe/ROX qPCR Master Mix (Invitrogen) and TaqMan® specific probes (Thermofisher) following the manufacturer’s protocol. The result was analyzed with the ΔΔCT method and normalized geometric mean of 2 internal normalization controls (Gapdh and Rplp0) for SYBR green qPCRs and U6 expression for TaqMan® miRNA and pri-miRNA. TaqMan® probes and sequence of the SYBR Green oligonucleotide primers is available in the Supplementary Table S8.

Lentivirus vectors and virus generation

Lentiviral vectors LV1-eGFP (control) or LV1-eGFP-miR-7 were generated by subcloning inserts from pAAV_hSYN1-eGFP-miR-7 and pAAV_hSYN1-eGFP (provided by Thomas B. Hansen) inside LV1 (immunodeficiency virus 1 (HIV-1)-based LV-PGK-GFP) backbone by GenScript Biotech Corporation. The generated construct contained HIV-1-LV backbone with hSYN1-eGFP-miR-7 insert instead of PGK-GFPN inserted creating a terminal SmaI (CCCGGG) and a C-terminal Apal (GGGCCC) flanking restriction sites. The same was performed for the control vector with hSYN1-eGFP insert only. 3rd generation lentiviral particles were produced by the BioCenter Kuopio National Virus Vector Laboratory in Kuopio, Finland. The viral titer was assessed through qPCR serial dilution quantification using eGFP ReadyMade™ Primers (IDT) (Supplementary Table S8). Work with the virus vectors was carried out under permission from Finnish National Supervisory Authority for Welfare and Health, Valvira. Cells were infected at day 2 post isolation with MOI 0.5.
achieving 80% positive infected cells assessed by GFP expression in fluorescent microscope (Supplementary Figure 5A) at day 7. The amount of overexpression of miR-7 was assessed by qPCR as above specified (Supplementary Figure 5B).

**Library preparation**

All the samples RNA were isolated with TRIzol™ Reagent (Invitrogen) as specified above. RNA samples were treated with TURBO DNA-free™ Kit (Ambion) following the manufacture’s instruction. RNA integrity was assessed through Agilent Bioanalyzer 2100 system with the Agilent RNA 6000 Nano. The concentration of the samples was established with Qubit™ RNA Extended range kit (Invitrogen). From cortical neurons subjected to OGD we generated 1) library to detect circularRNAs and mRNA and lncRNAs using SMARTer® Stranded Total RNA Sample Prep Kit (Takara Bio USA, Inc.) and 2) library for miRNAs detection utilizing NEBNext® Small RNA Library Prep Set for Illumina (New England Biolabs (UK) Ltd) quality checked and size selected using pippin prep method following manufacturer’s protocol. We generated a library from ciRS-7 WT and KO tMCAo animals contralateral and peri-ischemic cortices to detect mRNAs changes using CORALL total RNA-Seq Library Prep Kit (Lexogen GmbH) after ribosomal RNA depletion of 600ng of RNA with RiboCop rRNA Depletion Kit V1.2 (Lexogen GmbH). All the libraries were generated following the manufacturer’s protocol. After generation, the libraries were quantified with Qubit™ High Sensitivity DNA kit (Invitrogen) and by qPCR using KAPA Library Quantification Kit for Illumina® Platforms (Roche). Library size was determined with Agilent Bioanalyzer 2100 system using the Agilent High Sensitivity DNA Kit. Cortical neurons samples to detect circularRNAs, mRNA, and lncRNAs were sequenced paired-end 100 cycles on NovaSeq™ 6000 platform (Illumina) and single-read 75 cycles on NextSeq™ 500 system (Illumina) to detect microRNAs. Animal samples were sequenced single-read 75 cycles on NextSeq™ 550.
Calcium imaging

Calcium imaging was performed with murine E15 cortical neurons. The neurons were plated onto PDL-coated circular plastic coverslips (13 mm diameter) in 12-well plate at a density of 30,000 cells/cover and kept for 5 days in Neurobasal medium supplemented with B27. To quantify and compare the functional expression of glutamate and GABA receptors in neuronal cultures, we used calcium-imaging technique as previously described. Briefly, neuronal cultures were loaded with the cell-permeable indicator Fluo-4am (Life Technologies, F10471) for 30 min at 37 °C, followed by 10 min washout, and placed in the perfusion chamber mounted on the stage of Olympus IX7010 microscope. Neurons were continuously perfused by basic salt solution (BSS) 3 ml/min containing in mM: 152 NaCl, 10 HEPES, 10 glucose, 2.5 KCl, 2 CaCl2, 1 MgCl2 and pH adjusted to 7.4. Test compounds diluted in the BSS to final concentrations were applied through fast perfusion system (Rapid Solution Changer RSC-200, BioLogic Science Instruments). Cells were imaged with 10x objective using Olympus IX-7010. Excitation wavelength was set as 494 nm, sampling frequency 2 FPS. Glutamate (100 µM with the co-agonist glycine 10 µM promoting activation of NMDA receptors subtype) or GABA (100 µM) were applied for 2 s. Finally, KCl (50 mM) application for 2 s was used to distinguish excitatory neurons from possible non-neuronal cells. Fluorescence was detected with the Till Photonics imaging system (FEI GmbH) equipped with a 12-bit CCD Camera (SensiCam) with a light excitation wavelength of 494 nm. Calcium responses to neurotransmitters were evaluated from changes in fluorescence intensity of individual neurons. To this end, regions of interest (ROI) of round shape around the cell body were selected from the whole image with the TILL vision Imaging Software (TILL Photonics GmbH). To distinguish from non-neuronal cells, ROI was taken at the time point corresponding to KCl-induced activation of neurons. The intensity values in each ROI were averaged at each time point to form a fluorescence signal for each neuron and normalized to the baseline level. Signals above 5% of the baseline were included in the analysis.
N2a Cell culture

