Biological Psychiatry: GOS

Archival Report

MicroRNA Regulates Early-Life Stress–Induced Depressive Behavior via Serotonin Signaling in a Sex-Dependent Manner in the Prefrontal Cortex of Rats

Lauren Allen McKibben and Yogesh Dwivedi

ABSTRACT

BACKGROUND: The underlying neurobiology of early-life stress (ELS)-induced major depressive disorder is not clearly understood.

METHODS: In this study, we used maternal separation (MS) as a rodent model of ELS and tested whether microRNAs (miRNAs) target serotonin genes to regulate ELS-induced depression-like behavior and whether this effect is sex dependent. We also examined whether environmental enrichment prevents susceptibility to depression- and anxiety-like behavior following MS and whether enrichment effects are mediated through serotonin genes and their corresponding miRNAs.

RESULTS: MS decreased sucrose preference, which was reversed by enrichment. Males also exhibited greater changes in forced swim climbing and escape latency tests only following enrichment. *Slc6a4* and *Htr1a* were upregulated in the frontal cortex following MS. In male MS rats, enrichment slightly reversed *Htr1a* expression to levels similar to control rats. miR-200a-3p and miR-322-5p, which target *SLC6A4*, were decreased by MS, but not significantly. An *HTR1A*-targeting miRNA, miR-320-5p, was also downregulated by MS and showed slight reversal by enrichment in male animals. miR-320-5p targeting of *Htr1a* was validated in vitro using SHSY neuroblastoma cell lines.

CONCLUSIONS: Altogether, this study implicates miRNA interaction with the serotonin pathway in ELS-induced susceptibility to depression-related reward deficits. Furthermore, because of its recovery by enrichment in males, miR-320 may represent a viable sex-specific target for reward-related deficits in major depressive disorder.

https://doi.org/10.1016/j.bpsgos.2021.05.009

Various forms of early-life stress (ELS), including neglect, have been shown to increase susceptibility to major depressive disorder (MDD) (1), which also carries an increased risk of suicide, among other warning signs (2). MDD is the most prevalent psychiatric illness and has a particularly heavy financial and societal burden in the United States (3), yet a large percentage of patients do not respond to pharmacologic antidepressant treatment (4). MDD pharmacotherapies [such as duloxetine (5)] have classically targeted the serotonin system and been proven moderately successful at managing symptoms. However, individuals who report at least one previous occurrence of ELS are less responsive to antidepressant drugs, especially those that act on the serotonin system (i.e., selective serotonin reuptake inhibitors, serotonin antagonist and reuptake inhibitors, serotonin and norepinephrine reuptake inhibitors) and may be more responsive to psychotherapy (6). Furthermore, while there is little evidence of gender differences in the experience of ELS (7), women are more likely to experience MDD (8). A clearer understanding of the serotonergic neurobiology underlying depression susceptibility, including the biological basis of effective behavioral or environmental treatments, will be crucial to improving outcomes for those with MDD.

Both MDD and ELS are associated with disruptions in serotonin signaling [reviewed in (9)]. For example, individuals who possessed the *s/s* genotype for 5-HTTLPR and were exposed to ELS had the highest probability of developing MDD, followed by the *s/l* genotype (10). Early maternal separation (MS) increased serotonin receptor 1A (*Htr1a*) expression in the amygdala and decreased its expression in the dorsal raphe nucleus; the serotonin transporter (*Slc6a4*) was also decreased in the dorsal raphe (11). Prolonged MS also decreased serotonin receptor 2A (*Htr2a*) expression in the prefrontal cortex (PFC) during the MS period, as early as postnatal day 7 (12). The PFC is responsible for executive functions and plays a role in emotion regulation by appraising signals from other regions, detecting threats, and moderating the reactivity of limbic regions such as the amygdala (13). It comprises mostly postsynaptic serotonergic neurons projected by the dorsal raphe (14) as opposed to presynaptic neurons. Modern

ISSN: 2667-1743

antidepressants such as agomelatine target serotonin receptor 2C (HTR2C) and have been shown to improve neuroplasticity in the PFC (15). There is also a burgeoning field of research around the treatment and prevention of psychiatric disorders using nonpharmaceutical interventions. In a study comparing environmentally enriched animals to those in standard housing, the levels of serotonin in the PFC were significantly increased (16).

MicroRNAs (miRNAs), a subclass of noncoding RNAs, are estimated to regulate 20%–90% of genes in the genome (17,18). miRNAs are short sequences of nucleotides synthesized in the nucleus and then processed into mature miRNAs in the cytoplasm (19). Typically, miRNAs bind to messenger RNA and repress its ability to undergo translation into protein. While there are a few reports of miRNA expression changes in animal models of ELS (20–23), there is limited research on a direct link between changes in serotonin signaling and miRNAs in response to ELS. Also, several studies have demonstrated positive behavioral outcomes and changes in serotonin signaling following enrichment (16,24); there are no studies implicating miRNAs in serotonin gene regulation in the context of ELS.

In this study, we used MS as a rodent model of ELS and tested whether miRNAs target serotonin genes to regulate ELS-induced depression-like behavior and whether this effect is sex dependent. We also examined whether environmental enrichment prevents susceptibility to depression-like behavior following MS and whether enrichment effects are mediated through serotonin genes and their corresponding miRNAs. We also validated gene targets of significantly altered miRNA in vitro. Based on previous reports of serotonin signaling aberrations in MDD and preclinical stress models, we hypothesized that genes in the serotonin signaling pathway would be altered by ELS and potentially recovered by enrichment. We also hypothesized that miRNAs targeting these genes may be partially responsible for gene expression changes. These effects will be sex specific.

METHODS AND MATERIALS

Animals

Pregnant female Holtzman rats were purchased from Envigo and housed in standard conditions according to the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. A timeline of the experimental design is shown in Figure 1A. When the pups were born, the litter was assigned to the control or MS groups. Pups in the control litters were handled 5 minutes daily from postnatal day (PND) 1–14. Daily, MS animals were separated from the dam and individually housed for 180 minutes from PND 1–14. At PND 21, MS animals were assigned to enrichment (MS + environmental enrichment [Enr]) or no-Enr groups and then weaned

