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(54) **Title**: MICRO-RNAS AND COMPOSITIONS COMPRISING SAME FOR THE TREATMENT AND DIAGNOSIS OF SEROTONIN-, ADRENALIN-, NORADRENALIN-, GLUTAMATE-, AND CORTICOTROPIN-RELEASING HORMONE- ASSOCIATED MEDICAL CONDITIONS

(57) **Abstract**: Micro RNAs and compositions comprising same for the treatment and diagnosis of serotonin-, adrenalin-, noradrenalin-, glutamate-, and corticotropin-releasing hormone-associated medical conditions are provided.

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MICRO-RNAS AND COMPOSITIONS COMPRISING SAME FOR THE TREATMENT AND DIAGNOSIS OF SEROTONIN-, ADRENALIN-, NORADRENALIN-, GLUTAMATE-, AND CORTICOTROPIN-RELEASING HORMONE- ASSOCIATED MEDICAL CONDITIONS

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to microRNAs and, more particularly, but not exclusively, to the use of same for disease diagnosis, treatment and monitoring treatment.

Mood disorders such as major depression represent some of the most common and proliferating health problems worldwide effecting about 10 % of the population. Despite many decades of research, the mechanisms behind depression onset, susceptibility and available therapies are only partially understood. Currently only about a third of patients respond to available treatments, therefore, there is a great need for better understanding of the pathology. The current dogma regarding the etiology of depression is of a complex interaction between environmental factors and genetics predisposition, suggesting a mechanistic role for epigenetic processes.

Serotonin (5HT) is a monoamine neurotransmitter produced in the brain by the raphe nucleus (RN), which project extensively throughout the brain to modulate variety of cognitive, emotional and physiological functions. The link between disregulated serotonergic activity and depression is well established [Michelsen KA. et al., Brain Res Rev (2007) 55(2):329-42]. The levels of 5HT, as well as the genetic circuitry in charge of it production, secretion, reuptake and deactivating, are dysregulated in depression. Furthermore, most currently available antidepressant drugs target the function of 5HT system related proteins, resulting in increased 5HT levels in the synapse [Krishnan V and Nestler EJ, Nature (2008) 455: 894-902]. Available therapeutics require a long period of administration before relief of symptoms is observed.

MicroRNAs (miRs) are a subset of endogenous small (approximately 22 nucleotide) RNA molecules that repress gene expression post-transcriptionally. MiRs are transcribed as primary-miR molecules that are processed in the cell nucleus into precursor miRs with stem loop structures, which are exported to the cytoplasm where

they are further processed into the active mature miRs. The mature miR is subsequently incorporated into the RNA-induced silencing complex and function primarily by binding to the 3'untranslated regions (3'UTRs) of specific mRNA molecules. Binding occurs via the seed sequence, a 6–8 nucleotides sequence at the 5′ end of the miR, that base pairs to a complementary seed match sequence on the target mRNA 3' UTR. Binding of a miR leads to direct mRNA destabilization or translational repression, ultimately resulting in reduced protein levels of target gene.

MiRs are abundant in the nervous system, and initial research has mainly focused on neurons in the context of development, cancer and neurodegenerative disorders and normal process such as plasticity [Kosik KS. Nat Rev Neurosci (2006) 7:911-20]. Additionally, it has been suggested that miRs play a role in psychiatric disorders such as schizophrenia, autism and also depression and anxiety, both in humans and in mouse models [Miller BH and Wahlestedt C, Brain Res (2010) 1338: 89-99]. Several studies have recently demonstrated the involvement of miRs in regulating 5HT related genes [Millan MJ. Curr Opin Pharmacol (2011) 11(1):11-22] revealing the emerging role of miRs in the regulation of 5HT system and their potential association with depression related disorders.

U.S. Patent Application No. 20100222413 (to Stoffel M. et al.) disclose chemically modified oligonuceotides for modulating expression of microRNAs. U.S. 20100222413 further discloses methods for silencing microRNAs (e.g. miR-122, miR-16, miR-192 and miR-194) for the treatment of diseases of the central nervous system.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of treating a medical condition in which an elevation of serotonin level is therapeutically beneficial in a subject in need thereof, the method comprising administering to or expressing in a cell of the subject an exogenous polynucleotide encoding at least one microRNA or a precursor thereof, wherein the microRNA is selected from the group consisting of miR-135, miR-335, miR-26 and miR-182, thereby treating the medical condition.

According to an aspect of some embodiments of the present invention there is provided a use of an exogenous polynucleotide encoding at least one microRNA or a

precursor thereof, wherein the microRNA is selected from the group consisting of miR-135, miR-335, miR-26 and miR-182, for the manufacture of a medicament identified for treating a medical condition in which an elevation of serotonin level is therapeutically beneficial.

According to an aspect of some embodiments of the present invention there is provided a method of increasing a serotonin level in a synaptic cleft of a subject in need thereof, the method comprising administering to or expressing in a serotonergic neuron of the subject an exogenous polynucleotide encoding at least one microRNA or a precursor thereof, wherein the microRNA is selected from the group consisting of miR-135, miR-335, miR-26 and miR-182, thereby increasing the serotonin level in the synaptic cleft.

According to an aspect of some embodiments of the present invention there is provided an isolated neuroglia cell comprising a nucleic acid construct expressing at least one microRNA or a precursor thereof, wherein the microRNA is selected from the group consisting of miR-135, miR-335, miR-26 and miR-182 under a transcriptional control of a cis acting regulatory element.

According to an aspect of some embodiments of the present invention there is provided an isolated polynucleotide encoding at least one microRNA or a precursor thereof, wherein the microRNA is selected from the group consisting of miR-135, miR-335, miR-26 and miR-182, for treating a medical condition in which an elevation of serotonin level is therapeutically beneficial.

According to an aspect of some embodiments of the present invention there is provided a method of treating a medical condition in which a low adrenaline or noradrenaline level is therapeutically beneficial in a subject in need thereof, the method comprising administering to or expressing in a cell of the subject an exogenous polynucleotide encoding a miR-19 or a precursor thereof, thereby treating the medical condition.

According to an aspect of some embodiments of the present invention there is provided a use of an exogenous polynucleotide encoding a miR-19 or a precursor thereof for the manufacture of a medicament identified for treating a medical condition in which a low adrenaline or noradrenaline level is therapeutically beneficial.

According to an aspect of some embodiments of the present invention there is provided an isolated cell comprising a nucleic acid construct expressing a miR-19 or a precursor thereof under a transcriptional control of a cis acting regulatory element.

According to an aspect of some embodiments of the present invention there is provided an isolated polynucleotide encoding a miR-19 or a precursor thereof for treating a medical condition in which a low adrenaline or noradrenaline level is therapeutically beneficial.

According to an aspect of some embodiments of the present invention there is provided a method of treating a medical condition in which a low corticotropin-releasing hormone (CRH) level is therapeutically beneficial in a subject in need thereof, the method comprising administering to or expressing in a cell of the subject an exogenous polynucleotide encoding a miR-15 or a precursor thereof, thereby treating the medical condition.

According to an aspect of some embodiments of the present invention there is provided a use of an exogenous polynucleotide encoding a miR-15 or a precursor thereof for the manufacture of a medicament identified for treating a medical condition in which a low corticotropin-releasing hormone (CRH) level is therapeutically beneficial.

According to an aspect of some embodiments of the present invention there is provided an isolated cell comprising a nucleic acid construct expressing a miR-15 or a precursor thereof under a transcriptional control of a cis acting regulatory element.

According to an aspect of some embodiments of the present invention there is provided an isolated polynucleotide encoding a miR-15 or a precursor thereof for treating a medical condition in which a low corticotropin-releasing hormone (CRH) level is therapeutically beneficial.

According to an aspect of some embodiments of the present invention there is provided a method of treating a medical condition in which a low glutamate receptor level is therapeutically beneficial in a subject in need thereof, the method comprising administering to or expressing in a cell of the subject an exogenous polynucleotide encoding a miR-181 or a precursor thereof, thereby treating the medical condition.

According to an aspect of some embodiments of the present invention there is provided a use of an exogenous polynucleotide encoding a miR-181 or a precursor

thereof for the manufacture of a medicament identified for treating a medical condition in which a low glutamate receptor level is therapeutically beneficial.

According to an aspect of some embodiments of the present invention there is provided an isolated cell comprising a nucleic acid construct expressing a miR-181 or a precursor thereof under a transcriptional control of a cis acting regulatory element.

According to an aspect of some embodiments of the present invention there is provided an isolated polynucleotide encoding a miR-181 or a precursor thereof for treating a medical condition in which a low glutamate receptor level is therapeutically beneficial.

According to an aspect of some embodiments of the present invention there is provided a nucleic acid construct comprising a nucleic acid sequence encoding a microRNA or a precursor thereof, wherein the microRNA or a precursor thereof is selected from the group consisting of miR-135, miR-335, miR-26, miR-27, miR-181, miR-182, miR-19 and miR-15, the nucleic acid sequence being under a transcriptional control of a cis acting regulatory element.

According to an aspect of some embodiments of the present invention there is provided a pharmaceutical composition comprising the nucleic acid construct of the present invention and a pharmaceutically acceptable carrier or diluent.

According to an aspect of some embodiments of the present invention there is provided a method of regulating an expression of a tryptophan hydroxylase 2 (Tph2) gene in a neuroglia cell, the method comprising modulating an activity or expression of a microRNA or a precursor thereof in the neuroglia cell, wherein the microRNA is selected from the group consisting of miR-181 and miR27, thereby regulating the expression of the Tph2 gene.

According to an aspect of some embodiments of the present invention there is provided a method of regulating an expression of a glutamate receptor gene in a neuroglia cell, the method comprising modulating an activity or expression of miR-181 or a precursor thereof in the neuroglia cell, thereby regulating the expression of the glutamate receptor gene.

According to an aspect of some embodiments of the present invention there is provided an isolated polynucleotide comprising a nucleic acid sequence for downregulating an expression of miR-181, miR-27 or a precursor thereof.

According to an aspect of some embodiments of the present invention there is provided a nucleic acid construct comprising a nucleic acid sequence for downregulating an expression of a microRNA or a precursor thereof, wherein the microRNA or a precursor thereof is selected from the group consisting of miR-181 and the miR-27, the nucleic acid sequence being under a transcriptional control of a cis acting regulatory element.

According to an aspect of some embodiments of the present invention there is provided a method of regulating an expression of a serotonin transporter (Slc6a4) gene in a neuroglia cell, the method comprising modulating an activity or expression of a microRNA or a precursor thereof in the neuroglia cell, wherein the microRNA is selected from the group consisting of miR-135 and miR-335, thereby regulating the expression of the Slc6a4 gene.

According to an aspect of some embodiments of the present invention there is provided a method of regulating an expression of a serotonin inhibitory receptor 1a (Htr1a) gene in a neuroglia cell, the method comprising modulating an activity or expression of a microRNA or a precursor thereof in the neuroglia cell, wherein the microRNA is selected from the group consisting of miR-135, miR-335, miR-181, miR-182 and miR-26, thereby regulating the expression of the Htr1a gene.

According to an aspect of some embodiments of the present invention there is provided a method of regulating an expression of a Down Syndrome Cell Adhesion Molecule (Dscam) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-182 or a precursor thereof in the neuroglia cell, thereby regulating the expression of the Dscam gene.

According to an aspect of some embodiments of the present invention there is provided a method of regulating an expression of a Cell adhesion molecule L1 (L1cam) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-182 or a precursor thereof in the neuroglia cell, thereby regulating the expression of the L1cam gene.

According to an aspect of some embodiments of the present invention there is provided a method of regulating an expression of a Translin-associated protein X (Tsnax) gene in a neuroglia cell, the method comprising modulating an activity or

expression of a miR-182 or a precursor thereof in the neuroglia cell, thereby regulating the expression of the Tsnax gene.

According to an aspect of some embodiments of the present invention there is provided a method of regulating an expression of a monoamine hydroxylase (MaoA) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-27, thereby regulating the expression of the MaoA gene.

According to an aspect of some embodiments of the present invention there is provided a method of regulating an expression of a beta adrenergic receptor 1 (Adrb1) gene in a neuroglia cell or cardiac cell, the method comprising modulating an activity or expression of a miR-19, thereby regulating the expression of the Adrb1 gene.

According to an aspect of some embodiments of the present invention there is provided a method of regulating an expression of a canabinoid receptor 1 (CB1) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-19 or a precursor thereof in the neuroglia cell, thereby regulating the expression of the CB1 gene.

According to an aspect of some embodiments of the present invention there is provided a method of regulating an expression of a CRH type 1 receptor gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-15, thereby regulating the expression of the CRH type 1 receptor gene.

According to an aspect of some embodiments of the present invention there is provided a method of regulating an expression of a FK506 binding protein 5 (FKBP5) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-15 or a precursor thereof in the neuroglia cell, thereby regulating the expression of the FKBP5 gene.

According to an aspect of some embodiments of the present invention there is provided a method of regulating an expression of a syntaxin 1a (Stx1a) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-15 or a precursor thereof in the neuroglia cell, thereby regulating the expression of the Stx1a gene.

According to an aspect of some embodiments of the present invention there is provided a method of regulating an expression of a serum/glucocorticoid_regulated kinase (Sgk1) gene in a neuroglia cell, the method comprising modulating an activity or

expression of a miR-15 or a precursor thereof in the neuroglia cell, thereby regulating the expression of the Sgk1 gene.

According to an aspect of some embodiments of the present invention there is provided a method of regulating an expression of a beta 2 adrenergic receptor (Adrb2) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-15 or a precursor thereof in the neuroglia cell, thereby regulating the expression of the Adrb2 gene.

According to an aspect of some embodiments of the present invention there is provided a method of monitoring treatment of an anti-depressant drug, the method comprising: (a) treating a subject in need thereof with an anti-depressant drug; and (b) measuring an expression level of a miR-135 in the blood of the subject prior to and following the treatment, wherein a lower expression level of the miR-135 following to the treatment by the anti-depressant drug as compared to the expression level of the miR-135 prior to the treatment by the anti-depressant drug is indicative of the efficient treatment.

According to an aspect of some embodiments of the present invention there is provided a method of diagnosing a serotonin-related medical condition in a subject in need thereof, the method comprising measuring an expression level of a miR-135 in a blood of the subject, wherein a high expression level of the miR-135 as compared to that in a blood sample of a healthy subject is indicative of the serotonin-associated medical condition.

According to some embodiments of the invention, the cell is a neuroglia cell.

According to some embodiments of the invention, the neuroglia cell is a serotonergic neuron.

According to some embodiments of the invention, the miR-135 is as set forth in SEQ ID NO: 58-62.

According to some embodiments of the invention, the miR-335 is as set forth in SEQ ID NO: 63-64.

According to some embodiments of the invention, the miR-26 is as set forth in SEQ ID NO: 65-69.

According to some embodiments of the invention, the miR-182 is as set forth in SEQ ID NO: 70-71.

According to some embodiments of the invention, the medical condition is selected from the group consisting of a depression, an anxiety, a stress, a fatigue, an impaired cognitive function, a panic attack, a compulsive behavior, an addiction, a social phobia, a sleep disorder, a food related disorder, a growth disorder and a reproduction disorder.

According to some embodiments of the invention, when the microRNA is miR-135, the medical condition is depression or anxiety.

According to some embodiments of the invention, the cell is a neuroglia cell or a cardiac cell.

According to some embodiments of the invention, the miR-19 is as set forth in SEQ ID NO: 72-76.

According to some embodiments of the invention, the medical condition is selected from the group consisting of a stress, an anxiety, a memory impairment and a heart condition.

According to some embodiments of the invention, the miR-15 is as set forth in SEQ ID NO: 77-80

According to some embodiments of the invention, the medical condition is selected from the group consisting of a depression, an anxiety, a stress, a fatigue, an impaired cognitive function, a panic attack, a compulsive behavior, an addiction, a social phobia, a sleep disorder, a food related disorder, a growth disorder and a reproduction disorder.

According to some embodiments of the invention, the polynucleotide being under a transcriptional control of a cis acting regulatory element active in a neuroglia cell.

According to some embodiments of the invention, the polynucleotide being under a transcriptional control of a cis acting regulatory element active in a cardiac cell.

According to some embodiments of the invention, the miR-181 is as set forth in SEQ ID NO: 85-94.

According to some embodiments of the invention, the medical condition is selected from the group consisting of seizures, Huntington's disease, Schizophrenia, Fragile X syndrome, generalized anxiety disorder and cancer.

According to some embodiments of the invention, the cis acting regulatory element is active in a neuroglia cell or a cardiac cell.

According to some embodiments of the invention, the subject is a human subject.

According to some embodiments of the invention, when the regulating comprises upregulating the expression of the Tph2 gene, the modulating comprises downregulating the miR-181 and/or the miR-27 in the neuroglia cell.

According to some embodiments of the invention, the method further comprises measuring an expression of the Tph2 gene following the downregulating of the miR-181 and/or the miR-27 in the neuroglia cell.

According to some embodiments of the invention, the glutamate receptor gene is selected from the group consisting of glutamate receptor metabotropic 1 (Grm1), glutamate receptor ionotropic, kainate 3 (Grik3), glutamate receptor metabotropic 5 (Grm5), glutamate receptor ionotropic kainate 2 (Grik2) and glutamate receptor metabotropic 7 (Grm7).

According to some embodiments of the invention, when the regulating comprises downregulating the expression of the Slc6a4 gene, the modulating comprises upregulating the miR-135 and/or miR-335 in the neuroglia cell.

According to some embodiments of the invention, the method further comprises measuring an expression of the Slc6a4 gene following the upregulating the miR-135 and/or miR-335 in the neuroglia cell.

According to some embodiments of the invention, when the regulating comprises downregulating the expression of the Htr1a gene, the modulating comprises upregulating the miR-135, miR-335, miR-181, miR-182 and/or miR-26 in the neuroglia cell.

According to some embodiments of the invention, the method further comprises measuring an expression of the Htr1a gene following the upregulating the miR-135, miR-335, miR-181, miR-182 and/or miR-26 in the neuroglia cell.

According to some embodiments of the invention, when the regulating comprises downregulating the expression of the MaoA gene, the modulating comprises upregulating the miR-27 in the neuroglia cell.

According to some embodiments of the invention, the method further comprises measuring an expression of the MaoA gene following the upregulating the upregulating the miR-27 in the neuroglia cell.

According to some embodiments of the invention, when the regulating comprises downregulating the expression of the Adrb1 gene, the modulating comprises upregulating the miR-19 in the neuroglia cell or the cardiac cell.

According to some embodiments of the invention, the method further comprises measuring an expression of the Adrb1 gene following the upregulating the miR-19 in the neuroglia cell or the cardiac cell.

According to some embodiments of the invention, when the regulating comprises downregulating the expression of the CB1 gene, the modulating comprises upregulating the miR-19 in the neuroglia cell.

According to some embodiments of the invention, the method further comprises measuring an expression of the CB1 gene following the upregulating the CB1 in the neuroglia cell.

According to some embodiments of the invention, when the regulating comprises downregulating the expression of the CRH type 1 receptor gene, the modulating comprises upregulating the miR-15 in the neuroglia cell.

According to some embodiments of the invention, the method further comprises measuring an expression of the CRH type 1 receptor gene following the upregulating the miR-15 in the neuroglia cell.

According to some embodiments of the invention, when the regulating comprises downregulating the expression of the FKBP5 gene, the modulating comprises upregulating the miR-15 in the neuroglia cell.

According to some embodiments of the invention, the method further comprises measuring an expression of the FKBP5 gene following the upregulating the miR-15 in the neuroglia cell.

According to some embodiments of the invention, the method further comprises obtaining a blood sample from the subject prior to the treatment.

According to some embodiments of the invention, the anti-depressant drug is selected from the group consisting of selective serotonin reuptake inhibitors (SSRI), tricyclic antidepressants and noradrenaline reuptake inhibitors (NRI).

According to some embodiments of the invention, the serotonin-associated medical condition is a psychiatric condition

According to some embodiments of the invention, the psychiatric condition is selected from the group consisting of a depression, an anxiety, a stress, a fatigue, an impaired cognitive function, a panic attack, a compulsive behavior, an addiction, a social phobia, a sleep disorder and a food related disorder.

According to some embodiments of the invention, the miR-135 comprises miR-135a or miR-135b.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIGs. 1A-I depict microRNA expression in serotonin (5HT) neurons. Figure 1A is a graphic illustration of differentially expressed miRNAs in 5HT neurons. Lowess normalized values are depicted as ln2 fold change of spot intensity plotted against average log intensities (MA plot); Figure 1B is a validation of array results in miRs real time PCR indicating increased levels of miR-375 in the 5HT neurons compared to control. n = 5 5HT cells, n = 4 non 5HT. Bars represent mean ± s.e.m. **P=0.0071; Figure 1C is a validation of array results in miRs real time PCR indicating decreased levels of miR-135a in the 5HT neurons compared to control. N = 5 5HT cells, n = 4 non 5HT. **P=0.0075; Figure 1D is a van diagram representing crossing bioinformatics predictions for Slc6a4 with 5HT microarray results and listing miRs chosen for *in vitro*

testing; Figure 1E is a van diagram representing crossing bioinformatics predictions for Htr1a with5HT microarray results and listing miRs chosen for *in vitro* testing; Figure 1F is a van diagram representing crossing bioinformatics predictions for Tph2 with 5HT microarray results and listing miRs chosen for *in vitro* testing; Figure 1G is a van diagram representing crossing bioinformatics predictions for MaoA with 5HT microarray results and listing miRs chosen for *in vitro* testing; Figure 1H is a graph illustrating luciferase reporter assay results indicating that miR-181c and miR-27b may target Tph2 3'UTR; and Figure 1I is a graph illustrating luciferase reporter assay results indicating that miR-27b may target Htr1a MaoA.

FIGs. 2A-H depict microRNA targeting of Slc6a4 3'UTR (SEQ ID NO: 25) and Htr1a 3'UTR (SEQ ID NO: 27). Figure 2A is an illustration of miR-135a and miR-135b (SEQ ID NOs: 24 and 26, respectively) targeting of Slc6a4 3'UTR; Figure 2B is an illustration of miR-135a and miR-135b (SEQ ID NOs: 24 and 26, respectively) targeting of Htr1a 3'UTR; Figure 2C is a graph illustrating luciferase reporter assay results indicating that miR-135a and miR-135b may target Slc6a4 3'UTR. Luciferase assay data depicts renilla luciferase activity normalized to the activity of a co-transfected firefly luciferase reporter in HEK293 cells transfected with 3'UTR of the gene described and an empty vector, or a vector over-expressing a specific miR. Bars represent mean ± s.e.m. *P = 0.014, ***P = 0.0002, for miR-16 #p < 0.0535, for miR-27 #P = 0.0967; Figure 2D is a graph illustrating luciferase reporter assay results indicating that miR-135a, miR-135b, miR-335, miR-181C and miR-26a may target Htr1a 3'UTR. ***P < 0.0001, **P = 0.0029; Figure 2E is an illustration of slc6a4 3'UTR conservation of the seed matches for miR-135 (SEQ ID NOs: 27-41); Figure 2F is an illustration of Htr1a 3'UTR seed matches for miR-135 (SEQ ID NOs: 42-54), indicating seed 1 appearing only in mouse 3' UTR, and seed 2 is highly conserved; Figure 2G is a graph illustrating that mutation in miR-135 seed match in slc6a4 3'UTR blocked the repressor effect of miR-135a and miR-135b. ***P < 0.0001, **P = 0.0032; and Figure 2H is a graph illustrating mutation in miR-135 seed matches in Htr1a 3' UTR individually and both together, indicating miR-135b targets Htr1a via both the seed matches and miR-135a only by seed 2. ***P < 0.0001.

FIGs. 3A-J depict miR-135a and miR-135b levels under different conditions. Figure 3A is a graph illustrating down-regulation of miR-135a levels in the RN

following acute stress. Bars represent mean ± s.e.m. (n=8 in the 0 group, n=10 in the 90 group and n=9 in the 24 group) ***P < 0.0001, *P = 0.0357; Figure 3B is a graph illustrating down-regulation of miR-135b levels in the RN following acute stress. ***P < 0.0001, **P = 0.0055; Figure 3C is a graph illustrating up-regulation of miR-135a levels in the RN following acute and chronic imipramine administration independently from whether the mice were exposed to social defeat. (n=8 in control chronic saline and control chronic imipramine, n=7 acute imipramine, n=11 social defeat chronic saline, n=9 in the social defeat chronic imipramine) **P = 0.003; Figure 3D is a graph illustrating up-regulation of miR-135b levels in the RN following acute and chronic imipramine administration independently from whether the mice were exposed to social defeat. **P = 0.0093; Figure 3E is a graph illustrating increase in miR-135a levels in the RN following acute or chronically administrated SSRI, and not NRI or saline. (n=8 in each group apart from acute saline n=7) ***P < 0.0001; Figure 3F is a graph illustrating an unaltered miR-135b levels in the RN following acute or chronically administered SSRI or NRI; Figure 3G is a graph illustrating decrease in miR-135a levels in the plasma of mice receiving chronic or acute SSRI as compared to controls. (n=8 in each group apart from chronic SSRI and NRI n=7) **P = 0.0004 for acute SSRI compared to acute saline and **P = 0.0006 for the chronic SSRI compared to the chronic saline; Figure 3H is a graph illustrating unchanged miR-135b levels in the plasma of mice receiving chronic or acute SSRI as compared to controls; Figure 3I is a scatter plot graph demonstrating individual mice miR-135a levels in the RN compared to the plasma indicating a reverse correlation in mice receiving SSRI or saline treatment; and Figure 3J is a scatter plot graph demonstrating no correlation between miR-135b levels in the RN to the plasma in mice receiving SSRI treatment compared to controls.

FIGs. 4A-H depict *in vivo* over-expression of miR-135b. Figure 4A is a schematic illustration of lentiviruses for over-expression of miR-135b; Figure 4B is a graph illustrating real time PCR results indicating over-expression of miR-135b *in vivo* in the dorsal raphae nucleus (DRN) of adult mice. Bars represent mean ± s.e.m. (n=5 GFP injected and n=3 miR-135 OE) P = 0.0032; Figures 3C-D are illustrations of a DRN injection site by demonstration of GFP staining at injections site. (Section map adopted from Paxinos); Figure 4E is a graph illustrating decreased immobility time in the forest swim test in mice over-expressing miR-135b in the RN compared to control

mice. (n=9 control n=9 miR-135) P = 0.0088 in minute 3 and P = 0.00330 for minute 4; Figure 4F is a graph illustrating decreased immobility time in the tail suspension test in mice over-expressing miR-135b in the RN compared to control mice. P = 0.07351; Figures 4G-H are graphs illustrating no difference in home cage locomotion in of mice over-expressing miR-135b in the RN compared to controls.

FIG. 5 depicts ADRb1 3'UTR cloned following the luciferase gene. Illustration of intact (top) ADRb1 3'UTR, harboring four miR-19 binding sites, and mutant (bottom) form of ADRb1 3'UTR, lacking all four miR-19 binding sites, cloned downstream to the luciferase gene in Psicheck2 plasmid.

FIGs. 6A-E depict that miR-19b targets ADRb1 3'UTR via seed matches on its 3'UTR; Figures 6A-B are graphs illustrating normalized luciferase levels measured in HT22 cells that express low endogenous miR-19 levels following transfection with (Figure 6A) GFP plasmid or (Figure 6B) pre-miR-19b overexpression (OE) plasmid; Figures 6C-E are graphs illustrating normalized luciferase levels measured in HEK293T cells that express high endogenous miR-19 levels. Transfection with (Figure 6C) control plasmid, (Figure 6D) miR-19b knockdown (KD) probe or scrambled probe as control, and (Figure 6E) transfection with miR-19b miArrest plasmid or control miArrest plasmid. *** $P < 0.005$. Renilla luciferase activity was normalized by firefly luciferase expression levels and presented as ratio of activity achieved by the mutant form of Adrb1-3'UTR (Adrb1-mut) at the presence of control treatment.

FIGs. 7A-D depict differential expression of miRNA in the amygdale. Figures 7A-B are graphs illustrating differential expression of miRNA in the amygdala 90 minutes following acute stress. Figure 7A illustrates agilent array results. Figure 7b illustrates affymetrix array results. Normalized values are depicted as log2 ratio (stress vs. control) of spot intensity plotted against average intensities across conditions (N=2,2). The intensity of each miRNA was calculated as the average normalized intensity across biological repeats. miR-15a and miR-15b are indicated in red. miR-124, a well-established neuronal marker not affected by the stress protocol is indicated in white; Figure 7C illustrates that miR-15a and miR-15b have a semi-conserved seed match on corticotropin releasing hormone type 1 receptor 3'UTR [CRHR1, adapted from targetscan(dot)org]; and Figure 7D is a graph illustrating luciferase activity measured in HEK293T cells co-transfected with miR-15b-EGFP over-expressing or

GFP expressing plasmid and a luciferase reporter plasmid controlled by CRFR1-3'UTR. Renilla luciferase activity was normalized by firefly luciferase expression levels.

FIG. 8 is a graph illustrating luciferase reporter assay results indicating that miR-182 probably targets Htr1a 3'UTR. Luciferase assays data depicts renilla luciferase activity normalized to the activity of a co-transfected firefly luciferase reporter in HEK293 cells transfected with 3'UTR of the gene described and an empty vector, or a vector over-expressing a specific miR.

FIG. 9 is a graph illustrating real time PCR results of miR-182 expression levels in adult mice DRN indicating a trend for decreased expression following chronic social defeat. Data represents mean ± SEM n= 7 controls and 18 mice in social defeat group, # = p = 0.1.

FIG. 10 is a van diagram representing *in silico* bioinformatics predictions for miR-182 targets in two algorithms, and list of potential target genes highly relevant for normal and pathological neuronal function appearing in this prediction.

FIGs. 11A-C depict over-expression or knockdown of miR-182. Figure 11A is a schematic illustration of lentiviruses for over-expression of miR-182; Figure 11B is a graph illustrating real time PCR results indicating over-expression of miR-182 *in vitro* in N2A cell line; and Figure 11C is a schematic illustration of lentiviruses for knockdown of miR-182.

FIGs. 12A-D depict miR-19 levels in the PFC following NRI administration. The NRI reboxetine was administrated either acutely (once) or chronically (for 18 days). Of note, miR-19a and miR-19b levels decreased in the PFC following acute administration of NRI (Figure 12A and Figure 12B, respectively) but increased following chronic administration of NRI (Figure 12C and Figure 12D, respectively).

FIGs. 13A-D depict miR-19 levels in the PFC and amygdala of mice subjected to social defeat. miR-19a and miR-19b levels were measured in samples from amygdala taken from mice that were subjected to social defeat paradigm. Of note, miR-19a and miR-19b levels in the PFC were elevated in mice categorized as being "Susceptible" to social defeat relative to control mice (Figure 13A and Figure 13B, respectively). miR-19 levels were also elevated in the amygdala of mice categorized as being "Susceptible" to social defeat relative to control mice (Figure 13C and Figure 13D, respectively).

FIG. 14 depicts miRNA-19b targeting CB1 3'UTR. Transfection of HT-22 cells with CB1 3` UTR and plasmids overexpressing either miR-19b or GFP control lead to a 50 % decrease in normalized luciferase levels.

FIGs. 15A-B are schematic illustrations of a coronal section of the mouse brain. Figure 15A shows several nuclei in the brain including the BLA (adapted from the mouse brain by Paxinos and Franklin); Figure 15B shows a CB1 distribution in the brain (adapted from Allen Brain Atlas www.mouse.brain-map.org/). Of note, it is evident by this distribution that CB1 is abundant in the BLA.

FIG. 16 is a schematic illustration of a proposed mechanism for memory consolidation in the basolateral nucleus of the amygdala (BLA). Corticosterone (CORT) binds to a yet-uncharacterized membrane-bound glucocorticoid receptor (mbGR) that activates the Gs–cAMP/PKA pathway to induce endocannabinoid (eCB) synthesis. Endocannabinoids are released into the synapse where they bind to CB1 receptors on GABAergic terminals inhibiting GABA release. This inhibition of GABA release disinhibits norepinephrine (NE) release and increases NE activation of postsynaptic β-adrenoreceptors, increasing the consolidation of emotionally-aversive memories.

FIGs. 17A-B illustrate Ago2 in the RISC complex. Figure 17A is a schematic illustration of Ago2 in the RISC complex, mediating the interaction between the miRNA and the mRNA; Figure 17B illustrates a western blot analysis performed with anti-Ago2 antibody. This IP was specific to the Ago2 protein as can be seen when comparing the total brain sample that was precipitated once with the Ago2 antibody and once with the IgG1 control. Of note, there was no detection of the Ago2 protein on the samples precipitated with the IgG1 control.

FIGs. 18A-D depict a social avoidance test. Mice were placed in a maze for 3 minutes alone for habituation (Figure 18A and Figure 18B) and their movement was recorded and plotted. After 3 minutes a noval ICR mouse was placed in the chamber next to the examined mouse (Figure 18C and Figure 18D) and the movement of the examined mouse was recorded and plotted again.

FIG. 19A depicts a heatmap illustration of selected miRNAs up regulated in the arrays.

FIG. 19B depicts a heatmap illustration of selected miRNAs down regulated in the arrays.

FIGs. 20A-B depict a log2 expression of miR-15a (Figure 20A) and FKBP5 (Figure 20B) from the microarray results. Each red dot refers to one repetition of an array. The control group (CNT) had 4 repetitions, the "Susceptible" group (SUSC) had 3 repetitions and the "Resilient" group (RESIL) had 3 repetitions. The black line showed the mean of the repetitions in each group.

FIG. 20C depicts a 3` UTR sequence of mouse FKBP5 (taken from targetscan.org).

FIGs. 21A-B depict the levels of amygdalar miR-15a (Figure 21A) and FKBP5 (Figure 21B) in "Susceptible" mice relative to control mice following social defeat. Of note, miR-15a levels were elevated in the amygdala of mice subjected to social defeat and characterized as "Susceptible" (Figure 21A). FKBP5 levels were decreased in the amygdala of mice subjected to social defeat and characterized as "Susceptible" (Figure 21B).

FIG. 22 is a schematic illustration of the 3'UTR of Stx1a, Sgk1 and Adrb2, each harboring a single miRNA-15 binding site.

FIG. 23 depicts the levels of amygdalar miR-181 in mice subjected to social defeat relative to control mice. Of note, miR-181 levels were elevated in the amygdala of mice subjected to social defeat.

FIG. 24 depicts Van diagrams representing crossing bioinformatics predictions for miR-181 and glutamate receptors.

FIG. 25 is a schematic illustration of intact 3'UTR of 6 potential targets of miR-181.

FIG. 26 depicts expression levels of miR182 in the raphe nucleus following stress. Of note, an acute 30 minute immobilization stress led to decreased expression levels of miR182 in the RN when tested 24 hours following the stress as measured by real time PCR. **=P<0.01; n=8 in each group.

FIGs. 27A-C depict results of a luciferase reporter assay indicating that miR182 targets DSCAM, L1CAM and TSNAX 3'UTR. Figure 27A illustrates data of luciferase assays depicting renila luciferase activity normalized to the activity of a co-transfected firefly luciferase reporter in N2a cells transfected with 3'UTR of the genes described and an empty vector, or a vector over-expressing a specific miR. Mutation in miR182 seed match in L1cam (Figure 27B) and Tsnax (Figure 27C) 3' UTRs blocked the

represoric effect of miR182. Bars represent mean ± s.e.m. *P<0.05, **P<0.01, ***P<0.001.

FIGs. 28A-D depict expression of miR135 in the amygdala (AMY) and the prefrontal cortex (PFC). Figure 28A illustrates that acute SSRI and NRI increased miR135a levels in the AMY; Figure 28B illustrates that miR135b levels in the AMY were upregulated by acute SSRI or NRI administration compared to saline; Figure 28C illustrates that chronic SSRI decreased miR135a levels in the PFC; and Figure 28D illustrates that miR135b levels in the PFC were upregulated by acute SSRI or NRI and decreased by chronic SSRI treatment. n=7-8 in each group *=P<0.05;**=P<0.01;***=P<0.0001.

FIGs. 29A-B depict increased miR135 levels in mice circulation system following social defeat. miR135a (Figure 29A) and miR135b (Figure 29B) levels in plasma of mice two weeks following social defeat were significantly increased compared to control mice (**=P<0.01 n=7-16 in each group).

FIGs. 30A-E depict validation of miR135 KD *in vitro* and *in vivo*. Figures 30A-B illustrate results of a luciferase reporter assay indicating miR135 targeting of Htr1a (Figure 30A) and slc6a4 (Figure 30B) was blocked by the miR135b KD construct; Figure 30C is a schematic illustration of miR135bKD and control viral vectors; and Figures 30D-E are illustrations of a DRN injection site (Figure 30D adopted from Paxinos), and Figure 30E is a GFP staining of DRN infected with miR135 KD lentiviruses.

FIGs. 31A-G depict increased anxiety-like behavior and attenuated response to SSRI in miR135KD mice. Figure 31A illustrates that the behavior of miR135KD mice was similar to control mice in the open field test; Figure 31B illustrates increased anxiety-like behavior in miR135KD mice compared to control mice in the elevated pulse maze; Figure 31C illustrates that in the dark light transfer test miR135KD mice spent more time in the light chamber compared to control mice under basal stress conditions, but not following acute stress; Figure 31D illustrates that miR135KD mice visited the light chamber more times compared to control mice, under basal stress conditions, but not following acute stress; Figure 31E illustrates that miR135KD mice traveled less distance in the light chamber compared to control mice, under basal stress conditions, but not following acute stress; Figure 31F illustrates no difference between

miR135KD mice and control mice in tail suspension test both in basal conditions and following SSRI administration, yet reduction in immobility time was observed following SSRI treatment compared to basal condition in both groups. (Figures 31F-G). Immobility time was reduced by SSRI in both groups, however the reduction was attenuated in miR135KD mice compared to controls in the last 2 minutes of the test.~=p<0.1 *=p<0.05;**=p<0.01; ***=p<0.001. n=10-11 in each group.

FIG. 32 is a schematic illustration of miR135 mice inducible overexpression system. Transgenic mice expressing floxed transactional stop before miR135a sequence and GFP reporter. Mutant transgenic mice express miR135a only in 5-HT ePet positive cells.

FIGs. 33A-C depict validation of a mice line overexpressing miR135 in 5-HT neurons. Figure 33A illustrates that miR135 was overexpressed in the RN of miR135OE mice compared to control mice. Figures 33B-C illustrate that miR 135 target genes mRNA were downregulated in miR135OE mice RN, both Slc6a4 (Figure 33B) and Htr1a (Figure 33C). #=p<0.1 *=p<0.05; n=4 in each group

FIGs. 34A-E depict decreased anxiety and depression-like behavior following social defeat in miR135OE mice. Figure 34A shows that miR135OE mice have a decreased anxiety-like behavior in the open field test; Figure 34B shows less anxiety like behaviors compared to control of miR135OE mice in a dark light transfer test; Figure 34C shows decreased anxiety-like behavior compared to control in elevated pulse maze of miR135OE mice; Figure 34D shows tendency towards decreased immobility time of miR135OE mice compared to controls in tail suspension test; and Figure 34E shows reduced immobility time in miR135OE mice compared to controls in the forced swim test.#=p<0.1 *=p<0.05;**=p<0.01 n=7-11 in each group.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to microRNAs and, more particularly, but not exclusively, to the use of same for disease, diagnosis, monitoring and treatment.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

The link between dysregulated serotonergic activity and psychiatric disorders such as anxiety and depression has been previously established, yet the molecular mechanisms underlying these pathologies are not fully understood. MicroRNAs (miRs) are a subset of small RNA molecules that regulate gene expression post-transcriptionally and are abundant in the brain.

While reducing the present invention to practice, the present inventors have uncovered that specific microRNAs (miRs) are involved in regulation of serotonin (5HT) neuro-glia related genes and are thus involved in modulating medical conditions associated with aberrant serotonin levels such as psychiatric disorders.

As is illustrated hereinbelow and in the Examples section which follows, the present inventors determined the miRs expression pattern in 5HT neurons, obtained from the raphe nucleus (RN) of 5HT reporter mice (ePET-YFP), using miRs microarray (see Tables 2A-B in the Examples section which follows). The unique miRs expression profile of serotonergic neurons obtained from the array was bioinformatically analyzed to indentify miRs that putatively target key serotonergic related genes, such as serotonin transporter (Slc6a4, Figure 1D), serotonin auto receptor (Htr1a, Figure 1E), tryptophan hydroxylase 2 (Tph2, Figure 1F) and monoamine hydroxylase (MaoA, Figure 1G). miRNA targeting of the 3'UTRs for these genes were further tested *in vitro* illustrating specific miRs (e.g. miR-135) that specifically target and regulate the 5HT neuronal genes (see Figures 1H-I and Figures 2C-D). The present inventors have further illustrated that miR-135 expression levels are altered in the RN and plasma following acute stress (Figures 3A-D) and following treatment with antidepressants (Figures 3E-J). *In vivo* miR-135 over-expression in the RN of adult mice reduced depression-like behaviors following social defeat (Figures 4A-H). Moreover, the present inventors have illustrated the activity of miR-182 as a regulator of neuronal activity (via direct repression of Htr1a, Figure 8) and of psychopathological behavior (Figure 9) and of

miR-15 as regulator of stress response [via direct repression of CRH1R (Figures 7A-B), FK506 binding protein 5 (FKBP5) (Figures 21A-B) and Stx1a, Sgk1 and Adrb2 (Figure 22)]. The present inventors have also illustrated the specific targeting of beta adrenergic receptor (Adrb1) and canabinoid receptor 1 (CB1) by miR-19. miR-19 over-expression repressed Adrb1 (Figures 6A-C) while knockdown of miR-19 enhanced Adrb1 expression (Figures 6D-E). miR-19 over-expression also repressed CB1 (Figure 14). The present inventors have also uncovered targets for miR-181. Specifically, the present inventors have illustrated that miR-181 specifically regulates glutamate receptors (Figures 24 and 25). Taken together, these results substantiate the use of miRNAs or sequences regulating same, such as miR-135, miR-335, miR-181, miR-182, miR-26, miR-27, miR-15 and miR-19, as therapeutic modalities.

Thus, according to one aspect of the present invention there is provided a method of treating a medical condition in which an elevation of serotonin level is therapeutically beneficial in a subject in need thereof, the method comprising administering to or expressing in a cell of the subject an exogenous polynucleotide encoding at least one microRNA or a precursor thereof.

According to a specific embodiment, for treating a medical condition in which an elevation of serotonin level is therapeutically beneficial, the microRNA comprises miR-135, miR-335, miR-26 and miR-182.

According to another aspect of the present invention there is provided a method of treating a medical condition in which a low adrenaline or noradrenaline level is therapeutically beneficial in a subject in need thereof, the method comprising administering to or expressing in a cell of the subject an exogenous polynucleotide encoding a microRNA or a precursor thereof.

According to a specific embodiment, for treating a medical condition in which a low adrenaline or noradrenaline level is therapeutically beneficial, the microRNA comprises miR-19.

According to another aspect of the present invention there is provided a method of treating a medical condition in which a low corticotropin-releasing hormone (CRH) level is therapeutically beneficial in a subject in need thereof, the method comprising administering to or expressing in a cell of the subject an exogenous polynucleotide encoding a microRNA or a precursor thereof.

According to a specific embodiment, for treating a medical condition in which a low corticotropin-releasing hormone (CRH) level is therapeutically beneficial, the microRNA comprises miR-15.

According to another aspect of the present invention there is provided a method of treating a medical condition in which a low glutamate receptor level is therapeutically beneficial in a subject in need thereof, the method comprising administering to or expressing in a cell of the subject an exogenous polynucleotide encoding a microRNA or a precursor thereof.

According to a specific embodiment, for treating a medical condition in which a low glutamate receptor level is therapeutically beneficial, the microRNA comprises miR-181.

The term "treating" refers to inhibiting or arresting the development of a disease, disorder or condition and/or causing the reduction, remission, or regression of a disease, disorder or condition or keeping a disease, disorder or medical condition from occurring in a subject who may be at risk for the disease disorder or condition, but has not yet been diagnosed as having the disease disorder or condition. Those of skill in the art will understand that various methodologies and assays can be used to assess the development of a disease, disorder or condition, and similarly, various methodologies and assays may be used to assess the reduction, remission or regression of a disease, disorder or condition.

As used herein, the term "subject" includes mammals, preferably human beings at any age which suffer from the pathology. Preferably, this term encompasses individuals who are at risk to develop the pathology.

As used herein the phrase "medical condition in which an elevation of serotonin level is therapeutically beneficial" refers to a disease or disorder in which increasing the level of serotonin can prevent an occurrence of a disease or medical symptoms associated therewith or halt disease progression or medical symptoms associated therewith (as further detailed hereinbelow).

As used herein, the term "serotonin" refers to the monoamine neurotransmitter [also referred to as 5-hydroxytryptamine (5-HT)]. Serotonin is set forth e.g. in CAS number 50-67-9.

According to one embodiment, there is provided a method of increasing a serotonin level in a synaptic cleft, the method comprising administering to or expressing in a neuroglia cell e.g., serotonergic neuron of the subject an exogenous polynucleotide encoding at least one microRNA or a precursor thereof.

As used herein, the term "synaptic cleft" refers to the area between two neurons through which electrical or chemical signals pass.

A “neuroglia cell” refers to a neuron or a glial cell (e.g., oligodendrocytes or astrocyte).

