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Short communication

Stress induces region specific alterations in microRNAs expression in mice

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ABSTRACT

Several studies have demonstrated that exposure to both acute and chronic aversive stimuli can affect neural activity in different brain areas. In particular it has been shown that stressful events can induce not only short-term changes in neural transmission and gene regulation, but also long-term changes that can lead to structural modification. In this study we investigated, in CD1 mice, the effects of single or repeated exposures to restraint stress (2 h for 1 or 5 consecutive days) in the frontal cortex on a crucial class of gene expression regulators, the microRNAs (miRs).First we performed a microarray profiling on RNA extracted from the frontal cortex of mice exposed to acute or repeated restraint stress. The results indicated a prominent increase in the expression levels of different miRs after acute stress while only minor changes were observed after repeated restraint. The Northern blot analysis on selected miRs confirmed an increase after acute restraint for let-7a, miR-9 and miR 26-a/b. Finally, Northern blot analysis of the selected miRs on RNA extracted from the hippocampus of stressed mice demonstrated that such changes were region specific, as no differences were observed in the hippocampus. These data suggest that control of mRNA translation through miRs is an additional mechanism by which stressful events regulates protein expression in the frontal cortex.

Acute and chronic stressful stimuli elicit a variety of behavioral responses that range from increased alertness and vigilance to effects on cognitive processes. When stressors exceed individual threshold, such responses can lead to maladaptive behaviors manifested in humans as depressive symptoms, anxious states or alterations in learning and memory [2,24,30]. In particular it has been suggested that while the effect induced by acute stress might be indicative of an adaptive change meant to restore normal physiological conditions, the response to repeated unpredictable and uncontrollable stressors leads to a long-lasting inadequate modification of brain function that could be the basis for stress-related disorders [12,20].

Restraint stress is one of the most commonly used animal models of stress and it has been described to modulate plasticity-related neuronal processes that might play a role in stress-induced vulnerability to psychiatric diseases. For example, acute restraint induces increased transcription of c-fos [7,22] and Arc in the brain [25]. Changes in the expression levels of adhesion molecules [27,32], neurotrophins [1], synaptobrevin [11], synapsin I [1] and synaptophysin [34] have been reported after chronic restraint. Interestingly, chronic restraint also induces inhibition of adult neurogenesis [26] and morphological changes, such as alterations in spine densities and dendritic branching [6,9,18], thus suggesting that the long-lasting behavioral consequences of repeated exposure to stressors might be related to structural plasticity [21].

In addition to control of transcription and proteins transport to synapses, local dendritic protein synthesis is emerging as a crucial mechanism of synaptic plasticity [14,28,29], thus its regulation might be critical for stress response. Recently a class of small non-coding RNA molecules, microRNA (miRs), with functions in the post-transcriptional regulation of gene expression, has been identified. Interestingly, many neuronal miRs are localized to actively translating polyribosomes in dendrites, where they may control translation of dendrite-specific mRNAs [15]. Despite the fact that miRs have proved to be important players in the control of diverse biological processes [4,16], studies of miRs functional role in the

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vertebrate nervous system are still limited and mainly centered on developmental processes. However miRs, by regulating protein translation, might play a pivotal role in experience dependent plasticity in the mature brain and therefore in the response to stressors. Recent reports provided interesting evidence in support of this hypothesis. Indeed it has been showed that miR-18a down-regulates the glucocorticoid receptor *in vitro* [31,33] and that stress hyper-responsive rats present higher expression of this miR and lower levels of glucocorticoid receptor than normal rats in the paraventricular nucleus of the hypothalamus [31].

The main purpose of the present study was to investigate the effects of restraint stress on miRs expression in the frontal cortex (FC), a key neuroanatomical substrate of stress response [5,8].

