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ARTICLE

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MicroRNA expression in rat brain exposed to repeated inescapable shock: differential alterations in learned helplessness *vs.* non-learned helplessness

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Abstract

MicroRNA (miRNA) expression was measured within frontal cortex of male Holtzman rats subjected to repeated inescapable shocks at days 1 and 7, tested for learned helplessness (LH) at days 2 and 8, and sacrificed at day 15. We compared rats that did *vs.* did not exhibit LH, as well as rats that were placed in the apparatus and tested for avoidance but not given shocks (tested controls, TC). Non-learned helpless (NLH) rats showed a robust adaptive miRNA response to inescapable shock whereas LH rats showed a markedly blunted response. One set of 12 miRNAs showed particularly large, significant down-regulation in NLH rats relative to tested controls (mir-96, 141, 182, 183, 183*, 298, 200a, 200a*, 200b, 200b*, 200c, 429). These were encoded at a few shared polycistronic loci, suggesting that the down-regulation was coordinately controlled at the level of transcription. Most of these miRNAs are enriched in synaptic fractions. Moreover, almost all of these share 5′-seed motifs with other members of the same set, suggesting that they will hit similar or overlapping sets of target mRNAs. Finally, half of this set is predicted to hit Creb1 as a target. We also identified a core miRNA co-expression module consisting of 36 miRNAs that are highly correlated with each other across individuals of the LH group (but not in the NLH or TC groups). Thus, miRNAs participate in the alterations of gene expression networks that underlie the normal (NLH) as well as aberrant (LH) response to repeated shocks.

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Introduction

Clinical studies have demonstrated that stress acts as a predisposing and precipitating factor in depression (Lloyd, 1980; Paykel, 1982). Onset of depression is most commonly associated with adverse life events that are often outside the control of affected individuals and may lead to feelings of helplessness. Parallel studies of the effects of uncontrollable stress have been performed in animals with results of proactive interference with the acquisition of escape/avoidance responding (Seligman & Maier, 1967). This phenomenon, termed learned helplessness (LH), has been used extensively as an animal model of stress-induced behavioural depression (Greenwood & Fleshner, 2008; Hajszan *et al*. 2009; Mironova & Rybnikova, 2008; Petty & Sherman, 1979; Sherman *et al*. 1982). Most LH studies have used the acute (single session) model. However, behavioural changes after a single session persist only for a short period of time (24 h) whereas, by definition, depression persists much longer after negative life events. To overcome this issue, we recently modified the LH model to include repeated-shock sessions, which greatly prolonged the duration of LH (Dwivedi *et al*. 2004*b*, 2005*a*, *b*). The neurochemical differences in these rats

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more closely resemble those reported in depressed subjects (Dwivedi *et al*. 2004*a*, *b*, 2005*a*; Shelton *et al*. 1996, 1999). Therefore, in the present study, we used the repeated-shock model.

Although several studies have performed gene expression profiling of rats exhibiting LH (Nakatani *et al*. 2004), to our knowledge, only a single study has directly compared LH *vs.* non-learned helpless (NLH) *vs.* tested controls (TC): Kohen *et al*. (2005) performed gene expression profiling of acute LH *vs.* NLH *vs.* TC *vs.* cage-control rats in hippocampus. Surprisingly, LH rats resembled the TC and cage-control rats in their gene expression profile, whereas the NLH rats showed numerous significant mRNA changes (mostly down-regulation). Thus, the NLH rats showed a robust adaptive response to inescapable shock (IS) whereas the LH rats failed to show this response.

In the past few years, it has become clear that besides the traditional transcriptional mechanisms (e.g. activation of genes by transcription factors and alternative splicing), gene expression in the adult mammalian brain is also regulated by a variety of non-coding RNA transcripts that generate microRNAs (miRNAs), antisense RNAs and other small RNAs, which are linked to several post-transcriptional and epigenetic mechanisms. Of these, the best investigated are the miRNAs, which comprise hundreds of distinct genes. miRNAs regulate translation of proteins within dendrites (Schratt, 2009) and participate in the altered gene expression that accompanies chemical long-term potentiation (Park & Tang, 2009) and learning (Smalheiser *et al*. 2010). Many miRNAs regulate, and are regulated by, activity-dependent transcription factors such as CREB, MEF2 and others. Recently, miRNA expression has been examined in mice (Rinaldi *et al*. 2010) and rats (Meerson *et al*. 2010) undergoing chronic immobilization stress. However, the repeated LH model has a distinct advantage in that we can compare rats that do (LH) *vs.* do not (NLH) show a depressive-like phenotype. In the present study, we have performed profiling of miRNA expression in the LH rat model, to learn whether miRNA expression is altered specifically in the LH *vs.* NLH condition, and whether this is part of a larger coordinated response to repeated shocks that is related to the depression-like phenotype.