As used herein, the term "serotonergic neuron" refers to a neuron which secretes serotonin or is capable of serotonin reuptake (i.e. by serotonin transporters expressed on their cell surfaces).

The medical condition in which an elevation of serotonin level is therapeutically beneficial may comprise, for example, any mood disorder including depression, anxiety, stress, fatigue, impaired cognitive function, panic attack, compulsive behavior, addiction, social phobia; sleep disorder, food related disorder, growth disorder and reproduction disorder.

According to a specific embodiment, the medical condition in which an elevation of serotonin level is therapeutically beneficial comprises depression.

According to one embodiment, when the medical condition is depression or anxiety, the microRNA is miR-135.

It will be appreciated that the depression or anxiety may not necessarily be related to serotonin.

As used herein the phrase "medical condition in which a low adrenaline or noradrenaline level is therapeutically beneficial" refers to a disease or disorder in which decreasing the expression or activity of adrenaline or noradrenaline can prevent an occurrence of a disease or medical symptoms associated therewith or halt disease progression or medical symptoms associated therewith (as further detailed hereinbelow).

As used herein, the term "adrenaline" refers to the hormone and neurotransmitter (also known as epinephrine). Adrenaline is set forth e.g. in CAS number 51-43-4.

As used herein, the term "noradrenaline" refers to the catecholamine acting as a hormone and neurotransmitter (also known as norepinephrine). Noradrenaline is set forth e.g. in CAS numbers (l) 51-41-2 (l) and 138-65-8(dl).

The medical condition in which a low adrenaline or noradrenaline level is therapeutically beneficial may comprise, for example, stress-related disorder, anxiety, memory impairment, heart conditions (e.g. palpitations, tachycardia, arrhythmia), headaches, tremors, hypertension, and acute pulmonary edema.

As used herein the phrase "medical condition in which a low corticotropin-releasing hormone (CRH) level is therapeutically beneficial" refers to a disease or disorder in which decreasing the expression or activity of CRH can prevent an occurrence of a disease or medical symptoms associated therewith or halt disease progression or medical symptoms associated therewith (as further detailed hereinbelow).

As used herein, the term "corticotropin-releasing hormone (CRH)" refers to the polypeptide hormone and neurotransmitter (also known as corticotropin-releasing factor (CRF) or corticoliberin). CRH is set forth e.g. in NP_000747.1.

The medical condition in which a low CRH level is therapeutically beneficial may comprise, for example, stress, depression, anxiety, stress, fatigue, impaired cognitive function, panic attack, compulsive behavior, addiction, social phobia, sleep disorder, food related disorder, growth disorder, reproduction disorder and obesity.

As used herein the phrase "medical condition in which a low glutamate receptor level is therapeutically beneficial" refers to a disease or disorder in which decreasing the expression or activity of a glutamate receptor can prevent an occurrence of a disease or medical symptoms associated therewith or halt disease progression or medical symptoms associated therewith (as further detailed hereinbelow).

As used herein, the term "glutamate receptor" refers to a synaptic receptor typically located on the membranes of neuronal cells (e.g. Grm1, Grik3, Grm5, Gria2, Grik2 and Grm7). Glutamate receptor is set forth e.g. in NP_000822.2 [glutamate receptor ionotropic kainate 3 (Grik3)]; NP_000817.2, NP_001077088.1, NP_001077089.1 [glutamate receptor ionotropic AMPA 2 (Gria2)]; NP_001159719.1, NP_068775.1, NP_786944.1 [glutamate receptor ionotropic kainate 2 (Grik2)]; NP_000833.1, NP_001137303.1 [glutamate receptor metabotropic 5 (Grm5)]; NP_000835.1, NP_870989.1 [glutamate receptor metabotropic 7 (Grm7)]; NP_000829.2, NP_001107801.1 [glutamate receptor metabotropic 1 (Grm1)].

The medical condition in which a low glutamate receptor level is therapeutically beneficial may comprise, for example, seizures (e.g. epilepsy), Huntigtons disease,

Schizophrenia, Fragile X syndrome, generalized anxiety disorder and cancer (e.g. melanoma).

As used herein, the term "microRNA or a precursor thereof" refers to the microRNA (miRNA) molecules acting as post-transcriptional regulators. MicroRNAs are typically processed from pre-miR (pre-microRNA precursors). Pre-miRs are a set of precursor miRNA molecules transcribed by RNA polymerase III that are efficiently processed into functional miRNAs, e.g., upon transfection into cultured cells. A Pre-miR can be used to elicit specific miRNA activity in cell types that do not normally express this miRNA, thus addressing the function of its target by down regulating its expression in a "gain of (miRNA) function" experiment. Pre-miR designs exist to all of the known miRNAs listed in the miRNA Registry and can be readily designed for any research. The microRNAs may be administered to the cell per se or encoded from a precursor molecule ligated into a nucleic acid construct, as further described hereinbelow.

It will be appreciated that the microRNAs of the present teachings may bind, attach, regulate, process, interfere, augment, stabilize and/or destabilize any microRNA target. Such a target can be any molecule, including, but not limited to, DNA molecules, RNA molecules and polypeptides, such as but not limited to, serotonin related genes, such as the serotonin transporter (i.e. SERT or Slc6a4), the serotonin inhibitory receptor 1a (Htr1a), tryptophan hydroxylase 2 (Tph2) and monoamine hydroxylase (MaoA); adrenaline or noradrenaline receptors (adrenergic receptors such as Adr1); Adenylate cyclase type 1 (ADCY1); CRH receptors such as Crh1R; or any other molecules e.g. FK506 binding protein 5 (FKBP5), canabinoid receptor 1 (CB1), Down Syndrome Cell Adhesion Molecule (Dscam), Translin-associated protein X (Tsnax) and Cell adhesion molecule L1 (L1cam), all described in further detail hereinbelow.

It will be appreciated that the microRNAs of the present invention can be identified via various databases including for example the micro-RNA registry (http://wwwdotsangerdotacdotuk/Software/Rfam/mirna/indexdotshtml).

The methods of the present invention may be effected by administering to or expressing in a cell of the subject an exogenous polynucleotide encoding a microRNA.

The term “polynucleotide” refers to a single-stranded or double-stranded oligomer or polymer of ribonucleic acid (RNA), deoxyribonucleic acid (DNA) or

mimetics thereof. This term includes polynucleotides and/or oligonucleotides derived from naturally occurring nucleic acids molecules (e.g., RNA or DNA), synthetic polynucleotide and/or oligonucleotide molecules composed of naturally occurring bases, sugars, and covalent internucleoside linkages (e.g., backbone), as well as synthetic polynucleotides and/or oligonucleotides having non-naturally occurring portions, which function similarly to respective naturally occurring portions.

The length of the polynucleotide of the present invention is optionally of 100 nucleotides or less, optionally of 90 nucleotides or less, optionally 80 nucleotides or less, optionally 70 nucleotides or less, optionally 60 nucleotides or less, optionally 50 nucleotides or less, optionally 40 nucleotides or less, optionally 30 nucleotides or less, e.g., 29 nucleotides, 28 nucleotides, 27 nucleotides, 26 nucleotides, 25 nucleotides, 24 nucleotides, 23 nucleotides, 22 nucleotides, 21 nucleotides, 20 nucleotides, 19 nucleotides, 18 nucleotides, 17 nucleotides, 16 nucleotides, 15 nucleotides, optionally between 12 and 24 nucleotides, optionally between 5-15, optionally, between 5-25, most preferably, about 20-25 nucleotides.

The polynucleotides (including oligonucleotides) designed according to the teachings of the present invention can be generated according to any oligonucleotide synthesis method known in the art, including both enzymatic syntheses or solid-phase syntheses. Equipment and reagents for executing solid-phase synthesis are commercially available from, for example, Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the capabilities of one skilled in the art and can be accomplished via established methodologies as detailed in, for example: Sambrook, J. and Russell, D. W. (2001), "Molecular Cloning: A Laboratory Manual"; Ausubel, R. M. et al., eds. (1994, 1989), "Current Protocols in Molecular Biology," Volumes I-III, John Wiley & Sons, Baltimore, Maryland; Perbal, B. (1988), "A Practical Guide to Molecular Cloning," John Wiley & Sons, New York; and Gait, M. J., ed. (1984), "Oligonucleotide Synthesis"; utilizing solid-phase chemistry, e.g. cyanoethyl phosphoramidite followed by deprotection, desalting, and purification by, for example, an automated trityl-on method or HPLC.

It will be appreciated that a polynucleotide comprising an RNA molecule can be generated using an expression vector as is further described hereinbelow.

Preferably, the polynucleotide of the present invention is a modified polynucleotide. Polynucleotides can be modified using various methods known in the art.

For example, the oligonucleotides or polynucleotides of the present invention may comprise heterocylic nucleosides consisting of purines and the pyrimidines bases, bonded in a 3'-to-5' phosphodiester linkage.

Preferably used oligonucleotides or polynucleotides are those modified either in backbone, internucleoside linkages, or bases, as is broadly described hereinunder.

Specific examples of preferred oligonucleotides or polynucleotides useful according to this aspect of the present invention include oligonucleotides or polynucleotides containing modified backbones or non-natural internucleoside linkages. Oligonucleotides or polynucleotides having modified backbones include those that retain a phosphorus atom in the backbone, as disclosed in U.S. Pat. Nos.: 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050.

Preferred modified oligonucleotide or polynucleotide backbones include, for example: phosphorothioates; chiral phosphorothioates; phosphorodithioates; phosphotriesters; aminoalkyl phosphotriesters; methyl and other alkyl phosphonates, including 3'-alkylene phosphonates and chiral phosphonates; phosphinates; phosphoramidates, including 3'-amino phosphoramidate and aminoalkylphosphoramidates; thionophosphoramidates; thionoalkylphosphonates; thionoalkylphosphotriesters; and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogues of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts, and free acid forms of the above modifications can also be used.

Alternatively, modified oligonucleotide or polynucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short-chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short-chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in

part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide, and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene-containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts, as disclosed in U.S. Pat. Nos.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439.

Other oligonucleotides or polynucleotides which may be used according to the present invention are those modified in both sugar and the internucleoside linkage, *i.e.*, the backbone of the nucleotide units is replaced with novel groups. The base units are maintained for complementation with the appropriate polynucleotide target. An example of such an oligonucleotide mimetic includes a peptide nucleic acid (PNA). A PNA oligonucleotide refers to an oligonucleotide where the sugar-backbone is replaced with an amide-containing backbone, in particular an aminoethylglycine backbone. The bases are retained and are bound directly or indirectly to aza-nitrogen atoms of the amide portion of the backbone. United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262; each of which is herein incorporated by reference. Other backbone modifications which may be used in the present invention are disclosed in U.S. Pat. No. 6,303,374.

Oligonucleotides or polynucleotides of the present invention may also include base modifications or substitutions. As used herein, "unmodified" or "natural" bases include the purine bases adenine (A) and guanine (G) and the pyrimidine bases thymine (T), cytosine (C), and uracil (U). "Modified" bases include but are not limited to other synthetic and natural bases, such as: 5-methylcytosine (5-me-C); 5-hydroxymethyl cytosine; xanthine; hypoxanthine; 2-aminoadenine; 6-methyl and other alkyl derivatives of adenine and guanine; 2-propyl and other alkyl derivatives of adenine and guanine; 2-thiouracil, 2-thiothymine, and 2-thiocytosine; 5-halouracil and cytosine; 5-propynyl uracil and cytosine; 6-azo uracil, cytosine, and thymine; 5-uracil (pseudouracil); 4-

thiouracil; 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl, and other 8-substituted adenines and guanines; 5-halo, particularly 5-bromo, 5-trifluoromethyl, and other 5-substituted uracils and cytosines; 7-methylguanine and 7-methyladenine; 8-azaguanine and 8-azaadenine; 7-deazaguanine and 7-deazaadenine; and 3-deazaguanine and 3-deazaadenine. Additional modified bases include those disclosed in: U.S. Pat. No. 3,687,808; Kroschwitz, J. I., ed. (1990),"The Concise Encyclopedia Of Polymer Science And Engineering," pages 858-859, John Wiley & Sons; Englisch et al. (1991), "Angewandte Chemie," International Edition, *30*, 613; and Sanghvi, Y. S., "Antisense Research and Applications," Chapter 15, pages 289-302, S. T. Crooke and B. Lebleu, eds., CRC Press, 1993. Such modified bases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines, and N-2, N-6, and O-6-substituted purines, including 2-aminopropyladenine, 5-propynyluracil, and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 ºC (Sanghvi, Y. S. et al. (1993), "Antisense Research and Applications," pages 276-278, CRC Press, Boca Raton), and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

According to a specific embodiment, the miRNA polynucleotide of the present invention has a nucleic acid sequence as set forth in SEQ ID NOs: 58-94 (see Table 1A).

Table 1A: miRNA polynucleotide sequences

Sequence	*miRNA*
SEQ ID NOs: 77-80	miR-15
SEQ ID NOs: 72-76	miR-19
SEQ ID NOs: 65-69	miR-26
SEQ ID NOs: 81-84	miR-27
SEQ ID NOs: 58-62	miR-135
SEQ ID NOs: 85-94	miR-181
SEQ ID NOs: 70-71	miR-182
SEQ ID NOs: 63-64	miR-335

As is mentioned hereinabove and is shown in the Examples section which follows, micro-RNAs are processed molecules derived from specific precursors (*i.e.*, pre-miRNA), upregulation of a specific miRNA function can be effected using a specific miRNA precursor molecule.

Also contemplated are sequences homlogous to the miRNAs and precursors thereof. The level of homology should be relatively high for the mature miRNA but more orders of freedom are allowed at the precursor level (e.g., at least 60 %, 70 %, 80 %, 85 %, 90 %, 95 % or more) as long as the sequence alterations are in the hair pin sequence and not in the nucleic acid segment corresponding to the mature miR.

Such precursor polynucleotide agents are typically administered to the target cells (e.g. neuroglia cells or cardiac cells) as part of an expression construct. In this case, the polynucleotide agent is ligated in a nucleic acid construct under the control of a cis-acting regulatory element (e.g. promoter) capable of directing an expression of the microRNA in the target cells (e.g. neuroglia cells or cardiac cells) in a constitutive or inducible manner.

Examples of microRNA polynucleotide agents of the present invention include, but are not limited to, miR-15 (e.g. GenBank accession no. NR_029485 RNA), miR-19 (e.g. GenBank accession no. NR_029489.1), miR-26 (e.g. GenBank accession nos. NR_029500 and NR_029499), miR-27 (e.g. GenBank accession no. NR_029501 RNA), miR-135 (e.g. GenBank accession no. NR_029677.1), miR-335 (e.g. GenBank accession no. NR_029899.1), miR-181 (e.g. GenBank accession no. NR_029611.1) and miR-182 (e.g. GenBank accession no. NR_029614).

Examples of neuron cell specific promoters include, but are not limited to, neuron-specific enolase gene promoter, synapsin promoter, enhanced synapsin promoter, calcium calmodulin promoter and Thy1 promoter.

Examples of cardiac cell specific promoters include, but are not limited to, cardiac NCX1 promoter and α-myosin heavy chain (αMHC) promoter.

The expression constructs of the present invention may also include additional sequences which render it suitable for replication and integration in eukaryotes (e.g., shuttle vectors). Typical cloning vectors contain transcription and translation initiation sequences (e.g., promoters, enhances) and transcription and translation terminators (e.g., polyadenylation signals). The expression constructs of the present invention can further

include an enhancer, which can be adjacent or distant to the promoter sequence and can function in up regulating the transcription therefrom.

Enhancer elements can stimulate transcription up to 1,000-fold from linked homologous or heterologous promoters. Enhancers are active when placed downstream or upstream from the transcription initiation site. Many enhancer elements derived from viruses have a broad host range and are active in a variety of tissues. For example, the SV40 early gene enhancer is suitable for many cell types. Other enhancer/promoter combinations that are suitable for the present invention include those derived from polyoma virus or human or murine cytomegalovirus (CMV) and the long tandem repeats (LTRs) from various retroviruses, such as murine leukemia virus, murine or Rous sarcoma virus, and HIV. See Gluzman, Y. and Shenk, T., eds. (1983). Enhancers and Eukaryotic Gene Expression, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference.

Polyadenylation sequences can also be added to the expression constructs of the present invention in order to increase the efficiency of expression of the detectable moeity. Two distinct sequence elements are required for accurate and efficient polyadenylation: GU- or U-rich sequences located downstream from the polyadenylation site and a highly conserved sequence of six nucleotides, namely AAUAAA, located 11-30 nucleotides upstream of the site. Termination and polyadenylation signals suitable for the present invention include those derived from SV40.

In addition to the embodiments already described, the expression constructs of the present invention may typically contain other specialized elements intended to increase the level of expression of cloned nucleic acids or to facilitate the identification of cells that carry the recombinant DNA. For example, a number of animal viruses contain DNA sequences that promote extra-chromosomal replication of the viral genome in permissive cell types. Plasmids bearing these viral replicons are replicated episomally as long as the appropriate factors are provided by genes either carried on the plasmid or with the genome of the host cell.

The expression constructs of the present invention may or may not include a eukaryotic replicon. If a eukaryotic replicon is present, the vector is capable of amplification in eukaryotic cells using the appropriate selectable marker. If the

construct does not comprise a eukaryotic replicon, no episomal amplification is possible. Instead, the recombinant DNA integrates into the genome of the engineered cell, where the promoter directs expression of the desired nucleic acid.

The nucleic acid construct may be introduced into the target cells (e.g. neuroglia cells or cardiac cells) of the present invention using an appropriate gene delivery vehicle/method (transfection, transduction, etc.) and an appropriate expression system.

Examples of mammalian expression vectors include, but are not limited to, pcDNA3, pcDNA3.1(+/-), pGL3, pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pSinRep5, DH26S, DHBB, pNMT1, pNMT41, and pNMT81, which are available from Invitrogen, pCI which is available from Promega, pMbac, pPbac, pBK-RSV and pBK-CMV, which are available from Strategene, pTRES which is available from Clontech, and their derivatives.

Expression vectors containing regulatory elements from eukaryotic viruses such as retroviruses can be also used. SV40 vectors include pSVT7 and pMT2, for instance. Vectors derived from bovine papilloma virus include pBV-1MTHA, and vectors derived from Epstein-Barr virus include pHEBO and p2O5. Other exemplary vectors include pMSG, pAV009/A^+, pMTO10/A^+, pMAMneo-5 and baculovirus pDSVE.

Lipid-based systems may be used for the delivery of these constructs into the target cells (e.g. neuroglia cells or cardiac cells) of the present invention.

Liposomes include any synthetic (i.e., not naturally occurring) structure composed of lipid bilayers, which enclose a volume. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. The liposomes may be prepared by any of the known methods in the art [Monkkonen, J. *et al.*, 1994, J. Drug Target, 2:299-308; Monkkonen, J. *et al.*, 1993, Calcif. Tissue Int., 53:139-145; Lasic D D., Liposomes Technology Inc., Elsevier, 1993, 63-105. (chapter 3); Winterhalter M, Lasic D D, Chem Phys Lipids, 1993 September;64(1-3):35-43]. The liposomes may be positively charged, neutral or negatively charged. For Mononuclear Phagocyte System (MPS) uptake, the liposomes can be hydrophobic since hydrophilic masking of the liposome membrane (e.g., by use of polyetheleneglycol-linked lipids and hydrophilic particles) may be less prone to MPS uptake. It is also preferable that the liposomes do not comprise sterically shielded lipids

such as ganglioside-GM_1 and phosphatidylinositol since these lipids prevent MPS uptake.

The liposomes may be a single lipid layer or may be multilamellar. If the therapeutic agent is hydrophilic, its delivery may be further improved using large unilamellar vesicles because of their greater internal volume. Conversely, if the therapeutic agent is hydrophobic, its delivery may be further improved using multilamellar vesicles. Alternatively, the therapeutic agent (e.g. oligonucleotide) may not be able to penetrate the lipid bilayer and consequently would remain adsorbed to the liposome surface. In this case, increasing the surface area of the liposome may further improve delivery of the therapeutic agent. Suitable liposomes in accordance with the invention are non-toxic liposomes such as, for example, those prepared from phosphatidyl-choline phosphoglycerol, and cholesterol. The diameter of the liposomes used can range from 0.1-1.0 microns. However, other size ranges suitable for phagocytosis by phagocytic cells may also be used. For sizing liposomes, homogenization may be used, which relies on shearing energy to fragment large liposomes into smaller ones. Homogenizers which may be conveniently used include microfluidizers produced by Microfluidics of Boston, MA. In a typical homogenization procedure, liposomes are recirculated through a standard emulsion homogenizer until selected liposomes sizes are observed. The particle size distribution can be monitored by conventional laser beam particle size discrimination. Extrusion of liposomes through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is an effective method for reducing liposome sizes to a relatively well defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome size distribution is achieved. The liposomes may be extruded through successively smaller pore membranes to achieve a gradual reduction in liposome size.

Any method known in the art can be used to incorporate micro-RNA polynucleotide agent into a liposome. For example, the micro-RNA polynucleotide agent may be encapsulated within the liposome. Alternatively, it may be adsorbed on the liposome's surface. Other methods that may be used to incorporate a pharmaceutical agent into a liposome of the present invention are those described by Alfonso *et al.*, [The science and practice of pharmacy, Mack Publishing, Easton Pa 19^{th} ed., (1995)] and those described by Kulkarni *et al.*,[J. Microencapsul.1995, 12 (3) 229-46].

The liposomes used in the methods of the present invention preferably cross the blood barriers. Thus, the liposomes of the present invention preferably do not comprise a blood barrier targeting polysaccharide (e.g. mannose) in their membrane portion. Preferably, the liposomes of the present invention do not comprise peptides in their membrane portion that target the liposomes to a receptor on a blood barrier. Examples of such peptides include but are not limited to transferrin, insulin, IGF-1, IGF-2 anti-transferrin receptor antibody, anti-insulin receptor antibody, anti-IGF-1 receptor antibody and anti-IGF-2 receptor antibody.

In order to determine liposomes that are especially suitable in accordance with the present invention a screening assay can be performed such as the assays described in U.S. Pat. Appl. No. 20040266734 and U.S. Pat. Appl. No. 20040266734; and in Danenberg *et al.*, Journal of cardiovascular pharmacology 2003, 42:671-9; Circulation 2002, 106:599-605; Circulation 2003, 108:2798-804

Other non-lipid based vectors that can be used according to this aspect of the present invention include but are not limited to polylysine and dendrimers.

The expression construct may also be a virus. Examples of viral constructs include but are not limited to adenoviral vectors, retroviral vectors, vaccinia viral vectors, adeno-associated viral vectors, polyoma viral vectors, alphaviral vectors, rhabdoviral vectors, lenti viral vectors and herpesviral vectors.

Retroviral vectors represent a class of vectors particularly suitable for use with the present invention. Defective retroviruses are routinely used in transfer of genes into mammalian cells (for a review, see Miller, A. D. (1990). Blood *76*, 271). A recombinant retrovirus comprising the polynucleotides of the present invention can be constructed using well-known molecular techniques. Portions of the retroviral genome can be removed to render the retrovirus replication machinery defective, and the replication-deficient retrovirus can then packaged into virions, which can be used to infect target cells through the use of a helper virus while employing standard techniques. Protocols for producing recombinant retroviruses and for infecting cells with viruses in vitro or in vivo can be found in, for example, Ausubel et al. (1994) Current Protocols in Molecular Biology (Greene Publishing Associates, Inc. & John Wiley & Sons, Inc.). Retroviruses have been used to introduce a variety of genes into many different cell types, including

neuronal cells, epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, and bone marrow cells.

According to one embodiment, a lentiviral vector, a type of retroviral vector, is used according to the present teachings. Lentiviral vectors are widely used as vectors due to their ability to integrate into the genome of non-dividing as well as dividing cells. The viral genome, in the form of RNA, is reverse-transcribed when the virus enters the cell to produce DNA, which is then inserted into the genome at a random position by the viral integrase enzyme. The vector (a provirus) remains in the genome and is passed on to the progeny of the cell when it divides. For safety reasons, lentiviral vectors never carry the genes required for their replication. To produce a lentivirus, several plasmids are transfected into a so-called packaging cell line, commonly HEK 293. One or more plasmids, generally referred to as packaging plasmids, encode the virion proteins, such as the capsid and the reverse transcriptase. Another plasmid contains the genetic material to be delivered by the vector. It is transcribed to produce the single-stranded RNA viral genome and is marked by the presence of the ψ (psi) sequence. This sequence is used to package the genome into the virion.

A specific example of a suitable lentiviral vector for introducing and expressing the polynucleotide sequences of the present invention in neuroglia cells or cardiac cells is the lentivirus pLKO.1 vector.

Another suitable expression vector that may be used according to this aspect of the present invention is the adenovirus vector. The adenovirus is an extensively studied and routinely used gene transfer vector. Key advantages of an adenovirus vector include relatively high transduction efficiency of dividing and quiescent cells, natural tropism to a wide range of epithelial tissues, and easy production of high titers (Russel, W. C. (2000) J Gen Virol *81*, 57-63). The adenovirus DNA is transported to the nucleus, but does not integrate thereinto. Thus the risk of mutagenesis with adenoviral vectors is minimized, while short-term expression is particularly suitable for treating cancer cells. Adenoviral vectors used in experimental cancer treatments are described by Seth et al. (1999). "Adenoviral vectors for cancer gene therapy," pp. 103-120, P. Seth, ed., Adenoviruses: Basic Biology to Gene Therapy, Landes, Austin, TX).

A suitable viral expression vector may also be a chimeric adenovirus/retrovirus vector combining retroviral and adenoviral components. Such vectors may be more

efficient than traditional expression vectors for transducing tumor cells (Pan et al. (2002). Cancer Letts *184,* 179-188).

When introducing the expression constructs of the present invention into target cells (e.g. neuroglia cells or cardiac cells) by viral infection the viral dose for infection is at least 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13}, 10^{14}, 10^{15} or higher pfu or viral particles.

Regardless of the method or construct employed, there is provided an isolated cell comprising the nucleic acid construct encoding a microRNA, as detailed above.

As used herein the term "isolated" refers to at least partially separated from the natural environment e.g., the human body.

According to one embodiment, there is provided an isolated cell comprising a nucleic acid construct expressing at least one microRNA or a precursor thereof, wherein the microRNA is selected from the group consisting of miR-135, miR-335, miR-15, miR-19, miR-26, miR-27, miR-181 and miR-182 under a transcriptional control of a cis acting regulatory element.

According to a specific embodiment, there is provided an isolated neuroglia cell comprising a nucleic acid construct expressing at least one microRNA or a precursor thereof, wherein the microRNA is selected from the group consisting of miR-135, miR-335, miR-26 and miR-182 under a transcriptional control of a cis acting regulatory element.

According to a specific embodiment, there is provided an isolated cell comprising a nucleic acid construct expressing a miR-19 or a precursor thereof under a transcriptional control of a cis acting regulatory element.

According to a specific embodiment, there is provided an isolated cell comprising a nucleic acid construct expressing a miR-15 or a precursor thereof under a transcriptional control of a cis acting regulatory element.

According to a specific embodiment, the cell is a neuroglia cell or a cardiac cell.

According to a specific embodiment, the neuroglia cell is a neuron such as a serotonergic neuron.

The microRNAs or precursors thereof are to be provided to the cells i.e., target cells (e.g. neuroglia cells or cardiac cells) of the present invention *in vivo* (*i.e.*, inside the organism or the subject) or *ex vivo* (e.g., in a tissue culture). In case the cells are

treated *ex vivo*, the method preferably includes a step of administering such cells back to the individual (*ex vivo* cell therapy).

For *ex vivo* therapy, cells are preferably treated with the agent of the present invention (e.g., a polynucleotide encoding a microRNA), following which they are administered to the subject in need thereof.

Administration of the *ex vivo* treated cells of the present invention can be effected using any suitable route of introduction, such as intravenous, intraperitoneal, intra-kidney, intra-gastrointestinal track, subcutaneous, transcutaneous, intramuscular, intracutaneous, intrathecal, epidural, and rectal. According to presently preferred embodiments, the *ex vivo* treated cells of the present invention may be introduced to the individual using intravenous, intra-kidney, intra-gastrointestinal track, and/or intraperitoneal administration.

The cells of the present invention (e.g. neuroglia cells or cardiac cells) can be derived from either autologous sources or from allogeneic sources such as human cadavers or donors. Since non-autologous cells are likely to induce an immune reaction when administered to the body several approaches have been developed to reduce the likelihood of rejection of non-autologous cells. These include either suppressing the recipient immune system or encapsulating the non-autologous cells in immunoisolating, semipermeable membranes before transplantation.

Encapsulation techniques are generally classified as microencapsulation, involving small spherical vehicles, and macroencapsulation, involving larger flat-sheet and hollow-fiber membranes (Uludag, H. et al. (2000). Technology of mammalian cell encapsulation. Adv Drug Deliv Rev *42*, 29-64).

Methods of preparing microcapsules are known in the art and include for example those disclosed in: Lu, M. Z. et al. (2000). Cell encapsulation with alginate and alpha-phenoxycinnamylidene-acetylated poly(allylamine). Biotechnol Bioeng *70*, 479-483; Chang, T. M. and Prakash, S. (2001) Procedures for microencapsulation of enzymes, cells and genetically engineered microorganisms. Mol Biotechnol *17*, 249-260; and Lu, M. Z., et al. (2000). A novel cell encapsulation method using photosensitive poly(allylamine alpha-cyanocinnamylideneacetate). J Microencapsul *17*, 245-521.

For example, microcapsules are prepared using modified collagen in a complex with a ter-polymer shell of 2-hydroxyethyl methylacrylate (HEMA), methacrylic acid (MAA), and methyl methacrylate (MMA), resulting in a capsule thickness of 2-5 μm. Such microcapsules can be further encapsulated with an additional 2-5 μm of ter-polymer shells in order to impart a negatively charged smooth surface and to minimize plasma protein absorption (Chia, S. M. et al. (2002). Multi-layered microcapsules for cell encapsulation. Biomaterials *23*, 849-856).

Other microcapsules are based on alginate, a marine polysaccharide (Sambanis, A. (2003). Encapsulated islets in diabetes treatment. Diabetes Thechnol Ther *5*, 665-668), or its derivatives. For example, microcapsules can be prepared by the polyelectrolyte complexation between the polyanions sodium alginate and sodium cellulose sulphate and the polycation poly(methylene-co-guanidine) hydrochloride in the presence of calcium chloride.

It will be appreciated that cell encapsulation is improved when smaller capsules are used. Thus, for instance, the quality control, mechanical stability, diffusion properties, and in vitro activities of encapsulated cells improved when the capsule size was reduced from 1 mm to 400 μm (Canaple, L. et al. (2002). Improving cell encapsulation through size control. J Biomater Sci Polym Ed *13*, 783-96). Moreover, nanoporous biocapsules with well-controlled pore size as small as 7 nm, tailored surface chemistries, and precise microarchitectures were found to successfully immunoisolate microenvironments for cells (See: Williams, D. (1999). Small is beautiful: microparticle and nanoparticle technology in medical devices. Med Device Technol *10*, 6-9; and Desai, T. A. (2002). Microfabrication technology for pancreatic cell encapsulation. Expert Opin Biol Ther *2*, 633-646).

Examples of immunosuppressive agents which may be used in conjunction with the ex vivo treatment include, but are not limited to, methotrexate, cyclophosphamide, cyclosporine, cyclosporin A, chloroquine, hydroxychloroquine, sulfasalazine (sulphasalazopyrine), gold salts, D-penicillamine, leflunomide, azathioprine, anakinra, infliximab (REMICADE.sup.R), etanercept, TNF.alpha. blockers, a biological agent that targets an inflammatory cytokine, and Non-Steroidal Anti-Inflammatory Drug (NSAIDs). Examples of NSAIDs include, but are not limited to acetyl salicylic acid, choline magnesium salicylate, diflunisal, magnesium salicylate, salsalate, sodium

salicylate, diclofenac, etodolac, fenoprofen, flurbiprofen, indomethacin, ketoprofen, ketorolac, meclofenamate, naproxen, nabumetone, phenylbutazone, piroxicam, sulindac, tolmetin, acetaminophen, ibuprofen, Cox-2 inhibitors and tramadol.

For *in vivo* therapy, the agent (e.g., a polynucleotide encoding a microRNA) is administered to the subject per se or as part of a pharmaceutical composition. Preferably such compositions are formulated to allow passage through the blood brain barrier (BBB).

Conventional approaches for drug delivery to the central nervous system (CNS) include: neurosurgical strategies (e.g., intracerebral injection or intracerebroventricular infusion); molecular manipulation of the agent (e.g., production of a chimeric fusion protein that comprises a transport peptide that has an affinity for an endothelial cell surface molecule in combination with an agent that is itself incapable of crossing the BBB) in an attempt to exploit one of the endogenous transport pathways of the BBB; pharmacological strategies designed to increase the lipid solubility of an agent (e.g., conjugation of water-soluble agents to lipid or cholesterol carriers); and the transitory disruption of the integrity of the BBB by hyperosmotic disruption (resulting from the infusion of a mannitol solution into the carotid artery or the use of a biologically active agent such as an angiotensin peptide).

Methods for drug delivery behind the BBB include intracerebral implantation (such as with needles) and convection-enhanced distribution. Mannitol can be used in bypassing the BBB. Likewise, mucosal (e.g., nasal) administration can be used to bypass the BBB.

The micro-RNA polynucleotide agents of the present invention can also be administered to an organism in a pharmaceutical composition where it is mixed with suitable carriers or excipients.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to the peptide accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intravenous, inrtaperitoneal, intranasal, or intraocular injections.

Alternately, one may administer the pharmaceutical composition in a local rather than systemic manner, for example, via injection of the pharmaceutical composition directly into a tissue region of a patient.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal

administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuos infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The pharmaceutical composition of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective

amount means an amount of active ingredients (peptide) effective to prevent, alleviate or ameliorate symptoms of a disorder (e.g., diabetes) or prolong the survival of the subject being treated.

According to an embodiment of the present invention, overexpression of miR-135 has an anti-depressant effect.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from in vitro and cell culture assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures in vitro, in cell cultures or experimental animals. The data obtained from these in vitro and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide sufficient plasma levels of the active ingredient to induce or suppress the biological effect (minimal effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration,

the judgment of the prescribing physician, etc. The dosage and timing of administration will be responsive to a careful and continuous monitoring of the individual changing condition.

It will be appreciated that animal models exist by which the agents of the present invention may be tested prior to human treatment. For example, animal models of depression, stress, anxiety such as learned helplessness model (LH), chronic mild stress (CMS) model, social defeat stress (SDS) model and maternal deprivation model may be used.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

It will be appreciated that the therapeutic compositions of the invention may comprise, in addition to the micro-RNA polynucleotide agents, other known medications for the treatment of depression, stress, anxiety, sleep deprivation, etc. such as, but not limited to, selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), noradrenergic and specific serotonergic antidepressants (NaSSAs), norepinephrine (noradrenaline) reuptake inhibitors (NRIs), norepinephrine-dopamine reuptake inhibitors, selective serotonin reuptake enhancers, norepinephrine-dopamine disinhibitors, tricyclic antidepressants (e.g. Imipramine), monoamine oxidase inhibitors (MAOIs). These medications may be included in the article of manufacture in a single or in separate packagings.

The present inventors have shown that overexpression of miR-27 results in suppression of MaoA (see Example 1, hereinbelow), overexpression of miR-135 results in suppression of Slc6a4 (see Example 1, hereinbelow), overexpression of miR-135, miR-335, miR-26, miR-181 or miR-182 results in suppression of Htr1a (see Example 1, hereinbelow), overexpression of miR-19 results in suppression of Adr1 (see Example 2, hereinbelow) and in suppression of CB1 (see Example 3B, hereinbelow), and that overexpression of miR-15 results in suppression of Crh1R (see Example 4, hereinbelow) and in suppression of FKBP5 (see Example 4B, hereinbelow).

Thus, according to one embodiment of the present invention, there is provided a method of regulating an expression of a serotonin transporter (Slc6a4) gene in a neuroglia cell, the method comprising modulating an activity or expression of a microRNA or a precursor thereof, wherein the microRNA is selected from the group consisting of miR-135 and miR-335.

As used herein, the term "serotonin transporter (Slc6a4)" refers to the monoamine transporter protein (also named SERT) involved in reuptake of serotonin from the synaptic cleft. An exemplary Slc6a4 is set forth in NP_001036.1.

According to another embodiment, there is provided a method of regulating an expression of a serotonin inhibitory receptor 1a (Htr1a) gene in a neuroglia cell, the method comprising modulating an activity or expression of a microRNA or a precursor thereof in the neuroglia cell, wherein the microRNA is selected from the group consisting of miR-135, miR-335, miR-181, miR-182 and miR-26.

As used herein, the term "serotonin inhibitory receptor 1a (Htr1a)" refers to the G protein-coupled receptor that functions as an autoreceptor in the presynaptic neuron and mediated inhibition of serotonin release. An exemplary Htr1a is set forth in NP_000515.2.

According to another embodiment, there is provided a method of regulating an expression of a monoamine hydroxylase (MaoA) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-27 or a precursor thereof.

As used herein, the term "monoamine hydroxylase (MaoA)" refers to the enzyme that degrades amine neurotransmitters, such as dopamine, norepinephrine, and serotonin. An exemplary MaoA is set forth in NP_000231.1.

According to one embodiment of the present invention, there is provided a method of regulating an expression of a tryptophan hydroxylase 2 (Tph2) gene in a neuroglia cell, the method comprising modulating an activity or expression of a microRNA or a precursor thereof in the neuroglia cell, wherein the microRNA is selected from the group consisting of miR-181 and miR27.

As used herein, the term "tryptophan hydroxylase 2 (Tph2)" refers to the enzyme which catalyzes the first and rate limiting step in the biosynthesis of serotonin. In exemplary Tph2 is set forth in NP_ NP_775489.2.

According to another embodiment, there is provided a method of regulating an expression of a beta adrenergic receptor 1 (Adrb1) gene in a neuroglia cell or cardiac cell, the method comprising modulating an activity or expression of a miR-19 or a precursor thereof.

As used herein, the term "beta adrenergic receptor 1 (Adrb1)" refers to the receptor that mediates the physiological effects of adrenaline and noradrenaline. An exemplary Adrb1is set forth in NP_000675.1.

According to another embodiment, there is provided a method of regulating an expression of a beta 2 adrenergic receptor (Adrb2) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-15 or a precursor thereof.

As used herein, the term "beta 2 adrenergic receptor (Adrb2)" refers to the receptor that is directly associated with the class C L-type calcium channel Ca(V)1.2. Adrb2 is set forth e.g. in NP_000015.1.

According to another embodiment, there is provided a method of regulating an expression of a CRH type 1 receptor gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-15 or a precursor thereof.

As used herein, the term "CRH type 1" refers to the receptor which binds corticotropin-releasing hormone (CRH). CRH type 1 is set forth e.g. in NP_001138618.1, NP_001138619.1, NP_001138620.1 and NP_004373.2.

According to another embodiment, there is provided a method of regulating an expression of a glutamate receptor gene in a neuroglia cell, the method comprising modulating an activity or expression of miR-181 or a precursor thereof.

According to another embodiment, the glutamate receptor gene comprises glutamate receptor metabotropic 1 (Grm1), glutamate receptor ionotropic kainate 3

(Grik3), glutamate receptor metabotropic 5 (Grm5), glutamate receptor ionotropic kainate 2 (Grik2) and glutamate receptor metabotropic 7 (Grm7), as described in further detail above.

According to another embodiment, there is provided a method of regulating an expression of a Down Syndrome Cell Adhesion Molecule (Dscam) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-182 or a precursor thereof.

As used herein, the term "Down Syndrome Cell Adhesion Molecule (Dscam)" refers to the cell adhesion molecule that plays a role in neuronal self-avoidance. Dscam is set forth e.g. in NP_001380.2.

According to another embodiment, there is provided a method of regulating an expression of a Cell adhesion molecule L1 (L1cam) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-182 or a precursor thereof.

As used herein, the term "Cell adhesion molecule L1 (L1cam)" refers to the neuronal cell adhesion molecule. L1cam is set forth e.g. in NP_000416.1, NP_001137435.1, NP_076493.1.

According to another embodiment, there is provided a method of regulating an expression of a Translin-associated protein X (Tsnax) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-182 or a precursor thereof.

As used herein, the term "Translin-associated protein X (Tsnax)" refers to the protein which specifically interacts with translin. Tsnax is set forth e.g. in NP_005990.1.

According to another embodiment, there is provided a method of regulating an expression of a canabinoid receptor 1 (CB1) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-19 or a precursor thereof.

As used herein, the term "canabinoid receptor 1 (CB1)" refers to the of cell membrane receptor (also known as CNR1). CB1 is set forth e.g. in NP_001153698.1, NP_001153730.1, NP_001153731.1, NP_057167.2, NP_149421.2.

According to another embodiment, there is provided a method of regulating an expression of a FK506 binding protein 5 (FKBP5) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-15 or a precursor thereof.

As used herein, the term "FK506 binding protein 5 (FKBP5)" refers to the protein which specifically binds to the immunosuppressants FK506 and rapamycin. FKBP5 is set forth e.g. in NP_001139247.1, NP_001139248.1, NP_001139249.1, NP_004108.1.

According to another embodiment, there is provided a method of regulating an expression of a syntaxin 1a (Stx1a) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-15 or a precursor thereof.

As used herein, the term "syntaxin 1a (Stx1a)" refers to the nervous system-specific protein. Stx1a is set forth e.g. in NP_001159375.1, NP_004594.1.

According to another embodiment, there is provided a method of regulating an expression of a serum/glucocorticoid regulated kinase (Sgk1) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-15 or a precursor thereof.

As used herein, the term "serum/glucocorticoid regulated kinase (Sgk1)" refers to serine/threonine protein kinase. Sgk1 is set forth e.g. in NP_001137148.1, NP_001137149.1, NP_001137150.1, NP_005618.2.

The present teachings contemplate upregulating (i.e. increasing) or downregulating (i.e. decreasing) the expression levels of the aforementioned genes.

Downregulation of gene expression according to the present teachings is typically carried out by administering to or expressing in the target cells (e.g. neuroglia cell or cardiac cell) a microRNA polynucleotide (as depicted in further detail hereinabove).

According to a specific embodiment, when the regulating comprises downregulating the expression of the Slc6a4 gene, the modulating comprises upregulating the miR-135 and/or miR-335.

According to a specific embodiment, when the regulating comprises downregulating the expression of the Htr1a gene, the modulating comprises upregulating the miR-135, miR-335, miR-181, miR-182 and/or miR-26.

According to a specific embodiment, when the regulating comprises downregulating the expression of the MaoA gene, the modulating comprises upregulating the miR-27.

According to a specific embodiment, when the regulating comprises downregulating the expression of the Adrb1 gene, the modulating comprises upregulating the miR-19.

According to a specific embodiment, when the regulating comprises downregulating the expression of the CRH type 1 receptor gene, the modulating comprises upregulating the miR-15.

According to a specific embodiment, when the regulating comprises downregulating the expression of the CB1 gene, the modulating comprises upregulating the miR-19.

According to a specific embodiment, when the regulating comprises downregulating the expression of the FKBP5 gene, the modulating comprises upregulating the miR-15.

Alternatively, according to another embodiment of the present invention, upregulating gene expression is affected by administering to or expressing in the target cells (e.g. neuroglia cell or cardiac cell) an agent capable of downregulating an expression of a microRNA.

Downregulation of microRNAs can be effected on the genomic and/or the transcript level using a variety of molecules which interfere with transcription and/or translation (e.g., RNA silencing agents, Ribozyme, DNAzyme and antisense).

Methods of downregulating microRNA expression are known in the art.

Nucleic acid agents that down-regulate miR activity include, but are not limited to, a target mimic, a micro-RNA resistant gene and a miRNA inhibitor.

The target mimic or micro-RNA resistant target is essentially complementary to the microRNA provided that one or more of following mismatches are allowed:

(a) a mismatch between the nucleotide at the 5' end of the microRNA and the corresponding nucleotide sequence in the target mimic or micro-RNA resistant target;

(b) a mismatch between any one of the nucleotides in position 1 to position 9 of the microRNA and the corresponding nucleotide sequence in the target mimic or micro-RNA resistant target; or

(c) three mismatches between any one of the nucleotides in position 12 to position 21 of the microRNA and the corresponding nucleotide sequence in the target

mimic or micro-RNA resistant target provided that there are no more than two consecutive mismatches.

The target mimic RNA is essentially similar to the target RNA modified to render it resistant to miRNA induced cleavage, e.g. by modifying the sequence thereof such that a variation is introduced in the nucleotide of the target sequence complementary to the nucleotides 10 or 11 of the miRNA resulting in a mismatch.

Alternatively, a microRNA-resistant target may be implemented. Thus, a silent mutation may be introduced in the microRNA binding site of the target gene so that the DNA and resulting RNA sequences are changed in a way that prevents microRNA binding, but the amino acid sequence of the protein is unchanged. Thus, a new sequence can be synthesized instead of the existing binding site, in which the DNA sequence is changed, resulting in lack of miRNA binding to its target.

According to a specific embodiment, the target mimic or micro-RNA resistant target is linked to the promoter naturally associated with the pre-miRNA recognizing the target gene and introduced into the plant cell. In this way, the miRNA target mimic or micro-RNA resistant target RNA will be expressed under the same circumstances as the miRNA and the target mimic or micro-RNA resistant target RNA will substitute for the non-target mimic/micro-RNA resistant target RNA degraded by the miRNA induced cleavage.

