Elevation of *Il6* is associated with disturbed let-7 biogenesis in a genetic model of depression

YB Wei1,2, JJ Liu1,2, JC Villaeuscus2–4, E Åberg4, S Brené4, G Wegener5,6, AA Mathé7 and C Lavebratt1,2

Elevation of the proinflammatory cytokine IL-6 has been implicated in depression; however, the mechanisms remain elusive. MicroRNAs (miRNAs) are small non-coding RNAs that inhibit gene expression post-transcriptionally. The lethal-7 (let-7) miRNA family was suggested to be involved in the inflammation process and IL-6 was shown to be one of its targets. In the present study, we report elevation of *Il6* in the prefrontal cortex (PFC) of a genetic rat model of depression, the Flinders Sensitive Line (FSL) compared to the control Flinders Resistant Line. This elevation was associated with an overexpression of LIN28B and downregulation of let-7 miRNAs, the former an RNA-binding protein that selectively represses let-7 synthesis. Also DROSHA, a key enzyme in miRNA biogenesis was downregulated in FSL. Running was previously shown to have an antidepressant-like effect in the FSL rat. We found that running reduced *Il6* levels and selectively increased let-7i and miR-98 expression in the PFC of FSL, although there were no differences in LIN28B and DROSHA expression. Pri-let-7i was upregulated in the running FSL group, which associated with increased histone H4 acetylation. In conclusion, the disturbance of let-7 family biogenesis may underlie increased proinflammatory markers in the depressed FSL rats while physical activity could reduce their expression, possibly through regulating primary miRNA expression via epigenetic mechanisms.

Translational Psychiatry (2016) 6, e869; doi:10.1038/tp.2016.136; published online 16 August 2016

INTRODUCTION

In the past two decades, clinical evidence has linked inflammatory responses with psychiatric disorders including major depressive disorder (MDD). Cytokines, chemical messengers between immune cells, have been shown to have an important role in mediating behavioral, neuroendocrine and neurochemical features of MDD. Elevated levels of proinflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α) and IL-6 have been found in serum/plasma and cerebrospinal fluid of depressed patients, also in the absence of comorbid medical illness; the most consistent result being an increase in IL-6. In addition, stimulation of the immune system with lipopolysaccharide can elicit symptoms of depression in humans with no previous episodes of depression. Several findings also indicated that IL-6 has a pathophysiological role in depression, especially in patients who fail to respond to selective serotonin reuptake inhibitors. Physical exercise has been shown to have antidepressant effects and to reduce the risk for elevated levels of proinflammatory markers. Thus, although accumulated evidence shows increased IL-6 in MDD, the mechanisms underlying these alterations have not been clarified.

MicroRNAs (miRNAs) are small non-coding RNAs that typically function as key post-transcriptional repressors of gene expression. MiRNAs control a variety of developmental and cellular processes and evidence has linked altered miRNA expression with psychiatric disorders, for example, MDD. The classic miRNA biogenesis begins with transcription of primary transcripts (pri-miRNAs) by RNA-PolyII. In the cell nucleus, pri-miRNAs are processed by DROSHA and its cofactor DGCR8, releasing the 60–80 nucleotides (nt) precursors (pre-miRNAs). After transfer to the cytoplasm, pre-miRNAs are further cleaved by DICER to generate approximately 22 nt double-stranded mature miRNAs. One strand of the mature miRNA is incorporated into the RNA-induced silencing complex (RISC), whereas the other strand is degraded. The miRNA-RISC regulates target mRNA expression through mRNA degradation and/or translational repression.

Lethal-7 (let-7) is one of the most studied miRNA families and is highly conserved between species. In human, the let-7 family consists of 12 genes encoding nine distinct miRNAs (let-7a to let-7i and miR-98). There is increasing evidence suggesting the involvement of the let-7 family in inflammation and immune response. A previous study showed that the let-7 family directly inhibited IL-6 expression in breast cancer cell lines, and thereby may act as an immunorepressor. Let-7 is abundant in adult brain and has been implicated in neuronal proliferation and differentiation and synaptic plasticity, but it is not known whether it has a role in the pathophysiology of depression. In cancer research, coordinated downregulation of multiple let-7 family members was found in many tumor types. This reduction was associated with an overexpression of LIN28 (including paralogous LIN28A and LIN28B in mammals), an RNA-binding protein that selectively represses let-7 maturation. Importantly, a recent study showed that LIN28B and LIN28A inhibited let-7 expression by different mechanisms, that is, LIN28B directly binds the primary let-7 (pri-let-7) transcripts and prevents...
DROSHA-mediated cleavage. `{34} Besides, heteronuclear ribonucleoprotein A1 (hnRNPA1) was shown to negatively regulate let-7 biogenesis in cells lacking LIN28 expression. `{35}