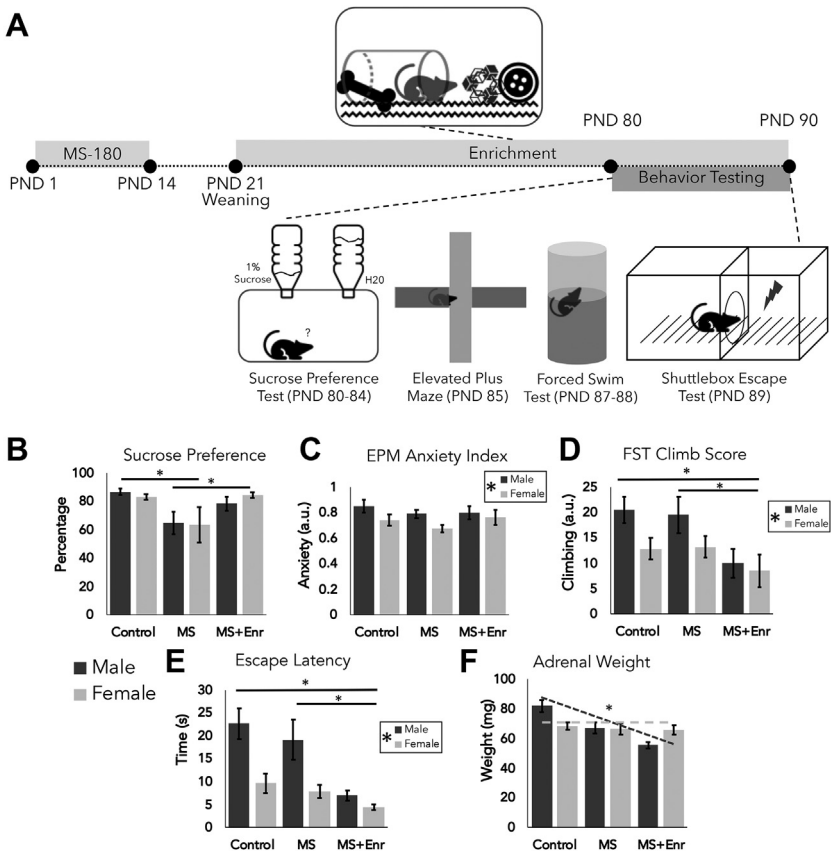


Figure 1. Behavior changes associated with MS and Enr. Mean behavior resulting from MS and MS+Enr are shown as bar diagrams. Data are mean ± SEM. Dark gray bars indicate male animals and light gray bars indicate female animals. **(A)** A schematic diagram shows the timeline of behavioral experiments. **(B)** Sucrose preference was significantly reduced by MS and recovered by enrichment. **(C)** Females exhibited decreased anxiety-like behavior in the EPM, and **(D)** FST climb score was reduced by enrichment compared with control and MS animals. Females climbed less than males (as shown by * in the key). **(E)** Enrichment also reduced escape latency compared with control and MS animals, and females showed reduced escape latency compared with males. **(F)** MS and enrichment significantly reduced adrenal weight in males but had no effect on female animals; dotted lines indicate a significant interaction effect. Significance was determined by two-way analysis of variance and follow-up independent samples *t* tests ($n = 6$ per group, $N = 36$); *$p < .05$. a.u., arbitrary units; Enr, environmental enrichment; EPM, elevated plus maze; FST, forced swim test; MS, maternal separation; PND, postnatal day.

and group housed. MS+Enr animals received 4–5 additional pieces of enrichment each week beginning on PND 21. Environmental enrichment included chewable wooden sticks, cotton or paper shreddable objects, colorful plastic toys, polyvinyl chloride tubing, and metal platforms for climbing; objects were sanitized weekly and randomly replaced to maintain novelty. Each group consisted of 6 males and 6 females (N = 36), each of which were tested for depression- and anxiety-related behaviors as follows: sucrose preference test on PND 80–84, elevated plus maze (EPM) on PND 85, forced swim test (FST) on PND 87–88, and shuttle box escape test on PND 89. At 24 hours following behavior testing, the animals were sacrificed and tissues collected. Behavior testing and tissue collection were initiated between 8:00 AM and 11:00 AM.

Behavior Testing

Sucrose Preference Test. Rats were tested for reward-related deficits (i.e., anhedonia) using the sucrose preference test, adapted from Dwivedi *et al.* (25). Initially, the animals were acclimated to two 500-mL bottles of 1% sucrose water for 24 hours, followed by a bottle each of sucrose water and tap water. Food and water were then removed for 24 hours. Finally, each animal was individually housed and given food and tightly sealed 500-mL bottles of tap water and 1% sucrose water. After 24 hours, the volume of sucrose and tap water consumed was measured. Sucrose preference was calculated as the percent of sucrose water consumed out of the total liquid consumed (i.e., mL sucrose water/[mL sucrose + mL tap water] × 100).

Elevated Plus Maze. After 30-minute acclimation to the testing room, each animal was individually placed in the center of a 100-cm EPM. The animal's movement was tracked for 5 minutes (EthoVision XT 11.5; Noldus). A single anxiety-related value (called Anxiety Index) was calculated based on the number of open arm entries, time spent in the open arm, total arm entries, and total test time (26).

Forced Swim Test. At 24 hours before testing, each animal was acclimated in a Plexiglas (Rohm and Haas) cylinder (~30 cm diameter × ~40 cm tall) filled ~20 cm deep with room temperature for 15 minutes. At testing, the subjects swam under the same conditions as the previous day for 6 minutes, which was video recorded and analyzed by a rater blinded to conditions using Kinoscope software (27); climb, swim, and immobility scores were extracted.

Shuttle Box Escape Testing. At 24 hours after FST, escape testing was conducted as described earlier (28) in a shuttle box (70 × 20 × 20 cm) (Med Associates) containing an electrified grid floor. Footshock was delivered through the grid floor by a shock generator (0.6 mA on a variable interval schedule) (model no. ENV-413; Med Associates). The testing montage began with 5 trials during which a single crossing terminated the shocks, followed by 25 trials in which crossing and then returning to the initial side was required to terminate the shocks; shocks terminated automatically after 30 seconds. Escape latency was recorded.

Tissue Collection

At 24 hours after escape testing, female animals were tested for estrous timing via vaginal lavage and then returned to their home cage for at least 1 hour. Then, each animal was anesthetized using isoflurane, and blood was collected in EDTA tubes via thoracotomy and cardiac puncture. Whole blood was centrifuged (1400 rpm, 4 °C for 15 min) to collect plasma. Adrenal glands were collected and weighed. The PFC was dissected and frozen on liquid nitrogen for storage.

Plasma-Based Corticosterone Enzyme-Linked Immunosorbent Assay and Rat Brain RNA Isolation, Complementary DNA Synthesis, and Relative Gene Expression

Corticosterone was measured in rat plasma collected at the time of sacrifice using an enzyme-linked immunosorbent assay (Enzo Life Sciences).

Total RNA was isolated from frozen PFC tissue using Trizol (Invitrogen) as previously described (29). After phase separation with chloroform, the aqueous phase was collected, and RNA was precipitated using isopropanol and 20 μg of glycogen. Following overnight incubation, the RNA pellet was collected by centrifugation, washed with 70% ethanol, and dried before resuspension in water. The RNA purity ratios (260/280 nm and 260/230 nm) were assessed by NanoDrop (Thermo Scientific, Waltham, MA).

For quantitative polymerase chain reaction (qPCR)–based gene expression testing, complementary DNA (cDNA) was synthesized following an oligonucleotide dT–based priming method using 1 μg of total RNA. For miRNA-specific expression testing, cDNA was synthesized by poly A tailing and oligonucleotide dT adapter primer as previously described (29).

qPCR-Based Gene Expression

Relative gene expression was tested by qPCR using BrightGreen chemistry. We selected serotonin signaling genes, including *Slc6a4*, *Htr1a*, *Htr1b*, *Htr2a*, *Htr2b*, *Htr2c*, *Tph1*, *Tph2*, *Maoa*, and *Maob*. Gene primer sequences are provided in Table 1. Gene expression values were normalized to the geometric mean of *GAPDH*, *β-actin*, and *18S* ribosomal RNA. Relative expression was determined following Livak's ΔΔCT calculation.

miRNA Target Prediction

miRGate (30) and miRWalk (31) were used to identify targeting miRNAs. miRGate included results based on other databases, such as Targetscan, miRanda, and Rnahybrid. The predicted target list was narrowed to include miRNAs predicted by more than 1 database (e.g., miRWalk, Targetscan, miRanda) or those predicted to target both genes of interest. Top candidate miRNAs were selected for expression testing based on a literature search focused on stress, depression, or psychiatric disorders. Although miR-135, miR-200a-3p, and miR-320-3p were not detected in target prediction, they were included in expression testing based on literature search findings. To assess the potential for common regulatory mechanisms due to proximity (by long noncoding RNA, for example), miRNA and their gene targets were visualized on the rat genome using a phenogram (http://visualization.ritchielab.org/phenograms/

