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Intracranial self-stimulation modulates levels of SIRT1 protein and neural plasticity-related microRNAs

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Abstract:	Deep brain stimulation (DBS) of reward system brain areas, such as the medial forebrain bundle (MFB), by means of intracranial self-stimulation (ICSS), facilitates learning and memory in rodents. MFB-ICSS has been found capable of modifying different plasticity-related proteins, but its underlying molecular mechanisms require further elucidation. MicroRNAs (miRNAs) and the longevity-associated SIRT1 protein have emerged as important regulatory molecules implicated in neural plasticity. Thus, we aimed to analyze the effects of MFB-ICSS on miRNAs expression and SIRT1 protein levels in hippocampal subfields and serum. We used OpenArray to select miRNA candidates differentially expressed in the dentate gyrus (DG) of ICSS-treated (3 sessions: 45' session/day) and sham rats. We further analyzed the expression of these miRNAs, together with candidates selected after bibliographic screening (miR-132-3p, miR-134-5p, miR-146a-5p, miR-181c-5p) in DG, CA1 and CA3, as well as in serum, by qRT-PCR. We also assessed tissue and serum SIRT1 protein levels by Western Blot and ELISA, respectively. Expression of miR-132-3p, miR-181c-5p, miR-495-3p and SIRT1 protein was upregulated in DG of ICSS rats ($P<0.05$). None of the analyzed molecules was regulated in CA3, while miR-132-3p was also increased in CA1 ($P=0.011$) and serum ($P=0.048$). This work shows for the first time that a DBS procedure, specifically MFB-ICSS, modulates the levels of plasticity-related miRNAs and SIRT1 in specific hippocampal subfields. The mechanistic role of these molecules could be key to the improvement of memory by MFB-ICSS. Moreover, regarding the proposed clinical applicability of DBS, serum miR-132 is suggested as a potential treatment biomarker.

Dear Benedict C. Albensi

According to the email received on February 27 and following your request, we are resubmitting the latest version of our manuscript (submitted on February 6), with reference number MOLN-D-19-00951R2, for your consideration. In this version, we had taken into consideration the reviewer's comments received on February 3 concerning to the revised manuscript (MOLN-D-19-00951R1). We hope our adjustments to the manuscript are satisfactory and will make this manuscript suitable for publication in Molecular Neurobiology.

All the changes made are clearly marked in green. Line numbers refer to the "Manuscript revision_R2_ MOLN-D-19-00951" document.

Yours sincerely,

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Reviewers' comments

Reviewer #4:

In particular, the authors changed the setting of the manuscript avoiding to focus it on Alzheimer's disease (AD) due to the lack of experiments on an animal model of AD.

I have the following further concern:

- **The Result section should describe the results obtained performing the experiments relative to the model used in the present work. Therefore, any mention of AD (including Table 2) should be removed from the Results to be inserted in the Discussion section.**

Response: We appreciate this suggestion. In accordance with it, we have removed the column regarding AD relation from Table 2, as well as its mention in the Results text body (lines 299-300 and 302).

TITLE:

Intracranial self-stimulation modulates levels of SIRT1 protein and neural plasticity-related microRNAs

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Conflict of Interest: The authors declare that they have no conflict of interest.

Author's contributions: PS, LA and SG performed MFB-ICSS procedures. IP performed molecular assays and data analyses. NF contributed in SIRT1 molecular analysis and CG in microRNA analyses. IP, EK and GH designed the experiments and interpreted data. IP and EK were the major contributors in writing the manuscript. EK, GH, LA and PS revised it critically. All authors read and approved the final manuscript and agreed to be accountable in ensuring appropriate answer to questions related to the accuracy and integrity of any part of the work.

Abstract

Deep brain stimulation (DBS) of reward system brain areas, such as the medial forebrain bundle (MFB), by means of intracranial self-stimulation (ICSS), facilitates learning and memory in rodents. MFB-ICSS has been found capable of modifying different plasticity-related proteins, but its underlying molecular mechanisms require further elucidation. MicroRNAs (miRNAs) and the longevity-associated SIRT1 protein have emerged as important regulatory molecules implicated in neural plasticity. Thus, we aimed to analyze the effects of MFB-ICSS on miRNAs expression and SIRT1 protein levels in hippocampal subfields and serum.

We used OpenArray to select miRNA candidates differentially expressed in the dentate gyrus (DG) of ICSS-treated (3 sessions: 45' session/day) and sham rats. We further analyzed the expression of these miRNAs, together with candidates selected after bibliographic screening (miR-132-3p, miR-134-5p, miR-146a-5p, miR-181c-5p) in DG, CA1 and CA3, as well as in serum, by qRT-PCR. We also assessed tissue and serum SIRT1 protein levels by Western Blot and ELISA, respectively. Expression of miR-132-3p, miR-181c-5p, miR-495-3p and SIRT1 protein was upregulated in DG of ICSS rats ($P<0.05$). None of the analyzed molecules was regulated in CA3, while miR-132-3p was also increased in CA1 ($P=0.011$) and serum ($P=0.048$).

This work shows for the first time that a DBS procedure, specifically MFB-ICSS, modulates the levels of plasticity-related miRNAs and SIRT1 in specific hippocampal subfields. The mechanistic role of these molecules could be key to the improvement of memory by MFB-ICSS. Moreover, regarding the proposed clinical applicability of DBS, serum miR-132 is suggested as a potential treatment biomarker.

