microRNAs as novel antidepressant targets: converging effects of ketamine and electroconvulsive shock therapy in the rat hippocampus

Richard M. O’Connor¹, Susan Grenham¹, Timothy G. Dinan²,³ and John F. Cryan¹,²

¹ Department of Anatomy and Neuroscience, University College Cork, Ireland
² Alimentary Pharmabiotic Centre, University College Cork, Ireland
³ Department of Psychiatry, University College Cork, Ireland

Abstract
Early-life stress is a main contributory factor to the onset of depression. Treatments remain inadequate and as such, a large unmet medical need for novel therapeutics remains. Impeding advancement is the poor understanding of the molecular pathology. microRNAs (miRNAs) are novel regulators of gene expression. A paucity of information regarding their role in depressive pathology and antidepressant action remains. This study investigated changes to hippocampal miRNA levels induced via early-life stress in Sprague–Dawley rats and whether antidepressant treatments could reverse these changes. Investigated were the selective serotonin reuptake inhibitor fluoxetine, the rapid acting N-methyl-D-aspartate receptor antagonist ketamine and electroconvulsive shock therapy (ECT). Microarray analysis revealed early-life stress affected the expression of multiple hippocampal miRNAs. Antidepressant treatments reversed some of these effects including a stress-induced change to miR-451. Ketamine and ECT possessed the highest number of common targets suggesting convergence on common pathways. Interestingly all three treatments possessed miR-598-5p as a common target. This demonstrates that changes to hippocampal miRNA expression may represent an important component of stress-induced pathology and antidepressant action may reverse these.

Received 13 December 2012; Reviewed 11 January 2013; Revised 28 March 2013; Accepted 2 April 2013; First published online 20 May 2013

Key words: ECT, ketamine, miRNAs, treatment-resistant depression.

Introduction
Current antidepressant treatments remain inadequate, burdened by a delayed onset of action, a significant percentage of non-responders and side-effects (Wong and Licinio, 2004; Covington et al., 2010). The majority of antidepressant medications elicit their primary pharmacological effects by increasing monoamine neurotransmission; a concept discovered in the 1950s (Wong and Licinio, 2004; Kessler and Wang, 2008). Impeding efforts towards developing novel therapeutics is the poor understanding of the underlying molecular pathology of depression. Stress, particularly in early life, is a major contributory factor to the onset of mood disorders such as depression (Charney and Manji, 2004). However, the underlying stress-induced molecular disturbances remain poorly understood. In recent years, studies focusing on novel gene-regulatory systems including epigenetic changes have revealed the potential of these mechanisms to produce long-term changes to gene expression and as such, may potentially underlie some of the aforementioned molecular pathological changes (Tsankova et al., 2007).

One such mechanism is via microRNAs (miRNAs) which have emerged as major players in the regulation of eukaryotic gene expression. They are a class of non-coding RNA (~22nts) that regulate the translation of mRNAs through binding to the 3’ untranslated region (UTR) of mRNAs in a sequence specific manner. More recently it has emerged that many miRNAs bind to the 5’ UTR of mRNAs increasing the complexity of their
regulatory capacity (Lee et al., 2009). miRNAs regulate key processes in the central nervous system (CNS) including neuronal development, neurogenesis and synaptic plasticity (O’Connor et al., 2012); disruptions to these processes have been linked to the development of depression (Manji et al., 2003). Furthermore, miRNAs are intimately involved in the adaptive (and potentially maladaptive) response to a psychological stressor (Meerson et al., 2010; Uchida et al., 2010) and moreover, appear to be therapeutically relevant effectors of currently used pharmacotherapies including the mood stabilizers lithium and sodium valproate (Zhou et al., 2009) and the antidepressant fluoxetine (Baudry et al., 2010). By targeting this aberrant miRNA functioning therapeutic benefits may be possible.

In the context of searching for novel more efficacious antidepressants, the N-methyl-D-aspartate receptor antagonist, ketamine, has generated much excitement producing, at sub-anaesthetic doses, a rapid and sustained antidepressant effect in treatment-resistant depression (Zarate et al., 2010). Electroconvulsive shock therapy (ECT) is also a clinically validated method for alleviating symptoms in treatment-resistant depression (Greenhalgh et al., 2005). A large paucity of information regarding the molecular effects of ketamine administration and ECT remains. To date their effects on expression levels of miRNAs remain completely unexplored. To redress this deficit we employed the maternal separation (MS) procedure, a well validated stress model (Clarke et al., 1996; O’Mahony et al., 2008; Gosselin et al., 2010; Uchida et al., 2010) to induce early-life stress in Sprague-Dawley rats. We then investigated if the antidepressant treatments of chronic fluoxetine, repeated ECT and acute ketamine were able to reverse these changes in a convergent manner.