Methods

Animals

Virus-free male Holtzman rats (Harlan Sprague–Dawley Laboratories) were housed under standard laboratory conditions [temperature 21 ± 1 °C, humidity 55 ± 5%, 14-h/10-h light/dark cycle (lights on 05:00–19:00 hours)]. Animals were provided free access to food and water and adapted to the laboratory environment for 3 wk before the experiment. Body weights were 325–375 g at the start of the experiment. Rats were housed in groups of three prior to experimentation, after which they were housed individually. All the experiments were performed between 08:00 and 10:00 hours. Experimental procedures were approved by the Animal Care Committee of the University of Illinois at Chicago.

Behavioural procedures

A total of 24 rats were included in this study. LH induction (IS) and escape test (ET) behavioural paradigms are provided in greater detail in our earlier publications (Dwivedi *et al*. 2004*b*, 2005*a*, *b*). Rats were given IS on day 1 and tested for escape behaviour on day 2; these animals were given another IS on day 7 and tested for escape behaviour on day 8. After first IS, rats were placed in individual home cages. All the animals were decapitated on day 15 (between 08:00 and 10:00 hours). Rats were kept in a separate room and brought individually to the room where decapitation was performed.

IS treatment

The rats were placed in Plexiglas tubes with the tail extending from the rear of the tube. Shocks were delivered by means of a computer-controlled constant current shock generator (Model ENV-410B, Med Associates, USA) to electrodes augmented with electrode paste to the rat's tail. The IS consisted of 100 random shocks delivered for 5 s at the rate of 1.0 mA, with a mean interval of 60 s. Another group of rats was placed in Plexiglas tubes but not subjected to shocks (TC).

Shuttle escape testing

Footshock (0.6 mA) was delivered through the grid floor by a shock generator (Model no. ENV-413, Med Associates). Shuttle escape testing began with five trials (FR-1) during which a single crossing would terminate the shocks, followed by 25 trials (FR-2) in which a rat had to cross from one side of the shuttle box to the other and come back to terminate the shocks. Shocks were terminated automatically after 30 s if there was no response within that time. Shuttle escape latencies were recorded automatically by a computer attached to the generator and shuttle box.

Based on the mean latency observed after FR-2, rats were divided into two groups: (1) LH showing mean latency $\geqslant 20$ s and (2) NLH showing mean latency <20 s. We found that about 50% of rats became LH.

Rats were decapitated on day 15. Brains were removed quickly. The various brain areas were dissected on ice and immediately stored at $-80\,^{\circ}$C until required. For the present study, frontal cortices from eight rats in each group were examined individually.

miRNA analysis

RNA was isolated in samples of frontal cortex using a protocol designed to optimize recovery of small RNAs (Lugli *et al*. 2008). Expression of miRNAs was determined by methods described previously (Smalheiser *et al*. 2010). Reverse transcription was performed with the TaqMan® MicroRNA Reverse Transcription kit (ABI, USA) and the multiplex RT for TaqMan® MicroRNA Assays that consists of eight predefined reverse transcription primer pools following the manufacturer's protocol. For each RT pool, 100 ng total RNA was used and the product was diluted 1:62.5 and 55 μl mixed with 55 μl TaqMan® Universal PCR Master Mix, No AmpErase® UNG. Then 100 μl of each mix was dispensed in the appropriate well in the TaqMan® Rodent MicroRNA Array v. 2.0 (TLDA, ABI) and run for 40 cycles according to the manufacturer's protocol on a ABI 7900HT. MicroRNAs were assayed on two plates (A and B); plate A contained many of the canonical miRNA sequences in miRBase, whereas plate B primarily contained minor or star* miRNA sequences arising from the opposite arm of the pre-miR hairpin precursor. Because the biology of plate A and B miRNAs might differ, they were analysed separately, although the same trends were observed on both plates. An equal number of LH, NLH and TC samples were processed and assayed in parallel. A sample processed without reverse transcription showed no detectable miRNA values.

Using samples run on duplicate plates to monitor inter-plate reliability, we observed that C_t values >35 were markedly less reliable, and so $C_t = 35$ was set as the threshold of detectability. C_t values were normalized using U6 RNA, whose mean value did not vary across groups and showed low variability. It should be noted that C_t values cannot be compared directly across different miRNAs, but when comparing the same miRNA in different samples, a higher C_t value indicates lower abundance. Fold-differences in miRNA expression across groups were calculated (using U6-normalized C_t values) using the formula $2^{(C_{t1}-C_{t2})}$.

Statistical analysis

Statistical significance was calculated using both the non-parametric Wilcoxon paired sign-rank test, two-tailed and the paired *t* test, two-tailed. (Paired tests were conducted to match rats that were subjected to repeated shocks in the same experiments and samples that were prepared and analysed for miRNA abundance in the same runs.) Both methods produced similar results. To correct for multiple testing, SAM analysis (Significance Analysis of Microarrays, Stanford University) was performed using 500 perturbations.