Non-functional miRNA alleles or miRNA resistant target genes may also be introduced by homologous recombination to substitute the miRNA encoding alleles or miRNA sensitive target genes.

Recombinant expression is effected by cloning the nucleic acid of interest (e.g., miRNA, target gene, silencing agent etc) into a nucleic acid expression construct under the expression of a plant promoter.

In other embodiments of the invention, synthetic single stranded nucleic acids are used as miRNA inhibitors. A miRNA inhibitor is typically between about 17 to 25 nucleotides in length and comprises a 5' to 3' sequence that is at least 90 % complementary to the 5' to 3' sequence of a mature miRNA. In certain embodiments, a miRNA inhibitor molecule is 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length, or any range derivable therein. Moreover, a miRNA inhibitor has a sequence (from 5' to 3') that is or is at least 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.1, 99.2, 99.3, 99.4, 99.5,

99.6, 99.7, 99.8, 99.9 or 100 % complementary, or any range derivable therein, to the 5' to 3' sequence of a mature miRNA, particularly a mature, naturally occurring miRNA.

The miRNA inhibitors may be contacted with the cells using transient transfection techniques. miRNA inhibitors are commercially available from Companies such as Applied Biosystems.

Alternatively, the miRNA inhibitors may be part of an expression vector, as described herein above. In this case, cells may be transiently or stably transfected with the vector.

According to a specific embodiment, when the regulating comprises upregulating the expression of the Tph2 gene, the modulating comprises downregulating the miR-181 and/or miR-27.

According to one embodiment, downregulating the expression of a microRNA is effected by the use of a nucleic acid sequence which specifically binds and downregulates the expression of the microRNA. An exemplary nucleic acid sequence which may be used in accordance with the present invention may be purchased from any manufacturer, as for example, from Genecopoeia (miArrest, microRNA vector based inhibitors).

Thus, according to another embodiment, there is provide an isolated polynucleotide comprising a nucleic acid sequence for downregulating an expression of miR-181, miR-182, miR-26, miR-27, miR-135, miR-335, miR-15 and miR-19 or a precursor thereof.

Exemplary polynucleotides which may be used in accordance with the present invention to downregulate the expression of miR-181 include, but are not limited to, those set in SEQ ID NOs: 134-137 and SEQ ID NOs: 154-157.

Exemplary polynucleotides which may be used in accordance with the present invention to downregulate the expression of miR-182 include, but are not limited to, those set in SEQ ID NOs: 138-141 and SEQ ID NO: 147.

Exemplary polynucleotides which may be used in accordance with the present invention to downregulate the expression of miR-26 include, but are not limited to, those set in SEQ ID NOs: 126-129 and SEQ ID NOs: 145-146.

Exemplary polynucleotides which may be used in accordance with the present invention to downregulate the expression of miR-27 include, but are not limited to, those set in SEQ ID NOs: 130-133 and SEQ ID NOs: 152-153.

Exemplary polynucleotides which may be used in accordance with the present invention to downregulate the expression of miR-135 include, but are not limited to, those set in SEQ ID NOs: 110-113 and SEQ ID NOs: 142-143.

Exemplary polynucleotides which may be used in accordance with the present invention to downregulate the expression of miR-335 include, but are not limited to, those set in SEQ ID NOs: 114-117 and SEQ ID NO: 144.

Exemplary polynucleotides which may be used in accordance with the present invention to downregulate the expression of miR-15 include, but are not limited to, those set in SEQ ID NOs: 118-121 and SEQ ID NOs: 150-151.

Exemplary polynucleotides which may be used in accordance with the present invention to downregulate the expression of miR-19 include, but are not limited to, those set in SEQ ID NOs: 122-125 and SEQ ID NOs: 148-149.

Such nucleic acid sequences may be further comprised in an expression vector as described in further detail hereinabove.

The present invention further contemplates assessing the expression of the target gene (e.g. transcript or polypeptide) following downregulating or upregulating the microRNA level in the cell (e.g. neuroglia cell or cardiac cell).

Thus, the presence and/or level of a target gene (e.g. Slc6a4, Htr1a, MaoA, Adrb1, Adrb2,_CRH type 1 receptor, CB1, FKBP5, Tph2, Grm1, Grik3, Grm5, Grik2, Grm7, Gria2, Dscam, L1cam, Tsnax, Sgk1 and/or Stx1a) nucleic acid sequence (e.g. transcript) can be determined using an isolated polynucleotide (e.g., a polynucleotide probe, an oligonucleotide probe/primer) capable of hybridizing to a target gene's nucleic acid sequence (e.g. Slc6a4 as set forth in e.g. NM_001045.4 or a portion thereof; Htr1a as set forth in e.g. NM_000524.3 or a portion thereof; MaoA as set forth in e.g. NM_000240.3 or NM_001270458.1 or a portion thereof; Adrb1 as set forth in e.g. NM_000684.2 or a portion thereof; Adrb2 as set forth in e.g. NM_000024.5 or a portion thereof; CRH type 1 receptor as set forth in e.g. NM_001145146.1, NM_001145147.1 or a portion thereof; CB1 as set forth in e.g. NM_001160226.1, NM_033181.3 or a portion thereof; FKBP5 as set forth in e.g. NM_001145775.1, NM_001145777.1 or a portion

thereof; Tph2 as set forth in e.g. NM_173353.3 or a portion thereof; Grm1 as set forth in e.g. NM_000838.3, NM_001114329.1 or a portion thereof; Grik3 as set forth in e.g. NM_000831.3 or a portion thereof; Grm5 as set forth in e.g. NM_000842.3, NM_001143831.2 or a portion thereof; Grik2 as set forth in e.g. NM_001166247.1, NM_021956.4 or a portion thereof; Grm7 as set forth in e.g. NM_000844.3, NM_181874.2 or a portion thereof; Gria2 as set forth in e.g. NM_000826.3, NM_001083619.1 or a portion thereof; Dscam as set forth in e.g. NM_001389.3 or a portion thereof; L1cam as set forth in e.g. NM_000425.3, NM_001143963.1, NM_024003.2 or a portion thereof; Tsnax_as set forth in e.g. NM_005999.2 or a portion thereof; Sgk1 as set forth in e.g. NM_001143676.1, NM_001143677.1, NM_001143678.1 or a portion thereof and/or Stx1a as set forth in e.g. NM_001165903.1, NM_004603.3 or a portion thereof). Such a polynucleotide can be at any size, such as a short polynucleotide (e.g., of 15-200 bases), and intermediate polynucleotide (e.g., 200-2000 bases) or a long polynucleotide larger of 2000 bases.

The isolated polynucleotide probe used by the present invention can be any directly or indirectly labeled RNA molecule (e.g., RNA oligonucleotide, an *in vitro* transcribed RNA molecule), DNA molecule (e.g., oligonucleotide, cDNA molecule, genomic molecule) and/or an analogue thereof [e.g., peptide nucleic acid (PNA)] which is specific to the target gene RNA transcript of the present invention.

Oligonucleotides designed according to the teachings of the present invention can be generated according to any oligonucleotide synthesis method known in the art, as described in detail hereinabove.

The oligonucleotide of the present invention is of at least 17, at least 18, at least 19, at least 20, at least 22, at least 25, at least 30 or at least 40, bases specifically hybridizable with sequence alterations described hereinabove.

The oligonucleotides of the present invention may comprise heterocylic nucleosides consisting of purines and the pyrimidines bases, bonded in a 3' to 5' phosphodiester linkage.

Preferably used oligonucleotides are those modified in either backbone, internucleoside linkages or bases, as is broadly described hereinabove.

The isolated polynucleotide used by the present invention can be labeled either directly or indirectly using a tag or label molecule. Such labels can be, for example,

fluorescent molecules (e.g., fluorescein or Texas Red), radioactive molecule (e.g., ^{32}P-γ-ATP or ^{32}P-α-ATP) and chromogenic substrates [e.g., Fast Red, BCIP/INT, available from (ABCAM, Cambridge, MA)]. Direct labeling can be achieved by covalently conjugating a label molecule to the polynucleotide (e.g., using solid-phase synthesis) or by incorporation via polymerization (e.g., using an *in vitro* transcription reaction or random-primed labeling). Indirect labeling can be achieved by covalently conjugating or incorporating to the polynucleotide a non-labeled tag molecule (e.g., Digoxigenin or biotin) and subsequently subjecting the polynucleotide to a labeled molecule (e.g., anti-Digoxigenin antibody or streptavidin) capable of specifically recognizing the non-labeled tag.

The above-described polynucleotides can be employed in a variety of RNA detection methods such as Northern blot analysis, reverse-transcribed PCR (RT-PCR) [e.g., a semi-quantitative RT-PCR, quantitative RT-PCR using e.g., the Light Cycler™ (Roche)], RNA *in situ* hybridization (RNA-ISH), *in situ* RT-PCR stain [e.g., as described in Nuovo GJ, et al. 1993, Intracellular localization of polymerase chain reaction (PCR)-amplified hepatitis C cDNA. Am J Surg Pathol. 17: 683-90, and Komminoth P, et al. 1994, Evaluation of methods for hepatitis C virus detection in archival liver biopsies. Comparison of histology, immunohistochemistry, *in situ* hybridization, reverse transcriptase polymerase chain reaction (RT-PCR) and *in situ* RT-PCR. Pathol Res Pract., 190: 1017-25] and oligonucleotide microarray analysis [e.g., using the Affymetrix microarray (Affymetrix®, Santa Clara, CA)].

The presence and/or level of the target gene (e.g. Slc6a4, Htr1a, MaoA, Adrb1, Adrb2, CRH type 1 receptor, CB1, FKBP5, Tph2, Grm1, Grik3, Grm5, Grik2, Grm7, Gria2, Dscam, L1cam, Tsnax, Sgk1 and/or Stx1a) amino acid sequence (e.g. protein) can be determined using, for example, a specific antibody via the formation of an immunocomplex [*i.e.*, a complex formed between the target gene antigen (an amino acid sequence) present in the biological sample and the specific antibody].

The immunocomplex of the present invention can be formed at a variety of temperatures, salt concentration and pH values which may vary depending on the method and the biological sample used and those of skills in the art are capable of adjusting the conditions suitable for the formation of each immunocomplex.

The term "antibody" as used in this invention includes intact molecules as well as functional fragments thereof, such as Fab, F(ab')2, Fv or single domain molecules such as VH and VL to an epitope of an antigen. These functional antibody fragments are defined as follows: (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) (Fab')2, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')2 is a dimer of two Fab' fragments held together by two disulfide bonds; (4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; (5) Single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule; and (6) Single domain antibodies are composed of a single VH or VL domains which exhibit sufficient affinity to the antigen.

Methods of producing polyclonal and monoclonal antibodies as well as fragments thereof are well known in the art (See for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

Antibody fragments according to the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in E. coli or mammalian cells (e.g. Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')2. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab'

fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647, and references contained therein, which patents are hereby incorporated by reference in their entirety. See also Porter, R. R. [Biochem. J. 73: 119-126 (1959)]. Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

Fv fragments comprise an association of VH and VL chains. This association may be noncovalent, as described in Inbar et al. [Proc. Nat'l Acad. Sci. USA 69:2659-62 (19720]. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise VH and VL chains connected by a peptide linker. These single-chain antigen binding proteins (scFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as E. coli. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing scFvs are described, for example, by Whitlow and Filpula, Methods 2: 97-105 (1991); Bird et al., Science 242:423-426 (1988); Pack et al., Bio/Technology 11:1271-77 (1993); and U.S. Pat. No. 4,946,778, which is hereby incorporated by reference in its entirety.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick and Fry [Methods, 2: 106-10 (1991)].

Antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies

(Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)]. Similarly, human antibodies can be made by introduction of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., Bio/Technology 10,: 779-783 (1992); Lonberg et al., Nature 368: 856-859 (1994); Morrison, Nature 368 812-13 (1994); Fishwild et al., Nature Biotechnology 14, 845-51 (1996); Neuberger, Nature Biotechnology 14: 826 (1996); and Lonberg and Huszar, Intern. Rev. Immunol. 13, 65-93 (1995).

Exemplary antibodies which may be used in accordance with the present invention include e.g. anti-Slc6a4 antibody available e.g. from Abnova Corporation, Abgent and MBL International; anti-Htr1a antibody available e.g. from Novus Biologicals, Acris Antibodies GmbH and Abnova Corporation; anti-MaoA antibody available e.g. from Abnova Corporation, Proteintech Group, Inc. and Abgent; anti-Adrb1 antibody available e.g. from Biorbyt, Abgent and antibodies-online; anti-Adrb2 antibody available e.g. from Tocris Bioscience, Abnova Corporation and antibodies-online; anti-CRH type 1 receptor antibody available e.g. from MyBioSource.com, Abcam and Novus Biologicals; anti-CB1 antibody available e.g. from Santa Cruz Biotechnology, Inc. and Epitomics, Inc.; anti-FKBP5 antibody available e.g. from BD Biosciences and Abnova Corporation; anti-Tph2 antibody available e.g. from Novus Biologicals and Acris Antibodies GmbH; anti-Grm1 antibody available e.g. from Novus Biologicals and Biorbyt; anti-Grik3 antibody available e.g. from Acris Antibodies GmbH and Atlas Antibodies; anti-Grm5 antibody available e.g. from Biorbyt and Acris Antibodies GmbH; anti-Grik2 antibody available e.g. from Proteintech Group, Inc., Aviva Systems Biology and Abgent; anti-Grm7 antibody available e.g. from Acris Antibodies GmbH and antibodies-online; anti-Gria2 antibody available e.g. from Proteintech Group, Inc. and Abnova Corporation; anti-Dscam antibody available e.g. from Novus Biologicals and R&D Systems; anti-L1cam antibody available e.g. from GeneTex, Novus Biologicals and Acris Antibodies GmbH; anti-Tsnax antibody

available e.g. from BD Biosciences and GenWay Biotech, Inc.; anti-Sgk1 antibody available e.g. from Epitomics, Inc. and Acris Antibodies GmbH; and/or anti-Stx1a antibody available e.g. from MBL International and Spring Bioscience.

Various methods can be used to detect the formation of the immunocomplex of the present invention and those of skills in the art are capable of determining which method is suitable for each immunocomplex and/or the type of cells used for diagnosis.

The specific antibody (e.g. anti-Slc6a4 antibody; anti-Htr1a antibody; anti-MaoA antibody; anti-Adrb1 antibody; anti-Adrb2 antibody; anti-CRH type 1 receptor antibody; anti-CB1 antibody; anti-FKBP5 antibody; anti-Tph2 antibody; anti-Grm1 antibody; anti-Grik3 antibody; anti-Grm5 antibody; anti-Grik2 antibody; anti-Grm7 antibody; anti-Gria2 antibody; anti-Dscam antibody; anti-L1cam antibody; anti-Tsnax antibody; anti-Sgk1 antibody and/or anti-Stx1a antibody) used in the immunocomplex of the present invention can be labeled using methods known in the art. It will be appreciated that the labeled antibodies can be either primary antibodies (*i.e.*, which bind to the specific antigen, e.g., a target gene-specific antigen) or secondary antibodies (e.g., labeled goat anti rabbit antibodies, labeled mouse anti human antibody) which bind to the primary antibodies. The antibody can be directly conjugated to a label or can be conjugated to an enzyme.

Antibodies of the present invention can be fluorescently labeled (using a fluorescent dye conjugated to an antibody), radiolabeled (using radiolabeled e.g., ^{125}I, antibodies), or conjugated to an enzyme (e.g., horseradish peroxidase or alkaline phosphatase) and used along with a chromogenic substrate to produce a colorimetric reaction. The chromogenic substrates utilized by the enzyme-conjugated antibodies of the present invention include, but are not limited to, AEC, Fast red, ELF-97 substrate [2-(5'-chloro-2-phosphoryloxyphenyl)-6-chloro-4(3H)-quinazolinone], p-nitrophenyl phosphate (PNPP), phenolphthalein diphosphate, and ELF 39-phosphate, BCIP/INT, Vector Red (VR), salmon and magenta phosphate (Avivi C., et al., 1994, J Histochem. Cytochem. 1994; 42: 551-4) for alkaline phosphatase enzyme and Nova Red, diaminobenzidine (DAB), Vector(R) SG substrate, luminol-based chemiluminescent substrate for the peroxidase enzyme. These enzymatic substrates are commercially available from Sigma (St Louis, MO, USA), Molecular Probes Inc. (Eugene, OR, USA),

Vector Laboratories Inc. (Burlingame, CA, USA), Zymed Laboratories Inc. (San Francisco, CA, USA), Dako Cytomation (Denmark).

Detection of the immunocomplex in a biological sample, such as blood sample or serum, which may contain soluble (e.g., secreted, shedded) target gene polypeptide can be performed using fluorescence activated cell sorting (FACS), enzyme linked immunosorbent assay (ELISA), Western blot and radio-immunoassay (RIA) analyses, immunoprecipitation (IP) or by a molecular weight-based approach.

For Western blot the proteins are extracted from a cell sample and are subjected to electrophoresis (e.g., SDS-PAGE) and blotting to a membrane (e.g., nylon or PVDF). The membrane is then interacted with a specific antibody (e.g. anti-Slc6a4 antibody; anti-Htr1a antibody; anti-MaoA antibody; anti-Adrb1 antibody; anti-Adrb2 antibody; anti-CRH type 1 receptor antibody; anti-CB1 antibody; anti-FKBP5 antibody; anti-Tph2 antibody; anti-Grm1 antibody; anti-Grik3 antibody; anti-Grm5 antibody; anti-Grik2 antibody; anti-Grm7 antibody; anti-Gria2 antibody; anti-Dscam antibody; anti-L1cam antibody; anti-Tsnax antibody; anti-Sgk1 antibody and/or anti-Stx1a antibody) which can be either directly labeled or further subjected to a secondary labeled antibody. Detection may be by autoradiography, colorimetric reaction or chemiluminescence. This method allows both quantitation of an amount of substrate and determination of its identity by a relative position on the membrane which is indicative of a migration distance in the acrylamide gel during electrophoresis.

In case the concentration of the antigen in the biological sample is low, detection of the antigen (target gene amino acid sequence) can be performed by immunoprecipitation (IP). For immunoprecipitation analysis the specific antibody (e.g. anti-Slc6a4 antibody; anti-Htr1a antibody; anti-MaoA antibody; anti-Adrb1 antibody; anti-Adrb2 antibody; anti-CRH type 1 receptor antibody; anti-CB1 antibody; anti-FKBP5 antibody; anti-Tph2 antibody; anti-Grm1 antibody; anti-Grik3 antibody; anti-Grm5 antibody; anti-Grik2 antibody; anti-Grm7 antibody; anti-Gria2 antibody; anti-Dscam antibody; anti-L1cam antibody; anti-Tsnax antibody; anti-Sgk1 antibody and/or anti-Stx1a antibody) may directly interact with a sample (e.g., cell lysate) including the target gene polypeptide and the formed complex can be further detected using a secondary antibody conjugated to beads (e.g., if the specific antibody is a mouse monoclonal antibody, the secondary antibody may be an anti-mouse antibody

conjugated to e.g., Sepharose beads). The beads can be then precipitated by centrifugation, following which the precipitated proteins (e.g., target gene polypeptide and specific antibodies) can be detached from the beads (e.g., using denaturation at 95 °C) and further subjected to Western blot analysis using antibodies. Alternatively, the specific antibody and the beads-conjugated secondary antibody may be added to the biological sample containing the antigen (target gene polypeptide) to thereby form an immunocomplex. Alternatively, if the target gene polypeptide is a highly glycosilated protein, it can be also precipitated using a substrate capable of binding glycosilated polypeptides such Concavalin A (GE Healthcare Bio-Sciences, Uppsala, Sweden) which may be also conjugated to beads, followed by Western blot analysis specific antibodies as described above.

FACS analysis enables the detection of antigens present on cell membranes. Briefly, specific antibodies, as described above, are linked to fluorophores and detection is performed by means of a cell sorting machine which reads the wavelength of light emitted from each cell as it passes through a light beam. This method may employ two or more antibodies simultaneously.

The presence and/or level of target gene polypeptide can be also determined using ELISA. Briefly, a sample containing the target gene antigen is fixed to a surface such as a well of a microtiter plate. An antigen specific antibody (e.g. anti-Slc6a4 antibody; anti-Htr1a antibody; anti-MaoA antibody; anti-Adrb1 antibody; anti-Adrb2 antibody; anti-CRH type 1 receptor antibody; anti-CB1 antibody; anti-FKBP5 antibody; anti-Tph2 antibody; anti-Grm1 antibody; anti-Grik3 antibody; anti-Grm5 antibody; anti-Grik2 antibody; anti-Grm7 antibody; anti-Gria2 antibody; anti-Dscam antibody; anti-L1cam antibody; anti-Tsnax antibody; anti-Sgk1 antibody and/or anti-Stx1a antibody) coupled to an enzyme is applied and allowed to bind to the antigen. Presence of the antibody is then detected and quantitated by a colorimetric reaction employing the enzyme coupled to the antibody. Enzymes commonly employed in this method include horseradish peroxidase and alkaline phosphatase. If well calibrated and within the linear range of response, the amount of substrate present in the sample is proportional to the amount of color produced. A substrate standard is generally employed to improve quantitative accuracy.

The presence and/or level of a target gene polypeptide can be also determined using radio-immunoassay (RIA). In one version, this method involves precipitation of the desired antigen (target gene polypeptide) with a specific antibody and radiolabeled antibody binding protein (e.g., protein A labeled with I^{125}) immobilized on a precipitable carrier such as agarose beads. The number of counts in the precipitated pellet is proportional to the amount of antigen.

In an alternate version of the RIA, a labeled antigen and an unlabelled antibody binding protein are employed. A sample containing an unknown amount of antigen is added in varying amounts. The decrease in precipitated counts from the labeled antigen is proportional to the amount of antigen in the added sample.

The presence and/or level of a target gene polypeptide can be also determined using molecular weight-based approach. Since the immunocomplex exhibits a higher molecular weight than its components, methods capable of detecting such a change in the molecular weight can be also employed. For example, the immunocomplex can be detected by a gel retardation assay. Briefly, a non-denaturing acrylamide gel is loaded with samples. A shift in the size (molecular weight) of the protein product as compared with its components is indicative of the presence of an immunocomplex. Such a shift to a higher molecular weight can be viewed using a non-specific protein staining such as silver stain or Commassie blue stain.

In situ detection of the target gene polypeptide in a biological sample such as a tissue section (e.g., paraffin embedded or cryosection) can be performed using immunological staining methods which detects the binding of antibodies on the cells in situ. Examples of immunological staining procedures include but are not limited to, fluorescently labeled immunohistochemistry (using a fluorescent dye conjugated to an antibody), radiolabeled immunohistochemistry (using radiolabeled e.g., ^{125}I, antibodies), and immunocytochemistry [using an enzyme (e.g., horseradish peroxidase or alkaline phosphatase) and a chromogenic substrate to produce a colorimetric reaction]. It will be appreciated that the enzymes conjugated to antibodies can utilize various chromogenic substrates as described hereinabove.

Preferably, the immunological staining used by the present invention is immunohistochemistry and/or immunocytochemistry.

Immunological staining is preferably followed by counterstaining the cells using a dye, which binds to non-stained cell compartments. For example, if the labeled antibody binds to antigens present on the cell cytoplasm, a nuclear stain (e.g., Hematoxylin -Eosin stain) is an appropriate counterstaining.

According to one embodiment, the method comprises measuring an expression of the Tph2 gene following the downregulating of the miR-181 and/or the miR-27.

According to one embodiment, the method comprises measuring an expression of the Slc6a4 gene following upregulating the miR-135 and/or miR-335.

According to one embodiment, the method comprises measuring an expression of the Htr1a gene following upregulating the miR-135, miR-335, miR-181, miR-182 and/or miR-26.

According to one embodiment, the method comprises measuring an expression of the MaoA gene following upregulating the upregulating the miR-27.

According to one embodiment, the method comprises measuring an expression of the Adrb1 gene following upregulating the miR-19.

According to one embodiment, the method comprises measuring an expression of the CB1 gene following upregulating the CB1.

According to one embodiment, the method comprises measuring an expression of the CRH type 1 receptor gene following upregulating the miR-15.

According to one embodiment, the method comprises measuring an expression of the FKBP5 gene following upregulating the miR-15.

The present inventors have further realized that mR135 is upregulated in subjects having a serotonin-associated medical condition (described above).

Thus, there is provided a method of diagnosing a serotonin-related medical condition in a subject in need thereof, the method comprising measuring an expression level of a miR-135 in a blood of the subject, wherein a high expression level of the miR-135 as compared to that in a blood sample of a healthy subject is indicative of the serotonin-associated medical condition.

Methods of analyzing miR in blood samples are well known in the art and are described hereinbelow.

Diagnosis can be further assessed and established using Gold-standard methods. Typically, at least one of a full patient medical history, physical assessment, and

thorough evaluation of symptoms helps determine the cause of the depression. Standardized questionnaires can be helpful such as the Hamilton Rating Scale for Depression, and the Beck Depression Inventory.

The present inventors have further shown that miR-135a plasma levels are decreased in subjects treated with an anti-depressant drug, such as Fluoxetine (an anti-depressant of the SSRI class), while brain miR-135a levels are increased in these same subjects (see Figures 3E-J).

Thus, according to another embodiment of the present invention, there is provided a method of monitoring treatment of an anti-depressant drug, the method comprising: (a) treating a subject in need thereof with an anti-depressant drug; and (b) measuring an expression level of a miR-135 in the blood of the subject prior to and following the treatment, wherein a lower expression level of the miR-135 following to the treatment by the anti-depressant drug as compared to the expression level of the miR-135 prior to the treatment by the anti-depressant drug is indicative of an efficient treatment.

As used herein, the term "anti-depressant drug" refers to any medication used to alleviate mood disorders, such as major depression and dysthymia, and anxiety disorders, such as social anxiety disorder. Exemplary anti-depressant drugs include, but are not limited to, Selective serotonin reuptake inhibitors (SSRIs, such as Citalopram, Escitalopram, Fluoxetine, Fluvoxamine, Paroxetine and Sertraline); Serotonin-norepinephrine reuptake inhibitors (SNRIs, such as Desvenlafaxine, Duloxetine, Milnacipran and Venlafaxine); Noradrenergic and specific serotonergic antidepressants (such as Mianserin and Mirtazapine); Norepinephrine (noradrenaline) reuptake inhibitors (NRIs, such as Atomoxetine, Mazindol, Reboxetine and Viloxazine); Norepinephrine-dopamine reuptake inhibitors (such as Bupropion); Selective serotonin reuptake enhancers (such as Tianeptine); Norepinephrine-dopamine disinhibitors (NDDIs such as Agomelatine); Tricyclic antidepressants (including Tertiary amine tricyclic antidepressants and Secondary amine tricyclic antidepressants); and Monoamine oxidase inhibitor (MAOIs).

According to a specific embodiment, the anti-depressant drug comprises selective serotonin reuptake inhibitors (SSRI) or noradrenaline reuptake inhibitors (NRI).

Measuring the expression level of miR-135 is typically effected in a blood sample obtained from the subject.

As used herein, the term "blood sample" refers to fresh whole blood, fractionated whole blood and blood plasma. The blood sample is typically obtained from the subject following to treatment with an anti-depressant drug, however, a blood sample may also be obtained from the subject prior to treatment for further comparison of miR-135 levels

An efficient anti-depressant treatment is determined when a lower expression level of the miR-135 is obtained following to the treatment as compared to the miR-135 expression level prior to the treatment.

According to another embodiment, there is provided a method of monitoring a psychiatric condition in a subject in need thereof, the method comprising measuring an expression level of a miR-135 in a blood of the subject, wherein a high expression level of the miR-135 as compared to a healthy subject is indicative of the psychiatric condition.

According to another embodiment, the psychiatric condition comprises a depression, an anxiety, a stress, a fatigue, an impaired cognitive function, a panic attack, a compulsive behavior, an addiction, a social phobia, a sleep disorder and a food related disorder.

According to a specific embodiment, miR-135 comprises miR-135a.

Measuring an expression level of a miR-135 may be carried out by any method known to one of ordinary skill in the art, as for example, by northern analysis, RNase protection assay, and PCR (e.g. real-time PCR).

Monitoring treatment may also be effected by assessing the patient's well being, and additionally or alternatively, by subjecting the subject to behavioral tests, MRI or any other method known to one of skill in the art.

It is expected that during the life of a patent maturing from this application many relevant inhibitors of miRNAs or alternatively miRNA modifications will be developed and the scope of the term microRNAs is intended to include all such new technologies *a priori*.

As used herein the term "about" refers to ± 10 %.

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term “consisting of means “including and limited to”.

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases “ranging/ranges between” a first indicate number and a second indicate number and “ranging/ranges from” a first indicate number “to” a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination

in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219;

5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

Differential expression of miRs in serotonin neurons

MATERIALS AND EXPERIMENTAL PROCEDURES

5HT neurons MicroRNA microarray

Hindbrain cells from embryonic day 12 of ePET YFP mice were cultured and sorted to distinguish 5HT neurons from surrounding non-5HT neurons. Total RNA including the miRNA population was purified, labeled and hybridized on Agilent Mouse miRNA Microarray (Agilent Tech, Mississauga, ON, Canada) design number 021828 based on Sanger miRBase release 12.0 according to manufactures instructions. The microarrays were scanned and the data was extracted and processed using the Feature Extraction Software (Agilent Technologies). Following scanning, intensity output data of the GeneView.txt files was analyzed to quantify differential relative expression of microRNAs using the the Partek® Genomics Suite (Partek Inc., St. Louis, MO). The data was log2 transformed, quantile normalized and filtered according to the flag "gIsGeneDetected" in the GeneView file. Of 666 murine miRs, 198 remained for further analysis upon this filtering step. Differentially expressed miRs were then identified by using a threshold of a 1.5 fold change with significance according to ANOVA. Contrasts were calculated within the ANOVA test. The Benjamini and Hochberg correction was used for false-positive reduction (multiple testing correction).

Cloning of 3' UTRs into Psicheck2 luciferase expression plasmid

3'UTR sequences of Slc6a4, Htr1a, MaoA and Tph2 were PCR amplified from mouse genomic DNA, or total brain cDNA. 3'UTR PCR fragments were ligated into pGEM-T easy vector (Promega) according to the manufacturer's guidelines, and further subcloned into a single *NotI* site at the 3' end of luciferase in the Psicheck2 reporter plasmid (Promega). Mutated 3' UTR sequences, lacking miR-135 seed sequences, were synthesized with primers overhangs across the seed match sequence. Cloning orientation was verified by diagnostic cuts and by sequencing.

Transfections and luciferase assay

HEK293T cells were grown on poly-L-lysine in 48-well format to a 70-85 % confluence and transfected using Polyethyleneimine with the following plasmids: 5 ng of Psicheck2-3'UTR plasmid and 215 ng of over-expressing vector for a specific miRNA, or empty-miR-vec over-expression plasmids. 24 hours following transfection cells were lysed and luciferase reporters activity were assayed as previously described [Chen A. et al. Mol Endocrinol (2005) 19: 441-58]. Renilla luciferase values were normalized to control firefly luciferase levels (transcribed from the same vector but not affected by 3'UTR tested) and averaged across six well repetitions per condition.

Animals and housing

Adult C57BL/6J male mice, 10 weeks old (Harlan, Jerusalem, Israel) were housed in a temperature-controlled room (22 ± 1 °C) on a reverse 12 hour light/dark cycle. Food and water were available *ad libitum*. All experimental protocols were approved by the Institutional Animal Care and Use Committee of The Weizmann Institute of Science.

Acute immobilization stress paradigms

Adult mice were introduced into a 50 ml ventilated tube for 30 minutes during their dark cycle.

Chronic Social defeat

Mice were subjected to a social defeat protocol as previously described [Krishnan V. et al. Cell (2007) 131: 391-404]. Briefly, the mice were placed in a home cage of an aggressive ICR mouse and they physically interacted for five minutes. During this time, the ICR mouse attacked the intruder mouse and the intruder displayed subordinate posturing. A perforated clear plexiglass dividers were then placed between

the animals and the mice remained in the same cage for 24 hours to allow sensory contact. The procedure was then repeated with an unfamiliar ICR mouse for each of the next 10 days.

Antidepressant treatment

Mice received i.p. injection of tritricyclic- Imipramine, or SSRI- Fluoxetine, or NRI- Reboxetine (20 mg/kg in saline) or saline. Chronic injections were carried out for 18-21 consecutive days, and an acute injection was performed 24 hours prior to brain microdissections.

Microdissection of the raphe nucleus and plasma collections

Brain samples were taken from mice raphe nucleus (RN) after removing the brain and placing it on acryl brain matrix (Stoelting). Slices were taken using standard razor blades (GEM) based on designated anatomical markers. Blunted 14G syringes were used to extract the RN region from 3 mm slices removed from the matrix. Additionally, trunk blood was collected in EDTA containing tubes to avoid coagulation. After centrifugation in 3,500 g for 30 minutes at 4 °C, plasma was separated and kept at -70 °C until RNA purification.

microRNA purification and Quantitative RT-PCR Expression Analysis

mRNAs, including microRNAs, were isolated from sorted neurons, frozen brain punches and plasma using miRNeasy mini kit (Qiagen) according to the manufacturer instructions, and treated using miScript Reverse transcription kit miRNA to generate cDNA. cDNA samples were then analyzed using SYBR®Green PCR kit (Qiagen) according to the manufacturer's guidelines in AB 7500 thermocycler (Applied Biosystems). Specific primers for each miR were used together with the commercial universal primer, while U6 snRNA was used as internal control.

Table 1B: Primers sequences used for real time PCR

SEQ ID NO.	Primer sequence	Gene
1	TATGGCTTTTTATTCCTATGTGA	miR135a
2	TATGGCTTTTCATTCCTATGTGA	miR135b
3	TTTGTTCGTTCGGCTCGCGTGA	miR375
4	GATGACACGCAAATTCGTGAA	U6
5	TAAGGCACGCGGTGAATGCC	miR124

Table 1C: Primers sequences used for molecular cloning

Primer Sequence	Orientation	Product size	Gene	
AGTTCTGCCGCTGATGATG (SEQ ID NO: 6)	sense	2600 with 2	Htr1a 3' UTR	**1**
GCACAAATGGAGAGTCTGATT AAA (SEQ ID NO: 7)	antisense		Htr1a 3' UTR	**2**
TGCCTTTAATGCAAAACAGC (SEQ ID NO: 8)	sense	2000 with 4	MaoA 3'UTR	**3**
CCAAGTTTACAACCATCAAGC A (SEQ ID NO: 9)	antisense		MaoA 3'UTR	**4**
ATCCGCATGAATGCTGTGTA (SEQ ID NO: 10)	sense	760 with 6	Slc6a4 3'UTR	**5**
GTGGGTGGTGGAAGAGACAC (SEQ ID NO: 11)	antisense		Slc6a4 3'UTR	**6**
CCTACACGCAGAGCATTGAA (SEQ ID NO: 12)	sense	870 with 8	Tph2 3' UTR	**7**
ACATCCCTGTGGGATTTGAG (SEQ ID NO: 13)	antisense		Tph2 3' UTR	**8**
TGTCTTGCTTATATTTTCTCAGT AG (SEQ ID NO: 14)	sense	320 with 6	Slc6a4 3'UTR mutated	**9**
GAAAATATAAGCAAGACATCC CTGTT (SEQ ID NO: 15)	antisense	440 with 5	Slc6a4 3'UTR mutated	**10**
AAAGATCCCTTTCCCCAATG (SEQ ID NO: 16)	sense	1400 with 12	Htr1a 3' UTR short	**11**
CAGTGCGTCTTCTCCACAGA (SEQ ID NO: 17)	antisense		Htr1a 3' UTR short	**12**
ATAAGCAAGGGCCCAAAAGGA AGA (SEQ ID NO: 18)	sense	1300 with 12	Htr1a 3' UTR mutated seed 1	**13**
TTTTGGGCCCTTGCTTATAAGT CC (SEQ ID NO: 19)	antisense	120 with 11	Htr1a 3' UTR mutated seed 1	**14**
CTGCCCTGCCACATGTGTTTTT AT (SEQ ID NO: 20)	sense	170 with 12	Htr1a 3' UTR mutated seed 2	**15**
TAACAAATAAAAACACATGTG GCA (SEQ ID NO: 21)	antisense	1260 with 11	Htr1a 3' UTR mutated seed 2	**16**
ACCGGTCATATGATTCCCCAGT TTCCTGCTTT (SEQ ID NO: 22)	sense	199 with 18	Pre-mmu-miR135b	**17**
ACCGGTCCTCTGTGGCTGGTCC TTAG (SEQ ID NO: 23)	antisense		Pre-mmu-miR135b	**18**

Cloning of miR135b over expression viral vector

Pre-miR-135b was amplified by PCR from mouse genomic DNA with primers adding restriction enzyme AgeI sites and then was inSlc6a4ed to pGEM-T Easy vector (Promega, Madison, WI). After sequencing of pGEM-T Easy and digestion of both pGEM-T Easy and pEGFP vector (Clontech laboratories Inc., Mountain View, CA) with the AgeI, the premature miR-135b sequence was ligated to the pEGFP vector to

construct the expression plasmid pEGFP-miR-135b. Afterwards, pEGFP-miR-135b was cut by BamHI and BsrGI in parallel to cutting pCSC-E/Syn-eGFP plasmid with the same enzymes, and the miR-135b-eGFP sequence was ligated to pCSC-E/Syn to construct pCSC-eSNY-pre-miR-135b-eGFP plasmid which was confirmed by restriction endonuclease analysis and DNA sequencing.

Production of lentiviral vectors

Recombinant lentiviruses were produced by transient transfection in HEK293T cells, as previously described [Naldini L et al., Proc Natl Acad Sci U S A (1996) 93:11382-8]. Briefly, infectious lentiviruses were harvested at 48 and 72 hours post-transfection, filtered through 0.45 μm-pore cellulose acetate filters and concentrated by ultracentrifugation.

Intracerebral injections of lentiviruses

To provide precision control over the stereotaxic surgery and site of lentiviral delivery, inventors used a computer-guided sterotaxic instrument and a motorized nanoinjector (Angle Two™ Stereotaxic Instrument, myNeurolab). As previously described [Singer O. et al. Nat Neurosci (2005).8, 1343-9] mice were placed on a stereotaxic apparatus under general anesthesia, and coordinates were determined as defined by the Franklin and Paxinos atlas. The lentiviral preparation was delivered using a Hamilton syringe connected to the motorized nanoinjector system and solution injected at a rate of 0.2 μl every 1 min. Following two weeks recovery period, mice were subjected to behavioral and physiological studies and afterwards anesthetized and perfused with phosphate buffered 4 % paraformaldehyde. The fixed brains were serially sectioned to 30 μ slices in order to confirm the preciseness of the injection site, using immunohistochemistry.

Immunohistochemistry

The procedure used for immunohistochemistry was carried out as previously described [Chen A et al. J Neurosci (2006) 26: 5500-10]. For GFP immunostaining, inventors used biotinylated anti GFP antibody raised in rabbit as primary antibody (Abcam, Cambridge, UK), and streptavidin conjugated Cy2 as secondary antibody (Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA).

Behavioral assessments

All behavioral assessments were performed during the dark phase following habituation to the test room for 2 hours prior each test.

Tail suspension test

The tail suspension test was performed in the TSE Tail Suspension Monitor (TSE Systems, Bad Homburg, Germany). Each mouse was taped by the tip of its tail, and suspended from the force sensor for 10 minutes. Time spent immobile and time spent struggling were calculated and recorded by the software based on pre-set thresholds.

Modified forced swim test

The tail suspension test was performed as previously described [Krishnan V and Nestler EJ, Nature (2008) 455: 894-902]. In short, the apparatus used was a plastic bucket, 18 cm of diameter, filled with 25 ^{o}C water to a depth of 15 cm. Each mouse was placed in the center of the bucket to initiate a 6 minutes video recorded test session. The duration of time spent immobile during the 2-6 minute of testing was automatically scored using EtoVision XT (Noldus, Wageningen, Netherlands).

Locomotor activity

To control for the possibility of behavioral effects originating from differences in ambulatory movement, locomotor activity of mice was examined over a 48 hours period, which proceeded a few days of habituation. Mice were single housed in specialized home cages and locomotion was measured using the InfraMot system (TSE Systems, Bad Hamburg, Germany).

Statistical analysis

Data were expressed as means +/- SEM. To test for statistical significance, student's *t test* was used in cases where only two groups were compared, such as between microarray validation qPCR. One way ANOVAs was used to compare between multiple groups such as between the different treatments in the luciferase assay. Two way ANOVAs was used in the cases of 2 independent variable, such as the SSRI NRI injection, both in acute and chronic durations. Post hoc *t test*s were used when necessary to reveal statistical significance. Differences between groups were considered significant when $P < 0.05$.

RESULTS

5HT neurons were isolated from the RN of ePET YFP embryos, and their miR expression profile was compared to non-5HT neurons, obtained from the same nucleus, using miR microarray (Figure 1A). Fourteen miRs were found to be upregulated and twenty-seven downregulated by more than 2 fold in 5HT neurons compared to the non-5HT neurons (see Tables 2A-B, below). Representative validation of array results was performed using real time PCR for miRs upregulated in 5HT neurons such as miR-375 (P=0.0071; Figure 1B) and downregulated such as miR-135a (P=0.0075; Figure 1C). In order to further study the role of miRs as modulators of 5HT neurons, extensive bioinformatic analysis was performed in a hypothesis driven manner. Targeting prediction of known serotonin related genes that have been previously demonstrated to be associated with psychopathologies, were crossed with the microarray results. The following four protein coding target genes expressed in 5HT neurons in the RN were chosen for testing: serotonin transporter, responsible for 5HT reuptake (also known as SERT or Slc6a4); serotonin inhibitory receptor 1a (also known as Htr1a); tryptophan hydroxylase 2 (Tph2), the rate limiting enzyme of 5HT synthesis in the brain; and monoamine hydroxylase (MaoA), which deactivates 5HT. MicroRNA targeting predictions for these genes was performed using two different web-based algorithms: Target Scan [www(dot)targetscan(dot)org] and Miranda [www(dot)microrna(dot)org] and were crossed with the list of 91 miRs altered by at least ± 1.5 in the 5HT neurons miRs array, compared to non-5RH cells. Based on the miRs array data and the bioinformatic analysis, eight miRs were chosen for further *in vitro* studies (Figure 1D-G).

Table 2A: List of miRs upregulated in 5HT neurons compared to non-serotonergic (by more than 2 fold).

Fold change	microRNA name
20.72	mmu-miR-375
11.73	mmu-miR-376c
4.44	mmu-miR-7a
2.87	mmu-miR-137
2.79	mghv-miR-M1-2
2.61	mmu-miR-709
2.51	mmu-miR-291b-5p
2.40	mmu-miR-1224
2.37	mmu-miR-1892
2.31	mmu-miR-702
2.25	mmu-miR-139-3p
2.24	mmu-miR-762
2.10	mmu-miR-671-5p
2.04	mmu-miR-483*

Table 2B: List of miRs downregulated in 5HT neurons compared to non-serotonergic (by more than 2 fold).

Fold change	microRNA name
-5.10	mmu-miR-691
-4.11	mmu-miR-466l
-3.95	mmu-miR-17
-3.18	mmu-miR-376b
-3.13	mmu-miR-124
-3.08	mmu-miR-218
-2.99	mmu-miR-128
-2.92	mmu-miR-140*
-2.86	mmu-miR-148a
-2.86	mmu-miR-340-5p
-2.82	mmu-miR-181c
-2.72	mmu-miR-210
-2.69	mmu-miR-135a
-2.66	mmu-miR-27a
-2.45	mmu-miR-452
-2.20	mmu-miR-370
-2.19	mmu-miR-300
-2.17	mmu-miR-376a
-2.13	mmu-miR-127
-2.12	mmu-miR-15b
-2.07	mmu-miR-101a
-2.06	mmu-miR-16
-2.05	mmu-miR-324-5p
-2.05	mmu-miR-434-5p
-2.03	mmu-miR-92a
-2.00	mmu-miR-669i

In vitro luciferase assays were performed to test the miR-target interactions between the 3'UTR of the tested 5HT related gene and the miRs predicted to putatively target it. Inventors found that Tph2 3'UTR was mildly repressed (by approximately 20 %) by miR-27b ($P = 0.0051$) and miR-181C ($P = 0.0305$, Figure 1H) and MaoA 3'UTR was also repressed by miR-27b ($P = 0.0008$, Figure 1I). miR-135 targeting of Slc6a4 3'UTR (Figure 2A and 2C) and Htr1a 3'UTR (Figure 2B and 2D) resulted in robust repression of translation of these transcripts. While miR-135a lead to approximately 30 % repression to Slc6a4 ($P = 0.014$) and Htr1a ($P < 0.0001$), miR-135b caused approximately 50 % repression to Slc6a4 ($P = 0.0002$) and Htr1a ($P < 0.0001$). Additionally significant repression of Htr1a 3'UTR was generated by miR-335 ($P<0.0001$), miR-181c ($P=0.0029$) and miR-26a ($P<0.0001$, Figure 2D). Further genomic approach bioinformatic analysis revealed a strong conservation of miR-135 seed match in the slc6a4 3'UTR (Figure 2E) and in one out of the two identified seed matches in the Htr1a 3'UTR (Figure 2F). Mutation studies in the 3'UTR of the Slc6a4 transcript, which removed the miR seed match of miR-135, revealed that both miR-135a and miR-135b targeting of Slc6a4 was mediated via its seed match sequence. The repression induced by the miR-135 was fully blocked by the mutation in Slc6a4 3'UTR (Figure 2G). Mutating the Htr1a miR-135 seed matches individually or both revealed that miR-135a was repressing Htr1a 3'UTR via the distal and not the proximal seed match while miR-135b act via both predicted sites (Figure 2H).

