Supplementary Figure and Legend

**Supplementary Fig. 1.** Physiological data of non-stressed controls and rats that underwent CVMS. (A) In the forced swim test, CVMS exposed rats (black bars) spent a significantly increased amount of time immobile (left panel) and a decreased amount of time mobile (right panel) as compared to the control rats (white bars). (B) Right and left adrenal gland weight relative to total body weight was not significantly different between control and CVMS rats. (C) Corticosterone (CORT) levels do not significantly differ for control and CVMS rats. (D) Total body weight gain per week for control and CVMS rats. (E) Thymus gland weight relative to total body weight was significantly decreased in CVMS compared to control rats. The forced swim test was performed directly after completion of the CVMS protocol. Rats were sacrificed directly after the forced swim test and CORT levels, adrenal and thymus weight were measured. Total body weight was measured each week, adrenal and thymus weights are relative to the last measured total body weight. Bars represent mean ± SEM of 8 animals in each condition. *** P<0.001 One-way or repeated measures of ANOVA.
Supplementary Table 1. Clinicopathological information of suicide victims and control subjects

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age (years)</th>
<th>Body composition</th>
<th>Diagnosis</th>
<th>Post-mortem delay (h)</th>
<th>Antidepressive medication in the last 2 months</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>51</td>
<td>average</td>
<td>control</td>
<td>5</td>
<td>none</td>
<td>cardiac arrest; arrhythmia</td>
</tr>
<tr>
<td>M</td>
<td>42</td>
<td>average</td>
<td>control</td>
<td>3,5</td>
<td>none</td>
<td>acute respiratory failure; cardiac ischemia</td>
</tr>
<tr>
<td>M</td>
<td>53</td>
<td>average</td>
<td>control</td>
<td>5</td>
<td>none</td>
<td>pulmonary embolism</td>
</tr>
<tr>
<td>M</td>
<td>55</td>
<td>below average</td>
<td>control</td>
<td>1</td>
<td>none</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>M</td>
<td>47</td>
<td>below average</td>
<td>control</td>
<td>2</td>
<td>none</td>
<td>acute cardiovascular failure; pulmonary embolism</td>
</tr>
<tr>
<td>M</td>
<td>63</td>
<td>average</td>
<td>control</td>
<td>2</td>
<td>none</td>
<td>acute cardiovascular failure</td>
</tr>
<tr>
<td>M</td>
<td>56</td>
<td>average</td>
<td>control</td>
<td>2</td>
<td>none</td>
<td>acute cardiovascular failure; coronary ischemia</td>
</tr>
<tr>
<td>M</td>
<td>47</td>
<td>average</td>
<td>MD/suicide</td>
<td>2,5</td>
<td>none</td>
<td>hanging</td>
</tr>
<tr>
<td>M</td>
<td>52</td>
<td>average</td>
<td>MD/suicide</td>
<td>2</td>
<td>none</td>
<td>hanging</td>
</tr>
<tr>
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<td>43</td>
<td>average</td>
<td>MD/suicide</td>
<td>3</td>
<td>none</td>
<td>hanging</td>
</tr>
<tr>
<td>M</td>
<td>42</td>
<td>average</td>
<td>MD/suicide</td>
<td>4</td>
<td>none</td>
<td>hanging</td>
</tr>
<tr>
<td>M</td>
<td>32</td>
<td>below average</td>
<td>MD/suicide</td>
<td>6</td>
<td>none</td>
<td>hanging</td>
</tr>
</tbody>
</table>

Abbreviations: HBB-Human Brain Bank Budapest Hungary; M-male; MD-major depression; CSF-cerebrospinal fluid

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**Supplementary Table 2. An overview of PCR primers used for this study.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5' → 3')</th>
</tr>
</thead>
</table>
| Rat Ucn1      | Forward CCCTTTCCCCACCTACCCCG  
Reverse GGGTAACTCTGGGCGCGGCT   |
| Rat miR-325   | Forward GGTTCCTAGTAGGTGCTCAG  
Reverse Universal primer (Qiagen miRScript kit)  |
| Rat miR-326   | Forward CCCCCAAGCTTCAGGTG TGACTTCAG  
Reverse Universal primer (Qiagen miRScript kit)  |
| Rat U6        | Forward GCTTCGGCACGACATATA  
Reverse CGCTTCACGAATTTCGT   |
| Rat -Actin    | Forward CCAGATCATGTTTGAGACCTTC  
Reverse AGGATCTTCATGAGTAGTCTG   |
| Human Ucn1    | Forward TCCCCAAGCCGTCTTCAG  
Reverse TGCCTCATGGTGCCGC   |
| Human CART    | Forward CCGAGCCCCTGGACATCTACTC  
Reverse AGCGCTTCGATCAGTCTCCT   |
| Human miR-325 | Forward GGTTCCTAGTAGGTGCTCAG  
Reverse Universal primer (Qiagen miRScript kit)  |
| Human miR-326 | Forward CCCCCAAGCTTCAGGT GAGCTTCAG  
Reverse Universal primer (Qiagen miRScript kit)  |
| Human U6      | Forward CTGC GCTCGCACAGCATATACT  
Reverse ACGCTTCACGAATTTCGTGC   |
| Human -Actin  | Forward TCCCTGGAGAGAGCTACGA  
Reverse AGCACTGTGTGGGCGTAGCA   |
Supplemental Experimental Procedures

Bioinformatics analysis

The miRanda and Targetscan algorithms were used to investigate the 3’UTR sequence of Ucn 1 and cocaine and amphetamine regulated transcript peptide (CART) mRNA and for putative binding sites of miRNAs across different animals. miRNAs were selected for further analyses as judged by the low predicted free energy of hybridization with the cognate mRNA target, as well as the degree of evolutionary conservation for the miRNA-binding site. Constructs and primers used in this report were designed using VectorNTI (Life Technologies, Carlsbad, CA, USA).