Keywords:

Intracranial self-stimulation, microRNA, SIRT1, neural plasticity, hippocampus

Abbreviations:

CA, Cornu Ammonis; DBS, deep brain stimulation; DG, dentate gyrus; HP, hippocampus; ICSS, intracranial self-stimulation; LH, lateral hypothalamus; MFB, medial forebrain bundle; miRNA, microRNA; SIRT1, sirtuin1

Introduction

Research on the use of deep brain stimulation (DBS) therapies as a mean to enhance learning and memory has increased in the recent times [1,2]. In our laboratory, stimulation of the medial forebrain bundle (MFB) in the lateral hypothalamus (LH), by means of intracranial self-stimulation (ICSS), has been shown to facilitate learning and memory in rats [3-5]. This type of DBS, which ensures a correct physiological rewarding activation, has also been able to revert memory deficits caused by brain lesions and normal aging [6-8]. However, the molecular mechanism of DBS is not yet fully understood.

On the way to deciphering the underlying biological mechanisms of ICSS-induced cognitive improvements, some studies performed by our group and others have assessed cellular and molecular changes resulting from ICSS in memory-related areas. This treatment has been reported to induce structural changes in the complexity of dendritic branching and synapse density in the CA3 [9] and CA1 [10] hippocampal subfields. Moreover, ICSS has been found to induce the expression of CREB-dependent genes related to synaptic plasticity and neurogenesis, including *Arc* and *Bdnf*, in the hippocampus [11-13].

Nevertheless, neural plasticity relies on a plethora of coordinated molecules. In this scenario, microRNAs (miRNAs) have emerged as central regulators of gene expression that could have a key role in orchestrating plasticity mechanisms [14]. Some miRNAs have been described to be enriched or specifically expressed in the brain in relation to cognitive function. miR-132 and miR-134 are two of the best-studied miRNAs in the context of learning and memory. Their pathway has been linked to key synaptic plasticity-related proteins such as CREB and BDNF [15,16]. Recent studies have also shown their relation to SIRT1 [17-19], a deacetylase important for neuronal health and plasticity during normal aging [20,21]. Moreover, microRNAs

are present in body fluids, so they are being intensively studied due to their potential as disease and/or treatment biomarkers.

From this evidence we can speculate that MFB-ICSS could induce neural plasticity in part through changes in miRNA expression in both brain tissue and circulatory fluids. In this work we studied the effects of MFB-ICSS, using the same stimulation parameters that have been shown to facilitate learning and memory [4], on the regulation of miRNAs in dentate gyrus (DG), CA1 and CA3 hippocampal subfields and serum, as well as changes induced in SIRT1 protein levels.

Materials and methods

Animals

A total of 40 adult male Wistar rats (Harlan Laboratories, Horst, The Netherlands) with a mean age of 15.46 weeks (SD±1.42) at the beginning of the experiments and a mean weight of 480.07 g (SD±46.25) at the time of surgery, were used in this study, which was approved by the University Animal Welfare committee. The rats were housed individually in a controlled environment (21 ± 1 °C; humidity, 60%; lights on from 8:00 A.M. to 8:00 P.M.; food and water available ad libitum). All experiments were carried out in compliance with the European Community Council Directive for care and use of laboratory animals (CEE 86/609) and the Generalitat de Catalunya decree (Departament de Medi Ambient; Generalitat de Catalunya, 1995; protocol number 2023R).

Experimental groups

Animals were divided into two groups: electrode-implanted rats that received intracranial self-stimulation treatment (ICSS group, n=19) and electrode-implanted rats that did not receive ICSS treatment (sham group,

n=21). For OpenArray analysis, 12 samples of each group were pooled in 4 pools of 3 rats each. For other analyses we used individual samples.

Chronic electrode implantation

Both ICSS and sham rats were chronically implanted with an ICSS electrode aimed at the MFB in the LH. Animals were general anesthetized using 110 mg/Kg Ketolar® *Ketamine chlorhydrate* (Parke-Davis S.L. Pfiezer. Madrid) and 0.08 ml/100 g Rompun® *Xylazin 23 mg/ml*; i.p. (Bayer, Barcelona) and set on a stereotactic apparatus. A little hole was drilled in the skull, at AP=-2.3 mm and L=-1.8 mm from Bregma, in the right hemisphere, according to stereotaxic atlas Paxinos and Watson (1997). A monopolar stainless steel electrode (150 µm in diameter) was implanted at P=-8.8 mm. Electrodes were anchored to the skull with jeweler's screws and dental cement, leaving the connector protruding outside.

Intracranial self-stimulation behavior and treatment

After the post-surgery recovery period, animals were randomly assigned to one of the two experimental groups. Rats in the ICSS group were taught to self-stimulate in an ICSS behavior-establishment session, by pressing a lever in a conventional Skinner box (25×20×20 cm). Electrical brain stimulation consisted of 0.3s trains of 50 Hz sinusoidal waves at intensities ranging from 10 to 250 µA. In the ICSS establishment session, the optimum-current intensity (OI) of ICSS was established for each rat as previously described [22].

MFB-ICSS treatment was administered once daily during 45 minutes for the three following days after the ICSS establishment session (**Fig. 1**). ICSS rats were free to self-administer electrical stimulation at their OI by pressing the lever, while sham rats were handled and allowed to explore the ICSS box but did not receive any electrical current through the electrode.

Sample collection

Blood samples were collected from the lateral tail vein 90 minutes after last ICSS/sham treatment session, using Microvette tubes (Microvette® CB 300, SARSTEDT Sau). Tubes were maintained 45 min at room temperature and centrifuged 10 minutes at 3000 rpm to collect serum. Immediately after blood extraction, animals were sacrificed by decapitation and brains were dissected to isolate the hippocampus. Hippocampal subfields CA1, CA3 and DG were dissected as previously described by Lein et al. [23]. Both serum and tissue samples stored at -80ºC until use.

Protein and RNA isolation

Both total RNA and protein were extracted from tissue using mirVana PARIS Kit (Ambion). Briefly, samples were homogenized in Cell Disruption Buffer using a Heidolph DIAX900 homogenizer. Half of the homogenate was further processed for protein analysis and the other half was used for RNA extraction. For serum, 100 µL of sample were used to obtain RNA extracts employing the same kit. After the extraction process, RNA and protein extracts were stored at -80ºC until use.