Co-expression analysis

MicroRNA pairs were filtered according to the following criteria: (*a*) miRNAs were detectably expressed ($C_t < 35$ in all samples in all groups). (*b*) miRNA pairs showed significant pairwise correlations ($r > 0.707$) across individuals in the LH group. (*c*) Pairwise correlations in NLH and TC groups were significantly less than in the LH group (rLH-rNLH and rLH-rTC > 0.707). (*d*) The miRNA pairs are not negatively correlated significantly in the NLH or TC groups (rNLH > -0.707 and rTC > -0.707). Further filtering was performed for the dataset shown in Fig. 1; namely, only miRNAs are shown that are co-expressed significantly with at least three other miRNAs in the dataset. This ensures that the data shown in the visualized network has extremely high confidence.

Results

Escape latencies

Escape latencies were significantly higher ($p < 0.001$) in LH rats compared to TC or NLH rats on day 2 (TC $= 7.6 \pm 3.1$, NLH $= 5.6 \pm 1.5$, LH $= 25.4 \pm 3.1$) and day 8 (TC $= 4.8 \pm 2.4$, NLH $= 6.4 \pm 3.1$, LH $= 25.9 \pm 3.1$). In our earlier studies, we have repeatedly demonstrated that the rats that were tested on day 8 and showed LH behaviour after second IS (escape latency >25), remained LH on day 14 (Dwivedi *et al*. 2004*b*, 2005*a*, *b*). To avoid the effect of stress on miRNA expression that may have arisen due to shuttle escape testing, in the present experiment, we did not determine the escape latency on day 14.

MicroRNA analysis

MicroRNA alterations in frontal cortex were computed for each of three comparisons: NLH *vs.* TC; LH *vs.* TC; and LH *vs.* NLH. In total, 590 RNAs were

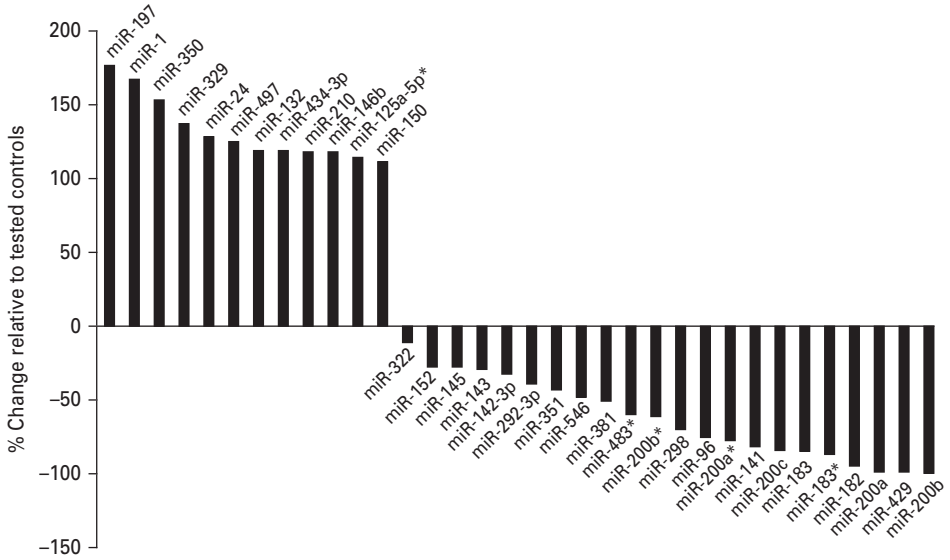

Fig. 1. miRNAs that are significantly up- or down-regulated in the NLH *vs.* TC comparison. See Supplementary Table S1 for C_t and p values for each miRNA.

measured across the TLDA A and B plates; most (585) were miRNAs, but U6 RNA, Y1 RNA, and a few snoRNAs were also included. A total of 371 (256 RNAs on plate A, 115 on plate B) were expressed at >2 times threshold (i.e. mean C_t value <34) in at least one treatment group and were examined further.

Global analysis of miRNAs

The most striking miRNA response was observed in the NLH *vs.* TC comparison. NLH rats showed miRNA changes in both directions, but down-regulated miRNAs predominated. This can be demonstrated in several different ways: (*a*) Across all expressed miRNAs, the NLH group showed levels that were significantly lower on average than in the TC group (plate A: 9.4% decrease, $p=0.0047$; plate B: 9.5% decrease, $p=0.0022$). (*b*) Among the miRNAs that exhibited relatively large changes in expression ($\Delta C_t>0.5$), 14 miRNAs were up-regulated *vs.* 44 down-regulated (plate A: 12 up, 31 down; plate B: two up, 13 down). (*c*) For a set of 371 miRNAs one would expect to observe nine miRNAs showing changes in each direction at $p=0.05$ simply by chance. Among the miRNAs that showed significant changes at $p<0.05$ by *t* test, 12 were up-regulated and 22 down-regulated [Fig. 1; Supplementary Table S1 (available online)].

