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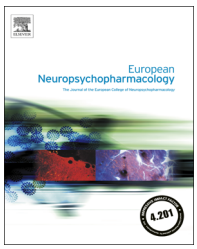

Time-dependent miR-16 serum fluctuations together with reciprocal changes in the expression level of miR-16 in mesocortical circuit contribute to stress resilient phenotype in chronic mild stress – An animal model of depression

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Abstract
MicroRNAs (miRNAs) are involved in stress-related pathologies. However, the molecular mechanisms underlying stress resilience are elusive. Using chronic mild stress (CMS), an animal model of depression, we identified animals exhibiting a resilient phenotype. We investigated serum levels of corticosterone, melatonin and 376 mature miRNAs to find peripheral biomarkers associated with the resilient phenotype. miR-16, selected during screening step, was assayed in different brain regions in order to find potential relationship between brain and peripheral alterations in response to stress. Two CMS experiments that lasted for 2 and 7 consecutive weeks were performed. During both CMS procedures, sucrose consumption levels were significantly decreased in anhedonic-like animals ($p<0.0001$) compared with unstressed animals, whereas the drinking profiles of resilient rats did not change despite the rats being stressed. Serum corticosterone measurements indicated that anhedonic-like animals had blunted hypothalamic-pituitary-adrenal (HPA) axis activity, whereas resilient animals exhibited dynamic responses to stress. miRNA profiling revealed that resilient animals had elevated serum levels of miR-16 after 7 weeks of CMS (adjusted p-value <0.007). Moreover, resilient animals exhibited reciprocal changes in miR-16 expression level in mesocortical pathway after 2 weeks of CMS ($p<0.008$). A bioinformatic analysis showed that miR-16 regulates genes involved in the

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functioning of the nervous system in both humans and rodents. Resilient animals can actively cope with stress on a biochemical level and miR-16 may contribute to a "stress-resistant" behavioral phenotype by pleiotropic modulation of the expression of genes involved in the function of the nervous system.

1. Introduction

Prolonged stress is considered to be one of the major triggers of depression. Nevertheless, many people and laboratory animals exposed to challenging conditions maintain normal behavior and, in the case of humans, cognitive flexibility and optimism (Krishnan et al., 2007; Haglund et al., 2007). These subjects are described as being resilient to stress. Numerous scientific and clinical studies have been performed to explain the pathological changes underlying depression. However, only a few studies have focused on the molecular and neurobiological mechanisms of the stress-resilience phenomenon (McEwen et al., 2015; Feder et al., 2009; Charney, 2004; Issler et al., 2014). Still, little is known about the genetic, epigenetic and biochemical factors responsible for the maintenance of normal behavior and allostatic balance in the face of stress in resilient subjects. Recent advances have revealed that microRNA (miRNA) transcripts play an important role in the physiology of the central nervous system (Jin et al., 2013; Higa et al., 2014) and may be engaged in the pathophysiology of a wide range of neuropsychiatric disorders, including anxiety, depression (Dwivedi, 2014; Mouillet-Richard et al., 2012; Smalheiser et al., 2012) and schizophrenia (Santarelli et al., 2013). Nevertheless, the role of miRNAs in depressed and resilient phenotypes is elusive. miRNAs are short (18–23 nucleotides), non-coding, single-stranded RNA transcripts that are highly evolutionarily conserved among mammals. miRNAs post-transcriptionally regulate gene expression patterns via the down-regulation of their specific targets based on complementarity between the mature miRNA and the 3′ untranslated region (3′UTR) of the targeted mRNA transcript. Impairments of miRNA function in schizophrenic, bipolar (Moreau et al., 2011) and depressed patients (Smalheiser et al., 2012, 2014; Lopez et al., 2014) as well as in stress-vulnerable animals in animal models of depression (Bai et al., 2012; Meerson et al., 2010; Liu et al., 2015a) suggest that miRNAs may play important roles in the regulation of neuronal functioning and in the adaptation of the brain (Dwivedi, 2014). Haramati et al. have shown that acute and chronic stress induce up-regulation of the expression of miR-34c in central amygdala (CA) in mice. This is natural response to a challenging conditions because overexpression of miR-34c in CA caused anxiolytic behavior after challenge (Haramati et al., 2011). A few reports have shown that serum levels of several miRNAs are changed in depressed patients (Li et al., 2013) and that naturalistic stress in healthy persons causes time-dependent changes in miRNA levels in the blood (Honda et al., 2013). Moreover, it has been shown that 12 weeks of antidepressant treatment with escitalopram significantly altered the expression of 30 miRNAs in the blood of depressed patients (Bocchio-Chiavetto et al., 2013). Issler and co-workers have revealed that miR-135a which can negatively regulate the expression of serotonin transporter (SERT) and serotonin receptor 1a (5-HT1a) is involved in antidepressant response in mice. Acute and chronic administration of SSRIs, but not NRIs up-regulated the expression of miR-135a in serotonergic neurons in mice. They also have shown that results from mouse model can be extrapolated into humans since depressed suicides had downregulated the expression levels of miR-135a and miR-16 in the brains. Additionally, patients who suffer from depression had decreased blood level of miR-135a and after 3 months of cognitive behavioral therapy (CBT) they showed significant increase in total blood miR-135a levels as compared to patients receiving only SSRI medication (Issler et al., 2014). Despite the existence of the blood–brain barrier, circulating miRNAs specific for brain tissue can be found extracellularly in biological fluids, such as serum, plasma or CSF (Rong et al., 2011; Liu et al., 2015b), where they exhibit remarkable resistance to degradation (Chen et al., 2008) and may play a potential role as noninvasive biomarkers of mental illness as well as of antidepressant treatment and individual stress responses. Chronic mild stress (CMS), a well-established animal model of depression, can be exploited to investigate the stress-related dysregulation of miRNA functioning, and the results have the appealing potential to be extrapolated to humans and clinical studies because miRNAs are highly conserved among mammals (Mouillet-Richard et al., 2012). CMS uses multiple variable stressors that do not elicit technical habituation and do mimic natural stressors. CMS produces anhedonia, a core symptom of depression that can be measured as decreased sucrose consumption or preference. Moreover, it has been observed that there is a proportion of animals that do not respond to challenging conditions by decreasing their sucrose consumption and instead, maintain their normal behavior (Zurawek et al., 2013). These animals are classified as stress-resilient. Although increasing evidence indicates that peripheral and brain miRNAs are involved in the pathophysiology of depression, little is known about their role in the stress-resilience phenomenon. Moreover, there is still sparse information available on the relationship between central and peripheral changes in miRNA expression resulted in response to stress. Thus, the goal of our work was to investigate time-dependent alterations in a set of 376 mature miRNAs in the serum of rats exposed to a CMS procedure to identify potential, non-invasive, peripheral markers that differentiate the stress-resilient phenotype from the anhedonic-like phenotype. Next, the expression of selected peripheral miRNA markers was examined in ventral tegmental area (VTA), nucleus

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accumbens septi (NAcc), medial prefrontal cortex (mPCx) and hippocampus (Hip) of rats subjected to 2 and 7 weeks of CMS in order to delineate potential interplay between peripheral and brain miRNA function. Dopaminergic projections from VTA to NAcc and mPCx create mesolimbic and, respectively, mesocortical pathways which are crucially implicated in anhedonia and other reward-associated deficits. Hippocampus-via its rich afferent and efferent connectivities with e.g. VTA and mPCx-is equally as important in pathophysiology of depression as mesolimbic and mesocortical pathways.