Inventors further tested the regulation of RN-miR-135 expression *in vivo* following different environmental challenges or pharmacological treatments. Following manipulation of the mice (i.e. acute immobilization stress) RN was removed, RNA was extracted and miR-135 levels were tested using real time PCR. Since 5HT levels are known to be alerted by acute stress, inventors tested miR-135 levels in different time points after acute restraint stress, and found that both miR-135a and miR-135b were downregulated 90 minutes following acute stress ($P < 0.0001$). The reduced levels of these miRs still remained 24 hours after stress, compared to control mice ($P = 0.0357$ for miR-135a, Figure 3A; $P=0.0055$ for miR-135b, Figure 3B). Furthermore, since 5HT neuronal functions and Slc6a4 and Htr1a expression levels are known to be strongly affected in depressed patients and following anti-depressants medications, inventors tested the levels of the two miR variants in mice exposed to environmental model for

induction of depression-like behaviors (chronic social defeat model) and to the tricyclic antidepressant, Imipramine. Interestingly, chronic social defeat stress did not alter miR-135 levels in the raphe nucleus, however, Imipramine administered acutely or chronically, both in stressed and non stressed mice, increased miR-135a (P=0.003; Figure 3C) and miR-135b (P=0.0093; Figure 3D) expression levels in the RN. Since Imipramine is not a specific 5HT reuptake inhibitor, inventors further tested the affect of both acute and chronic selective serotonin reuptake inhibitors (SSRI), Fluoxetine, and the noradrenaline reuptake inhibitors (NRI), Reboxetine, and found a robust increase in miR-135a levels following both acute and chronic SSRI treatment (P<0.0001, Figure 3E), and not in miR-135b levels in the RN (Figure 3F). Intrigued by the change in miR-135 levels in the RN following SSRI treatment, inventors tested the levels of circulating miR-135 in mice plasma, and found a robust decrease in miR-135a levels both following acute and chronic SSRI administration (main effect for drug P<0.0001, Figure 3G) and no effect in circulating miR-135b levels (Figure 3H), suggesting a strong reverse correlation between miR-135a levels in the RN and the plasma following SSRI administration (Figure 3I and 3J).

To further explore the importance of miR-135 levels in the whole animal context inventors manipulated miR-135 levels *in vivo* specifically in the RN of adult mice and tested its effects on the mice depression-like behaviors. To this end, inventors constructed recombinant lentiviruses over-expressing miR-135b specifically in neurons using the enhanced synapsin promoter, which also co-expressed the GFP reporter (see materials and experimental procedures section above and Figure 4A). Inventors tested the lentiviruses *in vivo* by injecting them into the RN of adult mice, and compared miR-135b levels in RN to control lentiviruses injected mice. Real time PCR analysis of miR-135b levels revealed a 10 fold induction compared to control lentiviruses injected mice (P=0.0032, Figure 4B). Adult mice injected with miR-135b over-expression were exposed to chronic social defeat, to initiate depression-like behaviors, and were subsequently tested behaviorally. Following behavioral testing, mice were perfused and brains were analyzed for location of injection site (Figures 4C-D). RN miR-135 over-expressing mice demonstrated reduced immobility time in the forced swim (P=0.0088 in minute 3 and P=0.00330 for minute 4; Figure 4E) and in the tail suspension tests (P=0.07356 in the last 5 min of the test, Figure 4F) without any observed change in their

home cage locomotion (Figures 4G-H), suggesting an antidepressant effect for miR-135 over-expression.

Taken together, the present inventors determined the specific miRs expression fingerprint of the RN serotonergic and non-serotonergic neurons. The present inventors crossed this unique data set with bioinformatics prediction for miRs targeting of 5HT related genes. The present inventors tested *in vitro* the targeting prediction for Tph2, MaoA, Slc6a4 and Htr1a using 3'UTR's luciferase assays and in mutation studies and reveled, among other miR-target interactions, a strong inhibitory effect for miR-135 both on Sl6a4 and Htr1a 3'UTR. Furthermore, the inventors demonstrated that miR-135 in the RN is down-regulated by acute stress, and upregulated by antidepressant administration, specifically by SSRI drugs. Furthermore, the present inventors identified a reverse correlation between miR-135a levels in the RN to its levels in the plasma following SSRI administration. Finally, the present inventors demonstrated that site-specific over-expression of miR-135 in the adult mice RN leads to decreased depression-like behaviors following social defeat.

EXAMPLE 2

miR-19 specifically targets type one beta adrenergic receptor (Adrb1)

MATERIALS AND EXPERIMENTAL PROCEDURES

Cloning of 3' UTRs into Psicheck2 luciferase expression plasmid

3'UTR sequence of ADRb1 was PCR amplified from mouse genomic DNA. Mutated 3' UTR sequences, lacking all four miR-19 seed matches, was synthesized by Epoch Biolabs, Inc. (TX, USA). 3'UTR PCR fragments were ligated into pGEM-T easy vector (Promega) according to the manufacturer's guidelines, and further subcloned into a single *NotI* site at the 3' end of luciferase in the Psicheck2 reporter plasmid (Promega). Cloning orientation was verified by diagnostic cuts and by sequencing.

Transfections and luciferase assay

HEK293T cells or HT22 neuronal cells were grown on poly-L-lysine in 48-well format to a 70-85 % confluence and transfected using Polyethyleneimine with the following plasmids: Psicheck2-3'UTR plasmid, pre-mmu-miR-19b over-expression in pEGFP plasmid or pEGFP plasmid alone (clontech), miR-19b knockdown (KD)

plasmid (Genecopoeia) or control-KD plasmid (Genecopoeia). 24 hours following transfection cells were lysed and luciferase reporters activity were assayed as previously described [Chen A. et al. Mol Endocrinol (2005) 19: 441-58]. Renilla luciferase values were normalized to control firefly luciferase levels (transcribed from the same vector but not affected by 3'UTR tested) and averaged across six well repetitions per condition.

RESULTS

Bioinformatic analysis for stress related genes with a distinct, evolutionary conserved miRNA target sequences that contain several repeats in their 3'UTR revealed miR-19 as a strong candidate for the targeting of type one beta adrenergic receptor (Adrb1), with three strongly conserved and one less conserved miR-19 seed match on Adrb1 3'UTR. Adrb1 is an adrenergic receptor that is expressed in various regions of the brain including the amygdala, hippocampus and paraventricular nucleus (PVN). Amygdalar Adrb1 was previously described as affecting anxiety-like behavior [Fu A et al., Brain Res (2008) 1211: 85-92; Rudoy CA and Van Bockstaele EJ, Prog Neuropsychopharmacol Biol Psychiatry (2007) 31: 1119-29] and fear memory [Roozendaal B et al., J Neurosci (2004) 24: 8161-9; Roozendaal B et al., Neuroscience (2006) 138: 901-10]. Intriguingly, Adrb1 was found on CRF positive cells of the amygdala and is a G-protein coupled receptor (GPCR) that exert its effect via Gs further activating adenylate cyclase (AC). There are 10 known genes encoding for AC, namely ADCY1-10. Three of these (ADCY1, ADCY7 and ADCY9) were bioinformaticly predicted to be targeted by miR-19. ADCY1 has a brain-specific expression and it was previously shown that over-expression of same in the mouse forebrain enhances recognition memory and LTP [Wang H et al., Nat Neurosci (2004) 7: 635-42].

In order to investigate whether miR-19 indeed regulates Adrb1 or ADCY1 expression through its presumed target sequences on Adrb1-3'UTR or ADCY1-3'UTR, an intact, or mutated forms of Adrb1-3'UTR (Figure 5) or ADCY1-3'UTR were cloned downstream of the luciferase gene in the Psicheck2 expression plasmid. In the mutated form of ADRb1-3'UTR all 4 seed matches for miR-19b were absent (Figure 5). In the mutated form of the partial ADCY1-3'UTR, only the conserved seed-match (out of 3) was absent.

Luciferase assay was used to determine the nature of interaction between miR-19 and Adrb1-3'UTR and also between miR-19 and ADCY1-3'UTR. In HT22 cells, that

endogenously express low levels of miR-19, no difference was found between luciferase levels controlled by either intact or mutated form of ADRb1-3'UTR (Figure 6A). However, when miR-19b was over-expressed in HT22 cells, luciferase levels were significantly (approximately 2 fold) lower when driven by the intact form relative to the mutated form of ADRb1-3'UTR (in addition to a general, seemingly non-specific reduction in normalized luciferase expression) (Figure 6B). In HEK293T cells that endogenously express high levels of miR-19b, luciferase expression levels regulated by ADRb1-3'UTR were 2-4 times lower than those expressed when regulated by the mutated form of ADRb1-3'UTR (Figures 6C).

MiRs knockdown (KD) system was used in order to manipulate miR-19 levels in HEK293T cells. Namely, (1) miRCURY LNA KD probes (Exiqon, MA, USA Figure 6D), and (2) plasmid based knockdown sequence miArrest (Genecopoeia, Rockville, MD, USA, Figure 6E). LNA-Anti-miR-19b enhanced luciferase levels expressed when regulated under ADRb1-3'UTR at about 20 % relative to control scrambled KD probe and had no effect on the mutated form of ADRb1-3'UTR (Figure 6D). Whereas, plasmid based miR-19b KD, caused up to 2 fold enhancement in luciferase expression regulated by the intact form of ADRb1-3'UTR relative to Control KD sequence (Figure 6E). No full rescue of luciferase levels relative to that driven by the mutant form of ADRb1-3'UTR was achieved. This may be explained either by miR-19b specificity of the probe/genomic sequence (spearing miR-19a regulation), the high miR-19 levels in HEK293T cells that may be difficult to fully down-regulate, or the effect of other possible miRNAs expressed in HEK293T cells that may bind to the same seed-match sequences on ADRb1-3'UTR.

EXAMPLE 3A

MiR-19a and MiR-19b are upregulated in the PFC and amygdala following chronic stress

MATERIALS AND EXPERIMENTAL PROCEDURES

Animals and housing

miR 17~92 *flx/flx* Mice [Ventura A et al, Cell (2008) 875-86:(5)132;7], are cross-bred with CamKIIa-Cre mice [Dragatsis I et al Genesis. (2000) 26(2):133-5]. Transgenic Mice or Adult C57BL/6J male mice are housed in a temperature-controlled

room (22 ± 1 °C) on a reverse 12 hour light/dark cycle. Food and water available *ad libitum*. All experimental protocols were approved by the Institutional Animal Care and Use Committee of The Weizmann Institute of Science.

Generating lentiviruses for miR-19b manipulation in adult brain

MiR-19b KD sequence was cloned into a lentiviral plasmid following the RNA polymerase III - H1 promoter. In addition, Pre-miR-19b sequence was cloned following a neuronal specific promoter (Enhanced synapsin, ESyn) in a lentiviral plasmid. Lentiviruses are generated for both i*n-vivo* miR-19b-KD and Pre-miR-19b-overexpression (OE) experiments. These lentiviruses are used to manipulate miR-19b levels in target regions where miR-19 levels are found to be altered following a behavioral/pharmacological challenge.

Generating mice lacking miR-19 in the forebrain

In order to generate mice lacking miR-19 in the forebrain, inventors are breading mice carrying the gene encoding for Cre recombinase under the CamKIIa promoter, with mice carrying a conditional form of the miRs cluster miR17~92. MiR-19 family includes miR-19a and miR-19b. In the mouse genome miR-19b has two identical copies, miR-19b-1 and miR-19b-2. MiR19a and miR-19b-1 are located on the same miRNA cluster, namely miR17~92, whereas miR-19b-2 is located at a different genomic locus, miR106a~363. The latter seems to have little or no expression in mouse tissues and therefore the knockout of miR17~92 cluster is expected to be enough to enable a profound effect on miR-19a and miR-19b expression levels in the forebrain.

Behavioral/pharmacological challenges

Mice lacking miR-17~92 cluster in the forebrain, or mice where miR-19 was specifically manipulated (overexpressed or down-regulated (KD) in specific brain regions) will be examined for expression levels of ADRb1, ADCY1 and other transcripts and gene products. These animals will be also tested for anxiety like behavior, locomotor activity and memory performance. Furthermore, the levels of expression of miR-19a and miR-19b are examined in different regions of interest (E.G the hippocampus, amygdala and forebrain) following an acute and chronic systemic treatment with the Noradrenaline reuptake inhibitor Reboxetine in WT mice.

RESULTS

The physiological link between miRNA-19 and Adrb1 was studied by assessing the level of miR-19a/b in the prefrontal cortex (PFC) of mice that were injected with Reboxetine, a noradrenalin reuptake inhibitor (NRI), either acutely or chronically (Figures 12A-D). As shown in Figures 12A-D, miR-19 a/b levels were down regulated following acute administration of Reboxetine (Figure 12A,B) and upregulated following chronic administration of Reboxetine (Figure 12C,D).

Next, the levels of miR-19 were assessed following stress by measuring the levels of miR-19 a and b in the PFC and amygdale of mice subjected to social defeat protocol (Figures 13A-D). As shown in Figures 13A-D, the levels of miR-19 a and b increased both in the PFC and amygdala following chronic stress. These results illustrate the involvement of miR-19 in the regulation of the central stress response.

EXAMPLE 3B

miRNA-19 and canabinoid receptor 1 (CB1)

MATERIALS AND EXPERIMENTAL PROCEDURES

Animals and housing

As described in Example 3A, above.

Generating lentiviruses for miRNA-19b manipulation in adult brain

As described in Example 3A, above.

RESULTS

CB1 is one of the most abundantly expressed GPCRs in the brain and is particularly enriched in the cortex, amygdala, hippocampus, basal ganglia, and cerebellum (Figures 15A-B) [Herkenham M. et al., The Journal of neuroscience: the official journal of the Society for Neuroscience (1991) 11:563-583; Mackie, K. Handbook of experimental pharmacology (2005) 299-325]. CB1 receptors are highly expressed on axons and axon terminals, where they are well positioned to modulate neurotransmission. Inventors found that CB1 contains 2 seed sites that are compatible with miRNA-19.

A luciferase assay was used to determine the nature of interaction between miRNA-19 and CB1-3'UTR. When miRNA-19b was over-expressed in HT22 cells

along with the 3`UTR of CB1, luciferase levels were significantly (50 %) lower when compared to GFP over expressed with the same 3`UTR (Figure 14), supporting a possible role for miR-19 in the regulation of CB1 levels. Additional mutation experiments are performed to verify the role of the predicted miR-19 seed sequence to the observed regulation (as described for Adrb1 above).

Interestingly, previous studies have convincingly demonstrated that the consolidation of aversive memories is facilitated by cross-talk between glucocorticoids, noradrenergic and cannabinoid signaling in the basolateral nucleus of the amygdala (BLA) [Roozendaal, B. et al. Neurobiology of learning and memory (2006) 86:249-255]. A model proposed by Hill and McEwen [Hill M.N. and McEwen B.S. Proc of the Nat Acad of Sci of the USA (2009) 106:4579-4580] shows a possible mechanism of action in the BLA for memory consolidation (Figure 16).

As shown in the present results, MiRNA-19 appears to regulate both Adrb1 and CB1 *in vitro*. Over-expression and knockdown of miR-19 using e.g. lentiviruses delivered specifically to the BLA where it may alter the levels of Adrb1 and CB1, are carried out as well as tests examining the mice's performance in learning and memory paradigms such as fear conditioning with and without exposure to stressful challenges.

EXAMPLE 3C

Identification of differentially expressed miRNAs in mice subjected to chronic stress

MATERIALS AND EXPERIMENTAL PROCEDURES

Immunoprecipitation of Ago2 protein

Pools of 3 amygdalae from 3 animals that are part of the same group ("Susceptible", "Resilient" or Control) were homogenized in NP40 buffer which was supplemented with RNase inhibitor, protease inhibitor and phosphates inhibitor. The samples were maintained on constant agitation for 2 hours at 4 °C. Samples were then centrifuged for 20 min at 12,000 rpm at 4 °C in a micro centrifuge, the supernatant was placed in a fresh tube kept on ice and the pellet was discarded. Magnetic protein G beads (Dynabeads, Invitrogen) were incubated with the Ago2 monoclonal antibody (WAKO) with rotation at room temperature for 10 minutes. After several washes the samples were added to the Ago2 coated protein G beads and incubated over night at 4

°C under agitation. The following day the beads were washed 3 times with PBS. For RNA purification the beads were homogenized in RLT buffer (RNeasy kit, miRNA supplementary protocol). For western blot analysis the beads were boiled in sample buffer to release the protein from the beads.

RNA purification and microarray

RNA from the Ago2 immunoprecipitation samples was isolated using the RNeasy plus kit (Qiagen) following Qiagen supplementary Protocol 1: Purification of total RNA containing miRNA. RNA for all other purposes was isolated from frozen brain punches using miRNeasy mini kit (Qiagen) according to the manufacturer recommendation, and RNA integrity was evaluated using the Agilent 2100 bioanalyzer. RNA derived from tissues of stressed mice following Ago2 immunoprecipitation was further analyzed on Affymetrix miRNA 2.0 arrays (enriched RNA protocol) and Affymetrix Mouse Gene 1.0 ST array.

RESULTS

In order to identify and study differentially expressed miRNAs isolated from the amygdala of mice subjected to chronic stress paradigm and/or associated with "Resilient" or "Susceptible" behavioral phenotype, the social defeat protocol was used (see Methods section).

In order to identify a genuine connection between miRNAs and their target gene`s 3` UTR following the social defeat paradigm, an imuunoprecipitation (IP) of the Ago2 complex was performed and the population of miRNAs and mRNAs co-precipitated was analyzed. When a mature miRNA was formed it was incorporated to the RISC complex. While in the RISC complex, Ago2 facilitates the interaction between a specific miRNA and its target mRNA 3` UTR [Meister G. et al., Molecular cell (2004) 15:185-197] (Figure 17A).

In order to verify that the Ago2 complex can indeed be precipitated with its bound RNA, the IP was performed on the amygdala of naive mice. The IP was performed using protein G magnetic beads which were reacted with monoclonal Ago2 antibody. As shown in Figure 17B, a specific Ago2 band was precipitated from an extract of NIH 3T3 cells (Figure 17B, lane 1) or from an extract of amygdala tissue (Figure 17B, lane 2).

To demonstrate the specificity of the IP, a total brain sample was divided into two, where one was precipitated with anti Ago2 and the other with a control IgG1 nonspecific antibody. A specific Ago2 band was present only in the Ago2 precipitate (Figure 17B, lanes 3, 4).

Therefore, by pulling down the Ago2 complex and analyzing the miRNA as well as the mRNA populations in the precipitated material there was a greater chance to discover a correct connection between a given miRNA and its targeted mRNA 3` UTR in specific brain regions.

Isolation of Ago 2 associated RNA from mice amygdala subjected to social defeat paradigm

Next, based on the specific results of the Ago2 IP experiment, the same strategy was implemented in order to reveal potential differences in miRNA and their target mRNAs in the brain of mice that were subjected to social defeat protocol.

After 10 days of the social defeat paradigm, mice were categorized into 3 groups: Control, "Susceptible" and "Resilient". A mouse was characterized as "Susceptible" when it exhibited social avoidance when it encountered a new mouse from the same strain that attacked him during the social defeat paradigm. A mouse was characterized as "Resilient" if it does not avoid the new aggressive mouse and interacts with it. Most of the mice subjected to social defeat typically exhibit social avoidance and therefore would be classified as "Susceptible". Approximately only 10-20 % of the mice in an experiment are expected to be "Resilient". Shown below is an example of the social avoidance test conducted.

As demonstrated in Figure 18A, the mouse was placed alone in the social maze for 3 minutes for habituation. The camera tracked the mouse movements throughout the maze. In Figure 18C, the same mouse was exposed to a novel ICR mouse that was placed beyond a divider. The camera tracked the mouse in the farthest corner of the arena distant from the location of the novel mouse. This response was considered as social avoidance and therefore this mouse was classified as "Susceptible". In contrast, in Figure 18B and Figure 18D the mouse did not exhibit social avoidance and therefore was classified as "Resilient".

Forty mice underwent the social defeat paradigm and forty mice served as control. Following the social avoidance test 9 "Resilient" mice, 9 "Susceptible" mice

and 12 control mice were selected for brain microdissection. Brain samples were collected 8 days after the social avoidance test from the amygdala, BNST, PFC, dorsal raphe and hippocampus along with trunk blood.

Pools of 3 amygdala punches obtained from 3 different mice were combined and the immunoprecepitation with anti Ago2 was performed. Following the IP, RNA was extracted from the precipitated material. After the pulling of 3 amygdalae from each group there were 3 RNA samples from the "Resilient" mice, 3 RNA samples from the "Susceptible" mice and 4 RNA samples from the control mice – a total of 10 RNA samples. Each sample was tested in a mouse ST microarray as well as in miRNA array (both Affymetrix). Genes and miRNAs that were up or down regulated in each of the 2 groups: "Susceptible" or "Resilient" relative to the control group, were examined. If an interaction between a certain miRNA and a target gene takes place invnetors expected for an opposite correlation in their total levels. However, mRNA present in the RISC complex (precipitated with the anti Ago2) were expected to be in high levels because they have not yet been fragmented, therefore while looking at the array data inventors examined miRNAs and potential mRNA targets that were both either elevated or down regulated relative to the control sample because this was an indication that they interacted in the RISC complex.

Microarray results

Table 3, hereinbelow, illustrated the preliminary array results analyzed using conventional filters.

Tables 3A-B: List of amygdalar miRNAs up regulated (Table 3A) or down regulated (Table 3B) following IP with Ago2.

Upregulated	Fold-Change Susceptible	Fold-Change Resilient
mmu-miR-301a_st	1.96	2.11
mmu-miR-15a_st	1.66	1.87
mmu-miR-29a_st	1.42	1.82
mmu-miR-19b_st	1.97	2.34
mmu-miR-146b_st	1.55	1.94
mmu-miR-181d_st	1.54	1.64
mmu-miR-146a_st	1.41	1.60
mmu-miR-27b_st	1.45	1.91
mmu-miR-20a_st	1.57	1.52
mmu-miR-30a_st	1.34	1.65
mmu-miR-100_st	1.41	1.55
mmu-miR-153_st	1.44	1.92
mmu-miR-194_st	1.57	1.78
mmu-miR-30c_st	1.40	1.66
mmu-miR-23a_st	1.51	1.70
mmu-miR-106a_st	1.62	1.61
mmu-miR-30b_st	1.43	1.70
mmu-miR-195_st	1.59	1.98
mmu-miR-30e_st	1.36	1.56
mmu-miR-126-3p_st	1.58	1.76
mmu-let-7i_st	1.49	1.57
mmu-miR-434-5p_st	1.30	1.55
mmu-miR-376b_st	1.64	1.99
mmu-miR-495_st	1.45	1.82
mmu-miR-369-5p_st	1.60	1.77
mmu-miR-421_st	1.71	1.53
mmu-miR-543_st	1.52	1.69
mmu-miR-410_st	1.44	1.76
mmu-miR-34b-5p_st	2.18	1.53

(Table 3A)

Downregulated	Fold-Change Susceptible	Fold-Change Resilient
mmu-miR-210_st	-1.59	-2.13
mmu-miR-298_st	-1.75	-2.08
mmu-miR-423-5p_st	-1.68	-1.94
mmu-miR-346_st	-1.74	-1.96
mmu-miR-139-3p_st	-1.71	-2.13
mmu-miR-320_st	-1.74	-2.03
mmu-miR-485_st	-1.53	-1.88
mmu-miR-491_st	-1.53	-2.01
mmu-miR-31_st	-1.30	-1.53
mmu-miR-92b_st	-1.20	-1.53
mmu-miR-93_st	-1.36	-1.50
mmu-miR-125a-3p_st	-1.32	-1.55
mmu-miR-134_st	-1.47	-1.63
mmu-miR-323-5p_st	-1.43	-1.76
mmu-miR-345-5p_st	-1.30	-1.62
mmu-miR-341_st	-1.36	-1.89
mmu-miR-370_st	-1.33	-2.04
mmu-miR-433_st	-1.49	-1.75
mmu-miR-455_st	-1.40	-1.61

(Table 3B)

*For both Tables 3A-B, the data was presented as fold change for "Susceptible" or "Resilient" mice compared with Control. Values in bold are significantly altered.

Several miRNAs, which have been significantly upregulated in the "Susceptible" and "Resilient" groups of mice, have been selected and illustrated in a heatmap (see Figures 19A-B).

Gene expression array (mRNA)

Table 4: List of amygdalar mRNAs up regulated following IP with Ago2.

Upregulated	Fold-Change Susceptible	Fold-Change Resilient
Tnrc18	1.36	1.23
Ifi30	1.34	1.21
Adamts9	1.79	1.52
Fkbp5	1.35	1.26
Adh1	1.42	1.05
Pxdn	1.32	1.19
Impdh2	1.41	1.02
Pdzd2	1.31	1.31
Csmd3	1.33	1.44
Usf1	1.33	1.20
A2m	1.71	1.09
Ccnd3	1.34	1.10
Rrh	1.33	1.02
Wfikkn2	1.40	1.07
Fras1	1.48	1.34
Notch2	1.50	1.22
Fam38a	1.33	1.18
Hist1h3f	1.31	1.19
Fam167a	1.31	1.05
Calml4	1.68	1.11
Tspan4	1.30	1.21
Dnahc6	1.38	1.07
Jag2	1.31	1.19
Shank2	1.60	1.42
Dock6	1.33	1.10
Mamdc2	1.30	1.20
Sgms2	1.39	1.13
Iqub	1.51	1.11
Ubxn11	1.36	1.06
Wfdc2	1.53	1.11
Spef2	1.33	1.16
Fggy	1.31	1.14
Pcolce2	1.37	1.16
Thbs1	1.32	1.13
Dnahc7b	1.40	1.13
Nt5dc2	1.41	1.12
Slc4a2	1.34	1.07

Adamts17	1.40	1.35
Plscr2	1.34	1.21
Clic6	1.43	1.13
St6galnac2	1.38	1.08
Amigo2	1.33	1.06
Trio	1.33	1.15
Lamb1-1	1.35	1.20
Sema3b	1.40	1.01
Fap	1.39	1.10
Frem1	1.51	1.20
Pon1	1.34	1.03
Plin4	1.43	1.24
Steap1	1.36	1.10
Rdh5	1.52	1.13
Cldn2	1.56	1.11
Frrs1	1.37	1.10
Spef2	1.36	1.07
Slco1a5	1.31	1.13
Ltc4s	1.35	1.17
Mfsd7c	1.37	1.14
Acss3	1.32	1.16
Hif3a	1.36	1.17
Serpinb8	1.40	1.18
Pcolce	1.36	1.16
Dnmt3a	1.20	1.19
GILZ (Tsc22d3)	1.19	1.15
Sdk2	1.29	1.36
Prg4	1.16	1.72
Fbn1	1.24	1.10
Slitrk6	1.11	1.28
Plxna1	1.30	1.16
Plxnb2	1.25	1.10
Sema4b	1.29	1.14

(Table 4, cont.)

* Data is presented as fold change for “Susceptible” or “Resilient” mice compared with Control. Values in bold are significantly altered.

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Table 5: List of amygdalar mRNAs down regulated following IP with Ago2.

Downregulated	Fold-Change Susceptible	Fold-Change Resilient
Cyp2d10	-1.22	-1.34
Lonrf1	-1.32	-1.31
Btnl5	-1.64	-1.54
B2m	-1.33	-1.20
Tekt5	-1.36	-1.10
Prp2	-1.51	-1.02
Krtap5-1	-1.34	-1.10
Krtap5-4	-1.33	-1.10
Klhl38	-1.38	-1.07
Th	-1.42	-1.03
Pcsk9	-1.33	-1.20
Dnahc3	-1.39	-1.22
Sgpp2	-1.37	-1.03
Opalin	-1.49	-1.28

Several potential miRNAs and their putative targets in the brain are analyzed.

EXAMPLE 4A

miR-15a and miR-15b as regulators of the stress response

MATERIALS AND EXPERIMENTAL PROCEDURES

Total RNA extraction

Amygdala tissue was dissected 90 minutes following acute stress procedure. Total RNA was isolated using miRNeasy kit (Qiagen) in order to preserve miRNAs. Frozen brain punches were transferred into lysis buffer and immediately homogenized. Neuronal primary cultures or N2a cell cultures were lysed in-well, on ice. Further processing was done according to the manufacturer's recommendation. RNA extracts were stored at -80 °C until use.

miRNA Array

miRNA differential expression was assayed by Agilent (Agilent, Santa Clara, CA, USA) or Affymetrix (Affymetrix, Santa Clara, CA, USA) miRNA microarrays, according to the manufacturer's instructions. For the assessment of miRNA differential

expression using the Agilent array, 100 ng total RNA per sample (3 control samples and two acute stress samples) were each labeled and hybridized according to the manufacturer's instructions. Arrays were scanned using an Agilent microarray scanner. The data was extracted using the Agilent Feature Extraction software v9 and analyzed using Partek® Genomics Suite (Partek Inc., St. Louis, Missouri, USA). Data from the GeneView.txt files were subject to log transformation and quantile normalization. For the assessment of miRNA differential expression using the Affymetrix array, 1 µg total RNA per sample (two control samples and two acute stress samples) were each labeled and hybridized according to the manufacturer's instructions. Arrays were scanned using an Affymetrix microarray scanner. The data was extracted using the Affymetrix scanner software and normalized using the default parameters of the Affymetrix miRNAQCtool software (background adjustment, quantile normalization, log transformation and threshold determination). The normalized data from the four files were imported into Partek Genomics software. Genes not presented in any of the microarrays were filtered out. Due to the difference in miRNA distribution, different log ratio cutoffs (corresponding to about 1 standard error for each array) were chosen for each array: 0.2 for Agilent and 0.4 for Affymetrix. miRNAs with log ratios greater than the cutoff were compared between arrays and the common miRNAs are reported.

Cloning of 3' UTRs into Psicheck2 luciferase expression plasmid

3'UTR sequence of CRFR1 was PCR amplified from mouse genomic DNA. 3'UTR PCR fragments were ligated into pGEM-T easy vector (Promega) according to the manufacturer's guidelines, and further subcloned into a single *NotI* site at the 3' end of luciferase in the Psicheck2 reporter plasmid (Promega). Cloning orientation was verified by diagnostic cuts and by sequencing.

Transfections and luciferase assay

HEK293T cells were grown on poly-L-lysine in 48-well format to a 70-85 % confluence and transfected using Polyethyleneimine with the following plasmids: Psicheck2-3'UTR plasmid, pre-mmu-miR-15 over-expression in pEGFP plasmid or pEGFP plasmid alone (clontech). 24 hours following transfection, cells were lysed and luciferase reporters activity were assayed as previously described [Chen A. et al. Mol Endocrinol (2005) 19: 441-58]. Renilla luciferase values were normalized to control

firefly luciferase levels (transcribed from the same vector but not affected by 3'UTR tested) and averaged across six well repetitions per condition.

RESULTS

miR-15a and miR-15b emerged as up regulated 90 minutes following acute restraint stress (Figure 7A-B). Both miR-15a and miR-15b were bioinformatically predicted to target CRFR1-3'UTR (Figure 7C). In-Vitro overexpression of miR-15b in HEK293T cells significantly reduced the levels of luciferase expression controlled by CRFR1-3'UTR (Figure 7D).

EXAMPLE 4B

The effect of miR15 on FKBP5

MATERIALS AND EXPERIMENTAL PROCEDURES

As illustrated in Example 4A, hereinabove.

RESULTS

According to the array results, miR-15a and FK506 binding protein 5 (also known as FKBP5) were both up regulated in the "Susceptible" and "Resilient" mice relative to the control group (Figures 20A-B), suggesting their up regulation in the RISC complex as a result of chronic stress.

Genetic studies have identified a role for FKBP5 in posttraumatic stress disorder, depression and anxiety. For example, single nucleotide polymorphisms (SNPs) in FKBP5 have been found to interact with childhood trauma to predict severity of adult posttraumatic stress disorder (PTSD) [Binder, E.B. et al., Nature genetics (2004) 36:1319-1325]. These findings suggest that individuals with these SNPs who are abused as children are more susceptible to PTSD as adults. FKBP5 has also been found to be less expressed in individuals with current PTSD [Yehuda, R. et al., Biological psychiatry (2009) 66:708-711]. The FKBP5 gene has been found to have multiple polyadenylation sites and is statistically associated with a higher rate of depressive disorders [Binder et al. supra].

Further analysis of the 3` UTR of FKBP5 revealed that it has one conserved seed match sequence to miR-15 (Figure 20C).

If indeed miR-15a regulates FKBP5 mRNA, it was expected that while both miR-15a and FKBP5 would be up regulated in the Ago-2 precipitate (as shown by the microarray results, Fig. 20B), the total levels of either mRNA or protein of FKBP5 in the amygdala sample would be decreased.

In order to examine whether the interaction between miR-15a and FKBP5 takes place in the amygdale, a real time PCR analysis on total RNA sample obtained from the amygdala of "Susceptible" and control mice was performed. As shown in Figures 21A-B, miR-15a levels were increased in total RNA extract taken from susceptible mice whereas FKBP5 levels were decreased. These results indicated that miR-15a represses FKBP5 levels in the amygdala following chronic stress condition.

Cloning the intact and mutated 3` UTR forms of FKBP5 for luciferase assay analysis are performed in order to find whether a direct interaction between miR-15a and FKBP5 occurs *in vitro*.

In addition to FKBP5, miR-15 can potentially regulate a number of genes that are involved in the stress response including Stx1a (syntaxin 1a), Sgk1 (serum/glucocorticoid regulated kinase) and Adrb2 (Figure 22).

EXAMPLE 4C

miR-181 regulates glutamate receptors

MATERIALS AND EXPERIMENTAL PROCEDURES

Cloning of 3' UTRs into Psicheck2 luciferase expression plasmid

3'UTR sequences of Grm1, Grik3, Grm5,Grik2 and Grm7 were PCR amplified from mouse genomic DNA. 3'UTR PCR fragments were ligated into either pGEM-T easy vector (Promega) or pJET1.2 vector (Fermentas) according to the manufacturer's guidelines, and further subcloned into a single *NotI* or *XhoI* site at the 3' end of luciferase in the Psicheck2 reporter plasmid (Promega). Cloning orientation was verified by diagnostic cuts and by sequencing.

Chronic Social defeat

Mice were subjected to a social defeat protocol as previously described [Krishnan V. et al. Cell (2007) 131: 391-404]. Briefly, the mice were placed in a home cage of an aggressive ICR mouse where they physically interacted for five minutes.

During this time, the ICR mouse attacked the intruder mouse and the intruder displayed subordinate posturing. Perforated clear plexiglass dividers were then placed between the animals and the mice remained in the same cage for 24 hours to allow sensory contact. The procedure was then repeated with an unfamiliar ICR mouse for each of the next 10 days.

RESULTS

miR-181d levels were significantly increased in mice suffering from chronic stress (Figure 23). In an attempt to find interactions between miR-181 and potential mRNA targets, Inventors discovered that miR-181 can potentially regulate many types of glutamate receptors. In general, glutamate receptors can be divided into two groups, Ionotropic glutamate receptors (iGluRs), which form the ion channel pore that activates when glutamate binds to the receptor, and Metabotropic glutamate receptors (mGluRs), which indirectly activate ion channels on the plasma membrane through a signaling cascade that involves G proteins.

Of the many specific subtypes of glutamate receptors, it is customary to refer to primary subtypes by a chemical which binds to it more selectively than glutamate. The research, though, is ongoing, as subtypes are identified and chemical affinities measured. Several compounds are routinely used in glutamate receptor research and associated with receptor subtypes:

Table 6: Glutamate receptors categorized into subgroups

Name	Type
NMDA receptor	Ionotropic
Kainate receptor	
AMPA receptor	
mGluR	Metabotropic

As illustrated in Figures 24 and 25, out of all the conserved predicted targets of miR-181, there are 6 glutamate receptors (Grm1, Grik3, Grm5, Gria2, Grik2 and Grm7).

It has been shown previously that miR-181a controls Gria2 surface expression in hippocampal neurons [Saba. R. et al., Molecular and Cellular Biology (2012) 32(3):619-32]. Luciferase assays are being performed in order to verify the miRNA-mRNA

interaction. Furthermore, a conditional miR-181 KO mice line are crossed with a specific cre line thereby obtaining a deletion of miR-181 in specific brain nuclei.

EXAMPLE 5A

MiR-182 a fine tuner of normal neuronal activity and of psychopathological behavior

MATERIALS AND EXPERIMENTAL PROCEDURES

Cloning of 3' UTRs into Psicheck2 luciferase expression plasmid

3'UTR sequence of Htr1a was PCR amplified from mouse genomic DNA. 3'UTR PCR fragments were ligated into pGEM-T easy vector (Promega) according to the manufacturer's guidelines, and further subcloned into a single *NotI* site at the 3' end of luciferase in the Psicheck2 reporter plasmid (Promega). Cloning orientation was verified by diagnostic cuts and by sequencing.

Transfections and luciferase assay

HEK293T cells were grown on poly-L-lysine in 48-well format to a 70-85 % confluence and transfected using Polyethyleneimine with the following plasmids: Psicheck2-3'UTR plasmid, pre-mmu-miR-182 over-expression in pEGFP plasmid or pEGFP plasmid alone (clontech). 24 hours following transfection cells were lysed and luciferase reporters activity were assayed as previously described [Chen A. et al. Mol Endocrinol (2005) 19: 441-58]. Renilla luciferase values were normalized to control firefly luciferase levels (transcribed from the same vector but not affected by 3'UTR tested) and averaged across six well repetitions per condition.

Chronic Social defeat

Mice were subjected to a social defeat protocol as previously described [Krishnan V. et al. Cell (2007) 131: 391-404]. Briefly, the mice were placed in a home cage of an aggressive ICR mouse and they physically interacted for five minutes. During this time, the ICR mouse attacked the intruder mouse and the intruder displayed subordinate posturing. Perforated clear plexiglass dividers were then placed between the animals and the mice remained in the same cage for 24 hours to allow sensory contact. The procedure was then repeated with an unfamiliar ICR mouse for each of the next 10 days.

Microdissection of the raphe nucleus and plasma collections

Brain samples were taken from mice raphe nucleus (RN) after removing the brain and placing it on acryl brain matrix (Stoelting). Slices were taken using standard razor blades (GEM) based on designated anatomical markers. Blunted 14G syringes were used to extract the RN region from 3 mm slices removed from the matrix.

microRNA purification and Quantitative RT-PCR Expression Analysis

mRNAs, including microRNAs, were isolated from sorted neurons, frozen brain punches and plasma using miRNeasy mini kit (Qiagen) according to the manufacturer instructions, and treated using miScript Reverse transcription kit miRNA to generate cDNA. cDNA samples were then analyzed using SYBR®Green PCR kit (Qiagen) according to the manufacturer's guidelines in AB 7500 thermocycler (Applied Biosystems). Specific primers for each miR were used together with the commercial universal primer, while U6 snRNA was used as internal control.

Cloning of miR182 over expression viral vector

Pre-miR-182 was amplified by PCR from mouse genomic DNA with primers adding restriction enzyme AgeI sites and then was inSlc6a4ed to pGEM-T Easy vector (Promega, Madison, WI). After sequencing of pGEM-T Easy and digestion of both pGEM-T Easy and pEGFP vector (Clontech laboratories Inc., Mountain View, CA) with the AgeI, the premature miR-182 sequence was ligated to the pEGFP vector to construct the expression plasmid pEGFP-miR-182. Afterwards, pEGFP-miR-182 was cut by BamHI and BsrGI in parallel to cutting pCSC-E/Syn-eGFP plasmid with the same enzymes, and the miR-182-eGFP sequence was ligated to pCSC-E/Syn to construct pCSC-eSNY-pre-miR-182-eGFP plasmid which was confirmed by restriction endonuclease analysis and DNA sequencing.

Production of lentiviral vectors

Recombinant lentiviruses were produced by transient transfection in HEK293T cells, as previously described [Naldini L et al., Proc Natl Acad Sci U S A (1996) 93:11382-8]. Briefly, infectious lentiviruses were harvested at 48 and 72 hours post-transfection, filtered through 0.45 μm-pore cellulose acetate filters and concentrated by ultracentrifugation.

RESULTS

To date miR-182 was reported mainly in cancer related studies such as an human lung adenocarcinoma cells, glioma , breast cancer, bladder cancer, melanoma and DNA repair. Additionally, miR-182 was found to be involved in developmental processes such as inner ear and retinal development, and in the immune system in activation of T lymphocytes, and in lupus disease. In the nerves system mi-R182 was implied in sensory organ-specific rat dorsal root ganglia, and as a circadian clock modulator, while a correlation between genetic variants of pre-miR-182 were found in major depression patients [Saus E et al., Hum Mol Genet. (2010) 19(20):4017-25]. Additionally, miR-182 was listed among other 12 miRs as down-regulated in resilient to learned helpless behaviors male rats prefrontal cortex [Smalheiser NR et al., Int J Neuropsychopharmacol. (2011) 1-11].

Bioinforamtical analysis of Htr1a 3'UTR performed as part of the 5HT miRs microarray analysis implied a possible targeting of this gene by miR-182. Therefore, inventors performed *in vitro* testing via a luciferase assay, which revealed a strong repression of Ht1a 3'UTR by miR-182 (Figure 8). Two conserved seed matches sequence for miR-182 appeared in Htr1a mouse 3' UTR.

Regulation studies indicated a strong tendency of down-regulation of miR-182 expression levels in the RN of adult male mice exposed to chronic social defeat compared to controls (Figure 9) suggesting involvement of miR-182 in the molecular response to environmental stimulus known to induced depression-like behaviors.

Further bioinformatics analysis generating targeting predictions for miR-182 in two databases revealed a long list of potential targets, including genes related to neuronal activity both in normal and in pathological conditions (Figure 10).

In order to further test miR-182 *in vitro* for identification of specific miR target interactions, and to reveal miR-182 role in regulation of normal and pathological behaviors *in vivo*, plasmid and lentiviral systems for manipulation of miR-182 were developed. Neuronal specific over-expression lentiviruses were manufactures (Figure 11A) and tested *in vitro* in the neuronal cell line N2a. These results demonstrated increased miR-182 levels in cells infected with miR-182 over-expression lentiviruses compared to control (Figure 11B). Knockdown plasmid sequence specific for miR-182 named miArrest (Genecopoeia, Rockville, MD, USA, Figure 11C) was purchased and

sub-cloned to viral constructs (Figure 11C). These systems are tested in cell culture and by site specific injection to adult mice brains.

Null mice for miR-182 are developed in order to investigate the miRs role in retina development. Recently, inventors obtained breading pairs for this line, and upon a generation a colony, miR-182 KO and their WT liter mates are being phenotyped behaviorally and physiologically.

EXAMPLE 5B

Regulation of miR182 expression levels by acute stress

MATERIALS AND EXPERIMENTAL PROCEDURES

As described in Example 5A, hereinabove

RESULTS

The effect of acute stress on miR182 level was examined. As illustrated in Figure 26, acute immobilization stress led to decreased miR182 expression levels in mice raphe nucleus (RN) 24 hours following induction of stress ($P<0.01$). miR182 demonstrated reduced expression levels in the raphe nucleus both following acute and chronic stress, suggesting it has a role in modulation the molecular responses to stress in the raphe nucleus, possibly by effecting its target gene Ht1a modulating 5-HT levels in the synapse.

<u>miR-target interaction assay for miR182 predicted target genes</u>

Using a luciferase assay, eleven predicted target genes of miR182, chosen after extensive bioinformatics, were examined (Figure 27A). 3'UTRs of the target genes were tested *in vitro* to check if miR182 has a represoric effect as measured by the activity of the conjugated reporter gene luciferase. Out of the eleven genes tested three genes: Dscam (Down Syndrome Cell Adhesion Molecule), L1cam (Cell adhesion molecule L1) and Tsnax (Translin-associated protein X) had demonstrated represoric effect by miR182 as in luciferase assay (Figure 27A). When testing the 3'UTR of the listed above target gene of miR182 a conserved seed match sequence for miR182 was observed both in Tsnax, L1cam and Dscam, suggesting this miR-target interaction had a functional role (data not shown).

Next, the direct represoric effect of miR182 on these three genes was verified. Therefore, the 3'UTRs was mutated to remove miR182 seed match sequence and compared the regular 3'UTRs to the mutated one *in vitro* by luciferase assays. miR182 represoric effect on L1cam 3'UTR was abolished when mutated its seed match sequence (Figure 27B), and similarly the effect of miR182 on Tsnax was abolished in the mutated 3'UTR (Figure 27C) indicating miR182 targeted this gene directly. Similar verification for Dscam and Htr1a with mutated 3'UTR are performed.

A mice model lacking miR182 is used to study the interaction between miR182 and its target genes *in vivo*. Inventors are examining the behavioral phenotype of miR182KO mice in tests for social behavior, learning and memory, and schizophrenia-like behaviors.