Table 1. Primer Sequences for Real-Time Polymerase Chain Reaction Experiments

Gene/miRNA	Primer Sequence
rno_5HTR1A_F	TTTCTCATCTCCATCCCGCC
rno_5HTR1A_R	AGCGGGATATAGAAAGCGCC
rno_5HTR2A_F	GGTTTCCTTGTCATGCCTGTG
rno_5HTR2A_R	TGGATGCCGTAGAAAAGAGCA
rno_GAPDH_F	CACTGAGCATCTCCCTCACAA
rno_GAPDH_R	TGGTATTCGAGAGAAGGGAGG
rno_5HTR2B_F	GACGCAAGCAAACCAAGGAA
rno_5HTR2B_R	GGAACTACTATACCCGTGCGT
rno_Slc6a4_F	GCATACGTGGTGACTCTGCT
rno_Slc6a4_R	AAGCCCAGCATCTCCTTCAC
rno_HTR1B_F	CGCCTTTGTAATCGCTACGG
rno_HTR1B_R	GATGGACACGAGCAGGTCAG
rno_HTR2C_F	CCCCTCTAATGTGGTCAGCG
rno_HTR2C_R	TCACAGGAAAATCACGGGGG
rno_TPH1_F	TCATGCTTCTTTTCAAGCCCAC
rno_TPH1_R	TTACACTCTCTCGACCGGCT
rno_TPH2_F	TAGAGGATGTGCCGTGGTTC
rno_TPH2_R	CCTTGAATCCTGGGTGGTCG
rno_MAOB_F	AGTGCCATCCCACCTGTTTT
rno_MAOB_R	AACCCAAAGGCACACGAGTA
rno_MAOA_F	AGAGATCCCCGTTGATGCAC
rno_MAOA_R	TACGCAAATTCCCGAGCAGT
rno_mir-320-5p	GCCTTCTCTTCCCGGTTCTTCC
rno_mir-320-3p	AAAAGCTGGGTTGAGAGGGCGA
rno_mir-181a-2-5p	AACATTCAACGCTGTCGGTGAGT
rno_mir-181c	ATTCAACCTGTCGGTGAGT
rno_mir-200a-3p	TAACACTGTCTGGTAACGATGT
rno_mir-322-5p	AGCAGCAATTCATGTTTTGGA
rno_mir-322-3p	CATGAAGCGCTGCAACAA
rno_mir-185-3p	TTTCCTCTGGTCCTTCTCTAAAA
rno_mir-224-5p	AAGTCACTAGTGGTTCCGTTT
rno_mir-3572	CTTGCCCTTTTTTCCCCAG
rno_mir-16-5p	GCAGCACGTAAATATTGGCG
rno_mir-135	TATGGCTTTTCATTCCTATGTGAAAAAA
U6_F	CTCGCTTCGGCAGCACA
U6_R	AACGCTTCACGAATTTGCGT
hsa_GAPDH_F	CCACATCGCTGAGACACCAT
hsa_GAPDH_R	AGTTAAAAGCAGCCCTGGTGA
hsa_SLC6A4_F	CAGTTGGAATGTCAGGTGGT
hsa_SLC6A4_R	ACTTGCATTGGTGGTAGAGCA
hsa_HTR1A_F	TGGCTCCCCCAGTAAAACCT
hsa_HTR1A_R	AAATGCAGCGAGTGTTGGGA
hsa_miR-200a-3p	AACACTGTCTGGTAACGATGT
hsa_miR-322-5p	CAGCAGCAATTCATGTTTTGAAAA
hsa_miR-320-3p	GGTTGAGAGGGCGAAAAAAA
hsa_miR-320-5p	TTCTCTTCCCGGTTCTTCC
Universal Reverse	GCGAGCACAGAATTAATACGAC

Forward and reverse primer sequences are listed for genes and microRNAs tested using real-time quantitative polymerase chain reaction.

F, forward; hsa, *Homo sapiens*; R, reverse; rno, *Rattus norvegicus*.

plot). miRNA expression was tested using qPCR (primers are listed in Table 1) and normalized to U6.

SHSY-5Y Cell Transfection

To validate miRNA regulation of target genes, SHSY-5Y neuroblastoma cells were transfected with mimic and antisense fluoroarabinonucleic acid antisense oligonucleotides for miR-200a-3p, miR-320-3p, miR-320-5p, and miR-322-5p (AUM BioTech) in triplicate. A third group with no oligonucleotide was used as a control. After 72-hour incubation, total RNA was isolated from each lysate following the Trizol method. Gene- and miRNA-specific cDNA were synthesized, and qPCR was used to test for miRNA and gene expression changes using BrightGreen chemistry (primers listed in Table 1). Gene expression was normalized to *GAPDH* and miRNA to *U6*.

Statistical Analysis

A two-way analysis of variance (ANOVA) between sex and group was used to determine behavior and gene/miRNA expression differences in vivo. Tukey's post hoc tests were used to parse significant group differences.

RESULTS

Animal Behavior

Two-way ANOVA showed a significant effect of group on sucrose preference ($F_{2,30} = 5.786$, $p < .01$), and Tukey's post hoc tests revealed that sucrose preference was significantly decreased in MS animals compared with control animals ($p < .01$) or animals with enrichment ($p < .05$) (Figure 1B). There were no significant differences in latency to enter the open arm of the EPM or the number of entries to the open arms ($p > .05$) (Supplement). However, there was a significant effect of sex on EPM anxiety index ($F_{1,30} = 5.244$, $p < .05$), which incorporates several EPM values into a single metric; females showed lower scores (Figure 1C) than males. There were significant main effects of sex ($F_{1,30} = 5.192$, $p < .05$) and group ($F_{2,30} = 4.551$, $p < .05$) on climbing in the FST (Figure 1D). Male animals showed greater climb scores than females, and a Tukey's post hoc test revealed that enrichment was associated with significantly reduced climbing compared with control and MS animals ($p < .05$). There were no significant main effects of either sex or group in FST swim or immobility scores ($p > .05$) (Supplement). There were significant main effects of both sex ($F_{1,30} = 18.27$, $p < .001$) and MS ($F_{2,30} = 8.954$, $p < .005$) on escape latency in the shuttle box escape test (Figure 1E). Male animals exhibited greater latency to escape than females. Post hoc tests revealed that enrichment significantly reduced escape latency compared with both control ($p < .005$) and MS ($p < .05$) animals. There were no significant differences in animal behavior based on estrous timing ($p > .05$); therefore, we did not use this as a variable of interest for subsequent assays.

Adrenal Weight and Corticosterone Level

There was a significant interaction between group (control, MS, and MS+Enr) and sex on adrenal weight ($F_{2,30} = 6.045$,

$p < .01$), with adrenal weight decreasing after MS only in male animals (Figure 1F). There was no significant effect of MS on corticosterone levels ($p > .05$; not shown).

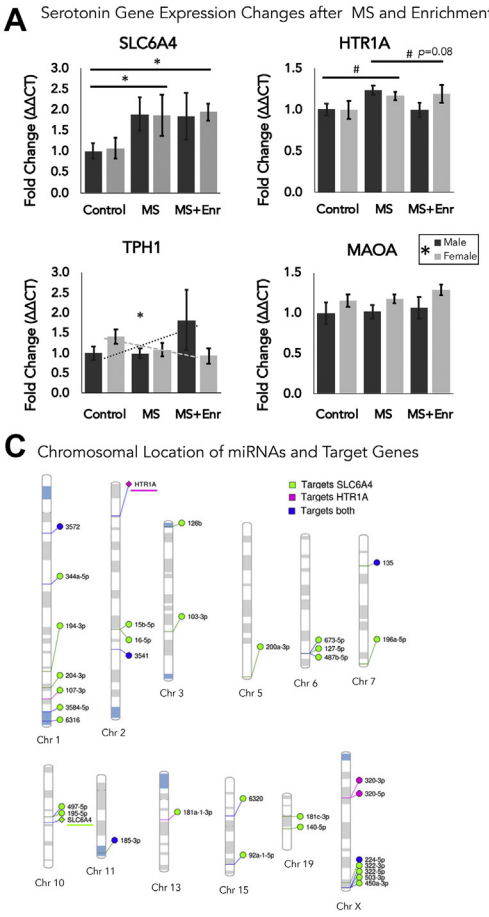
Expression Analysis of Serotonin Genes

qPCR was used to test the expression of genes associated with serotonin signaling in the PFC (Figure 2A). Two-way ANOVA revealed a main effect of group on *Slc6a4* expression ($F_{2,30} = 5.82$, $p < .01$). MS significantly increased *Slc6a4* expression, which was not recovered by enrichment (Tukey's post hoc test, $p < .05$). *Htr1a* was also increased by MS but it did not reach significance ($F_{2,30} = 2.76$, $p = .08$). There was a significant interaction between sex and group on *Tph1* expression ($F_{2,30} = 3.98$, $p < .05$). In male rats, *Tph1* was increased only in the MS+Enr group compared with the control group, while in females, *Tph1* was decreased in both the MS and MS+Enr groups compared with the control group. MS and enrichment did not significantly alter *Maoa* expression, yet females exhibited significantly increased expression compared with males regardless of group ($F_{1,30} = 4.48$, $p < .05$). There were no main or interaction effects on expression of *Htr1b*, *Htr2a*, *Htr2b*, *Htr2c*, *Tph2*, or *Maob* (data not shown).