Corticosterone is widely considered as rodent stress-related hormone. The increase in serum level of corticosterone is linked to response of the organism to stressful conditions (Christiansen et al, 2012). Melatonin is a pineal hormone which regulates biological circadian rhythm, sleep and mood (Singh and Jadhav, 2014). A numerous studies indicate that alterations in melatonin system may be one of physiological parameters associated with sleep disturbances and diurnal changes in patients suffering from depressive disorder (McClung, 2011; Hansen et al., 2014). Thus, additionally to miRNA profiling, we investigated alterations in serum levels of corticosterone and melatonin in resilient and anhedonic-like animals in order to obtain more complex view of biochemical changes in rats showing different coping effectiveness in CMS model.

Understanding the molecular basis of stress resilience is of prime importance and may lead to the development of new therapeutic strategies based on the restoration of a resilient molecular phenotype among maladapted depressed individuals.

2. Experimental procedures

2.1. Animals and training procedure

Male Wistar Han rats (Charles River, Germany) were trained for 6 consecutive weeks to consume a palatable 1% sucrose solution. Training consisted of several 1-h tests (twice per week) during which sucrose solution was presented to the rats following overnight food and water deprivation. Sucrose consumption was measured by re-weighing the pre-weighed bottles containing the sucrose solution after the test. All of the behavioral experiments were performed in accordance with the guidelines of the ethical use of animals set by the European Community Council Directive and were approved by the Local Bioethics Commission.

2.2. CMS procedure

The CMS procedure was performed according to the method described in detail by Zurawek et al. (2015). In brief, after the training procedure, rats (n=445) were randomly divided into the control (n=245) and stressed (n=200) groups. Control animals were housed undisturbed in a separate room. The stressed group of animals was subjected to the weekly CMS regimen for 2 or 7 consecutive weeks. Each weekly stress regimen consisted of a period of water deprivation, two periods of food deprivation, two periods of 45° cage tilt, two periods of intermittent illumination (lights on and off every 2 h), one period of a soiled cage (250 ml of water in the sawdust bedding), one period of paired housing, two periods of low-intensity stroboscopic illumination (150 flashes/min) and two periods of no stress. All of the stress and no-stress periods had durations of 10–14 h. Sucrose intake was monitored in all of the groups of rats once per week. Distinctions between anhedonic-like (n=143) and resilient animals (n=57) were made based on a retrospective statistical analysis of the sucrose consumption of the rats under the stressful conditions relative to the median split of the baseline sucrose consumption. Of all of the rats subjected to the CMS procedure, 28.5% were resilient. For our further biochemical experiments, we randomly chose 14–16 representative animals from each group after 2 and 7 weeks of CMS. The remaining animals were used for another experiment and were not included in this study.

2.3. Serum and brain tissue sample collection

Before the start (baseline) and during each week of the 7 weeks of CMS, 24 h after sucrose consumption test, 0.5 ml of tail-vain blood was collected to the Eppendorf tubes from each rat. Next, after both CMS procedures, rats were sacrificed by decapitation 24 hours after the last sucrose test. Trunk blood and brains were collected. Tail-vein and trunk blood samples were kept at room temperature for 30 min to clot. Then, the blood was centrifuged for 20 min at 1500g at 4 °C. The supernatant was collected in a new Eppendorf tube and once again centrifuged for 15 min at 1500g at 4 °C to remove remaining cell contamination. Non-hemolyzed serum was frozen and stored at −80 °C until use. To minimize the effect of circadian rhythm on serum corticosterone and melatonin concentrations, trunk and tail-vein blood samples were collected between 9 a.m. and 2 p.m. After decapitation the rat brains were rapidly removed from the skull and cut into 1 mm thick coronal tissue slices using cutting edge. Next, ventral tegmental area (Bregma, −5.80 to −6.30 mm), nucleus accumbens septi (Bregma, 1.60–0.70 mm), medial prefrontal cortex (Bregma, 3.70–2.70 mm) were punched out, using tissue puncher, from tissue slices containing the above structures, according to The Rat Brain in Stereotaxic Coordinates Atlas (Paxinos and Watson, 1998). Hippocampus (Bregma, −3.14 to −4.80 mm) was dissected from the brains according to standard procedure. All tissue samples were frozen on dry ice in Eppendorf tubes and stored at −80 °C until use.

2.4. Measurement of corticosterone and melatonin concentrations in rat serum

Hormones were measured according to the Rat Stress Hormone Magnetic Bead Panel Kit (MILLIPLEX® MAP, Merck KGaA, Darmstadt, Germany) protocol, which allows the parallel quantification of corticosterone and melatonin in the same sample. Serum samples were analyzed on a MAGPIX® (Luminex, Texas, USA). Median fluorescence intensity (MFI) data, obtained using a weighted 5-parameter logistic curve-fitting method, was used to calculate analyte concentrations. Measurements of serum samples after 2 and 7 weeks of CMS were performed twice in triplicate. The inter-assay variation for both analytes was less than 12%.

2.5. miRNA purification from serum samples

Automated purification of total RNA, including miRNAs, from the serum was performed in accordance with the miRNeasy Serum/Plasma protocol (Qiagen, Germany) and it was combined with the use of a QIAcube robotic workstation (Qiagen, Germany) for automated RNA purification. Yeast tRNA (Sigma-Aldrich, Germany) was added to the QIAzol Lysis Reagent (Qiagen, Germany) to a final concentration of 10 μg/ml to increase miRNA recovery from the serum and to minimize technical variation between samples (Andreasen et al, 2010). Additionally, the synthetic miRNA mimic ath-miR159a mirVana® (Life Technologies, CA, USA) was spiked into the QIAzol Lysis Reagent to a final concentration of 4 fmol/ml. Synthetic ath-miR-159a is not expressed in animals and does not exhibit complementarity with any of the mature miRNAs assayed.

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Thus, it was used as a spike-in control to monitor the quality of the RNA purification, cDNA synthesis and PCR amplification in the qPCR experiment. The purity of the extracted RNA was measured with a NanoDrop spectrophotometer (Thermo Scientific, MA, USA). The determination of the concentration of the miRNA fraction isolated from biological fluids by spectrophotometric reading is often unreliable due to the very small amounts of circulating miRNAs as well as the addition of tRNA and the synthetic spike-in control template. Therefore, we used the same biofluid input amount combined with qPCR monitoring of the quantity of spike-in control among all of the samples as a reference for the RNA concentration. Samples with a difference in threshold cycle (Ct) <1 for ath-miR159a were classified as being of good quality and equally amplified and were included in the miRNA profiling study.

2.6. miRNA purification from brain tissue samples

Automated purification of total RNA, including miRNAs, from HIP and mPCx were performed in accordance with the miRNeasy Mini Kit (Qiagen, Germany) and from NAcc and VTA were performed in accordance with the miRNeasy Micro Kit (Qiagen, Germany) protocols. It was combined with the use of a QIAcube robotic workstation (Qiagen, Germany). The purity and the concentration of the extracted RNA samples were measured with a NanoDrop spectrophotometer (Thermo Scientific, MA, USA). Only RNA samples with good quality were taken for further analysis.