In contrast, few global miRNA changes were observed in the LH *vs.* TC comparison: (*a*) Across all expressed miRNAs, the LH group showed mixed trends relative to the TC group (plate A: 5% decrease, $p=0.022$; plate B: 6.3% increase, $p=0.090$). (*b*) Among the expressed miRNAs that exhibited relatively large changes in expression ($\Delta C_t>0.5$), 22 were up-regulated *vs.* 22 down-regulated (plate A: nine up, 16 down; plate B: 13 up, six down). (*c*) Again, for a set of 371 miRNAs one would expect to observe nine miRNAs showing changes in each direction at $p=0.05$ simply by chance. Among the miRNAs that showed significant changes at $p<0.05$ by *t* test, only eight were up-regulated and four down-regulated (plate A: five up, two down; plate B: three up, two down) (Fig. 2; Supplementary Table S2). (*d*) Note that the changes in miRNA expression observed relative to TC rats were highly correlated in NLH and LH rats across all expressed miRNAs (plate A: $r=0.87$; plate B: $r=0.45$). However, miRNAs showed a much smaller average decrease in the LH-TC comparison compared to the NLH-TC comparison (plate A: $\Delta C_t=0.092$ *vs.* 0.159; plate B: $\Delta C_t=-0.088$ *vs.* 0.145).

All of these measures indicate that globally, the NLH rats showed a vigorous miRNA response to repeated IS, whereas the overall miRNA response of LH rats was similar but markedly blunted. Thus, miRNA changes in this paradigm can be viewed as part of a normal response to stress that is adaptive and homeostatic, but that is deficient in LH rats.

The most salient comparison for understanding the biological basis of LH is between LH *vs.* NLH rats, since these are exposed to the same repeated shocks,

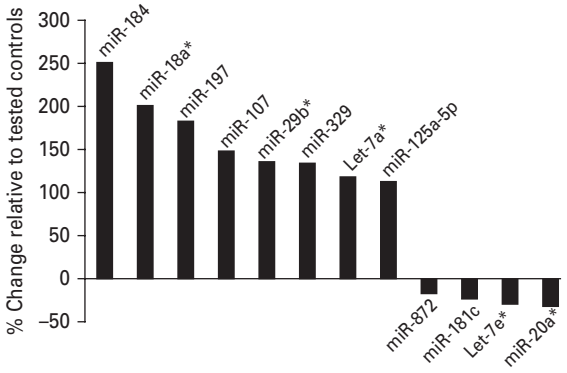

Fig. 2. miRNAs that are significantly up- or down-regulated in the LH *vs.* TC comparison. See Supplementary Table S2 for C_t and p values for each miRNA.

but exhibited different phenotypic outcomes. Globally, LH rats exhibited a definite up-regulation of miRNA levels relative to NLH rats: (*a*) Across all expressed miRNAs, the LH group showed levels that were significantly higher on average than in the NLH group (plate A: 4.8% increase, $p=0.012$; plate B: 17.5% increase, $p=1.4\times10^{-5}$). (*b*) Among the miRNAs that exhibited relatively large changes in expression ($\Delta C_t>0.5$), 42 miRNAs were up-regulated *vs.* 11 down-regulated (plate A: 21 up, eight down; plate B: 21 up, three down). (*c*) For a set of 371 miRNAs one would expect to observe nine miRNAs showing changes in each direction at $p=0.05$ simply by chance. Among the miRNAs that showed significant changes at $p<0.05$ by t test, 20 were up-regulated and two down-regulated (Fig. 3; Supplementary Table S3).

Analysis of individual miRNAs

The miRNAs significantly altered in the NLH-TC comparison (Fig. 1) should give insight into the participation of miRNAs in the adaptive stress response. When tested individually, 30 miRNAs measured on the A plate achieved significance at $p=0.05$ or better (Fig. 1; Supplementary Table S1). Perturbation analysis (SAM, Stanford University), which takes testing of multiple miRNAs into account, identified ten of these miRNAs as significant at a very stringent false discovery rate of 0% (mir-96, 141, 182, 183, 298, 200a, 200b, 200c, 322, 429).