Method

Maternal separation

All experiments were conducted in full accordance with the European Community Council Directive (86/609/EEC) and approved by the ethics committee of University College Cork. MS was performed as described previously (Gosselin et al., 2010; O’Mahony et al., 2010). Briefly, male and female rats (250–300 g; Harlan Laboratories, UK) were mated in the local animal unit. Food and water was available ad libitum and animals were maintained on a 12:12-h dark–light cycle (lights on 07:00 hours) with temperature at 20°C±1°C. Male pups were separated from their dams as a whole litter for a period of 180 min between post-natal days (PD) 2 and 12. Separations were conducted between 09:00 and 12:00 hours in plastic cages placed on top of heater pads (30–33°C) in a room separate from the main holding room. Control rats consisted of non-handled pups, left untouched with their respective dams. Following weaning, rats were left undisturbed (4–5 per cage) except for routine cage cleaning every 2 d and a weekly body weight measurement. The lack of essential maternal care results in an alteration to CNS functioning resulting in a phenotype reminiscent of depression in adulthood (Caldji et al., 2000) and has been consistently replicated in our laboratory (Desbonnet et al., 2010; Gosselin et al., 2010).

Antidepressant treatment

Once the animals reached maturity (8–10 wk) MS rats and non-stressed (NS) controls began one of three antidepressant treatments or received sham-only treatments. Individual groups consisted of rats from multiple litters to account for any confounding litter effects. Treatments consisted of: (1) repeated ECT treatment (85 mA for 0.5 ms/d for 10 d; Chen et al., 2001; O’Connor et al., 2013) to induce a classic grande mal seizure lasting ~15 s; (2) acute ketamine treatment (a single dose of 10 mg/kg in saline; Li et al., 2010; Autry et al., 2011); (3) chronic (21 d) fluoxetine treatment (10 mg/kg/d in saline; Cryan and Lucki, 2000; Cryan et al., 2004). ECT treatment was administered via ear clips (UgoBasille, Italy).

Each animal was handled exactly the same as every other animal in the study except for the specific treatment it was receiving. Ketamine and ECT treated animals received vehicle injections for the preceding 20 d while fluoxetine treated animals were being treated. Furthermore, animals not receiving ECT received sham treatments i.e. they were handled in the same way and had the electrodes placed on their ears throughout the course of treatment. As such, all animals received identical treatments except for the ‘active’ component of the treatment. This allowed antidepressant treatment groups to be compared against each other. Twenty-four hours after the final treatment rats were killed by decapitation and the hippocampus was dissected out and immediately placed in RNALater® (Applied Biosystems). A time-point of 24 h was chosen to ensure any ketamine-mediated effects observed would be independent of any of the potential dissociative effects of ketamine treatment. Ketamine produces a rapid and persistent antidepressant effect which can be seen up to and beyond 24 h both in a
clinical setting (Zarate et al., 2006) and in animal models (Li et al., 2010). Samples were left in RNALater® for 24 h at 4 °C to allow the solution to fully penetrate the tissue. Following this, the RNALater® was removed and the tissue was frozen at −80 °C until further processing.

RNA isolation

Total RNA was isolated from hippocampi using the miRVana, miRNA isolation kit as per manufacturer’s instructions (Ambion, USA). Briefly, tissue was homogenized using a denaturing lysis solution. The lysate was then extracted using phenol-chloroform; ethanol was then added to the solution before being filtered over a glass-fibre filter. The filter was then washed and finally the RNA was eluted using a low-ionic strength solution. Isolated RNA was stored at −80 °C until further processing. RNA concentration was quantified using the ND-1000 spectrophotometer (NanoDrop®, Thermo Scientific, USA) and RNA quality was assessed using the Agilent 2100 Bioanalyzer.

Microarray analysis

miRNA samples were labelled before being run on an Agilent miRNA expression miRNA microarray spotted with probes for 350 miRNAs obtained from miRBase data base release 10. Samples were pooled from three subjects in the same group in triplicate. Comparative analysis was performed using the Rosetta Resolver Agilent/miRNA-Ratio Builder (Patron et al., 2012). This pipeline builds ratio experiments from Agilent/miRNA intensity experiments. Two-sided p values are calculated for the ratios, by calculating the difference of the numerator and denominator intensities and scaling the difference error. Log-ratios and their errors are derived from these differences. The level of significance for the microarray analysis was set at a p value of 0.05 and/or a fold change of 1.3.

Selection of miRNAs for follow-up analysis using quantitative real-time PCR

miRNAs selected for follow-up analysis were selected on the basis of being common targets to two or more antidepressant treatments and/or undergoing an antidepressant induced reversal of altered expression due to stress.