2.7. Real-time qPCR analysis and data normalization for screening assay from serum samples after 2 and 7 weeks of CMS

Purified RNA (3 μl) from each sample was reverse transcribed into cDNA using the TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies, CA, USA) and the Megaplex™ RT Primer Pool set A (Life Technologies, CA, USA), which contains a set of 380 predefined stem-loop reverse transcription primers, in accordance with the manufacturer's protocol. Afterwards, 2.5 μl of RT product was pre-amplified with the TaqMan® PreAmp Master Mix (2x) and the Megaplex™ PreAmp Primer Pool set A (Life Technologies, CA, USA) according to the manufacturer's protocol. The pre-amplification reaction increases the sensitivity of the qPCR method using TaqMan® hydrolysis probes, and it has been shown that it does not result in significant variation of the Ct values (Chen et al., 2009). Next, reverse-transcribed and pre-amplified cDNA product from each sample (9 μl) was mixed with 450 μl of TaqMan® Universal PCR MasterMix, No Amperase® UNG (Life Technologies, CA, USA) and 441 μl of nuclease-free water (Sigma-Aldrich, Germany) to a final volume of 900 μl. Then, the cDNA mixture was loaded into the TaqMan® Low Density Array Rodent MicroRNA Cards (TLDA) A set v 3 (Life Technologies, CA, USA). RT-qPCR was conducted on the QuantStudio™ 12K Flex system (Life Technologies, CA, USA) using the default cycling conditions and the automatic baseline and threshold values in accordance with the manufacturer's protocol. Amplification quality and Ct values were checked, filtered and extracted from data files using ExpressionSuite software (Life Technologies, CA, USA). A Ct value >30 was set as the cut-off point for undetectable miRNAs. These results were not included for further analysis. It has been shown that pre-amplified miRNAs that are very low in abundance, above Ct 30, are more sensitive to changes in the overall mean of all of the miRNAs. In effect, these miRNAs exhibit larger variations in Ct values that are caused more by variation in the initial sample size than by true changes in the expression level under the experimental conditions (Chen et al., 2009; Qureshi and Sacan, 2013). Raw and filtered Ct values were analyzed using qubase^{PLUS} software (Biogazelle, Belgium). Ct values were normalized using the delta-delta Ct method and converted to relative quantities for NormFinder and geNorm analysis (Mestdagh et al., 2009). Each TLDA card contained five proposed reference genes: U6 snRNA in quadruplicate and snoRNA135, snoRNA202, U87 snRNA and Y1 RNA, which have been used to normalize miRNA measurements in many studies. However, we found that none of the proposed reference genes was suitable for data normalization in this experiment because only U6 snRNA was present in the serum samples. geNorm analysis showed that the U6 snRNA did not meet the reference gene criteria and could not be used as an unbiased normalizer. geNorm analysis revealed that miR-106b, miR-500 and let-7i met the criteria for reference genes. Thus, the relative quantities of target miRNAs were normalized using qubase^{PLUS} software to the geometric means of miR-106b, miR-500 and let-7i.

2.8. Time-dependent analysis of selected miRNA in serum samples during 7 weeks of CMS and in brain structures after 2 and 7 weeks of CMS with the use of real-time qPCR method

3 μl of purified RNA from serum samples and 1 μg of purified RNA from brain tissue samples were reverse transcribed into cDNA using

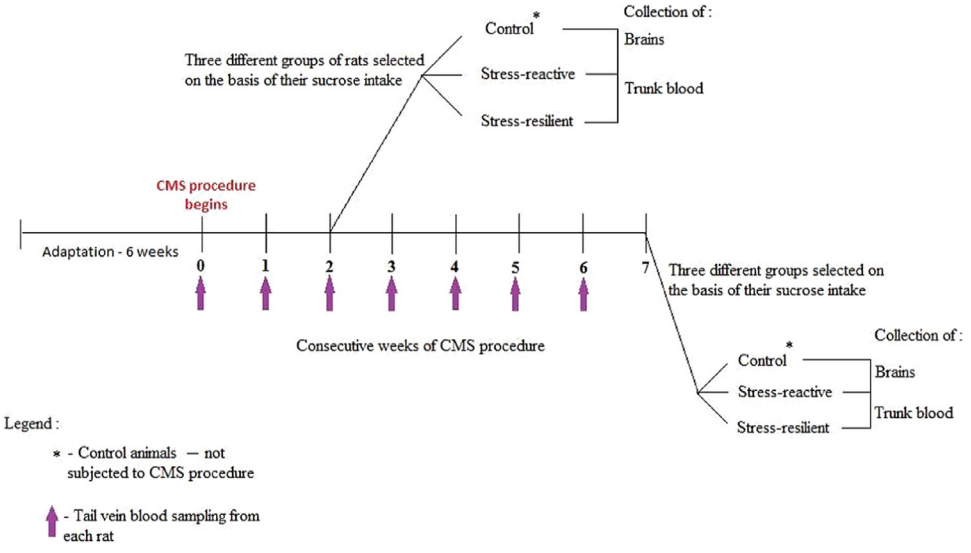

Figure 1 Schematic representation of behavioral experimental design.