Predicted Targeting miRNAs and Their Expression Levels

Because *Slc6a4* expression was altered by MS, its targeting miRNAs were predicted using mirWalk and miRGate; we also predicted targeting miRNAs for *Htr1a* because its expression was increased regardless of sex, although nonsignificantly. Figure 2B lists all targeting miRNAs; bolded miRNAs were tested for relative expression changes using qPCR. A total of 26 miRNAs were found to target *Slc6a4*, while 1 miRNA was predicted to target *Htr1a*; these two genes shared five targeting miRNAs. A phenogram of the targeting miRNAs' chromosomal localization (Figure 2C) revealed that seven *Slc6a4*-targeting miRNAs grouped onto chromosome 1, while another five grouped tightly on the X chromosome. *Htr1a*, located on chromosome 2, was not closely localized near any of its targeting miRNAs. *Slc6a4* localized closely with miR-195-5p and miR-497-5p on chromosome 10.

Expression of miRNAs miR-16, -181a, -181c, -200a, -322-3p, -322-5p, -135, -185, -224, -3572, -320-3p, and -320-5p were tested in MS and MS+Enr rat PFCs (Figure 3A–L). No significant changes were detected in miRNAs miR-16 or -322-3p. Using two-way ANOVA, miR-181a was shown to be significantly altered by group ($F_{2,30} = 7.76$, $p < .005$), with



B

Predicted miRNAs Targeting SLC6A4 and HTR1A Genes			
Input Gene	Transcript ID	Targeting MiRNA	Prediction Databases
HTR1A	ENSRNOT00000013618	***rno-mir-135**	*Issler *et al.* (40)
HTR1A	ENSRNOT00000013618	**rno-mir-185-3p**	Miranda
HTR1A	ENSRNOT00000013618	**rno-mir-224-5p**	Miranda
HTR1A	ENSRNOT00000013618	***rno-mir-320-3p**	*Zurawek *et al.* (46)
HTR1A	ENSRNOT00000013618	**rno-mir-320-5p**	Miranda, Rnahybrid
HTR1A	ENSRNOT00000013618	rno-mir-3541	Rnahybrid
HTR1A	ENSRNOT00000013618	**rno-mir-3572**	Miranda
SLC6A4	ENSRNOT00000004717	rno-mir-103-3p	mirWalk, Miranda, Targetscan
SLC6A4	ENSRNOT00000004717	rno-mir-107-3p	mirWalk, Miranda, Targetscan
SLC6A4	ENSRNOT00000004717	rno-mir-126b	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	rno-mir-127-5p	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	***rno-mir-135**	*Issler et al. (40)
SLC6A4	ENSRNOT00000004717	rno-mir-140-5p	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	rno-mir-15b-5p	Miranda, Targetscan
SLC6A4	ENSRNOT00000004717	**rno-mir-16-5p**	Miranda, Targetscan
SLC6A4	ENSRNOT00000004717	**rno-mir-181a-1-3p**	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	**rno-mir-181c-3p**	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	**rno-mir-185-3p**	Targetscan
SLC6A4	ENSRNOT00000004717	rno-mir-194-3p	mirWalk, Rnahybrid
SLC6A4	ENSRNOT00000004717	rno-mir-195-5p	Miranda, Targetscan
SLC6A4	ENSRNOT00000004717	rno-mir-196a-5p	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	***rno-mir-200a-3p**	*Hou *et al.* (45)
SLC6A4	ENSRNOT00000004717	rno-mir-204-3p	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	**rno-mir-224-5p**	Targetscan
SLC6A4	ENSRNOT00000004717	**rno-mir-322-3p**	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	**rno-mir-322-5p**	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	rno-mir-344a-5p	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	rno-miR-3541	mirWalk
SLC6A4	ENSRNOT00000004717	rno-mir-3572	Miranda
SLC6A4	ENSRNOT00000004717	rno-mir-3584-5p	mirWalk, Rnahybrid
SLC6A4	ENSRNOT00000004717	rno-mir-450a-3p	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	rno-mir-487b-5p	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	rno-mir-497-5p	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	rno-mir-503-3p	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	rno-mir-6316	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	rno-mir-6320	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	rno-mir-673-5p	mirWalk, Miranda
SLC6A4	ENSRNOT00000004717	rno-mir-92a-1-5p	Miranda, Rnahybrid

Figure 2. Serotonin pathway genes altered by MS and Enr in the prefrontal cortex and miRNA target prediction. **(A)** Relative rat gene expression of *Slc6a4*, *Htr1a*, *Tph2*, and *Maoa* is shown as mean ± SEM. MS and MS+Enr expression are shown relative to control. Data were normalized to the geometric mean of *GAPDH*, *β-Actin,* and *18S* ribosomal RNA. Dotted lines on *Tph1* indicate a significant interaction. miRWalk was used to predict miRNAs targeting *Slc6a4* and *Htr1a*. **(B)** Targeting miRNAs are shown as a table with quantitative polymerase chain reaction–tested miRNAs bolded. Starred miRNAs were selected based on literature search but did not appear in miRWalk prediction. **(C)** Chromosomal localization of genes and miRNAs were extracted from Rat Genome Database and miRbase, respectively. Localization was visualized using PhenoGram. miRNAs in green target *Slc6a4*, magenta target *Htr1a*, and blue target both genes. Significance was determined using two-way analysis of variance ($n = 6$ per group, $N = 36$); $^{*}p < .05$; $^{\#}p < .10$. Enr, environmental enrichment; miRNA, microRNA; MS, maternal separation.

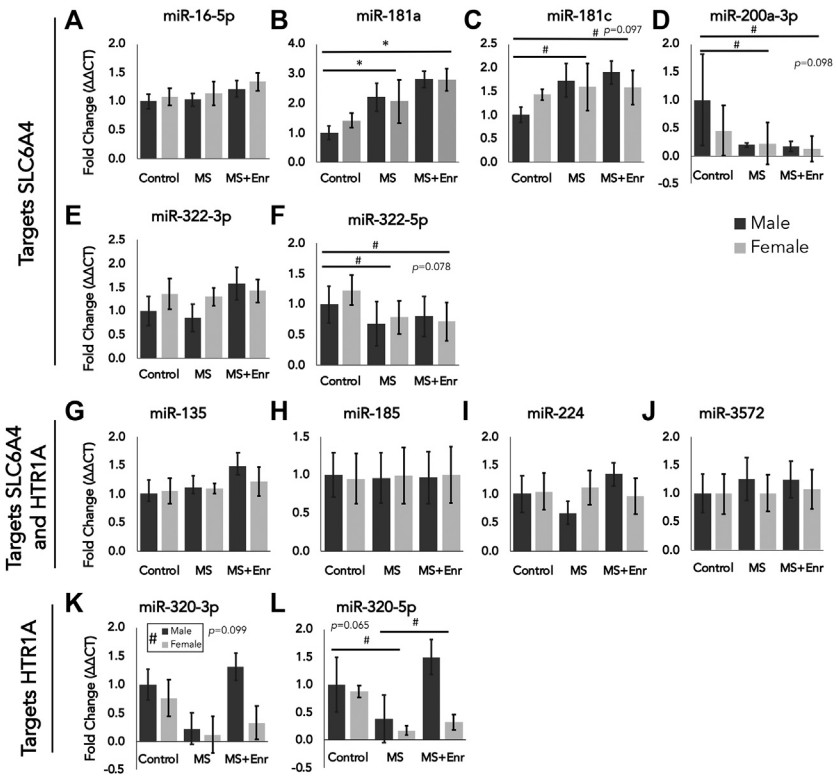


Figure 3. *Slc6a4*- and *Htr1a*-targeting miRNAs altered by MS and Enr. Relative mature rat miRNA expression targeting *Slc6a4*: **(A)** miR-16-5p, **(B)** miR-181a, **(C)** miR-181c, **(D)** miR-200a-3p, **(E)** miR-322-3p, and **(F)** miR-322-5p. miRNAs targeting both *Slc6a4* and *Htr1a*: **(G)** miR-135, **(H)** miR-185, **(I)** miR-224, and **(J)** miR-3572. miRNAs targeting *Htr1a*: **(K)** miR-320-3p and **(L)** miR-320-5p. Data are mean ± SEM; MS and MS+Enr expression are shown relative to control; data were normalized to U6 expression; significance was determined using two-way analysis of variance (n = 6 per group, N = 36); $^{*}p < .05$; $^{\#}p < .10$. Enr, environmental enrichment; miRNA, microRNA; MS, maternal separation.