The Flinders Sensitive Line (FSL) was selectively bred for increased sensitivity to the anticholinesterase agent diisopropyl fluorophosphate and exhibits behaviors that resemble a number of symptoms in human depression. `{36,37} FSL and their controls, the Flinders Resistant Line (FRL) are widely used to explore putative pathophysiology of human depression and test antidepressant-like effects of both pharmacological and non-pharmacological interventions such as serotonin reuptake inhibitor, `{38} histone deacetylase inhibitor, `{39} deep brain stimulation, `{40} electroconvulsive stimuli `{41} and voluntary wheel running. `{42} Previously, we have shown altered expression of the inflammatory markers both centrally and in the periphery in the FSL depression model. `{43,44}

We hypothesized that Il6 expression was elevated in the prefrontal cortex (PFC) of the FSL strain compared with FRL, and that this elevation would associate with a downregulation of the let-7 family, in turn influenced by alterations in miRNA biogenesis. Second, we hypothesized that physical exercise would lower the elevated Il6 levels in the PFC of the FSL rats, by normalizing let-7 expression.

MATERIALS AND METHODS

Tissue samples and wheel running experiments

The PFC from 3-month-old male FRL/FSL rats (n = 7 in each group) was dissected according to the method of Glowinski and Iversen `{45} and stored at –80 °C until subsequent analyses. Wheel running experiments were performed as previously described. `{46} In brief, 3-month-old male FSL rats were individually housed, randomly assigned with either free access (FSL-fl) or no access (FSL-control, n = 7) to a running wheel during a period of 35 days. The sample sizes were chosen on the basis of previous publications and literatures in relevant field. Running data were sampled 48 times per day using a computer-based data system with customized software. The PFC region was dissected following wheel running experiments. The investigators were blinded to group allocation during all the procedures. All the animals had access to food and water ad libitum, and were subjected to a controlled 12-h light/dark cycle (lights on at 0700 h). All the experiments were approved by the Danish National Committee for Ethics in Animal Experimentation and the Ethical Committee for Protection of Animals at the Karolinska Institutet.

RNA extraction and reverse transcription

Total RNA was extracted using miRNA Universal Kit (Qiagen, Hilden, Germany) followed by treatment with DNase I (Qiagen) to digest contaminating DNA. Concentrations were determined using the NanoDrop ND-1000 (NanoDrop Technologies, Waltham, MA, USA). Reverse transcription of miRNA was performed using Universal cDNA Synthesis Kit II for RT-PCR (Exiqon, Vedbaek, Denmark). Unisp6 RNA was spiked in for monitoring conversion efficiency. The reaction were incubated with the enzyme mix at 42 °C for 60 min followed by termination at 95 °C for 5 min. Complementary DNA (cDNA) to pri-miRNA and miRNA was synthesized using the SuperScript III First-Strand Synthesis Kit for Real-time polymerase chain reaction (RT-PCR) (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA). In brief, equal amounts of RNA were random-hexamer primed at 25 °C for 10 min, followed by an incubation with SuperScript III RT at 50 °C for 50 min, and termination of the reaction at 85 °C for 5 min. cDNA of the miRNA and the mRNA was stored at −20 °C and RNA at −80 °C until further processing. All the experimental procedures were according to the manufacturer’s protocols.

Gene expression analyses

Amplification of cDNA corresponding to miRNAs, pri-miRNAs and mRNAs was assessed using RT-PCR. All RT-PCR amplifications were performed in triplicates using Power SYBR Green (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA) on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), with the following conditions: 95 °C for 10 min, followed by 40 repeats of 95 °C for 15 s (10 s for miRNA amplification), 60 °C for 1 min and a final dissociation stage to monitor amplification specificity. The microRNA LNA PCR primers (Exiqon) were used to amplify let-7 miRNA and the data were normalized to Rnu5g. Pri-let-7 and mRNA expression data were normalized to two reference genes (Gapdh, glyceraldehyde-3-phosphate dehydrogenase; and Ppia, cyclophilin A). Relative quantification was calculated using the qBase software (version 1.3.4). `{47} The tested genes and corresponding primer sequences are listed in Supplementary Table S1.

Protein expression

Protein levels for LIN28B and DROSHA were quantified in naive FRL/FSL PFC tissue using a modified western blot protocol, based on Lindfors et al.`{48} Briefly, following sample homogenization and centrifugation, the protein concentration of the lysates was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). After denaturing at 95 °C for 5 min, equal amounts of protein (60 μg) were loaded on a NuPAGE Novex 4–12% Bis-Tris Gel (Invitrogen). The separated protein was transferred to Amersham Hybond ECL Nitrocellulose Membrane (GE Healthcare UK, Buckinghamshire, UK) for 1.5 h at room temperature, and then blocked with 5% nonfat milk for 1 h at room temperature. Immunoblotting was performed overnight at 4 °C with a polyclonal rabbit anti-LIN28B antibody (1:500 dilution; #5422, Cell Signaling Technology, Danvers, MA, USA) or with a polyclonal goat anti-DROSHA antibody (1:1000 dilution; ab58589; Abcam plc, Cambridge, UK), separately, and with a mouse monoclonal anti-β-actin antibody (1:10 000; Santa Cruz Biotechnology) for 60 min at room temperature. Finally, immunoreactive bands were visualized using the Amersham ECL Plus Western Blotting Detection System (GE Healthcare), exposed to Amersham Hyperfilm ECL (GE Healthcare) and optical densities were quantified using the NIH ImageJ software (1.47 version). LIN28B and DROSHA expression levels were normalized to the expression levels of β-actin and the data were presented as relative levels.