EXAMPLE 6

Regulation of miR135 levels in the plasma and brain of adult mice

MATERIALS AND EXPERIMENTAL PROCEDURES

Cloning of miR135 overexpression viral vectors and of miR135 KD viral vector

miR135b KD plasmid pEZX-H1-miR135KD-CMV-mCherry and control pEZX-H1-control KD-CMV-mCherry were purchased from GeneCopeia (USA). H1 promoter and the KD sequence were amplified using primers with flanking NheI site and ligated to pGEM-T Easy. After sequencing of pGEM-T Easy and digestion of both pGEM-T Easy and p156- pRRL-CMV-GFP with the NheI site, H1-KD miR and nicked p156 were ligated to generate p156-pRRL-H1-miR135bKD-CMV-GFP and p156-pRRL-H1-control KD-CMV-GFP.

Behavioral assessments

All behavioral assessments were performed during the dark phase following habituation to the test room for 2 hours before each test.

Light/dark transfer test

The light/dark transfer test apparatus and experimental conditions were as previously described. Briefly, the apparatus contained 2 chambers, a dark covered one, in which the mice were placed in the beginning of the test, and brightly lighted

chamber, to which it can transfer freely during the 5 minutes test. Time spent in the light compartment, distance traveled in light, latency to visit the light chamber and number of light-dark transitions were quantified with a video tracking system (VideoMot2; TSE Systems, Bad Hamburg, Germany).

Open-field test

The open field test was performed in a 50x50x22 cm white box, lighted in 120 lux, in which the mice were put for a 10 minutes test. Time spent in the center, number of visits in the center, latency to visit the center, number of rearing and total distance traveled were quantified using a video tracking system (VideoMot2; TSE Systems, Bad Hamburg, Germany).

Elevated plus maze test

This test apparatus had a plus shape and contained 2 barrier walls and 2 open very low lighted (6 lux) arms. The number of entries, distance traveled and the time spent in the open arms was automatically scored using video tracking system (VideoMot2; TSE Systems, Bad Hamburg, Germany) during the 5 minutes test.

RESULTS

The effects of antidepressant administration on miR135 levels were tested in brain sites known to be innervated by serotonergic neurons from the RN and involved in mood regulation, the amygdala (AMY) and the prefrontal cortex (PFC). In the AMY both miR135 variants were upregulated by acute serotonin reuptake inhibitors (SSRI) and the noradrenaline reuptake inhibitors (NRI) but not by chronic administration of these drugs (P=0.0001 for SSRI, p=0.003 for NRI for miR135a, Figure 28A; p=0.0001 for SSRI and p=0.003 for NRI for miR-135b, Figure 28B). At the PFC, miR135b levels were upregulated by acute SSRI and NRI (P=0.0183 for SSRI and 0.0013 for NRI Figure 28c) but miR135a levels were not significantly altered (Figure 28D). Additionally, chronic SSRI led to decreased miR135a and miR135b levels in the PFC (P=0.0241 for miR135a Figure 28C, and P= 0.0067 for miR135b Figure 28D).

Additionally, miR135 levels in the circulation were tested following the social defeat paradigm. miR135a (P=0.0089; Figure 29A) and miR135b (P=0.0033; Figure 29B) levels were increased in the plasma of mice exposed to chronic social defeat compared to control mice as measured in real time PCR. Thus, the present results demonstrated that miR135 in plasma was upregulated following chronic stress, known

to induce depression-like behaviors in mice, and robustly decreased by antidepressant administration. These finding suggest miR135 levels in the plasma as a biomarker for serotonergic-related depressive states.

EXAMPLE 7

Establishment of miR135 knockdown system; cloning, lentiviruses generation and *in vitro* and *in vivo* validations

MATERIALS AND EXPERIMENTAL PROCEDURES

Cloning of miR135 KD viral vector

miR135b KD plasmid pEZX-H1-miR135KD-CMV-mCherry and control pEZX-H1-control KD-CMV-mCherry were purchased from GeneCopeia (USA). H1 promoter and the KD sequence were amplified using primers with flanking NheI site and ligated to pGEM-T Easy. After sequencing of pGEM-T Easy and digestion of both pGEM-T Easy and p156- pRRL-CMV-GFP with the NheI site, H1-KD miR and nicked p156 were ligated to generate p156-pRRL-H1-miR135bKD-CMV-GFP and p156-pRRL-H1-control KD-CMV-GFP.

RESULTS

To evaluate the effect of decreased miR135 levels in RN on mice 5-HT-related behaviors, a plasmid based miR135b inhibitor was utilized and its efficiently was tested in a luciferase assay. In this assay, HEK293T cells were co-transected with miR135OE, miR135KD and 3'UTR plasmids, and the ability of miR135bKD plasmid to block the repressing effect of miR135 on Slc6a4 and Htr1a 3' UTR was tested. miR135b represoric effect of Htr1a 3'UTR was blocked by miR135KD plasmid (Figure 30A). Similarly, miR135b effect on Slac6a4 3'UTR was blocked by miR135KD (Figure 30B). These results indicate that miR135KD plasmid indeed blocks the biological activity of miR135.

miR135KD sequence and a control sequence were sub-cloned to a viral vector (Figure 30C) and lentiviruses expressing the different knockdown (KD) sequence were generated. In order to test the lentiviruses' ability to infect brain tissue, mice *RN* were infected with either one of the lentiviruses. Indeed, infection caused expression of GFP (Figures 30D-E) demonstrating the ability of miR135bKD lentiviruses to infect brain tissue.

EXAMPLE 8

Behavioral effects of miR135 knock down in adult mice RN

MATERIALS AND EXPERIMENTAL PROCEDURES

Behavioral assessments

mice were behaviorally characterized by using tests for anxiety and depression-like behaviors as described in Example 6 above.

RESULTS

Following the *in vitro* and *in vivo* validation of miR135KD lentiviruses, they were used to manipulate miR135 levels in the RN and to test their effect on mice behavior. Adult mice were injected either with miR135KD lentiviruses, or KD control lentiviruses to RN and following recovery period were tested for anxiety and depression-like behaviors. Since miR135 represses negative regulators of 5-HT, we expected miR135KD to lead to decrease 5-HT levels in the synapse and by that to increased anxiety and depression-like behaviors.

In the open field test no differences were observed between the groups (Figure 31A), however in the elevated pulse maze test, miR135KD mice demonstrated higher anxiety-like behavior by demonstrating a tendency to spend less time in the open arms (P=0.0644) and to visit less times in the open arms (P=0.0572 Figure 31B). Additionally miR135KD mice walked significantly less distance in the open arms (P=0.0433) and had a longer tendency to visit in open arms (P=0.0124 Figure 31B). Similarly, in the dark light test performed under basal stress conditions, miR135KD mice demonstrated a significant increased anxiety-like behavior compared to the controls by spending less time in the light (P=0.0454 Figure 31C), visiting less times in the light chamber (P=0.0107 Figure 31D) and walking a smaller distance in the light chamber (P=0.0402s Figure 31E). The results illustrated a decrease in miR135 levels 40 min and 24 hours after acute stress (Figure 30A-B), therefore, the present theory was that stressed miR135KD mice would not differ from their controls in anxiety-like behaviors when tested following acute stress, since the control mice would also have a decreased miR135 levels due to the stress. Indeed, there was no difference between the groups when re-tested in the dark light transfer test in any of the parameters, both when tested 40 minutes or 24 hours after acute stress (Figure 31C-E).

Depression-like behaviors of miR135KD were tested both under basal conditions and following pharmacological manipulation. Since miR135 levels were showed to increase in the RN following SSRI administration (Figure 31E), the speculation was that the reduction of miR135 levels may lead to reduced response to SSRI. In the tail suspension test performed both in basal levels and after SSRI administration, there was no difference between miR135b KD mice and control KD mice in immobility time (Figure 31F), and the expected decrease in immobility time due to SSRI treatment was observed ($P<0.0008$). However, in the forced swim test, additionally to the main effect for SSRI injection ($P<0.0001$), miR135KD mice injected with SSRI were more immobile in the last 2 minutes of the test compared to control KD mice ($P=0.0141$ 5 minute, $P=0.0404$ 6 minute; Figure 31G suggesting attenuation of SSRI antidepressant effects by reducing miR135 levels in the RN. This result implies that miR135 is part of the endogenous alternation leading to behavioral changes caused by SSRI.

EXAMPLE 9

miR135 overexpression in 5-HT neurons

MATERIALS AND EXPERIMENTAL PROCEDURES

Mice over expressing miR135a in 5-HT neurons were compared to there littermates controls both in expression levels of miR135 and its target genes and behaviorally.

RESULTS

The effects of manipulating miR135 levels specifically in 5-HT neurons in the RN of mice was tested for anxiety and depression-like behaviors. For that purpose, a genetic system was developed using the Cre-loxP system. Specifically, the 5-HT specificity was obtained using the ePet Cre mice expressing Cre recombinase specifically in the 5-HT RN positive neurons and miR135 overexpression was performed by crossing the 5-HT-specific Cre line (ePet Cre) with transgenic mouse line with conditional overexpression for miR135a (Figure 32).

miR135 expression level in the RN of mice overexpressing miR135 specifically in 5-HT neurons (miR135OE) was tested by real time PCR for miR135 and compared to control mice, positive for the miR135 conditional overexpression allele but negative for ePet CRE. miR135OE mice demonstrated near to 2 fold overexpression compared to control mice (Figure 33A; $P<0.05$). Overexpression levels of miR135 were similar to levels measured in the RN of mice following SSRI administration, suggesting this mice line was a good model for studying miR135 antidepressant characteristics. Additionally, miR135 target gene mRNA, Slc6a4 (Figure 33B; $P<0.05$) and Htr1a (Figure 33C; $P<0.1$) were down-regulated in the RN of miR135OE mice compared to control demonstrating *in vivo* repression by miR135 of its target genes.

In order to test miR135 overexpression specifically in 5-HT neurons, miR135OE mice and their littermates controls were exposed to chronic social defeat paradigm, a procedure know to induce depression and anxiety-like behaviors, and subsequently were tested for anxiety and depression-like behaviors.

miR135OE mice demonstrated increased anxiety-like behaviors following social defeat compared to control liter mates. In the open field, a tendency for increased anxiety was observed in miR135OE mice time and visit number to the center ($P<0.1$, Figure 34A). While in the dark light transfer test miR 135OE mice spent more time in light ($P<0.05$, Figure 34B) and spent less time in the light chamber ($P<0.01$, Figure 34B). Similar results were observed in the elevated pulse maze ($P<0.05$, Figure 34B) while miR135OE mice spent more time in the open arms ($P<0.05$, Figure 34C) and traveled larger distance in the open arms ($P<0.05$, Figure 34C).

Depression-like behaviors of miR135OE mice following social defeat were lower than of the control litter mates. A tendency towards decreased immobility time of the miR135OE mice compared to controls was observed in the tail suspension test ($P<0.1$, Figure 34D), along with a significant decreased immobility time in the forces swim test ($P<0.05$, Figure 34E).

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all

such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

WHAT IS CLAIMED IS:

1. A method of treating a medical condition in which an elevation of serotonin level is therapeutically beneficial in a subject in need thereof, the method comprising administering to or expressing in a cell of said subject an exogenous polynucleotide encoding at least one microRNA or a precursor thereof, wherein said microRNA is selected from the group consisting of miR-135, miR-335, miR-26 and miR-182, thereby treating the medical condition.

2. Use of an exogenous polynucleotide encoding at least one microRNA or a precursor thereof, wherein said microRNA is selected from the group consisting of miR-135, miR-335, miR-26 and miR-182, for the manufacture of a medicament identified for treating a medical condition in which an elevation of serotonin level is therapeutically beneficial.

3. A method of increasing a serotonin level in a synaptic cleft of a subject in need thereof, the method comprising administering to or expressing in a serotonergic neuron of said subject an exogenous polynucleotide encoding at least one microRNA or a precursor thereof, wherein said microRNA is selected from the group consisting of miR-135, miR-335, miR-26 and miR-182, thereby increasing said serotonin level in the synaptic cleft.

4. An isolated neuroglia cell comprising a nucleic acid construct expressing at least one microRNA or a precursor thereof, wherein said microRNA is selected from the group consisting of miR-135, miR-335, miR-26 and miR-182 under a transcriptional control of a cis acting regulatory element.

5. An isolated polynucleotide encoding at least one microRNA or a precursor thereof, wherein said microRNA is selected from the group consisting of miR-135, miR-335, miR-26 and miR-182, for treating a medical condition in which an elevation of serotonin level is therapeutically beneficial.

6. The method of claim 1, wherein said cell is a neuroglia cell.

7. The isolated neuroglia cell of claim 4 or method of claim 6, wherein said neuroglia cell is a serotonergic neuron.

8. The method of claim 1 or 3, use of claim 2, isolated neuroglia cell of claim 4 or polynucleotide of claim 5, wherein said miR-135 is as set forth in SEQ ID NO: 58-62.

9. The method of claim 1 or 3, use of claim 2, isolated neuroglia cell of claim 4 or polynucleotide of claim 5, wherein said miR-335 is as set forth in SEQ ID NO: 63-64.

10. The method of claim 1 or 3, use of claim 2, isolated neuroglia cell of claim 4 or polynucleotide of claim 5, wherein said miR-26 is as set forth in SEQ ID NO: 65-69.

11. The method of claim 1 or 3, use of claim 2, isolated neuroglia cell of claim 4 or polynucleotide of claim 5, wherein said miR-182 is as set forth in SEQ ID NO: 70-71.

12. The method of claim 1, use of claim 2, or polynucleotide of claim 5, wherein said medical condition is selected from the group consisting of a depression, an anxiety, a stress, a fatigue, an impaired cognitive function, a panic attack, a compulsive behavior, an addiction, a social phobia, a sleep disorder, a food related disorder, a growth disorder and a reproduction disorder.

13. The method of claim 1, wherein when said microRNA is miR-135, said medical condition is depression or anxiety.

14. A method of treating a medical condition in which a low adrenaline or noradrenaline level is therapeutically beneficial in a subject in need thereof, the method comprising administering to or expressing in a cell of said subject an exogenous polynucleotide encoding a miR-19 or a precursor thereof, thereby treating the medical condition.

15. Use of an exogenous polynucleotide encoding a miR-19 or a precursor thereof for the manufacture of a medicament identified for treating a medical condition in which a low adrenaline or noradrenaline level is therapeutically beneficial.

16. An isolated cell comprising a nucleic acid construct expressing a miR-19 or a precursor thereof under a transcriptional control of a cis acting regulatory element.

17. An isolated polynucleotide encoding a miR-19 or a precursor thereof for treating a medical condition in which a low adrenaline or noradrenaline level is therapeutically beneficial.

18. The method of claim 14 or isolated cell of claim 16, wherein said cell is a neuroglia cell or a cardiac cell.

19. The method of claim 14, use of claim 15, isolated cell of claim 16 or polynucleotide of claim 17, wherein said miR-19 is as set forth in SEQ ID NO: 72-76.

20. The method of claim 14, use of claim 15, or polynucleotide of claim 17, wherein said medical condition is selected from the group consisting of a stress, an anxiety, a memory impairment and a heart condition.

21. A method of treating a medical condition in which a low corticotropin-releasing hormone (CRH) level is therapeutically beneficial in a subject in need thereof, the method comprising administering to or expressing in a cell of said subject an exogenous polynucleotide encoding a miR-15 or a precursor thereof, thereby treating the medical condition.

22. Use of an exogenous polynucleotide encoding a miR-15 or a precursor thereof for the manufacture of a medicament identified for treating a medical condition in which a low corticotropin-releasing hormone (CRH) level is therapeutically beneficial.

23. An isolated cell comprising a nucleic acid construct expressing a miR-15 or a precursor thereof under a transcriptional control of a cis acting regulatory element.

24. An isolated polynucleotide encoding a miR-15 or a precursor thereof for treating a medical condition in which a low corticotropin-releasing hormone (CRH) level is therapeutically beneficial.

25. The method of claim 21 or isolated cell of claim 23, wherein said cell is a neuroglia cell.

26. The method of claim 21, use of claim 22, isolated cell of claim 23 or polynucleotide of claim 24, wherein said miR-15 is as set forth in SEQ ID NO: 77-80.

27. The method of claim 21, use of claim 22 or polynucleotide of claim 24, wherein said medical condition is selected from the group consisting of a depression, an anxiety, a stress, a fatigue, an impaired cognitive function, a panic attack, a compulsive behavior, an addiction, a social phobia, a sleep disorder, a food related disorder, a growth disorder and a reproduction disorder.

28. The polynucleotide of claim 5, 17 or 24, being under a transcriptional control of a cis acting regulatory element active in a neuroglia cell.

29. The polynucleotide of claim 17, being under a transcriptional control of a cis acting regulatory element active in a cardiac cell.

30. A method of treating a medical condition in which a low glutamate receptor level is therapeutically beneficial in a subject in need thereof, the method comprising administering to or expressing in a cell of said subject an exogenous polynucleotide encoding a miR-181 or a precursor thereof, thereby treating the medical condition.

31. Use of an exogenous polynucleotide encoding a miR-181 or a precursor thereof for the manufacture of a medicament identified for treating a medical condition in which a low glutamate receptor level is therapeutically beneficial.

32. An isolated cell comprising a nucleic acid construct expressing a miR-181 or a precursor thereof under a transcriptional control of a cis acting regulatory element.

33. An isolated polynucleotide encoding a miR-181 or a precursor thereof for treating a medical condition in which a low glutamate receptor level is therapeutically beneficial.

34. The method of claim 30 or isolated cell of claim 32, wherein said cell is a neuroglia cell.

35. The method of claim 30, use of claim 31, isolated cell of claim 32 or polynucleotide of claim 33, wherein said miR-181 is as set forth in SEQ ID NO: 85-94.

36. The method of claim 30, use of claim 31, or polynucleotide of claim 33, wherein said medical condition is selected from the group consisting of a seizure, a Huntington's disease, a schizophrenia, a fragile X syndrome, a generalized anxiety disorder and a cancer.

37. A nucleic acid construct comprising a nucleic acid sequence encoding a microRNA or a precursor thereof, wherein said microRNA or a precursor thereof is selected from the group consisting of miR-135, miR-335, miR-26, miR-27, miR-181, miR-182, miR-19 and miR-15, said nucleic acid sequence being under a transcriptional control of a cis acting regulatory element.

38. The nucleic acid construct of claim 37, wherein said cis acting regulatory element is active in a neuroglia cell or a cardiac cell.

39. A pharmaceutical composition comprising the nucleic acid construct of claims 37 or 38 and a pharmaceutically acceptable carrier or diluent.

40. The method of claims 1, 3, 14, 21 or 30, wherein said subject is a human subject.

41. A method of regulating an expression of a tryptophan hydroxylase 2 (Tph2) gene in a neuroglia cell, the method comprising modulating an activity or expression of a microRNA or a precursor thereof in said neuroglia cell, wherein said microRNA is selected from the group consisting of miR-181 and miR27, thereby regulating the expression of the Tph2 gene.

42. The method of claim 41, wherein when said regulating comprises upregulating the expression of the Tph2 gene, said modulating comprises downregulating said miR-181 and/or said miR-27 in said neuroglia cell.

43. The method of claim 42, further comprising measuring an expression of said Tph2 gene following said downregulating of said miR-181 and/or said miR-27 in said neuroglia cell.

44. An isolated polynucleotide comprising a nucleic acid sequence for downregulating an expression of miR-181, miR-27, or a precursor thereof.

45. A nucleic acid construct comprising a nucleic acid sequence for downregulating an expression of a microRNA or a precursor thereof, wherein said microRNA or a precursor thereof is selected from the group consisting of miR-181 and said miR-27, said nucleic acid sequence being under a transcriptional control of a cis acting regulatory element.

46. A method of regulating an expression of a glutamate receptor gene in a neuroglia cell, the method comprising modulating an activity or expression of miR-181 or a precursor thereof in said neuroglia cell, thereby regulating the expression of the glutamate receptor gene.

47. The method of claim 46, wherein said glutamate receptor gene is selected from the group consisting of glutamate receptor metabotropic 1 (Grm1), glutamate receptor ionotropic kainate 3 (Grik3), glutamate receptor metabotropic 5 (Grm5), glutamate receptor ionotropic kainate 2 (Grik2) and glutamate receptor metabotropic 7 (Grm7).

48. A method of regulating an expression of a serotonin transporter (Slc6a4) gene in a neuroglia cell, the method comprising modulating an activity or expression of a microRNA or a precursor thereof in said neuroglia cell, wherein said microRNA is selected from the group consisting of miR-135 and miR-335, thereby regulating the expression of the Slc6a4 gene.

49. The method of claim 48, wherein when said regulating comprises downregulating the expression of the Slc6a4 gene, said modulating comprises upregulating said miR-135 and/or miR-335 in said neuroglia cell.

50. The method of claim 49, further comprising measuring an expression of said Slc6a4 gene following said upregulating said miR-135 and/or miR-335 in said neuroglia cell.

51. A method of regulating an expression of a serotonin inhibitory receptor 1a (Htr1a) gene in a neuroglia cell, the method comprising modulating an activity or expression of a microRNA or a precursor thereof in said neuroglia cell, wherein said microRNA is selected from the group consisting of miR-135, miR-335, miR-181, miR-182 and miR-26, thereby regulating the expression of the Htr1a gene.

52. The method of claim 51, wherein when said regulating comprises downregulating the expression of the Htr1a gene, said modulating comprises upregulating said miR-135, miR-335, miR-181, miR-182 and/or miR-26 in said neuroglia cell.

53. The method of claim 52, further comprising measuring an expression of said Htr1a gene following said upregulating said miR-135, miR-335, miR-181, miR-182 and/or miR-26 in said neuroglia cell.

54. A method of regulating an expression of a Down Syndrome Cell Adhesion Molecule (Dscam) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-182 or a precursor thereof in said neuroglia cell, thereby regulating the expression of the Dscam gene.

55. A method of regulating an expression of a Cell adhesion molecule L1 (L1cam) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-182 or a precursor thereof in said neuroglia cell, thereby regulating the expression of the L1cam gene.

56. A method of regulating an expression of a Translin-associated protein X (Tsnax) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-182 or a precursor thereof in said neuroglia cell, thereby regulating the expression of the Tsnax gene.

57. A method of regulating an expression of a monoamine hydroxylase (MaoA) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-27, thereby regulating the expression of the MaoA gene.

58. The method of claim 57, wherein when said regulating comprises downregulating the expression of the MaoA gene, said modulating comprises upregulating said miR-27 in said neuroglia cell.

59. The method of claim 58, further comprising measuring an expression of said MaoA gene following said upregulating said upregulating said miR-27 in said neuroglia cell.

60. A method of regulating an expression of a beta adrenergic receptor 1 (Adrb1) gene in a neuroglia cell or cardiac cell, the method comprising modulating an activity or expression of a miR-19, thereby regulating the expression of the Adrb1 gene.

61. The method of claim 60, wherein when said regulating comprises downregulating the expression of the Adrb1 gene, said modulating comprises upregulating said miR-19 in said neuroglia cell or said cardiac cell.

62. The method of claim 61, further comprising measuring an expression of said Adrb1 gene following said upregulating said miR-19 in said neuroglia cell or said cardiac cell.

63. A method of regulating an expression of a canabinoid receptor 1 (CB1) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-19 or a precursor thereof in said neuroglia cell, thereby regulating the expression of the CB1 gene.

64. The method of claim 63, wherein when said regulating comprises downregulating the expression of the CB1 gene, said modulating comprises upregulating said miR-19 in said neuroglia cell.

65. The method of claim 64, further comprising measuring an expression of said CB1 gene following said upregulating said CB1 in said neuroglia cell.

66. A method of regulating an expression of a CRH type 1 receptor gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-15, thereby regulating the expression of the CRH type 1 receptor gene.

67. The method of claim 66, wherein when said regulating comprises downregulating the expression of the CRH type 1 receptor gene, said modulating comprises upregulating said miR-15 in said neuroglia cell.

68. The method of claim 67, further comprising measuring an expression of said CRH type 1 receptor gene following said upregulating said miR-15 in said neuroglia cell.

69. A method of regulating an expression of a FK506 binding protein 5 (FKBP5) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-15 or a precursor thereof in said neuroglia cell, thereby regulating the expression of the FKBP5 gene.

70. The method of claim 69, wherein when said regulating comprises downregulating the expression of the FKBP5 gene, said modulating comprises upregulating said miR-15 in said neuroglia cell.

71. The method of claim 70, further comprising measuring an expression of said FKBP5 gene following said upregulating said miR-15 in said neuroglia cell.

72. A method of regulating an expression of a syntaxin 1a (Stx1a) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-15 or a precursor thereof in said neuroglia cell, thereby regulating the expression of the Stx1a gene.

73. A method of regulating an expression of a serum/glucocorticoid_regulated kinase (Sgk1) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-15 or a precursor thereof in said neuroglia cell, thereby regulating the expression of the Sgk1 gene.

74. A method of regulating an expression of a beta 2 adrenergic receptor (Adrb2) gene in a neuroglia cell, the method comprising modulating an activity or expression of a miR-15 or a precursor thereof in said neuroglia cell, thereby regulating the expression of the Adrb2 gene.

75. A method of monitoring treatment of an anti-depressant drug, the method comprising:

(a) treating a subject in need thereof with an anti-depressant drug; and

(b) measuring an expression level of a miR-135 in the blood of said subject prior to and following said treatment, wherein a lower expression level of said miR-135 following to said treatment by said anti-depressant drug as compared to said expression level of said miR-135 prior to said treatment by said anti-depressant drug is indicative of the efficient treatment.

76. The method of claim 75, further comprising obtaining a blood sample from said subject prior to said treatment.

77. The method of claim 75, wherein said anti-depressant drug is selected from the group consisting of selective serotonin reuptake inhibitors (SSRI), tricyclic antidepressants and noradrenaline reuptake inhibitors (NRI).

78. A method of diagnosing a serotonin-related medical condition in a subject in need thereof, the method comprising measuring an expression level of a miR-135 in a blood of the subject, wherein a high expression level of said miR-135 as compared to that in a blood sample of a healthy subject is indicative of the serotonin-associated medical condition.

79. The method of claim 78, wherein said serotonin-associated medical condition is a psychiatric condition.

80. The method of claim 79, wherein said psychiatric condition is selected from the group consisting of a depression, an anxiety, a stress, a fatigue, an impaired cognitive function, a panic attack, a compulsive behavior, an addiction, a social phobia, a sleep disorder and a food related disorder.

81. The method of claim 75 or 78, wherein said miR-135 comprises miR-135a or miR-135b.

FIG. 1A

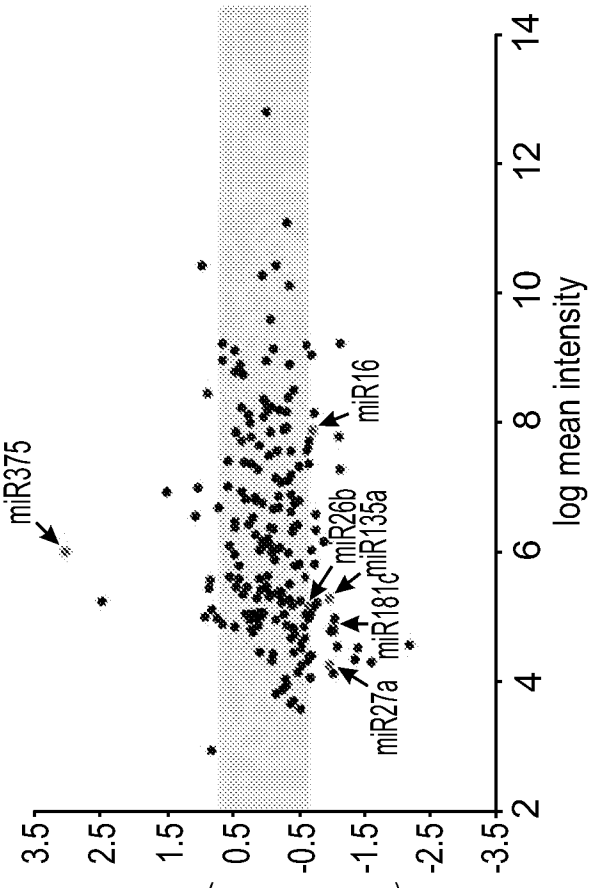


FIG. 1B

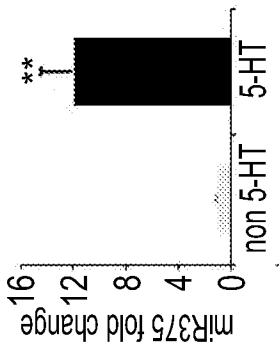


FIG. 1C

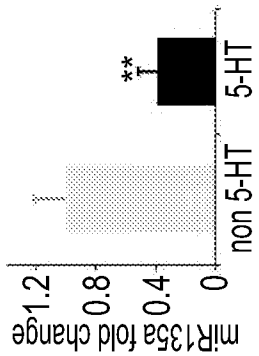


FIG. 1D

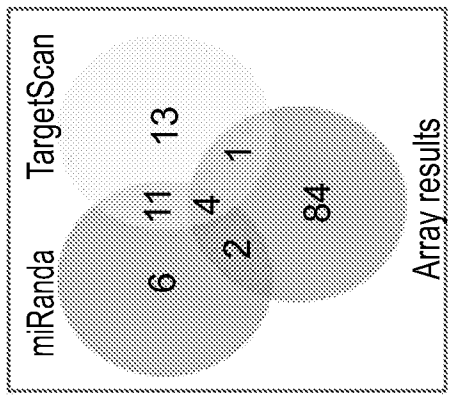


FIG. 1E

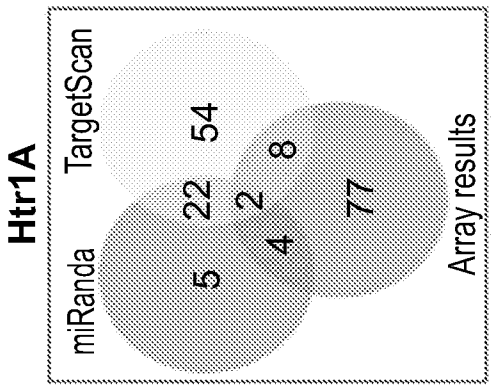


FIG. 1F Tph2

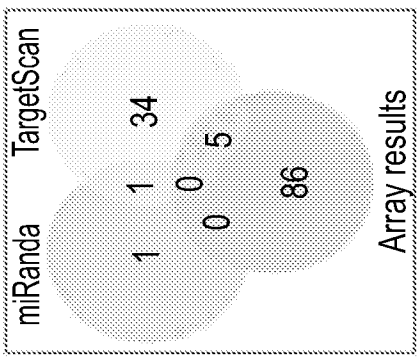


FIG. 1G MaoA

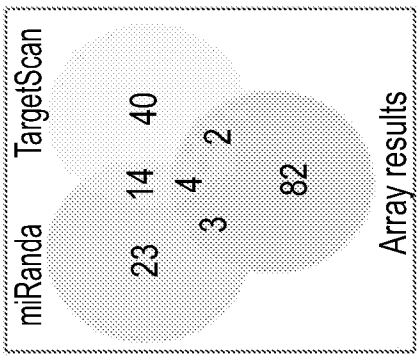


FIG. 1I

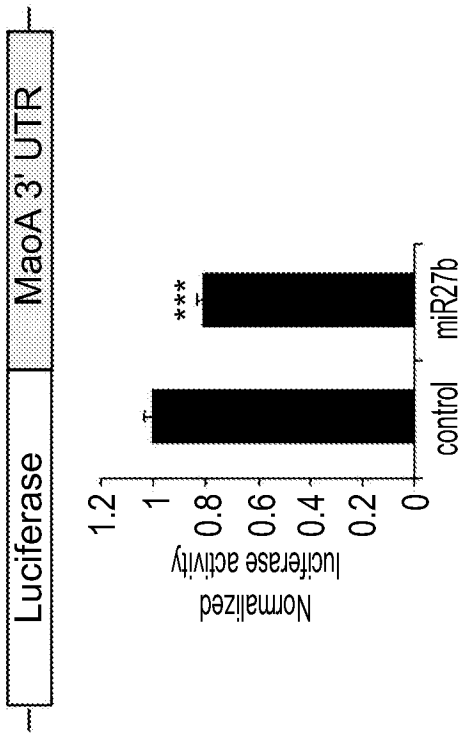


FIG. 1H

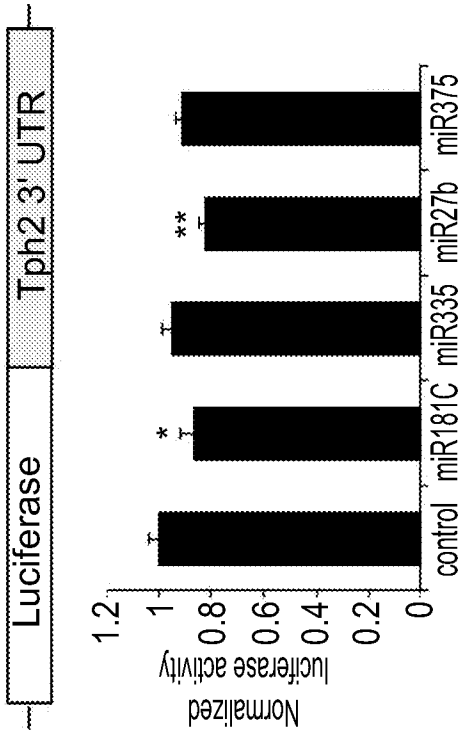


FIG. 2A

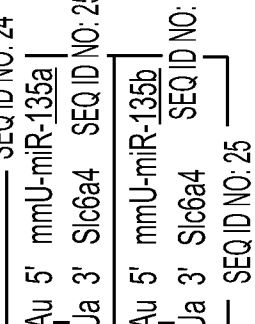


FIG. 2B

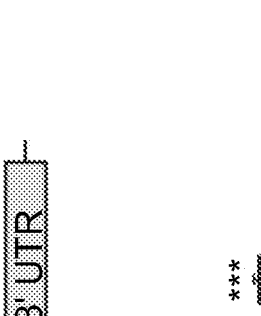


FIG. 2C

FIG. 2D

FIG. 2E

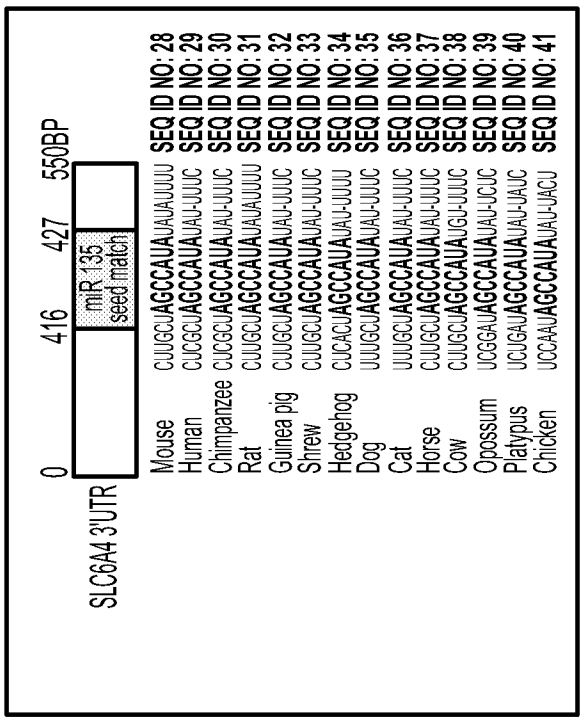


FIG. 2F

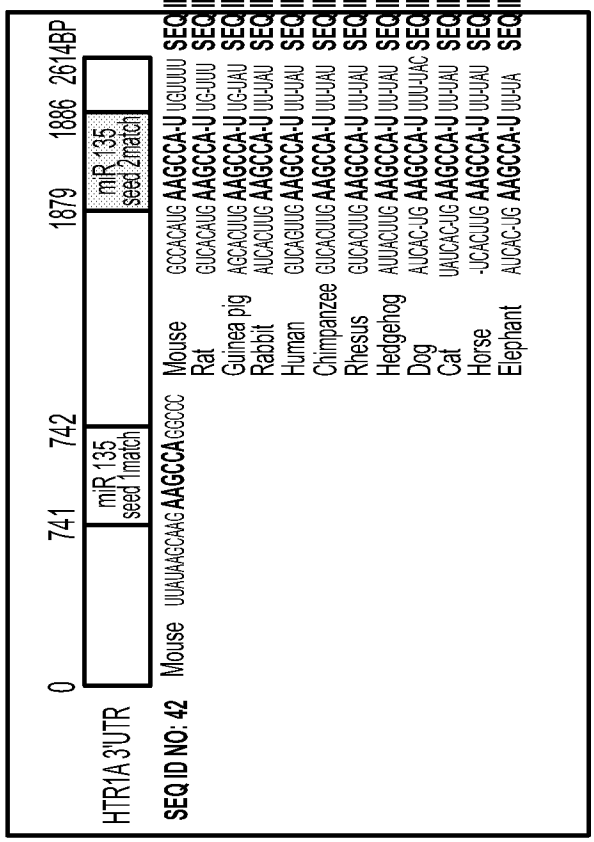


FIG. 2G

FIG. 2H

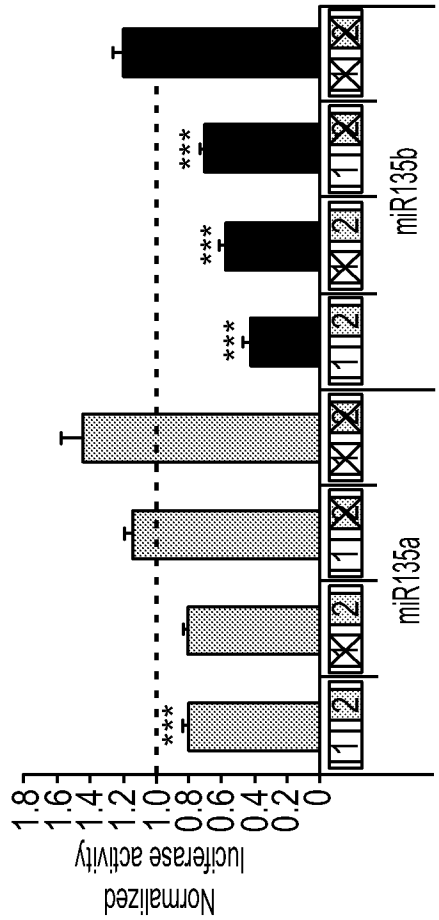


FIG. 3A

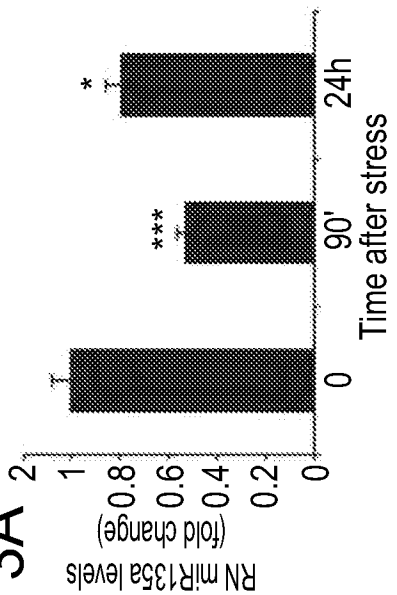


FIG. 3B

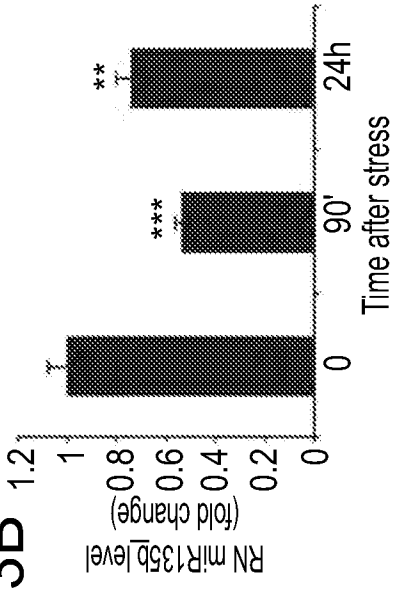


FIG. 3C

FIG. 3D

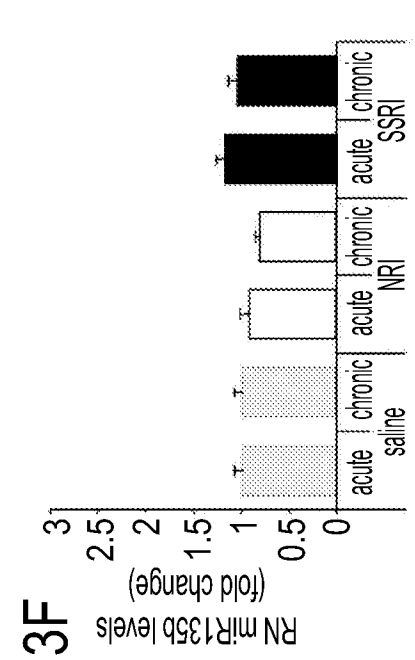


FIG. 3F

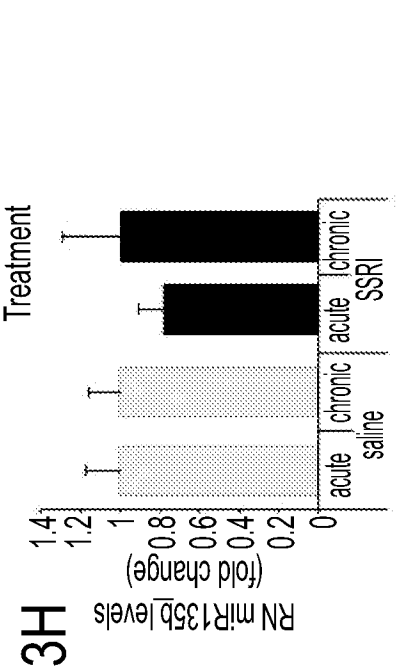


FIG. 3H

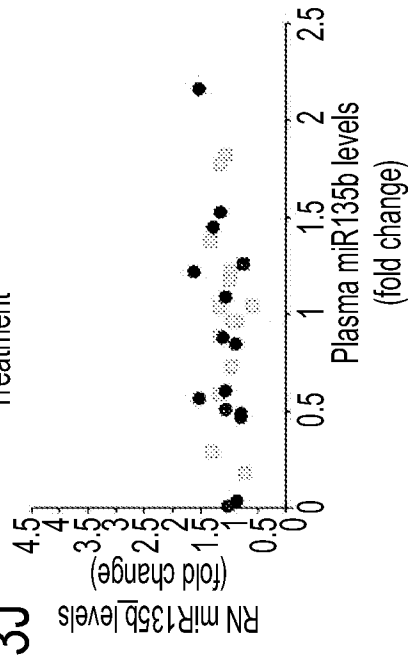


FIG. 3J

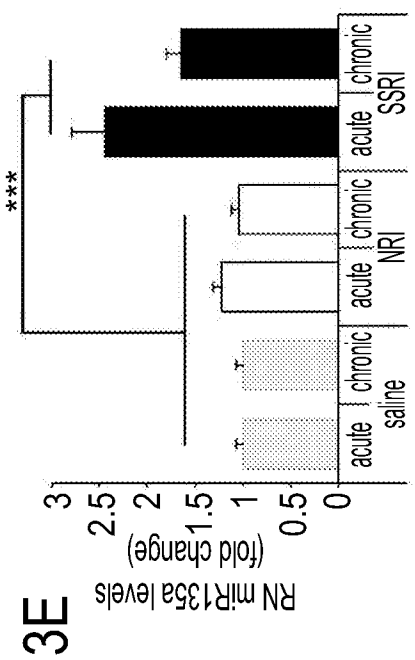


FIG. 3E

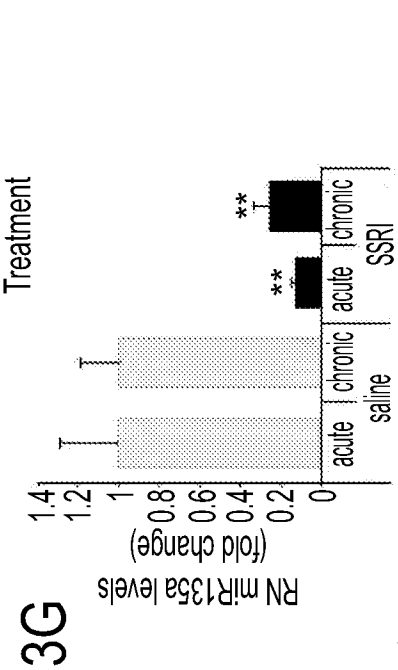


FIG. 3G

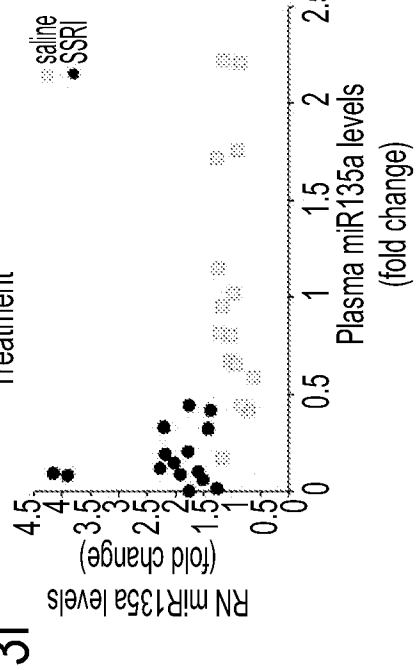


FIG. 3I

FIG. 4B

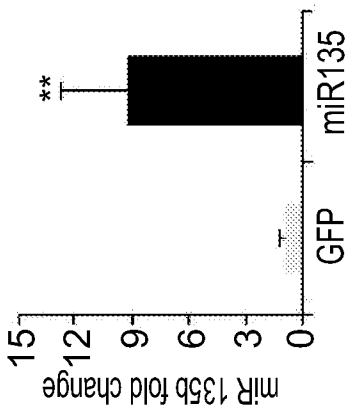

miR 135b fold change
15
12
9
6
3
0
**
GFP
miR135

FIG. 4A


ClaI
XhoI
eSny promoter
AgeI
BamHI
pre-mmu-miR135b
AgeI
eGFP
BsrGI

FIG. 4C

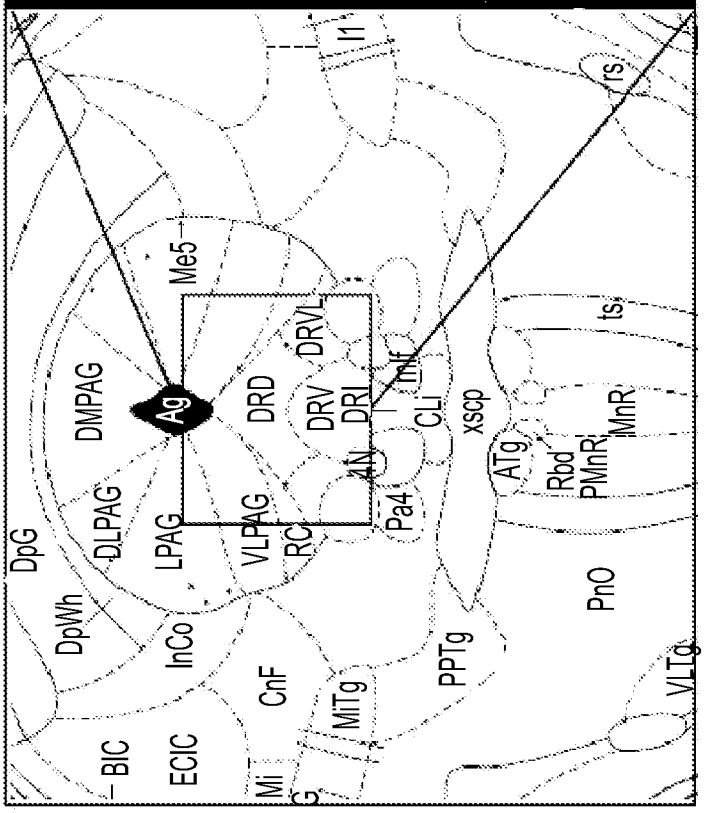

Ag
Me5
DMPAG
DLPAG
LPAG
VLPAG
DpG
DpWh
InCo
CnF
BIC
ECIC
Mi
MiTg
RC
Pa4
DRD
DRV
DRL
DRVL
4N
mlf
CLi
xscp
I1
rs
ts
ATg
Rbd
PMnR
MnR
PnO
PPTg
VLTg