23 CD1 male mice (Charles River, Calco, Italy), 10–12 weeks old were housed in groups of 3–5 in standard breeding cages and maintained in a temperature-controlled animal facility (21 ± 1 °C), with a light/dark cycle of 12 h (light on at 7.00 a.m.) and *ad libitum* access to food and water. All experiments were conducted in accordance with the guidelines on the ethical use of animals set by the European Community Council Directive. Mice were randomly assigned to four different conditions: *naive*, *acute stress*, *repeated stress*, *acute stress* + 5*d*. *Naive* mice were left undisturbed in their home cage until sacrifice. *Acute stress* mice were subjected to a single 2 h-restraint session in a black plastic tube (diameter: 3 cm, length: 10 cm, with several holes to allow breathing). *Repeated stress* mice were subjected to 2 h-restraint session/day, for 5 consecutive days. All mice were sacrificed 24 h after the last restraint session. A further group, *acute stress* + 5*d* mice was subjected to a single 2 h-restraint experience and sacrificed 5 days later. This latter group was added in order to verify whether the effects observed after acute stress were stable or only transient. All mice were tested and sacrificed between 10.00 a.m. and 2.00 p.m. and groups were counterbalanced across days. Immediately after sacrifice, brains were rapidly removed, and the structure of interest dissected. Samples were collected in Trizol (Invitrogen, Italy) and frozen in liquid nitrogen. Total RNA was isolated from mouse brain tissue using Trizol (Invitrogen) following the manufacturer's recommendations. RNA quantification was made by Nanodrop ND1000 spectrophotometer. RNA quality was assessed by gel electrophoresis.

As a first step we performed miRs microarray analysis on total RNA obtained from the FC of naive, acutely and repeatedly stressed mice, in order to identify candidate miRs regulated by stress. The microarray was run in four technical replicates on a single biological replication for each group (see supplemental material). We observed several alterations of miRs expression (Fig. 1 supplemental material). Overall wider changes were found after acute then repeated restraint stress. Moreover increases in miRs expression level were prevalent compared with decreases.

Based on the expression changes revealed by the microarray, we selected a subset of miRs for further quantitative analysis on a larger sample of mice. In particular we selected four miRs (out of seven) showing a variation with log ratio higher than 1.2 (9, 26b, 30b and let-7a) and a miR showing only a marginal increase (125a) (Table 1 supplemental material). Consistently with previous report [31], miR-18a expression levels in the FC were below detection limits both in basal conditions as well as after stress.

Northern blot analysis of miRs was performed as previously described [10]. Shortly, RNA samples (10 μg each) were separated on a 10% polyacrylamide, 7 M urea and TBE gel, electroblotted onto a Hybond-N + membrane (Amersham Biosciences, Piscataway, NJ, US) and UV-crosslinked. Oligonucleotide probes correspond to miR sequences at the miR registry (http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml). As miR-26a and miR-26b differ by only one nucleotide, the corresponding probes cannot reliably distinguish between the two miRs. The same holds true for miR-30b and miR-30c. Thus in the present study we referred to them as miR-26a/b and miR-30b/c respectively.

Probes were labeled using T4 polynucleotide kinase and [a-32P]ATP. Hybridization was done at 37 °C in 0.1% SDS, 5× Denhardt and 6× SSPE overnight. Membranes were washed once at RT with 6× SSPE and then at 37 °C for 30 min. Signals were read by Amersham Typhoon 9200 phosphoimager and densitometry was performed with IMAGEQUANT software (Amersham). Membranes were stripped by boiling in 0.1% SDS and rehybridized up to 6 times without observing decreases in quality or intensity of radioactive signals. U2 and U6 RNA were used as loading controls. miRs signals were normalized to corresponding U2 signals for each sample. Samples whose U2 levels were not reliably distinguishable from background were excluded from further analysis.

As reported in Fig. 1 the Northern blot analysis confirmed a significant modulation of the expression of most of the selected miRs after exposure to acute stress. Repeated measure ANOVA revealed a significant main effect for the repeated factor miR ($F_{(4,64)} = 13.026$, $P < 0.0001$) and a miR by stress condition interaction ($F_{(12,64)} = 3.463$, $P = 0.0006$), but no main effect for the factor