The concentration of RNA was determined using a NanoDrop 1000 Spectrophotometer (ThermoFisher) and quality was assessed using Agilent Bioanalyzer 2100, showing RNA integrity ranking between RIN 7,0 and RIN 8,6.

Protein quantification was performed using Pierce BCA Protein Assay kit (ThermoScientific), following kit instructions.

TaqMan OpenArray procedure

Four sham samples and four ICSS samples were obtained by pooling extracted RNA from DG of 3 rats/pool, with a total of 12 rats per group being used. cDNA was synthesized from 100 ng total RNA, using Megaplex

Primer pools A and B and the MicroRNA Reverse Transcription kit (Life Technologies). Pre-amplified samples (1:40 dilution) were loaded onto TaqMan® OpenArray® Rodent MicroRNA Panel (ThermoFisher), using the AccuFill System, to be run on QuantStudio™ 12K Flex Real-time PCR system (Applied Biosystems).

miRNA profiling data were analyzed using Expression Suite Analysis Software v1.1 (ThermoFisher). Some assays were omitted from the analysis based on the quality of the amplification curve. Maximum allowed CT was fixed to 28.0 to avoid false positive results. Relative quantification value for each miRNA was obtained by the algorithms implemented in the software for the comparative (ΔΔ) CT method, using sham group as the reference biological group and a global normalization method. A list of differential expressed miRNAs between sham and ICSS pools was obtained using a FoldChange Boundary of 1.3 and P-value Boundary of 0.05. No Benjamini-Hochberg false discovery rate was used to adjust P-values in order to avoid false negative results, because it would unnecessarily limit the set of candidate list for further validation. The differentially expressed miRNAs were functionally investigated using prediction tools accessible from DIANA mirPath v.3 and TargetScan v7.2, together with bibliographic research. A subset of miRBase-annotated miRNAs, which were also interesting on a functional basis, was selected as potential miRNA targets of ICSS for further qRT-PCR validation.

TaqMan miRNA qRT-PCR

Expression levels of several miRNAs of interest were determined in tissue and serum samples. cDNA was synthesized and preamplified from 10 ng total tissue RNA from individual subjects, using TaqMan® Advanced miRNA cDNA Synthesis Kit (Applied Biosystems), in an AB vecti 96 well thermocycler (Applied Biosystems). Slight modifications were included for serum samples regarding the initial RNA extract volume (3 µL) and the

number of cycles of the miR-Amp reaction (16). PCRs were run on an AB QuantStudio 7, using TaqMan Advanced miRNA qPCR assays (Applied Biosystems). Expression levels of the target miRNAs were determined in DG and serum samples using the assays listed in **Table 1**. miR-16-5p, let-7a-5p, let-7b-5p and miR-124-3p were included as reported potential endogenous controls, according to literature. In addition, we further analyzed miR-132-3p, miR-181c and miR-495-3p in CA1 and CA3 samples.

Relative quantity of each target miRNA was determined as $2^{-\Delta\Delta C_T}$ ($\Delta\Delta C_T$ = ΔCt sample − ΔCt reference sample; ΔCt= Ct target – Ct normalizer), using the mean in the sham group as the reference sample and miR-16-5p as hippocampal tissue normalizer or let-7a-5p as serum normalizer, being the most stable endogenous candidates according to NormFinder algorithm.

Western Blot

Total protein (30 µg) extracted from DG, CA1 and CA3 subfields was loaded onto a Criterion TGX Stain-Free PreCast Gels 18 well comb (Bio-Rad) under reducing conditions, and electrotransferred to PVDF membranes. After 1 h of blocking with 5% Bovine Serum Albumin (Sigma) in TBS-T (tris-buffered saline [100 mM NaCl, 10 mM Tris-HCl pH 7.5] containing 0.1% Tween-20), membranes were incubated with primary antibodies: rabbit anti-SIRT1 (1:2,000, no. 07-131, Millipore) and mouse anti-GAPDH (1:800,000, MAB374, Millipore) at 4ºC, overnight. Peroxidase-conjugated secondary antibodies were used for 1h at room temperature: goat anti-rabbit (1:20,000, no. 31460, ThermoScientific) and goat anti-mouse (1:20,000, no. 115-035-044, Jackson ImmunoResearch). Intensities of antibody reactive bands were detected using Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore) in a FluorChem luminometer, and quantified by densitometry using FluorChem SP software (AlphaEaseFC™). SIRT1 relative intensities were normalized

by GAPDH intensity, and sham group was used as normalizer for each membrane.

SIRT1 ELISA

Concentration of SIRT1 protein in diluted serum samples (1:15) were quantified by ELISA (LifeSpan BioSciences, LS-F21634), according to manufacturer's instructions.

Statistical analyses

All statistical analyses were performed using IBM SPSS Statistics 25. Normality analyses were performed for the data of each group using the Shapiro-Wilk normality test. Statistical differences on miRNA expression derived from qRT-PCR analyses and on SIRT1 protein levels were assessed using independent samples t-test, for parametric comparisons, or Mann–Whitney U test, for nonparametric comparisons. Correlations between variables were estimated using the Spearman correlation test. Statistical significant results were considered when $P<0.05$ using a 95% confidence interval.

Results

Synaptic plasticity-related miRNAs regulated by MFB-ICSS in DG

In order to find potential miRNA targets of MFB-ICSS we performed a TaqMan OpenArray profiling 750 well-characterized Rodent miRNAs. From the expression data, we identified 14 miRNAs as differentially expressed between sham and ICSS condition (FC±1.3; $P<0.05$), in DG subfield, as shown in **Fig. 2**. Functional analyses and bibliographic research on these targets reveals that some of the miRNAs are associated with neuroplasticity pathways and reportedly target synaptic plasticity-related proteins, such as SIRT1 and BDNF (**Table 2**).