Quantitative real-time PCR

Quantitative real-time (qRT)-PCR was carried out using probes (6 carboxy fluorescein- FAM) designed by Applied Biosystems™ (USA). RNA was first reverse-transcribed to cDNA using hairpin primers specific to each miRNA gene of interest on Applied Biosystem’s GeneAmp PCR System 9700. RT-PCR was carried out on the ABI7300 Real Time PCR machine (Applied Biosystems, UK). Samples were heated to 95 °C for 10 min, and then subjected to 40 cycles of amplification by melting at 95 °C and annealing at 60 °C for 1 min. Experimental samples were run in triplicate with 1.33 μl cDNA per reaction. To check for amplicon contamination, each run contained template free controls in triplicate for each probe used. Cycle threshold (Ct) values were recorded. Data were normalized using small nucleolar-RNAs and transformed using the 2−ΔΔCt method (Simen et al., 2006). Reverse transcription took place on the GeneAmp PCR System 9700 and the PCR was carried out using the ABI7300 Real Time PCR machine.

Bioinformatic analysis

miRNAs selected for follow-up analysis were also put through mRNA target prediction using the TargetScan website (www.targetscan.org; Lewis et al., 2003). This program generates a list of mRNA targets predicted to undergo regulation by the miRNA in question. This predictive algorithm is based on sequence information from both sets of RNA molecules as well as sequence conservation (Lewis et al., 2003). Notable targets were selected on an ad hoc basis based on potential interest.

Statistics

Data was analysed using a two-way analysis of variance (ANOVA) and Fisher’s least significant difference post hoc tests using the statistical software package SPSS 16.0 (SPSS Inc., USA). A p value of 0.05 was selected as the threshold of statistical significance.

Results

Microarray

Microarray analysis revealed early-life stress and antidepressant treatment affected the expression of multiple hippocampal miRNAs (Fig. 1a). In NS animals chronic fluoxetine treatment, repeated ECT and acute ketamine treatment significantly altered the expression levels of two, 10 and 14 miRNAs respectively (Fig. 1b, d). Interestingly, one of these increases was common to all three antidepressants, namely miR-598-5p. MS significantly altered levels of 24 hippocampal miRNAs. Chronic fluoxetine treatment
Fig. 1. Altered hippocampal micro RNA (miRNA) expression following maternal separation (MS) stress and antidepressant treatment. (a) Heat map representing alterations to hippocampal miRNA expression. Green represents a reduction in expression and red represents increased expression when compared to non-separated (NS) controls (Con). (b) Venn diagram illustrating common miRNA targets of antidepressant treatments in NS animals: fluoxetine (Flu) altered two miRNAs;
significantly decreased four miRNAs following MS with three of these representing a partial normalization of stress-induced changes. Repeated ECT altered 86 miRNAs (48 decreased, 38 increased); 16 of these were a normalization of stress-induced changes. Acute ketamine treatment altered 55 miRNAs (32 decreased, 23 increased); 11 of these changes were a reversal to stress-induced changes (Fig. 1c, e, f). ECT and ketamine treatment share 43 common miRNA targets following MS with seven being a reversal to stress-induced changes. All three antidepressants share one common miRNA target in MS animals, miR-451, which is a reversal to a stress-induced change.

Selection of miRNA targets for follow-up analysis

miR-598-5p was selected on the basis of having an altered expression due to all three antidepressants with a similar magnitude. miR-451 displayed an altered expression due to MS which was reversed by all three antidepressant treatments.

qRT-PCR

miR-598-5p

A two-way ANOVA revealed a significant antidepressant treatment effect on miR-598-5p expression and no effect of MS nor any interaction effect (MS $F_{1, 66} = 2.56$ $p > 0.05$; antidepressant $F_{3, 66} = 5.20$ $p < 0.005$; interaction $F_{3, 66} = 0.51$ $p > 0.05$; Fig. 2a). Post hoc analysis revealed fluoxetine and ECT significantly increased expression levels of miR-598-5p compared to NS controls (fluoxetine $p < 0.005$; ECT $p < 0.05$). A ketamine-mediated effect on miR-598-5p was not found using qRT-PCR which is at odds with the microarray data. No other treatment group was found to be significantly different from controls ($p > 0.05$; Fig. 2a).