Chromatin immunoprecipitation

In vivo chromatin immunoprecipitation (ChIP) was performed according to Melas et al.`{48} Rat prefrontal cortex was homogenized and cross-linked with 1% formaldehyde for 10 min at room temperature followed by addition of glycine to stop the reaction. The samples were lysed and sonicated to shear the DNA into fragment sizes of 200–500 bp. Sonicated DNA was either aliquoted as genomic input DNA or immunoprecipitated using an anti-acetyl-histone H3 antibody (#06-599; EMD Millipore, Billerica, MA, USA), anti-acetyl-histone H4 antibody (#06-598; Millipore) or normal rabbit IgG (negative control; Millipore) at 4 °C overnight with rotation. Protein A Sepharose beads (Sigma-Aldrich) were used to catch the chromatin–antibody complexes. Immunoprecipitates containing protein–DNA complexes were washed and eluted followed by reverse crosslinking. DNA was purified using QIAquick PCR Purification Kit (Qiagen) and quantified by RT-PCR using Power SYBR Green (Applied Biosystems). PCR primers were designed to target within the pri-let-7i promoter (approximately 510–681 bp upstream of the putative transcription start site). `{49} The primer sequences are listed in Supplementary Table S1. The amount of immunoprecipitated pri-let-7i promoter DNA in each sample was calculated as percentage of genomic input DNA (% Input) as follows: % Input = 2−ΔCt sample−ΔCt (sample)−ΔCt (input).

RNA immunoprecipitation

In vivo RNA immunoprecipitation (RIP) was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit according to the manufacturer’s protocol (Millipore). In brief, rat PFC was lysed and immunoprecipitated overnight at 4 °C with a LIN28B antibody (3 μg per immunoprecipitation; #5422, Cell Signaling Technology) or normal rabbit IgG (negative control, 3 μg per immunoprecipitation; Millipore) bound to protein A/G magnetic beads. Ten percent of the lysate was saved as RIP input and stored at −80 °C until starting RNA purification. After washing, the immunoprecipitate was digested by proteinase K followed by RNA purification. cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) followed by RT-PCR for pri-let-7 expression. The primer sequences are listed in Supplementary Table S1.
Statistical analyses
Data in the bar graphs are presented as mean values ± s.e.m. Normality of the data and the homogeneity of the variance were tested using Shapiro–Wilk and Levene’s tests, respectively. The difference in mean between two groups was assessed using two-tailed Student’s t test. The threshold for statistical significance was set at $P < 0.05$. All analyses were performed using IBM SPSS Statistics version 22 (IBM, Armonk, NY, USA).

RESULTS
Increased Il6 levels are associated with downregulation of let-7 family expression in the prefrontal cortex of the FSL rats
First, we measured Il6 mRNA levels in the PFC from FSL and FRL rats. The FSL had higher Il6 mRNA levels compared to the FRL ($P = 0.045$, Figure 1a). Second, we examined whether the increased Il6 levels in the FSL associated with downregulation of let-7 expression. Several of the let-7 family members displayed reduced levels in the FSL compared to FRL. Specifically, let-7b, let-7c, let-7f, let-7i and miR-98 were significantly reduced in FSL ($P = 0.011$, $P = 0.024$, $P = 0.004$ and $P = 0.006$, respectively, Figure 1b). These results suggested that elevation of Il6 in the FSL PFC is associated with a deficiency of let-7 family expression, possibly linked with a disturbed let-7 biogenesis.