Immortalized murine neuroblastoma cells (N2a) were maintained in DMEM, high glucose, GlutaMAX™ Supplement (Gibco, 31966021) media supplemented with supplemented with 10% Fetal Bovine Serum (Gibco, 10270106) and 1% penicillin and streptomycin in temperature controlled humidified incubator (37 °C, 5% CO₂). As previously published⁹, for differentiation induction 100,000 cells were plated in a 6-well plate and maintained in complete media supplemented with 20μM retinoic acid (Sigma-Aldrich, R2625) in dimethyl sulfoxide (DMSO) with or without serum starvation (2% FBS instead of 10%) changing half of the media every second day for 5 days before collection.

MOUSE STRAINS AND ANIMAL PROCEDURES

All experiments follow the Helsinki Declaration and guidelines set by the European Commission (European Communities Council Directive, 86/609/EEC) and were approved by the National Animal Experiment Board of Finland.

Animals were housed with same sex siblings, in controlled temperature, humidity and light (12 hours light/dark cycle) conditions. Animals had access to ad libitum food. Before the beginning of the animal study mice were divided in single cages. Mice were randomized and all the participants were blinded to the genotype (surgery, MRI acquisition, behavioral test, sample collection and data analysis). Prior to the surgery, we use random number generator (GraphPad Prism quick calcs https://www.graphpad.com/quickcalcs/randomN1/) to randomize the mice into treatment groups.

This numbering has been used in crescent order on 3-4 months old male mice that were divided into groups. pMCAo study was performed on 3-4 months old Balb/cOlaHsd male mice (n = 6-8 per timepoint). Cdr1as (ciRS-7) KO tMCAo study was performed on 3-4 months old C57BL/6N-Cdr1asem1Nikr⁹ (Cdr1as KO mice and the WT counterpart), provided by Prof. Dr. Nikolaus Rajewsky, MDC, Berlin, Germany (n = 22-24 per genotype). miR-7 inducible KO tMCAo study was
performed on 3-4 months old B6.Cg-miR7a1tm1(fl/fl)ms miR7a2tm1(fl/fl)ms miR7btm1(fl/fl)ms 39
Ndor1Tg(UBC-cre/ERT2)1Ejb/Stf and UBC-cre negative counterpart39 provided by Prof. Dr. 39
Markus Stoffel, ETH, Zurich, Switzerland (n = 20-26 per genotype).

Genotyping

Before the animal studies, ciRS-7 KO and miR-7 KO mice were genotyped as before9,39 except for 39
the tissue dissociation step. Briefly, ear puncture samples were digested in 50 mM Sodium Hydroxide 39
solution for one hour at 95 °C. Once equilibrated to room temperature the samples were neutralized 39
with 1M Tris Hydrochloride pH 8. Two microliters of digested samples were used to run the reaction 39
of PCR with Taq 2X Master Mix (New England Biolabs (UK) Ltd) following manufacturer protocol 39
and using the oligonucleotide primers as specified in Supplementary Table S8. The PCR products 39
were separated on a stained 2.5% or 1.5% Agarose gel for ciRS-7 and miR-7 KO respectively and 39
run in an electrophoresis chamber. Images were acquired with ChemiDoc Imaging Systems (Bio-Rad 39
Laboratories, Inc).

Permanent middle cerebral artery surgery (pMCAo)

Permanent middle cerebral artery occlusion (pMCAo) was performed in 3-4 months old 39
Balb/cOlaHsd male mice as previously described50. Briefly, mice were anesthetized using 5% 39
isoflurane and anesthesia was maintained with 2% isoflurane. Temperature of the animals was 39
controlled during the surgery with heating blanket and rectal probe (Harvard apparatus). After the 39
skin incision, the left temporal bone was exposed under the temporal muscle, and 1 mm hole was 39
drilled on top of bifurcation in the middle cerebral artery (MCA). The dura was gently removed, and 39
the MCA was lifted and permanently occluded under the bifurcation using a thermocoagulator (Aaron 39
Medical Industries Inc.). Success of the occlusion was confirmed by cutting the MCA above the 39
occlusion site. After the occlusion, the temporal muscle was replaced, and the wound was sutured.
The surgery was performed on a total of 20 mice, 3 mice (one per timepoint) died during the surgery and was then not included.