The levels of 6 of the 14 altered miRNAs in DG hippocampal subfield by MFB-ICSS related to neural plasticity (4 upregulated and 2 downregulated, highlighted in **Table 2**), were further analyzed by qRT-PCR in individual samples, in order to validate OpenArray results from pools. Moreover miR-132-3p, miR-134-5p, miR-146a-5p and miR-181c-5p, selected according to their reported role in learning and memory function, were also analyzed (see **Table 1**). Results from qRT-PCR analyses are shown in **Fig. 3a**. Expression of miR-495-3p, miR-132-3p and miR-181c-5p was significantly increased in the DG of ICSS rats relative to sham ($P=0.041$, $P=0.005$ and $P=0.020$, respectively). miR-196a-5p, miR-485-3p, miR-185-5p, miR-154-5p, miR-197-3p, miR-134-5p and miR-146a-5p did not appear to be significantly different between ICSS and sham groups.

Specific hippocampal subfield expression of miR-495, miR-132 and miR-181c after MFB-ICSS

To assess the region-specificity of miR-495-3p, miR-132-3p and miR-181c-5p upregulation, we further evaluated their levels in CA1 and CA3 hippocampal subfields. Only miR-132-3p showed a significant increase in CA1 ($P=0.011$) (**Fig. 3c**), even though a tendency is also seen for miR-181c-

5p (P=0.082) (**Fig. 3d**). Statistically significant changes were not found in CA3, for any of the three miRNAs (**Fig. 3b-d**).

MFB-ICSS-regulated miRNAs in serum

We assessed the changes of the 10 MFB-ICSS-regulated miRNA candidates in serum samples. Levels of miR-485-3p, miR-495-3p, miR-154-5p and miR-134-5p were under the detection limit for many serum samples, belonging to both sham and ICSS group. No differences were found between the two conditions in the detected miRNAs miR-196a-5p, miR-185-5p, miR-197-3p, miR-146a-5p and miR-181c-5p (**Fig. 4a**). Only miR-132-3p was found to be significantly upregulated (P=0.048) in ICSS compared to sham rats' serum (**Fig. 4b**).

Moreover, the correlation analysis of miR-132-3p levels in DG and serum showed a significant negative correlation in the ICSS group (ρ=-0.615, P=0.033), being non-significant in the sham group (**Fig. 4c**). Significant correlations were not observed in CA1 and in CA3.

SIRT1 protein levels in hippocampal subfields and serum after MFB-ICSS

SIRT1 relative protein levels in DG were found to be significantly increased in the ICSS group compared with sham (P=0.033). However, SIRT1 levels did not change in CA1 and CA3 subfields (**Fig. 5a**).

In serum, SIRT1 was detected in all samples. There were no differences in the levels between the two groups (**Fig. 5b**). However, we found a significant positive correlation (ρ=0.650, P=0.022) between SIRT1 serum levels and miR-132 serum levels, specifically in the ICSS group (**Fig. 6**).

Discussion

The main findings of the present study show that rewarding electrical stimulation of the MFB, a treatment with demonstrated ability to improve memory, induces subfield-specific hippocampal upregulation of miR-495, miR-132 and miR-181c, three miRNAs linked to neural plasticity. Importantly, MFB-ICSS also increases serum levels of miR-132, one of the best-studied miRNAs in the context of cognition. In addition, MFB-ICSS modifies SIRT1 protein levels, a deacetylase putatively targeted by all three affected miRNAs, specifically in the DG subfield of hippocampus.

Different studies, including the latest research in our lab, have established that memory-enhancing DBS to different targets induces increased hippocampal expression of synaptic plasticity protein markers, which are prominent molecular correlates of memory consolidation [11,27]. However, this is the first study showing that a DBS procedure, specifically MFB-ICSS, induces miRNA changes in the rat hippocampus, 90 min after stimulation. The time selected for the present study was chosen based on the induction time described for different miRNAs after various learning or plasticity-related stimuli [28,29]. Moreover, miRNA changes were separately evaluated in DG, CA1 and CA3 subfields, since these regions are known to differ in terms of neural plasticity mechanisms [30,31]. DG subfield is one of the few regions that preserve neurogenesis in the adult life, which is crucial for maintaining synaptic connectivity [32]. This fact emphasizes DG relevance when studying the effects of MFB-ICSS on memory.

Out of the 6 miRNA candidates to be regulated by MFB-ICSS in DG, which were preselected from a first OpenArray screening using pooled samples, only miR-495 was found to be significantly upregulated in ICSS-treated rats, revealing the need to validate the array results using a complementary technique and an increased sample size. Additionally, the increased

expression of miR-495 was not observed in CA1 and CA3 subfields. The specific role of miR-495 in the brain, as well as its function regarding learning and memory, has not yet been well characterized. However, it may be involved in activity-dependent remodeling of synaptic plasticity [33] and both *Bdnf* and *Arc* mRNAs have been identified among its putative targets [34,35]. We have previously reported an increase in levels of these mRNAs in extracts of the whole hippocampus at 90 min after MFB-ICSS, which apparently contradicts the putative role of this miRNA. However, the association between increased or decreased levels of *Bdnf* mRNA and hippocampal miR-495 regulation has been shown to vary depending on the region after ethanol treatment, revealing the existence of a complex regulation of the hippocampal subfields [34].