LIN28B elevation co-occurred with downregulation of the let-7 family in the FSL PFC
First, we tested the hypothesis that overexpression of LIN28A and LIN28B, acting as let-7 repressors, was associated with the let-7 family deficiency in the FSL. We found detectable levels of Lin28b mRNA but not of Lin28a (Ct > 35) expressed in the PFC of adult FSL and FRL. Specifically, FSL rats exhibited increased LIN28B both at the mRNA and protein levels compared to the FRL (mRNA: $P = 0.021$; protein: $P = 0.042$, Figure 1a). We also tested...
whether Hnrnpa1, a negative regulator of let-7 that is independent of LIN28 regulation, was associated with the decreased let-7 expression in the FSL. Hnrnpa1 expression levels in PFC were not different between the FSL and the FRL \((P=0.38, \text{Figure 1a})\). Second, to gain further support that LIN28B directly associated with pri-let-7 to block mature let-7 synthesis \(\text{in vivo}\), we performed RIP analysis. The RIP results revealed that in the PFC of FSL there was an \(\sim 3\) to 20-fold enrichment of the pri-let-7 transcripts \((\text{pri-let-7c-1, pri-let-7i, pri-mir-98})\) associated with LIN28B compared with the FRL \((\text{pri-let-7c-1: fold change 2.96, } P<0.001; \text{pri-let-7i: fold change: 4.43, } P=0.019; \text{pri-mir-98: fold change 20.34, } P<0.001, \text{respectively, Figure 1c})\). Third, we compared the total expression levels of pri-let-7b, pri-let-7c-1, pri-let-7f-1, pri-let-7i and pri-mir-98 between the FSL and FRL PFC; none of those pri-let-7 transcripts showed different expression between the rat strains \((P>0.5, \text{Figure 1d})\), indicating that reduction in mature let-7 expression in the FSL did not originate from decreased levels of pri-let-7 transcripts. Fourth, we assessed whether the expression levels of key enzymes \((\text{Drosha and Dicer})\) involved in miRNA biogenesis could potentially influence let-7 expression. The DROSHA levels were significantly reduced in the FSL compared with FRL \((\text{mRNA: } P<0.001; \text{protein: } P=0.032; \text{Figure 1a})\). Thus, the downregulation of let-7 family members in FSL compared with FRL \((\text{mRNA: } P<0.001; \text{protein: } P=0.080)\). The individual let-7 family members may compete with each other when exerting their repressive function since each member uses the same seed \((5’-\text{GAGGUA}-3’)\) sequence of the let-7 as a template for recognizing complementary sites in the \(3’\)-untranslated region of \(\text{let-7}\) as a template for recognizing complementary sites in the \(3’\)-untranslated region of \(\text{let-7}\), which was associated with downregulation of let-7 family members in the PFC of FSL \((\text{mRNA: } P<0.001; \text{protein: } P=0.004 \text{ and } P=0.093 \text{ respectively, Figure 2c})\). In accord with the pri-let-7i upregulation, FSL-runners showed an increased histone H4 acetylation \((P=0.030)\) but not histone H3 acetylation \((P=0.99)\) within the pri-let-7i promoter \((\text{Figure 2d})\). Thus, physical activity reduced \(\text{Il6}\) levels and rescued let-7 expression in the FSL PFC.

**Discussions**

In the present study, we demonstrate increased \(\text{Il6}\) levels and in parallel decreased levels of let-7 miRNA family in the PFC of a well-established model of depression, the FSL rat.\(^{26,37}\) Since the let-7 family is known to target \(\text{Il6}\), the results suggest that the let-7 family dysregulation contributes to the overexpression of \(\text{Il6}\) in the PFC of FSL. The results also suggest that this decrease of let-7 miRNA levels is in part due to a disturbed LIN28B-mediated miRNA biogenesis, and possibly in part due to a dysregulated DROSHA. In addition, we show that physical activity normalizes \(\text{Il6}\) levels and could rescue let-7 expression. The let-7 upregulation by physical activity appeared not to be associated with miRNA biogenesis processes but rather with epigenetic changes upstream pri-let-7 mRNA.

Elevation of \(\text{Il6}\) is associated with downregulation of let-7 miRNAs in the PFC of FSL, a rat model of depression.\(^{28,30}\) Elevation of peripheral proinflammatory cytokine IL-6 has been reported in clinical depression by a number of studies.\(^{5-7}\) Also, a recent report demonstrated increased IL-6 levels in the brain from rats that exhibited a depression-like phenotype after chronic stress paradigms.\(^{10}\) In line with these results, we found upregulation of \(\text{Il6}\) in the PFC of the FSL. However, according to our previous study, serum IL-6 levels were not different between the FSL and FRL.\(^{34}\) The let-7 family was previously shown to directly inhibit IL-6 expression in breast cancer cell lines, to be abundant in the adult brain and to be implicated in the regulation of neural stem cell proliferation, differentiation and synaptic plasticity.\(^{22-26}\) We showed that the \(\text{Il6}\) elevation in PFC of FSL was associated with a reduced let-7 miRNAs expression. Thus, five of the eight studied let-7 family members were significantly downregulated in the PFC, and the two of the remaining three showed a tendency to downregulation \((\text{let-7a: } P=0.058; \text{let-7e: } P=0.080)\). The individual let-7 family members may compete with each other when exerting their repressive function since each member uses the same seed \((5’-\text{GAGGUA}-3’)\) sequence of the let-7 as a template for recognizing complementary sites in the \(3’\)-untranslated region of \(\text{let-7}\) which was associated with downregulation of let-7 family members in the PFC of FSL \((\text{mRNA: } P<0.001; \text{protein: } P=0.004 \text{ and } P=0.093 \text{ respectively, Figure 2c})\). In accord with the pri-let-7i upregulation, FSL-runners showed an increased histone H4 acetylation \((P=0.030)\) but not histone H3 acetylation \((P=0.99)\) within the pri-let-7i promoter \((\text{Figure 2d})\). Thus, physical activity reduced the elevated \(\text{Il6}\) levels in PFC region of the FSL rats and increased expression of certain let-7 family members present already at primary transcript stage, possibly through epigenetic mechanisms.