FIG. 4D

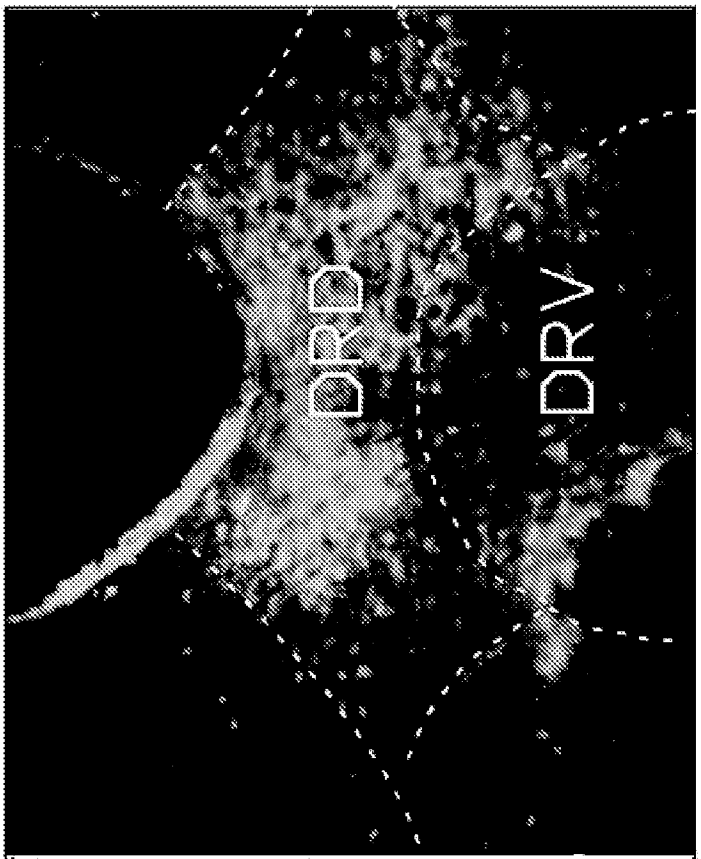

DRD
DRV

FIG. 4E

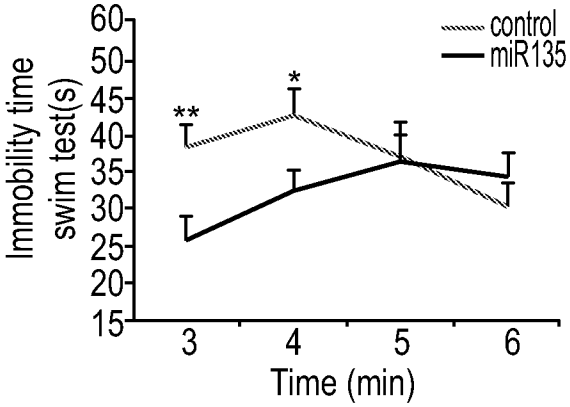


FIG. 4F

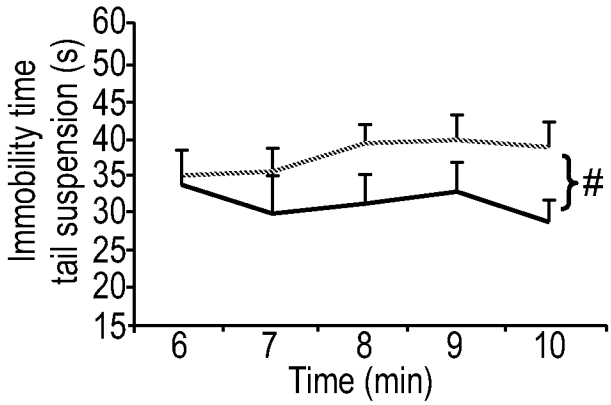


FIG. 4G

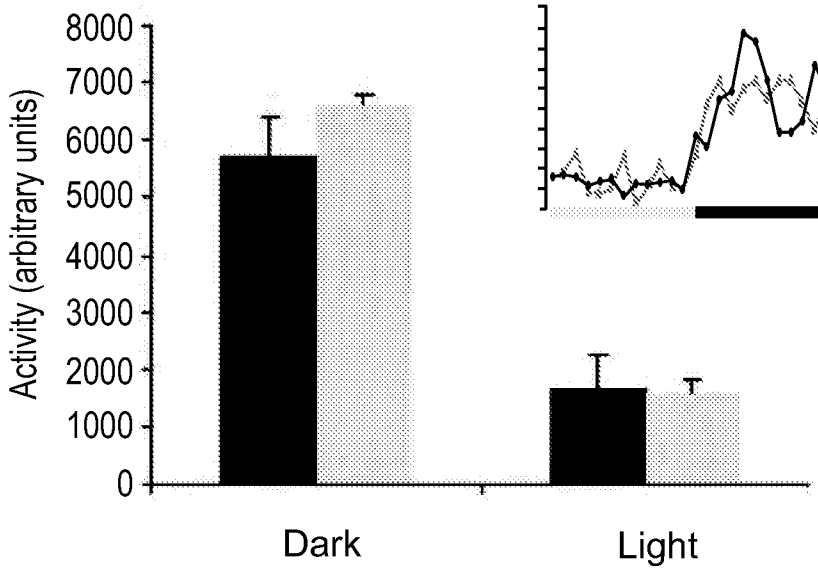
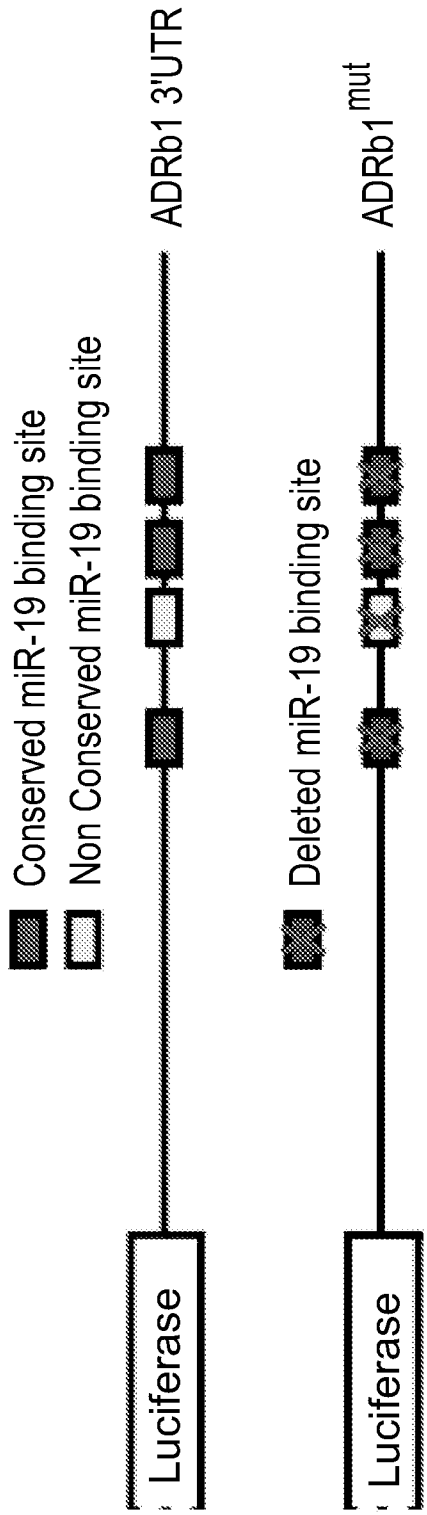


FIG. 4H

FIG. 5


Conserved miR-19 binding site
Non Conserved miR-19 binding site
ADRb1 3'UTR
Luciferase
Deleted miR-19 binding site
ADRb1mut
Luciferase

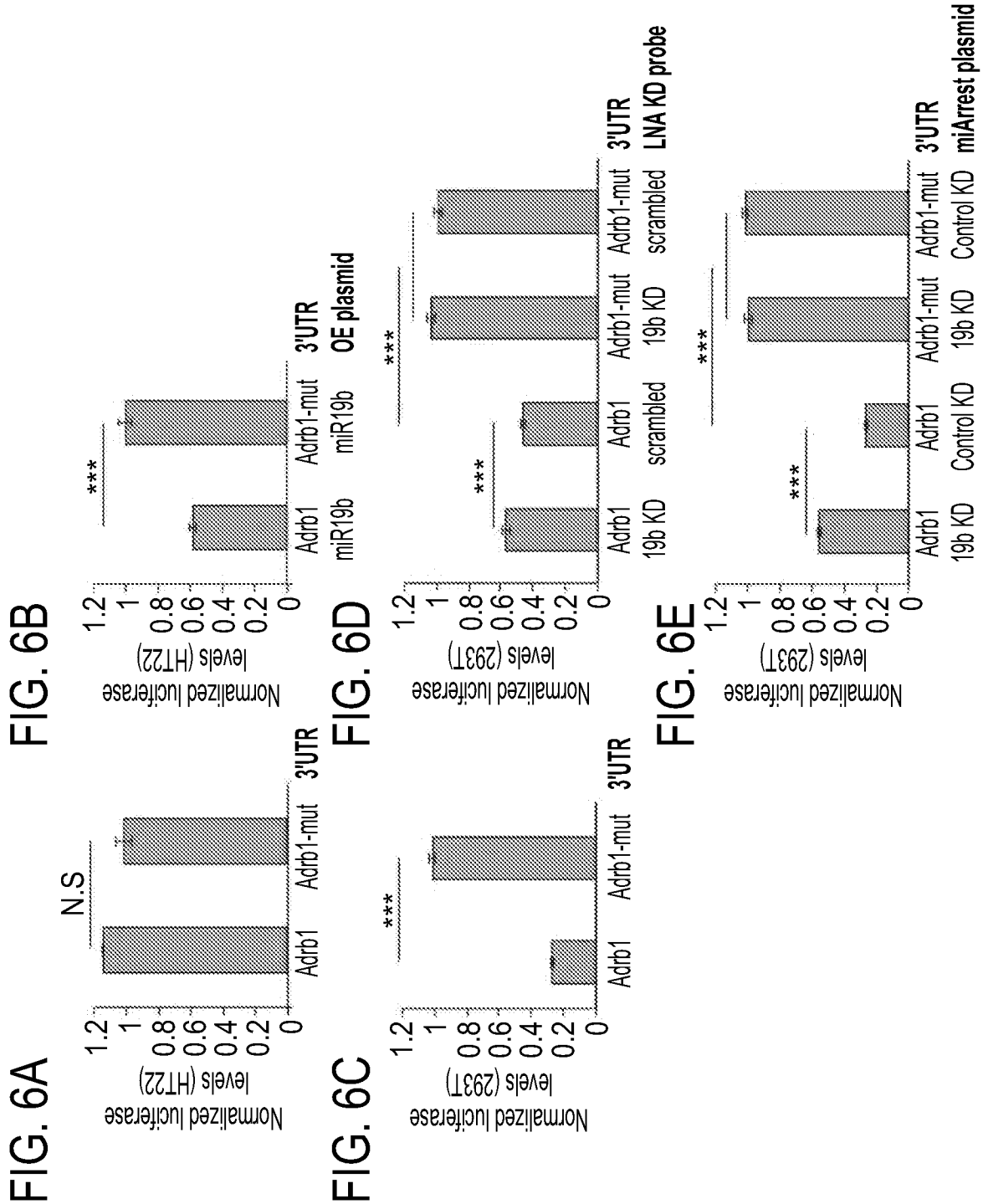


FIG. 6A
N.S
Normalized luciferase levels (HT22)
1.2
1
0.8
0.6
0.4
0.2
0
Adrb1
Adrb1-mut
3'UTR
FIG. 6B
***
Normalized luciferase levels (HT22)
Adrb1
miR19b
Adrb1-mut
miR19b
3'UTR
OE plasmid
FIG. 6C
***
Normalized luciferase levels (293T)
Adrb1
Adrb1-mut
3'UTR
FIG. 6D
***
***
Normalized luciferase levels (293T)
Adrb1
19b KD
Adrb1
scrambled
Adrb1-mut
19b KD
Adrb1-mut
scrambled
3'UTR
LNA KD probe
FIG. 6E
***
***
Normalized luciferase levels (293T)
Adrb1
19b KD
Adrb1
Control KD
Adrb1-mut
19b KD
Adrb1-mut
Control KD
3'UTR
miArrest plasmid

FIG. 7A

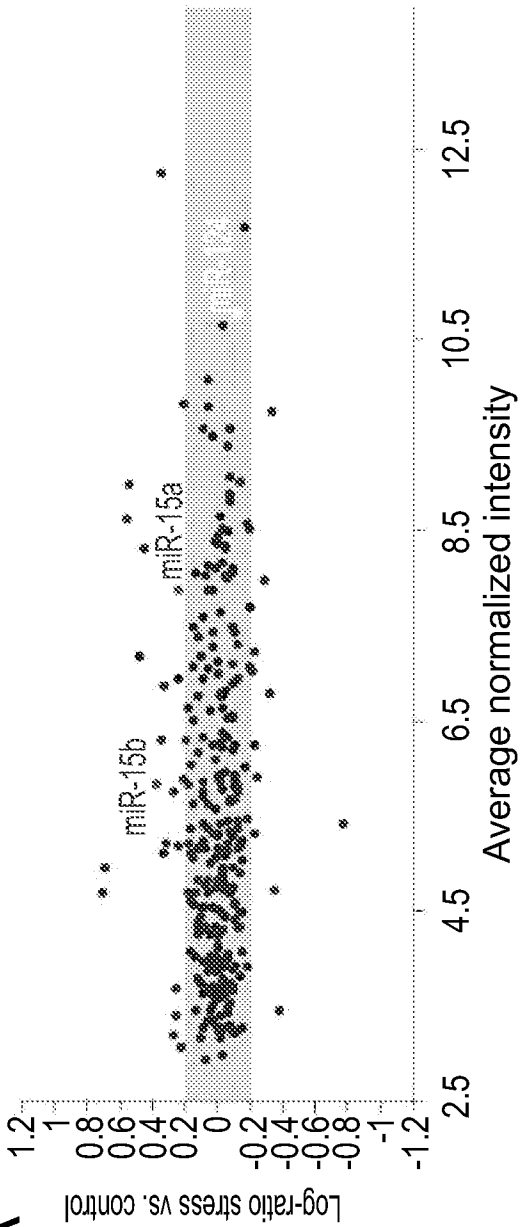

miR-15b
miR-15a
Log-ratio stress vs. control
Average normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
2.5
4.5
6.5
8.5
10.5
12.5

FIG. 7B

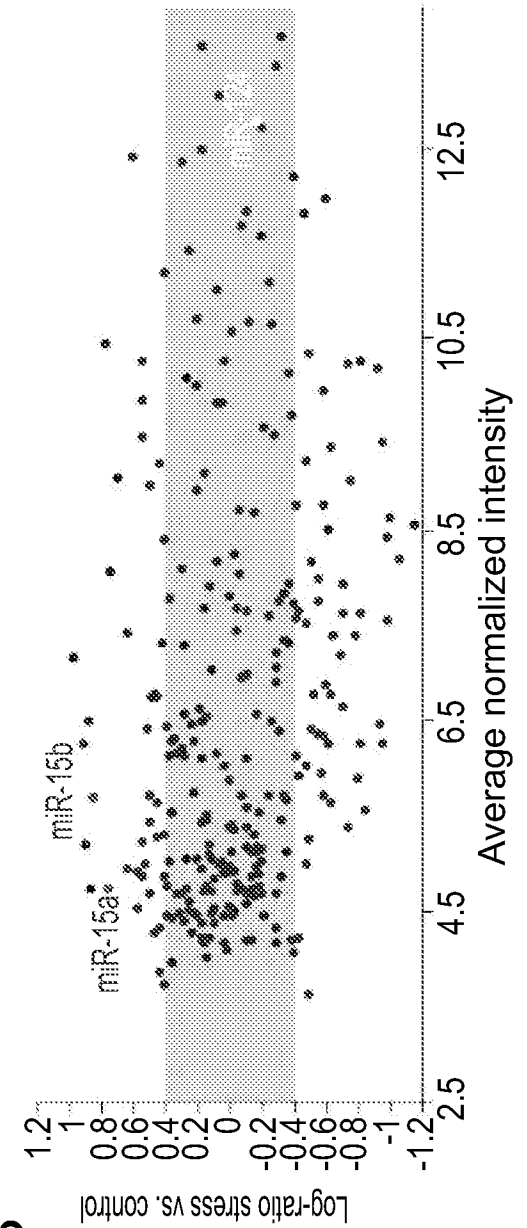

miR-15b
miR-15a
Log-ratio stress vs. control
Average normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
2.5
4.5
6.5
8.5
10.5
12.5

FIG. 7C

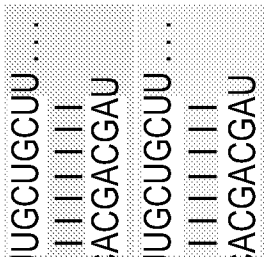

Position 554-560 of Crhr1 3' UTR 5' . . . UGGAAAGCCCUGCCUUGCUGCUU . . . SEQ ID NO: 55
mmu-miR-15a 3' GUGUUUGGUAAUACACGACGAU SEQ ID NO: 56
Position 554-560 of Crhr1 3' UTR 5' . . . UGGAAAGCCCUGCCUUGCUGCUU . . . SEQ ID NO: 55
mmu-miR-15b 3' ACAUUUGGUACUACACGACGAU SEQ ID NO: 57

FIG. 7D

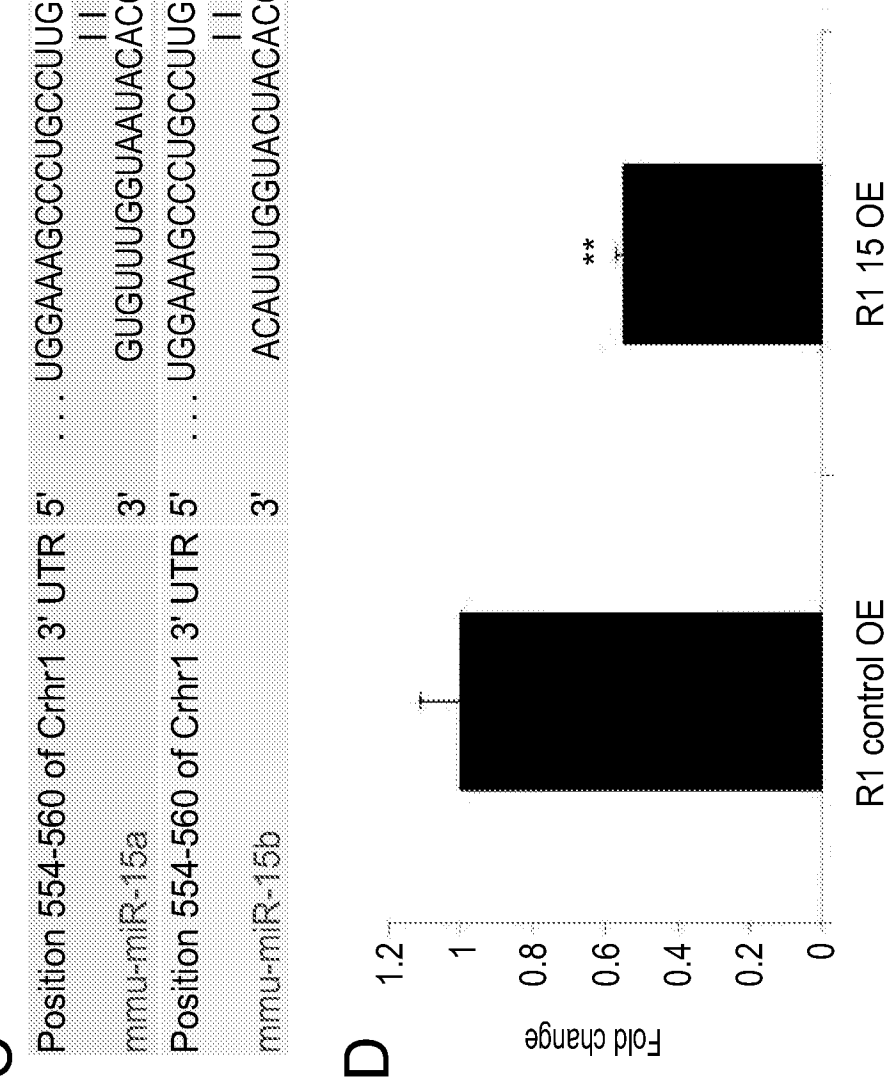

Fold change
1.2
1
0.8
0.6
0.4
0.2
0
**
R1 control OE
R1 15 OE

FIG. 8

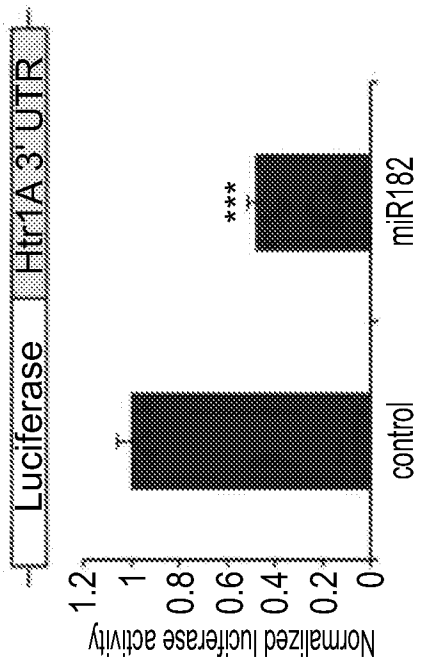

Luciferase
Htr1A 3' UTR
Normalized luciferase activity
1.2
1
0.8
0.6
0.4
0.2
0
***
control
miR182

FIG. 9

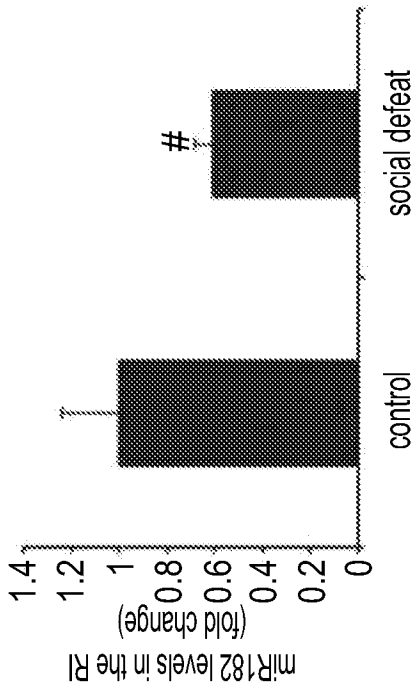

miR182 levels in the RI
(fold change)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
#
control
social defeat

FIG. 10

SLC33A1	solute carrier family 33 (acetyl-CoA transporter), member 1
FOXP2	forkhead box P2
ADCY2	adenylate cyclase 2 (brain)
ADCY6	adrenergic, alpha-2C-, receptor
BDNF	brain-derived neurotrophic factor
GRIA1	glutamate receptor, ionotropic, AMPA 1
GRIA3	glutamate receptor, ionotrophic, AMPA 3
GRIK3	glutamate receptor, ionotropic, kainate 3
GRM5	glutamate receptor, metabotropic 5
NUFIP2	nuclear fragile X mental retardation protein interacting protein 2
CACNB4	calcium channel, voltage-dependent, beta 4 subunit
SLC1A1	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1
GDNF	glial cell line derived neurotrophic factor
KCNH5	potassium voltage-gated channel, subfamily H (eag-related), member 5
CREB1	cAMP responsive element binding protein 1
FXR1H	fragile X mental retardation gene 1, autosomal homolog
GAD2	glutamic acid decarboxylase 2
FMR1	fragile X mental retardation syndrome 1 homolog
HTR2C	5-hydroxytryptamine (serotonin) receptor 2C
HDAC9	histone deacetylase 9

FIG. 11A

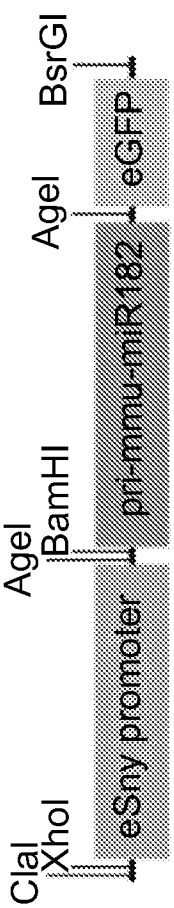


FIG. 11B

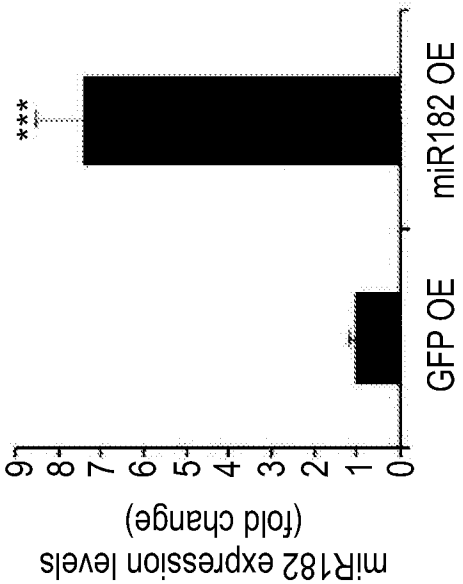


FIG. 11C

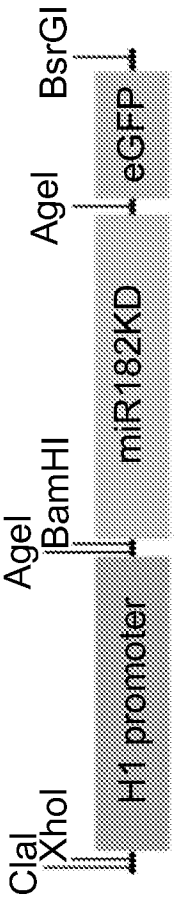


FIG. 12A

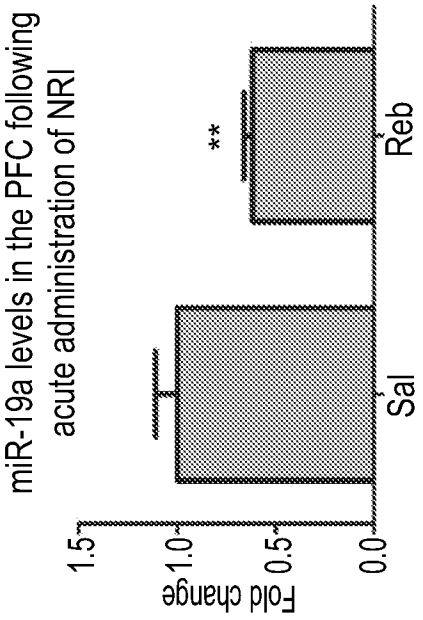


FIG. 12B

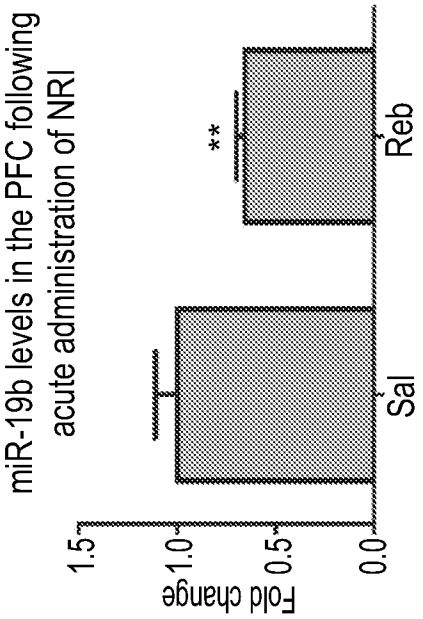


FIG. 12C

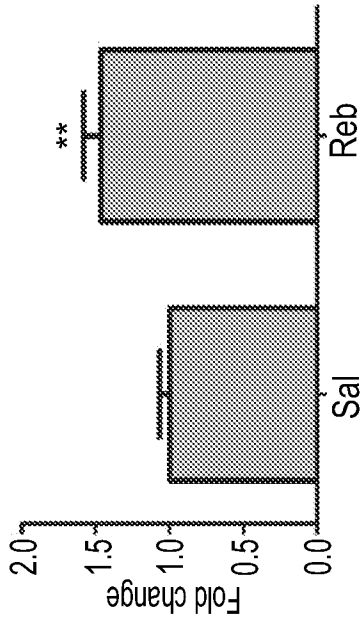


FIG. 12D

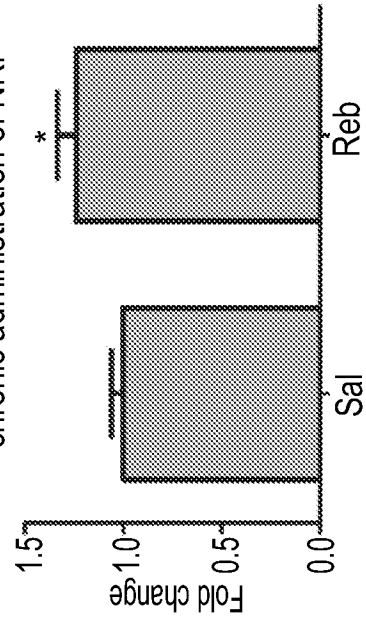


FIG. 13A

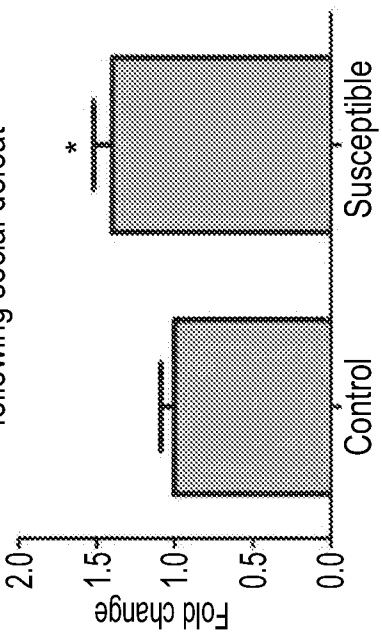


FIG. 13B

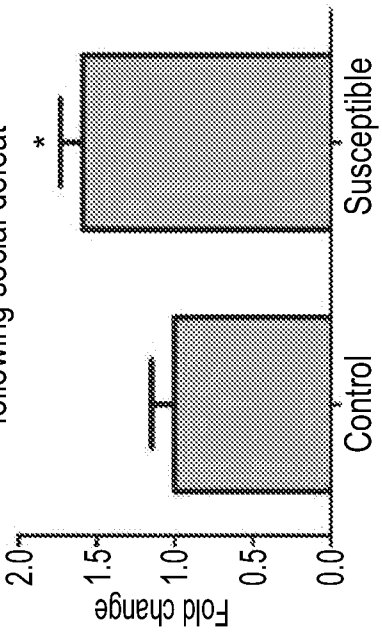


FIG. 13C

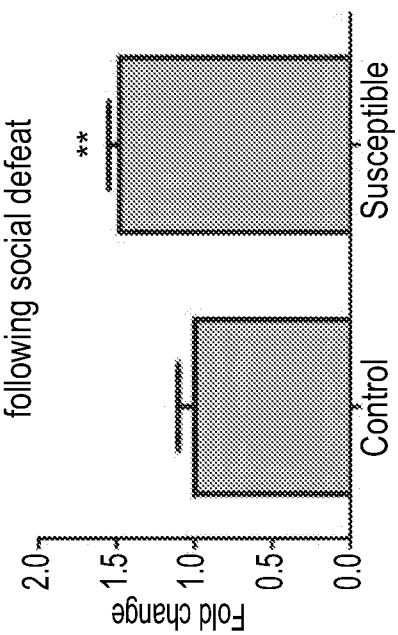


FIG. 13D

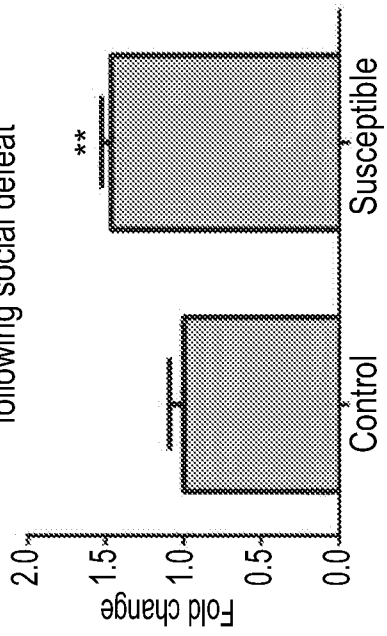
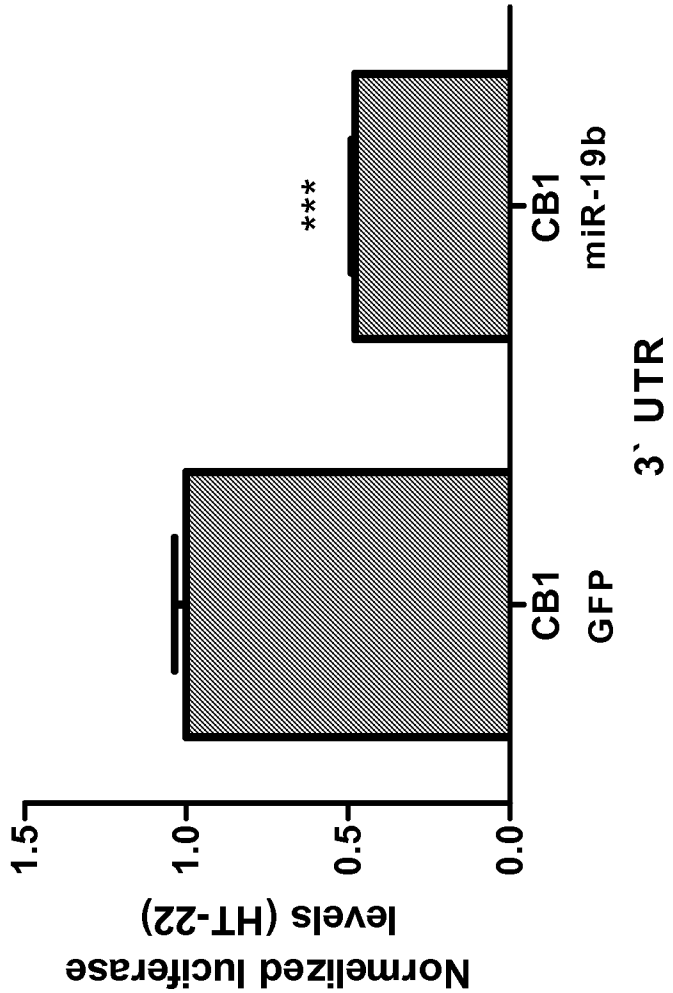


FIG. 14


Normelized luciferase
levels (HT-22)
1.5
1.0
0.5
0.0
***
CB1
GFP
CB1
miR-19b
3` UTR

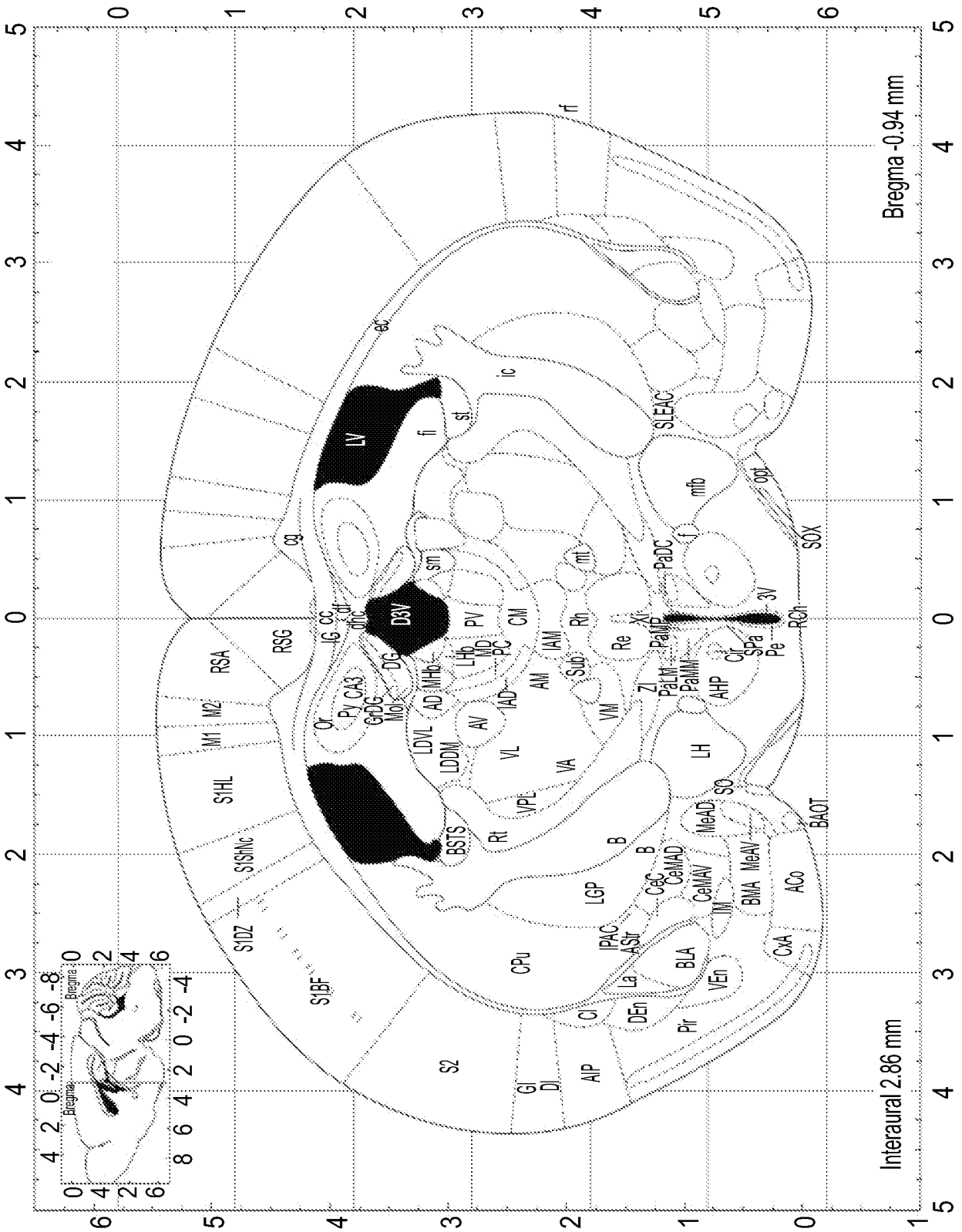

Bregma -0.94 mm
Interaural 2.86 mm
LV
DSV
fi
st
ic
ec
rf
cg
sm
mt
SLEAC
mfb
opt
SOX
3V
RCh
PaDC
Xi
PaMP
PaMM
Cir
SPa
Pe
AHP
Re
Rh
CM
PV
IAM
Sub
PC
MD
LHb
MHb
DG
CA3
Py
Or
GrDG
Mol
AD
AM
IAD
AV
VM
ZI
PaLM
LDVL
LDDM
VL
VA
LH
VPL
SO
MeAD
MeAV
BAOT
BSTS
Rt
B
CeMAD
CeMAV
CeC
IM
BMA
ACo
LGP
IPAC
AStr
CxA
CPu
BLA
VEn
La
DEn
Cl
Pir
AIP
GI
DI
S2
S1BF
S1DZ
S1ShNc
S1HL
M1
M2
RSA
RSG
IG
cc
dhc
dtr
Bregma
Bregma