In this regard, we report an upregulation of miR-132 and miR-181c 90 minutes after MFB-ICSS, which was also dependent on the analyzed hippocampal subfield. Increased expression of these two miRNAs in hippocampus has been linked to learning and memory [36-38]. In this work, we report a significant increase of miR-132 in both DG and CA1, but not in CA3 subfield. Regarding the adult DG, miR-132 has been reported to coordinate the integration of newborn neurons [39]. ICSS has been found to upregulate neurogenesis-related genes [13] and to increase the number of DCX-positive cells and functional newly generated cells in DG [40, 41]. This evidence, together with our results, suggests that one of the possible functions of miR-132 could be mediating the increase in neurogenesis after ICSS. Furthermore, it has been reported that miR-132 is linked to spinogenesis, spine enlargement and dendritic growth in cultured neurons [42,43], and we have previously demonstrated that ICSS induces an increase in dendritic arborization and synaptic density in CA1 measured at three and 20 days post-ICSS [11]. Since increased CREB function in CA1 is able to rescue dendritic complexity and spine density alterations [44], the increment we report in miR-132, a CREB-regulated miRNA [43] may facilitate the

structural plasticity observed in CA1 after ICSS. Moreover, it has been reported that LTP can produce the simultaneous induction of *Arc* mRNA and miR-132 [45]. In agreement with this study, the regional regulation of miR-132, induced by MFB-ICSS, matches results from our previous studies reporting hippocampal *Arc* mRNA increases at 90 min, as well as increased ARC protein at 4.5 hours after ICSS in DG and CA1, but not in CA3 [11]. Similarly, present results show that miR-181c is upregulated in DG, and reveal a tendency to significance regarding upregulation in CA1. Even though its specific role in these regions is not completely clear, it has been suggested that miR-181c can modulate dendritic branching and synaptogenesis in vitro [46,47,38]. The region-specific effects of MFB-ICSS on miR-495, miR-132 and miR-181c could indicate that these miRNAs may be orchestrating the regional plasticity mechanisms of MFB-ICSS.

In order to further elucidate the molecular pathways leading to synaptic plasticity effects of MFB-ICSS-upregulated miRNAs, the study of their putative targets acquires paramount importance. miR-495, miR-132 and miR-181c all have been suggested to target *Sirt1* [34,21,48]. SIRT1 protein plays a key role in learning and memory functions, by deacetylating a variety of substrates critical for signal transduction cascades. SIRT1 knockout mice exhibit deficits in cognitive abilities associated with impaired synaptic plasticity [20]. In hippocampal neurons, SIRT1 has been reported to regulate dendritic development and axonal elongation [49,50]. We found SIRT1 to be significantly upregulated in DG subfield 90 minutes after MFB-ICSS. Ma et al. reported an increase in SIRT1 expression during neural stem cell differentiation in DG, and they suggested that SIRT1 is an important regulator of the differentiating/self-renewal balance of adult neural stem cells [51]. Thus, the specific overexpression of SIRT1 in DG, where a neurodevelopmental environment exists, could suggest that this protein is involved in promoting the neurogenesis found after ICSS. In

accordance with our results, parallel changes of SIRT1 and miR-132 at the hippocampus [21], and even positive correlations between them in brain tissue [52], have been described. Salta et al. presented a bimodal regulatory network by which an increase in miR-132 results in increased SIRT1 expression in glial progenitors during development [17]. Thus, taking these observations into account, once induced by MFB-ICSS, both molecules could work together, improving neurogenesis and synaptic plasticity in DG.

In our study, MFB-ICSS-treated rats showed increased miR-132 serum levels, consistent with changes in DG miR-132. However, this resulted in a negative correlation, specifically for the ICSS group. This negative correlation could be explained by the timeframe between the start of the clearance of miR-132 from brain tissue to blood, which would result in the progressive decrease of its levels in the tissue coupled with an increase in its serum levels. Although SIRT1 serum levels were not found to be different in ICSS and sham condition, we observed a positive correlation between serum levels of miR-132 and SIRT1 in the ICCS-treated rats, indicating once more that these two molecules might cooperate to enhance neural plasticity.

Interestingly, miR-495, miR-132, miR-181c and SIRT1 have been mechanistically related to the main molecular hallmarks of Alzheimer's disease and are all found to be significantly reduced in patients' brain [21,53,54,55]. miR-132 is dysregulated at early stages of the disease, even before neuronal loss occurs [56], and higher hippocampal levels of miR-132 correlate with better cognition scores in Alzheimer's disease patients [21]. Moreover, miR-132 has been suggested to play a rather upstream role in the pathogenic cascade of Alzheimer's disease [57], and to be also related to amyloid pathology [58,52]. SIRT1 is also associated with attenuated amyloid production, which it achieves by promoting the non-amyloidogenic processing of APP [59]. miR-181c was found to be downregulated in

hippocampal neurons treated with amyloid beta peptides, as well as in the hippocampus of APP23 transgenic mice [60], and negative correlations between amyloid beta and miR-181c were observed in Alzheimer's disease patients' brains [54]. Moreover, miR-132, miR-181c and SIRT1 have all been related to tau expression, phosphorylation or aggregation [61- 63], suggesting protective roles for them in the context of tauopathies [64,65]. The situation of these molecules at an intersection between MFB-ICSS effects and Alzheimer's-related dysregulations is, at least, intriguing, and encourages further research of MFB-ICSS in an Alzheimer's-modeling context.

Conclusions

Our results show for the first time that a DBS procedure, in this case MFB-ICSS, induces miRNA changes in the rat hippocampus, 90 min after stimulation. These changes include the upregulation of three important neural plasticity related miRNAs (miR-495, miR-132, miR-181c) with a specific hippocampal subfield pattern. This study also reports DG upregulation after MFB-ICSS of the SIRT1 protein, a deacetylase that plays a role in synaptic plasticity and aging-associated neuronal protection. The functional pathways regulated by these molecules could be key to the memory-improving effects of MFB-ICSS. Moreover, the changes in miR-132 levels in serum after MFB-ICSS serve as preliminary evidence to suggest its future potential use as DBS treatment biomarker.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Ethics Committee at the Universitat Autònoma de Barcelona, with order number 3942).