**miR-7 KO induction with tamoxifen**

Before tMCAo surgery, miR-7 inducible KO mice were injected with 2mg of Tamoxifen intraperitoneally once per day for 5 consecutive days at the age of 3 to 4 weeks as before39. Recombination was induced in both Cre+ and Cre- littermates. Tamoxifen (Sigma-Aldrich, T5648) was resuspended in 90% corn oil (Sigma-Aldrich, C8267) and 10% pure ethanol and dissolved at 56°C for 30 minutes in the dark. After injection the mice were put on soft food diet and their weight was monitored daily. Mice showing a successful genomic recombination in ear puncture samples two weeks after injection were included in the study (Supplementary Figure S4A). At the end of the study the animals were analyzed for miR-7 abrogation in the brain through RT-qPCR (Supplementary Figure S4B).

**Transient middle cerebral artery surgery (tMCAo)**

Transient middle cerebral artery occlusion (tMCAo) was performed in 3-4 months old male Cdr1as and miR-7 KO and WT mice as previously described51. Briefly, induction of anesthesia was performed with 3.5-4% isoflurane in 0.5 L/min of 100% O2-enriched air and maintained at 1-1.5% isoflurane during the surgery. The body temperature maintained at 36 ± 1°C during surgery with a heating pad attached to a rectal probe. A Doppler probe was fixed on the temporal bone over the MCA to monitor blood flow. The right common, external, and internal carotid arteries (CCA, ECA, and ICA) were exposed through an incision in the neck. The arteries were carefully dissected, and a 6-0 silicon coated monofilament of 0.21 mm of diameter (Doccol Corporation) was inserted into the right CCA or left ECA (Cdr1as and mir-7 KO respectively) and led through the ICA until blocking the origin of the MCA. Blood flow blockage was monitored with the Doppler probe, and animals
with less than 80% of blood flow decrease were discarded. After one hour of occlusion, the monofilament was withdrawn allowing blood reperfusion. Animals that did not show a correct reperfusion measured by laser doppler were discarded from the study. Incisions were permanently sutured, and the animals were allowed to recover in a temperature-controlled environment for 24 hours. The surgery was performed on a total of 32 WT and 25 Cdr1as KO mice, and 36 WT and 26 miR-7 KO. In the ciRS-7 animal study a total of 7 mice was excluded as: 2 KO and 2 WT mice lacked reperfusion upon surgery, 2 WT and 1 KO mice died during the study. In the miR-7 animal study a total of 12 mice was excluded as: 3 WT and 2 KO lacked reperfusion upon surgery, 4 WT and 3 KO died during the study.

**Magnetic resonance imaging (MRI)**

MRI was performed at 1, 3, and 7dpi using a vertical 9.4 T/89 mm magnet (Oxford instrument PLC) upon anesthesia with 1.8% isoflurane in 30% O2/70% N2O. We acquired twelve slices of 0.8mm thickness per mouse (echo time 40ms, repetition time of 3000ms, matrix size of 128 × 256 and field of view 19.2 × 19.2 mm²) and analyzed the first seven images using the Aedes software (http://aedes.uef.fi/) for MatLab program (Math-works). Upon definition of the region of interest (ROI) of contralateral, ipsilateral, and lesion, the lesion volume normalized on oedema was calculated on the first 7 section as: 

\[
\text{Corrected lesion size} = \text{lesion size} \times \left(1 - \frac{\text{ipsilateral size} - \text{contralateral size}}{\text{contralateral size}}\right)
\]

The lesion size is expressed has percentage of lesion on the total brain size.

**Neurological severity score (NSS)**

Mice were examined for neurological deficits at baseline and dpi 1, 3, and 7 using a severity scale comprising the following tests: postural reflex, circling, falling to contralateral side, placement of the contralateral forelimb during motion, and general state of alertness or consciousness. Deficits were
graded from 0 (normal) to 2 or 3 (severe). A sum of these scores were used for statistics. Behavioral assessment was performed by an experimenter blinded to the genotype of mice.

Transcardiac perfusion and sample collection

After anesthesia, mice were perfused transcardially with cold saline solution with heparin 2500 IU/l (Leo Pharma A/S). In the pMCAo study, the brains were collected and dissected into contralateral and peri-ischemic cortical regions. In the tMCAo study the brains were cut in 6 coronal sections and stained with 1% 2,3,5-Triphenyltetrazolium Chloride (TTC) (Sigma-Aldrich) in PBS solution for 5 minutes at 37 °C in agitation before dissection of contralateral and peri-ischemic cortical regions. For immunohistochemistry staining, brains were collected and fixed in 4% paraformaldehyde solution in 0.1 M phosphate buffer (PB) pH 7.4. After 22 hours of fixation, the brains were transferred in 30% sucrose in PB buffer solution for 48 hours and then frozen in liquid nitrogen before being stored in -70 °C.