expression in the MS and MS+Enr groups increased compared with the control group ($p < .05$). miR-181c was upregulated in MS and MS+Enr animals, but it did not reach significance ($F_{2,30} = 2.53$, $p = .097$). miR-200a-3p expression was reduced but not significant in MS and MS+Enr male animals compared with control animals ($F_{2,30} = -2.51$, $p = .098$). Similarly, miR-322-5p expression was reduced by MS and MS+Enr compared with control animals but did not reach the threshold for significance ($F_{2,30} = -2.86$, $p = .078$).

There were no significant group differences in expression of miR-135, -185, -224, or -3572 (Figure 3G–J). miR-320-3p expression was decreased in females compared with males but was not significant ($F_{1,30} = 2.90$, $p = .099$). Similarly, miR-320-5p expression was decreased in MS animals compared with control animals, but it did not reach significance ($F_{2,30} = 3.00$, $p = .065$).

In Vitro Validation of miRNAs and Target Genes

miRNAs were selected for transfection if their pattern of regulation was opposite of their target gene (*Slc6a4* or *Htr1a*), which suggested that these miRNAs might be responsible for gene expression changes after MS. Following transfection (schematic diagram in Figure 4A) with miR-200a-3p mimic, miR-200a-3p expression was increased 10.17-fold compared with nontransfected control ($t_7 = 8.29$, $p < .001$); however, its target, *Slc6a4*, was not increased ($p > .05$) (Figure 4B). Transfection with anti–miR-200a-3p decreased miR-200a-3p expression, but it did not reach significance ($t_7 = -2.04$, $p = .08$). Conversely, anti–miR-200a-3p increased *Slc6a4* expression 1.55-fold, but it also did not reach significance ($t_7 = 2.17$, $p = .06$) (Figure 4B). SHSY cells transfected with miR-322-5p mimic and anti–miR-322-5p did not show changes in miR-322-5p or *SLC6A4* expression ($p > .05$) (Figure 4C).

Transfection with miR-320-3p mimic and anti–miR-320-3p did not significantly alter expression of miR-320-3p or its target, *Htr1a* (Figure 4D). miR-320-5p mimic significantly increased miR-320-5p expression by 1.73-fold ($t_7 = 3.57$, $p < .01$) and decreased *Htr1a* expression ($t_7 = -6.02$, $p < .001$) (Figure 4E). Anti–miR-320-5p significantly reduced expression of miR-320-3p ($t_7 = -3.57$, $p < .01$) but also reduced expression of *Htr1a* ($t_7 = -6.90$, $p < .001$) (Figure 4E).

DISCUSSION

This study aimed to assess PFC expression changes in serotonin pathway genes and their targeting miRNAs following MS and environmental enrichment. Enrichment showed some promise as a preventive measure in that enriched MS animals showed sucrose preference levels similar to control animals; however, MS and enrichment did not clearly affect anxiety-like behaviors. *Slc6a4* was significantly upregulated by MS but not recovered by enrichment, while *Htr1a* was also upregulated (at trend level, $p = .08$) by MS and subsequently recovered by enrichment, most notably in male animals. Both miR-200a and miR-322-5p were decreased by MS regardless of enrichment, although it did not reach significance; miR-320-5p, which targets *Htr1a*, was significantly reduced by MS. To test if their

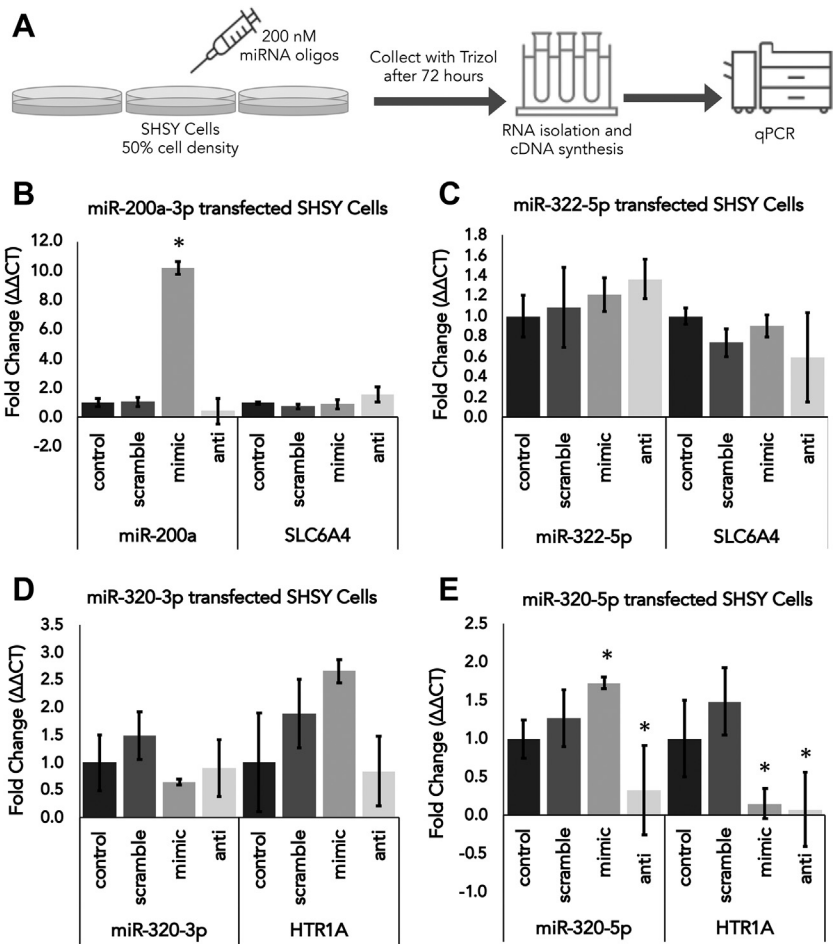


Figure 4. miRNA and gene expression changes following miRNA transfection in vitro. **(A)** A schematic diagram of transfection using SHSY cells. miRNA oligonucleotides (200 nM) were transfected on adherent SHSY cells at 50% density in triplicate (n = 3). After 72-hour incubation at 37 °C, the cells were collected in Trizol and RNA was isolated. miRNA and gene expression were tested using qPCR. Expression of **(B)** miR-200a-3p and **(C)** miR-322-5p were tested in miR-200a-3p– and miR-322-5p–transfected cells, respectively, along with their target, *SLC6A4*. Bars represent fold change relative to control and SEM. Expression of **(D)** miR-320-3p and **(E)** miR-320-5p were tested in miR-320-3p– and miR-320-5p–transfected cells, as well as their target, *HTR1A*. Significance was determined by independent samples t test comparing mimic or antisense to the two control groups (control and scramble); $^{*}p < .05$. anti, antisense; cDNA, complementary DNA; miRNA, microRNA; oligo, oligonucleotide; qPCR, quantitative polymerase chain reaction.

reduction might have enabled the translation of *Slc6a4* and *Htr1a* after ELS, SHSY cells were transfected with miRNA oligonucleotides. *HTR1A* was validated as a target of miR-320-5p. Transfection did not confirm miR-200a-3p and miR-322-5p as targeting *SLC6A4*. Based on these data, miR-320-5p is an intriguing miRNA that may confer resilience to reward-related behavioral deficits after ELS through its effects on expression of *HTR1A*. Although a direct connection between *SLC6A4* and miR-200a-3p and miR-322-5p was not established, their expression was affected by MS. Future studies might explore other gene and miRNA regulatory mechanisms that might explain changes.