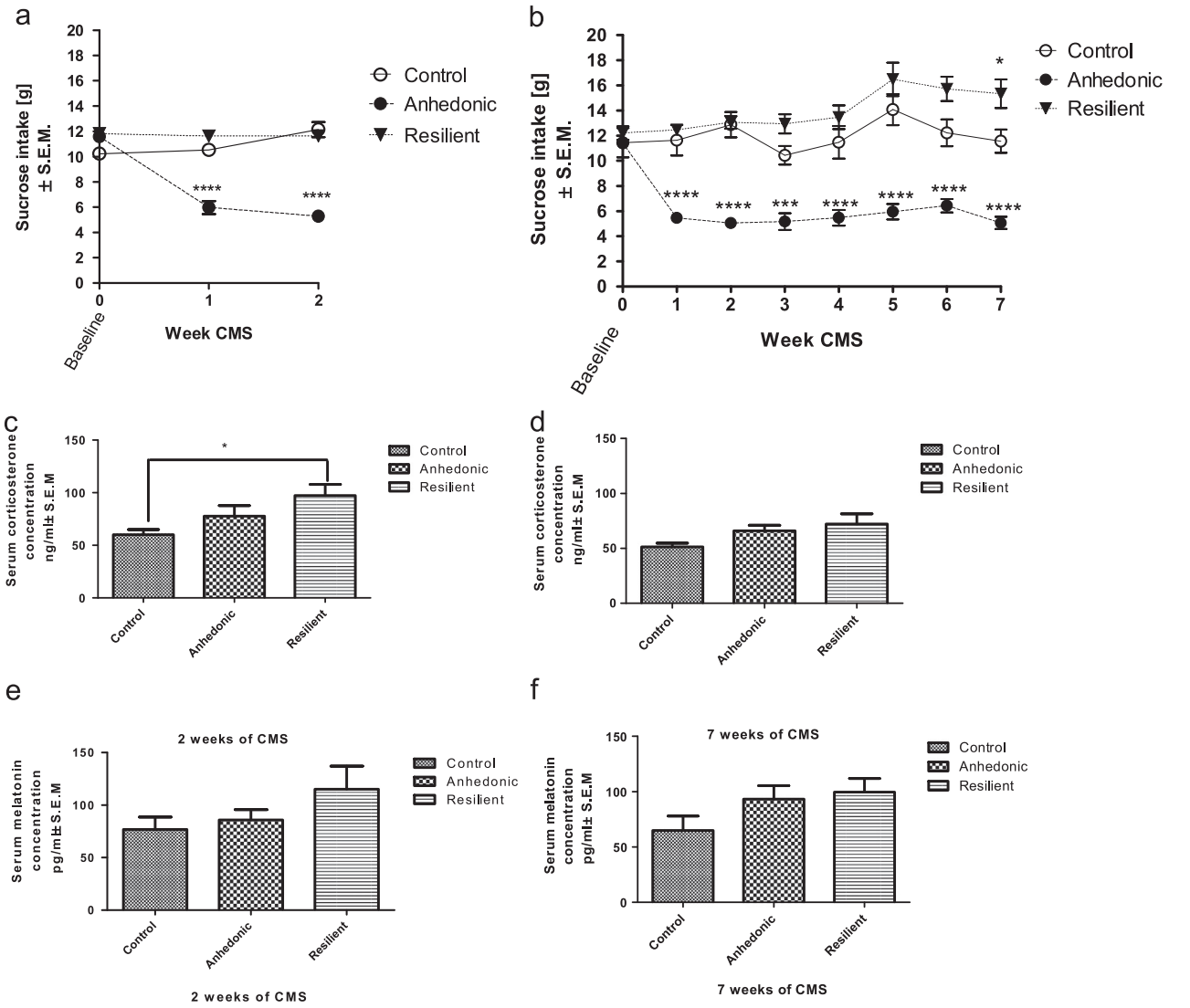

Figure 2 The effect of exposure to the chronic mild stress paradigm on the consumption of 1% sucrose solution (a-b), serum corticosterone (c-d) and melatonin (e-f) levels by the tested animals. Left column represents results obtained from animals tested for 2 weeks of CMS. Right column represents results obtained from animals tested for 7 weeks of CMS. Based on the sucrose intake of the controls, anhedonic and resilient groups of rats were selected. (a) 2 weeks of CMS; (b) 7 weeks of CMS, (c) One-way ANOVA analysis of variances showed significantly increased serum level of corticosterone in resilient animals after 2 weeks of CMS. Data represent the mean ± SEM. $n=11$-16; *$p<0.05$., ***$p<0.001$., ****$p<0.0001$ vs control group.

the TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies, CA, USA) and custom made TaqMan® MicroRNA Assay mixture (Life Technologies, CA, USA) according to the Procedure for multiplexing the RT step without pre-amplification while using TaqMan® MicroRNA Assays. Then, 6 μl of RT product was mixed with 50 μl of TaqMan® Universal PCR MasterMix, No Amperase® UNG (Life Technologies, CA, USA) and 44 μl of nuclease-free water (Sigma-Aldrich, Germany) to a final volume of 100 μl. Then, the cDNA mixture was loaded into the Custom-made TaqMan® Low Density Cards (TLDA) (Life Technologies, CA, USA). RT-qPCR was conducted on the QuantStudio™ 12 K Flex system (Life Technologies, CA, USA) using the default cycling conditions and the automatic baseline and threshold values in accordance with the manufacturer's protocol. Raw and filtered Ct values were analyzed using qubase^{PLUS} software (Biogazelle, Belgium). Ct values were normalized using the delta-delta Ct method and converted to relative quantities for NormFinder and geNorm analysis. The relative quantities of target miRNAs were normalized using qubase^{PLUS} software to the geometric means of miR-106b and let-7i.

Figure 1 presents the scheme of the behavioral experiment together with time points in which blood and tissues were collected for further biochemical experiments (Figure 1).

2.9. Bioinformatic miR-16 target gene prediction and biological pathway analysis

miR-16 target genes were predicted using myMIRsite software (http://www.itb.cnr.it/micro/index.html), which allows one to perform target meta-prediction based on the integration, filtering and re-ranking of data from the most widely used miRNA target prediction algorithms (Corrada et al., 2011). Target prediction was performed on a genome-wide 3′UTR dataset obtained from the *Homo sapiens* (hg19) and *M. musculus* (mm9) genomes. miR-16

target genes in *R. norvegicus* genome (rn5) were predicted using miRWalk2.0 software (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/index.html), which documents miRNA binding sites within the complete sequence of rat genome and combines this information with a comparison of binding sites resulting from existing miRNA-target prediction programs such as miRanda, mir Bridge, miRNAMap, RNAhybrid2.1 and Targetscan6.2 (Dweep and Gretz, 2015; Dweep et al., 2011). GeneCodis3.0 software (http://genecodis.cnb.csic.es/analysis) was used to identify biological net works that were significantly associated with predicted miR-16 target genes in both organisms (Carmona-Saez et al., 2007; Tabas-Madrid et al., 2012). The mature miR-16 nucleotide sequence in the different species was obtained from the miRBase v21 database (Kozomara and Griffiths-Jones, 2014).

2.10. Statistical analysis

Independent repeated-measures ANOVA followed by Tukey's post-hoc test was used to analyze the data from the 2- and 7-week CMS as well as data from time-dependent changes in miR-16 level in serum. Differences in corticosterone and melatonin levels as well as differences in the relative quantity of miRNA in serum and brain structures between the control, anhedonic and resilient groups of rats after 2 and 7 weeks of CMS were analyzed using one-way ANOVA and Tukey's post-hoc test. *P*-values were also corrected for multiple comparisons using the correction method of Benjamini and Hochberg, controlling the false discovery rate (FDR) at an alpha level of 5% (Benjamini and Hochberg, 1995). Correlation between miR-16 expression level in VTA and mPCx in rats after 2 weeks of CMS was analyzed using Pearsons's correlation test. Correlation between miR-16 level in serum and brain areas in the same animal after 2 and 7 weeks of CMS was analyzed using Pearson's correlation test. Singular enrichment analysis of the genome ontology-based (GO) biological processes and pathways listed in the Kyoto Encyclopedia of Genes and Genomes (KEGG) was performed for miR-16 target genes following the hypergeometric distribution statistic with FDR correction of the *p*-values. Only pathways with a corrected *p*-value <0.001 were considered significant.

3. Results

3.1. The effect of stress on sucrose consumption levels

At the beginning of the stress procedures, all of the animals exhibited the same sucrose consumption level. After 2 and 7 weeks of CMS, a retrospective analysis of individual drinking profiles enabled the separation of the stressed group of animals into two different subgroups: anhedonic-like and resilient. Repeated-measures ANOVA revealed significant effects of stress ($F_{2,\ 129}=72.42$; $p<0.0001$), time ($F_{2,\ 129}=14.57$; $p<0.0001$) and stress × time ($F_{4,\ 129}=26.57$; $p<0.0001$) on sucrose consumption after 2 weeks of CMS (Figure 2a). After 7 weeks of CMS, we observed statistically significant effects of stress ($F_{2,\ 352}=179.55$; $p<0.0001$), time ($F_{7,\ 352}=3.7$; $p<0.0007$) and stress × time ($F_{14,\ 352}=4.01$; $p<0.0001$) on sucrose consumption (Figure 2b). The post-hoc analysis showed that the anhedonic-like animals exhibited a significant decrease in sucrose consumption compared with the control group of animals in both behavioral procedures. The stress-resilient group of rats did not change their drinking behavior under stressful conditions compared to control animals after 2 and 7 weeks of CMS. Post-hoc analysis revealed that even the resilient group of rats exhibited increased sucrose consumption level compared with the control animals during the 7th week of CMS.

3.2. The effect of stress exposure on serum corticosterone and melatonin levels

One-way ANOVA followed by Tukey's post-hoc test revealed that only the resilient group of animals responded to the stress paradigm with elevated serum level of corticosterone after 2 weeks of CMS (Figure 2c) compared with the control animals ($F_{2,\ 32}=4.425$; $p<0.02$). After 7 weeks of the CMS procedure, there was no difference in the serum level of corticosterone among all three of the groups of rats (Figure 2d). No changes in serum melatonin levels were observed in either of the behavioral experiments (Figure 2 e–f).

3.3. The effect of stress exposure on serum miRNA levels after 2 and 7 weeks of CMS

After 2 and 7 weeks of CMS, 160 miRNAs were detected in the serum of the rats. After 2 weeks of CMS, serum levels of 7 miRNAs were affected by stress; however, after correcting for multiple comparisons, the adjusted *p*-values were not statistically significant. After 7 weeks of CMS, serum levels of 14 miRNAs were affected by stress. After correcting for multiple comparisons, the serum level of miR-16 was significantly altered by stress ($F_{2,\ 21}=16.25$, adjusted *p*-value <0.007) (Figure 3). Tukey's post-hoc test showed that the stress-resilient animals (after 7 weeks of CMS) exhibited significantly increased serum levels of miR-16 (fold change-1.4) compared with the control and anhedonic-like animals.