GFAP immunostaining

Each brain was then cut using a cryostat (Leica Microsystems) into six 20 μm coronal sections 400 μm apart, collected on Superfrost™ Plus Microscope Slides (ThermoFisher Scientific) and stored in -70°C until immunostaining. Sections were then rehydrated with phosphate-buffered saline (PBS) pH 7.4 for 10 min and PBS with 0.05% Tween-20 (PBST) (Sigma-Aldrich) for 5 min. Endogenous peroxidase was blocked by using 0.3 % hydrogen peroxide (H₂O₂) in MeOH for 30 min after which sections were washed 3 x 5 min in PBST. Non-specific binding was blocked with 10 % normal goat serum (NGS)(Vector, S-1000) in PBST for 1 h at RT Sections were incubated overnight at RT in primary antibody rabbit anti-GFAP (Agilent, Dako Z0334,1:500 in 5 % NGS-PBST) and then with biotinylated secondary antibody anti-rabbit IgG (H+L) (Vector, BA-1000, 1:200 in 5 % NGS-PBST) for 2 h at RT followed by incubation in ABC reagent (Vector Elite Kit) for 2 h at RT. Sections were
washed 3 x 5 min in PBST before and after the incubations. Nickel-3,3'-diaminobenzidine (Ni-DAB) solution (0.175M Sodium acetate, 1% Nickel ammonium sulphate, 50mg DAB (Sigma-Aldrich, D-5905)) with 0.075 % H₂O₂ was used to develop the colour for 6 min stopping the reaction by washing the sections 2 x 5 min in dH₂O. Sections were then dehydrated in 50 % EtOH, 70 % EtOH, 95 % EtOH, 100 % EtOH for 2 min in each and 3 x 5 min in xylene followed by mounting the coverslips with Depex.

**Image acquisition & analysis**

Six GFAP immunoreactivity light microscope images per mouse were acquired by Leica DM6B-Z Thunder Imager microscope (Leica Microsystems CMS GmbH) equipped with DMC2900 camera using 10x magnification. Images were captured using LAS X software (Leica Microsystems CMS GmbH) with exposure time 5 ms, color-gain mode: R:0-G:0-B:25. The images were quantified from the peri-ischemic cortex next to the lesion border (1mm) and the corresponding area of the healthy contralateral hemisphere from 10x images using ImageJ software function “Measure particles”. The results were presented as relative immunoreactive area to the total area analyzed.

**RNA-seq data analysis**

Bulk RNA sequences of mRNA and miRNA have been aligned and quantified to the mouse genome of reference mm10 with the Cdr1as annotation of “chrX:61183248|61186174|.+” using the nf-core workflow (3.0 version of “rnaseq” and 1.1.0 version of “smrnaseq” applied without clipping and the three prime adapter “AGATCGGAAGAGCACACGTCT”), while circular sequences using 1.1 CIRIquant set to Read 1 matching the sense strand. Count data were prepared following the workflow defined by Law et al. We filtered out lowly expressed molecules in any condition using the function “filterByExpr” to increase the reliability of the mean-variance relationship. We removed the differences between samples due to the sequencing depth normalizing the count using the trimmed
mean of M-values (TMM)\textsuperscript{57} method and applied a log transformation minimizing sum of sample-specific squared difference to enhance the true positive and negative ratio in the downstream analysis\textsuperscript{58}. We checked for batch effect due to different timings in biological replicates preparation by performing a principal component analysis and unsupervised consensus clustering with Cola\textsuperscript{59}. We identified a batch effect due to different timings in biological replicates preparation influencing the grouping of the normoxic and OGD samples in the dataset ciRS-7 KO/WT cortical neurons (Supplementary Table S5). We corrected the batch effect of this dataset using the negative binomial regression from Combat\textsuperscript{60}. We adjusted the variance between the samples as before\textsuperscript{61} through winsorization\textsuperscript{62}. We finally created the design matrix for each pair of conditions to compare (contrast) and performed the differential expression analysis using limma/edgeR model\textsuperscript{56} controlling for the false discovery rate with Benjamini-Hochberg Procedure\textsuperscript{63}.

**Ingenuity Pathway Analysis (IPA) and Functional enrichment analysis**

We uploaded the differentially expressed genes of each contrast to QIAGEN IPA (QIAGEN Inc., https://digitalinsights.qiagen.com/IPA)\textsuperscript{64} and Metascape\textsuperscript{65} for Ingenuity pathway analysis and functional enrichment analysis, respectively. The analysis has been performed with default parameters and IPA’s background was composed of non-differentially expressed genes.

**Deconvolution analysis with scRNAseq**

We exploited cell-type specific gene expression from external single-cell RNA sequencing (scRNA-seq) data to define the cell subpopulations composing our cortical neuron culture (Supplementary Table 2) and ischemic stroke animal tissue (Supplementary Table 6) bulk RNA sequencing datasets. The scRNA-seq dataset of Loo et al.\textsuperscript{66} of the mouse cerebral cortex at embryonic day 14.5 was provided to SCDC\textsuperscript{67} for performing the deconvolution of our gene expression matrix with cortical neuron samples in normoxic conditions. The scRNA-seq data of Zeisel at al.\textsuperscript{68} composed of murine
cerebral cortex samples from p25 to p60 was instead provided to MuSiC\textsuperscript{69} to perform the deconvolution of our gene expression matrix with contralateral and peri-ischemic samples of wild-type and ciRS-7 KO ischemic stroke animals (Supplementary Table 6) as this method is designed to work with multi-subject scRNA-seq dataset.