Very strong patterns were observed among the affected miRNAs, indicating that the NLH response occurred in a coordinated manner. Five groups of significantly affected miRNAs were encoded at adjacent genomic positions, and presumably arose from the same primary miRNA gene transcript or even from the same pre-miR hairpin precursor (Fig. 1; Supplementary Table S1): mir-200a, 200a*, 200b, 200b* and 429 were encoded at 4qE2; mir-96, 182, 183 and 183* at 6qA3.3; mir-141 and 200c at 6qF2; mir-143 and 145 at 18qE1; and mir-322 and 351 at XqA5. The miRNAs encoded at the same locus showed the same direction and similar magnitude of change as expected, if the changes were due to decreased transcription or processing. Fiore *et al*. (2009) have shown that a cluster (mir-379-410) of miRNAs encoded at 12F1 are stimulated by neuronal activity and mediated by MEF2-dependent transcription; however, two miRNAs within this locus (mir-329, 381) showed changes in opposite directions, indicating that other events may be occurring as well, such as transcription of individual miRNAs from local promoters or differential turnover of miRNAs. Four of the affected miRNAs (mir-322, 329, 350, 351) were first identified in rat cortical pyramidal neurons (Kim *et al*. 2004) and at least nine showed synaptic enrichment ratios of $\geqslant2$ (mir-182, 183, 200a, 200b, 200c, 322, 350, 351, 429; Lugli *et al*. 2008); these miRNAs, at least, are likely to be expressed primarily in neurons. However, the affected miRNAs covered a wide range of synaptic enrichment ratios and evolutionary patterns of vertebrate expression.

There were also numerous examples in which miRNAs expressed the same 5′-seed sequences (defined here as bases 2–8 of the miRNA sequence listed in miRBase), which are thought to be utilized for target recognition (Fig. 1; Supplementary Table S1). For example, mir-322 and 497 have the same seed agcagca; mir-125a-5p and 351 share cccugag; mir-141 and 200a share aacacug; mir-200b, 200c and 429 share aauacug; and mir-200a* and 200b* share aucuuac. Others shared five or six bases within the seed: e.g. mir-96, 182 and 183 share uggca. This suggests that the affected miRNAs will exhibit extensive overlap in their target mRNAs (see below).

Of the relatively few miRNAs that were significantly altered in the LH-TC comparison (Fig. 2; Supplementary Table S2), several showed very similar changes, in direction and magnitude, in both NLH and LH rats relative to TC rats (mir-125a-5p, 197, 329). A single miRNA (mir-184) showed a different trend (elevated in the LH *vs.* TC comparison but not in the NLH *vs.* TC comparison). However, this finding could not be replicated when mir-184 was re-analysed individually (see below).

The miRNAs significantly altered in the LH-NLH comparison (Fig. 3; Supplementary Table S3) include five of the miRNAs encoded within the mir-379-410 cluster discussed above, including sequences arising

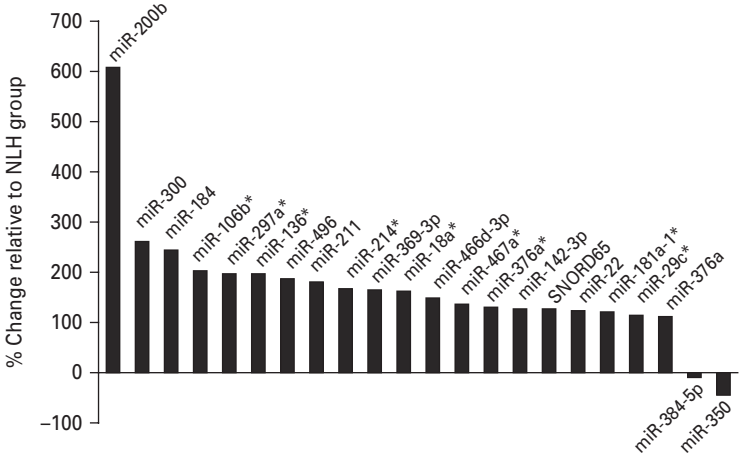

Fig. 3. The set of miRNAs that are significantly up- or down-regulated in the LH *vs.* NLH comparison. See Supplementary Table S3 for C_t and p values for each miRNA.

from the same pre-miR hairpin (mir-376a, 376a*) and these are likely to be co-regulated on the level of transcription. Another three miRNAs are encoded in a large mouse-specific cluster of miRNA genes at 2qA1. However, the affected miRNAs included those expressed in fish as well as those that were mammal-specific. One snoRNA, SNORD65, was also significantly up-regulated. Two affected miRNAs (mir-297a*, 466d-3p) had identical 5′-seed sequences (auacaua) and a third (mir-467a*) was nearly identical (uauacau). Two miRNAs (mir-200b, 369-3p) shared the 5-mer motif aauac; two (mir-136*, 181a-1) shared caucg; and two (mir-376a, 376a*) shared guaga. Again, shared 5′-seed motifs suggest similarity or overlap in their mRNA targets.

As mentioned above, most of the changes observed in the LH group relative to TC rats were similar to, or blunted in magnitude relative to, the NLH *vs.* TC comparison. This was also true for most of the miRNAs that showed significant changes in the LH *vs.* NLH comparison, whether they were up-regulated or down-regulated (Fig. 3). To look more generally for miRNAs that responded quite differently between LH and NLH groups, a complementary quantile rank analysis was performed for A plate miRNAs. First, quantile ranks were assigned for LH and NLH groups (i.e. the most highly expressed miRNA in each group was assigned rank 1, the next most highly expressed miRNA was given rank 2, and so on), and then the difference in quantile ranks was computed for the LH *vs.* NLH comparison. The quantile rank differences followed a normal distribution (mean = 0, S.D. = 7.61). Those miRNAs whose quantile rank differences were $\geqslant 23$ (i.e. >3 S.D. from the mean) were examined further. This method identified mir-182, 184, 200a and 200b (whose changes were also significant based on one or more t tests; Figs 1–3; Supplementary Tables S1–S3) as well as two additional miRNAs (mir-20b, 135a) that were down-regulated (22–46%) in the LH-TC comparison but up-regulated about 2.5-fold in the NLH-TC comparison.