3.4. The effect of stress exposure on serum miR-16 level – the analysis of time-dependent changes during 7 weeks of CMS

Repeated measures ANOVA revealed significant effects of stress ($F_{2,\ 159}=14.72$; $p<0.0001$), time ($F_{2,\ 159}=6.479$; $p<0.0001$) and stress × time ($F_{2,\ 159}=2.410$; $p<0.004$) on the level of miR-16 in serum of rats after 7 weeks of CMS (Figure 4a). Tukey's post-hoc test showed that at the beginning of the CMS procedure (before the stress) there was no difference in basal serum level of miR-16 amongst all animals (Figure 4a – "Baseline" time-point). Stress resilient animals exhibited elevated level of serum miR-16 after the first week of CMS (fold change 1.46) as compared to non-stressed controls and anhedonic-like animals. Moreover, after 6th and 7th week of CMS resilient animals showed elevated serum level of miR-16 (fold changes 1.91 and 1.58 respectively) as compared to control group of rats (Figure 4a).

3.5. The effect of stress exposure on the expression level of miR-16 in limbic structures in rats after 2 and 7 weeks of CMS

The expression level of miR-16 was affected by stress in the VTA ($F_{2,29}=17.33$, $p<0.0001$) and mPCx ($F_{2,29}=13.14$, $p<0.0001$) of rats after 2 weeks of CMS. Tukey's post-hoc

List of miRNAs affected by 2 weeks of CMS						List of miRNAs affected by 7 weeks of CMS					
target	p-value	adjusted p-value	Group	FC	95% CI values	target	p value	adjusted p value	Group	FC	95% CI values
mmu-miR-195	0.01451	0.735784	Anhedonic	1.4	1.01-1.95	mmu-miR-16	0.000049	0.007436	Anhedonic	1.18	1.10-1.26
			Control	1,00	0.90-1.11				Control	1,00	0.9-1.11
			Resilient	1.45	1.14-1.84				Resilient	1.4	1.26-1.56
mmu-miR-370	0.016549	0.735784	Anhedonic	0.53	0.37-0.76	mmu-miR-191	0.001761	0.131229	Anhedonic	1.3	1.12-1.50
			Control	1,00	0.44-2.26				Control	1,00	0.79-1.27
			Resilient	0.95	0.8-1.12				Resilient	1.5	1.34-1.67
mmu-miR-15b	0.034067	0.735784	Anhedonic	1.26	1.00-1.58	mmu-miR-743a	0.006307	0.313291	Anhedonic	0.48	0.3-0.76
			Control	1,00	0.91-1.09				Control	1,00	0.7-1.43
			Resilient	1.06	0.93-1.22				Resilient	0.53	0.41-0.68
mmu-miR-26b	0.040696	0.735784	Anhedonic	1.46	1.05-2.03	mmu-miR-320	0.012976	0.359394	Anhedonic	1.15	1.04-1.26
			Control	1,00	0.76-1.31				Control	1,00	0.9-1.11
			Resilient	1.35	1.12-1.62				Resilient	1.2	1.09-1.31
mmu-miR-301a	0.043313	0.735784	Anhedonic	1.25	0.99-1.58	mmu-miR-34a	0.015304	0.359394	Anhedonic	0.7	0.49-0.99
			Control	1,00	0.87-1.15				Control	1,00	0.66-1.52
			Resilient	1.22	1.06-1.40				Resilient	0.53	0.41-0.69
mmu-miR-92a	0.043631	0.735784	Anhedonic	1.42	1.14-1.76	mmu-miR-331-3p	0.015512	0.359394	Anhedonic	0.76	0.63-0.91
			Control	1,00	0.90-1.12				Control	1,00	0.83-1.21
			Resilient	1.4	0.94-2.09				Resilient	0.69	0.55-0.87
mmu-miR-224	0.046905	0.735784	Anhedonic	1.73	0.77-3.88	mmu-let-7g	0.018697	0.359394	Anhedonic	0.87	0.77-0.98
			Control	1,00	0.81-1.23				Control	1,00	0.92-1.08
			Resilient	1.25	1.00-1.57				Resilient	1,00	0.94-1.06
						mmu-miR-202-3p	0.020606	0.359394	Anhedonic	0.57	0.37-0.98
									Control	1,00	0.53-1.98
									Resilient	1.39	0.92-2.09
						mmu-miR-186	0.022578	0.359394	Anhedonic	1.16	1.11-1.22
									Control	1,00	0.81-1.23
									Resilient	1.34	1.11-1.60
						mmu-miR-143	0.02412	0.359394	Anhedonic	0.87	0.69-1.09
									Control	1,00	0.83-1.20
									Resilient	1.25	1.01-1.55
						mmu-miR-367	0.031292	0.530107	Anhedonic	0.58	0.49-0.68
									Control	1,00	0.48-2.08
									Resilient	0.73	0.04-12.19
						mmu-miR-24	0.04648	0.530107	Anhedonic	1.26	1.04-1.53
									Control	1,00	0.87-1.15
									Resilient	1.11	1.00-1.24
						mmu-miR-145	0.046581	0.530107	Anhedonic	0.68	0.37-1.26
									Control	1,00	0.60-1.68
									Resilient	1.64	0.94-2.87
						mmu-miR-494	0.048886	0.530107	Anhedonic	0.53	0.34-0.83
									Control	1,00	0.6-1.66
									Resilient	0.78	0.58-1.05

Figure 3 List of miRNAs affected by the two different periods of CMS. Differentially expressed miRNAs in the serum of the anhedonic-like and the resilient group of rats relative to the control group after 2 weeks of CMS (n=5-7) and 7 weeks of CMS (n=7-8)., expressed as fold-change (FC) with the 95% confidence interval (95% CI value). Statistical significance was analyzed by one-way ANOVA (p-value) and controlled for multiple comparisons (false discovery rate; FDR) at a level of 5% (adjusted p-value).

analysis showed that resilient animals exhibited up-regulated expression of miR-16 in the VTA (fold change 1.67) as compared to control and anhedonic-like groups of animals (Figure 4b). Interestingly, at the same time in the same animals we observed, opposite to VTA, changes in miR-16 expression level in the mPCx (Figure 4e) i.e. down-regulation of expression of miR-16 in resilient (fold change 0.70) and anhedonic-like animals (fold change 0.83) as compared to non-stressed control group. Pearson's correlation analysis revealed significant and reverse correlation ($r=-0.5029$, $p<0.008$) between up-regulation of miR-16 in VTA and down-regulation of miR-16 in mPCx of rats subjected to short period of stress (Figure 5). After 7 weeks of CMS the expression of miR-16 was affected in the VTA ($F_{2,29}=12.62$, $p<0.0001$), the NAcc ($F_{2,29}=4,87$, $p<0.01$) and the Hip ($F_{2,29}=5\,287$, $p<0.01$). Tukey's post-hoc test