DNA constructs

The full 3’UTR from the Ucn 1 gene was amplified from a rat brain cDNA library using primers described in Table 1. The sense and antisense strands of oligonucleotides coding for the rat Ucn 1 3’UTR lacking the 7bp-seed sequence (CCCAGAG, putative miR-326-targeting site, mutated targeting site (MTS)) were synthesized (Sigma Aldrich, St. Louis, MO, USA). Full-length or MTS Ucn 1 3’UTR were ligated into the PmirGLO Dual-Luciferase miRNA target expression vector (Promega, Madison, WI, USA), using the SacI and XbaI restriction sites. The entire miR-326 gene including its 100 nt flanking region was amplified from (Wistar) rat brain genomic DNA using primers designed with vector NTI and described in Table 1. Polymerase chain reaction (PCR) products were cloned into the pmR-mCherry vector (Clontech, Mountain view, CA, USA) using the HindIII and BamHI restriction sites.

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Transfection of DNA constructs and short RNA oligonucleotides

Transfection of the DNA plasmids was performed using lipofectamine 2000 reagent (Life Technologies Carlsbad, CA, USA), according to the manufacturer’s protocol. Cells were transfected with mcherry or mcherry-rno-miR-326 plasmid DNA (400 ng), diluted in 50 μl Opti-Mem (Life Technologies, Carlsbad, CA, USA). Transfection of miRNA mimics and anti-miR’s (Qiagen, Hilden, Germany) was performed using siPORT Neofx (Life Technologies, Carlsbad, CA, USA) according to manufacturer’s protocol. Cells were transfected with 20 pM of either miR-326 mimic or anti-miR-326, (Qiagen, Hilden, Germany), diluted in 50 μl Opti-Mem (Invitrogen, Carlsbad, CA, USA). Transfection was carried out 24 hr after cell plating in either 24 or 48 well plates. Lipofectamine 2000 and siPORT Neofx reagent containing medium was removed 4 hours after transfection.

Immunocytochemical analysis

Primary neurons were fixed 72 hr following transfection using 4% paraformaldehyde (PFA) in PBS. Glass coverslips harbouring cells were washed thrice for five minutes with 50 mM NH₄Cl and thrice for five minutes with 0.1% triton x-100 in PBS. Coverslips were incubated overnight at 4°C with the primary antibody Ucn 1 anti-rabbit (Sigma Aldrich, St. Louis, MO, USA) at a 1:2000 dilution, with 2% normal donkey serum. Following primary antibody incubation, coverslips were washed thrice for five minutes with PBS, and incubated for 2 hr with the secondary antibody, Donkey-anti-rabbit Cy2 (Jackson immunoresearch, West Grove, PA, USA) at 1:100 dilution. Glass coverslips were

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washed thrice for five minutes with PBS followed by a five-minute incubation with 50 mM DAPI in PBS. Glass coverslips where mounted using Fluorsave reagent (Merck, Whitehouse Station, NJ, USA). Images were taken using a TCS SP2 confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany). Image analysis was performed using the ImageJ (National Institutes of Health) application Fiji (ww.fiji.sc). Quantification of Ucn1 protein levels was performed on cells showing both mcherry and Ucn 1-cy2 signal. The Ucn 1-cy2 mean pixel intensity signal was measured and subtracted from the background, according to quantification analysis previously performed by de Chevigny, and Peng.

Reverse transcription and quantitative Real-Time PCR (qRT-PCR)

The isolation of total RNA from postmortem human brain samples was completed as previously described. Following rat EWcp dissection, total RNA was isolated from this brain region using TRIzol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol. microRNA from human brain samples and Serum and brain miRNAs from CVMS rats were isolated using the mirVana-PARIS kit according to the manufacturer’s protocol (Life Technologies Carlsbad, CA, USA). The purity of all isolated RNA samples was determined by agarose gel electrophoresis and UV-spectrophotometric analysis, respectively. The mean ± S.D. of the 260/280 nm ratios was 2.0 ± 0.05. The integrity of the RNA isolated was further examined using the BioAnalyzer (Agilent). cDNA was synthesized from 0.5 - 1 μg RNA according to the protocol provided with the revertAid First Strand cDNA Synthesis Kit (Fermentas, St.
Leon-rot, Germany). cDNA synthesis for miRNA expression detection was performed using the miScript Reverse transcription kit (Qiagen, Hilden, Germany) as previously described\(^7\). Semi-quantitative real-time PCR (qRT-PCR) was performed with 1/10 diluted cDNA using the Maxima SYBR green kit (Fermentas, St. Leon-rot, Germany) according to manufacturer’s protocol for detection of mRNA using primers designed using vector NTI software. Relative mature miRNA expression levels were analysed using the miScript SYBR green PCR kit (Qiagen, Hilden, Germany), according to manufacturer’s protocol. qRT-PCR was performed using a Corbett Rotor-gene 6000 Real-time PCR machine (Qiagen, Hilden, Germany). Relative gene expression values were calculated using the delta Ct method.\(^8\) The relative expression levels of both housekeeping genes snRNA U6 and -actin were used to normalize for miRNAs, CART, and Ucn 1 levels. The housekeeping genes were stably expressed in all sample examined, suggesting that they could serve endogenous references for the quantifications of miRNAs and coding transcripts using qPCR assays. A list of the primers used is depicted in supplemental Table 2.

References


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