Serotonin Receptor 1A and Stress Susceptibility

We are cautious to interpret changes to *Htr1a* expression after MS and enrichment because these findings did not reach significance. However, direct manipulation of miR-320-5p did affect *Htr1a* expression in vitro. In support of our findings, previous MS (32) and stress-related (33) studies have found increased *Htr1a*. The serotonergic system has been heavily implicated in MDD pathophysiology, yet MS only induced anhedonia-related behavior changes. Thus, *Htr1a* may not have been as significantly affected as animals exhibiting behavioral despair or learned helplessness. Moreover, anxiety has been associated with reductions in *Htr1a* expression (34). Thus, it is possible that we did not observe overt anxiety-related behavior because MS increased *Htr1a* expression. In male animals, enrichment returned *Htr1a* expression to that of control animals without the use of pharmacological agents. Considering that even recovered patients with MDD can exhibit increased *Htr1a* expression, indicating this phenotype as a trait of MDD susceptibility (35), environmental enrichment shows even greater promise for its ability to protect against this susceptibility phenotype. A different study of rodent ELS found that social enrichment in adulthood did not reflect in changes to *Htr1a* expression (36), highlighting the importance of intervention prior to adulthood. In adolescents, reduced activation of reward-related brain regions predicts later onset of depression symptoms (37). Similarly, our findings reflect coexisting reward-related deficits and *Htr1a* expression increases, which may promote further susceptibility to depression. Furthermore, enrichment prior to adulthood might negate this susceptibility through miR-320-5p regulation of *Htr1a*.

The Serotonin Transporter and Stress Susceptibility

In MS animals who also exhibited anhedonic behavior, *Slc6a4* expression was significantly increased in the PFC. Generally, increased *Slc6a4* binding is associated with increases in MDD symptomology (38). While our findings are consistent with this pattern of *Slc6a4* expression in MDD, we were unable to confirm regulation by miRNAs. Various other epigenetic changes to *Slc6a4* have been associated with ELS. In humans, prenatal exposure to maternal MDD has been shown to alter methylation of *Slc6a4* in a sex-dependent manner, with males exhibiting increased methylation compared with females (39); other loci in the *Slc6a4* promoter showed increased methylation regardless of sex (39). There is also the potential that *Slc6a4* is responsive to synaptic serotonin concentration changes (38), which are also affected by *Htr1a*.

miRNA Regulation of Serotonergic Genes in ELS

Following the canonical regulatory relationship between miRNAs and their gene targets, miRNAs miR-200a-3p, -322-5p, and -320-5p were significantly reduced in MS animals. A few previous studies have explored miRNA regulation of the serotonergic system, including miR-135 effects on *Slc6a4* and *Htr1a* (40), and miR-26a targeting of *Htr1a* and its upregulation by antidepressants in male mice (41). Only one study has implicated miR-322 in ELS. O'Connor *et al.* (42) found that miR-322 was decreased in MS rodents following treatment with electroconvulsive therapy. Our findings showed that miR-322 was also decreased in MS+Enr animals compared with control animals; however, MS-only animals also exhibited this same decreased expression. miRNA-322 is implicated in several cellular processes that are relevant to stress, such as plasticity and cell differentiation (43). While some studies have elucidated miR-200 expression changes in ELS models (23,44), to our knowledge, this study represents the first to connect miR-200 with serotonergic changes following ELS. Although we found opposite directions of expression change for miR-200a and *Slc6a4* in our rat MS model, we could not confirm miR-200a inhibition of *SLC6A4* in human neuroblastoma SHSY cells. A previous report validated that miR-200a inhibits *Slc6a4* in the rat colon (45), which could indicate that this targeting occurs outside of the brain.

miR-320-5p was previously unidentified relative to ELS, yet in vitro transfection with miR-320-5p mimic significantly increased miR-320-5p expression and decreased *Htr1a* expression. Rodent chronic mild stress increased expression of miR-320-3p in the ventral tegmental area but only in animals that exhibited resilience (no change relative to control animals) in the sucrose preference test (46); *Slc6a4* was subsequently reduced in the ventral tegmental area of resilient animals. We validated that miR-320-5p targets *Htr1a* and showed slight expression reduction after MS. Reduced endogenous expression of miR-320-5p allows more *Htr1a* mRNA to be translated into functional receptors without its interference. Tentatively, we speculate that MS leads to reduced miR-320-5p, which increases postsynaptic *Htr1a* expression (Figure 5A), thus reducing serotonin availability (Figure 5B). While it is not clear how *Slc6a4* is increased, it may upregulate in response to reduced extracellular serotonin availability. In the PFC, *Htr1a* is a heteroreceptor primarily responsible for inhibition of downstream activity (47), which may partially explain behavior changes subsequent to increased *Htr1a* expression. In male animals, *Htr1a* and miR-320-5p expression changes are reversed by enrichment, although *Htr1a* changes were not significant. miR-320-5p is one of many X-linked miRNAs that may partially explain sex-based differences in its response following enrichment. There have been a few studies on X-linked miRNAs in other disorders (48,49); however, no studies have explored this potential mode of sex differences and epigenetic heritability in MDD or ELS. This is the first study, to our knowledge, to validate miR-320-5p targeting of *Htr1a* in a central nervous system cell line.

Timing of neurobiological assays and stress interventions, such as MS, also contribute to differences in gene expression. One study of MS found that adolescent male animals who

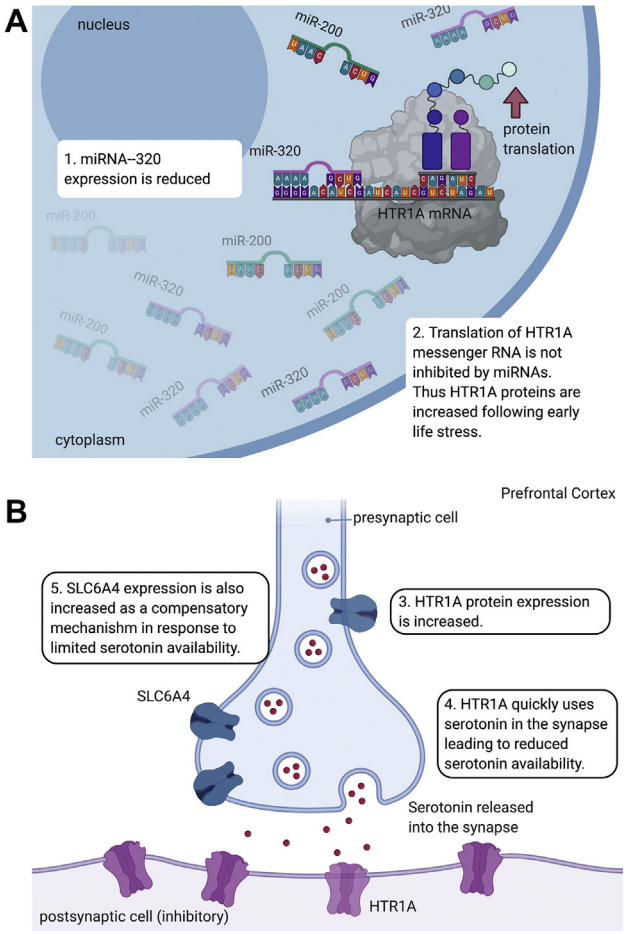


Figure 5. Maternal separation reprograms miRNA expression and its regulation of *Slc6a4* and *Htr1a* in the prefrontal cortex. **(A)** Early-life stress reduces the expression of miR-320 in the prefrontal cortex such that it cannot inhibit the translation of *Htr1a* into proteins. Increased HTR1A protein is likely reflected as **(B)** a higher density of postsynaptic HTR1A in maternal separation animals. Once serotonin is released into the synapse, HTR1A quickly expends serotonin, thus reducing its availability. Putatively, *Slc6a4* expression is increased presynaptically to compensate for limited availability of serotonin. In male animals, enrichment reverses these *Htr1a* expression changes. Created with BioRender.com. miRNA, microRNA.

received MS exhibited increased *Slc6a4* expression immediately following the MS period, but this expression was reduced in adulthood (50). It has been frequently reported that male rodents exhibit more stress-induced behavior change (51); yet, women are considered more susceptible to MDD with an almost twofold incidence compared with men (8). Differences across species may underlie some disparities in sex-based stress susceptibility between humans and rodents. For example, distinct from humans, rodents do not exhibit different alleles of the *Slc6a4* gene (52).