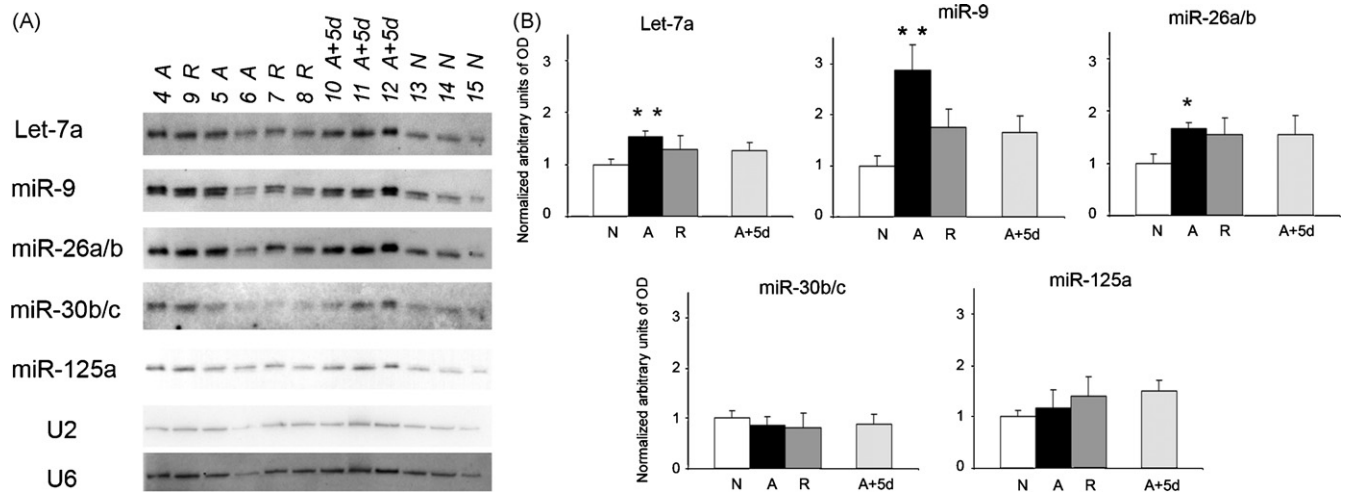

Fig. 1. miRs expression in the frontal cortex after acute and repeated stress. (A) Representative Northern blot analysis of let-7a, miR-9, miR-26a/b, miR-30b/c, miR-125a, U2 and U6 RNA expression levels in acute stress [A], repeated stress [R], acute stress + 5d [A + 5d] and naive [N] mice. (B) Quantitative analysis of miRs expression levels ($n = 5$ per group). Data were normalized to U2 RNA levels and expressed as fold change relative to naive. Bars represent mean ± SE. $^{*}P < 0.05$ *vs* N; $^{**}P < 0.01$ *vs* N.

stress condition ($F_{(3,16)} = 1{,}513$, $P = 0.25$). In particular a significant increase of the expression of let-7a ($t_8 = 3.376$, $P = 0.01$), miR-9 ($t_8 = 3.457$, $P = 0.009$) and miR26a/b ($t_8 = 3.023$, $P = 0.016$) was observed in acutely stressed mice as compared to naive mice. After repeated restraint or 5 days after acute stress miR-9, let-7a and miR-26a/b expression was not significantly different from basal level, though the latter still slightly increased. These results suggest that acute stress modulates quickly miRs expression, but its effects are not long lasting. Finally the Northern blot analysis confirmed a marginal change in the expression level of miR-125a, more evident five days after acute stress ($t_8 = 2.248$, $P = 0.055$). Among the selected miRs only miR-30b/c expression was not modulated by stress.

The increased expression of miRs we observed in the present study is consistent with the genomic response observed by different authors after stress [1,23] and further suggests that stress-induced neural plasticity might depend not only upon transcriptional changes, but also on miRs mediated post-transcriptional regulatory processes, conceivably at the dendritic level.

Then we sought to investigate whether the alterations of the selected miRs were specific for the FC. In light of the pivotal role of the hippocampal formation in modulating stress response, we measured the effects of single or repeated restraint events on the expression of the selected miRs in this region. Surprisingly no major differences were found in the level of these miRs among the different experimental groups (Fig. 2), as confirmed by statistical analysis (ANOVA: stress condition $F_{(3,16)} = 0.789$, $P = 0.518$; miR $F_{(4,12)} = 8.688$, $P < 0.0001$; stress condition by miR interaction $F_{(4,64)} = 1.203$, $P = 0.301$). We observed a slight increase in the expression of miR-9 in acutely stressed mice as compared to naive, but this was only marginally significant ($t_8 = 2.274$, $P = 0.053$). It should be noted that we looked at changes in a specific population of miRs and at a specific time point, therefore, it can not be excluded that the analysis of different time windows or miRs could reveal miR expression changes also in the hippocampal complex. Nevertheless these results indicate that stress-induced alterations of the selected miRs were not only temporally, but also spatially defined.