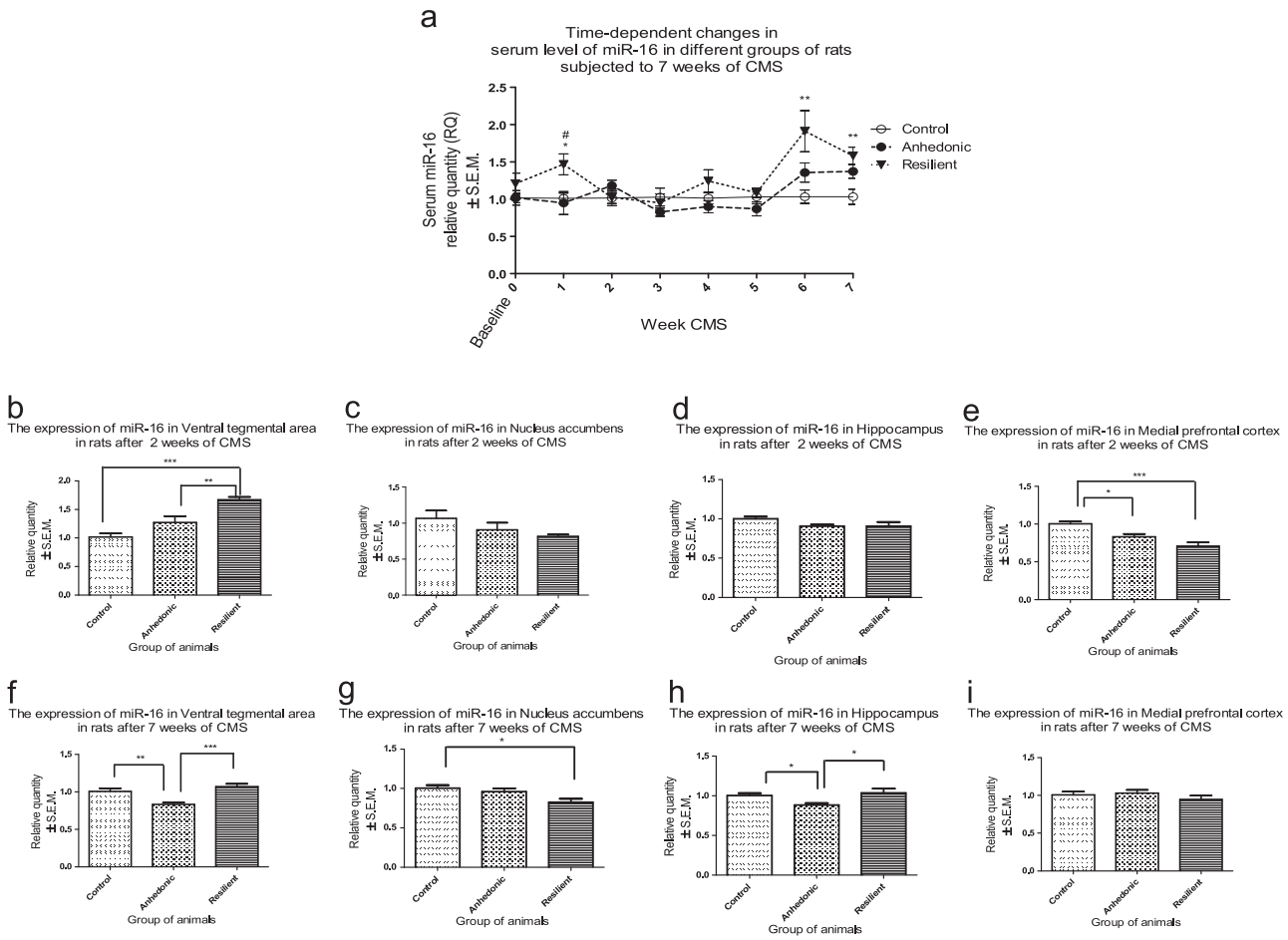

Figure 4 Time-dependent changes in serum level of miR-16 in rats with different response to stress (a) and the effect of stress on the expression level of miR-16 in different brain structures in rats after 2 (b-e) and 7 (f-i) weeks of CMS. Repeated measures ANOVA followed by Tukey's post-hoc test showed statistically significant time-dependent fluctuations in serum level of miR-16 in response to stress. (a) data represent the mean ± SEM. n=5-11; *$p<0.05$, **$p<0.01$ resilient vs control group, $p<0.05$ resilient vs anhedonic-like group. One-way ANOVA followed by Tukey's post-hoc test showed significant changes in miR-16 expression level in different brain structures of rats showing different stress response. (b-i) data represent the mean ± SEM n=9-10; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

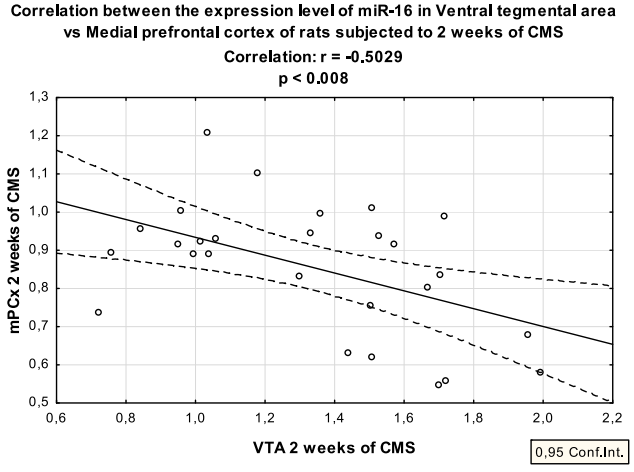

Figure 5 Pearson's correlation analysis between changes in the expression level of miR-16 in VTA and mPCx of rats after 2 weeks of CMS procedure; $r=-0.5029$, $p<0.008$.

revealed that after 7 weeks of CMS anedonic-like animals showed significantly down-regulated expression of miR-16 in the VTA (fold change 0.83; Figure 4f) and hippocampus (fold change 0.88; Figure 4h) as compared to control and resilient groups of rats. We observed also that resilient animals after 7 weeks of CMS exhibited decreased level of miR-16 (fold change 0.82) in the NAcc (Figure 4g) as compared to non-stressed and anhedonic-like animals.

3.6. Bioinformatic analysis of miR-16 target prediction and biological pathways

Based on the miRBase21 database, we determined that the nucleotide sequence of the mature miR-16 is the same across different mammals. Using target prediction analysis, we found that 1254 mouse, 3734 rat and 2339 human genes were potentially regulated by miR-16. In the Supplemental Material, we present the 100 most probable targets from *H. sapiens* (**S1A**), *M. musculus* (**S1B**) and *R. norvegicus* (**S1C**).

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Table 1 List of the biological processes and KEGG pathways related to nervous system function that were significantly enriched by the putative miR-16 target genes in rodents and humans.

Biological process					
Rat		**Mouse**		**Human**	
Annotation	Name	Annotation	Name	Annotation	Name
GO:0007399	Nervous system development	GO:0007399	Nervous system development	GO:0007399	Nervous system development
GO:0007411	Axon guidance	GO:0007411	Axon guidance	GO:0007411	Axon guidance
GO:0007420	Brain development	GO:0007420	Brain development	GO:0048011	Nerve growth factor receptor signaling pathway
GO:0007612	Learning	GO:0007409	Axonogenesis		
KEGG pathway					
KEGG 04360	Axon guidance	KEGG 04360	Axon guidance	KEGG 04360	Axon guidance
KEGG 04722	Neurotrophin signaling pathwy	KEGG 04722	Neurotrophin signaling pathwy		
KEGG 04730	Long-term depression	KEGG 04730	Long-term depression		
KEGG 04720	Long-term potentiation	KEGG 04370	VEGF signaling pathway		
KEGG 04080	Neuroactive ligand-receptor interaction				

Functional analysis using GeneCodis3 software identified many different downstream biological processes in humans and rodents that involve genes regulated by miR-16. Moreover, we showed that the majority of these pathways are commonly regulated by miR-16 in all investigated species. The Supplemental Material includes the list of significantly enriched biological processes and KEGG pathways for *H. sapiens* (**S2A**), *M. musculus* (**S2B**) and *R. norvegicus* (**S2C**) together with the statistical results. In addition, our analysis demonstrated a significant enrichment of the miR-16 target genes in several biological processes related to the function of the nervous system in both humans and rodents (Table 1).