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Figure captions

Fig. 1 Schematic representation of the experimental design. Rats in the ICSS group were treated with three MFB-ICSS sessions (45'/session/day on days 1, 2 and 3), with a previous session for ICSS establishment (on day 0). Rats in the sham group were exposed to sham sessions (days 1, 2 and 3). 90 minutes after last ICSS/sham session, serum samples were obtained and rats were sacrificed in order to obtain hippocampal subfield samples to perform molecular analyses

Fig. 2 ICSS differential miRNA expression in DG. Volcano plot representing comparative miRNA expression of ICSS versus sham group in DG subfield, using TaqMan OpenArray MicroRNA Rodent Panel. X-axis represents difference in expression level on a $\log_2$ scale, whereas y-axis corresponds to the P-values on a negative $\log_{10}$ scale. Black dots depict miRNAs with significant differential expression in ICSS group relative to sham, both down- and upregulated. Grey dots represent miRNAs with no significant differential expression between the two groups. n=4 pools of 3 rats per group. Fold-Change Boundary=1.3; P-value Boundary=0.05

Fig. 3 Relative expression of miRNA candidates in hippocampal subfields. Expression of **a** 10 miRNA candidates in DG subfield and **b** miR-495-3p, **c** miR-132-3p and **d** miR-181c-5p in CA1 and CA3 subfields, in sham and ICSS groups. Levels of miRNAs were detected by qRT-PCR and calculated as $2^{-\Delta\Delta Ct}$, using miR-16-5p as endogenous normalizer, and sham group mean as the reference sample. Data are presented as mean±SD, n=12-17 rats/group for DG and CA1 subfields and 6 rats/group for CA3 subfield. Statistically significant differences analyzed using independent samples t-test or Mann–Whitney U test. *P<0.05 relative to sham

Fig. 4 Relative expression of miRNA candidates in serum. a-b Relative expression of the 6 miRNA candidates detected by qRT-PCR in serum in

ICSS and sham group, calculated as $2^{-\Delta\Delta Ct}$, using let-7a-5p as endogenous normalizer, and sham group mean as the reference sample. Data are presented as mean±SD, n=11-12 rats/group. Statistically significant differences analyzed using independent samples t-test or Mann–Whitney U test. *P<0.05 relative to sham **c** Scatter plot showing the relation between miR-132 levels in DG and serum in sham and ICSS group. Spearman correlation test was used to determine significance, being non-significant in sham group (P=0.519; Spearman's correlation coefficient (ρ)=-0.218) and significant for ICSS group (P=0.033; Spearman's correlation coefficient (ρ)=-0.615). n=11 rats/sham group; 12 rats/ICSS group

Fig. 5 SIRT1 protein levels in hippocampal subfields and serum. a SIRT1 relative expression in ICSS and sham groups in DG, CA1 and CA3 subfields, detected by Western Blot and calculated as SIRT1 band intensity normalized against GAPDH band intensities. Data are presented as mean±SD, n=14-21 rats/group for DG and CA1 subfields and 6 rats/group for CA3 subfield. Statistically significant differences analyzed using independent samples t-test. *P<0.05 relative to sham. **b** SIRT1 concentration in serum, analyzed by ELISA, in sham and ICSS group. n=12 rats/group

Fig. 6 Correlation between SIRT1 protein levels and miR-132 in serum. Scatter plot showing the relation between SIRT1 protein levels and miR-132 levels ($2^{-\Delta\Delta Ct}$) in serum in sham and ICSS group. Spearman correlation test was used to determine significance, being non-significant in sham group (P=0.347; Spearman's correlation coefficient (ρ)=-0.333) and significant for ICSS group (P=0.022; Spearman's correlation coefficient (ρ)= 0.650). n=10 rats/sham group; 12 rats/ICSS group