The pattern of basal expression of miRs in the brain could provide useful information on their specific regulatory function in the different brain regions in adult mice [3,13]. Thus we analyzed the relative expression levels of the selected miRs in the FC and the hippocampus as well as in structures that have not been associated with stress, such as the striatum, the cerebellum and the superior colliculus, in naive mice. In this experiment we verified also the expression level of miR-20 as negative control, being not expressed in the brain [17]. Northern blot analysis showed that miRs basal expression levels vary considerably among the different areas (Fig. 3), confirming recent characterizations of miRs expression in adult mouse brain [3,13]. Overall miRs expression appears to be higher in the cerebellum and the superior colliculus than in all other regions. On the contrary, the FC shows low levels of miRs, while intermediate levels were observed in the hippocampus and in the striatum (Fig. 3). This pattern was particularly clear for let-7a, miR-9, and miR-30b/c, while no major regional differences were observed for the other miRs. We found very low levels of expression for miR-26a/b and miR-125a. Indeed their hybridization signal in some regions was very close to detection levels. This result differs from previous reports [3], however different methods of detection, the limited population of mice used in this experiment or strain differences could partially account for this discrepancy. Finally, miR-20 was barely detectable in all regions, consistently with previous reports showing very low expression levels of this miR in the brain [17].

To our knowledge this is the first study reporting stress-induced modulation of miRs expression *in vivo*. Overall the most interesting finding was that a stressful experience induces a transient increase of selected miRs expression in the FC. Moreover such changes were region specific, as no alteration of miRs levels were observed in the hippocampus in the same mice.

The effects found in the FC after acute stress confirm the prominent role played by this structure in the response to aversive events [5,8]. In light of the possible role of miRs in the inhibition of dendritic protein synthesis, the low level of miRs expression found in basal condition in the FC might indicate a lose control over localized protein synthesis in this region. In agreement with the executive functions attributed to the FC [19], changes of miRs levels after restraint stress might allow frontal neurons to quickly respond to external stimuli by changing their synaptic efficacy through regulation of localized protein translation.

The physiological meaning of these results will need further investigations correlating these findings with mRNA and protein