**GRO-seq data analysis**

A summary of all GRO-seq samples used to quantify pri-miRNA expression levels are presented in Supplementary Table S3. Raw reads for public GRO-seq data were acquired from the GEO database. GRO-Seq reads were trimmed using the HOMER v4.3 (http://homer.salk.edu/homer)\textsuperscript{70} software to remove A-stretches originating from the library preparation. From the resulting sequences, those shorter than 25 bp were discarded. The quality of raw sequencing reads was controlled using the FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc)\textsuperscript{71} and bases with poor quality scores were trimmed using the FastX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Reads were aligned to mouse mm9 reference genome using the Bowtie\textsuperscript{72} version bowtie-0.12.7. Up to two mismatches and up to three locations were accepted per read and the best alignment was reported. The data was used to create Tag Directories using HOMER. To optimize coverage, a combined tag directory representing all samples of a given cell type (under one or several GSE numbers) was created and used for pri-miRNA quantification using the ‘analyzeRepeats.pl’ command and ‘-rpkm-strand +’ options”. The dataset used in this analysis are reported in Supplementary Table S3.

**miRWalk analysis**

We associated the significantly deregulated miRNAs and differentially expressed genes of wild-type and ciRS-7 KO cortical neurons subjected to OGD (Supplementary Table 2, Supplementary Table 4). We filtered miRNA-Target interactions obtained from 3.0 miRWalk\textsuperscript{73,74} database with 99% probability and located in the 3-UTR region and included Oip5os1 (Cyrano) as target of mmu-miR-
We linked the differentially expressed miRNAs and genes of each contrast by anticorrelation (e.g., miRNA with positive log fold change and significant adjusted probability value is associated to genes with negative log fold change, significant adjusted probability value and targets in miRWalk of the miRNA) focusing on mmu-miR-7a-5p.

**CDF generation**

We tested the assumption of anticorrelation between mmu-miR-7a-5p and its miRWalk gene targets using the empirical Cumulative Distribution Function (eCDF). For each contrast, we compared the eCDF (control function) obtained from the values of log fold change of the non-target genes against the one of the miRNA’s targets and tested their equality with the Kolmogorov–Smirnov test. To determine the magnitude and direction of the shift of the eCDF of the targets in respect of the non-targets, we measured the area between the two curves following this formula: \[ \int \{ F_Y(t) - F_X(t) \} \, dt \] and the Wasserstein distance. Both the considered targets and non-targets genes passed the filtering by expression, count normalization and participated at the differential expression analysis.

**CLIP-seq analysis**

We collected the HITS-CLIP dataset of Argonate 2 in pyramidal excitatory neurons produced by Tan et al. (Supplementary Table S9) and replicated the original analysis to map and annotate the genomic regions with a significant read cluster (peak). Briefly, we performed a quality control with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and applied trimming to remove bases after 50 base pairs due to low quality. We applied Cutadapt to remove adapters and over-represented sequences. We selected reads of at least 24 nucleotides in length and quality score higher than 20 for each nucleotide. Reads were aligned to the mouse reference genome mm10 using Burrows-Wheeler aligner with default parameters, then the mapped reads were expanded by 30
nucleotides on each side. We considered only reads present in at least 2 experimental replicates and clusters composed of at least 10 overlapping reads. Bowtie\textsuperscript{79} was used to map all occurrences of sequences complementary to the 6mer seed sequence (positions 2-7 of the mature miRNA) of mouse miRNAs from miRBase V21 (https://www.mirbase.org/) to the mouse genome mm10, allowing no mismatches. Strand specific intersection was done with the filtered clusters in the 3'UTR, 5'UTR, coding sequence (CDS) and intron regions from RefSeq gene definitions, as well as antisense matches to RefSeq genes\textsuperscript{80}. Strand specific intersection was also done to circRNAs from circBase\textsuperscript{81}. This generated a genome-wide list with predicted miRNA target sites backed by the detected Ago2 HITS-CLIP clusters for all mouse miRNAs. A subset including all miR-7a-5p targets is supplied as Supplementary Table S7. Only 3'UTR target sites were used to define miRNA mediated regulation. The full list of all predicted miRNA target sites was uploaded to GEO, as described in the Data Availability section.

Statistical analysis

Graphs and statistical analysis were performed in GraphPad Prism 9. Every Figure legend reports parameters of replicates (n), statistical test and p-value obtained. Where not specified, p-value was not statistically significant (p-value > 0.05). We refer to n in animal study as single biological replicate (mouse) and in cortical neuron as technical replicates in the same batch, the experiment has been performed in three independent biological replicates.