To replicate the findings obtained using TLDA plate technology, we re-analysed seven selected miRNAs individually that showed significant changes in one or more comparisons (mir-182, 184, 300, 329, 350, 429, rno-mir-1). The normalizer U6 was also re-measured. The re-analysed changes were very similar to the TLDA results (Supplementary Table S4), except for mir-184, which failed to show similar changes across groups as detected by TLDA. The reason for the difference in mir-184 results between TLDA and individual TaqMan probes is unclear, although the latter reagent was synthesized by a different supplier.

Target analysis

MicroRNAs are generally thought to function via inhibiting translation and stability of target mRNAs (and perhaps other non-coding RNAs); recent studies indicate that they can modulate RNAs both up and down, and can act at the level of transcription as well (Place *et al*. 2008). It lies beyond the scope of this article to correlate globally the miRNA and mRNA changes associated with LH (or the normal homeostatic response to repeated shocks). Rather, we would like to focus on Creb1 as a potential target mRNA, since this is known to be down-regulated in LH rats (but not

NLH rats), and since Creb1 is a prominent 'hub' protein that both regulates, and is regulated by, dozens of miRNAs. We employed TargetScan Mouse 5.1, a leading and relatively conservative prediction server maintained by the Whitehead Institute (http://www.targetscan.org/mmu_50/), employing mouse predictions (as well as rat) because of the better coverage of the mouse genome. Creb1 was predicted to be hit by 55 different miRNAs (among families that are conserved across vertebrates or across mammals). Of the 11 miRNAs that were significantly different in the LH-NLH comparison and that were included in the TargetScan server, four (mir-22, 200b, 211, 300) were predicted to hit Creb1 – and all were up-regulated. Conversely, of the 30 miRNAs that were significantly different in the NLH-TC comparison and included in the TargetScan server, six (mir-96, 182, 200b, 200c, 351, 429) were predicted to hit Creb1 – and all were down-regulated. There is no reason to believe that miRNA changes are the primary determinants of Creb levels in rats exposed to repeated shocks. However, if up-regulation of miRNAs should tend to down-regulate levels of the target protein and/or mRNA, then the miRNA changes in LH rats would tend to reinforce the decreased Creb1 levels observed in these rats. Conversely, the miRNA changes in NLH rats would have the opposite effect of de-repressing, and hence preserving, Creb levels in these rats.

Co-expression analysis

In contrast to identifying miRNAs that show significant mean differences in expression across treatment groups, pairwise correlation analysis identifies pairs of miRNAs whose expression varies up or down in parallel across individuals of the *same* treatment group (Smalheiser *et al*. 2010). Pairs of miRNAs may be co-expressed because they are encoded by the same pri-miR or pre-miR precursor, or because they are driven by the same transcription factor or regulated by the same cellular mechanism(s). [Two miRNAs may also be correlated if they are both *negatively* correlated with the same (third) miRNA.] Here, we identified pairs of miRNAs that are significantly and positively correlated across individuals in the LH group, but *not* significantly correlated in the NLH or TC groups. This provides a novel means of detecting coordinated changes in miRNA expression that are specifically associated with LH.

A total of 278 pairs of miRNAs were correlated only in the LH group (only A plate miRNAs were analysed; see Methods section for details). Figure 4 displays a network visualization for the core set of 36 miRNAs that are each pairwise highly correlated with at least three other miRNAs in this set. Although the factor(s) that co-regulate the miRNAs in this core module are unknown, at least five miRNAs are transcribed in an activity-dependent fashion (CREB: mir-9, 132, 218; MEF2: mir-369-5p, 409-3p). As well, the core module includes six of the 12 miRNAs that were significantly up-regulated in the NLH *vs.* TC comparison (mir-24, 125a-5p, 132, 150, 210, 434-3p), but none of the 18 miRNAs down-regulated in the same comparison. A few of the miRNAs in the core module are encoded near each other at the same genomic locus (e.g. mir-191, 425; Fig. 4); insofar as these two miRNAs are co-expressed in controls, there is *no* direct link between them, but they exhibit shared relationships with other miRNAs (including some that may be negatively correlated and thus do not show up in the diagram itself).