We have provided the first evidence that miRNAs are involved in MS-induced PFC gene expression changes and reversal following environmental enrichment. Future studies should explore other brain regions and enrichment paradigms. Our enrichment montage was relatively simple yet produced robust changes in behavior and miRNA expression changes in MS animals that were not observed with MS alone. As our study did not include a control group that received enrichment, we cannot attribute these changes solely to enrichment. Studies of enrichment in control animals may reveal unique mechanisms of effect or additional positive benefits beyond prevention of disease. It will also be of particular interest to compare enrichment to antidepressants that block *SLC6A4* from binding serotonin, such as duloxetine (5), in addition to their combination. This information will provide critical insights for the development of pharmacological treatments or preventions for depression. Future studies should consider altering the expression of these miRNAs in vivo to determine if their direct manipulation conveys resilience to stress.

Limitations

Because of the complex targeting networks formed by miRNAs in which a single miRNA may target hundreds of genes (19), expression changes in a single miRNA may not translate to opposite changes in its gene target. Other targeting miRNAs may exhibit an opposite change, counteracting a single miRNA's effects. This may partially explain why we did not find consistent depression-related behaviors such as learned helplessness (increased escape latency), behavioral despair (increased FST immobility), or anxiety. It is also clear that small differences in stress paradigms can significantly alter animal behavior at a later time point (53). It is critical to the study of ELS to develop models that more closely replicate their intended human psychiatric disorders.

Conclusions

Serotonin signaling and metabolism have long been associated with psychiatric disorders such as MDD. While a few studies have explored miRNA regulation of the serotonergic system related to stress, none have considered it in the context of MS or its treatment with enrichment. Not only does MS induce significant behavior phenotypes resembling anhedonia, but we also showed a reduction of miR-320-5p following MS, with sex differences in its response to enrichment. Its predicted target, *Htr1a*, showed opposite expression changes, and we validated this targeting in vitro. We also found significant increases in *Slc6a4* expression in the PFC. Altogether, this study showed that enrichment may be a viable tool for prevention of anhedonia following ELS via miRNA and serotonergic gene targets.

ACKNOWLEDGMENTS AND DISCLOSURES

The research was partly supported by the National Institute of Mental Health (Grant Nos. MH082802, MH101890, MH100616, MH107183, MH112014, and MH118884 [to YD]) and the American Foundation for Suicide Prevention (Grant No. DIG-0-041-18 [to YD]).

We thank Bhaskar Roy for his help troubleshooting throughout this project. We also thank Kevin Prall, Genelle Samson, Emma Jones, and Grant Schell, who each helped with some aspects of behavior data and animal tissue collection.

The authors report no biomedical financial interests or potential conflicts of interest.

ARTICLE INFORMATION

From the Department of Psychiatry and Behavioral Neurobiology, University of Alabama at Birmingham, Birmingham, Alabama.

Address correspondence to Yogesh Dwivedi, Ph.D., at ydwivedi@uab.edu.

Received Mar 4, 2021; revised May 6, 2021; accepted May 23, 2021.

Supplementary material cited in this article is available online at https://doi.org/10.1016/j.bpsgos.2021.05.009.

REFERENCES

1. Carr CP, Martins CM, Stingel AM, Lemgruber VB, Juruena MF (2013): The role of early life stress in adult psychiatric disorders: A systematic review according to childhood trauma subtypes. J Nerv Ment Dis 201:1007–1020.
2. Bachmann S (2018): Epidemiology of suicide and the psychiatric perspective. Int J Environ Res Public Health 15:1425.
3. Greenberg PE, Fournier AA, Sisitsky T, Pike CT, Kessler RC (2015): The economic burden of adults with major depressive disorder in the United States (2005 and 2010). J Clin Psychiatry 76:155–162.
4. Rush AJ, Kraemer HC, Sackeim HA, Fava M, Trivedi MH, Frank E, *et al.* (2006): Report by the ACNP Task Force on response and remission in major depressive disorder. Neuropsychopharmacology 31:1841–1853.
5. Girardi P, Pompili M, Innamorati M, Mancini M, Serafini G, Mazzarini L, *et al.* (2009): Duloxetine in acute major depression: Review of comparisons to placebo and standard antidepressants using dissimilar methods. Hum Psychopharmacol 24:177–190.
6. Nemeroff CB, Heim CM, Thase ME, Klein DN, Rush AJ, Schatzberg AF, *et al.* (2003): Differential responses to psychotherapy versus pharmacotherapy in patients with chronic forms of major depression and childhood trauma. Proc Natl Acad Sci U S A 100:14293–14296.
7. Middlebrooks J, Audage N (2008): The Effects of Childhood Stress on Health Across the Lifespan. Atlanta: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Injury Prevention and Control.
8. Center for Behavioral Health Statistics and Quality (2017): 2016 National Survey on Drug Use and Health: Methodological Summary and Definitions. Rockville: Substance Abuse and Mental Health Services Administration.
9. Syed SA, Nemeroff CB (2017): Early life stress, mood, and anxiety disorders. Chronic Stress (Thousand Oaks) 1:2470547017694461.
10. Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, *et al.* (2003): Influence of life stress on depression: Moderation by a polymorphism in the 5-HTT gene. Science 301:386–389.
11. Bravo JA, Dinan TG, Cryan JF (2014): Early-life stress induces persistent alterations in 5-HT1A receptor and serotonin transporter mRNA expression in the adult rat brain. Front Mol Neurosci 7:24.
12. Ohta K, Miki T, Warita K, Suzuki S, Kusaka T, Yakura T, *et al.* (2014): Prolonged maternal separation disturbs the serotonergic system during early brain development. Int J Dev Neurosci 33:15–21.
13. Etkin A, Egner T, Kalisch R (2011): Emotional processing in anterior cingulate and medial prefrontal cortex. Trends Cogn Sci 15:85–93.
14. Albert PR (2012): Transcriptional regulation of the 5-HT1A receptor: Implications for mental illness. Philos Trans R Soc Lond B Biol Sci 367:2402–2415.