4. Discussion

Using chronic mild stress (CMS) – animal behavioral model of anhedonia – the present study has shown that under chronic stressful conditions rats developed different behavioral responses that were measured by sucrose intake. Anhedonic-like animals significantly decreased their sucrose intake as compared to the level of sucrose drinking before the start of the stress procedure, whereas resilient rats did not change their drinking profile despite being stressed for 2 or 7 weeks. Thus, changes in sucrose intake are very good marker for anhedonia, a core symptom of depression, which has been described as diminished interest in response to stimuli that were perceived as rewarding or pleasurable before the impact of stress. Decreased sucrose consumption by anhedonic-like rats is specific response since intake of plain water is not affected by stress (Wiborg, 2013). This observation indicates that CMS has potential as a behavioral paradigm that allows the study of the resilience phenomenon (Feder et al., 2009). Recent research has indicated that dealing with stress is more an active, dynamic process, strategy or lifestyle rather than a passive, endogenous resistance to stressful influences (Krishnan et al., 2007; Zurawek et al., 2013; de Kloet et al., 2005). This idea has also been confirmed in the present study. Using two behavioral experiments that lasted for different periods of time (2 and 7 weeks), we observed that resilient animals exhibited elevated serum concentration of corticosterone after a short period of stress, whereas after a long period of stress exposure, serum corticosterone concentration returned to control level. However, anhedonic-like animals did not exhibit time-dependent changes in hypothalamic-pituitary-adrenal (HPA) axis activity in response to stress (Azpiroz et al., 1999). The fast activation of HPA axis activity among the resilient animals reflected by the elevated concentration of corticosterone after the shorter exposure to stress represents a primary defensive reaction that aims to maintain the homeostatic balance of the organism. Then, after a longer period of stress, the hormonal response is dampened to protect the organism from the damaging influence of prolonged elevated level of glucocorticoids (Schumann et al., 2014). Behavioral changes related to anhedonia and anxiety have been reported to be more prevalent in rats with blunted HPA axis responses and lower concentrations of corticosterone in plasma (Cohen et al, 2006). Nevertheless, many studies have reported that susceptible, but not resilient, animals showed elevated level of corticosterone under stressful conditions (Blugeot et al., 2011, Taliaz et al., 2011). This discrepancy may result from different approach in measuring corticosterone level: usually it is measured only in one time point during behavioral experiment. This approach shows only a snapshot of what happens in the organism during continuous stress influence and response.

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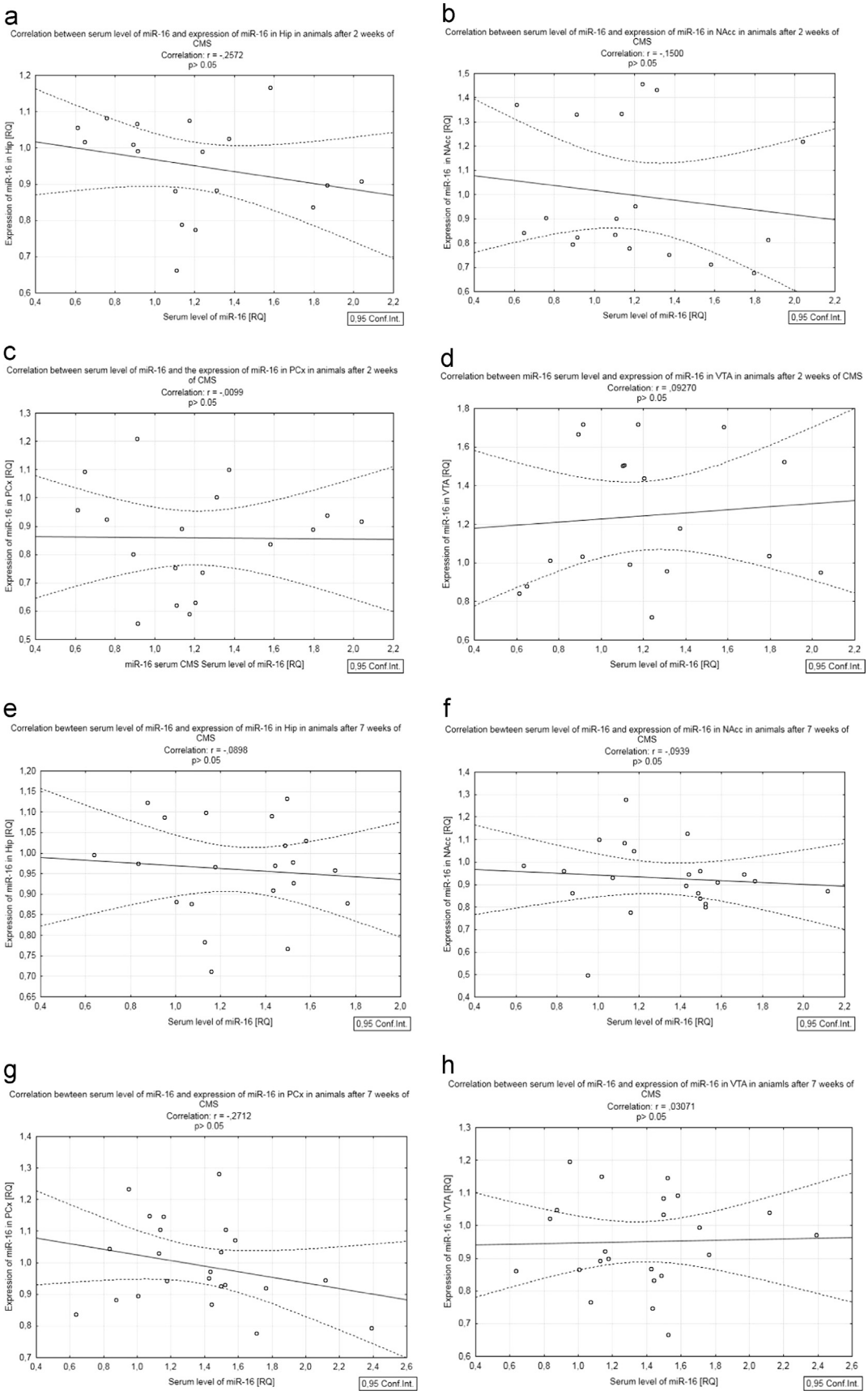

Figure 6 Pearson's correlation analysis between changes in serum level of miR-16 and brain areas in the same animal after 2 (a–d) and 7 (e–h) weeks of CMS.

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Christiansen and co-workers have reported that stress resilient animals responded much faster and more adequately to stress than anhedonic-like animals what was manifested as shorter habituation time measured as difference in alteration in fecal corticosterone metabolites (FCMs). They found, that after 2 weeks of CMS the level of FCMs in resilient animals was similar to control despite earlier slight increase (Christiansen et al, 2012; Wiborg, 2013). We observed that resilient animals after 2 weeks of CMS showed significantly elevated serum level of corticosterone. This discrepancy might result from completely different approach to measure HPA axis activity. Christiansen et al. have measured fecal level of corticosterone metabolites which, despite being less stressful to the animals than blood collection, it is indeed an indirect approach to measurement of glucocorticoid response to stress. Results obtained from FCMs approach might depend on many factors that can influence on the final results such as the rate of corticosterone metabolism, delay in time between serum corticosterone peak and FCMs production etc. Nevertheless, our and Christiansen's observations have led to the same important conclusion that resilient animals in CMS show more dynamic and effective coping strategies at the level of corticosterone response, than anhedonic-like animals.