Discussion

There are many similarities between the LH animal model and human depression. For example, uncontrollable stressful events, which precipitate depression-like behaviours observed in rodents, similarly precede the onset of some clinical depressions in humans (Gold *et al*. 1988; Lloyd, 1980). Moreover, exposure of animals to similar but controllable events does not produce relevant behavioural changes (Corum & Thurmond, 1977; Weiss *et al*. 1982). Several of the principal symptoms that characterize clinical depression are seen in stressed animals: decreased motor activity (Seligman & Maier, 1967; Weiss *et al*. 1980), decreased eating and drinking, weight loss/lack of weight gain (Ritter *et al.* 1978), decreased grooming (Zacharko *et al*. 1983), decreased competitive behaviour (Corum & Thurmond, 1977; Peters & Finch, 1961), increased errors in a choice/discrimination task (Sherman *et al*. 1982), decreased response to rewarding brain stimulation (Zacharko *et al*. 1983), and sleep disturbances (Weiss & Simson, 1985). These symptoms closely correspond to those typically used for the diagnosis of depression as listed in DSM-IV. In summary, exposure of animals to highly stressful uncontrollable events produces a model of depression characterized by aetiology and symptomatology that resembles clinical depression in humans and makes this an interesting and reliable model to explore the pathophysiology of depression. One of the major attractions of this model is that it is derived from the cognitive view of depression in which events are viewed negatively and interpreted as not controllable, leading to feelings of anxiety and helplessness when dealing with them.

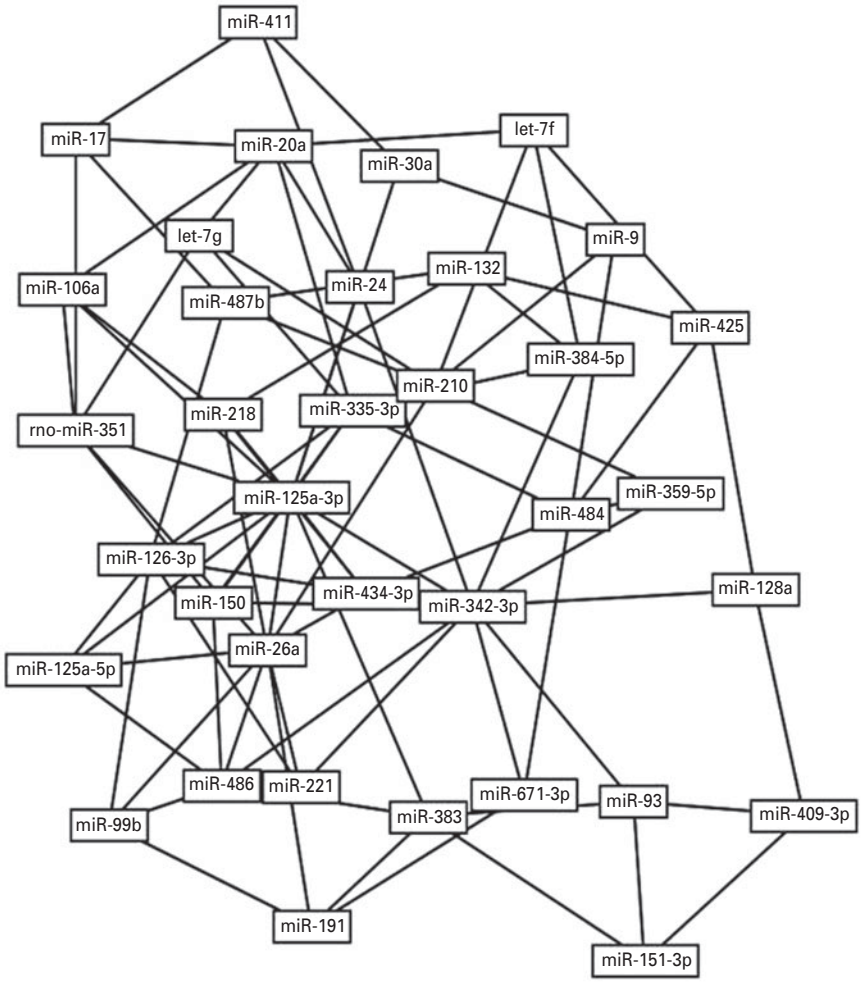

Fig. 4. A core module of miRNAs that are selectively and highly co-expressed within the LH group but not in the NLH or TC groups. Although the miRNA changes in the LH *vs.* sham comparison was generally blunted compared to the NLH *vs.* sham comparison (see Results section), co-expression analysis reveals that active and coordinated changes in miRNA regulation did occur selectively in the LH group. The network visualization shows 36 miRNAs that were significantly correlated with at least three other miRNAs in the LH group, but not in the NLH or sham groups (see Methods section for details). The observation that these miRNAs are strongly co-expressed and interconnected in the LH group, but not in the NLH or sham groups, indicates that specific transcription factors or other miRNA regulatory factors are operative selectively in the LH group.