**LISTEN**

In the present study, we demonstrate increased \(\text{Il6}\) levels and in parallel decreased levels of let-7 miRNA family in the PFC of a well-established model of depression, the FSL rat.\(^{26,37}\) Since the let-7 family is known to target \(\text{Il6}\), the results suggest that the let-7 family dysregulation contributes to the overexpression of \(\text{Il6}\) in the PFC of FSL. The results also suggest that this decrease of let-7 miRNA levels is in part due to a disturbed LIN28B-mediated miRNA biogenesis, and possibly in part due to a dysregulated DROSHA. In addition, we show that physical activity normalizes \(\text{Il6}\) levels and could rescue let-7 expression. The let-7 upregulation by physical activity appeared not to be associated with miRNA biogenesis processes but rather with epigenetic changes upstream pri-let-7 mRNA.
binding to the 3′-untranslated region target sites, creating a double-negative feedback loop. Noteworthy, let-7 could also be regulated in an LIN28B-independent fashion, for example, through epigenetic mechanisms such as DNA methylation and histone modifications, which is supported by our data from FSL rats under physical exercise (discussed in the next section). In addition to LIN28B changes, we observed that FSL PFC had a decreased DROSHA expression, suggesting a disturbed miRNA biogenesis probably not only in let-7 but also in a variety of other miRNAs. In line with this hypothesis, a recent study has demonstrated a general reduction of miRNA expression in the PFC from depressed suicidal subjects.

**Figure 2.** Gene and miRNA expression levels were measured in the prefrontal cortex of FSL with/without access to running wheel (FSL-runners versus FSL-controls) using RT-PCR. (a) Physical activity normalized *Il6* levels in the FSL-runner group but had no effect on *Lin28b* and *Drosha* mRNA levels. (b) In line with an *Il6* reduction in the FSL rats that were running, let-7i and miR-98 showed significantly increased expression. Specifically, upregulation of let-7i was associated with primary let-7i overexpression (c). (d) Chromatin immunoprecipitation (ChIP) showed an increased histone H4 acetylation (H4ac) but not histone H3 acetylation (H3ac) within the pri-let-7i promoter of the FSL-runners. Gene expression data were presented as relative quantifications (R.Q.), two reference genes (*Gapdh* and *Ppia*) were used for normalization of *Il6*, *Lin28b*, *Drosha* and the primary miRNA genes. *Rnu5g* was used for normalization of mature miRNAs. ChIP data are presented as percentage of genomic input DNA. Data are presented as group means ± s.e.m. For all figures: n = 5–7 animals per group. *P < 0.05, **P < 0.01, ***P < 0.001. FSL, Flinders Sensitive Line; mRNA, messenger RNA; miRNA, microRNA; RT-PCR, real-time polymerase chain reaction.

Physical activity rescued let-7 expression independent of LIN28B regulation

Previous studies demonstrated that physical activity in the form of wheel running exerts antidepressant-like effect and affects histone acetylation levels in the FSL rat. In the present study, we found that FSL runners had reduced *Il6* levels in the PFC compared with FSL rats without access to a running wheel. This *Il6* decrease associated with an increased let-7i expression. Consistently, a recent study reported that physical exercise was able to induce let-7i in the mouse hippocampus. These results suggest that the antidepressant-like effect of physical activity may, in part, be due to brain let-7i expression.

In agreement, let-7 miRNAs were also
found to respond actively to antidepressant drug treatment. For instance, in a rat model of learned helplessness, enoxacin exerted an antidepressant-like effect coupled with a let-7a elevation in the frontal cortex. Further, in blood samples from depressed patients, a number of let-7 family members were upregulated after a 3-month treatment with escitalopram. Collectively, these results may suggest a role for let-7 as a therapeutic target in depression. We showed that the exercise-induced upregulation of let-7 miRNAs in FSL was independent of Lin28B and Drosha changes, implying that other mechanisms are involved in regulating let-7 expression in response to physical activity. In agreement with an effect upstream the primary miRNA transcripts, we found pri-let-71 transcript to be upregulated in the FSL runners, which was associated with an increase in histone H4 acetylation. Supported by ours and others’ findings, physical activity increased histone acetylation in Npy and Bdnf genes and decreased histone deacetylase expression in the brain, suggesting that pri-let-71 may share an upstream epigenetic regulation with stress resilience genes that is independent of LIN28B processing.

Cholinergic dysregulation in the FSL rats

The FSL rats have increased sensitivity to cholinergic agonists compared with the control line FRL, which is associated with the increased density of muscarinic acetylcholine receptors and α7 nicotinic acetylcholine receptors, but not of α5 nicotinic acetylcholine receptors in the FSL brain. The α7 nicotinic receptor is implicated in the production of cytokines including IL-6. Consistent with the previous report, we found no statistically significant difference in expression level of Chrna7 encoding α7 nicotinic receptor in the PFC between FSL and FRL (FSL: 0.89 ± 0.13, FRL: 0.94 ± 0.13, P = 0.79; Relative quantification using RT-PCR, data are presented as group means ± s.e.m.). This suggests that the elevated Il6 levels in the FSL PFC was not due to an α7 nicotinic receptor disturbance. Moreover, previous studies demonstrated that the acetylcholinesterase activity was similar in FSL and FRL suggesting that the increased cholinergic sensitivity in the FSL was not due to the changes in the acetylcholinesterase activity. However, one should keep in mind that alternative splicing and expression of AChE, encoding acetylcholinesterase, is regulated also epigenetically induced by external exposures. For example, the histone deacetylase inhibitor sodium butyrate, known to have an antidepressant effect, affected histone modifications that regulated AChE.