Data availability

The data produced and analyzed in this publication have been deposited in NCBI's Gene Expression Omnibus\textsuperscript{82} and are accessible through GEO Series accession number GSE213179, GSE213067, GSE213177, GSE211552 and GSE215210 as presented in the Supplementary Table S9. The code...
developed to analyze the data produced in this study is available upon request at

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AUTHOR CONTRIBUTIONS

F.S. and T.M. conceived and planned the study; F.S., T.M., T.H., J.J., R. Giniatullin, and D.T. provided intellectual contribution and contributed to the interpretation of the results; F.S., V.S., P.K., I.U., J.J, H.D., C.P., N.V and J. Koistinaho contributed to the in vivo experiments; F.S., V.S., D.M.T., M.G.B., R. Giniatullina, N.K., S.YH. contributed to the in vitro experiments; F.S., A. H.S., J.S., and J. Kjems planned and performed the libraries for the RNA-seq experiments; L.G. planned and
generated the code for the bioinformatic analysis and analyzed RNA-seq data; M.L., M.P., M.S., N.R.
provided the transgenic animals and assisted in the animal study on these models; M.V. and A.S.
produced and analyzed the HITS-CLIP-seq data; R.H. coordinated and supported the sample
preparation in Oulu University by F.S., V.S., S.K. and A.H; M.K. analyzed the GRO-seq data; F.S.
and T.M. wrote the manuscript in consultation with T.H., J.J. R. Giniatullin, J. Kjems with input from
all authors. All authors provided critical feedback and helped shape the research, analysis and
manuscript.

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Figure 1. ciRS-7 is the most abundant circRNA in mouse cortical neuron cultures and downregulated by in vitro OGD.

(A) Box plot of bulk RNA-seq deconvolution scores. Cell types are sorted based on the median of the universal semantic groups which belong to (e.g. Int1, Int2 in Interneurons). (B) Calcium imaging analysis of the cellular response to glutamate and GABA with representative fluorescence images (n = 11 biological replicates; signal included > 5% of baseline; data as mean ± SD). (C) Bar plot of
Ingenuity pathway analysis (IPA) conducted on bulk RNA-seq data of cortical neurons treated with OGD. Z-score indicates IPA prediction of transcription factor activation (positive value) or inhibition (negative value). Green color highlights HIF1α activation (|z| > 2 is considered significant). (D) Schematic representation of CIRIquant algorithm. Gene composed by three exons (dark blue, light blue, purple) generating a circular molecule through back-splicing or a linear transcript. In green specific circular reads on the back-splice junction (BSJ), in gray reads generated from forward splicing junctions (FSJ) in the same back-splicing region. Junction ratio score formula calculates a circularization score, higher the score and higher percentage of circular form of the gene of interest. (E) Heat map of logCPM of detected circularRNAs between normoxic (N) and oxygen and glucose deprived (OGD) murine cortical neuron culture. Blue color represents low expression and purple color represents high expression. Significant differentially expressed circularRNA grouped with a bar (* = adj. p-value < 0.05, n = 5 biological replicates). (F) (left) bar plot of CPM of the BSJ and FSJ of differentially expressed circularRNAs between normoxic and oxygen and glucose deprivation conditions, BSJ represents circular specific reads. (right) bar plot of the Junction ratio score calculated by CIRIquant algorithm. The score is a ratio of BSJ reads and FSJ reads mapped to BSJ site and represents percentage of circularization in respect of the linear transcript.
**Figure 2.** ciRS-7 network is dynamically altered in ischemic stroke like conditions.

(A) Schematic illustration of the relationships between the molecules of the regulatory network composed by ciRS-7, miR-7, Cyrano and miR-671. (1) Hansen et al. EMBO J, 2011; Hansen et al. Nature, 2013; (2) Piwecka et al. Science, 2017; (3) Kleaveland et al. Cell, 2018. (B) Volcano plot of differentially expressed microRNAs in cortical neuron cultures treated with OGD. Highlighted significantly upregulated (pink) and downregulated (green) microRNAs, miR-7 variants (red), miR-671 passenger strands (white), miR-671 (black) (n = 5 biological replicates; p-adj. <0.05 and |log2FC| > 0.3). (C) Schematic illustration of the murine genomic loci from which miR-7 primary transcripts...
(pri-miR) are transcribed, processed in precursor molecules (pre-miR) and finally into mature miR-7. Highlighted in red the seed sequence shared between miR-7a-5p and miR-7b-5p, underlined in black the non-seed mismatch in position 10 between the variants. (D) Bar plot of reads per kilobase million (RPKM) of miR-7 genomic loci in different cell type obtained from GRO-seq datasets. (E) Bar plot of the expression of miR-7 pri-mirna in cortical neuron after OGD treatment quantified by quantitative PCR. Relative expression normalized on normoxic condition (n = 4; * = p-value < 0.05, ** = p-value < 0.01 calculated with paired t-test; data as mean ± SD). (F) Plot of the cumulative distribution functions (CDFs) of the log fold changes of genes compared between OGD treated wild-type cortical neurons for 12h and normoxic conditions. Red curve is the CDF obtained with only miR-7 targets, while black curve with non-targets (n = 5 biological replicates; p-value calculated with Kolmogorov-Smirnov test). (G) Volcano plot of differentially expressed transcripts in cortical neuron cultures treated with OGD. Highlighted significantly upregulated (pink) and downregulated (green) transcripts, Cyrano (purple), miR-7 hosting gene HnrnpK (black) (n = 5 biological replicates; p-adj. <0.05 and |log2FC| > 0.3). (H) Bar plot of the quantification by quantitative PCR of ciRS-7 (blue), miR-7 (magenta), Cyrano (purple) in cortical neurons OGD treated at different timepoints (1 hour, 6 hours, 12 hours and 24 hours). Relative expression normalized on normoxic condition (normoxia n = 2-4, OGD n=5-6; * = p-value < 0.05, ** = p-value < 0.001, **** = p-value < 0.0001 calculated with unpaired t-test; data as mean ± SD).
Figure 3. ciRS-7 knock-out genotype exhibit differential OGD response without affecting the OGD induced changes in miR-7 and Cyrano.