FIG. 15A

FIG. 15B

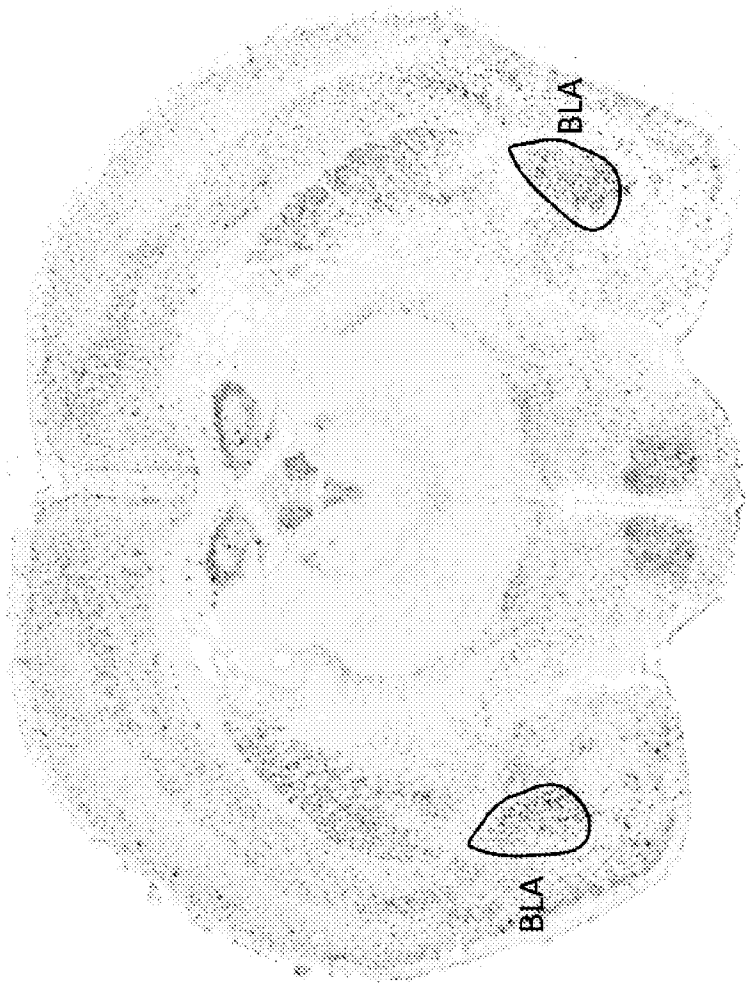
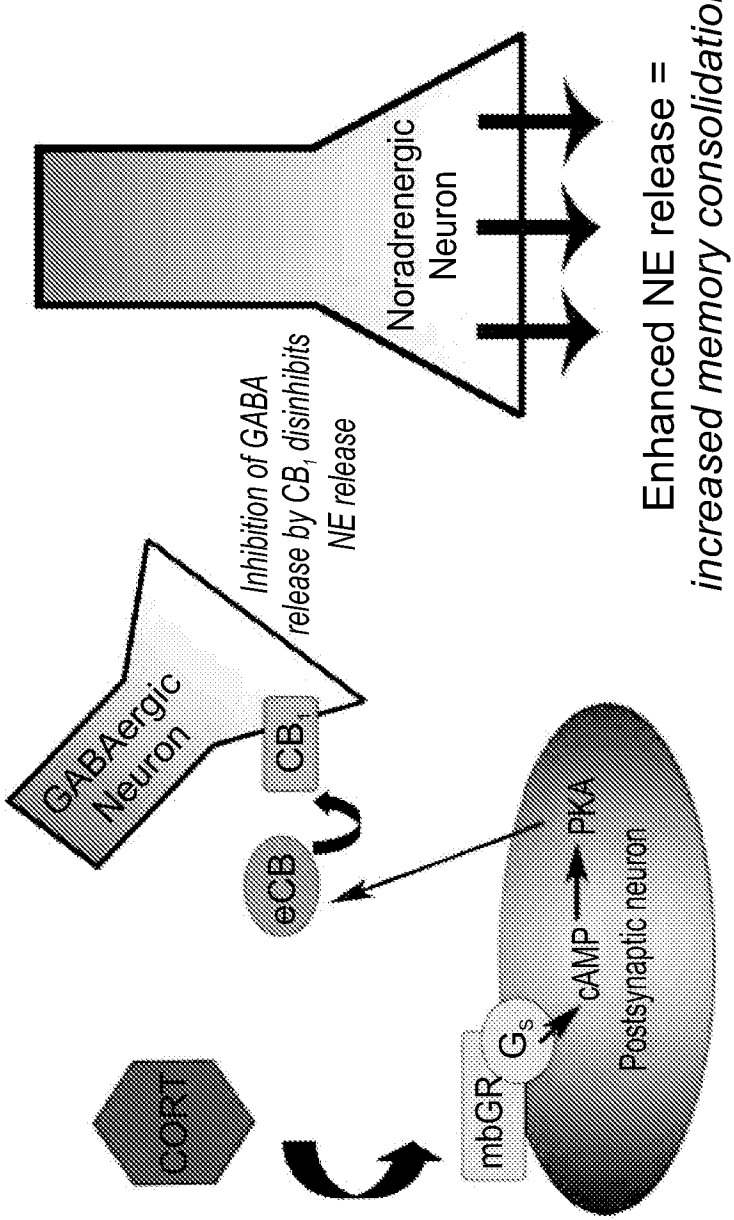
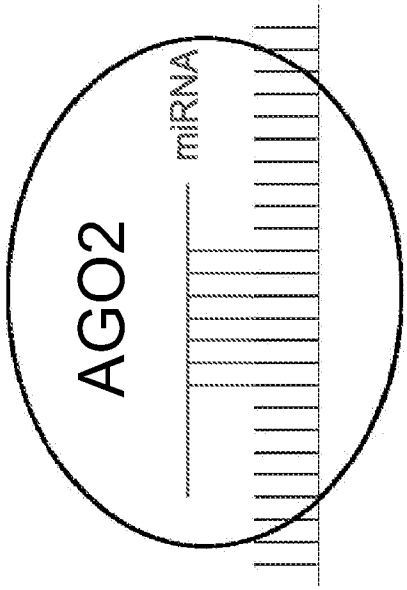


FIG. 16



Incorporated from Hill M.N. and McEwen B.S. Proc of the Nat Acad of Sci of the USA (2009) 106:4579-4580

FIG. 17A



Incorporated from Meister G. et al., Molecular cell (2004) 15:185-197

FIG. 17B

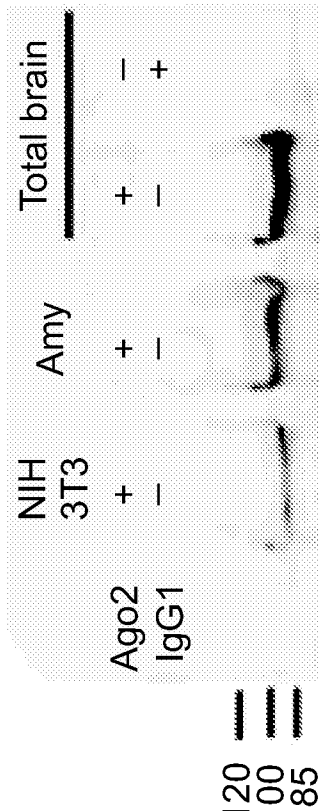
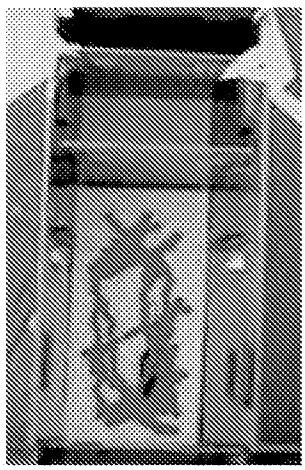


FIG. 18A

Susceptible



Habituation

FIG. 18B

Resilient

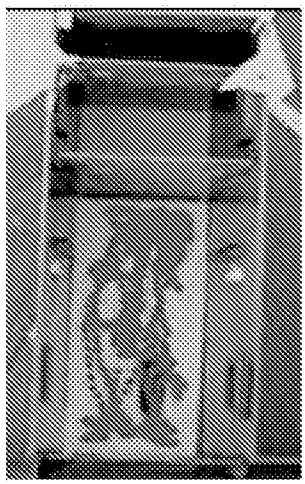
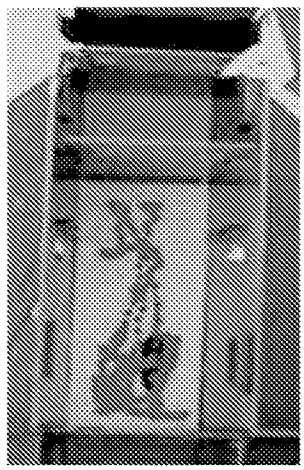


FIG. 18C



Unfamiliar mouse

FIG. 18D

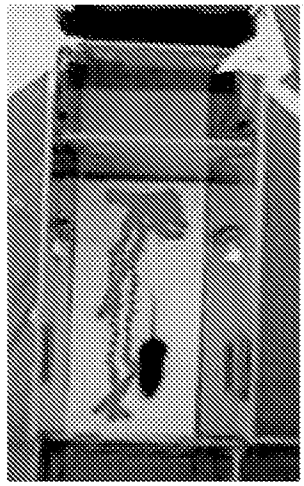


FIG. 19A

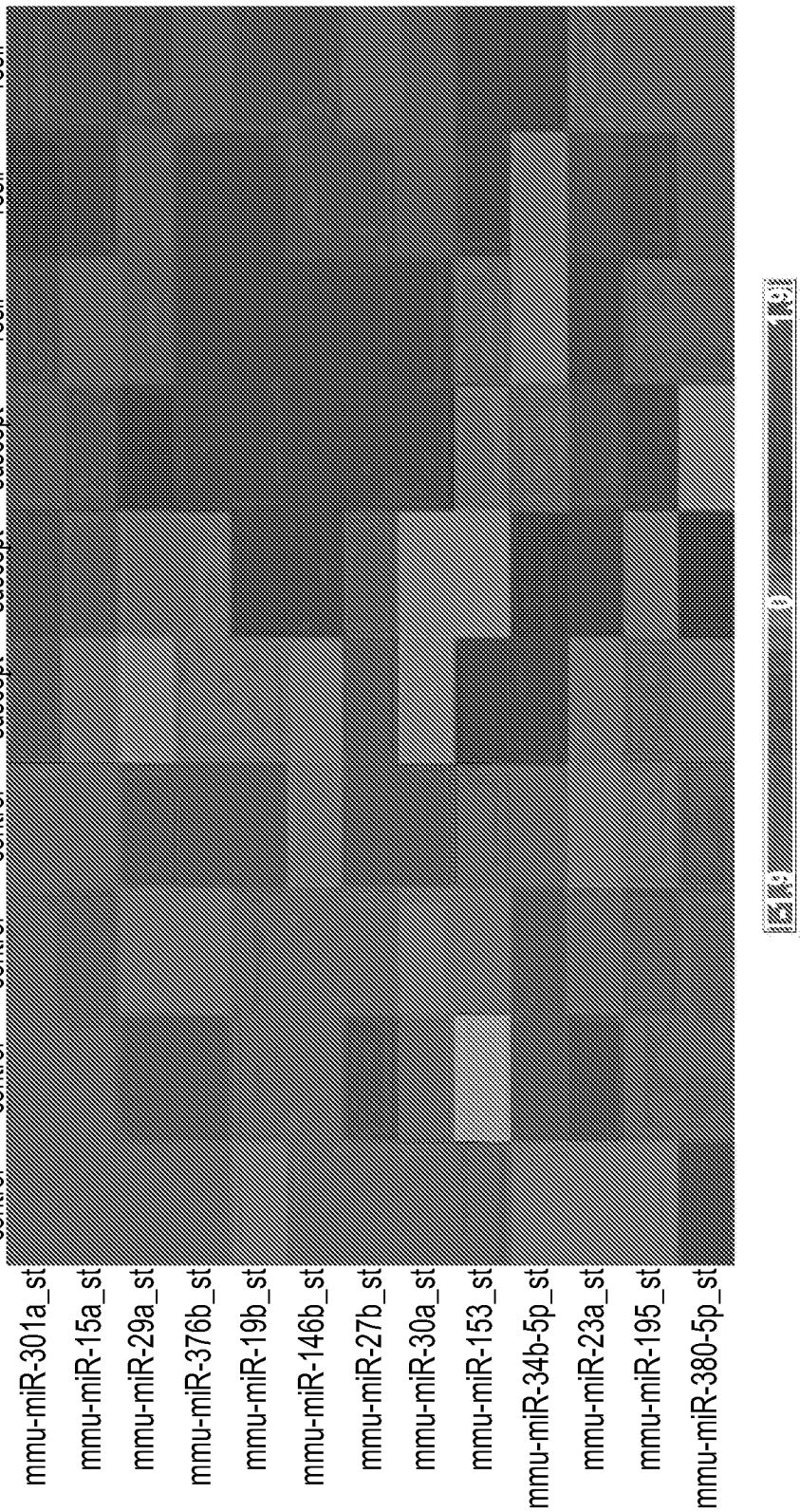

control
control
control
control
suscept
suscept
suscept
resil
resil
resil
mmu-miR-301a_st
mmu-miR-15a_st
mmu-miR-29a_st
mmu-miR-376b_st
mmu-miR-19b_st
mmu-miR-146b_st
mmu-miR-27b_st
mmu-miR-30a_st
mmu-miR-153_st
mmu-miR-34b-5p_st
mmu-miR-23a_st
mmu-miR-195_st
mmu-miR-380-5p_st

FIG. 19B

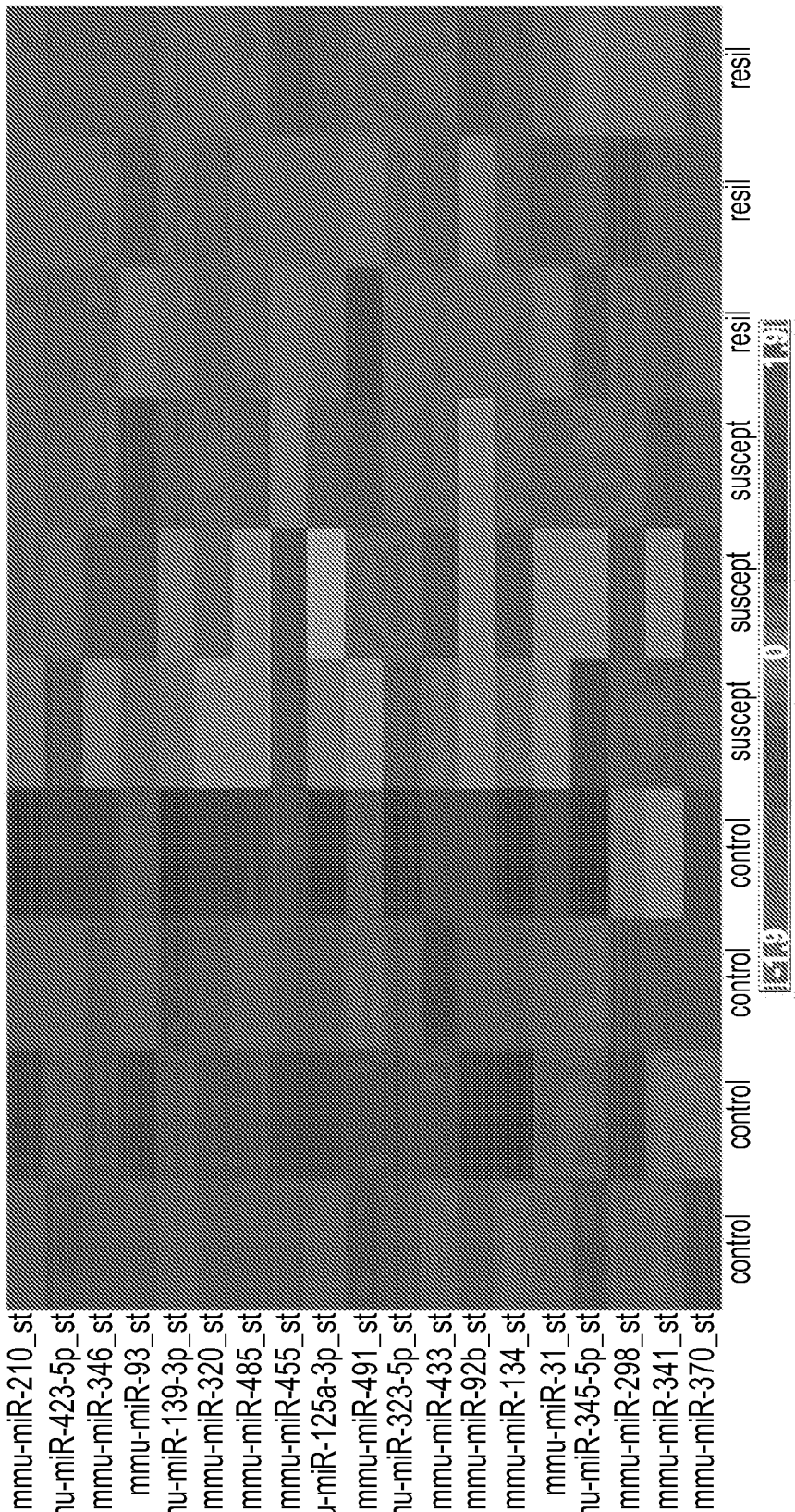

mmu-miR-210_st
mmu-miR-423-5p_st
mmu-miR-346_st
mmu-miR-93_st
mmu-miR-139-3p_st
mmu-miR-320_st
mmu-miR-485_st
mmu-miR-455_st
mmu-miR-125a-3p_st
mmu-miR-491_st
mmu-miR-323-5p_st
mmu-miR-433_st
mmu-miR-92b_st
mmu-miR-134_st
mmu-miR-31_st
mmu-miR-345-5p_st
mmu-miR-298_st
mmu-miR-341_st
mmu-miR-370_st
control
control
control
control
suscept
suscept
suscept
suscept
resil
resil
resil
resil

FIG. 20A

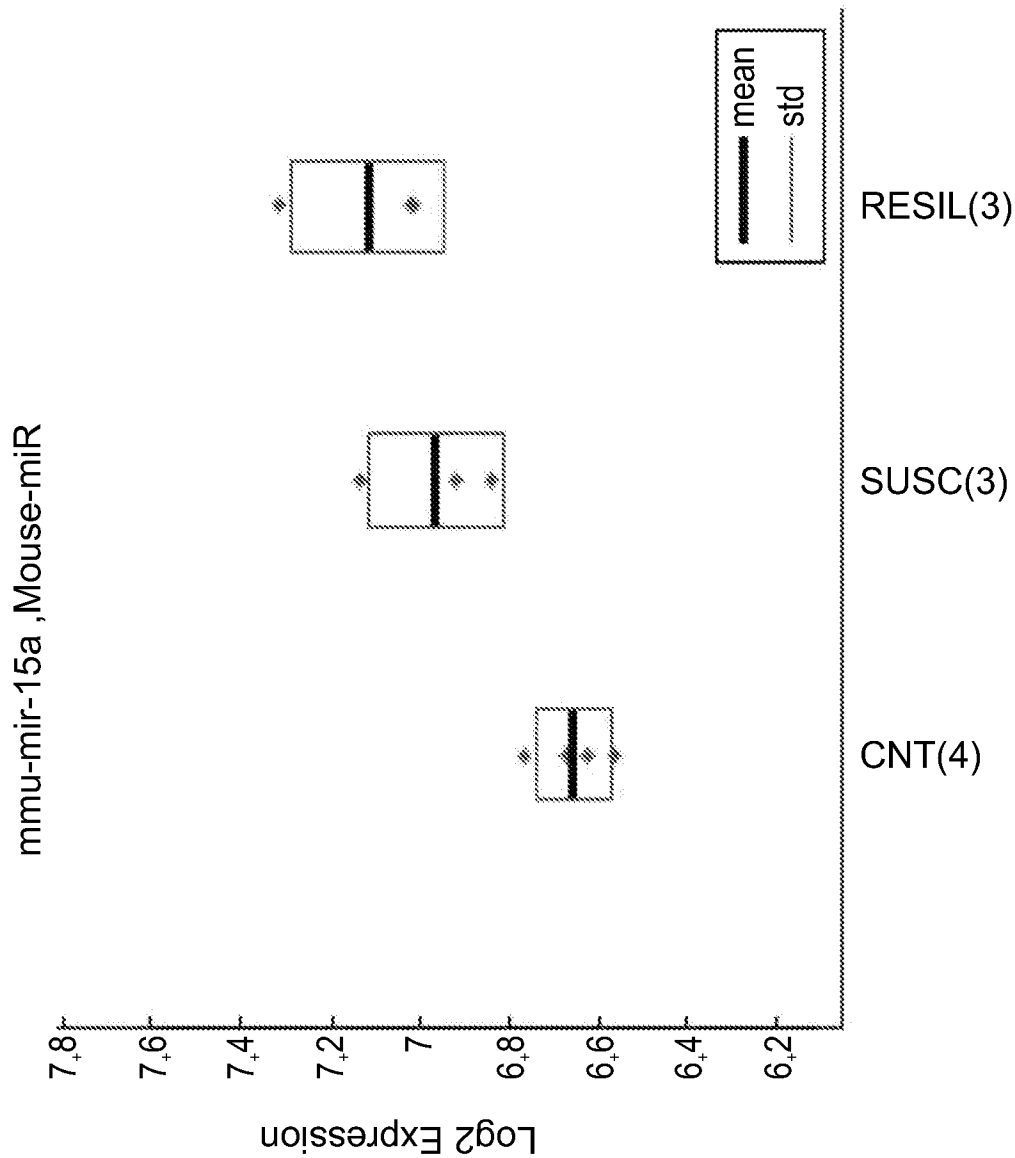

mmu-mir-15a ,Mouse-miR
Log2 Expression
7.8
7.6
7.4
7.2
7
6.8
6.6
6.4
6.2
CNT(4)
SUSC(3)
RESIL(3)
mean
std

FIG. 20B

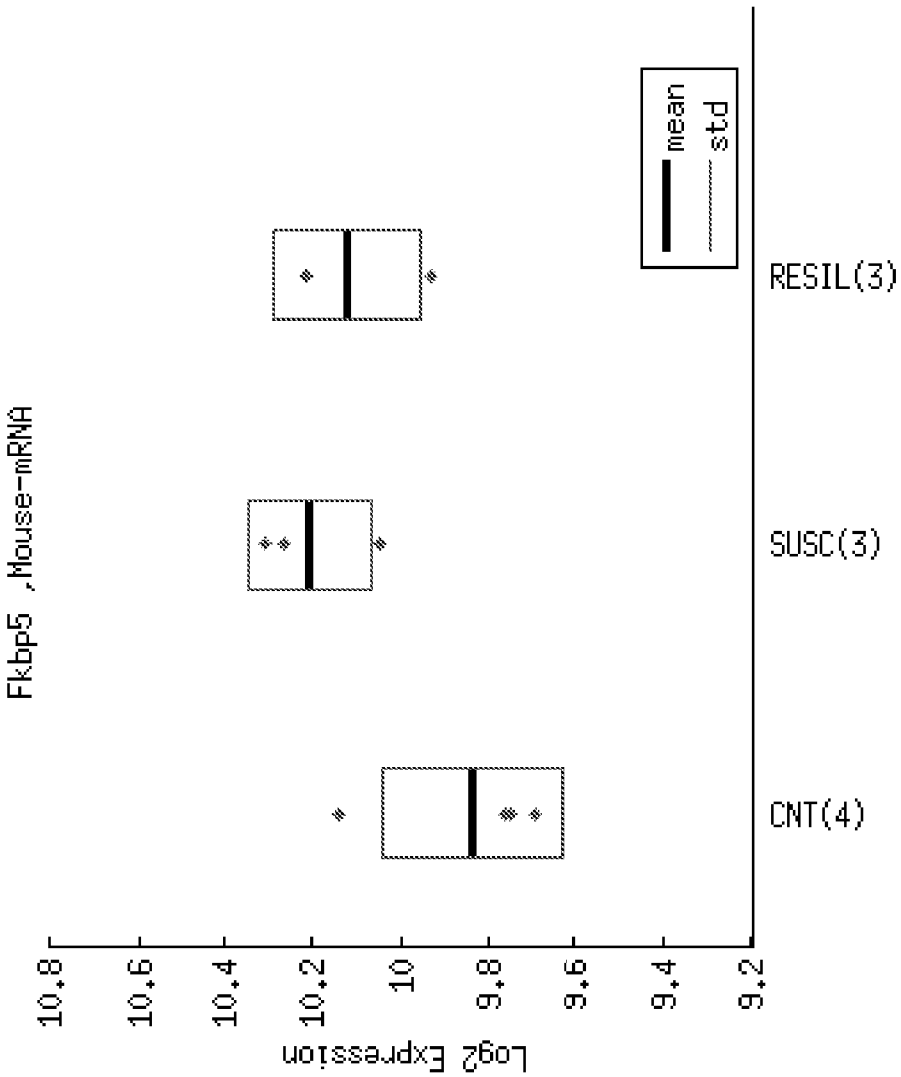

Fkbp5 ,Mouse-mRNA
Log2 Expression
10.8
10.6
10.4
10.2
10
9.8
9.6
9.4
9.2
CNT(4)
SUSC(3)
RESIL(3)
mean
std

FIG. 20C

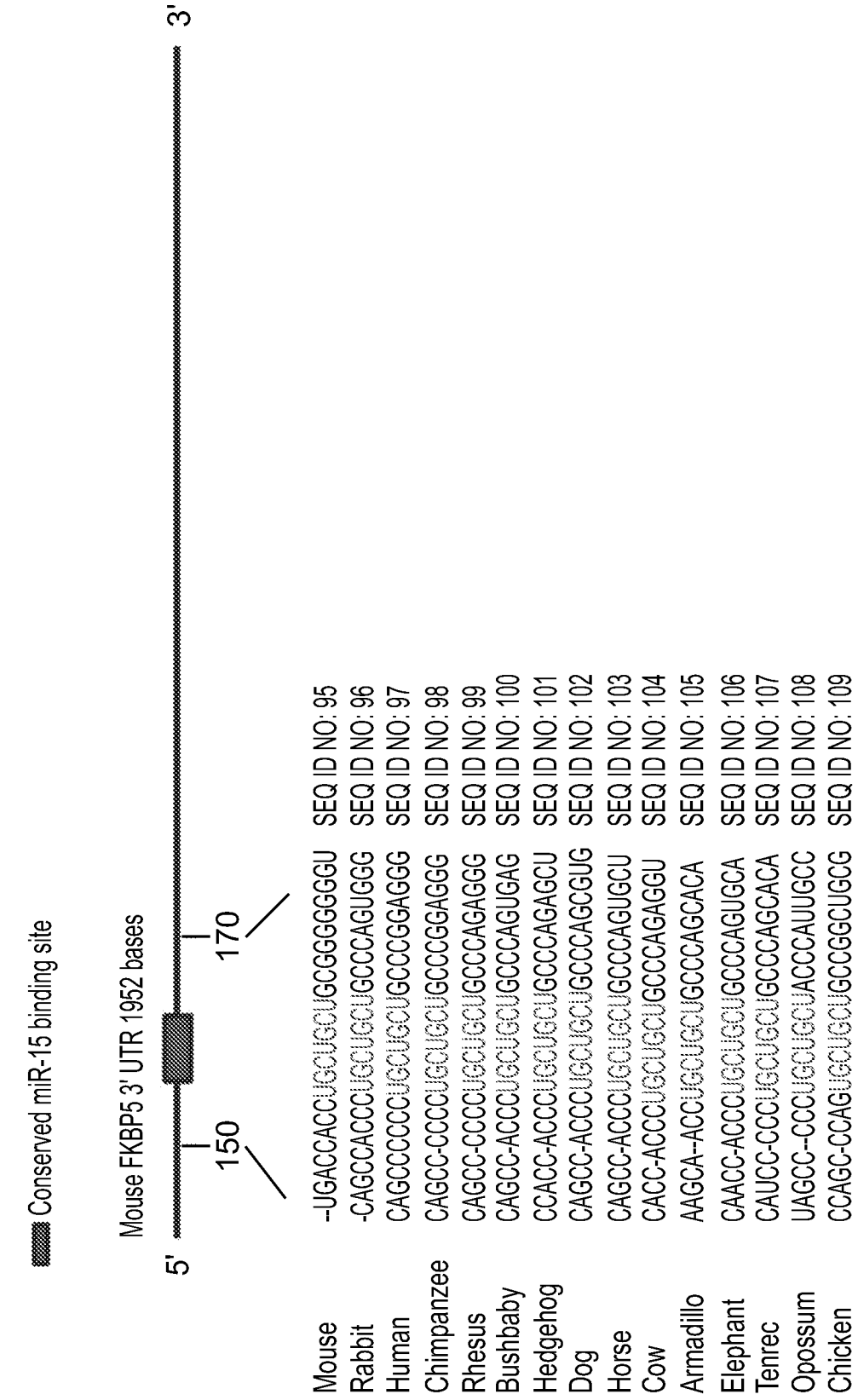

Conserved miR-15 binding site
Mouse FKBP5 3' UTR 1952 bases
5'
3'
150
170
Mouse -UGACCACCUGCUGCUGCGGGGGGGU SEQ ID NO: 95
Rabbit -CAGCCACCCUGCUGCUGCCCAGUGGG SEQ ID NO: 96
Human CAGCCCCCCCUGCUGCUGCCCGGAGGG SEQ ID NO: 97
Chimpanzee CAGCC-CCCCUGCUGCUGCCCGGAGGG SEQ ID NO: 98
Rhesus CAGCC-CCCCUGCUGCUGCCCAGAGGG SEQ ID NO: 99
Bushbaby CAGCC-ACCCUGCUGCUGCCCAGUGAG SEQ ID NO: 100
Hedgehog CCACC-ACCCUGCUGCUGCCCAGAGCU SEQ ID NO: 101
Dog CAGCC-ACCCUGCUGCUGCCCAGCGUG SEQ ID NO: 102
Horse CAGCC-ACCCUGCUGCUGCCCAGUGCU SEQ ID NO: 103
Cow CAC-ACCCUGCUGCUGCCCAGAGGU SEQ ID NO: 104
Armadillo AAGCA--ACCUGCUGCUGCCCAGCACA SEQ ID NO: 105
Elephant CAACC-ACCCUGCUGCUGCCCAGUGCA SEQ ID NO: 106
Tenrec CAUCC-CCCUGCUGCUGCCCAGCACA SEQ ID NO: 107
Opossum UAGCC--CCCUGCUGCUACCCAUUGCC SEQ ID NO: 108
Chicken CCAGC-CCAGUGCUGCUGCCGGCUGCG SEQ ID NO: 109

FIG. 21B

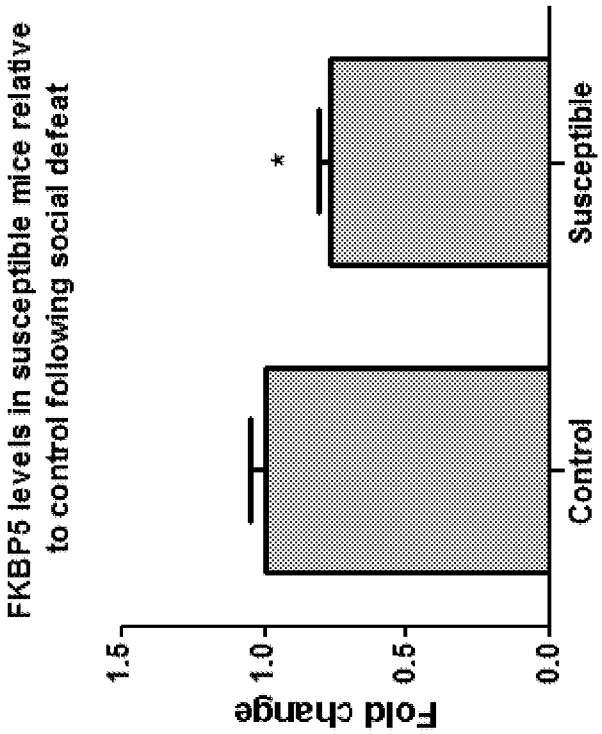


FIG. 21A

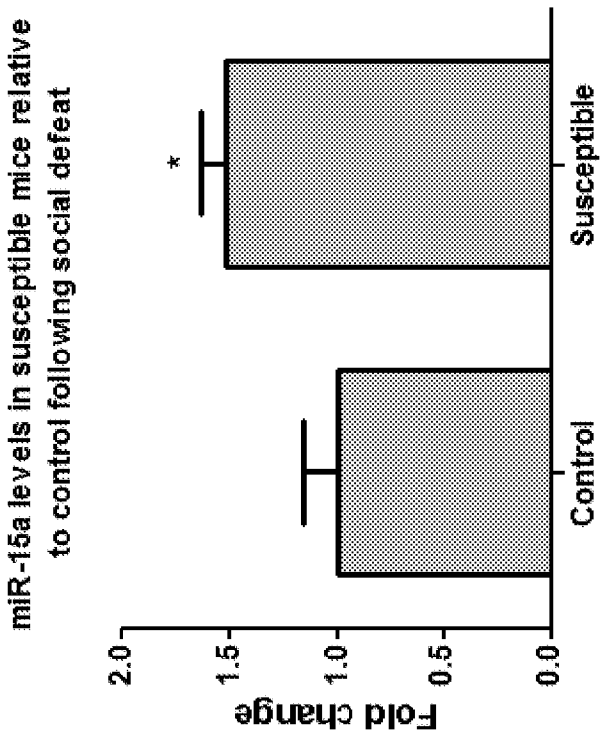


FIG. 22

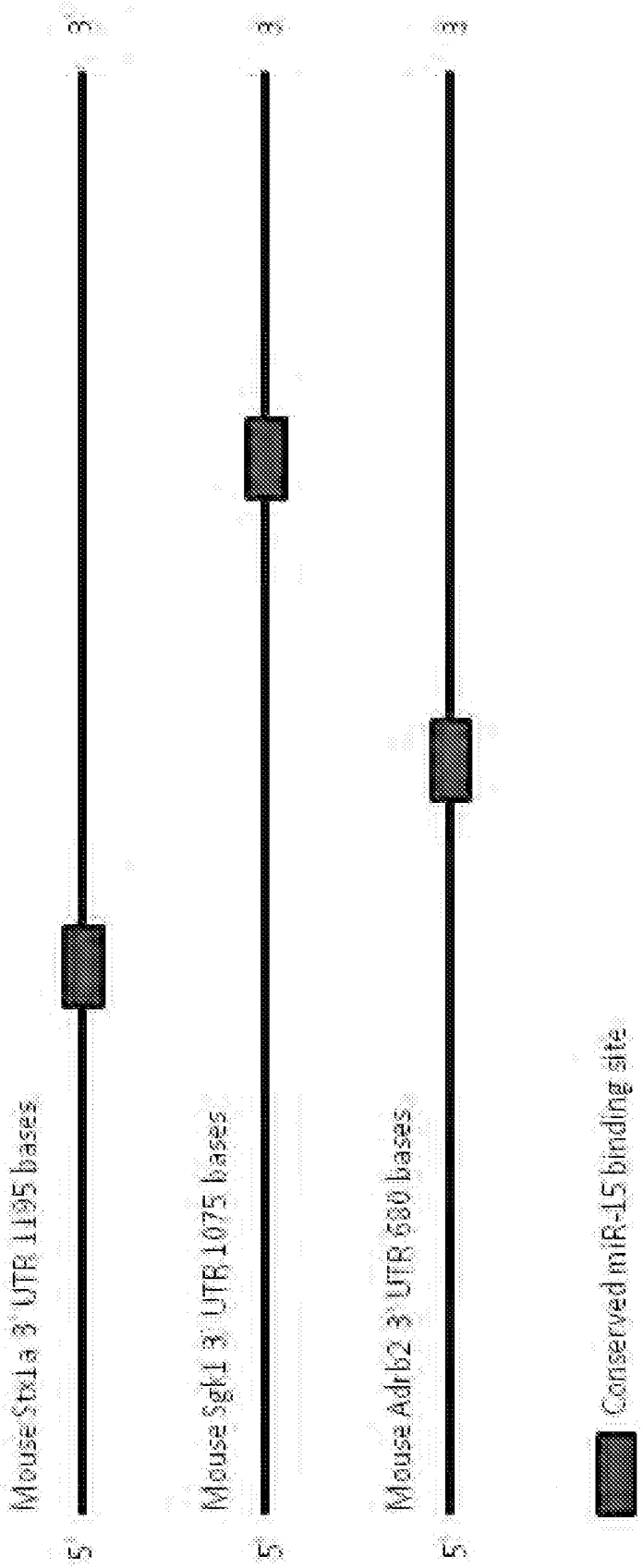

Mouse Strla 3' UTR 1195 bases
Mouse Sgtl 3' UTR 1075 bases
Mouse Adrb2 3' UTR 660 bases
5'
3'
Conserved miR-15 binding site

FIG. 23

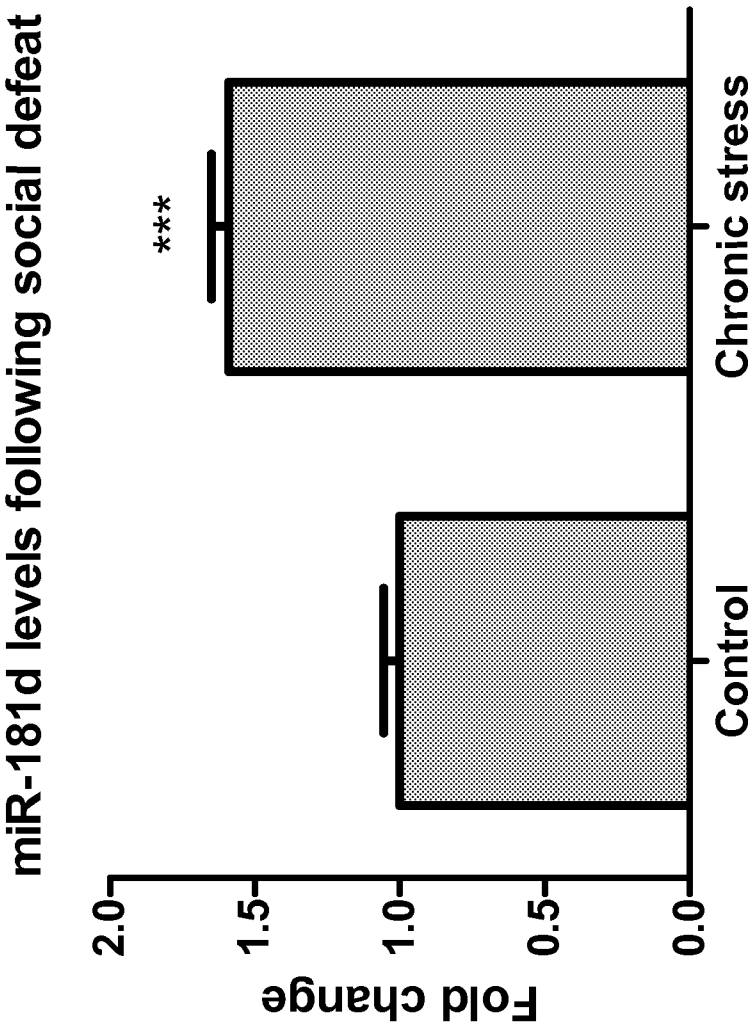

miR-181d levels following social defeat
***
Fold change
2.0
1.5
1.0
0.5
0.0
Control
Chronic stress

FIG. 24

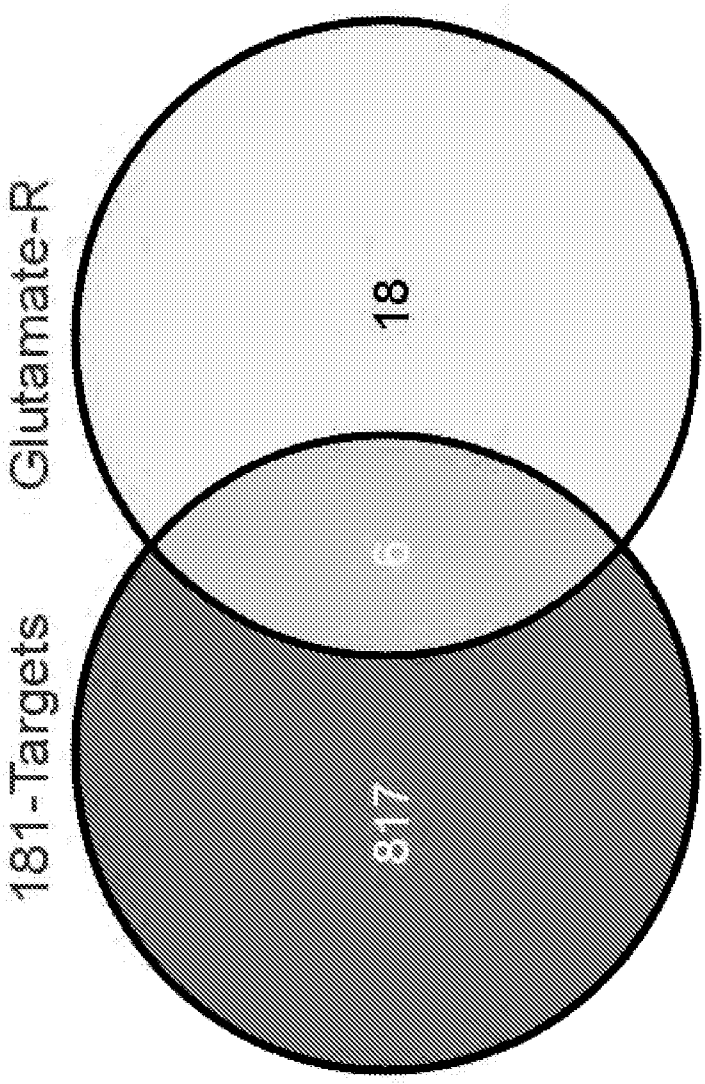


FIG. 25

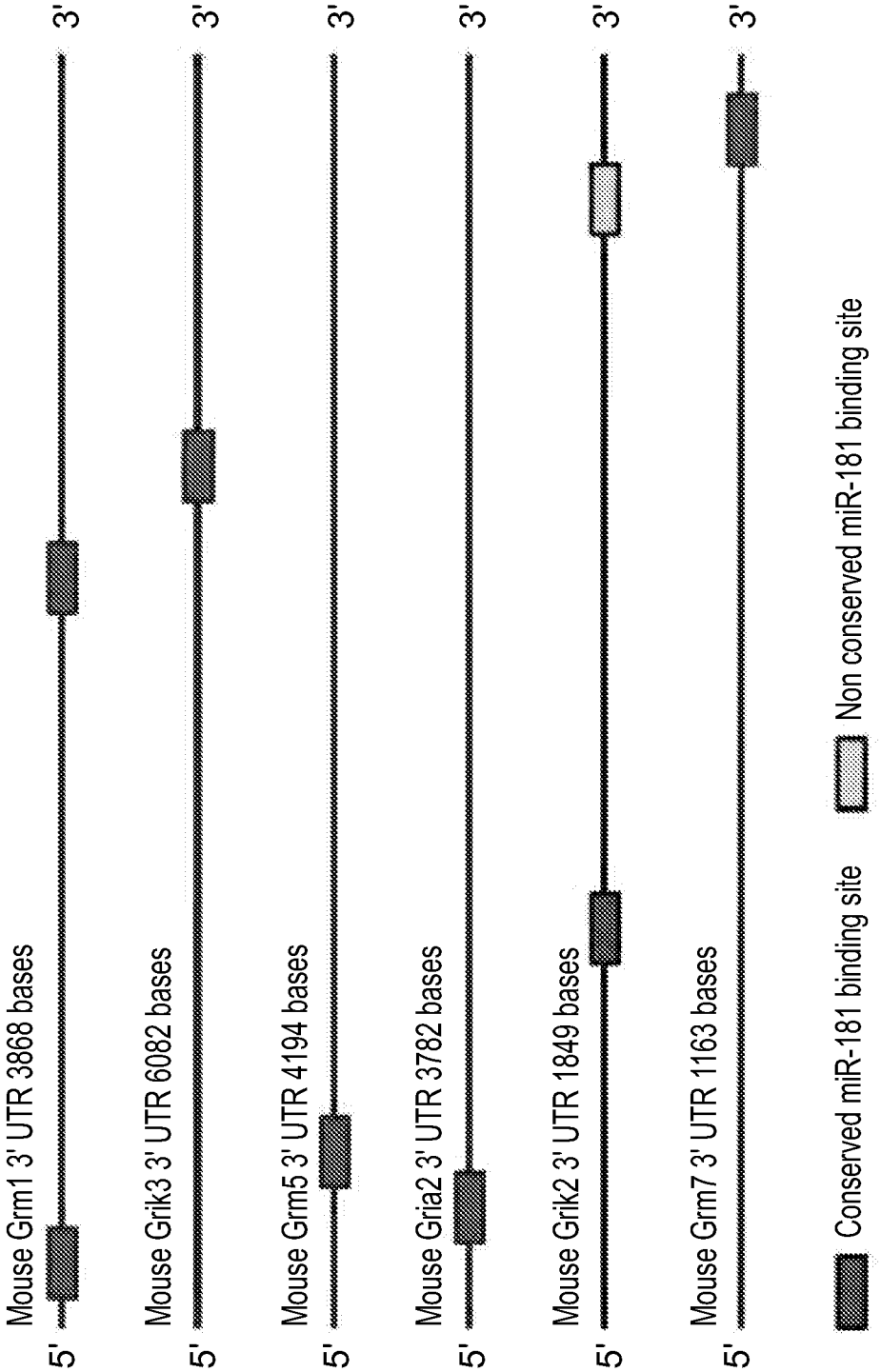
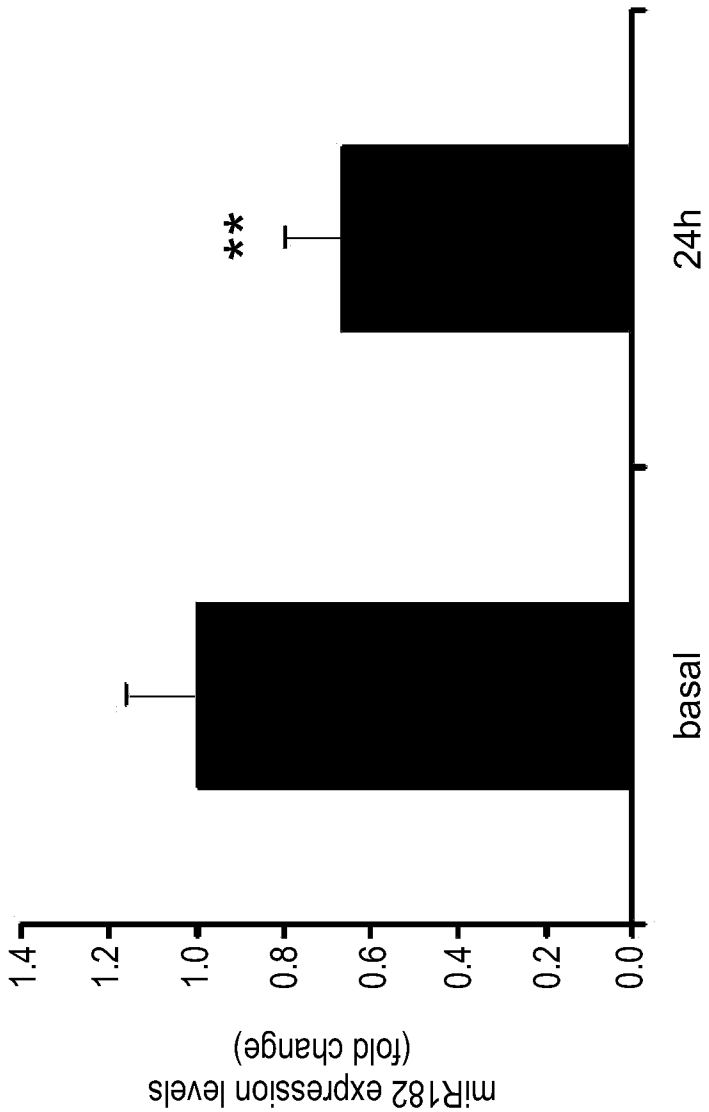