Limitations

There are limitations of this study: (1) Only one reference gene was used for the miRNA expression analysis: Rnu5g. The results from the FRL/FSL rats were consistent when adding also reference gene U6. However, as U6 was affected by physical activity, it was not used as reference in this report. (2) IL-6 protein levels were not studied due to the lack of material. (3) The data are derived from male rats of one age group only. Previously, a difference of Bdnf expression was found between the female and male FSL after escitalopram treatment. There is also report showing increased proinflammatory cytokine production in healthy elderly people. (4) We did not investigate DNA methylation within the promoter of pri-let-71 in FSL-runners, but only histone H3 and H4 acetylation. As reported by O’Hara et al., DNA methylation was not detectable in the pri-let-71 promoter region in a cell line with high let-71 expression. However, DNA methylation variability in the pri-let-71 promoter of the rat PFC cannot be ruled out. (5) The correlation of gene expression with the time spent on wheel running or quantitative depressive behavior in individual rats was not assessed due to incomplete data. Behavior test was not performed on the FSL runners in this particular study, however, a similar wheel-running protocol had antidepressant-like effect in the FSL tested using forced swimming test. (6) We did not have the possibility to analyze Il6 and let-7 in specific cell types in this experiment. However, Il6 and let-7 are expressed throughout the central nervous system, including both neuron and glia cells.

CONCLUSION

We provide results demonstrating for, we believe, the first time that elevated proinflammatory Il6 in the depressed brain is associated with let-7 deficiency. We show that the low levels of let-7 may be a result of disturbed LIN28B-mediated miRNA biogenesis and DROSHA dysregulation. Physical activity was found to normalize the Il6 and let-7 levels through epigenetic regulations upstream primary miRNA transcription. Our results warrant further studies on let-7 regulation in depression. As modulators of IL-6 have been suggested to have a therapeutic potential for treatment-resistant depression, further investigation of let-7 is indicated to test this hypothesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank Drs Andreas Lennartsson and Joëlle Rüegg for technical guidance. This work was supported by the Karolinska Institutet’s Faculty Funds, the Swedish Research Council (grant nos 2010-3631 (CL), 2011-4732 (AAM)), the Fredrik and Ingrid Thürings Foundation, the regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet (CL), Danish Medical Research Council and the Lundbeck Foundation. These funding sources had no involvement in the study design, analysis and interpretation of the data, in the writing of the manuscript or in the decision to submit the report for publication.

REFERENCES

1. Maes M. Evidence for an immune response in major depression: a review and hypothesis. Prog Neuropsychopharmacol Biol Psychiatry 1995; 19: 11–38.
2. Raison CL, Capuron L, Miller AH. Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends Immunol 2000; 21: 24–31.
3. Schiepers OJ, Wichers MC, Maes M. Cytokines and major depression. Prog Neuropsychopharmacol Biol Psychiatry 2005; 29: 201–217.
4. Simon NM, McNamara K, Chow CW, Maser RS, Papakostas GI, Pollack MH et al. A detailed examination of cytokine abnormalities in major depressive disorder. Eur Neuropsychopharmacol 2008; 18: 230–233.
5. Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK et al. A meta-analysis of cytokines in major depression. Biol Psychiatry 2010; 67: 446–457.
6. Lindqvist D, Janelidse S, Hagell P, Erhardt S, Samuelsson M, Minthon L et al. Interleukin-6 is elevated in the cerebrospinal fluid of suicide attempters and related to symptom severity. Biol Psychiatry 2009; 66: 287–292.
7. Loftis JM, Huckans M, Morasco BJ. Neuroimmune mechanisms of cytokine-induced depression: current theories and novel treatment strategies. Neurobiol Dis 2010; 37: 519–533.
8. Dantzer R, O’Connor JC, Lawson MA, Kelley KW. Inflammation-associated depression: from serotonin to kynurenic. Psychoneuroendocrinology 2011; 36: 426–436.
9. Dantzer R, O’Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci 2008; 9: 46–56.
10. Sukoff Rizzo SJ, Neal SJ, Hughes ZA, Beyna M, Rosenzweig-Lipson S, Moss SJ et al. Evidence for sustained elevation of IL-6 in the CNS as a key contributor of depressive-like phenotypes. Transl Psychiatry 2012; 2: e199.
11. Yoshimura R, Hori H, Ikenouchi-Sugita A, Umeno-Nakano W, Ueda N, Nakamura J. Higher plasma interleukin-6 (IL-6) level is associated with SSRIs- or SNRI-refractory depression. Prog Neuropsychopharmacol Biol Psychiatry 2009; 33: 722–726.
12. O’Brien SM, Scully P, Fitzgerald P, Scott LV, Dinan TG. Plasma cytokine profiles in depressed patients who fail to respond to selective serotonin reuptake inhibitor therapy. J Psychiatr Res 2007; 41: 326–331.
13. Lanquillon S, Krieg JC, Bening-Abu-Shach U, Yedder H. Cytokine production and treatment response in major depressive disorder. Neuropsychopharmacology 2000; 22: 370–379.
14 Hamer M, Sabia S, Batter GD, Shipley MJ, Tabak AG, Singh-Manoux A et al. Physical activity and inflammatory markers over 10 years: follow-up in men and women from the Whitehall II cohort study. *Circulation* 2012; 126: 928–933.