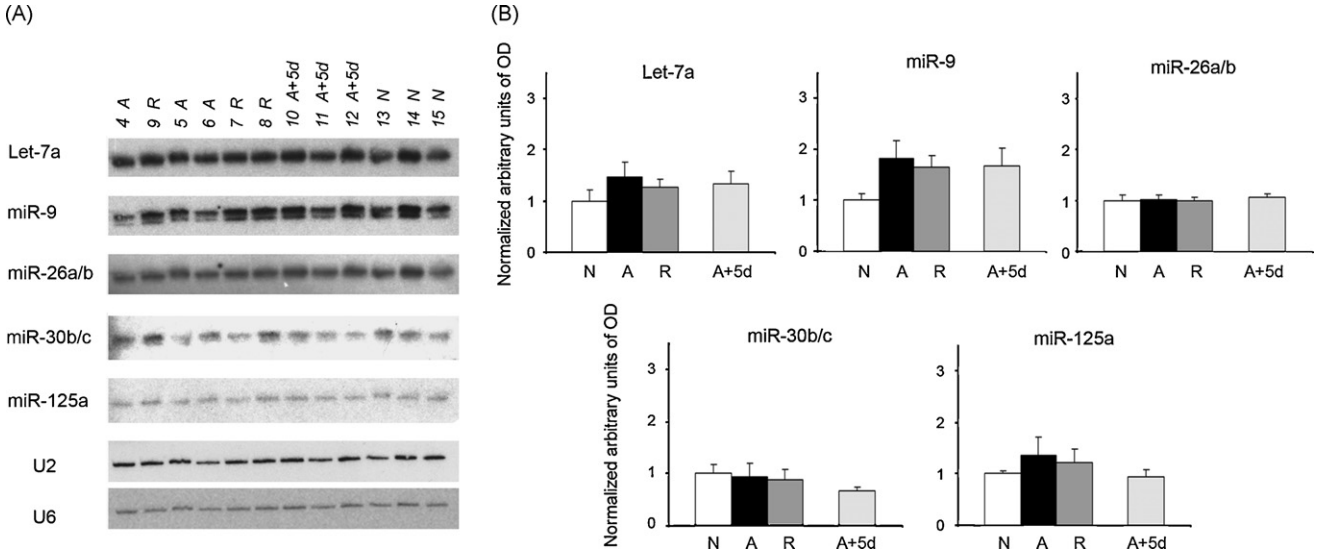

Fig. 2. miRs expression in the hippocampus after acute and repeated stress. (A) Representative Northern blot analysis of let-7a, miR-9, miR-26a/b, miR-30b/c, miR-125a, U2 and U6 RNA expression levels in acute stress [A], repeated stress [R], acute stress + 5d [A + 5d] and naive [N] mice. (B) Quantitative analysis of miRs expression levels ($n = 5$ per group). Data were normalized to U2 RNA levels and expressed as fold change relative to naive. Bars represent mean ± SE.

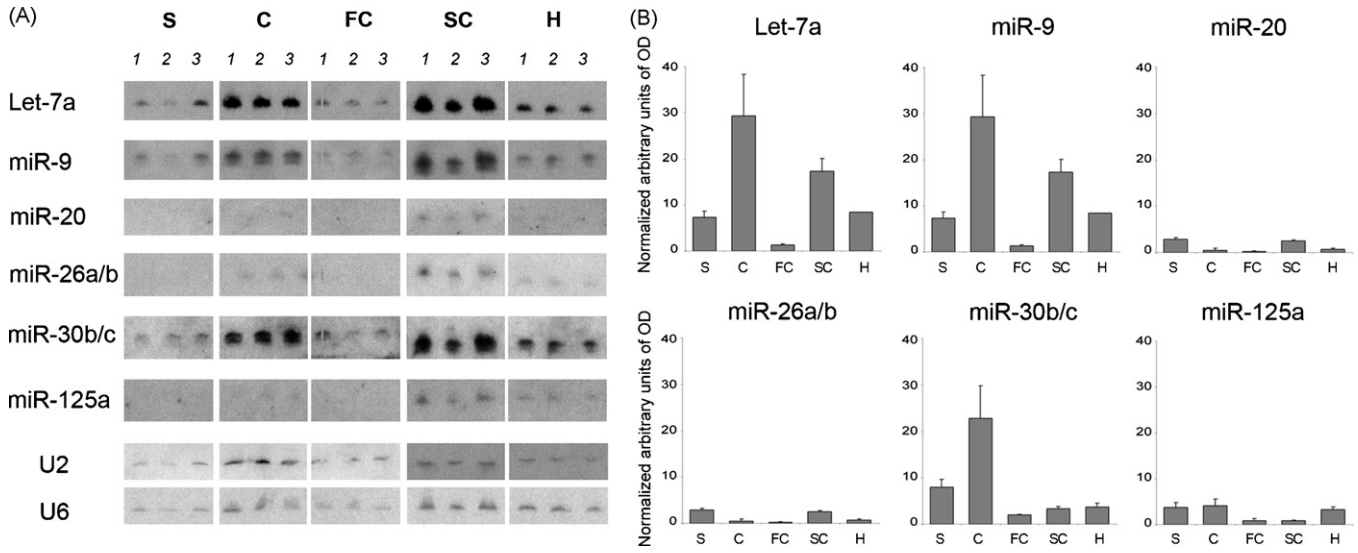

Fig. 3. Differential expression of selected miRs in different brain regions. (A) Representative Northern blot analysis of let-7a, miR-9, miR-26a/b, miR-30b/c, miR-125a, U2 and U6 RNA expression levels in the striatum [S], the cerebellum [C], the frontal cortex [FC], the superior colliculus [SC] and the hippocampus [H] of naive mice. (B) Quantitative analysis of miRs expression levels ($n = 3$ per group). Data were normalized to U2 RNA levels. Bars represent mean ± SE.

expression levels, nevertheless, the modulation of this novel class of non-coding RNAs by aversive experiences provides an interesting perspective for the study of these molecules as new pharmacological targets for stress-induced pathophysiological responses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbr.2009.11.012.

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