miR-451

A two-way ANOVA revealed a significant antidepressant treatment effect on miR-451 expression; no effect of MS but there was an MS × antidepressant interaction effect (MS $F_{1, 67} = 1.32$ $p > 0.05$; antidepressant $F_{3, 67} = 4.01$ $p < 0.05$; interaction $F_{3, 67} = 4.64$ $p < 0.001$; Fig. 2b). However post hoc analysis revealed levels of miR-451 to be significantly reduced in MS animals compared to NS animals ($p < 0.001$). Fluoxetine treatment reversed this stress-induced change to miR-451 levels ($p < 0.001$). Ketamine also significantly reduced levels of miR-451 in NS animals ($p < 0.005$). Ketamine and ECT treatment were not found to reverse the stress-induced perturbation to miR-451 expression.
Discussion

In the present study we investigated the effects of early-life stress-induced changes to hippocampal miRNAs in Sprague-Dawley rats. Moreover, we also probed the influence three different antidepressant treatments, chronic fluoxetine, repeated ECT and acute ketamine, would have on miRNAs in the hippocampus of both control animals and in those which had undergone MS. This strategy possesses the potential to not only uncover stress-induced changes to miRNAs which could be important components of stress-induced pathology, but also antidepressant-induced therapeutic effects which may only become apparent following an environmentally induced insult such as early-life stress. This, to our knowledge, is the first time the capacity of antidepressant treatments to reverse stress-induced changes to miRNA levels has been investigated.

Employing the use of a microarray we found MS significantly altered the expression levels of 24 different miRNAs in the hippocampus (Fig. 1). Interestingly, in control animals, antidepressant treatments produced relatively modest effects on miRNA levels when compared to MS animals; all three antidepressant treatments altered the levels of substantially more miRNAs following MS than in naive animals with many reversing MS-induced changes (three for fluoxetine, 16 for ECT and 11 for ketamine) to miRNA expression. Thus, many antidepressant treatment-induced changes to miRNA levels only become apparent following a pathological insult such as stress and perhaps may represent a means by which these treatments allow an organism to overcome stress-induced pathological changes. However, it is worth noting that miR-598-5p represents a common hippocampal target of all three treatments in NS animals and as such may have an important role in antidepressant action. A further point of interest (Fig. 1) is antidepressant treatment in NS adult animals produced a similar pattern, albeit smaller in magnitude, of hippocampal miRNA perturbation to that of early-life stress. As such, one can speculate that antidepressant administration, independent of an underlying pathology, may in fact produce stress-like changes, at least as far as miRNAs are concerned. This is an interesting avenue worthy of further research.

The most striking feature of this data is the fact that ECT and ketamine shared 43 miRNA targets following MS. It is tempting to speculate that the antidepressant effect of these strategies is mediated via common pathways converging on the same miRNAs. Conceptually thus, by directly targeting relevant miRNAs a higher antidepressant efficacy may be possible; however, it is worth noting there are many challenges involved in altering miRNA levels in the CNS for therapeutic benefit (O’Connor et al., 2012).

miRNAs of most interest (miR-451 and miR-598-5p) have also been validated by qRT-PCR (Fig. 2). Early-life stress-induced changes to miR-451 levels were attenuated by antidepressant treatment. Interestingly, bioinformatic analysis (www.targetscan.org) demonstrated that this miRNA is predicted to regulate a number of important genes including those linked to the CREB pathway (CREB5) as well as GABAergic (GABA_A receptor associated protein) and cholinergic neurotransmission (muscarinic cholinergic receptor 5). Ketamine and ECT treatment possessed the ability to reverse several stress-induced changes to miRNAs which fluoxetine did not (miR-217, miR-203, miR-211, miR-152, miR-1, miR-204). These changes could contribute to therapeutic capabilities of these two treatments. Of course ECT and ketamine are far from being selective treatments and as such many changes mediated by these two strategies will be unrelated to their antidepressant effect. Thus, more research is required to further inform whether these miRNAs are related to depressive pathology and/or the antidepressant effect of these treatments.

Overall these studies can be viewed as preliminary yet exciting, demonstrating the potential involvement of hippocampal miRNAs in early-life stress-induced pathology and in antidepressant treatments. More research will need to be conducted in order to more fully inform on what exactly the role of these small RNA molecules are in depression-related pathology and whether these miRNAs are worth pursuing as therapeutic targets.

Acknowledgements

J.F.C. and T.G.D. were supported in part by Science Foundation Ireland in the form of a Centre Grant (grant nos. 02/CE/B124 and 07/CE/B1368). The Alimentary Pharmabiotic Centre is a research centre funded by Science Foundation Ireland (SFI), through the Irish Government’s National Development Plan.

Statement of Interest

None.
References


Chen AC, Shin KH, Duman RS, Sanacora G (2001) ECS-Induced mossy fiber sprouting and BDNF expression are attenuated by ketamine pretreatment. J ECT 17:27–32.