15 Cooney GM, Dwan K, Greig CA, Lavelor DA, Rimer J, Waugh FR et al. Exercise for depression. *Cochrane Database Syst Rev* 2013; 9: CD004366.

16 Knol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet* 2010; 11: 597–610.

17 Mouillet-Richard S, Baudry A, Launay JM, Kellermann O. MicroRNAs and depression. *Neurobiol Dis* 2012; 46: 272–278.

18 Dwivedi Y. Evidence demonstrating role of microRNAs in the etiopathology of major depression. *J Chem Neuroanat* 2011; 42: 142–156.

19 Kocerha J, Dwivedi Y, Brennan KJ. Noncoding RNAs and neurobehavioral mechanisms in psychiatric disease. *Mol Psychiatry* 2015; 20: 677–684.

20 Pasquonelli AE, Reinhardt F, Slack F, Martindale MQ, Kuroda M, Maller B et al. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* 2000; 408: 86–89.

21 Polkkepad S, Knight JM, Naghavi AO, Oplt T, Creighton CJ, Shaw C et al. Pri-miRNA profiling for let-7 microRNAs in experimental asthma. *J Biol Chem* 2010; 285: 30139–30149.

22 Teng GG, Wang WH, Dai Y, Wang SJ, Chu YX, Li J. Let-7b is involved in the inflammation and immune responses associated with Helicobacter pylori infection by targeting Toll-like receptor 4. *PLoS One* 2013; 8: e56709.

23 Kumar M, Ahmad T, Sharma A, Mabalirajan U, Kulshreshtha A, Agrawal A et al. Regulation of opioid tolerance by let-7 family microRNA targeting the mu opioid receptor. *Neuron* 2010; 68: 73–84.

24 Rybak A, Fuchs H, Smirnova L, Brandt C, Pohl EE, Nitsch R et al. microRNA-mediated regulation of IL-13 and allergic airway inflammation. *J Allergy Clin Immunol* 2011; 128: 1077–1085 e1071-1010.

25 Chandrasekar V, Dreyer JL. microRNAs miR-124, let-7d and miR-181a regulate signaling. *Cancer Res* 2010; 70: 357–368.

26 Hamer M, Sabia S, Batty GD, Shipley MJ, Tabak AG, Singh-Manoux A et al. Enhanced microRNA levels in rat hippocampus after electroconvulsive stimuli selectively bred putative animal model of depression. *Electroconvulsive Therapy* 2012; 18: 393–403.

27 Carboni L, Becchi S, Piubelli C, Mallei A, Giambelli R, Razzoli M et al. Early-life stress and antidepressants modulate peripheral biomarkers in a gene–environment rat model of depression. *Prog Neuropsychopharmacol Biol Psychiatry* 2010; 34: 1037–1048.

28 Glowinski J, Iverson LL. Regional studies of catecholamines in the rat brain. I. The disposition of 3Hnorepinephrine, 3Hdopamine and 3Hdopa in various regions of the brain. *J Neurochem* 1966; 13: 655–669.

29 Kellar J, Mortier G, De Paeppe A, Speelman F, Vandesompele J. dbase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* 2007; 8: R19.

30 Linford C, Nilsson IA, Garcia-Roves PM, Zuberi AR, Karimi M, Donahue LR et al. Hypothalamic mitochondrial dysfunction associated with anorexia in the anax/a mouse. *Proc Natl Acad Sci USA* 2011; 108: 18108–18113.

31 Melas PA, Lennartsson A, Valkafahmetoglu-Norberg H, Wei Y, Aberg E, Werme M et al. Allele-specific programming of Npy and egf epigenetic effects of physical activity in a genetic model of depression. *Transl Psychiatry* 2013; 3: e255.

32 O’Hara SP, Splinter PL, Gadgos GB, Trussoni CE, Fernandez-Zapico ME, Chen XM et al. HkappaBp50–CCAAT/enhancer-binding protein beta (C/EBPbeta)-mediated transcriptional repression of microRNA let-7i following microbial infection. *J Biol Chem* 2010; 285: 216–225.