15. Pompili M, Serafini G, Innamorati M, Venturini P, Fusar-Poli P, Sher L, *et al.* (2013): Agomelatine, a novel intriguing antidepressant option enhancing neuroplasticity: A critical review. World J Biol Psychiatry 14:412–431.
16. Brenes JC, Rodríguez O, Fornaguera J (2008): Differential effect of environment enrichment and social isolation on depressive-like behavior, spontaneous activity and serotonin and norepinephrine concentration in prefrontal cortex and ventral striatum. Pharmacol Biochem Behav 89:85–93.
17. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, *et al.* (2005): Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 433:769–773.
18. Lewis BP, Burge CB, Bartel DP (2005): Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120:15–20.
19. Bartel DP (2018): Metazoan microRNAs. Cell 173:20–51.
20. Xu J, Wang R, Liu Y, Wang W, Liu D, Jiang H, Pan F (2019): Short- and long-term alterations of FKBP5-GR and specific microRNAs in the prefrontal cortex and hippocampus of male rats induced by adolescent stress contribute to depression susceptibility. Psychoneuroendocrinology 101:204–215.
21. Xu J, Wang R, Liu Y, Liu D, Jiang H, Pan F (2017): FKBP5 and specific microRNAs via glucocorticoid receptor in the basolateral amygdala involved in the susceptibility to depressive disorder in early adolescent stressed rats. J Psychiatr Res 95:102–113.
22. Liu Y, Liu D, Xu J, Jiang H, Pan F (2017): Early adolescent stress-induced changes in prefrontal cortex miRNA-135a and hippocampal miRNA-16 in male rats. Dev Psychobiol 59:958–969.
23. Morrison KE, Narasimhan S, Fein E, Bale TL (2016): Peripubertal stress with social support promotes resilience in the face of aging. Endocrinology 157:2002–2014.
24. Kokras N, Sotiropoulos I, Besinis D, Tzouveka EL, Almeida OFX, Sousa N, Dalla C (2019): Neuroplasticity-related correlates of environmental enrichment combined with physical activity differ between the sexes. Eur Neuropsychopharmacol 29:1–15.
25. Dwivedi Y, Roy B, Lugli G, Rizavi H, Zhang H, Smalheiser NR (2015): Chronic corticosterone-mediated dysregulation of microRNA network in prefrontal cortex of rats: Relevance to depression pathophysiology. Transl Psychiatry 5:e682.
26. Cohen H, Matar MA, Joseph Z (2013): Animal models of post-traumatic stress disorder. Curr Protoc Neurosci Chapter 9:Unit 9.45.
27. Kokras N, Baltas D, Theocharis F, Dalla C (2017): Kinoscope: An open-source computer program for behavioral pharmacologists. Front Behav Neurosci 11:88.
28. Dwivedi Y, Zhang H (2016): Altered ERK1/2 signaling in the brain of learned helpless rats: Relevance in vulnerability to developing stress-induced depression. Neural Plast 2016:7383724.
29. Roy B, Dunbar M, Shelton RC, Dwivedi Y (2017): Identification of microRNA-124-3p as a putative epigenetic signature of major depressive disorder. Neuropsychopharmacology 42:864–875.
30. Andrés-León E, González Peña D, Gómez-López G, Pisano DG (2015): miRGate: A curated database of human, mouse and rat miRNA-mRNA targets. Database (Oxford) 2015:bav035.
31. Sticht C, De La Torre C, Parveen A, Gretz N (2018): miRWalk: An online resource for prediction of microRNA binding sites. PLoS One 13: e0206239.
32. de Souza JA, da Silva MC, Costa FCO, de Matos RJB, de Farias Campina RC, do Amaral Almeida LC, *et al.* (2020): Early life stress induced by maternal separation during lactation alters the eating behavior and serotonin system in middle-aged rat female offspring. Pharmacol Biochem Behav 192:172908.
33. Le François B, Soo J, Millar AM, Daigle M, Le Guisquet AM, Leman S, *et al.* (2015): Chronic mild stress and antidepressant treatment alter 5-HT1A receptor expression by modifying DNA methylation of a conserved Sp4 site. Neurobiol Dis 82:332–341.
34. Piszczek L, Piszczek A, Kuczmanska J, Audero E, Gross CT (2015): Modulation of anxiety by cortical serotonin 1A receptors. Front Behav Neurosci 9:48.
35. Hesselgrave N, Parsey RV (2013): Imaging the serotonin 1A receptor using [11C]WAY100635 in healthy controls and major depression. Philos Trans R Soc Lond B Biol Sci 368:20120004.
36. Bodden C, van den Hove D, Lesch KP, Sachser N (2017): Impact of varying social experiences during life history on behaviour, gene expression, and vasopressin receptor gene methylation in mice. Sci Rep 7:8719.
37. Telzer EH, Fuligni AJ, Lieberman MD, Galván A (2014): Neural sensitivity to eudaimonic and hedonic rewards differentially predict adolescent depressive symptoms over time. Proc Natl Acad Sci U S A 111:6600–6605.
38. Spies M, Knudsen GM, Lanzenberger R, Kasper S (2015): The serotonin transporter in psychiatric disorders: Insights from PET imaging. Lancet Psychiatry 2:743–755.
39. Stonawski V, Frey S, Golub Y, Rohleder N, Kriebel J, Goecke TW, *et al.* (2019): Associations of prenatal depressive symptoms with DNA methylation of HPA axis-related genes and diurnal cortisol profiles in primary school-aged children. Dev Psychopathol 31:419–431.
40. Issler O, Haramati S, Paul ED, Maeno H, Navon I, Zwang R, *et al.* (2014): MicroRNA 135 is essential for chronic stress resiliency, antidepressant efficacy, and intact serotonergic activity. Neuron 83:344–360.
41. Xie L, Chen J, Ding YM, Gui XW, Wu LX, Tian S, Wu W (2019): MicroRNA-26a-2 maintains stress resiliency and antidepressant efficacy by targeting the serotonergic autoreceptor HTR1A. Biochem Biophys Res Commun 511:440–446.
42. O'Connor RM, Grenham S, Dinan TG, Cryan JF (2013): microRNAs as novel antidepressant targets: Converging effects of ketamine and electroconvulsive shock therapy in the rat hippocampus. Int J Neuropsychopharmacol 16:1885–1892.
43. Wang F, Liang R, Tandon N, Matthews ER, Shrestha S, Yang J, *et al.* (2019): H19X-encoded miR-424(322)/-503 cluster: Emerging roles in cell differentiation, proliferation, plasticity and metabolism. Cell Mol Life Sci 76:903–920.
44. Cattane N, Mora C, Lopizzo N, Borsini A, Maj C, Pedrini L, *et al.* (2019): Identification of a miRNAs signature associated with exposure to stress early in life and enhanced vulnerability for schizophrenia: New insights for the key role of miR-125b-1-3p in neurodevelopmental processes. Schizophr Res 205:63–75.
45. Hou Q, Huang Y, Zhang C, Zhu S, Li P, Chen X, *et al.* (2018): MicroRNA-200a targets cannabinoid receptor 1 and serotonin transporter to increase visceral hyperalgesia in diarrhea-predominant irritable bowel syndrome rats. J Neurogastroenterol Motil 24:656–668.
46. Zurawek D, Kusmider M, Faron-Gorecka A, Gruca P, Pabian P, Solich J, *et al.* (2017): Reciprocal microRNA expression in mesocortical circuit and its interplay with serotonin transporter define resilient rats in the chronic mild stress. Mol Neurobiol 54:5741–5751.
47. Albert PR, Zhou QY, Van Tol HH, Bunzow JR, Civelli O (1990): Cloning, functional expression, and mRNA tissue distribution of the rat 5-hydroxytryptamine1A receptor gene. J Biol Chem 265:5825–5832.
48. Pinheiro I, Dejager L, Libert C (2011): X-chromosome-located microRNAs in immunity: Might they explain male/female differences? The X chromosome-genomic context may affect X-located miRNAs and downstream signaling, thereby contributing to the enhanced immune response of females. Bioessays 33:791–802.
49. Khalifa O, Pers YM, Ferreira R, Sénéchal A, Jorgensen C, Apparailly F, Duroux-Richard I (2016): X-linked miRNAs associated with gender differences in rheumatoid arthritis. Int J Mol Sci 17:1852.
50. Oreland S, Pickering C, Gökturk C, Oreland L, Arborelius L, Nylander I (2009): Two repeated maternal separation procedures differentially affect brain 5-hydroxytryptamine transporter and receptors in young and adult male and female rats. Brain Res 1305 Suppl:S37–S49.
51. Cohen H, Yehuda R (2011): Gender differences in animal models of posttraumatic stress disorder. Dis Markers 30:141–150.
52. Murphy DL, Lesch KP (2008): Targeting the murine serotonin transporter: Insights into human neurobiology. Nat Rev Neurosci 9:85–96.
53. Peña CJ, Nestler EJ, Bagot RC (2019): Environmental programming of susceptibility and resilience to stress in adulthood in male mice. Front Behav Neurosci 13:40.