Figure 1

Click here to access/download;Figure;Fig1.docx

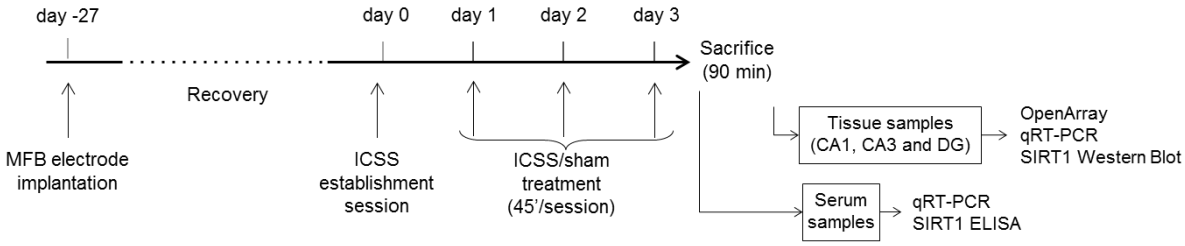

Figure 2

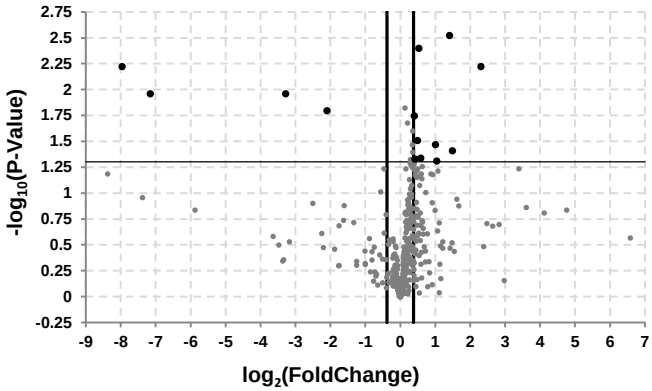

Figure 3

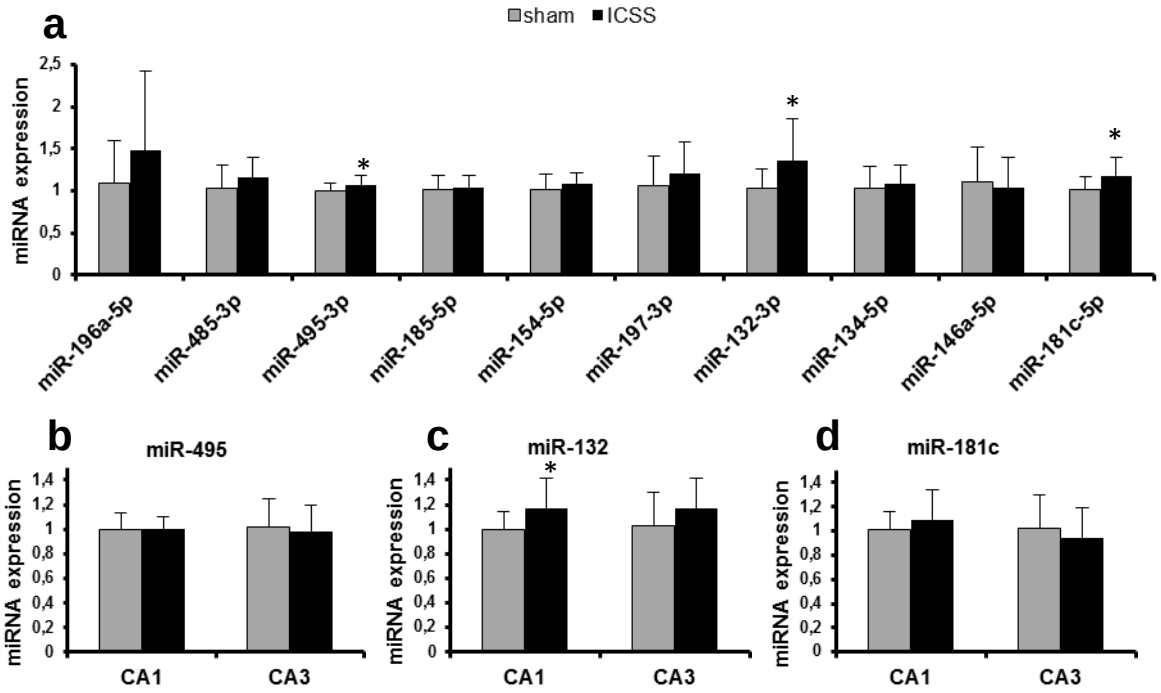

Figure 4

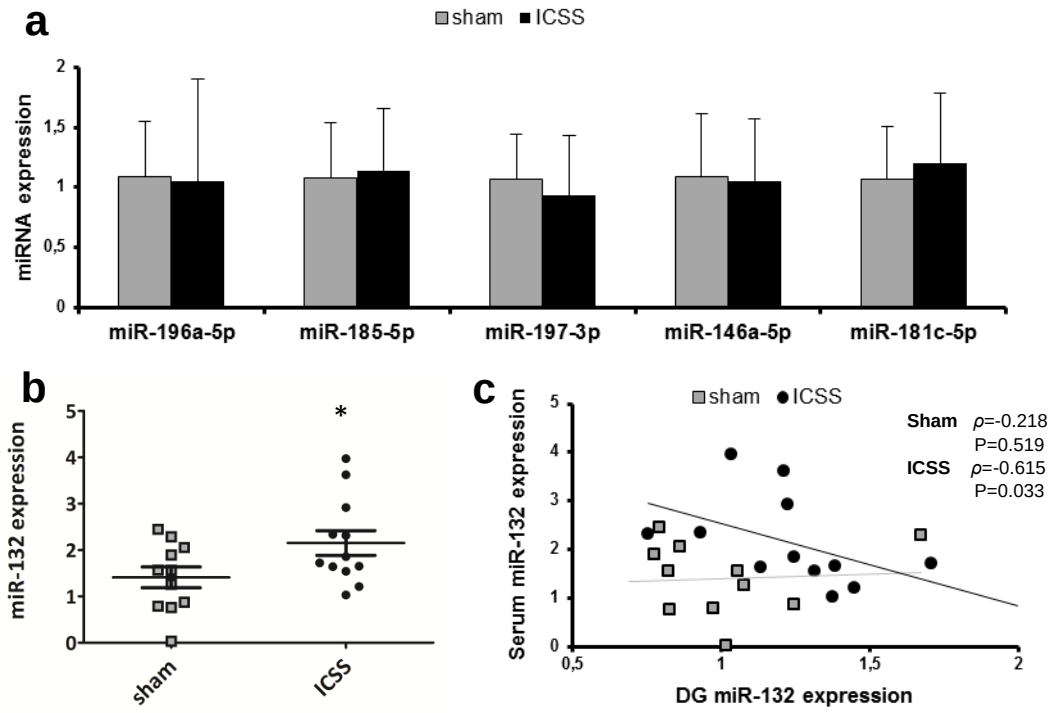

Figure 5

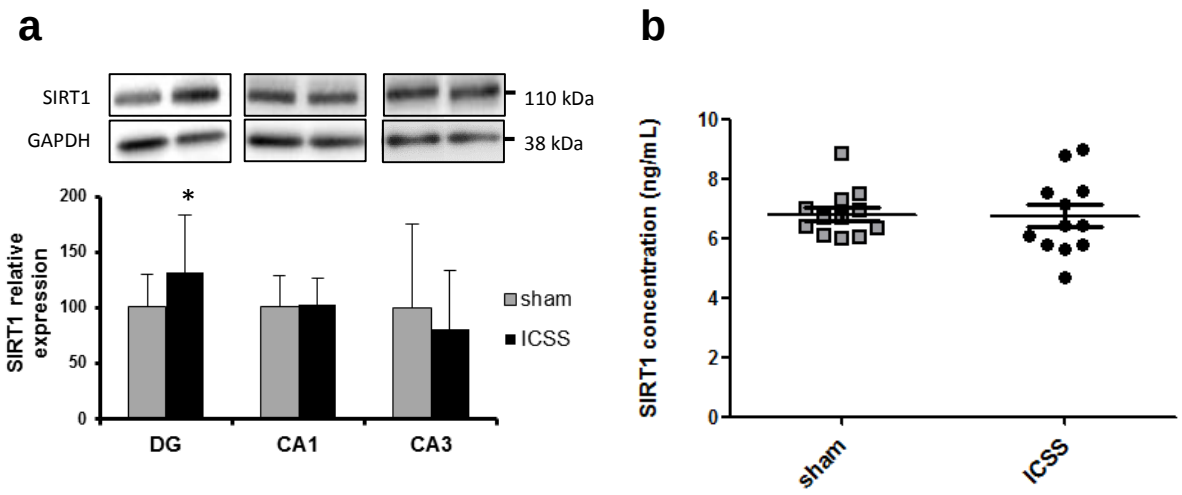

Figure 6

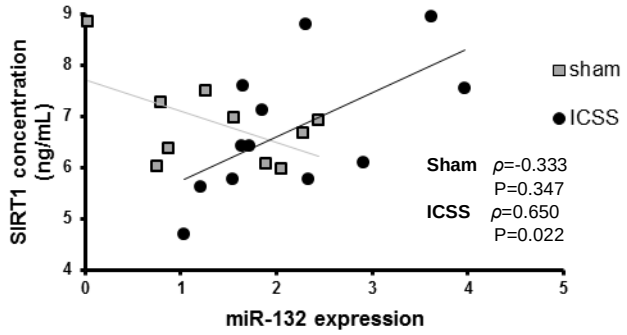

Table 1. MicroRNAs analysed by qRT-PCR, using TaqMan Advanced Assays.

Selection	Type	MicroRNA symbol	TaqMan Advanced miRNA Assay name	TaqMan Advanced miRNA Assay ID
OpenArray-based	Target	miR-196a-5p	hsa-miR-196a-5p	478230_mir
		miR-485-3p	mmu-miR-485-3p	mmu481854_mir
		miR-495-3p	mmu-miR-495-3p	mmu482634_mir
		miR-185-5p	hsa-miR-185-5p	477939_mir
		miR-154-5p	hsa-miR-154-5p	477925_mir
		miR-197-3p	hsa-miR-197-3p	477959_mir
Literature-based		miR-132-3p	rno-miR-132-3p	rno480919_mir
		miR-134-5p	rno-miR-134-5p	rno480922_mir
		miR-146a-5p	rno-miR-146a-5p	rno481451_mir
		miR-181c-5p	rno-miR-181c-5p	rno481295_mir
	Endogenous candidate	miR-16-5p	hsa-miR-16-5p	rno481312_mir
		miR-124-3p	rno-miR-124-3p	rno480901_mir
		let-7a-5p	hsa-let-7a-5p	478575_mir
		let-7b-5p	hsa-let-7b-5p	478576_mir

Table 2. MFB-ICSS-regulated miRNA candidates.

TaqMan OpenArray Assay	miRNA	FC	UP- or DOWN-REGULATED	Interesting pathways/putative pathways	Interesting putative targets
hsa-miR-196A	**miR-196a-5p**	4,964	UP	beta-tubulin polymerization [24] cytoskeleton remodelling [24] [25], ABC transporters [25]	APPB, BACE
mmu-miR-1959	miR-1959	2,805	UP	-	-
rno-miR-24-1#	miR-24-1-5p	2,665	UP	-	BACE
mmu-miR-300	miR-300-3p	2,055	UP	axon guidance, glutamatergic synapse	APP, APPB, BDNF, TTBK