33 Zhao C, Sun G, Ye P, Li S, Shi Y. MicroRNA let-7d regulates the TLX/microRNA-9 cascade to control neural cell fate and neurogenesis. *Sci Rep* 2013; 3: 1329.

34 He M, Liu Y, Wang X, Zhang MQ, Hannon GJ, Huang ZJ. Cell-type-based analysis of microRNA profiles in the mouse brain. *Neuron* 2012; 73: 35–48.

35 Shinohara Y, Yahagi K, Mihara K, Chihara D, Suzuki N et al. The microRNA profile of rat hippocampal CA3 by deep sequencing. *Biochem Biophys Res Commun* 2011; 409: 293–298.

36 Meersoon A, Cachea L, Goosens KA, Sapolisk RM, Soreq H, Kaufer D. Changes in brain MicroRNAs contribute to cholinergic stress reactions. *J Mol Neurosci* 2010; 40: 47–55.

37 Sangiao-Alvarelos S, Manfredi-Lozano M, Ruiz-Pino F, Navarro VM, Sanchez-Garrido MA, Leon S et al. Changes in hypothalamic expression of the Lin28/let-7 system and related microRNAs during postnatal maturation and after experimental manipulations of puberty. *Endocrinology* 2013; 154: 942–955.

38 Hayashi Y, Tsuji M, Wang J, Kondo J, Akatsuka T, Jin Y et al. CagA mediates epigenetic regulation to attenuate let-7 expression in Helicobacter pylori-related carcinogenesis. *Gut* 2013; 62: 1536–1546.

39 Smalley NR, Lugli G, Ricavi HS, Torvik VI, Turecki G, Dwivedi Y. MicroRNA expression is down-regulated and reorganized in prefrontal cortex of depressed suicide subjects. *PLoS One* 2012; 7:e33201.

40 Cosin-Tomas M, Alvarez-Lopez MJ, Sanchez-Roige S, Lalanza JF, Bayod S, Sanfelici C et al. Epigenetic alterations in hippocampus of SAMP8 senescent mice and modulation by voluntary physical exercise. *Front Aging Neurosci* 2014; 6: 51.

41 Smallheiser NR, Zhang H, Dwivedi Y. Exonuclease elevates MicroRNA levels in rat frontal cortex and prevents learned helplessness. *Front Psychiatry* 2014; 5: 6.

42 Socci-Ciavolitto L, Maffettoli E, Bettinelli P, Giovannini C, Bignotti S, Tardito D et al. Blood microRNA changes in depressed patients during antidepressant treatment. *Eur Neuropsychopharmacology* 2013; 23: 602–611.

43 Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL, Nestler EJ. Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nat Neurosci* 2006; 9: 519–525.

44 Tizabi Y, Overstreet DH, Rozvani AH, Louis VA, Clark EJ, Jr., Janowsky DS et al. Antidepressant effects of nicotine in an animal model of depression. *Psychopharmacology* 1999; 142: 193–199.

45 Fujiy X, Fujigaya H, Moriwaki Y, Misawa H, Kasahara T, Grando SA et al. Enhanced serum antigen-specific IgG1 and proinflammatory cytokine production in nicotinic acetylcholine receptor alpha7 subunit gene knockout mice. *J Neurommunol* 2007; 189: 69–10.

46 Sihtotang O, Kallio J, Overstreet DH. Studies of a possible relationship of brain proteins to changes in behavioral sensitivity to DFP. *Life Sci* 1983; 32: 413–420.

47 Overstreet DH, Russell RW, Crocker AD, Schiller GD. Selective breeding for differences in cholinergic function: pre- and post synaptic mechanisms.
involved in sensitivity to the anticholinesterase, DFP. *Brain Res* 1984; **294**: 327–332.

65 Sailaja BS, Cohen-Carmon D, Zimmerman G, Soreq H, Meshorer E. Stress-induced epigenetic transcriptional memory of acetylcholinesterase by HDAC4. *Proc Natl Acad Sci USA* 2012; **109**: E3687–E3695.

66 Hansson AC, Rimondini R, Heilig M, Mathe AA, Sommer WH. Dissociation of antidepressant-like activity of escitalopram and nortriptyline on behaviour and hippocampal BDNF expression in female rats. *J Psychopharmacol* 2011; **25**: 1378–1387.

67 Fagiolo U, Cossarizza A, Scala E, Fanales-Belasio E, Ortolani C, Cozzi E et al. Increased cytokine production in mononuclear cells of healthy elderly people. *Eur J Immunol* 1993; **23**: 2375–2378.

68 Schobitz B, de Kloet ER, Sutanto W, Holsboer F. Cellular localization of interleukin 6 mRNA and interleukin 6 receptor mRNA in rat brain. *Eur J Neurosci* 1993; **5**: 1426–1435.

69 Ni J, Wang X, Chen S, Liu H, Wang Y, Xu X et al. MicroRNA let-7c-5p protects against cerebral ischemia injury via mechanisms involving the inhibition of microglia activation. *Brain Behav Immun* 2015; **49**: 75–85.

Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)