Genetic and epigenetic factors also profoundly contribute to dynamic responses to stress. Using animal models of neuropsychiatric disorders, it has been shown that maladaptive responses to stress are associated with changes in the expression of many different miRNAs in various brain regions, such as the hippocampus (Meerson et al., 2010; O'Connor et al., 2013, Liu et al., 2015a), frontal cortex (Rinaldi et al., 2010) and amygdala (Meerson et al., 2010). Recent evidence suggests that miRNA functions are impaired in the brains of depressed individuals who commit suicide (Smalheiser et al., 2012, 2014). Moreover, it has been shown that elevated serum levels of miR-132 and miR-182 negatively correlate with serum levels of brain derived neurotrophic factor (BDNF) in depressed patients (Li et al., 2013). Twelve weeks of antidepressant treatment with escitalopram has been shown to significantly alter the expression of 30 miRNAs in the blood of depressed patients. Many of the altered miRNAs examined may be involved in the regulation of gene expression in the brain (Bocchio-Chiavetto et al., 2013). Our analysis revealed that the serum level of miR-16 is associated with the stress-resilient phenotype. Resilient animals exhibited higher serum level of miR-16 compared with unstressed and anhedonic-like animals after 7 weeks of CMS. In the next step we investigated fluctuations in serum level of miR-16 at different time points of the 7 weeks of the CMS procedure. This approach allowed the examination of dynamism in serum level of miR-16 in order to check whether miR-16 may be a good marker that differentiate resilient from anhedonic-like animals in broader time perspective. Increased serum level of miR-16 during the first week of CMS as well as after 6th and 7th week of CMS, observed only in resilient group of rats, suggests that resilient phenotype is strongly related to an active, time-dependent and efficient coping strategy which is associated with up-regulated miR-16 level. In resilient animals dynamic regulation of serum miR-16 level in response to stress consists of two stages. Initially, we can observe the early response to short stress (after the very first week of CMS procedure), and then – constantly elevated level of miR-16 during the last two consecutive weeks of CMS, which may reflect homeostatic balance obtained by resilient animals under long-lasting stressful conditions. Anhedonic-like animals did not show alterations in serum miR-16 level during CMS procedure what can be interpreted as inadequate or inefficient coping strategy as compared to resilient rats. Serum miR-16 fluctuations in resilient animals suggest that performing scientific investigations on biochemical response to stress different time points should be taken into account. Considering only one time point it is more likely to lose many valuable information about complex body response to a challenging conditions which often last for a long period of life.

We also assayed the expression level of miR-16 in mesolimbic and mesocortical circuits of rats after 2 and 7 weeks of CMS in order to find potential relationship between altered levels of miR-16 in periphery and the expression of miR-16 in various brain regions (Figure 4). Based on the data from brain and serum we can conclude that there is no straightforward relationship between peripheral and brain changes in miR-16 in response to stress (Figure 6a–h). We observed that, in general, resilient animals showed much more prevalent and dynamic changes in miR-16 expression in the brain and in the serum as compared to anhedonic-like animals. Moreover, we observed very interesting reverse relationship between miR-16 expression level in mesocortical circuit of rats being stressed. Alterations in miR-16 expression level in VTA and mPCx structures showed reciprocal and dynamic response to stressful conditions. After short period of stress influence the expression of miR-16 was up-regulated in VTA of resilient animals while in the same time we observed down-regulation of miR-16 in mPCx of all stressed animals. This observation shows that miR-16 might play an important role in allostatic regulation of mesocortical pathway – brain circuit strongly involved in processing of positive as well as negative events and mood regulation. It can be seen that brain circuits respond strongly to short stress. After longer stress-period (i.e. 7 weeks) perturbations in miR-16 expression level in mesolimbic and mesocortical circuits returned to control level in resilient animals. It shows that resilient phenotype has more efficient coping strategies at the level of miR-16 action. Anhedonic-like animals did not show such dynamic responses and after longer stress they showed even more disturbed miR-16 expression in VTA and Hip. Smallheiser and co-workers have shown that non-learned helpless (NLH) rats showed a robust adaptive miRNA response to inescapable shock what was manifested by down-regulation of different miRNAs in the frontal cortex whereas learned helpless (LH) rats showed a markedly blunted response (Smalheiser et al., 2011). We observed consistent effects in stressed animals: after short period of stress downregulation of miR-16 in mPCx occurred which was accompanied by an increase in miR-16 expresison in VTA. Thus, adaptive changes in miR-16 expression in mPCx may depend on activation of VTA region. These data, together with our observation of the dynamic response in serum corticosterone levels, support the hypothesis that the resilient phenotype exhibits tightly regulated time-dependent physiological changes at the level of epigenetic modulation and hormonal activity. miRNAs may serve as good biomarkers that reflect an individual's capacity to counteract

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challenging conditions. However, the interaction between central and peripheral action of miRNAs is still elusive and ambiguous. Honda et al. have shown that naturalistic stress caused time-dependent changes in nine miRNAs, including miR-16, in the peripheral blood of healthy Japanese students. They proposed that blood levels of miR-16 and miR-144 may play a role in the down-regulation of inflammatory cytokine responses during naturalistic stressors (Honda et al., 2013). This finding indicates that miR-16 may be associated with the regulation of the natural capacity to cope with stress and this capacity may result from the sum of different, specific to a particular tissue region, and dynamic actions of miRNAs. This work supports our observations and the hypothesis that the stress-resilient phenotype is associated with the systemic, time-dependent actions of miR-16. The lack of temporal changes in the hormonal responses and diminished brain and serum miR-16 level in the anhedonic-like animals may reflect their individual loss of resilience. miR-16 has been shown to play an important role as a micromanager of hippocampal neurogenesis and as a negative regulator of serotonin transporter levels in raphe neurons after fluoxetine treatment (Launay et al., 2011; Baudry et al., 2010). Decreased level of miR-16 after 7 weeks of CMS in hippocampi of anhedonic-like rats may thus refer to maladapted changes to stress by these animals. Therefore, the conclusion that miR-16 is involved in the stress response and the pathophysiology of depression and antidepressant treatment is justified. Our bioinformatic analysis indicates that miR-16 may be involved in the regulation of many biological processes related to the functioning of the nervous system, such as axon guidance, axonogenesis, neuroactive ligand-receptor interaction, brain development, nerve growth factor receptor signaling and others. All of these processes are responsible for the "well-being" of neuronal networks; therefore, one may suggest that they are important for the stress-resilience phenotype. As our analysis revealed, many of these pathways are comparably regulated by miR-16 in humans and rodents. miRNA regulation and the phenomenon of whole-RNA interference are evolutionarily conserved. Thus, miRNAs may play an important role in translational studies that, based on animal models. Our results indicate a new aspect that may be used to identify new biological bases for mental illnesses in which there is a relationship between behavior and a physiological condition of the body that is reflected by altered systemic action of miR-16.

Conflict of interest

All authors declare no conflict of interest.

Role of the funding source

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All of Institutions had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Contributors

Prof. M. Dziedzicka-Wasylewska, D. Zurawek and M. Kolasa designed the study and managed the literature.

A. Faron-Gorecka, M. Kusmider, Zurawek D, P. Pabian, J. Solich and K. Szafran-Pilch, performed serum collection and participated in behavioral experiment.

M. Kusmider and Zurawek D performed serum hormone and miR concentration measurements, miR-16 expression analysis in different brain structures and analysed the data.

Prof. M. Papp and P. Gruca designed and performed behavioral experiments.

D. Zurawek, M. Kolasa optimized and performed RT-qPCR-based miRNA profiling, undertook bioinformatic and statistical analysis and interpreted the data.

D. Zurawek and Prof. M. Dziedzicka-Wasylewska wrote the manuscript. All authors accepted the manuscript for publication.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.euroneuro.2015.11.013.

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