(A) (left) Representative trace of calcium induced fluorescence in response to glutamate treatment in wild-type and ciRS-7 KO cortical neurons. (right) Scatter dot plot of calcium imaging analysis of the cellular response to glutamate of wild-type and ciRS-7 KO cortical neurons. (n = 8 biological replicates; signal included > 5% of baseline; * = p-value < 0.05 calculated with unpaired t-test; central
bar represents median value). **(B)** Bar plot of relative absorbance in MTT viability assay of wild-type and ciRS-7 KO cortical neurons treated with vehicle or glutamate 250μM. Data expressed as survival % normalized on wild-type vehicle (n = 4 biological replicates; * = p-value < 0.05, **** = p-value < 0.0001 calculated with one-way ANOVA corrected with Tukey's post-hoc test; data as mean ± SD).

**(C)** Venn diagram of the differentially expressed genes in normoxic versus OGD condition of wild-type and ciRS-7 KO cortical neurons (n = 5 biological replicates; p-adj. <0.05 and |log2FC|> 0.3).

**(D)** Bar plot of functional enrichment analysis top 20 significant Metascape clusters performed on differentially expressed genes in wild-type normoxic versus OGD cortical neurons. **(E)** Bar plot of functional enrichment analysis top 20 significant Metascape clusters performed on differentially expressed genes in ciRS-7 KO normoxic versus OGD cortical neurons. **(F) (left)** Bar plot of log2FC of ciRS-7 (blue), miR-7 (magenta) and Cyrano (purple) obtained from RNA-seq of wild-type and ciRS-7 KO cortical neurons in normoxic conditions versus OGD. **(right)** Bar plot of log2FC of miR-7 (magenta) obtained from RNA-seq showing differences in the expression in normoxic conditions (N) or OGD between the wild-type and ciRS-7 KO genotype (n = 5 biological replicates; p-adj. <0.05 and |log2FC|> 0.3).

**(G)** Plot of the cumulative distribution functions (CDFs) of the log fold changes of genes compared between OGD treated ciRS-7 KO cortical neurons for 12h and normoxic conditions. Red curve is the CDF obtained with only miR-7 targets, while black curve with non-targets (n = 5 biological replicates; p-value calculated with Kolmogorov-Smirnov test).
Figure 4. Lack of ciRS-7 ameliorates ischemic stroke outcome in vivo.

(A) Bar plot of the quantification by quantitative PCR of ciRS-7 (blue), miR-7 (magenta), Cyrano (purple) in 3-4 months old BALB/c mice subjected to pMCAo surgery. Peri-ischemic (PI) and contralateral (CL) cortices were collected at different timepoints (6 hours, 1 day and 5 days). Relative expression normalized on the average expression of CL (n = 6-7; * = p-value < 0.05, ** = p-value < 0.001, *** = p-value < 0.0001 calculated with paired t-test; data as mean ± SD).