mmu-miR-196a#	miR-196a-3p	2,007	UP	beta-tubulin polymerization [24] cytoskeleton remodelling [24] [25], ABC transporters [25], long term depression	APP, APPB, BACE, BDNF, TTBK
rno-miR-345-3p	miR-345-3p	1,499	UP	-	APPB, BACE, DBN
mmu-miR-485-3p	**miR-485-3p**	1,447	UP	dendritic spine development [26], axon guidance	APPB, SIRT1
mmu-miR-495	**miR-495-3p**	1,404	UP	axon guidance, dopaminergic cholinergic and GABAergic synapses, prion diseases, long-term potentiation	APPB, ARC, BDNF, SIRT1
hsa-miR-185	**miR-185-5p**	1,337	UP	axon guidance, BDNF pathway	APP
mmu-miR-187	miR-187-3p	1,319	UP	synaptic vesicle cycle, axon guidance	-
hsa-miR-197	**miR-197-3p**	0,233	DOWN	synaptic vesicle cycle	APP, BACE, DBN, SYN
rno-miR-333	miR-333	0,103	DOWN	-	-
hsa-miR-154	**miR-154-5p**	0,007	DOWN	GABAergic synapses, BDNF pathway	APPB
mmu-miR-2182	miR-2182	0,004	DOWN	-	-

List of assays showing differential expression between ICSS and sham pools in DG subfield, using TaqMan OpenArray MicroRNA Rodent Panel, and their functional relations according to literature and data base research. miRNAs in bold indicate those selected as MFB-ICSS-regulated candidates for further qRT-PCR analysis. n=4 pools of 3 rats per group. Fold-Change Boundary =1.3; P-value Boundary=0.05.

Abbreviations: APP: amyloid beta precursor protein; APPB: APP binding family; ARC: activity-regulated cytoskeleton-associated protein; BACE: beta-site APP-cleaving enzyme; BDNF: brain derived neurotrophic factor; DBN: drebrin family; SIRT1: sirtuin1; SYN: synapsin; TTBK: tau tubulin kinase.