Mouse Grm1 3' UTR 3868 bases
Mouse Grik3 3' UTR 6082 bases
Mouse Grm5 3' UTR 4194 bases
Mouse Gria2 3' UTR 3782 bases
Mouse Grik2 3' UTR 1849 bases
Mouse Grm7 3' UTR 1163 bases
5'
3'
Conserved miR-181 binding site
Non conserved miR-181 binding site

FIG. 26


miR182 expression levels
(fold change)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
basal
**
24h

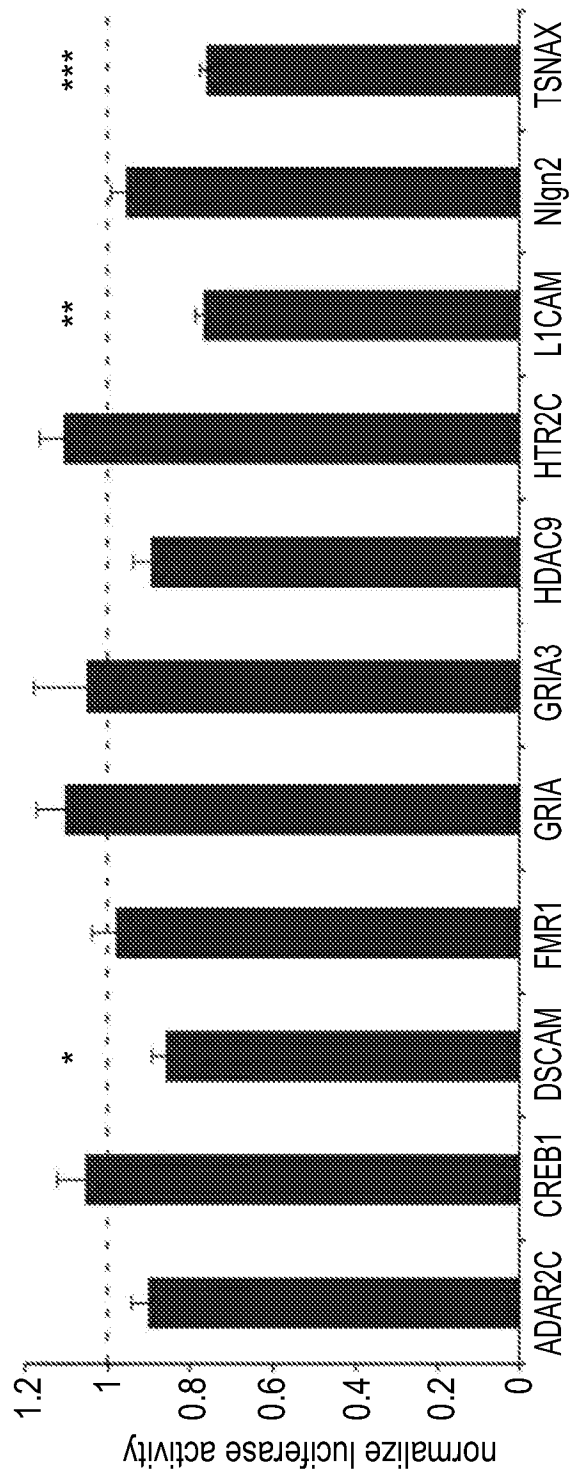


FIG. 27A

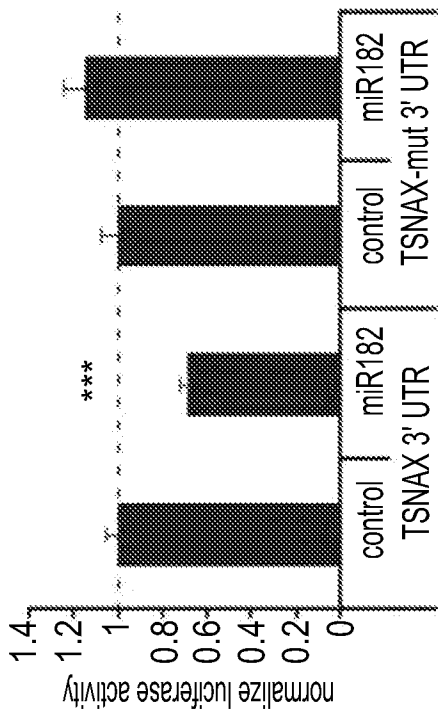


FIG. 27C

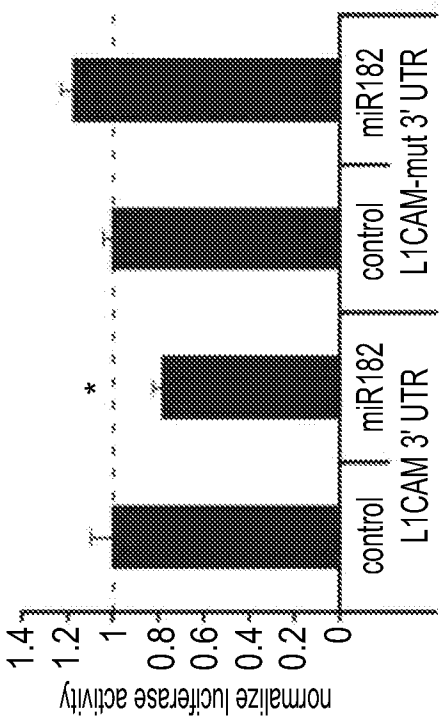


FIG. 27B

FIG. 28A

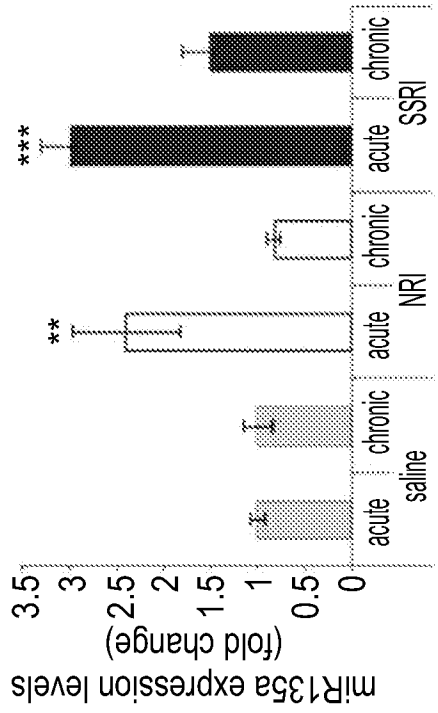


FIG. 28B

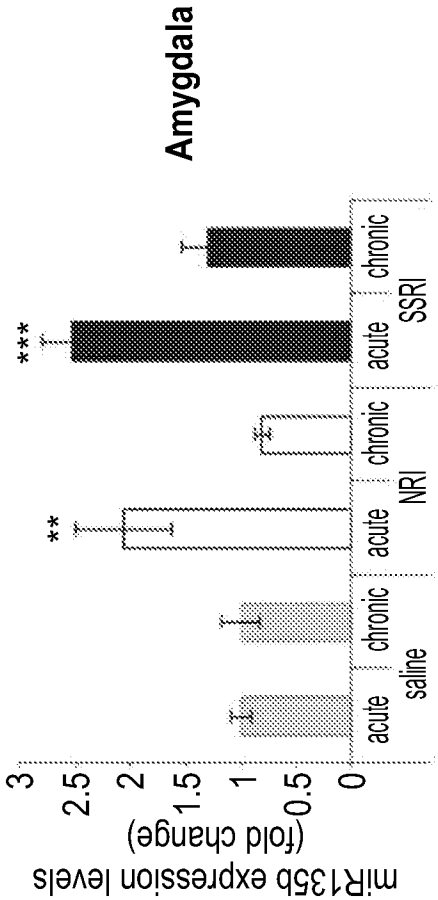


FIG. 28C

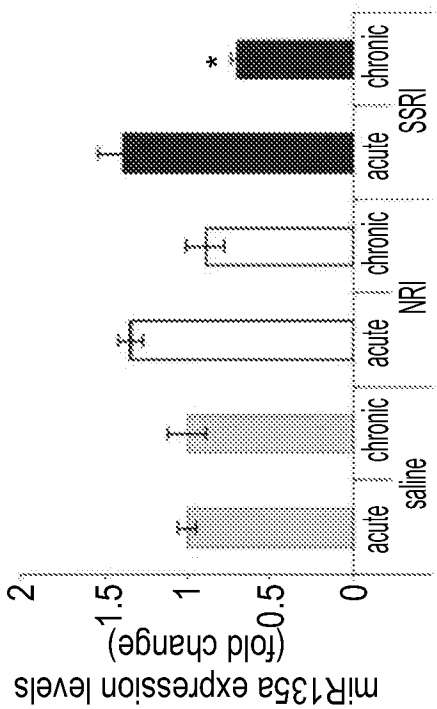


FIG. 28D

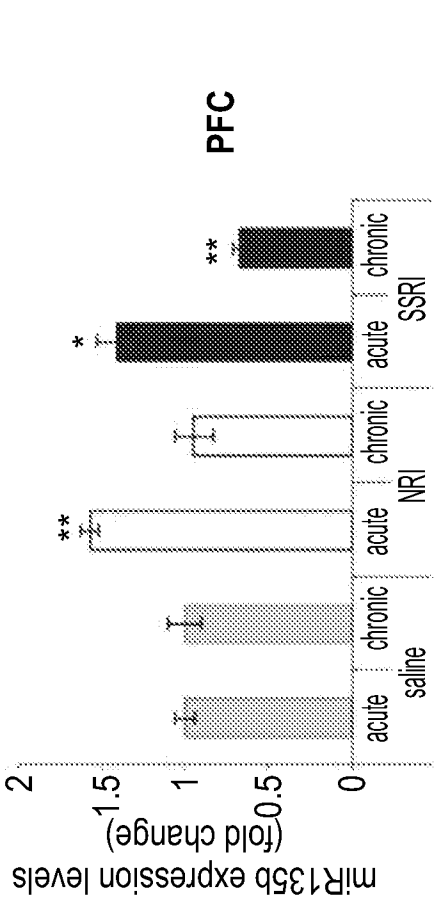


FIG. 29A

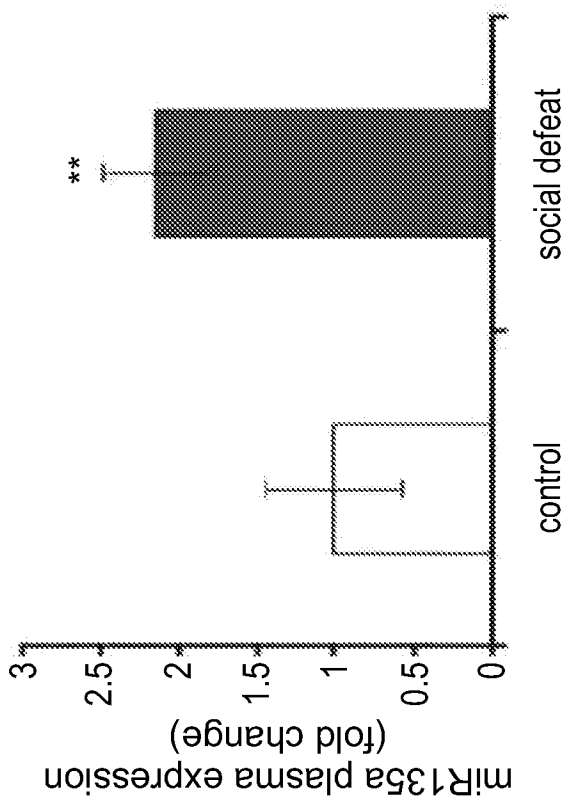


FIG. 29B

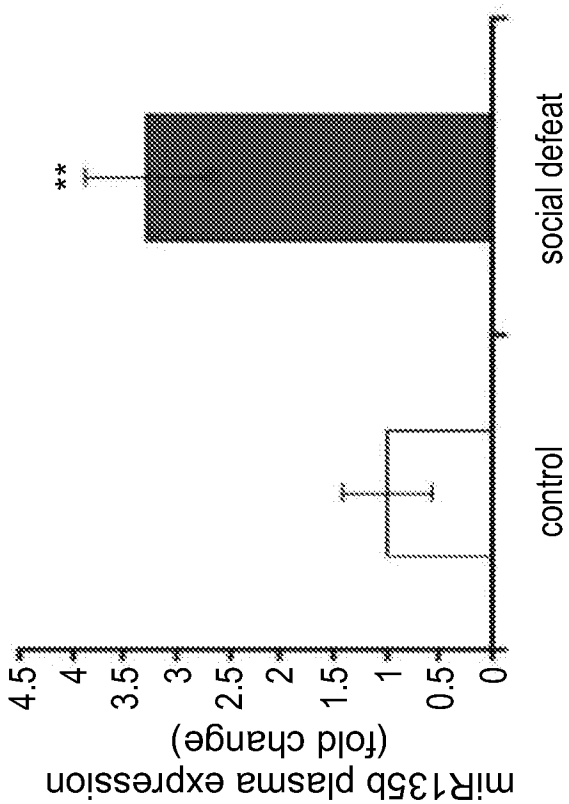


FIG. 30B

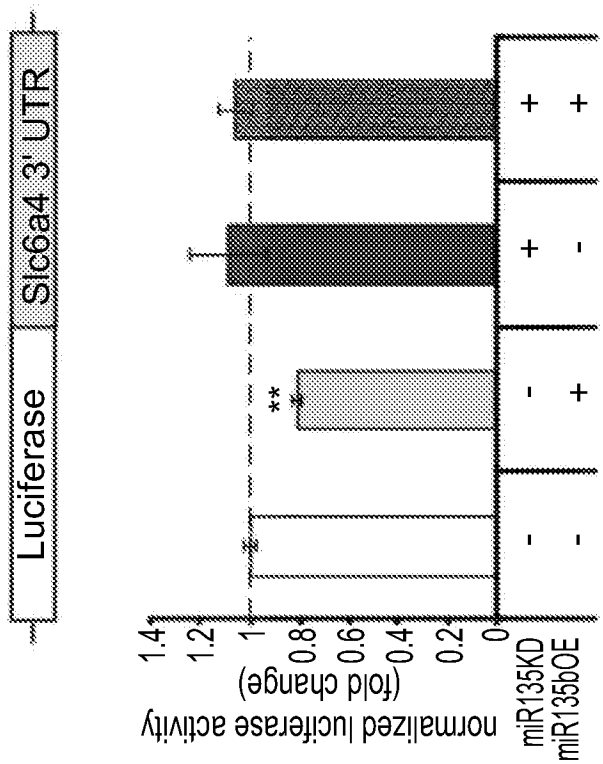


FIG. 30A

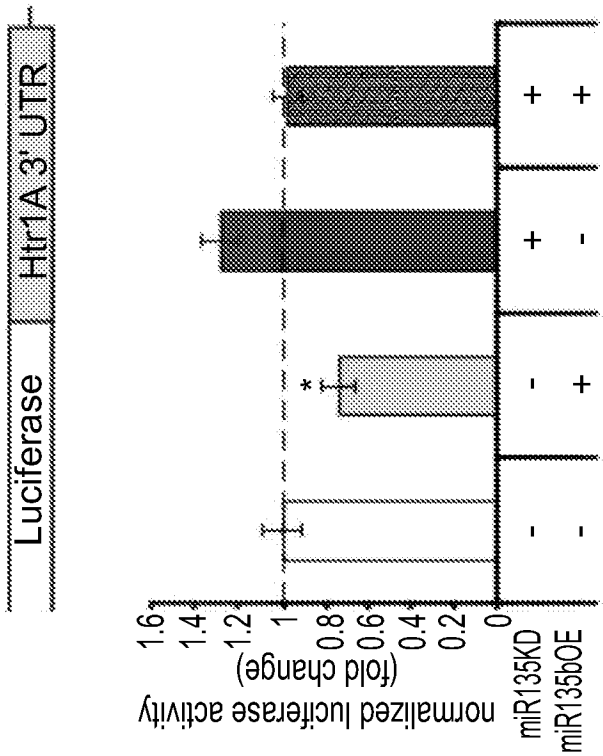


FIG. 30C

- H1 – control KD – CMV - eGFP -

- H1 – miR135 KD – CMV - eGFP -

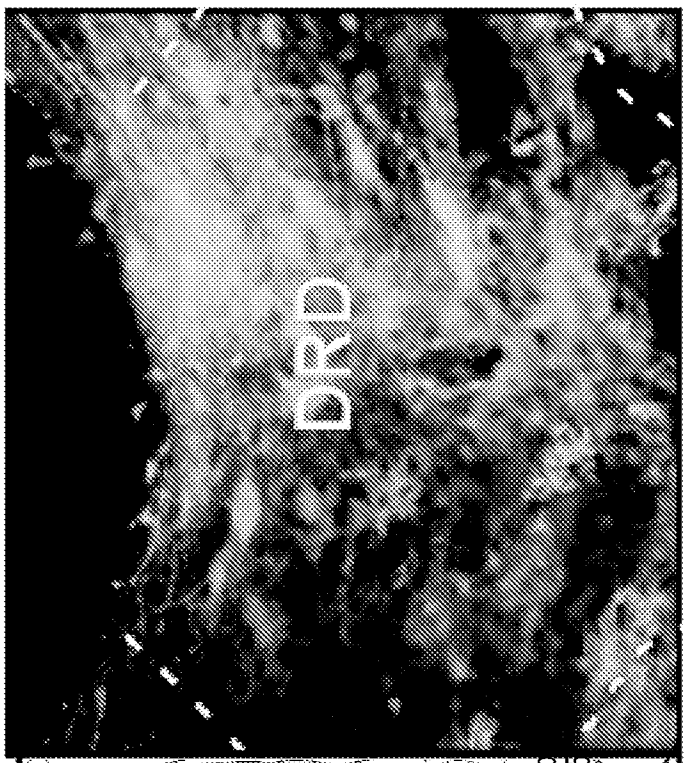


FIG. 30E

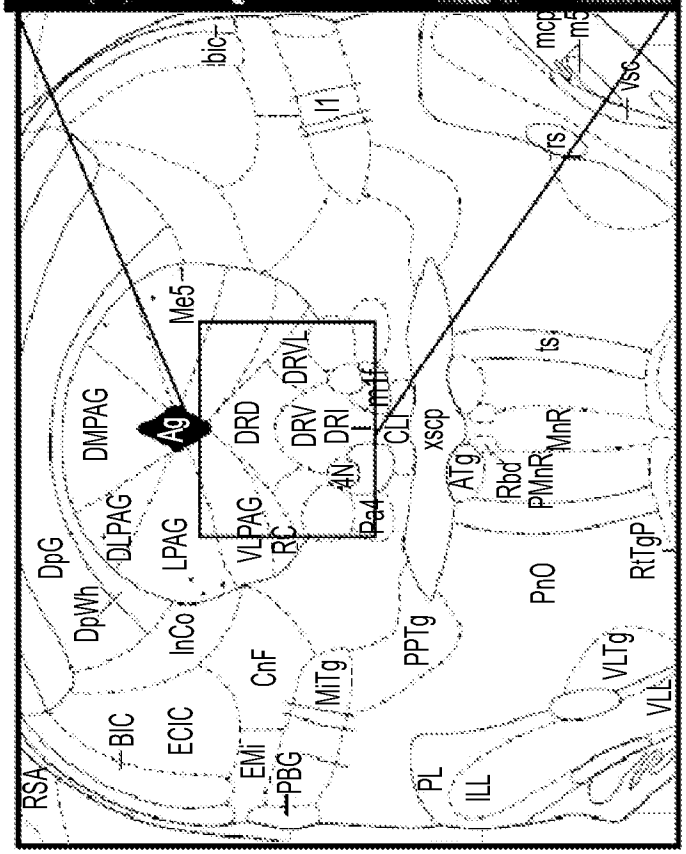


FIG. 30D

FIG. 31A

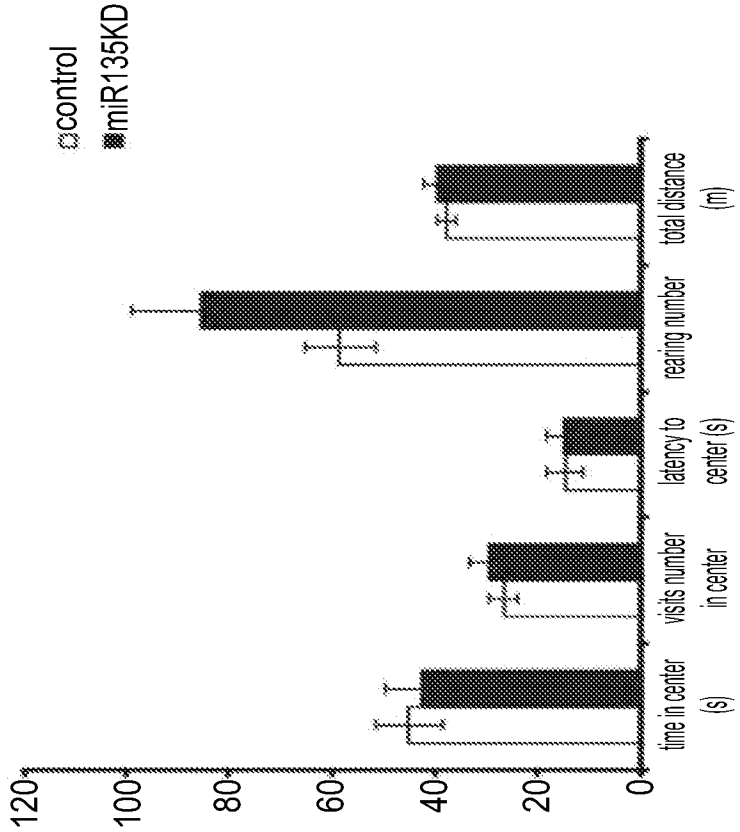


FIG. 31B

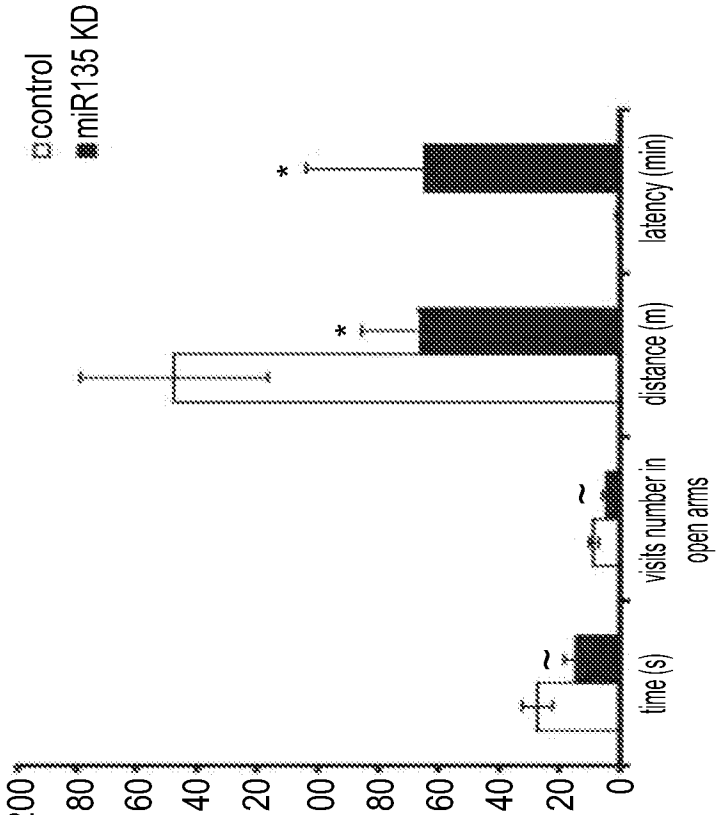


FIG. 31C

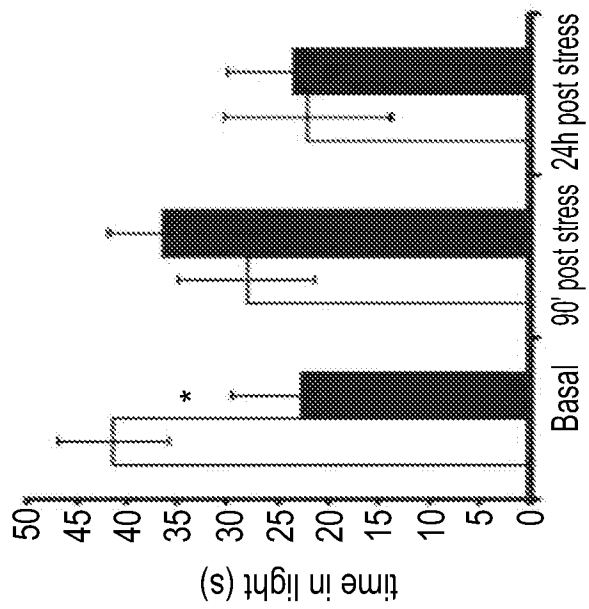


FIG. 31D

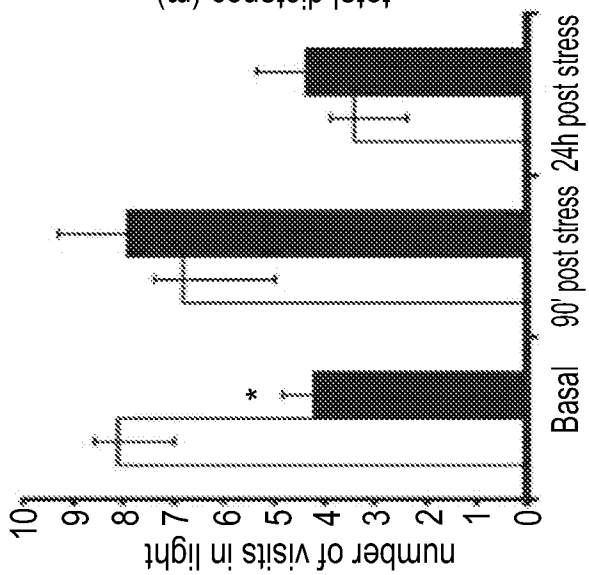


FIG. 31E

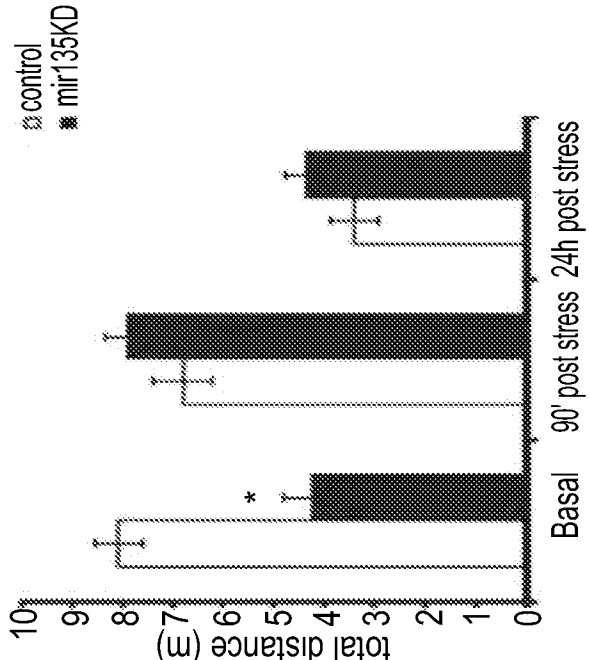


FIG. 31G

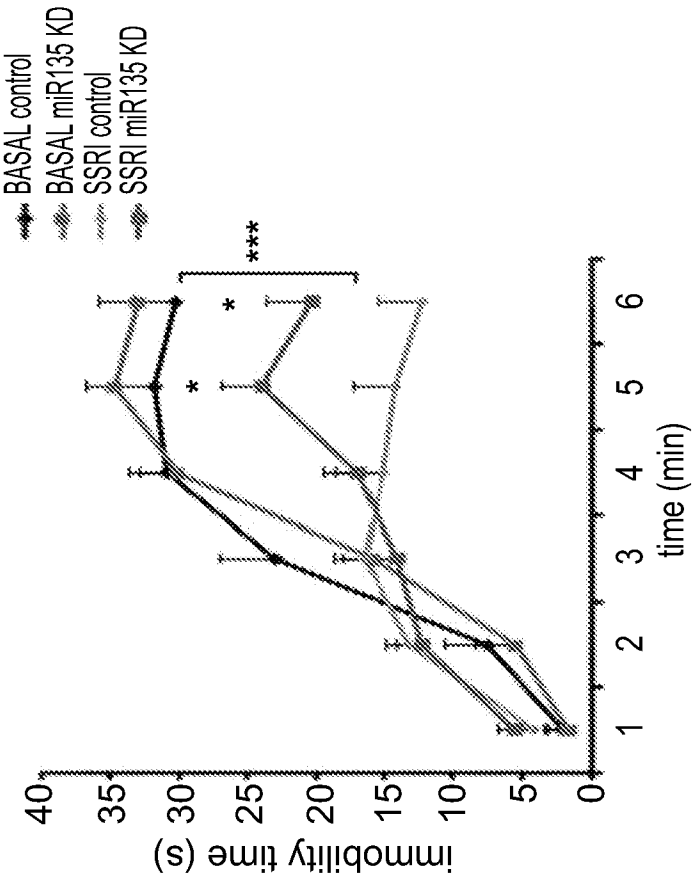


FIG. 31F

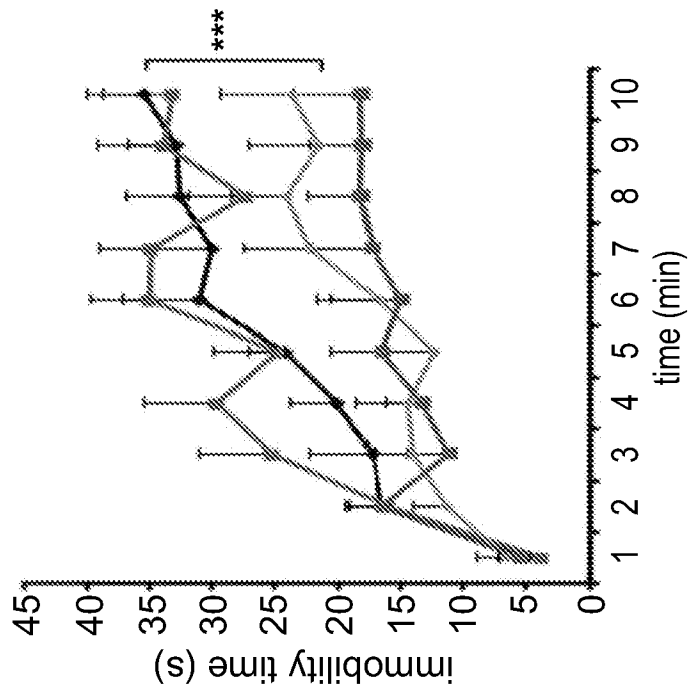
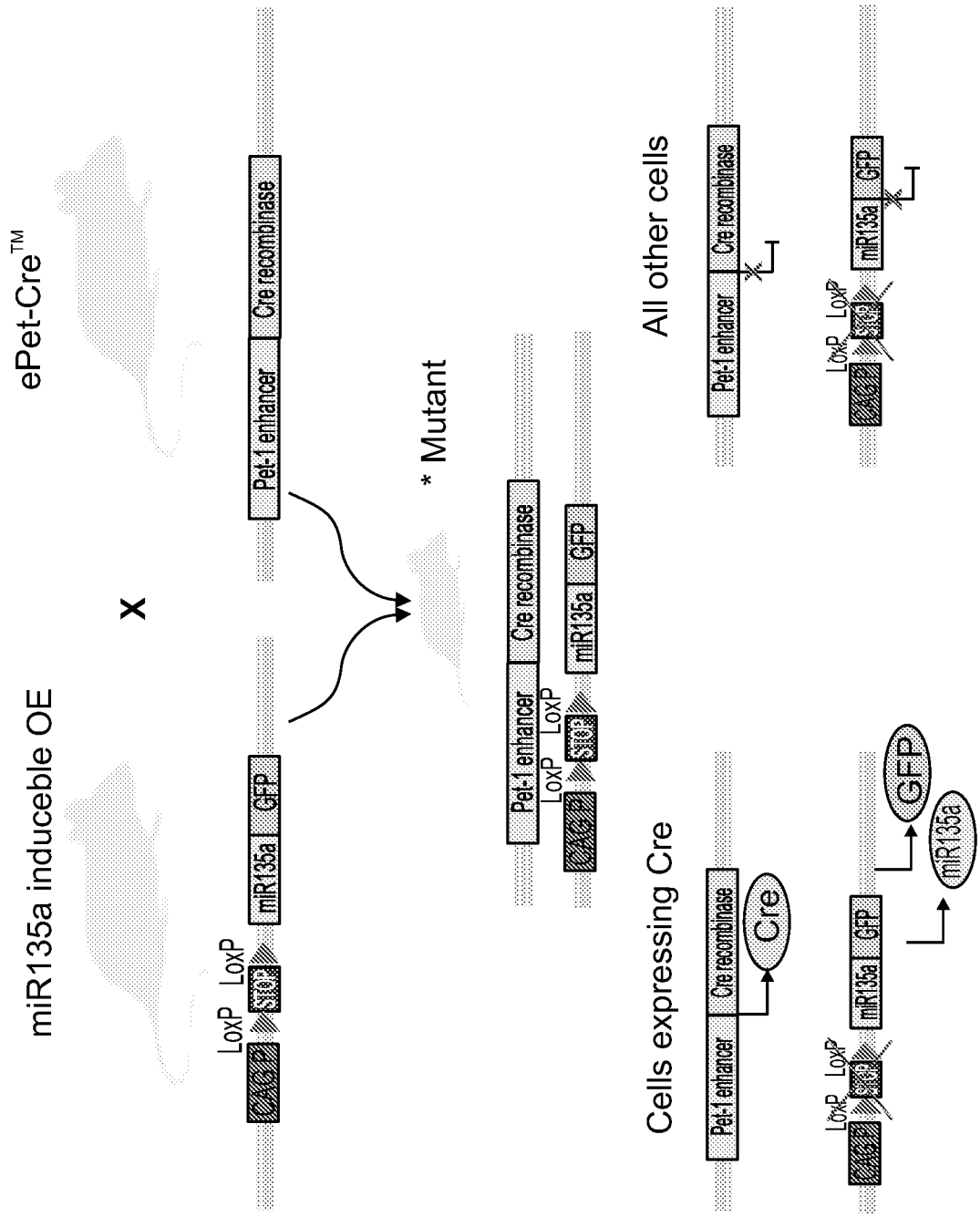


FIG. 32


miR135a inducible OE
x
ePet-Cre™
Pet-1 enhancer
Cre recombinase
LoxP
LoxP
miR135a
GFP
* Mutant
Pet-1 enhancer
Cre recombinase
LoxP
LoxP
miR135a
GFP
All other cells
Pet-1 enhancer
Cre recombinase
Cells expressing Cre
Pet-1 enhancer
Cre recombinase
Cre
GFP
miR135a

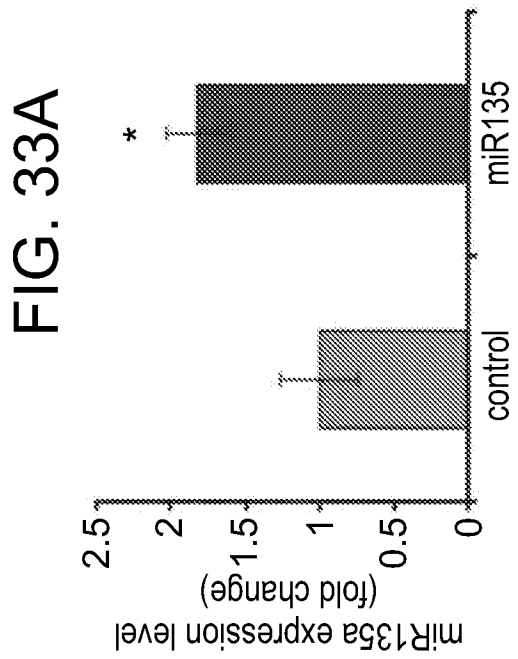


FIG. 33A

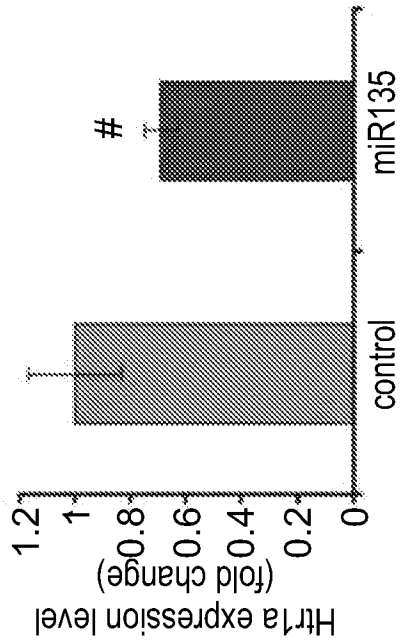


FIG. 33C

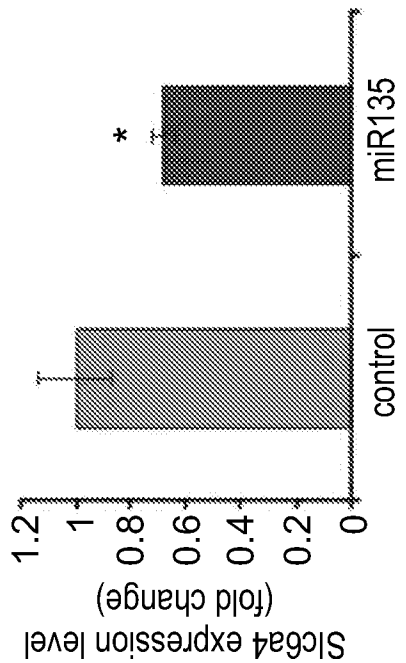


FIG. 33B

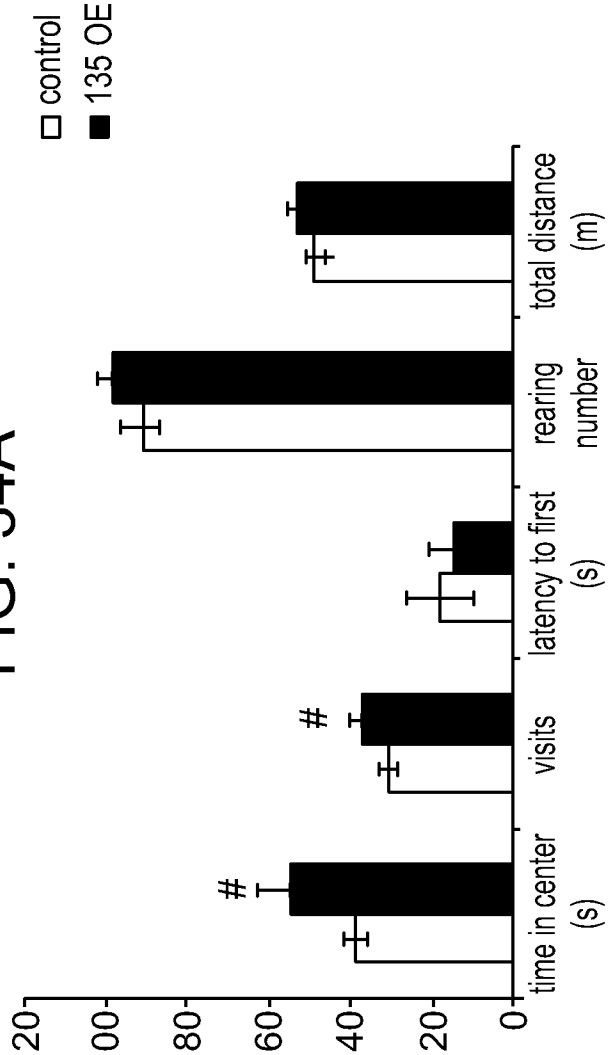


FIG. 34A

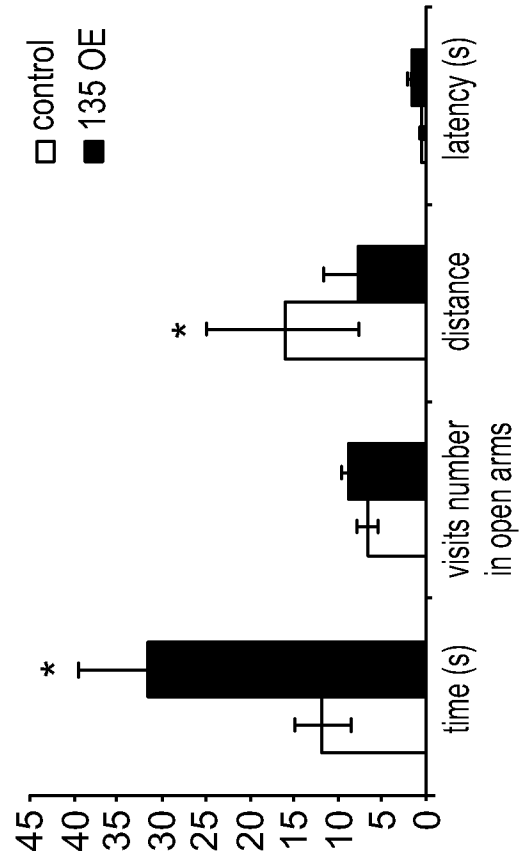


FIG. 34C

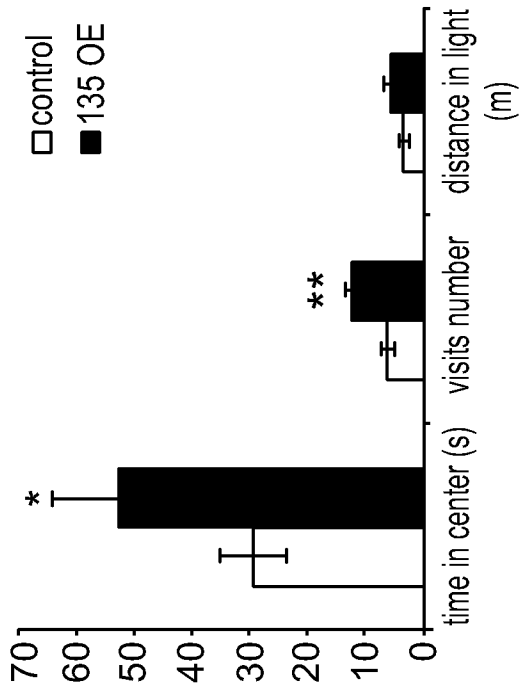


FIG. 34B

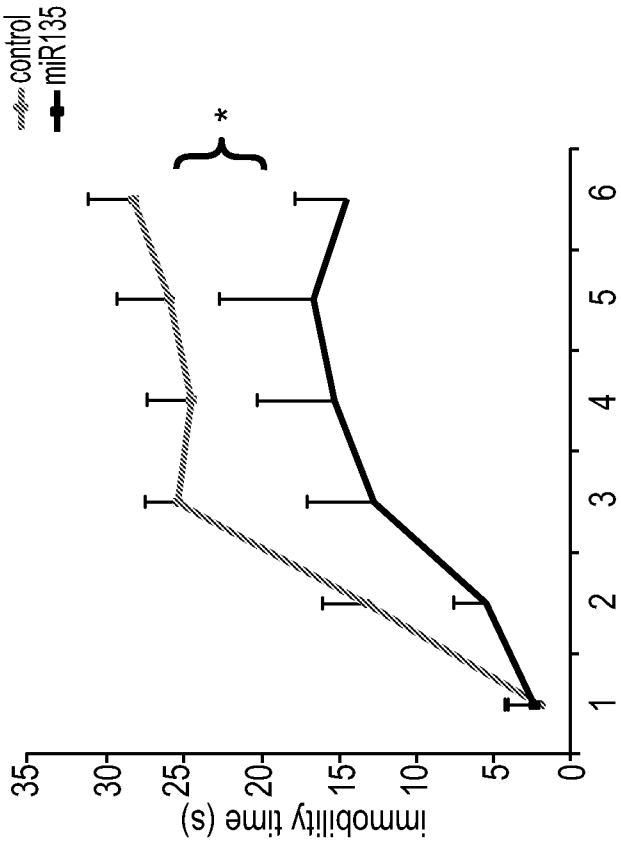


FIG. 34E

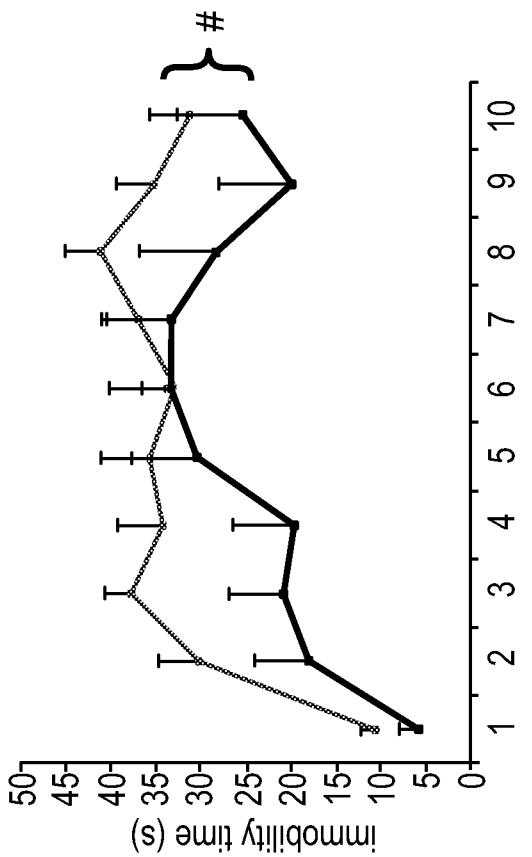


FIG. 34D