(B) Schematic illustration of the experimental design: wild-type and ciRS-7 KO mice were subjected to tMCAo
surgery and monitored with neuroscore testing and MRI at 1 day, 3 days and 7 days post-surgery (dpi). Six mice were used at 1dpi to obtain peri-ischemic and contralateral cortices to perform RNA-sequencing. Eight to nine mice per group were used at dpi post ischemia for immunohistochemistry analysis. (C) (left) Bar plot of MRI quantification of the lesion size of wild-type and ciRS-7 KO mice subjected of tMCAo 1 day post-surgery. Data expressed as lesion percentage on total brain size adjusted for oedema (n = 25-28 per group; ** = p-value < 0.01 calculated with Mann–Whitney test; data as median with 95% confidence interval). (right) Representative image of lesion perimeter (blue) changes measured by MRI between wild-type (WT) and ciRS-7 KO animals. (D) Scatter dot plot of sensorimotor deficits measured by neuroscore in tMCAo wild-type and ciRS-7 KO mice at 1dpi (data expressed as total neuroscore; n = 22-25 per group; * = p-value < 0.05 calculated with Mann–Whitney test; data as median with 95% confidence interval). (E) Bubble plot of Ingenuity Pathway Analysis of differentially expressed genes between wild-type and ciRS-7 KO peri-ischemic cortices obtained through RNA-seq. Z-score indicates IPA prediction of pathway activation (positive value) or inhibition (negative value) in ciRS-7 KO. Color bar expresses p-value significance (yellow to purple), size expresses the number of genes contained in the pathway (|z| > 2 is considered significant, pathway containing >10 genes were included). (F) (left) Spiderweb plot of wild-type (gray) and ciRS-7 KO (blue) contralateral and peri-ischemic cortices bulk RNA-seq deconvolution scores. (right) Bar plot of neurons and astrocyte contribution based on deconvolution scores in wild-type (gray) and ciRS-7 KO (blue) contralateral and peri-ischemic regions. (G) (left) Bar plot of quantification of GFAP DAB (3,3'-Diaminobenzidine) immunostaining of wild-type and ciRS-7 KO tMCAo mice at 7dpi. (right) representative images of the staining highlighting GFAP+ cells. Relative expression normalized on the average expression of contralateral of wild-type mice (n = 8; * = p-value < 0.05, ** = p-value < 0.01, **** = p-value < 0.0001 calculated with one-way ANOVA corrected with Tukey's post-hoc test; data as mean ± SD). (H) Bar plot of the quantification by quantitative PCR of ciRS-7 (blue), miR-7 (magenta), Cyrano (purple) in contralateral (CL) and peri-ischemic (PI) cortices of ischemic wild-type and ciRS-7 KO mice at 1dpi. Schematic representation correlating it to MRI lesion size at the same timepoint. Data are normalized on the average expression of contralateral of wild-type mice (n = 5 per group; * = p-value < 0.05, ** = p-value < 0.01 calculated with paired t-test; data as mean ± SD).
Figure 5. Lack of miR-7 exaggerates ischemic stroke outcome regulating glutamatergic response.

(A) Schematic illustration of Cre-LoxP miR-7 KO animal model. Mice transgenic genome with LoxP sequences (blue) flanking all the three miR-7 loci (magenta) and tamoxifen (orange) inducible Cre recombinase (purple). Tamoxifen activation of the Cre recombinase results in miR-7 loci deletion. Control mice are not transgenic for Cre recombinase. (B) (left) Bar plot of MRI quantification of the lesion size of wild-type and miR-7 KO mice subjected of tMCAo one day post-surgery. Data expressed as lesion percentage on total brain size adjusted for oedema (n = 8-11 per group; * = p-
value < 0.05 calculated with Mann–Whitney test; data as median with 95% confidence interval). (right) Representative image of lesion perimeter (blue) changes measured by MRI between wild-type (WT) and miR-7 KO animals. (C) Schematic illustration of recombinant mice utilized in to generate Ago2 CLIP-seq data in excitatory neurons. FLAG-Ago2 (blue) is translated only in Cam2Ka+ (red) excitatory neurons in which Cre recombinase (yellow) expression lead to FLAG-Ago2 translation due to the removal of a stop codon (gray). After cross-linking (thunder) FLAG-Ago2-miRNA-target complex is immunoprecipitated with anti-FLAG antibody (purple) and the miRNA-target complex output is sequenced. (D) Pie chart of the distribution of miR-7 binding sites obtained by CLIP-seq analysis. The 3779 identified binding sites are divided into protein coding sequences (CDS, purple), 5' untranslated regions (5' UTR, blue), 3' untranslated regions (3' UTR, dark green), intronic sequences (Intron, light green). (E) Venn diagram of the miR-7 binding sites in the 3' UTR identified by CLIP-seq in excitatory neurons (645) and the downregulated genes in ciRS-7 KO tMCAo animals peri-ischemic region (3286), in which miR-7 is upregulated. The genes resulting from the overlap of these datasets (276) represent miR-7 physical targets potentially affected in ischemic stroke. (F) Bar plot of functional enrichment analysis top 20 significant Metascape clusters performed of the genes identified as miR-7 physical targets potentially affected in ischemic stroke. (G) (left) Representative trace of calcium induced fluorescence in response to glutamate treatment in wild-type and ciRS-7 KO neurons infected with miR-7 or control lentivirus. (right) Scatter dot plot of the calcium imaging analysis quantifying the cellular response to glutamate of wild-type and ciRS-7 KO cortical neurons infected with control (GFP) or miR-7 overexpressing (GFP + miR-7) virus (n = 5 biological replicates; signal included > 5% of baseline; * = p-value < 0.05, **** = p-value < 0.0001 calculated with Kruskal–Wallis test; central bar represent median value). (H) (left) Bar plot of neurite length quantified through live imaging of wild-type type and ciRS-7 KO cortical neurons infected with control (GFP) or miR-7 overexpressing lentivirus (GFP + miR-7). Data normalized on average neurite length of wild-type GFP infected neurons (n = 3 biological replicates, n = 8 technical replicates; ** = p-value < 0.01 calculated with one-way ANOVA test corrected with Tukey's post-hoc test; data as mean ± SD). (right) Representative image of ciRS-7 KO cortical neurons infected with control (GFP) or miR-7 overexpressing virus (GFP + miR-7).