MicroRNAs regulate the expression and translation of mRNAs within neural tissue, including near synaptic regions. We and others have recently shown that miRNA levels can be rapidly altered by behavioural, sensory and contextual cues such as those that accompany learning. Therefore, we hypothesized that miRNA changes may be observed in repeated-shock paradigms. In the present study, we measured miRNA levels within frontal cortex of rats subjected to repeated IS (at days 1 and 7), tested for LH at days 2 and 8, and sacrificed at day 14. We compared rats that did (LH) *vs.* did not (NLH) exhibit LH, as well as rats that were placed in the apparatus and tested for avoidance but were not given shocks (TC).

Kohen *et al*. (2005) previously performed gene expression profiling of acute LH *vs.* NLH *vs.* sham (tested controls) *vs.* cage-control (naive) rats in hippocampus. They reported that, surprisingly, LH rats resembled the TC and cage-control rats in their gene expression profile, whereas the NLH rats showed numerous significant mRNA changes (mostly down-regulation). Thus, the NLH rats showed a robust adaptive response to IS whereas the LH rats failed to show this response. As a group, the down-regulated

mRNAs tended to be correlated with each other across individuals, and they shared certain AT-rich motifs in their 3′-UTR regions, which they suggested could represent potential miRNA target sites. The present study differs from Kohen *et al*. (2005) in numerous ways. We characterized changes in miRNAs, rather than mRNAs; we subjected rats to repeated shocks rather than a single session; and we examined frontal cortex, rather than hippocampus. Nevertheless, our findings are congruent with theirs: NLH rats showed numerous significant miRNA changes whereas apart from a few affected miRNAs, LH rats generally resembled TC rats in their miRNA expression profile.

One set of miRNAs showed particularly large, significant, consistent and interesting alterations in NLH rats, consisting of mir-96, 141, 182, 183, 183*, 298, 200a, 200a*, 200b, 200b*, 200c, and 429. All were down-regulated in NLH rats relative to TC rats, and all showed a blunted response in LH rats. All were encoded at a few shared polycistronic loci suggesting that their down-regulation was coordinately controlled at the level of transcription. In preliminary studies, levels of pri-mir-182 primary transcript were decreased 4- to 5-fold in the NLH and LH groups relative to TC rats, which supports this idea further (Y. Dwivedi, unpublished observations). Most of these miRNAs have previously been shown to be enriched in synaptic fractions (Lugli *et al*. 2008). Moreover, almost all of these share 5′-seed motifs with other members of the same set, suggesting that they will hit similar or overlapping sets of target mRNAs. Several of the miRNAs in this set have experimentally validated targets; notably, mir-200a,b,c and 429 are known to target the transcription factor Zeb1 and Zeb2 and in turn, Zeb1-binding sites are present upstream of mir-96, 141, 182, 183 and 200c that stimulate their expression, thus forming a complex feedback loop (Bracken *et al*. 2008). Finally, half of this set (but no other miRNAs affected in NLH rats) are predicted to hit Creb1 as a target (see Results section), and binding sites for CREB lie upstream of mir-96, 182, 183, 200a, b, c, 220a* and 200b* (UCSC Genome Browser Conserved TFBS track; data not shown). This suggests that a similar feedback loop arrangement may also exist for CREB as well, similar to what has been described for other CREB-stimulated miRNAs and target genes (Klein *et al*. 2007; Wu & Xie, 2006). Since these miRNAs are down-regulated in NLH rats (which maintain normal CREB levels) but not in LH rats (which show lower CREB levels), this can be speculatively interpreted as a homeostatic response intended to minimize repressive effects on Creb1.

Besides characterizing miRNAs that showed changes in mean expression levels across treatment groups, we also identified a large core co-expression module consisting of miRNAs that are strongly correlated with each other across individuals of the LH group, but not in either NLH or TC groups. The presence of such a module implies that the normal homeostatic miRNA response to repeated IS is not merely absent or blunted in LH rats; rather, gene expression networks (presumably including both mRNAs and miRNAs) are actively reorganized in LH rats which may support their distinctive persistent (albeit pathological) phenotype. In this regard, it is interesting to note that five of the miRNAs in this core module (mir-125a-5p, 132, 151-3p, 191, 486-5p) were also included in a co-expression module of miRNAs expressed in human post-mortem prefrontal cortex (pairwise correlated in depressed subjects but not in control subjects; Y. Dwivedi, unpublished data).

In conclusion, the present study indicates that miRNAs participate in alterations of gene expression networks that underlie the normal (NLH) response to repeated shocks as well as the aberrant (LH) response. This sets the stage for future studies to investigate experimentally whether altering individual miRNAs, or individual components of the miRNA biogenesis machinery (e.g. dicer), will alter the susceptibility of rats to exhibit the LH phenotype, or affect their responsiveness to agents (e.g. antidepressants) which reverse the LH phenotype (Chen *et al*. 2006).

Note

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org/pnp).

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Statement of Interest

None.

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